Involvement of the p38 Mitogen-activated Protein Kinase Pathway in Transforming Growth Factor-β-induced Gene Expression*

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Transforming growth factor-β (TGF-β)-activated kinase 1 (TAK1), a member of the mitogen-activated protein kinase kinase kinase family, is suggested to be involved in TGF-β-induced gene expression, but the signaling mechanism from TAK1 to the nucleus remains largely undefined. We have found that p38 mitogen-activated protein kinase, and its direct activator MKK6 are rapidly activated in response to TGF-β. Expression of dominant negative MKK6 or dominant negative TAK1 inhibited the TGF-β-induced transcriptional activation as well as the p38 activation. Constitutive activation of the p38 pathway in the absence of TGF-β induced the transcriptional activation, which was enhanced synergistically by coexpression of Smad2 and Smad4 and was inhibited by expression of the C-terminal truncated, dominant negative Smad4. Furthermore, we have found that activating transcription factor-2 (ATF-2), which is known as a nuclear target of p38, becomes phosphorylated in the N-terminal activation domain in response to TGF-β, that ATF-2 forms a complex with Smad4, and that the complex formation is enhanced by TGF-β. In addition, expression of a nonphosphorylatable form of ATF-2 inhibited the TGF-β-induced transcriptional activation. These results show that the p38 pathway is activated by TGF-β and is involved in the TGF-β-induced transcriptional activation by regulating the Smad-mediated pathway.

Members of the transforming growth factor-β (TGF-β) superfamily regulate cell proliferation, differentiation, and apoptosis. They exert their effects through heteromerization of receptor complexes consisting of type I and type II serine/threonine kinase receptors. Following ligand binding, the type II receptor phosphorylates the type I receptor to activate it (1, 2). Intracellular signaling downstream of these receptor complexes is mediated by the recently identified Smad family. To date, at least nine vertebrate Smad proteins have been identified (1–5). Each member of the Smad family has different roles in signal transduction. For example, the receptor-regulated Smads, Smad1, -2, -3, -5, and -8, are phosphorylated within a conserved C-terminal SS(V/M)S motif by the specific type I receptors (6–14) and then associate with the common Smad, Smad4, which in turn translocates into the nucleus (6, 7, 10, 12, 15–20). Following nuclear translocation, Smads induce transcriptional activation of specific target genes through cooperation with other transcriptional factors. The Smad2/Smad4 complex interacts with the Mix.2 promoter through FAST1, a transcription factor that binds to an activin response element, and interacts with the goosceoid promoter through FAST2/FAST1 (21–24). Similarly, at AP-1 binding sites in the promoter of TGF-β-responsive genes, the Smad3/Smad4 complex interacts with c-Jun/c-Fos (25). Other transcription factors and coactivators such as TFE3, cAMP-response element-binding protein-binding protein/p90, and cAMP-response element-binding protein have been shown to interact with Smad complexes (25–31). Thus, the mechanism of transcriptional activation by Smads is likely, at least partly, based on the interaction with other transcription factors.

Although previous studies indicated that TAK1, a member of the MAP kinase kinase kinase family, is involved in the TGF-β signaling pathway (32, 33), the signaling mechanism to the nucleus remains largely undefined. TAK1 is a potent activator of the p38 pathway and the SAPK/JNK pathway (34–36). However, it is not known whether TAK1 actually acts as a physiological activator of these signaling pathways in vivo. Recently, it was reported that the SAPK/JNK pathway is required for TGF-β-mediated signaling (36–38). But the activation of SAPK/JNK by TGF-β is maximal 12 h after stimulation, whereas TGF-β induces the activation of TAK1 within 15 min. These results seemed to imply that TAK1 is not a direct activator of the SAPK/JNK pathway in TGF-β signaling. Thus we have investigated which pathway plays a pivotal role downstream of TAK1 in TGF-β signaling. Furthermore, we have examined the possible functional link between the Smad and TAK1 pathways in TGF-β signaling. Here we report that the MKK6-p38 kinase cascade appears to lie downstream of TAK1 in TGF-β signaling, and the transcription factor ATF-2 functions as one of targets of this pathway. ATF-2 can associate with Smad4 in response to TGF-β. Our results suggest that TGF-β activates the Smad and TAK1 pathways, resulting in the formation of an active transcription complex containing Smad4 and ATF-2.

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The abbreviations used are: TGF-β, transforming growth factor-β; MAP, mitogen-activated protein; TAK1, TGF-β-activated kinase 1; SAPK, stress-activated protein kinase; JNK, c-Jun N-terminal kinase; ATF-2, activating transcription factor-2; TAB1, TAK1-binding protein; HA, hemagglutinin; TJαRi, TGF-β type I receptor; TJβRII, TGF-β type II receptor; Mv1Lu, mink lung epithelial.
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EXPERIMENTAL PROCEDURES

Antibodies, Immunoblotting, and Immunoprecipitation—For assaying endogenous p38, SAPK/JNK, MKK6, and ATF-2 activities, 2 × 10⁶ cells were lysed in 150 µl of lysis buffer containing 20 mM Tris-HCl (pH 7.5), 12.5 mM 2-glycerophosphate, 150 mM NaCl, 1.5 mM MgCl₂, 2 mM EGTA, 10 mM NaF, 0.5% Triton X-100, 2 mM diithiothreitol, 1 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride, and 20 µg/ml aprotinin. Cell lysates (20 µl/lane) were subjected to immunoblotting with the indicated anti-phospho-specific antibodies. For anti-MyC immunoprecipitations, cell lysates were incubated with anti-MyC 9E10 antibody and protein G-Sepharose beads (Amersham Pharmacia Biotech) for 2 h at 4 °C with rocking. The beads were washed three times with ice-cold phosphate-buffered saline and subjected to kinase assays or association assays.

Cell Cultures and Transfection—C2C12 cells were cultured in Dulbecco’s modified Eagle’s medium containing 15% fetal bovine serum. mink lung epithelial (MvILu) cells were cultured in Dulbecco’s modified Eagle’s medium/F-12 containing 5% fetal bovine serum. These cells were transfected using LipofectAMINE according to the manufacturer’s instructions (Life Technologies, Inc.). For protein kinase assays, we prepared cell lysates from 5 × 10⁶ cells that were transiently transfected with the indicated constructs (~20–50% transfection efficiencies).

Protein Kinase Assays—Immunocomplex kinase reactions of Myc-MKK6 and Myc-p38 were performed in a final volume of 15 µl containing 20 mM Tris-HCl (pH 7.5), 2 mM EGTA, 15 mM MgCl₂, 100 µM [γ-³²P]ATP, and 3 µg of His-tagged kinase-negative MPK-2 or glutathione S-transferase-tagged ATP-2 as substrate. Substrate phosphorylations were detected and quantified by autoradiography and image analysis (Bio-Rad).

Luciferase Assays—For luciferase reporter assays, cells were transiently transfected with p3TP-Lux, which contains TGF-β-responsive elements (40), pCMV-β-gal, and the indicated constructs or with empty vector alone. The total amount of DNA for each transfection was kept constant using empty vector. Cells were treated for 15–20 h with or without 5–10 ng/ml TGF-β, and luciferase activity in cell lysates was measured using the luciferase assay system (Promega) in a Berthold

FIG. 1. Activation of MKK6 and p38 by TGF-β treatment. A, TGF-β stimulates the kinase activity of p38, but not SAPK/JNK, rapidly. C2C12 cells were exposed to TGF-β (20 ng/ml) or NaCl (0.7 M) for the indicated times. Cell lysates were immunoblotted with polyclonal antibody to phospho-p38 (αP-p38) (Thr180/Tyr182) or phospho-SAPK/JNK (αP-SAPK/JNK) (Thr183/Tyr185) respectively (Biolabs). That the amounts of p38 and SAPK/JNK proteins were unchanged was confirmed by immunoblotting total cell lysates using anti-p38 antibody (αp38) and anti-SAPK/JNK (αSAPK/JNK) antibody (Biosales). Essentially the same results were obtained in three independent experiments. B, activation of MKK6 by TGF-β is shown. C2C12 cells were exposed to TGF-β (20 ng/ml) or NaCl (0.7 M) for the indicated times. Cell lysates were immunoblotted with polyclonal antibody to phospho-MKK3/6 (αP-MKK3/6) (Ser189/Ser207) (Biolabs). That the amount of MKK6 or MKK3 protein was unchanged was confirmed by immunoblotting total cell lysates using anti-MKK6 monoclonal antibody (αMKK6) (39) or anti-MKK3 polyclonal antibody (αMKK3) (Santa Cruz Biotechnology), respectively. Essentially the same results were obtained in two independent experiments. C, activation of MKK6 and p38 by TGF-β treatment is shown. C2C12 cells were transiently transfected with pSRα-Myc-MKK6 (2.0 µg). After 24 h, the cells were stimulated with TGF-β (10 ng/ml) or NaCl (0.7 M) for the indicated times prior to harvesting. Cell lysates were immunoprecipitated with anti-Myc 9E10 antibody (αMyC), and immunoprecipitates were subjected to in vitro kinase assay using His-tagged kinase-negative MPK-2 (KN-MPK2) as substrate. The phosphorylated proteins were resolved by SDS-polyacrylamide gel electrophoresis and visualized by autoradiography (upper panel). C2C12 cells transiently transfected with pSRα-Myc-p38 (2.0 µg) were stimulated with TGF-β (10 ng/ml), and the anti-Myc immunoprecipitations were subjected to in vitro kinase assay using glutathione S-transferase-tagged ATF-2 as substrate (lower panel). That an equal amount of MKK6 or p38 was immunoprecipitated was confirmed by immunoblotting of the immunoprecipitations. Essentially the same results were obtained in three independent experiments. D, effect of the dominant negative forms of various kinases on TGF-β-induced MKK6 and p38 activation is shown. C2C12 cells were transiently transfected with pSRα-Myc-MKK6 (1.0 µg) together with an empty vector or PEF TAK1/K63W kinase-dead mutant (each 1.0 µg). After 24 h, the cells were stimulated with TGF-β (10 ng/ml) for 45 min and then were subjected to in vitro kinase assay as described above (upper left panel). C2C12 cells were transiently transfected with pSRα-Myc-p38 (1.0 µg) in the presence of expression vectors encoding TβRI(K232R), TAK1(K63W), MKK6(AA), or pSRα vector alone (each 1.0 µg). After 24 h, the cells were left untreated or treated with TGF-β (10 ng/ml) for 1 h prior to harvesting. Kinase activities of p38 were measured as described above (upper right panel). C2C12 cells were transiently transfected with indicated expression vectors encoding TβRI(K232R), TAK1(K63W), MKK6(AA), or pSRα vector alone (each 2.0 µg). After 24 h, the cells were stimulated with TGF-β (10 ng/ml) for 1 h and then the cell lysates were immunoblotted with the polyclonal antibody to phospho-p38 (αP-p38) (Thr180/Tyr182) (lower panel). Nearly the same results were obtained in three independent experiments.
Lumat LB 9507 luminometer. To determine transfection efficiency in each assay, β-galactosidase activity was measured according to the protocol of Promega, and the data were normalized for β-galactosidase activity.

**Smad Association Assays**—C2C12 cells cotransfected with hemagglutinin (HA)-tagged XSmad2, Myc-tagged XSmad4, and the indicated plasmids were treated with TGF-β for 60 min and then were lysed in TNE buffer containing 10 mM Tris-HCl (pH 7.8), 150 mM NaCl, 1 mM EDTA, 1% (v/v) Nonidet P-40, 2 mM dithiothreitol, 1 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride, and 20 μg/ml aprotinin. The total amount of DNA for each transfection was kept constant using empty vector. Cell lysates were subjected to anti-Myc immunoprecipitation as described above. XSmad complexes in the immunoprecipitates and in total lysates were separated by SDS-polyacrylamide gel electrophoresis and detected by immunoblotting as indicated.

**RESULTS AND DISCUSSION**

Recent studies have shown that TAK1 can activate the p38 and SAPK/JNK pathways in vitro and when overexpressed in cells (34–36), but the physiological significance of this reaction is unclear. Because TAK1 has been shown to function as a mediator of the TGF-β-induced transcriptional activation (32), we investigated whether p38 or SAPK/JNK also functions in TGF-β signaling. To determine whether p38 or SAPK/JNK is activated in response to TGF-β, we first tested these kinase activities using antibodies specific for the phosphorylated forms of p38 or SAPK/JNK, respectively. The immunoblotting of whole cell extracts showed that slight activation of p38 was detected within 15 min of TGF-β stimulation, and marked activation was observed at 30–60 min in C2C12 cells (Fig. 1A, TGF-β, aP-p38). The level of the activation was about one-fourth or one-fifth of the maximal activation that was attained by osmotic shock (Fig. 1A, NaCl). The total amount of p38 was unchanged during stimulation (Fig. 1A, αP-p38). In contrast to p38, there was no apparent activation of SAPK/JNK within 60 min of TGF-β stimulation (Fig. 1A, αP-SAPK/JNK). Essentially identical results were obtained in Mv1Lu cells and Ha-
CaT cells (data not shown). Then we focused on the p38 pathway. We have previously demonstrated that MKK6, a member of the MAP kinase kinase family, can act as a strong activator of p38 (34, 39). We tested whether MKK6 is also activated by TGF-β in C2C12 cells. The anti-phospho immunoblotting of whole cell extracts showed that activation of MKK6 was observed within 15 min of TGF-β stimulation and peaked at 45 min (Fig. 1B). The slight activation of MKK3, another activator of p38, was also observed (Fig. 1B). The total amounts of MKK6 and MKK3 did not change during stimulation (Fig. 1B). Next, Myc epitope-tagged MKK6 or p38 was transiently transfected into C2C12 cells. After treatment of the cells with TGF-β, their activities were determined by in vitro kinase assays using kinase-negative MPK2 and ATF-2, respectively, as substrates. Both the transfected MKK6 and the transfected p38 were activated in response to TGF-β, with the time courses of their activation being the same as those of endogenous MKK6 and p38 (Fig. 1C, cf. Fig. 1, A and B). The TGF-β-induced activation of MKK6 was blocked by expression of dominant negative TAK1, TAK1(K63W) (Fig. 1D, upper left panel). The TGF-β-induced activation of p38 was also blocked by expression of TAK1(K63W). It was inhibited by expression of dominant negative TGF-β type I receptor, TjRI(K232R) or by that of dominant negative MKK6, MKK6(AA) (Fig. 1D, upper right panel). The anti-phospho immunoblotting of whole cell extracts showed that the activation of endogenous p38 by TGF-β was also inhibited by expression of TjRI(K232R), TAK1(K63W), or MKK6(AA) (Fig. 1D, lower panel). In these experiments, transfection efficiencies of dominant negative constructs were ~50% and endogenous p38 in whole cells was assayed, so it is reasonable that only partial inhibitions were seen, and about 50% inhibitions seen might mean almost complete inhibition. These results demonstrate that the MKK6-p38 cascade is activated in response to TGF-β through TAK1.

To test possible involvement of the p38 pathway in the induction of gene expression by TGF-β, we examined the effect of dominant negative mutants of TAK1 and MKK6 on TGF-β-induced transcriptional activation. We used the p3TP-Lux reporter construct containing a luciferase gene controlled by a TGF-β-inducible promoter (40). Transient transfection of p3TP-Lux into Mv1Lu cells resulted in a strong induction of luciferase activity in response to TGF-β (Fig. 2A). Cotransfection of an expression plasmid encoding TAK1(K63W) or MKK6(AA) inhibited activation of 3TP promoter by TGF-β in Mv1Lu cells (Fig. 2A). Similar results were obtained with C2C12 cells, and the inhibitory effect of MKK6(AA) on transcriptional activation by TGF-β was dose-dependent (Fig. 2A). Cotransfection of a plasmid encoding CL100, which is a dual-specificity phosphatase acting on members of the MAP kinase superfamily including p38, also resulted in inhibition of the transcriptional activation by TGF-β (data not shown). Moreover, expression of a constitutively active MKK6, MKK6(DE), induced the transcriptional activation in the absence of TGF-β, and coexpression of p38 enhanced this transcriptional activation (Fig. 2B). These results indicate that a kinase cascade consisting of TAK1, MKK6, and p38 is involved in the induction of gene expression by TGF-β.

Because it has been shown that Smad proteins play an essential role in TGF-β signaling, we examined the relationship between the Smad and p38 pathways. As previously reported (12, 16, 17), overexpression of Smad2 and Smad4 induced activation of the 3TP promoter in the absence of TGF-β. Although expression of either TAK1(WT) or MKK6(DE) induced some modest increase of transcriptional activation, coexpression with Smad2 and Smad4 resulted in a strong induction of 3TP promoter activation (Fig. 3). Coexpression of Smad2 and Smad4 along with TAK1 and TAB1, which is an activator of TAK1, resulted in further synergistic activation of 3TP promoter, and this activation was enhanced by expression of MKK6 (data not shown). As shown in Fig. 4A, the MKK6(AA)-induced transcriptional activation was effectively inhibited by the C-terminal truncated type of Smad4 (Smad4ΔC), which is known as the dominant interfering type of Smad4. On the other hand, TAK1(K63W) and MKK6(AA) scarcely inhibited the transcriptional activation induced by overexpression of Smad2 and Smad4 (Fig. 4B). In a control experiment, coexpression of Smad4ΔC completely abolished the Smad2/4-induced transcriptional activation (Fig. 4B). These results suggest that the TAK1-MKK6-p38 pathway interacts cooperatively with the Smad pathway to mediate signaling of TGF-β to the nucleus and that the signaling of the p38 pathway requires the Smad pathway.

To investigate the possibility that p38 might regulate Smad proteins directly, we tested the effect of the TAK1-MKK6-p38 pathway on association of Smad2 and Smad4 in response to TGF-β. Coexpression of Smad2 and Smad4 in C2C12 cells resulted in formation of a heteromeric complex in a TGF-β stimulation-dependent manner (Fig. 4C). This association was significantly decreased in the presence of TjRI(KR), the dominant interfering TjRI mutant. In contrast, coexpression of TAK1(K63W) or MKK6(AA) did not affect the association (Fig. 4C). A constitutively active TAK1, ΔNTAK1, did not affect it either (Fig. 4C). These results suggest that the TAK1-MKK6-p38 pathway does not directly regulate the association of Smads.

As the other likely mechanism by which the TAK1-MKK6-p38 pathway regulates the TGF-β-induced transcriptional activation, we then hypothesized the possibility of p38-mediated phosphorylation of transcription factors. The 3TP promoter contains three consecutive 12-O-tetradecanoylphorbol-13-acetate response elements and a portion of the plasminogen activator inhibitor 1 promoter region that contains putative AP-1 sites (40). Among the transcription factors known to bind to the AP-1 element, ATF-2 has been shown to be phosphorylated by p38 on Thr69 and Thr71 (41–43). Phosphorylation of ATF-2 on
Fig. 4. Relationship between the TAK1-MKK6-p38 pathway and Smad proteins in TGF-β signaling. A, effect of the C-terminal truncated type (ΔC) of Smad4 on the activated MKK6(DE)-induced reporter activity is shown. C2C12 cells were transfected with p3TP-Lux (0.2 μg) together with plasmids encoding MKK6(DE) (0.3 μg) and Smad4ΔC (0.3 μg) or an empty vector plasmid. After 24 h, cells were harvested and assayed for luciferase activity. The relative luciferase activities (means ± S.D.; n = 3) are presented. The experiment was repeated three times with similar results. B, effect of dominant negative TAK1 or dominant negative MKK6 on Smad-induced reporter activity is shown. C2C12 cells were transfected with p3TP-Lux (0.2 μg) and the indicated combinations of Smad2 and Smad4 (Smad2/4), Smad4ΔC, TAK1(K63W), and MKK6(ΔA). After 24 h, cells were harvested and assayed for luciferase activity. The relative luciferase activities (means ± S.D.; n = 3) are presented. The experiment was repeated three times with similar results. C, effect of the TAK1-MKK6-p38 pathway on TGF-β-induced interaction of Smad2 with Smad4 is shown. C2C12 cells were transfected with SR-α-HA-Xenopus Smad2 (a gift from Dr. D. A. Melton) and SRα-Myc-Xenopus Smad4 together with TβRII(K232R) (TβRII(KR)), TAK1(K63W), or MKK6(AA) or the activated forms of TAK1(ΔN) (ΔNTAK1) as indicated. Cell lysates were subjected to immunoprecipitation (IP) with anti-Myc 9E10 antibody and then immunoblotted (IB) using anti-HA Y11 antibody or anti-Myc 9E10 antibody. To confirm equivalent levels of Smad2 expression, aliquots of total lysates were immunoblotted with anti-HA antibody. The experiment was repeated three times with similar results.

these sites causes an increase in transcriptional activity in vivo (41–43). To determine whether TGF-β stimulates phosphorylation of ATF-2 at these threonine residues, we analyzed phosphorylation at these sites using an antibody specific for the Thr71-phosphorylated form of ATF-2. Little, if any, phosphorylation at these sites using an antibody specific for the phosphorylation of ATF-2 at these threonine residues, we analyzed phosphorylation at these sites using an antibody specific for the Thr71-phosphorylated form of ATF-2 (Fig. 5A). We next analyzed the effect of the TAK1 pathway on the phosphorylation of ATF-2 in human embryonic kidney epithelial 293 cells, which lack any detectable expression of the endogenous TGF-β type II receptor (TβRII) (44). When 293 cells were transiently transfected with HA epitope-tagged ATF-2, TβRI, and TβRII and treated with TGF-β, we observed TGF-β-stimulated phosphorylation of ATF-2 (Fig. 5B, left). Some phosphorylation of ATF-2 in the absence of ligand is also observed and is likely caused by overexpression of TβRI and TβRII in 293 cells, which drives their ligand-independent association and consequent activation of TβRI. Activation of TAK1 by cotransfection of TAB1 and TAK1 caused enhanced phosphorylation of ATF-2 (Fig. 5B, right). These results suggest that ATF-2 phosphorylation in response to TGF-β signaling is mediated by the TAK1 pathway. This is consistent with the observation that TAK1 regulates the MKK6-p38 cascade. Because ATF-2 is localized in the nucleus, it is likely that TGF-β stimulation induces the nuclear accumulation of Smads and consequent association with ATF-2. To examine this possibility, we tested the interaction between ATF-2 and Smad4. Coexpression of ATF-2 and Smad4 in COS7 cells resulted in formation of a complex, and this association was enhanced in response to TGF-β (Fig. 5C). Furthermore, overexpression of ATF-2(Ala69/Ala71), a nonphosphorylated form of ATF-2 in which Thr69 and Thr71 are replaced by alanine residues, inhibited transcriptional activation induced by TGF-β (Fig. 5D). Taken together, these observations suggest that Smad complexes and phosphorylated ATF-2 participate in a complex that binds to DNA sequences present in the region of p3TP-Lux, resulting in its transcriptional activation.

The present and previous studies have demonstrated that the TGF-β signal activates two independent pathways, the TAK1-mediated and the Smad-mediated pathways (2, 32, 34, 35, 45). In the Smad pathway, TGF-β stimulation leads to the direct phosphorylation of Smad2 and Smad3 by the type I receptor kinase, consequent hetero-oligomer formation with Smad4, and accumulation in the nucleus (9, 11–13, 46, 47). Our results indicate that in the TAK1 pathway TGF-β activates the TAK1-MKK6-p38 kinase cascade leading to the phosphorylation of ATF-2, and ATF-2 associates with Smad4 in response to
TGF-β. Therefore, Smad complexes and phosphorylated ATF-2 may interact in a nucleoprotein complex that associates with DNA and activates transcription of TGF-β-responsive genes.

Two papers reporting a similar conclusion appeared after submission of this article (48, 49). In our study, dominant negative Smad4 could inhibit the p38 pathway-dependent transcriptional activation efficiently, although dominant negative TAK1 or dominant negative MKK6 could not inhibit the Smad2/Smad4-induced transcriptional activation. Thus, the Smad pathway is essential. It is likely that the affinity of Smad2 and Smad4 for DNA in the absence of TGF-β is low or insufficient, and other transcription factors such as ATF-2 may enhance or stabilize Smad DNA complexes in a ligand-dependent manner. Overexpression of Smad2 and Smad4 may be sufficient for efficient binding to DNA even in the absence of TGF-β to activate transcription of target genes. On the other hand, expression of a nonphosphorylated form of ATF-2 could inhibit the TGF-β-induced transcriptional activation completely, whereas dominant negative MKK6 could inhibit it significantly but not completely. It is possible that other MAP kinase-related pathways such as JNK/SAPK and classical MAP kinase pathways are involved in the transcriptional activation through phosphorylation of ATF-2 or ATF-2-related transcriptional factors.

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