ATF-2 Is a Common Nuclear Target of Smad and TAK1 Pathways in Transforming Growth Factor-β Signaling*

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Upon transforming growth factor-β (TGF-β) binding to its cognate receptor, Smad3 and Smad4 form heterodimers and transduce the TGF-β signal to the nucleus. In addition to the Smad pathway, another pathway involving a member of the mitogen-activated protein kinase kinase family of kinases, TGF-β-activated kinase-1 (TAK1), is required for TGF-β signaling. However, it is unknown how these pathways function together to synergistically amplify TGF-β signaling. Here we report that the transcription factor ATF-2 (also called CRE-BP1) is bound by a hetero-oligomer of Smad3 and Smad4 upon TGF-β stimulation. ATF-2 is one member of the ATF/CREB family that binds to the cAMP response element, and its activity is enhanced after phosphorylation by stress-activated protein kinases such as c-Jun N-terminal kinase and p38. The binding between ATF-2 and Smad3/4 is mediated via the MH1 region of the Smad proteins and the basic leucine zipper region of ATF-2. TGF-β signaling also induces the phosphorylation of ATF-2 via TAK1 and p38. Both of these actions are shown to be responsible for the synergistic stimulation of ATF-2 trans-activating capacity. These results indicate that ATF-2 plays a central role in TGF-β signaling by acting as a common nuclear target of both Smad and TAK1 pathways.

Members of the Smad group of proteins mediate TGF-β,1 BMP (bone morphogenetic protein), and activin signaling from receptors to nuclei (for review, see Refs. 1 and 2). Smad2 and Smad3 are substrates and mediators of the related TGF-β and activin receptors in vertebrates (3–7). TGF-β first directly binds to the TGF-β type II receptor and leads to the formation of an oligomeric complex of the type I and type II receptors (8). Upon ligand binding, the C-terminal ends of these Smad proteins, which bind directly to the type I receptor, are phosphorylated by the type I receptor. This results in their release (7) and hetero-oligomerization with Smad4, a common-mediator of Smad (9–11). Hetero-oligomers of Smad move into the nucleus and directly participate in TGF-β- and activin-dependent transcriptional activation (12–14). Smad2 and Smad4 interact with FAST-1, a member of the winged-helix transcription factor family, and mediate activin-dependent transcriptional activation (13, 14). Recently, the N-terminal regions of Drosophila Mad and mammalian Smad3 and Smad4, which are conserved in the Smad gene family, were shown to interact with specific DNA sequences, and the direct binding of Smad3/4 to DNA is critical for the TGF-β-induced transcriptional activation (15–18).

In addition to the Smad group of proteins, another pathway involving a member of the MAPKKK family of kinases, TAK1 (TGF-β-activated kinase), is also known to be involved in TGF-β signaling (19). TAB1 and TAB2 were identified as proteins that directly bind to TAK1 (20). Overexpression of TAB1 enhances the activity of the plasminogen activator inhibitor 1 (PAI-1) gene promoter, which is regulated by TGF-β, and increases the kinase activity of TAK1, suggesting that TAB1 is an upstream regulator of TAK1. Furthermore, TAK1 activates stress-activated protein kinases (SAPKs), p38 through MKK6 or MKK3 (21) and c-Jun N-terminal kinases (JNKs) via MKK4 (22). Since MKK4 can also activate p38 (23, 24), TAK1 may activate p38 via MKK4. However, it is unknown how the Smad and TAK1 pathways function together to synergistically amplify TGF-β signaling.

Recently, the cAMP response element (CRE) in the Ultra-bithorax (Ubx) gene enhancer was shown to mediate transcriptional activation by Dpp, a Drosophila homologue of TGF-β/BMP (25). In addition, mutation of the AP-1 sites of the collagenase promoter eliminated TGF-β-dependent transcriptional activation (16). The sequences of the CRE and AP-1 sites (12-O-tetradecanoylphorbol-13-acetate response element,) are similar to each other, and ATF/CREB and members of the Jun family of proteins bind to these sites, respectively (26). So far, a number of transcription factors of the ATF/CREB family have been identified. All members of this family contain a DNA binding domain consisting of a cluster of basic amino acids and a leucine zipper region, the so-called b-ZIP (for review, see Ref. 27). They form homodimers or heterodimers through their leucine zipper regions and bind to CRE. Among many of the transcription factors of the ATF/CREB family, two factors, CREB (28, 29) and ATF-2 (also called CRE-BP1) (30–32), are the best characterized. CREB is activated via direct phosphorylation by cAMP-dependent protein kinase (33). On the other hand, SAPKs such as JNKs and p38 phosphorylate ATF-2 at Thr-69, Thr-71, and Ser-90 which lie close to the N-terminal transcriptional activation domain and stimulate its trans-activating capacity (34–36). Thus, these two groups of factors, CREB and ATF-2, are linked to distinct signaling cascades...
involved the cAMP-dependent protein kinase and SAPK pathways. ATF-2, ATF-3, and CRE-BP1 form a subgroup (30, 37, 38) and have a transcriptional activation domain containing the metal finger structure located in their N-terminal regions (38, 39). These factors bind to CRE with high affinity as a homodimer or heterodimer with c-Jun (26, 40). Among these three factors, ATF-2 has been more extensively studied, and shown to be ubiquitously expressed, with the highest level of expression being observed in the brain (41). Mutant mice generated by gene targeting exhibited lowered postnatal viability and growth, in addition to a defect in endochondrial ossification and a reduced number of cerebellar Purkinje cells (42).

The fact that ATF-2 activity is enhanced by SAPK whose activity in turn is stimulated by TAK1 allowed us to hypothesize that ATF-2 might play an important role in the TGF-β signal transduction pathway. Our results indicate that ATF-2 not only directly binds to Smad3/4 hetero-oligomers but also that ATF-2 is phosphorylated by TGF-β signaling via TAK1 and p38. The two pathways, Smad and TAK1, synergistically enhance the activity of ATF-2 which acts as their common nuclear target.

**EXPERIMENTAL PROCEDURES**

**In Vitro Binding Assay**—The plasmids used to express the GST-Smad fusion proteins containing various forms of Smad3/4 were constructed by using appropriate enzyme sites or the polymerase chain reaction (PCR)-based method with the use of the cytomegalovirus promoter-containing expression vector. The plasmids encoding the VP16-ATF-2 fusion protein containing the C-proximal region of ATF-2 (amino acids 291–414) were made by the PCR-based method with the use of the cytomegalovirus promoter-containing expression vector. The plasmids used to express the Gal4-ATF-2 fusion protein containing the Gal4 DNA-binding domain joined to the C-proximal region of ATF-2 (amino acids 291–414) were made by the PCR-based method with the use of the cytomegalovirus promoter-containing expression vector.

The plasmids used to express the GST-Smad fusion proteins were constructed by using appropriate enzyme sites or the polymerase chain reaction (PCR) method with the use of the cytomegalovirus promoter-containing expression vector. The plasmids encoding the GST-Smad3 or GST-Smad4 fusion proteins were expressed at various concentrations of the plasmid DNA. The plasmids used to express the GST-ATF-2 expression plasmid were constructed by using the previously reported CRE-CAT reporter plasmid (39). The plasmids used to express the GST-Smad3 or GST-Smad4 expression plasmids were constructed by inserting the corresponding cDNAs downstream of the cytomegalovirus promoter-containing expression vector. The plasmids used to express the GST-Smad3 or GST-Smad4 fusion proteins were expressed at various concentrations of the plasmid DNA. The plasmids used to express the GST-Smad3 or GST-Smad4 expression plasmids were expressed at various concentrations of the plasmid DNA. The plasmids used to express the GST-Smad3 or GST-Smad4 expression plasmids were expressed at various concentrations of the plasmid DNA.

**Co-transfection Assay**—The plasmids to express Smad3 and Smad4 were constructed by inserting the corresponding cDNA downstream of the cytomegalovirus promoter. The CRE-containing luciferase reporter was constructed using the previously reported CRE-CAT reporter plasmid (39). The plasmids used to express the ATM-β3-CAT reporter were constructed by using the previously reported CRE-CAT reporter plasmid (39). The plasmids used to express the ATM-β3-CAT reporter were constructed by using the previously reported CRE-CAT reporter plasmid (39).

**Detection of Phosphorylated Proteins**—To examine the phosphorylation of endogenous protein, 293 cells were serum-starved and incubated with TGF-β3 (3 ng/ml). The cells were disrupted in RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% deoxycholate, 0.1% SDS, 50 mM NaF, 2 mM Na3VO4, 0.1 μM okadaic acid, 25 mM β-glycerophosphate, and protease inhibitor mixture). After centrifugation, the supernatant was analyzed by SDS-PAGE, followed by Western blotting. The phosphorylation of ATF-2, p38, JNK1, and JNK2 were examined using PhosphoPlus ATF-2 (Thr-71), p38MAPK (Thr-180/Tyr-182), and JNK1/2/Tyr-183/Tyr-185) antibody kits, respectively (New England Biolabs). To inhibit p38 activity, 293 cells were treated with SB203580 (Calbiochem) for 45 min before the preparation of cell lysates. To analyze the phosphorylated state of ATF-2 expressed by the transfected DNA, 293 cells were transfected using LipofectAMINE (Life Technologies, Inc.) and a mixture made up of 1.5 μg of the plasmid expressing the wild type or C91 mutant lacking the C-terminal 91 amino acids and 1.5 μg of a plasmid expressing the activated form of TAK1 (TAK1ΔN) or no protein at all. About 45 h after transfection, cell lysates were prepared, and the phosphorylated state of ATF-2 was examined again as described above.

**RESULTS AND DISCUSSION**

**ATF-2 in TGF-β Signaling**

We examined the effect of dominant negative forms of ATP-2, Smad3, and Smad4, and the following mutants were used. The Ala mutant (ATF-2Ala) in which the three serine residues of the SS motif are mutated to alanine (Smad3AAVA) was also constructed using the PCR-based method. The N-truncated mutant of ATP-2 (ATF-2C107) lacking the N-terminal 107 amino acids was described previously (39). The C-truncated mutant of Smad3 (Smad3ΔC) or Smad4 (Smad4ΔC) lacking the C-terminal 40 or 38 amino acids were made by using the PCR-based method. The Smad3 mutant in which all the three serine residues of the SSX5 motif are mutated to alanine (Smad3AAVA) was also constructed using the PCR-based method. The dominant negative form of Smad3 (Smad3ΔC) or Smad4 (Smad4ΔC) lacking the C-terminal 40 or 38 amino acids were made by using the PCR-based method. The Smad3 mutant in which all the three serine residues of the SSX5 motif are mutated to alanine (Smad3AAVA) was also constructed using the PCR-based method. The dominant negative form of Smad3 (Smad3ΔC) or Smad4 (Smad4ΔC) lacking the C-terminal 40 or 38 amino acids were made by using the PCR-based method.
ATF-2 Binds to the MH1 Region of Smad3 and Smad4—To investigate whether ATF-2 functions in the Smad pathway, we first of all examined for a direct interaction between Smad3/4 and ATF-2 (Fig. 1). Protein affinity resins in which the GST, GST-Smad3, or GST-Smad4 fusion protein containing the full-length form of Smad3 or Smad4 was used as a ligand were prepared (Fig. 1, A and B). The full-length form of human ATF-2 was synthesized using the in vitro transcription/translation system and was mixed with this affinity resin. Approximately 17 and 20% of ATF-2 were bound to the resin containing the GST-Smad3 and the GST-Smad4 fusion protein, respectively, but none was bound by the GST resin alone (Fig. 1C). We further examined which region of Smad3 and Smad4 binds to ATF-2. In addition to the GST fusion protein containing the full-length form of Smad3 and Smad4, five fusion proteins containing a series of truncated Smad3 or Smad4 protein were prepared and used in the binding assays (Fig. 1, A and B). Among Smad proteins, there are two homologous regions, the N-terminal MH1 (mad homology domain 1) and the C-terminal MH2, which are conserved in Smad-related proteins in various species ranging from insects to vertebrates (Fig. 1A). The truncated mutants of Smad3 and Smad4 that lacked the region downstream of the MH1 region still retained the ability to interact with ATF-2 (Fig. 1, A and C). However, the mutants of Smad3 and Smad4 that lacked a part or the whole region of MH1 could not bind to ATF-2 (see CT1 and NT1 of Smad3 and CT1, NT1, and NT2 of Smad4). These results indicate that the N-terminal MH1 region binds to ATF-2.

**RESULTS**

**ATF-2 Binds to the MH1 Region of Smad3 and Smad4**—To determine whether region of ATF-2 interacts with Smad4, we made various mutants of ATF-2 by the in vitro transcription/translation system and used them in the GST pull-down assay (Fig. 2). Among the six mutants, the two mutants lacking the basic region (ΔBR) or containing a mutated leucine zipper (L34V), in which the third and fourth leucine residues were mutated to valine, failed to bind to GST-Smad4. In contrast, all the other ATF-2 mutants bound to GST-Smad4 with an efficiency similar...
The interaction between ATF-2 and Smad3/4 in mammalian cells was investigated by co-immunoprecipitation (Co-IP). Whole-cell lysates were prepared from 293 cells transfected with a mixture of plasmids to express ATF-2 and Flag-linked Smad3 and Smad4, and samples from the lysates were directly used for Western blotting with the anti-Flag or anti-ATF-2 antibodies (Direct Western). Whole-cell lysates were also immunoprecipitated by anti-ATF-2 antibody (Ab), and the immunocomplexes were analyzed by Western blotting using anti-Flag antibodies. In lanes 1 and 3, the plasmids to express the constitutively active TGF-β type I receptor and the C-proximal region of ATF-2 were co-transfected, and the transfected cells were stimulated with TGF-β for 1 h before preparation of cell lysates. In lane 1, normal IgG was used as a control for immunoprecipitation. B, mammalian two-hybrid interaction. Left, HepG2 cells were co-transfected with the Gal4 site-containing reporter, the plasmid to express Gal4-Smad3 containing the full length Smad3/4 or VP16 alone. These results indicate that the b-ZIP domain of ATF-2 interacts in mammalian cells with the MH1 region of Smad3 and Smad4.

TGF-β Signaling Induces Phosphorylation of ATF-2 via TAK1—We next examined whether phosphorylation of ATF-2 is enhanced by TGF-β treatment (Fig. 4). The TGF-β-responsive 293 cells were treated with TGF-β, and ATF-2 phosphorylated at Thr-71 was detected by the phospho-ATF-2-specific antibody at various intervals after TGF-β treatment (Fig. 4A). The Thr-71 residue is known to be the phosphorylation site of SAPK (34–36). The degree of phosphorylation of ATF-2 increased up to a maximum of 4-fold at 15 min after TGF-β treatment, whereas the amount of ATF-2 was not affected by TGF-β treatment. To confirm that TGF-β signaling phosphorylates ATF-2 at the same sites as SAPK, the ATF-2 mutant, whose three SAPK phosphorylation sites (Thr-69, Thr-71, and Ser-90) were replaced by alanine, was used. Since this alanine mutant cannot be recognized by the antibody raised against the peptide containing these phosphorylation sites, we used the C-truncated form of ATF-2 to discriminate from the endogenous protein, and we judged the phosphorylation status of the mutants by their altered migration during SDS-PAGE. The C-truncated form of ATF-2, which was phosphorylated by TGF-β signaling via SAPK, migrated more slowly during SDS-PAGE than the non-phosphorylated form (Fig. 4B, compare lanes 1 and 2). However, the migration of the alanine mutant was the same as that of the wild type even in the presence of TGF-β treatment, confirming that at least one of these three sites was phosphorylated by TGF-β signaling. To investigate further whether phosphorylation of ATF-2 is mediated by TAK1, we examined the effect of activated TAK1 on the phosphorylation of ATF-2 (Fig. 4C). Co-transfection of the plasmid to express the activated form of TAK1, which lacked its N-terminal 22 amino acids, with the C-truncated ATF-2 expression plasmid increased the amount of ATF-2 phosphorylated at Ser-71, as detected by the phospho-ATF-2-specific antibody (Fig. 4C, compare lanes 1 and 3). In addition, the activated form of TAK1 further enhanced the phosphorylation of ATF-2 in the presence of TGF-β treatment (Fig. 4C, compare lanes 2 and 4).
and non-phosphorylated forms are indicated, respectively. ATF-2 proteins phosphorylated at Thr-71, and both the phosphorylated and non-phosphorylated forms are indicated, respectively. The phosphorylation of exogenous ATF-2 at JNK/p38 phosphorylation sites by TGF-β signal. The plasmid to express the ATF-2 protein lacking the C-terminal 91 amino acids but containing either the normal three JNK/p38 phosphorylation sites or these sites mutated to alanines was transfected into 293 cells. Cell lysates were prepared, and the C-truncated ATF-2 was detected by Western blotting using anti-ATF-2 antibody which recognizes both the phosphorylated and non-phosphorylated forms. Since the phospho-ATF-2-specific antibody cannot react with the alanine mutant, the phosphorylated form was detected as a slower migrating band on a long SDS-PAGE gel. phosphorylation of ATF-2 through the action of TAK1 and p38. The two plasmids to express ATF-2 lacking the C-terminal 91 amino acids and the activated form of TAK1 (TAK1ΔN) or no protein were transfected into 293 cells. Phospho-ATF-2 and ATF-2 were detected as described in A.

These results suggest that TGF-β signaling induces the phosphorylation of ATF-2 at the SAPK phosphorylation sites via TAK1.

Involvement of p38 in TGF-β-induced Phosphorylation of ATF-2—The results of the ATF-2 phosphorylation assays and the fact that TAK1 activates SAPKs, JNKs, and p38 (21, 22) suggest that SAPKs phosphorylate ATF-2 upon TGF-β stimulation. To investigate which SAPK is activated by TGF-β signaling, we examined the phosphorylation of p38, JNK1, and JNK2. The 293 cells were treated with TGF-β, and p38 phosphorylated at Thr-180/Tyr-182 and JNK1/JNK2 phosphorylated at Thr-183/Tyr-185 were detected by the phosphorylated form-specific antibody at various intervals after TGF-β treatment (Fig. 5A). The degree of phosphorylation of p38, which displayed a timing similar to that of ATF-2, increased up to 4-fold. In contrast, the phosphorylation of JNK1 and JNK2 remained unchanged, suggesting that TGF-β signaling leads to phosphorylation of ATF-2 through mainly p38 rather than JNK. To confirm these results further, the effect of the specific inhibitor of p38, the pyridinyl imidazole derivative SB203580 which cannot inhibit JNKs (46, 47), on the TGF-β-induced phosphorylation of ATF-2 was examined (Fig. 5B). SB203580 almost completely blocked TGF-β-induced phosphorylation of ATF-2. These results indicate that TGF-β induces the phosphorylation of ATF-2 through the action of TAK1 and p38.

Synergistic Activation of ATF-2 Activity by Smad and TAK1 Pathways—To investigate whether the trans-activating capacity of ATF-2 is enhanced by Smad3/4 and TAK1 pathways, co-transfection assays were performed using a reporter plasmid containing four copies of the consensus CRE sequence (Fig. 6). This artificial promoter was weakly responsive to TGF-β in HepG2 cells (2-fold). When present separately, ATF-2, Smad3/4, and the activated form of TAK1 stimulated this promoter activity by 2-, 10-, and 2-fold, respectively, in the absence of TGF-β treatment, and by 5-, 17-, and 7-fold, respectively, in the presence of TGF-β treatment. The degree of activation of this promoter by ATF-2 was synergistically increased by co-expression of Smad3/4 or the activated form of TAK1. Furthermore, promoter activity could be strongly enhanced by co-expression of all the three effectors together, resulting in a 145- and 209-fold stimulation in the absence and presence of TGF-β treatment, respectively. These results support the idea that both the Smad3/4 pathway and TAK1 pathway synergistically activate ATF-2. CRE is recognized by the ATF-2/c-Jun heterodimer with high affinity and the c-Jun homodimer with lower affinity (40). To determine which of these actually contributes to CRE-dependent activation, we transfected the cells with plasmids expressing both ATF-2 and c-Jun or with a plasmid expressing c-Jun alone. As reported previously (39),
the ATF-2/c-Jun heterodimer activated more strongly the CRE-containing promoter (8-fold) compared with the ATF-2 homodimer. However, further stimulation of ATF-2/c-Jun heterodimer-dependent activation by co-expression of both Smad3/4 and the activated form of TAK1 was inefficient compared with that seen with ATF-2 alone. The trans-activating capacity of the c-Jun homodimer was also not so strongly enhanced by Smad3/4 and TAK1 compared with the marked increase in the capacity of the ATF-2 homodimer. These results suggest that the ATF-2 homodimer is the preferred target for the Smad and TAK1 pathways at least in HepG2 cells, although the activity of the ATF-2/c-Jun heterodimer and the c-Jun homodimer are also stimulated to some extent by both pathways.

**Involvement of ATF-2 in TGF-β-inducible Promoter Activation—**To examine the role of ATF-2 in the regulation of the TGF-β-inducible promoters, co-transfection experiments were performed using a fusion promoter (p3TP-Lux reporter) consisting of PAI-1 and collagenase promoters (48) (Fig. 7A). This promoter was highly responsive to TGF-β in HepG2 cells (38-fold). Smad3/4 stimulated this promoter activity by 251-fold in the absence of TGF-β treatment and by 409-fold in the presence of TGF-β treatment. The degree of activation of this promoter by Smad3/4 was slightly enhanced by co-expression of ATF-2 or the activated form of TAK1. Furthermore, promoter activity could be strongly enhanced by co-expression of all the three effectors together, resulting in a 791- and 1104-fold stimulation in the absence and presence of TGF-β treatment, respectively. These results support the idea that co-expression of ATF-2, Smad3/4, and the activated form of TAK1 synergistically activated this promoter activity. When these results are compared with those with the CRE-containing promoter described above, however, some difference is evident. Unlike the case of CRE-containing promoter, Smad3/4 strongly activated this promoter. In addition, ATF-2 alone did not enhance this promoter activity, and the synergism between ATF-2 and the activated form of TAK1 was not observed using this promoter. This could be due to the fact that Smad3/4 can activate this promoter not only via a complex formation with ATF-2 but also via direct binding to the specific sites in the PAI-I promoter (see “Discussion”).

To confirm that ATF-2 plays an important role for TGF-β-induced activation of the p3TP-Lux promoter, we used two ATF-2 mutants as follows: the Ala mutant (ATF-2Ala) in which
the three SAPK phosphorylation sites (Thr-69, Thr-71, and Ser-90) (34–36) were replaced by alanine and the N-truncated mutant (ATF-2Δ107) lacking the N-terminal 107 amino acids including the SAPK phosphorylation sites (Fig. 7B). These two mutants cannot be phosphorylated by TGF-β signaling via p38 and were expected to act as a dominant negative form. Cotransfection of either of these two mutants strongly inhibited the TGF-β-induced activity of the p3TP-Lux promoter, indicating that ATF-2 is involved in the activation of 3TP-Lux promoter by TGF-β signaling. In addition, the dominant negative form of TAK1, in which Lys-63 of the ATP-binding site was replaced by tryptophan (TAK1K63W), inhibited the TGF-β-induced activity of the p3TP-Lux promoter. To confirm the role of Smad3/4 in the TGF-β-induced activity of the p3TP-Lux promoter, we used two types of mutants. The C-truncated mutant of Smad3 (Smad3ΔC) or Smad4 (Smad4ΔC) lacking the C-terminal transcriptional activation domain was reported to act as a dominant negative form (6). The TGF-β type I receptor phosphorylates Smad2 at Ser-465 and Ser-467 in the SSXS motif, and the mutant in which all the three serine residues in the SSXS motif were replaced by alanines acts as a dominant negative form, because this mutant stably binds to the TGF-β type I receptor (7, 49, 50). In addition, the alanine mutant of Ser-464 of Smad2 also act as a dominant negative form, although this site is not directly phosphorylated. Therefore, we constructed second type of putative dominant negative form of Smad3 by replacing all the three serine residues of the corresponding SSXS motif to alanine (Smad3AAVA). Co-transfection of the C-truncated mutant of Smad3 (Smad3ΔC) or Smad4 (Smad4ΔC) or the Smad3 alanine mutant (Smad3AAVA) inhibited the TGF-β-dependent p3TP-Lux promoter activity. These results indicate that that both Smad and TAK1 pathway are required for the TGF-β-induced activation of the 3TP-Lux promoter.

To confirm the role of ATF-2 further, we examined the effect of two ATF-2 mutants (ATF-2Ala and ATF-2Δ107) on the Smad3/4- and/or activated TAK1-induced promoter activity of 3TP-Lux (Fig. 7C). Either of these two mutant significantly inhibited the 3TP-Lux promoter activity enhanced by Smad3/4, activated TAK1, or both Smad3/4 and activated TAK1. Thus, a dominant negative form of ATF-2 can inhibit the stimulatory effect of either Smad and TAK1 pathways on the 3TP-Lux promoter.

**DISCUSSION**

Our results indicate that ATF-2 is a common nuclear target of the Smad and TAK1 pathways (Fig. 8). Upon binding of TGF-β to the type II receptor, the TGF-β-bound type II receptor makes a heteromeric complex with the type I receptor, resulting in the activation of the latter’s serine/threonine kinase activity. The activated serine/threonine kinase of the type I receptor then phosphorylates the bound Smad3 or Smad2 protein, which results in its release from the type I receptor. The released Smad3 forms a hetero-oligomer with Smad4, which is thought to be localized in the cytosol in the absence of TGF-β stimulation, and the hetero-oligomer moves into the nucleus. This hetero-oligomer directly binds to ATF-2 through the MH1 region of Smad3/4 and the b-ZIP region of ATF-2, although the exact number of ATF-2 and Smad3/4 molecules in this complex remains unknown. Binding of the Smad3/4 complex to ATF-2 enhances ATF-2 activity, as suggested by the observation that overexpression of Smad3/4 enhances the trans-activating capacity of ATF-2 (Fig. 6). In this sense, Smad3/4 resembles adenovirus E1A, which stimulates CRE-dependent transcription via binding to the b-ZIP region of ATF-2 (51). In addition to this Smad pathway, another pathway, the TAK1 pathway, is required for TGF-β signal transduction. The expression of the dominant negative form of TAK1 inhibits the TGF-β-induced activation of the PAI-1 promoter (19). Upon TGF-β stimulation, the TAB1 protein is thought to be activated, an event that results in its binding to the serine/threonine kinase domain of TAK1 (20). However, the precise mechanism of signal transduction from the TGF-β receptor to TAB1 remains unknown. TAK1 is a member of the MAPKKK family and activates MKK3 and MKK6 of the MAPKK family, both of which share striking homology with each other (21). TAK1 also activates MKK4, another member of MAPKK (22). TAK1 activates p38, one member of the SAPK family via MKK6/MKK3 (21), and also possibly through MKK4 (22–24). p38 directly phosphorylates ATF-2 at Thr-69, Thr-71, and Ser-90, resulting in stimulation of its trans-activating capacity. In fact, co-expression of activated TAK1 enhanced this trans-activating capacity of ATF-2 (Fig. 6). Thus, the Smad and TAK1 pathways synergistically stimulate TGF-β-induced transcription by acting on the common nuclear target ATF-2. TGF-β has an important role in the regulation of genes involved in cell cycle control and genes encoding the extracellular matrix, and many of them have a CRE in their transcriptional control regions. Recently we found that the expression level of some TGF-β-inducible genes encoding extracellular matrix was decreased in mouse embryonic fibroblasts lacking ATF-2 and its related gene CRE-BPα2 supporting that the ATF-2 family is important for the TGF-β-mediated transcriptional activation. The identification of ATF-2 as a common nuclear target of Smad and TAK1 pathways may provide a clue as to how the signal transduction of TGF-β is regulated. Recent studies using the Xenopus system indicated that TAK1 and TAB1 also function in the BMP signal transduction pathway in Xenopus embryos (52). Therefore, ATF-2 could also play an important role in BMP signal trans-
The b-ZIP region of ATF-2 directly interacts with the N-terminal MH1 region of Smad3/4. The b-ZIP region of ATF-2 and the MH1 region of Smad3/4 can also directly bind to CRE and the recently identified Smad-recognition sequence, respectively (17, 30). Therefore, the interaction between ATF-2 and Smad3/4 is mediated by a different type of DNA binding domain. There exist numerous examples of DNA binding domains that can additionally serve as interaction sites for specific proteins. The adenosovirus 13SE1A protein binds to multiple DNA binding domains including not only the b-ZIP region of ATF-2 but also the metal finger of Sp1 and the basic-helix-loop-helix region of upstream factor (51). Similarly, the helical structure of the b-ZIP region of ATF-2, which would be exposed to the solvent, may serve as a protein surface for interaction with Smads. Several examples of protein-protein interaction being mediated via different DNA binding domains have also been reported (44, 53).

Two SAPK family members have been identified in mammalian cells, JNKs (54, 55) and p38 homologues (also termed p40, p38-1/p38-2, p38-α/p38-β/p38-δ, p38α, p38β, p38δ, and PIKK) (56–59). Among these two type of SAPKs, only p38 was activated up to a maximum at 15 min after TGF-β treatment via TAK1 in 293 cells (Fig. 5). In contrast to this, TAK1 stimulated by ceramide was reported to activate JNK in COS7 cells at the similar timing (22). Although JNK was also reported to be activated by TGF-β stimulation in 293T, this activation of JNK activity was observed at 12 h after TGF-β treatment (60). This contradiction could be due to the difference of cells used, possibly due to the cell type-specific expression of some co-factor(s). ATF-2 is a good substrate of both SAPKs, whereas c-Jun is phosphorylated by JNKs but not by p38 (61), suggesting that ATF-2 is a preferable nuclear signaling (62).

Two SAPK family members have been identified in mammalian cells, JNKs (54, 55) and p38 homologues (also termed p40, p38-1/p38-2, p38-α/p38-β/p38-δ, p38α, p38β, p38δ, and PIKK) (56–59). Among these two type of SAPKs, only p38 was activated up to a maximum at 15 min after TGF-β treatment via TAK1 in 293 cells (Fig. 5). In contrast to this, TAK1 stimulated by ceramide was reported to activate JNK in COS7 cells at the similar timing (22). Although JNK was also reported to be activated by TGF-β stimulation in 293T, this activation of JNK activity was observed at 12 h after TGF-β treatment (60). This contradiction could be due to the difference of cells used, possibly due to the cell type-specific expression of some co-factor(s). ATF-2 is a good substrate of both SAPKs, whereas c-Jun is phosphorylated by JNKs but not by p38 (61), suggesting that ATF-2 is a preferable nuclear signaling (62).

Synergistic activation of the CRE-containing promoter by ATF-2 and Smad3/4 suggests the interaction between ATF-2 bound to CRE and Smad3/4 on DNA in this case. Recently, however, direct binding of Smad3 and Smad4 to a specific DNA sequence was reported (17). In addition, the putative Smad-binding sites in the PAI-1 promoter were demonstrated to be critical for the TGF-β inducibility of the promoter activity (18, 63). Consistent with these reports, we observed that co-expression of Smad3/4 alone enhanced the 3TP-Lux activity (18, 63). Consistent with these reports, we observed that co-expression of Smad3/4 alone enhanced the 3TP-Lux activity (18, 63). Consistent with these reports, we observed that co-expression of Smad3/4 alone enhanced the 3TP-Lux activity (18, 63). Consistent with these reports, we observed that co-expression of Smad3/4 alone enhanced the 3TP-Lux activity (18, 63). Consistent with these reports, we observed that co-expression of Smad3/4 alone enhanced the 3TP-Lux activity (18, 63). Consistent with these reports, we observed that co-expression of Smad3/4 alone enhanced the 3TP-Lux activity (18, 63).
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