c-Jun interacts with the corepressor TG-interacting factor (TGIF) to suppress Smad2 transcriptional activity

Marcia Pessah*†, Céline Prunier*†, Jacqueline Marais*, Nathalie Ferrand*, Anne Mazars*, François Lallemand*, Jean-Michel Gauthier‡, and Azeddine Atfi*§

*Institut National de la Santé et de la Recherche Médicale U 482, Hôpital Saint-Antoine, 184 Rue du Faubourg Saint-Antoine, 75571, Paris Cedex 12, France; and ‡Hybrigenics, 180, avenue Daumesnil, 75012 Paris, France

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The Sma and Mad related (Smad) family proteins are critical mediators of the transforming growth factor-β (TGF-β) superfamily signaling. After TGF-β-mediated phosphorylation and association with Smad4, Smad2 moves to the nucleus and activates expression of specific genes through cooperative interactions with DNA-binding proteins, including members of the winged-helix family of transcription factors, forkhead activin signal transducer (FAST)-1 and FAST2. TGF-β has also been described to activate other signaling pathways, such as the c-Jun N-terminal Kinase (JNK) pathway. Here, we show that activation of JNK cascade blocked the ability of Smad2 to mediate TGF-β-dependent activation of the FAST proteins. This inhibitory activity is mediated through the transcriptional factor c-Jun, which enhances the association of Smad2 with the nuclear transcriptional corepressor TG-interacting factor (TGIF), thereby interfering with the assembly of Smad2 and the coactivator p300 in response to TGF-β signaling. Interestingly, c-Jun directly binds to the nuclear transcriptional corepressor TGIF and is required for TGIF-mediated repression of Smad2 transcriptional activity. These studies thus reveal a mechanism for suppression of Smad2 signaling pathway by JNK cascade through transcriptional repression.

Transforming growth factor-β (TGF-β) regulates a broad range of cellular functions, including proliferation, apoptosis, extracellular matrix production, and differentiation (1, 2). TGF-β initiates responses by contacting two distantly related transmembrane serine/threonine kinases called receptors I (TβRI) and II (TβRII), promoting activation of TβRI by the TβRII kinase (1). The activated TβRI then interacts with an adapter molecule Smad anchor for receptor activation (SARA), which facilitates the access of Sma and Mad related protein (Smad)-2 and Smad3 to activated TGF-β receptor (3). After phosphorylation, Smad2 and Smad3 associate with the shared partner Smad4 and translocate to the nucleus where Smad complexes participate in transcriptional activation of target genes (1, 4).

The Smad proteins have been shown to act as transcription factors through their ability to associate with DNA-binding factors (1, 2). For example, Smad2 interacts with the forkhead-containing DNA-binding proteins forkhead activin signal transducer (FAST)-1 and FAST2 to activate responsive elements in the *Xenopus mix.2* promoter and the mouse *goosecoid* promoter. In other cases, Smad proteins can achieve recognition of target promoters by associating with factors that are independently capable of DNA binding and transcriptional activation, such as c-Jun, activating transcription factor (ATF)-2, or TFE3 (1, 5). The ability of Smads to modulate transcription in response to ligand results also from a functional cooperativity with the general transcription coactivators CREB binding protein (CBP)/p300 or with the transcriptional corepressor TG-interacting factor (TGIF; ref. 1). Although the interaction of Smad2 with both the coactivators CBP/p300 and with the corepressor TGIF is important in Smad2 signaling, additional signaling pathways triggered by TGF-β may influence the ability of CBP/p300 and TGIF to modulate Smad-dependent transcription.

A number of reports support the existence of other effector pathways operating downstream of TGF-β receptors. The best characterized of these is the c-Jun N-terminal Kinase (JNK) family of cytoplasmic serine/threonine kinases (6–8). The activation of JNK by TGF-β can be mediated through mitogen-activated protein kinase (MAPK) kinase kinase-1 (MEKK1) and MAPK kinase 4 (MKK4). Once activated, JNK phosphorylates c-Jun, and, in turn, phosphorylated c-Jun homodimerizes with members of the Jun family or heterodimerizes with members of the Fos family. All these complexes named activating-protein-1 (AP-1) bind to AP1 sites and can control the expression of a number of genes, including c-Jun itself (9, 10). Previous data from several groups have indicated that Smad3 can interact with c-Jun at TGF-β-responsive AP-1-binding sites (11, 12). The cooperativity between AP1 and Smad3 results in TGF-β-induced transcriptional activation from these promoters. By contrast, c-Jun was shown to repress a TGF-β-inducible promoter containing the Smad3/4 binding element CAGA (13, 14). It is therefore likely that the interplay of JNK and Smad signaling pathways is key to a coordinated cellular response dependent on physiological context. Here, we report an additional and heretofore unexpected role for the JNK signaling pathway in the inhibition of Smad2 transcriptional activity, which appeared to be mediated by c-Jun association with the transcriptional corepressor TGIF. This association suggests a novel mechanism for regulating Smad2/FAST-dependent transcription by JNK signaling pathway through transcriptional repression.

Materials and Methods

Cell Lines and Expression Vectors. COS-7, HepG2, and Mv1Lu cells were maintained in DMEM supplemented with 10% heat-inactivated FCS and 5 mM glutamine.

Expression vectors for MEKK1.K432A, MEKK1.EE, MKK4.Ala, FAST1, and G5E1b-Lux were described previously (8, 15, 16). Expression vectors for activin response element (ARE)-Lux and wild-type or constitutively activated TGF-β type I receptor were a gift from Dr. J. Wrana. The p3TP-Lux reporter construct, GAL4-FAST1, and pCMV5-Flag-TGIF were a gift

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Abbreviations: ARE, activin responsive element; JNK, Jun N-terminal Kinase; MEKK1, mitogen-activated protein kinase (MAPK) kinase kinase1; MKK4, MAPK kinase 4; TGIF, TG-interacting factor; TGF-β, transforming growth factor β; Smad, Sma and Mad related protein; CBP, CREB binding protein; GST, glutathione *S*-transferase; HDAC, histone deacetylase; FAST, forkhead activin signal transducer.

†M.P. and C.P. contributed equally to this work.

§To whom reprint requests should be addressed. E-mail: atfi@adr.st-antoine.inserm.fr.

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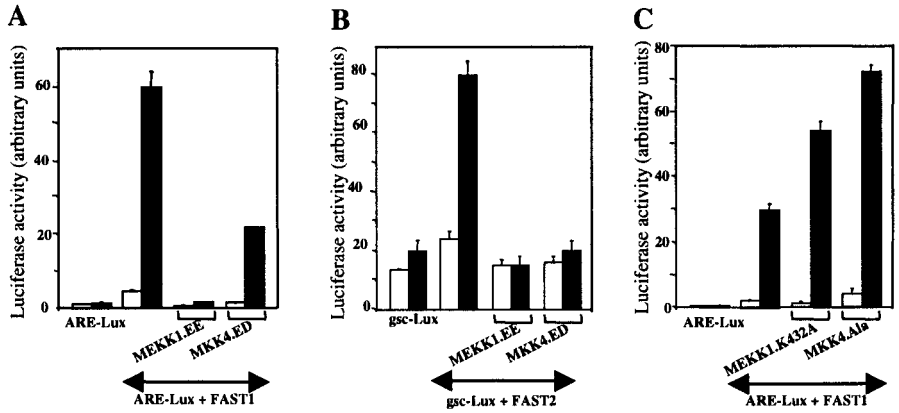

Fig. 1. Activation of JNK cascade inhibits Smad2-dependent transcription. (*A*) Mv1Lu cells were transfected with ARE-Lux alone or with FAST1 in the presence or absence of constitutively activated mutants of MEKK1 (MEKK1.EE) and MKK4 (MKK4.ED). (*B*) HepG2 cells were transfected with gsc-Lux and FAST2 in the presence or absence of MEKK1.EE and MKK4.ED. (*C*) Mv1Lu cells were transfected with ARE-Lux alone or with FAST1 in the presence of absence of dominant-negative mutants of MEKK1 (MEKK1.K432A) and MKK4 (MKK4.Ala). In all cases, cells were treated with (filled bars) or without (open bars) TGF-β for 16 h before lysis and then assayed for luciferase activity. Luciferase activity was normalized to β-galactosidase activity and was expressed as mean $\pm$ SD of triplicates from a representative experiment performed at least three times.

from Dr. J. Massagué (Memorial Sloan-Kettering Cancer Center). The *Bam*HI-*Eco*RI fragment containing full-length TGIF cDNA was subcloned from pCMV5-Flag-TGIF into pGEX4T-1 (Amersham Pharmacia). Expression vector for Myc-Smad2 was a gift from Dr. R. Janknecht (The Salk Institute). Expression vectors for FAST2 and gsc-Lux were a gift from Dr. L. Attisano (University of Toronto). Expression vectors for MKK4.ED, HA-c-Jun, and HA-c-JunbZip were a gift from Dr. D. Bohmann (European Molecular Biology Laboratory). The reporter construct AP1-Lux was purchased from Stratagene.

Gene Expression Analysis. For TGF-β-inducible luciferase reporter assays, cells were plated to semiconfluency and 24 h later transfected with expression vectors by the Lipofectamine (GIBCO) method as described previously (15). To induce the luciferase reporter, cells were treated with human TGF-β1 (Sigma) at 80 pM for 16 h. Luciferase activity was measured by using the luciferase assay system described by the manufacturer (Promega) and was normalized for transfection efficiency by using a β-galactosidase-expressing vector (pCMV5.LacZ) and the Galacto-Star system (Perkin–Elmer).

Immunoprecipitation and Immunoblotting. After transfection, cells were lysed at 4°C in lysis buffer (13). For the association of TGIF with c-Jun or Smad2, cells were resuspended in LSLD buffer and lysed by sonication. Lysates were subjected to immunoprecipitation with either monoclonal anti-Flag M2 (Sigma) or monoclonal anti-c-Myc (9E10) antibodies (Santa Cruz Biotechnology) for 2 h, followed by adsorption to Sepharose-coupled protein G for 1 h. Immunoprecipitates were separated by SDS/PAGE and analyzed by immunoblotting. For determination of total protein levels, aliquots of cell lysates were subjected to direct immunoblotting.

***In Vitro* Protein Interaction Assay.** The *in vitro* transcription and translational reactions were performed by using the TNT-coupled reticulocyte lysate system (Promega) following the manufacturer's instructions. Translation of c-Jun was carried out in the presence of [^{35}S]methionine, and labeled protein was incubated with purified glutathione *S*-transferase (GST)-TGIF, GST-Smad2, or GST in LSLD buffer for 2 h at 4°C and then washed five times with the same buffer. Samples were resolved by SDS/PAGE, and bound c-Jun was visualized by autoradiography.

Results

Repression of Smad2-Dependent Transcription by JNK Cascade. To examine the possible effect of JNK pathway on the TGF-β/Smad2 signal transduction, we focused our analyses on ARE-Lux, which contains a luciferase reporter gene under the control of three AREs. The ARE is stimulated by either TGF-β or activin signaling, which induces assembly of a DNA-binding complex that is composed of Smad2, Smad4, and a member of the FAST family of forkhead DNA-binding protein (17). Expression of ARE-Lux construct in Mv1Lu cells had minimal basal activity, but a strong TGF-β-dependent increase of transcriptional activity was detected in cells cotransfected with FAST1 (Fig. 1*A*). Interestingly, this TGF-β-dependent activation of ARE-Lux was markedly decreased in cells cotransfected with the constitutively activated mutants of MEKK1 (MEKK1.EE) and MKK4 (MKK4.ED) (Fig. 1*A*). A similar inhibition of Smad2-dependent transcription by MEKK1.EE and MKK4.ED was observed in HepG2 cells coexpressing FAST2, which is structurally related to FAST1 (ref. 18; data not shown). We also tested for this inhibitory effect on the *goosecoid* promoter (gsc-Lux) and found a similar repression by MEKK1.EE and MKK4.ED (Fig. 1*B*).

To provide further evidence that activation of JNK cascade leads to the repression of Smad2-dependent transcription, we transfected MEKK1.K432A and MKK4.Ala, which act as dominant-negative mutants with respect to JNK activation by TGF-β (6–8). As expected, expression of both MEKK1.K432A and MKK4.Ala increased the sensitivity of the Mv1Lu cells to TGF-β (Fig. 1*C*). A similar effect on ARE-Lux transcriptional activity was observed in transiently transfected HepG2 cells and in MDCK cells stably expressing MKK4.Ala (data not shown).

Together, these results provide strong evidence that activation of JNK cascade block TGF-β-dependent Smad2-mediated transcription in HepG2, Mv1Lu, and MDCK cells. In contrast, Brown *et al.* (19) reported that MEKK1 can specifically stimulate Smad2-mediated transcription in cultured epithelial cells. The basis for these differing observations on the crosstalk between Smad2 signaling pathway and JNK cascade is not clear; clarification of the conditions under which JNK cascade may support or antagonize Smad2-dependent transcription will require further investigation.

c-Jun Inhibits Smad2-Dependent Transcription. In an attempt to determine the mechanism underlying the inhibitory effect JNK

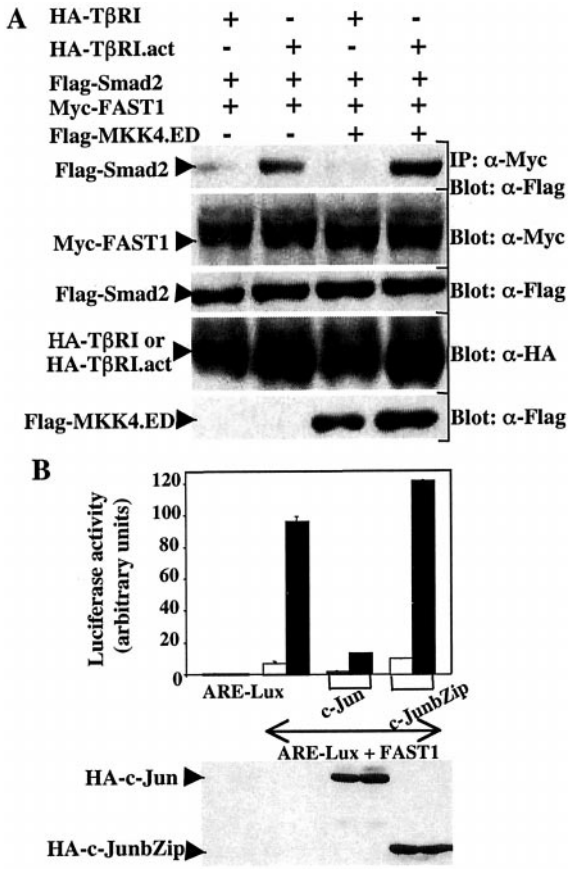

Fig. 2. c-Jun inhibits Smad2 transcriptional activity. (*A*) COS-7 cells were transfected with Myc-FAST1 and Flag-Smad2 in the presence or absence of MKK4.ED and wild-type (HA-TβRI), or constitutively activated (HA-TβRI.act) TGF-β type I receptor. Cell lysates were subjected to immunoprecipitation with anti-Myc antibody and then immunoblotted with anti-Flag antibody. The expression of transfected DNA was determined by immunoblotting whole cell extracts with anti-Myc or anti-Flag antibodies. (*B*) HepG2 cells were cotransfected with ARE-Lux, together with FAST1 and HA-c-Jun or HA-c-JunbZip, and cell lysates were assayed for luciferase activity. Cells were treated with (filled bars) or without (open bars) TGF-β for 16 h before lysis and then assayed for luciferase activity (*Upper*). Expression of HA-c-Jun or HA-c-JunbZip was assessed by Western blotting of cell lysates with anti-HA antibody (*Lower*).

cascade, we found that activation of JNK does not interfere with the phosphorylation of Smad2 by the activated type I receptor, its subsequent heterodimerization with Smad4, and its translocation to the nucleus (data not shown). Therefore, we tested whether the expression of the constitutively activated mutant MKK4.ED might interfere with the association of Smad2 with FAST1 in response to TGF-β signaling. COS-7 cells were transfected with Myc-FAST1 and Flag-Smad2 in the presence or absence of MKK4.ED and wild-type or a constitutively activated TβRI. Immunoprecipitation of Smad2, followed by immunoblotting for associated FAST1, revealed a low basal level of interaction between Smad2 and FAST1 that was enhanced significantly in the presence of the constitutively active TβRI. Interestingly, cotransfection of the constitutively active MKK4.ED with the activated TβRI failed to inhibit ligand-dependent association of Smad2 and FAST1 (Fig. 2*A*). Because the formation of Smad2/FAST1 complex in the nucleus recapitulates various signaling events, these results demonstrated that activation of JNK cascade can inhibit Smad2 transcriptional activity without preventing the ligand-dependent nuclear accumulation of Smad2 and its subsequent interaction with FAST1.

To determine the potential mechanism underlying the inhibitory activity of JNK cascade, we investigated whether overexpression of c-Jun could repress TGF-β-dependent induction of the ARE-Lux reporter. As shown in Fig. 2*B*, expression of c-Jun inhibited TGF-β-mediated activation of the ARE promoter, suggesting that Smad2 signaling was impaired. To provide further evidence that JNK inhibits Smad2 signaling through c-Jun, we investigated the function of a mutant version of c-Jun (c-JunbZip), which harbors a deletion in the N-terminus that includes the binding sites of JNK (20). In contrast to wild-type c-Jun, expression of c-JunbZip, which was expressed efficiently, resulted in a reproducible enhancement of ARE-Lux activity (Fig. 2*B*).

c-Jun Stabilizes the TGIF/Smad2 Complex. The CBP and the closely related protein p300, which have histone acetyltransferase (HAT) activity, act as coactivators of Smad2 through direct physical interactions with its MH2 domain (1, 5). The repression by c-Jun of Smad2-dependent transcription may be, at least in part, because of the ability of c-Jun to block TGF-β-mediated association of Smad2 protein with CBP/p300. To test this possibility, COS-7 cells were transfected with Myc-Smad2 and HA-c-Jun expression vectors, together with a deletion mutant Flag-p300(1892–2441), which lacks the c-Jun interaction domain but still associates with Smad2 (21, 22). Of note, the use of the truncation form p300(1892–2441) facilitates our analysis by ruling out a squelching mechanism involving a p300 titration by c-Jun. Consistent with published results (22), the interaction of Smad2 with p300(1892–2441) was strongly stimulated by the activated type I receptor (Fig. 3*A*). In contrast, in cells coexpressing c-Jun, the interaction of Smad2 and p300(1892–2441) was greatly reduced (Fig. 3*A*).

Recently, Smad2 has been shown to interact with the nuclear corepressor TGIF after TGF-β receptor activation. Repression of Smad2-dependent transcription by TGIF correlates with the recruitment of histone deacetylase (HDAC) instead of the transcriptional coactivator p300 (23). Because our biochemical analyses of p300/Smad2 complexes exclude the possibility that c-Jun prevents ligand-dependent association of Smad2 and p300 by binding and sequestering limiting amounts of p300 present within the cells, we hypothesized that it may do so by stabilizing the TGIF/Smad2 complex. To test this possibility, COS-7 cells were transfected with Myc-Smad2 and Flag-TGIF in the presence or absence of HA-c-Jun and wild-type or activated TβRI. As shown in Fig. 3*B*, association of Smad2 with TGIF was strongly increased by the activated type I receptor, similar to previous observations (23). Interestingly, cotransfection of c-Jun resulted in an increase in the amount of Smad2 present in Flag-TGIF immunocomplexes, suggesting that c-Jun can stabilize the Smad2/TGIF complex.

c-Jun Binds TGIF. In initial experiments, we observed that c-Jun can bind the Smad2/TGIF complex (Fig. 3*B*). To approach the question of how c-Jun stabilizes the Smad2/TGIF complex, we looked for possible interactions between c-Jun and TGIF or Smad2 through transient transfections. Immunoprecipitation of cell lysates from transfected COS-7 cells with an antibody directed against Flag-TGIF revealed the presence of HA-c-Jun, which was absent in a control transfection in which only HA-c-Jun was expressed (Fig. 4*A*). Similar results were obtained when Myc-Smad2 was used instead of Flag-TGIF (Fig. 4*A*). To further examine the association of c-Jun with TGIF and Smad2, we translated full-length c-Jun *in vitro* and tested for binding to bacterially expressed GST-Smad2 and GST-TGIF fusion proteins. As shown in Fig. 4*B*, c-Jun bound specifically to GST-TGIF, suggesting a direct interaction between c-Jun and TGIF. Interestingly, *in vitro* translated c-Jun failed to bind the purified recombinant GST-Smad2 protein, indicating that Smad2 must bind to c-Jun *in vivo* through interaction with yet-to-be-identified

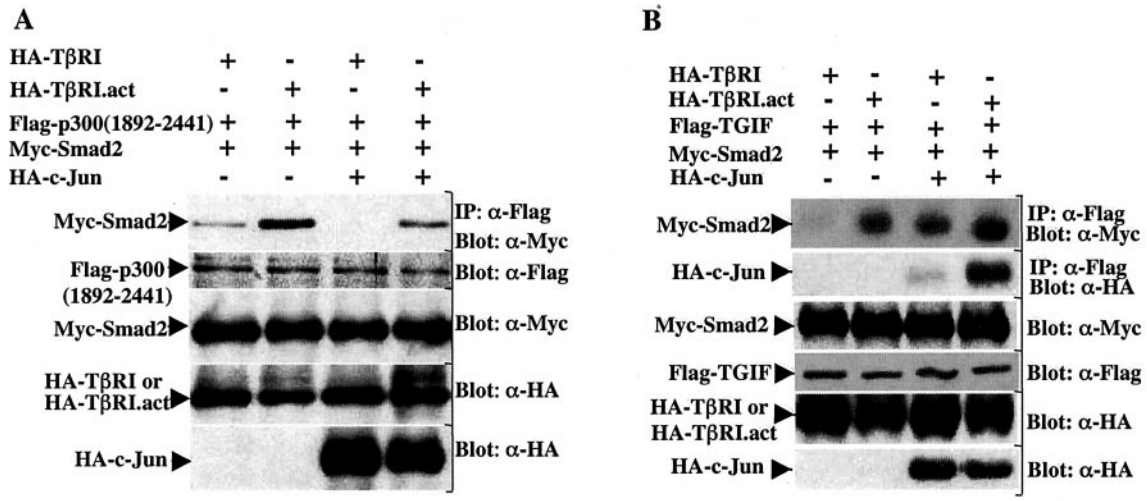

Fig. 3. c-Jun stabilizes the Smad2/TGIF complex. (*A*) COS-7 cells were transfected with Flag-p300(1892–2441) and Myc-Smad2 in the presence or absence of HA-c-Jun and HA-TβRI or HA-TβRI.act. Association of p300(1892–2441) with Smad2 was analyzed by blotting the Flag immunoprecipitates with the anti-Myc antibody. (*B*) COS-7 cells were transfected with various combinations of Flag-TGIF, Myc-Smad2, HA-c-Jun, and HA-TβRI or HA-TβRI.act as indicated. Flag immunoprecipitates were subjected to immunoblotting with anti-Myc or anti-HA antibodies.

partners, such as endogenous TGIF. Because we were able to detect an interaction between c-Jun and Smad2 in the absence of overexpressed TGIF, further detailed studies with cells deficient in TGIF would allow us to determine whether TGIF plays a role in bridging c-Jun and Smad2.

We next examined the effect of TGIF on c-Jun-mediated transcriptional activation of an AP1-Lux reporter, which contains AP1 sites and drives expression of a luciferase gene. As expected, expression of c-Jun strongly induced transcriptional activation of AP1-Lux (Fig. 4*C*). Interestingly, introduction of increasing amounts of TGIF cDNA resulted in a corresponding decrease in c-Jun-induced transactivation (Fig. 4*C*), providing support for a functional interaction between c-Jun and TGIF. In a control experiment, TGIF does not repress the transcription of a reporter construct containing the binding sites for β-catenin/T cell-specific factor (TCF) transcription factors (data not shown), indicating the specificity of the inhibition of c-Jun.

Stimulation with TGF-β Induces the Association of c-Jun with TGIF. To examine whether activation of TGF-β signaling might influence

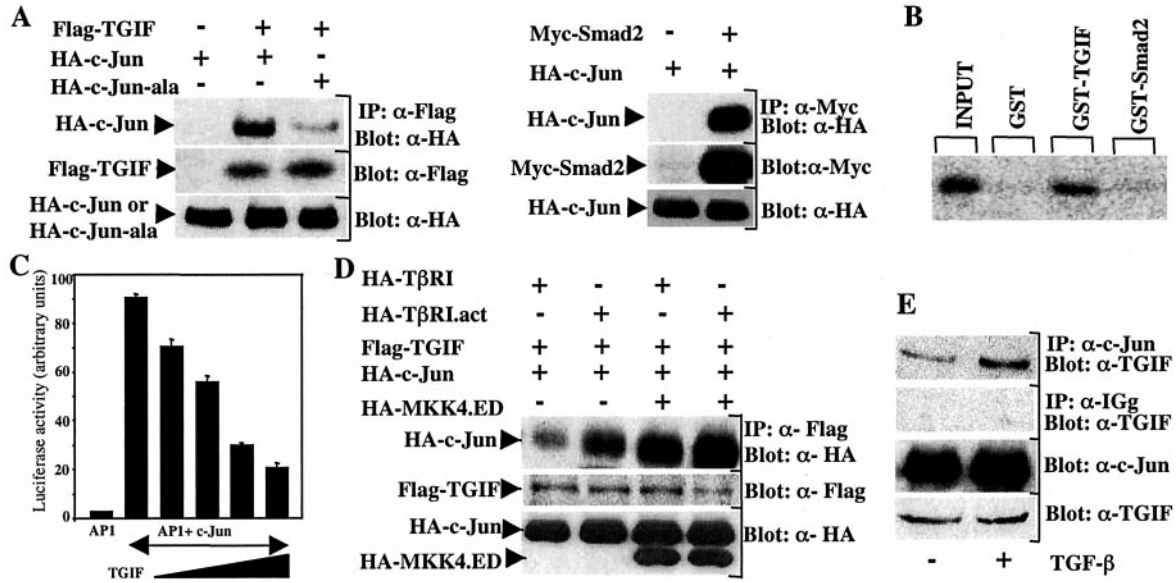

Fig. 4. Association of c-Jun with TGIF. (*A*) Cell lysates from transiently transfected COS-7 cells were subjected to immunoprecipitation with anti-Flag or anti-Myc antibodies and then immunoblotted by using anti-HA that recognizes HA-c-Jun or HA-c-Jun-ala. (*B*) *In vitro* interaction of c-Jun with TGIF or Smad2 was examined by incubating full-length [^{35}S]methionine-labeled c-Jun produced by *in vitro* transcription/translation with Sepharose-bound bacterially expressed GST-TGIF, GST-Smad2, or GST. Bound material was visualized by SDS and autoradiography. Ponceau staining of the membrane showed that similar amounts of GST, GST-Smad2, and GST-TGIF were used in this assay (data not shown). (*C*) HepG2 cells were cotransfected with AP1-Lux together with c-Jun and increasing amounts of TGIF. After 48 h, luciferase activity was determined and normalized to β-galactosidase activity. (*D*) COS-7 cells were transfected with wild-type Flag-TGIF and HA-c-Jun, together with HA-TβRI or HA-TβRI.act, either in the absence or the presence of MKK4.ED. Cell lysates were subjected to anti-Flag immunoprecipitation and then immunoblotted with anti-HA antibody. (*E*) Proteins were precipitated from Mv1Lu and from Mv1Lu cells treated with TGF-β for 1 h by using an anti-rabbit polyclonal antibody specific for c-Jun (Oncogene; *Top*) or normal rabbit antiserum (*Middle*). Precipitated proteins were analyzed by Western blotting with a goat antibody specific for TGIF (Santa Cruz). For comparison, a portion of cell lysates was probed with anti-c-Jun or anti-TGIF (*Bottom*) antibodies.

the interaction of c-Jun with TGIF, we transfected COS-7 cells with Flag-TGIF, HA-c-Jun, and either wild-type or activated TβRI. As shown in Fig. 4*D*, cotransfection of the activated type I receptor enhanced the interaction of TGIF with c-Jun. We concluded that c-Jun and TGIF can form physical complexes, the level of which can be enhanced by the activation of the TGF-β signaling pathway. A similar result was obtained with overexpression of the constitutively active mutant MKK4.ED (Fig. 4*D*), suggesting that activation of JNK cascade, which inhibits Smad2 transcriptional activity, can also stabilize the c-Jun/TGIF complex. Consistent with this notion, replacement of JNK phosphorylation sites (Ser-63 and Ser-73) with alanine in c-Jun dramatically reduced the interaction between c-Jun and TGIF (Fig. 4*A*). Further evidence was obtained by the ability of c-JunbZip or c-Jun-ala to interfere with the association of TGIF and wild-type c-Jun (data not shown).

We also determined whether the TGF-β-inducible TGIF/c-Jun complex occurred with physiological levels of these proteins. c-Jun-associated proteins were precipitated with a specific c-Jun antibody, and immunoprecipitates were analyzed by Western blotting with a specific anti-TGIF antibody. Similar to our previous observations in COS-7 cells, weak interaction between TGIF and c-Jun could be detected in unstimulated cells, and addition of TGF-β enhanced the interaction of TGIF with c-Jun (Fig. 4*E*).

The Interaction of c-Jun with TGIF Is Critical for the Repression of Smad2-Mediated Transcriptional Activity. Having shown an association between c-Jun and the corepressor TGIF, we set out to analyze its role in the repression of Smad2 transcriptional activity. We first examined whether the mutant c-JunbZip could interact with TGIF in COS-7 cells. Analysis of c-Jun interaction with TGIF showed that wild-type c-Jun interacts with TGIF, and this association was enhanced by the activated TβRI (Fig. 5*A*). In contrast, no interaction of TGIF with c-JunbZip was observed, despite efficient expression of the truncated protein (Fig. 5*A*). Interestingly, expression of c-JunbZip reduced the ligand-dependent association of TGIF and Smad2 to a lower level, as compared with cells transfected with empty control vector (Fig. 5*B*), suggesting that c-JunbZip plays a dominant-negative role. Because c-JunbZip is defective in its ability to be phosphorylated by JNK, these interaction experiments suggest that JNK function is required for the stabilization of the Smad2/TGIF complex. In support of this hypothesis, expression of c-Jun-ala blocked the majority of TGF-β-induced association of Smad2 and TGIF (Fig. 5*B*).

We also carried out experiments to determine whether c-JunbZip could interfere with the ability of TGIF to inhibit Smad2-dependent induction of the ARE-Lux construct. In control experiments, wild-type c-Jun synergizes dramatically with TGIF to induce a strong suppression of TGF-β-induced ARE-Lux activity (Fig. 5*C*). In contrast, expression of c-JunbZip, strongly relieved repression of ligand-dependent induction of luciferase activity by TGIF (Fig. 5*C*). A similar effect was observed when c-Jun-ala was used instead of c-JunbZip (data not shown). These results therefore provide compelling evidence that stable interaction of c-Jun with TGIF is critical for the repression of Smad2-mediated transcriptional activation.

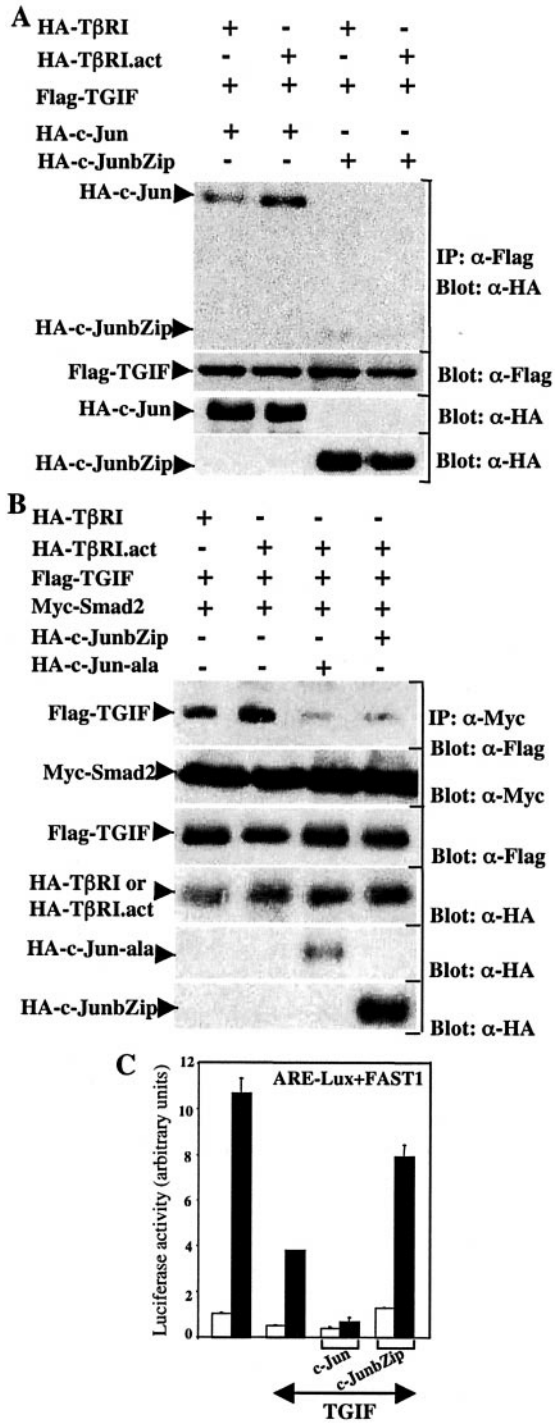

Fig. 5. Expression of the mutant c-JunbZip inhibits TGIF-mediated repression of Smad2 transcriptional activity. (*A*) HA-c-Jun or HA-c-JunbZip was cotransfected in COS-7 cells with Flag-TGIF and with HA-TβRI or HA-TβRI.act. Cell lysates were subjected to anti-Flag immunoprecipitation and then immunoblotted with anti-HA antibody. (*B*) Cell lysates from transiently transfected COS-7 cells were subjected to immunoprecipitation with anti-Myc antibody and then immunoblotted with anti-Flag antibody. (*C*) HepG2 cells were cotransfected with the indicated combination of ARE-Lux, FAST1, TGIF, HA-c-Jun, and HA-c-JunbZip. Cells were treated with (filled bars) or without (open bars) TGF-β for 16 h before lysis and then assayed for luciferase activity.

Discussion

In this study, we examined the relationships between the Smad2 and JNK signaling pathways in TGF-β-mediated transcriptional activation. We chose to focus our analysis on *Xenopus mix.2* and *goosecoid* promoters as targets of Smad2 because activation of either *mix.2* or *goosecoid* by TGF-β requires the formation of a Smad2–Smad4–FAST complex that binds to a sequence promoter known as TGF-β/ARE1. We have provided a mechanism of suppression of Smad2-dependent transcription by JNK signaling pathway.

Our results show that overexpression of constitutively active mutants of various components of JNK cascade, including MEKK1 and MKK4, inhibits the ARE-Lux transcriptional response to TGF-β, whereas overexpression of dominant negative mutants of these kinases had an opposite effect. The inhibition of Smad2 signaling pathway by JNK most likely takes place in the nucleus and directly involves a physical interaction between c-Jun and TGIF. The interaction between c-Jun and TGIF was increased on activation of JNK signaling pathway, suggesting that c-Jun may play a role in JNK-dependent repression of Smad2 signaling. Interestingly, overexpression of c-Jun enhances the association of Smad2 with TGIF, thereby interfering with the assembly of Smad2 and the coactivator p300 in response to TGF-β signaling. We are also struck by the importance of the c-Jun/TGIF complex in TGIF-mediated repression of Smad2 transcriptional activity, because the expression of the mutants c-JunbZip and c-Jun-ala, which fail to interact with TGIF, blocked the ability of TGIF to mediate repression of ligand-dependent induction of the ARE-Lux reporter. Therefore, in our proposed model, the activation of JNK pathway leads the association of c-Jun with TGIF, thereby stabilizing the Smad2/TGIF complexes, resulting in the repression of Smad2-mediated transcription. Such a regulatory mechanism might help cells to more finely tune the expression of genes regulated by Smad2.

The acetylation of chromosomal histones has long been known to correlate strongly with transcriptional status (24). Recently, it was demonstrated that TGIF functions to recruit HDAC to Smad2 and that histone deacetylase is required for TGIF-mediated repression of Smad2-dependent transcription (23, 25). Our finding that c-Jun can stabilize the interaction of Smad2 with the nuclear corepressor TGIF suggests that repression of Smad2-mediated transcription may involve deacetylation of nucleosomal histones. However, we cannot rule out the possibility that the repression of Smad2 transcriptional activity by c-Jun/TGIF is independent of HDAC activity, because TGIF appears to contain two separate repression domains, only one of which depends on HDAC (23, 25). Further studies will be required to determine the exact role of HDAC in c-Jun/TGIF-mediated repression of Smad2 transcriptional activity.

Several mechanisms have been proposed for the inactivation of Smad/TGF-β signaling pathway (1). For example, TGF-β signaling pathway can be blocked in Ras-transformed cells by MAP kinase phosphorylation of Smad2 and Smad3, which prevents their nuclear translocation. Smad signaling can also be limited by IFN-γ and tumor necrosis factor (TNF)-α, which induce the expression of Smad7. In addition, the zing finger protein Evi-1 interacts with Smad3 and represses its DNA binding activity, whereas the nuclear Ski and SnoN oncoproteins have been suggested to inhibit TGF-β signaling by recruitment of the transcriptional repressor N-CoR to TGF-β-responsive promoters through interaction with Smad proteins. The finding outlined in the present study that JNK signaling pathway suppresses the ability of Smad2 to mediate TGF-β transcriptional responses differs from these studies in one important and fundamental aspect, namely repression of Smad2 signaling by another downstream target of TGF-β receptors. This antagonistic crosstalk between two signaling pathways activated by TGF-β is very interesting because this mechanism would allow cells to display diverse patterns of transcriptional responses to TGF-β, depending on the relative activation of Smad proteins vs. JNK signaling.

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