c-Jun Inhibits Transforming Growth Factor β-mediated Transcription by Repressing Smad3 Transcriptional Activity*

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Transforming growth factor β (TGF-β) is a pleiotropic cytokine that exerts its effects through a heteromeric complex of transmembrane serine/threonine kinase receptors. At least two intracellular pathways are activated by TGF-β as follows: the SAPK/JNK, involving the MEKK1, MKK4, and JNK cascade, and the Smad pathway. Here, we report that the SAPK/JNK pathway inhibits the Smad3 pathway. Expression of dominant negative or constitutively active mutants of kinases of the SAPK/JNK pathway, respectively, activates or represses a TGF-β-induced reporter containing Smad3-binding sites. This effect is not dependent on blocking of Smad3 nuclear translocation but involves a functional interaction between Smad3 and c-Jun, a transcription factor activated by the SAPK/JNK pathway. Overexpression of constitutively active MEKK1 or MKK4 mutants stabilizes the physical interaction between Smad3 and c-Jun, whereas dominant negative mutants inhibit this interaction. Moreover, overexpression of wild-type c-Jun inhibits Smad3-dependent transcription. However, c-Jun does not inhibit Smad3 binding to DNA in vitro. The repression obtained with a c-Jun mutant unable to activate transcription through AP-1 sites indicates that the inhibitory mechanism does not rely on the induction of a Smad3 repressor by c-Jun, suggesting that c-Jun could act as a Smad3 co-repressor. The inhibition of the Smad3 pathway by the SAPK/JNK pathway, both triggered by TGF-β, could participate in a negative feedback loop to control TGF-β responses.

Members of the TGF-β superfamily (TGF-βs, activins, and BMPs) play pivotal roles in a wide range of biological processes including morphogenesis during development, extracellular matrix deposition, and cell cycle regulation. Signaling by these cytokines is transduced by heteromeric complexes of transmembrane Ser/Thr kinase receptors such as the Smad proteins. Eight mammalian Smad proteins have been identified and divided into three classes (2, 3). The first class includes pathway-restricted proteins such as Smad1, Smad5, and Smad8 that are specifically involved in BMP signaling and Smad2 and Smad3 that are restricted to TGF-β/activin pathway. The second class contains the common mediator Smad4 implicated in both BMP and TGF-β/activin pathways. The third class contains the inhibitory Smads, Smad6 and Smad7. After phosphorylation by TGF-β-activated type I receptor on their carboxyl-terminal SSXS sequence, pathway-restricted Smads form heteromeric complexes with Smad4 and then translocate to the nucleus where they control expression of diverse genes (4).

TGF-β has also been described to initiate other pathways such as the stress-activated protein kinase/c-Jun amino-terminal kinase (SAPK/JNK) pathway (5). This intracellular signaling proceeds through sequential activation of a mitogen-activated protein kinase kinase kinase (MEKK1), a MAPK kinase (MKK4 or MKK7), and a MAPK (JNK). JNK then translocates to the nucleus where it phosphorylates several transcription factors including c-Jun, ATF-2, and Elk-1 (6). c-Jun homodimerizes with members of the Jun family or heterodimerizes with members of the Fos family. These complexes named activating protein-1 (AP-1) bind to the AP-1 DNA-binding site and control expression of a number of genes including c-jun itself. TGF-β has recently been shown to induce the transcriptional activation of the c-Jun promoter through AP-1 and Smad3/Smad4-binding sites (7).

Smad proteins are key factors in TGF-β intracellular signaling, but the activity of Smad proteins is also modulated by other cytokines or growth factors. For example interferon-γ (IFN-γ) inhibits TGF-β signaling by transcriptionally inducing Smad7 expression through the Jaks/STATs signaling pathway (8). By activating Ras, epidermal growth factor (EGF) can also act as an inhibitor of TGF-β signaling. Oncogene ras or EGF treatment leads to the activation of members of the Erk subfamily of MAPKs through Raf and MEK1. Erk2 has been shown to phosphorylate Smad1, Smad2, and Smad3 at a different site than TGF-β type I receptor phosphorylation site. This EGF-induced phosphorylation prevents Smads translocation to the nucleus, thereby interrupting TGF-β signaling (9, 10). In contrast another report indicates that EGF and hepatocyte growth factor trigger Smad2 phosphorylation on its carboxyl-terminal SSXS sequence through kinase(s) downstream of MEK1, thus promoting Smad2 nuclear translocation and its transcriptional activity (11). Therefore Smad proteins may be...
phosphorylated by kinases of the MAPK pathway on different sites leading to either their activation or their repression. In endothelial cells, MEKK1, a kinase of the SAPK/JNK pathway, also positively regulates Smad2 activation. MEKK1-induced Smad2 phosphorylation does not require the carboxyl-terminal SSX sequence and promotes Smad2 association with Smad4 and the complex translocation to the nucleus (12). Therefore, Smad proteins integrate various extracellular signals leading to either their activation or their inhibition.

TGF-β treatment of HepG2 cells induces the formation of a Smad3/Smad4-containing complex that activates transcription from the Smad-binding sites called CAGA boxes (13). In these cells TGF-β has also been shown to activate the SAPK/JNK pathway (5). In this report, we show that these two TGF-β-activated pathways converge at the transcriptional level on CAGA sites. Surprisingly, whereas Smad3 synergizes with c-Jun on AP-1 site-mediated transcription, c-Jun represses Smad3 transcriptional activity on CAGA sites, suggesting that in this context, c-Jun may act as a Smad3 co-repressor.

**EXPERIMENTAL PROCEDURES**

**Plasmids**—The (CAGA), MLP-Luc reporter was described previously (13). pAP-1-Luc containing seven copies of an AP-1 site was purchased from Stratagene. Expression vectors for dominant negative MEKK1, constitutively active MEK1, and dominant negative MKK4 were described previously (5). Expression vectors for partially active MKK4, constitutively active MEK, and c-Jun were a gift from Dr. Bohmann. Smad3 in pGEX4T-1 (a gift from Dr. R. Derynck) was subcloned into pEGFP in frame with an amino-terminal green fluorescent protein (GFP) tag to generate pGFP-Smad3. Expression vector for human Smad3 was a gift from Dr. P. ten Dijke. Expression vector for constitutively active TGF-β type I receptor was a gift from Dr. G. Wraza. Expression vectors for human c-FOS and human c-JUN were polymerase chain reaction-amplified from a human liver cDNA library (Life Technologies, Inc.) and subsequently cloned into pcDNA3.1 (Invitrogen). Expression vectors for murine c-JUN and murine c-JUN CDL were gifts from Prof. M. Yaniv.

**Cell Culture, Transfection, and Gene Expression Analysis**—The human hepatoma cell line HepG2 was maintained in BME medium (Life Technologies, Inc.) supplemented with 10% fetal calf serum (FCS) or RPMI (Life Technologies, Inc.) containing 10% FCS. The human breast adenocarcinoma cell line MDA-MB468 was grown in a 7.5% CO2, 92.5% air atmosphere in Dulbecco’s modified Eagle’s medium/F12 (1:1) medium (Life Technologies, Inc.) with 10% FCS. COS-7 cells were maintained in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) containing 10% FCS. For gene expression analysis, cells were plated to approximately 50% confluence and transfected a day later with expression vectors by the LipofectAMINE method (Life Technologies, Inc.) or the Bio Reagent (Roche Molecular Biochemicals). Cells were subsequently serum-starved before induction with human TGF-β (2 or 5 ng/ml) for 16 h. Cell extracts were assayed for luciferase activity using a luciferase assay system (Promega). The luciferase activities were normalized on the basis of either β-galactosidase expression from pCMV5-LacZ control vector or Renilla luciferase expression from pRL-TK control vector. Experimental points were realized in triplicates in at least two independent transfections. Values given in figures are the arithmetic means, and the error bars represent standard deviations of a representative experiment.

**Immunoprecipitation and Immunoblotting**—COS-7 cells were transfected with expression vectors using the LipofectAMINE method, and 48 h post-transfection, cells were lysed at 4 °C in lysis buffer (20 mM Tris-HCl (pH 8), 150 mM NaCl, 5 mM MgCl2, 1% glycerol, 0.5 mg/ml aprotinin, and 20 mg/ml leupeptin). Lysates were then incubated with anti-c-Myc (9E10) monoclonal antibody (Santa Cruz Biotechnology) for 2 h, and immunocomplexes were immobilized on Sepharose-coupled protein G for 1 h. The beads were washed five times in lysis buffer, and samples were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Immunoblots were generated and used to direct immunoblotting to monitor transfection efficiency. Proteins were electrophoretically transferred to nitrocellulose membranes and probed with anti-HA (3F10; Roche Molecular Biochemicals). The bands were visualized by an enhanced chemiluminescent detection system according to the manufacturer’s instructions (ECL, Amersham Pharmacia Biotech).

**In Situ Fluorescence**—COS-7 cells were transiently transfected with GFP-Smad3 together with the indicated expression vectors. After 48 h, slides were washed with phosphate-buffered saline, fixed for 15 min with 4% paraformaldehyde at room temperature, and washed three times. To detect GFP staining, cells were observed under a fluorescence microscope.

**Gel Shift Experiments**—Nuclear extracts were prepared from control and 30-min TGF-β-treated HepG2 cells as described previously (13). c-Jun and Smad3 were also produced using the TNT T7 Quick-coupled Transcription/Translation System (Promega) according to the manufacturer’s protocol. Oligonucleotides were end-labeled with [α-32P]CTP and used as a probe to detect the Klenow fragment of DNA polymerase. For binding reactions 10 μg of nuclear extracts (and/or in vitro synthesized proteins) were preincubated with or without 200 ng of cold probe or 2 μl of antibody (Smad2/Smad3 or c-Jun antibodies from Santa Cruz Biotechnology) for 20 min at 37 °C in final 40 μl of binding buffer (final concentrations: 20 mM HEPS (pH 7.9), 30 mM KCl, 4 mM MgCl2, 0.1 mM EDTA, 0.8 mM NaF, 20% glycerol, 4 mM spermine, 5 μg of poly(dI-dC)). Then 2 ng of labeled oligonucleotides were added for another 20 min at 37 °C. Protein-DNA complexes were resolved in 5% polyacrylamide gels containing 0.5X TBE. The sequence of the double-stranded CAGA oligonucleotide used as a probe was 5’TCTGAGGCCAGACAAAGGCAAGCAGACAGCACACGACACGACACGACTAATGACTAATGACTAATGACTAATGACTAAC-3’ and its complementary strand. The sequence of the double-stranded AP-1 oligonucleotide used as a probe was 5’TCAAGTGGTATGATGACTAATGACTAATGACTAATGACTAC-3’ and its complementary strand.

**RESULTS**

**Activation of JNK Pathway Inhibits Smad Pathway**—We have previously shown that in HepG2 cells, TGF-β activates the p3TP-Lux reporter vector through the SAPK/JNK pathway (5). This vector contains three AP-1 sites from the collagenase-1 promoter. The activation of SAPK/JNK pathway by TGF-β promotes the activation of the c-Jun-containing factor AP-1. pTP-Lux also contains a plasmogen activator inhibitor-1 (PAI-1) promoter sequence with a CAGA box recognized by a TGF-β-activated Smad3/Smad4 complex (13). Therefore to dissociate the effects of TGF-β on AP-1 or Smad3/Smad4-binding sites, we used the CAGA reporter containing exclusively nine copies of the Smad-binding site derived from the PAI-1 promoter (13). We hypothesized that the SAPK/JNK pathway could modulate the TGF-β-activation of this reporter. To test this hypothesis, HepG2 cells were transfected with an expression vector for a dominant negative form of MEKK1, and the activity of the CAGA reporter vector by TGF-β was studied (Fig. 1B). MEKK1 is a member of the mitogen-activated protein kinase kinase kinase family and an upstream activator of the SAPK/JNK pathway (14). As observed with the dominant negative form of MKK4, this protein is dominant negative with respect to JNK activation by TGF-β (15-17). Expression of a constitutively active MEKK1 protein dramatically reduced the reporter activation by TGF-β (Fig. 1B). These results could indicate that the activation of JNK pathway may negatively regulate TGF-β-induced Smad pathway in HepG2 cells.

**MEKK1 phosphorylates MKK4 (Fig. 1A), and this dual specificity kinase then phosphorylates and activates JNK (6; Fig. 1A). To provide further evidence that the activation of JNK pathway by TGF-β abrogates the CAGA reporter activation, we transfected an expression vector encoding a dominant negative form of MKK4. This protein is dominant negative with respect to JNK activation by TGF-β (14). As observed with the dominant negative form of MEKK1, expression of this MKK4 mutant also abrogated the CAGA reporter activation by TGF-β (Fig. 1C). Expression of a partially active form of MKK4 reduced the reporter activation (Fig. 1C). Thus MKK4 can mimic the MEKK1 effect, suggesting that the inhibition of the CAGA reporter activation by TGF-β may directly involve MKK4 or a downstream protein.
Translocation—MKK4 belongs to the same kinase family as MEK1, a mitogen-activated protein kinase kinase of the MAPK pathway. MEK1 activates the Erk kinase which in turn phosphorylates Smad3, thereby preventing its nuclear translocation (10). Therefore, an attractive hypothesis is that MKK4 may inhibit Smad3 nuclear translocation leading to an inhibition of the TGF-β-induced activation of the CAGA reporter vector. To test this hypothesis, we studied Smad3 nuclear translocation in COS-7 cells in the presence or absence of partially active MKK4. As a control we expressed a constitutively active form of MEK1. In order to induce Smad3 nuclear translocation, we transfected the expression vector for the constitutively active TGF-β type I receptor that triggers intracellular cascade in the absence of TGF-β type II receptor and cytokine. As expected, expression of the activated type I receptor markedly increased Smad3 nuclear translocation, whereas expression of the activated MEK1 prevented this translocation (Fig. 2). We did not observe an inhibition of Smad3 nuclear translocation with the activated MKK4 but rather a stronger nuclear staining. Thus MKK4 inhibitory effect on the CAGA reporter activation by TGF-β cannot be explained by an abrogation of Smad3 nuclear translocation. Furthermore, these data suggest that the JNK pathway does not favor Smad7 expression as this protein would prevent Smad3 nuclear translocation. Indeed it has been shown that IFN-γ inhibits Smad7 expression (8).

**c-Jun Directly Inhibits Smad Pathway**—In the JNK pathway, MKK4 activates JNK which then translocates into the nucleus where it phosphorylates several transcription factors, in particular c-Jun. This factor is particularly interesting because it is able to form a complex with Smad3 (15, 16). To determine whether the inhibitory effect observed involves c-Jun, we transfected a wild-type c-Jun expression vector together with the CAGA reporter in HepG2 cells. As expected, c-Jun activated transcription from AP-1 sites, but under the same experimental conditions c-Jun markedly inhibited the
TGF-β-induced activation of the CAGA reporter in a dose-dependent manner (Fig. 3A). Thus TGF-β-initiated SAPK/JNK pathway activates the nuclear factor c-Jun which in turn inhibits the CAGA reporter activation by TGF-β. To test whether this repression could also be observed in a naturally occurring context, we studied a TGF-β-activated promoter. The PAI-1 promoter contains three CAGA sequences that are necessary for the TGF-β-dependent activation of the promoter (13) in addition to other Smad3-binding sites (17-19). As shown in Fig. 3B, overexpression of c-Jun mediated repression of this natural PAI-1 promoter, which is consistent with the hypothesis that activation of c-Jun by TGF-β can repress Smad3 transcriptional activity.

As c-Jun is a transcription factor that activates many AP-1-containing target genes, its effect on Smad3 transcriptional activity could be the consequence of a c-Jun-dependent expression of a Smad3 repressor. In order to determine whether the c-Jun effect is mediated by such a protein, we transfected a vector encoding a c-Jun CDL mutant which is defective in activating transcription from AP-1 sites. The CDL mutant is truncated in the leucine zipper, thus unable to form active dimers. As expected the CDL mutant did not activate the AP-1 reporter but inhibited TGF-β-activated CAGA reporter (Fig. 3C). This result indicates that the expression of a gene activated by c-Jun through AP-1 sites is not involved in this repression and rather suggests a direct effect of c-Jun on Smad3. Nevertheless, we cannot exclude that c-Jun represses the expression of a co-activator important for Smad3 transcriptional activity.

c-Jun Inhibits Smad3 Transcriptional Activity—We have previously reported that overexpression of Smad3 in the absence of TGF-β activates the CAGA-mediated transcription (13, 20). In order to assess directly the effect of c-Jun on Smad3-mediated transcription, we co-transfected Smad3 and c-Jun expression vectors along with the CAGA reporter in the absence of cytokine thus preventing any TGF-β-induced signals aside from Smad3 activation. As shown in Fig. 4A, exogenous c-Jun inhibited the Smad3-activated CAGA reporter. In line with other reports, Smad3 potentiated c-Jun transcriptional activity on AP-1 sites (7, 15). Previous studies have shown a direct interaction between Smad3 and c-Jun (15, 16). We investigated the possibility that JNK pathway activation could modulate Smad3/c-Jun interaction. COS-7 cells were transfected with tagged Smad3 and c-Jun expression vectors in the absence or the presence of dominant negative and constitutively active MEKK1 and MKK4 expression vectors. As shown in Fig. 4B, c-Jun co-precipitated with Smad3. Expression of constitutively active MEKK1 and MKK4 resulted in an increase of c-Jun co-precipitated with Smad3, indicating that JNK pathway activation might repress Smad3 transcriptional activity by stabilizing the c-Jun-Smad3 complex. Remarkably co-transfection of dominant negative MEKK1 and MKK4 mutants abrogated the Smad3-c-Jun complex formation. Overall these results suggest that the physical interaction between c-Jun and Smad3 is directly involved in the inhibitory effect observed on the Smad3-activated CAGA reporter.

As Smad3 forms heteromers with Smad4 and c-Jun heterodimerizes with c-Fos, we considered that Smad4 or c-Fos could modulate c-Jun inhibition of Smad3 transcriptional activity. Overexpression of Smad3 in Smad4 homozygous null MDA MB468 cells activated the CAGA reporter in the absence of TGF-β stimulation (Fig. 4C). As in HepG2 cells, overexpression of c-Jun reduced this activation indicating that Smad4 is not necessary for the c-Jun inhibition of Smad3-mediated transcription. We were next interested in determining whether c-Fos could synergize with c-Jun to inhibit CAGA activation. As expected c-Fos activated the AP-1 reporter in synergy with c-Jun. At doses where c-Fos activated the AP-1 reporter, we did not observe any effect of c-Fos alone on the TGF-β-induced CAGA reporter (Fig. 4D). Co-transfection of c-Jun and c-Fos expression vectors did not lead to a stronger repression than c-Jun alone indicating that, at low doses, c-Fos did not synergize with c-Jun to inhibit Smad3 transcriptional activity. However, at very high doses, c-Fos repressed the TGF-β-induced CAGA reporter and did not show a clear additive effect with c-Jun (Fig. 4D). It should be noted that this repression is observed at much higher doses than those required for AP-1 activation. This would suggest that c-Fos like c-Jun may inhibit TGF-β-induced transcription although c-Fos is a weaker inhibitor.
In an attempt to elucidate this inhibitory mechanism, we performed the following additional experiments. Direct interaction of c-Jun with Smad3 could prevent Smad3 transcriptional activity by interfering with Smad3-binding to DNA. As shown in Fig. 5 and already described before (13), a TGF-β-induced complex containing Smad3 bound specifically to an oligonucleotide containing CAGA sites. As expected, in vitro translated c-Jun was able to bind specifically to an AP-1 probe, whereas Smad3 did not bind (see the competition with cold probe and interference with c-Jun antibody in Fig. 5, right panel). However, we did not observe any effect of c-Jun on Smad3 DNA binding to CAGA boxes in gel shift experiments when we added c-Jun, whatever the source of proteins, i.e. in vitro translated or cell extracts (Fig. 5 and data not shown). These results would suggest that c-Jun does not inhibit Smad3 binding to its target sites in vitro but represses Smad3 transcriptional activity by a mechanism yet to be discovered.

**FIG. 3.** Effect of c-Jun on TGF-β-induced transcriptional activity. HepG2 cells were transfected in 24-well plates. A, cells were transfected either with 300 ng of pAP-1-Luc (left) or (CAGA)₉ MLP-Luc (right) together with various amounts of c-Jun expression vector (2, 4, 8, or 16 ng). B, cells were transfected with PAI-Luc reporter vector with the same amounts of c-Jun expression vector as in A. C, HepG2 cells were transfected either with pAP-1-Luc (left) or with (CAGA)₉ MLP-Luc (right) together with 20, 40, 60, or 80 ng of c-Jun CDL expression vector.

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**DISCUSSION**

TGF-β initiates intracellular signaling leading to transcriptional regulation of many genes (25). In several reports, AP-1 sites have been involved in TGF-β-transcriptional activation (7, 26–29). Several lines of evidence indicate that c-Jun, a transcription factor that binds to AP-1 site, is a downstream target of the TGF-β signaling pathway. First TGF-β induces c-Jun expression through transcriptional activation of the c-Jun promoter which contains both AP-1 and Smad3-binding sites (7). Second TGF-β initiation of the JNK pathway leads to c-Jun phosphorylation at Ser-63 and Ser-73 that increases its transcriptional activity (5). Finally, TGF-β induces the activation of Smad3 and its subsequent interaction and cooperation with c-Jun on AP-1 sites (15, 16). These last years have seen the emergence of the Smad2 and Smad3 transcriptional regulators as critical factors in TGF-β signaling. In particular, several TGF-β-induced promoters are regulated by Smad3, a transcription factor that binds to specific DNA sequences such as the CAGA box present in promoters including PAI-1, JunB, collagen, or Smad7. We therefore wondered whether the JNK pathway could modulate activation of the Smad pathway. In the present study we report that the activation of the SAPK/JNK pathway represses the Smad pathway. Overexpression of constitutively active mutants of MEKK1 and MKK4 inhibited TGF-β-induced transcription, whereas overexpression of dominant negative versions of these kinases had an opposite effect. We have obtained a stronger repression with the constitutively active MEKK1 mutant compared with the MKK4 mutant (see Fig. 1, B and C). This could be attributed to the fact that the
MKK4 mutant is partially active, leading to a weaker activation of the JNK pathway than the MEKK1 mutant (30). Alternatively, MEKK1 could initiate, in addition to the MKK4/JNK pathway, another signaling involved in Smad3 repression. We have not been able to observe a modulation of Smad3 transcriptional activity by overexpression of a wild-type or a kinase-inactive version of JNK (data not shown), but both JNK constructs were also inactive on AP-1-mediated transcription, contrary to data obtained for MEKK1 and MKK4 mutants.

This suggests that, in our systems, these JNK proteins do not have the potential to modulate c-Jun transcriptional activity. We are thus unable at present to test the ability of overexpressed wild-type JNK to inhibit CAGA reporter. Identification of a mutant that constitutively activates JNK protein would help clarify this issue.

Our data indicate that the cross-talk between Smad and JNK pathways occurs in the nucleus and that overexpression of c-Jun, which specifically interacts with Smad3, inhibits...
Smad3-mediated transcription. By using a transcriptionally inactive c-Jun mutant, we have excluded that c-Jun modulates Smad3 transcriptional activity by the neosynthesis of a Smad3 repressor. Remarkably, overexpression of the dominant negative MEKK1 and MKK4 mutants, which potentiate TGF-β-induced transcription, disrupts the physical interaction between c-Jun and Smad3. In contrast, constitutively active MEKK1 and MKK4 mutants inhibit TGF-β-induced transcription and stabilize the c-Jun-Smad3 complex. These results indicate that interaction between Smad3 and c-Jun may repress Smad3 transcriptional activity. Although we have not addressed the phosphorylation state of c-Jun in the present paper, it will be interesting to study the effect of phosphorylation on this interaction. c-Fos, which heterodimerizes with c-Jun, seems able to repress TGF-β-induced transcription, although we observed a repression only at much higher doses than those required to activate an AP-1 reporter. Taken together with published reports, our results show that whereas Smad3 promotes c-Jun transcriptional activity on AP-1 sites, c-Jun inhibits Smad3-mediated transcription.

Brown et al. (12) have previously reported that MEKK1 could stimulate Smad2-mediated transcriptional activation in endothelial cells. These observations diverge from ours since we report a repression of Smad3 transcriptional activity by c-Jun and the JNK pathway in HepG2 cells. However, Smad2 and Smad3 may be differently regulated, and the different cellular contexts of the two studies may be important. Our data describing a repression of Smad3 activity fit with several recent papers revealing transmodulation of Smad pathway by other signaling pathways. As published by Kretzschmar and coworkers (9, 10), Smad3 transcriptional activity is repressed by EGF-induced phosphorylation of Smad proteins in the linker region. This phosphorylation by Erk kinases prevents Smad translocation to the nucleus (9, 10). Another paper (8) describes that Smad activation can be inhibited by IFNγ, the latter promoting Smad7 expression. Thus, both EGF and IFNγ prevent Smad translocation to the nucleus. In our experiments we have excluded the possibility that the repression of Smad activity is mediated by Smad3 retention in the cytoplasm.

Other mechanisms of transmodulation might also exist since a recent study indicates that ectopic viral integration site-1 (Evi-1), a zinc finger protein, interacts with Smad3 MH2 domain and represses Smad3 DNA binding activity (31). In the present study, c-Jun inhibition of Smad3 transcriptional activity does not seem to rely on the same mechanism since we have not detected a reduction of Smad3 binding to DNA in vitro. Smad3 has also been shown to interact with several transcription factors including c-Jun, c-Fos, ATF-2, and vitamin D receptor (32, 33). Comparable to what we report here, a recent paper (34) describes the inhibition of Smad3 transcriptional activity by the glucocorticoid receptor, involving a physical interaction between both transcription factors. Several proteins may thus modulate Smad3 transcriptional activity by various mechanisms. In particular, transcriptional activity of Smad proteins depends on co-activators such as CBP/p300 and co-repressors such as TGIF, Ski, or SnoN (32, 35–40). It has been proposed that these proteins control transcription by modifying the access to chromatin by acetylation (co-activator) or deacetylation (co-repressors) of core nucleosomal histones. Since c-Jun also interacts with CBP/p300, c-Jun may modify the interaction of Smad3 with its co-activators or co-repressors. However, it remains to be shown whether the acetylation state of histones may be involved in the c-Jun silencing of TGF-β transcriptional responses. Nevertheless, our results are the first revealing a repression of Smad3 transcriptional activity by a TGF-β-activated transcription factor. Although we cannot rule out the possibility that JNK pathway triggers additional components required for full Smad3 transcriptional activity, our data and the physical interaction between c-Jun and Smad3 favor the hypothesis that c-Jun may act as a direct co-repressor of Smad3 activity by a yet unknown mechanism.

In addition to our data obtained on the native PAI-1 promoter, c-Jun has already been reported to inhibit the TGF-β induction of the α2 type I collagen promoter (26). Since TGF-β activates both Smad3 and c-Jun, we propose a model in which Smad3 transcriptional repression by c-Jun may participate in a feedback control mechanism (Fig. 6). First TGF-β induces the activation of both Smad3 and c-Jun which modulate transcription of various genes including c-jun itself (7). As the activation of JNK pathway promotes the accumulation of c-Jun, Smad3 transcriptional activity is repressed leading to silencing of some TGF-β effects. Although the exact mechanism through
which c-Jun acts to repress Smad3 transcriptional activity and the kinetics of the interactions remain to be established, the finding that JNK pathway inhibits Smad3-mediated gene expression provides new insights into TGF-β-regulated gene expression.

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