Evidence for a Role of the JNK Cascade in Smad7-mediated Apoptosis*

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Smad proteins are central mediators of the transcriptional effects of transforming growth factor β (TGF-β) superfamily that regulate a wide variety of biological processes. Smad7, an inhibitory Smad protein that prevents TGF-β signaling by interacting with the activated type I TGF-β receptor, was recently shown to induce sensitization of cells to different forms of cell death. Here we examined the effect of Smad7 on the c-Jun N-terminal kinase (JNK) cascade and investigated the role of this cascade in both the inhibitory and apoptotic functions of Smad7. The transient and stable expression of Smad7 caused a strong and sustained activation of JNK. Expression of a dominant-interfering mutant of mitogen-activated protein kinase kinase 4, which completely abolished Smad7-induced activation of JNK, had no effect on Smad7-mediated inhibition of TGF-β signaling, indicating that the inhibitory function of Smad7 is independent of the JNK cascade. In contrast, expression of the dominant-interfering mutant of mitogen-activated protein kinase kinase 4 impaired the ability of Smad7 to promote cell death. These experiments reveal a novel link between Smad7 and the JNK cascade, which is essential for potentiation of cell death by this inhibitory Smad.

The transforming growth factor-β (TGF-β)1 is a member of a large family of cytokines that regulates a variety of biological functions, including proliferation, apoptosis, extracellular matrix production, and differentiation (1–3). Signaling by TGF-β is initiated by the heteromeric TGF-β cell surface receptor, composed of two distantly related transmembrane serine/threonine kinases called receptors I (TβRI) and II (TβRII). Primary binding of the ligand occurs with TβRII, promoting formation of a heterotetramer with TβRI and activation of TβRI by the TβRII kinase (4, 5). The activated TβRI then interacts with an adaptor molecule SARA (Smad anchor for receptor activation) (6), which facilitates the access of Smad2 and Smad3 to activated TGF-β receptors.

The Smad proteins are divided into three distinct classes based upon structure and function (7–9). Receptor-regulated Smads are phosphorylated on two serines at the C terminus, within a highly conserved SSXS motif by the type I receptors in a ligand-specific manner. Thus, Smad2 and -3 are phosphorylated by TGF-β or activin receptors, whereas Smad1, Smad5, and presumably Smad8 are substrates for bone morphogenic protein receptors (7–9). Following phosphorylation, receptor-regulated Smads associate with the shared partner Smad4 and translocate to the nucleus where Smad complexes participate in transcriptional activation of target genes (7–9). In contrast to receptor-regulated Smads and Smad4, the antagonistic Smads, which include Smad7, appear to negatively regulate these pathways by blocking ligand-dependent signaling (10–12). The expression of both Smad6 and Smad7 is increased in response to TGF-β, bone morphogenic protein, and activin, providing a mechanism for negative feedback regulation of the Smad signaling pathway (9). The expression of Smad7 can also be induced by interferon-γ and by TNF-α, raising the interesting possibility that Smad7 may fulfill other cellular functions independent of its inhibitory role (13, 14). Consistent with this, overexpression of Smad7 has been reported to sensitize various cell types to many forms of cell death (15, 16). However, the mechanism by which Smad7 induces cell death remains largely undefined.

There is growing evidence that TGF-β can activate various mitogen-activated protein kinase (MAPK) signaling pathways, most prominently the c-Jun N-terminal kinase (JNK) family of cytoplasmic serine/threonine kinases (17–19). The activation of JNK by TGF-β can be mediated through the small GTP-binding proteins Cdc42 and Rac1 and the downstream protein kinase they activate, such as MAPK kinase kinase 1 (MEKK1) (17, 19–21). In turn, MEKK1 phosphorylates and activates MAPK kinase 4 (MKK4), which activates JNK (17, 19–21). Once activated, JNK phosphorylates the transactivating domain of c-Jun protein at serine residues 63 and 73 and increases its transcriptional activating potential (22). The JNK pathway is also activated by TNF-α and other stress-activated signals (23). Dominant-negative components of the JNK pathway can block stress- and TNF-α-induced apoptosis in several cell lines, suggesting that activation of the JNK pathway is required for these apoptotic inducers (24). In other studies, it has been shown that apoptosis signal-regulating kinase 1, an activating kinase of the JNK pathways that functions in response to TNF-α, is sufficient to induce apoptosis and is required for TNF-α-induced cell death (25). Furthermore, expression of MEKK1, which activates JNK, resulted in the apoptotic death

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1 The abbreviations used are: TGF-β, transforming growth factor β; ARE, activin responsive element; JNK, c-Jun N-terminal kinase; MKK, mitogen-activated protein kinase kinase; TNF-α, tumor necrosis factor α; MAPK, mitogen-activated protein kinase; MEKK1, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase kinase 1; GST, glutathione S-transferase; HA, hemagglutinin; GFP, green fluorescent protein; FCS, fetal calf serum; MOPS, 4-morpholinepropanesulfonic acid; MDCR, Madin-Darby canine kidney.

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of fibroblasts, suggesting that under some circumstances JNK signaling alone might be sufficient to cause cell death (26).

To elucidate the mechanism by which Smad7 induces apoptosis, we examined the potential role of the JNK pathway in this process. In the current study, we demonstrate that expression of Smad7 specifically initiates a signaling cascade leading to JNK activation. Furthermore, using a dominant-negative mutant of MKK4, the kinase that phosphorylates and activates JNK, we show that forced expression of Smad7 triggers cell death through JNK activation. These data provide a novel property of Smad7 in regulating JNK cascade activity and point out a role for JNK in Smad7-mediated apoptosis.

**EXPERIMENTAL PROCEDURES**

**Plasmid Constructs and Cell Culture**—Expression plasmids for GST-Jun (1–79), HA-JNK1, Flag-Smad2, Flag-Smad3, Flag-Smad4, Flag-Smad7, Mv1Lu-S7 cells) (29) expression vector were generously provided by Dr. M. Karin. The ARE-Lux reporter plasmid. 48 h after transfection, cells were assayed for JNK activity in transient transfection. COS-7 or MDCK cells were propagated in Dulbecco's medium supplemented with 1% nonidet P-40, 20 mM EDTA, 50 mM Tris-HCl (pH 7.5). After centrifugation for 5 min at 1,600 × g, the supernatants were collected, and the extraction was repeated once. The supernatants were incubated with 2 μg/ml RNase A for 2 h at 56 °C and then 100 μg/ml proteinase K were added, and the incubation was continued for at least 2 h at 37 °C. DNA fragments were digested with 2 volumes of ethanol in the presence of 0.5 volume of 10 mM ammonium acetate at −20 °C overnight. After centrifugation, samples were washed with 70% ethanol and resuspended in loading buffer. Electrophoresis was performed in 0.5 × Tris-borate-EDTA (TBE) buffer on 15% agarose gels containing ethidium bromide. For the clonal growth assays, MDCK cells were cotransfected with pEM4 vector encoding a hygroycin resistance gene using LipofectAMINE, and hygromycin-resistant colonies were visualized with crystal violet 10–13 days after transfection.

**RESULTS AND DISCUSSION**

We first examined the effect of Smad7 overexpression on TGF-β-induced JNK activation using a well characterized cell line in which the level of Smad7 expression is controlled by the human inducible metallothionein II A promoter (see Ref. 29 and Fig. 1A). Mv1Lu cells stably transfected with a pMEP4 vector (Mv1Lu-pMEP4 cells) or a pMEP4-Smad7 (Mv1Lu-S7 cells) expression vector were treated with the inducer ZnCl2 and with or without TGF-β before being examined for JNK activity by an immune complex kinase assay using GST-Jun (1–79) as substrate. Surprisingly, we found that Smad7 synergizes dramatically with TGF-β to induce a strong increase in JNK enzymatic activity (Fig. 1B). To determine the basis of this synergism, we examined the effect of ectopic Smad7 expression on JNK activity. For this, Mv1Lu-S7 cells were treated with or without ZnCl2 for different times, and lysates were assayed for the in vitro phosphorylating activity of JNK and expression of the Flag-Smad7 by Western blotting. As shown in Fig. 1C, induction of Flag-Smad7 resulted in a marked stimulation of endogenous JNK activity, which is detectable after 3 h and increases with time. In contrast, control cells carrying an empty vector (Mv1Lu-pMEP4 cells) showed little or no activation of JNK in response to ZnCl2 (data not shown). Immunoblotting analysis with anti-JNK antibody of total cell lysates from Mv1Lu-S7 cells demonstrated that approximately equivalent amounts of JNK protein were examined (Fig. 1C). Together, these data indicate that Smad7 can effectively induce a signaling pathway leading to the activation of endogenous JNK.

To confirm the activation of JNK by Smad7, we cotransfected MDCK cells with increasing amounts of a Flag-Smad7 expression vector and a plasmid encoding an HA epitope-tagged version of JNK1. After 48 h, the transiently expressed HA-JNK1 was isolated by immunoprecipitation, its activity was measured using an immune complex kinase assay, and its expression level was determined by immunoblotting. Similar to the above observation in Mv1Lu cells, JNK activity was increased in a dose-dependent fashion by Smad7 overexpression (Fig. 1D). Anti-HA immunoblotting of total cell lysates confirmed that the observed increase in JNK activity was not because of high levels of HA-JNK1 expression in the Smad7-transfected cells (Fig. 1D). We also tested for this effect in transiently transfected COS-7 cells and found a similar activation by Smad7 (see Fig. 2, A and B, and see Fig. 3), which is consistent with the notion that the Smad7-dependent JNK activation can occur in multiple cell lines.

To determine whether the endogenous Smad7 protein might act as an activator of JNK, we attempted to reduce the level of endogenous Smad7 protein using an expression vector encod-
cells were exposed to ZnCl₂ (100 μM) and cell lysates were analyzed for Smad7 expression by immunoblotting using the anti-Flag M2 antibody. B, Mv1Lu-pMEP4 and Mv1Lu-S7 cells were treated for 24 h with ZnCl₂ (100 μM) and then exposed to TGF-β1 (10 ng/ml) for the indicated times. Cell lysates were immunoprecipitated with the polyclonal anti-JNK1 antibody (Santa Cruz Biotechnology), and immunoprecipitates were subjected to in vitro kinase assay using GST-Jun (1–79) as substrate. The phosphorylated proteins were resolved by SDS-polyacrylamide gel electrophoresis and visualized by autoradiography (top). Anti-JNK1 immunoblotting of whole cell extracts showed that similar amounts of JNK proteins were recovered in each sample (bottom). C, Mv1Lu-S7 cells were exposed to ZnCl₂ (100 μM) for the indicated times, and cell lysates were immunoprecipitated before being assayed for JNK activity (top). Expression of Flag-Smad7 (middle) or endogenous JNK (bottom) was monitored by direct immunoblotting. D, MDCK cells were transiently transfected with HA-JNK1 and increasing amounts of Flag-Smad7. Cell lysates were immunoprecipitated with anti-Flag (top) or anti-HA (bottom) antibodies. E, MDCK cells were transfected with HA-JNK1 and either an empty vector or a Flag-Smad7 expression vector, in the absence or presence of an antisense cDNA to Smad7 (AS.Smad7, bottom). The expression of transfected DNA was determined by immunoblotting total cell lysates with anti-Flag (middle) or anti-HA (bottom) antibodies. The phosphorylated proteins were resolved by SDS-polyacrylamide gel electrophoresis and visualized by autoradiography (top). Anti-JNK1 immunoblotting of whole cell extracts showed that similar amounts of JNK proteins were recovered in each sample (bottom). F, COS-7 cells were transfected with an expression vector containing the c-Jun activation domain (Fig. 1F). This stimulation is likely to depend on c-Jun phosphorylation, because overexpression of Smad7 failed to stimulate the activity of a GAL4-c-Jun (1–223; A63/73) fusion protein in which the serines that are phosphorylated by JNK were replaced by ala-
ines (Fig. 1F).

Three groups of MAPKs have been identified in mammalian cells, the extracellular signal-regulated kinases, referred to as p42/44 MAPKs, and the stress-activated protein kinases p38 and JNK. To test for the specificity of JNK activation by Smad7, COS-7 cells were transfected with an expression vector for Myc-Smad7, together with another vector encoding HA-JNK, HA-MAPK, or Flag-p38. The activity of immunopurified tagged MAPK was then measured by phosphorylation of GST-c-Jun, myelin basic protein, or GST-ATF2, respectively. In contrast to JNK, Smad7 failed to elevate MAPK and p38 activities, which were strongly stimulated by cotransfection of the constitutively active forms of Ras and MKK6, respectively (Fig. 2A).

To determine whether JNK activation was exclusively induced by Smad7, we tested for the effects of Smad2, Smad3, and Smad4. When transfected into COS-7 cells, Flag-Smad2, Flag-Smad3, and Flag-Smad4 were detectably expressed, as judged by their immunodetection with the anti-Flag M2 antibody. However, these Smad proteins failed to activate JNK, whereas Smad7 had a very potent effect under the same experimental conditions (Fig. 2B). The failure of these Smad proteins to activate JNK is in contrast to several previous observations demonstrating that the expression of Smad7 can

The efficient activation of the JNK cascade by Smad7 should result in stimulation of c-Jun transcriptional activity. Indeed, overexpression of Smad7 in MDCK cells stimulated the transcriptional activity of a GAL4-c-Jun (1–223) fusion protein containing the c-Jun activation domain (Fig. 1F). This stimulation is likely to depend on c-Jun phosphorylation, because overexpression of Smad7 failed to stimulate the activity of a GAL4-c-Jun (1–223; A63/73) fusion protein in which the serines that are phosphorylated by JNK were replaced by ala-
ines (Fig. 1F).
be increased by overexpression of Smad2, -3, and/or -4 (30–35). In several attempts, we were also unable to see an effect of overexpression of different combinations of Smad2, -3, and -4 on JNK enzymatic activity (data not shown). The apparent discrepancy between these findings and ours might be because of the sensitivity of the biological responses under study or could reflect the possibility that overexpression of these Smad proteins generates another signal that counteracts the effect of Smad7 on JNK activity. Identification of the mechanisms by which Smad7 activates JNK cascade will help clarify this issue.

One mechanism proposed to explain the inhibitory function of Smad7 in the TGF-β signaling pathway is based on the ability of Smad7 to bind the activated type I receptor, thereby preventing access and phosphorylation of receptor-regulated Smads. In contrast with its stimulatory effect on JNK, stable expression of Smad7 blocked the ability of TGF-β to induce growth arrest and expression of the endogenous PAI-1 gene (see Refs. 27 and 29, and data not shown), suggesting that Smad7 might stimulate JNK activity by a mechanism that is independent of its ability to inhibit TGF-β-mediated responses. To demonstrate this directly, we examined whether the expression of a nonfunctional truncated form of Smad7 (Smad7NCΔ), which no longer interacts with the TGF-β receptor (10), might be able to trigger JNK activation (Fig. 3). To test this possibility, we compared the effect of increasing amounts of expression vectors for Smad7 and Smad7NCΔ on JNK activity. Similar to overexpression of wild-type Smad7, overexpression of Smad7NCΔ consistently induced a marked increase in the in vitro phosphorylating activity of JNK (Fig. 3), thus providing direct evidence that Smad7 elevates JNK activity by a mechanism that is independent of its ability to interact with the TGF-β type I receptor and disruption of TGF-β signaling pathways. In support of this notion, we found that Smad6, which interacts stably with the TGF-β type I receptor to inhibit TGF-β signaling through Smad proteins (11), failed to stimulate JNK enzymatic activity (Fig. 3).

We have reported previously that the activation of the JNK cascade by constitutively activated mutants of MKK4 and MKK1 can suppress the ability of TGF-β to mediate gene expression by repressing Smad2 and Smad3 transcriptional activities (36, 37). To begin to investigate the role of the Smad7-dependent activation of JNK in TGF-β signaling, we examined whether the expression of a dominant-interfering mutant of MKK4 (MKK4.Ala), the kinase that phosphorylates and activates JNK, abrogated Smad7-induced JNK activation. As shown in Fig. 4A, expression of MKK4.Ala inhibited Smad7-dependent activation of JNK, indicating that Smad7 signaling was specifically blocked in the transfected cells. To test the hypothesis of a role for JNK activation in Smad7 inhibition of TGF-β signaling, we turned our attention on the luciferase CAGA reporter plasmid, which contains concatamizered CAGA elements shown previously to bind complexes of Smad3 and Smad4 and to be transactivated by both TGF-β and complex Smad3-Smad4 expression. As expected, TGF-β-induced expression of the reporter gene was markedly inhibited by Smad7 expression in HepG2 cells (Fig. 4B). Under these experimental conditions, we confirmed (36, 37) that expression of MKK4.Ala or a dominant-negative mutant of c-Jun (TAM67) increased the sensitivity of the cells to TGF-β (Fig. 4B). However, expression of MKK4.Ala or TAM67, together with Smad7, did not block the inhibitory effect of Smad7 even at the highest amounts of transfected cDNA (see Fig. 4B, and data not shown), suggesting that this inhibitory function of Smad7 is independent of JNK signaling pathway. A similar conclusion could be drawn when the ARE-Lux reporter, which contains three copies of the ARE from the Xenopus Mix.2 promoter linked to a basic TATA box and a luciferase reporter gene, was used in these analyses (Fig. 4C). This ARE is stimulated by TGF-β/activin signaling pathways, which induce assembly of a DNA-binding complex that is composed of the forkhead-containing DNA-binding protein Fast-1 and the complex Smad2-Smad4 (38).

In the course of these analyses, we also investigated the ability of a constitutively activated mutant of MKK4 (MKK4.ED) or wild-type c-Jun to modulate Smad7-dependent repression of TGF-β signaling. Transfection of HepG2 cells with the expression vectors encoding MKK4.ED or wild-type c-Jun, together with the reporters CAGA (Fig. 4D) or ARE-Lux (Fig. 4E), stimulated by themselves luciferase activity with an efficiency approaching that elicited by transfection of Smad7. Interestingly, MKK4.ED or wild-type c-Jun synergize with Smad7 to induce a strong suppression of TGF-β-induced luciferase activity, supporting the notion that Smad7 and JNK signaling are not acting through the same pathway to suppress TGF-β-dependent gene expression.

The JNK pathway has been widely studied in the context of stress activation and induction of programmed cell death. If Smad7-induced JNK activation leads to cell death, it would be expected that coexpression of the dominant inhibitory MKK4.Ala, which suppresses Smad7-dependent JNK activation, would also inhibit apoptosis induced by Smad7. To test this hypothesis, COS-7 cells were transiently transfected with...
an expression plasmid for GFP-Smad7, which allows specific detection of transfected cells by its green fluorescence. After 24 h, cells were transferred into medium containing 0.1% FCS and maintained in this medium for 24 h before being examined for apoptosis. Expression of GFP alone had no significant effect on cell viability, cell morphology, or nuclear structure. In clear contrast, COS-7 cells that express GFP-Smad7 fusion protein displayed morphological alterations that are characteristic of apoptotic cells, including cell shrinkage, chromatin condensation, and fragmentation of nucleus (Fig. 5A). Annexin V staining confirms that the death of COS-7 cells induced by overexpression of Smad7 occurred by an apoptotic mechanism (data not shown). Interestingly, coexpression of MKK4.Ala blocked the majority of Smad7-induced cell death, demonstrating that the JNK pathway is required for this process. To quantitate these results, cells were scored in a blinded manner as healthy or apoptotic by cell morphology. Over 35% of green cells arising from transfection of GFP-Smad7 showed morphological changes consistent with apoptosis, whereas only 8–10% of cells that had been cotransfected with GFP-Smad7 plasmid, in combination with MKK4.Ala, exhibited such a phenotype (Fig. 5B).

The transient transfection assays described above suggested that the apoptotic function of Smad7 was linked to its ability to promote JNK activation. To confirm these results, wild-type MDCK cells or MDCK cells overexpressing MKK4.Ala (39) were stably transfected with a Smad7 expression vector, and drug-resistant pools of transfected cells (Fig. 5C) were examined for the sensitivity to serum withdrawal-induced apoptosis. As expected from our previous studies, expression of Smad7 resulted in a marked increase in nuclear DNA fragmentation, and this effect was significantly lowered when the dominant-interfering mutant MKK4.Ala was cotransfected (Fig. 5D). To examine further the role of the JNK cascade in Smad7-mediated cell death, MDCK cells were cotransfected with a vector encoding a hygromycin resistance gene and maintained in 0.1% FCS medium for 48 h. Hygromycin-resistant colonies were visualized with crystal violet 10–13 days after transfection. As shown in Fig. 5E, expression of Smad7 reduced the number of viable MDCK cells by 80% or more, reinforcing the role of Smad7 as a positive mediator of cell death. This cytotoxic effect of Smad7 is lost in cells cotransfected with the dominant-interfering mutant MKK4.Ala, thereby providing strong evidence that Smad7 induces apoptotic cell death through activation of the JNK cascade.

We have reported previously that expression of Smad4, but not of Smad3, induces programmed cell death in MDCK cells (28, 40). However, coexpression of Smad3, along with Smad4, resulted in a dramatic decrease in the number of viable cells, suggesting that Smad4 and Smad3 may function in a cooperative way to induce cell death (28). Interestingly, expression of Smad3 and/or Smad4 under identical experimental conditions did not affect the activity of JNK (see Fig. 2B, and data not shown), which is consistent with the hypothesis that JNK cascade is not a component of the Smad3/Smad4-triggered signaling cascade resulting in apoptotic cell death.

To explore the possibility that Smad3/Smad4 could induce cell death via the induction of Smad7, we took advantage of the availability of the expression vector encoding the antisense cDNA to Smad7, which dramatically inhibits Smad7 protein synthesis (Fig. 1E). Using the clonal growth assay developed for our studies on Smad7 (Fig. 5E), we observed that expression of Smad3/Smad4 reduced the number of sur-
vival colonies by about 80%, consistent with our previous studies (28). In cells cotransfected with the antisense cDNA to Smad7, Smad3/Smad4 reduced the number of viable MDCK cells as strongly as it is in the absence of the Smad7 antisense (Fig. 5F), indicating that the Smad3/Smad4-induced apoptotic pathway is separate from the pathway that leads to induction of Smad7 expression.

The identification of Smad7 as essential downstream mediators of TGF-β transactivation responses has been a major advance in understanding how this cytokine exerts its biological effects. Upon phosphorylation on C-terminal serine residues by the activated type I receptor, Smad2 or Smad3 associate with Smad4, and the resulting complexes move into the nucleus to regulate expression of a large number of target genes, most of which remain to be identified. The Smad signaling pathway can be limited by Smad7, which is suggested to function by binding to the activated receptor complex to prevent phosphorylation of Smad2 and Smad3. Here, we report an additional and heretofore unexpected role for Smad7 in the activation of the JNK signaling pathway, which appears to play a critical role in mediating the apoptotic function of Smad7. At present, the molecular mechanism whereby Smad7 induces activation of the JNK cascade is unknown. One attractive possibility is that Smad7 might stimulate JNK activity by directly binding to an upstream kinase of the JNK cascade, such as MKK4, MEKK1, ASK1, or TGF-β-activated kinase (TAK1). However, we were unable to detect such an interaction using different techniques, including immunoprecipitation followed by Western blotting and in vitro binding assays with GST-Smad7 produced in bacteria (data not shown). Because association could be transient or unstable, these negative results do not definitively rule out the possibility of a direct interaction of Smad7 with a kinase of the JNK cascade. Therefore, the molecular framework that we proposed gives rise to a number of areas for future studies, including the identification of downstream targets of Smad7 in the biochemical route to JNK and the investigation of how these targets are regulated to initiate JNK activation and apoptotic cell death.

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