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Evidence for a Role of Rho-like GTPases and Stress-activated Protein Kinase/c-Jun N-terminal Kinase (SAPK/JNK) in Transforming Growth Factor β-mediated Signaling*

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Transforming growth factor β (TGF-β) is a multifunctional factor that induces a wide variety of cellular processes which affect growth and differentiation. TGF-β exerts its effects through a heteromeric complex between two transmembrane serine/threonine kinase receptors, the type I and type II receptors. However, the intracellular signaling pathways through which TGF-β receptors act to generate cellular responses remain largely undefined. Here, we report that TGF-β initiates a signaling cascade leading to stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) activation. Expression of dominant-interfering forms of various components of the SAPK/JNK signaling pathways including Rho-like GTPases, mitogen-activated protein kinase (MAPK) kinase kinase 1 (MEKK1), MAPK kinase 4 (MKK4), SAPK/JNK, and c-Jun abolishes TGF-β-mediated signaling. Therefore, the SAPK/JNK activation contributes to TGF-β signaling.

Members of the Ras superfamily of small GTPases play essential roles in the regulation of diverse cellular functions such as growth control, differentiation, vesicular transport, motility, and cytoskeletal organization (1–3). The Rho family of GTPases, which includes Rho, Rac, and CDC42, have been implicated in distinct dynamic processes involving the actin cytoskeleton: the formation of filopodia and lamellipodia by CDC42 and Rac, respectively, and the assembly of focal adhesions and stress fibers by Rho (3, 4). In addition to their effects on the actin cytoskeleton, Rho, Rac, and CDC42 also have a role in regulating cell proliferation, transcription, and cell transformation (5–10). Recently, downstream mediators linking Rho-like GTPase activation to nuclear events were identified (5–8). Rac1 and CDC42H were shown to play a critical role in activation of members of the mitogen-activated protein kinase (MAPK)[1] group, the stress-activated protein kinases (SAPKs also known as c-Jun N-terminal kinases (JNKs)), in response to growth factors, such as tumor necrosis factor-α or epidermal growth factor (6–8). The SAPK pathway involves sequential activation of MAPK kinase kinase (MEKK1), MAPK kinase 4 (MKK4), SAPK/JNK, and c-Jun (11–15).

Transforming growth factors β (TGFs-β) belong to a family of multifunctional cytokines that regulate cell proliferation, differentiation, motility, and extracellular matrix formation (16–19). TGF-β signals by simultaneously contacting two transmembrane serine/threonine kinases known as the type I and type II receptors (19–21). The type II receptor can directly bind TGF-β, but is incapable of mediating responses in the absence of a type I receptor (20, 21). Bound TGF-β is recognized by type I receptor, which is then phosphorylated by the receptor II kinase, thereby allowing propagation of the signal to downstream components (20, 21). To date, the postreceptor mechanisms of action of TGF-β and the TGF-β-related cytokines remains unresolved. Recently, TAK1, a potential component of both TGF-β and bone morphogenetic protein 4 (BMP4) signaling pathways, has been described as a member of the MAPKK kinase (MAPKKK) family (22). Thus, a MAPK cascade might be involved in signaling by TGF-β and TGF-β-related cytokines. In the current study, we provide strong evidence for the involvement of the JNK/SAPK pathway in TGF-β-mediated signaling. Furthermore, we demonstrate that Rho-like GTPase function is critical for the activation of gene expression by TGF-β.

EXPERIMENTAL PROCEDURES

Plasmids—pcDNA3CDC42H(QL), pcDNA3RhoA(QL), pcDNA3Rac1 (QL), pCEV29CDC42H(N17), pCEV29RhoA(N19), pCEV29Rac1(N17), and pcDNA3-HA-JNK1 were gifts from S. Gutkind. The p3TP-Lux reporter construct (a gift from Dr. Joan Massagué) contains three consecutive 12-*O*-tetradecanoylphorbol-13-acetate response elements, the plasminogen activator inhibitor (PAI-1) promoter, and a luciferase reporter gene. pXFTβR1R4(KR) and pXFTβRII(KR)15 were kindly provided by Dr. Rick Derynck. Expression plasmid for the dominant-negative mutant of Jun pCMVTAM67 was a gift from Dr. Michael Birrer. GST-Jun, the kinase-inactive MEKK1 mutant (pCMV5 MEKK1 (K432A)), and the dominant-interfering pcDNA3-Flag-MKK4 (Ala) and pcDNA3-Flag-JNK1 (Ala[183] and Phe[185]) mutants have been described previously (23–25). GST-Jun-(1–79) was expressed in *Escherichia coli* as described (13).

Cell Culture, Transfection, and Gene Expression Analysis—The hepatoma cells HepG2 were maintained in RPMI containing 10% heat-inactivated fetal calf serum (FCS), MDCK cells in RPMI containing 5% FCS, and CHO cells in RPMI containing 10% FCS. For gene expression analysis, cells were plated to semiconfluency and 24 h later transfected with expression vectors by the LipofectAMINE™ method (Life Technologies, Inc.). Cells were subsequently incubated in the presence or absence of human TGF-β1 (2 ng/ml) for 12 h. Extracts were then prepared and assayed for luciferase activity using the luciferase assay system described by the manufacturer (Promega). Light emission was measured during the initial 30 s of the reaction using a luminometer. The luciferase activities were normalized on the basis of CAT expression from pCAT-control vector (Promega) and protein content.

Protein Kinase Assay—For assaying JNK activity, cells were lysed at

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[1] The abbreviations used are: MAPK, mitogen-activated protein kinase; SAPK, stress-activated protein kinase; JNK, c-Jun N-terminal kinases; MEKK1, MAPK kinase kinase 1; MKK4, MAPK kinase 4; TGF-β, transforming growth factor-β; MAPKKK, MAPKK kinase; BMP4, bone morphogenetic protein 4; FCS, fetal calf serum; HA, hemagglutinin; GST, glutathione *S*-transferase; MOPS, 4-morpholinepropanesulfonic acid.

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4 °C in lysis buffer containing 25 mM HEPES (pH 7.5), 0.3 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol, 1% Triton X-100, 0.5% sodium deoxycholate, 20 mM β-glycerophosphate, 1 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride, 20 μg/ml aprotinin, and 20 μg/ml leupeptin, and lysates were clarified by centrifugation. Endogenous JNK was immunoprecipitated with polyclonal anti-JNK antibody (Santa Cruz). Immune complexes were collected by binding to protein G-Sepharose, washed extensively in lysis buffer, and resuspended in 30 μl of kinase reaction mixture containing 12.5 mM MOPS (pH 7.5), 12.5 mM β-glycerophosphate, 7.5 mM $MgCl_2$, 0.5 mM EGTA, 0.5 mM sodium fluoride, 0.5 mM sodium vanadate, 2 μg of GST-Jun, 20 μM unlabeled ATP, and 5 μCi of [γ-^{32}P]ATP. After incubation at 30 °C for 20 min, kinase reaction products were analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. The HA epitope-tagged JNK1 activity was also assayed by immunocomplex kinase assays after immunoprecipitation with the HA monoclonal antibody 12CA5.

Immunoblots—Proteins were separated on 10% SDS-polyacrylamide gels and electroblotted onto Hybond™ membranes (Amersham). After blocking, the membranes were probed with anti-JNK polyclonal antibody (Santa Cruz) or anti-HA (12CA5) as described elsewhere (26). The immunoblot was then developed using enhanced chemiluminescence detection according to the manufacturer's protocol (Amersham).

RESULTS AND DISCUSSION

We explored the possibility that the Rho family of GTPases are potential downstream effectors of TGF-β receptors because these proteins are involved in signaling to the nucleus leading to transcriptional activation (5–8). Members of this family function as binary switches by cycling between the active GTP-bound state and the inactive GDP-bound state. These GTPases can be activated through substitution of glutamine by a leucine residue in a position analogous to that of codon 61 of Ras. Such a mutation has been shown to inhibit the GTPase activity of most of these proteins (8, 27). To investigate whether Rho-like GTPases are intermediates in a TGF-β-initiated signaling pathway leading to transcriptional activation, we tested the ability of constitutively activated mutants of RhoA, Rac1, and CDC42Hs to signal transcriptional responses that are typical of TGF-β. A TGF-β reporter construct (p3TP-Lux) containing a luciferase gene controlled by a TGF-β-inducible promoter was used to monitor TGF-β-induced changes in gene expression in HepG2 cells (20, 21, 26). Transient transfection of p3TP-Lux into HepG2 cells resulted in a strong induction of luciferase activity in response to TGF-β1 (Fig. 1*A*). Cotransfection of expression plasmids encoding the constitutively activated small GTPases RhoA-QL, Rac1-QL, or CDC42H-QL stimulated by themselves luciferase activity with an efficiency approaching that of TGF-β stimulation in the case of Rac1-QL (Fig. 1*A*). Addition of TGF-β potentiated the responses of the reporter gene to activated Rho-related GTPases, although to a variable extent. Whereas CDC42H-QL did not enhance luciferase activity and activated RhoA caused only a very modest increase, overexpression of the activated form of Rac1 led to superinduction of luciferase activity (Fig. 1*A*). Cotransfection of increasing amounts of Rac1-QL expression plasmids potentiated TGF-β-induced reporter activity in a concentration-dependent manner (Fig. 1*B*). In a control experiment, expression of the dominant-negative forms of TGF-β types I (TβRI) and II (TβRII) receptors abolishes both control and TGF-β-induced transcriptional activation, indicating that this effect is specific to TGF-β (Fig. 1*A*).

To provide further evidence for the involvement of Rho-like GTPases in TGF-β signaling, we examined the effect of dominant-negative mutants of RhoA, Rac1, and CDC42H on transcriptional activation by TGF-β. Dominant-negative mutants were generated through substitution of threonine at position 17 to asparagine. The analogous mutation in p21*ras* increases its affinity for GDP. This results in sequestration of guanine nucleotide exchange factors making them unavailable for activation of endogenous p21*ras* and thereby blocking downstream signaling events (28). By analogy to the activity of N17Ras, the

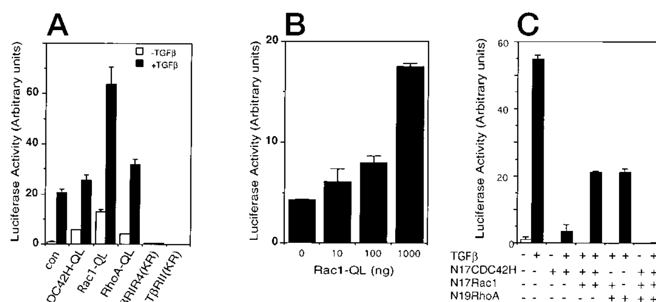

FIG. 1. **Effect of Rho family GTPases in TGF-β signaling.** HepG2 cells were transfected with p3TP-Lux (1 μg) together with the indicated expression plasmids and treated with TGF-β (2 ng/ml) in fresh medium containing 10% FCS. After 12 h, cells were harvested and assayed for luciferase activity. *A*, HepG2 cells were cotransfected with the control plasmid (*con*) or with plasmids encoding the indicated activated small G proteins (1 μg). As a control, cells were cotransfected with 1 μg of plasmids encoding dominant-negative mutants of TGF-β type I (*TβRIR4(KR)*) or type II (*TβRII(KR)*) receptors. *B*, dose-response effect of activated Rac1 on TGF-β-induced transcriptional activation. HepG2 cells were cotransfected with various amounts of Rac1-QL expression plasmids. DNA concentration was kept constant with pcDNA3. *C*, HepG2 cells were cotransfected with pcDNA3 (control) or with expression vectors encoding N19RhoA, N17Rac1, or N17CDC42H (1 μg) alone or in combination as indicated. Values represent luciferase activities relative to unstimulated cells transfected with vector and are shown as mean ± S.E. for representative experiments performed at least five times, with triplicates in each experiment.

mutants of CDC42H, Rac1, and RhoA have similarly been shown to function as dominant-negative molecules (6, 8).

As shown in Fig. 1*C*, TGF-β-induced transcription of the reporter gene was effectively inhibited by N17Rac1 expression. However, induction of transcription by TGF-β was also inhibited by expression of either N19RhoA or N17CDC42H (Fig. 1*C*). Hence, functional RhoA and CDC42H also appear to be required for TGF-β-mediated signaling, even though activated RhoA and CDC42H are not sufficient for activation (Fig. 1*A*). This situation is remarkably similar to that of the Ras-mediated signaling pathways. For example, activated Ras is not sufficient to activate serum response factor-linked signaling, yet expression of the dominant-interfering Ras derivative N17Ras inhibits activation of the serum response factor-linked pathway by extracellular stimuli that act through G protein-coupled receptors (5). Additional evidence that RhoA and CDC42H are components in the TGF-β signaling pathway is provided by our findings that overexpression of N19RhoA and N17CDC42H together with N17Rac1 abolishes completely transcriptional activation in response to TGF-β, whereas overexpression of N17Rac1 alone had a less inhibitory effect on reporter gene activity (Fig. 1*C*).

Together, these results provide strong evidence that RhoA, Rac, and CDC42H play an important role in TGF-β receptor signaling. However, our data differ significantly from that recently published by Mucsi *et al.* (29). They demonstrated that expression of the dominant-negative mutant of Rac, but not dominant-negative mutants of CDC42H and Rho, inhibited transcriptional activation by TGF-β in NIH3T3 cells. The apparent discrepancy between these findings and ours might be due to cell type differences or could reflect the possibility that receptor activation generates another signal that synergizes with RhoA and CDC42H to activate gene expression. In this context, recent studies have shown that RhoA, Rac1, and CDC42H signal to the nucleus in a cell type-specific manner (30). Furthermore, we have previously shown that TGF-β induces rapidly (2 min) the activity of a 78-kDa serine/threonine kinase p78 in HepG2 cells for which TGF-β can act as a growth-inhibitory factor (26). In contrast, there was no apparent in-

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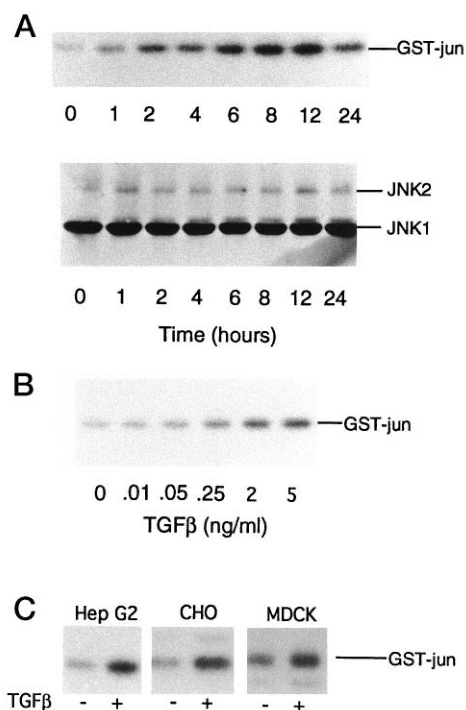

FIG. 2. **Activation of JNK by TGF-β.** *A*, *top*, HepG2 cells were exposed to TGF-β (5 ng/ml) for the indicated time. Cell lysates were immunoprecipitated with anti-JNK (Santa Cruz), and immunoprecipitates were subjected to *in vitro* kinase assay using GST-Jun-(1–79) as substrate. The phosphorylated proteins were resolved by SDS-polyacrylamide gel electrophoresis and visualized by autoradiography. *Bottom*, anti-JNK immunoblotting of whole cell extracts showed that similar amounts of JNK1 and JNK2 proteins were recovered in each sample. Results are representative of at least five experiments. *B*, HepG2 cells were treated with the indicated concentrations of TGF-β for 12 h, and endogenous JNK was immunoprecipitated and assayed for kinase activity as described in *A*. *C*, HepG2, MDCK, and CHO cells were exposed to TGF-β (5 ng/ml) for 12 h, and endogenous JNK was immunoprecipitated and assayed for kinase activity. The phosphorylated GST-Jun-(1–79) is indicated.

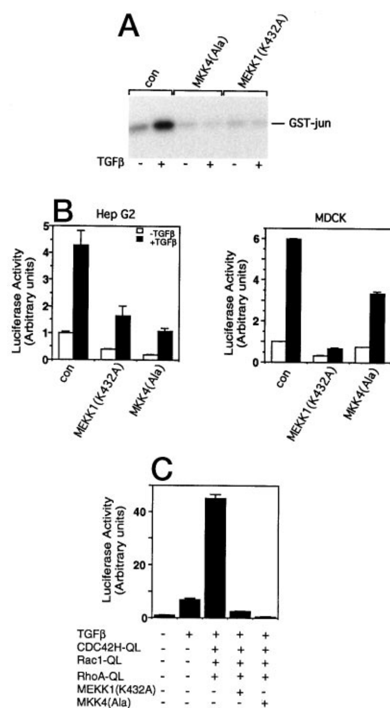

FIG. 3. **Dominant-negative mutants of MEKK1 and MKK4 abrogate TGF-β-induced transcriptional activation.** *A*, HepG2 cells were transfected with pcDNA3-HA-JNK1 (2 μg) together with an empty expression vector (*con*) or expression vectors carrying cDNAs for MEKK1(K432A) or MKK4(Ala) (2 μg per plate in each case). After 36 h, cells were treated with TGF-β1 for 12 h prior to harvesting. Lysates were immunoprecipitated with monoclonal antibody 12CA5 and assayed for JNK activity. *B*, HepG2 (*left panel*) or MDCK (*right panel*) cells were transfected with 1 μg of p3TP-Lux together with 1 μg of either an empty expression vector (*con*) or an expression vector encoding MEKK1(K432A) or MKK4(Ala). Cells were then incubated in the presence or absence of TGF-β (2 ng/ml) for 12 h, and cell lysates were assayed for luciferase activity. *C*, HepG2 cells were transfected with 1 μg of p3TP-Lux together with the indicated expression vector or empty vector (1 μg/plate in each case). Cells were treated with TGF-β, and cell extracts were processed as in *B*.

duction of p78 activity when the assay was done with NIH3T3 cells, a cell line that failed to undergo growth arrest in response to TGF-β. Identification of the mechanisms by which Rho, Rac, and CDC42H pathways link TGF-β-receptor activation to nuclear events would help clarify this issue.

Recently, Rac and CDC42H have been shown to activate the JNK signaling pathway leading to c-Jun transcriptional activation (6, 8). To determine whether SAPK/JNKs are activated in response to TGF-β, we tested SAPK/JNK activity in an immune-complex kinase assay using GST-Jun-(1–79) as substrate (6, 8). Fig. 2*A* shows that exposure of HepG2 to TGF-β caused a marked and persistent increase in SAPK/JNK activity, with maximal activation at 12 h. The effect of TGF-β on SAPK/JNK activity was dose-dependent (Fig. 2*B*). In comparison with the immediate and transient JNK activation induced by other stimuli such as tumor necrosis factor-α (5 min) and anisomycin (6, 8, and data not shown), we did not detect a significant increase of GST-Jun phosphorylation before 1 h. However, the time course of SAPK/JNK activation by TGF-β is similar to that induced by radiation (15, 31). Furthermore, using HepG2 cells transiently transfected with the hemagglutinin (HA)-tagged SAPK/JNK1 (p46) and SAPK/JNK2 (p54), the two forms recognized by the antibody used to detect the endogenous SAPK/JNKs (Fig. 2*A*), we found that TGF-β increased both SAPK/JNK1 and SAPK/JNK2 activities (data not shown). Identical results were obtained with the MDCK and CHO cell lines, suggesting similarities in stimulus-response coupling mechanisms of TGF-β receptors (Fig. 2*C*). In contrast, recent studies using NIH3T3 cells have shown that TGF-β stimulation did not significantly induce the activation of SAPK/JNK at any time up to 3 h, although the addition of TGF-β to these cells led to the increase in MAPK activity under these experimental conditions (29). This may simply represent a difference in the delay of JNK induction by TGF-β in NIH3T3 cells. Consistent with this hypothesis, JNK activation did not occur until 4 to 6 h after exposure of MDCK cells to TGF-β (data not shown). The activation of SAPK/JNK indicated by our observations agrees with recent studies showing that TGF-β activates a novel MAPKKK, known as TAK1, that may be involved in signal transduction by members of the TGF-β superfamily. Activated TAK1 phosphorylates and promotes activation of MKK4 (also termed SEK1 or JNKK), the kinase that controls activation of SAPK/JNK (22). Whether TAK1 is downstream of the TGF-β receptor in the biochemical route to JNK warrants further investigation.

To test the hypothesis that SAPK/JNK activation may contribute to the induction of gene expression by TGF-β, we examined the ability of the dominant-interfering mutants of MEKK1 and MKK4 to block SAPK/JNK activation and inhibit transcriptional responses following exposure of cells to TGF-β. Expression of either MEKK1(K432A) or MKK4(Ala) inhibited activation of co-transfected HA-tagged SAPK/JNK1 in response to TGF-β, indicating that TGF-β signaling was specifically blocked in the transfected cells (Fig. 3*A*). Interestingly, the dominant-negative MEKK1 and MKK4 mutants had sim-

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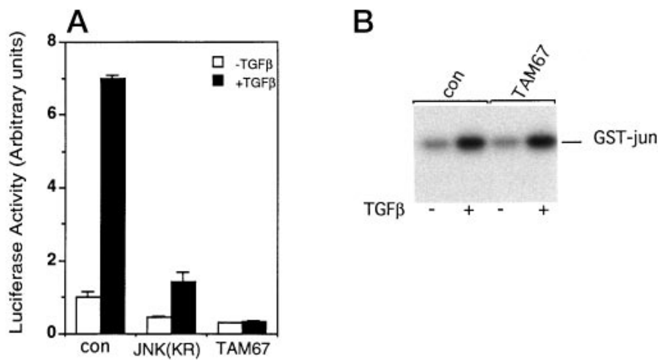

FIG. 4. **Effects of dominant-negative mutants of JNK1 and c-Jun on TGF-β-activated transcription.** *A*, HepG2 cells were transfected with 1 μg of p3TP-Lux together with 1 μg of either an empty expression vector (*con*) or an expression vector encoding JNK1(Ala^{183} and Phe^{185}) or c-JunTAM67. Cells were then treated with TGF-β as for Fig. 1, and cell lysates were assayed for luciferase activity. *B*, HepG2 cells were transfected with 2 μg of pcDNA3-HA-JNK1 together with 2 μg of either empty pCMV5 expression vector (*con*) or expression vector for TAM67 (2 μg per plate in each case). After 36 h, cells were treated with TGF-β1 for 12 h and processed for immunocomplex JNK assays.

ilar effects on TGF-β-mediated transcriptional activation (Fig. 3*B*), which is consistent with the hypothesis that activation of MEKK1-MKK4-SAPK/JNK signaling pathway plays a central role in mediating these transcriptional processes. Identical results were obtained with the MDCK cells indicating that the inhibitory effect of dominant-negative mutants of MEKK1 and MKK4 on transcriptional activation by TGF-β occurs in multiple cell types (Fig. 3*B*). Expression of MEKK1(K432A) or MKK4(Ala) is also sufficient to block the superinduction of the gene reporter activity by TGF-β in cells coexpressing the constitutively activated forms of RhoA, Rac1, and CDC42H (Fig. 3*C*). Taken together, these results support a model in which the SAPK/JNK cascade participates in a signaling pathway activated by TGF-β receptors through Rho family GTPases.

To further demonstrate a role of the SAPK/JNK cascade on transcriptional activation by TGF-β, we made the use of dominant-interfering mutants of SAPK/JNK1 and c-Jun. c-JunTAM67 acts as a dominant-interfering mutant because a deletion in the N-terminal transactivation domain of c-Jun that includes the binding site for SAPK/JNK1 (31, 32). As expected, expression of both dominant-negative mutants of SAPK/JNK1 (JNK1(Ala^{183} and Phe^{185})) and c-Jun (TAM67) inhibited the activation of the reporter gene by TGF-β (Fig. 4*A*). In a dose-response experiment, low levels (0.01 μg) of TAM67 construct were sufficient to inhibit TGF-β-induced gene expression significantly, and high levels (1 μg) completely blocked activation (data not shown). In contrast, overexpression of TAM67 had no effect on TGF-β-mediated HA-JNK activation (Fig. 4*B*). From these results, it is evident that inhibition of transcriptional responses to TGF-β occurs at a level downstream of SAPK/JNK and that c-Jun plays an important role in TGF-β signaling. In support of this interpretation are data showing that TGF-β may up-regulate the expression of c-Jun product (33). Moreover, SAPK/JNKs are thought to be responsible for phosphorylating the transactivating domain of c-Jun protein *in vivo*, and, in turn, phosphorylated c-Jun homodimers have potent AP-1 activity and can control the expression of a number of genes, including c-*jun* itself (34). We conclude that SAPK/JNK signaling pathways contribute to the intracellular relay of transcriptional signals originating from the TGF-β receptors.

Our present studies provide the first demonstration of the involvement of Rho and CDC42H in TGF-β-mediated signaling pathways and indicate a critical role of the SAPK/JNK cascade in delivering a signal to the nucleus leading to transcription activation. This signaling pathway may account for part of the genetic response of cells to TGF-β. In addition, this pathway may contribute to, or cooperate with, the activation of the MAD transcription factor by the TGF-β-related factor BMP-2 (19, 35–37). Our findings raise questions concerning the role of Rho-like GTPases and the SAPK/JNK signaling pathway in the biological actions of TGF-β (16–19, 26, 38). Substantial evidence has been accumulated demonstrating that Rho-like GTPases and the SAPK/JNK cascades play essential roles in the regulation of multiple physiological processes, including cell growth control, cell death, cell motility, embryonic morphogenesis, and regulation of the cytoskeleton (3, 5–8, 25, 39, 40). The identification of Rho family GTPases and the SAPK/JNK cascade as essential components in the TGF-β signaling pathway provide new insight into the mechanism by which TGF-β mediates its biological actions.

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