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*

(Received for publication, November 6, 1996)

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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 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) 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 factor β (TGF-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 morphogenenic protein 4 (BMP4) signaling pathways, has been described as a member of the MAPKK kinase (MAP3K4) 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—pCNA3CD42H(QL), pCNA3RhoA(QL), pCNA3Rac1 (QL), pCEV29CD42H(N17), pCEV29RhoA(N19), pCEV29Rac1(N17), and pCNA3-HA-JNK1 were gifts from S. Gutkind. The pB-P-Luc reporter construct (a gift from Dr. Joan Massague) contains a constitutive 12-O-tetradecanoylphorbol-13-acetate response elements, the plasminogen activator inhibitor (PAI-1) promoter, and a luciferase reporter gene. pXFTβRII(KR) and pXFTβRII(KR) were kindly provided by Dr. R. Derynck. Expression plasmid for the dominant-negative mutant of Jun pCMV4M6 was a gift from Dr. Michael Birrer. GST-Jun, the kinase-inactive MEKK1 mutant (pCMV5 MEKK1(R410A)) and the dominant-interfering pCNA3-Flag-MEK4(1-194) and pCNA3-Flag-JNK1 (Ala183 and Phe185) 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 CHOL cells in RPMI containing 10% FCS. For gene expression analysis, cells were plated to semiconfluent 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

1The abbreviations used are: MAPK, mitogen-activated protein kinase; SAPK, stress-activated protein kinase; JNK, c-Jun N-terminal kinase; MEKK1, MAPK kinase 1; MKK4, MAPK kinase 4; TGF-β, transforming growth factor-β; MAPKKK, MAPK kinase; BMP4, bone morphogenetic protein 4; FCS, fetal calf serum; HA, hemagglutinin; GST, glutathione S-transferase; MOPS, 4-morpholino-propanesulfonic acid.
GTPases in TGF-β signaling events (28). By analogy to the activity of N17 Ras, the HepG2 cells (20, 21, 26). Transient transfection of p3TP-Lux overexpression of the activated form of Rac1 led to superinduction of the gene to activated Rho-related GTPases, although to a variable amount. 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-β (Fig. 1A). 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. 1A). 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. 1A). Cotransfection of increasing amounts of Rac1-QL expression plasmids potentiated TGF-β-induced reporter activity in a concentration-dependent manner (Fig. 1B). 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. 1A).

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 p21ras increases its affinity for GDP. This results in sequestration of guanine nucleotide exchange factors making them unavailable for activation of endogenous p21ras and thereby blocking downstream signaling events (28). By analogy to the activity of N17Ras, the mutants of CDC42H, Rac1, and Rhoa have similarly been shown to function as dominant-negative molecules (6, 8).

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-β (Fig. 1A). 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. 1A). 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. 1A). Cotransfection of increasing amounts of Rac1-QL expression plasmids potentiated TGF-β-induced reporter activity in a concentration-dependent manner (Fig. 1B). 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. 1A).

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As shown in Fig. 1C, 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. 1C). 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. 1A). This situation is remarkably similar to that of the Ras-mediated signaling pathways. For example, activated Ras is not sufficient to activate reporter gene transcription. 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. 1C).

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-
and CDC42H pathways link TGF-β receptors to 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 transfected 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.

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 transfected 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.

Recently, Rac and CDC42H pathways link TGF-β-receptor activation to nuclear events would help clarify this issue.

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. 3A). Interestingly, the dominant-negative MEKK1 and MKK4 mutants had sim-
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 cascade 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.

REFERENCES

1. Bokoch, G. M., and Der, C. J. (1993) FASEB J. 7, 750–759
2. Downward, J. (1995) Trends Biochem. Sci. 15, 469–471
3. Hall, A. (1995) Opin. Cell Biol. 5, 265–268
4. Chant, J., and Stowers, L. (1995) Cell 81, 1–4
5. Hill, C. S., Wynne, J., and Treisman, R. (1995) Cell 81, 1159–1170
6. Minden, A., Lin, A., Clarlet, F. G., Abo, A., and Karin, M. (1995) Cell 81, 1147–1157
7. Vojtech, A., and Cooper, J. A. (1995) Cell 82, 527–529
8. Coo, O. A., Chiarriello, M., Y., J.-C., Teramoto, H., Crespo, P. X., Miki, T., and Gutkind, J. C. (1995) Cell 81, 1317–1346
9. Qiu, R. G., Chen, J., Kirk, D., McCormick, F., and Symons, M. (1995) Nature 374, 457–459
10. Olson, M. F., Awoodor, A., and Hall, A. (1996) Science 269, 1207–1212
11. Derijard, B., Raineaud, J., Barrett, T., Wu, I.-H., Han, J., Ulevitch, R. J., and Davis, R. J. (1995) Science 267, 682–685
12. Sanchez, I., Hughes, R. T., Mayer, B. J., Yen, K., Woodgett, J. R., Kyriakis, J. M., and Zon, L. I. (1994) Nature 372, 794–798
13. Minden, A., Lin, A., McMahon, M., Lange-Carter, C., Derijard, B., Davis, R. J., and Karin, M. (1994) Science 266, 1719–1723
14. Yan, M., Tai, X., Deak, J. C., Kyriakis, J. M., Zon, L. I., Woodgett, J. R., and Templeton, D. J. (1994) Nature 372, 798–800
15. Whitemarsh, A. J., and Davis, R. J. (1996) J. Mol. Med., in press
16. Massagué, J., Attisano, L., and Warna, J. L. (1994) Trends Cell Biol. 4, 172–178
17. Kolesnick, R. (1996) Cell 85, 875–885
18. Atfi, A., Lepage, K., Allard, P., Chapdelaine, A., and Chevalier, S. (1995) FASEB J. 9, 657–663
19. Chou, M. M., and Blenis, J. (1996) Cell 85, 489–500
20. Verheij, M. T., Bose, R., Lin, X. H., Yao, B., Jarvis, W. D., Grant, S., Bierer, M. J., Szabo, E., Zon, L. I., Kyriakis, J. M., Haimovitz-Friedman, A., Fuks, Z., and Kolesnick, R. (1996) Nature 380, 75–79