Activation of the Hematopoietic Progenitor Kinase-1 (HPK1)-dependent, Stress-activated c-Jun N-terminal Kinase (JNK) Pathway by Transforming Growth Factor β (TGF-β)-activated Kinase (TAK1), a Kinase Mediator of TGF β Signal Transduction*

(Received for publication, June 17, 1997)

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Transforming growth factor β (TGF-β)-activated kinase (TAK1) is known for its involvement in TGF-β signaling and its ability to activate the p38 mitogen-activated protein kinase (MAPK) pathway. This report shows that TAK1 is also a strong activator of c-Jun N-terminal kinase (JNK). Both the wild-type and a constitutively active mutant of TAK1 stimulated JNK in transient transfection assays. Mitogen-activated protein kinase kinase 4 (MKK4)/stress-activated protein kinase/extracellular signal-regulated kinase (SEK1), a dual-specificity kinase that phosphorylates and activates JNK, synergized with TAK1 in activating JNK. Conversely, a dominant-negative MKK4/SEK1 mutant inhibited TAK1-induced JNK activation. A kinase-defective mutant of TAK1 effectively suppressed hematopoietic progenitor kinase-1 (HPK1)-induced JNK activity but had little effect on germinal center kinase activation of JNK. There are two additional MAPK kinase kinases, MEKK1 and mixed lineage kinase 3 (MLK3), that are also downstream of HPK1 and upstream of MKK4/SEK1 mutant. However, because the dominant-negative mutants of MEKK1 and MLK3 did not inhibit TAK1-induced JNK activity, we conclude that activation of JNK1 by TAK1 is independent of MEKK1 and MLK3. In addition to TAK1, TGF-β also stimulates JNK activity. Taken together, these results identify TAK1 as a regulator in the HPK1 → TAK1 → MKK4/SEK1 → JNK kinase cascade and indicate the involvement of JNK in the TGF-β signaling pathway. Our results also suggest the potential roles of TAK1 not only in the TGF-β pathway but also in the other HPK1/JNK-mediated pathways.

To date, three related mitogen-activated protein kinase (MAPK) cascades that propagate signals from the plasma membrane to the interior of mammalian cells have been identified (1). These three groups of the MAP kinase family include the extracellular signal-regulated kinases (ERKs) (2), p38 MAPKs (3), and the c-Jun N-terminal kinases (JNKs), also known as the stress-activated protein kinases (SAPKs) (4, 5). The ERKs are the central elements in mitogenic signal transduction downstream of Ras (2). p38 MAPKs seem to participate in the signaling pathways of pro-inflammatory cytokines and environmental stresses, such as interleukin-1, tumor necrosis factor α, and osmotic shock (1, 3). JNKs/stress-activated protein kinases play a crucial role in the responses stimulated by pro-inflammatory cytokines, environmental stresses, and apoptotic agents (4–7).

Considerable progress has been achieved in identifying the upstream kinases of the JNK cascade. JNK can be phosphorylated and activated by its immediate upstream kinase MAPK kinase 1 (MEK1) which itself can be phosphorylated and activated by MEK1 (8, 9). Together with the recent findings from our laboratory that hematopoietic progenitor kinase-1 (HPK1) directly binds to and phosphorylates MEKK1, a simple JNK pathway paradigm can be drawn: HPK1 → MEKK1 → MAPK kinase 4/SEK1 → JNK (10). However, the JNK cascade, like other MAPK cascades, is complicated by the fact that several other kinases such as germinal center kinase (GCK) (11), MLK3 (12), p21-activated protein kinase (PAK) (13), and Tpl-2 (14) are capable of activating this pathway. In addition, some of the kinases are not involved exclusively in the JNK pathway such as SEK1 which also activates p38 MAPK (15). The specificities of various upstream kinases involved in activating JNK in different physiological processes remain unclear.

TAK1 was cloned and identified recently as a member of the MAP kinase kinase family (16). The TAK1 kinase activity can be activated by TGF-β but not by epidermal growth factor, indicating that TAK1 is involved in the TGF-β signaling pathway. In addition, TAK1 activates p38 MAPK, partly through SEK1 (16, 17). Since SEK1 is also an activator of JNK, we investigated whether TAK1 can induce JNK. In this study, we found that TAK1 was a potent activator of the JNK pathway and that JNK activation by TAK1 was through MAPK kinase 4/SEK1. A dominant-negative TAK1 mutant blocked the JNK activation by HPK1 but not by GCK. Like TAK1, two other MAP kinase kinase kinases, MEKK1 and MLK3, act downstream of HPK1 and upstream of MAPK kinase 4/SEK1. However, we found that the dominant-negative mutants of MEKK1 center kinase; SEK, stress-activated protein kinase/extracellular signal-regulated kinase; ERK, extracellular signal-regulated kinase; GST, glutathione S-transferase; HA, hemagglutinin; MOPS, 4-morpholinepropanesulfonic acid; PAGE, polyacrylamide gel electrophoresis.
and MLK3 did not inhibit TAK1-induced JNK activity, indicating that the activation of JNK1 by TAK1 is independent of MEK1 and MLK3. Finally, we also found that TGF-β stimulated JNK kinase activity. Altogether, these results identify TAK1 as a regulator in the HPK1 → TAK1 → MAPK kinase 4/SEK1 → JNK pathway and indicate the involvement of JNK in the TGF-β signaling pathway.

MATERIALS AND METHODS

Cells, Transfections, Antibodies, and Reagents—293T cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum and 1 × penicillin/streptomycin. 293T cells were plated at a density of 2 × 10^5 cells/100-mm dish (or 1.5 × 10^5 cells/35-mm plate as indicated) and transfected the next day using the calcium phosphate precipitation protocol (Specialty Media, Inc.).

The amounts of DNA used for transfection were as indicated, and the empty vectors were used to achieve equal amounts of DNA in each transfection. Polyethylen glycol rabbit anti-JNK1 antibody Ab101 was described previously (6). Monoclonal antibody against HA epitope, HA.11, was purchased from BABCO. TGF-β was purchased from R&D Systems, and phosphorl 12-myristate 13-acetate and ionomycin were purchased from Sigma.

Plasmids and Fusion Proteins—pCl-HPK1, pCl-HPK(M46), pCl-JNK1, pUna3-FL-MEKK-KR, pEBG-SEK1, pEBG-SEK1-AL, and GST-Jun(1–79) fusion constructs were described previously (10). pEF-TAK1, pEF-TAK1N, and pEF-TAK1-K63W were kindly given by Dr. K. Matsumoto (Nagoya University, Japan). The plasmid pCMV-BL44 containing the coding region of GC kinase was a gift from Dr. J. Kohl (National Institutes of Health, Bethesda, MD). pEBG-MLK3 and pEBG-MLK3-KE were kindly given by Dr. N. Lassam (University of Toronto, Canada). GST-ATP2 and pCEV-HA-ERK2 were kindly provided by Dr. J. S. Gutkind (National Institutes of Health, Bethesda, MD).

Immunocomplex Kinase Assay and Western Blot Analysis—Immunocomplex kinase assays were carried out as described previously (6). MAP kinases were precipitated by incubation with specific antibodies and protein A-agarose beads (Bio-Rad) in incubation buffer (20 mM HEPES (pH 7.4), 2 mM EGTA, 50 mM glycerophosphate, 1% Triton X-100, 10% glycerol, 1 mM dithiothreitol, 2 μg/ml leupeptin, 5 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na3VO4). The precipitates were washed twice with incubation buffer, twice with LiCl buffer (500 mM LiCl, 100 mM Tris-Cl (pH 7.6), 0.1% Triton X-100, 10% glycerol, 1 mM dithiothreitol, 2 μM ATP, and 10 μM MgCl2, 1 mM dithiothreitol, 0.1% Triton X-100, and 1 mM Na3VO4) and then mixed with 5 μg of the indicated substrates, 15 μM ATP, and 10 μM of γ[32P]ATP in 30 μl of kinase buffer. The kinase reaction was performed at 37 °C for 30 min and terminated with an equal volume of SDS sampling buffer. The reaction mixtures were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis. The Western blot analysis, antibody incubation, and ECL detection were described previously (10).

RESULTS

TAK1 Activates JNK and p38-MAPK Activity—To examine whether TAK1 can activate JNK activity, transient transfections of 293T cells with JNK1 and different forms of TAK1 were performed. Subsequently, JNK activity was analyzed using the immunocomplex kinase assay with anti-JNK1 antibody. Cotransfection of wild-type full-length TAK1 with JNK1 cDNA resulted in a 10–20-fold increase of JNK activity compared with that of JNK1 cDNA transfected alone (Fig. 1A, lanes 1 and 2). A similar fold increase of JNK activity was also found in the cotransfection of JNK1 with the constitutively activated TAK1 mutant (TAK1-ΔN) which lacks 22 amino acids at the N terminus (Fig. 1A, lane 3), as a control, cotransfection of the TAK1 kinase-defective mutant (TAK1-K63W) with JNK1 did not increase JNK activity (Fig. 1A, lane 4). This result indicates that TAK1 kinase activity is necessary for JNK1 activation. The HA-tagged TAK1, immunoprecipitated by anti-HA antibody, did not phosphorylate GST-Jun (Fig. 1B, lanes 3 and 4). This result indicates that the detected JNK induction was not due to an associated Jun kinase activity immunoprecipitated by anti-HA antibody. Consistent with a previous report (17), cotransfection of TAK1-ΔN with p38-MAPK resulted in a 3–10-fold p38-MAPK activity increase. In addition, we found that wild-type TAK1 also activated p38-MAPK activity (Fig. 2A). As for ERK2, neither TAK1-ΔN nor TAK1 can activate ERK2; whereas a positive control, HA-tagged ERK2-transfected cells treated with phosphorl 12-myristate 13-acetate and ionomycin, did show ERK2 activity increase (Fig. 2B). To demonstrate equivalent JNK, p38-MAPK, and ERK2 expression, an equal amount of each cell lysate was resolved by SDS-PAGE and immunoblotted with anti-JNK1 and anti-HA antibodies (Figs. 1 and 2, bottom panels).

TAK1 Activates JNK Activity through SEK1—TAK1 activates p38-MAPK activity through MAPK kinase 4/SEK1 by a coupled kinase assay (16). Besides activating p38-MAPK, SEK1 also activates JNK directly. Hence, we tested whether JNK activation by TAK1 is also through SEK1. Consistent with a previous report (8), SEK1 coexpression with JNK1 led to a marginal JNK activity increase (Fig. 3, lane 2). However, the ability of TAK1 to activate JNK was much stronger than that of SEK1 (lane 1). When SEK1 and TAK1 cotransfected with JNK1, a synergistic effect on JNK activation was observed (lane 3). As for ERK2, neither TAK1 nor TAK1-ΔN could activate ERK2; whereas a positive control, HA-tagged ERK2-transfected cells treated with phosphorl 12-myristate 13-acetate and ionomycin, did show ERK2 activity increase (Fig. 2B). To demonstrate equivalent JNK, p38-MAPK, and ERK2 expression, an equal amount of each cell lysate was resolved by SDS-PAGE and immunoblotted with anti-JNK1 and anti-HA antibodies (Figs. 1 and 2, bottom panels).

Activation of JNK by TAK1 Is Independent of MEKK1 and MLK3—At the MAP kinase kinase kinase level, there are several other kinases such as MEKK1 (8, 9), MLK3 (12), MAPK upstream kinase (MUK) (18), and tumor progression locus 2 (Tpl-2) (14) which also activate JNK. Like TAK1, both MEKK1 and MLK3 were activated by TAK1.
and MLK3 also activate JNK through SEK1. Hence, we investigated if activation of JNK by TAK1 is independent of other kinases such as MLK3 and MEKK1, or if TAK1 is either upstream or downstream of those kinases. To address this issue, transient transfections of 293T cells with different kinase combinations and subsequent immunocomplex JNK kinase assays were performed. When MLK3-KN and MEKK1-KR, two dominant-negative mutants of MLK3 and MEKK1, respectively, were cotransfected with TAK1 and JNK1, we found that the JNK activation by TAK1 was not significantly affected (Fig. 4, lanes 4–6). Furthermore, TAK1-K63W, a dominant-negative mutant of TAK1, was unable to block the activation of JNK by MLK3 (Fig. 4, lanes 2 and 3). A Western blot probed with anti-JNK1 antibody was performed to normalize the expression levels of the JNK protein (Fig. 4, bottom). The results indicate that JNK activation by TAK1 is independent of MLK3 and MEKK1.

TAK1 Mutant TAK1-K63W Can Block Activation of JNK by HPK1 but Not by GCK—A group of mammalian homologs of yeast Ste20 Ser/Thr kinases, including the p21-activated protein kinase (PAK) family (13), HPK1 (10, 19), and GCK (11) were recently shown to activate the JNK pathway. Human HPK1 is immediately upstream of MEKK1 because HPK1 interacts with and phosphorylates MEKK1. Also, HPK1 activation of JNK is blocked by the dominant-negative mutant, MEKK1-KR (10). To test whether TAK1 has any role in the HPK1-JNK or GCK-JNK pathway, transfections and immunocomplex JNK kinase assays were performed. To our surprise, when TAK1-K63W was cotransfected with HPK1 and JNK1, the JNK activation was effectively blocked (Fig. 5, lanes 2 and 6), whereas TAK1-K63W had little effect on the JNK activation by GCK (Fig. 5, lanes 2 and 4). This result indicates that TAK1 is involved in the HPK1-JNK pathway rather than in the GCK-JNK pathway. When HPK kinase-defective mutant HPK(M46) was cotransfected with TAK1 and JNK1, the JNK activation by TAK1 did not change (Fig. 5A, lanes 3 and 5), indicating that HPK1 is not downstream of TAK1 in the JNK kinase cascade. A Western blot of equal amounts of cell lysates using anti-JNK1 antibody was also performed to normalize the expression levels of the JNK protein (Fig. 5, bottom panels). Because TAK1 alone potently activated JNK1, we did not detect any significant synergistic JNK induction after the addition of HPK1 to TAK1 plus JNK1 (Fig. 5A, lane 4). Taken together, these results indicate that the ability of TAK1 to activate JNK1 is due to the participation of endogenous HPK1 or other TAK1-activating kinases and that TAK1 acts downstream of HPK1.

TGF-β Stimulates JNK Kinase Activity—Because TGF-β stimulates TAK1 kinase activity, we investigated whether TGF-β also stimulates JNK kinase activity. 293T cells were treated with 5 ng/ml TGF-β for various durations; cell lysates were prepared and subjected to immunocomplex kinase assays using anti-JNK antibody and GST-cJun as a substrate. We found that TGF-β indeed stimulated JNK kinase activity; the JNK kinase activity peaked at 12-h time point and slowly declined (Fig. 6). We also observed similar JNK induction by...
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**FIG. 5.** TAK1-K63W can suppress the JNK activation by HPK1 but not by GCK. A, 293T cells (1.5 × 10^7/35-mm plate) were cotransfected with JNK1 alone (lane 1), HPK1 plus JNK1 (lane 2), TAK1 plus JNK1 (lane 3), HPK1 plus TAK1 and JNK1 (lane 4), HPK1-M46 plus TAK1 and JNK1 (lane 5), as well as HPK1 plus TAK1-K63W and JNK1 (lane 6). 1 μg of each kinase DNA, except TAK1 (0.5 μg), was used. B, 293T cells were transfected with JNK1 (lane 1), GCK plus JNK1 (lane 2), GCK plus TAK1 and JNK1 (lane 3), and GCK plus TAK1-K63W and JNK1 (lane 4). 10 μg of each kinase plasmid were used. Empty vectors were used to normalize the total amount of transfected DNA. 48 h posttransfection, immunocomplex kinase assays were performed using anti-JNK1 antibody and GST-Jun (1–79) as a substrate. As a control for JNK1 expression, an equal amount of each cell lysate was resolved by 10% SDS-PAGE and immunoblotted with anti-JNK1 antibody (bottom panels).

**FIG. 6.** TGF-β stimulates JNK kinase activity in 293T cells. 293T cells (2.5 × 10^6) were plated into 6-well plates and 24 h later treated with TGF-β (5 ng/ml) for the indicated time periods. Cell lysates were immunoprecipitated with anti-JNK1 antibody (Ab101). Immunocomplex kinase assays were performed with GST-Jun (1–79) as a substrate. The phosphorylated proteins were resolved by SDS-PAGE and visualized by autoradiography.

TGF-β in breast cancer MCF-7 cells and lung carcinoma A549 cells (data not shown).

**DISCUSSION**

Here we demonstrate that TAK1, a TGF-β-activated Ser/Thr kinase, is a potent activator of JNK. An earlier study provided evidence that TAK1 activated p38-MAPK (17). In comparison, we found that the magnitude of JNK activation by TAK1 is higher than that of p38-MAPK activation in our assays and that the wild-type and the activated TAK1 mutant stimulated JNK and p38-MAPK almost equally, whereas in yeast only the activated TAK1 mutant complements the Ste11 mutant (16). However, this appears to be a common phenomenon shared by several other mammalian Ser/Thr kinases such as MEKK1,Tpl-2, MUK, and MLK3 that can activate the JNK pathway. The ability of TAK1 to activate JNK1 is likely due to the participation of endogenous SEK1, and the ability of SEK1 to stimulate JNK1 may be partly due to the activity of endogenous TAK1.

At the TAK1 (MAP kinase kinase kinase) level, there are several other mammalian Ser/Thr kinases such as MEKK1, Tpl-2, MUK, and MLK3 that can activate the JNK pathway. Some of the kinases like MEKK1 and MLK3 act through SEK1, but whether these kinases function linearly or independently remains unclear. We demonstrated that MLK3 and MEKK1 dominant-negative mutants, which can effectively block JNK induction by HPK1 (data not shown) (10), were ineffective in blocking TAK1-induced JNK activation. In a similar approach, Tibbles et al. (28) showed that a MLK3 dominant-negative mutant cannot block MEKK1-induced JNK activation. The result suggests that TAK1 is independent of MLK3 and MEKK1 in the JNK pathway. The redundancy of kinases at the same level in the JNK kinase cascade may be indicative of required stage- or tissue-specific regulations in controlling their designated functions. For example, TAK1, but not MLK3, is expressed abundantly in the brain (16, 29).

We also demonstrate that activation of the JNK pathway by one of the mammalian Ste20-like kinases, HPK1, was blocked by the dominant-negative mutant of TAK1 protein, TAK1-K63W. However, the same protein has little effect on the activation of the JNK pathway by another mammalian Ste20-like kinase, GCK. This result suggests that (i) TAK1 is downstream of HPK1 but not GCK and (ii) HPK1 rather than GCK may be involved in the TGF-β signaling pathway. Further study is required to understand the underlying mechanisms of TAK1 activation by HPK1. Taken together, our data identify TAK1 as a component in the HPK1 → TAK1 → MAPK kinase 4/SEK1 → JNK kinase cascade.

**Acknowledgments—**We thank Drs. K. Matsumoto, J. Kehrl, N. Lassam, J. Gutkind, and J. Woodgett for providing valuable reagents;
members of the Tan laboratory for their helpful discussions; A. Brown and S. Lee for technical assistance; and M. Lowe for secretarial assistance.

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