Functional Interactions of Transforming Growth Factor β-activated Kinase 1 with IκB Kinases to Stimulate NF-κB Activation*

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Several mitogen-activated protein kinase kinase kinases play critical roles in nuclear factor-κB (NF-κB) activation. We recently reported that the overexpression of transforming growth factor-β-activated kinase 1 (TAK1), a member of the mitogen-activated protein kinase kinase kinase (MAP3K) family, together with its activator TAK1-binding protein 1 (TAB1) stimulates NF-κB activation. Here we investigated the molecular mechanism of TAK1-induced NF-κB activation. Dominant negative mutants of IκB kinase (IKK) α and IKKβ inhibited TAK1-induced NF-κB activation. TAK1 activated IKKα and IKKβ in the presence of TAB1. IKKα and IKKβ were coimmunoprecipitated with TAK1 in the absence of TAB1. TAB1-induced TAK1 activation promoted the dissociation of active forms of IKKα and IKKβ from active TAK1, whereas the IKK mutants remained to interact with active TAK1. Furthermore, tumor necrosis factor-α-activated endogenous TAK1, and the kinase-negative TAK1 acted as a dominant negative inhibitor against tumor necrosis factor-α-induced NF-κB activation. These results demonstrated a novel signaling pathway to NF-κB activation through TAK1 in which TAK1 may act as a regulatory kinase of IKKs.

Transcription factor nuclear factor κB (NF-κB)1 is composed of homodimers and heterodimers of Rel family proteins and plays a pivotal role in the gene expression involved in inflammatory and immune responses (1–3). NF-κB is sequestered in the cytoplasm by inhibitory proteins such as IκBα, IκBβ, and IκBε, which mask the nuclear localization signal of NF-κB (4–8). The phosphorylation of two Ser residues at an N-terminal regulatory domain of IκB proteins triggers polyubiquitination of IκB proteins, which targets them for rapid degradation through a proteasome-dependent pathway, thereby releasing NF-κB to enter the nucleus (9–15). Diverse extracellular stimuli such as tumor necrosis factor (TNF)-α and interleukin-1β, phorbol esters, and environmental stresses lead to NF-κB activation utilizing the common mechanism for the IκB degradation, suggesting the diversity of the upstream signaling pathways for phosphorylation of IκB proteins.

Several regulatory kinases involved in the signal-induced phosphorylation of IκB proteins have recently been reported. Two closely related kinases designated IκB kinase (IKK) α and IKKβ have been identified as components of the multiprotein IKK complex (500–900 kDa) that directly phosphorylates the critical Ser residues of IκB proteins (16–20). Together, IKKα and IKKβ form a heterodimer through their C-terminal leucine zipper motifs, and the functional IKK complex contains both IKK subunits, NF-κB-inducing kinase (NIK) is a member of the mitogen-activated protein kinase kinase kinase (MAP3K) family, which was first identified as a TNF-α receptor-associated factor (TRAF) 2-interacting protein (21). The ligand-mediated trimerization of the TNF-α receptor triggers the recruitment of NIK to TRAF2, and this association results in the activation of NIK, which in turn phosphorylates and activates IKKs. NIK also interacts with TRAF6, another member of the TRAF family, which is required for interleukin-1β-induced NF-κB activation (22). In addition, MAPK/Extracellular signal-regulated kinase kinase kinase (MEKK1), another member of the MAP3K family, stimulates NF-κB activation by preferentially activating IKKβ over IKKα (23–25). These findings suggest that several MAP3Ks have a key role in the NF-κB activation pathway by regulating the kinase activity of the IKK complex. However, little is known about the regulatory molecular mechanisms of the kinase activity of the IKK complex induced by diverse extracellular stimuli.

Transforming growth factor (TGF)-β-activated kinase 1 (TAK1) was first identified as a MAP3K that can be activated by TGF-β and bone morphological protein (26). TAK1 activity is regulated by its activator, TAK1-binding protein 1 (TAB1) (27). TAK1 is suggested to act as a MAP3KK in the c-Jun N-terminal kinase (JNK)/stress-activated kinase (JNK) stress-activated protein kinase (SAPK) and the p38 MAPK cascades, in which TAK1 phosphorylates MAPK kinase (MKK) 4, MKK3, and MKK6 (28, 29). In addition, hematopoietic progenitor kinase 1 induces the activation of the JNK pathway mediated by TAK1 but not MEKK1 and mixed lineage kinase 3 (30). However, the biological role of TAK1 in the intracellular signaling pathways is poorly understood.

We recently reported that the overexpression of TAK1 together with TAB1 stimulates NF-κB activation (31). In the present study, we investigated the molecular mechanisms of TAK1-induced NF-κB activation. We found functional interactions of TAK1 with IKKα and IKKβ. In the activation of TAK1-induced IKKs, two Ser residues in the activation loop of the IKKs were critically involved.

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MATERIALS AND METHODS

Expression Vectors—in our previous study, three isoforms of human TAK1 cDNA were isolated (31). TAK1a is the most abundantly expressed in HeLa cells and was used in the present study. Full-length
TAK1 cDNA was subcloned into the EcoRI-Khol site in pFLAG-CMV2 mammalian expression vector (Kodak) and expressed as a Flag epitope-tagged protein. The expression vectors for TAB1 and NIK624-947 were described previously (31). IKKα and IKKβ cDNAs were obtained from human monocytic THP-1 cells by reverse transcription-polymerase chain reaction. The primers used were as follows: 5′-GGCCCGCTGGAA- TTTCCCGCCCCATGGA-3′ and 5′-TTTTCTGAAGATATCCCATACCG-3′ for the N terminus of IKKα, 5′-GAGGATGCAGGATGCTTCGAAAGA-3′ and 5′-CGAGGCTTACGTTTTGGA-3′ for the C terminus of IKKα, 5′-TGAGGGCGGACCTGACTCTCTAAGAAA-3′ and 5′-CCCCGCTGGCTGCTGGCTCGTCCTC-3′ for the C terminus of IKKβ. Full-length IKKα and IKKβ cDNAs were subcloned into the EcoRI-NoI and KpnI-NoI sites of pcDNA3.1 (+) and pcDNA3.1 (+) HisB (Invitrogen), respectively. Expression vectors encoding the dominant negative mutants (TAK1 (K63W), IKKα (K44M), IKKβ (K44M)) (SS177, 181AA), and IKKβ (K44M)) were constructed using a QuikChange site-directed mutagenesis kit (Stratagene). All of the mutations were verified by DNA sequencing analysis.

Cell Cultures and Transfection—HeLa cells were maintained in high-glucose Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C in 5% CO₂. Cells were transfected with expression vectors using Lipofectamine (Life Technologies, Inc.).

Gel Shift Assay and Luciferase Assay—Twenty-four h after transfection, the cells were harvested, and gel shift assays were performed with nuclear extracts as described previously (32). Luciferase reporter gene assay was performed by using pNFκB-Luc plasmid (Stratagene). pRSV-β-gal plasmid was kindly provided by Dr. M. Tsuda (Toyama Medical and Pharmaceutical University).

Immunocomplex Kinase Assay—Cell lysates were immunoprecipitated with the anti-Flag antibody (Kodak) on ice for 1.5 h and rotated with protein G-conjugated Sepharose (Pharmacia) at 4 °C for 1 h. The beads were washed five times with washing buffer (20 mM HEPES (pH 7.7), 150 mM NaCl, 0.5 mM EDTA, 0.1% Triton X-100, 20 mM β-glycerophosphate, 0.1 mM sodium orthovanadate, 0.5 mM phenylmethylsulfonfonyl fluoride, 1 mM diithiothreitol, 10 μg/ml aprotinin, and 10 μg/ml leupeptin). Cell lysates were diluted with 3 volumes of dilution buffer (20 mM HEPES (pH 7.7), 2.5 mM MgCl₂, 0.1 mM EDTA, 0.05% Triton X-100, 20 mM β-glycerophosphate, 0.1 mM sodium orthovanadate, 0.5 mM phenylmethylsulfonfonyl fluoride, 1 mM diithiothreitol, 10 μg/ml aprotinin, and 10 μg/ml leupeptin) and incubated on ice for 10 min. After centrifugation, the pellets were resuspended in 5 μl lysis buffer (25 mM HEPES (pH 7.6), 2 mM dithiothreitol, 20 μM ATP, 20 μM β-glycerophosphate, 20 μM disodium p-nitrophenylphosphate, 0.1 mM sodium orthovanadate, and 3 μCi of [γ-32P]ATP) at 30 °C for 30 min. The reaction mixture was resolved by SDS-PAGE, followed by autoradiography.

MAP Kinase Assay—The JNK activity was determined by an in vitro immunocomplex kinase assay. Immunoprecipitation was carried out using an anti-JNK1 (FL) antibody (Santa Cruz Biotechnology), and a kinase reaction with GST-IκBα as a substrate using the procedure described above. The GST-c-Jun expression plasmid was kindly provided by Dr. M. Hibi (Osaka University). Phospho-specific antibodies were obtained from New England Biolabs.

Phosphatase Treatment—Cell lysates were immunoprecipitated with the anti-Flag antibody. After washes, the beads were incubated with 2 units/μl calf intestinal alkaline phosphatase (Takara) at 37 °C for 30 min. Where indicated, sodium orthovanadate (1 μM) was added in the reaction mixture.

RESULTS

NF-κB Activation by TAK1—We previously demonstrated the ability of TAK1 to activate p50/p65 NF-κB in a TAB1-dependent manner (31). To investigate the molecular mechanism of TAK1-induced NF-κB activation, N-terminal Flag epitope-tagged wild-type TAK1 or a kinase inactive mutant (TAK1K63W) was transiently expressed in HeLa cells. A gel shift assay showed that wild-type TAK1 together with TAB1 induced the nuclear translocation of NF-κB, whereas TAK1K63W could not induce the translocation even when TAB1 was coexpressed (Fig. 1A). In contrast, the Oct-1 DNA binding activity was not affected by the overexpression of TAB1 and TAK1 (Fig. 1A). In addition, two major inhibitory proteins, IkBα and IkBβ, were degraded in cells expressing both wild-type TAK1 and TAB1 (Fig. 1B). The degradation of IkBα was blocked by a proteasome inhibitor, N-acetyl-leucyl-leucyl-norleucinal (data not shown), indicating that TAK1 may activate NF-κB through the ubiquitination-proteasome pathway.

Mechanism of TAK1 Activation by TAB1—TAB1 was first identified as a TAK1 activator in a yeast two-hybrid system (27). Here, we characterized the molecular mechanism of the TAK1 activation by TAB1 in mammalian cells. Flag-TAK1 and Flag-TAK1K63W were expressed with or without TAB1 in HeLa cells, and anti-Flag immunoprecipitates were analyzed for the coprecipitation of TAB1 by immunoblotting. TAB1 was communoprecipitated with wild-type and kinase inactive TAK1 (Fig. 2A). TAB1 migrated slowly on a SDS-polyacrylamide gel when coexpressed with TAK1, but not with TAK1K63W (Fig. 2A). Wild-type TAK1, but not TAK1K63W, also migrated slowly when coexpressed with TAB1 (Fig. 2A). In addition, TAB1 appeared to be stabilized as a consequence of the association with TAB1 (Fig. 2A). The reduced mobility of coexpressed TAK1 and TAB1 may reflect the phosphorylation of both proteins induced by their functional interaction, as has been described for several protein kinases including interleukin 1 receptor-associated kinase (33). To investigate this possibility, an in vitro kinase assay was conducted using the anti-Flag immunoprecipitates. The phosphorylation of TAK1 and TAB1 was detected in cells expressing TAK1 and TAB1 (Fig. 2B). Furthermore, treatment of the immunoprecipitated TAK1/TAB1 complex with calf intestinal alkaline phosphatase converted the slower-migrating forms to the faster-migrating forms (Fig. 2C). A phosphatase inhibitor, sodium orthovanadate, blocked this mobility shift of TAK1. The mobility of TAB1 was partially reduced by the inhibitor, suggesting multiple phosphorylation sites in TAB1. These results suggest that the association of TAB1 with TAK1 causes the activation of TAK1, during which TAK1 autophosphorylation and phosphorylation of TAB1 by TAK1 may be occurring.

Involvement of IκKs in TAK1-induced NF-κB Activation—The marked degradation of IκB proteins by TAK1 raises the possibility of the involvement of the IκK complex in TAK1-induced NF-κB activation. To investigate this possibility, the effects of dominant negative mutants of the IκKs were examined. The TAK1-induced nuclear translocation of NF-κB was inhibited by the kinase inactive mutants IKKα (K44M) and IKKβ (K44M) (Fig. 3A). In contrast, TAK1-induced JNK and p38 MAPK activation was not inhibited by these IκK mutants (Fig. 3B). These results suggest that TAK1-induced NF-κB activation is mediated by the IκK complex, but not through the MAPK signaling cascades.

The TAK1-induced regulation of IκK kinase activity was
investigated. First, the endogenous IKK kinase activity was determined by an in vitro anti-IKKα immunocomplex kinase assay using bacterially expressed GST-IκBα (1–54) as a substrate. The kinase activity was significantly increased when wild-type TAK1 and TAB1 were coexpressed, whereas TAK1K63W did not enhance the IKK activity (Fig. 4). The specificity of the IKK activity was confirmed by using a mutant substrate, GST-IκBα (1–54) (SS32 and 36AA), in which the critical Ser residues for IKKs were replaced with Ala (Fig. 4). The anti-IKKα antibody was able to recognize IKKβ as well as IKKα, suggesting that both IKK subunits contribute to the IKK activity. Similar results were obtained by an immunocomplex kinase assay using an anti-MAPK phosphatase-1 antibody (data not shown), which has been shown to precipitate the multisubunit IKK complex (17).

To further elucidate the contribution of the two IKK subunits, N-terminal Xpress epitope-tagged IKKα and IKKβ were coexpressed with TAK1 and TAB1, and the kinase activities of the IKKs were measured by an anti-Xpress immunocomplex kinase assay. TAK1, but not TAK1K63W, induced the kinase activity of IKKα when coexpressed with TAB1 (Fig. 5A). Similarly, IKKβ activity was enhanced by TAK1 plus TAB1, whereas IKKβ alone showed constitutive activity (Fig. 5B). In addition, TAK1K63W slightly inhibited the constitutive IKKβ activity (Fig. 5B). These results indicate that TAK1 acts as an activator for IKKα and IKKβ in the signaling pathway of NF-κB activation by TAK1. HeLa cells (1 × 10^6 cells/60-mm dish) were transfected with expression vectors for Flag-TAK1 (1 μg) or Flag-TAK1K63W (1 μg) with or without an expression vector for TAB1 (1 μg). The total amount of DNA was adjusted with an empty vector at 2 μg. A, 24 h after transfection, nuclear extracts were prepared, and gel shift assays were carried out with oligonucleotide probes containing a κB site or an octamer binding site. B, whole cell lysates were prepared, and immunoblotting was carried out with anti-IκBα and anti-IκBβ antibodies.

Fig. 2. TAB1-mediated activation of TAK1. HeLa cells were transfected with expression vectors for Flag-TAK1, Flag-TAK1K63W, and TAB1 as described in the Fig. 1 legend. Whole cell lysates were prepared 24 h after transfection. A, lysates were immunoprecipitated with an anti-Flag antibody and analyzed for coprecipitating TAB1 by immunoblotting with an anti-TAB1 antibody (top panel). The same blots were reprobed with an anti-TAK1 antibody (bottom panel). Similar results were obtained in the immunoblotting of lysates with an anti-Flag antibody. To monitor the expression of TAB1, lysates were immunoblotted with an anti-TAB1 antibody (middle panel). B, lysates were immunoprecipitated with an anti-Flag antibody and incubated with kinase buffer containing [γ-32P]ATP. The reaction mixtures were resolved by 7.5% SDS-PAGE, followed by autoradiography. C, the TAK1/TAB1 complex immunoprecipitated with anti-Flag antibody was treated with calf intestinal alkaline phosphatase (CIP). Mobility was analyzed by immunoblotting with the anti-TAK1 and the anti-TAB1 antibodies. Sodium orthovanadate (1 mM) was added in the reaction mixture, where indicated. P-TAK1 and P-TAB1 indicate their phosphorylated forms.
TAK1 as a Regulatory Kinase of IκB Kinases

**Fig. 3. Inhibition of TAK1-induced NF-κB activation by IKK mutants.** HeLa cells were transfected with expression vectors for Flag-TAK1 (1 μg) and TAB1 (1 μg) with or without expression vectors (1 μg each) for IKKα (K44M) or IKKβ (K44M). The total amount of DNA was adjusted with an empty vector at 3 μg. A, 24 h after transfection, gel shift assays were carried out with nuclear extracts. The shifted bands are shown. B, 24 h after transfection, whole cell lysates were analyzed for JNK and p38 MAPK activation. JNK activity was determined by immunoblotting with an anti-phospho p38 antibody.

**Fig. 4. Activation of endogenous IKK complex by TAK1.** HeLa cells were transfected with expression vectors (1 μg each) for Flag-TAK1, Flag-TAK1K63W, and TAB1. The total amount of DNA was adjusted with an empty vector at 2 μg. Twenty-four h after transfection, whole cell lysates were immunoprecipitated with an anti-IKKα antibody. The IKK kinase activity was measured by an in vitro immunocomplex kinase assay with GST-c-Jun (1–79) as a substrate.

**Fig. 5. Activation of IKKα and IKKβ by TAK1.** HeLa cells were transfected with expression vectors (1 μg each) for Flag-TAK1, Flag-TAK1K63W, TAB1, Xpress-IKKα, and Xpress-IKKβ. The total amount of DNA was adjusted with an empty vector at 3 μg. Twenty-four h after transfection, whole cell lysates were immunoprecipitated with an anti-Xpress antibody. Kinase activities of IKKα (A) and IKKβ (B) were measured by in vitro immunocomplex kinase assays with GST-IκBα (1–54) or GST-IκBα (1–54) (SS32 and 36AA) as a substrate. The expression level of IKKs is shown in Fig. 6.

**TAK1-induced NF-κB activation.**

*Interaction of TAK1 with IKKs—NIK* has been shown to directly associate with both IKKs and enhance their kinase activities (19, 20). Because TAK1 is also a member of the MAPKKK family, we investigated the interaction of TAK1 with IKKs. HeLa cells were transiently transfected with the expression vectors for Flag-TAK1 or Flag-TAK1K63W with Xpress-IKKα and Xpress-IKKβ. Anti-Flag immunoprecipitates were analyzed for the presence of IKKs by immunoblotting with the anti-Xpress antibody. The interaction of wild-type TAK1 with IKKα was detected in the absence of TAB1 (Fig. 6A). However, the interaction was not detected when TAK1 was activated by TAB1 (Fig. 6A). Similarly, the interaction of TAK1 with IKKβ was detected only in the absence of TAB1 (Fig. 6B). In contrast, interactions of NIK with both IKKα and IKKβ were detected through their active forms (data not shown). TAK1K63W interacted weakly with IKKα and IKKβ (Fig. 6, A and B), whereas this molecule had the potential to interact with TAB1 (Fig. 2A). The immunoblotting of cell lysates with the anti-Xpress antibody showed that IKKα and IKKβ migrated slowly on SDS-PAGE when cotransfected with both TAK1 and TAB1 (Fig. 6). These results indicate that TAK1 interacts with both IKK subunits to induce their kinase activities.

The **Significant Role of Ser Residues in the Activation Loop of IKKs**—Most of the interactions between activated protein kinases and phosphorylated substrates have been shown to be transient. However, a stable interaction could be detected when the kinase defective mutant or the mutated substrate that lacks the target residues for phosphorylation was used. To examine the features of TAK1-IKKs interactions, Xpress-tagged IKK mutants (KM and SSAA) were coexpressed with Flag-TAK1 in HeLa cells. The communoprecipitation assay showed that interactions between TAK1 and all IKK mutants were detectable in both the absence and presence of coexpressed TAB1 (Fig. 7A). These results indicate that the kinase activities of IKKs are necessary for the dissociation of TAK1 from IKKs, in which TAK1 may phosphorylate the Ser residues in the activation loop of the IKKs. In addition, the immunoblotting of cell lysates with the anti-Xpress antibody showed that all IKK mutants did not migrate slowly on SDS-PAGE even when in the presence of active TAK1, suggesting that the reduced mobility of wild-type IKKs reflects autophosphorylation. Furthermore, IKKα-SSAA and IKKβ-SSAA acted as dominant negative inhibitors in TAK1-induced NF-κB activation (Fig. 7B). These results indicate that the activation loop is critically involved in TAK1-induced IKKs activation.

**Selective Depletion of TAK1 and TAB1**—Interestingly, TAK1 and TAB1 appeared to be selectively depleted in cells cotransfected with IKKβ, whereas the expression of IKKα was not affected (Figs. 6B and 7A). Such a depletion was not observed in the presence of IKKα (Fig. 6A). The depletion of TAK1 and TAB1 was dependent on the kinase activities of TAK1 and IKKβ, because this was not observed in cells expressing kinase-negative mutants of TAK1 and IKKβ (Figs. 6B and 7A). This observation may indicate a novel regulatory mechanism of TAK1 kinase activity.

TNF-α-induced NF-κB Activation through TAK1—In A673 human rhabdomyosarcoma cells, endogenous TAK1 is activated by TNF-α in which the TAK1 activity was measured for
its ability to activate SEK1 (29). Here we investigated the effect of TNF-α on TAK1 activation in HeLa cells. The anti-TAK1 immunocomplex in vitro kinase assay using 6xHis-MKK6 as a substrate showed that TNF-α activated endogenous TAK1 transiently, and the maximal activation was observed at 2–5 min after stimulation (Fig. 8A). TAK1 activity was also detected together with its autophosphorylation and TAB1 phosphorylation (data not shown), which was similar to the data from the overexpression experiment (Fig. 2B). Interestingly, TAK1 activation was preceded by the activation of endogenous IKK complex, which was detected at 5–10 min after stimulation (Fig. 8A). In contrast, TGF-β did not induce TAK1 activation as well as IKK activation (Fig. 8A). These results suggest that TAK1/TAB1 might act as signal transducers of the NF-κB activation pathway through the TNF-α receptor. To clarify this possibility, we examined the effect of kinase-negative TAK1 on TNF-α-induced NF-κB activation. TAK1K63W inhibited κB-dependent luciferase gene expression (Fig. 8B). These results indicate that the TAK1/TAB1 complex plays a role in TNF-α-induced NF-κB activation.

Effect of the NIK Mutant on TAK1-induced NF-κB Activation—NIK plays a key role in TNF-α-induced NF-κB activation through IKK activation (19–21). Here we further investigated the effect of the NIK mutant. A truncated mutant NIK (NIK624–947) acted as a dominant negative inhibitor against the TNF-α-induced NF-κB activation (Fig. 9A). Furthermore, the NIK mutant partially inhibited TAK1-induced NF-κB activation (Fig. 9B).

**DISCUSSION**

TAK1 was first identified as a MAPKKK that can be activated by TGF-β and bone morphological protein (26) and was reported to play a role in bone morphological protein signaling in early Xenopus development (34). cDNA cloning of Xenopus TAK1 revealed that the amino acid sequence of the catalytic domain is highly conserved (98%) between Xenopus and human TAK1 (31, 34). Recent studies have shown that Smad proteins are critically involved in the signaling pathway from TGF-β and bone morphological protein receptors (35, 36). The injection of kinase-negative TAK1 mRNA into the Xenopus embryo reverses the Smad1- or Smad5-induced expression of ventral mesoderm markers, suggesting cooperation between TAK1 and Smad proteins (34). Here we demonstrated a novel function of TAK1 as an activator of the IKK complex to stimulate NF-κB activation. However, little is known about the functional relationship between TGF-β signaling and the NF-κB activation pathways. We previously reported that TGF-β could not induce the nuclear translocation of NF-κB in HeLa cells (31). In addition, we showed that TAK1 was not activated by TGF-β in HeLa cells. These results suggest that
TAK1 is involved in the NF-κB activation pathway induced by extracellular stimuli other than TGF-β. In this study, we demonstrated that TNF-α activated TAK1 to stimulate NF-κB activation. It has been shown that NIK plays a significant role in TNF-α-induced NF-κB activation. Our previous study showed that NIK624–947 did not inhibit the TAK1-induced nuclear translocation of NF-κB (31). In contrast, the truncated NIK mutant inhibits TAK1-induced NF-κB-dependent luciferase gene expression in human embryonal kidney 293 cells.\(^2\) We also observed the partial dominant negative effect of the NIK mutant in HeLa cells. These results suggest that TAK1 might be a regulatory kinase of NIK. Otherwise, TAK1 may regulate IKKs directly, when the NIK mutant could interact with and inactivate endogenous IKKs. Understanding the precise functional relationship between TAK1 and NIK in TNF-α-induced NF-κB activation requires further investigation.

Hematopoietic progenitor kinase 1 is a serine/threonine kinase with restricted expression in hematopoietic tissues (37, 38). It has been shown that hematopoietic progenitor kinase 1 activates the JNK pathway mediated by TAK1 (30). It is interesting to evaluate the ability of hematopoietic progenitor kinase 1 to stimulate NF-κB activation through TAK1, which may present a physiological function of TAK1-induced NF-κB activation in hematopoietic differentiation.

In the present study, we demonstrated that the recruitment of TAB1 to TAK1 may trigger both TAK1 autophosphorylation and phosphorylation of TAB1. The C-terminal 68 amino acids of TAB1 were shown to be sufficient for binding and activating TAK1 (27). In contrast, the N-terminal domain lacking the TAK1 binding domain acts as a dominant negative inhibitor in TGF-β signaling (27). In addition, the deletion of 20 amino acids from the N terminus of TAK1 renders the protein kinase constitutively active (26). These findings strongly suggest that TAK1 phosphorylates the C-terminal domain of TAB1 and the N-terminal domain of TAK1. In fact, these domains contain a Ser/Thr-rich sequence (26, 27). The identification of the phosphorylation sites of TAK1 will provide more information regarding the molecular mechanism of TAK1 activation by TAB1.

The functional implications of MAPK cascades in the signaling pathways to NF-κB activation have been characterized. The 90-kDa ribosomal S6 kinase (p90rsk) that lies downstream of the Raf-MAPK/extracellular signal-regulated kinase pathway is involved in phorbol ester-induced NF-κB activation by phosphorylating Ser\(^{32}\) but not Ser\(^{36}\) of IκBa (39). MAPK cascades that are sensitive to the MAPK/extracellular signal-regulated kinase inhibitor PD098059 and the p38 MAPK inhibitor SB203580 were shown to enhance the TNF-α-induced transactivation of the p65 NF-κB subunit (40). Several MAPKKKs including NIK (21), MEKK1 (41, 42), and MEKK3 (43) were recently shown to have the potential to activate NF-κB. NIK and MEKK1 preferentially activate IKKα and IKKβ, respectively (23, 44). Here we demonstrated that TAK1 is a new member of the MAPKKK family that activates IKKs. TAK1 as well as NIK interacts with both IKKα and IKKβ. In contrast, the interaction of MEKK1 with IKKs has not yet been demonstrated, although a MEKK1 catalytic subunit was copurified with the TNF-α-induced multiprotein IKK complex (17). A recent study attempting to

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\(^2\) Tsuji, N. J., Kishimoto, K., Hiyama, A., Inoue, J., Cao, Z., Matsumoto, K. (1999) Nature, in press.
isolate rat MEKK1 cDNA confirmed that MEKK1 is a 195-kDa protein with a large N-terminal regulatory domain (45), raising the possibility that the regulatory domain may play a role in the interaction with IKKs. In fact, the human T cell leukemia virus type I Tax protein binds to the regulatory domain of MEKK1 to stimulate IKK kinase activity (24). Thus, these observations indicate that MAPKKKs stimulate NF-κB activation through direct interactions with IKKs, but not through the MAPKK-MAP signaling pathways.

MAPKKKs activate MAPKKs by phosphorylating Ser residues in the activation loop (S-X-X-S) located between kinase subdomains VII and VIII (46). These Ser residues are conserved in both IKKα and IKKβ. The Ser residues in IKKβ were shown to be essential for NF-κB activation. In the signaling pathway to NF-κB activation, IKKβ mutants in which Ser177 and Ser181 are replaced with Ala or Glu act as a dominant negative inhibitor and a constitutively active mutant, respectively (17). In addition, NIK activates IKKs by phosphorylating Ser176 in the activation loop (44). In the present study, we demonstrated the functional significance of the Ser residues in the activation loop of both IKK subunits in TAK1-induced IKK activation. Collectively, these findings indicate that the molecular mechanism of the regulation of IKKs by TAK1 may be as follows. TAK1 interacts with IKKs in unstimulated cells. The recruitment of TAB1 to TAK1 activates the kinase activity of TAK1, where TAB1 phosphorylation by TAK1 activates the activation loop of both IKK subunits in TAK1-induced IKK activation. The activation loop of both IKK subunits in TAK1-induced IKK activation and the function of TAK1 is likely to have therapeutic value in treating inflammatory diseases, in which NF-κB may play significant pathogenic roles.

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