Receptor-interacting protein (RIP) plays a critical role in tumor necrosis factor-α (TNF-α)-induced IκB kinase (IKK) activation and subsequent activation of transcription factor NF-κB. However, the molecular mechanism by which RIP mediates TNF-α-induced NF-κB activation is not completely defined. In this study, we have found that TAK1 is recruited to the TNF-α receptor complex in a RIP-dependent manner following the stimulation of TNF-α receptor 1 (TNF-R1). Moreover, a forced recruitment of TAK1 to TNF-R1 in the absence of RIP is sufficient to mediate TNF-α-induced NF-κB activation, indicating that the major function of RIP is to recruit its downstream kinases to the TNF-R1 complex. Interestingly, we also find that TAK1 and MEKK3 form a functional complex, in which TAK1 regulates autophosphorylation of MEKK3. The TAK1-mediated regulation of MEKK3 phosphorylation is dependent on the kinase activity of TAK1. Although TAK1-MEKK3 interaction is not affected by overexpressed TAB1, TAB1 is required for TAK1 activation and subsequent MEKK3 phosphorylation. Together, we conclude that TAK1 is recruited to the TNF-R1 complex via RIP and likely cooperates with MEKK3 to activate NF-κB in TNF-α signaling.

The mitogen-activated protein kinases (MAPKs) are a family of serine/threonine kinases that have been shown to function in a wide variety of biological processes. Transforming growth factor-β-activated kinase 1 (TAK1) and mitogen-activated protein kinase kinase kinase 3 (MEKK3) are known to act as a MAP3K in the c-Jun N-terminal kinase and the p38 MAPK cascades. In addition, it has been shown that both kinases are involved in the nuclear factor κB (NF-κB) pathway. However, the molecular mechanism of this function is still unclear, mainly due to the poor characterization of MAP3K activation under the physiological conditions.

NF-κB is a family of transcription factors involved in inflammation and innate immunity. In unstimulated cells NF-κB is sequestered in the cytoplasm through an interaction with a family of inhibitory proteins, IκB. In response to extracellular stimuli, the IκB proteins are phosphorylated by the IκB kinase (IKK) complex, then ubiquitinated and rapidly degraded, which leads to the nuclear localization and activation of NF-κB.

One of the most potent NF-κB activators is tumor necrosis factor-α (TNF-α), a major proinflammatory cytokine. TNF-α functions through two distinct surface receptors, a 55-kDa receptor 1 (TNF-R1) and a 75-kDa receptor 2 (TNF-R2). TNF-R1 plays the predominant role in induction of cellular responses by soluble TNF-α. The binding of TNF-α to TNF-R1 leads to the recruitment of TNF-R1-associated death domain (TRADD), and TRADD further recruits TNF-receptor-associated factor 2 (TRAF2) and Receptor-interacting protein (RIP) directly with TRADD via its death domain. It has been demonstrated that TRAF2 plays an essential role in IKK recruitment to the TNF-R1 complex, but IKK activation requires the presence of RIP in the same complex. However, the kinase activity of RIP is not required for RIP to mediate TNF-α-induced NF-κB activation. Therefore, it has been proposed that IKK activation requires its phosphorylation by an upstream kinase(s) other than RIP. Indeed, genetic studies using murine embryonic fibroblasts deficient in MEKK3 demonstrate that TNF-α-induced NF-κB activation is severely impaired in these cells. Our previous studies showed that MEKK3, but not its homolog MEKK2, connects RIP to IKK complex and a direct recruitment of MEKK3 to TNF-R1 is sufficient to restore NF-κB activation in the absence of RIP. Recently, published data (7, 19, 20) also suggest that TAK1, another MAP3K, is critical for IKK activation in TNF-α-induced NF-κB activation. However, the mechanism by which TAK1 is involved in TNF-α signaling pathway remains to be determined. Therefore, we hypothesized that MEKK3 and TAK1 link RIP to IKK in TNF-α signaling. In this study, we found that TAK1 was recruited into TNF-R1 in a RIP-dependent manner. Moreover, TAK1 physically interacted with MEKK3 and modulated MEKK3 phosphorylation in a TAB1-dependent manner. Activation of both TAK1 and MEKK3 was necessary for NF-κB activation.

**MATERIALS AND METHODS**

*Reagents and Plasmids*—Antibodies specific for Myc (A14) or FLAG (M2) epitope tags and for TAK1 (C-9), TAB1 (N-19), IKKβ (H-470), and
β-tubulin (D-10) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho-IkBα monoclonal antibodies were purchased from Cell Signaling Technology (Beverly, MA). Anti-phospho-MEK2/3 antibodies were produced by immunizing rabbits with phospho-MEK2 peptide (CSGTGGMK(P)VTIWPY) (34). Antibodies specifically recognizing the Thr-187 phosphorylated form of TAK1 were described previously (21). Recombinant human TNF-specifically recognizing the Thr-187 phosphorylated form of TAK1 pho-MEK2 peptide (CSGTGMK(P)SVTGTPYW) (34). Antibodies MEKK2/3 antibodies were produced by immunizing rabbits with phospho-IκBα, MEKK2/3 antibodies were carefully removed. The nuclear pellets were then resuspended in 400 μl of extraction buffer (20 mM HEPES (pH 7.9), 0.4M NaCl, 10 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol, 0.5 mM PMSF, 1 mM NaF, 1 mM PMSF, 1 mM dithiothreitol, and 1% protease inhibitor mixture) and incubated on ice for 15 min. The nuclei were pelleted, and the cytoplasmic proteins were carefully removed. The nuclear pellets were then resuspended in 100 μl of extraction buffer (20 mM HEPES (pH 7.9), 0.4M NaCl, 1 mM EDTA, 1 mM dithiothreitol, 0.5 mM PMSF, and 1% protease inhibitor mixture). After vortexing for 30 min at 4 °C, the samples were centrifuged (13,000 × g, 10 min), and the nuclear proteins in the supernatant were collected. Protein concentrations of nuclear extracts were determined by the Bio-Rad protein assay (Bio-Rad) using bovine serum albumin as the standard. Nuclear extract (10 μg) was incubated with a radiolabeled, double-stranded, NF-κB-specific oligonucleotide probe or Oct-1 probe as a control (Promega) for 15 min at room temperature. After incubation, samples were fractionated on a 5% polyacrylamide gel and visualized by autoradiography.

**Yeast Two-hybrid Assay**—Yeast two-hybrid interaction assays were performed in yeast strain AH109 transformed by a standard lithium acetate method. Yeast plasmids were generated by inserting sequence encoding TAK1 downstream of the Gal4 DNA-binding domain in the pGBK-T7 vector and truncated forms of MEKK3 (1–367, MEKK3rd; 354–626, MEKK3kd) downstream of the Gal4 activation domain in the pGAD-T7 vector (Clontech).

**RESULTS**

**TAK1 Small Interference RNA (siRNA) Inhibits TNF-α-induced NF-κB Activation**—A previous study (7) suggests that TAK1 is involved in TNF-α-induced NF-κB activation. To verify this finding, we performed a siRNA experiment. HEK293 cells were transfected with TAK1 siRNA, which resulted in the effective knock-down of TAK1 (Fig. 1A).
and co-transfected with a NF-κB-dependent luciferase reporter. At 48 h post-transfection, cells were stimulated with TNF-α, and luciferase activity was determined. Consistent with previous findings, TAK1 knock-down resulted in a significant reduction of NF-κB activation in TNF-α-treated cells (Fig. 1B).

**Critical Role of RIP in TAK1 Recruitment to the TNF-R1 Complex**—It has been shown that RIP is required for TNF-α-induced NF-κB activation (16, 17). Our previous studies also showed that direct recruitment of the IKK complex to the TNF-R1 complex was not sufficient to bypass the requirement of RIP (18). These results suggest that the role of RIP is to recruit a downstream kinase that will, in turn, activate the IKK complex. To determine whether a recruitment of TAK1 to the TNF-R1 complex is dependent on RIP we performed a localization experiment using a confocal microscopy technique. Consistent with previous observations (23), TNF-R1 was recruited to lipid raft in Jurkat T cells upon stimulation with anti-TNF-R1 agonistic antibodies (Fig. 2, left top panels). Although TAK1 was translocated to the lipid raft in Jurkat cells (Fig. 2, left bottom panels), it failed to do so in RIP− cells (Fig. 2, middle bottom panels). In contrast, the translocation of TNF-R1 (Fig. 2, middle top panels) and TRAF2 (data not shown) was not affected. Moreover, reconstitution of RIP− cells with WT RIP restored TAK1 recruitment to the lipid raft (Fig. 2, right bottom panels). These results suggest that RIP plays a critical role in the recruitment of TAK1 to the TNF-R1 complex.

**TAK1-DD Fusion Protein Restores TNF-α-induced NF-κB Activation in RIP-deficient Cells**—Our previous studies (18) showed that a direct recruitment of MEKK3 to the TNF-R1 complex by a fusion protein of MEKK3 and the death domain (DD) of RIP (MEKK3-DD) effectively restored TNF-α-induced NF-κB activation in RIP-deficient cells. To examine whether the direct recruitment of TAK1 to the TNF-R1 complex can also bypass the requirement of RIP, we used the same strategy by constructing a fusion protein containing TAK1 and the death domain of RIP (TAK1-DD) and reconstituted RIP− cells with TAK1-DD (Fig. 3A). As shown in Fig. 3B, the expression of fusion protein did not alter the expression levels of endogenous TAK1 in TAK1-DD stable cell line. To confirm that TAK1-DD could be recruited into the TNF-R1 complex in a signal-dependent manner, the cells were either unstimulated or stimulated with TNF-α, and TNF-R1 complexes were immunoprecipitated with anti-TNF-R1 antibody-conjugated beads. We found that TAK1-DD fusion protein was recruited into TNF-R1 complexes following TNF-α stimulation (Fig. 3C). Next, TNF-α-induced NF-κB activation was examined by electrophoretic mobility shift assay. Indeed, we found that the expression of TAK1-DD in RIP-deficient cells fully restored TNF-α-induced NF-κB activation in the absence of RIP (Fig. 3D). Consistent with these results, IκBα was phosphorylated in the TNF-α-treated TAK1-DD cells (Fig. 3E). These data indicate that RIP-dependent recruitment of TAK1 is sufficient for TNF-α-induced NF-κB activation, which further support the hypothesis that the major role of RIP is to recruit the downstream kinases such as TAK1.

**TAK1 Regulates the Activity of MEKK3**—Since both TAK1-DD (Fig. 3) and MEKK3-DD (18) can effectively restore TNF-α-induced NF-κB activation in RIP-deficient cells, and both TAK1 (7, 20) and MEKK3 (6) are required for TNF-α-induced NF-κB activation, we postulated that TAK1 and MEKK3 might functionally interact. It has been shown that transient transfection of MEKK3 constitutively activates this kinase and results in MEKK3 phosphorylation by an unknown mechanism. This phosphorylation of MEKK3 is correlated with the activation of downstream MAPKs and NF-κB. To examine whether TAK1 regulates the function of MEKK3, we ectopically expressed MEKK3 and TAK1 in HEK293 cells. Expression of MEKK3 alone resulted in an autophosphorylation of MEKK3 (Fig. 4A, lane 2) and NF-κB activation (Fig. 4B). Surprisingly, co-transfected TAK1 or its kinase-deficient mutant, TAK1(K63A), together with MEKK3 prevented MEKK3 phosphorylation (Fig. 4A, lanes 4 and 6) and inhibited MEKK3-induced NF-κB activation (Fig. 4B). These effects were dose-dependent (Fig. 4C) and specific for TAK1, since co-expression of other MAP3Ks, such as Cot, MEKK2, and their kinase-defective mutants, did not affect the phosphorylation of MEKK3 (Fig. 4D). Consistent with previous observations that TAK1 did not exhibit kinase activity when expressed alone (4), the ectopic expression of TAK1 did not lead to the activation of NF-κB (Fig. 4B), suggesting that the ectopically expressed TAK1 is in its inactive form. Thus, our results suggest that the inactive TAK1 inhibits the function of MEKK3.

It has been shown that endogenous TAK1 is constitutively associated with TAK1-binding protein 1 (TAB1) (24), and this interaction is required for phosphorylation-dependent TAK1 activation (25). An overexpression study demonstrated that TAK1 was phosphorylated and activated when co-transfected with TAB1 and the TAB1-TAK1 complex mediated NF-κB activation (4). We therefore investigated whether co-expression of TAK1 with TAB1 affected MEKK3 phosphorylation.
We found that MEKK3 phosphorylation was inhibited by expression of TAK1 alone (Fig. 5A, lane 7), but importantly, this inhibition was reverted in the presence of TAB1 (Fig. 5A, lane 8). However, TAB1 could not restore TAK1(K63A)-mediated inhibition of MEKK3 phosphorylation (Fig. 5A, lane 12). In contrast, the expression of MEKK3 did not affect a TAB1-dependent phosphorylation of TAK1 (Fig. 5A, lane 8). The phosphorylation of MEKK3 and TAK1 was correlated with NF-κB activation (Fig. 5B), since NF-κB activation was observed when either MEKK3 or TAK1 was phosphorylated (Fig. 5A, lanes 3, 4, 6, and 8). Together, these results suggest that TAK1 regulates the activity of MEKK3, and this regulation is dependent on the kinase activity of TAK1.

To further determine how TAK1-TAB1 regulates MEKK3 phosphorylation, we constructed two truncated forms of TAK1: TAK1kd, which contains the N-terminal kinase domain, and TAK1rd, which contains the C-terminal regulatory domain (Fig. 6A). Interestingly, expression of
both TAK1d and TAK1rd inhibited MEKK3 phosphorylation (Fig. 6B, lanes 5 and 7). In contrast to the full-length TAK1 (Fig. 6B, lanes 2 and 3), TAK1d- and TAK1rd-mediated inhibition of MEKK3 phosphorylation could not be restored by expression of TAB1 (Fig. 6B, lanes 6 and 8). These results indicate that both kinase domain and regulatory domain of TAK1 are important for the regulation of MEKK3 phosphorylation.

**TAK1 Associates with MEKK3**—Since TAK1 functionally regulates MEKK3, we next examined whether TAK1 and MEKK3 could form a functional complex. HEK293 cells were transfected with Myc-tagged MEKK3 in the presence or absence of FLAG-tagged TAK1 or its kinase-defective mutant, TAK1(K63A). As shown in Fig. 7, MEKK3 was effectively co-precipitated with TAK1 and TAK1(K63A) (Fig. 7A, lanes 4 and 6 of the top panel). To examine whether TAK1 and MEKK3 directly interact, we performed a yeast two-hybrid interaction assay. We found that TAK1 directly associated with the N-terminal regulatory domain of MEKK3 (Fig. 6B). Together, these results suggest that the complex of TAK1 and MEKK3 may be involved in TNF-α-mediated activation of NF-κB.

**The Effect of TAB1 on MEKK3-TAK1 Association**—Since TAK1 associates with MEKK3 and co-expression of TAB1 reverted TAK1-mediated inhibition of MEKK3 activity (Fig. 5), we next determined whether the observed TAB1 effects on TAK1-mediated inhibition of MEKK3 phosphorylation was due to that TAB1 competitively associated with the MEKK3-binding site in TAK1. To address this question, we examined the effect of TAB1 on MEKK3-TAK1 association. We co-transfected HEK293 cells with Myc-MEKK3 and Flag-TAK1 or TAK1(K63A) in the presence or absence of HA-TAB1. The resulted complexes were immunoprecipitated using anti-Myc or anti-HA antibodies. We found that expression of MEKK3 effectively co-precipitated TAK1 and TAK1(K63A) (Fig. 7C, lanes 3 and 5 of the top panel). Although TAB1 associated with TAK1 when co-expressed (Fig. 7C, lanes 4 and 6 of the middle panels), it did not prevent the association of TAK1 and MEKK3 (Fig. 7C, lanes 4 and 6 of the top panel). However, we were not able to detect a phosphorylated form of TAK1 in the MEKK3 complexes. These results suggest that a portion of TAK1 that is phosphorylated in the presence of TAB1 can dissociate from MEKK3. This dissociation may be required for MEKK3 phosphorylation and subsequent activation of NF-κB.

**TAK1 Functions Upstream of MEKK3 and Downstream of RIP**—Since TAK1-DD restores TNF-α-induced NF-κB activation in RIP-deficient cells and TAK1 modulates MEKK3 phosphorylation, it is conceivable that TAK1 functions upstream of MEKK3 and downstream of RIP. If this model is correct, we would expect that deleting TAK1 will only affect RIP-induced, but not MEKK3-induced, NF-κB activation. To test this hypothesis, we performed a siRNA experiment. HEK293 cells were transfected with TAK1 siRNA or control siRNA. Western blot analysis indicated that TAK1 siRNA, but not the control, significantly reduced TAK1 protein levels (Fig. 8A and B). These cells were co-transfected with expression vectors encoding RIP or MEKK3 and a NF-κB-dependent luciferase reporter. We found that the knock-down of endogenous TAK1 significantly blocked NF-κB...
that MEKK3-deficient mouse embryo fibroblasts (MEFs) demonstrate that TAK1 indeed acts downstream of RIP but upstream of MEKK3 in TNF-α activation.

**DISCUSSION**

The results of this and previous studies (6–8, 18) demonstrate that both TAK1 and MEKK3 are critical mediators of TNF-α-induced NF-κB activation. To date, it is not clear why both of these kinases mediate cytokine-induced IKK activation. Genetic studies using MEKK3-deficient mouse embryo fibroblasts (MEFs) demonstrate that TNF-α-induced NF-κB activation is almost completely abolished in these cells (6). On the other hand, a knock-down of endogenous TAK1 by siRNAs also significantly impairs NF-κB activation in response to TNF-α treatment (Ref. 7 and Fig. 1 of this study). Furthermore, several studies confirmed that TAK1 mediates IL-1-induced activation of the NF-κB pathway (26–29), but a more recent study has demonstrated that MEKK3 to IL-1-induced NF-κB activation upon IL-1 treatment (8). During a reviewing process of our manuscript, Sato et al. (20) published the first genetic evidence that TAK1 was required for TNF-α and IL-1-induced NF-κB activation. They generated MEF cell line that is defective in TNF-α and IL-1-induced NF-κB activation (Fig. 4). These effects were also found that MEKK3-DD fusion protein could fully restore NF-κB activation in RIP−/− cells (18). These data suggest that RIP-dependent recruitment of TAK1, as well as MEKK3, is required for TNF-α-induced NF-κB activation. Since TAK1-DD and MEKK3-DD can bypass the requirement of RIP, we postulate that both kinases function downstream of RIP. However, how TAK1 and MEKK3 are activated after being recruited to the TNF-R1 complex remains unknown.

To date, upstream regulators of MEKK3 have not been identified, and little is known about the phosphorylation of MEKK3 in cells under physiological conditions. Transient transfection of MEKK3 constitutively forms a complex with its adaptor protein TAB2 (3), and TAB2 is capable to bind to polyubiquitinated RIP following TNF-α stimulation (30). In this study, we also demonstrated that TAK1 was recruited to the TNF-R1 complex in a RIP-dependent manner (Fig. 2). Moreover, we constructed a fusion protein composed of full-length TAK1 and death domain of RIP (TAK1-DD) and found that this fusion protein fully restored NF-κB activation in RIP−/− cells (Fig. 3), suggesting the major role of RIP is to recruit downstream kinases to the TNF-R1 complex. Using the same strategy, recently we also found that MEKK3-DD fusion protein could fully restore NF-κB activation in RIP−/− cells (18). These data suggest that RIP-dependent recruitment of TAK1, as well as MEKK3, is required for NF-κB activation. Since TAK1-DD and MEKK3-DD can bypass the requirement of RIP, we postulate that both kinases function downstream of RIP. However, how TAK1 and MEKK3 are activated after being recruited to the TNF-R1 complex remains unknown.

Our results demonstrate that TAK1 functions downstream of RIP (Fig. 8A), which is consistent with a recent report showing that TAK1 activation induced by RIP (Fig. 8A). In contrast, activation of NF-κB by overexpressed MEKK3 was comparable in cells transfected with mock and TAK1 siRNA (Fig. 8B). Together, these results suggest that TAK1 indeed acts downstream of RIP but upstream of MEKK3 in TNF-α signaling pathway.
assay using a kinase-inactive mutant of MEKK3, MEKK3(K391A), as a substrate for activated TAK1 or constitutively active TAK1-TAB1 fusion protein described earlier (31). In this experiment, we did not observed phosphorylation of MEKK3 by TAK1 (data not shown), suggesting that MEKK3 is not a direct substrate for TAK1.

Another potential mechanism to explain the effect of TAK1 on MEKK3 activation is that the activated TAK1 promotes an oligomerization of MEKK3, which could induce a kinase activity of MEKK3 leading to its autophosphorylation. Indeed, it has been reported that MEKK3 is able to form dimers, which lead to their self-phosphorylation and activation (32). We speculate that in resting cells, MEKK3 may be associated with a specific inhibitor that prevents a MEKK3 dimerization. A similar inhibitor has been described for MEKK2 (33). Upon stimulation of cells, the activated TAK1 could phosphorylate a hypothetical inhibitor resulting in MEKK3 activation. We will test this hypothesis in the future studies.

Our results demonstrate that TAK1 and MEKK3 form a functional complex (Fig. 7). Moreover, TAK1-MEKK3 interaction is not affected by overexpressed TAB1, but TAB1 is required for TAK1 activation and subsequent MEKK3 phosphorylation (Fig. 5). Interestingly, the expres-
sion of MEKK3 did not affect TAB1-dependent phosphorylation of TAK1 (Fig. 5A), but inactive TAK1 inhibited the function of MEKK3 (Fig. 4 and 5), suggesting that TAK1 acts upstream of MEKK3. To confirm this important finding, we performed a siRNA experiment to knock-down TAK1 in cells. We found that activation of NF-κB by overexpressed MEKK3 was comparable in cells transfected with mock and TAK1 siRNA. In contrary, TAK1-knockdown resulted in significant reduction of RIP-induced NF-κB activation (Fig. 8). These results suggest that TAK1 may function upstream of MEKK3 and downstream of RIP. However, overexpression of TAK1-TAB1 can still activate NF-κB in MEKK3-deficient MEF cells (data not shown). One explanation is that the overexpressed TAK1-TAB1 may activate other kinase such as NIK leading to activation of NF-κB as shown previously (26). Therefore, the exact mechanism by which TAK1 and MEKK3 cooperate to activate NF-κB will need to be further investigated.

In summary, our data show that both TAK1 and MEKK3 are critically involved in RIP-dependent NF-κB activation in the TNF-α signaling pathway. Moreover, we show that TAK1 physically interacts with MEKK3 and modulates MEKK3 phosphorylation. Further study is required to determine the exact mechanisms by which TAK1 regulates MEKK3.

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