Reciprocal Inhibition between the Transforming Growth Factor-β-activated Kinase 1 (TAK1) and Apoptosis Signal-regulating Kinase 1 (ASK1) Mitogen-activated Protein Kinase Kinases and Its Suppression by TAK1-binding Protein 2 (TAB2), an Adapter Protein for TAK1

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Background: The molecular mechanism underlies the inter-regulation between TAK1 and ASK1 MAP3Ks.

Result: TAK1-TAB1 complex negatively regulates ASK1-mediated signal, and TAB2, an adapter protein for TAK1, reciprocally regulates the TAK1- and ASK1-mediated signaling pathway through the TAK1-TAB2 interaction and the interferences of TAK1-ASK1 interaction.

Conclusion: TAK1 and its adapter protein, TAB2, reciprocally regulate both TAK1-and ASK1-mediated signaling pathways.

Significance: We propose a new insight on the reciprocal regulation of TAK1 and ASK1 MAP3Ks.

Mitogen-activated protein kinase kinase kinases (MAP3Ks) are activated by a wide spectrum of extracellular stimuli and are involved in various cellular events including proinflammatory and oxidative damage response through activations of two specific transcription factors, nuclear factor κB (NF-κB) and activator protein-1 (AP-1). Among members of the MAP3K family have both overlapping and distinct functions, the inter-regulatory mechanism of MAP3Ks remains largely unknown. In this study we demonstrated that transforming growth factor-β-activated kinase 1 (TAK1)-TAK1-binding protein 1 (TAB1) complex negatively regulates ASK1-mediated signaling, and TAB2 reciprocally regulates TAK1-induced NF-κB and apoptosis signal-regulating kinase 1 (ASK1)-mediated AP-1 activations through the TAK1-TAB2 interaction and the interferences of TAK1-ASK1 interaction. TAK1 interacted with the N or C terminus of ASK1 through the C-terminal TAB2 binding domain of TAK1, with resultant inhibition of ASK1-induced AP-1 activation. Interestingly, the interaction between TAK1 and TAB2 significantly attenuated the ASK1-TAK1 interaction through the competitive interaction with ASK1 to TAK1 and resulted in the activations of TAK1-induced activations of NF-κB and AP-1. More interestingly, H₂O₂- and TNF-α-induced apoptosis in TAK1-deficient mouse embryo fibroblast cells were dramatically enhanced by overexpression of ASK1, whereas the apoptosis was markedly inhibited by the overexpression of TAK1. Overall, these results demonstrate that TAK1 and its adapter protein, TAB2, reciprocally regulate both TAK1- and ASK1-mediated signaling pathways to direct the activations of NF-κB and AP-1.

Transforming growth factor-β-activated kinase 1 (TAK1) is a member of the mitogen-activated protein kinase kinase kinase (MAP3K) family. It was originally found to function in the transforming growth factor-β (TGF-β)-mediated MAPK activation (1). TAK1 has been demonstrated to be essential in TNFα- and IL-1β-mediated activation of nuclear factor κB (NF-κB), JNK, and p38 (2–5). Several binding proteins of TAK1, including TAK1-binding protein 1 (TAB1), TAB2, TAB3, and TAB4, have been implicated to play a role in the regulation of TAK1 activity in response to TNFα and IL-1β stimulation (2–5). The activated TAK1 triggers the activation of the 1kB kinase, JNK, and p38 MAPK and eventually leads to activation of transcription factors NF-κB and activating protein-1 (AP-1) and up-regulation of many genes encoding proinflammatory cytokines, chemokines, adhesion molecules, and proteolytic enzymes (3–5). However, the molecular regulation of TAK1 activation by various stimuli remains poorly understood.

Apoptosis signal-regulating kinase 1 (ASK1), a member of the mitogen-activated protein kinase kinase kinase (MAP3K) family, is an upstream activator of JNK/p38 MAPK cascades. ASK1 regulates the JNK and p38 MAPK pathways by directly phosphorylating and thereby activating their respective MAP2Ks, MKK4 (SEK1)/MKK7 and MKK3/MKK6 (7–9). ASK1 is activated by various stresses including oxidative stress,
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endoplasmic reticulum stress, and calcium overload and by receptor-mediated inflammatory signals such as tumor necrosis factor and lipopolysaccharide (10–14). It plays critical roles in apoptosis induced by oxidative stress, tumor necrosis factor, and endoplasmic reticulum stress as well as for the regulation of innate immune responses (11, 14, 15). ASK1 has multifunctional domains that are composed of an inhibitory N-terminal domain, an internal kinase domain, and a C-terminal regulatory domain (13, 16). The C-terminal domain of ASK1 binds to TRAF proteins such as TRAF2 and TRAF6, which are required for activation of ASK1 by TNF. The N-terminal inhibitory domain of ASK1 binds to thioredoxin, glutaredoxin, and 14-3-3, which are all involved in cytokine-, stress-induced ASK1 activation, and ASK1-induced apoptosis, respectively (13, 17). Nevertheless, the cellular and molecular mechanisms for ASK1 activation are not fully understood.

Recently, the biological significance of heteromeric complex formation between different MAP3Ks has received a great deal of attention and is supposed to have synergistic advantages or distinct regulatory effects for cells to cope up with various stimuli by fine regulation of cellular responses. Although the inhibitory role of ASK1 in interleukin-1-induced NF-κB activity through disruption of TRAF6-TAK1 interaction has previously been addressed (18), at least two different issues are still unresolved; (i) whether TAK1 is able to regulate the ASK1-mediated signaling pathway for AP-1 activation and (ii) whether TAK1-binding proteins such as TAB1, TAB2, and TAB3 are also involved in the ASK1-TAK1 reciprocal regulatory mechanism. In the present study we have addressed these two issues. We have shown that TAK1 inhibits ASK1-induced AP-1 activity through the interaction between the C-terminal domain of TAK1 and the N-terminal or C-terminal domain of ASK1. Using biochemical and reporter assays, we also identified that the TAB2 protein, which is capable of binding to C-terminal TAK1, is critically involved in the activation of ASK1-induced AP-1 through disruption of ASK1-TAK1 interaction. Furthermore, we found that TAK1 inhibits ASK1-mediated apoptosis by TNF-α and H₂O₂. Collectively, our results provide biochemical and functional evidence that TAK1 and its adapter protein, TAB2, can reciprocally regulate both TAK1- and ASK1-mediated signal pathways.

EXPERIMENTAL PROCEDURES

Cell Culture and Cytokines—HEK 293 cells were maintained in Dulbecco’s modified Eagle’s medium (Sigma) containing a high concentration of glucose (4.5 mg/ml) supplemented with 10% fetal bovine serum and 100 units/ml penicillin in a 5% CO₂ atmosphere at 37°C. TAK1+/− and TAK1−/− mouse embryonic fibroblasts (MEFs) were grown in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum (19). Recombinant human IL-1β and TNF-α were purchased from Roche Applied Science. LPS was purchased from Sigma.

AP-1- and NF-κB-dependent Luciferase Reporter Assay—HEK 293 cells were plated in 12-well culture plates. On the following day, the cells were transiently transfected with the indicated expression vectors using Lipofectamine LRX (Invitrogen). The total amount of DNA was kept constant by supplementation with empty vector, pcDNA3 (Invitrogen). Every transfection step included 100 ng of reporter plasmids together with 10 ng of Renilla for normalization of transfection efficiency. As reporter plasmids, AP-1 reporter plasmid and κ-luciferase reporter plasmid (pBIIx) were used (20). After 24–30 h cells were lysed in a luciferase lysis buffer (Promega). Where indicated, cells were treated with 10 ng/ml TNF-α or 10–50 ng/ml LPS for 6 h. The lysates were divided and analyzed for firefly luciferase and Renilla activities using a luminometer. All the luciferase experiments were performed in triplicate. To confirm an appropriate expression of transfected expression plasmids, the aliquots of whole cell lysates were subjected to Western blot analysis and analyzed by immunoblotting with anti-HA and anti-GAPDH (Roche Applied Science) antibodies.

p65/p50 (NF-κB) and c-Fos/c-Jun (AP-1) DNA Binding Assay by Enzyme-linked Immunosorbent Assay—Nuclear proteins from transfectants either treated with LPS and TNF-α or in an untreated form were prepared with CellLytic™ NuCLEAR™ Extraction kit in accordance with the manufacturer’s protocol (Sigma). Activities of transcription factors, p65, p50, c-Jun, and c-Fos were determined with TransAM NF-κB or AP-1 transcription factor assay kit according to the manufacturer’s instructions (Active Motif North America, Carlsbad, CA) (21, 22). Binding of transcription factors NF-κB-p65/p50 and AP-1-c-Jun/c-Fos to the related consensus sequence on the plate-bound oligonucleotide were studied in the nuclear extracts following the manufacturer’s procedure.

Plasmids—pcDNA3-HA-ASK1 wt was kindly provided by Dr. Hidenori Ichijo. pcDNA3-HA-, Myc-TAK1 wt, and FLAG-TAB2 were generated by PCR using HeLa cDNA library as a template and inserted into pcDNA3. HA-TAK1 mutants HA-TAK1 NT1–300 and HA-TAK1 NT300–578 were generated by PCR using HA-TAK1 wt as a template and inserted into pcDNA3 (23). FLAG-ASK1 mutants FLAG-ASK1 CT278–1375, FLAG-ASK1 CT385–1375, FLAG-ASK1 CT656–1375, FLAG-ASK1 CT946–1375, FLAG-ASK1 NT1–945, FLAG-ASK1 NT1–655, FLAG-ASK1 NT1–384, FLAG-ASK1NT1–277, and FLAG-ASK1 (278–945) were generated by PCR using HA-ASK1 wt as a template and inserted into pcDNA3.

Immunoprecipitation and Western Blot Analysis—To examine protein interaction in HEK 293 cells, transfected cells were lysed in a lysis buffer containing 150 mM NaCl, 20 mM Tris-HCl, pH 7.5, 10 mM EDTA, 1% Triton X-100, 1% deoxycholate, 1.5% aprotinin, and 1 mM phenylmethylsulfonyl fluoride. Cellular debris was removed by centrifugation. Co-immunoprecipitation procedures were followed as previously described (22–25). For immunoprecipitation and Western blotting, we used anti-HA (Roche Applied Science), anti-Myc (Calbiochem), anti-FLAG (Sigma), anti-phospho-TAK1 (Thr-187) (Cell signaling), anti-TAK1 (Cell signaling), anti-phospho-ASK1 (Thr-845) (Cell signaling), anti-ASK1 (Cell Signaling), anti-phospho-κB kinase αβ (Cell Signaling), anti-κB kinase αβ (Cell Signaling), anti-phospho-JNK (Cell Signaling), anti-JNK (Cell Signaling), anti-phospho-p38 (Cell Signaling), and anti-p38 antibodies (Cell Signaling). The proteins were detected by the enhanced chemiluminescence system (Amersham Biosciences).

Cell Cycle and Apoptosis Analysis—HEK 293 transfected or non-transfected cells were harvested, washed twice with PBS, and stained with BD Cyclence™ Plus-DNA reagent kit (BD
Biosciences) in accordance with the manufacturer's protocol. Samples were analyzed with FACSCalibur system and determined with CellQuest software and Modfit LT 3.0 software (BD Biosciences) (22–25). TAK1⁻/⁻ MEF transfectant cells were additionally treated or untreated for 24 h with 100 ng/ml TNF-α, harvested, and washed twice with PBS. The cells were stained with a FITC annexin V apoptosis detection kit (BD Biosciences) in accordance with the manufacturer's protocol. Samples were analyzed with FACSCalibur system and determined with CellQuest software (BD Biosciences). TAK1⁻/⁻ MEF transfectant cells were additionally treated or untreated for 24 h with 500 μM H₂O₂, harvested, and washed twice with PBS. The cells were stained with propidium iodide solution (40 μg/ml) and 100 μg/RNase A and analyzed with the FACSCalibur system, and apoptotic cells were determined with CellQuest software (BD Biosciences).

Measurement of Cytokines—pCDNA3.0 and HA-ASK1 plasmids (1 μg per 2 × 10⁵ cells) were electro-transfected into THP-1 cells by using MP-100 Microporator (Digital Bio). After 38 h the cells were divided and treated with or without 5 ng/ml TNF-α or 10 ng/ml LPS for 9 h. Levels of human TNF-α, IL-1β, and IL-6 cytokines were measured in the supernatants according to the manufacturer's protocol (R&D Systems, Minneapolis, MN).

Statistical Analysis—Statistical significance was analyzed using Prism 4.0 (GraphPad Software, San Diego, CA). A one-way analysis of variance test was performed, and the post test was used if overall p < 0.05. Data are expressed as the mean ± S.E.

RESULTS AND DISCUSSION

Effect of ASK1 on AP-1 and NF-κB Activation.—To explore the functional roles of ASK1 in AP-1 and NF-κB activation through proinflammatory stimuli, we performed a luciferase assay by using AP-1- and NF-κB-dependent reporter genes, respectively. Overexpression of ASK1 significantly activated AP-1 activity in a dose-dependent manner (Fig. 1A, columns 1–4). Moreover, TNF-α or LPS-induced AP-1 activities were markedly enhanced by the overexpression of ASK1 in a dose-dependent manner (Fig. 1A, columns 6–8, TNF-α and columns 10–12, LPS). Overexpression of ASK1 clearly reduced the basal activity of NF-κB (Fig. 1B, columns 2–4) as compared with that of control (Fig. 1B, column 1). Interestingly, TNF-α- or LPS-induced NF-κB activities were also significantly reduced by the overexpression of ASK1 in a dose-dependent manner (Fig. 1B, columns 6–8, TNF-α and columns 10–12, LPS). To rule out a possibility that overexpression of ASK1 affects the endogenous expression of TAK1, TAK1 expression was assessed. No significant changes in endogenous TAK1 expression could be detected (Fig. 1A, and B, IB:TAK1), indicating that the observed negative effect of ASK1 is not associated with the reduced expression of TAK1. To confirm luciferase assays, we further analyzed DNA binding activities of p65 and p50 for NF-κB and of c-Fos and c-Jun for AP-1, respectively, using a DNA binding ELISA-based method (21, 22). According to the stimulation of LPS or TNF-α, there was a marked increase in p65 and p50 DNA binding activities (Fig. 1, C, Mock+LPS and D, Mock+TNF-α), whereas a significant decrease was observed in ASK1-overexpressed cells (Fig. 1, C, ASK1+LPS, and D, ASK1+TNF-α). However, there was an increase in DNA binding activities of c-Fos and phosphorylated c-Jun with LPS and TNF-α stimulations (Fig. 1, E, Mock+LPS, and F, Mock+TNF-α), and a significant enhancement was observed in ASK1-overexpressed cells (Fig. 1, E, ASK1+LPS and F, ASK1+TNF-α). The results were consistent with those of AP-1- and NF-κB-dependent luciferase assays, shown in Fig. 1. These results suggest that ASK1 overexpression and LPS/TNF-α have additive effects on the activation of AP-1 but an inhibitory effect on the activation of NF-κB in response to proinflammatory stimulations.

TAK1 Is Negatively Involved in ASK1-induced AP-1 Activation.—A previous report has shown that ASK1 inhibits interleukin-1-induced NF-κB activity through disruption of TRAF6-TAK1 interaction (18). We further assessed whether TAK1 is able to regulate ASK1-induced AP-1 activity. In contrast to the activity of ASK1, the overexpression of TAK1 alone had no effect on the basal luciferase activity of AP-1 (Fig. 2A, Myc-TAK1 alone) and on DNA binding activities of c-Fos and phosphorylated c-Jun (Fig. 2B, Myc-TAK1 alone). Interestingly, ASK1-induced AP-1 activation was significantly reduced by the overexpression of TAK1 in a dose-dependent manner (Fig. 2A). Consistently, c-Fos and phosphorylated c-Jun DNA binding activities were also inhibited by the overexpression of TAK1 (Fig. 2B) compared with that of ASK1 alone (Fig. 2B, HA-ASK1 alone), indicating that TAK1 may be negatively involved in ASK1-induced AP-1 activation. We further tested whether the inducible AP-1 activities by TNF-α, IL-1β, and LPS are also inhibited by the overexpression of TAK1. TNF-α, IL-1β, and LPS stimulations resulted in increases in both AP-1-dependent luciferase and c-Fos and phosphorylated c-Jun DNA binding activities (Fig. 2, C and D, columns 2–4), and these activities were significantly enhanced in the presence of ASK1 overexpression (Fig. 2, C and D, column 7, 9, and 11). However, the overexpression of TAK1 markedly inhibited these inducible AP-1-dependent luciferase and c-Fos and phosphorylated c-Jun DNA binding activities (Fig. 2, C and D, column 8, 10, and 12). These results strongly suggest that TAK1 may be negatively involved in ASK1-induced AP-1 activation, although the molecular mechanism still remains to be addressed.

Molecular and Functional Interaction between ASK1 and TAK1-TAB1-TAB2 Complex—Subsequently, the molecular mechanism by which ASK1 activity is regulated by TAK1 was explored. TAK1 strongly interacted with ASK1 in the co-overexpression experiment (Fig. 3A, lane 3) and also endogenously co-immunoprecipitated with ASK1 in the unstimulated condition (Fig. 3, B and C, lanes 1 and 3). Additionally, TAK1-binding protein TAB1 was also co-precipitated with the TAK1-ASK1 complex (Fig. 3B, IB: anti-TAB1, lanes 1 and 3). To verify the TAK1-TAB1-ASK1 ternary complex, we performed co-overexpression experiment. As shown in Fig. 3D, TAK1 was significantly co-immunoprecipitated with ASK1 and TAB1, indicating that in resting conditions ASK1 may exist in a complex form as ASK1-TAK1-TAB1. Because these two MAP3Ks are inactive in the resting condition, we hypothesized that ASK1-TAK1 interaction may be dissociated to be involved in each MAP3K-induced signal pathways by exogenous stimuli. Upon stimula-
tions with TNF-α and LPS, phosphorylations of TAK1 (Thr-187) and ASK1 (Thr-845) were significantly induced, and other downstream signals, p38 and JNK, were also activated (Fig. 3E).

As expected, the interaction between ASK1 and TAK1 was significantly decreased by both TNF-α/H9251 and LPS stimulations as compared with the case of unstimulated cells (Fig. 3, B and C, and F). However, no significant changes could be detected in the interaction between TAK1 and TAB1 (Fig. 3B, IB: anti-TAB1, lanes 2 and 4) as compared to without stimulations (Fig. 3B, IB: anti-TAB1, lanes 1 and 3). These results propose that the inactive form of TAK1-TAB1-ASK1 complex might exist in the resting condition, whereas upon the stimulation, TAK1-ASK1 complex may be dissociated to be involved in each MAP3K-induced signal pathways.

ASK1 and TAK1 have different functional domains that are capable of inducing interactions with each regulatory protein for modulating ASK1 or TAK1 activity (2–5, 13, 16, 17). To identify the interacting domain of ASK1 and TAK1, we generated seven different truncated mutants of ASK1 based on the functional domains (Fig. 3F) and performed a co-immunoprecipitation assay with TAK1. As shown in Fig. 3, G–H, TAK1 interacted with the N-terminal thioredoxin binding domain and the C-terminal CC domain of ASK1. To verify the molecular interaction between ASK1 and TAK1, we further per-
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Upon stimulations with TNF-α, IL-1β, LPS, or LPS, interestingly, the interaction between TAK1 and TAB2, in a signal-dependent manner, we performed an endogenous immunoprecipitation assay with anti-TAK1 antibody. Endogenous TAK1 was significantly co-immunoprecipitated with ASK1 and TAB1 but not with TAB2 (Fig. 4D, lane 1). Upon stimulations with TNF-α or LPS, interestingly, the interaction between TAK1 and TAB2 markedly increased (Fig. 4D, IB: TAB2, lane 2 and lane 3), whereas the interaction between TAK1 and ASK1 dramatically reduced (Fig. 4D, IB: ASK1, lane 2 and 3), indicating a functional role of TAB2 in the ASK1-mediated signaling pathway through the interruption of ASK1-TAK1 interaction. However, there were no significant changes in the interaction between TAK1 and TAB1 as compared with that of without stimulation (Fig. 4D, IB: TAB1). It was consistent with that of Fig. 3B.

To address the functional role of TAB2 in ASK1-TAK1 reciprocal regulation, we further performed AP-1-dependent and NF-κB-dependent luciferase assays in the presence or absence of TAB2 protein. Overexpression of TAK1 alone had no effect on the basal activity of AP-1 and NF-κB (Fig. 4, E and F, TAK1 alone). However, both AP-1 and NF-κB activities were significantly enhanced during the co-overexpression of TAK1 and TAB2 (Fig. 4, E and F, TAK1 + TAB2), indicating that TAB2 may be a key regulator protein in the TAK1-induced NF-κB and AP-1 activations (26–28). Interestingly, an overexpression of ASK1 dramatically inhibited TAK1-TAB2-induced NF-κB and AP-1 activations (Fig. 4, E and F, ASK1 + TAK1 + TAB2). The inhibitory effect showed a dose-dependent manner in NF-κB and AP-1 activations (Fig. 4, G, columns 3–5, and H, columns 3–5). Moreover, co-expressions of TAK1 and TAB2 also significantly inhibited ASK1-induced AP-1 activation as compared with that of ASK1 alone (Fig. 4F, 1 h, and subunit-specific c-Jun/c-Fos ELISA assay were performed using 2 μg of nuclear extracts derived from each sample, as indicated. Absorbance was measured at 450 nm. The values indicated represent fold-increases when compared with that of mock transfectants. *, p < 0.05.

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**FIGURE 2. Inhibitory effect of TAK1 on ASK1-induced AP-1 activation.**

A, HEK 293 cells were transiently cotransfected with AP-1 reporter, Renilla, HA-ASK1, and different doses of Myc-TAK1 plasmids. After 24 h luciferase activities were determined by dual luciferase assay. The values indicated represent normalized luciferase activities and are shown as the mean ± S.E. based on triplicate assay. Three independent experiments produced similar results. Western blotting analyses of the same lysates from each transfection are shown in the lower panels. *, p < 0.01. B, HEK 293 cells were transiently cotransfected with HA-ASK1 or Myc-TAK1 as indicated. After 30 h, subunit-specific c-Jun/c-Fos ELISA assay was performed using 2 μg of nuclear extracts derived from each sample as indicated. Absorbance was measured at 450 nm. The values indicated represent fold-increases when compared with that of mock transfectants. *, p < 0.05. C, HEK 293 cells were transiently cotransfected with AP-1 reporter, Renilla, HA-ASK1, and Myc-TAK1 plasmids as indicated. After 24 h, cells were treated or untreated with TNF-α (10 ng/ml), IL-1β (10 ng/ml), and LPS (10 ng/ml) for 6 h, and the luciferase activities were determined by dual luciferase assay. The values indicated represent normalized luciferase activities and are shown as the mean ± S.E. based on triplicate assay. Three independent experiments produced similar results. *, p < 0.05. D, HEK 293 cells were transiently cotransfected with HA-ASK1 and Myc-TAK1 plasmids as indicated. After 30 h, cells were treated or untreated with TNF-α (10 ng/ml), IL-1β (10 ng/ml), and LPS (10 ng/ml) for 6 h, and the luciferase activities were determined by dual luciferase assay. The values indicated represent normalized luciferase activities and are shown as the mean ± S.E. based on triplicate assay. Three independent experiments produced similar results. *, p < 0.05.
According to the dose-dependent expression of TAB2, interestingly, the inhibitory effects of ASK1 on TAK1-induced NF-κB and AP-1 activations were significantly attenuated and induced gradual increases of NF-κB and AP-1 in a dose-dependent manner (Fig. 4, G, columns 7–9, and H, columns 7–9), as compared with that of co-expressions of ASK1 and TAK1 (Fig. 4, G, column 6, and H, column 6). These results indicate that TAB2 interrupts the ASK1-TAK1 interaction through the competitive interaction with ASK1 to the C-terminal domain of TAK1 and then TAK1-TAB2 complex, and free ASK1 induces AP-1 activation (see Fig. 6B).
FIGURE 4. Identification of molecular and functional interaction between ASK1 and TAK1-TAB2 complex. A, shown is a schematic representation of wild-type and mutant TAK1 proteins. Two truncated TAK1 mutants, NT1–3000 and CT300–578, as indicated were generated. B, HEK 293 cells were transiently cotransfected with HA-TAK1 wt (1 μg), HA-TAK1 NT1–300 mutant (1 μg), HA-TAK1 CT300–578 (1 μg), and Myc-ASK1 wt (1 μg) as indicated. After 36 h, transfected cells were extracted and immunoprecipitated (IP) with anti-HA antibody. The interaction was detected by Western blotting (IB) with anti-Myc antibody. The same lysates were verified with anti-HA and Myc antibodies. C, HEK 293 cells were transiently cotransfected with HA-TAK1 wt (1 μg), Myc-ASK1 (1 μg), and different concentrations of Flag-TAB2, as indicated. After 36 h, transfected cells were extracted and immunoprecipitated with anti-HA antibody. The interaction was detected by Western blotting with anti-Myc or anti-FLAG antibody. The same lysates were verified with anti-HA, anti-Myc, and anti-FLAG antibodies. D, HEK 293 cells were treated or untreated with TNF-α (10 ng/ml) and LPS (10 ng/ml) for 45 min as indicated. Cells were extracted and immunoprecipitated with anti-TAK1 antibody. The interaction was detected by Western blotting with anti-ASK1, anti-TAB2, or anti-TAB1 antibody. The same lysates were verified with anti-TAK1 antibody. The interaction was detected by Western blotting with anti-ASK1, anti-TAB2, or anti-TAB1 antibody. The same lysates were verified with anti-TAK1 antibody. The interaction was detected by Western blotting with anti-ASK1, anti-TAB2, or anti-TAB1 antibody. The same lysates were verified with anti-TAK1 antibody.

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We further tested whether the inducible AP-1 and NF-κB activities by TNF-α and LPS are also regulated by the overexpression of TAB2. Co-expressions of TAK1 and TAB2 significantly enhanced AP-1 and NF-κB activities in unstimulated condition (Fig. 5, A and B, column 5), and these activities were increased by TNF-α or LPS stimulations (Fig. 5, A and B, columns 11 and 17). In the presence of overexpression of ASK1, however, TAK1-TAB2-induced AP-1 and NF-κB activities were markedly attenuated in both the presence and absence of exogenous stimuli such as TNF-α and LPS (Fig. 5, A and B, columns 6, 12, and 18). These results demonstrate that, as depicted in Fig. 6B, the reciprocal regulation of ASK1, TAK1, and TAB2 occurs in the condition of exogenous stimuli. Based on our results, we propose possible models; as can be seen in Figs. 3–5, in resting conditions TAK1-ASK1 complexes may exist in at least three different inactive forms depicted in Fig. 6A as follows; (i) ASK1 binds with the TAK1-TAB1 complex through the C-terminal domain of ASK1, and (ii) ASK1 binds with the TAK1-TAB1 complex through the N-terminal domain of ASK1 and the C-terminal domain of ASK1. Additionally, ASK1 exerts inhibitory effects on TAK1-induced NF-κB and AP-1 activation through the competitive interaction with TAB2 to TAK1 molecules (Fig. 6D) and also TAK1 exerts inhibitory effects on ASK1-induced AP-1 activation through the interaction with either or both C-terminal or/and N-terminal domain of ASK1 (Fig. 6, C and D).

TAK1 or ASK1 Functionally Inhibits ASK1-mediated Apoptosis by Oxidative Stress or TAK1-mediated Signal, Respectively—The ASK1 and its downstream stress-activated kinases p38 and JNK constitute an important mammalian signaling pathway that can promote cell survival, apoptosis, proliferation, or differentiation depending on the cell type and/or cellular context (12, 13, 29–32). ASK1 is activated by various stresses including oxidative stress, endoplasmic reticulum stress, and calcium overload and by receptor-mediated inflammatory signals such as tumor necrosis factor and lipopolysaccharide (10–13). ASK1 is required for apoptosis induced by oxidative stress, tumor necrosis factor, and endoplasmic reticulum stress. Therefore, we finally investigated whether TAK1 is functionally able to regulate ASK1-induced apoptosis by TNF-α or H$_2$O$_2$. Overexpression of ASK1 in HEK 293 significantly induced apoptotic cell death when compared with that of mock-transfected cells (Fig. 7A, 25 ± 2% in mock versus 10 ± 2% in ASK1). Interestingly, apoptosis was significantly decreased during the overexpression of TAK1 (Fig. 7A, 25 ± 2% in ASK1 versus 14 ± 2% in ASK1+TAK1). These results demonstrate that TAK1 may negatively regulate ASK1-induced apoptosis, presumably through the interaction with ASK1 as shown in Fig. 3. To verify further the function of inter-regulation between TAK1 and ASK1, we performed TNF-α- or H$_2$O$_2$-induced apoptosis assay in TAK1$^{-/-}$ MEF cells. It is well known that TAK1-deficient cells are highly sensitive in response to TNF-α (5). Consistently, TNF-α treatment strongly induced apoptosis in TAK1$^{-/-}$ MEF cells when compared with that of without TNF-α (Fig. 7B, ~16% in mock without TNF-α versus ~30% in mock with TNF-α). However, overexpression of TAK1 significantly reduced TNF-α-induced apoptosis as compared with that of mock (Fig. 7B, ~22% in TAK1 with TNF-α versus ~30% in mock with TNF-α). Overexpression of ASK1 markedly enhanced the levels of TNF-α-induced apoptotic cells compared with that of mock transfectant (Fig. 7B, ~60% in ASK1 + with TNF-α versus ~30% in mock with TNF-α). Interestingly, apoptosis was significantly inhibited by co-expression of TAK1 when compared with that of ASK1 alone (~60% in ASK1+TNF-α versus ~40% in ASK1+TAK1 with TNF-α). In addition, treatment with 500 μM H$_2$O$_2$ markedly induced apoptotic cell death when compared with the condition without treatment (Fig. 7C, 4 ± 2% in mock + without versus 38 ± 4% in mock + with H$_2$O$_2$), and marked increase in the apoptotic cell death was seen during the overexpression of ASK1 (Fig. 7C, 50 ± 5). Consistently, apoptosis was inhibited by the co-overexpression of TAK1 as compared with that of ASK1 alone (Fig. 7C, 29 ± 4% in ASK1+TAK1 versus 50 ± 5% in ASK1). These results strongly demonstrate that TAK1 func-

![Figure 5. Inducible regulation to activate AP-1 or NF-κB between ASK1 and TAK1-TAB2. A, HEK 293 cells were transiently cotransfected with AP-1 reporter, Renilla, Myc-TAK1, FLAG-TAB2, and HA-ASK1 as indicated. After 30 h, cells were treated or untreated with TNF-α (10 ng/ml) and LPS (10 ng/ml) for 6 h, and the luciferase activities were determined by dual luciferase assay. The values indicated represent normalized luciferase activities and are shown as the mean ± S.E. based on triplicate assay. Three independent experiments produced similar results.

B, HEK 293 cells were transiently cotransfected with κ-luciferase reporter plasmid (pBIIx), Renilla, Myc-TAK1, FLAG-TAB2, and HA-ASK1 as indicated. After 30 h, cells were treated or untreated with TNF-α (10 ng/ml) and LPS (10 ng/ml) for 6 h, and the luciferase activities were determined by dual luciferase assay. The values indicated represent normalized luciferase activities and are shown as the mean ± S.E. based on triplicate assay. Three independent experiments produced similar results.

* indicates p < 0.05. **, HEK 293 cells were transiently cotransfected with κ-luciferase reporter plasmid (pBIIx), Renilla, Myc-TAK1, FLAG-TAB2, and HA-ASK1 as indicated. After 30 h, cells were treated or untreated with TNF-α (10 ng/ml) and LPS (10 ng/ml) for 6 h, and the luciferase activities were determined by dual luciferase assay. The values indicated represent normalized luciferase activities and are shown as the mean ± S.E. based on triplicate assay. Three independent experiments produced similar results.
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**Figure 6. Model of reciprocal regulation between ASK1 and TAK1-TAB2.** A, in resting conditions, three different TAK1-ASK1 complexes might have existed, ASK1-TAK1-TAB1 complex (i), TAK1-TAB1-ASK1 complex (ii), and TAK1-TAB1-ASK1-TAK1-TAB1 complex (iii). B–D, ASK1 may competitively interact with TAB2 through the C-terminal domain of TAK1. The preferential interaction of TAB2 to TAK1-TAB1 complex induces TAK1-mediated NF-κB and AP-1 activations (8). In contrast, the interaction of TAK1-TAB1 complex to C terminus of ASK1 (C) or the interaction of TAK1-TAB1 complex to N terminus of ASK1 (D) interrupts the interaction between TAK1-TAB1 complex and TAB2 and thereby inhibits ASK1-mediated AP-1 activation and TAK1-mediated NF-κB/AP-1 activations.

Conclusions and Remarks—So far there are at least 21 characterized MAP3Ks that activate downstream signal molecules through the various molecular clusters based on protein homology (33). Although these MAP3Ks provide specificity for stimulus-dependent activation of MAPK pathways through unique protein-protein interactions and phosphorylation of downstream signaling effectors, several previous reports have demonstrated either interpositive or -negative regulation in different MAP3Ks through heteromeric complex formation (6, 18). In terms of both functional specificity and biological significance, the interregulation of MAP3Ks has received a great deal of attention because of their divergent biological activities, which significantly affect the cell fate, differentiation, development, and tissue homeostasis (33).

In this study we provide evidence that the formation of functional heteromeric complex between TAK1 and ASK1 is important for the activation of the MAP3Ks by the extracellular stimuli. Our current results demonstrate that in resting conditions, the TAK1 complex is constitutively associated with its adapter protein TAB1 and forms a stable complex with ASK1 through the molecular interaction between C-terminal domain of TAK1 and N-terminal domain of ASK1. As depicted in Fig. 6A, the molecular interaction between TAK1-TAB1 and ASK1 might be possible to form three different heteromeric complexes. Indeed, we found that TAK1 endogenously co-immunoprecipitated with ASK1 and TAB1 in resting conditions (Figs. 3B and 4D), indicating that the molecular interaction might contribute in maintaining the preferential inactive forms of the respective MAP3Ks. As we propose possible models in Fig. 6, C and D, the molecular complexes between TAK1-TAB1 and ASK1 may reciprocally induce the inhibition of ASK1-induced AP-1 or TAK1-induced NF-κB and AP-1 activation. We further found that upon stimulations such as TNF-α and LPS, the TAK1-ASK1 complex was significantly dissociated from each other and resulted in the involvement of activations of specific MAP3K (Figs. 3, B and C, and 4D). Interestingly, TAB2, another TAK1-binding protein, preferentially interacted with TAK1 through the interacting domain with ASK1, with subsequent inhibition of TAK1-ASK1 interaction, and resulted in activations of both ASK1 and TAK1-mediated signaling (Figs. 4 and 5). These results indicate that TAB2 may be a potential regulator for the reciprocal regulation between TAK1 and ASK1 through the interruption of TAK1-ASK1 interaction.
depicted in Fig. 6B. In addition, we found that in terms of functional aspects, TAK1 or ASK1 functionally inhibited ASK1-mediated apoptosis by TNF-α/H9251 and H2O2 or TAK1-mediated signal, respectively (Fig. 7).

In conclusion, based on the previous report that ASK1 inhibits TAK1-mediated NF-κB activation through disruption of TRAF6-TAK1 interaction (18), our results demonstrate that TAK1 and its adapter protein, TAB2, can also reciprocally regulate both ASK1 and TAK1-mediated signaling for the activations of NF-κB and AP-1. However, a possibility that ASK1-associated proteins including thioredoxin, Prdx1, and other ASK family proteins may be involved in the reciprocal regulation of TAK1-ASK1 complex is not absolutely ruled out. Therefore, it is clear that additional biochemical and functional analysis will be necessary to fully elucidate the details as how the different MAP3Ks, especially TAK1 and ASK1, functionally interact and thereby regulate divergent biological activities.

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