ASK1 Inhibits Interleukin-1-induced NF-κB Activity through Disruption of TRAF6-TAK1 Interaction*

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Apoptosis signal-regulating kinase 1 (ASK1) is a member of the MAPKKK family in the JNK and p38 mitogen-activated protein kinase cascades and critically involved in stress- and cytokine-induced apoptosis. The transcription factor nuclear factor-κB (NF-κB) is a pivotal regulator of immune and inflammatory responses and exerts anti-apoptotic roles in various cells. Here we show that ASK1 directly interacts with transforming growth factor-β-activated kinase 1 (TAK1), another MAPKKK that has been identified as a signaling intermediate in the interleukin 1 (IL-1)-induced NF-κB pathway as well as the transforming growth factor-β superfamily-induced JNK/p38 pathway. Overexpression of ASK1 inhibits IL-1-, TRAF6-, or TAK1-induced, but not NF-κB-inducing kinase-induced, NF-κB activation. ASK1 dissociates TAK1 but not NF-κB-inducing kinase from TRAF6. Moreover, IL-1-induced complex formation of endogenous TAK1 and TRAF6 was blocked by ASK1 overexpression. It thus appears that the inhibition of NF-κB by ASK1 may result at least in part from the disruption of the TRAF6/TAK1 complex formation in the IL-1 signaling pathway. These results provide a new insight in the mode of action of MAPKKK family members; two distinct MAPKKKs in the same MAP kinase cascades directly interact and exert opposite effects in another signaling pathway, NF-κB.

In response to various extracellular stimuli, mitogen-activated protein kinases (MAPKs) are activated or inactivated and regulate a wide variety of cellular responses including gene expression, cell growth, differentiation, and apoptosis (1–4). In the MAPK signaling cascades, MAPK kinase (MAPKK) phosphorylates and activates MAPK, and MAPKKK is phosphorylated and activated by an immediately upstream kinase termed MAP kinase kinase (MAPKKK). The MAP cascade (MAPKKK-MAPKK-MAPK) is evolutionarily conserved and plays essential roles from yeast to metazoan (5–8). Apoptosis signal-regulating kinase 1 (ASK1) was identified as a member of the MAPKKK family that activated two different MAPK cascades, SEK1/MKK7-c-Jun N-terminal kinase (JNK; also called stress-activated protein kinase (SAPK)) and MKK3/MKK6-p38 pathways (9). Overexpression of wild-type or the constitutively active form of ASK1 has been reported to induce apoptosis in various cell types (9–11), and the kinase-inactive mutant of ASK1 inhibited apoptosis induced by tumor necrosis factor (TNF), Fas ligation, anti-cancer drugs, or withdrawal of neurotrophic factors (9, 11–14). ASK1 has thus been implicated in cytokine- and stress-induced apoptosis. On the other hand, we have recently found that in naive PC12 cells moderate expression of a constitutively active form of ASK1 induced neuronal differentiation or even survival (15). In addition, low and high expression of exogenous ASK1 in keratinocytes induced differentiation and apoptosis, respectively.3 These results suggest that ASK1 has a broad range of biological activities depending on cell types, cellular context, or the extent of ASK1 activation. Importantly, ASK1 expression was highly but transiently induced upon epithelial wounding (17) and spinal cord injury (18), indicating that regulation of ASK1 expression may also be an important step to control pathophysiological roles of ASK1 in vivo.

Transforming growth factor-β (TGF-β)-activated kinase 1 (TAK1), another MAPKKK family protein (19), can also be activated by a number of stimuli, including TGF-β, TNF, Fas, IL-1, ultraviolet, sorbitol, and ceramide (19, 20), and stimulates MKK1/MKK7-JNK and MKK3/MKK6-p38 pathways (19–21). In addition, TAK1 mediates the IL-1-induced nuclear factor-κB (NF-κB) activation (22); IL-1 induces recruitment of TAK1 to an adaptor protein known as TRAF6 and thereby activates TAK1 kinase activity. Activated TAK1 phosphorylates and activates another MAPKKK-like kinase named NIK (NF-κB-inducing kinase), which ultimately induces NF-κB activation through the IκB kinase-IκB-NF-κB cascade. NF-κB plays a

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1 The abbreviations used are: MAPK, mitogen-activated protein kinase; MAPKK, MAPKKK, MAP kinase kinase; ASK1, apoptosis signal-regulating kinase 1; SEK1, SAPK/extracellular signal regulated kinase 1; JNK, c-Jun N-terminal kinase; SAPK, stress-activated protein kinase; TNF, tumor necrosis factor; TGF-β, transforming growth factor β; TAK1, TGF-β-activated kinase; IL-1, interleukin-1; NF-κB, nuclear factor-κB; TRAF, TNF receptor-associated factor; NIK, NF-κB-inducing kinase; IκB, inhibitor protein of NF-κB; HA, hemagglutinin; TAB1, TAK1-binding protein 1; SDS, sodium dodecyl sulfate; MEKK1, mitogen-activated protein kinase/extracellular

2 K. Sayama, Y. Hanakawa, Y. Shirakata, K. Yasasaki, Y. Sawada, L. Sun, K. Yamanishi, H. Ichijo, and K. Hashimoto, submitted for publication.

3 Laser signal regulated kinase kinase kinase 1; WT, wild type.
broad range of roles in controlling gene expression such as inflammatory cytokines, cell adhesion molecules, chemokines, interferons, growth factors, and viruses (23). Interestingly, it has been suggested that the activation of NF-κB leads to protection of cells from apoptosis. Mice lacking RelA/p65, a member of the NF-κB family, die embryonically from extensive apoptosis in the liver (24). In addition, the sensitivity to TNF-induced apoptosis is enhanced in some cells expressing a dominant negative form of IkB (25, 26). These observations suggest a close link between death and survival signals involving MAPK and NF-κB cascades.

In the present study, we identified TAK1 as an interacting partner of ASK1. ASK1 inhibited IL-1-, TRAF6-, or TAK1- but not NIK-induced NF-κB activation. ASK1 dissociated TAK1 but not NIK from TRAF6, suggesting that the disruption of the IL-1-induced TRAF6-TAK1 complex may be one of the mechanisms how ASK1 inhibits IL-1-induced NF-κB activity.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Cytokine**—The human embryonic kidney 293 cells and IL-1 type I receptor-transfected 293 cells (293 IL-1 RI) 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. Recombinant human IL-1 β was purchased from Roche Molecular Biochemicals.

**Yeast Two-hybrid System**—A human fetal brain cDNA library in the pJG4–5 prey plasmid was screened for proteins that interact with ASK1-R709R using the EGY188 yeast host strain as described previously (10). Plasmids of positive clones were recovered; the cDNA inserts were sequenced, and TAK1 (347–579 amino acids) was identified. To assay the interaction between TAK1 and mutant ASK1, TAK1 and

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**FIG. 1**. Interaction of TAK1 with ASK1. A, interaction of TAK1 with wild-type and mutant ASK1 in yeast. The β-galactosidase reporter plasmid and the plasmid encoding full-length TAK1 fused to the DNA-binding domain were cotransformed into EGY48 yeast strain with the plasmids encoding the indicated forms of ASK1 fused to the transcriptional activation domain. Each transformant was streaked onto indicator plates. Positive interactions are shown by a +. B, interaction of TAK1 with wild-type and mutant ASK1 in mammalian cells. 293 cells were transiently cotransfected with Myc-TAK1 (2 μg; lanes 2–6) and HA-tagged wild-type and mutant ASK1 (2 μg; lanes 3–6). Transfected cells were extracted and immunoprecipitated (IP) with anti-HA antibody. The interaction was detected by Western blotting (WB) with anti-Myc antibody (top panel). The presence of HA-ASK1 (middle panel) and Myc-TAK1 (bottom panel) in the same lysates was verified by Western blot analysis. Markers of molecular mass are shown on the left. C, schematic representation of wild-type and mutant ASK1 proteins. The kinase domain is shown by the cross-hatched boxes. Positive interaction with wild-type TAK1 in yeast and 293 cells is shown by a +.
FIG. 2. Effect of ASK1 protein on NF-κB activation. A, ASK1 inhibits IL-1-induced NF-κB activation. 293 cells were transiently cotransfected with Ig-κ-luciferase reporter plasmid, Renilla plasmid, and HA-ASK1-WT as indicated. After 24 h, cells were left untreated or treated for 6 h with IL-1, and the luciferase activities were determined. The values indicated represent normalized luciferase activities and are shown as mean ± S.D. based on duplicate assays. Three independent experiments produced similar results. B, ASK1 inhibits TAK1-induced NF-κB activation. 293 cells were transiently transfected with Ig-κ-luciferase reporter plasmid, β-galactosidase plasmid, Myc-TAK1 (0.5 μg; lanes 7–22), TAB1 (0.5 μg; lanes 7–22), and HA-tagged wild-type and mutant ASK1 as indicated. Twenty-four hours after transfection, cells were harvested, and the luciferase activities were determined as in A. Western blotting of the same lysates from each transfection is shown in the lower panels. C, ASK1 inhibits TRAF6-induced NF-κB activation in a dose-dependent manner. 293 cells were transiently transfected with FLAG-TRAF6 (0.3 μg; lanes 7–20) and HA-tagged ASK1 as indicated. After 24 h, the luciferase activities were determined. Western blots of the same lysate from each transfection is shown in the lower panels. D, ASK1 has no inhibitory effects on NIK-induced NF-κB activity. Myc-NIK (0.5 μg; lanes 2 and 4–6) and HA-ASK1-WT were transiently transfected into 293 cells for 24 h before the measurement of the luciferase activities. The luciferase assays were performed as described in C.

ASK1 constructs were cotransformed along with the reporter plasmid pJK103 into EGY48 yeast strain. Then Ura" Trp" His" transformants were tested on 5-bromo-4-chloro-3-indolyl-D-galactopyranoside (X-gal; Calbiochem)-containing plates.

cDNA, Adenovirus, Antiserum, and Transfections—Hemagglutinin (HA) or Myc-tagged ASK1 cDNAs and adenovirus constructs encoding HA-tagged wild-type ASK1 or β-galactosidase have been described previously (9, 10, 11, 27). The full length of TAK1 yeast expression plasmid, HA- or Myc-tagged TAK1 expression plasmid, TAB1 expression plasmid, and the rabbit polyclonal antibody against TAK1, TAB1, and TRAF6 were previously described (22, 28, 29). FLAG- or Myc-tagged NIK and FLAG-tagged TRAF6 have been described (30, 31). Myc-tagged TRAF6 was constructed by polymerase chain reaction amplification. Transfection was performed with Tfx-50 (Promega) according to the manufacturer's instructions.

Immunoprecipitation and Western Blot Analysis—To examine protein interaction in 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 μg/ml phenylmethylsulfonyl fluoride. Cellular debris was removed by centrifugation, and the lysates were divided and incubated with 1 μg of the anti-HA antibody (Clone 12CA5, Roche Molecular Biochemicals), 5 μg of the anti-FLAG antibody (Clone M2, SIGMA), or 1 μg of the anti-Myc antibody (Clone 9E10, Calbiochem). After addition of protein A+, or protein G-Sepharose 4B conjugate (Zymed Laboratories Inc.), the lysates were incubated for an additional 30 min, and the beads were washed two or three times with the lysis buffer. Proteins bound to the beads were solubilized in SDS sample buffer (100 mM Tris-HCl, pH 8.8, 0.01% bromphenol blue, 36% glycerol, 4% SDS) in the presence of 10 mg/ml dithiothreitol, separated by SDS-polyacrylamide gel electrophoresis. The gel was transferred to nitrocellulose membrane (Hybond-C-super, Amersham Pharmacia Biotech) and analyzed by immunoblotting with anti-Myc, anti-FLAG, or anti-HA (Clone 3F10, Roche Molecular Biochemicals) antibody. The aliquots of whole cell lysates were subjected to Western blot analysis to confirm appropriate expression of transfected expression plasmids. The proteins were detected by the enhanced chemiluminescence system (Amersham Pharmacia Biotech).

NF-κB-dependent Luciferase Reporter Assay—The 293 cells were seeded in 6-well culture plates. On the following day, the cells were transiently transfected with the indicated expression vectors using Tfx-50 (Promega). The total amount of cDNA was kept constant by supplementation with empty vector, pcDNA3 (Invitrogen). Every transfection included 100 ng of the reporter plasmid, together with either 10 ng of pSV7-β-galactosidase or pEF-Renilla for normalization of transfection efficiency. As a reporter plasmid, the Ig-κ-luciferase reporter gene that contains three tandem repeats of κB motifs was used (22). After 24 h, cells were lysed in a luciferase lysis buffer (Promega). Where indicated, cells were treated with 50 ng/ml IL-1β for 6 h. The lysates were divided and analyzed for firefly luciferase and Renilla activities using a luminometer (LB953, EG & G Berthold). β-Galactosidase activities were determined by the β-galactosidase enzyme assay system (Promega) using a plate reader at 405 nm (Immuno-mini NJ-2900,
Intermed). All of the luciferase experiments were performed in duplicate. To confirm appropriate expressions of transfected plasmids, the lysates of duplicated wells were combined and subjected to Western blot analysis using the indicated antibodies. The expression of transfected plasmid was verified by Western blotting in the same lysates. Essentially identical results were obtained when an E-selectin promoter-derived NF-κB luciferase reporter was used in the same sets of experiment (data not shown).

RESULTS AND DISCUSSION

Interaction of TAK1 with ASK1—To explore the roles of ASK1, we have employed a yeast two-hybrid screening using kinase-inactive form of ASK1 as bait (10). Thioredoxin was recently isolated by this assay and was shown to be a physiological inhibitor of ASK1 (10). During the course of screening, we identified several positive clones that are unrelated to thioredoxin. DNA sequencing analysis revealed that one of them encoded a member of MAPKKK family known as TAK1. To localize the TAK1-interacting region in ASK1, we tested a set of ASK1 deletion mutants for TAK1 binding by a two-hybrid assay (Fig. 1A). Wild-type (ASK1-WT), N-terminal (ASK1-NT), and C-terminal (ASK1-CT) fragments of ASK1, but not a fragment of the kinase domain alone (ASK1-K), directly interacted with full-length TAK1 (Fig. 1A). We next determined whether the association between ASK1 and TAK1 occurs in mammalian cells. Myc-tagged wild-type TAK1 (Myc-TAK1) was transiently transfected in 293 cells together with expression plasmids encoding HA-tagged mutant forms of ASK1. Cells were extracted and immunoprecipitated with anti-HA antibody, and coimmunoprecipitated TAK1 was detected by immunoblotting with anti-Myc antibody (Fig. 1B, top panel). Consistent with the
two-hybrid assays, TAK1 can be coimmunoprecipitated with ASK1-WT, ASK1-NT, and ASK1-CT but not with ASK1-K (Fig. 1, B and C). These results indicate that ASK1 can form a complex with TAK1 through its N- and C-terminal noncatalytic domains.

**Effect of ASK1 on Nuclear Factor-κB Activation**—Since ASK1 and TAK1 have both been shown to activate JNK and p38 MAPK cascades, we first examined whether formation of ASK1-TAK1 complex within the cells may synergize to activate these MAPK cascades. To this end, expression vectors of JNK or p38 were cotransfected in 293 cells together with ASK1 and/or TAK1, and the JNK and p38 activity was determined, respectively. However, no synergistic activation of JNK or p38 was observed in the presence of ASK1 and TAK1 (data not shown). Certain MAPKKks such as TAK1, mitogen-activated protein kinase/ERK kinase 1 (MEKK1), and tumor progression locus 2 (Tpl-2) have been reported to be activated by the NF-κB signaling pathway as well (22, 32–35). Moreover, TAK1 was clearly shown to activate NF-κB in response to IL-1 (22). We thus examined whether ASK1 is involved in the IL-1-induced NF-κB pathway by using NF-κB-dependent reporter gene assays. Without IL-1 treatment, overexpression of ASK1-WT had no effect on the basal activity of the reporter gene (Fig. 2A, columns 1–4); however, IL-1-induced NF-κB activity was significantly reduced by the expression of ASK1-WT in a dose-dependent manner (Fig. 2A, columns 5–8). In the IL-1 signaling pathway, TRAF6-TAK1-NIK cascade has been reported to constitute an essential axis (22), in that IL-1-induced TRAF6 was detected in TAK1 immunoprecipitates (Fig. 3D, top panel). TRAF6 bound to TAK1 became undetectable. In contrast, ASK1 was coprecipitated with TAK1 (Fig. 3C, second panel, lanes 7–9). These results strongly suggest that overexpression of ASK1 takes TRAF6 and TAK1 away from the TRAF6-TAK1 complex and thereby inhibits TRAF6-TAK1 signaling complex leading to NF-κB activation. To confirm that this mechanism is operating indeed when ASK1 inhibits IL-1-induced NF-κB activation, IL-1-induced complex formation between endogenous TAK1 and TRAF6 was determined in the presence of ASK1 (Fig. 3D). Adenovirus-mediated expression of ASK1 but not control β-galactosidase blocked the IL-1-induced coimmunoprecipitation of TRAF6 with TAK1 (Fig. 3D, second panel), indicating that ASK1 inhibits IL-1-induced NF-κB activation at least in part via disruption of TRAF6-TAK1 signaling complex.

It has been reported that TRAF6 interacts with NIK as well (39). We thus also tested whether ASK1 has a similar effect on TRAF6-NIK binding. FLAG-tagged NIK (FLAG-NIK) and Myc-tagged TRAF6 (Myc-TRAF6) were transiently cotransfected, and NIK was immunoprecipitated with anti-FLAG antibody. Coimmunoprecipitated TRAF6 with NIK was detected by immunoblotting with anti-Myc antibody. TRAF6 associated with NIK (Fig. 3E, lane 7); in contrast to TRAF6-TAK1 interaction, however, TRAF6-NIK interaction was unaffected by the coexpression of ASK1 (Fig. 3E, lanes 8–10). These results again confirmed that ASK1 specifically targets TRAF6-TAK1 complex in the IL-1-induced NF-κB signaling pathway.

Several MAPKKks or MAPKK-like molecules, including TAB1, NIK, MEKK1, and tumor progression locus 2/COT, have been demonstrated to activate NF-κB with distinct molecular mechanisms (22, 30, 32–35, 40, 41). In contrast, we show here for the first time that another MAPKKk, ASK1, negatively regulates NF-κB activity. ASK1 had no effect on the basal activity of NF-κB. ASK1 inhibited, however, IL-1-induced NF-κB activity (Fig. 2A), suggesting that ASK1 may play important roles in the negative feedback regulation of NF-κB signaling. Although NF-κB is a central mediator of immune response and required for induction of various inflammatory pathways, it may also serve a negative role in immune responses.
certain pathological situations. In support of this notion, strong induction of ASK1 expression has been observed in certain pathological situations, including epithelial wound healing and spinal cord injury.

Finally, this is the first demonstration that a MAPKKK directly interacts with another MAPKKK and influences signals responsible for cell activation. It will be interesting to examine whether other MAPKKs or MAPKK-like molecules also cross-talk to each other. Such studies will shed light on how the highly divergent biological activities can be elicited by the limited number of MAP kinase superfamily members.

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