Recruitment of Tumor Necrosis Factor Receptor-associated Factor Family Proteins to Apoptosis Signal-regulating Kinase 1 Signalosome Is Essential for Oxidative Stress-induced Cell Death*

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Apoptosis signal-regulating kinase 1 (ASK1) plays a pivotal role in oxidative stress-induced cell death. Reactive oxygen species disrupt the interaction of ASK1 with its cellular inhibitor thioredoxin and thereby activates ASK1. However, the precise mechanism by which ASK1 freed from thioredoxin undergoes oligomerization-dependent activation has not been fully elucidated. Here we show that endogenous ASK1 constitutively forms a high molecular mass complex including Trx (1,500–2,000 kDa), which we designate ASK1 signalosome. Upon H2O2 treatment, the ASK1 signalosome forms a higher molecular mass complex at least in part because of the recruitment of tumor necrosis factor receptor-associated factor 2 (TRAF2) and TRAF6. Consistent with our previous findings that TRAF2 and TRAF6 activate ASK1, H2O2-induced ASK1 activation and cell death were strongly reduced in the cells derived from Traf2−/− and Traf6−/−/− mice. A novel signaling complex including TRAF2, TRAF6, and ASK1 may thus be the key component in oxidative stress-induced cell death.

Apoptosis signal-regulating kinase 1 (ASK1)2 is a mitogen-activated protein (MAP) kinase kinase kinase (MAPKKK) family member that activates the JNK and p38 MAP kinase pathways and is activated by various stresses including oxidative stress, TNFα, calcium overload, and endoplasmic reticulum stress (1–7). Recent analyses of ASK1-deficient mice have revealed that ASK1 is required for cell death induced by oxidative stress, TNFα, and endoplasmic reticulum stress (4, 8).

Among various stimuli tested, oxidative stress is one of the most potent activators of ASK1 (3). Thioredoxin (Trx), a reduction/oxidation (redox) regulatory protein, inhibits the kinase activity of ASK1 by its direct binding to the N-terminal noncatalytic region of ASK1 (6, 9). Reactive oxygen species (ROS) such as H2O2 dissociate Trx from ASK1 and thereby activate ASK1 (6). Following the dissociation of Trx, autophosphorylation-dependent activation of ASK1 occurs most likely through mechanisms involving homo-oligomerization (10, 11); however, precise mechanisms of the oligomerization-dependent activation of ASK1 remained unknown.

TNF receptor-associated factor 2 (TRAF2) has been shown to bind to and thereby activate ASK1 (5). TRAF2 is not only a pivotal intermediate in TNFα-induced NF-κB activation (12–16), but it also activates JNK in TNFα signaling through the association with MAPKKKs such as ASK1 and MEKK1 (5, 17, 18). A recent report has shown that AIP1/DAB2IP, a novel member of Ras-GAP family, regulates TRAF2-dependent activation of ASK1 in TNFα-treated endothelial cells (19). In accordance with the findings that ROS function as second messengers in TNFα signaling (20, 21), TNFα-induced association of ASK1 with TRAF2 and subsequent activation of ASK1 have been shown to depend largely on intracellular ROS production by TNFα (6, 17).

In addition to TRAF2, we have previously shown that among extensively characterized six members of the TRAF family (TRAF1–TRAF6) (22–24), TRAF5 and TRAF6 also interact with and activate ASK1 upon overexpression (5). TRAF5 has been implicated in NF-κB and JNK activation in signaling through lymphotoxin-β receptor, CD40, and CD27 (25–27). Analyses of TRAF5-deficient mice indicated that TRAF5 is required for CD40- and CD27-mediated lymphocyte activation (28). TRAF6 is a critical regulator of NF-κB and MAP kinases in the signaling pathways through the TNF receptor superfamily and Toll/interleukin-1 receptor family, as has been demonstrated by many studies using TRAF6-deficient mice (29–33). Activation of NF-κB and MAP kinase pathways by TRAF6 has been reported to be induced through the association with MAPKKKs such as TAK1 and MEKK3 (34, 35).

In this study, we show that ASK1 constitutively forms a high molecular mass complex (~1,500–2,000 kDa). Upon H2O2 treatment, ASK1 was dissociated from Trx but reciprocally associated with TRAF2 and TRAF6. The recruitment of TRAF2 and TRAF6 but not TRAF5 to ASK1 signaling complex was required for H2O2-induced activation of ASK1 and cell death. These results propose a novel signaling mechanism
involve TRAF family proteins and ASK1 in oxidative stress-induced cell death.

MATERIALS AND METHODS

Cell Culture and Plasmids—HEK293 cells and mouse embryonic fibroblasts (MEFs) were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, 4.5 g/liter glucose, and 100 units/ml penicillin G in a 5% CO2 atmosphere at 37 °C. HA-tagged wild-type ASK1 (ASK1WT-HA) and HA-tagged C-terminal coiled-coil domain deletion mutant of ASK1 (ASK1Δcoil-HA) have been described previously (11).

Reagents and Antibodies—Transfection was performed with FuGENE 6 (Roche Applied Science) according to the manufacturer’s instructions. Human recombinant TNFα was obtained from WAKO. The antibody to HA (clone 3F10) was from Roche Applied Science. Phospho-JNK (Thr183/Tyr185) and phospho-p38 (Thr180/Tyr182) antibodies were from Cell Signaling Technology. Anti-ASK1, anti-Trx, anti-TRAF2, anti-TRAF5, and anti-TRAF6 antibodies were from Santa Cruz Biotechnology, Inc. Affinity-purified rabbit polyclonal antibody raised against phospho-ASK1 (Thr835) was provided by Dr. J. Engelman (19). To generate anti-TRAF6 antibody, the synthetic peptide KREGFQPRSTD was used as immunogen, and the antiserum obtained from immunized rabbit was affinity-purified by the synthetic peptide (36).

Preparation of S-100 Fraction—To purify the ASK1 complex, HEK293 cells were stimulated with 0.5 mM H2O2, or 100 ng/ml TNFα. The cells (5 × 106 cells/ml) were washed twice with ice-cold phosphate-buffered saline and were lysed by using a glass Dounce homogenizer in buffer A (50 mM HEPES-KOH, pH 7.5, 10 mM KCl, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1.5 mM MgCl2, and 10% glycerol) plus 0.1% Chaps and 0.01% Brij35, supplemented with 1 mM diithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 5 μg/ml aprotinin. The homogenate was centrifuged at 10,000 × g for 30 min at 4 °C. The supernatant was recentrifuged at 105,000 × g for 90 min at 4 °C. The supernatant (S-100) was flash-frozen and stored at −135 °C.

Gel Filtration Chromatography—S-100 fraction from HEK293 cells was loaded onto a Superose 6 10/300 GL column pre-equilibrated with buffer A plus 0.1% Chaps and 0.01% Brij35. The proteins were eluted at 0.3 ml/min. Each fraction (0.5 ml/fraction) was precipitated using acetone/ethanol (1:4). Samples from one or four column runs were pooled and analyzed by Western blotting for the indicated antibodies.

Immunoblotting—The cells were lysed in buffer A plus 0.2% Nonidet P-40, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 5 μg/ml aprotinin. The cell extracts were resolved by SDS-PAGE and transferred to polyvinylidene difluoride membranes. After blocking with 5% skim milk in TBS-T (150 mM NaCl, 50 mM Tris-HCl, pH 8.0, and 0.05% Tween 20), the membranes were probed with the indicated antibodies. The antibody-antigen complexes were detected using the ECL system (Amersham Biosciences).

Co-immunoprecipitation Analysis—The cells were lysed in buffer A plus 0.2% Nonidet P-40, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 5 μg/ml aprotinin. The cell extracts were immunoprecipitated with 20 μl of protein A- or protein G-Sepharose beads (Amersham Biosciences) for overnight at 4 °C with the indicated antibodies. The beads were washed four times with the same buffer before analysis by SDS-PAGE.

Preparation of Splenocyte-derived Macrophage—Because the number of bone marrow cells was reduced significantly in Traf6−/− mice because of severe osteoporosis, macrophages were generated from splenocytes of Traf6+/− and Traf6−/− mice (30). After 6 days of culture with macrophage colony-stimulating factor (M-CSF), almost equal numbers of adherent cells, which displayed typical macrophage morphology, were obtained from both Traf6+/− and Traf6−/− splenocytes. More than 95% of the adherent cells from both Traf6+/− and Traf6−/− mice expressed CD11b and F4/80 (data not shown), indicating that TRAF6 deficiency does not affect macrophage differentiation.

Trypan Blue Dye Exclusion Assay—For the trypan blue dye exclusion assay, cells (1 × 106 cells/well) were plated in 12-well plates. Twenty-four h after the plating, the cells were treated with 0.1 mM H2O2 for 10 h. The cells were then directly stained with 0.4% trypan blue dye or harvested with trypsin followed by staining with 0.4% trypan blue dye. The trypan blue-positive cells were counted by using a hemocytometer under phase contrast microscopy.

RESULTS

ASK1 Forms a High Molecular Mass Signaling Complex in the Cell—We previously demonstrated that phosphorylation of a threonine residue within the activation loop of ASK1 kinase domain (Thr838 and Thr465 of human and mouse ASK1, respectively) is required for activation of ASK1 in response to various stresses (11). We also showed that the activation state of ASK1 can be monitored by the phospho-ASK1 antibody that detects activating phosphorylation of this threonine residue (11). Immunoblotting analysis using the phospho-ASK1 antibody revealed that basal activity of a mutant form of ASK1 that lacked C-terminal coiled-coil domain (ASK1Δcoil) was much lower than that of wild-type ASK1 (ASK1WT) when each construct was expressed in HEK293 cells (Fig. 1, A and B). Overexpression of ASK1Δcoil was not able to induce the activation of JNK and p38 or inhibit the activity of co-transfected ASK1WT (data not shown). These results confirm our previous finding that homo-oligomerization through the C-terminal coiled-coil domain is required for the activation of ASK1 (11); however, regulatory mechanisms of ASK1 oligomer have been largely unknown.

To investigate how homo-oligomerization of ASK1 is involved in its activation, we first examined the potential complex formation of ASK1 in the cells using a gel filtration column chromatography by an approach similar to that used to characterize the apoptosome (37). The expected molecular mass of monomeric ASK1 is ∼160 kDa. When HA-tagged ASK1WT was expressed in HEK293 cells, we found that ASK1WT formed a high molecular mass (HMM) complex in fractions 1–8 (∼1,500–2,000 kDa), endogenous ASK1 formed a similar HMM complex (Fig. 2, a and b), and ASK1Δcoil also formed an HMM complex with certain kinase activity (Fig. 2, c).

A kinase-negative mutant of ASK1 (ASK1K709R) also formed an HMM complex (data not shown), suggesting that the kinase activity of ASK1 is an HMM formation-dependent formation of the HMM complex is required for the kinase activity of ASK1.

ROS Induce a Higher Molecular Mass Complex Formation of ASK1—We next examined the states of endogenous ASK1 by gel filtration analysis. Fractionation of the extracts from HEK293 cells through a gel filtration column revealed that although confined to fractions 5–8 (∼1,500–2,000 kDa), endogenous ASK1 formed a similar HMM complex as was formed by exogenously expressed ASK1WT (Fig. 2A, top panel). A similar pattern of HMM complex was detected in all of the cultured cells we tested, e.g. HeLa cells, HT1080 cells, and MEFs (data not shown), suggesting that the HMM complex formation is a general feature of endogenous ASK1. Under unstimulated conditions, the
kinase activity of ASK1 is negatively regulated by its association with Trx (6). To examine whether Trx is a component of the unstimulated HMM complex, ASK1-containing fractions (corresponding to lanes 5–8 in Fig. 2A, top panel) were collected and subjected to immunoprecipitation with anti-ASK1 antibody. Trx was found to be co-immunoprecipitated with ASK1 (Fig. 2B), indicating that endogenous ASK1 forms a static HMM complex containing Trx, which may function as a signalosome competent to ROS-dependent activation of ASK1.

When cells were treated with H$_2$O$_2$, the ASK1 signalosome was eluted in higher molecular mass fractions, i.e. fractions 1–4 (>30 kDa; Fig. 2A, second and third panels). This elution profile was similar to that of exogenously expressed ASK1WT (Fig. 1C), suggesting that a certain population of exogenous ASK1 comprises an activated complex without any stimuli. The shift of the ASK1-containing fractions was inhibited by the pretreatment of cells with anti-oxidants such as propyl gallate (Fig. 2A, bottom panel) and N-acetyl cysteine (data not shown). The changes in the elution pattern of the ASK1 complex correlated well with the activation states of ASK1 and its downstream targets, JNK and p38 (Fig. 2C, western blot with the indicated antibodies). Apparent molecular mass was evaluated after gel filtration analysis of ASK1WT and ASK1coil mutant. HEK293 cells were transiently transfected with HA-tagged wild-type ASK1 expression plasmid (ASK1WT-HA), C-terminal coiled-coil domain deletion mutant (ASK1coil-HA), or control vector (pcDNA3). After 36 h, the cell extracts were subjected to immunoblotting with anti-HA antibody (3F10) and phospho-specific ASK1 antibody (p-ASK). C, gel filtration analysis of ASK1WT and ASK1coil mutant. HEK293 cells were transiently transfected with ASK1WT-HA or ASK1coil-HA in a 100-mm-diameter dish. After 36 h, the cell extracts were prepared as described under "Materials and Methods." After fractionation on a Superose 6 10/300 GL column, each fraction (50 μl) was analyzed by immunoblotting with the indicated antibodies. Apparent molecular mass is shown as described above. The elution positions of these proteins are indicated at the top of the figure. WB, Western blot; WT, wild type.

ASK1. To examine the binding states between Trx and ASK1 after ROS exposure, nontransfected HEK293 cells lysate was subjected to the co-immunoprecipitation analysis. As previously demonstrated in ASK1-transfected HEK293 cells (6), we confirmed that H$_2$O$_2$ treatment disrupted the endogenous interaction between Trx and ASK1 in nontransfected HEK293 cells (Fig. 2D). When nontransfected HEK293
cells lysate was analyzed by gel filtration, endogenous Trx was found to exist in all fractions regardless of the presence of ASK1 (Fig. 2E, second and fourth panels). Co-immunoprecipitation analyses of individual fractions showed that the interaction of ASK1 and Trx in unstimulated cells was clearly detectable in the HMM fractions almost in parallel with the amount of ASK1 in each fractions (Fig. 2E, top panel, lanes 5–10; compare with Fig. 2A, top panel); however, Trx-ASK1 interaction was strongly reduced in H2O2-treated cells (Fig. 2E, third panel). This indicates that the H2O2-induced higher molecular mass complex of ASK1 (corresponding to lanes 1–4 of the second and third panels in Fig. 2A) does not contain Trx. These results suggest that dissociation of Trx from and reciprocal association of additional component(s) to the pre-existing HMM signaling complex of ASK1 may occur during ROS-induced activation of ASK1.

**TNFα Also Induces Higher Molecular Mass Complex Formation of ASK1**—We have previously demonstrated that TRAF2 interacts with and activates ASK1 in TNFα signaling (5). We have also reported that ROS-dependent prior dissociation of Trx is required for the TNFα-induced TRAF2-ASK1 interaction and activation of ASK1 (6, 17). Fig. 3A shows that TNFα activated endogenous ASK1 in HEK293 cells within 15 min and peaked at 30 min after the treatment with TNFα. The interaction between exogenously expressed ASK1 (ASK1-HA), and endogenous TRAF2 was also found to peak at 30 min after the treatment with TNFα in HEK293 cells, and ASK1 co-immunoprecipitated with TRAF2 was found to be phosphorylated as determined by immunoblotting with anti-ASK1 antibody (Fig. 3A). Spleenocyte-derived macrophages (2 × 10^5) from Traf6^-/- or Traf6^-/- (Traf6^-/- #1 and #2; independently prepared cells) mice were treated with 1.0 mU H2O2 for 30 min (E) or 20 μM thapsigargin (Tg) for 30 min (F). The cell extracts were subjected to immunoblotting with the indicated antibodies. WB, Western blot; IP, immunoprecipitation; WT, wild type.

**ASK1 Interacts with TRAF2 in a ROS-dependent Manner**—The similarity between H2O2- and TNFα-induced changes in the ASK1 signaling complex (Figs. 2A and 3C), together with the finding that TNFα-induced activation of ASK1 depends largely on ROS (6, 17) prompted us to examine whether TRAF2 may also be involved in the mechanism of ROS-induced activation of ASK1. In support of this idea, it has recently been reported that TRAF2 is required for H2O2-induced JNK activation.
and cell death (38). We therefore examined whether TRAF2 is involved in H$_2$O$_2$-induced activation of ASK1. In parallel with the time course of H$_2$O$_2$-induced activation of ASK1 (Fig. 3D), ASK1 was co-immunoprecipitated with TRAF2 in an H$_2$O$_2$-dependent manner, and co-immunoprecipitated ASK1 was phosphorylated and thus activated (Fig. 3E). These results strongly suggested that the molecular components responsible for H$_2$O$_2$-induced larger complex formation of the ASK1 signalosome include TRAF2.

**TRAF2 and TRAF6 but Not TRAF5 Are Required for ROS-induced Activation of the ASK1-JNK/p38 Pathways—**Among the TRAF family members, not only TRAF2 but also TRAF5 and TRAF6 activate ASK1 (5). Furthermore, our recent report demonstrated that ROS-dependent interaction of TRAF6 and ASK1 is crucial for lipopolysaccharide-induced activation of the ASK1-p38 pathway (36). We thus explored the possibility of whether TRAF2, TRAF5, and TRAF6 are involved in ROS-induced ASK1 activation. To examine the requirement of TRAFs for ROS-induced ASK1 activation, we analyzed MEFs deficient for TRAF2 (39), TRAF5 (28) and TRAF6 (29, 33). Although the expression of endogenous ASK1 was relatively low in MEFs, almost equivalent expression of endogenous ASK1 in $Traf2^{−/−}$, $Traf5^{−/−}$, $Traf6^{−/−}$, and wild-type MEFs was confirmed by immunoblotting following immunoprecipitation (Fig. 4A). H$_2$O$_2$-induced activation of ASK1 was strongly suppressed in TRAF2- and TRAF6-deficient but not TRAF5-deficient MEFs when compared with each of genotype-matched control MEFs (Fig. 4B). Consistently, H$_2$O$_2$-induced activations of JNK and p38 were also inhibited in TRAF2- and TRAF6-deficient MEFs (Fig. 4, C and D). These results suggested that TRAF2 and TRAF6 but not TRAF5 are required for H$_2$O$_2$-induced activation of the ASK1-JNK/p38 pathways. Nevertheless, the relatively late (60 or 90 min) responses of JNK and p38 to H$_2$O$_2$ in TRAF2- and TRAF6-deficient MEFs were only partially impaired (Fig. 4, C and D), suggesting that H$_2$O$_2$ evoked secondary signals, which induce TRAF2- or TRAF6-independent activations of the JNK and p38 pathways in the late phase. Impairment of ASK1 activation in TRAF2-deficient cells treated with H$_2$O$_2$ for 90 min was also limited probably for the same reason, whereas H$_2$O$_2$-induced ASK1 activation was abolished throughout the time course tested in TRAF6-deficient cells (Fig. 4B). This contrast in the extent of impairment of ASK1 activation suggests that TRAF6 is more critically required for H$_2$O$_2$-induced ASK1 activation than TRAF2.

We further examined whether TRAF6 is required for H$_2$O$_2$-induced ASK1 activation in primary cultured cells. In the macrophages independently prepared from two $Traf6^{−/−}$ mice (30), H$_2$O$_2$-induced acti-
viation of ASK1-JNK/p38 pathways were strongly suppressed as was seen in TRAF6-deficient MEFs (Fig. 4E). On the other hand, activation of ASK1 by the treatment with thapsigargin, which triggers endoplasmic reticulum stress by depletion of luminal calcium stores (4), was not impaired in Traf6−/− macrophages (Fig. 4F). Taken together with our previous finding that TRAF2 and TRAF6 activates ASK1 (5), TRAF2 and TRAF6 appear to be crucial components of the H2O2-induced activation of ASK1.

TRAF6 Is Required for ROS-induced Higher Molecular Mass Complex of ASK1—We next examined whether TRAF6-ASK1 interaction can be induced by H2O2. Transfected ASK1 was co-immunoprecipitated with TRAF6 in an H2O2-dependent manner, and co-immunoprecipitated ASK1 with TRAF6 was phosphorylated (Fig. 5A). H2O2-dependent endogenous interaction of TRAF6 and ASK1 was also readily detectable in HEK293 cells (Fig. 5B). These results suggested that TRAF2 and TRAF6 are likely candidates for the components of H2O2-induced higher molecular mass complex of ASK1. To confirm this possibility, MEFs transiently transfected with HA-tagged ASK1WT were treated or untreated with H2O2 and subjected to the gel filtration analysis as determined using HEK293 cells in Fig. 1C. Fig. 5C shows that H2O2 induced the shift of ASK1-containing fractions to the higher molecular mass fractions also in MEFs. Immunoblotting analysis of the same membrane with the phospho-ASK1 antibody revealed that H2O2-induced active fractions also in MEFs. This shift was probably because of the low affinity of TRAF2 antibody or the lesser amount of endogenous TRAF2 compared with TRAF6 in MEFs (data not shown). To examine the requirement of TRAF6 for H2O2-dependent formation of the higher molecular mass complex, TRAF6-deficient MEFs were analyzed. We could not detect H2O2-dependent shift of the ASK1 signalosome in Traf6−/− MEFs (Fig. 5F). These results suggested that recruitment of TRAF6 is required for H2O2-induced higher molecular mass complex formation of the ASK1 signalosome.

Different Susceptibilities of Traf2−/−, Traf5−/−, and Traf6−/− MEFs to H2O2-induced Cell Death—Given that ASK1 is required for H2O2-induced cell death (8), it is formally possible that Traf2 and Traf6 are also involved in such cell death. We therefore investigated the susceptibility of Traf2−/− and Traf6−/− MEFs to H2O2-induced cell death by trypan blue dye exclusion assay (Fig. 6). Traf2−/− MEFs were apparently resistant to H2O2-induced cell death as recently reported (38), whereas wild-type and Traf5−/− MEFs were similarly vulnerable to H2O2 treatment. Traf6−/− MEFs were even more resistant than Traf2−/− MEFs to H2O2-induced cell death, correlating well with the results that H2O2-induced activation of the ASK1-JNK/p38 pathways was more severely impaired in Traf6−/− than Traf2−/− MEFs (Fig. 4, C and D). These results demonstrated that TRAF2 and TRAF6 are novel components of the ROS-induced ASK1 signaling pathway and play an essential role in ROS-induced cell death.

DISCUSSION

The importance of the so-called signalosome has been emerging, in that cells may take advantage of the preformed multi-molecular complex to commence rapid as well as specific signaling events in response to the cellular input. For example, the IκB kinase (IKKs) complex, a signalosome composed of IKKα, IKKβ, and NEMO (IKKγ), forms an HMM complex and plays an essential role in NF-κB activation (40–45). Activations of certain caspases were also reported to require HMM complex formation, such as apoptosome for caspase-9 (37, 46) and inflammasome for caspase-1 and -5 (47–49). Apaf-1 and NALP1, the members of the nucleotide binding-site family, are the specific components to assemble large caspase complexes for apoptosome and inflammasome, respectively. Apoptosome, for example, which is formed at the recruitment of caspase-9 to Apaf-1, brings several caspase-9 molecules into close proximity, resulting in their efficient cross-activation (37, 46). These findings validate the comprehensive analysis of ASK1 signalosome to understand the ASK1-dependent signaling mechanisms of oxidative stress-induced cell death.

In the present study, we found that ASK1 forms a static HMM complex composed of homo-oligomeric ASK1, Trx, and yet unidentified component(s) in unstimulated cells. We also found that oxidative-stress-dependent dissociation of Trx and reciprocal recruitment of TRAF2 and TARF6 to the ASK1 signaling complex are required for the activation of ASK1. Because H2O2-induced activation of ASK1 was almost completely abolished in Traf6−/− cells (Fig. 4, B and E), TRAF6 appears to be the most important among TRAF family proteins for the activation of ASK1. However, how TRAFs mechanistically contribute to the activation of ASK1 is yet to be elucidated. In this regard, two possibilities, physical and/or chemical modifications of ASK1, might apply. Because TRAF family proteins appear to form trimers in vivo (50–52),
physical association of TRAF6 may facilitate multimeric complex formation, resulting in close proximity of ASK1 molecules and thereby autophosphorylation-dependent activation. Although ASK1Δcoil, a deletion mutant of the C-terminal oligomerization domain, exhibited very low kinase activity (Fig. 1A), it was shown that forced homo-oligomerization of ASK1Δcoil by chemical oligomerizer induced kinase activation of this mutant (11), suggesting that a close proximity of ASK1 molecules is sufficient to activate ASK1. Nevertheless, the endogenous ASK1 signaling complex appears to be present as a homo-oligomerized but still inactive form (Fig. 2). These findings suggest that the homo-oligomerization of ASK1 through the C-terminal coiled-coil domain is not sufficient for ASK1 activation. Thus, the endogenously homo-oligomerized ASK1 is likely to be kept inactive by the presence of Trx, a “safety lock” for ASK1. Dissociation of Trx from ASK1 signaling complex appears to allow the association of TRAF2 and TRAF6 to ASK1 and thereby induce the close proximity-dependent conformational changes in the interface of the kinase domain of ASK1. The tight oligomerization assisted by the associated molecules may be physiologically necessary and sufficient for the autophosphorylation-dependent activation of ASK1. Activation status of the ASK1 signaling complex in oxidative stress response may thus be switched by reciprocal changes in the components of the signalosome; from ASK1-Trx signaling complex (inactive form) to the ASK1-TRAF2/TRAF6 signaling complex (active form) (Fig. 7).

Another possible mechanism by which TRAFs activate ASK1 may include chemical modifications, such as ubiquitination. TRAF6 is a RING domain-containing ubiquitin ligase that synthesizes Lys63-linked polyubiquitin chains (53). The targets of Lys63-linked polyubiquitination include NEMO, TRAF2, and TRAF6 itself, and the ubiquitinated TRAF6 is implicated in the activation of downstream effectors such as NEMO and TAK1 (54, 55). Similar implications of TRAF2 are also reported (56, 57). It was also reported that RIP1 is ubiquitinated by TRAF2 in a TNFα-dependent manner, which induces the activation of NF-κB and p38 MAP kinase (58). Although the role of Lys63-linked polyubiquitination remains unclear, ASK1 might require the Lys63-linked polyubiquitination by TRAF2 or TRAF6 for the kinase activation.

Thus, further studies are required for the elucidation of mechanisms by which the TRAF family proteins activate ASK1. Uncovering the unidentified components in the ASK1 signaling complex may reveal precise mechanisms of the ASK1-dependent signaling cascade that links oxidative stress and cell death.

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REFERENCES
1. Takeda, K., Matsuzawa, A., Nishitoh, H., and Ichijo, H. (2003) Cell Struct. Funct. 28, 23–29
2. Ichijo, H., Nishida, E., Irie, K., ten Dijke, P., Saitoh, M., Moriyama, T., Takagi, M., Matsumoto, K., Miyazono, K., and Gotoh, Y. (1997) Science 275, 90–94
3. Matsukawa, J., Matsuzawa, A., Takeda, K., and Ichijo, H. (2004) J. Biochem. (Tokyo) 136, 261–265
4. Nishitoh, H., Matsuzawa, A., Tobiute, K., Saegusa, K., Takeda, K., Inoue, K., Hori, S., Kakizuka, A., and Ichijo, H. (2002) Genes Dev. 16, 1345–1355
5. Nishitoh, H., Saitoh, M., Mochida, Y., Takeda, K., Nakano, H., Rothe, M., Miyazono, K., and Ichijo, H. (1998) Mol. Cell 2, 389–395
6. Saitoh, M., Nishitoh, H., Fujii, M., Takeda, K., Tobiute, K., Sawada, Y., Kawabata, M., Miyazono, K., and Ichijo, H. (1998) EMBO J. 17, 2596–2606
7. Takeda, K., Matsuzawa, A., Nishitoh, H., Tobiute, K., Kishida, S., Ninomiya-Tsujii, J., Matsumoto, K., and Ichijo, H. (2004) EMBO Rep. 5, 161–166
8. Tobiute, K., Matsuzawa, A., Takahashi, T., Nishitoh, H., Morita, K., Takeda, K., Minowa, O., Miyazono, K., Noda, T., and Ichijo, H. (2001) EMBO Rep. 2, 222–228
9. Liu, Y., and Min, W. (2002) Circ. Res. 90, 1259–1266
10. Gotoh, Y., and Cooper, J. A. (1998) J. Biol. Chem. 273, 17477–17482
11. Tobiute, K., Saitoh, M., and Ichijo, H. (2002) J. Cell. Physiol. 191, 95–104
12. Chen, G., and Goeddel, D. V. (2002) Science 296, 1634–1635
13. Devin, A., Cook, A., Lin, Y., Rodriguez, Y., Kelliber, M., and Liu, Z. (2000) Immunity 12, 419–429
14. Hsu, H., Shu, H. B., Pan, M. G., and Goeddel, D. V. (1996) Cell 84, 299–308
15. Liu, Z. G., Hsu, H., Goeddel, D. V., and Kari, M. (1996) Cell 87, 556–576
16. Rothe, M., Sarma, V., Dixit, V. M., and Goeddel, D. V. (1995) Science 269, 1424–1427
17. Liu, H., Nishitoh, H., Ichijo, H., and Kyriakis, J. M. (2000) Mol. Cell. Biol. 20, 2198–2208
18. Baud, V., Liu, Z. G., Bennett, B., Suzuki, N., Xia, Y., and Karin, M. (1999) Genes Dev. 13, 1297–1308
19. Zhang, H., Zhang, R., Luo, Y., D’Alessio, A., Pober, J. S., and Min, W. (2004) J. Biol.
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Chem. 279, 44955–44965

20. Goosens, V., Grooten, J., De Vos, K., and Fiers, W. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 8115–8119

21. Lin, Y., Choksi, S., Shen, H. M., Yang, Q. F., Hur, G. M., Kim, Y. S., Tran, J. H., Nedopasov, S. A., and Liu, Z. G. (2004) J. Biol. Chem. 279, 10822–10828

22. Bradley, J. R., and Pober, J. S. (2001) Oncogene 20, 6482–6491

23. Chung, J. Y., Park, Y. C., Ye, H., and Wu, H. (2002) J. Cell Sci. 115, 679–688

24. Inoue, J., Ishida, T., Tsukamoto, N., Kobayashi, S., Naito, A., Azuma, S., and Yamamoto, T. (2000) Exp. Cell Res. 254, 14–24

25. Nakano, H., Oshima, H., Chung, W., Williams-Abbott, L., Ware, C. F., Yagita, H., and Okumura, K. (1996) J. Biol. Chem. 271, 14661–14664

26. Akiba, H., Nakano, H., Nishinaka, S., Shindo, M., Kobata, T., Atsuta, M., Morimoto, C., Ware, C. F., Malinin, N. L., Hall, D., Yagita, H., and Okumura, K. (1998) J. Biol. Chem. 273, 13353–13358

27. Ishida, T. K., Tojo, T., Aoki, T., Kobayashi, N., Ohishi, T., Watanabe, T., Yamamoto, T., and Inoue, J. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 9437–9442

28. Nakano, H., Sakon, S., Koseki, H., Takemori, T., Tada, K., Matsumoto, M., Munehika, E., Sakai, T., Shirasawa, T., Akiba, H., Kobata, T., Santee, S. M., Ware, C. F., Rennert, P. D., Taniguchi, M., Yagita, H., and Okumura, K. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 9803–9808

29. Naito, A., Azuma, S., Tanaka, S., Miyazaki, T., Takaki, S., Takatsu, K., Nakao, K., Nakamura, K., Katsuki, M., Yamamoto, T., and Inoue, J. (1999) Genes Cells 4, 353–362

30. Gohda, J., Matsumura, T., and Inoue, J. (2004) J. Immunol. 172, 2931–2917

31. Ishida, T., Mizushima, S., Azuma, S., Kobayashi, N., Tojo, T., Suzuki, K., Aizawa, S., Watanabe, T., Mostoslav, G., Kieff, E., Yagita, H., and Okumura, K. (1996) J. Biol. Chem. 271, 28745–28748

32. Wong, B. R., Besser, D., Kim, N., Arron, J. R., Vologodskiaia, M., Hanafusa, H., and Choi, Y. (1999) Mol. Cell 4, 1041–1049

33. Lomaga, M. A., Yeh, W. C., Sarosi, I., Duncan, G. S., Furlonger, C., Ho, A., Morony, S., Capparelli, C., Van, G., Kaufman, S., van der Heiden, A., Itie, A., Wakeham, A., Khoo, W., Sasaki, T., Cao, Z., Penninger, J. M., Paige, C. J., Lacey, D. L., Dunstan, C. R., Boyle, W. J., Goeddel, D. V., and Mak, T. W. (1999) Genes Dev. 13, 1015–1024

34. Ninomiya-Tsuji, I., Kishimoto, K., Hiyama, A., Inoue, J., Cao, Z., and Matsumoto, K. (1999) Nature 398, 252–256

35. Huang, Q., Yang, J., Lin, Y., Walk, C., Cheng, J., Liu, Z. G., and Su, B. (2004) Nat. Immunol. 5, 98–103

36. Matsuzawa, A., Saegusa, K., Noguchi, T., Sadamitsu, C., Nishitoh, H., Nagai, S., Ko- yasu, S., Matsumoto, K., Takeda, K., and Ichijo, H. (2005) Nat. Immunol. 6, 587–592

37. Zou, H., Li, Y., Liu, X., and Wang, X. (1999) J. Biol. Chem. 274, 11549–11556

38. Shen, H. M., Lin, Y., Choksi, S., Tran, J., Jin, T., Chang, L., Karin, M., Zhang, J., and Liu, Z. G. (2004) Mol. Cell. Biol. 24, 5914–5922

39. Yeh, W. C., Shahinian, A., Speiser, D., Krausus, J., Billia, F., Wakeham, A., de la Pompa, J. L., Ferrick, D., Hurn, B., Isco, N., Ohashi, P., Rothe, M., Goeddel, D. V., and Mak, T. W. (1997) Immunity 7, 715–725

40. DiDonato, J. A., Hayakawa, M., Rothwarf, D. M., Zandi, E., and Karin, M. (1997) Nature 388, 548–554

41. Rothward, D. M., Zandi, E., Natoli, G., and Karin, M. (1998) Nature 395, 297–300

42. Zandi, E., Rothward, D. M., Delhase, M., Hayakawa, M., and Karin, M. (1997) Cell 91, 243–252

43. Karin, M., and Ben-Neriah, Y. (2000) Annu. Rev. Immunol. 18, 621–663

44. Tanaka, M., Fuentes, M. E., Yamaguchi, K., Dunrin, M. H., Dalrymple, S. A., Hardy, K. L., and Goeddel, D. V. (1999) Immunity 10, 421–429

45. Karin, M., and Lin, A. (2002) Nat. Immunol. 3, 221–227

46. Achehan, D., Jiang, X., Morgan, D. G., Heuser, J. E., Wang, X., and Akey, C. W. (2002) Mol. Cell 9, 423–432

47. Tschopp, J., Martinon, F., and Burns, K. (2003) Nat. Rev. Mol. Cell. Biol. 4, 95–104

48. Martinon, F., Burns, K., and Tschopp, J. (2002) Mol. Cell 10, 417–426

49. Martinon, F., and Tschopp, J. (2004) Cell 117, 561–574

50. Pullen, S. S., Labadia, M. E., Ingraham, R. H., McWhirter, S. M., Everdeen, D. S., Alber, T., Crute, J. J., and Kehry, M. R. (1999) Biochemistry 38, 10168–10177

51. McWhirter, S. M., Pullen, S. S., Holton, J. M., Crute, J. J., Kehry, M. R., and Alber, T. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 8408–8413

52. Park, Y. C., Burkitt, V., Villa, A. R., Tong, L., and Wu, H. (1999) Nature 398, 533–538

53. Deng, L., Wang, C., Spencer, E., Yang, Z., Braun, A., You, J., Slaughter, C., Pickart, C., and Chen, Z. J. (2000) Cell 103, 351–361

54. Sun, L., Deng, L., Eas, C. K., Xie, Z. P., and Chen, Z. J. (2004) Mol. Cell 14, 289–301

55. Wang, C., Deng, L., Hong, M., Akkaraju, G. R., Inoue, H., and Chen, Z. J. (2001) Nature 412, 346–351

56. Habelhah, H., Takahashi, S., Cho, S. G., Kadoya, T., Watanabe, T., and Ronai, Z. (2004) EMBO J. 23, 322–332

57. Shi, C. S., and Kehrl, J. H. (2003) J. Biol. Chem. 278, 15429–15434

58. Lee, T. H., Shank, J., Cusson, N., and Kelliher, M. A. (2004) J. Biol. Chem. 279, 33185–33191