Molecular Mechanism of Reactive Oxygen Species-dependent ASK1 Activation in Innate Immunity

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Apoptosis signal-regulating kinase 1 (ASK1), a mitogen- activated protein kinase kinase kinase, plays pivotal roles in stress responses. In addition, ASK1 has emerged as a key regulator of immune responses elicited by pathogen-associated molecular patterns (PAMPs) and endogenous danger signals. Recent studies have demonstrated that reactive oxygen species (ROS)-dependent activation of ASK1 is required for LPS-stimulated cytokine production as well as extracellular ATP-induced apoptosis in immune cells. The mechanism of ROS-dependent regulation of ASK1 activity by thioredoxin and TRAFs has been well characterized. In this review, we focus on the molecular details of the activation of ASK1 and its involvement in innate immunity.
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INTRODUCTION

The mitogen-activated protein kinase (MAPK) signaling pathways consist of three classes of protein kinases: MAPK, MAPK kinase (MAP2K), and MAP2K kinase (MAP3K). MAP3K phosphorylates and thereby activates MAP2K, and activated MAP2K in turn phosphorylates and activates MAPK such as ERK, JNK and p38 (1).

Apoptosis signal-regulating kinase 1 (ASK1) was originally identified as an apoptosis-inducing MAP3K of MKK4 (SEK1)/MKK7-JNK and MKK3/MKK6-p38 MAPK signaling cascades (2). Subsequent studies have shown that ASK1 is activated by various stresses including oxidative stress, TNF α, calcium influx, and endoplasmic reticulum stress (2-6).

In mammalian innate immunity, pathogen-associated molecular patterns (PAMPs) are recognized by specific toll-like receptors (TLRs), culminating in activation of the ERK, JNK, and p38 MAPK pathways and the nuclear factor-kappa B (NF-κB) pathways (7). Recently, it has been demonstrated that reactive oxygen species (ROS)-dependent activation of the ASK1-p38 pathway is required for TLR4-mediated innate immune responses (8,9).

Even in the absence of PAMPs, endogenous factors released from infected and/or damaged cells can elicit inflammatory responses. Extracellular ATP is known to serve as one such endogenous danger signal (10). ASK1 has been shown to be involved in extracellular ATP-induced macrophage apoptosis downstream of $P2X_7$ receptor, a plasma membrane receptor for ATP (T. Noguchi, et al. unpublished data).

Thus, ASK1 appears to be a pivotal component of both stress and immune responses. Here, we review recent studies on the mechanism of ROS-dependent ASK1 activation and its roles in host defense.

ASK1 SIGNALOSOME

Human and mouse ASK1 consist of 1374 and 1380 amino acids, respectively. ASK1 possesses a serine/threonine kinase domain in the middle portion of the molecule flanked by the N- and C-terminal coiled-coil domains (11,12). The two coiled-coil domains play critical roles in regulation of ASK1 activity (Fig. 1).

Endogenous ASK1 is constitutively oligomerized through its C-terminal coiled-coil (CCC) domain and forms a high molec-

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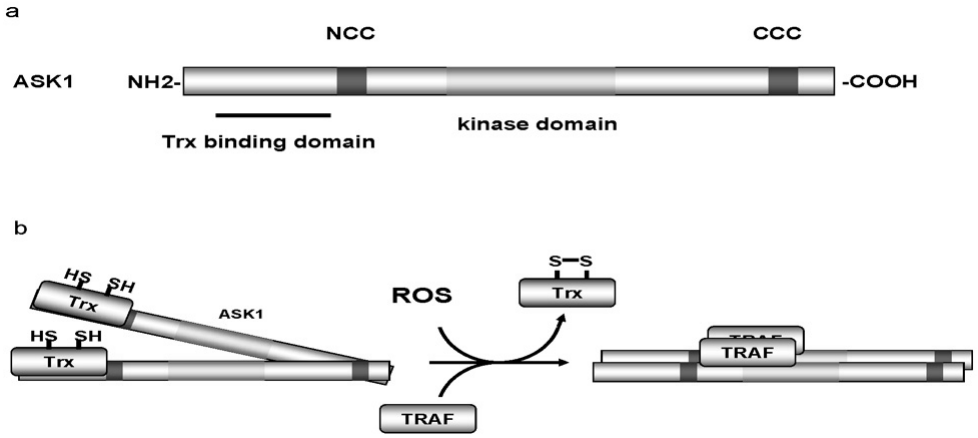

Figure 1. (a) Domain structure of ASK1. Human and mouse ASK1 consists of 1374 and 1380 amino acids, respectively. ASK1 has a serine/threonine kinase domain in the middle portion of the molecule flanked by the N- and C-terminal coiled-coil domains (NCC and CCC, respectively). Trx binds to the N-terminal region of ASK1. (b) ROS-dependent activation of ASK1. ASK1 constitutively forms high molecular mass complex including Trx. In response to ROS, Trx is oxidized and dissociate from ASK1, leading to the recruitment of TRAFs. TRAFs promote the NCC-dependent homophilic interaction of ASK1 and thereby activate ASK1.

ular mass complex (approximately 1,500～2,000 kDa) together with its regulatory proteins and unidentified factors. The high molecular mass complex appears to act as a molecular platform regulating basal activity and stimulus-induced activation of ASK1, which we designated ASK1 signalosome (13).

THIOREDOXIN

Thioredoxin (Trx) is a ubiquitously expressed reduction/ oxidation (redox) protein that is known to act as an antioxidant and a reductant cofactor (14,15). Trx is the first identified interacting protein of ASK1 and has been established to be a signaling intermediate of the ROS-ASK1 cascade. Trx possesses two redox-active cysteine residues in its active center (-Cys-Gly-Pro-Cys-), which form intramolecular disulfide bond in response to ROS. The reduced form of Trx [Trx-$(SH)_2$] directly binds to the N-terminal region of ASK1 and inhibits its kinase activity. Once oxidized, Trx (Trx-S_2) dissociates from ASK1 (16).

Our group recently found that the homophilic interaction of ASK1 through the N-terminal coiled-coil domain (NCC) was necessary for ROS-induced activation of ASK1. Trx disrupts this interaction by directly binding to ASK1. Therefore, the dissociation of Trx from ASK1 is prerequisite to the activation of ASK1 in response to ROS (17,18).

TRAFs

Dissociation of Trx from ASK1 is necessary but not sufficient for the activation of ASK1. Tumor necrosis factor receptor-associated factors (TRAFs) are the major signal transducers of the TNF receptor superfamily and the interleukin (IL)-1 receptor/TLR superfamily. By mediating the signaling pathways downstream of these receptors, TRAFs regulate various physiological processes including adaptive and innate immunity, embryonic development, bone metabolism and stress response (19). Among the TRAF family members, TRAF2 and TRAF6 have been established to play crucial roles in ASK1 activation (3,13).

Following the dissociation of Trx, the ASK1 signalosome forms a higher molecular mass complex (approximately more than 3,000 kDa), at least in part, due to the recruitment of TRAF2 and TRAF6 (13,18). In contrast to Trx, TRAF2 and TRAF6 promote the NCC-dependent homophilic interaction of ASK1, which results in autophosphorylation of the threonine residue in the activation loop and thereby activation of ASK1. Accordingly, H_2O_2-induced activation of ASK1 is strongly suppressed in TRAF2- and TRAF6-deficient mouse embryonic fibroblasts (MEFs) (13,17,20).

ASK1 IN TLR4-SIGNALING

Cells involved in the innate immune system include macro-

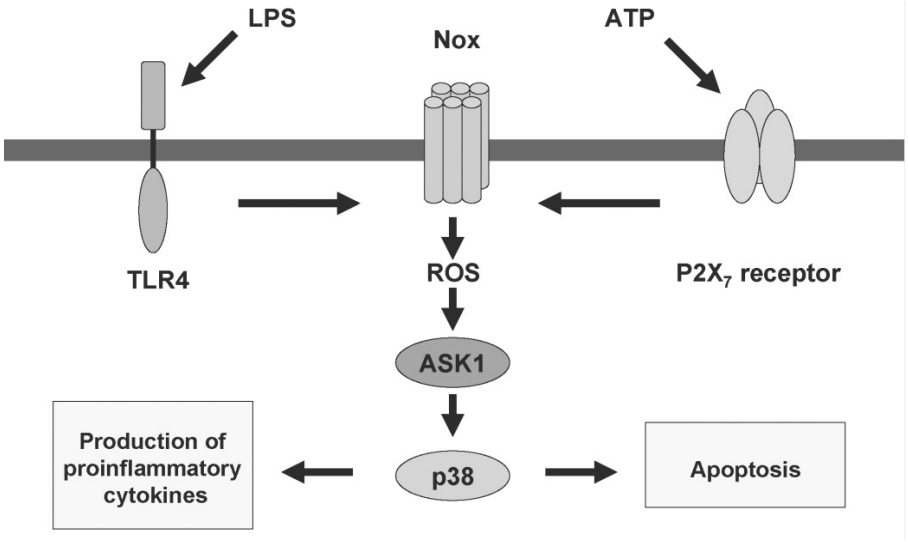

Figure 2. ROS-dependent activation of ASK1 in TLR4- and $P2X_7$ receptor-signaling.

phages, dendritic cells (DCs) and neutrophils, and these cells are responsible for the detection of pathogenic microorganisms following infection. The TLR family proteins recognize the molecular signature of invading pathogens, known as PAMPs, and make an important contribution to host defense. Each PAMP such as lipids, lipoproteins, proteins and nucleic acids is sensed by distinct TLRs, culminating in activation of the ERK, JNK and p38 MAPK pathways as well as the NF-κB pathways. In particular, TLR4 mediates the host cell responses toward the bacterial cell wall component lipopolysaccharide (LPS) (7,21).

Recent studies in mice have shown that many MAP3Ks are involved in TLR4-mediated inflammatory responses (22). For example, MEKK3 is required for the activation of JNK and p38, but not of ERK by TLR4. MEKK3-deficient MEF produces low level of IL-6 in response to LPS (23). Tpl2/Cot, another MAP3K, mediates the ERK pathway downstream of TLR4 and contributes to LPS upregulation of TNFα in splenocytes and macrophages (24).

ASK1 is also involved in mammalian innate immune responses mediated by TLR4 (Fig. 2). LPS activates ASK1 in RAW 264.7 murine macrophage cell line. In DCs and splenocytes from ASK1-deficient mice, the activation of p38 following LPS stimulation is impaired, whereas the activation of JNK and NF-κB is not affected. LPS-induced production of proinflammatory factors including IL-6, TNFα, IL-1β, IL-12 and NO is reduced in these cells when compared with control cells. Consistently, ASK1-deficient mice produce small amounts of TNFα and NO after LPS injection and therefore are resistant to septic shock. These data indicate that the ASK1-p38 pathway is required for the induction of proinflammatory cytokines downstream of TLR4 in vivo (8).

Interestingly, LPS-induced activation of ASK1 is dependent on the generation of ROS. In RAW 264.7 cells, pretreatment of antioxidants abolishes the activation of ASK1 and the cytokine production in response to LPS. Furthermore, LPS stimulates the interaction of ASK1 and TRAF6 in a ROS-dependent manner. Given the importance of Trx as a redox-dependent regulator of ASK1, it is conceivable that Trx is involved in this process (8).

Although the precise molecular mechanism underlying ROS generation after LPS stimulation remains elusive, the involvement of Nox4 has been shown. Nox4 is a member of the NADPH oxydase family that catalyzes the NADPH-dependent reduction of oxygen to generate superoxide (25). In HEK293T cells, direct interaction of Nox4 with TLR4 was reported to be required for LPS-stimulated ROS generation and NF-κB activation (26). Nox4 was also suggested to be responsible for LPS-induced ASK1 activation in U373 astrocytoma cells (27). However, the physiological relevance of these findings in immune cells remains unclear.

ASK1 IN $P2X_7$ RECEPTOR-SIGNALING

In recent years, it has been shown that endogenous factors released from infected and/or damaged cells can elicit in-

flammatory responses even in the absence of PAMPs. Extracellular ATP has been reported to serve as one such endogenous danger signal by acting on cell-surface ATP receptors (10).

Nucleotides including ATP are usually present at high concentrations within the cytoplasm (5∼10 mM), whereas their extracellular concentrations are low (nanomolar). Acute cell injury or death are therefore thought to cause passive ATP release into extracellular milieu (10). Moreover, ATP is reported to be actively secreted via as yet unidentified pathways (28). Such extracellular ATP stimulates two classes of cell-surface receptors; the ionotropic P2X receptors and metabotropic P2Y receptors. P2X receptors and P2Y receptors are ligand-gated cation channels and G-protein-coupled receptors in the plasma membrane, respectively (29). Accumulating evidence has suggested that $P2X_7$ receptor, a P2X receptor expressed primarily in immune cells, plays important roles in extracellular ATP-induced inflammatory responses. For example, ATP-stimulated maturation and secretion of the proinflammatory cytokine IL-1β have been shown to be mediated by $P2X_7$ receptor (30,31).

Recently, our group demonstrated that the $P2X_7$ receptor-ASK1-p38 pathway was necessary for ATP-induced apoptosis in macrophages (Fig. 2). In RAW 264.7 cells, ASK1 is activated in response to ATP, which is mediated by ROS derived from Nox2/gp91phox, a component of phagocytic Nox complex. ROS generation is almost completely inhibited by selective $P2X_7$ receptor antagonist, suggesting that $P2X_7$ receptor is responsible for the activation of ASK1. In spleen-derived macrophages from ASK1−/− mice, ATP-induced p38 activation and consequent apoptosis are suppressed (T. Noguchi, et al. unpublished data).

Although physiological role of ATP-induced apoptosis in macrophages remains to be elucidated, it is reported that macrophages infected with certain bacteria undergo apoptosis, resulting in the death of the intracellular pathogens (32). Therefore, the $P2X_7$ receptor-ASK1-p38 pathway might play a critical role in host defense against invading pathogens.

CONCLUSION AND PERSPECTIVE

Although the molecular mechanism of ROS-dependent ASK1 activation has been well characterized, important questions remain unanswered. Previous studies have established that Trx is a redox-dependent negative regulator of ASK1 (13,16,17). After the dissociation of Trx in response to ROS, TRAFs are recruited to the ASK1 signalosome. TRAFs promote homophilic interaction of ASK1 through the NCC domain, leading to the activation of ASK1. It remains unknown how Trx and TRAFs negatively and positively regulate this interaction, respectively.

TRAF6 is a RING domain-containing E3 ubiquitin ligase that synthesizes a polyubiquitin chain linked through lysine-63 of ubiquitin. TRAF6 ubiquitination has been shown to be involved in TAK1 activation. TAK1 is a member of MAP3K family capable of activating both MAPKs and NF-κB pathways upon stimulation of IL-1β, TNFα and ligands of several TLRs (33-35). In addition, ubiquitin-conjugating enzyme Ubc13 has been demonstrated to be a crucial component of TRAF6-mediated inflammatory responses downstream of TLR4, suggesting that TRAF6 serves as a ubiquitin ligase in TLR4-signaling (36,37). Whether TRAFs ubiquitination is necessary for ASK1 activation is yet to be determined.

Recent structural analysis of ASK1 has demonstrated that ASK1 kinase domain forms a dimmer interacting in a head-to-tail orientation, which might play a pivotal role in endogenous ASK1 oligomerization (38). Structural analysis of full-length ASK1 or ASK1 complex with its regulatory proteins such as Trx, TRAF2, and TRAF6 will provide more insight into the molecular mechanism of ASK1 activation.

ASK1 appears to be important for various immune responses mediated by ROS. It has been demonstrated that ROS-dependent activation of ASK1 is required for TLR4-mediated inflammatory responses toward LPS. Furthermore, ROS-dependent $P2X_7$ receptor-ASK1-p38 pathway is necessary for ATP-induced apoptosis in macrophages. Nox4 and Nox2 have been suggested to be responsible for ROS generation in TLR4- and $P2X_7$ receptor-signaling, respectively. However, the precise mechanism of their activation remains elusive (8).

Although many MAP3Ks are identified to mediate TLR-initiated innate immune responses, their distinct roles are poorly understood (22). Notably, MEKK3 is involved in the activation of the p38 pathway downstream of TLR4 in MEFs as well as ASK1 in DCs and splenocytes (8,23). Further studies are required for elucidation of specific roles of MAP3Ks as TLR-signaling components.

Thus, we are still in the early stages of investigating ASK1-signaling in immunity. Continued research in this field will contribute to greater understanding of host defense.

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