

Roles of MAPKKK ASK1 in Stress-Induced Cell Death

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ABSTRACT. Apoptosis signal-regulating kinase 1 (ASK1) is a ubiquitously expressed mitogen-activated protein (MAP) kinase kinase kinase that activates the c-Jun N-terminal kinase (JNK) and p38 MAP kinase signaling cascades. Recent findings from analyses of ASK1-deficient mice have revealed that ASK1 is required for apoptosis induced by oxidative stress, TNF and endoplasmic reticulum (ER) stress. In addition, several lines of evidence have suggested that ASK1 has diverse functions in the decision of cell fate beyond its pro-apoptotic activity. Thus, ASK1 appears to be a pivotal component not only in stress-induced cell death but also in a broad range of biological activities in order for cells to adapt to or oppose various stresses.

Key words: MAPKKK/ASK1/JNK/p38/apoptosis

The mitogen-activated protein (MAP) kinase cascades are multifunctional signaling pathways that are evolutionally well conserved in all eukaryotic cells (Ichijo, 1999; Widmann, 1999; Kyriakis and Avruch, 2001). Three MAP kinase cascades that converge on ERKs, c-Jun N-terminal kinases (JNKs), and p38 MAP kinases have been extensively characterized, and each consists of three classes of serine/threonine kinases, MAP kinase, MAP kinase kinase (MAPKK, also referred to as MEK) and MAPKK kinase (MAPKKK). MAPKKK phosphorylates and thereby activates MAPKK, and activated MAPKK in turn phosphorylates and activates MAP kinase. Among the three MAP kinase cascades, two of them that converge on JNKs and p38 MAP kinases are preferentially activated by cytotoxic stresses such as UV radiation, X-ray, heat shock and osmotic shock, and by proinflammatory cytokines such as tumor necrosis factor (TNF) and interleukin-1 (Tibbles and

Woodgett, 1999). One of the important biological responses mediated through these stress-activated MAP kinase pathways appears to be the decision of cell fate by regulating apoptosis. The possible roles of the JNK pathway in pro-apoptosis signaling have been demonstrated by knockout mouse studies. Mice lacking the JNK3 gene were reported to exhibit marked reduction in excitotoxicity-induced apoptosis of hippocampal neurons (Yang *et al.*, 1997). JNK2 was shown to be required for apoptosis of immature thymocytes induced by anti-CD3 antibody but not for activation-induced cell death of mature T cells (Sabapathy *et al.*, 1999). Compound mutant mice lacking the JNK1 and JNK2 genes suggested that JNK1 and JNK2 regulate region-specific apoptosis during early brain development (Kuan *et al.*, 1999). Several lines of evidence have also suggested the pro-apoptotic roles of the p38 pathway (Xia *et al.*, 1995; Kawasaki *et al.*, 1997; Harper and LoGrasso *et al.*, 2001), although they have not yet been supported by data of mice deficient for the p38 genes.

Apoptosis signal-regulating kinase 1 (ASK1)/MAPKKK5 is a ubiquitously expressed MAPKKK that activates the JNK and p38 pathways by directly phosphorylating and thereby activating their respective MAPKKs, MKK4 (SEK1)/MKK7 and MKK3/MKK6 (Wang *et al.*, 1996; Ichijo *et al.*, 1997) (Fig. 1). Overexpression of wild-type or constitutively active ASK1 induces apoptosis in various cells through mitochondria-dependent caspase activation

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Abbreviations: ASK1, apoptosis signal-regulating kinase 1; EGF, epidermal growth factor; ER, endoplasmic reticulum; JNK, c-Jun N-terminal kinase; MAP, mitogen-activated protein; MEF, mouse embryonic fibroblast; Nac, N-acetyl-L-cysteine; NGF, nerve growth factor; PP5, protein serine/threonine phosphatase 5; ROS, reactive oxygen species; TNF, tumor necrosis factor; TRAF2, TNF receptor-associated factor 2; Trx, thioredoxin.

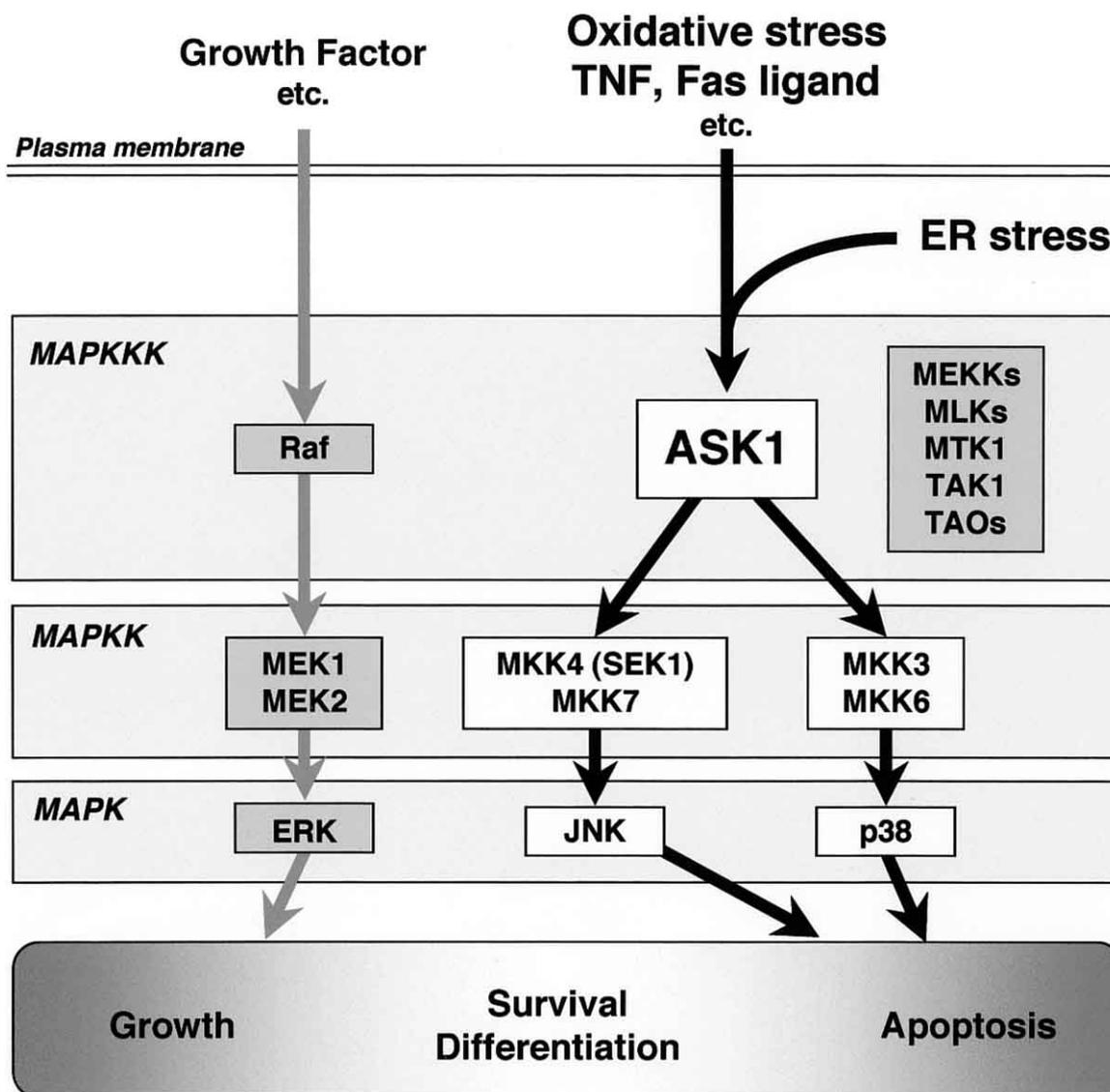


Fig. 1. ASK1 is a regulator of the JNK and p38 MAP kinase cascades in stress signaling. Three MAP kinase cascades that converge on ERKs, JNKs, and p38 MAP kinases have been extensively characterized, and each consists of three classes of serine/threonine kinases, MAP kinase (MAPK), MAPKK, and MAPKKK. MAPKKK phosphorylates and thereby activates MAPKK, and activated MAPKK in turn phosphorylates and activates MAP kinase. ASK1 is a MAPKKK that activates the JNK and p38 pathways by directly activating respective MAPKKs, MKK4 (SEK1)/MKK7 and MKK3/MKK6. ASK1 is essential for apoptosis induced by oxidative stress, TNF and ER stress. In Fas signaling, ASK1 appears to be required for JNK/p38 activation but not for Fas-induced apoptosis at least in certain types of cultured cell.

(Saitoh *et al.*, 1998; Kanamoto *et al.*, 2000; Hatai *et al.*, 2000), and ASK1 is required for apoptosis induced by oxidative stress, TNF and endoplasmic reticulum (ER) stress (Tobiume *et al.*, 2001; Nishitoh *et al.*, 2002). On the other hand, several lines of evidence have suggested that ASK1 has diverse functions in the decision of cell fate such as differentiation and survival (Sayama *et al.*, 2000; Takeda *et al.*, 2000; Sagasti *et al.*, 2001; Kim *et al.*, 2002). Thus, ASK1 appears to be a pivotal component not only in stress-induced cell death but also in a broad range of biological

activities. In this review, we focus on the activation mechanisms and physiological roles of ASK1 in the control of cell fate in response to stresses.

Activation mechanism of ASK1

Human and mouse ASK1 consist of 1375 and 1379 amino acids, respectively, and each possess a serine/threonine kinase domain in the middle part of the molecule with long NH₂- and COOH-terminal flanking regions (Ichijo *et*

al., 1997; Tobiume *et al.*, 1997). We first identified thioredoxin (Trx), a reduction/oxidation (redox)-regulatory protein, as an interaction partner of ASK1 by the yeast two hybrid screening (Saitoh *et al.*, 1998). Trx inhibits ASK1 kinase activity by direct binding to the N-terminal region of ASK1, and an ASK1 mutant that lacks N-terminal region (ASK1 Δ N) behaves as a constitutively active kinase. Trx has a redox active site, in which two cysteine residues provide the sulfhydryl groups involved in Trx-dependent reducing activity. Only the reduced form [Trx-(SH)₂], but not the oxidative form (Trx-S₂) or the mutant of the redox active site, of Trx binds to ASK1, suggesting that ASK1 activity depends on the redox status of Trx (Saitoh *et al.*, 1998; Liu and Min, 2002). In fact, reactive oxygen species (ROS) such as hydrogen peroxide (H₂O₂) dissociates Trx from ASK1 and thereby activates ASK1 by inducing oligomerization and subsequent phosphorylation of a critical threonine residue within the activation loop of ASK1 (Thr845 and Thr838 of mouse and human ASK1, respectively) (Gotoh and Cooper, 1998; Saitoh *et al.*, 1998; Tobiume *et al.*, 2002). The Trx-ASK1 system serves as a molecular switch that converts redox signal evoked by various oxidative stresses to the signaling through kinase cascades.

The Trx-ASK1 system is also involved in TNF signaling. TNF induces the dissociation of ASK1 and Trx, which is sensitive to the pretreatment with an antioxidant, N-acetyl-L-cysteine (Nac), suggesting that TNF activates ASK1 through redox signaling (Saitoh *et al.*, 1998; Liu *et al.*, 2000b). We have shown that TNF-dependent association of ASK1 with TNF receptor-associated factor 2 (TRAF2), an adaptor protein that couples TNF receptors to the JNK and p38 pathways, is required for the TNF-induced activation of ASK1 (Nishitoh *et al.*, 1998). Since the treatment with TNF or overexpression of TRAF2 has been reported to produce ROS (Goossens *et al.*, 1995; Liu *et al.*, 2000b), TNF-dependent production of ROS triggers the dissociation of Trx from ASK1 and the consequently liberalized ASK1 binds to TRAF2. Finally, TRAF2 appears to activate ASK1 by enhancing and stabilizing the oligomerization of ASK1 (Liu *et al.*, 2000b) (Fig. 2).

ASK1 activity is tightly regulated by another negative regulator, protein serine/threonine phosphatase (PP5). PP5 specifically binds to the activated form of ASK1 in response to oxidative stress and accomplishes dephosphorylation, thus inactivation, of ASK1 as a negative feedback mechanism (Morita *et al.*, 2001). Thus, Trx and PP5 negatively regulate ASK1 activity by distinct mechanisms, suggesting that ASK1 comprises an integrated system for oxidative stress signaling.

ASK1 is required for oxidative stress- and TNF-induced apoptosis

Previous studies demonstrated that overexpression of wild-type or constitutively active ASK1 induced apoptosis,

and that apoptosis induced by various stresses including TNF, anti-cancer drugs and deprivation of growth factors was inhibited by expression of a kinase-inactive mutant of ASK1 (Ichijo *et al.*, 1997; Saitoh *et al.*, 1998; Wang *et al.*, 1999; Kanamoto *et al.*, 2000; Hatai *et al.*, 2000). These findings suggest that ASK1 is a pivotal component in stress-induced apoptosis. We have recently disrupted the ASK1 gene in mice (Tobiume *et al.*, 2001). ASK1 $^{-/-}$ mice are born at the expected Mendelian frequency and are indistinguishable in appearance from their wild-type littermates. Histological analysis of ASK1-deficient mice also detected no developmental abnormalities, suggesting that ASK1 may not be necessary for apoptosis associated with fertility and embryogenesis. In ASK1 $^{-/-}$ mouse embryonic fibroblasts (MEFs), however, H₂O₂- and TNF-induced apoptosis is significantly impaired, indicating that ASK1 is required for oxidative stress- and TNF-induced apoptosis.

Concerning JNK and p38, the sustained but not the transient phase of activation of JNK and p38 in response to H₂O₂ or TNF is considerably impaired in ASK1 $^{-/-}$ MEFs (Tobiume *et al.*, 2001). ASK1-dependent sustained phase of JNK and/or p38 activation may mediate H₂O₂- and TNF-induced pro-apoptosis signals, although such activation of JNK/p38 may not be sufficient for the induction of apoptosis. The differential roles of transient versus sustained activation of MAP kinases have initially been proposed for the ERK pathway; transient activation of ERK induced by such as EGF causes proliferation of PC12 cells, whereas sustained activation induced by NGF induces neuronal differentiation (Marshall, 1995). In the cells treated with TNF (Guo *et al.*, 1998; Roulston *et al.*, 1998), UV-C, γ -irradiation (Chen *et al.*, 1996) or growth factor withdrawal (Xia *et al.*, 1995), transient and sustained activations of JNK/p38 have been implicated in cell survival and apoptosis, respectively. Duration of activation may thus be a critical factor in the cell fate decision through the MAP kinase pathways. In this regard, ASK1 $^{-/-}$ cells provide a good tool by which we can further investigate the physiological roles for the duration of JNK/p38 activation in oxidative stress- and TNF-induced apoptosis.

ASK1 is distinctively required for Fas signaling

Fas is a well-characterized member of the death receptor family. Engagement of Fas by Fas-ligand leads to the formation of a protein complex known as death-inducing signaling complex (DISC) and permits acute execution of apoptosis by caspase-8 activation (Nagata, 1999). Upon Fas activation, an alternative pathway involving the Fas-binding protein Daxx is also activated (Yang *et al.*, 1997). Daxx binds to N-terminal region of ASK1 in a ligand-dependent manner and thereby activates JNK, which may sensitize cells to apoptosis (Chang *et al.*, 1998). Consistently, Fas-induced JNK and p38 activities are decreased in ASK1 $^{-/-}$ thymocytes. However, Fas activation yields comparable

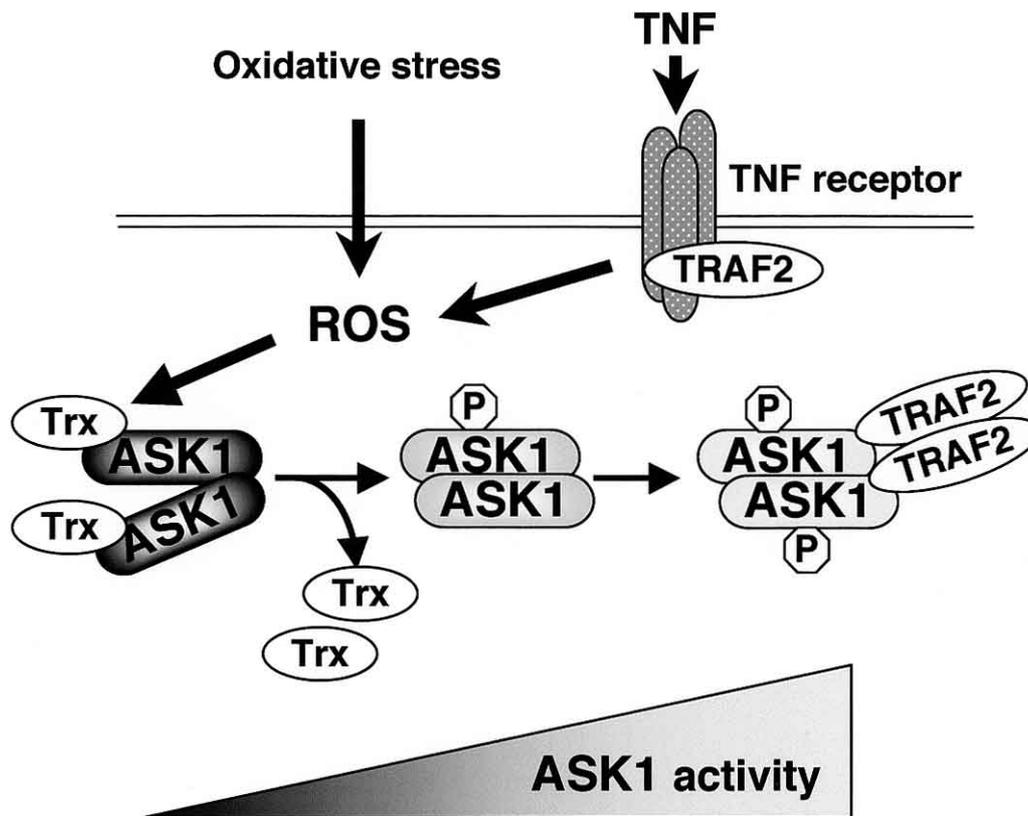


Fig. 2. Activation mechanism of ASK1. ASK1 constantly forms an inactive complex with Trx. ROS produced by various stresses or the treatment with TNF dissociates Trx from ASK1 and thereby activates ASK1 by inducing oligomerization and subsequent phosphorylation of a critical threonine residue within the activation loop of ASK1. In TNF signaling, the liberalized ASK1 from Trx binds to TRAF2, and TRAF2 appears to activate ASK1 by enhancing and stabilizing the oligomerization of ASK1.

apoptotic change in *ASK1*^{+/+} and *ASK1*^{-/-} thymocytes and MEFs (Tobiume *et al.*, 2001). These findings indicate that, although ASK1 serves as a critical regulator of the JNK and p38 pathways in Fas signaling, ASK1 is not required for Fas-induced apoptosis at least in certain types of primary cultured cells. These findings also indicate that activation of JNK and p38 is not necessarily required for execution of apoptosis, and suggest the unknown physiological function of the ASK1-JNK/p38 pathways in Fas signaling.

The differential requirement of ASK1 for TNF- and Fas-induced apoptosis can be attributable to the difference in dependency on the FADD-caspase-8 pathway, which is commonly required for TNF- and Fas-induced apoptosis (Nagata, 1997). In fact, caspase-8 activity induced by TNF was significantly lower than that induced by Fas when both stimuli induced similar magnitudes of apoptosis, suggesting that TNF is less dependent on the FADD-caspase-8 pathway than Fas (Tobiume *et al.*, 2001). Instead, the antioxidant-sensitive, i.e. ROS-dependent, pro-apoptotic signaling is involved in TNF-induced apoptosis as mentioned above; antioxidants such as NAC partially inhibits TNF-induced apoptosis (Schuize-Osthoff *et al.*, 1994; Saitoh *et al.*, 1998),

whereas Fas-induced apoptosis can occur in low oxygen and does not appear to require the generation of ROS (Jacobson and Raff, 1995). Interestingly, TNF-induced apoptosis in *ASK1*^{-/-} cells was still observed to some extent but was no longer sensitive to Nac (Tobiume *et al.*, 2001). These findings provide genetic evidence that the ASK1-JNK/p38 pathways are specifically required for ROS-dependent but not for caspase-8-dependent pro-apoptotic signals.

ASK1 is required for ER stress-induced apoptosis

Accumulation of unfolded and/or misfolded proteins within the ER lumen induces ER stress. ER stress triggers the expression of a number of molecular chaperones, such as Bip/GRP78, GRP94 and protein disulfide isomerase, which support cell survival by refolding of affected proteins. However, an excess extent of and/or a continuous exposure to ER stress eventually leads cells to apoptosis (Kaufman, 1999; Mori, 2000). Initial mediators of ER stress responses are ER-resident type I transmembrane serine/

threonine protein kinase, PERK and IRE1. Accumulation of unfolded proteins in ER induces oligomerization-dependent autophosphorylation of these kinases and thereby initiates cytoplasmic signal transduction (Bertolotti *et al.*, 2000; Liu *et al.*, 2000a). It has been shown that activated IRE1 on ER membrane recruits TRAF2 and ignites JNK activation, suggesting that the IRE1-TRAF2-JNK axis is a pivotal signaling component for responses to ER stress (Urano *et al.*, 2000). Recently, we have shown that ER stress activates the ASK1-JNK pathway through the IRE1-TRAF2-ASK1 complex formation. Importantly, JNK activation and apoptosis induced by the ER stress were considerably impaired in *ASK1*^{-/-} MEFs and primary neurons, indicating that ASK1 plays essential roles in ER stress-induced apoptosis (Nishitoh *et al.*, 2002). These results also indicate that the formation of the IRE1-TRAF2-ASK1 complex is critical for the ER stress-induced pro-apoptotic signaling through JNK activation.

ER stress has been implicated in human diseases such as amyloidosis, hypercholesterolemia, diabetes mellitus, and neurodegenerative disorders (Aridor and Balch, 1999; Kopito and Ron, 2000). It has been proposed that intracellular aggregation of insoluble proteins can be a common pathogenesis of various types of neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, and polyglutamine (polyQ) diseases (Kakizuka, 1998; Sherman, 2001). Recently, we have shown that ASK1 plays an important role in the neuropathological alterations in polyQ diseases (Nishitoh *et al.*, 2002). We found that forced expression of expanded and pathogenic length of polyQ triggered ASK1 activation through ER stress. One mechanism by which polyQ triggers ER stress appeared to be the impairment of the function of the ubiquitin-proteasome system, since the expression of expanded polyQ inhibited the proteasome activity and the treatment of cells with proteasome inhibitors triggered ER stress (Bush *et al.*, 1997; Bence *et al.*, 2001; Waelter *et al.*, 2001; Nishitoh *et al.*, 2002). The expression of expanded polyQ induced JNK activation and cell death, both of which were impaired in *ASK1*^{-/-} primary neurons (Nishitoh *et al.*, 2002). These findings strongly suggest that ASK1 is a key element in the control of cell fate in the pathological condition triggered by ER stress.

Emerging roles of ASK1 beyond the pro-apoptotic signaling intermediate

As we mentioned above in the section of Fas signaling, activation of the ASK1-JNK/p38 pathways do not necessarily induce cell death. Recently, strong evidence supporting novel roles of ASK1 has emerged from *C. elegans*. In *C. elegans*, calcium signaling through a voltage-dependent calcium channel and UNC-43 calcium/calmodulin-dependent protein kinase type II (CaMKII) determines asymmetric expression of the candidate odorant receptor gene *str-2* in a

specific bilateral pair of olfactory neurons (Troemel *et al.*, 1999). NSY-1, the *C. elegans* homolog of ASK1, has been found to function downstream of UNC-43 CaMKII in the control of asymmetric expression of *str-2*, odor discrimination and odor chemotaxis, suggesting the possible involvement of ASK1 in the functional differentiation of the nervous system (Sagasti *et al.*, 2001; Wes and Bargmann, 2001). NSY-1 has also been identified as a signaling component required for anti-bacterial response in *C. elegans*; *nsy-1* mutant shows enhanced susceptibility to killing by *Pseudomonas aeruginosa* (Kim *et al.*, 2002). This genetic evidence suggests that ASK1 may play a pivotal role in innate immune responses. Together with the findings that ASK1 induces neuronal differentiation and survival of PC12 cells and keratinocyte differentiation (Sayama *et al.*, 2000; Takeda *et al.*, 2000), ASK1 appears to mediate a broad range of biological activities as a determinant of cell fate.

Conclusion

ASK1 appears to act as a pro-apoptotic intermediate when cells receive strong stresses. In addition, ASK1 appears to possess various biological functions such as the control of differentiation status and the mediation of survival signals in order cells to adapt to or oppose various moderate stresses. Taken together, ASK1 functions as a multifunctional stress-sensing kinase to control cell fate in response to various kinds and strengths of stresses. Further investigations of the pathophysiological roles of ASK1 may suggest concrete strategies to overcome stress-related diseases.

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