The EMBO Journal Vol.17 No.9 pp.2596–2606, 1998

Mammalian thioredoxin is a direct inhibitor of apoptosis signal-regulating kinase (ASK) 1

Masao Saitoh, Hideki Nishitoh, Makiko Fujii, Kohsuke Takeda, Kei Tobiume, Yasuhiro Sawada, Masahiro Kawabata, Kohei Miyazono and Hidenori Ichijo[1,2]

Department of Biochemistry, The Cancer Institute, Tokyo, Japanese Foundation for Cancer Research, 1-37-1 Kami-Ikebukuro, Toshima-ku, Tokyo 170, Japan

[1]Present address: Department of Biomaterials Science, Faculty of Dentistry, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8549, Japan

[2]Corresponding author

M.Saitoh and H.Nishitoh contributed equally to this work

Apoptosis signal-regulating kinase (ASK) 1 was recently identified as a mitogen-activated protein (MAP) kinase kinase kinase which activates the c-Jun N-terminal kinase (JNK) and p38 MAP kinase pathways and is required for tumor necrosis factor (TNF)-α-induced apoptosis; however, the mechanism regulating ASK1 activity is unknown. Through genetic screening for ASK1-binding proteins, thioredoxin (Trx), a reduction/oxidation (redox)-regulatory protein thought to have anti-apoptotic effects, was identified as an interacting partner of ASK1. Trx associated with the N-terminal portion of ASK1 *in vitro* and *in vivo*. Expression of Trx inhibited ASK1 kinase activity and the subsequent ASK1-dependent apoptosis. Treatment of cells with *N*-acetyl-L-cysteine also inhibited serum withdrawal-, TNF-α- and hydrogen peroxide-induced activation of ASK1 as well as apoptosis. The interaction between Trx and ASK1 was found to be highly dependent on the redox status of Trx. Moreover, inhibition of Trx resulted in activation of endogenous ASK1 activity, suggesting that Trx is a physiological inhibitor of ASK1. The evidence that Trx is a negative regulator of ASK1 suggests possible mechanisms for redox regulation of the apoptosis signal transduction pathway as well as the effects of antioxidants against cytokine- and stress-induced apoptosis.

Keywords: apoptosis/ASK1/MAP kinase/redox/thioredoxin

Introduction

Apoptosis plays an essential role in animal development, homeostasis and the pathogenesis of multiple diseases including cancer, infectious and neurodegenerative disorders (reviewed by Steller, 1995; Thompson, 1995; Nagata, 1996; Kroemer *et al.*, 1997; Salvesen and Dixit, 1997). While numerous candidate genes playing important roles in the signaling of apoptosis have been identified, the mechanisms by and order in which they interact to transduce signals are poorly understood. Several lines of evidence have recently suggested that members of the mitogen-activated protein (MAP) kinase family are involved in apoptotic signaling, as well as in growth and differentiation control (reviewed by Errede and Levin, 1993; Nishida and Gotoh, 1993; Davis, 1994; Marshall, 1994; Waskiewicz and Cooper, 1995; Fanger *et al.*, 1997). Two different MAP kinase cascades that converge to c-Jun N-terminal kinase (JNK; also known as SAPK, stress-activated protein kinase) and p38 MAP kinase are preferentially activated in response to various cytotoxic stresses, including tumor necrosis factor (TNF)-α, hydrogen peroxide (H_2O_2), UV light, X-rays, heat shock and growth factor- or serum-withdrawal (Raingeaud *et al.*, 1995; Xia *et al.*, 1995; Kyriakis and Avruch, 1996; Verheij *et al.*, 1996). Apoptosis signal-regulating kinase (ASK) 1 is a recently identified MAP kinase kinase kinase (MAPKKK) that activates SEK1-JNK and MKK3/MKK6-p38 signaling cascades (Ichijo *et al.*, 1997). Overexpression of ASK1 in epithelial cells in low serum conditions induced cell death with apoptotic features and ASK1-K709R, a kinase-inactive mutant of ASK1, reduced TNF-α-induced apoptosis, suggesting that ASK1 is a pivotal component in the mechanism of cytokine- and stress-induced apoptosis (Ichijo *et al.*, 1997; Tobiume *et al.*, 1997). However, the molecular mechanism of regulation of ASK1 activity is unknown.

Reactive oxygen species (ROS) are thought to participate in a wide variety of cellular functions, including cell proliferation, differentiation and apoptosis (reviewed by Buttke and Sandstrom, 1994; Camhi *et al.*, 1995; Jacobson, 1996). ROS have been shown to act as essential intracellular second messengers for certain cytokines and growth factors (Meier *et al.*, 1989; Bulkley, 1994; Levine *et al.*, 1994; Ohba *et al.*, 1994; Lo and Cruz, 1995; Irani *et al.*, 1997). On the other hand, several observations have suggested that ROS might mediate apoptosis: (i) various cytotoxic stresses are sometimes accompanied by increases in intracellular ROS levels (Yamauchi *et al.*, 1989; Jacobson, 1996); (ii) addition of ROS or depletion of cellular antioxidants induces apoptosis (Buttke and Sandstrom, 1994); (iii) many antioxidants and free-radical scavengers including *N*-acetyl-L-cysteine (Nac) have been shown to inhibit or delay certain types of cell death induced, for example, by TNF-α, anticancer drugs, irradiation or survival factor withdrawal (Mayer and Noble, 1994; Cossarizza *et al.*, 1995); and (iv) anaerobic culture conditions inhibit TNF-α-induced cell death (Matthews *et al.*, 1987). Although the importance of ROS in the execution of apoptosis is controversial, ROS are likely to be involved in the signal transduction mechanism for apoptosis as signaling intermediates (Jacobson, 1996). However, the specific molecular targets of ROS in apoptosis signaling are largely unknown.

Thioredoxin (Trx) is a 12 kDa protein ubiquitously expressed in all living cells, which has a variety of biological functions related to cell proliferation and apoptosis (reviewed by Holmgren, 1985, 1995; Eklund *et al.*, 1991; Nakamura *et al.*, 1997). It is characterized by the reduction/oxidation (redox) active site sequence Trp-Cys-Gly-Pro-Cys-Lys, which is conserved through evolution. The two cysteine residues within the redox active center provide the sulfhydryl groups involved in Trx-dependent reducing activity. The oxidized form (Trx-S_2) contains a disulfide bridge in the active site that is reduced to a dithiol by NADPH and the flavoprotein Trx reductase. Thus, the Trx system is composed of Trx, Trx reductase and NADPH *in vivo* (Buchanan *et al.*, 1994; Holmgren, 1995). The reduced form [Trx-$(SH)_2$] acts as a potent protein disulfide oxido-reductase. Human Trx is identical to adult T cell leukemia-derived factor (ADF), which was originally characterized as a growth factor secreted by human T lymphotropic virus-I-transformed leukemic cell lines (Tagaya *et al.*, 1989). Trx has been found to modulate the DNA-binding activity of certain transcription factors including NF-κB, glucocorticoid receptor, and to indirectly modulate AP-1 activity through Ref-1 (Tagaya *et al.*, 1989; Hayashi *et al.*, 1993; Makino *et al.*, 1996; Hirota *et al.*, 1997). Trx has also been found to act as a powerful antioxidant by reducing ROS, and protects against hydrogen peroxide (H_2O_2)-, TNF-α- and *cis*-diamminedichloroplatinum (II) (CDDP)-induced cytotoxicity (Matsuda *et al.*, 1991; Nakamura *et al.*, 1994; Sasada *et al.*, 1996), in which the generation of intracellular ROS is thought to participate. On the other hand, Trx can be oxidized by various ROS molecules including H_2O_2, suggesting that Trx may be a molecular target of ROS.

Here we report that Trx is a physiological inhibitor of ASK1. We found that Trx bound directly to the N-terminal part of ASK1 and inhibited ASK1 kinase activity as well as ASK1-dependent apoptosis. The interaction between Trx and ASK1 was found to depend on the redox status of Trx, in that this interaction was observed only under reducing conditions, and the redox-inactive mutant form of Trx did not bind to ASK1. Moreover, inhibition of Trx activity by antisense oligonucleotides or by Trx reductase inhibitor resulted in activation of endogenous ASK1 activity. This evidence that Trx is a negative regulator of ASK1 suggests a possible mechanism for the redox-dependent apoptosis signal transduction pathway, which is presumably initiated by ROS-induced oxidation of Trx, followed by the dissociation of Trx from ASK1 and activation of ASK1.

Results and discussion

Two-hybrid screen for ASK1-interacting proteins

We employed the yeast two-hybrid system to search for proteins that bind to ASK1, using LexA DNA-binding domain ASK1-K709R (kinase-inactive mutant form of ASK1) as bait. In a screen of 1×10^6 human brain library clones, more than 500 clones specifically interacted with ASK1. DNA sequencing of the interacting clones revealed that a majority (>80%) of them encoded Trx fused in-frame to the transcriptional activation domain in the prey plasmid. The association of Trx with ASK1 was specific for but not dependent on the catalytic activity of ASK1, i.e. Trx interacted with ASK1-K709R as well as wild-type ASK1 (ASK1-WT) in yeast (Figure 1A and B). Trx interacted with an N-terminal non-catalytic fragment of ASK1 (ASK1-NT) but not with the N-terminus-deleted mutant (ASK1-ΔN) (Figure 1A and B). To verify that Trx associates with the N-terminal region of ASK1 in mammalian cells, we transiently cotransfected human 293 embryonal kidney cells with expression plasmids of Flag epitope-tagged Trx (Flag-Trx) and hemagglutinin (HA) epitope-tagged mutant forms of ASK1, and immunoprecipitated them with an anti-Flag monoclonal antibody. The immune complexes were subjected to immunoblotting with a monoclonal antibody to HA. ASK1-WT, C-terminus-truncated ASK1 (ASK1-ΔC) and ASK1-NT, but not ASK1-ΔN, co-immunoprecipitated with Trx, consistent with the finding for interaction in yeast (Figure 1C, top panel). Control immunoprecipitation and immunoblotting from the same lysates confirmed appropriate expression from transfected plasmids (Figure 1C, middle and bottom panels), indicating that Trx associates with the N-terminal region of ASK1 *in vivo*.

Trx inhibits the kinase activity of ASK1

Trx is known to control the redox state of proteins with its active site, which is composed of two redox active cysteines (Holmgren, 1989). Mammalian Trx is also known to scavenge ROS such as H_2O_2 and free radicals by its reducing activity (Mitsui *et al.*, 1992; Oblong *et al.*, 1994) and, most importantly, to protect against H_2O_2- and TNF-α-induced cytotoxicity (Matsuda *et al.*, 1991; Nakamura *et al.*, 1994). To determine whether Trx has a biochemical effect on ASK1, we transfected 293 cells with HA-tagged ASK1 and Flag-Trx and determined ASK1 kinase activity by an immune complex-coupled kinase assay using glutathione *S*-transferase (GST)–MKK6, GST–SAPK3/p38γ and the ATF2 peptide as a sequential substrate. Figure 2A shows the specificity of ASK1-dependent phosphorylation of ATF2 peptides in this assay. When increasing amounts of Trx plasmid were cotransfected, the kinase activity of ASK1-WT was inhibited in a dose-dependent manner (Figure 2B, left panel). Trx had no inhibitory effect on ASK1-ΔN, however (Figure 2B right), suggesting that Trx-induced inhibition of ASK1 activity depends on their interaction but is not due to a non-specific change in intracellular redox status. To examine whether co-immunoprecipitated Trx has an inhibitory effect on the kinase activity of GST–MKK6 or GST–SAPK3/p38γ other than that of ASK1, ASK1 activity was directly measured using myelin basic protein (MBP) as an exogenous substrate. Although less sensitive for measuring ASK1 activity in this case, ASK1 activity was also found to be reduced in the presence of Trx (Figure 2C). TNF-α-induced activation of ASK1 was also inhibited by Trx (Figure 2D).

ASK1-ΔN is a constitutively active kinase in vivo

Since Trx is ubiquitously expressed in mammalian cells and tissues (Holmgren, 1985), the kinase activity of ASK1 might be kept inactive at least in part by endogenous Trx *in vivo*; if this is the case, ASK1-ΔN should act as a constitutively active kinase. To test this hypothesis, various amounts of ASK1-WT and ASK1-ΔN plasmids were transfected into 293 cells and their relative kinase activities

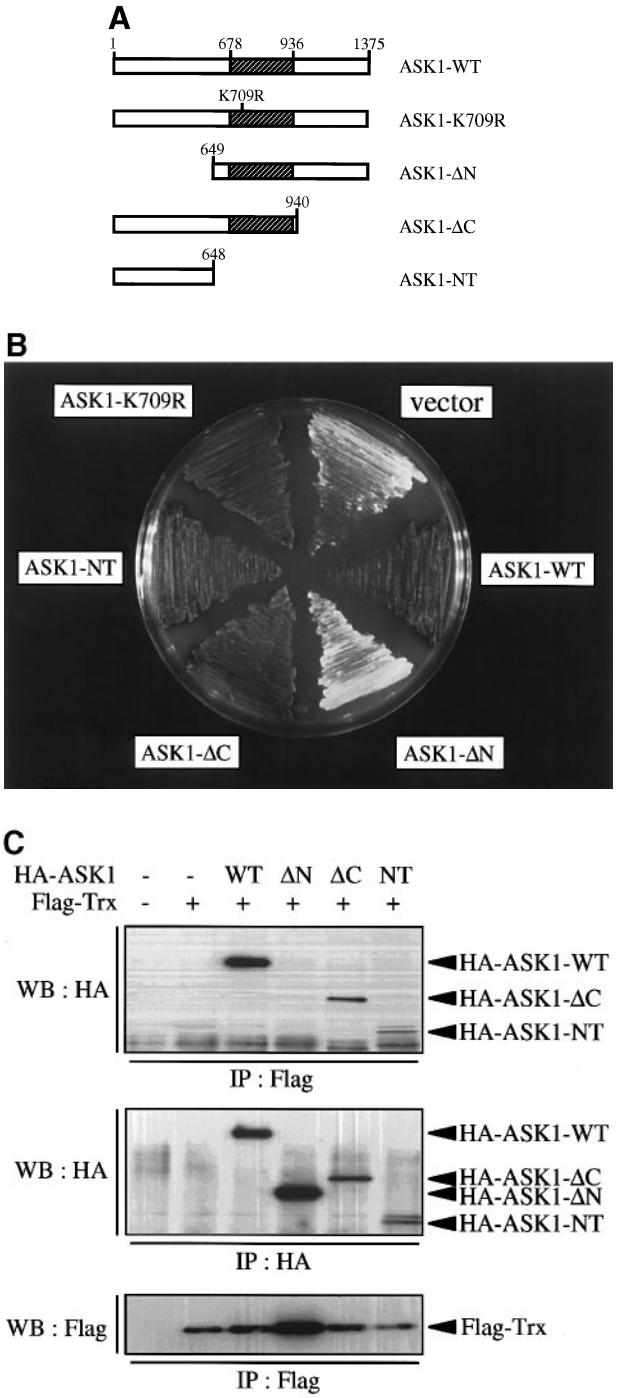


Fig. 1. Interaction of Trx with ASK1. (**A**) Schematic representation of wild-type and mutant ASK1 proteins. The kinase domain is shown by the hatched boxes. K709R represents a catalytically inactive mutant in which Lys709 has been replaced by Arg. (**B**) Interaction of Trx with wild-type and mutant ASK1 proteins in yeast. The β-galactosidase reporter plasmid and a plasmid encoding full-length Trx fused to the transcriptional activation domain were cotransfected into EGY188 yeast strains with plasmids encoding the indicated form of ASK1 fused to the DNA-binding domain. Each transformant was streaked onto an indicator plate. A positive interaction is seen for ASK1-K709R, ASK1-NT, ASK1-ΔC and ASK1-WT. (**C**) Interaction of Trx with wild-type and mutant ASK1 proteins in mammalian cells. 293 cells were transiently cotransfected with Flag-tagged Trx and HA-tagged ASK1 mutants. Lysates were divided and immunoprecipitated with anti-Flag antibody, and immunoblotted with anti-HA antibody (top). The presence of HA-ASK1 mutants (middle) and Flag-Trx (bottom) in the same lysates was verified by the indicated combination of immunoprecipitation (IP) and immunoblotting (WB).

were quantitatively determined by immune complex-coupled kinase assay. As expected, ASK1-ΔN exhibited much higher basal kinase activity than ASK1-WT (Figure 3A; >10-fold that of ASK1-WT at 0.5 μg ASK1 plasmids).

Apoptosis-inducing activity was also determined by morphology (Figure 3B, top panel) and viability (Figure 3B, bottom panel) in Mv1Lu epithelial cells, using a recombinant adenovirus system (Saito *et al.*, 1985; Miyake *et al.*, 1996). Transient expression of ASK1-ΔN, but not of ASK1-WT, ASK1-K709R or control β-galactosidase, induced marked apoptosis even in the presence of 10% fetal bovine serum (FBS), in which ASK1-WT is a poor inducer of apoptosis (Figure 3B; Ichijo *et al.*, 1997; see below). These findings suggest that association with the N-terminal region of ASK1 is required for Trx to inhibit ASK1 activity.

Redox control of apoptosis and ASK1 activity in vivo

ROS are reported to be generated and possibly to function as second messengers in response to cytotoxic stresses such as TNF-α and serum withdrawal (Camhi *et al.*, 1995; Satoh *et al.*, 1996). Since Trx is known to influence intracellular redox status by acting as an antioxidant to ROS or as a hydrogen donor for various proteins, we examined whether *in vivo* ASK1 activity is modulated by another thiol-based redox compound, Nac, which is also a potent antioxidant. To evaluate the effect of Nac on endogenous ASK1 activity, we used mouse L929 cells, which are sensitive to TNF-α-mediated apoptosis (Mayer and Noble, 1994); their expression of a large amount of endogenous ASK1 (Y.Sawada and H.Ichijo, unpublished observation) facilitates measurement of ASK1 activity. Treatment of L929 cells with H_2O_2 or TNF-α at a dose sufficient to induce apoptosis in these cells activated endogenous ASK1 (Figure 4A and B). Incubation of cells with Nac inhibited H_2O_2- or TNF-α-induced activation of ASK1 (Figure 4A). Moreover, no apparent cell death was observed in the presence of Nac (Figure 4B). These findings suggest that intracellular redox regulation might be involved in TNF-α-induced activation of ASK1 as well as apoptosis in L929 cells. However, the possibility that the intracellular redox status resulting from addition of H_2O_2 or TNF-α might induce cell death in a pathway independent of ASK1 activity is not excluded by these findings.

To determine more precisely the importance of intracellular redox status in ASK1-mediated apoptosis, we evaluated the effects of Trx and Nac in the system of activation-dependent apoptosis by ASK1. Apoptosis of Mv1Lu cells stably transfected with ASK1-WT under the control of the metallothionein promoter was rapidly induced in the presence of $ZnCl_2$, while cells transfected with the vector alone or ASK1-K709R remained intact (Figure 5A; Ichijo *et al.*, 1997). ASK1-dependent apoptosis was observed only under low serum conditions, but not in the presence of 10% FBS (Figure 5A; Ichijo *et al.*, 1997). Treatment of ASK1-transfected cells with serum-free medium after induction of ASK1 by $ZnCl_2$ resulted in the activation of ASK1 (Figure 5B). Therefore, the apoptosis observed in this system depends on the induction of the ASK1 activity by low serum conditions. This system thus enabled us to test whether redox regulation is involved

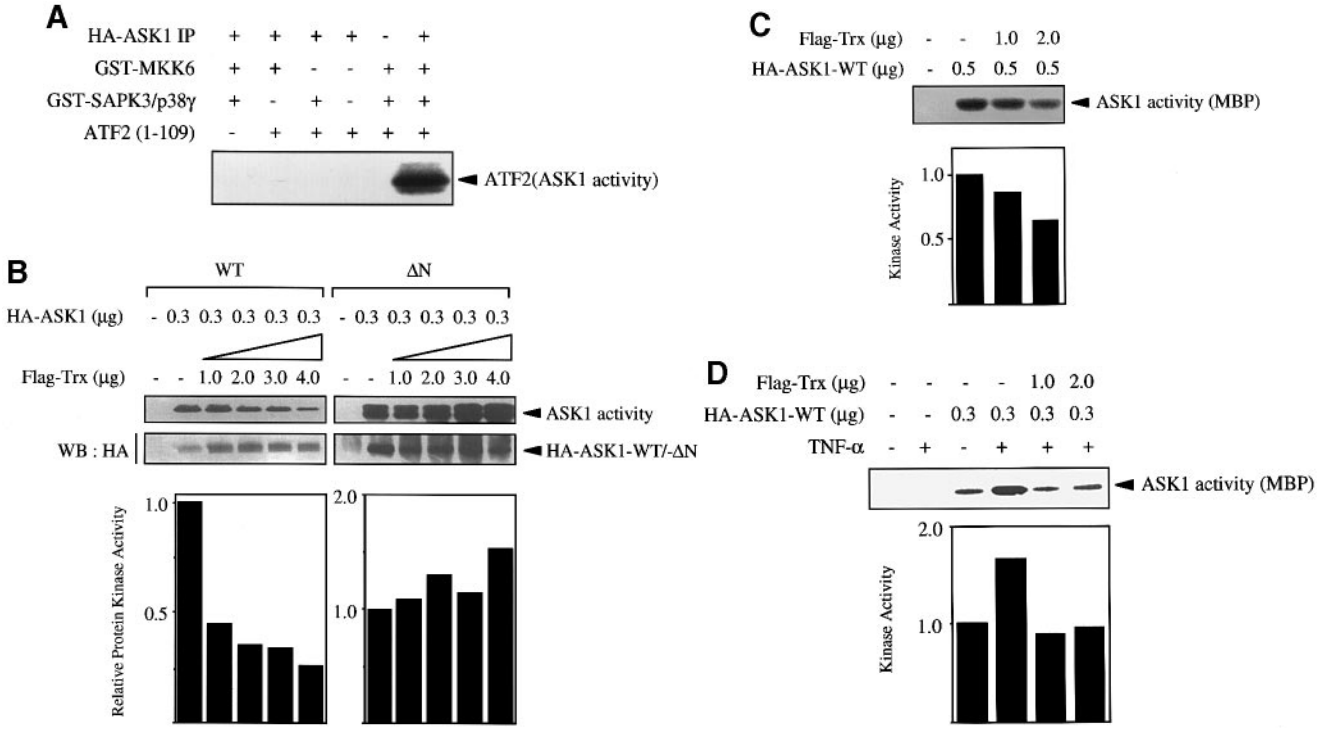
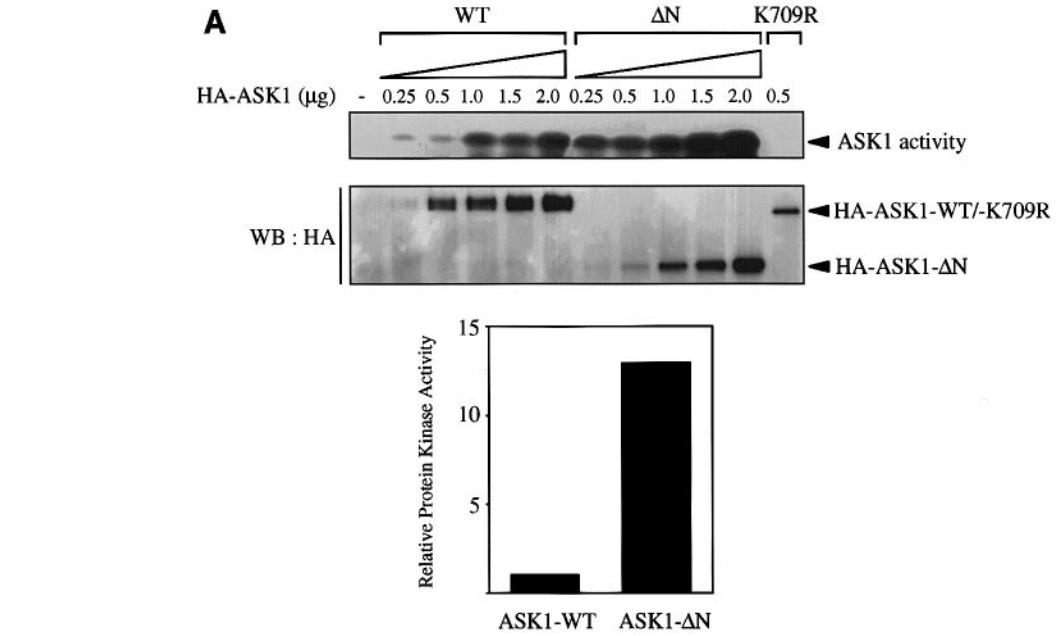


Fig. 2. Inhibition of ASK1 activity by Trx. (**A**) ASK1-dependent activation of the MKK6–SAPK3/p38γ–ATF2 pathway. 293 cells were transiently transfected with the HA-tagged ASK1 expression plasmid (pcDNA3-ASK1-WT-HA) and proteins were immunoprecipitated with anti-HA antibody. The immune complex was incubated with (+) or without (–) GST–MKK6 and GST–SAPK3/p38γ, and then kinase activity was measured with the substrate ATF2 peptides. (**B**) Inhibition of the kinase activity of ASK1 by co-expression of Trx. Indicated amounts of Flag-tagged Trx expression plasmid (pcDNA3-Flag-Trx) were transiently co-transfected into 293 cells with pcDNA3-ASK1-WT-HA or HA-tagged ASK1-ΔN expression plasmid (pcDNA3-ASK1-ΔN-HA). ASK1 was immunoprecipitated with anti-HA antibody and assayed for kinase activity as described in Figure 2A. Kinase activity relative to the amount of ASK1 protein was calculated, and the activity is shown as an X-fold increase relative to that of HA-ASK1 or HA-ASK1-ΔN from Flag-Trx-negative cells. This experiment was performed three times with similar results. (**C**) Inhibition of ASK1 by Trx in MBP kinase assay. 293 cells were transfected as described in Figure 2B. Immunoprecipitated HA-ASK1 kinase activity was measured by MBP as a direct substrate for ASK1. Samples were analyzed by SDS–PAGE (12%) with an image analyzer. (**D**) Inhibition of TNF-α-induced activation of ASK1 by Trx. 293 cells were transfected as described in Figure 2B. The cells were incubated with (+) or without (–) TNF-α (500 ng/ml) for 30 min. Thereafter, immunoprecipitated HA-ASK1 kinase activity was measured using MBP as a substrate.

in serum withdrawal-induced activation of ASK1 and ASK1-induced apoptosis. Nac inhibited ASK1-induced apoptosis (Figure 5A) as well as the activation of ASK1 by serum withdrawal (Figure 5B). When Trx activity against ASK1 was determined in this system by scoring apoptotic blue cells after transient cotransfection of expression plasmids of Trx and β-galactosidase, Trx was found to reduce ASK1-dependent apoptosis in a dose-dependent manner (Figure 5C). Moreover, neither Trx nor Nac inhibited ASK1-ΔN-induced apoptosis (data not shown), excluding the possibility that the target molecule(s) of Trx or Nac is a downstream effector of ASK1 in ASK1-dependent apoptosis. Together, these findings suggest that redox regulation may be involved in the control of ASK1 activity *in vivo*.

Redox status-dependent interaction of Trx and ASK1

We also examined the molecular mechanisms by which Trx inhibits ASK1 activity. There are three models for such mechanisms consistent with our observations: either (i) ASK1 itself is redox-sensitive and inactivated directly by Trx-catalyzed reduction; (ii) ASK1 is redox-sensitive and protected by Trx-mediated antioxidant activity from ROS-induced oxidation; or (iii) ASK1 is inactivated not by reduction but by a direct protein–protein interaction with Trx, which is sensitive to redox regulation. If the first two models are correct, the oxidized form of ASK1 may be the active form. However, we detected no *in vitro* activation of bacterially expressed GST fusion protein of ASK1 on direct incubation with H_2O_2 or diamide (a sulfhydryl-specific oxidant), in either the presence or absence of dithiothreitol (DTT) (data not shown). Moreover, since all the immunoprecipitations performed in the immune complex-kinase assay (Figures 2B, C, D and 3A) were performed in the presence of DTT, the immunoprecipitated ASK1 probably existed as a reduced form; therefore, it is hard to explain why additional reduction (and thus additional inhibition) was achieved by the addition of Trx. To test the third model, we examined the *in vitro* binding of ASK1 and Trx under various redox conditions. *In vitro*-translated ^{35}S-labeled ASK1 bound to immobilized GST fusion protein of Trx, but not to immobilized GST or GST-Trx(CS), a Cys-32 and Cys-35 Trx mutant which has lost reducing activity due to the mutation of cysteines in the redox active site (Figure 6A; Oblong *et al.*, 1994; Gallegos *et al.*, 1996). Notably, Trx–ASK1 interaction was highly dependent on the reducing conditions, i.e. binding of Trx was observed only in the presence of DTT (Figure 6A and B) or β-mercaptoethanol (β-ME) (Figure 6B). In addition, this interaction was reversibly inhibited by H_2O_2 or diamide (Figure 6C),



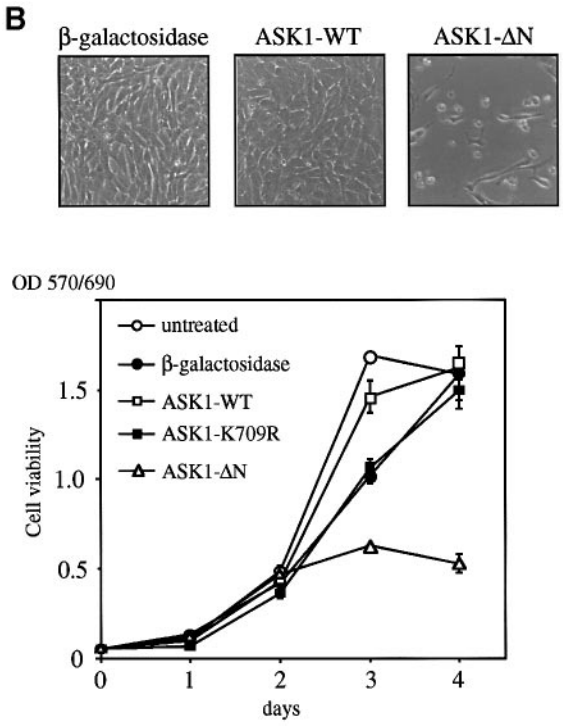


Fig. 3. ASK1-ΔN is a constitutively active mutant. (**A**) Comparison of the kinase activity of ASK1-ΔN with ASK1-WT. The indicated amount of pcDNA3-ASK1-WT-HA, pcDNA3-ASK1-ΔN-HA or HA-tagged ASK1-K709R expression plasmid (pcDNA3-ASK1-K709R-HA) was transfected into 293 cells, and the relative kinase activity was determined by the immune complex-kinase assay as described in Figure 2A. Representative relative kinase activities for ASK1-WT and ASK1-ΔN (at 0.5 μg DNA for each) are shown in the graph. (**B**) Induction of apoptosis by ASK1-ΔN. Mv1Lu cells were cultured with adenoviruses encoding β-galactosidase or HA-tagged ASK1-WT, ASK1-K709R or ASK1-ΔN. Representative cell morphology was examined by microscopy 3 days after infection (top; original magnification, ×82.5). The graph shows the time course of cell viability determined by MTT assay. Infection was performed on day 0. The values represent the means for triplicate wells ± SD.

confirming the dependence of this binding on reducing conditions. These findings do not completely exclude the possibility that reduction of ASK1 itself might also be required for the interaction; however, since the Trx–ASK1 interaction is highly dependent on the redox status of Trx, it appears that only a reduced form of Trx can bind ASK1, and that oxidation of Trx by certain oxidants including ROS may disrupt this interaction and consequently activate ASK1.

To verify the redox-dependent interaction of Trx and ASK1 *in vivo*, 293 cells transiently transfected with HA-ASK1-WT were treated with H_2O_2, and Trx–ASK1 complexes were then immunoprecipitated with an anti-Trx polyclonal antibody and analyzed by immunoblotting with anti-HA antibody. Anti-Trx but not control antibodies specifically co-immunoprecipitated ASK1 with endogenous Trx (Figure 6D). H_2O_2 markedly decreased the amount of co-immunoprecipitated ASK1 (Figure 6E), suggesting that redox-dependent interaction of Trx with ASK1 also occurs *in vivo*. Importantly, TNF-α also inhibited Trx–ASK1 interaction (Figure 6E), which was restored by the presence of Nac (Figure 6E and F), suggesting involvement of an ASK1 redox regulatory mechanism in TNF-α-induced apoptosis.

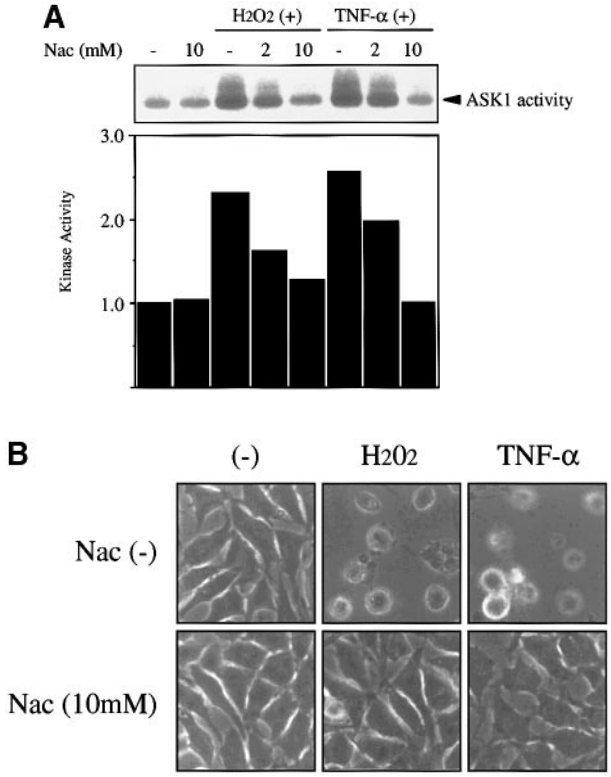


Fig. 4. Inhibition of ASK1 activity and apoptosis by Nac. (**A**) Inhibition of endogenous ASK1 activity by Nac. L929 cells were treated with H_2O_2 (1 mM) or TNF-α (400 ng/ml) for 15 min in the presence of indicated concentration of Nac. The endogenous ASK1 kinase activity was assayed using a rabbit anti-ASK1 antiserum (DAV). (**B**) Inhibition of H_2O_2- or TNF-α-induced apoptosis by Nac. L929 cells were treated with H_2O_2 (1 mM) or TNF-α (100 ng/ml) for 24 h in the presence of Nac (10 mM). Representative cell morphology was determined by phase-contrast microscopy (original magnification, ×82.5).

Trx is required for negative regulation of ASK1 in vivo

A targeted disruption of mouse Trx gene has recently been reported (Matsui *et al.*, 1996). Homozygous mutants died shortly after implantation, and the cells derived from pre-implantation embryos failed to grow, suggesting that Trx is essential for survival. In order to examine whether Trx is required for the inactivation of ASK1 *in vivo*, we attempted to inactivate endogenous Trx by treatment of cells with either an antisense oligonucleotide to Trx or a specific inhibitor of Trx reductase. Transfection of antisense oligonucleotides, but not of sense or mutated antisense oligonucleotides, in which eight out of 23 nucleotides were changed to different nucleotides decreased the amount of endogenous Trx protein as determined by immunoblotting (Figure 7A, bottom panel). When the same lysates were subjected to immune complex-coupled kinase assay using anti-ASK1 antibody, ASK1 was significantly activated only in antisense-treated cells (Figure 7A, top panel). In addition, incubation of cells with 1-chloro-2,4-dinitrobenzene (DNCB), an electrophilic compound which acts as a specific inhibitor of Trx reductase (Novogrodsky *et al.*, 1979; Arnér *et al.*, 1995) and thus increases the oxidized form of Trx *in vivo*, resulted in activation of ASK1 in a dose-dependent manner (Figure 7B). DNCB augmented the H_2O_2-induced activation of endogenous ASK1 as well (Figure 7B). Moreover, longer incubation of cells with DNCB induced cell death (data not shown). These findings strongly suggest that Trx is required for negative regulation of ASK1 activity *in vivo*.

Conclusion

Our findings suggest that Trx is a physiological inhibitor of ASK1. Figure 8 represents our current model of redox regulation of ASK1-mediated apoptosis signal transduction. Whereas the effects of Trx as a reductase against ROS and certain proteins have been emphasized in previous research, we suggest that Trx may also have an important role(s) as an effector of oxidants by acting as a signaling converter of redox changes into the kinase-mediated signal transduction cascade. Given that Trx is highly susceptible to ROS-induced oxidation and that ROS rapidly induce translocation of Trx into the nucleus (Tanaka *et al.*, 1997), ROS generated by cytokine or stress may oxidize and consequently remove Trx from pre-existing Trx–ASK1 complexes, leading to the activation of ASK1 and the subsequent ASK1-dependent apoptosis signaling cascade. Antioxidants including Nac might prevent apoptosis at least in part by inhibiting oxidation-induced dissociation of Trx from ASK1. It is still unclear whether serum withdrawal- and TNF-α-induced activation of ASK1 are primarily mediated by intracellular redox changes including generation of ROS. Intracellular redox changes might be the result of the initial apoptotic cellular dysfunction induced by alternative apoptosis signal transduction pathways. Once generated, however, ROS appear to act as important signaling intermediates in positive feedback in ASK1-dependent apoptosis signaling. It is important to distinguish between ROS molecules involved in the apoptosis signaling pathway and those mediating general cellular damage or necrosis. Although it has been thought that ROS may lack biological specificity as signaling intermediates, our model might associate ROS with signaling specificity in that ROS may target the specific interaction between Trx and ASK1. Currently, we do not know which type(s) of ROS molecule is specifically involved in inactivation of Trx and thus activation of ASK1; however, our model suggests a mechanism by which intracellular redox status, affected by a variety of cytotoxic stresses, meets the apoptosis signal transduction pathway, and suggests why antioxidants inhibit apoptosis.

Materials and methods

Reagents

MBP and DNCB were purchased from Sigma (St Louis, MO).

Cell culture

Mv1Lu and L929 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS and 100 units/ml penicillin in a 5% CO_2 atmosphere at 37°C. DMEM containing a higher concentration of glucose (4.5 mg/ml) was used for 293 cells.

Yeast two-hybrid system

A human fetal brain cDNA library in the pJG4-5 prey plasmid (a gift from Roger Brent) was screened for proteins that interact with ASK1-K709R as described (Kawabata *et al.*, 1995) using the EGY188 yeast reporter strain. The bait plasmid expressing ASK1-K709R protein was constructed in-frame with the LexA DNA-binding domain of the pEG202 bait plasmid. Plasmids of positive clones were recovered, and the cDNA inserts were sequenced. Plasmids encoding

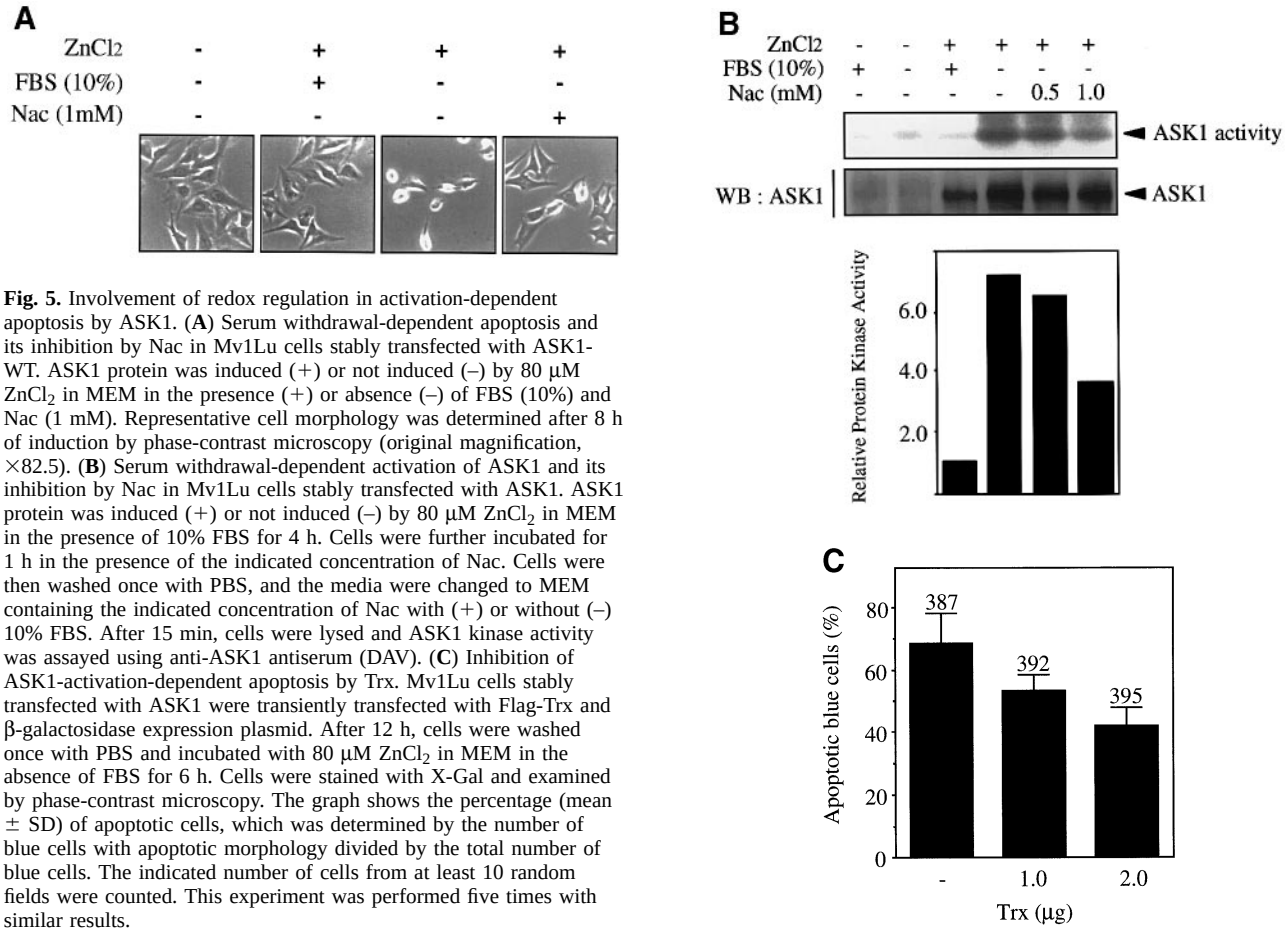


Fig. 5. Involvement of redox regulation in activation-dependent apoptosis by ASK1. (**A**) Serum withdrawal-dependent apoptosis and its inhibition by Nac in Mv1Lu cells stably transfected with ASK1-WT. ASK1 protein was induced (+) or not induced (–) by 80 μM $ZnCl_2$ in MEM in the presence (+) or absence (–) of FBS (10%) and Nac (1 mM). Representative cell morphology was determined after 8 h of induction by phase-contrast microscopy (original magnification, ×82.5). (**B**) Serum withdrawal-dependent activation of ASK1 and its inhibition by Nac in Mv1Lu cells stably transfected with ASK1. ASK1 protein was induced (+) or not induced (–) by 80 μM $ZnCl_2$ in MEM in the presence of 10% FBS for 4 h. Cells were further incubated for 1 h in the presence of the indicated concentration of Nac. Cells were then washed once with PBS, and the media were changed to MEM containing the indicated concentration of Nac with (+) or without (–) 10% FBS. After 15 min, cells were lysed and ASK1 kinase activity was assayed using anti-ASK1 antiserum (DAV). (**C**) Inhibition of ASK1-activation-dependent apoptosis by Trx. Mv1Lu cells stably transfected with ASK1 were transiently transfected with Flag-Trx and β-galactosidase expression plasmid. After 12 h, cells were washed once with PBS and incubated with 80 μM $ZnCl_2$ in MEM in the absence of FBS for 6 h. Cells were stained with X-Gal and examined by phase-contrast microscopy. The graph shows the percentage (mean ± SD) of apoptotic cells, which was determined by the number of blue cells with apoptotic morphology divided by the total number of blue cells. The indicated number of cells from at least 10 random fields were counted. This experiment was performed five times with similar results.

ASK1 mutants in pEG202 and full-length Trx in pJG4–5 were generated by PCR. All constructs were confirmed by sequencing.

In vivo binding of ASK1 and Trx

HA-tagged ASK1 expression plasmids in pcDNA3 (Invitrogen) were constructed by introducing an HA epitope sequence at the C-termini of ASK1 mutants by PCR. A Flag tag was inserted at the N-terminus of Trx in pcDNA3 by PCR. To examine protein interaction in 293 cells, plasmids of HA-tagged ASK1 (0.5 μg) and Flag-Trx (2 μg) were cotransfected into 293 cells in six-well culture plates by Tfx-50 (Promega) according to the manufacturer's instructions. For immunoprecipitation, 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 (PMSF), and the lysates were divided and incubated with either 5.1 μg/ml of anti-Flag mouse IgG1 (clone M2, Scientific Imaging Systems) or anti-HA rabbit antiserum (YPY, a gift from Carl-Henrik Heldin) for 1 h. After the addition of protein G–Sepharose (Zymed Laboratories), the lysates were incubated for an additional 30 min. The beads were washed twice with the lysis buffer, separated by SDS–polyacrylamide gel electrophoresis (PAGE), and immunoblotted with mouse anti-HA (clone 12CA5, Boehringer Mannheim) or anti-Flag antibodies. The proteins were detected with the ECL system (Amersham).

To examine the effects of H_2O_2 and TNF-α on the interaction of ASK1 with endogenous Trx, 293 cells were transfected with HA-tagged ASK1 (0.5 μg) by Tfx-50 and incubated overnight. Cells were treated with H_2O_2 and TNF-α for 20 min, lysed in buffer containing 150 mM NaCl, 20 mM Tris–HCl (pH 7.5), 10 mM EDTA, 0.5% Triton X-100, 1.5% aprotinin and 1 mM PMSF, and the lysates were immunoprecipitated with anti-human Trx goat antibody (American Diagnostica, Inc.) as described above. The samples were analyzed by immunoblotting using anti-HA (clone 3F10, Boehringer Mannheim) and anti-Trx antibodies. In some experiments, aliquots of whole cell lysates were subjected to immunoblotting analysis by anti-HA antibody (clone 3F10) to confirm appropriate expression of transfected HA-ASK1. The proteins were detected with the ECL system.

In vitro kinase assay

The plasmid of GST–human MKK6 for bacterial fusion protein was constructed in pGEX-4T-1 (Pharmacia Biotech, Inc.) by PCR, and GST–rat SAPK3 (Cuenda *et al.*, 1997) and ATF2 peptide (peptides 1–109) were kindly provided by Michel Goedert and Zhengbin Yao, respectively. Transfection to 293 cells and immune complex-kinase assay were performed as described (Ichijo *et al.*, 1997), except for the substrates and washing conditions of the immunoprecipitates. Briefly, cells were lysed in a buffer solution containing 20 mM Tris–HCl (pH 7.5), 12 mM β-glycerophosphate, 150 mM NaCl, 5 mM EGTA, 10 mM NaF, 1% Triton X-100, 0.5% deoxycholate, 3 mM DTT, 1 mM sodium orthovanadate, 1 mM PMSF and 1.5% aprotinin. Cell extracts were clarified by centrifugation, and the supernatants were immunoprecipitated with antibody to HA (12CA5) and protein A–Sepharose (Zymed Laboratories). The beads were washed twice with a solution containing 150 mM NaCl, 20 mM Tris–HCl (pH 7.5), 5 mM EGTA, 2 mM DTT and 1 mM PMSF, and subjected to kinase assays. To measure immune complex activity, 0.2 μg of GST–MKK6 was first incubated with the immune complexes for 10 min at 30°C in a final volume of 25 μl of a solution containing 20 mM Tris–HCl (pH 7.5), 20 mM $MgCl_2$ and 100 μM ATP, and subsequently with 1 μg of GST–SAPK3/p38γ for 10 min at 30°C. Thereafter, the activated complex was incubated with 0.3 μCi of [γ-^{32}P]ATP and 1 μg of ATF2 peptide in the same solution (final volume, 35 μl). Samples were subjected to SDS–PAGE (5–15% gradient gel), and the phosphorylation of ATF2 peptide was analyzed with an image analyzer (Fujix BAS2000). When ASK1 activity was measured using MBP or GST–MKK6(KN) as a direct substrate, immune complexes were incubated with MBP (40 μg/ml) or GST–MKK6(KN) (20 μg/ml) in buffer containing 20 mM Tris–HCl (pH 7.5), 20 mM $MgCl_2$ and 0.3 μCi of [γ-^{32}P] ATP. Samples were analyzed by SDS–PAGE with an image analyzer. To determine the amount of ASK1 protein

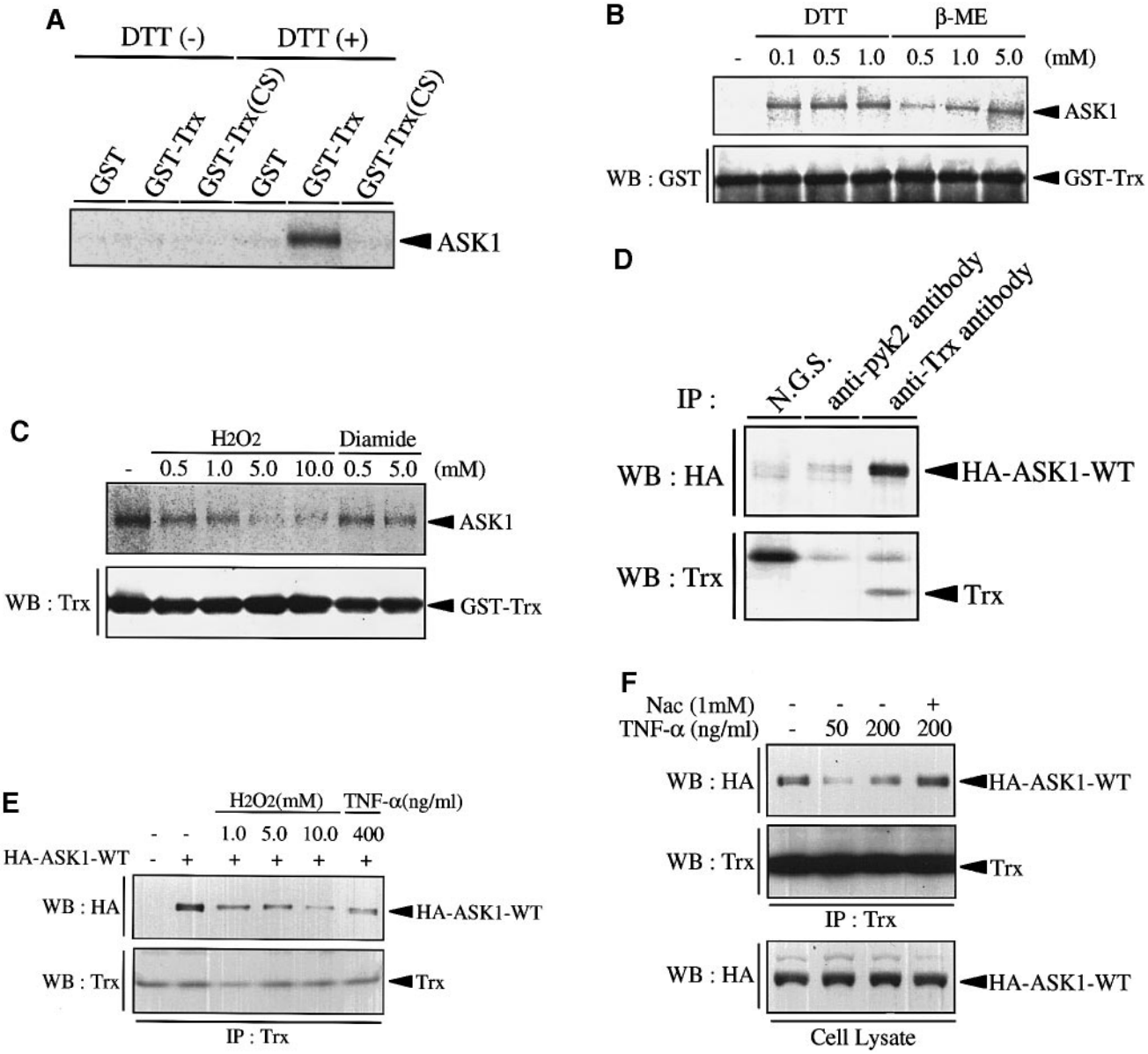


Fig. 6. Redox control of Trx–ASK1 interaction. (**A**) Binding of *in vitro*-translated ^{35}S-ASK1 to GST fusion proteins. Purified GST fusion proteins immobilized on the beads were treated or untreated with 1 mM DTT on ice for 30 min, washed with a buffer containing 20 mM Tris (pH 7.5), 150 mM NaCl, 10 mM EDTA, 1% Triton X-100 and 1% deoxycholate and subjected to *in vitro* binding assay as described in Materials and methods. (**B**) Reducing condition-dependent binding of Trx to ASK1. Purified GST–Trx immobilized on the beads was treated with the indicated concentration of DTT or β-mercaptoethanol (β-ME), and the binding was determined as described in (A) (top panel). To verify the amount of GST fusion proteins in the same sample, the lower part of the SDS–PAGE was cut out and immunoblotted with anti-GST antibody (lower panel). (**C**) Oxidation-induced dissociation of Trx from ASK1. Binding of *in vitro*-translated ^{35}S-ASK1 to DTT-treated GST–Trx and washing of beads were performed as described in (A). Thereafter, the beads were washed once with PBS, incubated in PBS with the indicated concentrations of H_2O_2 or diamide at 25°C for 15 min, washed twice with PBS, and then analyzed by SDS–PAGE with an image analyzer (top panel). To verify the amount of GST fusion proteins in the same sample, the lower part of the SDS–PAGE was cut out and immunoblotted with anti-Trx antibody (lower panel). (**D**) Specific co-immunoprecipitation of endogenous Trx and transfected HA-ASK1 by anti-Trx antibody in 293 cells. Cell lysates were immunoprecipitated with normal goat serum (NGS), anti-pyk2 goat antibody or anti-Trx goat antibody and analyzed by immunoblotting as described in Materials and methods. (**E**) Effects of H_2O_2 and TNF-α on the interaction of ASK1 with endogenous Trx. 293 cells transfected with HA-ASK1 were treated with the indicated concentration of H_2O_2 or TNF-α for 20 min and subjected to immunoprecipitation (by anti-Trx antibody)-immunoblotting analysis as in (D). (**F**) Inhibition of TNF-α-induced dissociation of Trx from ASK1 by Nac. 293 cells transfected with HA-ASK1 were pretreated or not with 1 mM Nac for 1 h. The cells were then treated with the indicated concentration of TNF-α for 20 min and subjected to immunoprecipitation (by anti-Trx antibody)-immunoblotting analysis as in (D). Top, copurified ASK1; middle, immunoprecipitated Trx; bottom, immunoblotting of aliquots of whole cell lysate by anti-HA antibody.

in the same sample, the upper part of the SDS–PAGE (>46 kDa) was cut out and immunoblotted with the mouse anti-HA antibody. The protein was detected with the ECL system, in which <10 min exposure to X-ray film did not detect any ^{32}P-radioactivity derived from autophosphorylation of ASK1. The amount of protein was quantified by densitometric analysis (Quantity One program: pdi, Inc.).

Adenovirus vectors

Recombinant adenoviruses were constructed as described (Saito *et al.*, 1985; Miyake *et al.*, 1996). Briefly, HA-tagged ASK1 cDNA mutants and β-galactosidase cDNA were subcloned into the *Swa*I site of pAxCAwt cassette cosmid, which is defective in adenovirus E1A, E1B and E3 regions. Each cosmid bearing the expression unit and

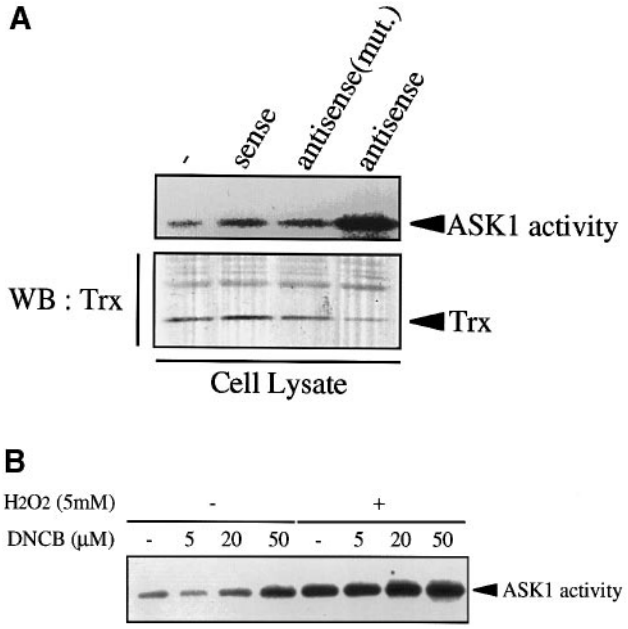


Fig. 7. Requirement of Trx for the inhibition of ASK1 activity *in vivo*. (**A**) 293 cells were treated with sense or antisense *S*-oligos (see Materials and methods). Endogenous ASK1 activity was analyzed by immune-complex-coupled kinase assay using GST–MKK6, GST–SAPK3/p38γ and the ATF2 peptide as a sequential substrate (top panel). A fraction of the whole cell lysate was analyzed by immunoblotting using anti-Trx antibody to determine the level of expression of Trx. (**B**) 293 cells were treated with the indicated concentration of DNCB for 30 min. Cells were further incubated with (+) or without (–) H_2O_2 for 30 min and subjected to immune complex-kinase assay using GST–MKK6 (KN) as a substrate.

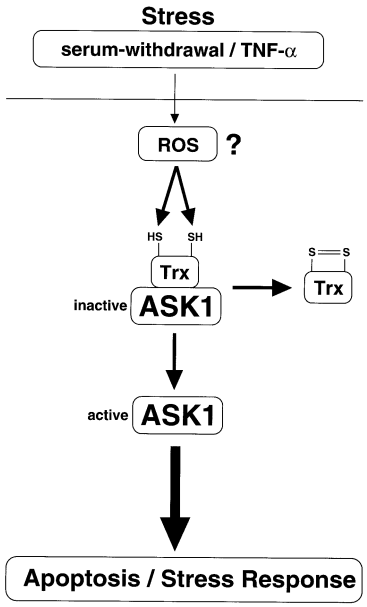


Fig. 8. Tentative model for the mechanism of redox-mediated ASK1 activation in stress-induced apoptosis.

adenovirus DNA-terminal protein complex was cotransfected into the E1 transcomplementing cell line 293. The recombinant adenoviruses generated by homologous recombination were isolated, and the insertion of ASK1 cDNAs was confirmed by restriction endonuclease digestion. High-titered stocks of recombinant adenoviruses were grown in 293 cells and purified. Infection of the recombinant adenoviruses into Mv1Lu cells was performed at a multiplicity of infection of 10^2 p.f.u./cell, in which adenovirus vectors can transduce nearly 100% of Mv1Lu cells as determined by β-galactosidase staining (M.Fujii and H.Ichijo, unpublished observation). The comparable level of protein expression for each ASK1 construct was determined by immunoprecipitation (data not shown). Cell viability was monitored by MTT assay using CellTiter96 kit (Promega) according to the manufacturer's instructions.

Apoptosis assay

Mv1Lu cells stably transfected with ASK1-WT under the control of metallothionein promoter (Ichijo *et al.*, 1997) were used in this assay. Cells were transiently cotransfected with pCMV β-gal (0.5 μg) and the pcDNA3 empty vector (1 μg) or pcDNA3–Trx (1 μg) by Tfx-50 reagent (Promega). After 12 h, cells were placed in serum-free minimal essential medium (MEM) and apoptosis was induced by 80 μM of $ZnCl_2$ (thus, by expression of ASK1) for 6 h. Cells were then fixed and stained with 5-bromo-4-chloro-3-indoxyl-β-D-galactosidase (X-gal) for 5 h and examined by phase-contrast microscopy. The percentage of apoptotic cells was determined by the number of blue cells with apoptotic morphology (cell shrinkage and nuclear fragmentation) divided by the total number of blue cells, as described (Hsu *et al.*, 1995).

Anti-ASK1 antiserum

Antiserum to ASK1 (DAV) was raised against the peptide sequence DAVATSGVSTLSSTVSHDSQ (amino acids 1216–1235). The peptide was coupled to keyhole limpet hemocyanin by the glutaraldehyde method, mixed with Freund's adjuvant, and used to immunize rabbits.

Antisense oligonucleotides

Antisense oligonucleotides, at nucleotide positions from –10 to +13 (relative to the translation initiation site) were designed to hybridize with human Trx. Nucleotide sequences were: antisense, 5′-TCTGCTT-CACCATCTTGGCTGCT-3′; sense, 5′-AGCAGCCAAGATGGTGAA-GCAGA-3′; mutant antisense in which eight (shown by bold face) of the 23 nucleotides were changed to different nucleotides, 5′-TC**GTTC**TCACCATCTTGG**TCCG**T-3′. All were synthesized as phosphorothionate oligonucleotides (*S*-oligo) and HPLC-purified by Greiner Japan (Chiang *et al.*, 1991; Takeuchi *et al.*, 1994). For transfection, 10 μM of each *S*-oligo was mixed with 2 μl of Tfx–50 in medium (800 μl) containing 10% FBS, and added to 293 cells in six-well plates and incubated for 60 h. Thereafter, endogenous ASK1 was immunoprecipitated by anti-ASK1 antiserum (DAV), and the ASK1 activity was determined by the immune complex-coupled kinase assay using GST–MKK6, GST–SAPK3/p38γ and the ATF2 peptide as a sequential substrate. A fraction of the whole cell lysate was analyzed by immunoblotting using anti-Trx antibody to determine the level of expression of Trx.

In vitro binding assay

GST constructs for Trx were prepared in pGEX-4T-1 vector (Promega). GST fusion proteins were purified as described (Smith and Johnson, 1988). *In vitro*-translated ^{35}S-labeled ASK1 was prepared with the TNT Reticulocyte Lysate System (Promega). ^{35}S-labeled ASK1 was incubated on ice for 30 min with each GST fusion protein in buffer containing 20 mM Tris (pH 7.5), 150 mM NaCl, 10 mM EDTA, 1% Triton X-100 and 1% deoxycholate, washed twice with the same buffer, and analyzed by SDS–PAGE with an image analyzer. To determine the amount of GST fusion protein in the same sample, the lower part of the SDS–PAGE was cut out and immunoblotted with anti-GST antiserum (a gift from Carl-Henrik Heldin) or anti-human Trx antibody. The protein was detected with the ECL system.

Acknowledgements

We thank T.Kitagawa for discussion. We also thank: B.Yao and K.Diener for the ATF2 peptide; M.Goedert for the GST-SAPK3 construct; J.Han and R.J.Ulevitch for the MKK6 cDNA; R.Brent for reagents used in the yeast interaction trap; I.Saito and J.Miyazaki for adenovirus vectors; C.-H.Heldin for the YPY and anti-GST antiserum; P.Horton for critical reading of the manuscript; and E.Nishida, Y.Gotoh, E.Oeda and Y.Eto for valuable comments. Supported by Grants-in-Aid for scientific research from the Ministry of Education, Science, Culture of Japan.

References

Arnér,E.S., Björnstedt,M. and Holmgren,A. (1995) 1-Chloro-2,4-dinitrobenzene is an irreversible inhibitor of human thioredoxin reductase. Loss of thioredoxin disulfide reductase activity is accompanied by a large increase in NADPH oxidase activity. *J. Biol. Chem.*, **270**, 3479–3482.

Buchanan,B.B., Schurmann,P., Decottignies,P. and Lozano,R.M. (1994) Thioredoxin: a multifunctional regulatory protein with a bright future in technology and medicine. *Arch. Biochem. Biophys.*, **314**, 257–260.

Bulkley,G.B. (1994) Reactive oxygen metabolites and reperfusion injury: aberrant triggering of reticuloendothelial function. *Lancet*, **344**, 934–936.

Buttke,T.M. and Sandstrom,P.A. (1994) Oxidative stress as a mediator of apoptosis. *Immunol. Today*, **15**, 7–10.

Camhi,S.L., Lee,P. and Choi,A.M. (1995) The oxidative stress response. *New Horiz.*, **3**, 170–182.

Chiang,M., Chan,H., Zounes,M.A., Freier,S.M., Lima,W.F. and Bennett,C.F. (1991) Antisense oligonucleotides inhibit intercellular adhesion molecule 1 expression by two distinct mechanisms. *J. Biol. Chem.*, **266**, 18162–18171.

Cossarizza,A. *et al.* (1995) Protective effect of N-acetylcysteine in tumor necrosis factor-α-induced apoptosis in U937 cells: the role of mitochondria. *Exp. Cell Res.*, **220**, 232–240.

Cuenda,A., Cohen,P., Buee-Scherrer,V. and Goedert,M. (1997) Activation of stress-activated protein kinase-3 (SAPK3) by cytokines and cellular stresses is mediated via SAPKK3 (MKK6); comparison of the specificities of SAPK3 and SAPK2 (RK/p38). *EMBO J.*, **16**, 295–305.

Davis,R.J. (1994) MAPKs: new JNK expands the group. *Trends Biochem. Sci.*, **19**, 470–473.

Eklund,H., Gleason,F.K. and Holmgren,A. (1991) Structural and functional relations among thioredoxins of different species. *Proteins*, **11**, 13–28.

Errude,B. and Levin,D.E. (1993) A conserved kinase cascade for MAP kinase activation in yeast. *Curr. Opin. Cell Biol.*, **5**, 254–260.

Fanger,G.R., Gerwins,P., Widmann,C., Jarpe,M.B. and Johnson,G.L. (1997) MEKKs, GCKs, MLKs, PAKs, TAKs, and tpls: upstream regulators of the c-Jun amino-terminal kinases? *Curr. Opin. Genet. Dev.*, **7**, 67–74.

Gallegos,A., Gasdaska,J.R., Taylor,C.W., Paine-Murrieta,G.D., Goodman,D., Gasdaska,P.Y., Berggren,M., Briehl,M.M. and Powis,G. (1996) Transfection with human thioredoxin increases cell proliferation and a dominant-negative mutant thioredoxin reverses the transformed phenotype of human breast cancer cells. *Cancer Res.*, **56**, 5765–5770.

Hayashi,T., Ueno,Y. and Okamoto,T. (1993) Oxidoreductive regulation of nuclear factor κB. Involvement of a cellular reducing catalyst thioredoxin. *J. Biol. Chem.*, **268**, 11380–11388.

Hirota,K., Matsui,M., Iwata,S., Nishiyama,A., Mori,K. and Yodoi,J. (1997) AP-1 transcriptional activity is regulated by a direct association between thioredoxin and Ref-1. *Proc. Natl Acad. Sci. USA*, **94**, 3633–3638.

Holmgren,A. (1985) Thioredoxin. *Annu. Rev. Biochem.*, **54**, 237–271.

Holmgren,A. (1989) Thioredoxin and glutaredoxin systems. *J. Biol. Chem.*, **264**, 13963–13966.

Holmgren,A. (1995) Thioredoxin structure and mechanism: conformational changes on oxidation of the active-site sulfhydryls to a disulfide. *Structure*, **3**, 239–243.

Hsu,H., Xiong,J. and Goeddel,D.V. (1995) The TNF receptor 1-associated protein TRADD signals cell death and NF-κB activation. *Cell*, **81**, 495–504.

Ichijo,H. *et al.* (1997) Induction of apoptosis by ASK1, a mammalian MAPKKK that activates SAPK/JNK and p38 signaling pathways. *Science*, **275**, 90–94.

Irani,K., Xia,Y., Zweier,J.L., Sollott,S.J., Der,C.J., Fearon,E.R., Sundaresan,M., Finkel,T. and Goldschmidt-Clermont,P.J. (1997) Mitogenic signaling mediated by oxidants in Ras-transformed fibroblasts. *Science*, **275**, 1649–1652.

Jacobson,M.D. (1996) Reactive oxygen species and programmed cell death. *Trends Biochem. Sci.*, **21**, 83–86.

Kawabata,M., Chytil,A. and Moses,H.L. (1995) Cloning of a novel type II serine/threonine kinase receptor through interaction with the type I transforming growth factor-β receptor. *J. Biol. Chem.*, **270**, 5625–5630.

Kroemer,G., Zamzami,N. and Susin,S.A. (1997) Mitochondrial control of apoptosis. *Immunol. Today*, **18**, 44–51.

Kyriakis,J.M. and Avruch,J. (1996) Protein kinase cascades activated by stress and inflammatory cytokines. *BioEssays*, **18**, 567–577.

Levine,A., Tenhaken,R., Dixon,R. and Lamb,C. (1994) H_2O_2 from the oxidative burst orchestrates the plant hypersensitive disease resistance response. *Cell*, **79**, 583–593.

Lo,Y.Y.C. and Cruz,T.F. (1995) Involvement of reactive oxygen species in cytokine and growth factor induction of c-*fos* expression in chondrocytes. *J. Biol. Chem.*, **270**, 11727–11730.

Makino,Y., Okamoto,K., Yoshikawa,N., Aoshima,M., Hirota,K., Yodoi,J., Umesono,K., Makino,I. and Tanaka,H. (1996) Thioredoxin: a redox-regulating cellular cofactor for glucocorticoid hormone action. Cross talk between endocrine control of stress response and cellular antioxidant defense system. *J. Clin. Invest.*, **98**, 2469–2477.

Marshall,C.J. (1994) MAP kinase kinase kinase, MAP kinase kinase and MAP kinase. *Curr. Opin. Genet. Dev.*, **4**, 82–89.

Matsuda,M. *et al.* (1991) Protective activity of adult T cell leukemia-derived factor (ADF) against tumor necrosis factor-dependent cytotoxicity on U937 cells. *J. Immunol.*, **147**, 3837–3841.

Matsui,M., Oshima,M., Oshima,H., Takaku,K., Maruyama,T., Yodoi,J. and Taketo,M.M. (1996) Early embryonic lethality caused by targeted disruption of the mouse thioredoxin gene. *Dev. Biol.*, **178**, 179–185.

Matthews,N., Neale,M.L., Jackson,S.K. and Stark,J.M. (1987) Tumour cell killing by tumour necrosis factor: inhibition by anaerobic conditions, free-radical scavengers and inhibitors of arachidonate metabolism. *Immunology*, **62**, 153–155.

Mayer,M. and Noble,M. (1994) N-acetyl-L-cysteine is a pluripotent protector against cell death and enhancer of trophic factor-mediated cell survival *in vitro*. *Proc. Natl Acad. Sci. USA*, **91**, 7496–7500.

Meier,B., Radeke,H.H., Selle,S., Younes,M., Sies,H., Resch,K. and Habermehl,G.G. (1989) Human fibroblasts release reactive oxygen species in response to interleukin-1 or tumour necrosis factor-α. *Biochem. J.*, **263**, 539–45.

Mitsui,A., Hirakawa,T. and Yodoi,J. (1992) Reactive oxygen-reducing and protein-refolding activities of adult T cell leukemia-derived factor/human thioredoxin. *Biochem. Biophys. Res. Commun.*, **186**, 1220–1226.

Miyake,S., Makimura,M., Kanegae,Y., Harada,S., Sato,Y., Takamori,K., Tokuda,C. and Saito,I. (1996) Efficient generation of recombinant adenoviruses using adenovirus DNA-terminal protein complex and a cosmid bearing the full-length virus genome. *Proc. Natl Acad. Sci. USA*, **93**, 1320–1324.

Nagata,S. (1996) Apoptosis: telling cells their time is up. *Curr. Biol.*, **6**, 1241–1243.

Nakamura,H. *et al.* (1994) Adult T cell leukemia-derived factor/human thioredoxin protects endothelial F-2 cell injury caused by activated neutrophils or hydrogen peroxide. *Immunol. Lett.*, **42**, 75–80.

Nakamura,H., Nakamura,K. and Yodoi,J. (1997) Redox regulation of cellular activation. *Annu. Rev. Immunol.*, **15**, 351–369.

Nishida,E. and Gotoh,Y. (1993) The MAP kinase cascade is essential for diverse signal transduction pathways. *Trends Biochem. Sci.*, **18**, 128–131.

Novogrodsky,A., Nehring,R.E.J. and Meister,A. (1979) Inhibition of amino acid transport into lymphoid cells by the glutamine analog L-2-amino-4-oxo-5-chloropentanoate. *Proc. Natl Acad. Sci. USA*, **76**, 4932–4935.

Oblong,J.E., Berggren,M., Gasdaska,P.Y. and Powis,G. (1994) Site-directed mutagenesis of active site cysteines in human thioredoxin produces competitive inhibitors of human thioredoxin reductase and elimination of mitogenic properties of thioredoxin. *J. Biol. Chem.*, **269**, 11714–11720.

Ohba,M., Shibanuma,M., Kuroki,T. and Nose,K. (1994) Production of hydrogen peroxide by transforming growth factor-β 1 and its involvement in induction of egr-1 in mouse osteoblastic cells. *J. Cell Biol.*, **126**, 1079–1088.

Raingeaud,J., Gupta,S., Rogers,J.S., Dickens,M., Han,J., Ulevitch,R.J. and Davis,R.J. (1995) Pro-inflammatory cytokines and environmental stress cause p38 mitogen-activated protein kinase activation by dual phosphorylation on tyrosine and threonine. *J. Biol. Chem.*, **270**, 7420–7426.

Saito,I., Oya,Y., Yamamoto,K., Yuasa,T. and Shimojo,H. (1985) Construction of nondefective adenovirus type 5 bearing a 2.8-kilobase hepatitis B virus DNA near the right end of its genome. *J. Virol.*, **54**, 711–719.

Salvesen,G.S. and Dixit,V.M. (1997) Caspases: intracellular signaling by proteolysis. *Cell*, **91**, 443–446.

Sasada,T. *et al.* (1996) Redox control of resistance to *cis*-diamminedichloroplatinum (II) (CDDP): protective effect of human thioredoxin against CDDP-induced cytotoxicity. *J. Clin. Invest.*, **97**, 2268–2276.

Satoh,T., Sakai,N., Enokido,Y., Uchiyama,Y. and Hatanaka,H. (1996) Survival factor-insensitive generation of reactive oxygen species induced by serum deprivation in neuronal cells. *Brain Res.*, **733**, 9–14.

Smith,D.B. and Johnson,K.S. (1988) Single-step purification of polypeptides expressed in *Escherichia coli* as fusions with glutathione *S*-transferase. *Gene*, **67**, 31–40.

Steller,H. (1995) Mechanisms and genes of cellular suicide. *Science*, **267**, 1445–1449.

Tagaya,Y. *et al.* (1989) ATL-derived factor (ADF), an IL-2 receptor/Tac inducer homologous to thioredoxin; possible involvement of dithiol-reduction in the IL-2 receptor induction. *EMBO J.*, **8**, 757–764.

Takeuchi,K., Sato,N., Kasahara,H., Funayama,N., Nagafuchi,A., Yonemura,S., Tsukita,S. and Tsukita,S. (1994) Perturbation of cell adhesion and microvilli formation by antisense oligonucleotides to ERM family members. *J. Cell Biol.*, **125**, 1371–1384.

Tanaka,T., Nishiyama,Y., Okada,K., Hirota,K., Matsui,M., Yodoi,J., Hiai,H. and Toyokuni,S. (1997) Induction and nuclear translocation of thioredoxin by oxidative damage in the mouse kidney: independence of tubular necrosis and sulfhydryl depletion. *Lab. Invest.*, **77**, 145–155.

Thompson,C.B. (1995) Apoptosis in the pathogenesis and treatment of disease. *Science*, **267**, 1456–1462.

Tobiume,K., Inage,T., Takeda,K., Enomoto,S., Miyazono,K. and Ichijo,H. (1997) Molecular cloning and characterization of the mouse apoptosis signal-regulating kinase 1. *Biochem. Biophys. Res. Commun.*, **239**, 905–910.

Verheij,M. *et al.* (1996) Requirement for ceramide-initiated SAPK/JNK signalling in stress-induced apoptosis. *Nature*, **380**, 75–79.

Waskiewicz,A.J. and Cooper,J.A. (1995) Mitogen and stress response pathway: MAP kinase cascades and phosphatase regulation in mammals and yeast. *Curr. Opin. Cell Biol.*, **7**, 798–805.

Xia,Z., Dickens,M., Raingeaud,J., Davis,R.J. and Greenberg,M.E. (1995) Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. *Science*, **270**, 1326–1331.

Yamauchi,N., Kuriyama,H., Watanabe,N., Neda,H., Maeda,M. and Niitsu,Y. (1989) Intracellular hydroxyl radical production induced by recombinant human tumor necrosis factor and its implication in the killing of tumor cells *in vitro*. *Cancer Res.*, **49**, 1671–1675.

Received January 27, 1998; revised and accepted March 6, 1998