P34SEI-1 inhibits ROS-induced cell death through suppression of ASK1

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Introduction

The serine/threonine protein kinase ASK1 (apoptosis signal-regulating kinase 1) is a key activator of an intracellular signal transduction pathway that regulates cell death.1,2 ASK1 is activated in response to various apoptotic stimuli, including tumor necrosis factor α (TNFα),3 endoplasmic reticulum (ER) stress,4 and reactive oxygen species (ROS) such as hydrogen peroxide (H2O2).5 ASK1 is regulated by multiple ways, including through dimerization, phosphorylation and protein-protein interactions.6 Under stress conditions, ASK1 undergoes self-dimerization, which leads to ASK1 autophosphorylation and consequent activation; in the absence of stresses, ASK1 binds to thioredoxin, which blocks the dimerization of ASK1, hence inhibiting its activation.7 Oxidative stressors, such as H2O2, promote the dissociation of thioredoxin from ASK1 and thereby activate ASK1.8 However, the mechanisms responsible for downregulation of ASK1 remain largely unexplored.

It was recently reported that proteasome-dependent degradation of ASK1 is facilitated by CHIP (C-terminal of Hsp70-associating protein), a co-chaperone with E3 ubiquitin ligase activity,9 and by c-IAP1 (cellular inhibitor of apoptosis proteins 1).10 CHIP binds the cytoplasmic molecular chaperones Hsp70 and Hsp90 through its N-terminal tetratricopeptide repeat (TPR) domain, and subsequently ubiquitinates them through its U-Box domain, which possesses ubiquitin ligase activity.11 ASK1, which contains a TPR-acceptor site, is ubiquitinylated by CHIP in a TPR-dependent manner12 and is a target of the ubiquitin ligase activity of c-IAP1, which promotes proteasome-dependent degradation of ASK1.10 Moreover, tumor necrosis factor receptor 2 (TNFR2) signaling induces c-IAP1-dependent ASK1 ubiquitination.13 Thus, CHIP and c-IAP1 both downregulate ASK1 activity by inducing its ubiquitination and subsequent proteasome-dependent degradation.

P34SEI-1, initially identified as a binding partner of cyclin-dependent kinase 4 (CDK4), positively controls the cell-division cycle.13 Recent studies have shown that p34SEI-1 directly binds to the BIR2 domain of XIAP (X-linked inhibitor of apoptosis protein) and prevents the ubiquitination of XIAP, thereby resulting in accumulation of XIAP and inhibition of apoptosis.14 It has also been reported that p34SEI-1 inhibits doxorubicin-induced senescence by suppressing ROS production.15 Interestingly, p34SEI-1 inhibits cellular senescence by inducing ubiquitination of protein kinase C (PKC)-δ and suppressing ROS production. These results suggest that p34SEI-1 plays an important role in the regulation of apoptosis and cellular senescence. However, the role of p34SEI-1 in ROS-inducible cellular events, especially cell death, is not clear.

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Here, we demonstrate that p34\textsuperscript{SEI-1} inhibits ROS-induced cell death by blocking ASK1 activity in human colon cancer cells, revealing a novel cell death-inhibitory mechanism for p34\textsuperscript{SEI-1}.

**Results**

P34\textsuperscript{SEI-1} inhibits H\textsubscript{2}O\textsubscript{2}-induced cell death through suppression of ASK1. Recently, we reported that human breast cancer cells display different sensitivities to various stimuli, including staurosporine, etoposide and cisplatin, depending on their level of p34\textsuperscript{SEI-1} protein.\textsuperscript{14} On the basis of these results, we tested the p34\textsuperscript{SEI-1} dependence of the sensitivities of human colon cancer cells to the cytotoxic stressor H\textsubscript{2}O\textsubscript{2}, which can induce cell death through an increase in intracellular ROS levels.

To determine whether p34\textsuperscript{SEI-1} promotes resistance to H\textsubscript{2}O\textsubscript{2} in human colon cancer cells, we transfected HCT116 and SW620 cells with GFP-tagged p34\textsuperscript{SEI-1} or vector control and treated them with different doses of H\textsubscript{2}O\textsubscript{2} (Fig. 1A). Unlike control cells, which were sensitive to H\textsubscript{2}O\textsubscript{2}, P34\textsuperscript{SEI-1}-expressing cells were resistant to H\textsubscript{2}O\textsubscript{2}. ASK1 phosphorylation is induced in an ROS-dependent manner and suppression of ASK1 expression inhibits apoptosis.\textsuperscript{16} Therefore, to further explore the inhibitory function of p34\textsuperscript{SEI-1} in H\textsubscript{2}O\textsubscript{2}-induced cell death, we examined the effect of p34\textsuperscript{SEI-1} on ASK1 phosphorylation. As shown Figure 1A, H\textsubscript{2}O\textsubscript{2}-induced ASK1 phosphorylation was significantly decreased in cells transfected with p34\textsuperscript{SEI-1} expression plasmids (Fig. 1B). Thus, overexpression of p34\textsuperscript{SEI-1} suppressed ASK1 activity and reduced H\textsubscript{2}O\textsubscript{2}-induced cell death, linking p34\textsuperscript{SEI-1} with resistance to H\textsubscript{2}O\textsubscript{2}-induced cell death.

The ROS-ASK1 signaling pathway plays an important role in inducing apoptosis in various human cancer cells, such as breast cancer, melanoma, neuroblastoma and lung adenocarcinoma.\textsuperscript{17-20} Thus, we next examined the effects of p34\textsuperscript{SEI-1} expression on ASK1-induced cell death in three cell lines: HCT116, HEK293 and HeLa. HeLa cells were chosen in addition to HCT116 and HEK293 cells because recent studies have shown that inhibition of Akt activation in HeLa cells results from ROS-mediated activation of ASK1.\textsuperscript{16} Co-transfection of an ASK1 expression construct in p34\textsuperscript{SEI-1}-expressing cells significantly decreased the population of dead cells in all three cell lines tested (Fig. 2). These results suggest that p34\textsuperscript{SEI-1} plays a key role in ASK1-dependent inhibition of cell death.

P34\textsuperscript{SEI-1} induces ASK1 degradation via a proteasome-dependent pathway. As shown in Figure 1, ASK1 phosphorylation was clearly decreased in p34\textsuperscript{SEI-1}-expressing cells. Thus, we next examined whether p34\textsuperscript{SEI-1} affected the level of ASK1 protein. First, we observed changes in ASK1 protein levels after transfection with GFP-tagged p34\textsuperscript{SEI-1} or vector control (Fig. 3). Expression of ASK1 was remarkably decreased by the expression of p34\textsuperscript{SEI-1} (Fig. 3A). To further study the inhibitory
expression by co-transfecting cells with GFP-tagged p34SEI-1 and HA-tagged ASK1. ASK1 was ubiquitinated in p34SEI-1-expressing cells, but not in cells that ectopically expressed ASK1 only (Fig. 3C). Collectively, these results suggest that p34 SEI-1 promotes ASK1 ubiquitination, resulting in proteasome-mediated degradation of ASK1 and subsequent inhibition of H2O2-induced cell death.

**Discussion**

ASK1 plays an important role in the induction of apoptosis in response to various stimuli, especially ROS.3-5 However, the identities of upstream regulators of ASK1 in ROS-induced apoptotic cell death are incompletely characterized. Here, we demonstrate that p34SEI-1, which positively regulates cell division by binding to CDK4,13 induces ASK1 ubiquitination and, as a consequence, inhibits ROS-induced cell death.

In response to H2O2, ASK1 is phosphorylated in various cancer cells17-20 and consequently induces apoptosis.16-21 Recent reports have shown that downregulation of ASK1 enhances anti-apoptotic activity.22,23 In this study, we found that p34 SEI-1 inhibited H2O2-induced cell death in two human colon cancer cell lines, HCT116 and SW620 (Fig. 1), and also inhibited H2O2-induced ASK1 phosphorylation. In addition, we found an inhibitory effect of p34 SEI-1 on ASK1-mediated signaling induced by...
ally decreased with increasing levels of p34 SEI-1, suggesting that p34 SEI-1 downregulates expression of ASK1 protein. Importantly, binding between p34 SEI-1 and ASK1; thus, the detailed molecular mechanism by which p34 SEI-1 mediates ASK1 ubiquitination (Fig. 4) and promotes proteasome-dependent ASK1 degradation.

We also found that p34 SEI-1 expression induced the degradation of ASK1 in cells transfected with cIAP1-siRNA or CHIP-siRNA (Sup. Fig. 1). Recent reports have shown that cIAP1 and CHIP are E3 ligases for ASK1.9-12 Our findings suggest that p34 SEI-1 induces ASK1 ubiquitination in a cIAP1- and CHIP-independent manner, suggesting that there may be additional E3 ligases in the ASK1 ubiquitination pathway that have yet to be discovered. We did not, however, obtain evidence for direct binding between p34 SEI-1 and ASK1; thus, the detailed molecular mechanism by which p34 SEI-1 induces ASK1 ubiquitination remains to be elucidated.

Collectively, the results of our studies suggest a novel function for p34 SEI-1, namely inhibition of ROS-induced apoptotic cell death by promoting ASK1 ubiquitination.

Materials and Methods

Cell culture, reagents, plasmid DNA and transfections. Human colon cancer cell lines, the HeLa cervical carcinoma cell line and HEK293 cells were cultured in 5% CO₂ at 37°C in Dulbecco’s modified Eagle’s medium (DMEM, GIBCO BRL, Grand Island, NY USA) containing high glucose and supplemented with 10% fetal bovine serum (Hyclone), penicillin (100 units/ml) and streptomycin (100 μg/ml). H₂O₂ was purchased from Sigma (St. Louis, MO USA). The GFP-tagged p34 SEI-1 plasmid and retroviral vectors were obtained by subcloning p34 SEI-1 cDNA (kindly provided by Dr. Rikiro Fukunaga) into the pEFP-N1 (Invitrogen, Carlsbad, CA) and pBabe-retroviral vectors, respectively. The HA-tagged ASK1 plasmid was kindly provided by Dr. Hidenori Ichijo (Graduate School of Pharmaceutical Sciences, University of Tokyo). Lipofectamine 2000 (Invitrogen) was employed for transient transfection into HEK293, HeLa and HCT116 cells.

RNA Interference. HEK293 cells were transiently transfected with small interfering RNAs (siRNAs; 150 pmol/60 mm dish) against cIAP1 (5'-GGA AGA AGG GUG UGG GCA A-3') or CHIP (5'-UUCC AAC ACU CUU CAG CUC AUC AUC C-3') using Lipofectamine 2000 (Invitrogen). Scrambled siRNAs for cIAP1 (5'-GGU AGA ACG GAG AGG GGU A-3') and CHIP (5'-GCU UUG GGA UAU CAU AGC GAV GAA U-3'), obtained from Proligo LLC (Boulder, CO, USA), were used as controls.

Cell viability analysis. Control cells or cells expressing ASK1 and p34 SEI-1 were seeded at 10⁵ cells/60 mm dish and cultured for 48 h before treating with H₂O₂ for 18 h. Cell viability was determined by trypan blue exclusion using at least 300–500 cells for each group.

Protein gel blot analysis. Total cell proteins (20 μg) were resolved by sodium dodecyl sulfate-PAGE (SDS-PAGE) and transferred to PolyScreen membranes (New England Nuclear, Boston, MA). Membranes were blocked with 5% non-fat dry milk in Tris-buffered saline containing 0.1% Tween-20 (TBS-T), and probed with primary antibodies against p34 SEI-1 ubiquitin (Santa Cruz Biotechnology, Santa Cruz, CA), ASK1, HA, GFP, anti-γ-tubulin (Santa Cruz Biotechnology, Santa Cruz, CA), and/or phospho-ASK1 (Cell Signaling Technology, Beverly, MA). Primary antibodies were detected with horseradish peroxidase-conjugated goat anti-mouse, goat anti-rabbit or donkey anti-goat secondary antibody, as appropriate and proteins were visualized using an enhanced chemiluminescence system (Amersham, Buckinghamshire, UK).

Immunoprecipitation and in vivo ubiquitination assay. Immunoprecipitation analyses were performed as previously described in reference 14. In preparation for in vivo ubiquitination assays, cells were transfected with either GFP-tagged p34 SEI-1 or HA-tagged ASK1. After immunoprecipitation with an anti-HA antibody, ubiquitin adducts were detected by immunoblot analysis using an anti-ubiquitin antibody.

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Figure 4. P34SEI-1 ubiquinates ASK1 and promotes its proteasome-dependent degradation. (A) Cells were transiently transfected with plasmids encoding empty vector or p34SEI-1-GFP. HEK293 transfectants were incubated for 48 h, and then MG132 (10 μM) was added 6 h before harvesting. Cell lysates were analyzed by immunoblotting with an anti-ASK1 antibody. (B) Cells were transfected with empty vector or p34SEI-1-GFP and, after incubating for 48 h, were treated with cycloheximide (25 μg/ml) for the indicated times. (C) HEK293 cells were transfected with GFP-tagged p34SEI-1 or HA-tagged ASK1 and cultured with 10 μM MG132 for 6 h. Cell lysates were immunoprecipitated with an anti-HA antibody and analyzed by Protein gel blotting with an anti-ubiquitin antibody.

Author Contributions

Note
Supplemental materials can be found at: www.landesbioscience.com/journals/cbt/article/15972

References


