SUMO-1 represses apoptosis signal-regulating kinase 1 activation through physical interaction and not through covalent modification

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This study examined whether small ubiquitin-related modifier-1 (SUMO-1) regulates apoptosis signal-regulating kinase 1 (ASK1). ASK1 interacted with SUMO-1 *in vitro* as well as in BOSC23 cells. Endogenous ASK1–SUMO-1 interaction was disrupted following H₂O₂ signal. SUMO-1 overexpression suppressed the self-oligomerization, kinase activity and apoptotic potential of ASK1, whereas SUMO-1 depletion potentiated such activities. SUMO-1(ΔC6), a sumoylation-incompetent mutant lacking carboxy-terminal six amino acids, suppressed ASK1 activation, implying that the suppressive effect of SUMO-1 on ASK1 is independent of sumoylation. ASK1(3M), an ASK1 mutant in which all three lysines in the ψKXE motif were substituted with alanines, still retained the kinase activity and activated the Jun amino-terminal kinase pathway. However, SUMO-1 failed to interact with ASK1(3M) and to suppress ASK1(3M) activation, indicating that the three lysines are important for regulation by SUMO-1. This study shows that SUMO-1 exerts a negative regulatory effect on ASK1 activation through physical interaction and not through covalent modification.

Keywords: ASK1; interaction; SUMO-1

EMBO reports (2005) 6, 949–955. doi:10.1038/sj.embor.7400511

INTRODUCTION

Apoptosis signal-regulating kinase (ASK1) is an upstream activator of Jun amino-terminal kinase (JNK) and p38 mitogen-activated protein kinase (MAPK) signalling cascades (Davis, 2000). Activation of ASK1 triggers diverse biological responses and has several binding partners. ASK1 binds to death-receptor-associated proteins, such as TRAFs, Daxx and hD53L1, and leads to MAPK activation (Cho *et al*, 2004). ASK1 activation can be inhibited by interactions with 14-3-3, thioredoxin, HSP-72, Nef and Raf-1 (Chen *et al*, 2001; Goldman *et al*, 2004).

Covalent modification by small ubiquitin-related modifier (SUMO)—sumoylation—regulates diverse cellular processes (Muller *et al*, 2001). Sumoylation is often targeted to lysines in the ψKXE motif (where ψ represents hydrophobic amino acids and X is any amino acid; Sampson *et al*, 2001). However, lysines that are not embedded in the ψKXE motif can also be targeted by SUMO (Johnson & Blobel, 1999; Kim *et al*, 1999). Sumoylation accumulates Ran GTPase-activating protein (RanGAP1) at the nuclear pore (Matunis *et al*, 1998) and transports Sp100 to promyelocytic leukaemia (PML) nuclear bodies (Zhong *et al*, 2000). Modification of transcription factors, including those in the p53, c-Jun, Sp3, c-Myb and C/EBP families, by SUMO-1 reduces its transcriptional activity (Gill, 2004). Sumoylation stabilizes CREB, MDM2 and IκBα by blocking ubiquitination (Muller *et al*, 2001). SUMO-1 also modulates cytokines, Wnt, AP-1 and steroid hormone signalling pathways by conjugating with STAT1, LEF-1, c-Jun, Elk-1 and androgen receptor (Muller *et al*, 2004).

SUMO-1 can regulate cellular processes without the characteristic conjugation. SUMO-1 inhibits RAD51-mediated homologous recombination by interaction with RAD51 (Li *et al*, 2000). SUMO-1 interacts with Fas and tumour necrosis factor receptor 1 (TNFR1) and provides protection against cell death, although covalent modifications of Fas and TNFR1 have not been observed (Okura *et al*, 1996). SUMO-1 can also inhibit dynamin-dependent endocytosis without the covalent modification of dynamin (Mishra *et al*, 2004).

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Fig 1 | Apoptosis signal-regulating kinase 1 interacts with Ubc9 and small ubiquitin-related modifier-1 in BOSC23 cells. (A) Apoptosis signal-regulating kinase 1 (ASK1) binds to small ubiquitin-related modifier-1 (SUMO-1) in BOSC23 cells. Cells were co-transfected with 2 μ g of indicated DNAs. At 48 h after transfection, cell lysates were immunoprecipitated with anti-haemagglutinin (HA) antibody and immunoblotted with anti-Xpress antibody (left panel). BOSC23 cells were untreated or treated for 1 h with 1 mM H_2O_2 . Cell lysates were immunoprecipitated with anti-mouse IgG₁ or with anti-ASK1 antibody and were immunoblotted with anti-SUMO-1 antibody (right panel). Whole-cell lysates (WCLs) were also immunoblotted with the indicated antibodies to show the expression. (B) ASK1 binds to Ubc9 in BOSC23 cells. A similar set of experiments as in (A) was carried out using Flag-Ubc9 instead of Xpress-SUMO-1 (left panel) and Ubc9 antibody instead of SUMO-1 antibody (right panel). IP, immunoprecipitation; WB, western blot.

In the present study, we explored the involvement of SUMO-1 in ASK1-mediated signalling. SUMO-1 interacts with ASK1 and inhibits ASK1 activation. Results show that physical interaction is sufficient for SUMO-1 to suppress ASK1 activation.

RESULTS

ASK1 interacts with SUMO-1 and Ubc9

We examined whether ASK1 interacted with SUMO-1 *in vitro*. As Ubc9 binds to the SUMO-1 acceptor site and efficiently transfers SUMO-1 to targets, we also examined whether ASK1 interacted with Ubc9. ³⁵S-labelled ASK1 was produced by *in vitro* translation and was incubated with purified glutathione S-transferase (GST), GST-Ubc9 or GST-SUMO-1 immobilized on GST-agarose beads (supplementary Fig S1 online). SUMO-1 and Ubc9 proteins interacted with ASK1 *in vitro*. Interactions of ASK1 with SUMO-1 and Ubc9 were also examined in BOSC23 cells. Xpress-SUMO-1 protein was detected in the immunoprecipitated haemagglutinin (HA)-ASK1 complexes, showing that ASK1-SUMO-1 interaction could occur in BOSC23 cells (Fig 1A, left panel). Anti-ASK1 antibody immunoprecipitated both ASK1 and SUMO-1 from BOSC23 cell lysates, indicating endogenous interaction (Fig 1A, right panel). However, the endogenous ASK1-SUMO-1 interaction was disrupted markedly by H_2O_2 treatment, implying that the interaction could be regulated physiologically. A similar set of experiments was carried out for ASK1-Ubc9 interaction. Flag-Ubc9 protein was also detected in the immunoprecipitated HA-ASK1 complexes (Fig 1B, left panel). Endogenous interaction between ASK1 and Ubc9 was also observed (Fig 1B, right panel) and the interaction was sustained by H_2O_2 treatment. Therefore, the interaction of ASK1 with SUMO-1 is regulated by oxidative stress, whereas that of Ubc9 with BOSC23 cells is not.

ASK1 sumoylation was not detected in BOSC23 cells

The possibility that ASK1 might be covalently modified by SUMO-1 was examined because ASK1 contains three lysine residues (K535, K1084 and K1115) embedded in ψ KXE, the sumoylation consensus motif (Fig 2A). BOSC23 cells were transiently transfected with HA-ASK1, Flag-Ubc9, MaxA-Xpress-SUMO-1 and MaxA-Xpress-SUMO-1(Δ C6), sumoylation-incompetent mutant lacking carboxy-terminal six amino acids, with the indicated combinations (Fig 2B). Sumoylated Flag-PML proteins (marked with asterisks) were clearly observed when SUMO-1 was co-transfected. SUMO-1(Δ C6) failed to show PML sumoylation as expected (Fig 2B, right panel). Both anti-ASK1 and anti-Xpress antibodies failed to detect putative sumoylated forms of ASK1 under our experimental conditions (Fig 2B, left panel).

ASK1(3M) retains kinase activity

To know whether lysines in the ψ KXE motif affect the kinase activity of ASK1, ASK1(3M)—in which lysines at 535, 1084 and 1115 were substituted with alanines—was analysed for its kinase activity and self-oligomerization potential. Similar to wild-type ASK1, ASK1(3M) phosphorylated known substrates MAPK kinase 6 (MKK6) and SAPK/ERK kinase 1 (SEK1), as well as itself following H_2O_2 challenge, implying that kinase activity was not impaired (Fig 3A). The oligomerization potential of ASK1(3M) also remained intact (Fig 3B). Thus, lysines in the ψ KXE motif did not affect the kinase activity of ASK1.

ASK1(3M) fails to interact with SUMO-1

As the three lysines did not affect ASK1 kinase activity, we next examined whether they influenced ASK1-SUMO-1 interaction. The three lysines in ASK1 were involved in the binding of ASK1 to

Fig 2 | Covalent modification of apoptosis signal-regulating kinase 1 by small ubiquitin-related modifier-1 is not observed. (A) Schematic representation of apoptosis signal-regulating kinase 1 (ASK1) indicating the amino-terminal domain (N), the kinase domain (K), the carboxy-terminal domain (C) and the ψ KXE motif (indicated by arrows). The K's marked with asterisks are the lysines in the ψ KXE motifs, and are located at amino-acid numbers 535, 1084 and 1115, respectively. (B) BOSC23 cells were co-transfected with 2 μ g of indicated plasmids. At 48 h after transfection, cell lysates were immunoprecipitated with anti-haemagglutinin (HA) antibody and immunoblotted with anti-ASK1 antibody and anti-Xpress antibody. Whole-cell lysates (WCLs) were immunoblotted with the indicated antibodies to show the expression. As a control, an experiment similar to that shown in the left panel was performed, except that promyelocytic leukaemia (PML) protein was used instead of ASK1 (right panel). IP, immunoprecipitation; WB, western blot.

SUMO-1 (supplementary Fig S2 online). GST pull-down assay showed that SUMO-1 and SUMO-1(ΔC6) could interact with ASK1 but barely interacted with ASK1(3M) (Fig 4A). However, Ubc9 could interact with both ASK1 and ASK1(3M). These results indicate that the three lysines of ASK1 are indispensable, whereas the C terminus of SUMO-1, which is essential for sumoylation, is dispensable for ASK1-SUMO-1 binding. The interaction was also investigated in the mammalian cellular environment (Fig 4B). Both Xpress-SUMO-1 and Xpress-SUMO-1(ΔC6) proteins were detected in the pull-down HA-ASK1 complexes, but not in the pull-down HA-ASK1(3M) complexes. These results show that lysine residues are essential in the *in vivo* interaction between ASK1 and SUMO-1.

SUMO-1 inhibits ASK1 activation

We examined whether ASK1-SUMO-1 interaction affected ASK1 kinase activity. The efficiency of SUMO-1 depletion is shown using short interfering RNA (siRNA; Fig 5A). siRNA-SUMO-1 depleted SUMO-1 in a time-dependent manner, whereas β -tubulin remained unchanged. The control small cytoplasmic RNA (scRNA), a 21-nucleotide RNA oligonucleotide with no significant homology to any mammalian gene sequence (Zhang *et al*, 2004b), did not affect SUMO-1 expression. The anti-HA antibody pulled down both HA-ASK1 and Flag-ASK1, indicating the oligomerization of ASK1 (Fig 5B). However, this oligomerization was disrupted by transfection of either SUMO-1 or SUMO-1(ΔC6). This implies that inhibition of ASK1 oligomerization occurs independently of the covalent modification by SUMO-1. Such inhibition of ASK1 oligomerization by SUMO-1 and SUMO-1(ΔC6) was not observed in the pull-down ASK1(3M) complexes.

The oligomerization potential of ASK1 was enhanced by SUMO-1 depletion.

Next, the catalytic ability of ASK1 was examined (Fig 5C). Transfection of both ASK1 and ASK1(3M) enhanced self-phosphorylation and also phosphorylation of MKK6 and SEK1. The kinase activity was attenuated significantly by the coexpression of SUMO-1 and SUMO-1(ΔC6). However, ASK1(3M) kinase activity was not suppressed significantly by either SUMO-1 or SUMO-1(ΔC6). The kinase activity of ASK1 was significantly increased by SUMO-1 depletion, but not by scRNA.

This study examined whether the interaction between ASK1 and SUMO-1 influenced JNK signalling. As shown in Fig 5D, JNK activation was enhanced by transfections of ASK1, ASK1(3M) and siRNA-SUMO-1, but not by scRNA. ASK1-mediated JNK activation was significantly attenuated by the coexpression of SUMO-1(ΔC6) and SUMO-1. However, ASK1(3M)-mediated JNK activation was not suppressed efficiently by either SUMO-1 or SUMO-1(ΔC6).

Collectively, our data show that SUMO-1 suppresses ASK1 oligomerization and subsequently inhibits the catalytic function of ASK1. The negative regulation of ASK1 activation by SUMO-1 occurs independently of the covalent modification of ASK1.

ASK1-mediated cell death was inhibited by SUMO-1

As ASK1 can initiate cell death, we questioned whether SUMO-1 protected cells from ASK1-mediated death. Transfection of both ASK1 and ASK1(3M) enhanced cell death (Fig 6). Both SUMO-1 and SUMO-1(ΔC6) were able to suppress ASK1-mediated cell death. However, both SUMO-1 and SUMO-1(ΔC6) failed to suppress ASK1(3M)-mediated apoptosis. siRNA-SUMO-1

Fig 3 | Apoptosis signal-regulating kinase 1 lysines in the ψ KXE motifs are not essential for the kinase activity and oligomerization potential. (A) BOSC23 cells were transfected for 48 h with 2 μ g of either haemagglutinin (HA)-apoptosis signal-regulating kinase 1 (ASK1) or HA-ASK1(3M) (an ASK1 mutant in which all three lysines in the ψ KXE motif were substituted with alanines). Cells were treated for 1 h with 1 mM H₂O₂ and cell lysates were immunoprecipitated with anti-HA antibody and assayed for the kinase activity *in vitro*. First panel, autophosphorylation assay of ASK1; second and third panels, *in vitro* kinase assay using glutathione S-transferase (GST)-mitogen-activated protein kinase kinase 6 (MKK6) and GST-SEK1 as substrates, respectively; fourth panel, whole-cell lysates (WCLs) were immunoprecipitated and immunoblotted with anti-HA antibody. (B) A 2 μ g portion of Flag-ASK1, HA-ASK1 and HA-ASK1(3M) was transfected with the indicated combinations in BOSC23 cells. At 48 h after transfection, cell lysates were immunoprecipitated with anti-Flag antibody and the resulting precipitates were immunoblotted with anti-HA antibody (top panel). WCLs were immunoblotted with the indicated antibodies to show the expression. IP, immunoprecipitation; WB, western blot.

transfection potentiated cell death, indicating that SUMO-1 depletion relieved ASK1 from suppressive regulation by SUMO-1.

DISCUSSION

This study presents a novel function of SUMO-1, which is mediated by interaction with the nonconjugated form of ASK1. The ASK1-SUMO-1 interaction was specific because substitutions of the three ASK1 lysines in the ψ KXE motifs impaired the binding. However, substitutions of the ASK1 lysines did not compromise the interaction with Ubc9, indicating that the lysines in the ψ KXE motifs have specific roles in ASK1-SUMO-1 interaction. The ASK1-SUMO-1 interaction inhibited the oligomerization of ASK1 and phosphorylation of downstream targets, resulting in the suppression of ASK1-mediated apoptotic cell death.

Inhibition of oligomerization by SUMO-1 is not unique to ASK1. Sumoylation of translocation E26 leukemia (TEL) protein inhibits

Fig 4 | Lysines in the ψ KXE motifs of apoptosis signal-regulating kinase 1 are important for the interaction with small ubiquitin-related modifier-1. (A) Glutathione S-transferase (GST) fusion proteins immobilized on glutathione-Sepharose 4B beads were incubated with ³⁵S-labelled apoptosis signal-regulating kinase 1 (³⁵S-ASK1) and ³⁵S-labelled ASK1(3M) (³⁵S-ASK1(3M), an ASK1 mutant in which all three lysines in the ψ KXE motif were substituted with alanines). The pull-down complexes were separated on an SDS-polyacrylamide gel electrophoresis gel and analysed by autoradiography (upper panel). The lower panel shows Coomassie staining of the GST fusion proteins. (B) BOSC23 cells were transfected with 2 μ g of indicated plasmids. At 48 h after transfection, cell lysates were immunoprecipitated with anti-Xpress antibody and the resulting precipitates were immunoblotted with anti-haemagglutinin (HA) antibody (top panel). Whole-cell lysates (WCLs) were immunoblotted with the indicated antibodies to show the expression. IP, immunoprecipitation; WB, western blot.

oligomerization and disrupts nuclear body formation (Zhang *et al*, 2004a). However, ASK1 is different from TEL in that ASK1 does not require sumoylation. There are proteins that are regulated by SUMO-1 by physical interactions without covalent modifications. SUMO-1 inhibits homologous recombination by RAD51 independently of conjugation, and prevents Fas- and TNFR-mediated apoptosis without conjugating death receptors. Therefore, ASK1 can be a new addition to the proteins that are regulated by SUMO-1 without covalent modification. In addition, ASK1 does not have the known SUMO-1 interaction motif, hhXSXS/Taaa (where h represents a hydrophobic residue and a an acidic residue), which is observed in some sumoylated proteins (Minty *et al*, 2000). Thus, diverse modes seem to exist for SUMO-1 interaction.

The ASK1 lysines in ψ KXE do not seem to affect the enzymatic function of ASK1. ASK1(3M) retained its self-oligomerization ability as well as autophosphorylation potential and successfully phosphorylated target proteins. ASK1-mediated apoptotic potential was also sustained. The fact that three lysines outside the kinase domain can downregulate ASK1-mediated signalling

Fig 6 Small ubiquitin-related modifier-1 suppresses cell death mediated by apoptosis signal-regulating kinase 1. BOSC23 cells were transfected with 2 μ g of plasmids with or without short interfering RNA (siRNA)–small ubiquitin-related modifier-1 (SUMO-1) with the indicated combinations. At 72 h after transfection, cells were fixed and stained with 4',6'-diamidino-2-phenylindole dihydrochloride (DAPI). DAPI-stained nuclei of green fluorescent protein (GFP)-positive cells were examined for apoptotic morphology by fluorescence microscopy and the percentages of apoptotic cells in five randomly chosen fields were determined. Data are mean $\pm$ s.d. of triplicates from one experiment that is representative of three independent experiments (top panel). Whole-cell lysates (WCLs) were also immunoblotted with the indicated antibodies to show the expression. WB, western blot.

suggests that domains outside the kinase domain might have intramolecular regulatory roles.

In the case of RanGAP1, a well-known sumoylation target, the ψ KXE motif was required for the establishment of Ubc9–RanGAP1 interaction as well as for the sumoylation of RanGAP1 (Sampson *et al*, 2001). The lysine acceptor in the ψ KXE motif is not essential for the ASK1–Ubc9 interaction. The dissimilarity in requiring the ψ KXE motif for Ubc9 interaction with RanGAP1 but not for that with ASK1 might be owing to differences in the amino-acid sequences surrounding the ψ KXE motif. The apparent uncoupling between Ubc9 binding and SUMO-1 binding in the case of ASK1 might provide some insight into failure of the covalent modification. It is tempting to speculate that the physical proximity between the Ubc9 interaction site and the sumoylation site is required for Ubc9 to catalyse the covalent modification. In the case of RanGAP1, the Ubc9 interaction site and the sumoylation site are in such close proximity that changes in the ψ KXE motif would affect the interaction with Ubc9. In the case of ASK1, however, the Ubc9 interaction site and the ψ KXE motif might not be close enough for this effect.

Our data clearly show that SUMO-1 downregulates c-Jun activity. However, it is not clear whether SUMO-1 modulates the affinity to DNA or the interaction with transcription cofactors. Phosphorylation of c-Jun inhibits sumoylation and as a result, SUMO-1 and c-Jun seem to regulate mutually. A reciprocal regulation has also been observed in the case of Elk-1 (Yang *et al*, 2003). Phosphorylation of Elk-1 prevents sumoylation, and SUMO-1 modification represses Elk-1 activation. Thus, important pathways such as MAPK pathways seem to be carefully

counterbalanced between two different mechanisms—activation by phosphorylation and repression by sumoylation. Moreover, this study shows the presence of another upstream target point regulated by SUMO-1 in c-Jun activation signalling. It is worthwhile to speculate on the benefit of having more than one target in the same signalling pathway. Redundant downregulation would guarantee the fidelity of the c-Jun activation further.

METHODS

Cell culture and transfections. BOSC23 cells were grown at 37 °C in 5% CO₂ in Dulbecco's modified Eagle's medium (DMEM; Jeil Biotechservices Inc., Daegu, Korea) supplemented with 10% fetal bovine serum (FBS) and antibiotics (Invitrogen, San Diego, CA, USA). BOSC23 cells (1×10^6) were transfected with the indicated plasmids using the calcium phosphate method or the Lipofectamine transfection reagent (Invitrogen).

Plasmids and mutagenesis. The ASK1(3M) mutant was generated using a QuikChange kit (Stratagene, La Jolla, CA, USA). Constructions of Ubc9 and SUMO-1 vectors were reported previously (Ryu *et al*, 2000; Hwang *et al*, 2002). pcDNA3-HA-SUMO-1 was kindly provided by Dr R.T. Hay (St Andrews University, St Andrews, UK). The SUMO-1(ΔC6) (amino acids 1–95) was amplified by PCR and subcloned into the pcDNA-MaxA and pGEX-4T-1 plasmids. The siRNA-SUMO-1 used in this study was reported previously (Kishi *et al*, 2003).

Antibodies. Mouse anti-Xpress (Invitrogen), mouse anti-Flag (M2) and mouse anti- β -tubulin antibodies and horseradish peroxidase-conjugated antibodies against rabbit IgG and mouse IgG were purchased from Sigma (St Louis, MO, USA). Rabbit anti-HA, mouse anti-ASK1 and goat anti-Ubc9 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Mouse anti-JNK (PharMingen, San Diego, CA, USA) and rabbit anti-SUMO-1 (LabFrontier Co., Seoul, Korea) antibodies were also purchased.

In vitro binding and co-immunoprecipitation analysis. ³⁵S-labelled ASK1 and ASK1(3M) proteins were made with the *in vitro* T_NT system (Promega, Madison, MD, USA). The expression and purification of GST fusion proteins and GST pull-down assays were carried out as described previously (Ryu *et al*, 2000). For co-immunoprecipitation experiments, lysates were subjected to immunoprecipitation by a 2 h incubation at 4 °C with appropriate antibodies and then by a 2 h incubation at 4 °C in the presence of protein A/G-Sepharose beads (Santa Cruz Biotechnology). The resulting immunoprecipitates were subjected to SDS–polyacrylamide gel electrophoresis (SDS-PAGE) and analysed by immunoblotting with the indicated antibodies.

Immunocomplex kinase assay. Cells were washed with phosphate-buffered saline (PBS) and lysed in the mammalian lysis buffer (Park *et al*, 2004). Supernatants containing 200 μ g of protein were subjected to immunoprecipitation by a 2 h incubation at 4 °C with appropriate antibodies. Immune complexes were recovered with the addition of protein A/G-Sepharose beads. The beads were washed three times with lysis buffer and once with kinase buffer (Cho *et al*, 2004) and resuspended in 50 μ l of the same kinase buffer. The beads were then incubated with GST–c-Jun, GST–MKK6, GST–SEK1 and 10 μ M [γ -³²P]ATP with 20 μ M cold ATP for 30 min at 30 °C. Phosphorylated proteins were analysed by SDS-PAGE and autoradiographed.

Apoptotic cell death. Cultured cells were transfected with pEGFP-C2 (Clontech, Palo Alto, CA, USA) and indicated plasmids.

At 72 h after transfection, cells were washed twice with PBS, fixed with 4% formaldehyde and stained with 4',6'-diamidino-2-phenylindole dihydrochloride (DAPI; Molecular Probes, Eugene, OR, USA). The DAPI-stained nuclei in green fluorescent protein (GFP)-positive cells were examined for apoptotic morphology by fluorescence microscopy (Bio-Red, Richmond, CA, USA).

Supplementary information is available at *EMBO reports* online (<http://www.emboreports.org>).

ACKNOWLEDGEMENTS

This work was supported by grant number FG04-21-03 of 21C Frontier Functional Human Genome Project from MOST of Korea.

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