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# Regulation of NF-κB signaling by ASK1 through interaction with NIK

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Apoptosis signal-regulating kinase 1 (ASK1) is mitogen-activated protein kinase kinase kinase 5 (MAP3K5) that activates the MAP2K-JNK/p38 signal cascades.[1] Overexpressed ASK1 has been reported to induce apoptosis.[2] ASK1 is a serine/threonine kinase that regulates Fas and tumor necrosis factor-α (TNF-α) pathways.[3] ASK1 is activated by various stresses such as oxidative stress, endoplasmic reticulum (ER) stress, calcium influx, TNF-α, and lipopolysaccharide (LPS).[4,5] Nuclear factor κ-B (NF-κB) inducing kinase (NIK) is another member of the MAP3K family, which plays a critical role in constitutive activation of NF-κB. NIK phosphorylates and activates inhibitor of NF-κB kinase (IKK)-α/β. Activated IKK-α/β lead to phosphorylation and degradation of inhibitor of NF-κB-α (IκB-α) followed by NF-κB activation.[6,7] NIK contributes to the cell survival and transformation.[8] Other MAP3Ks, including MEKK3 and TAK1 have been known to activate NF-κB with cellular mechanisms.[9-13] In addition, ASK1 has been reported to negatively regulate IL-1-induced NF-κB activity through disruption of TRAF6-TAK1 interaction.[2]

To investigate whether ASK1 regulates NIK-dependent NF-κB transactivation activity, BOSC 23 cells were transiently co-transfected with different concentration of the HA-tagged ASK1 (0.05 ~ 1.0 μg), NF-κB-Luc reporter, and NIK expression plasmids (Fig. 1). NIK has been shown to promote the activities of NF-κB promoters studied with luciferase reporter plasmids.[9,10] We examined the effect of ASK1 on NF-κB promoter-luciferase reporter activity in BOSC 23 cells. Whereas expression of NIK alone strongly stimulated NF-κB activity, co-expression of ASK1 apparently reduced NIK-mediated NF-κB transactivation activity in a dose-dependent manner.

We then investigated whether ASK1 associates with NIK in cells. Full-length HA-tagged ASK1 and FLAG-tagged NIK were overexpressed in BOSC 23 cells. ASK1 was immuno-

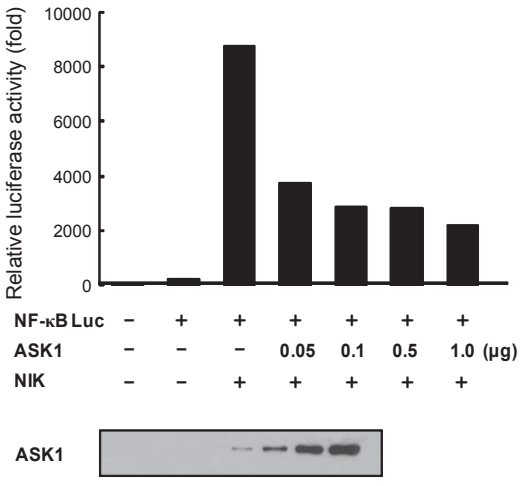


**Figure 1. ASK1 represses NIK-dependent NF-κB transcriptional activity.** BOSC 23 cells were transfected with the pNF-κB luciferase reporter and pcDNA-NIK and pcDNA-ASK1, as indicated. Immunoblot controls are provided for the expression of ASK1 proteins in co-transfected cells (bottom panel).

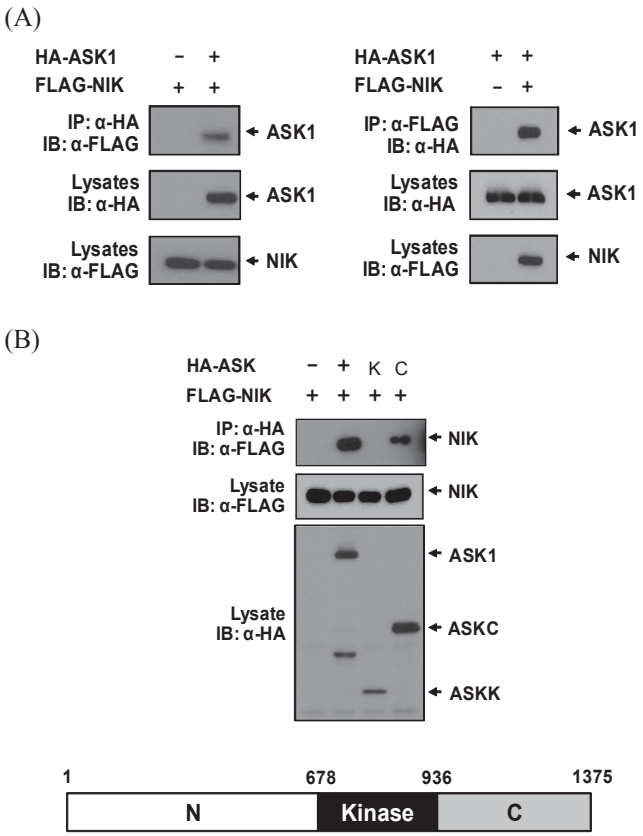


**Figure 2. NIK interacts with ASK1.** (A) Overexpressed NIK and ASK1 proteins bind directly. HA-tagged ASK1 and FLAG-NIK were transiently co-transfected into BOSC 23 cells, and the lysates were immunoprecipitated with appropriate antibodies. Samples were subjected to Western blotting analysis using appropriate antibodies. The expression of transfected plasmids were indicated in the same lysates. IP, immunoprecipitation; IB, immunoblot. (B) NIK binds to the C-terminal domain of ASK1. BOSC 23 cells were transiently co-transfected with FLAG-tagged NIK and HA-tagged ASK1 wild-type or mutant expression plasmids. Transfected cells were extracted and immunoprecipitated with an anti-HA antibody. The interaction was detected by Western blotting with an anti-FLAG antibody.

precipitated by anti-HA antibody from cell lysates and NIK in the immunoprecipitates was detected with anti-FLAG antibody (Fig. 2A, left panel). To test if NIK could bring down ASK1 in a reciprocal manner, we performed co-immunoprecipitation assays with expressed HA-ASK1 and FLAG-NIK proteins and found HA-ASK1 in the immunoprecipitated FLAG-NIK complex (Fig. 2A, right panel).

To identify the region of ASK1 responsible for interaction with NIK, we constructed a series of deletion mutant ASK1 proteins consisting of the kinase domain (ASKK, residues 649-940), the C-terminal domain (ASKC, residues 941-1375). These deletion mutant constructs were used in protein binding assay. Expression plasmids of HA-tagged ASK1 wild-type or mutants were co-transfected with FLAG-NIK into BOSC 23 cells. Total cell lysates were immunoprecipitated using anti-HA antibody and the resulting immunoprecipitates were analyzed by immunoblotting with an anti- FLAG antibody (Fig. 2B). Immunoblot data revealed that the C-terminal domain of ASK1 physically associates with NIK.

Since ASK1 interacts with and inhibits NIK, we investigated whether the kinase activity of ASK1 could regulate the transcriptional activity of NIK in the cells. Human ASK1 consists of 1375 amino acids and has a kinase domain in the middle of protein. BOSC 23 cells were transfected with HA-tagged ASK1 wild-type or catalytically inactive mutant (K709R) expression plasmid.[1] The inhibitory effect of ASK1 on NIK-induced NF-κB signaling was determined by NF-κB-luciferase reporter assays. While NIK activated NF-κB-dependent reporter activity and was repressed by wild-type ASK1, the inactive mutant (K709R) of ASK1 had no effect on the NF-κB transactivation activity (Fig. 3A). These results indicate that ASK1 kinase activity is necessary for regulation in NIK-mediated NF-κB transactivation activity. We then examined whether ASK1 could phosphorylate NIK. FLAG-ASK1 was transiently transfected into BOSC 23 cells and immunoprecipitated with anti-FLAG antibody. ASK1 kinase activities were determined by *in vitro* kinase assays using deletion mutants of GST-NIK as substrates (Fig. 3B). The C-terminal regulatory domain (residues 624 - 947) of NIK was phosphorylated by ASK1, but the N-terminal domain (residues 1 - 623) was not. NIK has protein binding domain in the C-terminal region, which binds to TRAF proteins and induces NF-κB activation by a pathway mediated by NIK. The N-terminal domain contains a negative-regulatory domain (NRD), which is composed of a basic region (BR) and a proline-rich repeat (PRR) motif.[8] Taken together, these results suggest that ASK1 regulates NIK through protein phosphorylation.

In summary, we first identified that ASK1 directly interacts with NIK and influences cellular signal cascade for NIK-mediated NF-κB activity *via* kinase activity. On the basis of our findings, we propose that proteins involved in cell survival and death signaling may communicate each other to determine the cell fate.

## Experimental Section

**Plasmids and recombinant proteins.** FLAG-tagged and HA-tagged ASK1 wild type and ASK1 mutants (K709R, ASKK, and ASKC) were constructed in pcDNA3.1 plasmid (Invitrogen, Carlsbad, CA). FLAG-NIK was generated in pcDNA3.1 plasmid. GST-NIKs (residues 1 - 623 or 624 - 947) were constructed in pGEX 6p-1 plasmid (Amersham Biosciences, Little Chalfont, UK) for *in vitro* kinase assays.

**Cell culture and transfection.** BOSC 23 cells, a derivative of the 293T human epithelial cell line, were maintained at 37 $^{o}$C in Dulbecco's modified Eagle's medium (DMEM, Invitrogen, Carlsbad, CA) containing a high concentration of glucose (4.5 mg/mL), supplemented with 10% fetal bovine serum (FBS, Invitrogen) and penicillin/streptomycin in the presence of 5% $CO_2$ atmosphere. For transient transfection, $1.4 \times 10^6$ cells were plated in each 60-mm cell culture plate, grown overnight, and

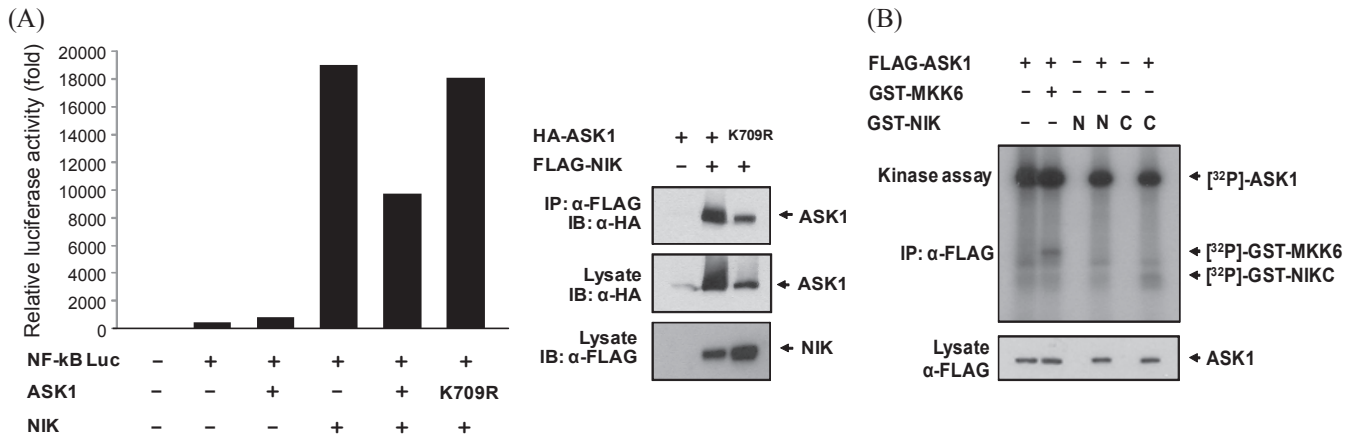


**Figure 3. ASK1 represses NIK-dependent transactivation *via* ASK1 kinase activity-dependent pathway.** (A) The catalytic domain of ASK1 controls NIK-dependent transactivation. BOSC 23 cells were transfected with wild-type or mutant (K709R) ASK1 and NF-κB luciferase activity was measured (left panel). Wild-type and mutant ASK1 proteins bind to NIK. Overexpressed proteins were immunoprecipitated with anti-FLAG antibody, and bound proteins were detected *via* immunoblotting analysis using an anti-HA antibody (right panel). (B) ASK1 has kinase activity to C-terminal of NIK protein. BOSC 23 cells were transfected with FLAG-ASK1. Cell lysates were immunoprecipitated with anti-FLAG M2-agarose. NIK phosphorylation was performed with the immunoprecipitated protein in the presence of [$^{32}$P]ATP. The kinase assay performed in the absence or presence of GST-MKK6 and truncated GST-NIKs (N; 1-623 amino acids or C; 624-947 amino acids). The radioactivity of individual GST-NIK mutants was determined from the phosphorimager.

transfected with DNA using LipofectAMINE (Invitrogen).

**Transfection and luciferase assay.** BOSC 23 cells were transfected by LipofectAMINE with and 0.5 μg of pCMV/β-gal and 1 μg each of NF-κB-Luc reporter, pcDNA-NIK in the absence or presence of pcDNA-ASK1. Total amounts of DNA were equalized with empty plasmids. Cells were harvested and assayed for luciferase activity using the luciferase assay system (Promega, San Diego, CA) as previously described.[14] The relative fold induction of luciferase activity was determined using an assay system (Promega) with a luminometer and normalized to β-galactosidase activity.

**Binding assays and immunoblotting analysis.** BOSC 23 cells were co-transfected with ASK1 and NIK expression plasmids. After 48 h of transfection, cells were washed twice with phosphate buffered saline (PBS) buffer and lysed in lysis buffer (0.5% NP-40, 0.5% Triton X-100, 150 mM NaCl, 20 mM Tris-HCl pH 8.0, 1 mM EDTA, 1% glycerol, 1 mM phenylmethylsulfonyl fluoride and 1 μg/mL aprotinin) for 30 min at 4 $^{o}$C, followed by centrifugation at 13,000 rpm for 30 min. The soluble fractions were incubated with 20 μL of anti-FLAG M2-agarose (Sigma, St. Louis, MO) or anti-HA affinity agarose (Santa Cruz Biotechnology, Santa Cruz, CA) at 4 $^{o}$C for 4 h with rotation. After binding, the beads were collected by centrifugation at 5,500 rpm for 2 min and washed five times in lysis buffer. The bound proteins were eluted with the SDS-PAGE sample buffer and then separated by SDS-PAGE, followed by immunoblotting with anti-FLAG antibody (1:10,000 dilution; Sigma) or anti-HA antibody (1:1,000 dilution; Santa Cruz Biotechnology). The protein bands were visualized by the ECL detection system (PIERCE, Rockford, IL, USA).

**Immunoprecipitation and *in vitro* kinase assay.** For the immune complex kinase assays, BOSC 23 cells were transiently transfected with FLAG-ASK1 expression plasmid. and lysed with the lysis buffer containing 0.5% NP-40, 0.5% Triton X-100, 150 mM NaCl/20 mM Tris-HCl pH 8.0, 1 mM EDTA, 1% glycerol, 1 mM phenylmethylsulfonyl fluoride and 1 μg/mL aprotinin. Cell lysates were subjected to centrifugation at 13,000 rpm for 30 min at 4 $^{o}$C. The soluble fraction was then subjected to immunoprecipitation with anti-FLAG M2-agarose by incubation at 4 $^{o}$C for 4 h. Immunocomplex kinase assays were performed as previously described.[15] The immune complexes were then resuspended in kinase reaction buffer (20 mM Tris-HCl (pH 7.5), 20 mM $MgCl_2$, 1 mM DTT) containing 20 μM ATP and 0.3 μCi of [γ-$^{32}$P]ATP with 1 μg of GST-NIK for 30 min at 30 $^{o}$C. MKK6, an ASK1 substrate, was used as a positive control. The product of kinase reactions was separated by SDS-PAGE. The gels were dried and exposed to film.

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## References

1. Ichijo, H.; Nishida, E.; Irie, K.; ten Dijke, P.; Saitoh, M.; Moriguchi, T.; Takagi, M.; Matsumoto, K.; Miyazono, K.; Gotoh, Y. *Science* **1997**, *275*, 90.
2. Mochida, Y.; Takeda, K.; Saitoh, M.; Nishitoh, H.; Amagasa, T.; Ninomiya-Tsuji, J.; Matsumoto, K.; Ichijo, H. *J. Biol. Chem.* **2000**, *275*, 32747.
3. Geleziunas, R.; Xu, W.; Takeda, K.; Ichijo, H.; Greene, W. C. *Nature* **2001**, *410*, 834.
4. Nagai, H.; Noguchi, T.; Takeda, K.; Ichijo, H. *J. Biochem. Mol. Biol.* **2007**, *40*, 1.
5. Chiang, E.; Dang, O.; Anderson, K.; Matsuzawa, A.; Ichijo, H.; David, M. *J. Immunol.* **2006**, *176*, 5720.
6. Malinin, N. L.; Boldin, M. P.; Kovalenko, A. V.; Wallach, D. *Nature* **1997**, *385*, 540.
7. Rangaswami, H.; Bulbule, A.; Kundu, G. C. *J. Biol. Chem.* **2004**, *279*, 38921.
8. Xiao, G.; Sun, S. C. *J. Biol. Chem.* **2000**, *275*, 21081.
9. Dhawan, P.; Richmond, A. *J. Biol. Chem.* **2002**, *277*, 7920.
10. Zhao, Q.; Lee, F. S. *J. Biol. Chem.* **1999**, *274*, 8355.
11. Channavajhala, P. L.; Rao, V. R.; Spaulding, V.; Lin, L. L.; Zhang, Y. G. *Biochem. Biophys. Res. Commun.* **2005**, *334*, 1214.
12. Schmidt, C.; Peng, B.; Li, Z.; Sclabas, G. M.; Fujioka, S.; Niu, J.; Schmidt-Supprian, M.; Evans, D. B.; Abbruzzese, J. L.; Chiao, P. J. *Mol. Cell.* **2003**, *12*, 1287.
13. Takaesu, G.; Surabhi, R. M.; Park, K. J.; Ninomiya-Tsuji, J.; Matsumoto, K.; Gaynor, R. B. *J. Mol. Biol.* **2003**, *326*, 105.
14. Cho, S.; Ko, H. M.; Kim, J. M.; Lee, J. A.; Park, J. E.; Jang, M. S.; Park, S. G.; Lee, D. H.; Ryu, S. E.; Park, B. C. *J. Biol. Chem.* **2004**, *279*, 16050.
15. Song, M.; Cho, S. *Bull. Korean Chem. Soc.* **2009**, *30*, 1190.