Phosphorylation of SAV1 by mammalian ste20-like kinase promotes cell death

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The mammalian ste20-like kinase (MST) pathway is important in the regulation of apoptosis and cell cycle and emerges as a novel tumor suppressor pathway. MST-induced phosphorylation of Salvador homolog 1 (SAV1), which is a scaffold protein, has not been evaluated in detail. We performed a mass spectrometric analysis of the SAV1 protein that was co-expressed with MST2. Phosphorylation was detected at Thr-26, Ser-27, Ser-36 and Ser-269. Although single or double mutations had little effects, the mutation of all four residues in SAV1 to Ala (SAV1-4A) had inhibitory effects on the MST pathway. MST2-mediated induction of SAV1-4A protein levels, SAV1-4A interaction with MST2 and the self-dimerization of SAV1-4A were weaker compared to those of wild-type SAV1. SAV1-4A inhibited MST2- and K-RasG12V-induced cell death of MCF7 cells. These results suggest that MST-mediated phosphorylation of four residues within SAV1 may be important in the induction of cell death by the MST pathway.

INTRODUCTION

The mammalian ste20 kinase (MST) pathway, which is known as the Hippo pathway in Drosophila, is a potent regulator of organ size, and deregulation of this pathway leads to tumorigenesis (1). The MST pathway negatively regulates proliferation and promotes cell death (1). The MST pathway is composed of a Ser/Thr protein kinase MST1/2, the scaffolding protein Salvador homolog 1 (SAV1 or WW45) and a Ser/Thr protein kinase Large tumor suppressor (LATS), which are homologs of the Drosophila proteins Hippo, Salvador and Warts, respectively.

There are two mammalian MST genes, MST1 and MST2, which are almost identical in their kinase domains and exhibit a high degree of homology throughout the proteins (2). Although MST1 is known to activate apoptosis in cell cultures (3, 4), MST1-knockout mice showed only a mild phenotype in T cell physiology (5, 6). However, the MST1/2 double knockout is embryonic lethal, suggesting a functional redundancy of MST1 and MST2 (7).

Recent studies have uncovered several downstream effectors of the MST signaling pathway (2). MST phosphorylates and activates LATS, which, in turn, phosphorylates and inactivates Yes-associated protein 1 (YAP1). YAP1 is a transcriptional co-activator that is responsible for the expression of multiple apoptosis-related genes (8-10). MST1, which is activated by oxidative stress phosphorylates FOXO1/3a and inhibits the Akt-induced nuclear exit of FOXO1/3a (5, 11, 12). Additionally, the MST-induced phosphorylation of histone H2B regulates chromatin compaction during apoptosis (13, 14).

Studies in Drosophila and mammalian systems have reported that SAV1 recruits LATS to MST to regulate MST-mediated phosphorylation of LATS (15, 16) and that SAV1 is required for the correct cellular localization and function of MST (17). SAV1 has domains that permit protein-protein interaction, including 2 WW domains and a coiled-coil motif in its C-terminus, which suggest that SAV1 functions as a scaffold in a multimeric complex. MST and SAV1 interact through this coiled-coil domain, which is called the SARAH domain (18). LATS binds to the WW domain of SAV1 (19). Disruption of SAV1 in mice results in embryonic lethality with epithelial hyperplasia that is accompanied by defects in the terminal differentiation in various organs (17). SAV1 has been reported to be phosphorylated by MST (20), and several phosphorylation residues of SAV1 were reported by using a large-scale proteomic approach (21, 22). However, the exact MST phosphorylation sites within SAV1 and the effects of MST phosphorylation on SAV1 function in the MST pathway have not been previously studied.

In the current report, we identified four Ser/Thr residues in SAV1 that were phosphorylated by MST2, and using a SAV1 mutant in which all these residues were mutated to Ala, we showed that the phosphorylation of these residues within SAV1 are required for the induction of cell death by the MST pathway.

RESULTS AND DISCUSSION

Identification of MST2 phosphorylation sites within SAV1

MST has been reported to phosphorylate SAV1, however, this phosphorylation has not been previously investigated in detail (20). MST2 increased the level of SAV1 protein and the inter-
Phosphorylation of SAV1 by MST2
Byoung Hee Park and Yong Hee Lee

Fig. 1. Identification of MST phosphorylation sites within SAV1. (A) The catalytic activity of MST2 was required for the stimulation of SAV1 expression and MST2-SAV1 interaction. HEK 293 cells were transfected with Myc-SAV1 and HA-tagged wild-type (WT) or inactive (KD) MST2. The interaction between SAV1 and MST2 was detected by immunoprecipitation with an anti-Myc antibody followed by immunoblotting with an anti-HA antibody. The purified SAV1 protein was analyzed using ion-trap mass spectrometry. The results show that Thr-26, Ser-27, Ser-36 and Ser-269 in SAV1 are phosphorylated by MST2 (Fig. 1B). Phosphorylation at Thr-26 and Ser-27 within SAV1 has been reported in a large-scale proteomic analysis; however, the other sites are novel (21, 22). Thr-26, Ser-27 and Ser-36 are located very close to one another, and a phospho-peptide with phosphorylated Thr-26 and Ser-27 was identified. These results indicate that Thr-26 and Ser-27 may be simultaneously phosphorylated by MST2. Ser-269 is very close to the end of the second WW domain in SAV1, and therefore, the phosphorylation at Ser-269 may have some effect on the interaction of SAV1 with other proteins that have the PPXY motif.

Single or double mutations of the phosphorylated residues into Ala or Glu had little effect on MST2-induced increases in SAV1 protein levels (Fig. 1D). Therefore, a SAV1 mutant, SAV1-4A, was created by mutating all four residues to Ala to completely inhibit phosphorylation. MST2-mediated increases in SAV1-4A protein expression was significantly lower compared to MST2-mediated increases in wild-type SAV1 protein expression. In addition, MST2 bound to SAV1-4A more weakly than to wild-type SAV1 (Fig. 1E). The data suggest that the phosphorylation at these four residues may have additive effects. These results clearly show that the MST2-mediated phosphorylation of SAV1 is essential for SAV1 function.

Phosphorylation of SAV1 is required for MST2-induced increases in protein level and self-dimerization of SAV1
To evaluate the phosphorylation effects of SAV1, we mutated the four residues of interest into Glu to create a SAV1 mutant, SAV1-4E that mimics the phosphorylated state of SAV1. As shown above, MST2 slightly increased SAV1-4A protein but
Phosphorylation of SAV1 by MST2
Byoung Hee Park and Yong Hee Lee

Fig. 2. Phosphorylation of SAV1 is required for MST2-induced increases in SAV1 protein level and self-dimerization of SAV1. (A) Phosphorylation of SAV1 was required for MST2-induced increases in SAV1 expression. HEK 293 cells were transfected with HA-MST2 and various amounts (μg) of Myc-tagged SAV1 mutants. The expression profiles of SAV1 and MST2 were detected by immunoblotting. (B) The phosphorylation of SAV1 was required for the MST2-mediated self-dimerization of SAV1. HEK 293 cells were co-transfected with HA-MST2, Flag-SAV1 and Myc-tagged wild-type SAV1 (WT) or mutant SAV1 (4A or 4E). The dimerization of SAV1 was detected by immunoprecipitation with an anti-Flag antibody followed by immunoblotting with an anti-Myc antibody.

Phosphorylation of SAV1 is required for MST2-induced cell death of MCF-7 cells
It is well known that the MST pathway promotes cell death. Therefore, we postulated that MST-induced phosphorylation of SAV1 may be required for the induction of cell death by the MST pathway. We measured the cell viability of MCF-7 cells, which were transiently co-transfected with SAV1-WT or -4A and MST2-WT or -KD. The catalytic activity of MST2 was critical in promoting the cell death of MCF-7 cells, especially in cooperation with SAV1 (Fig. 3A). MST2-stimulated cell death was almost completely inhibited with the co-expression of SAV1-4A (Fig. 3B). These results clearly show that phosphorylation of SAV1 at the four residues of interest is essential for the induction of cell death by the MST pathway.

Phosphorylation of SAV1 is required for K-RasG12V-induced cell death
MST was reported to mediate the pro-apoptotic activity of active Ras via binding to Nore1 or Rasff1 (25, 26). To investigate the role of SAV1 phosphorylation in Ras-induced cell death, we analyzed the effect of an active Ras mutant, K-RasG12V, on the cell death of MCF-7 cell. K-RasG12V augmented the MST2-induced increase in wild-type SAV1 protein (Fig. 4A). However, this effect was much weaker in SAV1-4A (Fig. 4A), suggesting that the phosphorylation of SAV1 by Ras-stimulated MST may be required for the stabilization of SAV1 protein. The co-expression of K-RasG12V resulted in a significant increase in the cell death of MCF-7 cells that was induced by MST2 and SAV1 (Fig. 4B). The co-expression of SAV1-4A with K-RasG12V significantly inhibited this increase in cell death (Fig. 4B), indicating that MST2-induced phosphorylation of SAV1 is required for the K-RasG12V-mediated cell death.

Our results suggest that MST2-mediated phosphorylation of SAV1 has dual functions as follows: to stabilize SAV1 protein and to increase the interaction of SAV1 with MST2 or SAV1 itself. These results, together with a previous report showing that the interaction of SAV1 and MST increased their protein levels (20), suggest that the phosphorylation-induced increase of SAV1 protein may be a secondary effect of the increased interaction of SAV1 with MST2. However, SAV1 phosphorylation may directly inhibit ubiquitin ligases and proteasomes.
Phosphorylation of SAV1 by MST2
Byoung Hee Park and Yong Hee Lee

Fig. 3. Phosphorylation of SAV1 is required for MST2-induced cell death in MCF-7 cells. (A) The catalytic activity of MST2 was required for the stimulation of cell death in MCF-7 cells. MCF-7 cells were co-transfected with SAV1 and wild-type MST2 or catalytically inactive MST2 mutant (KD). After 72 h, the cell viability was detected using trypan blue exclusion assay. (B) The phosphorylation of SAV1 was required for the MST2-mediated cell death in MCF-7 cells. MCF-7 cells were co-transfected with MST2 and wild-type SAV1 or SAV1 mutant (4A). After 72 h, the cell viability was detected using trypan blue exclusion assay.

Fig. 4. Phosphorylation of SAV1 is required for K-RasG12V-induced cell death. (A) The phosphorylation of SAV1 was required for K-RasG12V-induced increases in SAV1 protein levels. HEK 293 cells were co-transfected with HA-K-RasG12V, MST2 and Flag-SAV1-WT or -4A. After 48 h, the protein expression was detected by immunoblotting. (B) The phosphorylation of SAV1 was required for MST2- and K-RasG12V-mediated cell death in MCF-7 cells. MCF-7 cells were co-transfected with HA-K-RasG12V, MST2 and Flag-SAV1-WT or -4A. After 72 h, the cell viability was detected using trypan blue exclusion assay.

A previous report showed that SAV1 is phosphorylated by MST1 or MST2 and that the stabilization of SAV1 is independent of its phosphorylation (20). However, a careful re-examination of their data revealed that MST2-KD but not MST1-KD failed to increase SAV1 protein levels as efficiently compared to wild-type MST2, which is consistent with our results concerning MST2. These data suggest that MST2-mediated phosphorylation of SAV1 may function differently compared to MST1-mediated phosphorylation of SAV1.

SAV1 can interact with several target proteins other than members of the MST pathway, such as MST and LATS. Therefore, the dimerization or multimerization of SAV1 may bring MST or LATS in contact with their downstream substrate proteins, which are bound to SAV1. We showed that SAV1 phosphorylation is required for MST2-induced increases in SAV1 dimerization (Fig. 2B). Therefore, SAV1 phosphorylation increases SAV1 dimerization, which may facilitate the signal transduction of the MST pathway.

A single SAV1 protein may interact with MST and a downstream protein simultaneously. The phosphorylation of SAV1 increased the binding affinity of SAV1 with MST2 (Fig. 1E). The affinity of MST-bound SAV1 for a downstream target protein may be increased by phosphorylation to facilitate the signal transduction of the MST pathway.

In the current report, we identified four Ser/Thr residues within SAV1 that were phosphorylated by MST2, and showed that these phosphorylations are required for the activation of SAV1 and the induction of cell death by the MST pathway.

MATERIALS AND METHODS

Plasmids
Full length cDNAs for human SAV1 and human MST2 were obtained from the 21C Human Gene Bank (KRIBB, Korea) and subcloned into pCS4-3XMyr, -3XFlag or -3XHA mammalian expression vectors. MST2-K56R (MST2-KD), SAV1-T26A/S27A/S36A/S269A (SAV1-4A) and SAV1-T26E/S27E/S36E/S269E (SAV1-4E) mutants were generated using site-directed mutagenesis and verified by sequencing.
Antibodies
Antibodies against Myc (9E10; Santa Cruz Biotechnology, USA), HA (12CA5; Roche Applied Science, Germany), and Flag (M2; Sigma, USA) were used.

Cell culture and transfection
HEK 293 cells and MCF-7 cells were maintained in Dulbecco’s modified Eagle’s medium (Welgene, Korea) that was supplemented with 10% fetal bovine serum (Invitrogen, USA) and 100 units/ml of penicillin-streptomycin (Sigma, USA) at 37°C in a humidified atmosphere with 5% CO2. Transient transfections were performed using the Lipofectamine Plus reagent in a humidified atmosphere with 5% CO2. Transient transfections were performed using the Lipofectamine Plus reagent (Invitrogen, USA) or Welfect reagent (Welgene, Korea).

Identification of phosphorylation sites by mass spectrometry
The 6XHis-SAV1 and MST2 or MST2-KD constructs were over-expressed in HEK 293 cells, and the 6XHis-SAV1 proteins were purified using Ni2+ column. The purified protein was over-expressed in HEK 293 cells and the 6XHis-SAV1 proteins were purified using Ni2+ column. The purified protein was separated by 10% SDS-PAGE, and the corresponding SAV1 band was in-gel digested using trypsin and analyzed using tandem mass spectrometry (MS/MS) with an LTQ linear ion trap mass spectrometer (help of Dr. Edward P. Feener at Joslin Diabetes Center, Boston, USA). The assignment of MS/MS data was performed using SEQUEST software (Thermo Electron). Resultant matches were entered and compiled into a MySQL database, and proteomic computational analyses were performed using a Hypertext Preprocessor (PHP)-based program.

Immunoprecipitation and western blot analysis
The cells were lysed with cold lysis buffer (50 mM Tris-HCl (pH 7.4), 120 mM NaCl, 1% NP-40, 12 mM β-glycerophosphate, 10 mM NaF, 0.5 mM PMSF, 5 μg/ml leupeptin, 5 μg/ml aprotinin, 1 μg/ml pepstatin, and 100 μM Na3VO4]. Cell lysates were incubated with antibodies at 4°C for 2 h and complexes were subsequently retrieved with protein G-Sepharose beads (Amersham, UK). The immunoprecipitates were resolved by SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Millipore, US). The membrane was immunoblotted with the indicated primary antibodies and visualized by ECL (Elipis Biotech, Korea).

Cell death assay: MCF-7 cells were transiently transfected with plasmids. After 72 h, the cells were harvested and the viability was determined using the trypan blue exclusion method.

Acknowledgements
This work was supported by a research grant (2010-0006967) to YHL from National Research Foundation of Korea (NRF) and a research grant of the Chungbuk National University (2006) to YHL.

REFERENCES


