Biochemical and Biophysical Research Communications 433 (2013) 538–541

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

A conserved serine residue regulates the stability of *Drosophila* Salvador and human WW domain-containing adaptor 45 through proteasomal degradation

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ARTICLE INFO

Article history:
Received 5 March 2013
Available online 21 March 2013

Keywords:
Salvador
Stability
Proteasome
Hippo
WW domain-containing adaptor 45

ABSTRACT

The Hippo pathway is a conserved tumor suppressor pathway that controls organ size through the coordinated regulation of apoptosis and proliferation. *Drosophila* Salvador (Sav), which limits organ size, is a core component of the Hippo pathway. In this study, Ser-17 was shown to be important for the stability of Sav. Alanine mutation of Ser-17 promoted the proteasomal degradation of Sav. Destabilization and stabilization of the Sav protein mediated by alanine mutation of Ser-17 and by Hippo (Hpo), respectively, were independent of each other. This implies that the stability of Sav is controlled by two mechanisms, one that is Ser-17-dependent and Hpo-independent, and another that is Ser-17-independent and Hpo-dependent. These dual mechanisms also regulated the human counterpart of *Drosophila* Sav, WW domain-containing adaptor 45 (WW45). The conservation of this regulation adds to its significance in normal physiology and tumorigenesis.

1. Introduction

The *Drosophila* Salvador (Sav) protein controls organ size through the coordinated regulation of apoptosis and proliferation, and mutation of the gene leads to increased body mass and cell number [1,2]. The mammalian homologue of Sav, WW domain-containing adaptor 45 (WW45), is widely conserved and functions as a tumor suppressor [1,3]. Downregulation of WW45 leads to the development of lung cancer in mice [4] and high-grade clear cell renal cell carcinoma in humans [5].

Sav is a core component of the Hippo tumor suppressor pathway, in which the Hippo (Hpo) kinase phosphorylates and activates Warts (Wts), which itself phosphorylates Yorkie in a Mob as tumor suppressor (Mats)-dependent manner [6–11]. Yorkie is a transcriptional coactivator of several apoptosis- and cell cycle-related genes [12,13]. Phosphorylated Yorkie is retained in the cytoplasm, and so cannot interact with its target transcription factors and promoters, and is thereby inactivated [11]. In the Hippo pathway, Sav promotes Hpo-mediated phosphorylation of Wts by associating with these proteins and thereby promoting an interaction between them [6]. The components and functions of the Hippo pathway are highly conserved between *Drosophila* and humans [14,15].

Decreased *Drosophila* Sav and human WW45 levels are associated with tumorigenesis *in vivo* [4,5]; therefore, it is important to understand how the stabilities of these proteins are regulated. A conserved mechanism involving phosphorylation and stabilization by Hpo and mammalian sterile 20-like kinase-2 (Mst2, Hpo ortholog) regulates the stability of Sav and WW45, respectively [6,16]. However, other related mechanisms and factors remain to be elucidated.

In this study, a conserved serine site was shown to be key for the stability of *Drosophila* Sav and human WW45; mutation of this site to alanine led to proteasomal degradation of the proteins. Our findings provide new insights into how the stabilities of Sav and WW45 are regulated.

2. Materials and methods

2.1. Plasmids construction

The Sav and Hpo coding sequences were obtained as described previously [6], and cloned into the pAc-V5-HisB vector (Invitrogen) at EcoRI/NotI (for Sav) or EcoRI/XhoI (for Hpo) sites. The WW45 coding sequence was purchased from Addgene and cloned into the pCDNA-4/TO (Invitrogen) vector at EcoRI/XhoI sites. All the first methionine codons were replaced with an N-terminal epitope (Myc for Sav and WW45, HA for Hpo) by PCR. The Sav-S17A, Sav-S17D and WW45-S3A mutants were generated by site-directed

Abbreviations: Sav, Salvador; Hpo, Hippo; Wts, Warts; Mats, Mob as tumor suppressor; WW45, WW domain-containing adaptor 45; Mst2, mammalian sterile20-like kinase-2.

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http://dx.doi.org/10.1016/j.bbrc.2013.03.023

mutagenesis and confirmed by sequencing. Sequences of all PCR primers were listed in Supplementary Table 1. pCDNA-Flag-Mst2 plasmid was described previously [17].

2.2. Cell culture and transfection

Drosophila S2 cells were cultured in serum-free insect cell medium (Hyclone) at room temperature with no additional CO_2. Human A549 cells were cultured in RPMI 1640 medium (Hyclone) with 10% fetal bovine serum (Hyclone) at 37 °C with 5% CO_2. Plasmids were transfected with Roche FuGENE HD reagent following the manufacturer's instructions.

2.3. Western blot of Phos-tag™ SDS–PAGE gels

Phos-tag™ acrylamide was purchased from Wako and used in accordance with the manufacturer's instructions. Phos-tag™ SDS–PAGE gels were prepared by adding Phos-tag™ acrylamide and $MnCl_2$ to the SDS–PAGE gel solution at a final concentration of 150 mg/L and 10 mg/L, respectively. After electrophoresis, Phos-tag™ SDS–PAGE gels were soaked in transfer buffer containing 1 mM EDTA for 10 min to remove Mn^{2+}. Gels were transferred to PVDF membranes by wet transfer at 200 mA for 8 h. Other procedures were the same as those used for general Western Blot.

2.4. Antibodies

The antibodies used were as followed: anti-Myc, Abmart (China) or Jackson ImmunoResearch; anti-HA, Abmart; anti-Flag, Sigma–Aldrich; anti-β-Tubulin, Abmart; and horseradish peroxidase secondary antibodies, GE Healthcare.

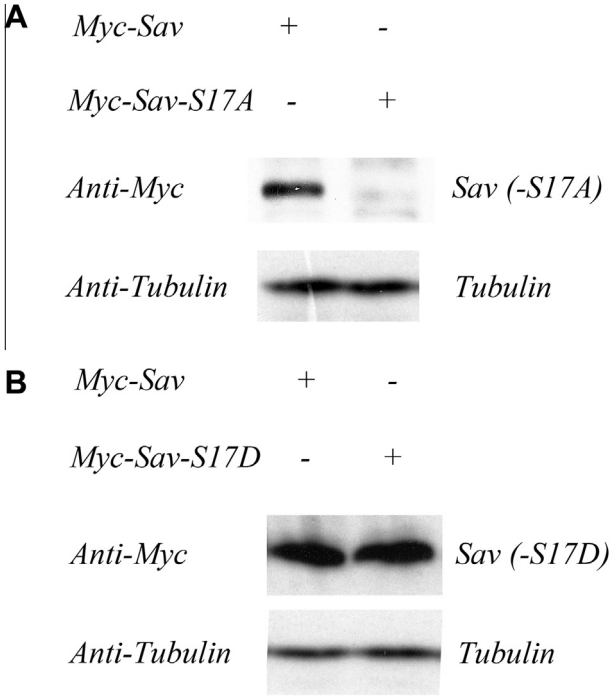

Fig. 1. Ser-17 is a key residue for the regulation of Sav stability. *Drosophila* S2 cells were transfected with pAC-Myc-Sav and the mutants (A, Sav-S17A and B, Sav-S17D) as indicated. At 36 h after transfection, total cell lysates were subjected to Western Blot analyses.

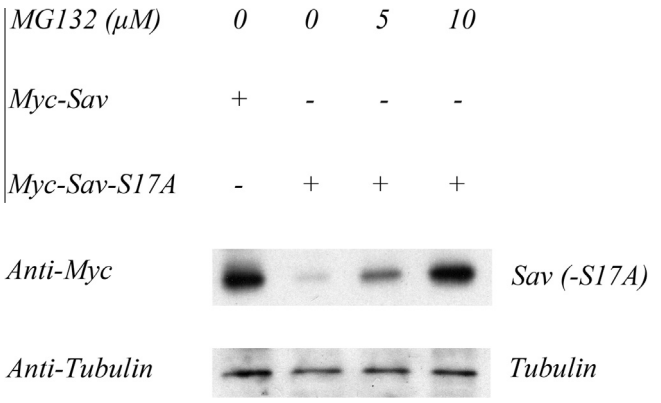

Fig. 2. Proteasome inhibitor MG132 blocks the degradation of Sav-S17A mutant. *Drosophila* S2 cells were transfected with pAC-Myc-Sav and Myc-Sav-S17A, respectively. At 12 h after transfection, cells were treated with a higher dose of MG132 for 24 h, and harvested for Western Blot experiments.

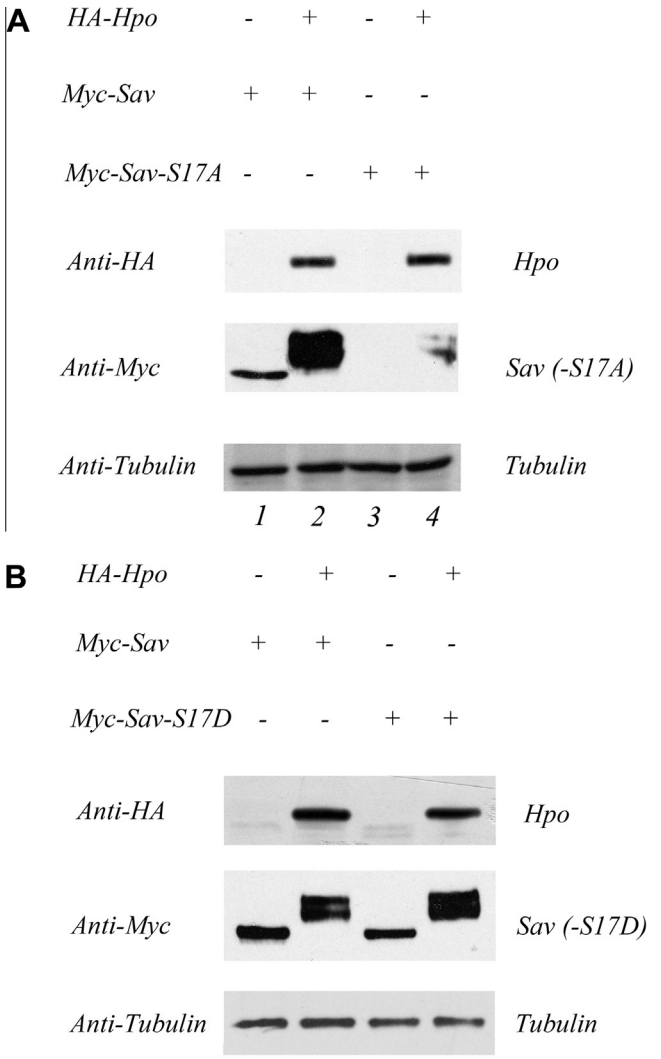

Fig. 3. Sav-S17 mutants are stabilized and phosphorylated by Hpo. *Drosophila* S2 cells were transfected with pAC-Myc-Sav and the mutants (A, Sav-S17A and B, Sav-S17D) with vector control or HA-Hpo as indicated. At 36 h after transfection, total cell lysates were subjected to Western Blot analyses.

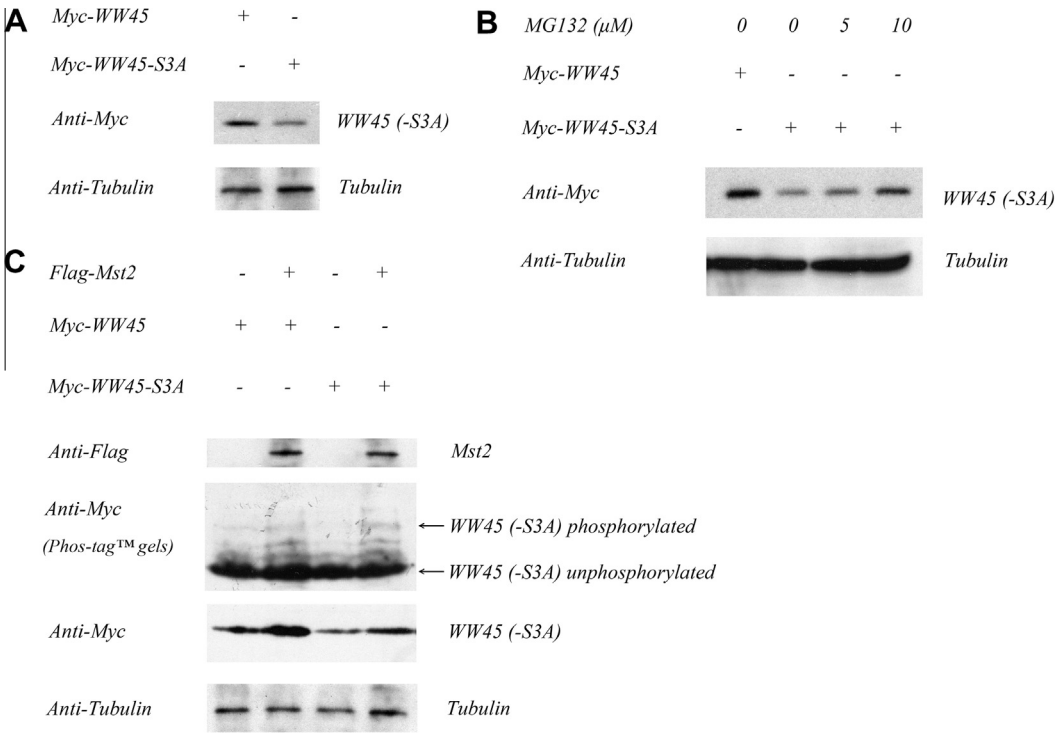

Fig. 4. The stability of human WW45 is regulated in a similar manner. (A) WW45-S3A mutant is unstable. Human A549 cells were transfected with pCDNA-Myc-WW45 and Myc-WW45-S3A mutants as indicated. At 36 h after transfection, total cell lysates were subjected to Western Blot analyses. (B) Proteasome inhibitor MG132 blocks the degradation of WW45-S3A. Human A549 cells were transfected with pCDNA-Myc-WW45-S3A. At 12 h after transfection, cells were treated with a higher dose of MG132 for 24 h, and harvested for Western Blot experiments. (C) WW45-S3A mutant is phosphorylated and stabilized by Mst2. Human A549 cells were transfected with pCDNA-Myc-WW45 and WW45-S3A mutant with vector control or Flag-Mst2 as indicated. At 36 h after transfection, total cell lysates were subjected to Western Blot analyses.

3. Results

3.1. Ser-17 is key for the stability of Sav

A systematic screening, in which each conserved serine and threonine residue of Sav was mutated to alanine, identified Ser-17 as a key residue in the regulation of Sav stability. Sav-S17A, a phosphorylation-deficient mutant, was destabilized so that it was undetectable (Fig. 1A). By contrast, the expression level of the phosphomimetic mutant, Sav-S17D, was similar to that of wild-type Sav (Fig. 1B). These results suggest that phosphorylation of Sav-S17 stabilizes the protein. This site may be constitutively phosphorylated *in vivo* and its dephosphorylation may lead to the degradation of Sav.

3.2. Sav-S17A is degraded via proteasome

To examine the molecular mechanism of Sav-S17A degradation, we assessed its stability in the presence of MG132, a proteasome inhibitor. Treatment of S2 cells expressing Myc-Sav-S17A with MG132 resulted in a dose-dependent stabilization of the protein (Fig. 2A), indicating that Sav-S17A degradation is mediated at least partially by the proteasome.

3.3. Sav-S17 mutants are stabilized following phosphorylation by Hpo

Next, the effect of phosphorylation of Ser-17 in Hpo-mediated stabilization of Sav was investigated. Sav is a well-known substrate of Hpo. Indeed, the electrophoretic mobility of Sav by SDS–PAGE was shifted when it was co-expressed with Hpo, indicating Sav was phosphorylated by Hpo [6]. The electrophoretic motilities of Sav-S17A and Sav-S17D were also shifted when they were co-expressed with Hpo (Fig. 3A and B), indicating that they were phosphorylated by Hpo. These results raise the possibility that Sav-S17 is not phosphorylated by Hpo, or conclude that at least Ser-17 is not the only residue of Sav that is phosphorylated by Hpo.

The Sav-S17 mutants were stabilized by Hpo; when co-expressed with Hpo, Sav-S17A was detectable (Fig. 3A) and the expression level of Sav-S17D was markedly increased (Fig. 3B). This indicates that phosphorylation of Ser-17 is not required for Hpo-mediated stabilization of Sav.

3.4. The stability of human WW45 is regulated in a similar manner

To determine whether this mechanism is conserved, we used the human homologue of *Drosophila* Sav, WW45, in which Ser-3 corresponds to Sav-S17 (Supplementary Fig. 1).

The WW45-S3A mutant was unstable when expressed in human A549 cells (Fig. 4A). MG132 treatment stabilized the WW45-S3A mutant in a dose-dependent manner (Fig. 4B). Mst2 co-expression did not cause any marked electrophoretic shift in both wild type WW45 and WW45-S3A mutant in conventional SDS–PAGE gels (Fig. 4C). This is in contrast to *Drosophila* Sav and consistent with previous reports [16]. Phos-tag™ SDS–PAGE gels are a useful tool for detecting protein phosphorylation through electrophoretic shift. An obvious electrophoretic mobility shift in WW45-S3A was detected using these gels when the mutant was co-expressed with Mst2, which indicates that WW45-S3A was phosphorylated by Mst2 (Fig. 4C). Mst2-mediated stabilization of WW45-S3A was detected by immunoblotting (Fig. 4C).

These results show that the stabilities of Sav and WW45 are connected with a conserved key serine residue. This indicates that a mechanism to regulate the stabilities of these proteins is conserved in *Drosophila* and humans, and that this mechanism may have functional significance in normal physiology and during tumorigenesis.

4. Discussion

Ser-17 and Hpo are key to the regulation of Sav stability. The relationship between them was investigated. It has been suggested that Sav-S17 has no role in Hpo-mediated stabilization of Sav (Fig. 3). On the other hand, with Hpo co-transfection, the protein level of Sav-S17A was still lower than that of wild-type Sav (compare lanes 2 and 4 in Fig. 3A), indicating that Hpo has little effect on the destabilization of Sav caused by Ser-17A mutation. Consistently, Mst2 and the Ser-3 residue of human WW45 function independently of each other in the regulation of the stability of human WW45 (Fig. 4D). This conclusion is supported by other studies [18]. Four serine/threonine residues in WW45 are Mst2 phosphorylation sites but Ser-3, which corresponds to Sav-S17, is not one of them. Mutation of these four sites to Ala abolishes Mst2-induced stabilization of WW45. Our results, together with those of previous studies, suggest that the stability of Sav and WW45 is controlled by two mechanisms, one is Ser-17 (Ser-3)-dependent and Hpo (Mst2)-independent, and the other is Ser-17 (Ser-3)-independent and Hpo (Mst2)-dependent. Such dual regulation would enable the level of Sav and WW45 to be accurately and dynamically modulated *in vivo*.

Many intriguing questions remain unanswered. It is unknown which kinase(s) phosphorylates the Sav-S17 and WW45-S3 residues. Furthermore, it remains to be determined whether ubiquitination mediates the degradation of the Sav-S17A and WW45-S3A mutants. Clarification of these issues will lead to a better understanding of the mechanism underlying how the stabilities of Sav and WW45 are regulated.

Notably, the strength of this regulatory mechanism differs between *Drosophila* and humans. Mutation of Sav-S17 to alanine destabilized the protein to a barely detectable level (Fig. 1A), and MG132 treatment markedly stabilized the mutant (Fig. 2A). The WW45-S3A mutant was detectable (Fig. 4A) and was stabilized to a much lesser extent that Sav-S17A by MG132 treatment (Fig. 4B). These observations are consistent with previous studies, which reported that *Drosophila* Sav is stabilized by Hpo to a much greater extent than human WW45 is stabilized by Mst2 [6,16]. In general, it appears that the stability of WW45 is more moderately regulated than the stability of Sav. We speculate that diversities between Sav and WW45 underlie these differences, which implies there are additional complexities in the conserved mechanisms that regulate the stabilities of Sav and WW45.

Acknowledgments

The authors thank Xinqi Liu, Xiaojing Wu and Xiaoyu Hu for providing *Drosophila* S2 cells, human A549 cells and pCDNA4/TO vector plasmid, respectively. The authors also thank Dr. Jacques Pouyssegur for critical reading and comments on the manuscript. This work was supported by the National Science Foundation of China (Grant No. 31171411).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.03.023.

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