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Insights into the regulation of heat shock transcription factor 1 SUMO-1 modification☆

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Abstract

The transcriptional regulatory protein HSF1 is the key mediator of induced heat shock protein gene expression in response to elevated temperature and other stresses. Our previous studies identified stress-induced SUMO-1 modification of HSF1 as an important regulator of the DNA-binding activity of this factor. The underlying molecular mechanism by which stress leads to sumoylation of HSF1 was unknown. Prompted by previous studies indicating stress-induced phosphorylation at serine 307 of HSF1, a site very near the sumoylation site at lysine 298, we examined the role of this phosphorylation event in regulating SUMO-1 modification of HSF1. Using a combination of transfection and in vitro phosphorylation/sumoylation experiments, our results indicate that phosphorylation at serine 307 stimulates sumoylation of HSF1. Our results also reveal a role for a conserved leucine zipper sequence in the C-terminal region of HSF1 in inhibiting its SUMO-1 modification. Based on these data, we postulate that phosphorylation at serine 307 could stimulate HSF1 sumoylation by causing a conformation change that relieves the inhibitory effect of the C-terminal leucine zipper.

Keywords: Sumoylation; HSF1; Phosphorylation

Exposure of cells to many different environmental stresses such as heat and heavy metals induces the transcription of heat shock genes. The transcription of heat shock genes is mediated by heat shock transcription factor 1 (HSF1), which exists in the absence of stress in a non-DNA-binding form. Heat shock converts HSF1 to the trimeric DNA binding-form that then interacts with promoters of heat shock protein (hsp) genes to up-regulate transcription [1,2]. We have previously shown that heat stress causes HSF1 to become covalently modified by SUMO-1, a small ubiquitin-like modifying protein, and that this modification activates the DNA binding ability of HSF1 [3]. Sumoylation of HSF1 occurs at lysine residue 298 which lies within a sumoylation consensus sequence ψKXE (where ψ represents a hydrophobic amino acid and X represents any amino acid) [3–5]. However, the underlying molecular mechanism by which stress treatment triggers SUMO-1 modification of the HSF1 protein is unknown.

Heat shock causes an increase in phosphorylation of certain serine residues of HSF1 [6–10]. One of these sites of phosphorylation that is increased by stress is serine 307, which is immediately adjacent to the sumoylation site at lysine 298. It was noted from in vivo phospholabeling studies that a small proportion of the HSF1 polypeptides present in the cell could be phosphorylated in the absence of stress treatment, but the very large increase in signal of this phosphopeptide after heat treatment in the absence of increase in total HSF1 protein level clearly supports a role for phosphorylation at serine 307 in mediating/regulating stress-induced function of HSF1 [6]. Serine 307 is phosphorylated by the MAP kinases ERK1/ERK2, whose activities are increased in cells after stress treatment [6,11].

☆ *Abbreviations:* HSF1, heat shock transcription factor 1; hsp, heat shock protein; PBS, phosphate-buffered saline; SUMO-1, small ubiquitin-related modifier; Erk-1/2; extracellular regulated kinase-1/2.

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Phosphorylation has been found to regulate the sumoylation of several proteins including Iκβ and mdm2, and thus in the present study we tested the hypothesis that stress-induced phosphorylation of serine 307 could be involved in regulating sumoylation of HSF1 at the nearby lysine 298 [12,13].

The results of our experiments indicate that phosphorylation at serine 307 does stimulate HSF1 sumoylation at lysine 298. The results also implicate a conserved C-terminal leucine zipper of HSF1 in negatively regulating its sumoylation. This zipper sequence was previously shown to be important for maintenance of HSF1 in the non-DNA-binding form [14]. Our results suggest that stress-induced phosphorylation of HSF1 at serine 307 is an important event leading to sumoylation of this protein, possibly by relieving inhibition of the C-terminal leucine zipper sequence.

Materials and methods

Mutagenesis of HSF1. A pEGFP HSF1β Δ378–407 mutant of HSF1 was generated using the QuickChange mutagenesis kit (Stratagene) according to manufacturer's protocol. Mutations were confirmed by DNA sequencing. Mutations of pcDNA HSF1β serine residues to alanine were also done by QuickChange mutagenesis. The serine residues that were changed were Ser303 and Ser307.

In vitro SUMO-1 modification assay. HSF1β wild-type and mutant proteins were in vitro translated using the Promega TNT T7 Quick for PCR DNA rabbit reticulocyte and then subjected to in vitro SUMO-1 modification assay essentially as previously described [15]. Modification reactions were terminated using SDS–sample buffer containing β-mercaptoethanol and fractionated by SDS–PAGE (10%) followed by gel fixation in 20% isopropanol:10% acetic acid for 15 min. After fixation gels were soaked in Amplify (Pharmacia) according to manufacturer's instructions and then dried. The dried gels were analyzed by autoradiography with quantification using phosphorimaging (Molecular Dynamics Storm model 860, Imagequant software).

Phosphorylation of in vitro translated HSF1β serines 303 and 307. Phosphorylation of HSF1 serines 303 and 307 was modified from [16]. Briefly, purified active recombinant Erk2 from *Escherichia coli* and GSK3β from rabbit muscle were obtained from Upstate Biotechnology Inc. Phosphorylation of HSF1 serine 307 was done using 8 μl of in vitro translated HSF1 and incubating it with 25 ng Erk2 in GSK3β buffer (8 mM Mops, pH 7.3, 200 μm EDTA, 10 mM $MgC_4H_6O_4$, 500 μm ATP, and 20 mM $MgCl_2$) in a final volume of 16 μl. Samples were then incubated at 30 °C for 20 min and then used for in vitro SUMO-1 modification assays. Phosphorylation of HSF1 serine 303 was done using 8 μl of in vitro translated HSF1 and incubating it with 40 mU GSK3β in GSK3β buffer in a final volume of 16 μl at 20 °C for 30 min. Sequential phosphorylation of Erk2/GSK3β was done as described previously [16]. Phosphorylated HSF1 proteins were subjected to in vitro SUMO-1 modification assay immediately following phosphorylation. For analysis of the effect of S307A mutation on enhancement of HSF1 sumoylation by Erk2 phosphorylation, ^{35}S-labeled in vitro translated wild-type and mutant HSF1 proteins were subjected to Erk2 phosphorylation followed by the sumoylation reaction as described above. The sumoylated versions of these proteins were then purified by Ni–NTA affinity chromatography based on the 6× His tag present on the SUMO-1 protein used for the in vitro sumoylation reaction, and then subjected to SDS–PAGE followed by autoradiography to detect the ^{35}S-labeled proteins.

Transient transfection of HeLa cells and immunoprecipitation analysis. HeLa cells were transfected with pcDNA3-wild-type HSF1, pcDNA3-Ser303 Ser307 HSF1 as described previously [3]. Immunoprecipitation analysis was performed as previously described [3].

Results

Mutational analysis of the role of Ser303 and Ser307 in HSF1 sumoylation

Previous studies demonstrated that phosphorylation of serine 307 increases after heat shock treatment [6]. The proximity of serine 307 to the SUMO-1 modification site, lysine 298, prompted us to hypothesize that phosphorylation at this site could play a role in regulating stress-induced HSF1 sumoylation. Since phosphorylation of serine 303 occurs after phosphorylation of serine 307, we predicted that this serine residue could also be involved in this mechanism [16,17]. The location of the sumoylation site with respect to the functional domains of HSF1 and its proximity to serines 307 and 303 are shown in Fig. 1. To test our hypothesis we first transfected HeLa cells with a wild-type myc-his HSF1 expression plasmid or a corresponding construct in which serines 303 and 307 were mutated to alanine, subjected the cells to heat shock, and then measured heat-induced sumoylation of the transfected proteins by immunoprecipitation with myc antibodies followed by SUMO-1 Western blot. The results indicate that the HSF1 S303/307A mutant exhibited a significant decrease in sumoylation in response to heat stress relative to the wild-type HSF1 (Fig. 2). This suggests that phosphorylation of one or both serine residues is important for the ability of HSF1 to be sumolated in response to stress.

In vitro sumoylation of HSF1 following phosphorylation by Erk-2 and GSK-3β

To directly test the role of phosphorylation of serine 303 or 307 in regulating HSF1 sumoylation we utilized

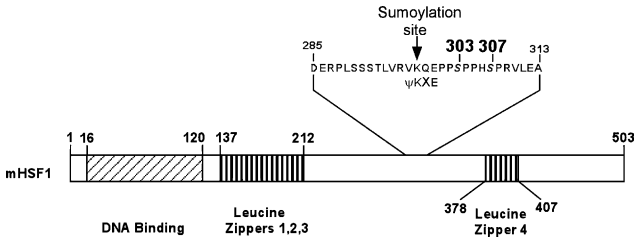

Fig. 1. Organization of DNA-binding domain and leucine zipper motifs in HSF1. The proximity of serines 303 and 307 (indicated in bold) to the HSF1 sumoylation site at lysine 298 is shown, as well as the location of this region of HSF1 with respect to the domains responsible for DNA-binding, trimerization (leucine zippers 1, 2, and 3), and the leucine zipper 4 motif whose function is not well understood.

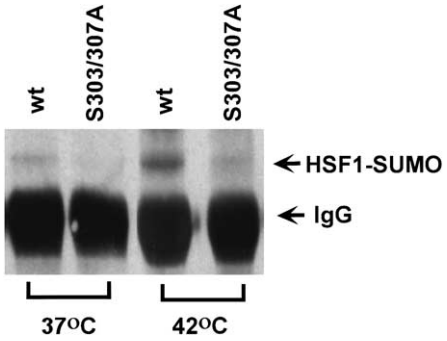

Fig. 2. Mutation of Ser303 and Ser307 results in loss of HSF1 SUMO-1 modification during heat shock. HSF1-myc fusion protein was immunopreciptated from extracts of transfected pcDNA HSF1- or pcDNA-HSF1-S303A and S307A-transfected HeLa cells after heat shock and then subjected to Western blotting using anti-SUMO-1 antibodies.

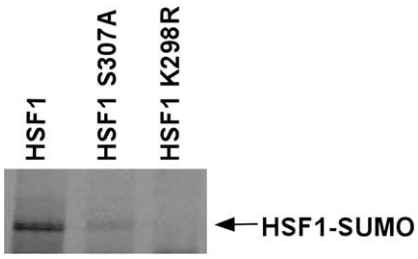

Fig. 4. Mutation of serine 307 to alanine prevents Erk-2-enhanced HSF1 sumoylation. In vitro translated ^{35}S-labeled HSF1 wild-type, HSF1 S307A, and non-sumolatable HSF1 K298R proteins were subjected to Erk-2 phosphorylation reaction followed by the in vitro sumolation assay. Using the 6× His tag on the SUMO-1 protein, we then purified the sumolated forms of these proteins using Ni–NTA affinity chromatography, followed by SDS–PAGE/autoradiography to visualize the ^{35}S-labeled proteins.

in vitro phosphorylation and sumoylation assays. In vitro translated HSF1 was incubated in the absence or presence of recombinant Erk-2, GSK-3β, or both, and then subsequently analyzed for the ability to be sumolated in a reconstituted in vitro sumoylation reaction. The results showed that SUMO-1 modification of HSF1 is enhanced by prior phosphorylation of the protein by Erk-2 (Fig. 3). Phosphorylation with GSK-3β did not have a similar effect on enhancing HSF1 sumoylation, and HSF1 subjected to phosphorylation by both kinases exhibited levels of sumoylation lower than that observed for Erk-2 alone. Previous studies demonstrated that Erk-2 phosphorylates HSF1 at serine 307 while GSK-3β phosphorylates serine 303 [16]. Thus, our results point to the involvement of phosphorylation at serine 307 in regulating HSF1 sumoylation at lysine 298. To confirm the importance of phosphorylation at serine 307 by Erk-2, as opposed to phosphorylation at other residues, for the effect on HSF1 sumoylation, we performed the following experiment. In vitro translated wild-type HSF1, HSF1 with serine 307 mutated to alanine as well as non-

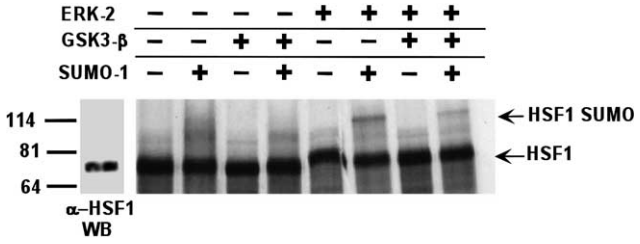

Fig. 3. Phosphorylation by Erk-2 increases HSF1 SUMO modification in vitro. In vitro translated ^{35}S-labeled HSF1 protein was incubated with Erk-2, GSK3β, or both, and then subjected to SUMO modification by addition of sumoylation reaction master mix with or without SUMO-1, followed by SDS–PAGE and autoradiography. The positions of unmodified and SUMO-modified HSF1 are indicated to the right of the panel. The first lane shows the size of unsumolated HSF1 in HeLa cells as identified by Western blot using polyclonal HSF1 antibodies.

sumolatable HSF1 K298R mutant were subjected to phosphorylation with Erk-2, followed by the sumoylation reaction. The SUMO-1 protein used for the in vitro sumoylation reaction has a 6× His tag at its N-terminus, and so we next isolated the sumolated HSF1 proteins from the sumoylation reaction by Ni–NTA affinity chromatography, followed by SDS–PAGE and autoradiography to detect the ^{35}S-labeled proteins. The results, shown in Fig. 4, indicate that mutation of serine 307 to alanine blocks the effect of Erk-2 on enhancing sumoylation of HSF1. The lack of signal in the lane containing the HSF1 K298R mutant protein supports the identity of the band in the wild-type HSF1 lane as the HSF-SUMO conjugation product.

Role of leucine zipper 4 in negative regulation of HSF1 SUMO modification

Previous data indicated that a conserved leucine zipper motif present between amino acids 378 and 407 of HSF1 is important for maintenance of this protein in the inactive non-DNA-binding form [14]. Since sumoylation triggers conversion of HSF1 to the DNA-binding form, we hypothesized that this zipper motif could play a role in regulating sumoylation of HSF1. To test this hypothesis, we constructed two HSF1 mutants, mHSF1 Δ378–407 and mHSF1 1–378 (shown in Fig. 5A), and then subjected these HSF1 mutants to the in vitro sumoylation assay. As shown in Fig. 5B and quantitated in Fig. 5C, deletion of this leucine zipper sequence along with the rest of the C-terminal region of HSF1 in the mHSF1 1–378 mutant results in approximately 22% of the in vitro translated protein being converted to the sumolated form, compared to negligible levels of modification observed for the wild-type HSF1. The mHSF1 Δ378–407 mutant, which has lost the zipper motif but retains the rest of the C-terminal region, shows an even larger increase in sumoylation efficiency, with approximately 57% of this protein being converted to the

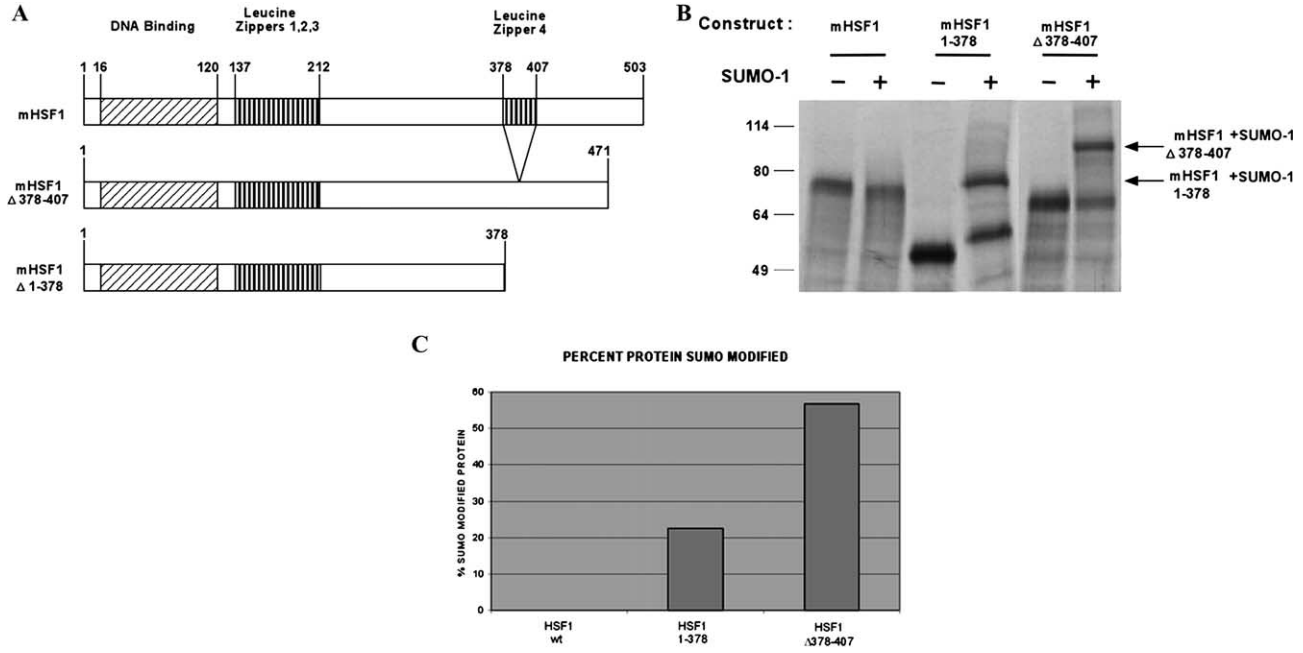

Fig. 5. Leucine zipper 4 negatively regulates SUMO-1 modification of HSF1 in vitro. (A) Schematic showing the HSF1 deletion mutants used in the in vitro SUMO modification assay compared to the wild-type HSF1. (B) In vitro translated ^{35}S-labeled wild-type HSF1 protein and the mutants HSF1 1–378 and HSF1 Δ378–407 were subjected to the in vitro sumoylation assay followed by SDS–PAGE/autoradiography to detect the ^{35}S-labeled proteins. (C) The bands in panel B corresponding to the sumolated forms of the wild-type and mutant HSF1 proteins were quantitated using a Molecular Dynamics phosphorimager.

SUMO-modified form. These results indicate that HSF1 leucine zipper 4 appears to play a role in negatively regulating the SUMO-1 modification of HSF1.

Discussion

We previously demonstrated that HSF1 undergoes stress-induced SUMO-1 modification and that modification stimulates DNA binding of this transcription factor [3]. However, these studies did not reveal the underlying molecular events that regulate HSF1 sumoylation in response to stress. The results in this paper indicate that phosphorylation of HSF1 at serine 307 by Erk1/Erk2, which has been shown to be stimulated by stress treatment, positively regulates sumoylation at the nearby lysine 298 [9,11]. The results also indicate that sumoylation of lysine 298 is under negative control by the conserved leucine zipper found between amino acids 378 and 407. Taking these results together, we postulate that sumoylation of HSF1 in response to stress may involve the following series of events: (1) prior to stress the leucine zipper 4 exists in a conformation that blocks the ability of HSF1 to be sumoylated, possibly by preventing access of the SUMO-1 E2 enzyme ubc9 to the lysine 298 modification site and (2) stress-induced phosphorylation of HSF1 at serine 307 may cause a conformational change that moves the leucine zipper 4 sequence from its blocking position, thus allowing sumoylation at lysine 298 to proceed.

Stress-induced phosphorylation of HSF1 at other residues has previously been implicated in stimulating function of the transactivation domain of this transcription factor [10,18]. Thus, phosphorylation of the HSF1 polypeptide occurring in response to stress appears to be involved in regulating a number of events important for HSF1 function as a transcriptional regulatory protein. Many of these other phosphorylation sites have yet to be determined, and future studies identifying and characterizing the functional effects of modification at these sites are likely to reveal even more complexity in HSF1 regulation by phosphorylation.

Acknowledgments

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