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BAG3 affects the nucleocytoplasmic shuttling of HSF1 upon heat stress

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ABSTRACT

Bcl2-associated athoanogene (BAG) 3 is a member of the co-chaperone BAG family. It is induced by stressful stimuli such as heat shock and heavy metals, and it regulates cellular adaptive responses against stressful conditions. In this study, we identified a novel role for BAG3 in regulating the nuclear shuttling of HSF1 during heat stress. The expression level of BAG3 was induced by heat stress in HeLa cells. Interestingly, BAG3 rapidly translocalized to the nucleus upon heat stress. Immunoprecipitation assay showed that BAG3 interacts with HSF1 under normal and stressed conditions and co-translocalizes to the nucleus upon heat stress. We also demonstrated that BAG3 interacts with HSF1 via its BAG domain. Over-expression of BAG3 down-regulates the level of nuclear HSF1 by exporting it to the cytoplasm during the recovery period. Depletion of BAG3 using siRNA results in reduced nuclear HSF1 and decreased Hsp70 promoter activity. BAG3 in MEF($hsf1^{-/-}$) cells actively translocalizes to the nucleus upon heat stress suggesting that BAG3 plays a key role in the processing of the nucleocytoplasmic shuttling of HSF1 upon heat stress.

1. Introduction

Heat shock transcription factor 1 (HSF1) is responsible for the activated expression of a large class of heat shock proteins (Hsps) that protect cells from damage incurred from cellular insults [1–3]. HSF1 is activated in response to various pathophysiologic stresses. Normally, HSF1 largely localizes to the cytoplasm. Upon exposure to stressful conditions, HSF1 translocalizes to the nucleus where it activates the transcription of target genes, including Hsps [4–6]. Hsps contribute to protection against conditions that generate cellular damage by functioning as molecular chaperones that accelerate refolding of denatured proteins and promote the degradation of damaged proteins during stressful conditions [6–8]. However, precise molecular mechanisms by which HSF1 translocalizes to the nucleus are poorly understood.

Bcl2-associated athanogene 3 (BAG3) is a member of BAG family of co-chaperones, and it interacts with Bcl2 and Hsp70 through its BAG domain [9]. In addition, BAG3 contains a WW domain, two IPV motifs, and a proline-rich repeat (PXXP) through which BAG3 interacts with other partners, such as Hsp22 and phospholipase C [9,10]. BAG3 is the only member of the family to be induced by stressful stimuli, including heat shock and heavy metals [11,12]. Interestingly, it has been recently reported that the induction of BAG3 in response to stress is mainly through the activation of HSF1 [13]. Growing evidence has shown that BAG3 regulates cellular adaptive responses against stressful stimuli by regulating apoptosis, development, cytoskeleton organization and autophagy [10,14–19]. However, the underlying molecular mechanisms are still largely unknown.

Considering the association of BAG3 with cellular stress, we hypothesized that BAG3 might be associated with HSF1 regulation in response to cellular stress. Therefore, in this study, we investigated the molecular action mechanism of BAG3 under stress conditions. We first demonstrated that under heat stress, BAG3 rapidly co-translocalized to the nucleus with HSF1. Down-regulation of BAG3 reduced the level of nuclear HSF1. The response of $hsf^{-/-}$ mouse embryonic fibroblast (MEF) cells showed that the translocation of BAG3 upon heat stress is not affected by the absence of HSF1, suggesting that BAG3 may act as an HSF1 regulator by nucleocytoplasmic shuttling upon heat stress.

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2. Materials and methods

2.1. Cell culture and treatment

HeLa and MEF cells were cultured at 37 °C in a humidified atmosphere with 5% CO_2. DMEM supplemented with 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin was used as a growth medium for all cell lines. MEF cells derived from wild-type mice and *hsf1*$^{-/-}$ mice were a gift from Dr. Ivor Benjamin (Southwestern Medical Center, University of Texas). For the heat-shock treatment, culture plates were wrapped with Parafilm™ and immersed in a 42 °C water bath for 1 h.

2.2. Plasmids and transfection

The open reading frame of BAG3 (1728 nucleotides) corresponding to GenBank® accession number NM_004281 was amplified by PCR using human skeletal muscle cDNA as a template (Clontech). The mammalian expression vectors for the N-terminally FLAG-tagged full length BAG3 (BAG3-WT, amino acids 1–575), Δ-BAG (BAG3-ΔBAG, amino acids 1–423), Δ-PXXP/BAG (BAG3-ΔPXXP/BAG, amino acids 1–309), Δ-WW (BAG3-ΔWW, amino acids 100–575), and Δ-WW/IPV (BAG3-ΔWW/IPV, amino acids 212–575) BAG3 proteins were created by inserting cDNA fragments into the 5′ *KpnI* and 3′ *XbaI* sites of pCDNA3.1-NF. Cells were transiently transfected using FuGENE® HD (Promega) according to the manufacturer's instructions. All constructs were confirmed by DNA sequencing.

2.3. Subcellular fractionation

To obtain cytoplasmic and nuclear fractions, cells were washed with PBS, resuspended in solution A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF) and incubated on ice for 5 min. Nonidet P-40 was added to a final concentration of 0.6% and the mixture was centrifuged at 4 °C, 12,000 rpm for 1 min. The supernatant (cytosolic fraction) was removed and the pellet (nuclear fraction) was resuspended in buffer B (20 mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF). After 15 min incubation on ice, samples were centrifuged at 4 °C, 13,000 rpm for 5 min, and the supernatant was used as the nuclear extract.

2.4. Immunoprecipitation and immunoblotting

For immunoprecipitation, HeLa cells were transiently transfected using FuGENE® HD (Promega) as described above. After 30 h, cells were treated heat shock at 42 °C for 1 h and lysed in RIPA buffer (PBS supplemented with 1% Nonidet P-40, 0.5% sodium deoxycholate, 1 mM PMSF, 1 μg/ml aprotinin, and 1 mM sodium orthovanadate). Cell lysates were harvested, incubated at 4 °C for 30 min and then centrifuged at 10,000 × g for 10 min. The supernatant was incubated with agarose-conjugated anti-FLAG antibody (Sigma–Aldrich) for 3 h. Immunoprecipitates were washed 4 times with RIPA buffer containing 0.05% SDS and boiled in SDS-PAGE sample buffer. For immunoblotting, proteins were resolved by SDS-PAGE and immunoblotted using the following antibodies: anti-FLAG (Sigma–Aldrich), anti-HSF1 (Santa Cruz Biotechnology), anti-BAG3 (Abcam), anti-Hsp70 (Santa Cruz Biotechnology), anti-actin (Sigma–Aldrich), and anti-TBP (Abcam).

2.5. Luciferase reporter gene assay

To assess the effects of BAG3 on HSF1 activity, HeLa cells were co-transfected with pGL3-Hsp70-Luc, pCDNA3.1-FLAG-BAG3, and pCH110 using FuGENE® HD (Promega) as described above. After 30 h, the cells were lysed with the Reporter Lysis Buffer (Promega) and luciferase activity was measured using the Luciferase Activity Assay kit (Promega) according to the manufacturer's instructions. β-galactosidase activities were determined to normalize the luciferase activities. All experiments were performed in triplicate, and the mean ± SD values were determined.

2.6. siRNA experiment

HeLa cells (2×10^5 cells/ml) were seeded on 12-well plates and transfected with 1.5 μg/ml of siGENOME BAG3 siRNA (Dharmacon) using DharmaFECT transfection reagent (Dharmacon) according to the manufacturer's instruction. siGENOME Non-Targeting siRNA Pool was used as control. After 48 h, cells were harvested and cell lysates were separated by SDS-PAGE. The expression level of BAG3 was analyzed by immunoblotting as described above.

2.7. Immunofluorescence microscopy

HeLa cells were seeded on 35 mm plates at a density of 1×10^5 cells/plate. The cells were cultured overnight and given a heat shock at 42 °C for 1 h, followed by recovery at 37 °C for 1 h. The cells were then washed twice with PBS and fixed with 4% paraformaldehyde for 10 min. After being washed twice with PBS, the cells were incubated with methanol for 2 min. For immunostaining, the cells were pretreated with 5% BSA for 30 min and then incubated with anti-BAG3 antibody or anti-HSF1 antibody in 2.5% BSA containing PBS for 1 h. After being washed with PBS for 10 min, the cells were incubated for 30 min with FITC-conjugated anti-rabbit secondary antibody (Vector Laboratories) or Alexa Fluor 594-conjugated anti-mouse secondary antibody (Invitrogen) in 2.5% BSA containing PBS. Finally, the cells were washed twice with PBS for 10 min and then mounted using crystal/mount (Biomeda Corporation). Nuclei were stained with 4′,6-diamidino-2-phenylindole (Invitrogen). The fluorescence analysis was performed by conventional fluorescence microscopy (Axio Observer D1, Carl Zeiss) and by confocal microscopy (LSM700, Carl Zeiss).

3. Results

3.1. BAG3 translocates to the nucleus upon heat stress

To investigate whether heat stress can modulate BAG3 expression, HeLa cells were given a heat shock, and the expression level of BAG3 was examined. When the cells were treated with heat shock for 1 h, HSF1 phosphorylation, an indicator of heat activation, was detected (Fig. 1A). Following the activation of HSF1, Hsp70 expression began to be induced after approximately 3 h of recovery. Like Hsp70 expression, BAG3 expression was induced by heat stress (Fig. 1A). Previous studies have shown that the expression of BAG3 is stimulated to protect cells from stressful stimuli, including heat shock, heavy metals, and proteasome inhibitors [11,12,20]. Our data also showed that BAG3 is induced by heat stress in HeLa cells.

To assess the distribution of BAG3 upon heat stress, HeLa cells were given a heat shock at 42 °C for 1 h and allowed to recover for the indicated time periods. The cytosolic and nuclear proteins were fractionated, and the localization of BAG3 was detected by Western blot analysis. HSF1 mainly localized to the cytosolic fraction under normal conditions and rapidly translocated to the nucleus upon heat treatment. Under non-stressed conditions, BAG3 was also predominantly found in the cytosolic fraction. Interestingly, the heat shock treatment also induced BAG3 translocation to the nucleus (Fig. 1B). To confirm the translocation of BAG3 to the nucleus, we examined the subcellular localization of endogenous BAG3 and