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Research Article

Sequential interplay between BAG6 and HSP70 upon heat shock

A. Corduan, S. Lecomte, C. Martin, D. Michel* and F. Desmots

Université de Rennes1. Interactions Cellulaires et Moléculaires UMR6026 CNRS-Hip-IFR140 GFAS Bâtiment 13. Campus de Beaulieu. 35042 Rennes cedex (France), Fax : +33 2 23235052,
e-mail: denis.michel@univ-rennes1.fr

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Abstract. BAG6/Scythe/Bat3 is a cochaperone of the heat shock protein HSP70 and is involved in various developmental processes, cellular stress and viability. BAG6 interferes with the protein-refolding activity of HSP70 but its precise involvement in proteotoxic stresses remains unknown. We show that BAG6 is required for the accumulation of HSP70 upon heat shock and that conversely, once accumulated, HSP70 leads to the massive and CHIP-independent degradation of BAG6 through the ubiquitin-proteasome system. These reciprocal influences between BAG6 and HSP70 upon heat shock suggest that BAG6 is a central regulator of the cellular content of HSP70. The HSP70-driven degradation of BAG6, following the BAG6-dependent accumulation of HSP70, could allow the protein-refolding activity of HSP70 and limit the extent of its induction.

Keywords. BAG6/Scythe/Bat3, heat shock, HSP70, protein degradation, retroaction loop.

Introduction

The heat shock response has been identified as a stereotyped transcriptional activity leading to the coordinated upregulation of a set of genes [5] encoding heat shock proteins (HSP), which are involved in the repair and clearance of proteins misfolded after thermal denaturation. The different molecular mechanisms underlying the cellular responses to heat shock have been progressively elucidated and shown to involve many levels of gene expression cascade, including primarily transcription initiation, triggered in eukaryotes by the heat shock factor HSF1 [6], transcription elongation [7], mRNA translation and protein stability, ensuring the final degradation of the transiently overexpressed HSP. A central member of the heat shock gene family is Hsp70, encoding an evolutionarily conserved protein closely related to DNA-K from E coli. HSP70 can refold disordered proteins in an ATP-dependent manner with the help of accessory proteins such as Hip, Hop and BAG1. HSP70, in conjunction with the E3 ubiquitin ligase CHIP (carboxyl terminus of HSC70-interacting protein), can also target irreversibly denatured proteins for proteasomal degradation [8]. The induction and cessation of the heat shock response depend on the presence or absence of altered proteins respectively. The heat shock response is mainly triggered by the accumulation of misfolded proteins with exposed hydrophobic domains, supposed to divert chaperone proteins from the HSF1 complex, thus allowing it to bind DNA and activate transcription of the Hsp genes [6]. Conversely, when damaged proteins are no longer

* Corresponding author.

present in the cell, the high HSP70 content then returns to ground level, after CHIP-mediated ubiquitination [9]. The participation of a novel actor in the heat shock stress, namely the cochaperone BAG6, is described in the present report. BAG6, also know as Scythe or Bat3, belongs to the so-called Bag family endowed with a BAG protein domain. BAG6 ensures multiple functions in development [4] and cell viability, through interaction with various apoptosis regulators [2, 10–12] and often related to its nucleocytoplasmic shuttling activity [2, 12, 13]. Besides, BAG6 interferes negatively with the refolding activity of Hsp70 [14]. BAG6/Bat3 has recently been shown to regulate the stability of Hsp2A in the context of spermatogenesis [15]. We show here that BAG6 is involved in heat shock stress at multiple levels. BAG6 is strictly required for the initial induction of HSP70 upon heat shock, mainly through protein stabilization and conversely, once accumulated, HSP70 induces the degradation of BAG6. This downregulation of BAG6 can allow the refolding activity of HSP70 and facilitate recovery from the heat shock response.

Materials and methods

Cells and culture conditions. COS-7 cells were maintained in complete DMEM (Dulbecco's modified Eagle's medium) supplemented with 10 % fetal calf serum, 4 mM glutamine, and 100 units/ml penicillin and streptomycin in a 5 % CO_2 humidified incubator. MEFs were obtained from E13.5 WT and Bag6$^{-/-}$ embryos and cultured in DMEM supplemented with 10 % fetal calf serum, 4 mM glutamine, 100 units of penicillin and streptomycin, 25 mM Hepes and non-essential amino acid in a 5 % CO_2 humidified incubator. Immortalization of these cells was efficiently established *in vitro* upon expression of the SV40 large T antigen driven by a promoter sequence derived from the human vimentin gene [1]. Heat-shock treatments were performed at 45 °C for 20 minutes, followed by a recovery period of 6, 24 or 48 hours at 37 °C as indicated. In some experiments, the inhibitor of proteasome, the MG132 (BIOMOL), was added to the cultures to a final concentration of 5 μM for 16 hours before harvesting cells.

DNA constructs and transient transfection. The CHIP expression plasmid pRKIM-MYC-CHIP, the HSP70 and HA-HSP70 expression plasmid were kind gifts from Zhijie Chang (Tsinghua University Beijing, China) and Yair Argon (The Children's Hospital of Philadelphia) respectively. Full-length BAG6 and truncated BAG6 variants (N380, N595, N595, C482 and C235; all HA-Tagged), pEGFP-BAG6 (full-length), pcDNA-HSP70 (not tagged or HA-tagged), prKIM-MYC-CHIP and pcDNA-BAG1 were transiently transfected in either COS-7 cells or MEFs cells using Fugene HD transfection reagent (Roche) and JetPei DNA transfection reagent (Polyplus Transfection) respectively, according to the manufacturer's procedures. Due to differential translation initiation codon usage, the pcDNA-BAG1 expression vector used in Figure 3 generated both the small and large BAG1 isoforms. The BAG6 constructs are described in [2]. For transient expression assays, pCMV-βgal expression vector and pTAL-luc reporter plasmid containing the HSV-TK promoter were from Clontech. HSE-Luc derived from pTAL contains a synthetic heat shock element (HSE) made of six consecutive and inverted AGAAC pentamers.

Transient expression assays. 24 hours after transfection, cells were harvested and the luciferase activity was then measured and normalized with β-galactosidase activity. The luciferase and β-galactosidase activities were determined with a luciferase assay system kit (Promega, Madison, Wi, USA) and a fluorimetric method respectively.

Real Time RT-PCR analysis. One million of each iMEF cell line were plated onto a 10 cm plate and harvested after 24 h of culture. Total RNAs were extracted with Trizol reagent (Invitrogen) according to the manufacturer's procedure. Then 5 μg of RNA were retrotranscribed using M-MLV RT (Invitrogen). Real time PCR was performed using 12.5 ng of cDNA, 300 nM of Hsp70-specific primers (forward: 5'-GGCCACATTGTTGATACATGC; reverse: 5'-CTACAGTGCAACCACCATGC) and 1X Sybr-Green, on MiniOpticon Real Time PCR (Bio-Rad). Amplification of each gene studied was normalized using amplification of three housekeeping genes Gapdh (Glyceraldehyde-3-phosphate dehydrogenase), Hprt1 (Hypoxanthine guanine phosphoribosyl transferase 1) and Tbp (TATA-binding protein). Data were analysed using qBase software [3].

Immunoblotting and immunoprecipitation. Cells were homogenized in 200 μl of lysis buffer (50 mM Tris pH 8; 150 mM NaCl; 5 mM EDTA; 5 mM EGTA; 0.5 % NP40, 15 mM $MgCl_2$, 1 mM DTT, 60 mM β-glycerophosphate, 0.1 mM sodium fluoride, 0.1 mM sodium orthovanadate, 0.1 mM PMSF and protease inhibitor cocktails (Roche)). Proteins were separated on SDS-PAGE and transferred to PVDF membranes (Millipore) and detected using antibodies to Scythe/BAG6 [4], HA (12CA5, Boehringer Mannheim), GFP (Molecular probes), MYC (Cell Signaling), HSC70 (stressgen), HSP70 (Stressgen), BAG1 (Sc-939 Santa-

Cruz), ERα (HC20, Santa-Cruz), β-actin (SIGMA). To study protein-protein interactions, COS-7 cells or MEFs were trypsinized 48 h after transfection, washed with PBS, and cell pellets were suspended in lysis buffer (described above) and sonicated. Lysates were immunoprecipitated overnight at 4 °C with 5 μg of HA antibody, followed by 2 h incubation at 4 °C with Protein A-sepharose beads and washed three times in lysis buffer. Immune complexes were boiled in 2x sample buffer, separated on an SDS-PAGE and then electroblotted to PVDF membranes. To detect ubiquitinated forms of BAG6, COS-7 cells were transiently transfected with HA-tagged BAG6 and/or pCDNA-HSP70 and/or FLAG-tagged ubiquitin and/or pcDNA-FLAG vector for 48 h using Fugene (Roche) according to manufacturer's guidelines. Before being harvested, cells were pretreated or not with an inhibitor of proteasome MG132 (5 μM) for 16 hours as indicated. Then, cells were lysed in lysis buffer (50 mM Tris pH 8; 150 mM NaCl; 1 % NP40, protease inhibitor cocktails (Roche) and 1X of 15X N-ethylmaleimide solution (50 mg/ml ethanol)). 400 μg of each lysate were immunoprecipitated using 30 μl of anti-Flag M2 affinity gel (sigma) at 4 °C for 4 hours. Resin was briefly centrifuged, washed three times with wash buffer (50 mM Tris pH 8; 400 mM NaCl; 1 % NP40, protease inhibitor cocktails (Roche) and 1X of 15X N-ethylmaleimide solution (50 mg/ml ethanol)) and resuspended in 2x SDS loading buffer. Proteins were separated by electrophoresis and transferred to PVDF membrane. Blots were probed for ubiquitinated forms of BAG6 using anti-HA antibody.

Results

BAG6 is required for Hsp70 up-regulation after heat shock. Functional relationships have been reported between BAG6 and the chaperone activity of HSP70, which led to the classification of BAG6 as a co-chaperone of HSP70. In this study, we wanted to check if, in addition, BAG6 could influence the expression of HSP70 in response to heat shock. WT and Bag6-deficient mouse embryonic fibroblasts (MEFs) were submitted in parallel to the same heat shock treatment and the resulting levels of HSP70 were measured. As shown in Figure 1A, HSP70 induction was not detectable in BAG6-deficient cells. To determine if this result was due to the absence of BAG6 or was an indirect consequence of gene inactivation, we re-introduced BAG6 in BAG6-deficient cells. As shown in Figure 1B, the heat shock induction of HSP70 was restored, demonstrating that the only parameter responsible for the difference of behaviour between the WT and mutant cells, with respect to the accumulation of HSP70, is the presence of BAG6. In addition, this experiment shows that the Hsp70 gene remains fully functional in Bag6-deficient cells. Moreover, we found that another way to restore the accumulation of HSP70 upon heat shock in Bag6-deficient cells is to inhibit proteasome even without re-introducing BAG6 (Fig. 1C), suggesting that BAG6 primarily influences HSP70 stability after heat shock. But since the action of MG132 can be mediated by both protein stabilization and by transcriptional activation in the case of heat shock genes [16], we also wanted to test the role of Bag6 in Hsp70 mRNA accumulation and Hsp70 gene transcription initiation. Real time RT-PCR analysis of the Hsp70 mRNA content in WT and Bag6-mutant cells revealed that the Hsp70 mRNA level was increased in approximately the same ratio, indicating that the absence of Bag6 does not forbid the transcriptional heat shock response. But when compared to a series of control genes, the steady state content of the Hsp70 mRNA appeared reproducibly lower in Bag6-ablated cells (Fig. 1D). We then performed transient expression assays using three different reporter plasmids. As shown in Figure 1E, the expression of luciferase driven by consensus heat shock elements (HSE-Luc) was increased to the same extent in WT and mutant cells, further supporting the conclusion that Bag6 is not required for the transcriptional, HSF1-regulated, heat shock response. However, when using the natural promoter of the mouse Hsp70 gene, heat shock induction was still obtained in Bag6-mutant cells, but reproducibly lower by about one-third. Comparison of the HSE-Luc and Hsp70-Luc constructs shows that the effect of BAG6 on the Hsp70 promoter activity is complex and seems not to be mediated by the classical heat shock elements. These experiments also show that the reduced level of Hsp70 mRNA in Bag6-mutant cells cannot explain the dramatic inhibition of HSP70 observed at the protein level. Together, these results suggest that the permissive action of BAG6 on HSP70 accumulation is mediated in part by unidentified transcriptional effects, but mainly by preventing a constitutive degradation of HSP70 occurring after heat shock.

Down-regulation of BAG6 upon HSP70 accumulation. Figure 1A shows that, in WT cells, the accumulation of HSP70 after heat shock is followed by a progressive decrease of the endogenous BAG6 content, obvious as soon as six hours after temperature shift. A decrease of the control protein, β-actin, was also observed at longer time periods and is likely to reflect a global decrease in gene expression following heat shock. But BAG6 decrease was more marked and clearly subsequent to HSP70 accumulation, suggesting that it is a secondary event, not required for HSP70

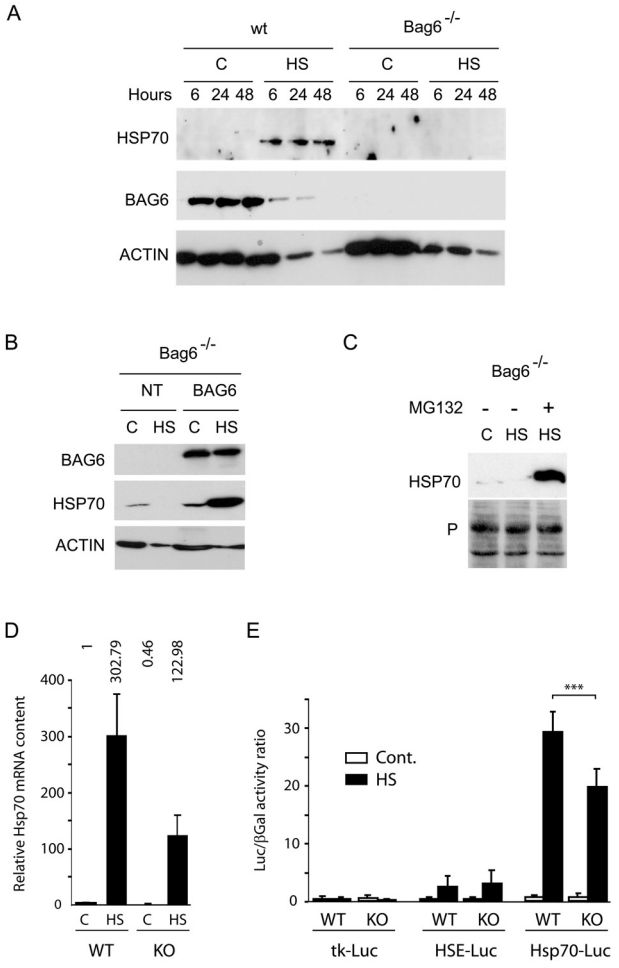

Figure 1. BAG6 is indispensable for HSP70 accumulation after heat shock. (*A*) The expression of BAG6 and HSP70 was analyzed by immunoblotting in WT and Bag6$^{-/-}$ iMEFs and at different times after heat shock (45 °C, 20 min). (*B*) Reintroduction of BAG6 in Bag6$^{-/-}$ cells restored a potent HSP70 induction. Bag6$^{-/-}$ cells were transfected with BAG6 and subjected or not to heat shock (45 °C, 20 min) and shifted back to 37 °C for 24 hours. (*C*) Proteasome inhibition is sufficient to recover the strong heat shock-induced HSP70 accumulation in Bag6-deficient cells. Protein loading was evaluated using β-actin immunoblotting or ponceau staining (P). (*D*) Real-time RT-PCR analysis of the Hsp70 mRNA content in WT and Bag6$^{-/-}$ iMEFs in normal condition (*C*) or after heat shock (HS). Data are expressed as fold change in mRNA Hsp70 levels after normalization to Gapdh, Tbp and Hprt1 mRNA levels. E) Transient expression assays. Luciferase reporter constructs and the CMV-β-gal plasmid used as a control were co-transfected in WT and Bag6$^{-/-}$ iMEFs and luciferase and β-gal activities were measured after heat shock (45 °C, 20 min) followed by a recovery step (37 °C, 4 hours). Values were normalized by calculating the ratio of luciferase activity on β-gal activity. The promoters used to drive luciferase expression were thymidine kinase (tk-Luc), synthetic promoter containing two consensus HSE (HSE-Luc) and the 470 bp-long upstream region of the human Hsp70 gene (Hsp70-Luc). Numerical values were expressed as mean ± S.D. of at least three different experiments (*** $p < 0.001$). The results shown are representative of three independent experiments.

induction. This conclusion is supported by the results in Figure 1B, showing that the forced expression of BAG6 did not forbid HSP70 accumulation. We then wanted to establish the causal relationships possibly existing between the intracellular contents of HSP70 and BAG6.

HSP70-triggered down-regulation of BAG6 proceeds through protein degradation. To determine if the accumulation of HSP70 and the following decrease in BAG6 content are causally related events or two independent consequences of heat shock, the status of BAG6 was examined as a function of HSP70 accumulation. To this end, COS-7 cells were transfected with HSP70 to mimic, in absence of heat shock, the accumulation of HSP70 observed after heat shock. As shown in Figure 2A (left panel), HSP70 overexpression was fully sufficient to cause a sharp reduction in the endogenous BAG6 content, and proteasome inhibition canceled this reduction (Fig. 2A, right panel), suggesting that the influence of HSP70 on BAG6 is mediated by the proteasome. The same effect of HSP70 was observed when using transfected GFP-BAG6 (Fig. 2B), ruling out the interpretation of this result by a genomic action. We also verified that the destabilizing effect of HSP70 was directed against the BAG6 moiety of the GFP-BAG6 fusion protein, since it was not obtained when using GFP only (Fig. 2C). To determine unambiguously if the ubiquitin-proteasome system is involved in the HSP70-mediated decrease of BAG6, we measured the ubiquitination status of BAG6 using the FLAG-ubiquitin immunoprecipitation technique. Intracellular conjugation of FLAG-ubiquitin to HA-tagged BAG6 was monitored by immunoprecipitation of protein extracts using the anti-FLAG antibody and subsequent immunoblotting using anti-HA antibody. As shown in Figure 2D, a ladder of high molecular weight forms of HA-BAG6 was clearly detectable after co-transfection of HSP70 and more visible after treatment with MG132, suggesting that it corresponds to a series of poly-ubiquitinated HA-BAG6 bands. Non-ubiquitinated forms also immunoprecipitated with FLAG-ubiquitin in this experiment. This seemingly paradoxical result did not result from the mere interaction with the FLAG epitope, since it was not obtained with FLAG alone (Fig. 2D). It could be related to the homodimeric interactions of BAG6 or to a direct interaction between ubiquitin and the ubiquitin-like domain of BAG6 [10]. According with this possibility, no HA-immunoreactive molecules were detected after transfection with FLAG only, whereas they were clearly present in the input protein extracts. The low amount of ubiquitin-conjugated forms, when compared to those non-ubiquitinated, is classical in the litterature, even upon proteasome inhibition. The very low ratio between ubiquitinated and deubiquitinated substrates remains unchanged upon protea-

some inhibition and is likely to result from the steady state ratio between ubiquitination and deubiquitination enzymatic activities in the cell. Whatever the interpretation of these features, the denaturing conditions used for the SDS-PAGE allow the definite conclusion that the modified forms observed are covalently conjugated to ubiquitin, consistent with the degradation of BAG6 by the 26S proteasome.

BAG6 degradation induced by HSP70 is not mediated by CHIP. CHIP is involved in heat shock stress at several levels, including the transcriptional activity of HSF1 [17], the processing of irreversibly damaged HSP70 clients [8] and the recovery period, through degradation of the accumulated pool of HSP70, no longer desirable after clearance of malfolded proteins [9]. The involvement of CHIP in the degradation of HSP70-associated substrates proposed in the literature [8] prompted us to check if the strong destabilization of BAG6 by HSP70 (Figs. 1 and 2) was also mediated by CHIP. We first observed that BAG6 and CHIP are indeed present in the same protein complexes, through co-immunoprecipitation (Fig. 3A). Given the transcriptional activity of CHIP [17], to determine if its overexpression can destabilize BAG6 we used fusion proteins to avoid possible transcriptional effects of CHIP on endogenous genes. As previously observed, co-transfection of BAG6 and HSP70 led to a decrease of GFP-BAG6 but, surprisingly, overexpression of CHIP prevented the HSP70-triggered degradation of BAG6 (Fig. 3B), while it has only little effect on BAG6 in the absence of HSP70 (Fig. 3A). This result, obtained in several independent experiments, was unexpected considering that CHIP is an E3 ubiquitin-ligase, generally involved in protein destabilization. To determine if this effect of CHIP is specific of BAG6, in the same experimental conditions we tested another member of the BAG family also capable of interacting with HSP70, namely BAG1. As shown in Fig. 3C, CHIP transfection did not change the level of both the short and long forms of BAG1. Finally, to verify that the enzymatic activity of the CHIP construct was maintained, we compared its action on GFP-BAG6 and on the estradiol receptor (ERα), which is an established substrate of CHIP [18], in the same experiment and starting the same protein inputs. As shown in Figure 3D, the level of ERα was decreased, whereas that of BAG6 was increased. This effect was obtained with transfected molecules and therefore did not involve possible secondary alterations of the genetic networks in mutant cells.

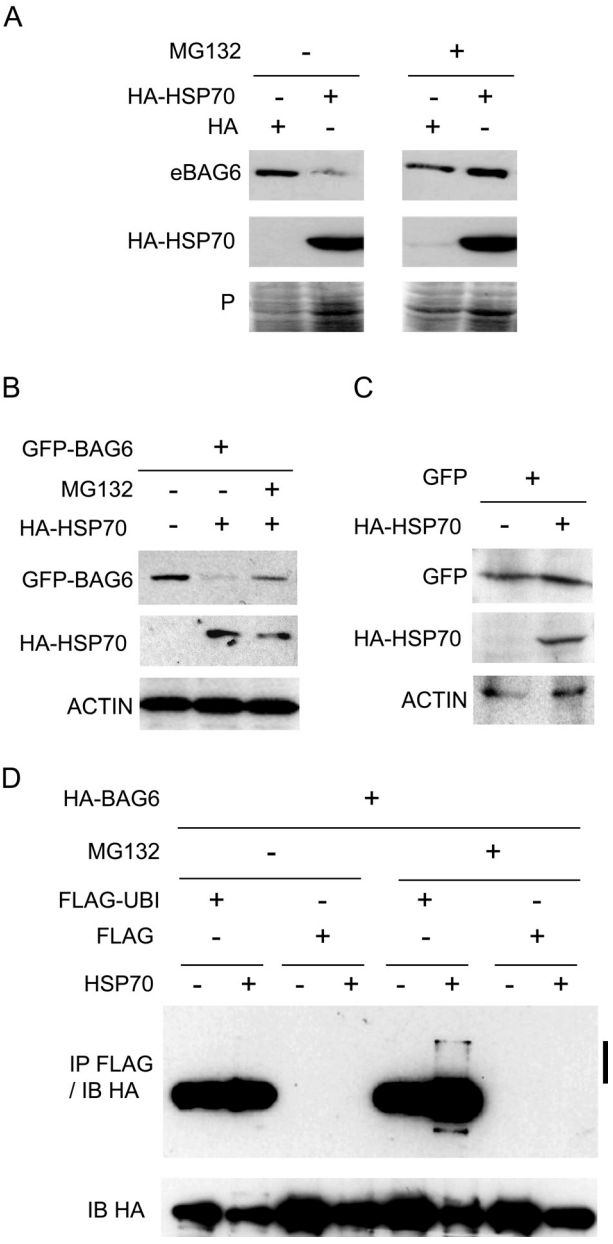

Figure 2. Overexpressed HSP70 downregulates BAG6. (*A*) COS-7 cells where transfected with either HA-tagged BAG6, HSP70 or both, and with or without FLAG-ubiquitin. Cellular proteins immunoprecipitated using the anti-FLAG antibody were probed by immunoblotting with anti-HA antibody or anti-BAG6 antibodies to visualize endogenous BAG6 (eBAG6). (*B*) Downregulation of transfected GFP-BAG6 by HA-HSP70 and partial reversion by the proteasome inhibitor MG132. (*C*) The effect of HSP70 on downregulation of GFP-BAG6 was not observed for GFP alone. (*D*) Immunoprecipitation of ubiquitin forms of HA-tagged BAG6 using anti-FLAG antibody; showing covalent conjugation between HA-BAG6 and FLAG-ubiquitin (black vertical bar).

Discussion

We show in this report strong and complex behaviours of BAG6 upon heat shock, first necessary to allow the

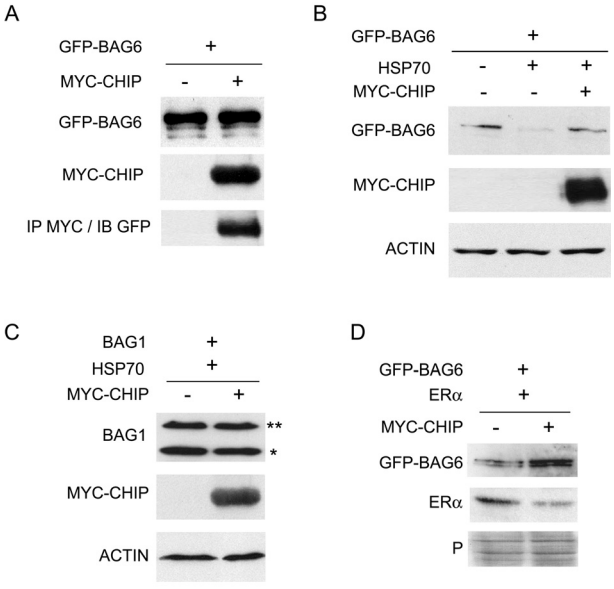

Figure 3. CHIP interacts with BAG6 and inhibits HSP70-induced degradation of BAG6. (*A*) GFP-BAG6 was transfected alone or co-transfected with MYC-CHIP in COS-7 cells. Cell extracts were immunoprecipitated with anti-GFP antibody and immune complexes were probed by immunoblotting with anti-MYC antibody. Expression of BAG6 and CHIP proteins was analyzed by western blot using anti-GFP and anti-MYC antibodies respectively. (*B*) COS-7 cells were transfected with combinations of HA-HSP70, GFP-BAG6 and MYC-CHIP and the proteins were detected using anti-HA, -GFP or -MYC antibodies respectively. (*C*) HSP70 and CHIP overexpression had no effect on both the short (*) and long (**) translation variants of BAG1, detected using anti-BAG1 antibody. (*D*) Inverse regulation of transfected GFP-BAG6 and ERα by CHIP. ERα was detected by western blotting using anti-ERα antibody.

upregulation of HSP70 after heat shock and then degraded because of HSP70 production (Fig. 4). The absence of BAG6 abolishes the burst of HSP70 classically observed after heat shock. BAG6/Bat3 deficiency has recently been shown, in the particular context of spermatogenesis, to accelerate the degradation of Hsp70–2/Hsp2A [15]. Our data reveal that this role of BAG6 in preventing the degradation of HSP70 proteins can be extended to the more general case of heat shock and to various cell type contexts. In addition, we show that, conversely, HSP70 strongly influences BAG6 ubiquitination and stability. The ubiquitin ligase involved in HSP70-mediated degradation of BAG6 is not CHIP and, more surprisingly, CHIP overexpression leads to a stabilization of BAG6 in HSP70 overexpressing cells. To our knowledge, this paradoxical result has not yet been obtained for other interacting partners of CHIP. A possible explanation of this observation could be a competing action for binding to CHIP, mutually exclusive with that of the unidentified ubiquitin ligase involved in BAG6 ubiquitination. Conversely, the association between BAG6 and CHIP can prevent CHIP to degrade HSP70, which could explain our observation that HSP70 accumulation is strongly reduced in the cells ablated for Bag6. From this perspective, one role of the degradation of BAG6 after HSP70 accumulation could be to allow a CHIP-mediated decrease of the high HSP70 content during recovery [9]. In addition, the HSP70-triggered degradation of BAG6, clearly shown in this report, could be functionally important to allow efficient protein repair, considering that BAG6 can inhibit the refolding activities of HSP70 [14]. It is worth noting that BAG1, which also interacts with HSP70, is not degraded upon heat shock. This differential behaviour is consistent with the proposed activities of these two members of the BAG family, which are both capable of interacting with the ATPase domain of HSP70, but with different outcomes. BAG6 inhibits its activity [14] while BAG1 is a potent nucleotide exchange factor for Hsc70, involved in the rapid and repetitive movements required for protein substrate refolding [19]. The present study reveals an unsuspected component of the regulatory network following heat shock, and puzzling mutual influences between BAG6 and HSP70. BAG6 is strictly required for HSP70 accumulation and, in turn, is thereafter rapidly destabilized by HSP70 once accumulated, thereby preventing further HSP70 production.

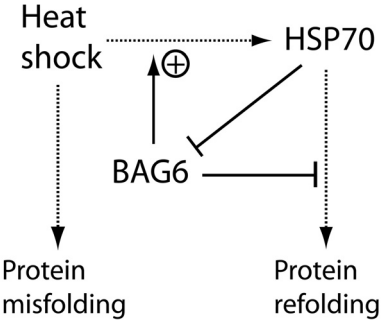

Figure 4. Functional relationships between HSP70 by BAG6 upon heat shock. BAG6 is required for the heat shock induction of HSP70 but, once accumulated, HSP70 induces the rapid degradation of BAG6. This step can be important for: i) avoiding sustained accumulation of HSP70 (present study) and ii) alleviating the the inhibitory action of BAG6 on the refolding activity and ATPase domain of HSP70 [14]).

The heat shock response is a vitally important but risky cellular activity, and sustained HSP70 production would seriously perturb normal cellular functions. Several mechanisms have been identified to shorten the effects of heat shock, including: i) chaperone accumulation, proposed to turn off HSF1 activity through a negative feedback mechanism [6, 20] and ii) active dissociation from DNA of the TATA-binding protein, as described in yeast [21]. The present report suggests the existence of an additional mechanism, through mutual influences between BAG6 and HSP70.

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