Cooperation of a ubiquitin domain protein and an E3 ubiquitin ligase during chaperone/proteasome coupling

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Background: Molecular chaperones recognize nonnative proteins and orchestrate cellular folding processes in conjunction with regulatory cofactors. However, not every attempt to fold a protein is successful, and misfolded proteins can be directed to the cellular degradation machinery for destruction. Molecular mechanisms underlying the cooperation of molecular chaperones with the degradation machinery remain largely enigmatic so far.

Results: By characterizing the chaperone cofactors BAG-1 and CHIP, we gained insight into the cooperation of the molecular chaperones Hsc70 and Hsp70 with the ubiquitin/proteasome system, a major system for protein degradation in eukaryotic cells. The cofactor CHIP acts as a ubiquitin ligase in the ubiquitination of chaperone substrates such as the raf-1 protein kinase and the glucocorticoid hormone receptor. During targeting of signaling molecules to the proteasome, CHIP may cooperate with BAG-1, a ubiquitin domain protein previously shown to act as a coupling factor between Hsc/Hsp70 and the proteasome. BAG-1 directly interacts with CHIP; it accepts substrates from Hsc/Hsp70 and presents associated proteins to the CHIP ubiquitin conjugation machinery. Consequently, BAG-1 promotes CHIP-induced degradation of the glucocorticoid hormone receptor in vivo.

Conclusions: The ubiquitin domain protein BAG-1 and the CHIP ubiquitin ligase can cooperate to shift the activity of the Hsc/Hsp70 chaperone system from protein folding to degradation. The chaperone cofactors thus act as key regulators to influence protein quality control.

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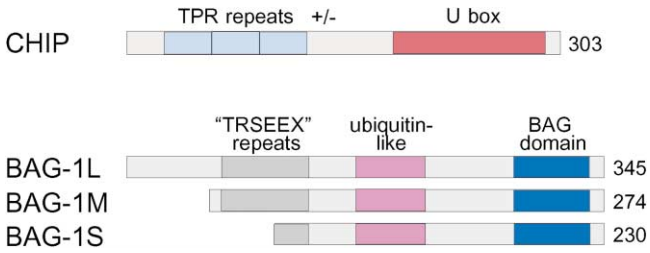
Background

The concerted effects of molecular chaperones and energy-dependent proteases appear to be required to mediate protein quality control [1–4]. Molecular chaperones recognize aberrantly folded proteins via associations with exposed hydrophobic stretches [5, 6]. By stabilizing the aberrant conformation, the chaperoning pathway promotes folding to the native state whenever possible. If the native state is not attainable, the damaged protein is targeted for degradation by energy-dependent proteases. In an analogous fashion, signaling molecules, such as steroid hormone receptors and some protein kinases, associate with molecular chaperones in their inactive state [7–10]. Interactions of these proteins with the chaperone machinery facilitate conformational changes that allow the signaling molecules to attain a folding state that is required for their activation. However, the chaperone-associated signaling molecules can also be diverted to a degradative pathway, suggesting an additional interplay between molecular chaperones and energy-dependent proteases [2, 3]. A major pathway for protein degradation in the eukaryotic cell involves the ubiquitin/proteasome system [11, 12]. Substrate proteins are tagged with the degradation marker ubiquitin through the action of multiple E2 ubiquitin-conjugating enzymes and E3 ubiquitin ligases, after ubiquitin activation mediated by the E1 enzyme. Multiubiquitinated proteins are recognized by the 26S proteasome, a proteolytic complex that catalyzes their degradation [13].

The CHIP protein was recently shown to stimulate the degradation of chaperone substrates by the ubiquitin/proteasome system [3, 4]. CHIP binds to the constitutively expressed Hsc70, the heat-inducible Hsp70, and also to Hsp90 through multiple tetratricopeptide (TPR) repeats and inhibits the folding activity of the chaperones [3, 14] (Figure 1). CHIP possesses a U box motif, which was originally identified in the yeast multiubiquitination factor Ufd2 [15]. Ufd2 cooperates with an E2 ubiquitin-conjugating enzyme and an E3 ubiquitin ligase in the formation of multiubiquitin chains and was therefore termed E4. The presence of a U box within CHIP suggested that the cochaperone might participate in ubiquitination reactions, possibly as an E4 enzyme. In fact, CHIP promotes the ubiquitination of chaperone substrates and stimulates their degradation by the ubiquitin/proteasome pathway [3, 4]. However, until now, the enzymatic function of CHIP has been uncertain [16].

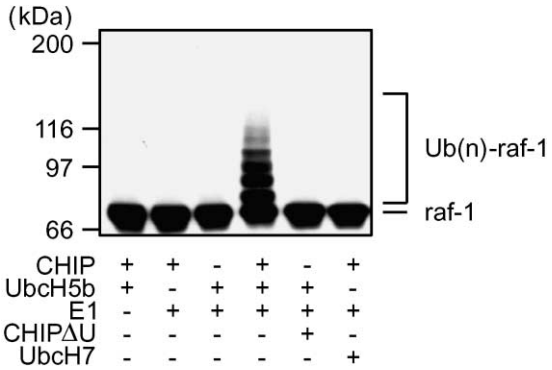
Besides CHIP, the chaperone cofactor BAG-1 has also been

Figure 1



A schematic presentation of the domain structure of CHIP and BAG-1. CHIP possesses an amino-terminal chaperone binding motif formed by three TPR repeats and an adjacent highly charged region. A U box required for ubiquitin ligase activity is present at the carboxyl terminus. The BAG-1 isoforms share a ubiquitin-like domain involved in proteasome binding and a BAG domain that mediates interaction with Hsp70. In addition, multiple repeats of the "TRSEEX" peptide motif of unknown function are found at the amino terminus of the BAG-1 isoforms.

Figure 2



CHIP acts as an E3 ubiquitin ligase to stimulate the ubiquitination of raf-1 kinase. In vitro ubiquitination of raf-1 was performed in the presence of CHIP or a deletion fragment of the cofactor lacking the U box (CHIPΔU), as indicated. For detection of raf-1 and ubiquitinated forms of the kinase (Ub(n)-raf-1), the immunoblot was probed with a specific anti-raf-1 antibody.

implicated to provide a link between chaperones and energy-dependent proteases. BAG-1 exists as multiple isoforms in the mammalian cell and acts as a nucleotide exchange factor for Hsc/Hsp70, stimulating the release of protein substrates from these chaperones [17–21]. BAG-1 binds to Hsc/Hsp70 via its carboxy-terminal BAG domain, which associates with the ATPase domain of the chaperones [22] (Figure 1). In addition, BAG-1 possesses a ubiquitin-like domain at its amino terminus that is utilized, at least in part, to target the chaperone cofactor to the 26S proteasome [23]. This targeting also induces an association of Hsc/Hsp70 with the proteasome, revealing BAG-1 as a coupling factor linking the chaperones and the proteolytic complex.

Here, we provide evidence that BAG-1 and CHIP cooperate in targeting chaperone substrates for degradation by the proteasome. In addition, we report that CHIP functions as an E3 ubiquitin ligase for chaperone substrates. Despite its structural relationship to the E4 multiubiquitination factor Ufd2, CHIP directly cooperates with E2 conjugation enzymes in ubiquitination reactions. Our study sheds light onto molecular mechanisms regulating the interplay of molecular chaperones and energy-dependent proteases.

Results

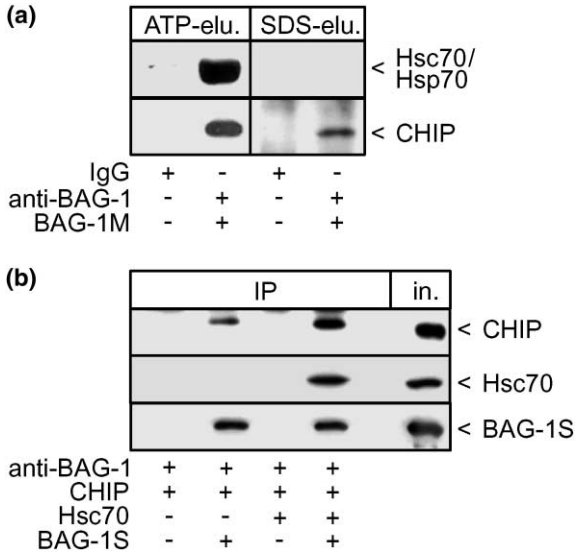
CHIP is a chaperone-associated ubiquitin ligase

Although a role for CHIP in the ubiquitination of chaperone substrates has been previously demonstrated [3, 4], the mechanism by which CHIP promotes ubiquitination of target proteins has so far been elusive. We therefore established an in vitro ubiquitination reaction using purified components, including rabbit E1, the human E2 proteins UbcH5b and UbcH7, and CHIP (or a deletion mutant of CHIP lacking the U box). UbcH5b was chosen because members of the Ubc4/5 family of ubiquitin-conjugating enzymes mediate the degradation of nonnative proteins and are therefore likely to cooperate with chaperone systems [24]. The capacity of these components to mediate ubiquitination of bacterially expressed raf-1 in the presence of purified ubiquitin and ATP was investigated. Raf-1 can be presented to the ubiquitin/proteasome system by Hsp90 and may thus be affected by the function of the chaperone cofactor CHIP [2]. In fact, CHIP, together with UbcH5b, formed a conjugation system that efficiently mediated the attachment of ubiquitin moieties to the raf-1 protein kinase (Figure 2). This activity was dependent on the U box of CHIP, revealing the essential role of this protein motif for ubiquitin conjugation. In contrast to UbcH5b, the ER-associated UbcH7 was unable to cooperate with CHIP, indicating specificity with regard to E2 coupling in this reaction. The CHIP protein is thus a chaperone-associated E3 ubiquitin ligase that cooperates with a specific E2 enzyme in the ubiquitination of signaling molecules.

BAG-1 interacts with the CHIP ubiquitin ligase

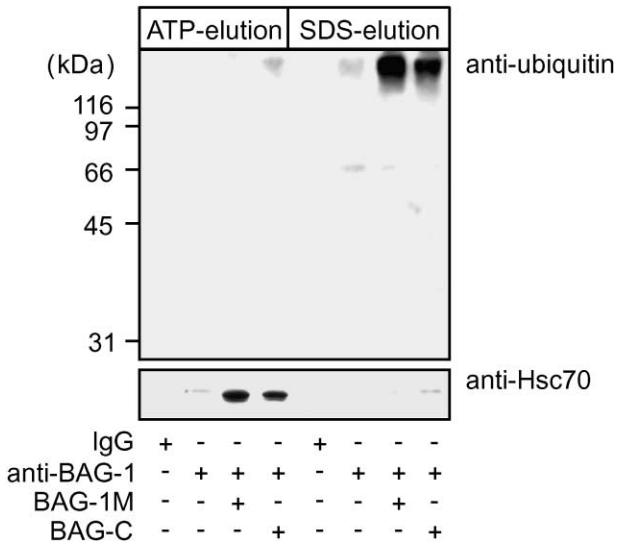
BAG-1 was recently shown to act as a coupling factor between Hsc/Hsp70 and the proteasome [23]. It was therefore conceivable that BAG-1 cooperates with CHIP during the targeting of chaperone substrates for degradation. As a first step to verify this notion, we investigated whether BAG-1 associates with the CHIP protein. Purified BAG-1M was added to a HeLa cell extract, followed by immunoprecipitation using an anti-BAG-1 antibody, to identify cofactor-associated proteins. Significant amounts of CHIP were detected in the BAG-1 immunocomplexes (Figure 3a). Notably, a major portion of the BAG-1-associated CHIP was released during ATP treatment of the immunocomplexes, which also stimulated a quantitative release of BAG-1-bound Hsc/Hsp70. As BAG-1 and CHIP bind to different domains of Hsc/Hsp70 [14, 17, 22], an interaction

Figure 3



BAG-1 associates with the CHIP ubiquitin ligase. **(a)** BAG-1 immunocomplexes were isolated from HeLa cell extracts after the addition of purified BAG-1M to the extract. The immunocomplexes were treated with ATP (ATP-elu.) prior to boiling in SDS-PAGE sample buffer (SDS-elu.). Immunoblots were probed with anti-Hsc/Hsp70 and anti-CHIP antibodies. **(b)** BAG-1S was incubated with purified CHIP and Hsc70 as indicated, and BAG-1-associated proteins were isolated by immunoprecipitation (IP). Input (in.) corresponds to 20% of the total amount of added protein. Hsc70 and CHIP were detected by subsequent immunoblotting.

Figure 4



BAG-1 binds multiubiquitinated proteins. Immunoprecipitations were performed from HeLa cell extracts using an anti-BAG-1 antibody or control IgG. When indicated, the antibody was saturated with purified BAG-1M and the carboxy-terminal fragment BAG-C, respectively. The purified immunocomplexes were treated with ATP to release BAG-1-bound Hsc/Hsp70 (ATP-elution), prior to boiling in SDS-PAGE sample buffer (SDS-elution). Ubiquitinated proteins and chaperones present in BAG-1 immunocomplexes were detected by immunoblotting with specific antibodies.

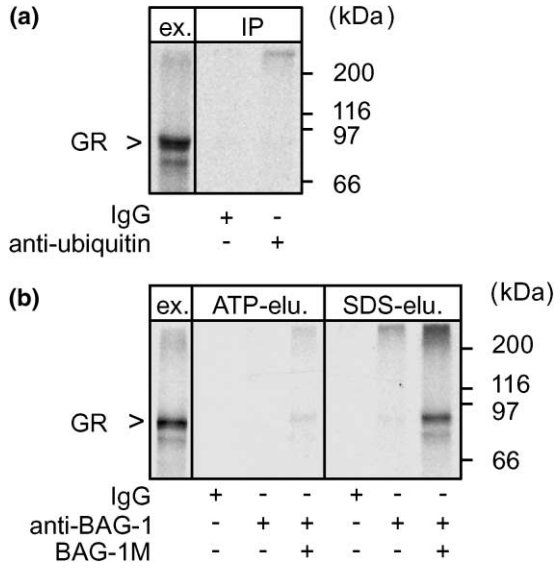
of BAG-1 with CHIP may actually be mediated through Hsc/Hsp70. Accordingly, disruption of the BAG-1/chaperone interaction by the addition of ATP is sufficient to induce corelease of CHIP (Figure 3a). A ternary complex, comprising Hsc/Hsp70 and its cofactors BAG-1 and CHIP, apparently exists in mammalian cells. However, this ternary complex is likely to be further stabilized by direct interactions between BAG-1 and CHIP, because a significant amount of CHIP remained associated with BAG-1 even when Hsc/Hsp70 was quantitatively released during ATP treatment (Figure 3a). We therefore investigated whether BAG-1 directly interacts with CHIP using purified components. BAG-1S was incubated with CHIP in the presence or absence of Hsc70, followed by BAG-1 immunoprecipitation. Although the presence of Hsc70 increased the amount of CHIP present in the BAG-1 immunocomplexes, significant amounts of CHIP were associated with BAG-1, even in the absence of the chaperone, revealing a direct interaction of the two cofactors (Figure 3b). BAG-1 and CHIP may thus act in a cooperative fashion to determine the function of Hsc/Hsp70 in protein degradation.

BAG-1 binds multiubiquitinated proteins

In immunoprecipitation experiments from HeLa cell extracts, we also observed an association of high-molecular mass multiubiquitinated proteins with BAG-1 (Figure 4, lane 6). As the endogenous levels of BAG-1 in HeLa cell extracts are low [17], the efficiency of coprecipitation was enhanced when the purified human isoform BAG-1M or a carboxy-terminal fragment (BAG-C) was added to the extract (Figure 4, lane 7 and 8). (Notably, the monoclonal anti-ubiquitin antibody did not recognize the ubiquitin-like domain of BAG-1.) Apparently, the chaperone cofactor associates with multiubiquitinated proteins through its carboxy-terminal domain. This association was maintained during ATP treatment of the purified immunocomplexes, whereas BAG-1-bound Hsc/Hsp70 was released (Figure 4). Therefore, a role for Hsc/Hsp70 as a mediator of the interaction can be excluded. These results link BAG-1 with ubiquitination events, perhaps by direct binding of BAG-1 to multiubiquitinated proteins.

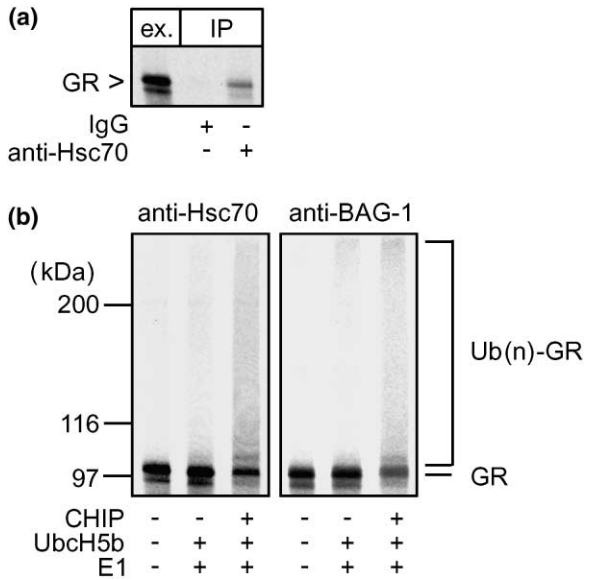
BAG-1 is known to interact with signaling molecules such as the raf-1 protein kinase and the glucocorticoid hormone receptor (GR) [25, 26]. Both proteins are substrates of the CHIP ubiquitination machinery ([3]; Figure 2). We therefore investigated whether these signaling molecules associate with BAG-1 in ubiquitinated form. To test this, in vitro transcription/translation was performed to obtain radiolabeled GR. Following translation, the receptor was detected, as were modified forms of higher molecular

Figure 5



Glucocorticoid hormone receptor associates with BAG-1 in multiubiquitinated form. **(a)** Following in vitro translation of glucocorticoid hormone receptor (GR), the receptor and high-molecular mass forms were detected in the lysate (ex.). The latter could be coprecipitated with an anti-ubiquitin antibody (IP). **(b)** Immunoprecipitation of in vitro-translated GR was performed using an anti-BAG-1 antibody or control IgG. When indicated, purified BAG-1M was added to the samples after translation. Purified immunocomplexes were treated with ATP (ATP-elution), followed by boiling in SDS-PAGE sample buffer (SDS-elution).

Figure 6



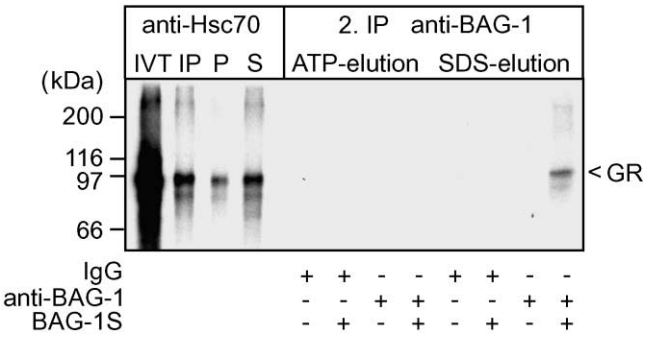
The CHIP conjugation machinery recognizes signaling molecules presented by Hsc/Hsp70 and BAG-1. **(a)** Immunoprecipitation of in vitro-translated GR (ex.) was performed with an anti-Hsc/Hsp70 antibody and control IgG (IP). **(b)** Immunocomplexes containing in vitro-translated GR were isolated with an anti-Hsc/Hsp70 (anti-Hsc70) or anti-BAG-1 antibody (anti-BAG-1). In the latter case, purified BAG-1S was added to the samples after translation. BAG-1 immunocomplexes were treated with ATP prior to ubiquitination to remove BAG-1-associated Hsc/Hsp70. Ubiquitination of coprecipitated GR was performed in the presence of purified ubiquitin, ATP, and components of the conjugation machinery as indicated. GR and ubiquitinated forms were detected by autoradiography.

mass that could be immunoprecipitated with an anti-ubiquitin antibody (Figure 5a). Apparently, a significant amount of GR becomes ubiquitinated during the translation reaction. When BAG-1 isoforms present in the GR-translating lysate were precipitated with an anti-BAG-1 antibody, multiubiquitinated forms of GR were cosedimented (Figure 5b, lane 6). In contrast, the unmodified receptor was only barely detectable in BAG-1 immunocomplexes. The efficiency of immunoprecipitation could be increased when purified BAG-1M was added to the lysate. In this situation, unmodified receptor coprecipitated with the chaperone cofactor, but again, an enrichment of multiubiquitinated forms was observed (Figure 5b, lane 7). The hormone receptor apparently accumulates in association with BAG-1 in multiubiquitinated form. Similar findings were obtained for the raf-1 protein kinase (data not shown). Remarkably, BAG-1 did not bind to a multiubiquitinated form of glutathione S-transferase generated in vitro (data not shown). It thus appears unlikely that BAG-1 acts as a ubiquitin chain receptor. Accordingly, an accumulation of multiubiquitinated proteins in BAG-1 immunocomplexes may arise from an association of the cofactor with the CHIP ubiquitin conjugation machinery (see above).

CHIP recognizes substrate proteins presented by Hsc/Hsp70 and BAG-1

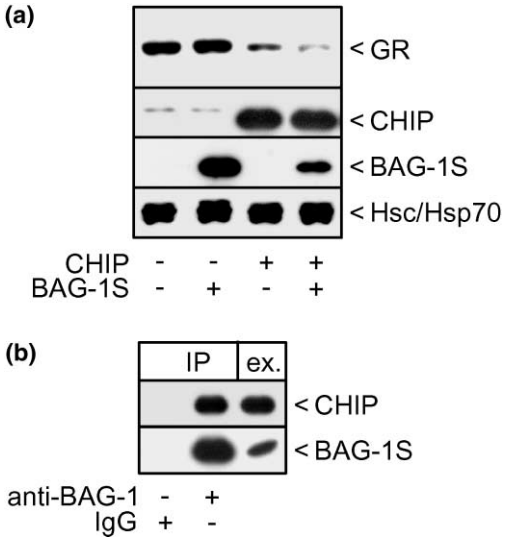
We investigated whether the CHIP conjugation machinery recognizes Hsc/Hsp70- and BAG-1-associated GR. Following its translation, GR could be coprecipitated using an anti-Hsc/Hsp70 antibody (Figure 6a). When the CHIP/UbcH5b conjugation system was added to the purified chaperone/receptor complexes, GR was efficiently ubiquitinated (Figure 6b). In a similar experiment, GR that was bound to the cofactor BAG-1 was isolated. Prior to the addition of the ubiquitin conjugation machinery, the BAG-1 immunocomplexes were treated with ATP to remove BAG-1-bound Hsc/Hsp70. The cofactor-bound hormone receptor was efficiently modified by UbcH5b and CHIP (Figure 6b). Apparently, CHIP in conjunction with UbcH5b was able to recognize GR when presented by Hsc/Hsp70 and BAG-1. Recognition of signaling molecules by the conjugation machinery when associated with different chaperones and cofactors may provide a means to efficiently divert the signaling molecules from a chaperone-dependent activation pathway to a proteasome-dependent degradation pathway.

Figure 7



BAG-1 accepts hormone receptor released from Hsc/Hsp70. In vitro translation of GR was performed (IVT), followed by immunoprecipitation with anti-Hsc/Hsp70 antibody (IP). The purified immunocomplexes were incubated in the presence of ATP and Hsp40 to induce cycles of substrate release and rebinding. Following the removal of protein G-sepharose-bound immunocomplexes, GR was detected in the pellet (P) and supernatant fractions (S). Supernatant fractions were subjected to a second immunoprecipitation with anti-BAG-1 antibody or control IgG (2. IP). When indicated, substrate release from Hsc/Hsp70 was performed in the presence of BAG-1S. BAG-1 immunocomplexes were treated with ATP (ATP-elution) prior to boiling in SDS-PAGE sample buffer (SDS-elution).

Figure 8



BAG-1 stimulates CHIP-induced degradation of the glucocorticoid hormone receptor (GR). **(a)** COS-7 cells were transfected to express human GR, BAG-1, and CHIP as indicated. Each lane represents 25 μg cellular extract. Labeled proteins were detected by immunoblotting. **(b)** Following coexpression, CHIP is readily detectable in association with BAG-1. BAG-1 immunocomplexes were isolated from COS-7 cells expressing BAG-1S and CHIP, and the two cofactors were detected by immunoblotting in immunocomplexes (IP) and total lysate (ex., 10 μg protein was loaded).

BAG-1 accepts substrate proteins from Hsc/Hsp70

Binding of BAG-1 to multiubiquitinated proteins suggests that one role for the cofactor is to accompany substrate proteins during transfer from Hsc70 to the proteasome. To substantiate this notion, we investigated whether BAG-1 is able to accept substrate proteins from Hsc/Hsp70. Chaperone/GR complexes were isolated by immunoprecipitation with an anti-Hsc/Hsp70 antibody and were subsequently incubated with ATP and Hsp40 (Figure 7). In this situation, the substrate protein started to cycle on and off the chaperones. Accordingly, released GR was detectable in the supernatant fraction after antibody-bound Hsc/Hsp70 was removed by centrifugation (Figure 7, lane 4). When cycling was induced in the presence of purified BAG-1S, significant amounts of chaperone-released GR were specifically coprecipitated in a second immunoprecipitation with an anti-BAG-1 antibody (Figure 7). As expected for a direct interaction of GR with BAG-1, the receptor remained BAG-1-associated during ATP treatment of the immunocomplexes and was eluted in the presence of SDS. Apparently, BAG-1 is able to accept substrate proteins released from Hsc/Hsp70.

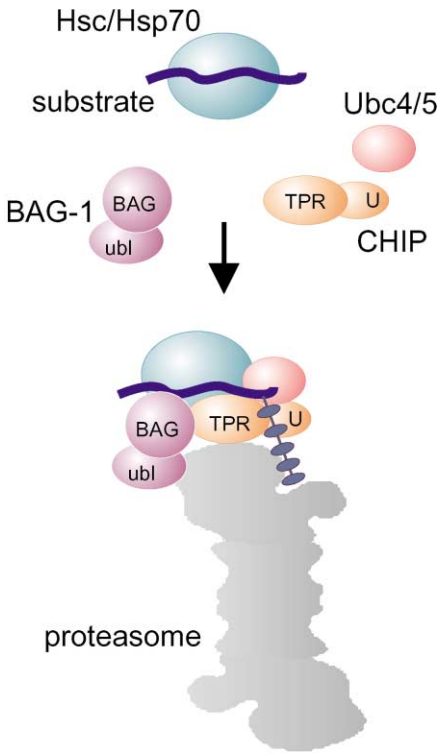
BAG-1 promotes CHIP-induced degradation

CHIP was recently shown to induce the degradation of GR in cell culture experiments, whereas a similar effect was not observed following BAG-1 expression [3]. CHIP-mediated ubiquitination thus appears to be rate limiting for the degradation of chaperone substrates. To elucidate the significance of CHIP/BAG-1 cooperation for substrate targeting to the proteasome, BAG-1 was coexpressed with CHIP, and the effects on GR degradation were analyzed. While BAG-1S expression alone did not promote the degradation of the hormone receptor, BAG-1S significantly affected CHIP-induced degradation of GR (Figure 8a). Coexpression of the two proteins reduced the amount of GR below the level detectable in CHIP-expressing cells. When BAG-1 immunocomplexes were isolated from coexpressing cells, CHIP was readily detectable in association with BAG-1 (Figure 8b). BAG-1 and CHIP can apparently team up to regulate the sorting of chaperone substrates to the proteasome.

Discussion

The CHIP protein is the first identified ubiquitin ligase that associates with molecular chaperones. Binding of CHIP to Hsc/Hsp70 and Hsp90 apparently shifts the chaperones from a protein folding to a degradation mode by tagging associated substrates with the degradation marker ubiquitin. The ubiquitin ligase activity of CHIP depends on its U box, a protein motif previously identified in the yeast Ufd2 protein. Ufd2 cooperates in a U box-dependent manner with an E2/E3 conjugation machinery during multiubiquitin chain formation and was therefore characterized as an E4 enzyme [15]. In contrast, CHIP cooperates directly with an E2 component and itself acts

Figure 9



A model for the cooperation of CHIP and BAG-1 in coupling Hsc/Hsp70 to the ubiquitin/proteasome system. CHIP associates with Hsc/Hsp70 via its TPR chaperone adaptor (TPR) and, at the same time, recruits E2 ubiquitin-conjugating enzymes of the Ubc4/5 family to the chaperone complex. This may involve binding of the ubiquitin-conjugating enzyme to the U box of the cofactor (U). In conjunction with E2, CHIP mediates ubiquitin attachment to the chaperone substrate and induces its targeting to the proteasome for degradation. The targeting process is facilitated by the ubiquitin domain protein BAG-1. BAG-1 binds to Hsp70 via its BAG domain (BAG) and utilizes its ubiquitin-like domain (ubl) for proteasomal association.

as an E3 ubiquitin ligase (Figure 9). Notably, the U box is structurally related to RING fingers that are present in diverse proteins, many of which were shown to possess ubiquitin ligase activity [27, 28]. The RING finger seems to activate E2 enzymes during ubiquitin transfer to substrate proteins. CHIP apparently utilizes the related U box to cooperate with specific E2s in ubiquitination reactions. Similar molecular mechanisms may thus underlie E3 and E4 function.

During sorting of chaperone substrates to the proteasome, CHIP can cooperate with the cochaperone BAG-1. BAG-1 was previously identified as a coupling factor that recruits Hsc/Hsp70 to the 26S proteasome [23]. Through its ubiquitin-like domain, BAG-1 associates with the proteasome while the BAG-domain simultaneously binds Hsc/Hsp70 (Figure 9). In addition, BAG-1 was shown to stimulate the release of protein substrates from Hsc/Hsp70 through its nucleotide exchange activity [18, 20, 21]. The dual function as a coupling factor at the proteasome and as a substrate release factor of Hsc/Hsp70 prompted speculations that BAG-1 may facilitate the degradation of chaperone substrates [23]. Dissociation of the chaperone/substrate complex stimulated by BAG-1 in the vicinity of the proteasome may promote efficient substrate transfer to the proteolytic complex. However, experimental evidence for this notion remained elusive. Moreover, cellular functions of BAG-1 that were identified in overexpression studies, such as stimulation of raf-1 and Bcl-2 activity [26, 29], were difficult to reconcile with an exclusive role of BAG-1 in proteasomal sorting. The data presented here may reconcile these discrepancies. In fact, elevating the cellular concentration of BAG-1 alone did not induce GR turnover [3] (Figure 8). Only coexpression with CHIP revealed a degradation-stimulating activity of BAG-1 (Figure 8). Multiubiquitination of the chaperone-bound substrate mediated by CHIP is apparently required for subsequent degradation and represents the rate-limiting step during the sorting process in the cellular milieu. The proteasome-recruiting and chaperone-regulating activity of BAG-1 does not circumvent substrate ubiquitination as a prerequisite for proteasomal degradation, but it facilitates degradation once the multiubiquitin chain has been assembled.

Transfer of chaperone substrates to the proteasome may actually occur in a preassembled chaperone/proteasome complex (Figure 9). Both BAG-1 and CHIP were shown to bind to the proteasome [3, 23]. In the case of BAG-1, binding is apparently mediated by the integrated ubiquitin-like domain. BAG-1 can thus be added to a growing list of ubiquitin domain proteins (UDPs) found to be associated with the proteasome [30]. In addition to proteasome association, interaction with ubiquitin ligases also appears to be a widespread feature among UDPs. For example, the human UDPs PLIC-1 and PLIC-2 associate with ubiquitin ligases and direct them to the proteasome [31]. A general role for UDPs in coordinating components of the ubiquitination machinery and the proteasome emerges. Remarkably, BAG-1 is not the only UDP that modulates Hsc/Hsp70 chaperone function. The apoptotic regulator Scythe possesses an amino-terminal ubiquitin-like domain and was recently shown to inhibit the protein refolding activity of Hsc/Hsp70 [32]. Scythe may thus participate in the regulation of molecular chaperones during protein degradation.

Association of CHIP with Hsc/Hsp70 and Hsp90 appears to enable CHIP to gain access to a broad spectrum of chaperone-bound substrates for subsequent ubiquitination and targeting to the proteasome [3, 4]. On the other hand, CHIP-affected signaling molecules, such as GR, sequentially associate with both the Hsc/Hsp70 and Hsp90 chaperone systems during their activation [7–10]. CHIP would therefore be able to divert signaling molecules to the degradation machinery at multiple stages of the activation

process. Interaction of CHIP with the cochaperone BAG-1 apparently further extends the capacity of the ubiquitin ligase to target chaperone substrates for proteasomal degradation. Yet, substrate selection may also involve direct binding of the CHIP ubiquitin ligase to the substrate protein. Mammalian chaperones that CHIP could cooperate with are absent in the reconstituted ubiquitination reaction. Nevertheless, CHIP efficiently mediates raf-1 ubiquitination, indicating direct binding of the ubiquitin ligase to raf-1. The capacity to directly associate with a subset of chaperone substrates may provide a molecular basis for substrate selection. Similarly, BAG-1 is able to associate with chaperone-released GR and presents the receptor to the CHIP conjugation machinery (Figure 7), but it does not interact with nonnative luciferase upon release from Hsc70 [18, 20]. BAG-1 may therefore participate in the selection process.

The data presented here identify chaperone cofactors as major regulators during protein quality control. Association of CHIP and BAG-1 with Hsc/Hsp70 directly turns the molecular chaperones into degradation factors. Remarkably, CHIP and BAG-1 both compete with other cofactors in chaperone binding [3, 17, *22*]. The intracellular balance of competing and cooperating cofactors may thus determine the folding and degradation activities of the Hsc/Hsp70 chaperone system.

Materials and methods

Protein expression and purification

Human BAG-1M, BAG-1S, and BAG-C (covering amino acids 129–274 of BAG-1M) were purified as described following recombinant expression in baculovirus-infected Sf9 cells [18, 20]. CHIP and the U box deletion form of CHIP (comprising amino acids 1–197) were expressed as His-tagged fusion proteins in *E. coli* BL21 (DE3) cells as previously described [14]. Human c-raf-1 and human Hsp40 (Hdj-1), UbcH5b, and UbcH7 were expressed in *E. coli* BL21 (DE3) cells using corresponding pET plasmids. For ubiquitination reactions, bacterial extracts containing raf-1 and UbcH7 were used after lysis of cells in a French pressure cell and centrifugation of the lysate at 100,000 × g for 30 min. Hsp40 was purified as described [17]. UbcH5b was purified to homogeneity by chromatography on DEAE-sepharose (Pharmacia) and Bio-Gel HT hydroxyapatite (Bio-Rad). Protein concentrations were determined using the Bio-Rad Bradford reagent with purified IgG (Sigma) as the standard.

In vitro transcription/translation

The cDNA for human GR was subcloned from pRK7-GR into pGEM-11Zf (Pharmacia), the resultant construct was linearized with BamHI, and in vitro transcription was performed using the SP6 RiboMax system according to the protocol of the manufacturer (Promega). Obtained RNA was used for in vitro translation of GR in nuclease-treated rabbit reticulocyte lysate (40% of total volume) as described by the manufacturer (Promega).

Immunoprecipitations

HeLa cells were grown in minimal essential medium (MEM) (GIBCO) supplemented with 10% fetal calf serum, penicillin, streptomycin, and glutamine. At about 90% confluency, attached cells were washed two times with phosphate-buffered saline (PBS) and were collected. HeLa cells were lysed in 25 mM MOPS (pH 7.2), 100 mM KCl, and 0.5% Tween 20 (buffer A) containing 10 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, and 0.5 μg/ml leupeptin. The lysate was centrifuged at 35,000 × g for 20 min at 4°C, and the supernatant fraction was used as the cell extract for immunoprecipitation. When indicated, purified human BAG-1M was added to the extract (4 μg purified protein/1 mg extract protein), followed by incubation at 4°C for 1 hr and the subsequent addition of 1.2 μg anti-BAG-1/C-16 antibody or 1.2 μg control IgG. The samples were incubated for 1 hr at 4°C, followed by the addition of protein G-sepharose and another incubation for 1 hr at 4°C. The sepharose was collected by centrifugation and washed four times with 1 ml buffer A and once with buffer A without detergent. The immunocomplexes were subsequently incubated for 15 min at 30°C in 500 μl detergent-free buffer A containing 2 mM $MgCl_2$, 1 mM ATP, and protease inhibitors. The protein G-sepharose was collected by centrifugation, and ATP-eluted proteins were precipitated from the supernatant fraction by the addition of trichloroacetic acid. The sepharose beads were washed again using buffer A, followed by elution of immunocomplexes through boiling in SDS-PAGE loading buffer. BAG-1-associated Hsc/Hsp70 and CHIP were detected by immunoblotting.

To test for a direct interaction between BAG-1 and CHIP, 2 μg purified BAG-1S was incubated with 1 μg CHIP in the presence or absence of 2 μg Hsc70 in 100 μl buffer A containing 1 mM EDTA for 2 hr at 4°C. Anti-BAG-1/C-16 (6 μg) was added together with 200 μl buffer A, and samples were incubated for an additional hour, followed by the addition of protein G-sepharose. Samples were incubated for 1 hr at 4°C, and the sepharose was collected by centrifugation and washed six times with 1 ml buffer A. Associated proteins were eluted by boiling in SDS-PAGE sample buffer and were detected by immunoblotting.

For the detection of BAG-1-associated multiubiquitinated proteins, HeLa cells were lysed in RIPA buffer (25 mM Tris [pH 8.0], 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% Nonidet P-40, 10% glycerol, 2 mM EDTA) containing Complete protease inhibitors (Boehringer), the lysate was centrifuged for 30 min at 30,000 × g, and the supernatant was used as a soluble extract. For immunoprecipitation assays, protein G-sepharose (Pharmacia) was used, to which affinity-purified rabbit anti-BAG-1/C-16 antibody (Santa Cruz Biotechnology) or an unrelated control antibody (rabbit anti-chicken IgG [Sigma]) was covalently attached at a protein concentration of 0.5 mg/ml. When indicated, the antibody resin was saturated with purified BAG-1M and BAG-C, respectively, by incubation for 2 hr at 4°C in the presence of 1.3 mg purified protein/mg immobilized antibody prior to the addition of soluble cell extract. After incubation for an additional 2.5 hr, absorbed immunocomplexes were washed eight times with 1 ml RIPA, followed by two washing steps with 20 mM MOPS (pH 7.2), 50 mM KCl, and 0.1 mM dithiothreitol (buffer B). The immunocomplexes were subsequently incubated for 15 min at 30°C in 500 μl buffer B containing 2 mM $MgCl_2$, 1 mM ATP, and protease inhibitors. The protein G-sepharose was collected by centrifugation, and ATP-eluted proteins were precipitated from the supernatant fraction by the addition of trichloroacetic acid. The sepharose beads were washed again using RIPA buffer, followed by elution of immunocomplexes through boiling in SDS-PAGE loading buffer. BAG-1-associated ubiquitinated proteins were detected by immunoblotting using a monoclonal anti-ubiquitin antibody (Zymed).

For immunoprecipitation of in vitro-translated proteins, translation reactions were stopped by the addition of 2 mM puromycin, 2 mM unlabeled methionine, and 10 mM EDTA, and samples were centrifuged at 30,000 × g for 10 min. To the supernatants, purified BAG-1M or BAG-1S (4 μg protein/125 μl translation reaction) was added as indicated, and samples were incubated for 1 hr at 4°C. Samples were diluted with 8 volumes of buffer A, prior to the addition of anti-ubiquitin (Sigma), anti-Hsc/Hsp70 (Stressgen), anti-BAG-1/C-16, or control IgG to a final concentration of 18 μg/ml as indicated. After incubation at 4°C for 1 hr, protein G-sepharose was added, followed by an additional incubation at 4°C for 1 hr. The sepharose was collected and washed eight times with buffer A and once with buffer A without detergent. ATP elution was performed as described above.

Ubiquitination reactions

Bacterially expressed human c-raf-1 (100,000 × g bacterial lysate at a protein concentration of 2 mg/ml) was incubated in the presence of 8 μM E2 enzyme, 6 μM CHIP or the U box deletion fragment, 0.1 μM purified rabbit E1 (Affiniti), and 2.5 mg/ml ubiquitin (Sigma) in 20 mM MOPS (pH 7.2), 100 mM KCl, 5 mM $MgCl_2$, 5 mM ATP, 10 mM DTT, and 1 mM phenylmethylsulfonyl fluoride (buffer C) for 2 hr at 30°C. Samples were analyzed by SDS-PAGE and immunoblotting with an anti-raf-1 antibody (Santa Cruz Biotechnology).

For ubiquitination of Hsc70- and BAG-1-associated GR, coimmunoprecipitations of in vitro-translated GR were performed as described above. The purified BAG-1 immunocomplexes were treated with ATP to remove BAG-1-bound Hsc/Hsp70 (see above). To the sepharose-bound immunocomplexes, 20 μl buffer C containing 8 μM UbcH5b, 3 μM CHIP, 0.1 μM E1, and 2.5 mg/ml ubiquitin was added as indicated. Samples were incubated for 2 hr at 30°C and were subsequently analyzed by SDS-PAGE and autoradiography.

Transfer reaction

To monitor the transfer of GR between Hsc/Hsp70 and BAG-1, chaperone/GR complexes were isolated by immunoprecipitation after in vitro translation of the hormone receptor (see above). The purified immunocomplexes were resuspended in 25 mM MOPS (pH 7.2), 50 mM KCl, 2 mM $MgCl_2$, and 1 mM ATP. Hsp40 was added to a final concentration of 0.02 μM, and, when indicated, BAG-1S was added to a final concentration of 0.95 μM. Samples were incubated at RT for 30 min, followed by the addition of 6 volumes of 20 mM MOPS (pH 7.2), 100 mM KCl, and 0.5% Tween 20. The samples were centrifuged to remove sepharose-bound Hsc/Hsp70 immunocomplexes, and 28 μg/ml anti-BAG-1/C-16 antibody or control IgG was added to the supernatant fraction. Immunoprecipitations were performed as described above.

GR-degradation assay

The degradation of human GR in COS-7 cells was analyzed as previously described [3]. COS-7 cells were transfected using the CalPhos transfection kit (Clontech) according to the protocol of the manufacturer. When indicated, 1.6 μg pcDNA3.1-*hbag-1S* and pcDNA3.1-*chip* was added per 35-mm well. The total amount of DNA was kept constant by the addition of pcDNA3.1-*myc/his-lacZ*. After 48 hr, cells were lysed in RIPA buffer and centrifuged for 20 min at 30,000 × g at 4°C. The resultant supernatant was used as cellular extract. For analysis of GR expression, the receptor was detected with a specific antibody (Santa Cruz Biotechnology). For detection of BAG-1/CHIP complexes after coexpression, transiently transfected COS-7 cells were lysed in buffer D (20 mM MOPS [pH 7.2], 50 mM KCl, and 0.5% Tween 20) containing 10 mM EDTA and Complete protease inhibitors. Extract preparation and immunoprecipitation were performed as described above using buffer D for washing.

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