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Ubiquitylation of BAG-1 Suggests a Novel Regulatory Mechanism during the Sorting of Chaperone Substrates to the Proteasome*

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BAG-1 is a ubiquitin domain protein that links the molecular chaperones Hsc70 and Hsp70 to the proteasome. During proteasomal sorting BAG-1 can cooperate with another co-chaperone, the carboxyl terminus of Hsc70-interacting protein CHIP. CHIP was recently identified as a Hsp70- and Hsp90-associated ubiquitin ligase that labels chaperone-presented proteins with the degradation marker ubiquitin. Here we show that BAG-1 itself is a substrate of the CHIP ubiquitin ligase *in vitro* and *in vivo*. CHIP mediates attachment of ubiquitin moieties to BAG-1 in conjunction with ubiquitin-conjugating enzymes of the Ubc4/5 family. Ubiquitylation of BAG-1 is strongly stimulated when a ternary Hsp70·BAG-1·CHIP complex is formed. Complex formation results in the attachment of an atypical polyubiquitin chain to BAG-1, in which the individual ubiquitin moieties are linked through lysine 11. The noncanonical polyubiquitin chain does not induce the degradation of BAG-1, but it stimulates a degradation-independent association of the co-chaperone with the proteasome. Remarkably, this stimulating activity depends on the simultaneous presentation of the integrated ubiquitin-like domain of BAG-1. Our data thus reveal a cooperative recognition of sorting signals at the proteolytic complex. Attachment of polyubiquitin chains to delivery factors may represent a novel mechanism to regulate protein sorting to the proteasome.

The control and maintenance of the three-dimensional structure of proteins are prerequisites for cell survival and involve a cooperation of molecular chaperones and energy-dependent proteases (1–4). Molecular chaperones recognize hydrophobic regions exposed on unfolded proteins and stabilize non-native conformations. As a consequence formation of insoluble protein aggregates is prevented, and folding to the native state is promoted. On the other hand, energy-dependent proteases, such as the eukaryotic 26 S proteasome, degrade irreversibly damaged proteins that fail to be folded properly.

Selection of proteins for degradation by the proteasome involves ubiquitin conjugation (5–7). A polyubiquitin chain is attached to a protein substrate through the concerted action of a ubiquitin-activating enzyme (E1),[1] a ubiquitin-conjugating enzyme (E2), and a ubiquitin-protein isopeptide ligase (E3). In contrast to the presence of only one type of E1 enzyme in the eukaryotic cytosol, E2 and E3 enzymes are recruited from large protein families and mediate a specific recognition of a large repertoire of protein substrates. A polyubiquitin chain generated through the linkage of lysine 48 residues of successive ubiquitin moieties is usually sufficient to target a protein substrate to the proteasome, where finally deubiquitylation, unfolding, and degradation occur.

Recent studies shed light onto molecular mechanisms underlying the cooperation of molecular chaperones with the ubiquitin/proteasome system during protein quality control. Two co-chaperones, CHIP and BAG-1, are of central importance in this regard. The CHIP protein was shown to act as a chaperone-associated ubiquitin ligase that directs chaperone substrates to the proteasome (8–12). CHIP interacts with the constitutively expressed Hsc70, the stress-inducible Hsp70, or Hsp90 via its amino-terminal tetratricopeptide repeat domain. This interaction negatively regulates ATPase and folding activities of the Hsc/Hsp70 chaperone and induces remodeling of Hsp90 heterocomplexes into a folding incompetent state (8, 13). In addition, CHIP possesses a U box domain at its carboxyl terminus which is structurally related to RING domains but lacks the Zn^{2+}-chelating residues (4, 14). The U box domain provides the structural basis for the ubiquitin ligase activity of CHIP (9, 10, 12). In conjunction with ubiquitin-conjugating enzymes of the Ubc4/5 family CHIP mediates polyubiquitylation of protein substrates presented by Hsc/Hsp70 and Hsp90. As a consequence, CHIP induces the degradation of known chaperone substrates such as the glucocorticoid hormone receptor or immature endoplasmic reticulum-localized forms of the cystic fibrosis transmembrane conductance regulator in cell culture experiments (8, 11). The ubiquitylation factor CHIP apparently mediates the removal of chaperone-selected proteins from the productive folding pathway and targets them for degradation by the proteasome.

The Hsc/Hsp70 cofactor BAG-1 acts as a coupling factor between molecular chaperones and the proteasomal complex (15). BAG-1 belongs to a family of proteins with an integral

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[1] The abbreviations used are: E1, ubiquitin-activating enzyme; E2, ubiquitin-conjugating enzyme; E3, ubiquitin-protein isopeptide ligase; CHIP, carboxyl terminus of Hsc70-interacting protein; HA, hemagglutinin; HEK, human embryonic kidney; Hsc70, 70-kDa heat shock cognate protein; Hsp40, Hsp70, Hsp90, 40-, 70-, and 90-kDa heat shock proteins, respectively; 4K-R, mutant form of BAG-1M with four lysine to arginine substitutions within its ubiquitin-like domain; MOPS, 4-morpholinepropanesulfonic acid; RIPA, radioimmune precipitation assay buffer.

ubiquitin-like domain, so called ubiquitin domain proteins (6). The referred domain appears to serve as a proteasomal targeting signal and enables BAG-1 to recruit Hsc/Hsp70 to the proteasome (15). In addition, BAG-1 is able to stimulate the release of chaperone substrates from Hsc/Hsp70 because of its nucleotide exchange activity (16–18). This chaperone-regulating activity may stimulate a transfer of chaperone substrates to the proteasome. In fact, evidence for a participation of BAG-1 in proteasomal targeting was recently provided when BAG-1 was shown to promote the degradation of the glucocorticoid hormone receptor in conjunction with CHIP (9). Cooperation of the two cofactors appears to reflect their physical interaction. BAG-1 and CHIP are able to associate simultaneously with Hsc/Hsp70 because of binding to different domains of the chaperone, and formation of the ternary chaperone-cofactor complex is promoted further through a direct interaction between the two cofactors (9). Accordingly, a model for the cooperation of Hsc/Hsp70 with the ubiquitin-proteasome system was proposed which integrated BAG-1 and CHIP activity (3, 4, 19). Association with CHIP seems to convert the Hsc/Hsp70 chaperone into a substrate recognition factor of a functional ubiquitin ligase complex, whereas BAG-1 supports binding of the ligase complex to the proteasome and triggers the release of ubiquitylated substrates from Hsc/Hsp70 for their transfer to the proteasome. However, central mechanistic and regulatory aspects of the proposed cooperation remained elusive.

Here, we show that BAG-1 itself is a substrate of the CHIP ubiquitin ligase *in vitro* and *in vivo*. CHIP cooperates with ubiquitin-conjugating enzymes of the Ubc4/5 family during ubiquitylation of BAG-1. The efficiency of ubiquitylation is increased strongly upon formation of the ternary Hsc70·BAG-1·CHIP complex. In this situation, a single polyubiquitin chain with linkages involving lysine 11 is assembled on BAG-1. Notably, CHIP-mediated ubiquitylation does not induce the degradation of BAG-1 but stimulates binding to the proteasome in a degradation-independent fashion. We provide evidence that the integrated ubiquitin-like domain of BAG-1 and the attached polyubiquitin chain are recognized by the proteasome in a cooperative manner. Our data reveal a novel regulatory step during the sorting of chaperone substrates to the proteasome.

MATERIALS AND METHODS

Antibodies and Recombinant Proteins—The following antibodies were used: mouse anti-Hsc/Hsp70 (Stressgen), rabbit anti-BAG-1 (C-16, Santa Cruz Biotechnology), mouse anti-ubiquitin (Zymed Laboratories Inc.), rabbit anti-ubiquitin (Sigma), and mouse anti-human C-8 (Affiniti). Purified bovine ubiquitin was purchased from Sigma, and the ubiquitin K48R mutant, *N*-methylated ubiquitin, and rabbit E1 were from Affiniti. Other point mutants of ubiquitin were expressed recombinantly in *Escherichia coli* BL21 (DE3) pJY2 cells using corresponding pET3a plasmids (20). Cells were lysed by sonication in 20 mM MOPS, pH 7.2, 100 mM KCl containing Complete protease inhibitor (Roche Molecular Biochemicals). The lysate was centrifuged for 30 min at 30,000 × *g*, and the resulting supernatant was used as a soluble extract. Rat Hsc70, human BAG-1M, and BAG-1S were purified as described after recombinant expression in baculovirus-infected Sf9 cells (16, 21, 22). Wheat E1 was also expressed recombinantly in insect cells and purified as described for bacterially expressed E1 (23). The C130 fragment of BAG-1 (covering the carboxyl-terminal 130 amino acids of the co-chaperone) was expressed in *E. coli* using a corresponding pTYB2 construct and purified as described via its self-cutting intein/chitin binding domain (22). Human Hsp40 (Hdj-1) and human UbcH5b were expressed in bacteria and purified as described (9, 16). A mutant form of BAG-1M with arginine substitutions of all lysine residues within the ubiquitin-like domain of the co-chaperone (4K-R) was generated by PCR using corresponding primers, subcloning into pET3a (Novagen), and expression in *E. coli* BL21 (DE3). The ATPase domain of Hsc70 was purified and immobilized as described previously (16). For initial ubiquitylation experiments His-tagged CHIP was used, which was purified as described (13). For purification of untagged CHIP the *chip* cDNA was subcloned into pET3a (Novagen). The protein was expressed in *E. coli* BL21 (DE3) pLysS cells. Cells were lysed by sonication in buffer A (20 mM MOPS, pH 7.2, 50 mM KCl, 0.5 mM EDTA) containing 1 mM β-mercaptoethanol and Complete protease inhibitor. After centrifugation of the lysate at 100,000 × *g* for 30 min at 4 °C, the supernatant was applied to a DEAE-Sepharose column (Amersham Biosciences). Bound protein was eluted by a linear gradient of 50–300 mM KCl. CHIP-containing fractions were pooled, and the potassium concentration was adjusted to 50 mM. The fractions were loaded onto an HT Gel hydroxyapatite column (Bio-Rad), and elution of bound protein occurred in a linear gradient from 20 to 200 mM potassium phosphate at pH 7.0. CHIP-containing fractions were pooled, and the potassium concentration was adjusted below 120 mM. The fractions were applied to a Source 30Q column (Amersham Biosciences), and bound CHIP was eluted by a linear gradient of 120–300 mM KCl. CHIP-containing fractions were pooled and stored at −80 °C. 26 S proteasomes were purified from human red blood cells as described previously (24). Protein concentrations were determined using the Bio-Rad Bradford reagent with purified IgG (Sigma) as the standard.

Transfection of Mammalian Cells—Mammalian cells were transiently transfected with a CalPhos transfection kit (Clontech) using pcDNA3.1-*hbag-1S*, pcDNA3.1-*hbag-1M*, pcDNA3.1-*chip*, and pcDNA3.1-*HA-ubiquitin* according to the protocol of the manufacturer. As control, plasmid pcDNA3.1-*myc*/*his-lacZ* was used. Usually, cell extracts were prepared 48 h post-transfection. Expression of BAG-1 isoforms in HeLa cells was analyzed after lysis of cells in RIPA buffer (25 mM Tris-HCl, 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 inhibitor for 15 min on ice. Samples were centrifuged at 30,000 × *g* at 4 °C for 20 min, and the soluble fraction was used as cell lysate. Effects of CHIP overexpression on the ubiquitylation of BAG-1 in HEK 293 cells were analyzed with stably transfected cells. For this purpose the CHIP-expressing plasmid was linearized before transfection, and stably transfected cells were finally selected in the presence of 1,200 μg/ml Geneticin (Invitrogen). After selection stably transfected cells were cultivated in medium containing 600 μg/ml Geneticin.

Immunoprecipitations—For immunoprecipitations of BAG-1 isoforms under denaturing conditions, HEK 293 cells were resuspended in buffer B (50 mM Tris-HCl, pH 7.4, 5 mM EDTA, 1% SDS, 5 mM dithiothreitol). The cell suspension was boiled for 5 min and diluted with 9 volumes of buffer C (50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 5 mM EDTA, 1% Triton X-100) containing Complete protease inhibitor. The diluted lysate was passaged repeatedly through a thin needle to reduce the viscosity of the solution and cleared by ultracentrifugation at 100,000 × *g* at 4 °C for 30 min. To the resultant supernatant 2 μg of anti-BAG-1 antibody or control IgG was added, and samples were incubated for 1 h at 4 °C. After the addition of protein G-Sepharose, incubation was continued for an additional hour. Protein G-Sepharose-absorbed immunocomplexes were collected by centrifugation and washed five times with RIPA buffer followed by elution of immunocomplexes in SDS-PAGE loading buffer. Samples were analyzed by SDS-PAGE and immunoblotting.

For coimmunoprecipitation of Hsc/Hsp70-associated BAG-1, transiently transfected HeLa cells were lysed in 25 mM Tris-HCl, pH 7.5, 100 mM KCl (buffer D) containing 0.5% Tween 20, 2 mM EDTA, and Complete protease inhibitor. Lysates were centrifuged for 10 min at 30,000 × *g* at 4 °C, and to the supernatant fractions 0.04 μg/μl anti-Hsc/Hsp70 or control IgG was added. Samples were incubated for 1 h at 4 °C, followed by the addition of protein G-Sepharose and further incubation for 1 h. Immunocomplexes were collected by centrifugation, washed four times with buffer D containing 0.5% Tween 20, once with buffer D lacking detergent, and bound BAG-1 was eluted by incubation for 15 min at 30 °C in buffer D containing 1 mM ATP, 2 mM $MgCl_2$, and protease inhibitors. Protein G-Sepharose was collected by centrifugation, and ATP-eluted proteins were precipitated from the supernatant fraction by the addition of trichloroacetic acid. Precipitated protein was analyzed by SDS-PAGE and immunoblotting.

In Vitro Ubiquitylation—Purified BAG-1M (0.6 μg in a 10-μl reaction) was incubated for 2 h at 30 °C in the presence of 20% rabbit reticulocyte lysate (Promega) in 20 mM MOPS, pH 7.2, 50 mM KCl, 0.1 mM dithiothreitol, containing 10 mM $MgCl_2$, 5 mM ATP, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml aprotinin, 0.5 μg/ml leupeptin. When indicated, 10 μg of purified ubiquitin and 0.4 μg of ubiquitin aldehyde (Affiniti) were added. In initial ubiquitylation reactions that explored the ubiquitylation activity of CHIP, histidine-tagged CHIP was used. Purified human BAG-1M and BAG-1S (each 0.6 μg/10-μl reaction) were incubated in the presence of 0.1 μM rabbit E1, 4 μM UbcH5b, 2 μM His-CHIP, and 2.5 μg/μl ubiquitin in 20 mM MOPS, pH 7.2, 100 mM KCl, 5 mM $MgCl_2$, 10 mM dithiothreitol, and 1 mM phenylmethylsulfonyl

fluoride (buffer E) for 2 h at 30 °C. Samples were analyzed by SDS-PAGE and immunoblotting with anti-BAG-1 antibody. To analyze ubiquitylation of the 4K-R mutant of BAG-1M, a soluble extract from BL21 (DE3) cells expressing the recombinant protein was prepared by sonication in buffer A and centrifugation at 100,000 × *g* for 30 min. 30 μg of the bacterial extract was used in the ubiquitylation assay. For ubiquitylation reactions that involved formation of Hsc70·BAG-1·CHIP complexes untagged CHIP was used to avoid steric hindrance. Purified human BAG-1S (2 μM) was incubated in the presence of 0.1 μM wheat E1, 8 μM UbcH5b, 3 μM CHIP, and 1.25 μg/μl ubiquitin (Sigma) in buffer E for 2 h at 30 °C. When indicated, ubiquitin was replaced by K48R ubiquitin at a concentration of 1.25 μg/μl, and purified Hsc70 and Hsp40 were added at a concentration of 3 and 0.3 μM, respectively. To analyze the linkage of the BAG-1-attached ubiquitin chain in more detail, ubiquitylation was performed as described above in the presence of soluble bacterial extracts containing point mutants of ubiquitin. Before the experiment, the ubiquitin concentration within the extracts was analyzed by immunoblotting, and the amount of extract added to the ubiquitylation reactions was adjusted so that similar amounts of ubiquitin and mutant ubiquitin were present during the reaction (about 0.3 μg/μl final concentration). Ubiquitylation in the presence of *N*-methylated ubiquitin (1.25 μg/μl final concentration) was performed at an increased concentration of conjugation components to counteract the reduced conjugation efficiency of the methylated protein, *i.e.* 0.3 μM wheat E1, 24 μM UbcH5b, and 6 μM CHIP were used. Samples were analyzed by SDS-PAGE and immunoblotting.

Hsc70 ATPase Domain Binding Assay—Binding of ubiquitylated BAG-1M to the ATPase domain of Hsc70 was assayed by previously established ATPase domain affinity chromatography (15). Prior to ATPase domain binding, *in vitro* ubiquitylation of BAG-1M was performed as described above in the presence of reticulocyte lysate and purified ubiquitin. After incubation for 2 h, a 5-fold volume of 20 mM MOPS, pH 7.2, 150 mM KCl, 5 mM EDTA (buffer F) was added, and samples were incubated in the presence of the ATPase domain affinity resin for 30 min at 4 °C. The affinity resins were washed four times with buffer F followed by boiling in SDS-PAGE sample buffer.

Pulse-Chase Experiments—COS-7 cells were transiently transfected with 1.5 μg of pcDNA3.1-*hbag-1S* and 2.5 μg of pcDNA3.1-*chip* or pcDNA3.1-*myc*/*his-lacZ*/35-mm dish. 24 h post-transfection cells were washed with phosphate-buffered saline and incubated for an additional hour at 37 °C in 1 ml of methionine-free medium. Metabolic labeling was performed for 1 h by the addition of 50 μCi/ml [^{35}S]methionine. The labeled cells were washed twice with phosphate-buffered saline and incubated further in medium supplemented with 25 μg/ml cycloheximide and 1 mM unlabeled methionine. At the indicated time points the cells were harvested, frozen immediately in fluid N_2, and stored at −80 °C. For immunoprecipitation, cells were thawed on ice and lysed in RIPA buffer supplemented with Complete protease inhibitor. Cell lysates were cleared by centrifugation at 30,000 × *g* at 4 °C for 20 min. Protein concentration of the lysates was determined, and equal amounts of lysate protein were incubated with 1 μg of anti-BAG-1 antibody at 4 °C for 1 h followed by the addition of protein G-Sepharose and an additional incubation period for 1 h at 4 °C. The Sepharose was collected by centrifugation and washed four times with RIPA buffer, prior to elution of bound proteins by boiling in SDS-PAGE loading buffer. Samples were separated on an SDS-polyacrylamide gel and analyzed using a PhosphorImager. Quantification of bands was carried out with OptiQuant software version 3.0 (Packard Instrument).

Proteasome Binding Assay—To analyze the effects of BAG-1 ubiquitylation on the association of the cofactor with the proteasome, 30-μl ubiquitylation reactions were performed in the presence of 6 μM BAG-1S, 6 μM C130, 0.1 μM wheat E1, 8 μM UbcH5b, 6 μM CHIP, 0.6 μM Hsp40, 6 μM Hsc70, and 2.5 μg/μl ubiquitin as indicated in buffer E for 2 h at 30 °C. Samples were cooled on ice followed by the addition of purified human 26 S proteasomes (16 μg/sample) and incubation for 1 h at 4 °C. Samples were diluted in 400 μl of 20 mM MOPS, pH 7.2, 100 mM KCl, 3 mM $MgCl_2$, 2 mM ATP, 1 mM phenylmethylsulfonyl fluoride, and split into two equal aliquots. One aliquot received 5 μg of anti-BAG-1 antibody, the other one the same amount of control IgG. Aliquots were incubated at 4 °C for 1 h, protein G-Sepharose was added, and incubation was continued at 4 °C for an additional hour. The Sepharose was collected by centrifugation and washed four times with buffer G (20 mM MOPS, pH 7.2, 100 mM KCl, 0.5% Tween 20) and once with buffer G lacking detergent. Bound proteasomes were dissociated from the immunocomplexes by the addition of detergent-free buffer G containing 2 mM $MgCl_2$, 1 mM ATP, and 1 mM phenylmethylsulfonyl fluoride and incubation at 30 °C for 20 min. The protein G-Sepharose was sedimented by centrifugation, and ATP-eluted proteins were precipitated from the supernatant fraction by the addition of trichloroacetic acid. Sepharose beads were washed again using buffer G followed by elution of BAG-1 through boiling in SDS-PAGE loading buffer. Samples were analyzed by SDS-PAGE and immunoblotting with specific antibodies.

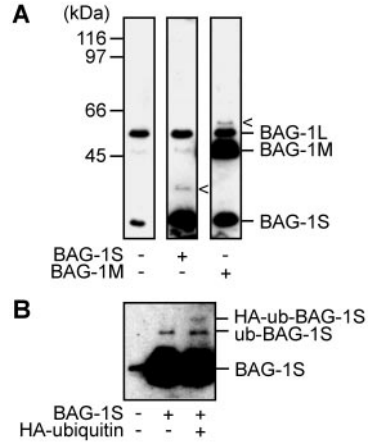

FIG. 1. **Ubiquitylated forms of BAG-1 can be detected *in vivo*.** *A*, HeLa cells were transiently transfected with vector constructs expressing β-galactosidase (*left lane*), BAG-1S (*center lane*), and BAG-1M (*right lane*), and BAG-1 isoforms were detected in cell lysates with an anti-BAG-1 antibody. Elevation of the intracellular levels of BAG-1S and BAG-1M allows the detection of covalently modified forms of the co-chaperones, consistent with an attachment of a single ubiquitin moiety (*arrowheads*). 40 μg of soluble protein extract was loaded per *lane*. *B*, coexpression of BAG-1S and hemagglutinin-tagged ubiquitin (*HA-ub*) results in the appearance of two modified forms of the co-chaperone caused by conjugation of endogenous and tagged ubiquitin to BAG-1S.

RESULTS

Ubiquitylated Forms of BAG-1 Are Detectable in Vivo—BAG-1 exists as multiple isoforms in human HeLa cells (25). The most abundant isoforms are the nuclear BAG-1L (~55 kDa apparent molecular mass) and the cytosolic BAG-1M (~46 kDa) and BAG-1S (~36 kDa) (Fig. 1*A*). However, after transient expression of BAG-1M and BAG-1S in human HeLa cells, the anti-BAG-1 antibody specifically detected additional polypeptides that possibly arose from covalent modification of the isoforms (Fig. 1*A*, *arrowheads*). Modification led to an increase of the apparent molecular mass by about 10 kDa. It thus appeared conceivable that the isoforms were modified by attachment of a ubiquitin moiety. To verify this hypothesis a tagged form of ubiquitin carrying a hemagglutinin (HA) epitope was coexpressed with BAG-1S. Coexpression led to the appearance of a second modified form of BAG-1S, consistent with the attachment of both HA-tagged and endogenous ubiquitin (Fig. 1*B*). Apparently, the chaperone cofactor can be covalently modified by attachment of ubiquitin.

To exclude the possibility that BAG-1 ubiquitylation was only a consequence of transient overexpression, endogenous BAG-1 was immunoprecipitated from human HEK 293 cells and analyzed for the presence of ubiquitylated isoforms by subsequent immunoblotting using an anti-ubiquitin antibody. The immunoprecipitation was performed under denaturing conditions to remove BAG-1-associated ubiquitylated proteins (9). In this situation high molecular mass ubiquitin conjugates were still detectable in BAG-1 immunoprecipitates (Fig. 2). The data reveal modification of endogenous BAG-1 isoforms by multiple ubiquitin attachment *in vivo*.

The Ubiquitin Ligase CHIP Can Mediate Ubiquitylation of BAG-1 in Conjunction with UbcH5b—We sought to reconstitute BAG-1 ubiquitylation *in vitro*. As a first step toward this goal rabbit reticulocyte lysate was used as a source for the ubiquitin-activating enzyme E1, ubiquitin-conjugating enzymes, and ubiquitin ligases. In the presence of reticulocyte

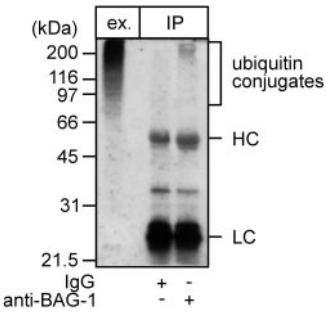

FIG. 2. **Polyubiquitin chains are attached to endogenous BAG-1.** To verify ubiquitylation of endogenous BAG-1, BAG-1 isoforms were immunoprecipitated from HEK 293 cell lysates under denaturing conditions. High molecular mass ubiquitin conjugates of the co-chaperone were specifically detected in anti-BAG-1 immunoprecipitates using an anti-ubiquitin antibody (*ex.* represents 30 μg of cellular lysate; *IP*, immunoprecipitates; *HC*, immunoglobulin heavy chain, *LC*, immunoglobulin light chain).

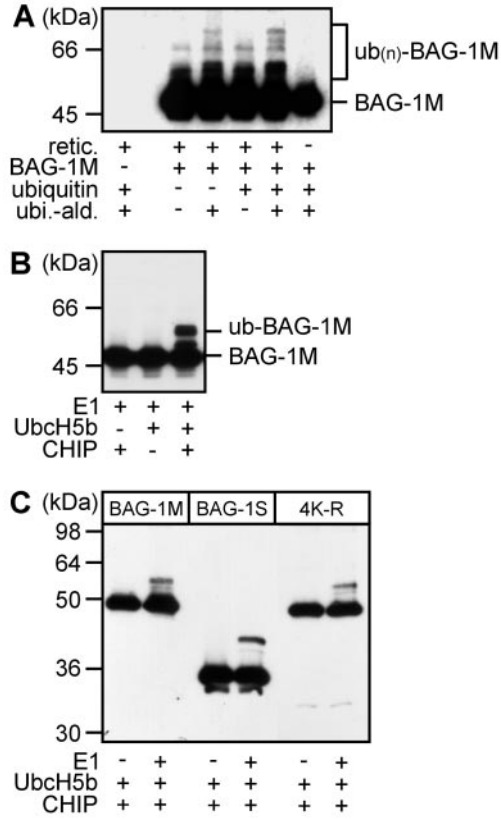

FIG. 3. **BAG-1 can be ubiquitylated in the presence of reticulocyte lysate and by the CHIP/UbcH5b ubiquitin conjugation system.** *A*, purified BAG-1M was incubated in the presence of rabbit reticulocyte lysate (*retic.*) as a source of ubiquitin conjugation components and ubiquitin, and covalently modified forms of the chaperone cofactor were detected using a BAG-1-specific antibody. Endogenous BAG-1 isoforms present in reticulocyte lysate are not detectable under the chosen conditions. Purified ubiquitin (*ubiquitin*) and ubiquitin aldehyde (*ubi.-ald.*) were added when indicated. *B*, ubiquitylation of BAG-1M was reconstituted with purified rabbit E1, human UbcH5b, and human CHIP. *C*, the CHIP/UbcH5b conjugation system mediated ubiquitin attachment to BAG-1M, BAG-1S, and 4K-R. The different forms of BAG-1 were detected using a specific antibody.

lysate, purified BAG-1M was modified by the attachment of ubiquitin moieties, and ubiquitylation was enhanced by the addition of purified ubiquitin and ubiquitin aldehyde, an inhibitor of ubiquitin isopeptidases (Fig. 3*A*). With experimental conditions for BAG-1 ubiquitylation established, we tested whether CHIP participates in the ubiquitylation process. CHIP was identified previously as a ubiquitin ligase that cooperates with Ubc4/5 ubiquitin-conjugating enzymes and binds BAG-1 (9, 10, 12, 26). Indeed, CHIP was able to mediate ubiquitin attachment to different BAG-1 isoforms and mutant forms of the co-chaperone in conjunction with UbcH5b (Fig. 3, *B* and *C*). Singly modified forms of BAG-1 were detectable under the chosen experimental conditions. However, ubiquitylation efficiency was increased strongly in the presence of Hsc/Hsp70, as will be shown later in this study.

Ubiquitin Attachment to BAG-1 Does Not Involve the Integrated Ubiquitin-like Domain of the Co-chaperone—Conceivably, the integrated ubiquitin-like domain of BAG-1 may function as an acceptor site for ubiquitin attachment. The domain comprises four lysine residues, one of which corresponds to lysine 48 in ubiquitin that is used for polyubiquitin chain formation. We therefore constructed a mutant form of BAG-1M, 4K-R, which lacked all four lysine residues within its ubiquitin-like domain because of lysine to arginine substitutions. Unexpectedly, the mutant form was still modified by ubiquitin attachment when incubated with the CHIP/UbcH5b conjugation machinery in the presence of E1 (Fig. 3*C*). Apparently, lysine residues outside the ubiquitin-like domain are utilized by the conjugation machinery.

Ubiquitylation of BAG-1 Does Not Interfere with Binding of the Cofactor to Hsc/Hsp70—We analyzed whether ubiquitylation of BAG-1 affects binding of the cofactor to Hsc/Hsp70. To address this question, ubiquitylated BAG-1M was generated *in vitro*, and binding to the ATPase domain of Hsc70 was analyzed by affinity chromatography. Ubiquitylated BAG-1M was retained on the affinity resin (Fig. 4*A*). It thus appears unlikely that the ubiquitylation of BAG-1 alters the interaction of the cofactor with Hsc/Hsp70. This conclusion was supported further by immunoprecipitation experiments. When Hsc/Hsp70 immunocomplexes were isolated from HeLa cells overexpressing BAG-1S, ubiquitylated cofactor was detectable in association with the chaperones (Fig. 4*B*). Ubiquitylation of BAG-1 may thus affect not only the cofactor itself, but may have functional consequences for the cofactor-chaperone complex that exists in mammalian cells.

CHIP Promotes Ubiquitylation of BAG-1 in Vivo—The experiments described above suggested a role of CHIP in mediating ubiquitylation of BAG-1 in the cellular milieu. We therefore investigated how CHIP expression affects BAG-1 ubiquitylation in cell culture experiments. For this purpose, CHIP was overexpressed in HEK 293 cells. Overexpression did not alter the amount of ubiquitylated proteins detectable in cellular extracts (Fig. 5). However, a significant increase of ubiquitylated BAG-1 isoforms was detectable after immunoprecipitation of the cofactor under denaturing conditions. Consistent with the *in vitro* data shown above, the CHIP ubiquitin ligase promotes ubiquitylation of BAG-1 *in vivo*.

Hsc70 Increases the Processivity of BAG-1 Ubiquitylation—We noted that CHIP primarily induced the formation of mono- or diubiquitylated BAG-1 under *in vitro* conditions, whereas overexpression of CHIP in cell lines stimulated polyubiquitylation of the co-chaperone (Figs. 3 and 5). This suggested a participation of additional proteins during ubiquitylation. In this regard it is noteworthy that BAG-1 and CHIP can associate simultaneously with Hsc/Hsp70 (9). We hypothesized that formation of the ternary chaperone-cofactor complex promotes recognition of BAG-1 by the CHIP ubiquitin ligase and may thus increase the efficiency of ubiquitylation. To verify this hypothesis, Hsc70 and the co-chaperone Hsp40 were added to *in vitro* ubiquitylation reactions. Hsp40 was included because BAG-1 preferentially binds to the ADP-bound state of Hsc70, which, in the presence of ATP, is attained through

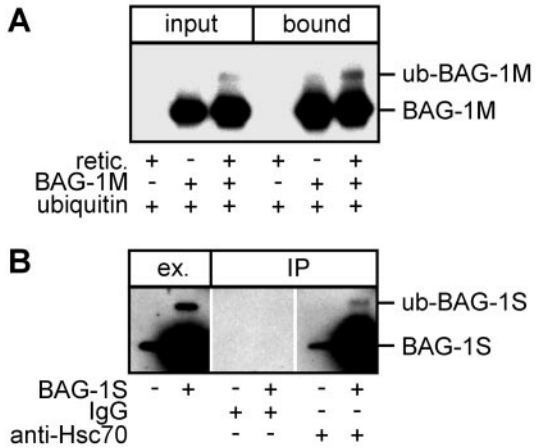

FIG. 4. **Ubiquitin attachment to BAG-1 does not interfere with Hsc/Hsp70 binding.** *A*, after *in vitro* ubiquitylation of BAG-1M in rabbit reticulocyte lysate (*retic.*), an association with the ATPase domain of Hsc70 immobilized on activated Sepharose beads was investigated. Ubiquitylated BAG-1M was found associated with the Hsc70 ATPase domain (*input* corresponds to 3.3% of the volume of the samples after the ubiquitylation reaction; *bound* represents 6.6% of the volume of the SDS-eluates from the ATPase domain affinity resin). *B*, after overexpression of BAG-1S in HeLa cells (*BAG-1S*), ubiquitylated BAG-1S (*ub-BAG-1S*) was coprecipitated with an anti-Hsc/Hsp70 antibody (*anti-Hsc70*). Immunoprecipitated proteins were eluted from Hsc/Hsp70 with ATP, and ATP-eluates were probed with an anti-BAG-1 antibody. When indicated, mouse IgGs were used as control antibody (*IgG*).

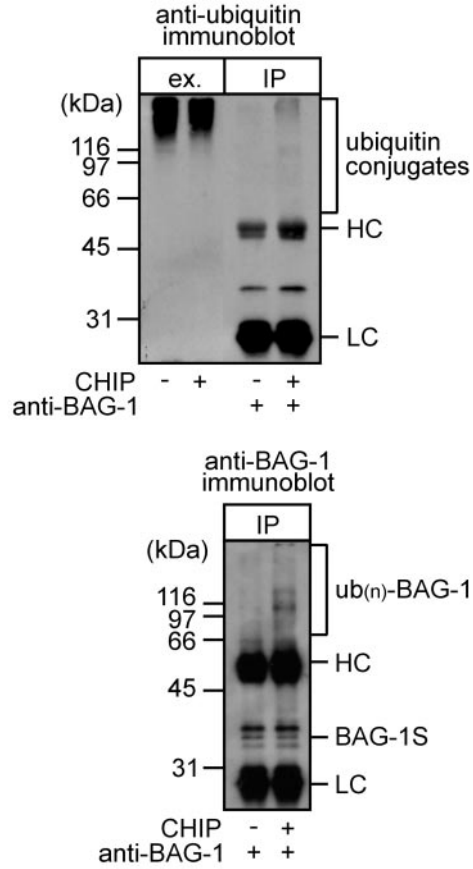

FIG. 5. **Overexpression of CHIP induces the polyubiquitylation of endogenous BAG-1 *in vivo*.** CHIP-overexpressing HEK 293 cells were lysed under denaturing conditions and BAG-1 isoforms immunoprecipitated from soluble lysates. Ubiquitylated forms of BAG-1 were detected in immunoprecipitates using an anti-ubiquitin (*upper panel*) and an anti-BAG-1 antibody (*lower panel*) (*ex.* represents 30 μg of soluble lysate; *IP*, anti-BAG-1 immunoprecipitates; *HC*, immunoglobulin heavy chain; *LC*, immunoglobulin light chain).

stimulation of Hsc70 ATPase activity by Hsp40. Indeed, addition of Hsc70 and Hsp40 resulted in a strong stimulation of BAG-1 ubiquitylation mediated by CHIP, and the attachment of multiple ubiquitin moieties to BAG-1 became apparent (Fig. 6*A*).

CHIP Mediates the Formation of a Lysine 11-linked Polyubiquitin Chain on BAG-1, Which Does Not Serve as a Degradation Signal—We wanted to test whether multiple lysine residues within BAG-1 are used for ubiquitin attachment. Therefore, ubiquitin was replaced by *N*-methylated ubiquitin that is unable to form polyubiquitin chains. In this situation, only a singly modified form of BAG-1 was detectable even after prolonged exposure (Fig. 6*B*). This indicates polyubiquitylation of BAG-1 at a single lysine residue. The architecture of the BAG-1-attached polyubiquitin chain was analyzed using several point mutants of ubiquitin. Replacement of ubiquitin by a mutant form with a lysine to arginine substitution at position 48 did not inhibit polyubiquitin chain formation (Fig. 6, *A* and *C*). Similar results were obtained when a triple mutant with arginine substitutions at positions 29, 48, and 63 was used (Fig. 6*C*). CHIP apparently mediates the formation of a noncanonical polyubiquitin chain on BAG-1 with linkages involving lysine residues other than lysines 29, 48, and 63. This was confirmed when ubiquitin mutants engineered to carry only a single lysine residue were utilized. The addition of lysines 29, 48, and 63, respectively, did not result in polyubiquitylation of BAG-1 (Fig. 6*C*). In contrast, in the presence of a mutant form of ubiquitin in which only lysine 11 was maintained, efficient polyubiquitylation of BAG-1 was observed. Apparently, ubiquitin moieties attached to BAG-1 by the CHIP conjugation machinery are mainly linked through lysine 11. Although the function of lysine 11-linked chains is largely enigmatic, a previous report revealed a role of such chains in proteasomal degradation when attached to an artificial model substrate (27). However, we observed that overexpression of CHIP in COS-7 cells did not significantly alter the stability of BAG-1 (Fig. 7). CHIP-mediated attachment of a lysine 11-linked ubiquitin chain to BAG-1 apparently serves functions other than targeting the chaperone cofactor for degradation.

CHIP-mediated Polyubiquitylation of BAG-1 Stimulates the Association of BAG-1 with the Proteasome—It was revealed previously that BAG-1 associates with the proteasome via its integrated ubiquitin-like domain (15). We reasoned that CHIP-mediated ubiquitylation of BAG-1 provides an additional sorting signal to stimulate proteasomal association of the chaperone cofactor in a degradation-independent manner. Therefore, the effects of CHIP on the association of BAG-1 with the proteasome were analyzed. Because CHIP is also able to induce the ubiquitylation of Hsc70- and BAG-1-bound proteins (9), *in vivo* experiments were considered inconclusive, and thus binding assays with purified components were performed. In these experiments a deletion fragment of BAG-1, C130, was also used. C130 covers the carboxyl-terminal 130 amino acids of the cofactor and thus lacks the integrated ubiquitin-like domain. Yet, the fragment retains the ability to bind and regulate Hsc/Hsp70 (22). Both BAG-1 and C130 were ubiquitylated efficiently in the presence of the Hsc70/Hsp40 chaperone system and the ubiquitin conjugation machinery comprising E1, CHIP, and UbcH5b (Fig. 8*A*). Using point mutants of ubiquitin we confirmed that the ubiquitin moieties of the C130-attached chain are mainly linked through lysine 11, as observed for full-length BAG-1 (data not shown). Association with the proteasome was monitored after the addition of purified human proteasomes and subsequent isolation of BAG-1 complexes by immunoprecipitation. In the absence of ubiquitylation (minus

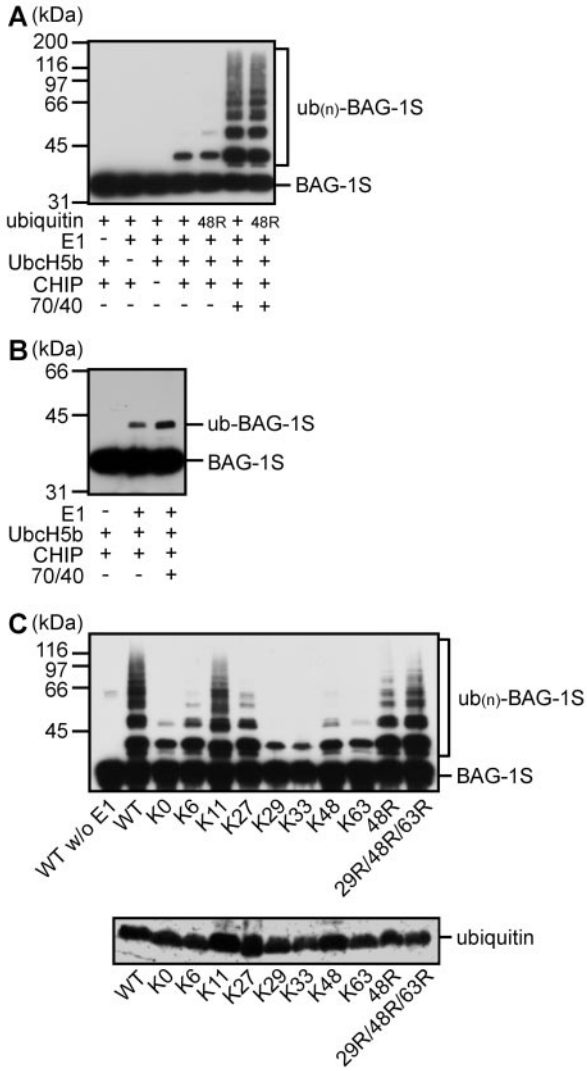

FIG. 6. **Hsc70 stimulates CHIP-mediated ubiquitylation of BAG-1, resulting in the attachment of a single lysine 11-linked polyubiquitin chain to the co-chaperone.** *A*, *in vitro* ubiquitylation reactions were performed using purified BAG-1S as a substrate. When indicated, purified ubiquitin, a lysine 48 to arginine mutant form of ubiquitin (*48R*), purified wheat E1, human UbcH5b, human CHIP, rat Hsc70 (*70*), and human Hsp40 (*40*) were added. Ubiquitylation of BAG-1S was analyzed by immunoblotting with an anti-BAG-1 antibody. *B*, *in vitro* ubiquitylation assays were performed using *N*-methylated ubiquitin that blocks polyubiquitin chain formation. *C*, point mutants of ubiquitin were used in *in vitro* ubiquitylation assays, which completely lacked all lysine residues (*K0*) or possessed only a single lysine residue (*K6*, *K11*, *K27*, *K29*, *K33*, *K48*, and *K63*). *29R/48R/63R* represent a triple mutant of ubiquitin with lysine to arginine substitutions at the indicated positions. A sample that did not receive the ubiquitin-activating enzyme E1 was used as a control (*WT w/o E1*). Ubiquitylated forms of BAG-1S were detected by immunoblotting. The *lower panel* shows the amount of ubiquitin and its mutant forms present during the ubiquitylation reaction. Similar amounts of the different forms were added. Ubiquitin was detected with a specific antibody.

E1 controls), proteasome was found associated with BAG-1 but not with C130 (Fig. 8*B*). This is consistent with our previous observation that the integrated ubiquitin-like domain of the cofactor is required for proteasome binding (15). The amount of proteasome detectable in association with BAG-1 was increased significantly after CHIP-mediated ubiquitylation of the chaperone cofactor (Fig. 8*B*, compare the *last two lanes*). The lysine 11-linked polyubiquitin chain on BAG-1 apparently stimulates binding of the cofactor to the proteolytic complex. Remarkably, however, proteasome binding was not observed

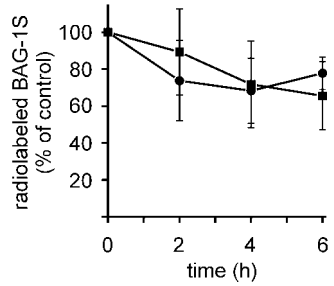

FIG. 7. **CHIP does not induce BAG-1 degradation.** COS-7 cells expressing BAG-1S and CHIP (*circles*), or BAG-1S alone (*squares*) were labeled with [^{35}S]methionine and chased for the indicated time. BAG-1S was immunoprecipitated using anti-BAG-1 antibody, and the amount of radiolabeled co-chaperone was quantified with a PhosphorImager. Data represent the means of four experiments ± S.D. The amount of BAG-1 detectable at time point zero was set to 100%.

for ubiquitylated C130. The polyubiquitin chain alone is apparently unable to induce detectable binding of the BAG-1 mutant form to the proteolytic complex, but it enhances binding to the proteasome when presented together with the integrated ubiquitin-like domain in the full-length protein. Our data thus suggest a cooperative recognition of the two sorting signals during proteasomal targeting of BAG-1.

It has to be pointed out that Hsc70 was also ubiquitylated efficiently by CHIP and UbcH5b under the chosen experimental conditions (Fig. 8*A*). Similar to our findings for BAG-1, CHIP-mediated ubiquitylation of Hsc70 results in the attachment of a noncanonical ubiquitin chain to the chaperone (10). It was therefore conceivable that ubiquitylation of Hsc70 might contribute to the formation of BAG-1·proteasome complexes in our binding assay. However, ubiquitylated Hsc70 did not induce an association of C130 with the proteasome (Fig. 8*B*). The polyubiquitin chain attached to Hsc70 is apparently unable to induce detectable binding of BAG-1 to the proteasome on its own. Yet, our data do not exclude a possible stimulatory role of the Hsc70-attached chain when presented to the proteasome together with the integrated ubiquitin-like domain of BAG-1 within a ternary Hsc70·BAG-1·CHIP complex. A cooperative binding of sorting signals by the proteasome may thus occur between the integrated ubiquitin-like domain of BAG-1 and noncanonical polyubiquitin chains attached to BAG-1 and Hsc/Hsp70.

DISCUSSION

The chaperone cofactors BAG-1 and CHIP were shown previously to team up to switch the activity of Hsc70 and Hsp70 from protein folding to protein degradation (9). The data presented here reveal a new level of functional cooperation between the two cofactors by demonstrating CHIP-mediated ubiquitylation of BAG-1 and subsequent increased targeting to the proteasome that does not result in BAG-1 degradation. In conjunction with ubiquitin-conjugating enzymes of the Ubc4/5 family, CHIP mediates attachment of a lysine 11-linked polyubiquitin chain to BAG-1. Although polyubiquitin chains linked through lysine 48 serve as degradation signals, the function of noncanonical chains with other linkages is less clear (7). There is some evidence for a role of lysine 29-linked chains in proteasomal degradation (for review, see Ref. 7), whereas lysine 63-linked chains apparently mediate nondegradative functions during DNA repair, the stress response, and endocytosis (7, 28, and references therein) and affect ribosome activity in a degradation-independent fashion (29). So far the physiological role of lysine 11-linked chains has not been analyzed in detail. Baboshina and Haas (27) have shown that the ubiquitin-conjugating enzyme $E2_{EPF}$ is able to attach lysine 11-linked chains to itself in an *in vitro* autoubiquitylation reaction and targets

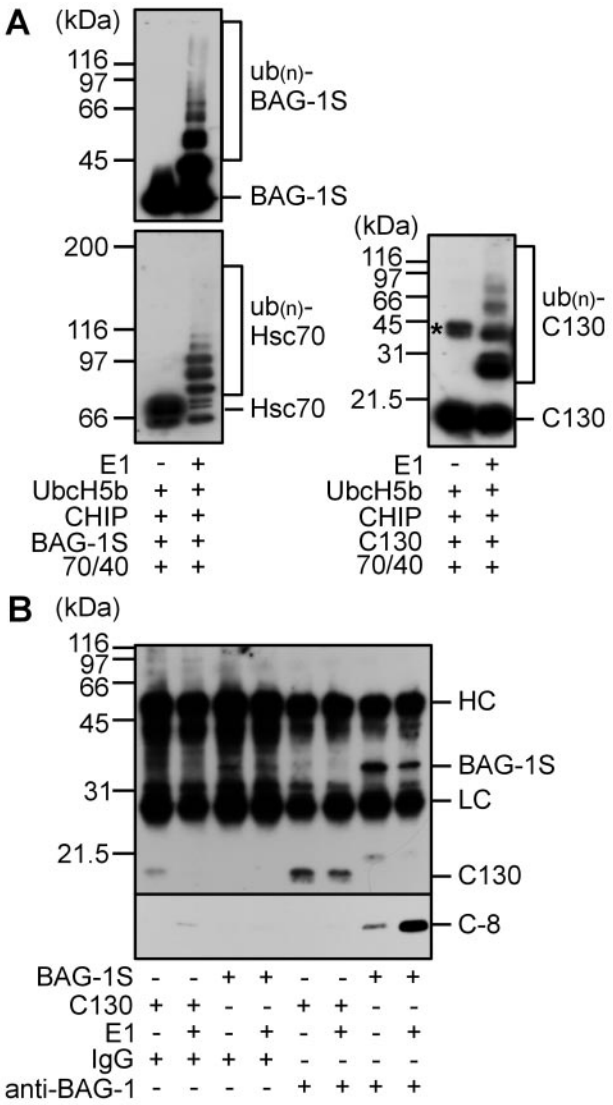

FIG. 8. **CHIP-mediated polyubiquitylation stimulates the association of BAG-1 with the proteasome.** *A*, *in vitro* ubiquitylation reactions were performed using purified BAG-1S, C130, Hsp40 (*40*), Hsc70 (*70*), E1, UbcH5b, CHIP, and ubiquitin as indicated. Samples that did not receive E1 were used as controls. Ubiquitin conjugation to BAG-1S, C130, and Hsc70 was analyzed by SDS-PAGE and immunoblotting with anti-BAG-1 and anti-Hsc/Hsp70 antibody. The *asterisk* marks a dimeric form of C130 which was not resolved during SDS-PAGE. *B*, *in vitro* ubiquitylation reactions were incubated with purified human proteasomes, and BAG-1S- and C130-associated proteins were immunoprecipitated using an anti-BAG-1 antibody. Unrelated rabbit IgG was used as control antibody. Associated proteasomes were detected in ATP-eluates from isolated immunocomplexes using an antibody against the catalytic core subunit C-8 (*C-8*). Immunoprecipitated BAG-1S and C130 were detected by immunoblotting with an anti-BAG-1 antibody. Ubiquitylated forms of BAG-1S and C130 are masked by the light and heavy chain of the antibody (*LC* and *HC*, respectively).

reduced carboxymethylated bovine serum albumin for proteasomal degradation by attachment of a lysine 11-linked chain in an *in vitro* assay. Our data confirm that such a noncanonical chain is involved in proteasomal targeting. However, when attached to BAG-1 the lysine 11-linked chain does not act as a degradation signal but participates in the degradation-independent sorting of BAG-1 to the proteasome.

Degradation-independent sorting mediated by CHIP is not restricted to BAG-1. The chaperones Hsc70 and Hsp70 are major cellular targets of CHIP, and also in this case the ubiquitin ligase mediates attachment of a noncanonical polyubiquitin chain to the chaperones without inducing degradation (10). Although the linkages of the Hsc/Hsp70-attached polyubiquitin chain remain to be determined in detail, BAG-1 and Hsc/Hsp70 are apparently affected similarly by the ubiquitylation activity of CHIP. This is even more remarkable as the chaperones and the two cofactors assemble to form a cellular complex that mediates the sorting of chaperone substrates to the proteasome (9). In fact, efficient ubiquitylation of BAG-1 was only observed upon complex assembly, illustrating the tight cooperation of Hsc/Hsp70, BAG-1, and CHIP (Fig. 6). We would therefore like to postulate that CHIP-induced attachment of noncanonical polyubiquitin chains to BAG-1 and Hsc/Hsp70 is an integral part of the sorting process mediated by the ternary chaperone-cofactor complex. In support of this hypothesis, we show here that CHIP-induced ubiquitylation stimulates an association of BAG-1 with the proteasome.

It remains to be elucidated why Hsc/Hsp70 and BAG-1 are not degraded when sorted to the proteasome through CHIP-induced polyubiquitylation, in contrast to chaperone substrates like the glucocorticoid hormone receptor (8). Possibly structural features of the chaperone and the cofactor may render the two proteins rather resistant to proteasomal degradation. On the other hand, the ubiquitin ligase Siah was shown recently to induce the proteasomal degradation of BAG-1 in the olfactory neuroepithelium (30). This illustrates that BAG-1 can be degraded by the proteasome in principle. It thus appears likely that a combination of structural features of the modified protein and of the attached type of polyubiquitin chain determine the degradation efficiency.

We used a deletion fragment of BAG-1, C130, to elucidate mechanistic aspects of the observed proteasome association. C130 covers the carboxyl-terminal 130 residues of the chaperone cofactor. It lacks the integrated ubiquitin-like domain but retains the ability to bind and regulate Hsc70 (22). In contrast to BAG-1, C130 did not bind to the proteasome (Fig. 8*B*). This is in agreement with the previously proposed essential role of the integrated ubiquitin-like domain of BAG-1 in proteasomal sorting (15). Surprisingly, however, ubiquitylation of C130 did not induce detectable binding to the proteasome. The data emphasize the functional importance of the integrated ubiquitin-like domain of BAG-1 for proteasomal targeting of the chaperone cofactor. Simultaneous recognition of the ubiquitin-like domain and the cofactor-attached polyubiquitin chain is apparently necessary to obtain an increased association with the proteasome upon CHIP-induced ubiquitylation.

On a molecular level such a cooperative recognition of proteasomal sorting signals might be best explained if one assumes two distinct but communicating binding sites within the 19 S regulatory complex of the proteasome. Different subunits of the 19 S regulatory complex are currently discussed as ubiquitin receptors of the proteasome (31–37). The S5a/Rpn10 subunit was initially identified as a polyubiquitin receptor and was later shown to bind also integrated ubiquitin-like domains for example of the human ubiquitin domain proteins hHR23 and hPLIC-2 (31, 32, 34, 35). Moreover, two distinct ubiquitin binding domains were identified in S5a, of which only one is used for ubiquitin domain protein recognition (32, 34). However, the physiological significance of these findings is currently a matter of debate. Yeast S5a is not an essential protein (36), and Pickart and colleagues (33) revealed that S5a in the context of the assembled 19 S complex does not recognize polyubiquitin chains efficiently. They provided evidence for a function of the S6′/Rpt5 subunit of the regulatory complex as a ubiquitin chain receptor. In addition, a different subunit of the regulatory complex, Rpn1, was identified recently as a receptor for integrated ubiquitin-like domains (37). The existence of distinct binding sites for ubiquitin-like domains and polyubiq-

uitin chains within the 19 S regulatory particle may thus provide the molecular basis for the observed cooperative recognition of sorting signals at the proteasome.

What might be the advantage of attaching polyubiquitin chains to delivery factors with regard to the proteasomal degradation of the delivered substrate protein? Notably, degradation mediated by the proteasome is considered a delicately timed process (7, 38). The polyubiquitin chain on the substrate protein is subjected to deubiquitylation by ubiquitin isopeptidases present in the regulatory complex of the proteasome. If the chain is trimmed before the substrate is unfolded and inserted into the catalytic core, it is likely to escape unharmed. It is therefore not surprising that cellular mechanisms have evolved to increase degradation efficiency. For example, several ubiquitin ligases were recently found associated with the proteasome, suggesting tight coupling of ubiquitylation and degradation (38–40). Ubiquitin ligase-mediated binding of substrates in the vicinity of the proteasome is likely to increase the time available for substrate processing at the proteasome. On the other hand, a reduction of the necessary processing may be achieved by the delivery of non-native substrates that can be inserted directly into the proteasomal core. The Cdc48 AAA ATPase apparently fulfills such a chaperone-like function during proteasomal sorting (41–45), and also the chaperone·BAG-1·CHIP complex is ideally suited for the delivery of non-native substrates. Attachment of polyubiquitin chains to the delivery factors Hsc/Hsp70 and BAG-1 may be considered as another means to increase the efficiency of the degradation process. Additional ubiquitin chains in close proximity to the modified substrate may reduce deubiquitylation activity on the substrate and may consequently prevent premature release of the substrate. Multiple polyubiquitin chains presented by a delivery complex and the bound substrate may thus ensure efficient degradation.

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REFERENCES

1. Wickner, S., Maurizi, M. R., and Gottesman, S. (1999) *Science* **286,** 1888–1893
2. McClellan, A. J., and Frydman, J. (2001) *Nature Cell Biol.* **3,** E1–E3
3. Höhfeld, J., Cyr, D. M., and Patterson, C. (2001) *EMBO Rep.* **2,** 885–890
4. Cyr, D. M., Höhfeld, J., and Patterson, C. (2002) *Trends Biochem. Sci.* **27,** 368–375
5. Varshavsky, A. (1997) *Trends Biochem. Sci.* **22,** 383–387
6. Jentsch, S., and Pyrowolakis, G. (2000) *Trends Cell Biol.* **10,** 335–342
7. Pickart, C. M. (2000) *Trends Biochem. Sci.* **25,** 544–548
8. Connell, P., Ballinger, C. A., Jiang, J., Wu, Y., Thompson, L. J., Höhfeld, J., and Patterson, C. (2001) *Nature Cell Biol.* **3,** 93–96
9. Demand, J., Alberti, S., Patterson, C., and Höhfeld, J. (2001) *Curr. Biol.* **11,** 1569–1577
10. Jiang, J., Ballinger, C. A., Wu, Y., Dai, Q., Cyr, D. M., Höhfeld, J., and Patterson, C. (2001) *J. Biol. Chem.* **276,** 42938–42944
11. Meacham, G. C., Patterson, C., Zhang, W., Younger, J. M., and Cyr, D. M. (2001) *Nature Cell Biol.* **3,** 100–105
12. Murata, S., Minami, Y., Minami, M., Chiba, T., and Tanaka, K. (2001) *EMBO Rep.* **2,** 1133–1138
13. Ballinger, C. A., Connell, P., Wu, Y., Hu, Z., Thompson, L. J., Yin, L.-Y., and Patterson, C. (1999) *Mol. Cell. Biol.* **19,** 4535–4545
14. Aravind, L., and Koonin, E. V. (2000) *Curr. Biol.* **10,** R132–R134
15. Lüders, J., Demand, J., and Höhfeld, J. (2000) *J. Biol. Chem.* **275,** 4613–4617
16. Höhfeld, J., and Jentsch, S. (1997) *EMBO J.* **16,** 6209–6216
17. Sondermann, H., Scheufler, C., Schneider, C., Höhfeld, J., Hartl, F.-U., and Moarefi, I. (2001) *Science* **291,** 1553–1557
18. Gassler, C. S., Wiederkehr, T., Brehmer, D., Bukau, B., and Mayer, M. P. (2001) *J. Biol. Chem.* **276,** 32538–32544
19. Wiederkehr, T., Bukau, B., and Buchberger, A. (2002) *Curr. Biol.* **12,** R26–R28
20. You, J., Cohen, R. E., and Pickart, C. M. (1999) *BioTechniques* **27,** 950–954
21. Lüders, J., Demand, J., Schönfelder, S., Frien, M., Zimmermann, R., and Höhfeld, J. (1998) *Biol. Chem. Hoppe-Seyler* **379,** 1217–1226
22. Lüders, J., Demand, J., Papp, O., and Höhfeld, J. (2000) *J. Biol. Chem.* **275,** 14817–14823
23. Scheffner, M., Huibregtse, J. M., Vierstra, R. D., and Howley, P. M. (1993) *Cell* **75,** 495–505
24. Emmerich, N., Nussbaum, A. K., Stevanovic, S., Priemer, M., Toes, R. E., Rammensee, H. G., and Schild, H. (2000) *J. Biol. Chem.* **275,** 21140–21148
25. Takayama, S., Krajewski, S., Krajewska, M., Kitada, S., Zapata, J. M., Kochel, K., Knee, D., Scudiero, D., Tudor, G., Miller, G. J., Miyashita, T., Yamada, M., and Reed, J. C. (1998) *Cancer Res.* **58,** 3116–3131
26. Hatakeyama, S., Yada, M., Matsumoto, M., Ishida, N., and Nakayama, K. I. (2001) *J. Biol. Chem.* **276,** 33111–33120
27. Baboshina, O. V., and Haas, A. L. (1996) *J. Biol. Chem.* **271,** 2823–2831
28. Hofmann, R. M., and Pickart, C. M. (2001) *J. Biol. Chem.* **276,** 27936–27943
29. Spence, J., Gali, R. R., Dittmar, G., Sherman, F., Karin, M., and Finley, D. (2000) *Cell* **102,** 67–76
30. Sourisseau, T., Desbois, C., Debure, L., Bowtell, D. D. L., Cato, A. C. B., Schneikert, J., Moyse, E., and Michel, D. (2001) *J. Cell Sci.* **114,** 1409–1416
31. Deveraux, Q., Ustrell, V., Pickart, C., and Rechsteiner, M. (1994) *J. Biol. Chem.* **269,** 7059–7061
32. Young, P., Deveraux, Q., Beal, R. E., Pickart, C. M., and Rechsteiner, M. (1998) *J. Biol. Chem.* **273,** 5461–5467
33. Lam, Y. A., Lawson, T. G., Velayutham, M., Zweier, J. L., and Pickart, C. M. (2002) *Nature* **416,** 763–767
34. Hiyama, H., Yokoi, M., Masutani, C., Sugasawa, K., Maekawa, T., Tanaka, K., Hoeijmakers, J. H. J., and Hannoka, F. (1999) *J. Biol. Chem.* **274,** 28019–28025
35. Walters, K. J., Kleijnen, M. F., Goh, A. M., Wagner, G., and Howley, P. M. (2002) *Biochemistry* **41,** 1767–1777
36. van Nocker, S., Sadis, S., Rubin, D. M., Glickman, M., Fu, H., Coux, O., Wefes, I., Finley, D., and Vierstra, R. D. (1996) *Mol. Cell. Biol.* **16,** 6020–6028
37. Elsasser, S., Gali, R. R., Schwickart, M., Larsen, C. N., Leggett, D. S., Müller, B., Feng, M. T., Tübing, F., Dittmar, G. A. G., and Finley, D. (2002) *Nature Cell Biol.* **4,** 725–730
38. Xie, Y., and Varshavsky, A. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 2497–2502
39. Kleijnen, M. F., Shih, A. H., Zhou, P., Kumar, S., Soccio, R. E., Kedersha, N. L., Gill, G., and Howley, P. M. (2000) *Mol. Cell* **6,** 409–419
40. You, J., and Pickart, C. M. (2001) *J. Biol. Chem.* **276,** 19871–19878
41. Koegl, M., Hoppe, T., Schlenker, S., Ulrich, H. D., Mayer, T. U., and Jentsch, S. (1999) *Cell* **96,** 635–644
42. Ye, Y., Meyer, H. H., and Rapoport, T. A. (2001) *Nature* **414,** 652–656
43. Braun, S., Matuschewski, K., Rape, M., Thoms, S., and Jentsch, S. (2002) *EMBO J.* **21,** 615–621
44. Jarosch, E., Taxis, C., Volkwein, C., Bordallo, J., Finley, D., Wolf, D. H., and Sommer, T. (2002) *Nature Cell Biol.* **4,** 134–139
45. Rabinovich, E., Kerem, A., Fröhlich, K. U., Diamant, N., and Bar-Nun, S. (2002) *Mol. Cell. Biol.* **22,** 626–634