**BAG-6 is essential for selective elimination of defective proteasomal substrates**

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**B**AG-6/Scythe/BAT3 is a ubiquitin-like protein that was originally reported to be the product of a novel gene located within the human major histocompatibility complex, although the mechanisms of its function remain largely obscure. Here, we demonstrate the involvement of BAG-6 in the degradation of a CL1 model defective protein substrate in mammalian cells. We show that BAG-6 is essential for not only model substrate degradation but also the ubiquitin-mediated metabolism of newly synthesized defective polypeptides. Furthermore, our in vivo and in vitro analysis shows that BAG-6 interacts physically with puromycin-labeled nascent chain polypeptides and regulates their proteasome-mediated degradation. Finally, we show that knockdown of BAG-6 results in the suppressed presentation of MHC class I on the cell surface, a procedure known to be affected by the efficiency of metabolism of defective ribosomal products. Therefore, we propose that BAG-6 is necessary for ubiquitin-mediated degradation of newly synthesized defective polypeptides.

**Introduction**

The significance of protein quality control is demonstrated by a number of human disorders, such as cystic fibrosis and Parkinson’s disease, which can result from the accumulation of misfolded proteins (Ward et al., 1995; Meacham et al., 1999; Ardley et al., 2003; Olzmann et al., 2007; Metzger et al., 2008; Nakamura and Lipton, 2009). Therefore, understanding the mechanisms of misfolded protein metabolism, as well as cotranslational degradation of defective proteins in mammalian cells (Qian et al., 2006), is of primary importance. Hydrophobic residues that are normally buried in a variety of natively folded proteins may be exposed by protein misfolding and act as degrons to deliver the misfolded protein mainly to the ubiquitin–proteasome system for degradation (Metzger et al., 2008). Thus, ubiquitin-mediated protein quality control is essential for monitoring protein folding and endowing misfolded and/or defective proteins for degradation/aggregation (Johnston et al., 1998; Schubert et al., 2000; Lelouard et al., 2004). In the ubiquitin system, recognition and recruitment of defective proteins to the degradation machinery is a key step in the selective elimination of aberrant proteins (Finley et al., 2004; Hartmann-Petersen and Gordon, 2004; Elsasser and Finley, 2005). Some cytosolic cochaperone proteins, such as CHIP, are known to be protein quality-control ubiquitin ligases that selectively target aberrant proteins for proteasomal degradation by promoting their ubiquitination (Meacham et al., 2001). In addition, targeting of polyubiquitinated substrates to the 26S proteasome is important because this process is thought to determine the final fate of defective proteins involved in various physiological and pathological reactions (Voges et al., 1999; Hershko et al., 2000; Madura, 2004; Hoeller et al., 2006). Previous studies have shown that the Rpn10 subunit of the 26S proteasome can recognize polyubiquitinated substrates, and it is thought to play a role as a ubiquitin receptor for the 26S proteasome (Deveraux et al., 1994; van Nocker et al., 1996; Kawahara et al., 2000; Wilkinson et al., 2001; Elsasser et al., 2004; Verma et al., 2004). Although it was believed that substrate recognition is an essential step in the proteasome-mediated degradation process, the role of Rpn10 remained obscure after the observation that its deletion in yeast and nematodes does not influence the viability of cells and causes...
Figure 1. BAG-6 is essential for CL1 degron-dependent proteasomal degradation. (A) Schematic representation of the 3xFlag-tagged EGFP protein fused with CL1 degron used in this study. (B) CL1 degron-associated proteins identified in this study. After transfection of a 3xFlag-tagged EGFP-CL1 expression vector, HeLa cells were treated with 5 µM MG132 for 4.5 h. Proteins immunoprecipitated with antibody against Flag were subjected to SDS-PAGE and PMF analysis. 3xFlag-tagged EGFP immunoprecipitates were used as a negative control. (C) Knockdown of BAG-6 suppressed the degradation of the CL1 degron substrate. 3xFlag-tagged EGFP-CL1 was expressed in HeLa cells with two distinct shRNA vectors for BAG-6 (siRNA-1 and siRNA-2) or control siRNA. After 60 h of shRNA treatment, whole-cell extracts were prepared and subjected to immunoblot analysis with antibodies against Flag, BAG-6, and actin. (D) Expression patterns of endogenous BAG-6 protein in various adult mouse tissues (top). The anti-Hsp70/Hsc70 blot confirmed equal protein loading (bottom). (E) MG132 treatment stimulated the formation of a larger BAG-6 complex. Extracts of NIH3T3 cells were subjected to gel filtration with Superose 6, and the fractions were subjected to Western blotting with specific antibodies against BAG-6.
only mild or highly specific phenotypes (van Nocker et al., 1996; Shimada et al., 2006). These observations suggested the existence of functionally redundant routes in the substrate recruitment system. Indeed, several proteins, such as Rad23p and Dsk2p, are involved in the delivery of polyubiquitinated substrates to the proteasome because they can bind simultaneously with polyubiquitinated substrates and the 26S proteasome (Kleijnen et al., 2000; Wilkinson et al., 2001; Chen and Madura, 2002; Elsasser et al., 2004). Accordingly, these gene products in the substrate delivery pathway may play a largely redundant role (Elsasser et al., 2004; Madura, 2004; Elsasser and Finley, 2005; Shimada et al., 2006). However, owing to this complex redundancy, a complete picture of substrate recognition and recruitment to the 26S proteasome in the ubiquitin system has not been adequately presented to date.

**BAG-6–Scythe–BAT3** was originally identified as a novel gene located within the human major histocompatibility complex (Banerji et al., 1990) that encodes the anti-apoptotic ubiquitin-like protein (Thress et al., 1998, 1999; Desmots et al., 2005; Kikukawa et al., 2005; Sasaki et al., 2007), although its function and mechanism of action remain largely obscure. Our previous analysis revealed that Scythe, a BAG-6–homologous protein expressed in *Xenopus* embryos (Thress et al., 1998), binds to the proteasomal Rpn10c subunit via the N-terminal 436 residues and that this region is required for apoptotic control in *Xenopus* embryos (Kikukawa et al., 2005; Minami et al., 2007). Furthermore, our recent analysis indicated that the N terminus of Scythe regulates ubiquitin-mediated proteolysis of a pro-apoptotic protein (Minami et al., 2007), although how BAG-6 is involved in the ubiquitin-mediated pathway remains a mystery to be clarified. In this study, we provide a set of evidence that BAG-6 is a novel proteasomal substrates-associated protein. Importantly, BAG-6–associated polyubiquitinated substrates are newly synthesized defective ribosomal products (DRiPs) and BAG-6 plays a crucial role in their metabolism. BAG-6 offers a protective role against cell death induced by the accumulation of aberrant proteins. Furthermore, we suggest that BAG-6 collaborates with immunoproteasomes to generate MHC class I presented antigenic peptides via targeted degradation of DRiPs and might play crucial roles in antigen presentation. These findings support the hypothesis that BAG-6 is a novel tethery factor that mediates selective elimination of defective nascent chain polypeptides in mammalian cells.

**Results**

**BAG-6 is essential for the CL1 degron-dependent degradation pathway**

The hydrophobic CL1 peptide is known to function as an unstable degron that can be targeted to the 26S proteasome in a ubiquitin-dependent manner in yeast (Gilon et al., 1998; Metzger et al., 2008). We prepared a chimeric protein that fused the CL1 degron to the C terminus of EGFP (designated EGFP-CL1) (Fig. 1 A) and confirmed that this fusion protein is highly unstable in HeLa cells and stabilized by proteasome inhibitors, such as MG132 (unpublished data), indicating that EGFP-CL1 is indeed metabolized by the ubiquitin–proteasome-dependent degradation pathway in mammalian cells. Our peptide MS fingerprint analysis of EGFP-CL1 immunoprecipitates from MG132-treated HeLa cell extracts revealed a series of CL1-associated proteins including various chaperones, translation-related proteins, subunits of the 26S proteasome, and BAG-6 (Fig. 1 B), suggesting that they might cooperatively participate in CL1 peptide metabolism. Among these proteins, we focused on BAG-6 as a novel key molecule for protein degradation because the *Xenopus* homologue of mammalian BAG-6 (called Scythe) has been reported to regulate apoptosis via the control of XEF1AO proteolysis in *Xenopus* embryos (Minami et al., 2007). To examine the function of mammalian BAG-6 in CL1 metabolism, we suppressed endogenous BAG-6 expression by shRNA. As shown in Fig. 1 C, only a partial knockdown of BAG-6 blocked EGFP-CL1 degradation, indicating that BAG-6 is essential for the ubiquitin–proteasome-mediated metabolism of EGFP-CL1 in HeLa cells (see also Fig. S1, A and B).

**BAG-6 is a novel 26S proteasome-associated protein**

To detect the endogenous BAG-6 protein, we prepared a specific antibody and found that mammalian BAG-6 was mainly expressed in the brain and lymphoid tissues (Fig. 1 D). With this antibody, we examined whether BAG-6 forms complexes with other cellular proteins. We found that the endogenous BAG-6 protein was eluted from gel filtration at a molecular weight of ~450 kD (Fig. 1 E, top). Unexpectedly, we found that treatment with MG132 stimulated the formation of a soluble, but much larger BAG-6 complex in HeLa cells (Fig. 1 E, bottom), and it apparently comigrated with 26S proteasomes, as determined by both gel filtration (Fig. S1 C) and glycerol density gradient fractionation (Fig. S1 D). Because we previously found that the BAG-6 homologue in *Xenopus* interacted with a proteasomal subunit, we further examined whether endogenous BAG-6 protein interacted with the 26S proteasomal complex. Our results clearly showed that the 26S proteasome associated with anti-BAG-6 immunoprecipitates (Fig. 1 F). Conversely, endogenous BAG-6 protein could be communoprecipitated by antibody against the Rpt6 subunit of the 26S proteasome (Fig. 1 G). In both precipitation experiments, the association between BAG-6 and the 26S proteasome was strengthened by treatment with MG132 (Fig. 1, F and G). These observations suggest that mammalian BAG-6 is a novel member of 26S proteasome-associated proteins.
cells (Fig. 2 A and Fig. S2 A). The precipitation of polyubiquitinated proteins was specific because preabsorption with an excess of BAG-6 antigen completely abolished the coimmunoprecipitation (Fig. 2 A). Immunoprecipitation of BAG-6 after denaturation with SDS never coprecipitated polyubiquitinated moieties (Fig. S2 B), revealing polyubiquitinated identity as BAG-6–bound proteins but not BAG-6 itself. Thus, BAG-6 appears not to be directly polyubiquitylated. Indeed, BAG-6 itself was stable in HeLa cells (Fig. S2, C–F).

**BAG-6 associates with polyubiquitinated proteasomal substrates**

To examine the function of BAG-6 in the ubiquitin-mediated protein degradation pathway, we next immunoprecipitated endogenous BAG-6 protein from HeLa cell extracts and blotted it with an antibody against ubiquitin. We found that a much larger amount of polyubiquitinated proteins coimmunoprecipitated with endogenous BAG-6 protein from extracts of MG132-treated cells (Fig. 2 A and Fig. S2 A). The precipitation of polyubiquitinated proteins was specific because preabsorption with an excess of antigen completely abolished the coimmunoprecipitation (Fig. 2 A). Immunoprecipitation of BAG-6 after denaturation with SDS never coprecipitated polyubiquitinated moieties (Fig. S2 B), revealing polyubiquitinated identity as BAG-6–bound proteins but not BAG-6 itself. Thus, BAG-6 appears not to be directly polyubiquitylated. Indeed, BAG-6 itself was stable in HeLa cells (Fig. S2, C–F). It has been reported that proteins of the BAG family interact with heat-shock proteins (HSPs) via the C-terminal BAG domain (Demand et al., 2001;
Takayama and Reed, 2001). It is conceivable that HSP substrate proteins might be modified by ubiquitin (Jiang et al., 2001) and thus coprecipitated with BAG-6. To determine whether polyubiquitinated species that coimmunoprecipitated with BAG-6 were proteins that bound with HSP, BAG-6 immunocomplexes were eluted by ATP (a procedure that stimulated the quantitative dissociation of Hsp70 from BAG-6; see Fig. 2 B, ATP elute). We found that polyubiquitinated proteins remained associated with BAG-6 after ATP elution but were released in the presence of SDS (Fig. 2 B, SDS elute). These results indicate that the majority of precipitated high molecular mass polyubiquitinated polypeptides are not Hsp70-associated client proteins or HSP itself. The results also suggest that a region other than the BAG domain is responsible for polyubiquitin coprecipitation.

We next determined the region of BAG-6 required for interaction with polyubiquitinated proteins (Fig. 2, C and D). A mutant form of BAG-6 lacking either the N-terminal UBL domain (by deletion of the N-terminal 148 residues, designated ΔUBL, Fig. 2 C) or the C-terminal BAG domain bound polyubiquitinated proteins as efficiently as did full-length (FL) BAG-6 (Fig. 2 D and unpublished data), indicating that neither the UBL domain nor the BAG domain is essential for binding polyubiquitinated proteins. In contrast, a mutant BAG-6 (designated AN) with truncation of the N-terminal 471 amino acids (the region corresponding to the N-terminal 436 amino acids of Xenopus Scythe; Minami et al., 2007) did not bind polyubiquitinated proteins (Fig. 2 D). Consistent with this result, a fragment that exclusively encodes the N-terminal 471 amino acids (N471) coprecipitated polyubiquitinated proteins as efficiently as did the full-length form of BAG-6 (Fig. 2 C) or the C-terminal BAG domain bound polyubiquitinated proteins as efficiently as did full-length (FL) BAG-6 (Fig. 2 D). These results indicate that the N-terminal 471 residues of BAG-6 are necessary and sufficient for its binding to polyubiquitinated substrates.

We then attempted to determine the fate of polyubiquitinated proteins associated with BAG-6 by “chasing” experiments. HeLa cells were treated with MG132 (5 µM) for 4.5 h, the inhibitor was washed out (this time point being defined as time zero), and the cells were cultured in fresh medium without the inhibitor was washed out (this time point being defined as time zero), and the cells were cultured in fresh medium without the

EGFP-CL1 was degraded after incubation with ATP, which stabilizes/supports 26S proteasome function (Fig. S3 B). In contrast, polyubiquitinated EGFP-CL1 was stabilized when ATP was absent. When the active site within the 20S core particle was blocked by MG132, EGFP-CL1 degradation was inhibited, even in the presence of ATP (Fig. S3 B). Thus, these findings support the hypothesis that BAG-6 provides a platform that connects the 26S proteasome and its ubiquitinated substrates to promote their efficient degradation.

BAG-6 is essential for metabolism of newly synthesized defective polypeptides Identification of the client proteins of BAG-6 is critical to understanding the function of BAG-6. We observed only a slow degradation of ubiquitin conjugates in the lysate (not bound with BAG-6) after the removal of MG-132 (Fig. 3 A, input panel), in contrast to the rapid disappearance of polyubiquitinated substrates on BAG-6 (Fig. 3 A, top IP panel). These observations indicate that polyubiquitinated substrates that associate with BAG-6 might be only a part of the proteasomal substrates and that BAG-6 substrates degrade much faster than those existing as free from BAG-6. To elucidate the endogenous client proteins of BAG-6, we analyzed the characteristics of polyubiquitin conjugates associated with BAG-6. We found that treatment with cycloheximide abolished most of the BAG-6–associated polyubiquitinated species in MG132-treated cells (Fig. 3 C; top IP panel), whereas there was only a small reduction of ubiquitin conjugates in the lysate (existing as free from BAG-6) after addition of cycloheximide (Fig. 3 C, input panel). These observations suggest that a significant part of BAG-6–associated polyubiquitinated species is cycloheximide-sensitive (and thus newly synthesized) polypeptides that are stabilized with MG132 treatment in cells. It has been reported that more than 30% of newly synthesized polypeptides are recognized as defective proteins by the ubiquitin-dependent degradation pathway (Schubert et al., 2000). Thus, we suspect that the primary target of BAG-6 might be defective ribosomal protein products (DRIPs) and that BAG-6 has a crucial function to support their efficient removal.

Inhibition of the proteasome-dependent proteolytic pathway by treatment with MG132 induced gradual cell death (Fig. 3 D) with accumulation of polyubiquitinated defective proteins. Although BAG-6 knockdown itself (up to 80%) did not significantly affect cell viability and growth (Fig. S3, C–E; and unpublished data), we found that knockdown of BAG-6 significantly enhanced the cytotoxicity of MG132 (and any other proteasome inhibitors; Fig. 3 D). This result indicates that BAG-6 knockdown renders cells more susceptible to MG132-induced death and that BAG-6 has an essential protective function against cell death induced by the accumulation of proteasomal substrates. Importantly, we found that the cytotoxicity enhanced by BAG-6 siRNA was also drastically suppressed by treatment with cycloheximide (Fig. 3 D). This observation further supports our conclusion that the effect of BAG-6 siRNA was mainly due to the excess accumulation of newly synthesized defective proteins. Thus, our data indicate that BAG-6 has a crucial function to metabolize defective ribosomal polypeptides.
ubiquitin-positive cytoplasmic aggregates called ALIS (Lelouard et al., 2004). Interestingly, we also found that BAG-6 could be detected as cytoplasmic dots (Fig. 4 B, top panels) exclusively in puromycin-treated cells, and these dots looked similar to puromycin-labeled protein aggregates (Fig. 4 B, bottom panels). Indeed, we confirmed that the BAG-6–positive aggregates formed by puromycin treatment were co-ubiquitin stained (Fig. 4 C). These observations indicate that BAG-6 is intimately associated with cytoplasmic aggregates that are composed of polyubiquitinated nascent chain polypeptides in puromycin-treated cells.

To estimate the impact of BAG-6 knockdown on formation of puromycin-induced aggregates, we compared aggregates with or without BAG-6 siRNA (Fig. 4, D–F). We found that cells with BAG-6 knockdown contain enlarged cytoplasmic aggregates compared with control siRNA cells (Fig. 4, D and E). In contrast, BAG-6 interacts with puromycin-labeled defective polypeptides

To investigate the relationship between BAG-6 and defective ribosomal products more directly, we examined whether BAG-6 recognized newly synthesized defective polypeptides. Puromycin is an analogue of the 3′ terminus of aminoacyl-tRNA that can be incorporated into newly synthesized polypeptides at their C termini, blocking further peptide elongation and thus producing truncated translational products (Fig. 4 A, bottom; Zhang et al., 1997; Lelouard et al., 2004). We found that BAG-6 could be co-immunoprecipitated by antibody against puromycin (Fig. 4 A, top IP panel). This result indicates that BAG-6 physically interacts with puromycin-labeled nascent chain polypeptides. Results of our immunocytochemical analyses of BAG-6 after puromycin treatment further support this notion. It has been reported that puromycin-labeled truncated proteins tend to form ubiquitin-positive cytoplasmic aggregates called ALIS (Lelouard et al., 2004). Interestingly, we also found that BAG-6 could be detected as cytoplasmic dots (Fig. 4 B, top panels) exclusively in puromycin-treated cells, and these dots looked similar to puromycin-labeled protein aggregates (Fig. 4 B, bottom panels). Indeed, we confirmed that the BAG-6–positive aggregates formed by puromycin treatment were co-ubiquitin stained (Fig. 4 C). These observations indicate that BAG-6 is intimately associated with cytoplasmic aggregates that are composed of polyubiquitinated nascent chain polypeptides in puromycin-treated cells.

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BAG-6–mediated degradation of nascent chain polypeptides in vitro

We then established a reticulocyte lysate-based in vitro degradation assay for artificial DRiPs. In this system, we used a puromycin-labeled, truncated luciferase (Luc163) that had been synthesized de novo as a model nascent chain polypeptide (Fig. S4 A). The Luc163 substrate, which was newly synthesized in a rabbit reticulocyte lysate, was unstable in the extract.

The number of ubiquitin-positive aggregates was decreased with BAG-6 siRNA (Fig. 4 F). These results indicate that BAG-6 controls the dynamics of aggregate metabolism. In addition, we found that BAG-6 knockdown accelerated puromycin-induced cell death (Fig. 4 G), indicating that BAG-6 has protective roles against nascent chain polypeptide-induced cell toxicity. Collectively, the results indicate that BAG-6 might be an essential element for the metabolism of nascent chain polypeptides.
detected the accumulation of polyubiquitinated forms of Luc163 in a MG132-treated rabbit reticulocyte lysate (Fig. S4, D and G). With this system, we showed that addition of antibody against (Fig. S4 B) and was stabilized by the addition of proteasome inhibitors (Fig. S4 C), indicating that Luc163 was metabolized via a ubiquitin-dependent pathway. In accordance with this, we

**Figure 5.** BAG-6 provides a platform that is necessary for linking puromycin-labeled defective protein with degradation machinery. (A) Addition of antibody against BAG-6 inhibited the degradation of puromycin-labeled, truncated luciferase in vitro. A messenger RNA encoding the 3xFlag-tagged N-terminal 163 residues of luciferase (Luc163) was incubated in a rabbit reticulocyte lysate chasing with 2 mM puromycin addition. After addition of 50 µg/ml anti–BAG-6 antibody, cycloheximide (CHX) chase analysis was performed, and lysates were harvested at the indicated times and probed with antibodies against Flag and Rpt6 to evaluate the stability of puromycin-labeled Luc163 (Luc163). Non-immune rabbit IgG or antibody against BAG-6 that was preabsorbed with an excess amount of recombinant antigen was used as a negative control. (B) Addition of antibody against BAG-6 inhibited ubiquitination of puromycin-labeled, truncated luciferase. Blot with Flag-Luc163 and Rpt6 are indicated as loading controls. (C) Immunoprecipitation of in vitro translated Luc163 with anti-Flag M2 beads coprecipitated endogenous BAG-6 and Hsp70 from lysates. (D) BAG-6 provided a platform for the targeted degradation of Luc163. Endogenous BAG-6 was immunoprecipitated from a rabbit reticulocyte lysate that was translating 3xFlag-tagged Luc163. The precipitated immunocomplex was further incubated in the presence (+) or absence (−) of 5 mM ATP, ubiquitin, and 25 µM MG132 at 37°C for 2 h as indicated. After incubation, the precipitated complexes were subjected to Western blot analysis with an antibody against Flag to examine the stability of Luc163 on BAG-6 during the incubation periods. (E) Endogenous BAG-6 was immunoprecipitated (first IP) as in D, and the precipitated complex was incubated with MG132 under the conditions indicated. After incubation, the complexes were denatured by SDS and diluted samples were further subjected to precipitation with anti-Flag M2 agarose (second IP). Precipitated, Flag-tagged Luc163 was blotted with antibody against ubiquitin to estimate the extent of its modification with ubiquitin.
the N terminus of BAG-6 to the extracts blocked both degradation (Fig. 5 A) and polyubiquitin modification (Fig. 5 B and Fig. S4 E) of Luc163. In the reticulocyte lysate, Luc163 was associated with BAG-6 (Fig. 5 C). After isolation of the BAG-6 complex from lysates by anti–BAG-6 immunoprecipitation, the immunocomplex was incubated at 37°C in the presence or absence of MG132 and/or ATP in vitro and the stability of BAG-6–associated Luc163 during the incubation periods was evaluated (Fig. 5 D). We found that isolated Luc163 in the complex was degraded after incubation with ATP (which stabilizes/supports 26S proteasome function) and ubiquitin. In contrast, Luc163 was stabilized when ATP was absent. When the 26S proteasome was blocked by MG132, Luc163 degradation was inhibited, even in the presence of ATP (Fig. 5 D). The situation is similar in polyubiquitination of Luc163. Incubation of the BAG-6–Luc163 immunocomplex in vitro with ATP, ubiquitin, and MG132 enhanced polyubiquitin modification of Luc163 on BAG-6 (Fig. 5 E and Fig. S4 F). Thus, these findings support the idea that BAG-6 provides a platform that connects degradation machinery and nascent chain polypeptides to promote their efficient degradation.

**BAG-6 knockdown blocks the cell surface presentation of MHC class I**

All of our observations support the idea that BAG-6 is essential for selective elimination of defective ribosomal products. It is reported that degradation products of DRiPs are the major source of peptide ligands presented on the cell surface MHC class I molecules of the major histocompatibility complex (Reits et al., 2000; Schubert et al., 2000; Khan et al., 2001; Yewdell and Bennink, 2001; Yewdell et al., 2003). If BAG-6–mediated processing of DRiPs is a major source of peptide ligands for MHC class I molecules, then knockdown of BAG-6 function in vivo should rapidly decrease the peptide supply and should block the cell surface presentation of MHC class I molecules because peptide binding is required for the rapid export of class I molecules. To directly examine whether BAG-6 knockdown affected the presentation of MHC class I molecules, we performed live-cell flow cytometric analysis with FITC-labeled anti–HLA-ABC antibody (W6/32). As our positive controls, we confirmed that treatment of HeLa cells with 10 µM MG132 increased the populations of FITC-negative cells (Fig. 6, A–C). Treatment with 10 µg/ml cycloheximide (CHX) also reduced the mean of fluorescence intensity of FITC-HLA-ABC on the surface of HeLa cells (Fig. 6, A and C). These results support a previous hypothesis that proteasome-mediated degradation of newly synthesized protein is essential for MHC class I presentation (Yewdell et al., 1996; Rock and Goldberg, 1999; Reits et al., 2000; Khan et al., 2001). In the case of BAG-6 knockdown, we found that the populations of FITC-negative cells increased significantly (Fig. 6, A and B). A quantitative evaluation of the mean of fluorescence intensity of FITC-HLA-ABC on the surface of HeLa cells indicated that the fluorescence decreased less than half in BAG-6 knockdown samples compared with the negative control (Fig. 6 C). These results suggest that knockdown of BAG-6 results in the suppression of MHC class I cell surface presentation. In addition, we found that an excess of exogenously supplied antigenic peptides partially rescued MHC class I cell surface expression (Fig. 6 C), suggesting that peptide production by the proteasome is limited after BAG-6 down-regulation.

To further verify the expression of MHC class I with another independent method, we performed an experiment to label the cell surface with biotin. In this experiment, cell surface proteins were biotinylated after siRNA, affinity purified with avidin beads, and blotted with antibody against MHC class I. As shown in Fig. 6 D, the abundance of biotinylated MHC class I molecules was clearly reduced with BAG-6 siRNA. The reduction of cell surface MHC molecules is not caused by simple defects in the peptide transport system inside cells, because the amount of EGF receptor protein (EGFR) was not affected by BAG-6 knockdown but was reduced by brefeldin A, a drug that is known to block anterograde protein transport from the ER to the Golgi. Thus, all of our experiments showed that BAG-6 is essential for supplying antigenic peptides to the immune system.

Finally, we also provided evidence that BAG-6 could be associated with immunoproteasomes, the principal cytosolic proteasomes used for generating MHC class I peptide ligands (Monaco and Nandi, 1995; Tanaka and Kasahara, 1998; Rock and Goldberg, 1999), after induction by treatment with interferon-γ (Fig. S5 A). Although our results showed that BAG-6 could be associated with constitutive 26S proteasome as well (Fig. 1, F and G), these observations suggest that BAG-6 collaborates with immunoproteasomes to generate antigenic peptides via targeted degradation of defective ribosomal products and might play a crucial role for antigen presentation in immune response.

**Discussion**

The CL1 degron used in this study was originally identified in a screen for a genomic sequence that destabilized the cytosolic protein Ura3p by targeting it for degradation via Ubc6p/Ubc7p in the yeast Saccharomyces cerevisiae (Gillon et al., 1998). Metzger et al. (2008) reported that CL1 represents a frame-shifted region of the yeast PMDI gene and that it contains a strongly hydrophobic region and thus may resemble a misfolded protein when it is exposed. Therefore, a study using a CL1 degron might reveal mechanisms for the targeted removal of improper translational products. A previous study indicated that Ura3p–CL1 degradation in yeast is dependent on the molecular chaperones Ydj1p (a DnaJ homologue) and Ssa1p (Hsp70 homologue) as well as the proteasome (Metzger et al., 2008). In accordance with the results of that study, we found that the CL1 degron could interact with human homologues of DnaJ, Hsp70, and 26S proteasome subunits in HeLa cells (Fig. 1 B). Furthermore, we identified BAG-6 as a novel mammalian CL1-associated protein. This observation suggested that BAG-6 may participate in the metabolism of misfolded proteins for proteasomal degradation. Indeed, we found in this study that BAG-6 recognized a CL1 degron substrate and supported its proteasomal degradation. We showed evidence that not only a CL1 model substrate but also newly synthesized polyubiquitinated polypeptides were associated with BAG-6 after proteasome inhibition (Fig. 3 C). All of our data suggest that BAG-6 provides a transient platform that is necessary for linking the 26S proteasome and its defective substrates for targeted degradation.
Because we found that BAG-6 interacted with polyubiquitinated proteins (Fig. 2; and Fig. 3) and that a part of BAG-6 moved to the insoluble fractions after treatment with MG132 (Fig. S5 B), we investigated whether BAG-6 colocalized with an aggresome. As shown in Fig. S5 C, confocal microscopic observation revealed that the treatment of MG132 resulted in the formation of a large perinuclear structure and that BAG-6 accumulated on this aggregate. Importantly, we found that BAG-6–positive aggregates were coimmunostained with either polyubiquitin or the intermediate filament protein vimentin, markers for aggresome formation (Johnston et al., 1998), in the presence of MG132 (Fig. S5 C).

It has been reported that 25% of rapidly degraded polyubiquitinated peptides (RDPs) lose their solubility within an hour of blocking proteasome activity (Qian et al., 2006). The expression of BAG-6 was diffuse and soluble in normal HeLa cells, whereas treatment with MG132 significantly increased the amount of BAG-6 in the detergent-insoluble fraction (Fig. S5 B). These results support the notion that the BAG-6 protein in the soluble fraction moves to and accumulates in the insoluble aggregates associated with ubiquitinated RDPs during proteasomal dysfunction. It has been reported that polyubiquitinated, aggregation-prone misfolded proteins are transported on microtubules to the MTOC and then form large perinuclear insoluble aggregates/aggresomes (Johnston et al., 1998; Bence et al., 2001; Ardley et al., 2003). Because we found that BAG-6 interacted with polyubiquitinated proteins (Fig. 2; and Fig. 3) and that a part of BAG-6 moved to the insoluble fractions after treatment with MG132 (Fig. S5 B), we investigated whether BAG-6 colocalized with an aggresome. As shown in Fig. S5 C, confocal microscopic observation revealed that the treatment of MG132 resulted in the formation of a large perinuclear structure and that BAG-6 accumulated on this aggregate. Importantly, we found that BAG-6–positive aggregates were coimmunostained with either polyubiquitin or the intermediate filament protein vimentin, markers for aggresome formation (Johnston et al., 1998), in the presence of MG132 (Fig. S5 C).
Our observations suggest that BAG-6 is functionally linked with the aggresome at the time of proteasome inhibition (Fig. S5 D).

Schubert et al. (2000) reported that 30% or more of newly synthesized proteins are destroyed by proteasomes of their synthesis. These unstable nascent polypeptides that emerge from the ribosome into the cytosol were designated as defective ribosomal products (Yewdell et al., 1996). DRiPs are polypeptides that fail to attain a stable conformation because of errors in translation, folding, and post-translational modification as well as errors in transcription and mRNA processing. Puromycin is mistakenly inserted during protein synthesis by the ribosome in place of normal amino acids, resulting in truncated DRiPs containing the drug at their C termini (Vazquez, 1974; Lelouard et al., 2004). Our immunocytologic and immunoprecipitation experiments provided evidence that BAG-6 could associate with puromycin-labeled defective nascent polypeptides in vivo. We also showed that BAG-6 controlled in vivo formation of puromycin-induced aggregation structures. Furthermore, our in vitro analysis clearly showed that puromycin-labeled truncated luciferase, a model DRiP substrate, was ubiquitinated and degraded on BAG-6 in rabbit reticulocyte lysates. All of these observations strongly support our conclusion that mammalian BAG-6 is essential for selective elimination of defective nascent polypeptides.

It has been shown that newly synthesized proteins are the major source of peptide ligands presented by MHC class I molecules of the major histocompatibility complex on the cell surface (Townsend et al., 1986; Anton et al., 1997; Khan et al., 2001). It has been reported that blocking protein synthesis for 30 min is sufficient to deplete cells of most TAP-transported antigenic peptides (Reits et al., 2000). Because a similar degree of depletion is reported to be achieved within 15 min of blocking proteasomes, the primary source of peptides is from proteins in the first 15 min of their synthesis (Reits et al., 2000). To date, there is considerable evidence that a significant source of self and viral peptides is DRiPs, which consist of prematurely terminated and/or misfolded polypeptides (Yewdell et al., 1996, 2003; Yewdell, 2002; Princiotta et al., 2003). If BAG-6–mediated processing of DRiPs is a major source of peptide ligands for MHC class I molecules, then blocking BAG-6 function should rapidly decrease the peptide supply and should subsequently slow the cell surface presentation of MHC class I molecules because peptide binding is required for the rapid export of class I molecules. That is the reason why we examined the cell surface presentation of MHC molecules in this study (Fig. 6). Both our FACS and biochemical analyses clearly showed that knockdown of BAG-6 resulted in the suppression of MHC class I cell surface presentation without affecting the intracellular protein transport system. These results also support our hypothesis that BAG-6 is essential for supplying antigenic peptides to the immune system. In good agreement with this observation, we found that BAG-6 was associated with immunoproteasome complexes after treatment with interferon-γ (Fig. S5 A). The initial evidence implicating immunoproteasomes in antigen processing was the discovery that the MHC region contains genes that encode two proteasome subunits and that their expression is controlled by cytokines released by activated T cells (Michaëlik et al., 1993; Monaco and Nandi, 1995; Tanaka and Kasahara, 1998). When induced, these subunits replace constitutively expressed subunits in newly assembled proteasomes to create immunoproteasomes, which appear to be better at producing peptides favored by MHC class I molecules (Tanaka and Kasahara, 1998). It is worth recalling that BAG-6 was originally described as an MHC-encoded gene product (Banerji et al., 1990) and that its expression is apparently enriched in lymphoid tissues (this paper; Fig. 1 D). Although we have not yet detected obvious induction of BAG-6 by interferon-γ, we present here an interesting possibility that BAG-6 is a novel factor that modulates immune responses via DRiP-mediated antigen presentations.

The role of BAG-6 in apoptotic cell death appears to be an area of controversy. In several previous studies, BAG-6 has been shown to be required for the induction of apoptosis in response to a variety of stimuli, and the loss of BAG-6 is associated with protection against apoptosis induced by calcium overloading in the ER as well as by menadione and thapsigargin (Desmots et al., 2008). Currently, we do not exactly know how the reported apoptotic function of BAG-6 is linked to the proteolytic function identified in this study. However, the region that is required for apoptotic control (N-terminal 436 residues in Xenopus Scythe–BAG-6; Minami et al., 2007) superficially overlaps with the region of substrate recognition in mammalian BAG-6. In addition, our previous study indicated that the N-terminal region of Scythe–BAG-6 interacts with XEF1AO, a maternal form of polypeptide elongation factor that was suggested to be a potential inducer of apoptosis in vertebrates (Minami et al., 2007). The binding stimulates polyubiquitin modification and subsequent degradation of XEF1AO in Xenopus embryos. In addition, we found in this study that BAG-6 provided protection against cell death induced by MG132 and puromycin treatment in mammalian cells. These observations imply that BAG-6–mediated modification of protein degradation is, at least in part, important for apoptotic control caused by the accumulation of aggregation-prone defective proteins. Because aggregated proteins with polyubiquitin have been proposed to be central to the pathogenesis of a number of neurological diseases (Bence et al., 2001; Taylor et al., 2002; Ardley et al., 2003), we are also interested in the possibility that BAG-6 is involved in protein quality control in the neural system.

In the ubiquitin-dependent protein degradation pathway, the substrate sorting process depends on the cooperation of chaperone machineries and ubiquitin chain recognition factors (Hartmann-Petersen and Gordon, 2004; Verma et al., 2004; Richly et al., 2005; Westhoff et al., 2005). These factors sequentially support the process through protein–protein interactions and thereby escort substrate recognition, ubiquitination, and ubiquitin–protein conjugate presentation to the proteasome (Richly et al., 2005). Although we do not fully know what kinds of ubiquitin chain recognition factors and ubiquitination machinery are associated with BAG-6 at present, we favor the idea that BAG-6 may possess roles in ubiquitin modification of tethered substrates. We observed that there was an inexplicable increase in BAG-6–bound ubiquitin conjugates for 1 h after MG132 removal (Fig. 4 B). In our previous study, we reported that Scythe–BAG-6 expression stimulates polyubiquitin modification of XEF1AO substrate (Minami et al., 2007). These observations suggest that the BAG-6–Scythe complex plays a role in
modification of polyubiquitin chains. We suggest that BAG-6 provides a transient platform that links the ubiquitinating machinery, the 26S proteasome, and its newly synthesized substrates to promote their efficient destruction. In any case, we have presented the first evidence that BAG-6–Scythe–BAT3 is a novel polyubiquitinated substrate-associated protein, and our results shed light on the importance of BAG-6 in the degradation of defective proteasomal substrates. Elucidation of the involvement of BAG-6 in the regulation of viral infections and/or onset of neurodegeneration caused by defects in the metabolism of DRiPs should be the next big challenge in understanding the significance of BAG-6 in the development of various human diseases.

Materials and methods

Plasmid construction

The cDNAs of BAG-6 were amplified by PCR from the library prepared from NIH3T3 and HeLa cells. The PCR products of BAG-6 were digested with SalI–NotI and inserted into the pCMV-3xFlag or pCMV-2S vectors for expression in mammalian cells. It should be noted that pCMV-3xFlag and pCMV-2S expression vectors contain three repeats of a Flag tag or two repeats of an S peptide sequences, respectively, at the N-terminal regions of their products. The truncated and mutated versions of BAG-6 were constructed by PCR and cloned into appropriate pCMV-neo vectors (Promega). The synthetic oligonucleotide encoding the CL1 peptide (ACKNWFSSH-FVH; Gilon et al., 1998) was inserted into the EcoRI site of pCMV-3xFlag-EGFP expression vector. Each vector was used for experiments after verification of the sequence of inserted DNA.

Mammalian cell culture and transfection

HeLa, Neuro2a, and NIH3T3 cells were cultured in DME (Sigma-Aldrich) supplemented with 10% heat-inactivated calf serum at 37°C under a 5% CO2 atmosphere. Transfection of HeLa cells was performed according to the standard calcium phosphate precipitation protocol. The total amount of plasmid DNA was adjusted to 2 µg with an empty vector. At 24 h after transfection, the cells were harvested and subjected to immunological analysis unless otherwise noted.

Immunological analysis

An anti-BAG-6 antibody was prepared as follows. 200 µg of bacterially produced Xenopus BAG-6 (N-terminal 436 amino acids) was mixed and emulsified with an equal amount of TiterMaxGold (TiterMax USA, Inc.) and then inoculated into a rabbit. The antibody was obtained after three rounds of immunization at 1-wk intervals and used after affinity purification. We then inoculated into a rabbit. The antibody was obtained after three rounds of immunization at 1-wk intervals and used after affinity purification. We then inoculated into a rabbit. The antibody was obtained after three rounds of immunization at 1-wk intervals and used after affinity purification. We then inoculated into a rabbit. The antibody was obtained after three rounds of immunization at 1-wk intervals and used after affinity purification.

For immunoprecipitation analysis, cultured cells were washed with ice-cold phosphate-buffered saline and lysed with immunoprecipitation (IP) buffer containing 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% Tween 20, 10% glycerol, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride (PMSF), 3 µg/ml pepstatin A, 5 µg/ml aprotinin, 10 mM N-ethylmaleimide, and 25 µM MG132. The lysate was sonicated for 1 s and centrifuged at 20,000 g for 20 min at 4°C, and the resulting supernatant was incubated with 3 µl of anti-Flag M2-agarose beads (Sigma-Aldrich), 5 µl of S-protein agarose beads (EMD), or anti-BAG-6 antibody stabilized to Protein A–Sepharose beads (GE Healthcare) for 2 h at 4°C. After the beads had been washed four times with the IP buffer, the precipitated immunocomplexes were subjected to SDS-PAGE. ATF elution was performed as described previously (Demand et al., 2001).

For Western blotting, the whole-cell lysate and immunoprecipitates were separated by SDS-PAGE and transferred onto nitrocellulose membranes (Bio-Rad Laboratories). The membranes were immunoblotted with specific antibodies as indicated and then incubated with horseradish peroxidase–conjugated antibody against mouse or rabbit immunoglobulin (GE Healthcare), followed by detection with ECL Western blotting detection reagents (GE Healthcare).

The following antibodies were used for immunological analyses in this study: anti-19S complex (Rpt1, Rpt5, and Rpt6 subunits; Thermo Fisher Scientific and Enzo Life Sciences, Inc.), anti-20S proteasome (α2 and β1i subunit; Thermo Fisher Scientific and Enzo Life Sciences, Inc.), anti-5 peptide (Santa Cruz Biotechnology, Inc.), anti-Flag tag (Sigma-Aldrich), anti-polyubiquitin FK2 (Nippon Bio-test Laboratories Inc.), anti-Hsp70/Hsc70 (MBI), anti-tubulin (ICN), anti-Chk2 (Santa Cruz Biotechnology, Inc.), anti-MHC class I (Santa Cruz Biotechnology, Inc.), anti-EGRF (Santa Cruz Biotechnology, Inc.), anti-GST (Santa Cruz Biotechnology, Inc.), anti-vimentin (Santa Cruz Biotechnology, Inc.), and anti–human HLA-ABC monoclonal antibody W6/32 (Becton Dickinson Co., Ltd.). Anti-puromycin antibody is a gift from Peter Walter at University of California, San Francisco (San Francisco, CA).

Identification of CL1-associated proteins

An expression vector encoding 3xFlag-tagged EGFP-CL1 was transfected into HeLa cells. After 4.5 h treatment with 5 µM MG132, cells were harvested and crushed with IP buffer. Using anti-Flag M2 agarose beads (Sigma-Aldrich), EGFP-CL1 were immunopurified and subjected to SDS-PAGE analysis. 3xFlag-tagged EGFP was used as a negative control. PMF analysis was performed with Voyager Biospectrometry Workstation DE-PRO (PerSeptive Biosystems) as described previously (Minami et al., 2007).

Gene knockdown experiments

For knockdown analysis of BAG-6 in Fig. 1 C, the oligonucleotides encoding two independent targeted sequences of BAG-6 (targeted sequence of siRNA-1: 5′-TGGGTCCCTATTACACG-3′, siRNA-2: 5′-TTTCTCCAAGAGCAGTATT-3′) were inserted into the pSUPER vector (Oligo Engine). The plasmids for siRNA were transfected into HeLa cells. 60 h after treatment of siRNA, cells were subjected to Western blot analysis. For other knockdown experiments, two independent duplex siRNAs encoding the targeted sequences of BAG-6 (BAG-6-1: 5′-TTTCTCCAAGAGCAGTATT-3′, BAG-6-2: 5′-ATAGTGCACATGAAATG-3′) were synthesized (SIGMA Genosys) and used with Lipofectamine 2000 (Invitrogen) according to the protocol provided by the manufacturer. Negative control siRNA was purchased from Invitrogen. The efficacies of each siRNA were verified by immunoblot and RTPCR (Fig. 53, D and E) or immunocytochemical observations with anti-BAG-6 antibody.

 Gel filtration analysis of the cell extracts of NIH3T3

Extracts of NIH3T3 cells were subjected to gel filtration on a column of Superose 6 HR 10/30 (GE Healthcare) in buffer A (10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 10 mM MgCl2, 5 mM EDTA, 1% Tween 20, 10% glycerol, 2 mM ATP, 1 mM dithiothreitol, 10% glycerol, 1 mM PMSF, 3 µg/ml pepstatin A, 5 µg/ml aprotinin, 10 mM N-ethylmaleimide, and 25 µM MG132) at a flow rate of 0.25 ml/min. Fractions of 0.5 ml were collected and subjected to SDS-PAGE, followed by Western blotting. Gel Filtration LMW/HMW Marker kits (GE Healthcare) were used as molecular mass standard proteins for gel filtration.

Microscopic observations

For immunocytochemical observations of cultured cells, HeLa cells were grown on micro coverglass (Matsunami), fixed by incubating in 4% paraformaldehyde, and were then permeabilized with 0.1% Triton X-100. Fixed cells were blocked with 1% bovine serum albumin in PBS and reactivated with a series of primary antibodies at room temperature for 1 h. Alexa 594–conjugated anti–rabbit IgG antibody (Invitrogen) and FITC-conjugated anti–mouse antibody (Jackson Immunoresearch Laboratories, Inc.) were used as secondary antibodies at dilutions of 1:800. To observe the nucleus, cells were stained with 2.5 µg/ml DAPI in PBS at the time of antibody staining. Immunofluorescent images were obtained with an invert confocal microscopy system (LSM510; Carl Zeiss, Inc.).

Cell viability assay

HeLa cells were treated with BAG-6–specific siRNA or negative control siRNA (Invitrogen). After 12 h, cells were plated in 96-well plates at a density of 104 cells/well and cultured for a further 72 h with or without treatment of 5 µM MG132 or 5 µg/ml puromycin for the indicated time. Cell viability was determined using a cell counting kit 8 (Dojindo) according to the protocol provided by the manufacturer. The absorbance of 450 nm was measured by a densitometer (CS-9300PC; Shimadzu).

Rabbit reticulocyte lysate-based preparation of model DRiP substrate

Flag-tagged luciferase gene in mammalian expression vector (pCI-neo; Promega) was digested with restriction enzyme BspI407I. BspI407I exclusively cut the middle of luciferase ORF at the position of 163 residue of its encoding luciferase protein. Using this linearized DNA as a template, mRNA was synthesized in vitro using mMESSAGE mMACHINE (Applied Biosystems). The synthesized mRNAs was added to rabbit reticulocyte lysate and incubated at 26°C for 40 min, followed by an additional 20-min incubation with 2 µM puromycin, resulting in a production of puromycin-labeled product.
truncated luciferase as a model nascent chain polypeptide. Flag-tagged full-length luciferase without puromycin treatment was used as a non-DRiP control polypeptide.

Assay for presentation of MHC class I on the cell surface
For FACS analysis, knockdown experiments were performed with two independent duplex siRNAs encoding the targeted sequences of BAG-6 (BAG-6 siRNA-1: 5′-TTCCTCAACAGAGGTATTTG-3′, BAG-6 siRNA-2: 5′-ATGATG-CACATGAACATCT-3′) with Lipofectamine 2000 (Invitrogen). After 72 h of siRNA transfection, 106 Hela cells were harvested and probed with FITC-labeled anti-human HLA-ABC monoclonal antibody W6/32 (Bayer Bioscience Co., Ltd.) in PBS (containing 10% FBS) at 4°C for 30 min. As positive controls, Hela cells were treated with 10 μg MGI32 10 μg/ml cycloheximide for 22 h (MG132) or 8 h (CHX), respectively, before harvesting. Exogenously supplied peptides (VYIKVSAR for HLA-A*6802, VQRL-NATGY for HLA-B*1503, CCFHCQVC for HLA-Cw*1203) were synthesized by Sigma Genosys and were pulsed with cells at 50 μM for 16 h before harvesting. Negative control siRNA was purchased from Invitrogen. Living-cell FACS analyses were performed with a FACSCalibur flow cytometer (Becton Dickinson) by ReproCELL, Inc.

Biochemical labeling experiments were performed as follows. After 72 h of siRNA transfection, Hela cells were harvested and the cell surface proteins were biotinylated. Immediately after cell lysis with biotin lysis buffer [20 mM Tris·HCl, pH 7.5, 150 mM NaCl, and 1% Triton X-100], cell surface–biotinylated proteins were affinity purified with avidin beads. The bound cell surface proteins were detected by antibodies against MHC class I and EGFR receptor (EGFR; control of cell surface proteins). Brefeldin A treatment at 1 μg/ml for 4 h was used as a positive control for the suppression of the transport of MHC class I and EGFR to the cell surface. Anti-actin blot was used as a loading control.

Online supplemental material
Fig. S1 shows our additional data that BAG-6 is essential for CL1 degradation-dependent proteasomal degradation. Fig. S2 demonstrates that BAG-6 is a stable protein that associates with polyubiquitinated substrates. Fig. S3 shows that polyubiquitinated proteins associated with BAG-6 are destined for degradation. Fig. S5 also shows the efficacies of BAG-6 knockdown. Fig. S4 describes our proteasome-dependent nascent polypeptide chain degradation system. Fig. S5 demonstrates that BAG-6 interacts with immunoproteasome and with aggresome. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200908092/DC1.

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Figure S1. **BAG-6 is essential for CL1 degron-dependent proteasomal degradation.** (A) Knockdown of BAG-6 stabilized the CL1 degron substrate. After 48 h of BAG-6 shRNA, 3xFlag-tagged EGFP-CL1 was expressed in HeLa cells for an additional 24 h, and then chased with protein synthesis inhibitor cycloheximide (CHX) at the concentration of 25 µg/ml. Whole-cell extracts were prepared at indicated times after CHX addition and subjected to immunoblot analysis with antibodies against Flag (for EGFP-CL1) and BAG-6. (B) Quantification of EGFP-CL1 protein in HeLa cells after the addition of CHX. Signal intensity at the point of CHX addition is defined as 100%. The half-life of EGFP-CL1 in control cells is 6.2 h, and partial knockdown of BAG-6 extended the half-life to 15.3 h. (C–F) BAG-6 is associated with the 26S proteasome in mammalian cells. To support our finding that mammalian BAG-6 interacts with the 26S proteasome, we performed gel filtration of NIH3T3 cell extracts. The cell extracts were subjected to Superose 6 gel filtration, and the fractions were subjected to Western blotting with specific antibodies to BAG-6, Hsp70/Hsc70, and 26S proteasome subunits Rpt1 and Rpt5. We found that endogenous BAG-6 protein comigrated with 26S proteasomal subunits Rpt1 and Rpt5. NIH3T3 cells were cultured with 20 µM MG132 before harvesting. (D) Glycerol density gradient sedimentation velocity analysis of HeLa cell extracts also supported comigration of BAG-6 and 26S proteasome. Extracts of HeLa cell cultured with 10 µM MG132 were fractionated by glycerol density-gradient centrifugation (10–40% glycerol from fractions 1–20). Proteins in each fraction (200 µl) were concentrated by aceton precipitation and subjected to Western blot analysis with antibodies to BAG-6, Rpt1 (19S complex), and α5 (20S proteosome). The arrows indicate the positions of 20S and 26S proteasomes. It should be noted that BAG-6 associates with large (and insoluble) polyubiquitylated aggregates (as shown in Fig. S5 A), although such insoluble aggregates precipitated to the bottom of glycerol density gradient tubes (not contained in the indicated fractions). (E) The association of BAG-6 with 26S proteasome complex was further supported by our pull-down experiments. 2S-tagged full-length form of BAG-6 was overexpressed in HeLa cells. Cells were cultured with (+) or without (−) 20 µM MG132 before harvesting. Cell extracts were subjected to affinity purification with S-protein agarose beads, and the precipitates were immunoblotted with antibodies to 20S proteasome α5 subunits, 19S complex (Rpt1 subunits), and S-peptide (confirming precipitations of recombinant BAG-6). This result clearly showed that the 2S-tagged BAG-6 coperiplated with endogenous 26S proteasome. All of these observations, as well as evidences provided in Fig. 1, E–G, support our notion that mammalian BAG-6 is a novel member of the family of the 26S proteasome-associated proteins. (F) Endogenous BAG-6 protein was coimmunoprecipitated with anti-polyubiquitin antibody from HeLa cell extracts. HeLa cells were treated with 10 µM MG132 for indicated times and harvested for immunoprecipitations. Immunoprecipitates of anti-polyubiquitin FK2 antibody were blotted with anti-BAG-6 and anti-ubiquitin antibodies. Immunoglobulins isolated from nonimmune mouse were used as a negative control for immunoprecipitations (Control).
Figure S2. **BAG-6 is a stable protein that associates with polyubiquitinated substrates.** (A) BAG-6 interacts with polyubiquitinated proteins accumulated in the cytoplasmic fraction of MG132-treated cells. MG132-treated (+) or nontreated (−) cells were fractionated into cytoplasmic soluble fractions and endogenous BAG-6 protein was affinity purified from the extract with anti–BAG-6 antibody. Immunocomplexes from the fraction were probed with anti-ubiquitin and anti–BAG-6 antibodies. Tubulin was used as a cytoplasmic loading control. Immunoglobulins isolated from nonimmune rabbit was used as a negative control for immunoprecipitations. Our results indicate that cell endogenous BAG-6 coprecipitates with polyubiquitin from MG132-treated cells. (B) We next asked whether BAG-6 itself polyubiquitylated. Our tandem immunoprecipitation analysis shows that BAG-6 never coprecipitated ubiquitin moieties after SDS denaturation. Flag-tagged BAG-6, BAG-1, and HR23a were expressed in MG132-treated cells and subsequently immunoprecipitated with anti–Flag antibody (first IP). The precipitates were eluted by 1% SDS denaturation at 90°C and eluted proteins were diluted with RIPA buffer that did not include...
SDS, and then the samples were affinity purified again with anti-Flag antibody (second IP). Bound proteins after second precipitations were subjected to Western blot analysis with anti-ubiquitin (left panels) and anti-Flag (right panels) antibodies. As reported previously, HR23a and BAG-1 are covalently modified, either polyubiquitinated (indicated by an arrowhead) or monoubiquitinated (indicated by arrows), respectively. In contrast, polyubiquitinated proteins that associated with BAG-6 completely disappeared from immunoprecipitates after a denaturation procedure with 1% SDS at 90°C that abolishes noncovalent protein–protein interactions. Thus, we confirmed that BAG-6 is not directly ubiquitinated at all, and we concluded that the polyubiquitin moiety coprecipitated with BAG-6 is associated with BAG-6 noncovalently. (C, D, and F) BAG-6 is a stable protein in mammalian cells. (C and D) HeLa cells were treated with cyclohexamide (CHX) for indicated times and harvested cells were subjected to Western blot with indicated antibodies. Although p27 is a highly unstable protein, BAG-6 did not significantly degrade for at least 24 h of CHX chase. Actin was used as a loading control. (F) Metabolic pulse-chase experiments of endogenous BAG-6 protein. 5 × 10^5 cells were pulse-labeled with ^35S-radioactive methionine for 24 h, and cells were chased with normal medium (not containing radioactive materials) for indicated periods. At indicated time points, cells from the whole dish (left) or a fixed number of cells (right, 10^6 cells/lane) were harvested and subjected to immunoprecipitation with anti–BAG-6 antibody. Precipitates were subjected to SDS-PAGE, and the radioactivities of BAG-6 were quantified. Note that cells in the whole dish continued to grow during chase periods, and thus the amounts of whole BAG-6 and actin loading control increased during chasing [left panel, Input]. As a control for equal protein loading, the results of anti-actin and anti–BAG-6 blot are shown in the right panel (Input). (E) The amount of BAG-6 is not affected by proteasome inhibitor treatment. Although p27 is stabilized by MG132, BAG-6 protein is not influenced in its amount by treatments of MG132 and/or CHX.
Figure S3. Polyubiquitin proteins associated with BAG-6 are destined for degradation, not simply dissociated from BAG-6. (A) MG132 is a reversible proteasome inhibitor that blocks active sites (threonine residue) of proteasomal chymotrypsin, trypsin and caspase-like activities. However, there is a possibility that the proteasome does not get reactivated rapidly enough after our MG132 wash-out conditions. To answer this question, we directly measured the proteolytic activity of the 26S proteasome with MG132-treated cells. As shown in A, chymotrypsin-like activity, as well as other peptidase activity of the proteasome, get rapidly reactivated within 4 h after MG132 removal. These observations are well consistent with our data in Fig. 3, A and B. (B) To support our idea that BAG-6 is a platform for protein degradation, we established a semi-vitro degradation assay system. 3xFlag-tagged EGFP-CL1 and 2S-tagged BAG-6 were coexpressed in HeLa cells with the proteasome inhibitor MG132 for 4 h before harvesting. EGFP-CL1 was immunoprecipitated with an anti-Flag antibody from cell extracts (first IP), and the immunocomplexes were eluted by 3xFlag peptide at 4°C. The eluted proteins were then affinity purified using S-protein beads (second precipitation) at 4°C, and precipitates were incubated in the presence (+) or absence (−) of 25 µM MG132 and/or 5 mM ATP at 37°C for 2 h. After incubation, the precipitated complexes were eluted with 1% SDS and probed with an anti-Flag antibody to examine the stability of 3xFlag-tagged EGFP-CL1 during incubation periods. The results show that substrates associated with BAG-6 degraded in an ATP-dependent manner. In addition, continued exposure of proteasome inhibitor never allows dissociation of substrates from the BAG-6 complex. These results suggest that polyubiquitinated species associated with BAG-6 are destined for degradation by an MG132-sensitive proteasome-dependent pathway in vivo, not simply dissoci-
Knockdown of BAG-6 did not significantly affect cell growth until 72 h of BAG-6 siRNA. After transfection of BAG-6 duplex siRNA into 10^5 HeLa cells (in 6-well multiwell dish), mean cell number was counted in every 24-h interval. We found that cell number increase more than 12-fold until 72 h of BAG-6 duplex RNA transfection, although cell growth rapidly terminated after 96 h of BAG-6 siRNA. The mean values of three independent experiments were indicated. At 72 h after siRNA, the protein level of BAG-6 is reduced less than one fifth of control siRNA. (D) Semi-quantitative estimation of the efficacies of BAG-6 knockdown. To appreciate the extent of our knock-down experiments, semi-quantitative Western blot analysis with several dilutions were performed with anti–BAG-6 antibody. After 72 h of BAG-6 siRNA transfection (BAG-6-1 duplex siRNA), we found the protein level of BAG-6 is almost equivalent to one-eighth dilution of control siRNA. This result indicates that more than 80% of BAG-6 protein was reduced within 72 h of siRNA treatment. As a control for protein loading and dilution, the result of anti-actin antibody was shown in under the panel. (E) RT-PCR analysis indicates that the transcript from BAG-6 siRNA cells is reduced to less than half compared with control siRNA. Total mRNA from BAG-6 siRNA and control siRNA cells were prepared with RNeasy® Mini kit (QIAGEN). After reverse transcription with oligo(dT) primer and SuperScript III (Invitrogen), RT-PCR was performed with BAG-6 primer pairs (sense, 5'-TCAGCTGAGAAGAACTTGG-3'; antisense, 5'-TCCCCTGATGAGGAAGGGCCA-3'). These primer pairs amplify a 423-bp fragment from 3' terminal region of BAG-6 transcript. The representative is the result of agarose gel electrophoresis with several dilutions after a 25-cycle amplification.
Figure S4. Establishment of proteasome-dependent nascent polypeptide chain degradation system. (A) Schematic representation of in vitro synthesis of DRiP model substrate. Expression vector encoding luciferase was digested with restriction enzyme Bsp1407I (these results in single cut at the middle of the luciferase gene at the site of amino acid 163 from N terminus), and corresponding in vitro–synthesized mRNA was incubated in rabbit reticulocyte lysate with puromycin. This procedure produces C-terminally truncated, puromycin-labeled model nascent chain polypeptide (Luc163). As a control, full-length normal luciferase was synthesized in the lysate without addition of puromycin (Luc FL). (B) Truncated luciferase (Luc163) but not full-length luciferase (Luc FL) is rapidly degraded in rabbit reticulocyte lysate. After in vitro translation of 3xFlag-tagged Luc FL or Luc163, the lysates were further incubated with 100 µg/ml CHX for indicated times. The stability of each luciferase in the lysates was evaluated by immunoblotting with anti-Flag antibody. Rpt6 (26S proteasome subunit) is used as an internal loading control. (C) Similar assay as in B was performed in the presence and absence of proteasome inhibitor. Addition of 200 µM MG132 stabilized Luc163 in the lysate. (D) Addition of 200 µM MG132 enhanced the accumulation of polyubiquitinated forms of Luc163 in a rabbit reticulocyte lysate. (E–G) Densitometric quantification of polyubiquitin smear signals in Fig. 5 B (E), Fig. 5 E (F), and Fig. S4 D (G), respectively. Error bars represent SD calculated from three experiments. *P < 0.01, by t test.
BAG-6 mediates proteasomal degradation (Minami et al.). Figure S5. BAG-6 interacts with immunoproteasome and with aggresome. (A) HeLa cells were treated with 150 units/ml interferon γ (IFN-γ) for indicated times with 20 µM MG132. After cell harvest, endogenous BAG-6 was immunoprecipitated and precipitates were subjected to Western blotting with anti-immunoproteasome (β1i), anti-20S (zeta), and anti-BAG-6 antibodies. (B) BAG-6 moves to insoluble aggregates after proteasome inhibition, although most of BAG-6 exists as soluble protein in normal HeLa cells. HeLa cells were treated with (+) or without (−) MG132 (10 µM) for 6 h. The cellular homogenates were fractionated to obtain the supernatant (Sup.) and pellet (Ppt.) fractions, and these fractions were subjected to immunoblotting with anti-BAG-6 and anti-polyubiquitin antibodies, respectively. (C) BAG-6 associates with insoluble aggresomes under the condition of MG132 treatment. HeLa cells were treated with MG132 (5 µM) or DMSO (as a negative control) for 24 h, and fixed cells were stained with anti-BAG-6 (red), anti-vimentin (green), and anti-ubiquitin (green) antibodies. Superimposed confocal images (indicated as “Merge”) show the colocalization of BAG-6 with aggresome markers, vimentin and ubiquitin. The positions of the nucleus are shown by DAPI staining. Arrowheads indicate the aggresome. Bar, 10 µm. (D) To determine whether BAG-6 is required for aggresome formation, we reduced BAG-6 levels by using a specific siRNA. Depletion of BAG-6 from neuroblastoma cells inhibits the formation of ubiquitin-positive aggresome after 5 µM MG132 treatment. Results of quantified aggresome formation in BAG-6–depleted cells compared with that in control depletion cells, which were assigned a value of 100. Error bars represent SD calculated from three experiments. P < 0.05, by t test. This observation suggests that BAG-6 is functionally linked with aggresome formation at the time of proteasome inhibition.