

HspB8 Chaperone Activity toward Poly(Q)-containing Proteins Depends on Its Association with Bag3, a Stimulator of Macroautophagy^{*[5]}

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Mutations in HspB8, a member of the B group of heat shock proteins (Hsp), have been associated with human neuromuscular disorders. However, the exact function of HspB8 is not yet clear. We previously demonstrated that overexpression of HspB8 in cultured cells prevents the accumulation of aggregation-prone proteins such as the polyglutamine protein Htt43Q. Here we report that HspB8 forms a stable complex with Bag3 in cells and that the formation of this complex is essential for the activity of HspB8. Bag3 overexpression resulted in the accelerated degradation of Htt43Q, whereas Bag3 knockdown prevented HspB8-induced Htt43Q degradation. Additionally, depleting Bag3 caused a reduction in the endogenous levels of LC3-II, a key molecule involved in macroautophagy, whereas overexpressing Bag3 or HspB8 stimulated the formation LC3-II. These results suggested that the HspB8-Bag3 complex might stimulate the degradation of Htt43Q by macroautophagy. This was confirmed by the observation that treatments with macroautophagy inhibitors significantly decreased HspB8- and Bag3-induced degradation of Htt43Q. We conclude that the HspB8 activity is intrinsically dependent on Bag3, a protein that may facilitate the disposal of doomed proteins by stimulating macroautophagy.

The intracellular aggregation of proteins, whether it occurs as a result of proteotoxic stress or genetic mutations, represents a major threat in the crowded environment of the cell. Consequently, efficient mechanisms of protein quality control exist involving molecular chaperones such as Hsp70 and Hsp90, which can recognize and bind to unfolded proteins thereby preventing their aggregation (1–4). The fate of the chaperone substrates is determined by associated co-chaperones. CHIP, a ubiquitin E3 ligase and Bag1, a member of the Bag family of proteins (Bag1–Bag6) that contains a ubiquitin-like domain, associate with Hsp70 and facilitate the targeting of Hsp70 sub-

strates to the ubiquitin-proteasome pathway (5, 6). In contrast, by associating with Hsp70, Bag2 hampers CHIP activity and favors substrate renaturation (7, 8), whereas Bag3 prevents Hsp70 substrate degradation and causes ubiquitylated substrate accumulation (9).

The role of chaperone complexes containing Hsp70 has been experimentally highlighted in a range of neurodegenerative disorders characterized by the accumulation of protein aggregates (10–14). However, it is within the HspB group of heat shock proteins (also known as small heat shock proteins) that mutations have first been associated with human diseases (15). We previously showed that HspB8 (H11/Hsp22), a member of this family, has chaperone activity *in vivo* toward Htt43Q, a pathogenic form of huntingtin that contains an expanded polyglutamine stretch making the protein prone to aggregation. Overexpression of HspB8 but not two closely related family members, HspB1 (Hsp27) or HspB5 (α B-crystallin), accelerated the degradation of Htt43Q and prevented both aggregation and formation of inclusion bodies (16). A similar chaperone activity was observed using as substrate the androgen receptor protein containing a 65 glutamine extension (16), suggesting that this chaperone activity could at least be generalized to polyglutamine-containing proteins.

Here, we have investigated the mechanism of action of HspB8. We found that HspB8 forms a stable stoichiometric complex with the co-chaperone Bag3. We further show that Bag3 stimulates macroautophagy, thus facilitating the degradation of mutated huntingtin.

EXPERIMENTAL PROCEDURES

Plasmids and Reagents—pHDQ43-HA³ encoding a HA-tagged Huntingtin exon 1 fragment with 43 CAG repeats was from Dr. Rubinsztein (10). Plasmids encoding human HspB8 and myc-tagged HspB8, Hsp70, and HspB1, were described previously (16, 17). The plasmid encoding His-tagged human Bag3 (pCINHisBag3) was from Dr. Kohn (9). pGFP-LC3 and pMyc-LC3 were a kind gift of Dr. T. Yoshimori (18). 3-Methyladenine (3-MA), wortmannin, Pepstatin, and E64d were from Sigma.

Antibodies—Anti-HspB8 and anti-Hu27 are rabbit polyclonal antibodies against human HspB8 and HspB1, respectively (17). Anti-LC3 was developed in rabbit against a recombinant protein made of glutathione *S*-transferase fused to rat

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[5] The on-line version of this article (available at <http://www.jbc.org>) contains supplemental Figs. S1 and S2.

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³ The abbreviations used are: HA, hemagglutinin; 3-MA, 3-methyladenine; Hsp, heat shock protein; siRNA, small interfering RNA.

LC3 lacking the 22 C-terminal residues (ending at Gly¹²⁰, the hypothetical cleavage site in LC3-II) (18). Anti-Hsc70 and anti-Hsp70 were from Santa Cruz and Stressgen Bioreagents, respectively. Anti-HA (HA.11) and anti- γ -tubulin were from Sigma, anti-myc (9E10) was from American Type Culture Collection, and anti-Bag1 was from BIOSOURCE. Anti-Bag3 was from GeneTex except for the data of Fig. 1*b*, where an in-house antibody was used, which was raised against the recombinant glutathione *S*-transferase-human Bag3 fusion protein.

Cell Culture, Transfection, and Immunofluorescence Cell Staining—All cell lines were grown in Dulbecco's modified Eagle's medium with high glucose (Invitrogen). HeLa (human cervical cancer), HEK-293T (human embryonal kidney), and COS-1 (African green monkey kidney) cells were grown in medium supplemented with 10% fetal bovine serum, whereas 5% fetal bovine serum was used for CCL39 (Chinese hamster lung fibroblast) cells and 10% bovine calf serum was used for NIH3T3 (mouse embryonic fibroblast) cells. Cells were transfected by calcium phosphate precipitation as previously described (16). Transfection of siRNA for HspB8 (target sequence: agagcaguuuacaacaga), HspB1 (target sequence: ugagacugccggcaagaa), Bag3 (target sequence: gcaugccagaacacuca), and a control sequence (Dharmacon's siCONTROL non-targeting siRNA) were performed using Lipofectamine (Invitrogen), according to the manufacturer's instructions. Immunocytochemistry was performed as previously described (16), except that the cells were fixed with 100% methanol for 30 min at -20°C . For statistical analysis samples from three independent experiments were analyzed for each condition using the one-way analysis of variance test.

L-[³⁵S]Methionine Metabolic Labeling and Co-immunoprecipitation—HeLa cells were incubated overnight in minimum essential medium (Invitrogen) containing 4% fetal bovine serum and 15 $\mu\text{Ci}/\text{ml}$ of L-[³⁵S]methionine (Amersham Biosciences). Cells were lysed in a buffer containing: 20 mM Tris-HCl, pH 7.4, 2.5 mM MgCl₂, 100 mM KCl, 0.5% Nonidet P-40, 3% glycerol, 1 mM dithiothreitol, complete EDTA-free (Roche). The cell lysates were centrifuged and cleared with protein A-Sepharose beads at 4°C for 1 h. Protein A-Sepharose beads complexed with anti-HspB8 antibody or non-immune rabbit serum were added to the pre-cleared lysates. After incubation for 2 h at 4°C , the immune complexes were centrifuged and the supernatants (output) collected. Beads were washed 4 times with the lysis buffer; both co-immunoprecipitated proteins and output fractions were resolved on SDS-PAGE. Dried polyacrylamide gel was exposed overnight using a Molecular Dynamic Storage Phosphor Screen (Kodak). Co-immunoprecipitations from non-labeled HeLa cells were performed as described above.

Tandem Mass Spectrometry—Immunoprecipitates from HeLa cells using anti-HspB8 antibody were separated by SDS-PAGE and stained with Coomassie Blue R-250. The polypeptides migrating at ~ 70 – 80 kDa were excised from the gel and send to the Southern Alberta Mass Spectrometry Centre for Proteomics (University of Calgary) for analyses by liquid chromatography-tandem mass spectrometry, using an ESI-TRAP instrument.

Filter Trap Assay and SDS-soluble and -insoluble Cell Extracts—SDS-insoluble proteins were analyzed either by filter trap assay or PAGE. For filter trap assay, the cells were scraped and homogenized in 2% SDS filter trap assay buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 50 mM dithiothreitol). Four different dilutions of each sample were heated at 98°C for 3 min and applied with mild suction onto a cellulose acetate membrane. The membrane was washed with 0.1% SDS filter trap assay buffer and processed for Western blot analysis. For PAGE analysis of SDS-soluble and SDS-insoluble proteins, the cells were scraped, homogenized, and heated for 10 min at 100°C in 2% SDS sample buffer supplemented with 50 mM dithiothreitol. The samples were centrifuged for 20 min at room temperature and the supernatants were used as the SDS-soluble fraction. The pellets (SDS-insoluble fraction) were incubated with 100% formic acid for 30 min at 37°C , lyophilized overnight, and finally resuspended in the same volume of 2% SDS sample buffer as the SDS-soluble fraction. Both SDS-soluble and SDS-insoluble fractions were processed for Western blotting.

Glycerol Gradient Centrifugation—NIH 3T3 cell extracts were prepared 24 h after transfection by sonication in 1 ml of a 3.33% glycerol HEPES buffer (25 mM HEPES, 1 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, pH 7.4). After centrifugation at $18,000 \times g$ for 10 min at 4°C , the supernatants were loaded on top of a 11-ml linear gradient of glycerol (10–40%) made in HEPES buffer. The tubes were centrifuged for 16 h at $34,000 \times g$ in a SW40 rotor (Beckman) at 4°C . The gradients were fractionated and the distribution of HspB8 and Bag3 was analyzed by Western blotting. The gradient was calibrated from the positions of known protein complexes (19).

RESULTS

HspB8 Forms a Stable Stoichiometric Complex with Bag3—Upon expression in cells, poly(Q) proteins such as Htt43Q forms small aggregates all over the cytoplasm, which gradually become SDS-insoluble and fuse to each other to form large amorphous inclusion bodies that accumulate in the juxtanuclear area of the cells (16). We previously described that the overexpression of HspB8 prevented the accumulation of HA-tagged Htt43Q (HA-Htt43Q), presumably by accelerating its degradation. This effect was not observed when overexpressing two other members of the HspB family, HspB1 or HspB5. In an initial attempt to determine the mechanism of action of HspB8 as a molecular chaperone, we investigated whether functional partners of HspB8 could be identified. Extracts prepared from HeLa cells metabolically labeled with [³⁵S]methionine were immunoprecipitated with specific anti-HspB8 antibodies. A protein with an apparent relative molecular mass of 80,000 abundantly co-immunoprecipitated with HspB8 and was identified as Bag3 by mass spectrometry (Fig. 1*a*). Co-immunoprecipitation experiments followed by Western blots performed in HeLa cells (Fig. 1*b*) and also in COS-1 cells (data not shown) confirmed the specificity of the interaction between HspB8 and Bag3. Bag3, but not Bag1, was co-immunoprecipitated by HspB8 antibodies, whereas HspB8, but not HspB1, was co-immunoprecipitated with Bag3 antibodies.

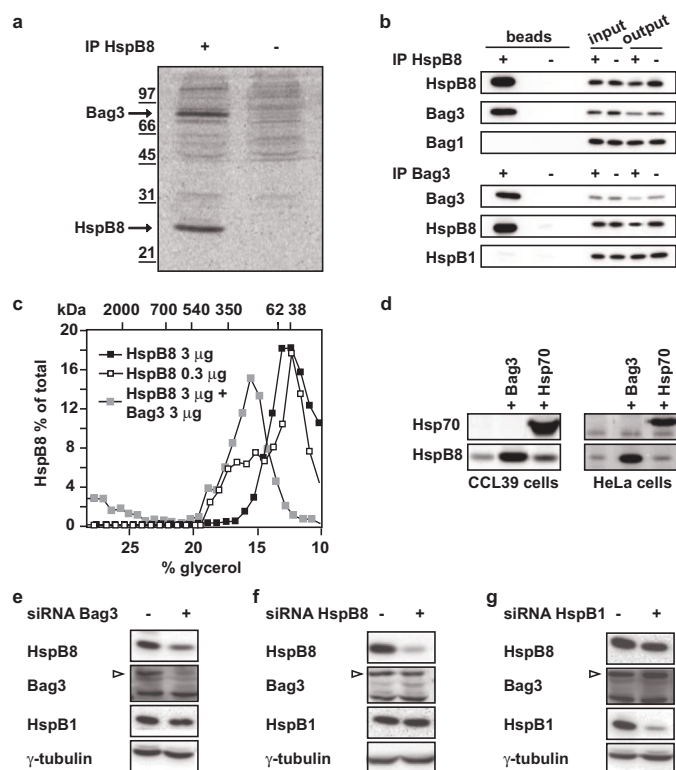


FIGURE 1. HspB8 forms a stable molecular complex with Bag3. *a*, extracts from HeLa cells were incubated overnight with 35 S-methionine and then immunoprecipitated using either anti-HspB8 (+) or a preimmune rabbit serum (–). Immunoprecipitated (IP) proteins were revealed by autoradiography. The bands corresponding to HspB8 and Bag3 are indicated. The numbers on the left correspond to the molecular mass of markers (kDa). *b*, specificity of the HspB8/Bag3 interaction. A protein extract from HeLa cells was immunoprecipitated with antibodies (+) against HspB8 (IP HspB8) or Bag3 (IP Bag3), or with a preimmune serum (–) and probed by Western with anti-Bag3, Bag1, HspB8, or HspB1 antibodies. Input and output correspond to 1/10 of the immunoprecipitated fraction. *c*, characterization of the HspB8-Bag3 complex. NIH-3T3 cells were transfected overnight with 0.3 or 3 μ g of plasmids coding for HspB8, alone or together with 3 μ g of plasmids coding for His-Bag3. Extracts were sedimented through 10–40% glycerol gradients. Fractions were analyzed by SDS-PAGE and Western blots to determine the concentration of HspB8 in each fraction relative to total. The position of known molecular markers is indicated on top. *d–g*, Bag3 stabilizes HspB8. *d*, HeLa and CCL39 cells were transfected for 44 h with myc-HspB8 alone (HspB8) or with myc-HspB8 and either His-Bag3 (+Bag3) or myc-Hsp70 (+Hsp70). Extracts were probed with the anti-myc antibody to measure the level of myc-HspB8 and myc-Hsp70. *e–g*, HeLa cells were transfected with mock solutions (–) or with 50 nM siRNA (+) targeting Bag3 (*e*), HspB8 (*f*), or HspB1 (*g*) and grown in complete medium for 48 h before harvesting. Bag3, HspB8, HspB1, and γ -tubulin protein levels were detected using specific antibodies. The empty arrowheads indicate the specific Bag3 signal.

HspB8 and Bag3 contain 5 and 10 methionine residues, respectively. From the amount of radioactivity associated with each of the two immunoprecipitated proteins, a stoichiometric ratio of 2:1 was determined for the HspB8-Bag3 complex. The complex was further characterized by glycerol gradient analysis in NIH3T3 cells, a cell line used here because it expresses very little endogenous HspB8. When expressed at high concentrations (transfecting 3 μ g of plasmid), HspB8 sedimented with an apparent molecular mass of about 50 kDa, as expected for a homodimer, the basic building unit of the HspB protein family (Fig. 1c) (19). However, when expressed at lower concentrations (transfecting 0.3 μ g of plasmid), a portion of the protein multimerized into large complexes sedimenting between the 62- and 350-kDa markers, consistent with the molecular mass

of 130 kDa predicted for the 2:1 HspB8-Bag3 complex. This suggested that upon transfection of HspB8, endogenous Bag3 becomes limiting for the formation of the HspB8-Bag3 complex. In agreement with this idea, co-expressing Bag3 with HspB8 allowed a higher proportion of HspB8 to incorporate into the approximate 130-kDa complex (Fig. 1c). We also determined that the presence of Bag3 was important for the stability of HspB8. In CCL39 and HeLa cells, overexpressing Bag3 together with HspB8 yielded a much higher expression of HspB8 than overexpressing HspB8 alone or HspB8 with Hsp70 (taken as control) (Fig. 1d). This was not caused by an increased transcriptional activity because HspB8 mRNA levels were not affected by Bag3 overexpression (supplemental Fig. S1). These findings suggested that the HspB8 proteins may be less stable when Bag3 is limiting. Accordingly, knocking down Bag3 expression by 75% using siRNA resulted in a 75% decrease in endogenous HspB8 expression, without affecting the expression of HspB1 (Fig. 1e). In contrast, reducing the HspB8 concentration below detectable levels caused little reduction in Bag3 (Fig. 1f). As an additional control, knocking down HspB1 caused no significant change in the expression level of either HspB8 or Bag3 (Fig. 1g). The reduction of HspB8 endogenous levels following treatment with Bag3 siRNA was observed not only in HeLa cells, where the complex has been first identified, but also in HEK-293T cells (see Fig. 4). All together these observations suggest that in different cell types, most HspB8 is associated with a pool of Bag3 and that this complex contributes importantly to HspB8 stability.

Bag3 Also Prevents Htt43Q Accumulation—We next investigated whether overexpression of Bag3 in cultured cells, like HspB8, prevents the accumulation of the polyglutamine protein Htt43Q. Cells were transfected with vectors encoding HA-Htt43Q alone or HA-Htt43Q and either Myc-HspB8, His-tagged Bag3 or both. The accumulation of Htt43Q aggregates was analyzed 44 h after transfection using the filter trap assay (filtration of the SDS lysates through a cellulose acetate membrane, which entraps the aggregates). Expression of Bag3, like HspB8, but not expression of Hsp70, dramatically reduced the accumulation of insoluble Htt43Q in both CCL39 cells (Fig. 2a), the cell line where the activity of HspB8 was initially characterized (16) and in HeLa cells (Fig. 2b). Similar results were also obtained in HEK-293T and COS-1 cells (see Figs. 3, 4, 6, and supplemental Fig. S2), suggesting that the activity was cell line-independent. Notably, an additive effect was observed when HspB8 and Bag3 were expressed together (Fig. 2, a and b). In control experiments in which green fluorescent protein was co-transfected with mutated huntingtin, HspB8 or Bag3 produced no reduction in the percentage of cells expressing green fluorescent protein nor in the survival of these cells, indicating that the reduction of HA-Htt43Q expression was not a consequence of toxicity (data not shown).

HspB8 Requires Bag3 to Exert Its Full Chaperone Activity—We then determined whether Bag3 was required for the activity of HspB8. HEK-293T cells were transfected with a control siRNA sequence or with a specific siRNA directed against Bag3. Twenty-four hours later, the cells were transfected with vectors encoding HA-Htt43Q alone or with myc-HspB8. Cells were incubated for an additional 48 h before being analyzed for the

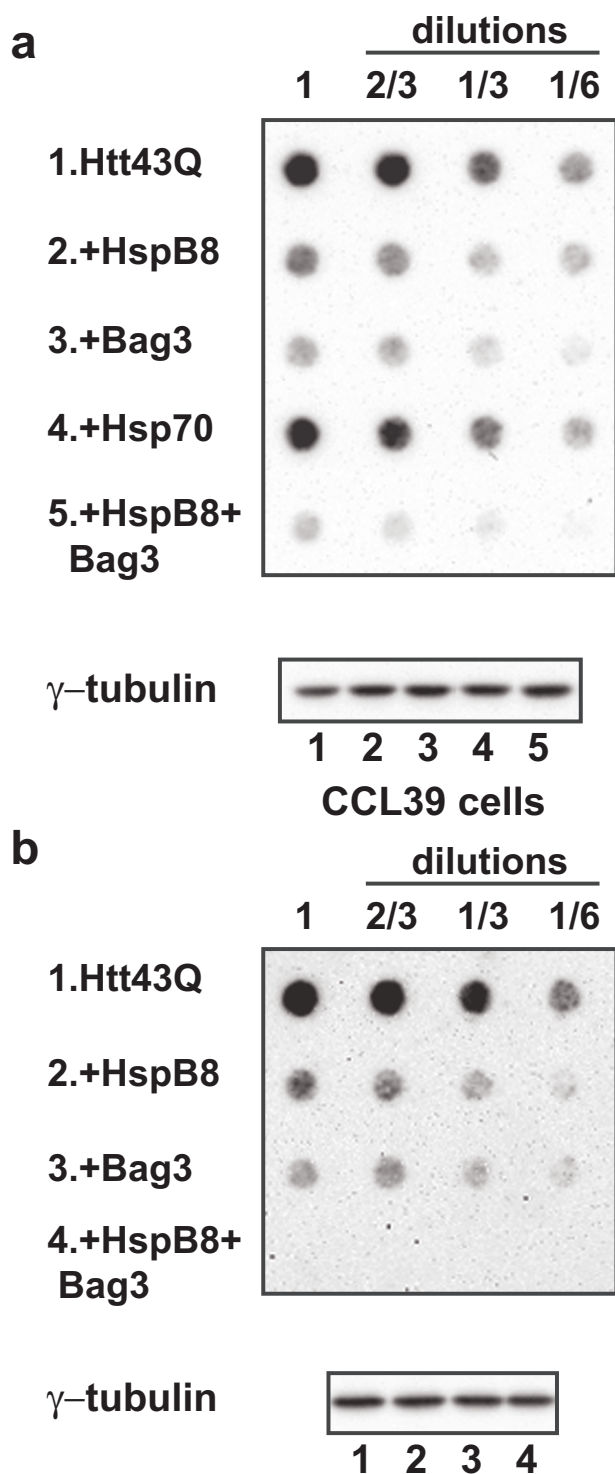


FIGURE 2. HspB8 and Bag3 prevent the accumulation of Htt43Q. CCL39 (a) or HeLa (b) were transfected with plasmids encoding HA-Htt43Q together with either an empty vector (Htt43Q) or Myc-HspB8 (+HspB8), His-tagged Bag3 (+Bag3), Hsp70 (+Hsp70), or both Myc-HspB8 and His-Bag3 (+HspB8+Bag3). Forty-four h later, the cellular extracts were filtered at 4 different concentrations (relative concentrations of 1, 2/3, 1/3, and 1/6) on a cellulose acetate membrane to trap SDS-insoluble materials and probed with anti-HA. As a loading control, aliquots of the extracts were analyzed by SDS-PAGE followed by Western blots using anti-γ-tubulin. In the γ-tubulin panels, track numbers refer to transfection group numbers as shown on the left side of the blots.

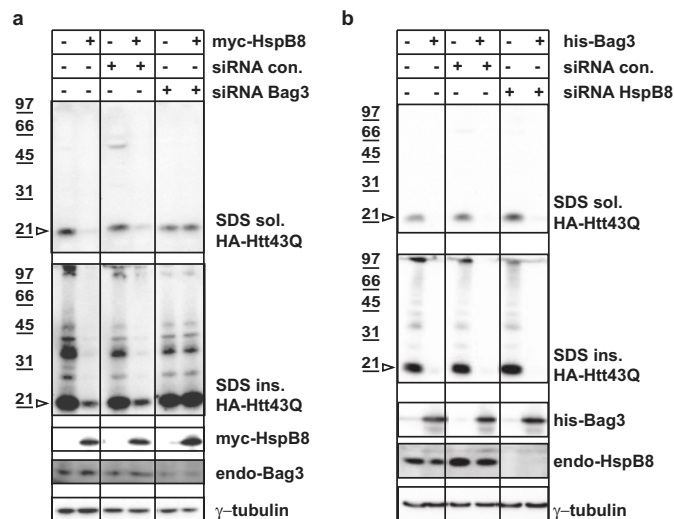


FIGURE 3. HspB8 requires Bag3 to efficiently prevent the accumulation of Htt43Q. HEK-293T cells were either sham-treated (–) or treated (+) with a 50 nM solution of control siRNA (siRNA con.), siRNA targeting Bag3 (siRNA Bag3), or siRNA targeting HspB8 (siRNA HspB8). Twenty-four h later, the cells were transfected with plasmids encoding HA-Htt43Q together with an empty vector (–), Myc-HspB8, or His-tagged Bag3 (+). SDS-soluble and SDS-insoluble fractions were prepared 44 h post-transfection. HA-Htt43Q levels were analyzed in both the SDS-soluble and SDS-insoluble fractions. The empty arrowheads show HA-Htt43Q migrating at ~21 kDa. The efficiency of the siRNA treatments or transfections were measured by probing in the SDS-soluble fraction the levels of endogenous Bag3 (endo-Bag3), endogenous HspB8 (endo-HspB8), transfected HspB8 (myc-HspB8), transfected Bag3 (his-Bag3), and γ-tubulin (a and b).

presence of SDS-soluble and SDS-insoluble HA-Htt43Q. Knocking down Bag3 expression efficiently blocked the ability of HspB8 to prevent Htt43Q accumulation (Fig. 3a). This was not due to a decreased expression of myc-HspB8 after the Bag3 siRNA treatment, thus suggesting that HspB8 requires Bag3 to exert its full chaperone function. A similar experiment was performed to investigate the impact of HspB8 knock-down on Bag3 activity. Fig. 3b shows that overexpressed Bag3 can block Htt43Q accumulation also in the absence of HspB8. All together these results suggested that Bag3 is an essential element of the HspB8-Bag3 complex and is responsible for the activity ascribed to HspB8 in decreasing Htt43Q accumulation.

Decreasing Endogenous Bag3 Expression Resulted in Enhanced Htt43Q Aggregation—Both Bag3 and HspB8 are up-regulated upon proteotoxic stress such as exposures to heavy metal or heat shock (20, 21). Therefore, HspB8 and/or Bag3 overexpression experiments mimic well the cellular stress response activated by misfolded proteins and aimed at their clearance. From the data presented in Fig. 3, however, it seemed that knocking down the expression of Bag3 or HspB8 alone had no significant effect on the accumulation of HA-Htt43Q. To better evaluate the role of endogenous HspB8 and Bag3 on Htt43Q accumulation, we repeated the experiment transfecting HEK-293T cells with a lower amount of cDNA encoding for HA-Htt43Q. Fig. 4a shows that pre-treatment with Bag3 and HspB8 siRNA together caused a significant increase in the amount of SDS-insoluble HA-Htt43Q as compared with cells transfected with control siRNA. This effect was quantified in two independent experiments and corresponded to a 2-fold increase in the amount of SDS-insoluble Htt43Q. Treatments

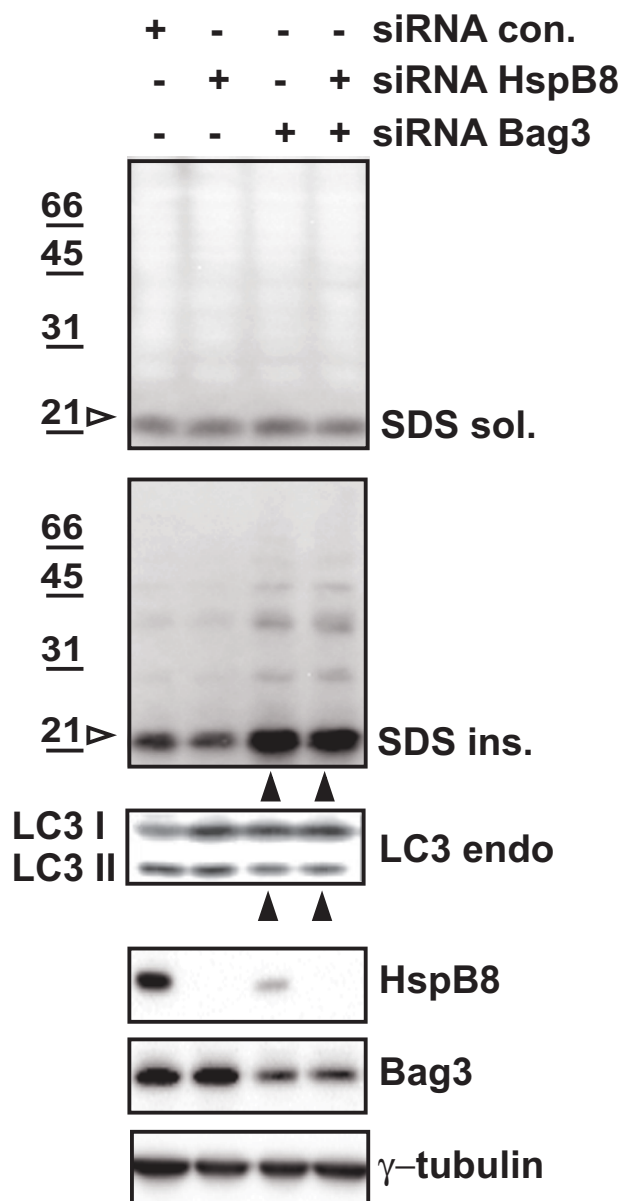


FIGURE 4. Knocking down Bag3 expression increases Htt43Q accumulation and decreases LC3 II accumulation. HEK-293T cells were transfected with 50 nM of either control siRNA (*siRNA con.*) or siRNA targeting HspB8, Bag3, or both HspB8 and Bag3, as indicated by the "+" and "-" signs. Forty-eight h later the cells were transfected with HA-Htt43Q using two times less plasmids than in Fig. 3, *a* and *b*. SDS-soluble and -insoluble fractions were prepared 48 h later and probed with anti-HA antibody. The empty arrowheads show HA-Htt43Q migrating at ~21 kDa. Levels of endogenous LC3 I and LC3 II, HspB8, Bag3, and γ -tubulin were also measured in the SDS-soluble fraction. The closed arrowheads highlight the increased levels of Htt43Q and decreased levels of LC3 II in samples pre-treated with siRNA targeting HspB8 and Bag3 or Bag3 alone.

with Bag3 siRNA alone (which, as described in Fig. 1e, also induced the depletion of HspB8) resulted in a similar increase of SDS-insoluble HA-Htt43Q. In contrast, depleting HspB8 alone had no effect. These results strongly suggested that Bag3 plays an active role in the basal capacity of the cells to eliminate badly folded proteins as they are produced. Whether or not Bag3 does it as part of the HspB8-Bag3 complex and uses alternative partners when HspB8 levels are decreased remains to be determined.

Htt43Q Accumulation in Cells Depleted for Bag3 Correlates with Decreased Macroautophagy Activity—A previous report indicated that Bag3 inhibits rather than stimulates the proteasomal degradation of proteins, suggesting that an alternative mechanism of protein degradation might be stimulated by Bag3 (9). Furthermore, macroautophagy is emerging as a significant proteolytic mechanism regulating levels of aggregated proteins, including expanded huntingtin (22–31). Macroautophagy is a bulk degradation process capable of degrading portions of cytoplasm and organelles (32, 33). On the basis of these findings we investigated the implication of macroautophagy in the Bag3 mechanism of action.

We first asked whether the accumulation of SDS-insoluble HA-Htt43Q observed following HspB8/Bag3 depletion correlated with an altered macroautophagy activity. This question was addressed by measuring the expression of the macroautophagy marker LC3. LC3, a mammalian homologue of yeast Atg8, undergoes complex post-translational modifications leading to 2 main forms: LC3 I, which has a diffuse cytoplasmic distribution and LC3 II, a lipidated form of LC3 I specifically found associated with macroautophagic vacuoles at all stages of formation (18, 34). Levels of LC3 II are considered a good indicator of macroautophagy activity. Intriguingly, we found that knocking down Bag3 alone or with HspB8, but not knocking down HspB8 alone, produced a decrease in the endogenous level of LC3 II (Fig. 4, *black arrowheads*). These results strongly suggested that macroautophagy is involved in the HspB8/Bag3 mechanism of action.

HspB8/Bag3 Stimulates Macroautophagy—To determine whether the accelerated degradation of Htt43Q observed upon overexpression of HspB8/Bag3 could be due to a stimulation of macroautophagy, we analyzed the effect of HspB8 or Bag3 overexpression on the levels of LC3 II. Myc-LC3 was transfected in HEK-293T cells alone or with either HspB8 or His-Bag3. Western blot analysis of the cell extracts using anti-Myc confirmed the accumulation of LC3 II upon expression of HspB8 or Bag3 (Fig. 5, *a* and *b*). The same results were obtained whether or not HA-Htt43Q was co-transfected with HspB8 or Bag3. This indicated that macroautophagy was activated independently of the presence of the misfolded protein. Pepstatin A and E64d, two lysosomal inhibitors that block the final step of macroautophagy leading to LC3 II degradation (35) did not induce a significant increase of LC3 II in control cells but did enhance LC3 levels in the presence of Bag3, indicating that macroautophagy was induced and LC3 turnover was higher in these cells than in control cells (Fig. 5*b*). A similar stimulation of Myc-LC3-II formation by HspB8 and Bag3 was obtained in COS-1 cells (Fig. 5, *c–e*). Bag1 used as a negative control caused no accumulation of LC3-II (Fig. 5*d*).

Macroautophagy Mediates HspB8/Bag3-stimulated Htt43Q Degradation—To confirm the role of macroautophagy in regulating the level of transfected HA-Htt43Q, COS-1 and HEK-293T cells were transfected overnight with a low concentration of plasmid encoding HA-Htt43Q and then incubated for 30 h in the presence of the macroautophagy inhibitors wortmannin and 3-MA. Wortmannin and 3-MA are two inhibitors of the class III phosphatidylinositol 3-kinase, the activity of which is required for the formation of the autophagic vacuoles (32, 33).

HspB8-Bag3, a Chaperone Complex for Macroautophagy

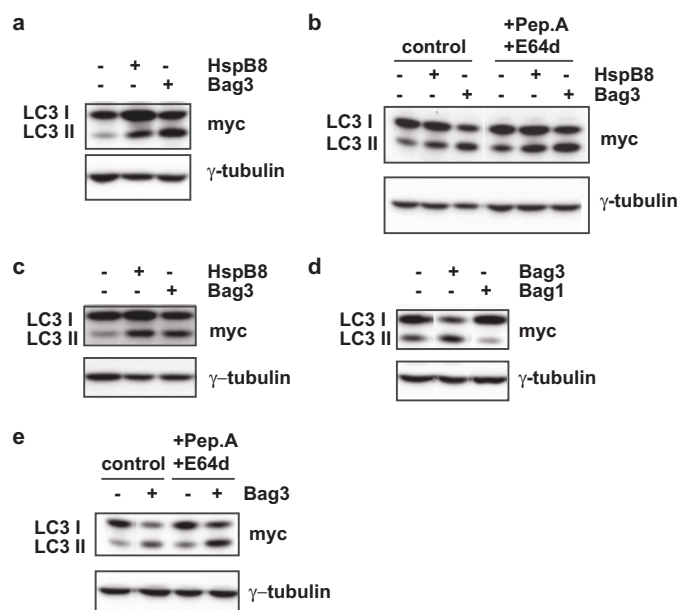


FIGURE 5. HspB8 and Bag3 stimulate the formation of LC3-II. HEK-293T (*a* and *b*) or COS-1 (*c–e*) cells were transfected with myc-LC3 or myc-LC3 plus either HspB8 or His-Bag3, as indicated by the “+” and “–” signs, in the presence (*b–e*) or absence (*a*) of HA-Htt43Q. Forty-four h (*a* and *c–e*) or 52 h later (*b*) extracts were prepared and analyzed by Western blotting using the anti-myc and anti- γ -tubulin antibodies. In *b* and *e*, the cells were either left untreated (control) or treated for 6 h with the lysosomal inhibitors Pepstatin A (10 μ g/ml) and E64d (10 μ g/ml) before extraction.

Transfection in the presence of the inhibitors led to a higher accumulation of insoluble Htt43Q than observed in untreated cells (Fig. 6*a*), thus confirming the implication of macroautophagy in HA-Htt43Q clearance.

We then evaluated the impact of blocking macroautophagy on the HspB8 and Bag3-stimulated degradation of HA-Htt43Q. Cells overexpressing HA-Htt43Q alone or HA-Htt43Q with either Myc-HspB8 or His-Bag3 were exposed to 3-MA and wortmannin for 30 h before being harvested and examined for Htt43Q accumulation. In cells transfected with HA-Htt43Q alone, treatments with the inhibitors induced a modest increase in the amount of HA-Htt43Q mainly accumulating as cross-linked HA-Htt43Q species (Fig. 6*b*). The effect was consistent with the presence of a basal level of macroautophagy activity directed against aggregated proteins. The efficacy of the macroautophagy inhibitor treatment on the accumulation of HA-Htt43Q overexpressed alone appears lower in the experiment reported in Fig. 6*b* than the one reported in Fig. 6*a*; this was due to the fact that 10 times more Htt43Q was expressed in the former. In contrast, the inhibitors of macroautophagy severely reduced HspB8 and Bag3 activities on Htt43Q degradation. These results support the hypothesis that Bag3 stimulates macroautophagy, which as a result, leads to the degradation of mutated huntingtin.

DISCUSSION

Molecular chaperones are at the front line of the cellular defense mechanisms against damaged or unstable proteins. They recognize and bind proteins that are in non-native conformations whether due to protein-denaturing stress or genetic mutations, and in collaboration with co-chaperones, either

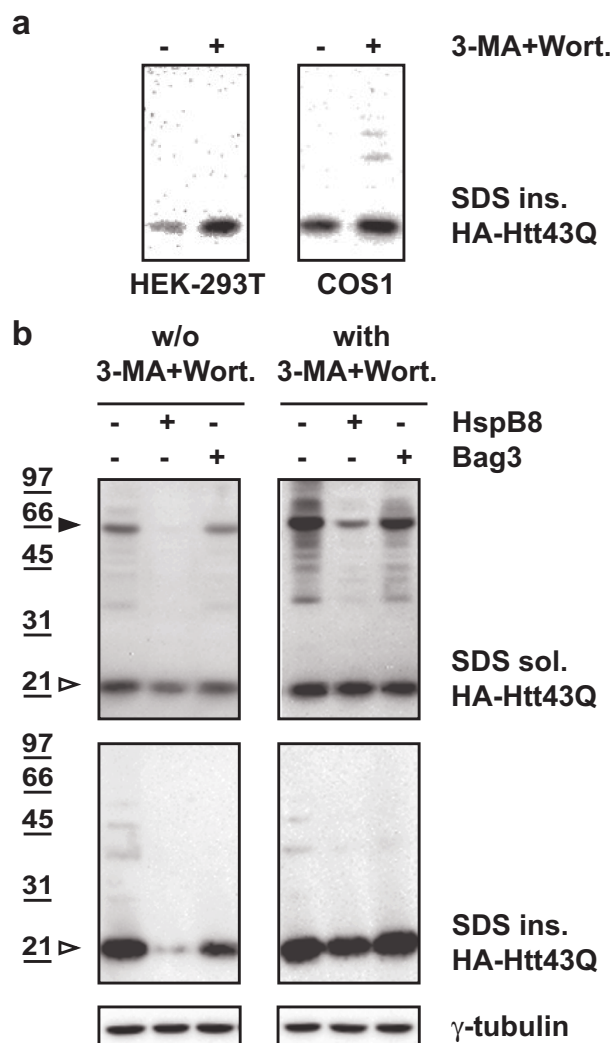


FIGURE 6. Basal and HspB8/Bag3-stimulated degradation of Htt43Q is regulated by macroautophagy. *a*, HEK-293T and COS1 cells were transfected with the plasmid encoding HA-Htt43Q. Twelve h post-transfection, cells were treated with 10 mM 3-MA and 0.2 μ M wortmannin (+) for 30 h or left untreated (–). SDS-insoluble extracts were then prepared and analyzed by Western blotting using the anti-HA antibody. *b*, COS1 cells were transfected with vectors encoding HA-Htt43Q and an empty vector (–), MycHspB8 (+HspB8), or HisBag3 (+Bag3). Fourteen h post-transfection, the cells were left untreated (w/o 3-MA and Wort.) or treated with 10 mM 3-MA and 0.2 μ M wortmannin (with 3-MA and Wort.) for 30 h. SDS-soluble and SDS-insoluble fractions were separated and analyzed by Western blotting using the anti-HA and the γ -tubulin antibodies.

assist them for refolding or target them for degradation (1, 2). Hence, molecular chaperone activity is extremely important in a number of conformational diseases characterized by the accumulation of misfolded and aggregated proteins. This has been well established for the molecular chaperone Hsp70 using both cellular and animal models of neurodegenerative disorders (10–14). One major activity of Hsp70 and co-chaperones is to target individual unfolded proteins to the proteasome. The tight cooperation between Hsp70, which can recognize unfolded proteins and the co-chaperones (*e.g.* Bag1, CHIP, and Hsp40), which regulate the targeting to the proteasome system, assures the selective degradation of the misfolded species, thus preventing or decreasing their aggregation (5, 36, 37). We previously showed that HspB8, member of the HspB family, has

chaperone activity in mammalian cells. In the present study, we searched for proteins that could interact with and mediate its chaperone function. We found that HspB8 forms a stable and stoichiometric complex with Bag3, a BAG domain-containing protein. We next showed that Bag3 is able to stimulate macroautophagy, thus facilitating the degradation of mutated Htt43Q.

Macroautophagy is an important nutrient-sensing process that regulates several biological pathways under both growth conditions and stress. Portions of cytoplasm and organelles are ensheathed by an isolation membrane to form double-membraned vacuoles, which acquire proteolytic capacity after fusion with lysosomes/endosomes to form the autophagolysosome (32, 33). In both cellular and animal models of protein conformational diseases, macroautophagy is emerging as an important proteolytic pathway capable of digesting microaggregated proteins (26, 30, 31). However, macroautophagy is considered as a rather unspecific proteolytic process and the mechanisms by which aggregated proteins can be selectively degraded by macroautophagy are not clearly understood. Recently it has been proposed that p62/SQSTM1, a polyubiquitin-binding protein that forms a shell around aggregated proteins, is involved in recruiting autophagosomal components to polyubiquitinated protein aggregates (24, 38). An additional mechanism increasing specificity was proposed to result from an active transport system that concentrates together autophagosomes, lysosomes, and protein microaggregates in the pericentriolar region. Such a transport occurs on microtubules and is mediated by HDAC6, a protein capable of binding both polyubiquitylated substrates and the dynein motors (23, 39). By showing a direct link between the macroautophagy machinery and a member of the heat shock protein family of molecular chaperones, our study provides yet another mechanism that could confer selectivity to macroautophagy. As discussed below, a likely scenario is that in the HspB8-Bag3 complex, HspB8 provides the substrate recognition capacity, whereas Bag3 mediates the interaction of the substrate-loaded complex with proteins involved in autophagosome formation and/or trafficking.

HspB8, is a member of the HspB group of Hsp, which, notwithstanding their lack of ATPase activity, are recognized as true molecular chaperones that can bind denatured substrates and keep them competent for further processing by additional chaperones and co-chaperones (40, 41). In particular, the capacity of HspB8 to act as a chaperone has been illustrated both with heat-denatured proteins in test tubes and mutated unstable proteins expressed in cultured cells (16, 17, 21). It is therefore likely that in the complex formed by HspB8 and Bag3, HspB8 is responsible for recognizing and binding misfolded proteins. In agreement with this model, overexpressing HspB8 in cells depleted of Bag3 as a result of siRNA pre-treatment was inefficient in degrading Htt43Q.

Bag3 is a multidomain protein likely interacting with many other polypeptides. A role as a platform from which macroautophagy could be stimulated is therefore plausible. Bag3 possesses three canonical protein-protein interaction domains, namely a WW domain mediating interaction with proline-rich sequences, a proline-rich domain mediating interactions with

WW-domain proteins or SH3-domain proteins such as phospholipase C γ -1, and a BAG domain known to mediate interactions with Bcl2 and Hsp70/Hsc70 (42, 43). One may speculate that the WW and the proline-rich domains provide localization cues to the substrate-loaded complex and/or ways to stimulate signalization cascades leading to the formation of autophagic vacuoles and the lipidation of LC3. A role for the BAG domain may be suggested. In addition to its role in apoptosis, Bcl2 was reported to bind Beclin 1, an evolutionarily conserved promoter of macroautophagy (44, 45). In theory, a recruitment of Hsp70 to the complex may at least in part explain the observed capacity of Bag3 to work in the absence of HspB8, in which case, Hsp70 could substitute to the chaperone activity of HspB8. This is also suggested by the fact that Hsp70 is known to participate in the chaperone activity of other proteins of the HspB family (41, 46). Nevertheless, except for HspB1 which, as demonstrated here, does not interact with Bag3, any of the 8 other members of the HspB family could participate in this activity. The characterization of the Bag3 domains should help uncover the mechanisms of action of this complex in stimulating macroautophagy.

Reducing the level of HspB8 alone had no effect on the capacity of the cells to eliminate the production of aggregated protein (as revealed by the accumulation of Htt43Q). However, Bag3 knockdown (which also drastically reduced HspB8 levels) did cause an increase in the accumulation of the aggregated proteins. Together with the result that depleting HspB8 had little effect on the capacity of Bag3 to stimulate macroautophagy and to degrade Htt43Q, it is suggested that Bag3 with or without HspB8, has a significant contribution to protein quality control, whereas HspB8 is dependable. It is possible that the HspB8-Bag3 complex does play a role in protein quality control but that in the absence of HspB8, another chaperone can substitute to HspB8. As discussed above, obvious candidates are Hsp70 and other proteins of the HspB family. Another possibility is that the HspB8-Bag3 complex plays yet unidentified functions in regulating specific proteins in resting cells, whereas it becomes a powerful regulator of protein quality in conditions of stress. Both HspB8 and Bag3 are up-regulated upon proteotoxic treatments such as heat shock or exposure to heavy metal (20, 21). During stress, the additional HspB8 and Bag3 produced may combine in active complexes to eliminate denatured and dangerous proteins.

Recently two mutations in HspB8 (K141E and K141N) have been associated with the development of hereditary peripheral neuropathies (47). However, although deregulated macroautophagy has been reported in a number of diseases affecting both neuronal and muscular cells, the specific role of macroautophagy in these neuropathies has not been documented (26–29). Moreover, it is not yet clear whether the participation of HspB8 in the progression of these neurodegenerative disorders is due to a loss of its chaperone activity or a gain of toxic function. Indeed, the Lys¹⁴¹ mutants of HspB8 and mutants at equivalent residues in two other proteins of this family, HspB1 and HspB5, are characterized by structural instability and tend to aggregate in the cells (17, 47–50). We previously demonstrated that mutations at Lys¹⁴¹ caused a small but significant decrease in HspB8 chaperone function. This was also accompanied by a

reduction in HspB8 protective activity, as demonstrated by the accumulation of Htt43Q in cellular debris (16). However, we found that the pathogenic HspB8 mutations have no apparent effect on the interaction of HspB8 with Bag3 (data not shown). As HspB8 mutations are associated with slowly developing diseases, a minor alteration in activity may with time suffice to significantly affect cell physiology. This is particularly true because the progressive aggregation of the unstable HspB8 mutants combined with the lost capacity to stimulate the autophagic vacuoles formation may act in an amplification loop leading to more and more aggregated proteins and cell death. In conclusion, our results show that HspB8 exerts its chaperone function through its association with Bag3, a stimulator of macroautophagy that promotes the disposal of mutated huntingtin.

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