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Regulation of the Cytoplasmic Quality Control Protein Degradation Pathway by BAG2*

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The cytoplasm is protected against the perils of protein misfolding by two mechanisms: molecular chaperones (which facilitate proper folding) and the ubiquitin-proteasome system, which regulates degradation of misfolded proteins. CHIP (carboxyl terminus of Hsp70-interacting protein) is an Hsp70-associated ubiquitin ligase that participates in this process by ubiquitylating misfolded proteins associated with cytoplasmic chaperones. Mechanisms that regulate the activity of CHIP are, at present, poorly understood. Using a proteomics approach, we have identified BAG2, a previously uncharacterized BAG domain-containing protein, as a common component of CHIP holocomplexes *in vivo*. Binding assays indicate that BAG2 associates with CHIP as part of a ternary complex with Hsc70, and BAG2 colocalizes with CHIP under both quiescent conditions and after heat shock. *In vitro* and *in vivo* ubiquitylation assays indicate that BAG2 is an efficient and specific inhibitor of CHIP-dependent ubiquitin ligase activity. This activity is due, in part, to inhibition of interactions between CHIP and its cognate ubiquitin-conjugating enzyme, UbcH5a, which may in turn be facilitated by ATP-dependent remodeling of the BAG2-Hsc70-CHIP heterocomplex. The association of BAG2 with CHIP provides a cochaperone-dependent regulatory mechanism for preventing unregulated ubiquitylation of misfolded proteins by CHIP.

Cell viability is constantly threatened by protein misfolding within the cytoplasm due to imprecise *de novo* protein folding and the consequences of oxidative and thermal stresses and conformational chain reaction events that affect protein structure. Because of the cellular inefficiencies and toxicities associated with off-pathway protein conformations, tightly regulated systems have evolved to minimize protein misfolding. The molecular chaperones constitute one component of the cytoplasmic protein quality control process. These proteins (including Hsp70, Hsp90, TRiC, and other associated proteins) assist in cotranslational folding, maintain metastable protein conformations, and repair proteins that are structurally defective. The molecular regulation and coordination of cytoplasmic folding and refolding are becoming increasingly clear (1).

In addition to promoting proper folding, a second requirement of protein quality control mechanisms is the efficient removal of proteins that are irreversibly damaged or extremely toxic. Degradation of misfolded proteins occurs predominantly through the ubiquitin-proteasome system. For proteins that are misfolded within the endoplasmic reticulum, the protein degradation pathways are well described (2). Less is known about degradation of misfolded proteins within the cytoplasm. Recently, the co-chaperone/ubiquitin ligase CHIP (carboxyl terminus of Hsp70-interacting protein) has been implicated in the degradation of a variety of chaperone-bound cytoplasmic proteins (3, 4). CHIP inhibits the ATPase activity of Hsp70 (5) and has U box-dependent ubiquitin ligase activity that targets a range of chaperone substrates for proteasome-dependent degradation (6, 7). The proteins that are marked for degradation by CHIP include substrates of Hsp70 (such as the cystic fibrosis transmembrane conductance regulator (CFTR)[3]), Hsp90 (ErbB2), and nonnative misfolded proteins that bind cytoplasmic chaperones (4, 8–10). Dimerization is a necessary step for this ubiquitin ligase activity, at least *in vitro* (11). A general model has been developed, which suggests that recruitment of CHIP to chaperone holocomplexes converts the chaperone machinery to a degradation-competent status to facilitate the removal of misfolded proteins (6, 7, 12). The recent demonstration that CHIP participates in the protein degradation pathway for misfolded, but not active, estrogen receptor (13) supports this model.

What remains unclear is how chaperone holocomplexes are remodeled to regulate the activity of CHIP, which, due to its constitutive expression, could be prone to ubiquitylate proteins on the proper folding pathway unnecessarily if its activity were unrestrained. In addition, it is also not certain how decisions are made by a chaperone complex to either refold or degrade a protein, a process referred to as "protein triage" (14). To resolve these questions, we have performed mass spectrometry to determine the components of endogenous CHIP complexes. We have found that BAG2 is present in cytoplasmic chaperone complexes that contain CHIP and is an endogenous inhibitor of CHIP ubiquitin ligase activity, at least partly, by interfering with CHIP-ubiquitin-conjugating enzyme interactions, thus providing a cochaperone-dependent mechanism for regulating CHIP activity.

MATERIALS AND METHODS

Antibodies—Rabbit polyclonal anti-CHIP and anti-BAG2 antibodies were described previously (5). Rabbit polyclonal anti-Hsc70 (SPA 816) was from Stressgen. Mouse anti-CHIP monoclonal antibody (colony 67) was produced in collaboration with the University of North Carolina Monoclonal Antibody Core Facility. Mouse CFTR NBD1-R domain-

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[3] The abbreviations used are: CFTR, cystic fibrosis transmembrane conductance regulator; GST, glutathione *S*-transferase; AMP-PNP, 5′-adenylyl-β,γ-imidodiphosphate; TOF, time of flight.

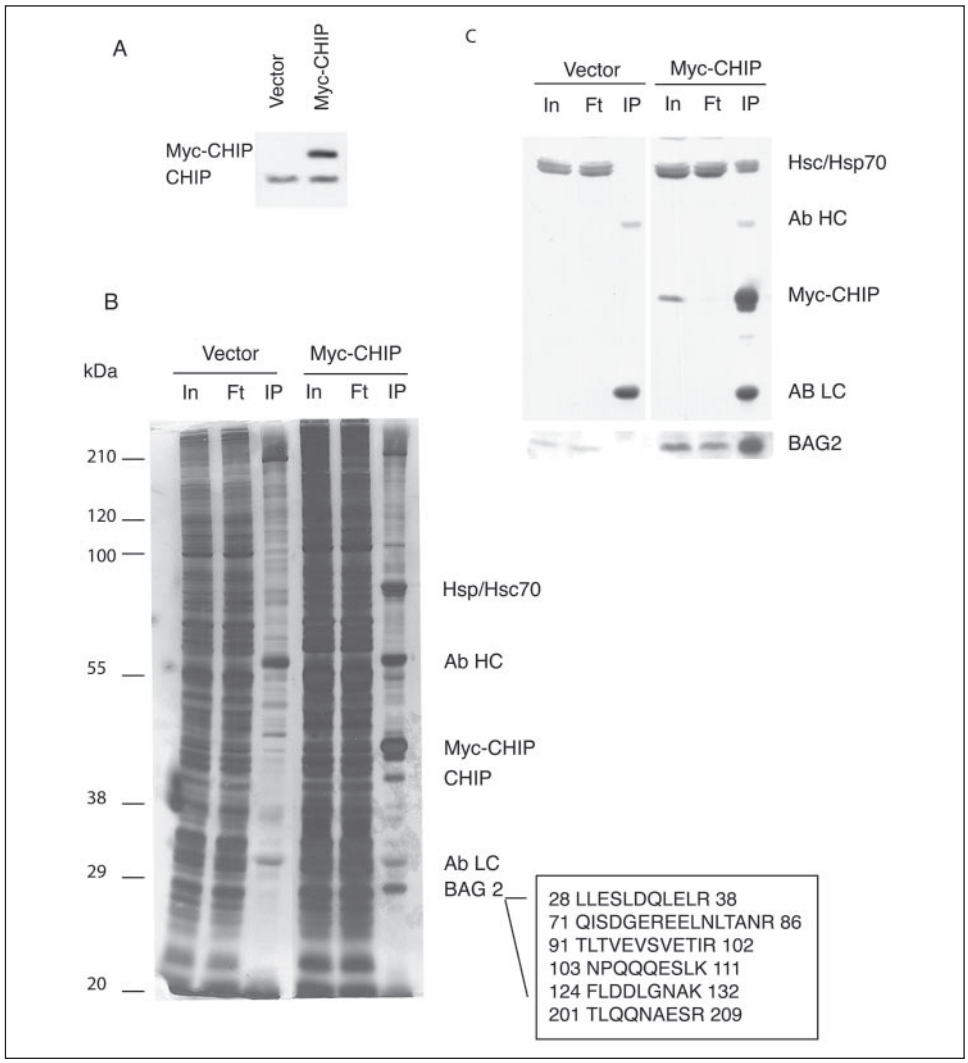

FIGURE 1. **Identification of BAG2 as a component of CHIP-containing complexes *in vivo*.** *A*, HeLa cell lines were stably transfected with a plasmid containing Myc-tagged CHIP or vector alone. Clones expressing Myc-CHIP at nearly endogenous levels, as confirmed by Western blot analysis, were selected and used to identify endogenous proteins present in CHIP complexes *in vivo*. *B*, silver staining of input (*In*), flow-through (*Ft*), and Myc immunoprecipitates (*IP*) from these HeLa cell lines indicates the proteins that co-purify with CHIP. A 26-kDa protein was sequenced by mass spectrometry, and the six indicated peptide sequences were identified, which led to the conclusion that this protein was BAG2. *C*, Western blot analysis with antibodies (*Ab*) against Hsp/Hsc70, CHIP, and BAG2 confirmed the identities of these proteins in immunoprecipitates from Myc-CHIP-expressing cells.

specific antibody (monoclonal antibody 1660) was from R&D Systems, and the MM13–4 anti-CFTR antibody was from Upstate Biotechnology, Inc. (Lake Placid, NY). Anti-rat fluorescein isothiocyanate and anti-mouse Texas Red fluorescent antibodies were from Molecular Probes, Inc. (Eugene, OR).

Cell Culture and Transfection—To generate stable transfectants, pcDNA3-Myc-CHIP or pcDNA3 alone was transfected into HeLa cells cultured on 100-mm plates. 48 h after transfection, cells were split, and G418 was added at a working concentration of 400 μg/ml. Two weeks after transfection, G418-resistant colonies were isolated with cloning rings and screened for expression by Western blotting. For transient transfections, HEK293 cells were transfected with equivalent concentrations of CFTRΔF508, CHIP, and BAG2 plasmids using Effectene (Qiagen) at a DNA/Effectene ratio of 1:5. Cells were lysed with radioimmune precipitation buffer 24 h after transfection, and CFTR protein levels were analyzed by SDS-PAGE followed by Western blotting.

Mass Spectrometry Detection of CHIP-associated Proteins—HeLa cells stably expressing Myc-CHIP were cultured in 150-mm plates to confluence. Cells were lysed with radioimmune precipitation buffer (50 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EGTA, 0.25% sodium deoxycholate, 1% Nonidet P-40, and 1 mM sodium orthovanadate). Anti-Myc-agarose (9E10; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was added to the cell lysates. Agarose beads were then spun down at 500 × *g* for 3 min and washed five times with radioimmune precipitation buffer. 2× SDS sample buffer was added to the beads and boiled. After pulse spinning, the supernatants were loaded onto SDS-PAGE. Silver staining was performed using the Invitrogen SilverQuest kit according to standard instructions. Individual gel bands were excised according to protocols described previously (15), and the proteins were digested in gel and analyzed by combined mass spectrometric approaches. Briefly, the gels were subjected to trypsin proteolysis using a ProGest automated digester (Genomics Solutions). The extracted peptides were analyzed on a matrix-assisted laser desorption/ionization-TOF/TOF spectrometer (Applied Biosystems) and an ESI Q-TOF spectrometer (API-US Micromass) equipped with a capillary LC system from Waters. Data were submitted to the MASCOT data base search engine (Matrix-Science) for protein identification by peptide mass fingerprinting and sequence tag approaches.

Preparation of Expression Constructs—pGEX-4T-1 BAG2 was previously described (16). Full-length BAG2 was subcloned into pcDNA3-HA for mammalian expression. PCR was performed to generate BAG2 4–90 and 91–211 fragments and cloned into pcDNA3-HA and pGEX-6P-1. His-Hsc70, Myc-CHIP, and GST-CHIP plasmids were described previously (5).

Protein Purification—Recombinant proteins were produced in BL21(DE3)RP cells (Stratagene). The cells were induced with 0.4 mM isopropyl 1-thio-β-D-galactopyranoside at room temperature for 5 h. The cultures were spun down at 4 °C, dissolved in GST lysis buffer (20

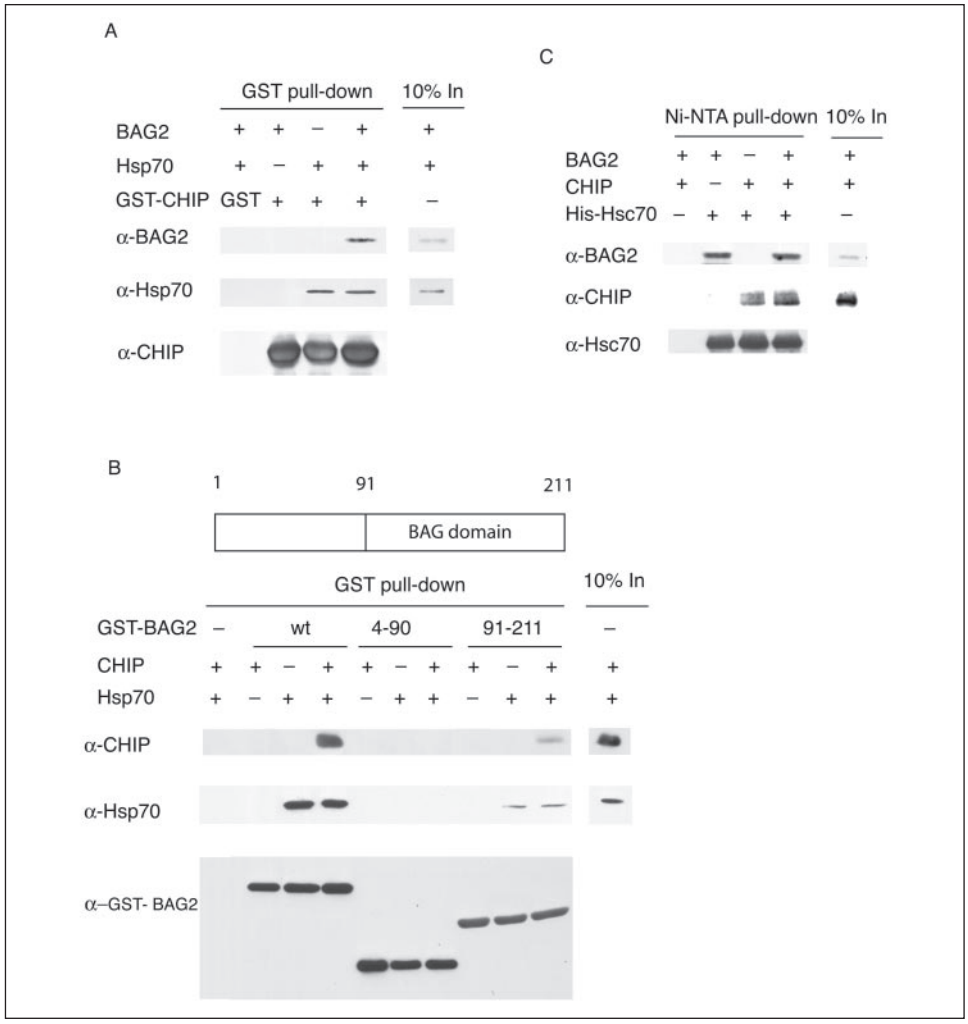

FIGURE 2. **Defining the interactions among BAG2, Hsp70, and CHIP.** *A, in vitro* pull-down assays with GST-CHIP (or GST alone) were used to test the associations of CHIP with BAG2 in the presence or absence of Hsp70. In these assays, CHIP efficiently interacted with BAG2, but only when Hsp70 was present in the binding assays. *B, in vitro* binding of recombinant CHIP and Hsp70 with full-length GST-BAG2 or BAG2 fragments containing amino acids 4–90 or 91–211 was tested in GST pull-down assays. The *schematic diagram* indicates the domains of BAG2 present in the fusion proteins. *C,* associations of proteins with Hsc70 (tagged with His), with or without BAG2 and/or CHIP, were tested in binding assays using immobilized nickel.

mM Tris, pH 8.0, 300 mM NaCl, 2 mM EDTA, and 2 mM dithiothreitol), sonicated, and cleared by centrifugation. Supernatants were incubated with glutathione-Sepharose 4B beads at 4 °C for 2 h and washed with GST lysis buffer with 0.5% Triton four times. In some experiments, the GST tag was cleaved with Precision protease (Amersham Biosciences) for pGEX-6P-1 constructs or thrombin for PGEX-4T-1 constructs. For purification of His-Hsc70, bacterial cultures were lysed in His lysis buffer (50 mM Tris, pH 8.0, 300 mM NaCl, 20 mM imidazole, 0.05% β-mercaptoethanol, and 1 mM benzamidine) and purified with Ni^{2+}-nitrilotriacetic acid-agarose beads (Qiagen). Recombinant NBD1-R, CHIP, Hsp70, Hdj2, and UbcH5a were purified as previously described (17).

In Vitro Ubiquitylation Reactions—In vitro ubiquitylation of CFTR NBD1-R domain was performed as described previously (17). Briefly, 0.5 μg of purified CFTR-NBD domain was incubated with 4 μM CHIP, 0.4–20 μM BAG1 or BAG2, 2 μM Hsp70, 4 μM Hdj2, 0.3 μg of purified rabbit E1 (Calbiochem), 1 mg/ml ubiquitin (Sigma), and 8 μM UbcH4 in 20 mM HEPES, pH 7.2, 100 mM KCl, 5 mM $MgCl_2$, 5 mM ATP, 10 mM dithiothreitol for 3 h at 37 °C. Samples were analyzed by SDS-PAGE and blotted with anti-CFTR NBD1-R domain or anti-Hsp70 antibodies.

Nucleotide Exchange Activity—Measurement of nucleotide exchange was determined using previously described methods (5). Briefly, 1 μg of Hsc70 was incubated with an equimolar concentration of recombinant BAG1, BAG2, or BAG2 deletion mutants in 25 μl of buffer (20 mM Hepes, 10 mM $(NH_4)_2SO_4$, 25 mM KCl, 2 mM $Mg(OAc)_2$, 0.1 mM EDTA, and 0.1 mM dithiothreitol) containing 1 μCi of [α-^{32}P]ATP (3000 Ci/mmol). Under these conditions, ATP is hydrolyzed, and ADP dissociation can be measured. At the indicated times, aliquots (2 μl) were analyzed by thin layer chromatography on Selecto Cellulose polyethyleneimine sheets using 1 M formic acid plus 1 M LiCl. Unlabeled ATP and ADP were run simultaneously as standards and identified by UV light. Sheets were dried and exposed to film, and binding of ATP and ADP was determined by densitometry. Experiments were performed in triplicate, and the results were expressed as bound ADP as a percentage of total.

Nucleotide Binding Assays—Measurement of nucleotide species bound to Hsp70, CHIP, or BAG2 was performed by [α-^{32}P]8-N_3ATP photolabeling. 0.5 μg of bovine serum albumin, 0.3 μg of CHIP, 0.4 μg of BAG2, or 0.5 μg of Hsp70 (to keep equimolar amounts of proteins in each reaction) was incubated with 1 μCi of [α-^{32}P]8-N_3ATP (10–15 Ci/mmol) in 20 μl of reaction buffer containing 20 mM Hepes, pH 7.4, 50 mM KCl, and 10 mM $MgCl_2$. After a 15-min incubation at room temperature, the reactions were irradiated with a UV transilluminator for 2 min. Nucleotides bound to proteins were then separated from the free nucleotides by size exclusion chromatography using a G50 spin column. 1 μl of the flow-through were analyzed by liquid scintillation counting.

GST Pull-down Assays—Glutathione-Sepharose-bound proteins were incubated with purified proteins for 2 h at 4 °C with continuous rotation in GST lysis buffer. The beads were washed four times with GST lysis buffer containing 400 mM NaCl. Beads were then boiled in 1× SDS sample buffer and loaded on SDS-PAGE. For His-Hsc70 binding

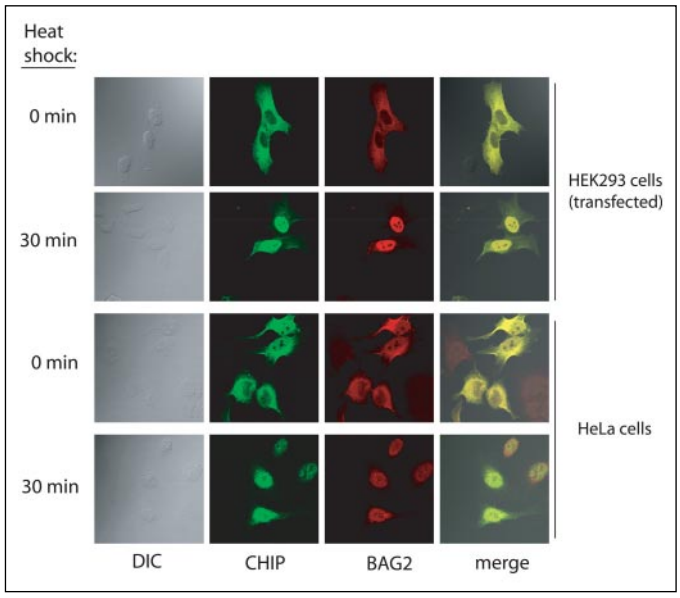

FIGURE 3. **Colocalization of BAG2 and CHIP.** HEK293 cells transiently transfected with plasmids expressing Myc-CHIP and HA-BAG2 and HeLa cells were analyzed by indirect immunofluorescence with antibodies against Myc or CHIP (*green*) and HA or BAG2 (*red*), respectively. Cells were examined under normal growth conditions and after heat shock at 42 °C for the indicated times. Expression was visualized by confocal microscopy, and *merged images* indicate colocalization before and after heat shock (*yellow*). *DIC*, differential interference contrast microscopy.

assays, binding reactions were performed in His lysis buffer with purified BAG2 and CHIP. When indicated, some experiments were performed in the absence or presence of ATP, ADP, or the nonhydrolyzable ATP analog AMP-PNP (5 mM).

Confocal Microscopy—HEK293 cells transiently transfected with HA-BAG2 and Myc-CHIP or HeLa cells stably transfected with Myc-CHIP at levels similar to endogenous protein were grown on coverslips. The cells were subjected to 30 min of heat shock at 42 °C, fixed with 3.7% paraformaldehyde, permeabilized with 0.5% Triton X-100, and then stained with appropriate antibodies to detect CHIP and BAG2 in the two cell types: mouse anti-Myc (9E10) and rabbit anti-HA in the HEK293 cells and mouse anti-Myc and rabbit anti-BAG2 antisera in HeLa cells. Secondary antibodies were Alexafluor goat anti-mouse 488 and Alexafluor goat anti-rabbit 568. Images were acquired on an Olympus 500 Fluoroview confocal microscope. All images are at ×60 magnification.

RESULTS

Identification of CHIP-associated Proteins by Mass Spectrometry—We created stable HeLa cell lines expressing Myc-tagged CHIP at levels approximating endogenous expression or cells transfected with the parent vector alone (Fig. 1*A*). Low level expression of the transgene avoided CHIP-dependent induction of heat shock factor-1 (18). Lysates from these cell lines were probed with an anti-Myc monoclonal antibody, and immunocomplexes were resolved by electrophoresis followed by silver staining to detect individual components. Representative immunocomplexes are shown in Fig. 1*B*. Individual bands were excised and analyzed by matrix-assisted laser desorption/ionization-TOF/TOF and ESI Q-TOF (15). Consistent with previous observations, peptides representing Hsc/Hsp70 and CHIP were identified in bands of the appropriate molecular weights, indicating that CHIP associates with chaperones and homodimerizes (5, 11). In addition, a 28-kDa band was identified that was present in approximate stoichiometry with Hsc70 in CHIP immunocomplexes. Six of six resolvable spectra coded fragments of BAG2. Using an anti-BAG2 antibody, we confirmed that BAG2 was specifically and highly enriched in CHIP immunocomplexes (Fig. 1*C*).

BAG2 is one of six proteins in mammals that contain the BAG domain. The BAG domain was originally identified in BAG1, a BCL2 partner protein that was subsequently confirmed to associate with Hsc70 (19, 20). Interestingly, BAG1 has also been implicated in CHIP-dependent protein degradation (8), although we did not discover BAG1 in these experiments or in other tests for endogenous CHIP interaction partners (data not shown). BAG domain proteins possess general nucleotide exchange activities toward Hsc/Hsp70 (16); therefore, they may each regulate chaperone activities under specific conditions. BAG2 contains the most divergent BAG domain among this family at its carboxyl terminus, and the 90 amino-proximal residues contain no motifs recognized by the SMART and PFAM algorithms. No cellular functions have been assigned to this protein to date.

We used several different approaches to characterize the interaction between CHIP and BAG2. Using GST-CHIP in pull-down assays with recombinant proteins, we found that BAG2 could be efficiently precipitated with CHIP, but only when Hsp70 was also present in the reaction (Fig. 2*A*). In converse experiments, GST-BAG2 precipitated CHIP in an Hsp70-dependent fashion (Fig. 2*B*). Deletions of either the BAG domain (amino acids 91–211) or the amino-terminal extension (amino acids 4–90) revealed that the BAG domain recruited both Hsp70 and CHIP, but with markedly lower efficiency than native BAG2, suggesting an additional role for the BAG2 amino terminus in efficient complex assembly. Finally, we used nickel chromatography to isolate recombinant proteins associating with His-tagged Hsc70. Both CHIP and BAG2 bound Hsc70 efficiently and without competition (Fig. 2*C*). For reasons discussed below, it is important to note that the preceding experiments were done in the absence of ATP. Taken together, they indicate the assembly of a CHIP-Hsp·Hsc70-BAG2 complex in which Hsp70 serves as a bridge linking CHIP and BAG2 and that depends on the BAG domain of BAG2. This complex appears to be further stabilized by interactions involving the amino terminus of BAG2 binding to other sites within this complex; these interactions were explored further in subsequent studies (see below).

Colocalization of BAG2 and CHIP in Vivo—CHIP has a characteristic and dynamic pattern of localization in cultured cells. Under quiescent conditions, it is localized in the cytoplasm and is strikingly concentrated at the outer membrane of the ER (5); after heat shock, a significant amount of CHIP undergoes nuclear import (18). If BAG2 regulates CHIP under endogenous conditions, then its expression and distribution after stress should mirror that of CHIP. BAG2 exhibited a similar pattern of distribution to CHIP in cultured HeLa cells and when transfected ectopically into HEK293 cells under quiescent conditions (Fig. 3). After heat shock, BAG2 translocated into the nucleus with temporal kinetics that mirror those of CHIP. The tight correlation of expression suggests that BAG2 and CHIP may undergo coordinated intracellular transport, perhaps as part of the same complex, and puts BAG2 in a position to regulate any of the activities so far assigned to CHIP.

BAG2 Inhibits the Ubiquitin Ligase Activity of CHIP—CHIP has two well defined functions in regulating cytoplasmic quality control; CHIP directly activates heat shock factor-1 (which transcriptionally regulates molecular chaperones) (18), and CHIP is a co-chaperone/ubiquitin ligase (21). Using a variety of assays, we did not identify any consistent affects of BAG2 on CHIP-dependent heat shock factor-1 activation (data not shown). However, we found that *in vitro* ubiquitylation of the NBD1 domain of CFTR by CHIP was efficiently inhibited by BAG2 (Fig. 4*A*). Half-maximal effects of BAG2 occurred at roughly stoichiometric concentrations of BAG2 relative to CHIP. Hsp70 itself is also a ubiqui-

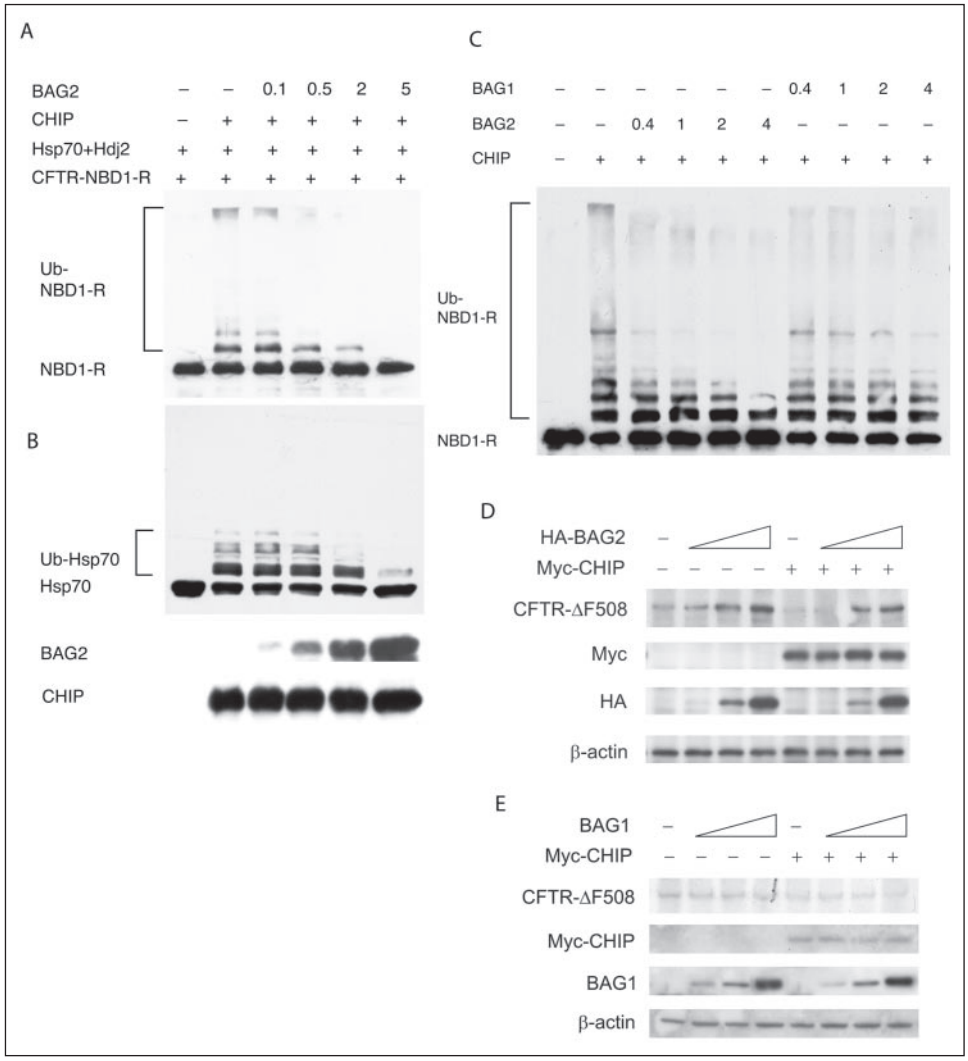

FIGURE 4. **Inhibition of CHIP ubiquitin ligase activity by BAG2.** *A*, *in vitro* ubiquitylation assays were performed in reactions containing E1, UbcH5a, CHIP, Hsp70, HDJ2, and the indicated molar ratios of BAG2 to CHIP. The NBD1-R domain of CFTR served as a substrate in these reactions. Western blotting for NBD1 was used to identify its ubiquitylated forms. *B*, similar *in vitro* reactions were performed with Hsp70 as the substrate. *C*, the antiubiquitylation activities of BAG1 and BAG2 were compared in *in vitro* ubiquitylation assays using CFTR NBD1-R as a substrate. *D* and *E*, plasmids expressing CFTRΔF508 and Myc-CHIP (0.5 μg each) were co-transfected with 0.1, 0.5, or 2.5 μg of HA-BAG2 (*D*) or BAG1 (*E*) expression plasmid in HEK293 cells. After 24 h, cell lysates were prepared and immunoblotted for the indicated proteins.

tylation target of CHIP (21), and BAG2 similarly blocked the addition of ubiquitin residues to this protein (Fig. 4*B*).

We tested the specificity of this inhibitory activity by deleting the BAG domain of BAG2, which totally abolished its inhibitory activity toward CHIP (data not shown). Since BAG1 has been placed in complexes with CHIP in *in vitro* studies (8), we compared the activity of BAG2 with BAG1 in these assays. Even at 10-fold higher concentrations compared with BAG2, BAG1 had negligible effects on CHIP ubiquitin ligase activity (Fig. 4*C*). These results are consistent with previous tests of BAG1 function (8) and indicate that the presence of a BAG domain alone is insufficient to inhibit CHIP activity. In addition, these experiments confirm the specificity of BAG2 as an inhibitor of CHIP ubiquitin ligase activity in this assay.

To explore the effects of BAG2 *in vivo*, we examined its activity on a known CHIP ubiquitylation target. CHIP efficiently reduces steady state levels of the CFTRΔF508 mutant by targeting it for ubiquitin-dependent proteasomal degradation (4). We therefore expressed CFTRΔF508 in HEK293 cells and tested the effects of CHIP and BAG2 on its expression. As has been demonstrated previously (4), CHIP reduced CFTRΔF508 levels (Fig. 4*D*). Consistent with its role in inhibiting CHIP activity, BAG2 overcame the effects of CHIP on CFTRΔF508 and markedly increased CFTRΔF508 expression, which is under constitutive control by endogenous CHIP, both in the presence and absence of ectopic CHIP expression. In contrast, overexpression of BAG1 under similar conditions did not rescue CFTRΔF508 expression, indicating specificity for BAG2 in these studies (Fig. 4*E*). These experiments are consistent with our *in vitro* observations and indicate that BAG2 prevents CHIP-dependent diversion of CFTRΔF508 to the proteasome-dependent quality control pathway. Interestingly, we did not identify any indication that BAG2 is itself a target for CHIP-dependent ubiquitylation under the conditions in which these *in vitro* and *in vivo* experiments were performed (data not shown).

To further characterize the participation of BAG2 in CHIP-dependent ubiquitylation, we examined the activity of the amino terminus and BAG domains of BAG2. First, we assessed the activities of BAG2 and the deletion mutants BAG2 4–90 and BAG2 91–211 in regulating nucleotide exchange with Hsp70. Consistent with previous observations, BAG2 inhibited nucleotide exchange with potency equivalent to BAG1 (22), an effect that was abolished by deletion of the BAG domain but not the amino terminus of BAG2 (Fig. 5*A*). We then tested the activity of the same proteins for their ability to inhibit CHIP-dependent ubiquitylation of CFTR NBD1. In contrast with its effect on nucleotide exchange, the BAG domain of BAG2 alone (BAG2 91–211) did not inhibit ubiquitylation of NBD1, nor did BAG1, even when present in 10-fold excess relative to CHIP (Fig. 5*B*). Taken together, these results indicate that the inhibitory effects of BAG2 are not strictly due to nucleotide exchange activity and that the amino terminus of BAG2 is required for its inhibitory effects on the ubiquitin ligase activity of CHIP.

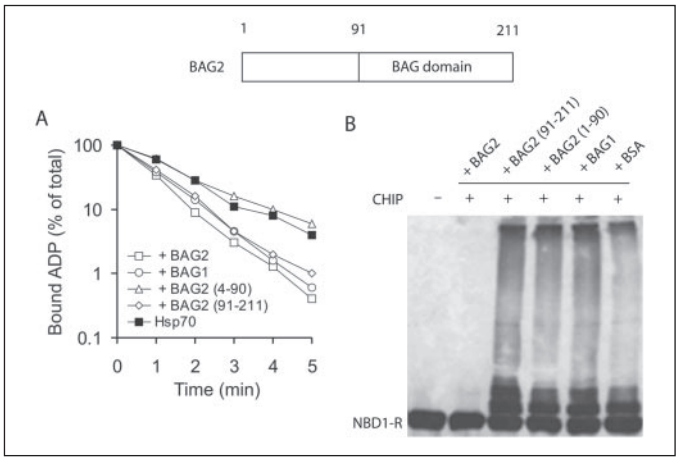

FIGURE 5. **Deletion analysis of BAG2 activity.** *A*, single turnover nucleotide exchange activity of Hsp70 alone or with the indicated proteins in equimolar concentrations was determined by measuring the kinetics of ADP release. The value of total nucleotide bound to Hsp70 after initial size exclusion chromatography was set at 100%, and results were expressed as the mean of three independent experiments. *B*, *in vitro* ubiquitylation assays were performed with CHIP and 10-fold excess molar ratios of the indicated BAG proteins. The NBD1-R domain of CFTR served as a substrate in these reactions. *BSA*, bovine serum albumin.

BAG2-dependent Remodeling of the CHIP Ubiquitin Ligase Complex—Based on our current understanding of CHIP activity, we considered several plausible mechanisms for the potent antagonistic effects of BAG2. Because CHIP dimerization is required for its ubiquitin ligase activity (11), we tested whether BAG2 disrupted CHIP-CHIP dimers. To do this, we co-expressed both Myc- and FLAG-tagged CHIP and used the different tags to assay CHIP dimerization by co-immunoprecipitation in cultured cells. CHIP dimers were easily detected under these conditions, and BAG2 was readily incorporated into complexes containing these dimers, but (even at saturating levels of BAG2 expression) CHIP dimerization was not disrupted (Fig. 6*A*). We next characterized the nucleotide dependence of the association of BAG2 and Hsp70 with CHIP by incubating GST-CHIP (or deletion mutants thereof) with recombinant BAG2 and Hsp70 (Fig. 6*B*). Consistent with our previous observations (5), Hsp70 binding required the tetratricopeptide repeat and adjacent charged domains of CHIP (amino acids 1–197). When binding reactions were performed in the absence of ATP, the binding of BAG2 to CHIP followed the same binding rules as does Hsp70, which is consistent with observations in Fig. 2. Surprisingly, in the presence of ATP, a lower affinity interaction of BAG2 with the U-box domain of CHIP was also observed that was independent of Hsp70 binding. Concomitant with this association, we found that BAG2 was released from Hsp70 in preformed Hsp70-BAG2-CHIP complexes by adenine nucleotides in binding assays of recombinant proteins using GST-BAG2; however, only ATP (and not ADP or the nonhydrolyzable ATP analog AMP-PNP) caused retention of CHIP in the Hsp70-depleted GST-BAG2 complexes (Fig. 6*C*). The nucleotide dependence of this reaction raised the question of which of these proteins bound to ATP. We tested this by 8-azido-ATP photolabeling, a sensitive index of ATP binding (Fig. 6*D*). As expected, ATP photoreacted with Hsp70 but not with CHIP or BAG2. Similar results were observed with ATP affinity chromatography (not shown). Taken together, these observations provided further evidence that direct interactions between BAG2 and CHIP occur. This remodeling of the Hsp70-BAG2-CHIP heterocomplex requires nucleotide binding to dissociate BAG2 from Hsp70 and (based on the effects of AMP-PNP) Hsp70-dependent nucleotide hydrolysis to facilitate the BAG2-CHIP interaction.

The release of Hsp70 from BAG2-CHIP complexes in the presence of ATP, as indicated in Fig. 6*C*, suggested that one mechanism to account for inhibition of CHIP by BAG2 is through dissociation of Hsp70 that is required for substrate presentation to CHIP. We therefore performed additional binding assays under the same conditions as our *in vitro* ubiquitylation reactions (Fig. 4) in an ATP-containing system. Under these conditions, and in contrast to the effects in the absence of ATP (see Fig. 2), BAG2 disrupted the association between CHIP and Hsp70 (Fig. 7*A*) in a concentration dependence that was analogous to its effect on CHIP ubiquitin ligase activity (Fig. 4*A*). In contrast, and despite the fact that it has equivalent effects on Hsp70 nucleotide exchange (Fig. 5*A*), BAG1 had little effect on CHIP-Hsp70 interactions, even at relatively high molar ratios (Fig. 7*B*). Consistent with this observation, BAG1 did not "rescue" CHIP from BAG2-dependent suppression of its ubiquitin ligase activity in *in vitro* assays (data not shown). We also found that BAG2 disrupted the association of CHIP and endogenous Hsc/Hsp70 *in vivo* (Fig. 7*C*). By disrupting the ability of Hsp70 to present substrates to CHIP, this ATP-dependent remodeling of the CHIP complex would have the expected effect of inhibiting its ubiquitin ligase activity toward substrates, as we observe in our *in vitro* and *in vivo* studies (Fig. 4).

BAG2 Disrupts CHIP E2-E3 Coupling—Given the indications of discrete contacts between BAG2 and the U-box of CHIP in these complexes (Fig. 6*B*), we further considered whether other aspects of ubiquitin ligase complex assembly were perturbed when BAG2 was present in these complexes. We have previously observed that stable interactions between CHIP and the ubiquitin-conjugating enzyme UBCH5a are necessary for ubiquitin ligase activity (21). Incorporation of BAG2 into CHIP complexes in the presence of ATP destabilized this interaction (Fig. 8*A*), preventing E2-E3 coupling that is required for ubiquitin chain assembly. Similarly, UbcH5a reversed BAG2-dependent stabilization of CFTRΔF508 and competitively released BAG2 from CHIP immunocomplexes in HEK293 cells (Fig. 8*B*), which supports a model in which UbcH5a and Bag2 compete (directly or indirectly) for binding to the U-box of CHIP. Given the proximity of BAG2 and CHIP within the chaperone-ubiquitin ligase holocomplex, this activity provides an additional mechanism for inhibition of chaperone substrate ubiquitylation. Taken together, our observations suggest that BAG2 exerts a checkpoint mechanism that may prevent dysregulated ubiquitylation of chaperone substrates.

DISCUSSION

Molecular chaperones require coordinated interactions with multiple co-chaperones to regulate the steps in protein folding. We have speculated that chaperone-dependent protein degradation is regulated with equivalent precision. Based on the growing appreciation for the role of CHIP in chaperone-dependent protein degradation, we immunopurified endogenous CHIP complexes with the goal of identifying the range of proteins that regulate the CHIP-Hsp70 complex. BAG2 is a prominent component of CHIP-containing holocomplexes, suggesting that it plays an endogenous role in regulating diversion of chaperone substrates to the ubiquitin-proteasome pathway for degradation.

BAG2 is one of six mammalian proteins containing carboxyl-terminal BAG domains. Many of the BAG domain proteins possess antiapoptotic activities (although presumably through nonoverlapping mechanisms) (16, 23), and all of them interact with the ATPase domain of Hsc70 in a BAG domain-dependent mechanism (22). It seems most likely that the principal biochemical effect of BAG-Hsc70 interactions is to facilitate nucleotide exchange (24, 25), although analyses of BAG1 suggest that nucleotide exchange may not comprise the entirety of BAG

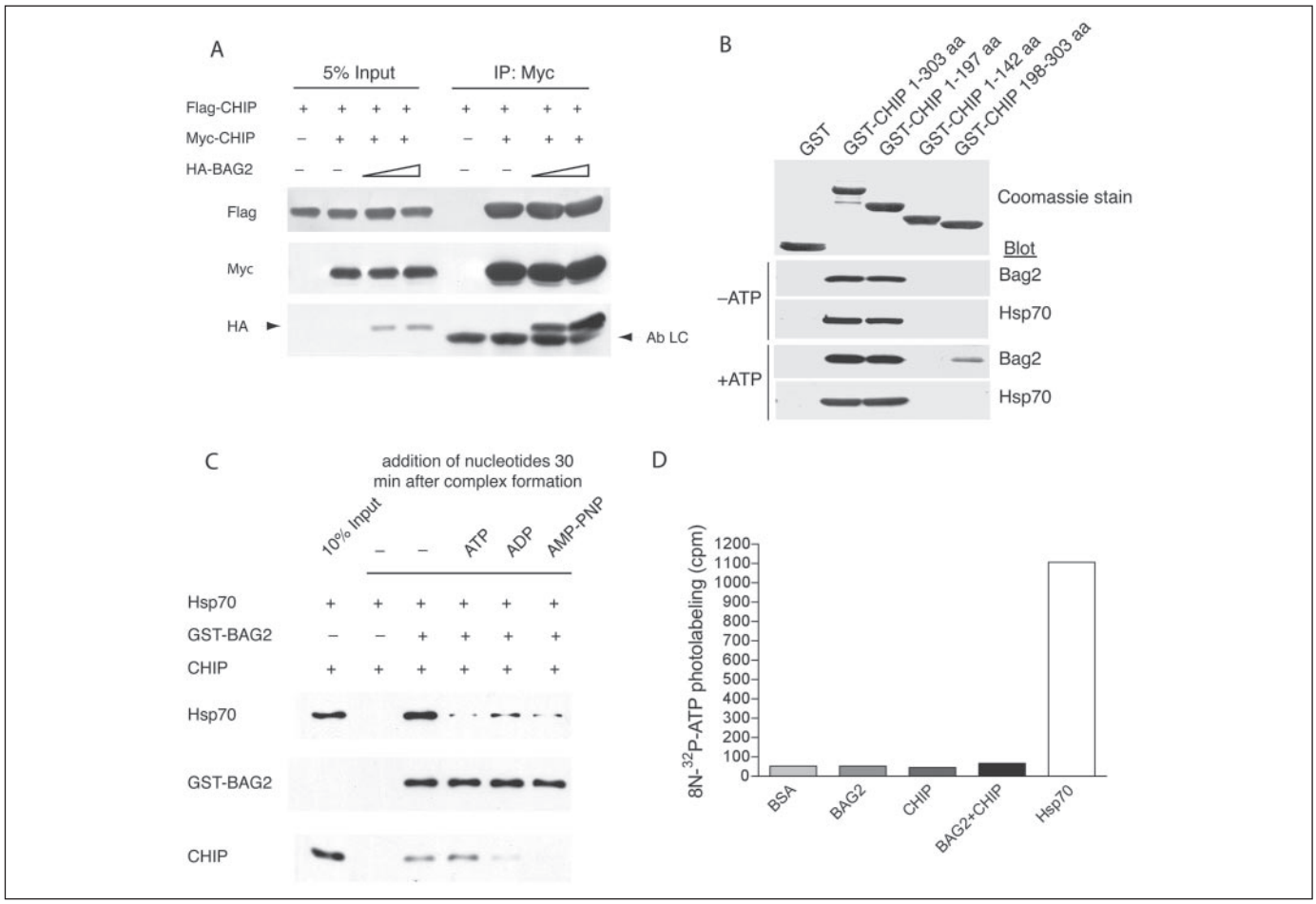

FIGURE 6. **Remodeling of CHIP-containing complexes by BAG2.** *A*, vectors expressing FLAG- and Myc-tagged CHIP (1 μg each) were co-transfected in HeLa cells along with increasing concentrations of a plasmid expressing HA-tagged BAG2 (1–5 μg). Cell lysates were immunoprecipitated (*IP*) with an anti-Myc antibody and then blotted to detect FLAG-CHIP and HA-BAG2 that associated with Myc-CHIP *in vivo*. *B*, an *in vitro* pull-down assay with GST-CHIP (and the indicated deletion mutants), followed by Western blotting, was used to test the associations of CHIP with recombinant BAG2 and Hsp70 in the presence or absence of ATP (5 mM). *C*, GST-BAG2, Hsp70, and CHIP were preassembled into complexes for 30 min and then incubated with ATP, ADP, or the nonhydrolyzable ATP analog AMP-PNP. BAG2-containing complexes were then precipitated with glutathione-Sepharose, and Western blotting was performed to detect Hsp70 and CHIP in these complexes. *D*, ATP binding was assayed by 8-azido-[^{32}P]ATP photolabeling of the indicated proteins. *BSA*, bovine serum albumin.

domain protein activities toward Hsp70 (26). Nevertheless, the consequences of BAG domain protein effects on Hsp70 can be modulated both by stoichiometric considerations and by structural features within the amino-terminal extension adjacent to the BAG domain (27, 28). It has been proposed that BAG domain proteins undergo conformational regulation that coordinates interactions among partner proteins (16), an effect that at least in some cases is ATP-dependent (28). Our data indicate that BAG2 is a major component of the Hsc70-CHIP chaperone-dependent ubiquitin ligase complex, and our observation that amino terminus sequences in BAG2 stabilize the ternary BAG2-Hsc70-CHIP complex (Fig. 2*B*) suggests that these sequences may form protein-protein interactions within this complex (in addition to the BAG-ATPase domain interaction) to tune the molecular functions of this complex. Consistent with this model, BAG2 directly interacts with the U-box domain of CHIP in an ATP-dependent fashion (Fig. 6*B*) and remodels these complexes such that Hsp70 is excluded (Fig. 7). The ability of BAG2 (but not BAG1) to suppress UbcH5a interactions with CHIP (Fig. 8) raises the interesting possibility that the amino terminus of BAG2 sterically hinders this association, although at the present time, we cannot exclude the possibility that BAG2 also has conformational effects on the complex that determine the rules of E2-E3 association (Fig. 9).

The present studies provide an important basis for beginning to understand how decisions about protein triage are made at the level of individual chaperone complexes. Until now, no mechanism has existed to explain how CHIP is prevented from ubiquitin-tagging proteins on the proper folding pathway for degradation. Our results indicate that BAG2 is likely to play a constitutive regulatory role in this decision. The close intracellular co-localization of CHIP and BAG2 under conditions of quiescence and thermal challenge suggests that coordination of CHIP activity by BAG2 is an ongoing process within the cell (Fig. 3). How this association is regulated is an open question at this time, although our data indicate that BAG2 interactions are clearly nucleotide-dependent. It is also likely that other events regulate the activity of this complex. In this regard, it is interesting to note that BAG2 was identified in an inductive proteomic screen for targets of p38 MAP kinase activity (29). However, we did not find that the effects of BAG2 on CHIP were altered by pharmacologic inhibitors or activators of p38 (data not shown). It is also plausible that other proteins compete for the ability of BAG2 to join CHIP-Hsp70 complexes; an analogous competition between BAG1, Raf-1, and Hsp70 has been proposed to coordinate stress signaling and mitogenic responses (30).

Interestingly, BAG2 is not the first BAG domain protein that has been implicated in CHIP-dependent protein degradation. BAG1 has been suggested to cooperate with CHIP by facilitating transfer of proteins

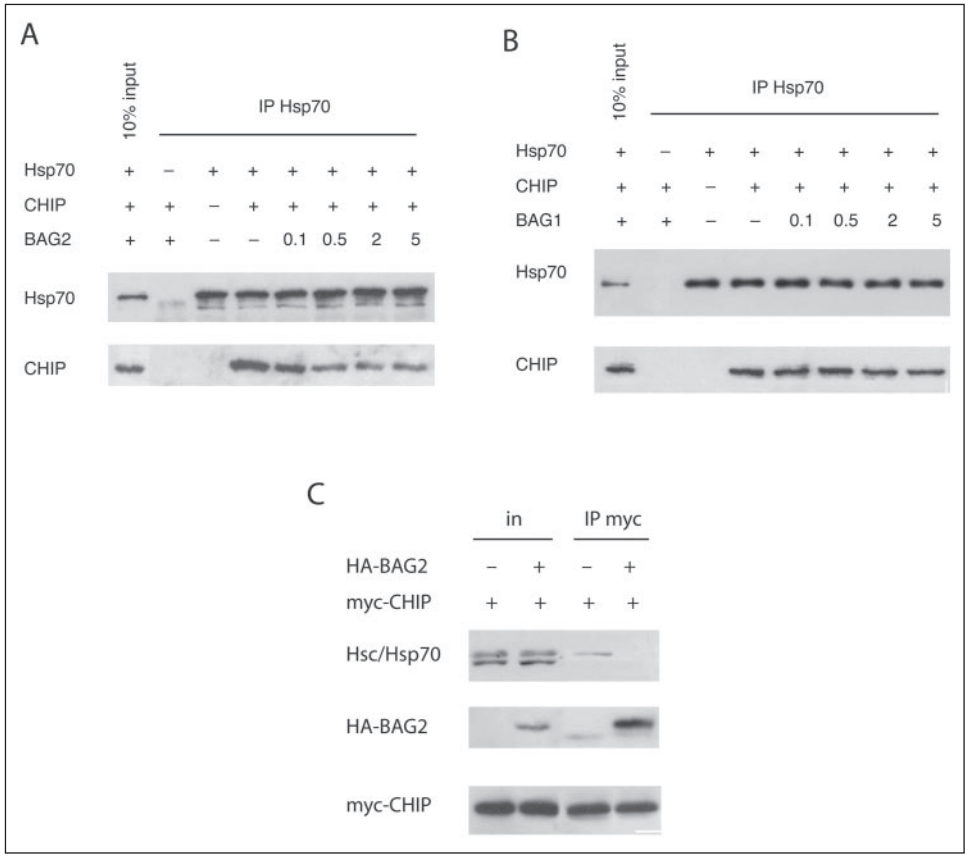

FIGURE 7. **ATP-dependent dissociation of Hsp70 and CHIP by BAG2.** *A* and *B*, *in vitro* binding reactions containing 4 μM CHIP, 2 μM Hsp70, 4 μM Hdj2, 0.3 μg of purified rabbit E1, 1 mg/ml ubiquitin, 8 μM UbcH4, and the indicated molar ratios of BAG2 (*A*) or BAG1 (*B*) were performed in 20 mM HEPES, pH 7.2, 100 mM KCl, 5 mM $MgCl_2$, 5 mM ATP, 10 mM dithiothreitol. Hsp70 immunocomplexes were precipitated (*IP*) and blotted for the presence of CHIP and BAG2. *C*, co-immunoprecipitation of Myc-tagged CHIP and endogenous Hsc/Hsp70 was performed in HEK293 cells transfected with or without HA-tagged BAG2. CHIP immunoprecipitates were blotted for the presence of BAG2 and Hsc/Hsp70 with specific antibodies.

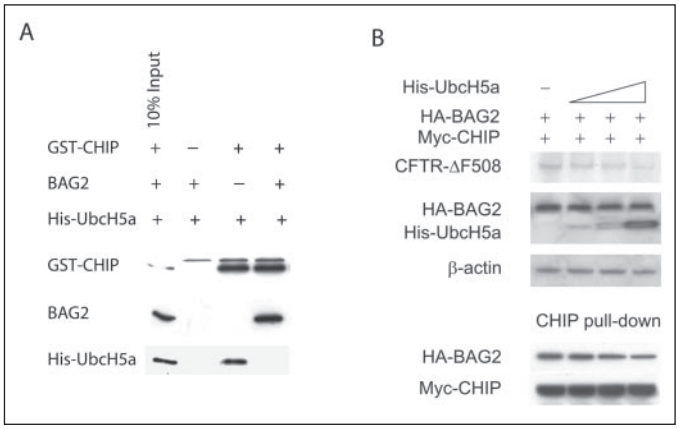

FIGURE 8. **Disruption of CHIP E2-E3 coupling by CHIP.** *A*, interactions between UbcH5a (expressed as a His-tagged protein) with GST-CHIP were tested in the absence or presence of equimolar concentrations of BAG2. These binding reactions were performed in the presence of ATP (5 mM). *B*, Myc-tagged CHIP, HA-tagged BAG2, and His-tagged UbcH5a were transfected as indicated into HEK293 cells along with CFTRΔF508. Cell lysates were subjected to immunoblotting to detect the indicated proteins either directly or after immunoprecipitation of CHIP.

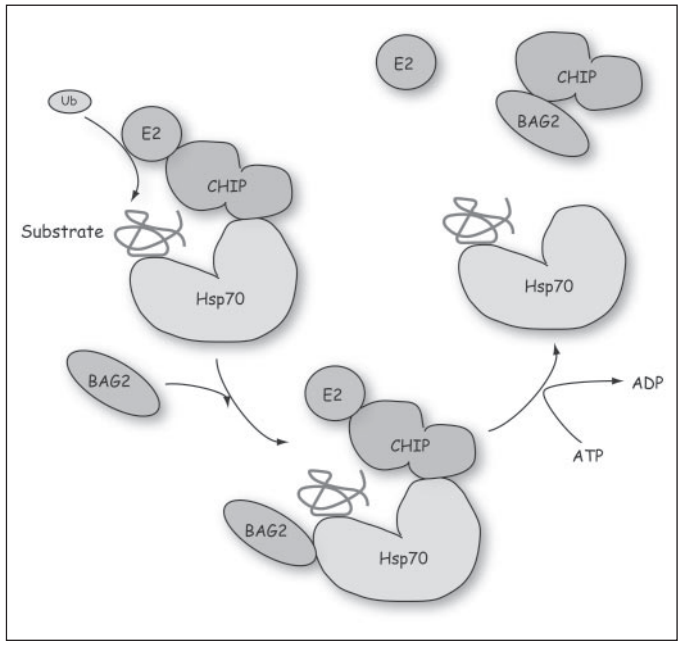

FIGURE 9. **A model of the mechanism underlying the inhibitory effects of BAG2 on CHIP ubiquitin ligase activity.** In the absence of BAG2, a holocomplex containing CHIP, Hsp70, an E2, and chaperone substrate assembles to facilitate substrate ubiquitylation. Through its BAG domain, BAG2 makes contact with the ATPase domain of Hsp70, and additional remodeling of the complex occurs in an ATP-dependent fashion that has two consequences. CHIP and BAG2 are dissociated from Hsp70, and binding of E2 to CHIP is uncoupled.

ubiquitylated by CHIP to the proteasome (8). This activity of BAG1 is dependent on associations of the proteasome with a ubiquitin-like domain at the amino terminus of BAG1 that is not present in BAG2. We have not detected endogenous complexes containing both CHIP and BAG1 in our studies, which may only indicate that this association is tightly regulated. It is interesting to speculate that serial interactions among BAG proteins regulate the CHIP-Hsp70 ubiquitin ligase. Associations of BAG2 inhibit the ubiquitin ligase and may instead prime the chaperone complex for ATP binding and subsequent rounds of folding. BAG1 binding would in turn relieve BAG2-dependent inhibition of ubiquitylation and would concomitantly facilitate transfer of chaperone substrates ubiquitylated by CHIP to the proteasome for ATP-dependent degradation, although the lower affinity of BAG1 compared with

BAG2 that we observed (Fig. 7) would argue that this might occur only under specific circumstances. In any event, our data indicate that BAG domain-containing proteins, and BAG2 specifically, provide a plausible mechanism to regulate the activity of Hsp70-CHIP complexes and therefore to govern the balance between folding and degradation of misfolded proteins.

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