Intraneuronal accumulation of phosphorylated Tau protein is a molecular pathology found in many forms of dementia, including Alzheimer disease. Research into possible mechanisms leading to the accumulation of modified Tau protein and the possibility of removing Tau protein from the system have revealed that the chaperone protein system can interact with Tau and mediate its degradation. Hsp70/Hsc70, a member of the chaperone protein family, interacts with Tau protein and mediates proper folding of Tau and can promote degradation of Tau protein under certain circumstances. However, because Hsp70/Hsc70 has many binding partners that can mediate its activity, there is still much to discover about how Hsp70 acts \textit{in vivo} to regulate Tau protein. BAG-1, an Hsp70/Hsc70 binding partner, has been implicated as a mediator of neuronal function. In this work we show that BAG-1 associates with Tau protein in an Hsc70-dependent manner. Overexpression of BAG-1 induced an increase in Tau levels, which is shown to be due to an inhibition of protein degradation. We further show that BAG-1 can inhibit the degradation of Tau protein by the 20 S proteasome but does not affect the ubiquitination of Tau protein. RNA-mediated interference depletion of BAG-1 leads to a decrease in total Tau protein levels as well as promoting hyperphosphorylation of the remaining protein. Induction of Hsp70 by heat shock enhanced the increase of Tau levels in cells overexpressing BAG-1 but induced a decrease of Tau levels in cells that were depleted of BAG-1. Finally, BAG-1 is highly expressed in neurons bearing Tau tangles in a mouse model of Alzheimer disease. This data suggests a molecular mechanism through which Tau protein levels are regulated in the cell and possible consequences for the pathology and treatment of Alzheimer disease.

A major molecular hallmark of many forms of dementia, including Alzheimer disease, is the accumulation of highly insoluble intraneuronal protein aggregates composing of the neuronal protein Tau. The Tau protein present in intracellular tangles is hyperphosphorylated and forms a misfolded structure (1). Tau protein normally acts as a microtubule-binding protein in the axon of the neurons, where it stabilizes the microtubule structure and, therefore, the neuronal morphology. Hyperphosphorylation of Tau protein inhibits its ability to bind to microtubules (2) and may lead to aggregation and ultimately cytotoxicity. Some research suggests that Tau protein accumulation, rather than hyperphosphorylation, may be a main culprit leading to toxicity (3, 4). Accumulation of Tau protein can lead to an inhibition of intracellular transport, leading to neurodegeneration (5, 6). In addition, accumulation of Tau may be a precursor to the buildup of phosphorylated Tau. Accumulation and aggregation of Tau protein may be triggered by a decreased ability to degrade Tau in the aging brain. Therefore, mechanisms that regulate the levels of Tau protein are an important area of research in Alzheimer disease.

Several groups have recently reported an association between Tau protein and chaperone proteins. Chaperone proteins, including the heat shock protein family, are crucial for the proper folding and maintenance of many cellular proteins. Their chaperone function can lead to a refolding of the substrate protein or, in some instances, to degradation of the substrate (7). An important member of this family of proteins is Hsp70/Hsc70. Hsp70 and Hsc70 have nearly identical structure and function, but Hsp70 is induced upon heat shock, whereas Hsc70 is constitutively expressed. Dou et al. (8) first demonstrated that Hsp70 and Hsp90 can associate with Tau protein and that induction of Hsp70 can induce a decrease in Tau phosphorylation and aggregation. Three separate groups further demonstrated that the Hsc70/Hsp70-binding protein CHIP is found within this protein complex and serves as a E3 ubiquitin ligase, therefore inducing 26 S proteasomal degradation of Tau protein (9–11). CHIP$^{-/-}$ mice exhibit a buildup of phosphorylated Tau protein (12). This suggests that CHIP may be necessary to induce degradation of aberrant Tau species. In addition, it was shown that induction of Hsp70 by antibiotic treatment can induce the degradation of Tau protein that is phosphorylated on specific amino acids (13). In light of the effects of chaperones and chaperone binding proteins on Tau protein, possible use of the chaperone system in therapeutics of Tauopathies have been proposed (14).

Bcl2-associated athanogene-1 (BAG-1),$^{2}$ an additional Hsc70/Hsp70-binding protein, has recently been implicated as an important molecule in the development and maintenance of neurons. BAG-1$^{-/-}$ mice die shortly after birth and have severe neurological phenotypes, including Alzheimer disease. Research into possible mechanisms leading to the accumulation of modified Tau protein and the possibility of removing Tau protein from the system have revealed that the chaperone protein system can interact with Tau and mediate its degradation. Hsp70/Hsc70, a member of the chaperone protein family, interacts with Tau protein and mediates proper folding of Tau and can promote degradation of Tau protein under certain circumstances. However, because Hsp70/Hsc70 has many binding partners that can mediate its activity, there is still much to discover about how Hsp70 acts \textit{in vivo} to regulate Tau protein. BAG-1, an Hsp70/Hsc70 binding partner, has been implicated as a mediator of neuronal function. In this work we show that BAG-1 associates with Tau protein in an Hsc70-dependent manner. Overexpression of BAG-1 induced an increase in Tau levels, which is shown to be due to an inhibition of protein degradation. We further show that BAG-1 can inhibit the degradation of Tau protein by the 20 S proteasome but does not affect the ubiquitination of Tau protein. RNA-mediated interference depletion of BAG-1 leads to a decrease in total Tau protein levels as well as promoting hyperphosphorylation of the remaining protein. Induction of Hsp70 by heat shock enhanced the increase of Tau levels in cells overexpressing BAG-1 but induced a decrease of Tau levels in cells that were depleted of BAG-1. Finally, BAG-1 is highly expressed in neurons bearing Tau tangles in a mouse model of Alzheimer disease. This data suggests a molecular mechanism through which Tau protein levels are regulated in the cell and possible consequences for the pathology and treatment of Alzheimer disease.

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$^{2}$ The abbreviations used are: BAG-1, Bcl2-associated athanogene-1; RNAi, RNA-mediated interference; HA, hemagglutinin; GFP, green fluorescent protein; GST, glutathione S-transferase; HEK cells, human embryonic kidney cells; MES, 4-morpholineethanesulfonic acid; WT, wild type; CHIP, C terminus of Hsc 70-interacting protein.
deficiencies in brain development, including massive apoptosis (15). BAG-1 is expressed as three isoforms in humans (50, 46, and 33 kDa) and two isoforms in mouse (50 and 29 kDa), all expressed from alternative start codons on the same mRNA transcript (16). BAG-1 associates with Hsc70 and can induce substrate release in vitro through activating the ATPase domain on Hsc70 (17). Recently, BAG-1 has been shown to up-regulate the refolding activity of Hsc70 in neuronal cells (18). Therefore, BAG-1 may be an important cofactor and regulator of Hsc70 function in neurons. Considering the important roles of BAG-1 in Hsc70 function and in neuronal development, we researched the possible interaction and regulation of Tau protein by BAG-1. In our work we demonstrate an Hsc70-dependent interaction between BAG-1 and Tau protein. We further demonstrate that BAG-1 functions to inhibit the proteasomal degradation of Tau protein, leading to an accumulation of Tau protein. Induction of Hsp70 augments the accumulation of Tau protein. We also demonstrate that BAG-1 co-localizes with aggregated Tau protein in an Alzheimer disease mouse model.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Transfections**—P19 mouse embryonic carcinoma cells were grown in minimal essential medium (Biological Industries, Beit-Haemek, Israel) supplemented with 5% heat-inactivated fetal calf serum in a 5% CO₂ incubator at 37 °C. Cells were induced toward neuronal differentiation by the addition of 1 μM retinoic acid as previously described (19). P19 cells were transfected using the JET PEI reagent according to the manufacturer’s instructions. To stably express plasmids in P19 cells, the cells were incubated with 700 μM neomycin for 1 month.

HEK-293 cells were grown in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal calf serum (Invitrogen). HEK-293 cells were transfected using the calcium phosphate precipitation method as described previously (19). Cells were lysed 48 h after transfection for analysis.

**Plasmids and Constructs**—pcDNA3/HA-BAG-1 was kindly provided by Prof. Harm H. Kampinga (University of Groningen, Netherlands). pEGFP-C1 construct (Clontech) was used to express GFP. To create GFP-BAG-1 fusion protein, BAG-1S was amplified by PCR from RNA extract from P19 cells using the primers 5’-CGGGATCCATGCGCAAGACCGAGGAGATGGTC and 3’-GAATTCTCATTCAGCCAGGGCCAAAGTTTGT. PCR product was digested with BamHI and EcoRI and then ligated into pEGFP-C1 vector that was digested with same enzymes. pGEX-4T-1/Bag1 and pGEX-4T-1/Bag1mut, for production of GST-BAG-1 fusion proteins, were kindly provided by Prof. Harm H. Kampinga (University of Groningen, Netherlands).

pGSHIN vector expressing GFP and containing H1 RNAi promoter was kindly provided by Prof. Kojima (Northwestern University) (20). Oligos containing previously published RNAi sequence against BAG-1 and control sequence (15) were synthesized (Sigma-Aldrich). The sequences synthesized are: for BAG-1 short hairpin RNAi, 5’-GATCCCCGGGCAACTAGCCAAATGTCTTCAGGAGATGGTC and 3’-AGCTAAAAAGGCAGAGATACCATATGCTTCTTGAGCATATGATTCAGGAGCCCGGG; for scramble, 5’-GATCCCCGGGCAAGATAACCATATGCTTCTTCAGGAGATGGTC and 3’-AGCTAAAAAGGCAGAGATACCATATGCTTCTTGAGCATATGATTCAGGAGCCCGGG. Oligos were annealed and ligated into pGSHIN vector that had been digested with BglII and HindIII restriction enzymes.

**Cell Lysis and Immunoblotting**—P19 or HEK-293 cells were lysed on the plate with lysis buffer (140 mM KCl, 3 mM MgCl₂, 1% Nonidet P-40, 1% glycerol, 20 mM β-glycerophosphate, pH 7.4, 20 mM β-mercaptoethanol) and then centrifuged at 10,000 × g for 5 min. Protein levels were determined using Bradford reagent. Equal amounts of protein were loaded on 10% SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked in nonfat milk (5% milk in Tris-buffered saline, 0.05% Tween) for 2 h, incubated with primary antibody overnight at 4 °C, washed, and then incubated with horseradish peroxidase-conjugated second antibody for 1 h. Membranes were then developed with enhanced chemiluminescence. Primary antibodies used included Tau-5, AT8 (Innogenetics, Gent, Belgium), glyceraldehyde-3-phosphate dehydrogenase (Ambion, Austin, TX), anti-β-actin (Sigma), tubulin (Sigma), phospho-extracellular signal-regulated kinase (ERK) and total ERK (kindly provided by Prof. R. Seger, Weizmann Institute of Science, Israel), phospho- glycogen synthase kinase 3β (GSK3β; kindly provided by Prof. G. Agam, Ben Gurion University, Israel), total GSK3β (kindly provided by Prof. H. Eldar-Finkelman, Tel-Aviv, University, Israel), anti-ubiquitin (kindly provided by Prof. Yossi Yarden, Weizmann Institute of Science, Israel), anti-BAG-1 (Santa Cruz, clone C-16), anti-neurofilament, 68, anti-HA, anti-ubiquitin (kindly provided by Prof. Yossi Yarden, Weizmann Institute of Science, Israel), anti-BAG-1 (Santa Cruz, clone C-16), anti-neurofilament, 68, anti-HA. Immunoblots were quantitated using the NIH imager program. Quantitated results are shown with “n” representing independent experiments performed with independent material. All experiments that were not quantitated and statistically analyzed were performed at least twice unless otherwise noted.

**Immunoprecipitation and GST Pulldown Assay**—P19 or HEK-293 cell extracts were prepared and adjusted to 2 mg of total protein in 1 ml of extract using the lysis buffer mentioned above. The appropriate monoclonal antibody was added, and the mixture was gently rotated for 2 h at 4 °C followed by an additional 2-h incubation with protein A/G-Sepharose beads (Santa Cruz Biotechnology). The immunocomplexes were centrifuged at 1000 × g, washed 5 times in cell lysis buffer, and then eluted from the beads with 40 μl of 0.2 mM glycine, pH 2.4. 1 μl Tris pH 8 was then added to neutralize the buffer. In the nucodazole experiment, to depolymerize microtubules, P19 neurons were incubated with 10 μM nucodazole for 1 h and 15 min before lysis.

Brain extract for immunoprecipitation was prepared by homogenizing hippocampus (tissue provided by Netherlands Brain Bank) in a 10-times volume of homogenization buffer (10 mM HEPES, pH 7.4, 0.32 m sucrose, 140 mM KCl). Extract was centrifuged at 14,000 × g for 10 min, and the supernatant was used for immunoprecipitation as described above.

To express GST-BAG-1 proteins, exponentially growing bacterial cultures were induced to express GST, GST-BAG-1,
BAG-1 Regulates Tau Stability

FIGURE 1. BAG-1 associates with Tau protein in a Hsc70-dependent manner. A, Tau protein and its associated proteins were immunoprecipitated (IP) from cell lysate of neuronally differentiated P19 cells using the Tau-5 antibody. Western blot analysis of the immunoprecipitate revealed a positive band for Hsc70 and BAG-1, whereas glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was absent from the precipitate. B, HEK-293 cells were transiently transfected with constructs expressing Tau and BAG-1-HA proteins. Cell extracts were immunoprecipitated with Tau-5 antibody and subsequently analyzed by Western blot. Hsc70 and BAG-1-HA were detected in the immunoprecipitate, whereas glyceraldehyde-3-phosphate dehydrogenase was absent. C, HEK-293 cells were transiently transfected with vector expressing Tau protein. Cell lysate was incubated with Sepharose beads attached to GST, GST-BAG-1, or GST-BAG-1 mutant. Bound proteins were eluted with 1 M KCl and analyzed by Western blot. Tau and Hsc70 were bound to GST-BAG-1, whereas neither bound to GST or GST-BAG-1 mutant. D, GST-BAG or GST beads were incubated with purified Hsc70 and Tau proteins. Bound proteins were eluted with 1 M KCl and analyzed by Western blot. Tau protein was found in association with GST-BAG-1 only when Hsc70 was added to the mixture. E, BAG-1 and its associated proteins were immunoprecipitated from human brain homogenate using an antibody to BAG-1. Western blot analysis revealed that all six isoforms of Tau were present in the BAG-1 immunoprecipitate.

FIGURE 2. BAG-1 associates with Tau protein in a Hsc70-dependent manner. A, Tau protein and its associated proteins were immunoprecipitated (IP) from cell lysate of neuronally differentiated P19 cells using the Tau-5 antibody. Western blot analysis of the immunoprecipitate revealed a positive band for Hsc70 and BAG-1, whereas glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was absent from the precipitate. B, HEK-293 cells were transiently transfected with constructs expressing Tau and BAG-1-HA proteins. Cell extracts were immunoprecipitated with Tau-5 antibody and subsequently analyzed by Western blot. Hsc70 and BAG-1-HA were detected in the immunoprecipitate, whereas glyceraldehyde-3-phosphate dehydrogenase was absent. C, HEK-293 cells were transiently transfected with vector expressing Tau protein. Cell lysate was incubated with Sepharose beads attached to GST, GST-BAG-1, or GST-BAG-1 mutant. Bound proteins were eluted with 1 M KCl and analyzed by Western blot. Tau and Hsc70 were bound to GST-BAG-1, whereas neither bound to GST or GST-BAG-1 mutant. D, GST-BAG or GST beads were incubated with purified Hsc70 and Tau proteins. Bound proteins were eluted with 1 M KCl and analyzed by Western blot. Tau protein was found in association with GST-BAG-1 only when Hsc70 was added to the mixture. E, BAG-1 and its associated proteins were immunoprecipitated from human brain homogenate using an antibody to BAG-1. Western blot analysis revealed that all six isoforms of Tau were present in the BAG-1 immunoprecipitate.

or GST-BAG-1 mutant (1–179) using 1 mM isopropyl β-D-galactopyranoside. Bacteria were lysed in GST lysis buffer (50 mM HEPES, pH 7.5, 150 mM KCl, 5 mM EDTA, 1 mM dithiothreitol) containing lysozyme and protease inhibitors. The cleared lysate was incubated with glutathione beads and washed 5 times with GST lysis buffer containing 1% Triton X-100 to remove nonspecifically bound bacterial proteins. Extract of P19 neurons (5 mg) was incubated with GST fusion protein-bound beads for 4 h at 4 °C. The complex was centrifuged and then washed five times in assembly buffer (100 mM MES, pH 6.9, 0.5 mM MgCl₂, 0.1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, and protease inhibitors as described in cell lysis above). Homogenate was centrifuged for 10 min at 10000 × g to separate nuclei. Supernatant was then cleared of other cellular debris by centrifugation at 16,000 × g for 10 min. Supernatant was collected and then adjusted to a final concentration of 2 mM GTP, 4 mM glycerol, and 20 μM taxol. The mixture was incubated for 30 min at 37 °C and then centrifuged at 100,000 × g for 30 min to pellet microtubules. Microtubule pellet was resuspended in SDS sample buffer. Non-microtubule supernatant was also recovered.

Phosphatase Treatment of Cell Lysates—Cell lysate of P19 neurons were diluted to a protein concentration of 0.5 μg/μl in phosphatase treatment buffer (50 mM Tris, pH 8.5, 2 mM MgCl₂, 1% SDS). Lysate was boiled for 5 min to denature the proteins, and then phenylmethylsulfonyl fluoride was added to final concentration of 1 mM. Lysate was incubated with 20 units/ml of alkaline phosphatase (Amersham Biosciences) for 2 h. SDS sample buffer was added, and the sample was analyzed by Western blot as described above.

Emitine Treatment—HEK-293 cells were transfected as mentioned above with plasmids for Tau alone or both Tau and BAG-1-HA. 48 h after transfection emitine was added to the medium to a final concentration of 50 μM for the incubation periods that are stated.

20 S Proteasomal Degradation Assay—20 S proteasomes were purified from mice livers as previously described (22). Purified proteins of interest were incubated with the 20 S proteasomes in degradation buffer (100 mM Tris pH 7.5, 150 mM NaCl) in 37 °C for 30 min. The degradation reaction was stopped with the addition of Laemmli sample buffer, heated at 95 °C for 5 min, and electrophoresed on SDS-PAGE. After electrophoresis, proteins were transferred to cellulose nitrate membranes and detected by immunoblot analysis.

Geldanamycin Treatment and Heat Shock—Geldanamycin (kindly provided by Prof. Yossi Shaul, Weizmann Institute of Science) was added to P19 cells in fresh medium on day 7 of neuronal differentiation at the concentrations indicated. Cells were lysed 24 h after the addition of geldanamycin, and immunoblot analysis was performed. For heat shock analysis, P19 cells differentiated into neurons. At day 8 of neuronal differentiation, cells were incubated at 41 °C for 1 h. Cells were then returned to 37 °C for an additional 12 h incubation until they were lysed, and immunoblot analysis was performed.

Immunohistochemistry—Paraformaldehyde-fixed brain slices from the 3XTg Alzheimer mouse model (23) were kindly provided by Ephraim Yavin (Weizmann Institute of Science, Israel) The appropriate primary antibody was applied overnight at 4 °C in Tris-buffered saline with 0.2% Tween. Slices were reacted with a secondary antibody conjugated to fluorescein isothiocyanate or CY3 as necessary. The slices were then
BAG-1 Regulates Tau Stability

for Tau and HA-BAG-1 proteins. Material immunoprecipitated with the Tau-5 antibody reacted with Hsc70 and HA antibodies in Western blot analysis (Fig. 1B). Therefore, this association could be repeated in a transfected non-neuronal system.

GST pulldown assays were used to further verify the association between these proteins and to determine whether BAG-1 associates with Tau directly or through its association with Hsc70. GST-BAG-1 and GST-BAG-1mut were incubated in the presence of P19 neuron cell extracts according to standard protocols for a GST pulldown. GST-BAG-1mut lacks a 57-amino acid segment of the C termini of the BAG-1 protein, which is mandatory for its binding to Hsc70. As visualized by Western blot, Hsc70 and Tau both were present in the eluate from GST-BAG-1 but were absent from the eluate from GST-BAG-1mut (Fig. 1C). Therefore, the segment of BAG-1 that is necessary for Hsc70 binding is also necessary for Tau association. Because this may indicate that BAG-1 associates with Tau indirectly through binding Hsc70, we performed an in vitro GST pull-down with purified Hsc70 and Tau proteins.

Tau protein is expressed as six isoforms in adult brain tissue as a result of alternative splicing. BAG-1 was immunoprecipitated from human brain extract to determine which Tau isoforms present in the human brain can associate with BAG-1. All isoforms of Tau in the human brain tissue were detected in the BAG-1 immunoprecipitate. Therefore, the BAG-1-Hsc70 complex can associate with all isoforms of Tau protein.

BAG-1 Interacts with Tau Protein in an Hsc70-dependent Manner—To determine whether Hsc70 and BAG-1 can physically associate with Tau protein, we performed immunoprecipitation and GST fusion protein assays. To detect an endogenous association between the proteins, we differentiated the P19 embryonic teratocarcinoma cell line into neuronal cells (P19 neurons) using retinoic acid. P19 neurons express neuronal markers such as neurofilaments, synaptophysin, and Tau protein and exhibit neuronal morphology, including distinct axons and dendrites. Using the Tau-5 antibody, both BAG-1 (29 kDa) and Hsc70 co-immunoprecipitated with Tau protein from extracts of P19 neurons (Fig. 1A). Only the 29-kDa BAG-1 isoform is expressed in the cytoplasm of P19 neurons. Nonspecific mouse IgGs were used as a control, and glyceraldehyde-3-phosphate dehydrogenase did not immunoprecipitate with Tau. In addition, we performed an immunoprecipitation experiment in HEK-293 cells that were transfected with constructs encoding Tau and HA-BAG-1 proteins. Material immunoprecipitated with the Tau-5 antibody reacted with Hsc70 and HA antibodies in Western blot analysis (Fig. 1B). Therefore, this association could be repeated in a transfected non-neuronal system.

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BAG-1 and Tau Expression and Localization in P19 Cells—To further understand the possible implications of the association between BAG-1 and Tau protein, we performed immunohistochemistry on P19 neurons with antibodies against BAG-1 and Tau to determine their cellular localization (Fig. 2). Therefore, this association could be repeated in a transfected non-neuronal system.

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BAG-1 regulates Tau stability.

**FIGURE 3.** Overexpression of BAG-1 induces increase in Tau protein levels. A, P19 cells were stably transfected with constructs expressing GFP-BAG-1 or GFP alone. The cell lines were differentiated to neurons and were lysed. Cell extracts were analyzed by Western blot using the specified antibodies. Tau levels were increased in GFP-BAG-1-expressing cells, whereas NF-68 and β-actin levels maintained stable. B, quantitative analysis of Tau protein levels in the cell lines studied in A, using NIH imager. Results are represented as the mean ± S.E. (n = 4; n represents independent experiments; *, p < 0.05, unpaired Student’s t test). C, HEK-293 cells were transfected with pcDNA-Tau and pcDNA-HA-BAG-1 constructs or with empty pcDNA vector. Cells were lysed 2 days after transfection, and the cell lysate was analyzed by Western blot analysis. Tau protein levels increased in cells that were co-transfected with pcDNA-BAG-1. D, quantitative analysis of Tau protein levels in the cell lysates studied in C, using NIH imager. Results are represented as the mean ± S.E. (n = 4; *, p < 0.05, unpaired Student’s t test). E, HEK-293 cells were transfected with pcDNA-Tau or pcDNA-Tau and pcDNA-BAG-1-HA. They were then treated with the translation inhibitor emitine at increasing intervals of time (1.5, 3, 4.5 h). Cells were lysed and analyzed by Western blot analysis. T, quantitative analysis of Tau protein levels in cell lysates studied in E, using NIH imager. Results are represented as the mean ± S.D. (n = 4; *, p < 0.05, unpaired Student’s t test). BAG-1 attenuated Tau protein degradation for 3 h after emitine treatment.

BAG-1 rose linearly throughout P19 neuronal differentiation, suggesting that it may perform important functions in the neuronal system (Fig. 2B). The p29 isoform of BAG-1 is also localized mainly in the cytosol, unlike the p50 isoform. Therefore, cytosolic interactions of BAG-1 may be more relevant in neuronal cells.

Tau protein exists mainly as a microtubule-associated protein or a soluble protein (24). Some reports have also suggested that Tau may be found in the nucleus. The functional consequence of the interaction between BAG-1, Hsc70, and Tau will depend on where this interaction takes place in the cellular environment. We found that BAG-1 and Hsc70 are not found in microtubule preparations from Tau-expressing HEK-293 cells. However, a significant fraction of Tau, BAG-1, and Hsc70 were found in the soluble cytosolic fractions. In addition, Hsc70 NF-68, did not change. Also of interest, the amount of phosphorylated Tau (as determined by AT8 antibody) increased by the same ratio as the increase in total Tau. Therefore, overexpression of BAG-1 had no direct effect on Tau phosphorylation but, rather, on levels of total Tau protein. In addition, overexpression of HA-BAG-1 in HEK-293 cells increased exogenous Tau levels similarly to the increase in P19 neurons (Fig. 3, C and D). Because the Tau construct is not under the control of the native Tau promoter and lacks the translational regulatory elements of Tau mRNA, this experiment shows that the increase in Tau protein is not due to a transcriptional or translational mechanism.

Based on the increase of Tau protein after overexpression of BAG-1, we examined if BAG-1 may affect the degradation of translated Tau protein. HEK-293 cells expressing Tau protein and BAG-1 were not detected when tubulin was immunoprecipitated from P19 neurons. Therefore, the interaction between Hsc70, BAG-1, and Tau is likely to occur in the soluble cytosolic environment and not on the microtubules. Disassembly of microtubules in P19 neurons by treatment with nocodazole induced an increase in the interaction between BAG-1 and Tau. This further suggests that non-microtubule-associated Tau binds to the BAG-1-Hsc70 complex. In summary, the BAG-1-Hsc70-Tau complex interacts in the soluble compartment of the cell body. Because the main function of Tau protein is to stabilize microtubules in the axons, the soluble Tau protein found in the cell body may need to be transported or degraded in order not to build up to levels that may form tangles. Therefore, BAG-1 may be involved in these mechanisms, as will be discussed below.

Overexpression of BAG-1 leads to increased total Tau protein—To determine the direct function of the association of BAG-1 and Tau, overexpression of BAG-1 was carried out both in the P19 neuronal system and in HEK-293 cells transfected with vector for Tau protein. P19 cells were stably transfected with vectors expressing either GFP-BAG-1 or GFP alone. P19 neurons expressing GFP-BAG-1 displayed a 2-fold increase in the level of Tau protein as compared with cells expressing only GFP or nontransfected cells (Fig. 3, A and B). Expression of another neuronal marker,
were treated with emetine to inhibit translation, and degradation of Tau protein was followed after increasing time intervals. HEK-293 cells that were co-transfected with both Tau and BAG-1 vectors showed a decreased degradation of Tau protein for up to 3 h of emetine treatment compared with cells transfected for only Tau (Fig. 3, E and F). Therefore, BAG-1 may stabilize Tau protein during the first few hours after translation of the nascent protein.

Inhibition of Proteasomal Degradation of Tau by BAG-1—Because previous work has shown that Hsc70 and its binding partners can regulate proteasomal degradation of Tau protein, we hypothesized that BAG-1 may attenuate the proteasomal degradation of Tau protein. First, we treated P19 neurons with MG132, a proteasomal inhibitor. After 24 h of inhibition, Tau levels in control P19 neurons reached similar levels as found in P19 neurons overexpressing BAG-1 (Fig. 4, A and B). Therefore, inhibition of the proteasome by chemical stimulation has the same effect on Tau protein as BAG-1 overexpression. In addition, MG132 had no significant effect on Tau protein in P19 neurons overexpressing BAG-1. This suggests that Tau protein in these cells is not being degraded by the proteasome.

Tau protein can be degraded either by the classic 26 S proteasome or the 20 S proteasome, which is a ubiquitin-independent mechanism (13, 25). The Hsc70-interacting protein CHIP can ubiquitinate Tau, leading to degradation by the 26 S proteasome. To check if BAG-1 affects ubiquitination of Tau, we examined if there are changes in the amount of ubiquitinated Tau in P19 neurons overexpressing BAG-1. Western blot analysis of immunoprecipitated Tau with an antibody for ubiquitin demonstrated no significant changes in the levels of ubiquitinated Tau in the BAG-1-overexpressing cells (Fig. 4C). Therefore, BAG-1 does not inhibit the ubiquitination of Tau protein and does not inhibit the degradation of ubiquitinated Tau.

Tau protein is also a particularly good substrate for the 20 S proteasome because it has little secondary structure. To determine whether BAG-1 affects the 20 S proteasomal degradation of Tau, we performed an in vitro 20 S proteasomal degradation assay. First, we incubated Tau with purified 20 S proteasome in solution with increasing amounts of Hsc70. Hsc70 alone induced a small inhibition of the degradation of Tau by 20 S proteasome (Fig. 4D). Next, we added increasing amounts of BAG-1 to the assay. BAG-1 at a high concentration attenuated 20 S proteasomal degradation of Tau with an efficiency much greater than Hsc70 alone (Fig. 4E). Therefore, BAG-1 can protect against 20 S proteasomal degradation of Tau. From these experiments, we learn that BAG-1 inhibits the degradation of Tau through a ubiquitin-independent pathway involving the 20 S proteasome.

Depletion of BAG-1 Leads to Hyperphosphorylation and Decreased Levels of Tau Protein—To further our functional analysis of the association between BAG-1 and Tau, we constructed small interfering RNA vectors against BAG-1 using previously published sequences (15). The small interfering RNA construct as well as a control construct containing the scramble sequence was stably expressed in P19 cell lines. According to Western blot, BAG-1 was knocked down by ~95% in undifferentiated P19 cells and was knocked down by ~85% after neuronal differentiation. Western blot analysis of Tau protein in the BAG-1 RNAi cell line revealed a significant upshift in the gel of a large proportion of Tau protein (Fig. 5A). The higher band also reacted strongly with AT8, an antibody against phosphorylated Tau. It is well documented that Tau protein often runs higher on SDS-PAGE gels after hyperphosphorylation. Cell lysates from the stable lines were treated with alkaline phosphatase and subsequently run on an SDS-PAGE gel. After phosphatase treatment, the higher band completely

**FIGURE 4.** BAG-1 inhibits proteasomal degradation of Tau protein. A, WT P19 neurons and P19 neurons overexpressing GFP-BAG-1 were treated with the proteasome inhibitor for 12 or 24 h. Cells were lysed, and the extracts were analyzed using specific antibodies against Tau. Proteasome inhibition increased Tau levels in WT P19 neurons but not in GFP-BAG-1 overexpressing P19 neurons. B-Actin levels were unaffected. B, quantitative analysis of Tau protein levels in the cell lysates studied in A, using NIH imager. Results are represented as the mean ± S.E. (n = 3, *, p < 0.05, unpaired Student’s t test). Con, control. C, Tau was immunoprecipitated from WT P19 neurons, GFP P19 neurons, and GFP-BAG-1 P19 neurons. The immunoprecipitate (IP) was analyzed by Western blot (WB) with antibodies against ubiquitin and Hsc70. Tau from all cell lines had the same level of ubiquitination and were associated with the same level of Hsc70. The experiment was carried out three times with the same results. D, Tau (400 ng) protein was incubated with purified 20 S proteasome and varying concentrations of Hsc70 (0, 50, 100 ng). Hsc70 provided a partial protection from proteasomal degradation of Tau. E, Tau protein was incubated with 20 S proteasome, Hsc70, and varying amounts of BAG-1 (0, 50, 100 ng). BAG-1 provided a robust protection from proteasomal degradation of Tau.

**BAG-1 Regulates Tau Stability**
BAG-1 Regulates Tau Stability

FIGURE 5. RNAi depletion of BAG-1 induces hyperphosphorylation and decreased levels of Tau protein. A, P19 cells were stably transfected with constructs expressing RNAi for BAG-1 or a scramble control RNAi. The cell lines were differentiated to neurons and were lysed for analysis by Western blot by various antibodies. B, cell lysate from P19 neurons and from the stable cell lines were treated with alkaline phosphatase and then analyzed with Western blot. The upper band visualized with Tau-5 antibody completely disappears after alkaline phosphatase treatment, and the lower band is strengthened. C, quantitative analysis of Tau levels after treatment of cell lysate with alkaline phosphatase from B (n = 3). NIH imager was used to measure band intensity, and results are represented as the mean ± S.E. (*, p < 0.05, unpaired Student's t test). D, cell lysate from P19 neurons and stable cell lines were analyzed with Western blot using antibodies for different Tau kinases and their regulatory phosphorylation sites. There was no change in these kinases in the cell lines. E, phospho-extracellular signal-regulated kinase; GSK3β, phospho-glycogen synthase kinase 3β (GSK3β) and extracellular signal-regulated kinase (ERK) were unaltered in RNAi cells by immunoprecipitation. Approximately 50% less Hsc70 was associated with Tau protein in the BAG-1 RNAi cells (Fig. 5E). Therefore, decreased association of Hsc70 with Tau may lead to the Tau hyperphosphorylation seen in the BAG-1 RNAi cells.

BAG-1 Regulates Function of Hsc70—In light of the fact that BAG-1 associates with Tau protein through Hsc70, we examined if overexpression of the heat shock protein could enhance the stabilizing effect of BAG-1 on Tau protein. Hsp70 was induced in control P19 neurons as well as in the BAG-1-overexpressing and BAG-1 RNAi cell lines. Hsp70 was induced using the antibiotic geldanamycin, which up-regulates the heat shock response (Fig. 6, A and B). Increasing concentrations of geldanamycin induced a slight increase of Tau protein in P19 neurons. However, geldanamycin induced a significantly larger increase of Tau protein levels in neurons overexpressing BAG-1. Interestingly, neurons expressing RNAi for BAG-1 exhibited a sharp decrease in Tau protein levels after treatment of geldanamycin. We repeated these experiments using heat shock treatment of P19 neurons in place of stimulation with geldanamycin (Fig. 6, C and D). Again, heat shock protein induction in BAG-1-overexpressing neurons resulted in increase in Tau protein, whereas the opposite result was observed in cells expressing RNAi for BAG-1. Therefore, BAG-1 acts as a switch between Tau protein stabilization and degradation in response to induction of Hsp70.

BAG-1 Co-localizes with Hyperphosphorylated Tau in 3XTg Mouse Model for Alzheimer Disease—In light of the fact that both overexpression and underexpression of BAG-1 leads to an increase in the amounts of hyperphosphorylated Tau in P19 neurons, we checked if an increase or a decrease in BAG-1 could be associated with neurons bearing hyperphosphorylated Tau in a mouse model for Alzheimer disease. We examined the 3XTg mouse model that contains mutations in the genes for amyloid precursor protein, Tau, and presenilin, which results in a robust Alzheimer disease-like pathology, including neurons bearing tangles of hyperphosphorylated Tau (23). Co-immunohistochemistry of brain sections from these mice revealed a strong correlation between neurons that strongly stained with the AT8 antibody (anti-phosphorylated Tau) and neurons that were positive for BAG-1 (Fig. 7). In particular, 95% of neurons containing AT8 staining were positive for BAG-1 compared with 30% BAG-1 staining for the overall cell population. These data suggest that BAG-1 expression may contribute to the accumulation of Tau in tangle-bearing neurons of Alzheimer disease.

DISCUSSION

In our study we have demonstrated a physical interaction between BAG-1 and Tau proteins. This interaction is Hsc70-dependent and occurs in the cytosolic fraction and independent of binding to microtubules. In the presence of excess BAG-1, Tau levels increased significantly, and degradation of the Tau protein was attenuated. The increase in Tau protein was shown to be due to an inhibition of proteasomal degradation as determined by experiments with a proteasome inhibitor and the 20S proteasome assay. In cells expressing small interfering RNA directed at BAG-1, Tau levels decreased and became hyperphosphorylated. The increase in phosphorylation...
of Tau protein that occurred in cells with BAG-1 RNAi is most likely due to the decreased association between Hsc70 and Tau. In contrast, BAG-1 overexpression did not affect the association between Hsc70 and Tau. As a result, BAG-1 overexpression did not directly affect Tau phosphorylation. Physiological concentrations of BAG-1 may be necessary to maintain the Hsc70-Tau complex, but overexpression of BAG-1 won’t increase this interaction. BAG-1 levels in P19 neurons regulate the effect of heat shock protein induction on Tau levels. Low BAG-1 levels favored a degradation of Tau protein, whereas BAG-1 overexpressing neurons exhibited a stabilization of Tau protein after heat shock. Finally, we witnessed a colocalization between Tau tangles and BAG-1 in Alzheimer mice model, suggesting that a correlation between these two proteins exists in a disease state.

The interaction between Tau and Hsc70/Hsp70 has gained attention as a possible target for therapeutic actions in Alzheimer disease and other Tauopathies. Hsp70 up-regulation has been shown to induce a decrease in Tau protein levels in primary neuronal culture. This would suggest that up-regulation of Hsp70 may be therapeutic in dementias caused by Tauopathy. Our work demonstrates that the regulation of Tau protein by Hsc70 is dependent on another member of the protein complex, BAG-1. Levels of BAG-1 may determine whether Hsc70 promotes degradation or stabilization of the Tau protein. This information would be important to keep in mind when designing therapies for Tauopathies based on heat shock up-regulation.

Another important member of the Hsc70-Tau complex is CHIP. The Hsc70 CHIP complex can bind to Tau and mediate ubiquitination, therefore leading to 26 S proteosomal degradation. In our work BAG-1 overexpression led to a buildup of Tau protein without affecting the ubiquitination of the protein. We further showed in vitro that BAG-1 can inhibit the degradation of Tau by the 20 S proteasome. Therefore, although CHIP may regulate the degradation of Tau by the 26 S proteasome, BAG-1 may regulate the degradation of Tau by the 20 S proteasome. According to this model, Hsc70
can modulate the degradation of Tau by recruiting multiple binding partners, which regulate multiple methods of degradation.

The exact mechanism through which BAG-1 may attenuate the degradation of Tau protein by 20 S proteasome is not completely clear. Allosteric interactions at the site of the proteasome may be involved, as BAG-1 has been shown to interact physically with the proteasome (26). Another likely explanation would be an enhancement of the refolding activity of Hsp70. Chaperones can enhance either refolding or degradation of client proteins. It has been demonstrated that BAG-1 can up-regulate the refolding activity of Hsp70 in neurons (18). Therefore, BAG-1 may act to up-regulate the refolding of Tau protein, consequently down-regulating the pathway leading to degradation of Tau protein. This theory would explain why induction of Hsp70 induced a stabilization of Tau protein in cells overexpressing BAG-1 but induced a decrease of Tau protein in cells lacking BAG-1.

An interesting outcome of the cellular experiments is the increase in total phosphorylated Tau that is induced by either up-regulation or down-regulation of BAG-1. Up-regulation of BAG-1 increases Tau levels, therefore causing a parallel increase in levels of phosphorylated Tau. Down-regulation leads directly to an increase in phosphorylated Tau. Therefore, theoretically, either an up-regulation or down-regulation of BAG-1 may be relevant to the progression of Tauopathy. To determine the relevance of BAG-1 expression to progression of Tauopathy, immunohistochemistry of Alzheimer disease model mouse was performed. Double immunostaining using antibodies against BAG-1 and phosphorylated Tau reveals that high levels of BAG-1 and phosphorylated Tau co-localize in high levels of phosphorylated Tau. Co-staining of high levels of BAG-1 and phosphorylated Tau suggests that overexpression of BAG-1 is more relevant to causation of Tauopathy under pathological conditions.

The colocalization of BAG-1 expression and Tau tangle bearing neurons suggests that BAG-1 may play a part in the pathogenesis of Tau protein and Alzheimer disease. BAG-1 may increase Tau levels, therefore increasing the amount of substrate available for Tau kinases such as extracellular signal-regulated kinase and glycogen synthase kinase 3β. Therefore, BAG-1 expression may possibly be an early event in a cascade of events leading to Tau tangle formation.

In conclusion, we report a novel role for BAG-1 as a modulator of Tau protein degradation. This role has implications for possible therapeutics of Tauopathies that up-regulate the chaperone apparatus of the cell. This role also has important implications for the pathogenesis of the disease. Further research will study the expression and role of BAG-1 in the human Alzheimer disease brain.

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REFERENCES

1. Grundke-Iqbal, I., Iqbal, K., Tung, Y. C., Quinlan, M., Wisniewski, H. M., and Binder, L. J. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 4913–4917
2. Xie, H., Liter斯基, J. M., Hartigan, J. A., Jope, R. S., and Johnson, G. V. (1998) Brain Res. 798, 173–183
3. Mandelkow, E. M., Stamer, K., Vogel, R., Thies, E., and Mandelkow, E. (2003) Neurobiol. Aging 24, 1079–1085
4. Thies, E., and Mandelkow, E. M. (2007) J. Neurosci. 27, 2986–2907
5. Mukrasch, M. D., von Bergen, M., Biernat, J., Fischer, D., Griesinger, C., Mandelkow, E., and Zweckstetter, M. (2007) J. Biol. Chem. 282, 12230–12239
6. Stamer, K., Vogel, R., Thies, E., Mandelkow, E., and Mandelkow, E. M. (2002) J. Cell Biol. 156, 1051–1063
7. Dickey, C. A., Kamal, A., Lundgren, K., Klosak, N., Bailey, R. M., Dunmore, J., Ash, P., Satabara, S., Zlatkovic, J., Eckman, C. B., Patterson, C., Dickson, D. W., Nahman, N. S., Jr., Hutton, M., Burrows, F., and Petrucelli, L. (2007) J. Clin. Invest. 117, 648–658
8. Dou, F., Netzer, W. J., Tanemura, K., Li, F., Hilti, F. U., Takashima, A., Gouras, G. K., Greengard, P., and Xu, H. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 721–726
9. Shimura, H., Schwartz, D., Gygi, S. P., and Kosik, K. S. (2004) J. Biol. Chem. 279, 4869–4876
10. Hatakeyama, S., Matsumoto, M., Kamura, T., Murayama, M., Chui, D. H., Panel, E., Takahashi, R., Nakayama, K. I., and Takashima, A. (2004) J. Neurochem. 91, 299–307
11. Petrucelli, L., Dickson, D., Kehoe, K., Taylor, J., Snyder, H., Grover, A., De Lucia, M., McGowan, E., Lewis, J., Prihar, G., Kim, J., Dillman, W. H., Brown, S. E., Hall, A., Voelmln, R., Tsuboi, Y., Dawson, T. M., Wolozin, B., Hardy, J., and Hutton, M. (2004) Hum. Mol. Genet. 13, 703–714
12. Dickey, C. A., Yue, M., Lin, W. L., Dickson, D. W., Dunmore, J. H., Lee, W. C., Zehr, C., West, G., Cao, S., Clark, A. M., Caldwell, G. A., Caldwell, K. A., Eckman, C., Patterson, C., Hutton, M., and Petrucelli, L. (2006) J. Neurosci. 26, 6985–6996
13. Dickey, C. A., Dunmore, J., Lu, B., Wang, J. W., Lee, W. C., Kamal, A., Burrows, F., Eckman, C., Hutton, M., and Petrucelli, L. (2006) FASEB J. 20, 753–755
14. Dickey, C. A., and Petrucelli, L. (2006) Expert Opin. Ther. Targets 10, 665–676
15. Gotz, R., Wiese, S., Takayama, S., Camarero, G. C., Rossoll, W., Schweizer, U., Troppmair, J., Jablonska, S., Hofmann, B., Reed, J. C., Rapp, U. R., and Sendtner, M. (2005) Nat. Neurosci. 8, 1169–1178
16. Takayama, S., Krajewsli, S., Krajewska, M., Kitada, S., Zapata, J. M., Kochel, K., Kne, D., Scudiero, D., Tudor, G., Miller, G. J., Miyashita, T., Yamada, M., and Reed, J. C. (1998) Cancer Res. 58, 3116–3131
17. Luders, I., Demand, J., Papp, O., and Hofeld, J. (2000) J. Biol. Chem. 275, 14817–14823
18. Liman, J., Ganesan, S., Dohn, C. P., Krajewska, S., Reed, J. C., Bahr, M., Wouters, F. S., and Kermer, P. (2005) Mol. Cell. Biol. 25, 3715–3725
19. Aronov, S., Aranda, G., Behar, L., and Ginzburg, I. (2001) J. Neurosci. 21, 6577–6587
20. Kojima, S., Vignjevic, D., and Borisy, G. G. (2004) Biotechniques 37(4), 64–79
21. Aranda-Abreu, G. E., Behar, L., Chung, S., Furneaux, H., and Ginzburg, I. (1999) J. Neurosci. 19, 6907–6917
22. Asher, G., Tsvetkov, P., Kahana, C., and Shaul, Y. (2005) Genes Dev. 19, 316–321
23. Oddo, S., Caccamo, A., Shepherd, J. D., Murphy, M. P., Golde, T. E., Kayed, R., Metherate, R., Mattson, M. P., Akbari, Y., and LaFerla, F. M. (2003) Neuron 39, 409–421
24. Cleveland, D. W., Hwo, S. Y., and Kirschner, M. W. (1977) J. Mol. Biol. 116, 207–225
25. David, D. C., Layfield, R., Serpell, L., Narain, Y., Goedert, M., and Spillantini, M. G. (2002) J. Neurochem. 83, 176–185
26. Luders, I., Demand, J., and Hofeld, J. (2000) J. Biol. Chem. 275, 4613–4617