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The Co-chaperone Carboxyl Terminus of Hsp70-interacting Protein (CHIP) Mediates α-Synuclein Degradation Decisions between Proteasomal and Lysosomal Pathways*

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Youngah Shin‡, Jochen Klucken‡, Cam Patterson§, Bradley T. Hyman‡, and Pamela J. McLean‡¶

From the ‡Alzheimer Disease Research Unit, Department of Neurology, Massachusetts General Hospital, Charlestown, Massachusetts 02129, the §Carolina Cardiovascular Biology Center and Departments of Medicine, Pharmacology, and Cell and Developmental Biology, University of North Carolina, Chapel Hill, North Carolina 27599

α-Synuclein is a major component of Lewy bodies, the pathological hallmark of Parkinson disease, dementia with Lewy bodies, and related disorders. Misfolding and aggregation of α-synuclein is thought to be a critical cofactor in the pathogenesis of certain neurodegenerative diseases. In the current study, we investigate the role of the carboxyl terminus of Hsp70-interacting protein (CHIP) in α-synuclein aggregation. We demonstrate that CHIP is a component of Lewy bodies in the human brain, where it colocalizes with α-synuclein and Hsp70. In a cell culture model, endogenous CHIP colocalizes with α-synuclein and Hsp70 in intracellular inclusions, and overexpression of CHIP inhibits α-synuclein inclusion formation and reduces α-synuclein protein levels. We demonstrate that CHIP can mediate α-synuclein degradation by two discrete mechanisms that can be dissected using deletion mutants; the tetratricopeptide repeat domain is critical for proteasomal degradation, whereas the U-box domain is sufficient to direct α-synuclein toward the lysosomal degradation pathway. Furthermore, α-synuclein, synphilin-1, and Hsp70 all coimmunoprecipitate with CHIP, raising the possibility of a direct α-synuclein-CHIP interaction. The fact that the tetratricopeptide repeat domain is required for the effects of CHIP on α-synuclein inclusion morphology, number of inclusions, and proteasomal degradation as well as the direct interaction of CHIP with Hsp70 implicates a cooperation of CHIP and Hsp70 in these processes. Taken together, these data suggest that CHIP acts a molecular switch between proteasomal and lysosomal degradation pathways.

α-Synuclein is a major component of Lewy bodies (LBs)[1] found in Parkinson disease, dementia with Lewy body disease (DLB), the Lewy body variant of Alzheimer disease, and glial cytoplasmic inclusions found in multiple system atrophy (1–3). In these neurodegenerative disorders, collectively referred to as synucleinopathies, LBs are characterized by abnormal fibrillar aggregates of α-synuclein protein in the cytoplasm of selective populations of neurons and glial cells (4–7). Three different missense mutations in the α-synuclein gene (7–9) as well as triplication of the α-synuclein gene (10) have been shown to cause dominant inherited Parkinson disease in a small subset of human patients. Moreover, overexpression of α-synuclein in transgenic mice, flies, and viral vector-transduced rodents leads to α-synuclein pathology accompanied by neuronal dysfunction, the loss of synaptic terminals, and/or neuronal cell loss (11–16). These studies implicate a molecular pathological role of α-synuclein in Lewy body-related neurodegenerative diseases.

In living cells, various stresses cause unfolded or misfolded proteins to accumulate. Heat shock proteins recognize misfolded proteins and aid refolding. In addition to chaperone activity, heat shock proteins have been shown to facilitate degradation of highly misfolded proteins by transferring them to the ubiquitin proteasome degradation system (17–21). Molecular chaperones and their functions in protein quality control have been implicated in several neurodegenerative diseases (22–25). It has recently been reported that overexpression of the molecular chaperone Hsp70 prevents dopaminergic neuronal loss induced by α-synuclein in *Drosophila* (16) and prevents accumulation of Triton X-100-insoluble α-synuclein in α-synuclein transgenic mice (26).

For proper function, chaperones rely on interactions with co-chaperones to control the cycle of ATP binding, hydrolysis, and substrate binding (27). The carboxyl terminus of Hsp70-interacting protein (CHIP) has been recently identified as a co-chaperone protein (28) and is composed of three major domains: an amino-terminal three tandem tetratricopeptide repeat (TPR) domain, a highly charged central domain, and a carboxyl-terminal U-box domain. Recent *in vitro* data demonstrate that CHIP interacts with Hsc70/Hsp70 through its TPR domain and adjacent charged domain and regulates chaperone activity (28). The U-box domain of CHIP has a modified ring finger motif similar to that found in ubiquitin ligases. This suggests that CHIP is involved in ubiquitin-dependent protein degradation pathways. In fact, CHIP has been reported to be a *bona fide* E3 ubiquitin ligase (29), and supporting data have shown that CHIP promotes ubiquitinylation of chaperone substrates like the glucocorticoid receptor (30), cystic fibrosis transmembrane conductance regulator (31), Raf-1 protein kinase (21), and ErbB2 (32) in a U-box-dependent manner and can direct them to degradation through the proteasome. Fur-

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¶ To whom correspondence should be addressed: Alzheimer Disease Research Unit, Dept. of Neurology, Massachusetts General Hospital, 114 16th St., Charlestown, MA 02129. Tel.: 617-724-1263; Fax: 617-724-1480; E-mail: pmclean@partners.org.

[1] The abbreviations used are: LB, Lewy body; DLB, dementia with Lewy body disease; CHIP, carboxyl terminus of Hsp70-interacting protein; TPR, tetratricopeptide repeat; E3, ubiquitin-protein isopeptide ligase; WT, wild type; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; LN, Lewy neurite.

thermore, CHIP has been demonstrated to cooperate with Hsp70/90 and induce ubiquitination of the microtubule-associated protein tau (33). Recent data have shown that CHIP interacts directly with Parkin and positively regulates Parkin E3 ubiquitin ligase activity, resulting in protection from neurotoxicity induced by unfolded protein stress in the endoplasmic reticulum of dopaminergic neurons (34, 35). Taken together, CHIP functions in protein quality control by shifting the molecular chaperone system between its refolding machinery and degradation.

In the current study, we provide evidence that CHIP plays a role in α-synuclein aggregation and degradation. CHIP colocalizes with α-synuclein and Hsp70 in Lewy bodies and also in a cell culture model of α-synuclein inclusions. Overexpression of CHIP reduces α-synuclein aggregation and increases α-synuclein degradation in cell culture. We also demonstrate that CHIP can mediate α-synuclein degradation via both the proteasomal degradation pathway and the lysosomal degradation pathway and that specific domains of CHIP are required to direct α-synuclein to mediate these degradation decisions.

EXPERIMENTAL PROCEDURES

Cell Culture, Transient Transfection, and Drug Treatment—Human H4 neuroglioma cells (HTB-148; ATCC) were maintained in Opti-MEM (Invitrogen) supplemented with 10% fetal bovine serum. H4 cells were plated in 4-well chamber slides or 60-mm dishes 24 h prior to transfection. Cells were transfected with an equimolar ratio of DNA constructs using Superfect (Qiagen, Chatsworth, CA) according to the manufacturer's instructions. 10 μg/ml *N*-acetyl-Leu-Leu-norleucinal (ALLN) (Sigma) in dimethyl sulfoxide was added to the culture medium for the last 16–18 h of a 48-h transfection, and 25 mM ammonium chloride was added for the last 22 h of a 24-h transfection.

Plasmid Constructs—The α-synuclein expression constructs used in this study have been described previously. Briefly, 93 amino acids of the N-terminal region of enhanced green fluorescent protein were fused to the carboxyl terminus of α-synuclein in pcDNA3.1, resulting in an α-synuclein enhanced green fluorescent protein deletion construct (WTSynEGFPΔ155, now called Syn-T), or the cDNA encoding WT α-synuclein was cloned into pSI expression vector (Promega) (36). Overexpression of α-synuclein was detected via immunofluorescence and not direct fluorescence. Synphilin-1 cDNA (37) was cloned into pcDNA 3.1/V5-His-Topo expression vector such that synphilin-1 has a C-terminal V5/His tag as described (38). Human Hsp70 cDNA was kindly provided by J.-C. Plumier (Massachusetts General Hospital) and subcloned into pcDNA3.1. For CHIP expression, pcDNA3-CHIP, pcDNA3-CHIPΔU (residues 196–303 deleted), and pcDNA3-CHIPΔTPR (residues 32–145 deleted) constructs were described previously (30). Myc-tagged CHIP constructs were produced by subcloning CHIP, CHIPΔU, or CHIPΔTPR into pcDNA3.1-Myc/His (B) (Invitrogen).

Immunohistochemistry/Immunocytochemistry and Confocal Microscopy—Human midbrain tissue from four subjects, with a pathological diagnosis of DLB was obtained along with temporal cortex of two cases of Alzheimer disease and two control cases from the Harvard Brain Tissue Resource Center in the Massachusetts Alzheimer Disease Research Center. The tissue was fixed in 4% paraformaldehyde and sectioned for free-floating, 40-μm sections. Transiently transfected cells were fixed in 4% paraformaldehyde for 10 min after 24- or 48-h transfection. After fixation, human brain sections or transfected cells were permeabilized with 0.5% Triton X-100 in Tris-buffered saline (pH 7.4) for 20 min at room temperature and blocked in 1.5% normal goat serum (Jackson ImmunoResearch, West Grove, PA) for 1 h at room temperature. DLB tissue was triple-stained with a mouse anti-α-synuclein antibody (H3C, monoclonal 1:3000; gift from Julia George, University of Illinois), rabbit anti-CHIP antibody (polyclonal, 1:500) (28), and biotinylated anti-Hsp70 antibody (polyclonal, 1:500; StressGen, Victoria, Canada). Transfected cells were stained with a mouse anti-α-synuclein antibody (syn-1, monoclonal, 1:1000; BD Transduction Laboratories), CHIP antibody and biotinylated Hsp70 antibody. Primary antibodies were incubated overnight at 4 °C followed by secondary antibodies (Cy3, 1:500 (Jackson ImmunoResearch); Bodipy, 1:500 (Molecular Probes, Inc., Eugene, OR); fluorescein isothiocyanate, 1:500 (Molecular Probes, Inc.); or Cy5, 1:500 (Jackson ImmunoResearch)) for 1 h at room temperature. Immunostaining was observed using a Bio-Rad MRC-1024 confocal microscope (Cy3: excitation at 568 nm, emission at 605 nm; Bodipy, fluorescein isothiocyanate: excitation at 488 nm, emission at 522 nm; Cy5: excitation at 647 nm, emission at 680 nm). Control experiments demonstrated no signal from any of the secondary antibodies in the absence of the corresponding primary antibody. For the preabsorption experiment, 30 μg of recombinant CHIP protein was incubated with anti-CHIP antibody (final dilution of 1:500) in PBS for 2 h at 37 °C and overnight at 4 °C. The mixture was centrifuged, and supernatant was used for immunostaining.

Quantitation of Lewy Bodies and Cells Containing Inclusions—α-Synuclein and CHIP immunoreactivity was observed using a ×20 objective and α-synuclein immunopositive Lewy bodies were counted. The percentage of Lewy bodies stained with CHIP was determined by assessing the number of CHIP-immunopositive Lewy bodies of 25 Lewy bodies immunopositive for α-synuclein in each case examined. The number of transfected H4 cells containing α-synuclein-immunopositive inclusions were assessed following immunocytochemistry using a fluorescence microscope with a ×20 objective as follows. Cells were assessed by an observer blind to the transfection conditions. A transfected cell containing inclusions was scored on the presence of detectable α-synuclein immunopositive inclusions compared with background (which in all cases was negligible). A cell was considered positive for inclusions regardless of the size or number of inclusions. Cells from two different wells of four-well slides were assessed, and 300–400 cells were counted for each experiment. The percentage of transfected cells containing inclusions was recorded.

Coimmunoprecipitation and Western Blot Analysis—For immunoprecipitation, H4 cells were harvested 24 h after transfection and lysed in lysis buffer: 50 mM Tris/HCl, pH 7.4, 150 mM NaCl, 0.3% CHAPS, 10 mM NaF, 100 μM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and protease inhibitor mixture (Roche Applied Science) for 1 h on ice. Cell lysates were centrifuged at 10,000 rpm for 10 min at 4 °C, and supernatant was used for immunoprecipitation using protein G-agarose and rat anti-Myc antibody (JAC6; Serotec, Oxford, UK) overnight at 4 °C. Immunoprecipitates were washed with lysis buffer three times and loaded onto 10–20% Tris/glycine gels (Invitrogen). Protein was transferred to Immobilon-P membrane (Millipore Corp.) and blocked in blocking buffer (Lycor, Lincoln, NE) for 1 h prior to the addition of primary antibody (mouse monoclonal anti-α-synuclein, syn-1, 1:1000; rabbit polyclonal anti-Hsp70, 1:10,000 (StressGen, Victoria, Canada); mouse monoclonal anti-V5, 1:2000 (Invitrogen); mouse monoclonal anti-Myc, 9E10, 1:1000 (Developmental Studies Hybridoma Bank, University of Iowa)) at room temperature for 1–2 h or overnight at 4 °C. Followed by three TBS-T washes, infrared fluorescent labeled secondary antibodies (IRDye 800 anti-rabbit or IRDye 700 anti-mouse, 1:2000, Rockland Immunochemicals (Gilbertsville, PA)) were incubated at room temperature for 1 h, and immunoblots were processed using the Odyssey infrared imaging system (Lycor).

For Western blot analysis, cells were washed with cold phosphate-buffered saline 24 or 48 h after transfection, lysed on ice in lysis buffer (50 mM Tris/HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, pH 8.0, 0.1% Triton X-100, protease inhibitor mixture), and sheared 10 times through a $30^{1/2}$-gauge needle. Protein concentration was determined using a Lowry protein assay. 30 μg of each cell lysate was loaded onto 10–20% Tris/glycine gels (Invitrogen), and mouse anti-α-synuclein, rabbit anti-Hsp70, and mouse anti-actin (AC-40, 1:500, Sigma) were used for Western blot analysis. Immunoblots were processed and quantified using the Odyssey infrared imaging system as described above (Lycor, Lincoln, NE) or processed using ECL (Amersham Biosciences) and quantified using ImageJ software.

Protein Degradation Assay—H4 tet-off cells stably overexpressing Syn-T were maintained in the absence of doxycycline (Syn-T expression "on"). 24 h following transfection of synphilin-1 and either empty vector or CHIP, cells were treated with 1 μg/ml doxycycline to turn off Syn-T expression. Cells were harvested at specific time points, and Syn-T expression was detected by Western blot analysis.

Statistical Analysis—Statistical analysis for comparison of groups was performed by analysis of variance, with Fisher's probability of least significant differences *post hoc* test for significance. The comparison of samples with a hypothesized mean of 1 was carried out using a one-group Student's *t* test with the statistical software, Statview (SAS Institute Inc., Cary, NC). For the protein degradation assay, linear regression analysis was performed to determine the independent effect of CHIP expression on Syn-T levels.

RESULTS

CHIP Localizes in Lewy Bodies—Several heat shock proteins, including Hsp70 and Hsp40, have been found to colocal-

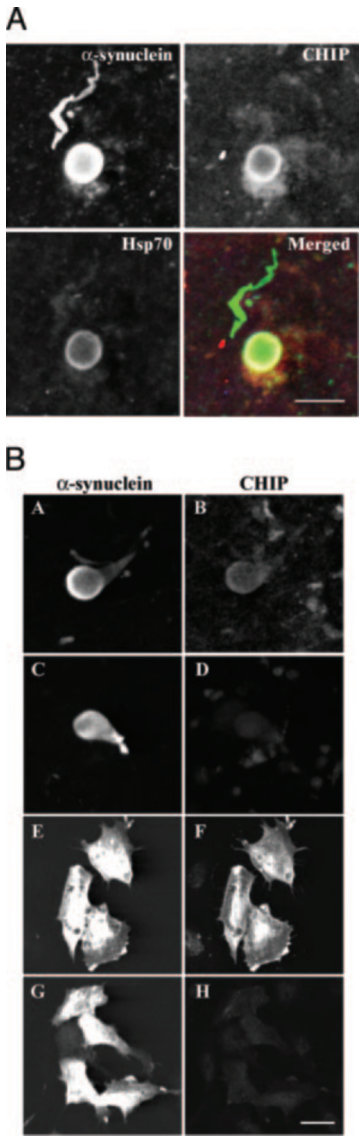


FIG. 1. **CHIP colocalizes with α-synuclein in Lewy bodies in DLB.** *A*, four pathologically confirmed cases of DLB were triple immunostained with α-synuclein, Hsp70, and CHIP. Approximately 87 ± 7.08% (S.D., $n = 4$) of LBs were immunopositive for CHIP. The *scale bar* represents 10 μm. *B*, preabsorption of anti-CHIP antibody with recombinant CHIP protein was used as a negative control for the antibody specificity. DLB tissue (*A–D*) and H4 cells (*E–H*) expressing SynT and CHIP was immunostained with anti-α-synuclein and either anti-CHIP (*A*, *B*, *E*, and *F*) or preabsorbed (*C*, *D*, *G*, and *H*) anti-CHIP antibody.

ize with α-synuclein in LBs and Lewy neurites (LNs) in Parkinson disease and DLB (16, 39). Taken together with the fact that ubiquitin is also a prominent component of the vast majority of LBs (40), this implicates protein refolding and degradation as a central process in the pathology of these diseases. In this study, we tested the hypothesis that CHIP also accumulates in LBs. To this end, we examined four pathologically confirmed cases of DLB for the presence of CHIP in LBs. LBs and LNs were positively identified using the monoclonal anti α-synuclein antibody H3C. Interestingly, ~87% ± 7.08 (S.D., $n = 4$) of nigral LBs were also immunopositive for CHIP using a polyclonal CHIP antibody (Fig. 1*A*). Consistent with our previous findings, ~70% of LBs were immunopositive for Hsp70 (39). By contrast, substantially less CHIP immunoreactivity was detected in LNs. In control brain and pathologically confirmed cases of Alzheimer disease, no LB-like inclusions were detected with either antibodies to α-synuclein or CHIP, although as previously reported, neurofibrillary tangles were CHIP-immunoreactive (33). The specificity of the CHIP antibody was affirmed by preabsorption with recombinant CHIP protein. Tissue sections and CHIP-overexpressing H4 cells were immunostained with preabsorbed CHIP antibody as well as secondary antibody alone in the absence of primary antibody. CHIP immunoreactivity was drastically reduced in those sections and cells receiving preabsorbed antibody, confirming antibody specificity (Fig. 1*B*).

Endogenous CHIP Localizes to α-Synuclein Inclusions in Cell Culture—Coexpression of a C-terminally tagged α-synuclein construct (Syn-T) with synphilin-1 in human H4 neuroglioma cells leads to the development of α-synuclein-immunopositive inclusions in ~50% of transfected cells (26, 36, 39). In the current study, we used immunofluorescence to examine the distribution of CHIP in Syn-T- and synphilin-1-transfected cells. All α-synuclein-immunopositive inclusions identified were immunoreactive for endogenous CHIP and endogenous Hsp70 (Fig. 2*A*). When CHIP, CHIPΔU, or CHIPΔTPR (Fig. 2*B*) was cotransfected with Syn-T and synphilin-1 we detected a robust up-regulation of endogenous Hsp70 expression in CHIP or CHIPΔU- but not CHIPΔTPR-transfected cells compared with control vector transfected cells with Western blot analysis (Fig. 2*C*). The fact that cotransfection of CHIP resulted in an up-regulation of endogenous Hsp70 is consistent with the idea that CHIP and Hsp70 are co-regulated (17, 21, 28).

CHIP Suppresses α-Synuclein Inclusion Formation and Changes the Morphology of α-Synuclein Inclusions in Cell Culture—We have previously shown that overexpression of Hsp70 and molecular chaperone-related proteins prevents α-synuclein inclusion formation in human H4 neuroglioma cells (39, 41). Since CHIP is known to be a co-chaperone that interacts with Hsp70 and regulates its chaperone activity (28), we tested the effect of CHIP on α-synuclein inclusion formation in H4 cells. CHIP or deletion mutant CHIP constructs were cotransfected with Syn-T and synphilin-1, and the number of cells containing α-synuclein-immunopositive inclusions was assessed 48 h after transfection. Interestingly, CHIP reduced the number of cells containing α-synuclein inclusions by 61.9% ($p < 0.05$) (Fig. 3*A*). Surprisingly, a decrease in the number of cells with α-synuclein inclusions was also seen following cotransfection with both CHIP deletion mutants, CHIPΔU (U-box deleted) and CHIPΔTPR (TPR domain deleted). Furthermore, cotransfection with CHIP also led to a dramatic change in the morphology of α-synuclein inclusions (Fig. 3*B*, *panels A* and *B*). Immunostaining revealed that cotransfection with CHIP resulted in a switch from several large inclusions (Fig. 3*B*, *panel A*) to numerous small inclusions/microaggregates throughout the cytoplasm of cells (Fig. 3*B*, *panel B*). This morphological change in inclusion type appeared to be TPR domain-dependent, because we observed the same tendency when CHIPΔU (containing the TPR domain) was cotransfected (Fig. 3*B*, *panel C*). However, when CHIPΔTPR (containing the U-box domain) was cotransfected, the majority of cells were indistinguishable from control (*i.e.* had larger inclusions) (Fig. 3*B*, *panel D*). Quantitation of inclusion size revealed that in SynT- and synphilin-1-transfected cells, 39% of cells with inclusions had predominantly large inclusions (≥2.5-μm diameter). Cotransfection of CHIP and CHIPΔU dramatically reduced the number of cells with large inclusions to 4.7 and 2.3%, respectively, whereas when CHIPΔTPR was cotransfected, 28.6% of cells with inclusions had large inclusions (Fig. 3*C*).

CHIP Degrades α-Synuclein via the Proteasomal and the Lysosomal Degradation Pathways—Recent data demonstrated that CHIP interacts with heat shock proteins and directs substrates of heat shock proteins away from a refolding pathway

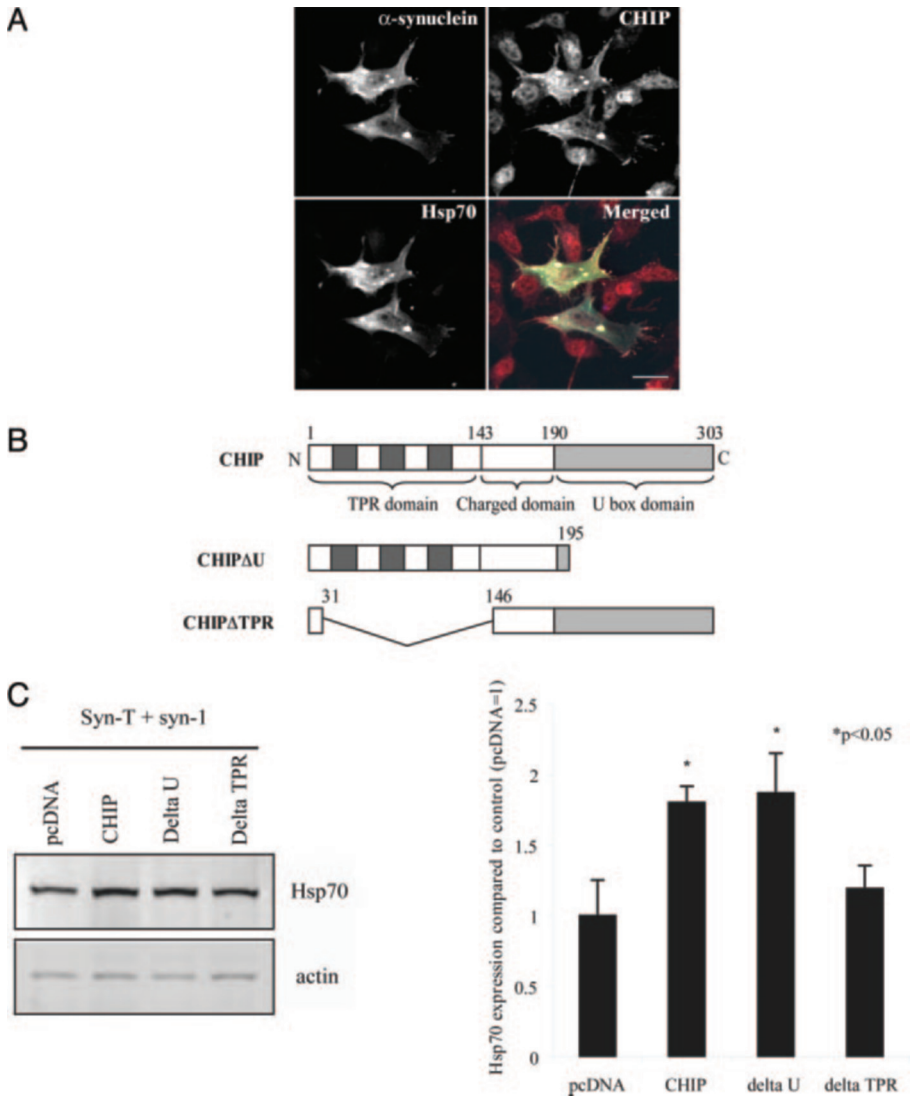


FIG. 2. **Endogenous CHIP colocalizes with α-synuclein inclusions in H4 cells and overexpression of CHIP induces endogenous Hsp70 expression.** *A*, H4 cells were cotransfected with Syn-T and synphilin-1 and immunostained for α-synuclein, Hsp70, and CHIP. Endogenous Hsp70 and CHIP colocalized with α-synuclein immunopositive inclusions. The *scale bar* represents 10 μm. *B*, schematic representation of the domain structure of CHIP and deletion mutant CHIP constructs. *C*, overexpression of CHIP and CHIPΔU induced endogenous Hsp70 expression. H4 cells were transfected with Syn-T, synphilin-1, and either control vector, CHIP, CHIPΔU, or CHIPΔTPR, and total cell lysate was analyzed for Hsp70. Band densities were quantified using the Odyssey infrared imaging system, normalized to actin to control for loading errors, and expressed as -fold above control. Data are presented as means ± S.E. of four independent experiments.

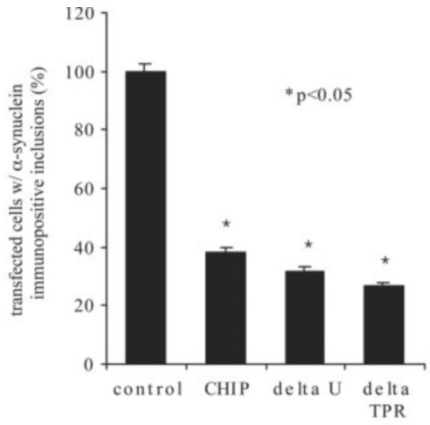
toward the ubiquitin-proteasome degradation pathway (20, 21, 30, 31). We next examined whether CHIP inhibition of α-synuclein inclusion formation is via enhanced protein degradation. H4 cells were cotransfected with Syn-T, synphilin-1, and either CHIP, CHIPΔU, or CHIPΔTPR, and total Syn-T expression was analyzed by quantifying the 27-kDa Syn-T band on SDS-PAGE (Fig. 4*A*). Cotransfection with CHIP significantly reduced Syn-T expression by 70 ± 5.7% (S.E., $n = 6$) compared with control. Cotransfection of CHIPΔU or CHIPΔTPR also decreased Syn-T protein level by 78 ± 7.0% (S.E., $n = 6$) and 65 ± 8.8% (S.E., $n = 6$), respectively. In parallel experiments, we also examined the impact of CHIP overexpression on WT α-synuclein, which does not form visible inclusions in H4 cells. Cotansfection with CHIP or either deletion mutant construct also reduced WT α-synuclein protein expression. These data suggest that CHIP reduces α-synuclein aggregation by enhancing degradation and that either the TPR domain or U-box domain is sufficient for the effect of CHIP.

To determine that the decrease in Syn-T protein levels is a result of enhanced degradation and not transcriptional downregulation, we used a tetracycline-regulated stably transfected cell line expressing SynT to assess the degradation kinetics. Stably transfected cells were maintained in the absence of doxycycline, which results in the overexpression of Syn-T. Following cotransfection of synphilin-1 and CHIP or control empty vector, the cells were treated with 1 μg/ml doxycycline to suppress α-synuclein expression. Cells were harvested at specific time points (0, 3, 6, and 12 h), and the level of Syn-T was assessed via Western blot analysis. When CHIP was co-expressed, we detected a significantly faster degradation of Syn-T compared with control transfected cells (Fig. 4*B*).

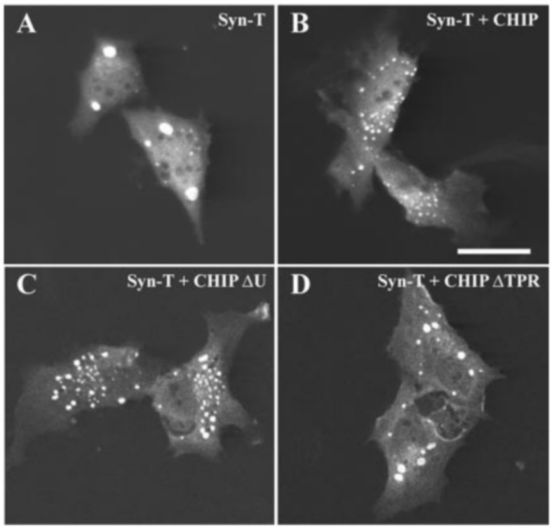
To determine the mechanism by which CHIP enhances α-synuclein degradation, we used a selective inhibitor of the 20 S proteasome, ALLN. Syn-T, synphilin-1, and CHIP were co-expressed in H4 cells and treated with 10 μg/ml ALLN for the last 16–18 h of a 48-h transfection. Interestingly, ALLN treatment prevented CHIPΔU-mediated degradation of α-synuclein, consistent with a critical role for the TPR domain (and hence CHIP-HSP interaction) in directing substrate toward the proteasome. However, we found that both full-length CHIP and CHIPΔTPR were still able to degrade α-synuclein, although the proteasomal degradation pathway was blocked by ALLN (Fig. 5*A*), suggesting a proteasome-independent mechanism of CHIP-mediated degradation.

Recent studies have suggested that lysosomal degradation may also play an important role in α-synuclein degradation and that mutant α-synuclein could prevent chaperone-mediated autophagy, a specific type of lysosomal degradation (42). To investigate whether CHIP can also mediate α-synuclein degradation via the lysosome, we used ammonium chloride (NH_4Cl), an inhibitor of lysosomal proteolysis (43). 25 mM NH_4Cl treatment prevented CHIPΔTPR-mediated degradation (Fig. 5*B*),

A



B



C

Cotransfection Condition	% large α-synuclein inclusions
pcDNA3.1	38.96±2.38
CHIP	4.72±1.34**
CHIPΔU	2.32±1.42**
CHIPΔTPR	28.63+4.74*

FIG. 3. **Overexpression of CHIP suppresses α-synuclein inclusion formation and dramatically changes the morphology of α-synuclein inclusions in H4 cells.** *A*, H4 cells were cotransfected with Syn-T, synphilin-1, and either CHIP, CHIPΔU, or CHIPΔTPR and immunostained for α-synuclein. 48 h after transfection, the number of cells containing α-synuclein-immunopositive inclusions were assessed as described in methods section. Data are presented as means ± S.E. of three independent experiments. *B*, cotransfection of SynT and synphilin-1 with CHIP (*B*) or CHIPΔU (*C*) resulted in a decrease in inclusion size, whereas CHIPΔTPR (*D*) resulted in inclusions with morphology similar to control (*A*). The *scale bar* represents 10 μm. *C*, quantitation of the number of cells containing large (≥2.5-μm diameter) α-synuclein-immunopositive inclusions following cotransfection with CHIP, CHIPΔU, or CHIPΔTPR. Data are presented as means ± S.E. of four independent experiments. **, $p < 0.0001$; *, $p < 0.02$ compared with control.

consistent with a role for the U-box domain of CHIP to direct α-synuclein toward the lysosome. However, both full-length CHIP and CHIPΔU were still able to degrade α-synuclein, although the lysosomal degradation pathway was blocked, consistent with our previous data indicating that CHIP can also mediate degradation via the proteasome.

Previous studies have found that CHIP directly interacts with Hsp70 via its TPR domain to regulate chaperone activity and directly interacts with Parkin to positively regulate its E3 ubiquitin ligase activity. To decipher the mechanism by which CHIP mediates α-synuclein degradation, we performed coimmunoprecipitation experiments whereby H4 cells were transfected with Myc-tagged CHIP, Syn-T, and synphilin-1 and immunoprecipitated using an anti-Myc antibody. Western blot analysis revealed that Syn-T, synphilin-1, and endogenous Hsp70 could all be coimmunoprecipitated with CHIP (Fig. 6). By contrast, CHIPΔTPR failed to coimmunoprecipitate Hsp70, confirming the fact that the TPR domain of CHIP is required for interactions with Hsp70 and suggesting that Hsp70 is not required for CHIP-mediated lysosomal degradation. Moreover, when H4 cells were transfected with Myc-tagged CHIP and Syn-T, without synphilin-1, CHIP could still immunoprecipitate Syn-T, suggesting the possibility of a direct CHIP-synuclein interaction, although facilitation by another member of the co-chaperone complex cannot be excluded.

DISCUSSION

In the present study, we tested the hypothesis that CHIP, a dual function protein (cochaperone and E3 ubiquitin ligase), can modulate α-synuclein inclusion formation. Here, we demonstrate that 1) CHIP is a component of Lewy bodies; 2) CHIP colocalizes with Hsp70 and α-synuclein in LB-like inclusions *in vitro*; 3) CHIP can reduce α-synuclein inclusions *in vitro*; 4) CHIP changes the morphology of α-synuclein inclusions *in vitro*; 5) CHIP enhances α-synuclein degradation by two mechanisms (via the proteasome in a TPR domain-dependent manner and via the lysosomal degradation pathway that is dependent on the U-box domain); and 6) CHIP, α-synuclein, synphilin-1, and Hsp70 exist as a complex.

Misfolding and aggregation of α-synuclein is thought to be a critical cofactor in the pathogenesis of LB related neurodegenerative diseases. Several proteins associated with misfolding and aggregation have been found to deposit in LBs in DLB and Parkinson disease brains, including chaperone-related proteins like 14-3-3 (44); heat shock proteins, including Hsp70 and Hsp40 (16, 39); and ubiquitin degradation pathway-related proteins like parkin (45, 46), UCH-L1 (47), ubiquitin (48, 49), and proteasomal subunits (50). This raises the possibility that the cellular machinery that recognizes and handles abnormally folded proteins may be involved in these diseases. Here, we demonstrate that CHIP accumulates in LBs in DLB brain. CHIP has been shown to interact with Hsp70/Hsc70 and facilitate the degradation of misfolded proteins (20, 21, 30, 31). Taken together, the presence of CHIP in LBs supports a role in protein misfolding and aggregation related to LB formation in the synucleinopathies. These data are consistent with the recent observation that CHIP is present in tau lesions in human postmortem tissue (33, 51), further implicating CHIP in neurodegenerative disease where protein misfolding is central to the disease pathogenesis.

Consistent with our observation that LBs contain CHIP, we also found that endogenous CHIP colocalized with α-synuclein and Hsp70 in intracellular inclusions in H4 human neuroglioma cells. In addition, we found that overexpression of CHIP or CHIPΔU up-regulated endogenous Hsp70. CHIP has been identified as an Hsp70-interacting protein (28), and overexpression of Hsp70 has been shown to decrease α-synuclein inclusion formation in H4 cells (26, 39) and reduce high molecular weight, aggregated α-synuclein *in vivo* and *in vitro* (26). These data suggest that a functional interaction exists between CHIP and Hsp70, which combines chaperone-mediated refolding and proteasomal and lysosomal degradation, to modulate α-synuclein aggregation.

Coexpression of Hsp70 reduces the number of cells containing α-synuclein inclusions (26, 39). Here, we found that CHIP not only reduced the number of cells containing α-synuclein

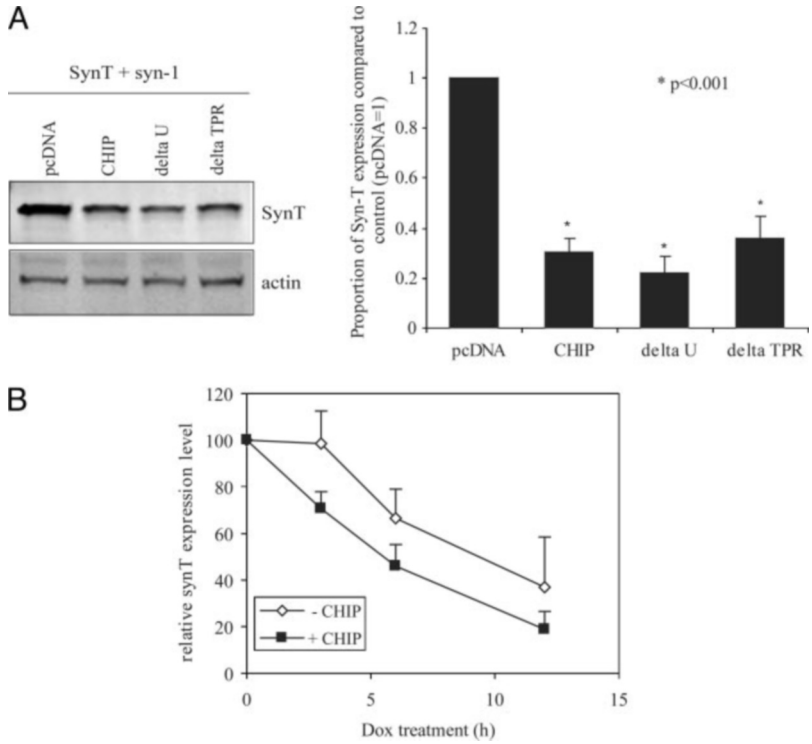


FIG. 4. **CHIP mediates α-synuclein degradation.** *A*, H4 cells were cotransfected with Syn-T, synphilin-1, and either empty vector, CHIP, CHIPΔU, or CHIPΔTPR. Total Syn-T expression was analyzed by quantifying the 27-kDa Syn-T band on SDS-PAGE compared with control transfected cells. Syn-T expression was normalized to actin expression. Band densities were quantified using Odyssey infrared imaging system and expressed as -fold above control. Data are presented as means ± S.E. of four independent experiments. Statistical analysis was done by one group *t* test for significance at the $p < 0.05$ level. *B*, Tet-regulated H4 cells stably overexpressing Syn-T were cotransfected with synphilin-1 and either empty vector or CHIP. 16 h after transfection, 1 μg/ml doxycycline was added in culture medium to turn off the Syn-T expression, and cells were harvested at the indicated time points. Syn-T expression was analyzed by Western blot analysis and quantified using imageJ analysis software. Data are presented as means ± S.E. of three independent experiments. Statistical analysis was done by linear regression analysis for significance at the $p < 0.05$ level.

inclusions but also produced a striking effect on inclusion morphology, shifting them from large inclusions to small inclusions throughout the cytoplasm. This change in morphology was TPR domain-dependent, which supports the idea that CHIP modulates the effect of Hsp70 on α-synuclein inclusions. This change in morphology was not concentration-dependent, because large inclusions were also observed when Syn-T expression was decreased using a tetracycline-regulated transfection system.[2] Recently, Lee *et al.* reported that overexpression of α-synuclein produces two distinct subtypes of inclusions: large fibrillar juxtanuclear inclusion bodies and small nonfibrillar punctuate aggregates scattered throughout the cytoplasm. They suggested that the inclusions appeared sequentially; small aggregates appeared first as a nucleation protofibril progressing to large fibrillar inclusions (52). It is not clear at this time if the small microaggregates of α-synuclein observed in the presence of CHIP represent nucleation protofibrils that have been blocked from proceeding to large inclusions or if they represent intermediate aggregates in the process of protein degradation. Further experiments will be required to clarify this process.

Our observation that overexpression of CHIP, CHIPΔU, or CHIPΔTPR could all decrease α-synuclein protein expression suggests that either the TPR domain or U-box domain alone is sufficient for CHIP to facilitate α-synuclein degradation. CHIP has been demonstrated to facilitate the degradation of chaperone substrates via the ubiquitin-dependent proteasomal degradation pathway in a U-box-dependent manner (30–32), so it is intriguing that CHIPΔU could also degrade α-synuclein when the E3 ubiquitin ligase activity conferring domain is deleted. This suggests that an additional degradation pathway for CHIP-mediated degradation of α-synuclein exists. This hypothesis is supported by the fact that the facilitation by CHIP of α-synuclein degradation was not reversed by proteasome inhibitor.

Most long lived proteins (half-life >5 h) are degraded in the lysosome and vacuole lytic compartments. Autophagy is a major cellular route to transport cytoplasmic proteins to the lysosome for degradation. Autophagic degeneration has been detected in the neurons of the substantia nigra in Parkinson disease patients (53) and Alzheimer disease (54). Here, we show that CHIP mediates α-synuclein degradation via the lysosome and that the U-box domain is required, suggesting that E3 ubiquitin ligase activity is involved in the lysosomal degradation pathway. Recently, several lines of evidence have emerged to suggest that ubiquitin-dependent protein trafficking usually requires the ligation of just one single ubiquitin to the substrate through ubiquitin Lys^{63} (55, 56). This monoubiquitination participates in targeting proteins to endosomal compartments either from the plasma membrane (57) or from the *trans*-Golgi network (58) and is also involved in protein sorting from endosome to multivesicular bodies and in delivery of transmembrane proteins into the lysosomal/vacuolar compartment (59).

That the TPR domain of CHIP is required for α-synuclein degradation via the proteasome indicates that a CHIP-Hsp70 interaction may be required to target α-synuclein to the proteasome. In support of this hypothesis, recent data have shown that CHIP cooperates with Hsc70/Hsp70 and Hsp90 for substrate targeting to the proteasome (31, 60, 61). CHIP has also been shown to inhibit Hsp70-mediated refolding of luciferase (28). Taken together, we propose that CHIP inhibits α-synuclein inclusion formation by redirecting Hsp70 from protein refolding to protein degradation. In Lewy bodies, this process may fail.

The fact that α-synuclein, synphilin-1, and Hsp70 coimmunoprecipitate with CHIP raises the possibility that CHIP may directly interact with α-synuclein or that a protein complex may exist. These data are consistent with the recent observation that CHIP specifically interacts with and degrades expanded polyglutamine proteins (62). Further studies will be required to determine the nature of the complex.

In sum, our data show that CHIP is associated with α-synuclein inclusions both *in vivo* and *in vitro* and that CHIP acts as a co-chaperone, altering α-synuclein aggregation and enhancing the degradation of misfolded α-synuclein. Importantly, we suggest for the first time that CHIP can direct degradation via either a TPR domain dependent proteasomal

[2] Y. Shin, J. Klucken, C. Patterson, B. T. Hyman, and P. J. McLean, unpublished observation.

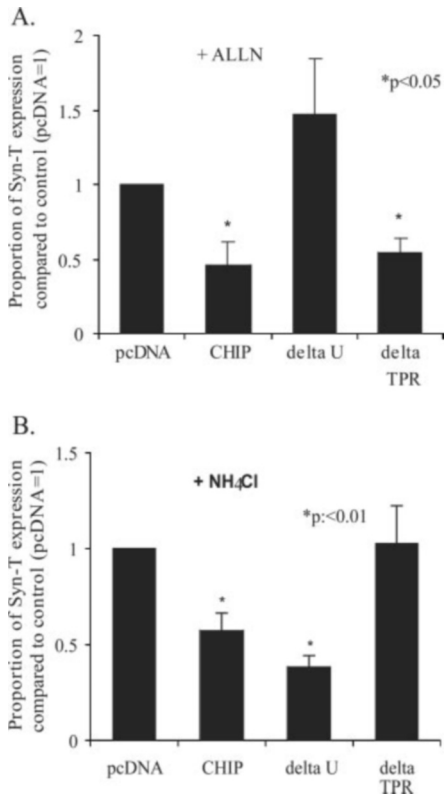


FIG. 5. **CHIP mediates α-synuclein degradation via proteasomal and lysosomal degradation pathways.** H4 cells were cotransfected with Syn-T, synphilin-1, and either empty vector, CHIP, CHIPΔU, or CHIPΔTPR. *A*, for proteasome inhibition, cells were treated with 10 μg/ml ALLN in dimethyl sulfoxide for the last 16–18 h of 48-h transfection. Data are presented as means ± S.E. of four independent experiments. *B*, cells were treated with 25 mM NH_4Cl for the last 22 h of 24 h transfection for lysosome inhibition. Data are presented as means ± S.E. of five independent experiments. Statistical analysis was done by one group *t* test for significance at the $p < 0.05$ level.

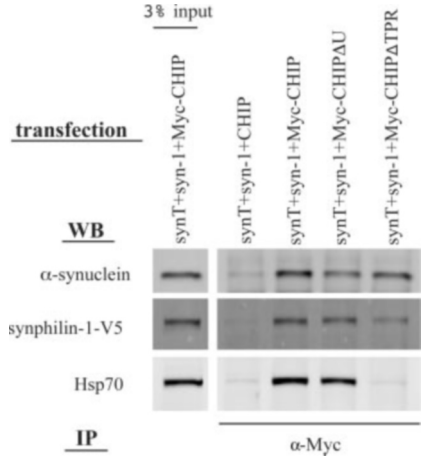


FIG. 6. **Syn-T coimmunoprecipitates with CHIP.** H4 cells were cotransfected with Syn-T, synphilin-1, and either CHIP, Myc-CHIP, Myc-CHIPΔU, or Myc-CHIPΔTPR. 24 h after transfection, cells were harvested and processed for immunoprecipitation with anti-Myc antibody. Immunoblot was probed with anti-α-synuclein, anti-V5 (synphilin-1), and anti-Hsp70 antibody.

pathway or a U-box dependent lysosomal pathway. Insofar as the balance of aggregation/degradation of misfolded α-synuclein may be critical in the synucleinopathies, CHIP and related heat shock protein co-chaperone molecules may play critical regulatory roles.

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