Hsp70 Reduces α-Synuclein Aggregation and Toxicity*

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Aggregation and cytotoxicity of misfolded α-synuclein is postulated to be crucial in the disease process of neurodegenerative disorders such as Parkinson’s disease and DLB (dementia with Lewy bodies). In this study, we detected misfolded and aggregated α-synuclein in a Triton X-100 insoluble fraction as well as a high molecular weight product by gel electrophoresis of temporal neocortex from DLB patients but not from controls. We also found similar Triton X-100 insoluble forms of α-synuclein in an α-synuclein transgenic mouse model and in an in vitro model of α-synuclein aggregation. Introducing the molecular chaperone Hsp70 into the in vivo model by breeding α-synuclein transgenic mice with Hsp70-overexpressing mice led to a significant reduction in both the high molecular weight and detergent-insoluble α-synuclein species. Concomitantly, we found that Hsp70 overexpression in vitro similarly reduced detergent-insoluble α-synuclein species and protected cells from α-synuclein-induced cellular toxicity. Taken together, these data demonstrate that the molecular chaperone Hsp70 can reduce the amount of misfolded, aggregated α-synuclein species in vivo and in vitro and protect it from α-synuclein-dependent toxicity.

α-Synuclein is a natively unfolded molecule that can self-aggregate to form oligomers and fibrillar intermediates (1–5) that accumulate to form Lewy bodies (LBs)1 and Lewy neurites in neurons at risk for degeneration in Parkinson’s disease and dementia with Lewy bodies (DLB) (6–14). For the most part, these α-synuclein aggregates are densely compact and can be immunostained for multiple additional components including ubiquitin, synphilin-1, and heat shock proteins (HSPs), which suggests that protein misfolding or degradation is altered in cells that develop LBs. Mouse models of α-synuclein aggregation exist that mimic the findings in human brains and show intracellular α-synuclein aggregates (15–17). Aggregated α-synuclein molecules are less detergent-soluble, and these detergent-insoluble species of α-synuclein can be detected in human brain, transgenic mouse models, and in vitro models (18–21). Although it is known that the conformation of α-synuclein in LBs is significantly different from that in the neuropil (22), it is unclear which conformation of α-synuclein contributes to inclusion formation. In addition to the formation of intracellular aggregates, α-synuclein is also cytotoxic. It has been postulated that α-synuclein oligomers found in Parkinson’s disease tissue by Western blot analysis represent the toxic species (5).

HSPs belong to the family of chaperone proteins and are important in both refolding misfolded proteins and directing proteins toward proteasomal degradation (23–25). HSPs can be protective in several neurodegeneration models (26–29), and recent data in the fly model suggest that overexpression of the molecular chaperone Hsp70 protects against α-synuclein-induced degeneration (26, 30). Hsp70 and its related co-chaperones may be important in α-synuclein misfolding. In fact, several HSPs and co-chaperones are associated with LBs (26, 31) and we previously reported that overexpression of Hsp70 and related molecules can prevent the formation of α-synuclein aggregates in vitro (31, 32). In this study, we establish biochemical assays to assess aggregated α-synuclein and investigate the influence of Hsp70 on these α-synuclein species. We demonstrate that Hsp70 reduces α-synuclein aggregation and toxicity via its refolding and degradation activities.

EXPERIMENTAL PROCEDURES

Human and Mouse Tissue—α-Synuclein transgenic animals (line D) have been described previously (16). Hsp70-overexpressing mice were a kind gift of W. H. Dillmann (University of California, San Diego, CA) (33). Fresh-frozen brain tissues (temporal cortex) from 12 subjects (10 men and 2 women) with a pathological diagnosis of DLB and 8 subjects (4 men and 4 women) without a neurodegenerative disease that served as control were obtained from the Harvard Brain Tissue Resource Center, The Massachusetts Alzheimer Disease Research Center, and the Massachusetts General Hospital-Massachusetts Institutes of Technology Uddal Center Brain Bank. The mean age of death for the DLB cases was 80.5 years (±6.4 S.D.) and 73 years (±12.1 S.D.) for controls. The mean post-mortem interval was 14.3 h (±9.9 S.D.) for DLB and 15.2 h (±6.5 S.D.) for control cases. Brain tissue was homogenized in 10 volumes (w/v) of cold lysis buffer (50 mm Tris/HCl, pH 7.4, 175 mm NaCl, 5 mm EDTA, pH 8.0, and protease inhibitor mixture (Roche Applied Science)) and sonicated for 10 s (total cell lysates).

Plasmid Construction—The constructs for human wild type untagged α-synuclein and its C-terminal tagged version (93 amino acid long tag referred to as Syn-T) and synphilin-1 have been described previously (31, 32). The C-terminal tagged α-synuclein is a truncated α-synuclein-enhanced GFP fusion protein that has 93 amino acids of enhanced GFP fused to the C terminus of α-synuclein. This fusion protein is no longer fluorescent but has a propensity to aggregate when overexpressed in H4 cells. Co-transfection with synphilin 1 further enhances this aggregation (32), and thus, co-transfection of Syn-T plus synphilin 1 functions as our α-synuclein inclusion model. cDNA-encoding genes were cloned into pcDNA3.1 or pEJ (Promega, Madison, WI) expression vectors. Human Hsp70 cDNA was kindly

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¶ The abbreviations used are: LBs, Lewy bodies; DLB, dementia with Lewy body; GFP, green fluorescent protein; PBS, phosphate-buffered saline; ANOVA, analysis of variance; HMW, high molecular weight.
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provided by J.-C. Plummer (Massachusetts General Hospital) and sub-
cloned in pcDNA3.1 (Clontech, Palo Alto, CA).

Cell Culture and Transfection—Human H4 neuroglioma cells (HTB-
148, ATCC, Manassas, VA) were maintained in Opti-MEM (Invitrogen)
supplemented with 10% fetal bovine serum. H4 cells were passaged 24 h prior to transfection and plated in four-well chamber slides for
immunocytochemistry (Labtek, Nalgen-Nunc, Naperville, IL) or 10-
well culture dishes for analysis of cell lysates (Corning, NY). Cells were transfected with equimolar ratios of plasmids using Superfect (Qiagen, Chatsworth, CA) according to the manufacturer’s instructions. Co-transfection with an empty pcDNA3.1 vector was used as control. After 48 h, cells were washed with cold PBS, harvested by scraping in cold lysis buffer without detergents (50 mM Tris/HCl, pH 7.4, 175 mM NaCl, 5 mM EDTA, pH 8.0, and protease inhibitor cocktail) and sheared once through a 30.5-G needle followed by sonication for 10 s (total cell lysate).

Detergent-solubility Fractionation and Gel Electrophoresis—Deter-
gent solubility was performed by adding Triton X-100 to total cell lysates (final concentration 1%) and incubating for 30 min on ice fol-
lowed by centrifugation (15,000 × g, 60 min, 4 °C). The supernant was designated Triton X-100 soluble fraction, and the pellet was redis-
solved in 2% SDS-containing lysis buffer and sonicated for 10 s (Triton X-100 insoluble fraction). Additional washing of the Triton X-100 insol-
uble pellet was found to not alter the α-synuclein expression in this fraction (data not shown) and was omitted from the experiments. Pro-
tein concentration was determined using a Lowry protein assay. 20–40 µg of each cell lysate was loaded onto 4–20% Tris-glycine gels (Invitrogen) for Western blot analysis. SDS-PAGE was performed with SDS containing running and sample loading buffer, whereas native-
PAGE (not containing any detergent) was performed with native (SDS-
free) running and sample loading buffer (Invitrogen). Protein was trans-
ferred to Immobilon-P membrane (Millipore, Bedford, MA) and blocked in blocking buffer (Lycor, Lincoln, NE) for 1 h prior to the addition of primary antibody, anti-α-synuclein (Syn-1, 1:1000, BD Transduction Laboratories) or anti-ubiquitin (SP240, 1:1000, Stressgen, Victoria, British Columbia, Canada), at room temperature for 1–2 h or overnight at 4 °C. Following three Tris-buffered saline with Tween
20 washes, infrared fluorescent-labeled secondary antibodies (IRDye
800 anti-rabbit or anti-mouse, Rockland Immunochemicals, Gilberts-
ville, PA, at 1:3000 or Alexa-880 anti-rabbit or anti-mouse, Molecular
Probes, Eugene, OR at 1:3000) were incubated at room temperature for
1 h and immunoblots were processed and quantified using the Odyssey infrared-imaging system (Lycor). Blots were also probed for actin (anti-
actin, AC40, Sigma), or proteins on the gels were stained with Coomas-
sie Blue (quantified using Odyssey infrared-imaging system as loading
controls). A mock-untransfected cell lysate was included in all of the cell culture experiments to control for nonspecific and endogenous signal.

Immunocytochemistry—Cells were plated into 4-well chamber slides (Nunc) and transfected as described above. 48 h later, the cells were washed with PBS and fixed with 4% paraformaldehyde for 10 min at room temperature. After washing with PBS, cells were permeabilized in Tris-buffered saline containing 0.1% Triton X-100 for 20 min at room temperature. After blocking in 1.5% normal goat serum containing Tris-buffered saline for 1 h, cells were incubated with primary antibody in blocking solution for 2 h at room temperature or overnight at 4 °C followed by washing with PBS and secondary antibody incubation for 1 h. As a control for antibody specificity, 1 µL of antibody solution was preabsorbed with 20 µg of recombinant protein (α-synuclein, Alpha-
Diagnostic, San Antonio, TX; Hsp70, Stressgen) in PBS for 2 h at 37 °C followed by 1 h at 4 °C. After centrifugation at 4 °C (15,000 rpm) for 15 min, the immune complex-free supernatant was diluted in blocking
solution and applied to the cells for 2 h followed by washing with PBS and secondary antibody incubation for 1 h. After a final wash, slides were coverslipped with aqueous mounting solution (GVA, Zymed Laboratories Inc., San Francisco, CA) and subjected to fluorescence microscopy using an Eclipse TE300 inverted microscope.

Toxicity Assay—Toxicity was analyzed 24 h after transfection by
measuring the release of adenylate kinase from damaged cells into the
culture medium using the ToxiLight™ (Cambrex, Walkersville, MD)
according to the manufacturer’s protocol.

Quantification of Cells Containing Inclusions—The number of cells containing α-synuclein-immunopositive inclusions was assessed by im-
munocytochemistry using a Nikon Eclipse TE300 inverted microscope with a ×20 objective as follows. Cells were assessed by an observer
blind to the transfection conditions (i.e., the co-transfected plasmid).
Approximately 300–400 cells from two wells were assessed for each
experiment. A total of four experiments were performed with each

condition. A positively transfected cell was scored based on the presence of significant α-synuclein immunostaining compared with background (which in all cases was negligible). A transfected cell containing inclu-
sions was scored based on the presence of a detectable aggregate of
α-synuclein immunostaining. A cell was considered positive for inclu-
sions independent of the size or number of inclusions. The percentage of cells containing inclusions compared with the total number of trans-
fected cells was recorded.

Statistical Analysis—Statistical analysis for comparison of groups
was performed by ANOVA with Fisher’s probability of least significant
differences post hoc test for significance.

RESULTS

Triton X-100 Insoluble Monomeric and High Molecular Weight Species (HMW) of α-Synuclein in DLB—The current understanding of the pathophysiology of α-synuclein is that α-synuclein can form oligomers that then further aggregate into protofibrils and fibrils and result in tightly packed α-synuclein in Lewy bodies. To assess this process in human brains, we performed biochemical assays using protein extracts from temporal neocortex of DLB cases and control brains. This brain area has only modest numbers of LBs and Lewy neurites in DLB (34). Detergent-solubility testing revealed a Triton X-100 insoluble fraction containing α-synuclein species that formed a HMW product with a molecular mass ranging from 60 to 250 kDa on SDS-PAGE (Fig. 1A). In addition to HMW-aggregated α-synuclein, we also detected SDS-soluble α-synuclein species in the Triton X-100 insoluble fraction that run at its expected monomeric mass on SDS-PAGE (~16 kDa) (Fig. 1B). Interestingly, quantification of these species from 12 DLB cases and 8 control brains on SDS-PAGE showed a sig-
nificant increase in both the HMW and the monomeric Triton X-100 insoluble fraction of α-synuclein in DLB brain compared with control brain. The HMW species and the monomeric α-synuclein found in the Triton X-100 insoluble fraction was ~2.5- or ~2.0-fold higher in DLB, respectively (Fig. 1C).

Analysis of α-synuclein levels in total protein fractions on SDS-
PAGE showed no significant difference in total (SDS-soluble) or Triton X-100 soluble monomeric α-synuclein between DLB and control brain (Fig. 1C). Thus, DLB differs from control brain because of the presence of increased α-synuclein in Triton X-100 insoluble inclusions, rather than a change in the total amount of α-synuclein.

FIG. 1. Triton X-100 insoluble and HMW α-synuclein species in
DLB patients. DLB patients have increased Triton X-100 (Tx) insol-
uble HMW (A) and monomeric 16-kDa α-synuclein species (B). Quanti-
fication of the expression level of different α-synuclein species in brain
tissue of temporal cortex of 12 DLB patients and 8 controls are shown
in C. Relative expression of HMW species are normalized to control (1.00). Arbitrary expression intensities of total SDS-soluble monomeric form of α-synuclein as well as Triton X-100 soluble and insoluble fraction
show only a significant increase of Triton X-100 insoluble mono-
meric α-synuclein level and portions.

| Species | Control | DLB | Fold Change |
|---------|---------|-----|-------------|
| HMW     | 0.25    | 0.60| 2.40        |
| Monomeric | 0.10   | 0.25| 2.50        |

| HMW | Monomeric |
|-----|-----------|
| 0.25| 0.10      |

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Fig. 2. Triton X-100 insoluble and HMW α-synuclein species in α-synuclein transgenic mice. Native HMW and Triton X-100 (Tx) insoluble monomeric species of α-synuclein are increased in the brain of α-synuclein transgenic mice (Masliah line D). Total protein from the cortices of two 6-month-old transgenic (Masliah line D and background) and two background mice were analyzed by native PAGE (A). Triton X-100 solubility testing was performed, and fractions were separated by SDS-PAGE (B).

Triton X-100 Insoluble and HMW α-Synuclein Species in α-Synuclein Transgenic Animals—We next asked whether similar α-synuclein species were present in a mouse model of α-synuclein pathology. As reported by Masliah et al. (16), α-synuclein transgenic mice (line D) have a substantial number of α-synuclein inclusions in the cortex that resemble some of the features of LBs found in human brain (16). To determine whether different aggregation states of α-synuclein led to the same biochemical features we observed in human DBL cortex, we examined total cell lysates from the cortex of 6-month-old α-synuclein transgenic and non-transgenic animals. Cell lysates were subjected to native PAGE and immunoblotted for α-synuclein. Interestingly, the HMW α-synuclein species of molecular masses between 90 and 150 kDa were detected in α-synuclein transgenic animals to a greater extent than in control animals (Fig. 2A). We next examined whether α-synuclein in the transgenic animals also displayed abnormal detergent insolubility. Indeed, ~2-fold more Triton X-100 insoluble α-synuclein species were present on SDS-PAGE in α-synuclein transgenic animals than in control animals (Fig. 2B). Under SDS-denaturing conditions, the Triton X-100 insoluble α-synuclein was resolved into only monomeric 16-kDa species, suggesting that aggregated α-synuclein species are not as tightly packed and detergent-resistant in transgenic animals as in DBL cases.

Prevention of HMW and Triton X-100 Insoluble α-Synuclein Species by Hsp70 in Mice—Recent data in the fly model suggest that overexpression of the molecular chaperone Hsp70 protects against α-synuclein-induced neuronal degeneration (26, 30). In addition, from our laboratory and related molecules can prevent α-synuclein aggregates from forming in vitro (31) and that LBs immunostain robustly for heat shock proteins (26, 31). Here we tested the hypothesis that overexpression of Hsp70 can reduce abnormal Triton X-100 insoluble α-synuclein species observed in the α-synuclein transgenic mice. We bred α-synuclein transgenic mice with mice overexpressing rat Hsp70 (33). Performing the same biochemical assays from five Hsp70 crossed with α-synuclein mice and three α-synuclein-only expressing mice (5-month-old), we found a significant ~5-fold (5.27 ± 1.51) reduction in the amount of HMW species of α-synuclein (Fig. 3A). In addition, the amount of α-synuclein in the Triton X-100 insoluble fraction was also decreased in the Hsp70 crossed with α-synuclein animals by ~2-fold (35.74 ± 3.40% compared with 17.40 ± 6.36% in Fig. 3C). The total amount of SDS-soluble monomeric α-synuclein did not differ between these mice (Fig. 3B), suggesting that Hsp70 specifically reduced abnormal conformations without changing total amount of α-synuclein.

Effect of Hsp70 on α-Synuclein Aggregation in Vitro—We previously reported that overexpression of C-terminally modified α-synuclein (Syn-T) as well as the naturally occurring rat syn-2 isoform (which has a different C terminus) led to the formation of intracellular α-synuclein-positive aggregates in H4 cells and that this phenomenon was increased by proteasome inhibition (31, 32). By contrast, transient expression of untagged wild type α-synuclein did not produce intracellular inclusions. Co-expression of Syn-T with synphilin-1 facilitation the formation of intracellular aggregates (32), but synphilin-1 fails to induce aggregation when co-transfected with wild type α-synuclein. We used co-expression of Syn-T and synphilin-1 as an in vitro α-synuclein inclusion model (Fig. 4) to study the protective function of Hsp70 on α-synuclein aggregation.

Interestingly, we observed α-synuclein species that were similar to the species detected in DBL cases and transgenic mice models in protein lysates of H4 cells transfected with Syn-T and synphilin-1. Native PAGE of total protein lysates revealed an α-synuclein-positive HMW product of molecular mass between 80 and 200 kDa (Fig. 5A, lane 1) that was not

Fig. 3. Reduced Triton X-100 (Tx) insoluble and HMW α-synuclein species in Hsp70-overexpressing α-synuclein transgenic mice. Breeding of α-synuclein transgenic mice with mice-overexpressing Hsp70 results in a reduction of HMW (A) with no change in total levels of monomeric α-synuclein (SDS-soluble) (B) and a corresponding reduction in Triton X-100 insoluble α-synuclein species (C). This suggests a specific reduction in higher aggregated and detergent-insoluble forms of α-synuclein (αSYN).

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detected in cells transfected with the untagged (non-aggregating) wild type α-synuclein (lane 3) or mock-transfected cells (lane 5). In addition, we detected an ∼55-kDa α-synuclein-positive band under native PAGE condition in Syn-T and wild type α-synuclein-transfected cells but no monomeric (16 kDa) form of α-synuclein (Fig. 5A). Triton X-100 solubility testing revealed Triton X-100 insoluble α-synuclein only if cells were transfected with both Syn-T and synphilin-1 (Fig. 5C). Wild type α-synuclein was found in Triton X-100 soluble fractions exclusively (Fig. 5C). Co-transfection of synphilin-1 did not alter this pattern compared with omitting synphilin-1 (data not shown).

We next asked whether Hsp70 can reduce the HMW and the Triton X-100 insoluble α-synuclein species in this model. Co-transfection of Hsp70 with Syn-T/synphilin-1 significantly reduced the HMW α-synuclein species (Fig. 5A, lane 2), consistent with the effect of crossing α-synuclein transgenic mice with Hsp70-overexpressing animals (Fig. 3). It also reduced the 55-kDa α-synuclein-positive band in Syn-T and wild type α-synuclein-transfected cells (Fig. 5A, lanes 2 and 4). Furthermore, quantification of the 16-kDa monomeric α-synuclein on SDS-PAGE in seven different experiments demonstrated that total α-synuclein expression levels were reduced by 45% by Hsp70 (Fig. 5B).

Co-transfection with Hsp70 not only decreased total α-synuclein levels, it also reduced the Triton X-100 insoluble α-synuclein species in Syn-T-transfected H4 cells (Fig. 5C). To assess the refolding activities of Hsp70 on misfolded α-synuclein, we determined the ratio of Triton X-100 insoluble α-synuclein compared with the soluble fraction in six independent experiments. This enabled us to measure changes in the Triton X-100 insoluble fraction independent of the total protein levels. A comparison of the Triton X-100 insoluble fraction in Hsp70 versus empty vector (control) co-transfected cells revealed that Hsp70 significantly reduced the percentage of α-synuclein in the Triton X-100 insoluble fraction by ∼50% (Fig. 5D). Thus, Hsp70 leads to a decrease in the total amount of (misfolded) Syn-T as well as to a specific decrease in the percentage of Syn-T found in detergent-insoluble fraction, suggesting either chaperone-related “refolding” and/or specific degradation of the misfolded detergent-insoluble fraction.

**Influence of Hsp70 on Cellular Toxicity of α-Synuclein Over-expression**—Because Hsp70 has been shown to be protective in α-synuclein-dependent toxicity in Drosophila, we examined the effect of Hsp70 on the toxicity of Syn-T, which forms macroscopic aggregates, and wild type α-synuclein, which does not form aggregates, in our cell culture model. Transfection with wild type α-synuclein alone increased the cellular toxicity by ∼2-fold (1.75 ± 0.16) compared with mock-transfected cells. Transfection with an irrelevant protein had no effect on toxicity (e.g., GFP, data not shown). Similarly, a ∼1.5-fold (1.56 ± 0.13-fold) increase in toxicity was observed by transfection with Syn-T. Interestingly, co-transfection with Hsp70 significantly reduced the toxicity of both wild type α-synuclein and Syn-T by ∼20% (Fig. 6). Thus, α-synuclein appears to be cytotoxic regardless of whether it might form aggregates. Hsp70 can protect in either case.

**DISCUSSION**

Our data suggest two major conclusions. First, we describe abnormal detergent-insoluble forms of α-synuclein in human DLB cortex, α-synuclein transgenic mice, and tissue culture models of α-synuclein aggregation. Although the exact biochemical characteristics vary among these preparations, it seems clear that Triton X-100 insolubility highlights a form of α-synuclein that is abnormal in the disease state. Second, we detected amelioration of this biochemical abnormality by overexpression of the molecular chaperone Hsp70 in two experimental systems. Hsp70 may have a role both in refolding and in degradation of misfolded α-synuclein molecules. Intriguingly, Hsp70 is also protective against α-synuclein toxicity. Thus, molecular chaperones may be involved in regulating the biochemical characteristics and toxicity of α-synuclein, supporting the idea that molecular chaperones play a role in protection against α-synuclein-mediated neurotoxicity (26).

In Parkinson’s disease and DLB, α-synuclein accumulates in aggregates that are densely compact and can be immunostained for multiple additional components including the α-synuclein-interacting protein, synphilin-1 (35, 36). The incremental aggregation process of α-synuclein involves several modifications (misfolding, dimer and oligomer formation, and self-aggregation) and is linked to Parkinson’s disease-associated mutations (5, 22, 37–50). In this study, we detected abnormal α-synuclein species based on their detergent solubility and electrophoretic mobility characteristics in three different systems: human DLB cortex, transgenic mice overexpressing wild type human α-synuclein, and a transiently transfected cell culture system. We found Triton X-100 insoluble HMW and monomeric α-synuclein species in tissue extracted from the temporal cortex of patients with DLB product. Both species were significantly increased in DLB patients compared with control brains. Total SDS and Triton X-100 soluble levels of α-synuclein were similar for DLB and control brains, suggesting that HMW and monomeric Triton X-100 insoluble/SDS-soluble species of α-synuclein specifically represent a portion of total α-synuclein that is abnormal in DLB. Because LBs and Lewy neurites are sparse in temporal neocortex, we speculate that the Triton X-100 insoluble fraction may represent a biochemical lesion that is independent of the morphological alteration seen in DLB.

We detected a similar increase in Triton X-100 insoluble and HMW α-synuclein species in the brains of α-synuclein transgenic mice compared with background mice. These mice develop intracellular α-synuclein inclusions that are associated with decrements of dopaminergic terminals in the striatum and result in motor and dopaminergic deficits (15, 16). However, in the Triton X-100 insoluble fraction, we detected only increased monomeric α-synuclein species and not the HMW

**Fig. 4. In vitro α-synuclein aggregation model. A,** transient transfection of H4 neuroglioma cells with C-terminal tagged α-synuclein (Syn-T) and synphilin-1 results in α-synuclein-immunopositive intracellular inclusions that also stain for endogenous Hsp70 (32). **B** and **C,** preabsorption of anti-α-synuclein antibody with recombinant α-synuclein protein (**B**) or anti-Hsp70 antibody with recombinant Hsp70 protein (**C**) served as negative controls for antibody specificity. Reflection images demonstrate the presence of cells in the absence of antibody staining. Syn-T is a truncation of α-synuclein-enhanced GFP with 93 amino acids of GFP fused to the C terminus of α-synuclein. The fusion protein is not fluorescent but has a propensity to form intracellular aggregates when co-transfected with synphilin-1 (32). Scale bars indicate 10 μm.

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**Fig. 5.** *Hsp70 reduces Triton X-100 insoluble and HMW α-synuclein species in vitro.* A, HMW α-synuclein species ranging in molecular mass from 50 to 200 kDa could be found by native PAGE (lane 1). Hsp70 reduced both HMW α-synuclein in the Syn-T inclusion model (Syn-T, lane 2) and the 55-kDa species of untagged wild type (WT) α-synuclein that appears with native PAGE (lanes 2 and 4). Endogenous α-synuclein in mock-transfected cells also runs at 55 kDa (enhanced intensity due to low expression levels, lane 5). B, quantification of total monomeric Syn-T (34 kDa on SDS-PAGE) was also reduced by Hsp70, suggesting enhanced degradation of α-synuclein. C, α-synuclein was found in the Triton X-100 insoluble fraction (Tx insol) in the Syn-T inclusion model, whereas WT α-synuclein only appeared in the Triton X-100 soluble fraction (Tx sol). Hsp70 reduced the amount of Triton X-100 insoluble α-synuclein in the inclusion model (Syn-T). D, changes in the proportion of Triton X-100 insoluble Syn-T compared with soluble Syn-T was normalized to control (100%) and demonstrated a significant reduction in Triton X-100 insoluble fraction of α-synuclein by co-transfection with Hsp70. Co-transfection with empty vector (EV) served as control.

**Fig. 6.** *Hsp70 protects from α-synuclein toxicity in vitro.* Both α-synuclein and Syn-T significantly increase cellular toxicity by 1.75- (±0.16 S.E.) or 1.56-fold (±0.13 S.E.), respectively (n = 9, p < 0.01, one sample Student’s t test). Hsp70 significantly reduced toxicity for both wild type α-synuclein (A) and Syn-T (B). Toxicity was measured by estimating the amount adenylate kinase released by dead cells into the growth medium 24 h after transfection of H4 cells.

α-synuclein species observed in DLB. This implies that the intracellular α-synuclein inclusions in 6-month-old α-synuclein transgenic mice (line D) recapitulate some features of LBs but lack the most highly aggregated α-synuclein species. Thus, the Triton X-100 insoluble HMW species in DLB brain could embody the typical fibrillar components of aggregated α-synuclein in LBs that are not present in α-synuclein transgenic mice (16). These data are in accord with other findings that show detergent-insoluble α-synuclein species in cases of DLB and Parkinson’s disease and transgenic mice (5, 21, 51). Different protein-processing techniques have been used by these groups, which make it likely that the detergent-insoluble fraction of α-synuclein consists of a mixture of different α-synuclein species.

We also found that overexpression of Hsp70 in the α-synuclein transgenic mice protects against the development of abnormal α-synuclein aggregation. When we crossed α-synuclein transgenic mice with Hsp70-overexpressing mice (33), we observed a significant reduction in HMW and Triton X-100 insoluble α-synuclein species. This observation is the first direct evidence for an influence of Hsp70 on the aggregation process of α-synuclein in a mammalian in vivo model. Several reports support the idea that heat shock proteins, in particular Hsp70, are protective in in vivo models of neurodegenerative diseases. Overexpression of Hsp70 leads to reduced toxicity in a model of SCA1 neurodegeneration (52), ameliorates phenotypes of the spinal and bulbar muscular atrophy transgenic mouse (53), protects mice from malonate and 3-nitropipionic acid-induced toxicity (54), and has some protective effect against cerebral ischemia (55–57). Modest protective effects were seen in a mouse model of Huntington’s disease (58), whereas Hsp70 clearly suppressed polyglutamine-mediated neurodegeneration in Drosophila (59). Recent data in a fly model of α-synuclein pathology (60) suggest that overexpression of Hsp70 also protects against α-synuclein-induced degeneration in Drosophila (26, 30). Because our results demonstrate that the α-synuclein protein levels in α-synuclein transgenic mice and Hsp70-overexpressing crossed with α-synuclein transgenic mice did not differ, we conclude that Hsp70 specifically affects the detergent-insoluble species of α-synuclein.

We then evaluated the influence of Hsp70 on α-synuclein aggregate formation in a cell culture model. In in vitro models, overexpression of HSPs provides protection against aggregation or toxicity of polyglutamine aggregates (24, 27, 29, 61–66) and polyglutamine aggregates (67) and diminishes aggregation and toxicity of mutant superoxide dismutase in cultured neurons (68). Moreover, we previously reported that endogenous Hsp70 stains α-synuclein aggregates in our in vitro model of α-synuclein aggregation and that overexpression of Hsp70 or its co-chaperones prevents aggregates from forming (31). In this study, we demonstrate that the biochemical characteristics of this α-synuclein aggregation model are comparable with the findings in Drosophila brains and in α-synuclein transgenic mice in that HMW α-synuclein-positive products and Triton X-100 insoluble/SDS-soluble monomeric α-synuclein species are formed. Similar to the transgenic mouse model, overexpression of Hsp70 reduced these Triton X-100 insoluble species.

Our data support the possibility that enhanced chaperone protein function can alter α-synuclein misfolding in vivo and toxicity in vitro. Hsp70 has activity both for refolding misfolded proteins and, in collaboration with co-chaperones such as C-terminal Hsp70-interacting protein (CHIP) and parkin-associ-

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**Table:**

| Treatment   | SDS soluble α-synuclein | Triton X-100 insoluble fraction |
|-------------|-------------------------|-------------------------------|
| Control     | 100%                    | 100%                          |
| Hsp70       | 54.94% (±16.75%)        | 52.54% (±15.87%)             |

* : p<0.01
Hsp70 Reduces α-Synuclein Aggregation and Toxicity

Hsp70 acts specifically upon a toxic abnormal species of these possibilities; however, the observation that (in San Diego) for the kind gift of the Hsp70 transgenic mice.

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