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A single amino acid substitution differentiates Hsp70-dependent effects on α-synuclein degradation and toxicity

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Abstract

α-Synuclein aggregation and toxicity play a major role in Parkinson's disease and dementia with Lewy bodies. Hsp70 is a multipurpose stress response chaperone protein that mediates both refolding and degradation of misfolded proteins. We have shown that Hsp70 is able to block both α-synuclein toxicity and aggregation. Here we introduce a mutation into the ATPase domain of Hsp70 (K71S) and demonstrate that this abolishes Hsp70 refolding activity. Nonetheless, Hsp70K71S continues to mediate α-synuclein degradation and blocks aggregate formation. In contrast to wild type Hsp70, the ATPase domain mutant mediates α-synuclein degradation through a non-proteasome inhibitor sensitive pathway. Although Hsp70K71S can diminish levels of α-synuclein to an even greater extent than Hsp70, HSP70K71S does not protect against α-synuclein toxicity. The Hsp70K71S mutant appears to dissociate the formation of aggregates, which it blocks, and toxicity, which it does not block. These data suggest that the ability of Hsp70 to prevent toxicity is distinct from degradation of α-synuclein and is dependent on its ATPase domain.

Keywords: Neurodegeneration; Parkinson's disease; Dementia with Lewy bodies; Chaperone; Protein aggregation

α-Synuclein aggregation and toxicity play a crucial role in neurodegenerative diseases such as Parkinson's disease and dementia with Lewy bodies. α-Synuclein aggregation and the formation of Lewy bodies in these diseases have been observed in parallel with neurotoxicity and neuronal loss. However, it remains unclear if α-synuclein aggregation and toxicity are directly related and if the toxic α-synuclein molecules are identical to the aggregating ones. An understanding of the underlying pathways that lead to these processes will be essential for developing therapeutic strategies to prevent disease progression.

Heat shock protein 70 (Hsp70) is a critical element of the cellular response to unfolded proteins and is involved in promoting proper protein folding, as well as promoting ubiquitination and degradation of misfolded proteins. Failure in either of these mechanisms can result in the accumulation of misfolded proteins and cellular toxicity. In vivo and in vitro models have shown that Hsp70 can efficiently prevent both the aggregation and toxicity of α-synuclein [1–3].

The chaperone function of Hsp70 requires the N-terminal ATPase domain in coordination with the C-terminal substrate-binding domain (Fig. 1). Hsp70 first binds to exposed stretches of hydrophobic residues in un- or misfolded proteins, followed by refolding that is regulated by ATP-hydrolysis induced conformational changes of Hsp70. The release of refolded proteins requires the binding of a new ATP molecule [4–8]. A single amino acid substitution (K71S) in the HSC4 protein (Hsc4p) ATP-binding domain in *Drosophila melanogaster* results in loss of function in an in vitro clathrin

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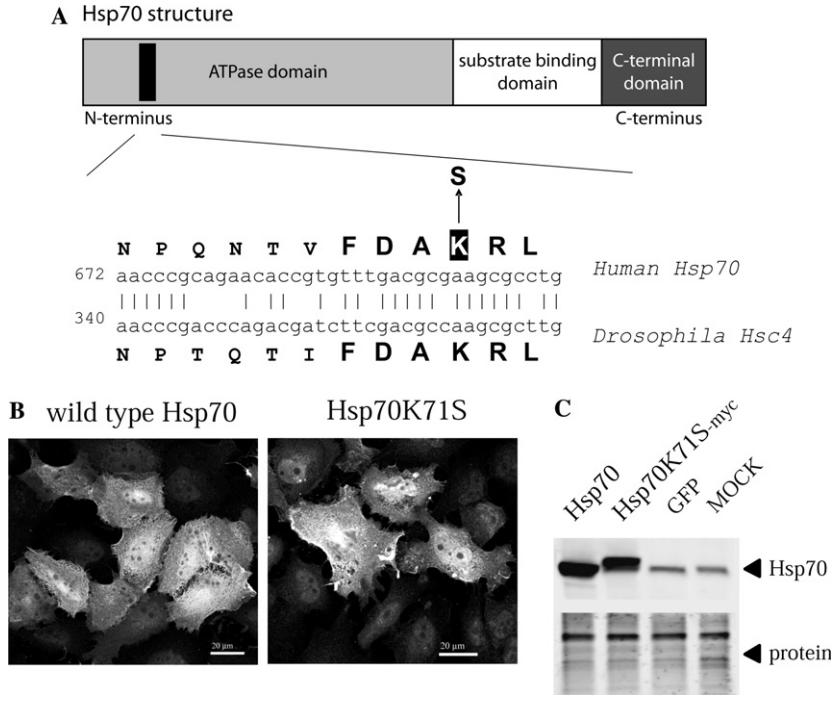

Fig. 1. (A) Schematic representation of Hsp70 with the N-terminal ATPase domain, the central substrate-binding domain, and the the C-terminal domain. Sequence alignment of the ATPase domain shows a high homology in amino acid sequence between the human Hsp70 (M11717—GenBank Accession No.) and its homolog in *Drosophila melanogaster* (NM_079632—GenBank Accession No.). The introduced mutation (K → S) is located in a completely conserved domain in the *D. melanogaster* and the human homolog of Hsp70 (bold letters). (B) Wild type Hsp70 and Hsp70K71S were recognized by immunostaining in transfected H4 neuroglioma cells. (C) Overexpression of Hsp70 and Hsp70K71S-myc was confirmed via Western blot analysis. H4 cells were transfected with Hsp70, Hsp70K71S-myc, GFP or no DNA (mock) and probed for Hsp70 (the myc-tag was used to differentiate Hsp70K71S overexpression from endogenous Hsp70. In all other experiments untagged Hsp70K71S was used). Coomassie blue staining for total protein confirms equal loading.

uncoating assay [9]. The HSC4 gene has substantial homology to human Hsp70 gene and the region around amino acid 71 is completely conserved at the protein level (Fig. 1).

Here we demonstrate that a single amino acid substitution (lysine to serine—K71S) in the ATPase domain of Hsp70 results in a loss of Hsp70 refolding activity and interferes with its ability to prevent toxicity. However, Hsp70K71S still mediates α-synuclein degradation and prevents aggregation, suggesting a dissociation between toxic and aggregating α-synuclein molecules.

Methods

Expression constructs and site-directed mutagenesis. Human Hsp70 cDNA was kindly provided by J.-C. Plumier, Massachusetts General Hospital, and subcloned into pcDNA3.1/V5-His-Topo (Invitrogen, Carlsbad, CA, USA). The K71S mutation was introduced using the Quickchange XL site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) and the construct was sequenced to verify the introduction of the mutation. For Western blot analysis Hsp70K71S was subcloned into pcDNA3.1/myc-His(−) (Invitrogen) to add a C-terminal myc-tag to the Hsp70K71S construct. 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 [2,3,10].

Luciferase refolding assay. Luciferase refolding assay was performed by heat-inactivating luciferase and measuring the re-activation of its activity after heat shock according to established protocols [8]. Briefly, H4 cells were transiently transfected with luciferase expression plasmid (pGL2-luciferase reporter vector, Promega, Madison, WI, USA) and either Hsp70 or Hsp70K71S. Twenty-four hours later cycloheximide was added to the media to block protein expression to measure only existing luciferase activity. Thirty minutes later the luciferase activity was blocked by heat shock (10 min incubation on 45 °C) followed by a recovery period at 37 °C for up to 60 min. Before and after heat shock, as well as 10 and 60 min after heat shock, cells were harvested and luciferase activity was estimated by luminometry according to the manufacturer's protocol (Luciferase Assay Kit, Promega).

Cell culture and transfection. Human H4 neuroglioma cells (HTB-148; ATCC, Manassas, VA, USA) were maintained in OPTI-MEM (Life Technologies, Grand Island, NY, USA) supplemented with 10% fetal bovine serum. H4 cells were passaged 24 h prior to transfection and plated in 4-well chamber slides for immunocytochemistry (Labtek, Nalgen-Nunc, Naperville, IL, USA) or 100 mm cell culture dishes for analysis of cell lysates (Corning, Corning, NY, USA). Cells were transfected with equimolar ratios of plasmids using Superfect (Qiagen, Chatsworth, CA, USA) according to the manufacturer's instructions. Co-transfection with an empty pcDNA3.1 vector was used as control. After 24 h cells were washed with cold PBS, harvested by scraping in cold lysis buffer without detergents (Tris/HCl 50 mM, pH 7.4, NaCl 175 mM, EDTA 5 mM, pH 8.0, and protease inhibitor cocktail (Roche, Basel, CH)) and sheared 1× through a $30\frac{1}{2}$ G needle followed by sonication for 10 s (*total cell lysates*). For inhibition experiments of proteasomal degradation ALLN (10 ng/μl; Sigma–Aldrich, St. Louis, MO, USA) was added to the cell culture 8 h prior to harvesting or DMSO as control.

Native and denaturing gel electrophoresis. Protein concentration was determined using a Lowry protein assay. Twenty to 40 μg of each cell lysate was loaded onto 4–20% or 10–20% 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 transferred to Immobilon-P membrane (Millipore, Bedford, MA, USA) and blocked in blocking buffer (Lycor, Lincoln, NE, USA) for 1 h prior to addition of primary antibody (anti-α-synuclein:Syn-1 1:1000; BD Transduction Lab, San Jose, CA, USA) at room temperature for 1–2 h or overnight at 4 °C. Following three TBS-Tween 20 washes, infrared fluorescent labeled secondary antibodies (IRDye 800 anti-rabbit or anti-mouse, Rockland Immunochemicals, Gilbertsville, PA, USA 1:3000 or Alexa-680 anti-rabbit or anti-mouse, Molecular Probes, Eugene, OR, USA 1:3000) were incubated at room temperature for 1 h and immunoblots were processed and quantified using the Odyssey infrared imaging system (Lycor, Lincoln, NE, USA). Blots were also probed for actin (anti-actin, AC40, Sigma–Aldrich), or proteins on the gels were stained with Coomassie blue (quantified using Odyssey infrared imaging system as loading controls). A mock/untransfected cell lysate was included in all cell culture experiments to control for non-specific and endogenous signal.

Immunocytochemistry. Cells were plated into 4-well chamber slides (Nunc, Naperville, IL, USA) and transfected as described above. Forty-eight hours later the cells were washed with PBS and fixed with 4% paraformaldehyde for 10 min at RT. After washing with PBS cells were permeabilized in TBS containing 0.1% Triton X-100 for 20 min at RT. After blocking in 1.5% normal goat serum containing TBS for 1 h cells were incubated with primary antibody (α-synuclein:Syn-1 1:1000, Hsp70:SPA812 1:500, Stressgen, San Diego, CA, USA; 20S proteasome subunits:PW8155 1:1000, Affinity, Mamhead, Exeter, UK) in blocking solution for 2 h at RT or overnight at 4 °C followed by washing with PBS and secondary antibody incubation for 1 h. After a final wash, slides were mounted with aqueous mounting solution (GVA, Zymed, San Francisco, CA, USA) and subjected to fluorescence microscopy using a Nikon Eclipse TE300 inverted microscope.

Toxicity assay. Toxicity was analyzed 24 h after transfection by measuring the release of adenylate kinase (AK) from damaged cells into the culture media using the ToxiLight (Cambrex, Walkersville, Maryland, USA) according to the manufacturer's protocol.

Quantification of cells containing inclusions. The number of cells containing α-synuclein immunopositive inclusions was assessed by immunocytochemistry 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 on the presence of significant α-synuclein immunostaining compared to background (which in all cases was negligible). A transfected cell containing inclusions was scored on the presence of a detectable aggregate of α-synuclein immunostaining. A cell was considered positive for inclusions independent of the size or number of inclusions. The percentage of cells containing inclusions compared to the total number of transfected cells was recorded.

Statistical analysis. Statistical analysis for comparison of groups was performed by ANOVA, with Fisher's probability of least significant differences (PLSD) post hoc test for significance.

Results

A comparison of the *Drosophila* HSC4 gene sequence and the human Hsp70 sequence reveals a high level of sequence identity. In particular, within the ATPase domain, the lysine residue at position 71 is conserved between the species (Fig. 1A). When lysine 71 is mutated to a serine residue in *Drosophila*, it results in a loss of function [9]. We hypothesized that the same amino acid substitution (K71S) in human Hsp70 would also result in a loss of function for the human protein. To test this, we introduced a single base pair substitution to produce Hsp70K71S. The resulting expression construct was sequenced to verify sequence integrity and transfected into H4 neuroglioma cells to test expression. Using antibodies that recognize Hsp70 we verified that Hsp70K71S resulted in the overexpression of a protein that could be detected with Hsp70 antibody (Figs. 1B and C—please note: Hsp70K71S-myc was used in WB analysis to confirm that Hsp70K71S overexpression could be detected and differentiated from endogenous Hsp70. In all subsequent experiments described herein, untagged Hsp70K71S was used).

To determine the effect of the K71S mutation on Hsp70 activity we employed a luciferase refolding assay. H4 cells were co-transfected with a luciferase expression construct and either empty vector, Hsp70 or Hsp70K71S. Following heat shock and a period of recovery from 10 to 60 min, cells were harvested and luciferase activity was estimated. As expected, Hsp70 enhanced luciferase refolding following heat shock in a time dependent manner. Interestingly, comparing the kinetics of refolding over time Hsp70K71S was unable to efficiently refold heat-inactivated luciferase after 10 min and even after 60 min the refolding activity was lower than that of Hsp70 and comparable to those of empty vector transfected cells, representing endogenous refolding activity (Fig. 2).

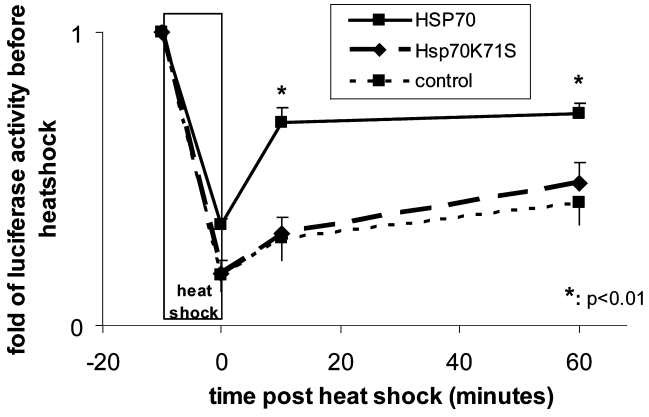

Fig. 2. Hsp70 supports refolding of heat-inactivated luciferase, whereas Hsp70K71S fails to enhance refolding. H4 cells transfected with luciferase as a reporter for protein misfolding and refolding were co-transfected with Hsp70, Hsp70K71S or empty vector (EV) as control. After 10 min heat shock (45 °C), cells were allowed to recover at 37 °C for a specified time. Co-transfection with Hsp70 substantially accelerates the kinetics of refolding of luciferase compared to control and Hsp70K71S.

We previously reported that Hsp70 reduces α-synuclein aggregation in vivo and in vitro, and also protects from α-synuclein toxicity [2,3]. We thus asked if α-synuclein aggregation is differently influenced by Hsp70K71S compared to wild type Hsp70. Overexpression of C-terminally modified α-synuclein (SynT) with synphilin-1 leads to the formation of intracellular α-synuclein positive aggregates in ~40% of transfected H4 cells 24 h after transfection [2,3,10]. Co-transfection with Hsp70 reduced the number of cells with inclusions by over 60% (from 42.54% to 15.93% ± 3.66% SD—Table 1) in accordance with our previous observations [2,3]. Surprisingly, co-transfection with Hsp70K71S leads to an equal reduction in the number of transfected cells with inclusions. Only ~10% (10.79% ± 0.39% SD) of the transfected cells had visible inclusions (Table 1). In addition, Hsp70K71S co-transfection caused a dramatic change in the morphology of the remaining α-synuclein positive inclusions (Fig. 3A). In particular, only one single or a small group of perinuclear aggregates is detected in the presence of Hsp70K71S, in contrast to the inclusions of various sizes which are the hallmark of this model.

The decrease in number and the change in morphology of inclusions led us to ask if Hsp70K71S altered clearance of α-synuclein. We found that α-synuclein inclusions immunostained for the 20S subunit of the proteasome [as do Lewy Bodies in DLB-11] suggesting proteasomal involvement in their degradation. Interestingly, although the α-synuclein inclusions contained 20S subunits, the expression pattern of 20S in the cytosol was not altered in transfected cells compared to untransfected neighboring cells. The same staining pattern for 20S was found for the inclusions still present in Hsp70 co-transfected cells, even though Hsp70 led to fewer cells with inclusions. By contrast, the remaining inclusions in cells co-transfected with Hsp70K71S were immunonegative for the 20S subunit (Fig. 3B, arrows), even though 20S co-localized with α-synuclein elsewhere in the cell.

We next asked if Hsp70K71S could also influence the appearance of high molecular weight (HMW) α-synuclein observed via Western blot and change in total α-synuclein expression levels. Native gel electrophoresis (without detergents) of lysates from H4 cells co-transfected with SynT, Synphilin 1, and either wild type Hsp70, Hsp70K71S or empty vector as control showed a dramatic reduction in HMW α-synuclein aggregates compared to control in the presence of Hsp70K71S (Fig. 4A). This reduction even exceeded the reduction

Table 1
Percentage of transfected cells with α-synuclein immunopositive inclusions

	% of cells with inclusions
Control	42.54 (±5.01)
Hsp70	15.93 (±0.366)
Hsp70K71S	10.79 (±0.39)

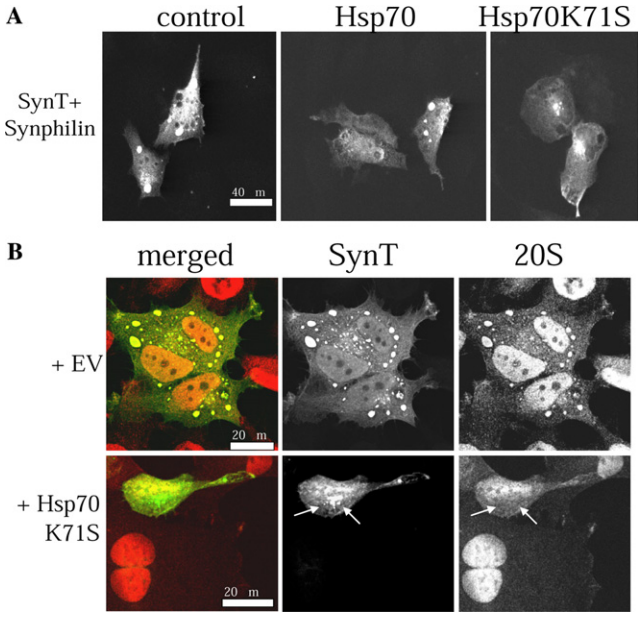

Fig. 3. H4 cells were co-transfected with Syn-T and Synphilin 1, and either with Hsp70 or Hsp70K71S. Immunostaining for α-synuclein (Syn1) revealed typical intracellular inclusions that are diminished by co-transfection with Hsp70. (A, left and middle panel) Co-transfection with Hsp70K71S leads to a unique and distinct morphological change of these inclusions, resulting in a single group of small perinuclear inclusion (A, right panel). The SynT inclusions are immunopositive for 20S proteasome subunits (B, top panel) which was found in control (EV) co-transfected cells and in the remaining inclusions in Hsp70 co-transfected cells. Intriguingly, the morphological distinct inclusions (arrows) observed in cells co-transfected with Hsp70K71S did not stain for 20S proteasome subunits, although 20S was observed to co-localize with α-synuclein expression elsewhere in the cell. Representative images from at least three reproducible experiments are shown.

seen for wild type Hsp70. Likewise, Hsp70K71S also diminished total protein levels to a greater extent than wild type Hsp70 (Fig. 4B). Degradation of α-synuclein by Hsp70 could be blocked by the proteasomal inhibitor ALLN (Fig. 5), suggesting that Hsp70 primarily degrades α-synuclein by proteasomal degradation. Intriguingly, the degradation observed for Hsp70K71S could not be blocked by proteasomal inhibition (Fig. 5) in agreement with the immunocytochemistry results for the 20S proteasome (Fig. 3B).

Hsp70 has been shown to be protective against α-synuclein toxicity, both in vivo and in vitro [2,3]. The K71S mutant form of Hsp70 has been reported not to confer protection in *D. melanogaster*. We examined the effects of Hsp70 and Hsp70K71S on α-synuclein cytotoxicity in H4 cells. For this assay we used both aggregating (Syn-T) and non-aggregating (wild type) forms of α-synuclein. Wild type Hsp70 reduced the toxicity of both wild type α-synuclein and SynT by ~20% (18.3% ± 18 SD for wild type and 19.2% ± .15 SD for SynT—$p < 0.05$—one group t test—Fig. 6). By contrast, Hsp70K71S had no effect on the toxicity of either wild type α-synuclein or Syn-T compared to control (empty

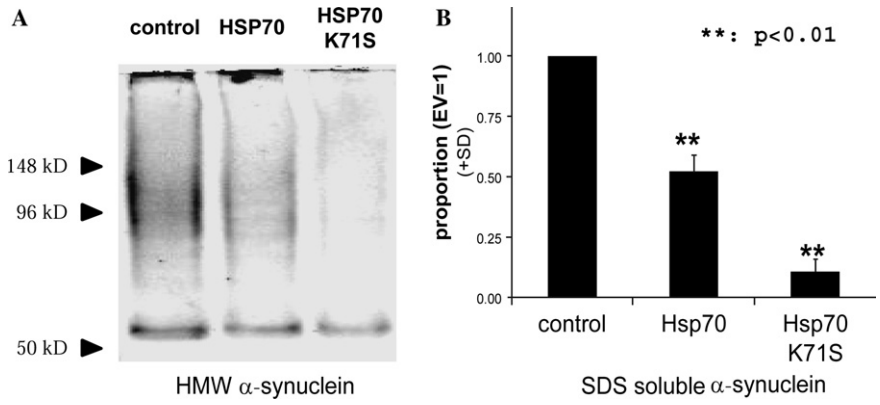

Fig. 4. (A) Hsp70 and Hsp70K71S reduce the amount of high-molecular-weight (HMW) α-synuclein (SynT) expression on native PAGE probed for α-synuclein compared to EV. (B) Quantification of SDS soluble SynT expression levels (as assessed by SDS–PAGE) showed a significant degradation of SynT by both Hsp70 and Hsp70K71S ($n = 11$ for EV and Hsp70, $n = 8$ for Hsp70K71S, one population t test, and double sided unpaired t test).

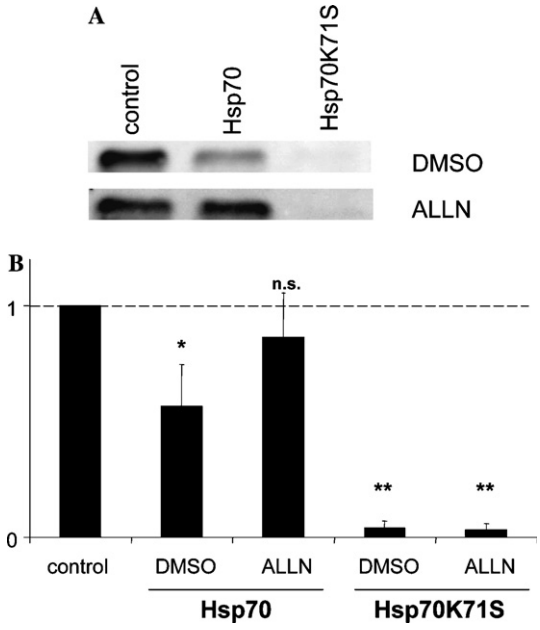

Fig. 5. ALLN treatment blocks Hsp70 mediated degradation of SynT. (A) Western blot demonstrating that Hsp70 mediated degradation of SynT can be blocked by ALLN treatment compared to control (DMSO-vehicle treated cells). (B) SynT expression levels were quantified from three experiments and normalized to protein (Coomassie blue staining). Values are expressed compared to control (empty vector co-transfection). The degradation observed with Hsp70K71S was not blocked by ALLN treatment suggesting a proteasomal *independent* degradation pathway.

vector transfected). Therefore, Hsp70K71S is unable to protect from α-synuclein induced toxicity compared to wild type Hsp70. Transfection with Hsp70K71S alone did not result in increased toxicity.

Discussion

We have previously shown that Hsp70 can protect from α-synuclein aggregation and toxicity in vivo and in vitro [2,3]. Here, we follow up on our previous observations investigating the association between α-synuclein aggregation and toxicity using a Hsp70 construct with a mutation in the ATPase domain. We provide evidence that the ATPase domain of Hsp70 is crucial for both (1) the refolding activity of Hsp70 and (2) prevention of α-synuclein dependent toxicity. By contrast, the K71S mutation in the ATPase domain of Hsp70 does not impair directing protein degradation, although the K71S mutation may instead support a novel, proteasome independent degradation pathway.

In the *D. melanogaster* homolog of Hsc70, HSC4, a single amino acid substitution (K71S) in the ATP-binding domain resulted in loss of function of HSC4 and increased lethality and protein aggregation in a dominant negative manner [9]. We introduced the corresponding mutation in mammalian Hsp70 protein (Fig. 1) and demonstrated that the K71S amino acid substitution results in a loss of Hsp70 refolding activity as measured by a luciferase refolding assay (Fig. 2). The process of refolding includes binding, refolding, and the release of the substrate protein. The ATP/ADP cycle is required for the release of the refolded protein [4,5] and the K71S mutation in the *D. melanogaster* homolog leads to a reduced release of the substrate from the Hsp70 molecule [9]. In addition, the ATP/ADP cycle can also be regulated by co-chaperones like Hip or Bag1 that competitively bind to this domain and thereby alter substrate release [6–8]. Thus, the change in refolding activity may be due to loss of the ATPase activity of Hsp70 directly or altered regulatory interaction with co-chaperones.

Hsp70 has been shown to reduce α-synuclein aggregation and toxicity in vitro [2,3]. It is thought that the ATPase dependent chaperone activity of Hsp70 is important for refolding and targeting proteins to degradation. However, our data support the hypothesis that degradation of α-synuclein is not dependent on the ATPase domain of Hsp70, because Hsp70K71S was still able to

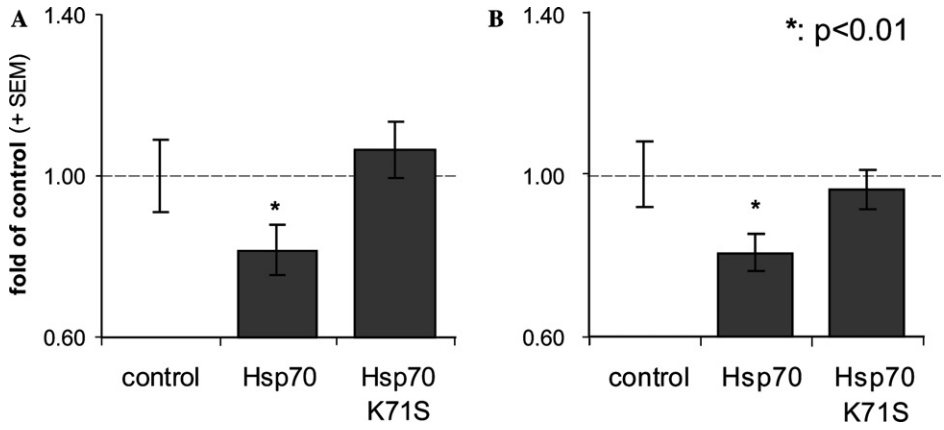

Fig. 6. Hsp70 protects but Hsp70K71S fails to protect from α-synuclein induced toxicity both for WT α-synuclein (A) and SynT (B). H4 cells were transfected with WT α-synuclein or SynT and co-transfected with Hsp70, Hsp70K71S or empty vector (EV) as control. Transfection with Hsp70K71S alone (1.43 ± 0.04 SD fold of MOCK transfection) gave comparable levels of basal toxicity compared to wild type Hsp70 (1.44 ± 0.03 SD fold of MOCK transfection). Toxicity was measured by adenylate kinase release into the conditioned media ($n = 7$ for Hsp70 and EV, $n = 4$ for Hsp70K71S co-transfection, double sided unpaired t test).

reduce α-synuclein aggregation. Nevertheless, the ATPase domain was found to be necessary for refolding activity of Hsp70 and prevention of α-synuclein toxicity. Similar results were reported in a *D. melanogaster* model for α-synuclein or polyglutamine associated degeneration where overexpression of Hsp70K71S enhanced degeneration, whereas wild type Hsp70 was protective [12]. Thus, Hsp70K71S does not protect against toxicity despite its preserved degradation ability. If Hsp70 is considered to have two independent functions—degradation and refolding—the Hsp70K71S mutant dissociates these functions. Moreover, the data indicate that degradation of α-synuclein molecules alone does not reduce toxicity, suggesting that the toxic species of α-synuclein in this model are distinct from the aggregating ones.

In H4 cells, α-synuclein inclusions robustly stain for the 20S proteasomal subunit (Fig. 3), consistent with the recent observation in LBs [11]. Even though 20S accumulated in the α-synuclein inclusions, soluble cytosolic α-synuclein did not co-stain with 20S proteasomal subunits suggesting a specific interaction of 20S proteasomal subunit with aggregated α-synuclein. These findings are in accordance with the notion that 20S binds exclusively to aggregated α-synuclein in an in vitro assay using recombinant monomeric α-synuclein and α-synuclein aggregates which resulted in an inhibition of the chymotrypsin-like activity of the 20S proteasome [11]. The authors demonstrated that this inhibitory effect could be reversed by the addition of recombinant Hsp70. In our hands, co-transfection with wild type Hsp70 reduced the number of cells with inclusions.

Of interest, Hsp70K71S also led to a dramatic change in the morphology of the remaining inclusions (Fig. 3). Only small perinuclear α-synuclein positive inclusions were detected, which were immunonegative for the 20S proteasomal subunit. Degradation of aggregated proteins is thought to be primarily via the chaperone mediated ubiquitin–proteasome system [13–15]. Our data show that wild type Hsp70 mediated α-synuclein degradation can be blocked by inhibition of the proteasomal degradation pathway (Fig. 5). Similar findings were reported by Liu et al. [16] in an in vitro assay for α-synuclein cleavage. Interestingly, the degradation observed with Hsp70K71S was not affected by proteasomal inhibition (Fig. 5) and additionally the inclusions in the Hsp70K71S transfected cells do not co-stain for 20S proteasomal subunit. Thus, we hypothesize that Hsp70K71S degrades α-synuclein aggregates by an alternative degradation pathway, uncovered by the K71S mutation.

Taken together, we show that degradation of aggregating α-synuclein species and prevention of α-synuclein induced toxicity are related but distinct pathways, and that structural components of Hsp70 are important to distinguish these pathways. The mutation in the N-terminal ATPase domain in Hsp70 might abolish the release of bound misfolded or aggregated α-synuclein molecules, thus failing to refold and detoxify them. Furthermore, we hypothesize that this complex of Hsp70 and α-synuclein might degrade α-synuclein via an alternative pathway from proteasomal degradation. This change in degradation pathways might also be structurally reflected by the presence of a C-terminally located interacting domain which binds to other co-chaperones like Hsp40 or CHIP (Fig. 1). Further studies will be required to identify these pathways and to describe the regulatory effects of Hsp70 and its co-chaperones on α-synuclein degradation and toxicity prevention.

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