Monoubiquitylation of α-Synuclein by Seven in Absentia Homolog (SIAH) Promotes Its Aggregation in Dopaminergic Cells*

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α-Synuclein plays a major role in Parkinson disease. Unraveling the mechanisms of α-synuclein aggregation is essential to understand the formation of Lewy bodies and their involvement in dopaminergic cell death. α-Synuclein is ubiquitylated in Lewy bodies, but the role of α-synuclein ubiquitylation has been mysterious. We now report that the ubiquitin-protein isopeptide ligase seven in absentia homolog (SIAH) directly interacts with and monoubiquitylates α-synuclein and promotes its aggregation in vitro and in vivo, which is toxic to cells. Mass spectrometry analysis demonstrates that SIAH monoubiquitylates α-synuclein at lysines 12, 21, and 23, which were previously shown to be ubiquitylated in Lewy bodies. SIAH ubiquitylates lysines 10, 34, 43, and 96 as well. Suppression of SIAH expression by short hairpin RNA to SIAH-1 and SIAH-2 abolished α-synuclein monoubiquitylation in dopaminergic cells, indicating that endogenous SIAH ubiquitylates α-synuclein. Moreover, SIAH co-immunoprecipitated with α-synuclein from brain extracts. Inhibition of proteasomal, lysosomal, and autophagic pathways, as well as overexpression of a ubiquitin mutant less prone to deubiquitylation, G76A, increased monoubiquitylation of α-synuclein by SIAH. Monoubiquitylation increased the aggregation of α-synuclein in vitro. At the electron microscopy level, monoubiquitylated α-synuclein promoted the formation of massive amounts of amorphous aggregates. Monoubiquitylation also increased α-synuclein aggregation in vivo as observed by increased formation of α-synuclein inclusion bodies within dopaminergic cells. These inclusions are toxic to cells, and their formation was prevented when endogenous SIAH expression was suppressed. Our data suggest that monoubiquitylation represents a possible trigger event for α-synuclein aggregation and Lewy body formation.

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3 The abbreviations used are: PD, Parkinson disease; 3-MA, 3-methyladenine; Me3SO, dimethyl sulfoxide; NH4Cl, ammonium chloride; SIAH, seven in absentia homolog; ubiquitin KO, lysine-less ubiquitin; shRNA, short hairpin RNA; HA, hemagglutinin; ATPγS, adenosine 5′-O-(thiotriphosphate); ESI-MS, electrospray ionization-mass spectrometry; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; Chaps, 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonic acid; E3, ubiquitin-protein isopeptide ligase; nano-LC-ESI-MS/MS, ESI-nano-liquid chromatography-tandem MS.

Parkinson disease (PD)3 is characterized by progressive degeneration of dopaminergic neurons in the substantia nigra and the presence of cytoplasmic inclusions called Lewy bodies in surviving neurons (1).

The majority of PD cases are sporadic, but mutations in different genes have been found to be responsible for familial PD (1, 2). α-Synuclein is believed to play a crucial role in the disease because it is mutated in some familial forms of the disease, and it is a major component of Lewy bodies in sporadic PD (3, 4). Three missense mutations in the α-synuclein gene, leading to A53T, A30P, and E46K substitutions at the protein level, have been described so far (5–7). Duplication and triplication of the α-synuclein gene were also shown to cause disease, suggesting that increase in the levels of α-synuclein can be pathogenic (8–10). In agreement, mouse models overexpressing α-synuclein revealed a correlation between α-synuclein accumulation and neuronal dysfunction (11–13). Drosophila models also recapitulate some features of the disease, such as aggregation of α-synuclein and death of dopaminergic neurons (14, 15). Nonetheless, the absence of classical Lewy bodies in some live models suggests that specific α-synuclein modifications may be involved in its efficient aggregation.

The role of α-synuclein in the formation of Lewy bodies is based on its ability to aggregate and to assemble into fibrils in vitro (16). Also, A53T and E46K mutations accelerate the α-synuclein fibrillation process (16, 17), suggesting that α-synuclein fibrillation may be implicated in the pathogenesis of the disease. Oligomerization of α-synuclein may also play a role in the disease because oligomerization, and not fibrillation, is common to all α-synuclein disease mutants (18). Understanding the mechanisms that trigger α-synuclein aggregation will provide the basis to better understand the disease and may
help determine which α-synuclein-aggregated form is responsible to promote dopaminergic cell death.

A possible dysfunction of the ubiquitin-proteasome system has been proposed to play a role in PD. The proteasome activity was found to be decreased in the substantia nigra of PD patients (19). Also, Parkin and UCH-L1, which are components of the ubiquitin-proteasome system, are mutated in familial forms of PD (20, 21). In addition, different proteins involved in PD, such as Parkin, synphilin-1, UCH-L1 and PINK1, were shown to form inclusions when they are not properly degraded by the proteasome (22–27).

Ubiquitylation of α-synuclein has potentially important implications for its normal and pathological functions. α-Synuclein purified from Lewy bodies was found to be monoubiquitylated (28–30). Parkin polyubiquitylates a rare glycosylated form of α-synuclein (31), whereas UCH-L1 was shown to polyubiquitylate α-synuclein in vitro (32). Recently, α-synuclein was shown to be monoubiquitylated by an unidentified E3 ubiquitin-ligase in HEK293 cells (33). However, the role of α-synuclein ubiquitylation has been mysterious.

Here we investigate the identity of the endogenous E3 ubiquitin-ligase for α-synuclein monoubiquitylation and its role in modulating α-synuclein aggregation. We present evidence that SIAH interacts with and monoubiquitylates α-synuclein in vivo. Also, we found in vivo and in vitro evidence that monoubiquitylated α-synuclein is more prone to aggregation. Our data indicate that monoubiquitylation of α-synuclein by SIAH plays an important role in α-synuclein aggregation and may contribute to the formation of Lewy bodies.

**EXPERIMENTAL PROCEDURES**

**Materials**—Ubiquitin aldehyde, purified ubiquitin-activating enzyme, UbcH5b, lactacystin, and 3-MA were purchased from Sigma.

**Site-directed Mutagenesis**—Full-length α-synuclein constructs mutated at lysine residues identified by mass spectrometry were generated by PCR using primers that contained arginine codons instead of lysine codons. Mutations of relevant lysines were confirmed by double strand sequencing.

**Cell Culture and Transfections**—SH-SY5Y cells were grown in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum in a 5% CO₂ atmosphere. Cells were transiently transfected with N-terminally tagged pRK5 and pFLAG-CMV-2 plasmids utilizing Lipofectamine 2000 (Invitrogen) and processed after 36 h. For experiments using shRNA, SH-SY5Y cells were transfected with shRNA sequences cloned into pSuper vector (34) using Lipofectamine 2000 and processed after 72 h. The shRNA sequences to suppress SIAH-1 and SIAH-2 expression, as well as to control shRNA, were described elsewhere (35).

**Western Blot Analysis**—Samples were homogenized as described (22). Blots were probed with antibodies mouse anti-HA (Covance), mouse anti-Myc (Oncogene), mouse anti-α-synuclein (BD Biosciences), rabbit anti-FLAG, rabbit anti-α-synuclein (Sigma), rabbit anti-Myc, rabbit anti-HA, goat anti-SIAH-1, goat anti-SIAH-2, goat anti-SIAH-1/2, and mouse anti-actin (Santa Cruz Biotechnology).

**Endogenous Co-immunoprecipitation Assays**—Rat brains were homogenized in buffer containing 50 mM Tris (pH 7.4), 140 mM NaCl, 1% Chaps, 30 μM MG132, and protease inhibitor mixture (Complete, Roche Diagnostics). Brain homogenates were clarified by centrifugation at 13,000 × g for 5 min. Antibodies to SIAH-1/2 or SIAH-1 (H-18 and N-15, respectively) (Santa Cruz Biotechnology) were coupled to protein G beads (22) and incubated for 7 h with brain homogenate (2 mg/ml). Immunoprecipitates were washed with lysis buffer containing 500 mM NaCl and detected by Western blot using mouse anti-α-synuclein (BD Biosciences).

**In Vitro Ubiquitylation Assays**—For the in vitro ubiquitylation assays, His-α-synuclein was purified from bacteria using TALON beads according to the manufacturer’s instructions (BD Biosciences). Recombinant α-synuclein was incubated in reaction medium containing 40 mM Tris (pH7.6), 5 mM MgCl₂, 2 mM dithiothreitol, 1 mM ATPγS, 7.5 μg of ubiquitin, 1 μM ubiquitin aldehyde, 100 ng of ubiquitin-activating enzyme, and 200 ng of UbcH5b, in the presence or absence of 500 ng of SIAH-2. The samples were incubated at 37 °C for 1 h, and ubiquitylated α-synuclein was determined by Western blot using anti-α-synuclein antibody.

**In Vitro Aggregation Assays**—The in vitro aggregation experiments were carried out after the in vitro ubiquitylation assays. To stop the in vitro ubiquitylation of α-synuclein, the samples were incubated at 65 °C for 10 min. To promote α-synuclein aggregation, the samples were further incubated at 65 °C for different time points as described previously (36). α-Synuclein aggregation was determined by Western blot analysis using anti-α-synuclein antibody.

**Transmission Electron Microscopy**—In vitro ubiquitylated and aggregated samples were added over 400-mesh carbon-coated copper grids and counterstained with 1% uranyl acetate. Samples were analyzed and photographed with JEOL JEM 100 SX electron microscope (Tokyo, Japan).

**Mass Spectrometry**—Fifty combined reactions of in vitro His-α-synuclein ubiquitylation assays were carried out as above in a volume of 300 μl. His-α-synuclein was then pulled down with TALON beads in buffer containing 30 mM Tris (pH 7.6), 500 mM NaCl, 2 mM imidazole, 6 μm urea, and 1% Triton X-100. The beads were extensively washed in pulldown buffer supplemented with 20 mM imidazole. Samples were run on 11% SDS-PAGE and stained with Coomassie Brilliant Blue, and protein bands were excised and destained using multiple washings with 50% acetonitrile in 50 mM ammonium bicarbonate. The protein bands were subsequently reduced, alkylated, and in-gel digested with bovine trypsin (sequencing grade, Roche Diagnostics) at a concentration of 12.5 ng/μl in 50 mM ammonium bicarbonate at 37 °C as described (37). An extracted peptide solution was dried for subsequent matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) and electrospray ionization (ESI) mass spectrometric analyses (MS). Aliquots of the extracted peptide mixture, dissolved in 0.1% trifluoroacetic acid or a mixture of formic acid/isopropanol alcohol/H₂O (1:3:2 v/v/v), were used for MALDI-MS using either fast evaporation (38, 39) or dry droplet (40) methods. α-Cyano-4-hydroxycin-
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namic acid and/or 2,5-dihydroxybenzoic acid were used as matrices for analysis. Sample purification and preparation for ESI-MS were performed as described previously (41). Micro-columns were prepared with Poros R2 reverse phase material (Perseptive Biosystem, Framingham, MA). Peptides were eluted with 60% methanol, 5% formic acid directly into a nano-electrospray capillary. Peptides were subjected to nano-ESI-MS/MS analysis and nano-LC-ESI-MS/MS (nano-liquid chromatography-ESI-MS/MS).

**Peptide Mass Mapping by MALDI-TOF/MS, Nano-ESI-MS/MS, and Nano-LC-MS/MS**—Mass spectrometric analysis was performed on a Bruker Reflex III™ MALDI-TOF mass spectrometer (Bruker, Bremen, Germany), equipped with a delayed extraction ion source, a reflector, and a 337 nm nitrogen laser, and on API QSTAR Pulsar® electrospray-quadrupole TOF tandem mass spectrometer (MDS-Sciex) equipped with a nano-electrospray source (MDS Proteomics, Odense, Denmark). Nano-LC-ESI-MS/MS for protein identification was carried out with a nano-liquid chromatography system incorporating UltiMate™ Capillary/Nano LC System, consisting of Famos™ Micro Autosampler, Switchos™ Micro Column Switching Module (LC Packings, Dionex) on line with API Q-STAR Pulser® electrospray-quadrupole TOF. A C18 nanocolumn inner diameter 75 μm, length 15 mm, particle size 5 μm (LC Packings, Dionex) was used. The flow rate through the column was 150 nl/min. A methanol/acetonitrile gradient was employed with a mobile phase containing 0.1 and 2% formic acid in buffers A and B, respectively. The gradient used was 5–50% acetonitrile over 45 min. The injection volume was 5 μl. In the nano-electrospray ionization source, the end of the capillary from the nano-LC column was connected to the emitter with pico-tip silica tubing, inner diameter 20 μm (New Objective) by stainless steel union, with a PEEK sleeve for coupling the nanospray with the on-line nano-LC. The voltage applied to the union to produce an electrospray was 2 kV, and the cone voltage was 3 V. Argon was introduced as a collision gas at a pressure of 1 p.s.i. The identity of the peptides and the exact ubiquitylation sites were concluded from the detected collision-induced dissociation products manually and by Mascot software (Matrix Science) and confirmed by manual inspection of the fragmentation series. For determination of sites of ubiquitylation, a modification of 114 Da was specified for lysine residues.

**In Vivo Ubiquitylation Assays**—Transfected SH-SY5Y cells were incubated in the absence or in the presence of 10 μM lactacystin, 10 mM NH₄Cl, and/or 10 mM 3-MA for 12 h. The transfected cells were then resuspended in buffer containing 50 mM Tris (pH 7.4), 140 mM NaCl, 1% SDS, boiled for 5 min, and then diluted 10-fold with buffer containing 50 mM Tris (pH 7.4), 140 mM NaCl, 1% Triton X-100, 30 μM MG132, 10 mM NH₄Cl, 10 mM 3-MA, and protease inhibitor mixture (Complete, Roche Diagnostics). Cell extracts were sonicated and then incubated for 16 h with anti-HA beads (Roche Diagnostics). Immunoprecipitates were washed with lysis buffer containing 500 mM NaCl and detected by Western blot.

**Immunocytochemistry Assays**—Transfected SH-SY5Y cells were incubated with or without 10 μM lactacystin, 10 mM NH₄Cl, and/or 10 mM 3-MA for 12 h, fixed with 4% paraformaldehyde for 15 min, and blocked in phosphate-buffered saline containing 0.2% Triton X-100 and 5% normal goat serum. Cells were labeled with anti-HA (Covance) and anti-Myc antibodies (Santa Cruz Biotechnology) as described (22). Immunolabeling was detected using fluorescein isothiocyanate- and Cy3-labeled secondary antibodies (The Jackson Laboratories). The percent of cells containing cytosolic inclusions was counted by an investigator unaware of the treatment groups. Statistics of the number of inclusion-containing cells was analyzed by Student’s t test or analysis of variance followed by Tukey’s post-hoc test.

**Cell Death Analysis**—Transfected SH-SY5Y cells were transfected and processed for immunocytochemistry as described above. Before mounting, nuclei were stained with Hoechst 33342. Cell death was determined by counting the number of α-synuclein/SIAH-transfected cells that were positive for nuclear condensation and fragmentation. Statistics of cell death were done by Student’s t test.

**RESULTS**

**SIAH Monoubiquitylates α-Synuclein in Vitro**—Preliminary evidence indicates that SIAH-2 is able to monoubiquitylate α-synuclein in vitro (22). We then carried out additional in vitro ubiquitylation experiments using solely purified recombinant proteins to ascertain whether α-synuclein is indeed monoubiquitylated by SIAH and not by contaminants from the in vitro translation reaction employed previously (22). We found that both recombinant SIAH-1 and SIAH-2 directly monoubiquitylate recombinant α-synuclein in the presence of purified components of the ubiquitin system (Fig. 1A).

We next sought to characterize the monoubiquitylation by SIAH and its effect on α-synuclein. Although both SIAH isoforms monoubiquitylate α-synuclein, the more robust effect of SIAH-2 prompted us to extend the characterization of α-synuclein monoubiquitylation using SIAH-2. To confirm the monoubiquitylation nature of α-synuclein post-translation modification, we carried out additional in vitro assays using methylated ubiquitin or lysine-less ubiquitin (ubiquitin K0), which do not allow polyubiquitylation to occur (Fig. 1B). Accordingly, α-synuclein was monoubiquitylated by methylated ubiquitin and ubiquitin K0 as well (Fig. 1B). Using these modified ubiquitin molecules, we observed a slight difference in the migration of monoubiquitylated α-synuclein, which may be related to differences in the arrangement of ubiquitin molecules conjugated to α-synuclein. α-Synuclein was not ubiquitylated by other E3 ubiquitin-ligases, such as Parkin, XIAP, and E6AP (Fig. 1C), indicating that the effect of SIAH is specific.

To identify the sites of ubiquitylation of α-synuclein by SIAH-2, we analyzed the ubiquitin-conjugated bands of our in vitro α-synuclein ubiquitylation reactions by mass spectrometry. After ubiquitylation, His-α-synuclein was purified by cobalt affinity column and subjected to SDS-PAGE (Fig. 1D). Ubiquitylated α-synuclein bands were cut, digested by trypsin, and analyzed by MALDI-TOF/MS, ESI-MS/MS, and LC-ESI-MS/MS. Ubiquitylation was identified by an increase in peptide mass of 114 Da, corresponding to diglycine-modified lysines upon trypsic cleavage. We observed diglycine-modified lysines at the positions 12, 21, and 23 (Table 1). These lysines were recently found to be ubiquitylated in Lewy bodies (30). Addi-
tionally, we identified lysines 10, 34, 43, and 96 to be ubiquitylated by SIAH as well (Table 1).

In an attempt to determine whether the absence of these identified lysines would prevent the ubiquitylation of α-synuclein by SIAH, we generated an α-synuclein construct where the lysines identified by mass spectrometry were mutated to arginines. Mutation of lysine 96 alone caused a change in the ubiquitylation pattern of α-synuclein, in which the upper ubiquitylated band almost disappeared (Fig. 2). However, mutations in all lysines modified by SIAH failed to abolish ubiquitylation, indicating that vicinal lysines are ubiquitylated when the preferred ones are mutated to arginine. Thus, when we tested a construct mutated in ½ of α-synuclein lysines (6, 10, 12, 21, 23, 32, 34, 43, 45 and 96), monoubiquitylation was not abolished and even exhibited an additional band at about 35 kDa (Fig. 2). This probably reflects the promiscuous ubiquitylation observed in several proteins in which mutation of the preferred lysines leads to ubiquitylation of adjacent lysine residues (42, 43).

α-Synuclein Interacts with and Is Monoubiquitylated in Vivo by SIAH—Because α-synuclein has been shown to be degraded by different proteolytic pathways (44–47), we first compared the effect of different proteolytic inhibitors on the steady-state levels of α-synuclein. For this, SH-SY5Y cells were transfected with full-length HA-α-synuclein and treated with 10 μM lactacystin, 10 mM ammonium chloride, or 10 mM 3-MA to inhibit the proteasomal, lysosomal, and autophagic pathways, respectively. We found that each inhibitor increased the steady-state levels of α-synuclein (Fig. 3A). When combined, the inhibitors promoted an additive increase in the steady-state levels of α-synuclein (Fig. 3A). Taken together, the data suggest that the three pathways, proteasomal, lysosomal, and autophagic, contribute to the degradation of α-synuclein to some extent. However, because 3-MA was the most effective in increasing the steady-state levels of α-synuclein (Fig. 3A), it is possible that the autophagic pathway may be the predominant pathway for α-synuclein degradation.

We next investigated if SIAH monoubiquitylates α-synuclein in vivo. For this, we carried out in vivo ubiquitylation assays in which SH-SY5Y cells were co-transfected with full-length HA-α-synuclein, myc-SIAH-2, and FLAG-ubiquitin. To increase the steady-state levels of α-synuclein, the cells were treated with a combination of 10 μM lactacystin, 10 mM ammonium chloride, and 10 mM 3-MA. α-Synuclein was then immunoprecipitated with an anti-HA antibody and its ubiquitylation determined by Western blot analysis. We found that SIAH-2 significantly increased α-synuclein monoubiquitylation compared with the control in the absence of SIAH-2 (Fig. 3B). This indicates that SIAH-2 monoubiquitylates α-synuclein in vivo. In agreement with the in vitro ubiquitylation assays, we found that SIAH-1 also ubiquitylates α-synuclein, but to a lesser extent (data not shown).

We also examined the ability of SIAH proteins to interact with α-synuclein by carrying out co-immunoprecipitation experiments using rat brain tissue. We found that α-synuclein

| TABLE 1 |

Ubiquitylated peptides of α-synuclein

Ubiquitylation was detected by an increase in 114 Da, corresponding to a diglycine-modified lysine (GlyGly(K)) generated after trypsin digestion. Modified lysines are in boldface and correspond to lysines 10, 12, 21, 23, 34, 43, and 96. Peptides were obtained by tryptic digestion of α-synuclein ubiquitylated as in Fig. 1D and detected by peptide fingerprinting by MALDI-TOF/MS, ESI-MS/MS, and LC-ESI-MS/MS.

| Position in the sequence | Sequence | \( M_\text{r} \) calculated | \( M_\text{r} \) measured |
|--------------------------|----------|--------------------------|--------------------------|
| 7–12                     | K.GLSK^16AK.E + GlyGly(K) | 716.28 | 716.32 |
| 11–23                    | K.AK^2EGVVA>EAK^17TK.O + 2GlyGly(K) | 1528.74 | 1528.68 |
| 13–32                    | K.EGVVAAAEK^20TK^23QGVEAAAAGK.E + 2GlyGly(K) | 2141.04 | 2140.9 |
| 33–45                    | K.TK^456GVIYVGSK^6K.T.E + 2 GlyGly(K) | 1522.78 | 1522.84 |
| 81–97                    | K.TVEGAGSIAAAATGFVK^33K.D + GlyGly(K) | 1719.86 | 1719.92 |

* Peptide fingerprinting was detected by MALDI-TOF/MS and LC-ESI-MS/MS.
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coop-precipitated with SIAH-1 and SIAH-2 (Fig. 3C). The co-immunoprecipitation of α-synuclein with SIAH proteins is specific because α-synuclein did not co-immunoprecipitate with control beads under the same experimental conditions (Fig. 3C). In addition, we found that α-synuclein also co-immunoprecipitates with SIAH-1, immunoprecipitated with an antibody specific to SIAH-1, and not with an unrelated protein (dynactin subunit, p150*Glued) (Fig. 3D).

We next investigated if endogenous SIAH monoubiquitylates α-synuclein. To suppress the expression of SIAH proteins, we used shRNAs to SIAH-1 and SIAH-2 that were previously characterized and shown to specifically suppress SIAH-1 and SIAH-2 expression (35). SH-SY5Y cells were co-transfected with HA-α-synuclein with control shRNA or shRNAs to SIAH-1 and -2. α-Synuclein was immunoprecipitated with an anti-HA antibody, and α-synuclein monoubiquitylation was determined by Western blot analysis. We found that suppression of SIAH proteins expression using shRNAs to SIAH-1 and -2 abolished α-synuclein monoubiquitylation (Fig. 4A, upper panel). This indicates that endogenous SIAH monoubiquitylates α-synuclein. We confirmed the suppression of endogenous SIAH-1 and -2 by Western blot analysis using anti-SIAH-1 and -2 antibodies (Fig. 4A, lower panels).

To ascertain that the modification in α-synuclein we observed in transfected cells was indeed ubiquitylation, we carried out in vivo ubiquitylation experiments where we immunoprecipitated FLAG-tagged ubiquitylated proteins from co-transfected cells using anti-FLAG antibody. By carrying out Western blot analysis of FLAG-immunoprecipitated proteins using anti-synuclein antibody, we found monoubiquitylated α-synuclein among the pool of ubiquitylated proteins (Fig. 4B). Thus, monoubiquitylation of α-synuclein revealed by FLAG immunoprecipitation was abolished when endogenous SIAH expression was suppressed by shRNA and significantly increased when SIAH-2 was overexpressed (Fig. 4B). In addition, in the presence of large excess of ubiquitin K0 (which prevents polyubiquitylation), SIAH-2 promoted the same pattern of monoubiquitylation to that obtained in cells transfected with wild-type ubiquitin (Fig. 5A), corroborating the finding that SIAH monoubiquitylates α-synuclein.

Deubiquitinases and Different Proteolytic Pathways Modulate α-Synuclein Monoubiquitylation—Deubiquitinases have been shown to regulate the levels of different monoubiquitylated proteins (48, 49). Thus, we raised the possibility that α-synuclein monoubiquitylation could be modulated by deubiquitinases as well. To test this possibility, we employed the ubiquitin G76A mutant to ubiquitylate α-synuclein, instead of the wild-type ubiquitin. The ubiquitin G76A mutant was shown to be less disassembled by deubiquitinases (50). Consequently, when the ubiquitin G76A mutant was used instead of the wild-type ubiquitin, we observed a significant increase in α-synuclein monoubiquitylation in SH-SY5Y-transfected cells (Fig. 5A), consistent with the role deubiquitinases modulating α-synuclein monoubiquitylation.

We next determined the contribution of the proteasomal, lysosomal, and autophagic pathways in affecting monoubiquitylated α-synuclein levels using the ubiquitin G76A mutant. For this, co-transfected SH-SY5Y cells were treated with 10 μM lactacystin, 10 mM ammonium chloride, or 10 mM 3-MA. We found that all inhibitors...
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FIGURE 4. Endogenous SIAH monoubiquitylates α-synuclein. A, SH-SYSY cells were transfected with HA-α-synuclein, FLAG-ubiquitin, in the absence or presence of shRNA to SIAH-1 and SIAH-2. Cells were incubated 12 h with 10 μM lactacystin, 10 mM NH₄Cl, and 10 mM 3-MA, HA-α-synuclein was immunoprecipitated (IP) with an anti-HA antibody, and monoubiquitylated α-synuclein was detected by Western blot using an anti-α-synuclein antibody. The lower panel shows the suppression of SIAH-1 and -2 expressions by the shRNAs using anti-SIAH-1 and -2 antibodies. B, monoubiquitylated α-synuclein is among the pool of ubiquitylated proteins. SH-SYSY cells were transfected with HA-α-synuclein, FLAG-ubiquitin, in the absence or presence of either myc-SIAH-2 or shRNAs to SIAH-1 and SIAH-2. Cells were incubated 12 h with 10 μM lactacystin, 10 mM NH₄Cl, and 10 mM 3-MA. FLAG-ubiquitylated proteins were immunoprecipitated with an anti-FLAG antibody, and monoubiquitylated α-synuclein was detected by Western blot using an antibody to α-synuclein. The figure panels are representative of three independent experiments.

FIGURE 5. Deubiquitinases and different proteolytic pathways modulate the amount of monoubiquitylated α-synuclein. A, SH-SYSY cells were transfected with HA-α-synuclein, FLAG-ubiquitin, in the presence of FLAG-ubiquitin,FLAG-ubiquitin K0, or FLAG-ubiquitin G76A mutant. Cells were incubated 12 h with 10 μM lactacystin, 10 mM NH₄Cl, and 10 mM 3-MA. HA-α-synuclein was immunoprecipitated (IP) with an anti-HA antibody and ubiquitylated α-synuclein was detected by Western blot using an anti-α-synuclein antibody. The lower panel shows the levels of SIAH-2 by Western blot using an anti-Myc antibody. The figures are representative of three independent experiments. WT, wild type.

Increased the levels of monoubiquitylated α-synuclein to some extent (Fig. 5B). 3-MA was the most effective in increasing α-synuclein monoubiquitylation levels, followed by lactacystin and then by ammonium chloride. As observed with the nonubiquitylated α-synuclein (Fig. 3A), the three inhibitors together promoted the largest increase in the levels of monoubiquitylation α-synuclein (Fig. 5B). The levels of α-synuclein monoubiquitylation did not depend exclusively on the levels of SIAH-2 because the use of all inhibitors together was not more efficient in increasing the steady-state levels of SIAH-2 than lactacystin alone (Fig. 5B, bottom panel). Together, the data indicate that different degradation pathways need to be simultaneously inhibited to maximize α-synuclein monoubiquitylation steady-state levels.

Analysis of α-synuclein steady-state levels and its degradation rate in pulse-chase experiments in the absence of proteolytic inhibitors provided no evidence for degradation of α-synuclein by SIAH-2 (data not shown). Thus, monoubiquitylation per se does not change the α-synuclein degradation rate, indicating that nonubiquitylated and monoubiquitylated α-synucleins are similarly degraded by the proteasomal, lysosomal, and autophagic pathways. This finding is compatible with the role in protein function and sorting attributed to monoubiquitylation, rather than accelerating protein degradation (51).

Monoubiquitylation by SIAH Increases α-Synuclein Aggregation in Vitro—It is recognized that accumulation of aggregated species of α-synuclein is implicated in the pathology of PD (4). However, the role of ubiquitylation in α-synuclein aggregation has not been investigated previously. We therefore sought to determine whether monoubiquitylation of α-synuclein could modulate its aggregation. For this, we first carried out in vitro aggregation experiments where α-synuclein was first monoubiquitylated in vitro by SIAH-2 and subsequently assayed for aggregation. We used an α-synuclein aggregation paradigm where α-synuclein was incubated for a few hours at 65 °C to induce aggregation (36). This aggregation paradigm did not allow the continuation of the ubiquitylation reaction because monoubiquitylation of α-synuclein was terminated after the reaction was incubated a few minutes at 65 °C (data not shown). α-Synuclein aggregation was determined by Western blot based on the presence of high molecular weight aggregates. Heating for 65 °C elicits the formation of nonubiquitylated α-synuclein dimer (Fig. 6A, asterisk). We found that monoubiquitylated α-synuclein aggregation was higher than in nonubiquitylated α-synuclein (Fig. 6A). The heat treatment promoted the formation of higher levels of high molecular weight aggregates of monoubiquitylated α-synuclein, indicating increased aggregation when compared with nonubiquitylated α-synuclein (Fig. 6A, compare lanes 3 and 4). The effect of monoubiquitylation on α-synuclein aggregation is not because of α-synuclein polyubiquitylation, because we obtained a similar increase in α-synuclein aggregation when monoubiquitylation was carried out with methylated ubiquitin, which prevents the formation of polyubiquitin chains (Fig. 6A,
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FIGURE 6. Monoubiquitylation of α-synuclein by SIAH increases its aggregation in vitro. A, His-α-synuclein was incubated with UbcH5b and the indicated components of the ubiquitin (Ubiq) system, in the absence or in the presence of recombinant SIAH-2. His-α-synuclein was then incubated at 65 °C for additional 10 h to promote its aggregation. His-α-synuclein ubiquitylation and aggregation were determined by Western blot using an antibody to α-synuclein. Asterisk indicates the position of α-synuclein dimer. B, His-α-synuclein was incubated with UbcH5b and indicated components of the ubiquitin system, in the absence or in the presence of recombinant SIAH-2. His-α-synuclein was incubated at 65 °C for 2 h to allow its aggregation and then separated into soluble (Sol) and insoluble (Insol) fractions by 16,000 × g centrifugation. The levels of His-α-synuclein monoubiquitylation and aggregation were determined by Western blot using an anti-α-synuclein antibody. The figures are representative of four independent experiments. C, electron micrographs showed increased α-synuclein aggregation by ubiquitylation. His-α-synuclein was incubated with recombinant SIAH-2, components of the ubiquitin system, in the absence (left panel) or in the presence of UbcH5b (middle and right panels). Reaction mixtures were then incubated at 65 °C for additional 16 h to promote α-synuclein aggregation. His-α-synuclein aggregation was determined by transmission electron microscopy analysis. Middle and right panels show the aggregation of α-synuclein monoubiquitylated at 30 and 10%, using 0.1 and 0.3 μg of SIAH, respectively. Magnification was 15,000 times. The figures are representative of three independent experiments. D, titration of α-synuclein monoubiquitylation by SIAH-2. His-α-synuclein was incubated with increasing amounts of SIAH-2, UbcH5b, and purified components of the ubiquitin system. Levels of His-α-synuclein ubiquitylation were determined by Western blot using an anti-α-synuclein antibody. E, densitometric quantification of the α-synuclein monoubiquitylation levels shown in D. Error bars represent standard error of four independent experiments. Highlighted condition in D and E represents 10% of monoubiquitylated α-synuclein. MetUbiq, methylated ubiquitin; E1, ubiquitin-activating enzyme.

To confirm that the high molecular weight species we observed with monoubiquitylated α-synuclein represent aggregated α-synuclein, we further separated the extracts into soluble and insoluble fractions. As expected, α-synuclein high molecular weight aggregates were higher in the presence of SIAH-2 and were mainly present in the insoluble protein fraction (Fig. 6B, compare lanes 2 and 4).

To further investigate the effect of monoubiquitylation on the aggregation of α-synuclein, we carried out electron microscopy experiments. For this, α-synuclein was first monoubiquitylated in vitro by SIAH-2, followed by incubation at 65 °C to induce aggregation as described in Fig. 6A, and then analyzed by transmission electron microscopy. We found a robust increase in the aggregation of monoubiquitylated α-synuclein relative to nonubiquitylated α-synuclein control (Fig. 6C, compare left and middle panels). Interestingly, we also observed that the aggregation of monoubiquitylated α-synuclein differs from that observed with nonubiquitylated α-synuclein. Although under our experimental conditions nonubiquitylated α-synuclein aggregated in the form of fibrils (Fig. 6C, left panel), monoubiquitylated α-synuclein promoted massive amounts of amorphous aggregates (Fig. 6C, middle panel).

Because monoubiquitylation of α-synuclein was found to represent only 10% of aggregated α-synuclein from α-synucleinopathy brains (28), we carried out electron microscopy experiments using conditions where only 10% of α-synuclein was monoubiquitylated by SIAH. To obtain 10% monoubiquitylation, we first titrated SIAH levels in our in vitro monoubiquitylation experiments (Fig. 6, D and E). Thus, a reduction of SIAH used in the ubiquitylation assays to 0.1 μg leads to monoubiquitylation of about 10% of the α-synuclein (Fig. 6E). We observed that even when only 10% of α-synuclein molecules are monoubiquitylated, there is a robust increase in α-synuclein aggregation, which appeared in the form of amorphous aggregates (Fig. 6C, right panel). Such increase in aggregation was caused by monoubiquitylation, as it only occurred when all the components of the ubiquitylation system were present.
Monoubiquitylation by SIAH Increases α-Synuclein Aggregation in Vivo—We next sought to determine whether monoubiquitylation of α-synuclein by SIAH-2 increases the formation of α-synuclein inclusions in vivo. Human dopaminergic SH-SY5Y cells were transfected with HA-α-synuclein and myc-SIAH-2. Transfected cells were treated with Me2SO or α-synuclein, lactacystin, ammonium chloride, and 3-MA, and the amount of α-synuclein inclusion bodies was determined by immunocytochemistry. We found that co-expression of α-synuclein with SIAH-2 in the presence of the proteolytic inhibitors significantly increased the formation of α-synuclein inclusions (Fig. 7, A and C), indicating that monoubiquitylation of α-synuclein indeed increases its ability to aggregate. The α-synuclein inclusions formed in the presence of SIAH-2 were observed in the soma and also adjacent to the membrane (Fig. 7A, bottom panels, arrows). We also observed formation of some cytosolic SIAH inclusions that are devoid of α-synuclein (Fig. 7A, bottom panels, arrowhead). This is compatible with the fact that SIAH can form inclusion bodies with other substrates, such as synphilin-1 (22).

We also investigated if endogenous SIAH can promote the aggregation of α-synuclein. For this, SH-SY5Y cells were transfected with α-synuclein, in the absence or presence of shRNAs to SIAH-1 and SIAH-2. Transfected cells were treated with lactacystin, ammonium chloride, and 3-MA to increase the levels of α-synuclein monoubiquitylation, and the amount of α-synuclein inclusion bodies was determined by immunocytochemistry. We found that suppression of SIAH expression by shRNAs inhibited the formation of α-synuclein inclusions (Fig. 7B and C). Moreover, when endogenous SIAH-1 and -2 expressions were suppressed by shRNAs, the addition of proteasome, lysosome, and autophagy inhibitors did not promote any increase in the formation of α-synuclein inclusions (Fig. 7C). This suggests that monoubiquitylation by SIAH, rather than a nonspecific effect of the inhibitors, is important for the formation of α-synuclein inclusions. Additional immunocytochemical experiments revealed that SIAH-2 increased the formation of α-synuclein inclusions even in the absence of further treatment (Fig. 7C), indicating that an increase in SIAH-2 steady-state levels also promotes α-synuclein inclusion formation.

The effects of proteasome, lysosome, and autophagy inhibitors on the α-synuclein inclusion formation, with and without myc-SIAH-2, were additive, because less inclusions were formed when each compound was used alone (Fig. 7C and data not shown). The data are in agreement with the additive effect we observed on the monoubiquitylation of α-synuclein in the presence of all three inhibitors (Fig. 5B).

To further confirm that monoubiquitylation of α-synuclein increases its aggregation, we generated a construct that encodes for an artificially monoubiquitylated α-synuclein, in which lysine-less ubiquitin was fused to the N terminus of α-synuclein (Fig. 8A). This strategy has been previously shown to faithfully mimic monoubiquitylation of several proteins (42, 49, 52, 53). To prevent the cleavage of the ubiquitin part by deubiquitinases, we mutated the last glycine residue of ubiquitin to valine. SH-SY5Y cells were transfected with either HA-α-synuclein or HA-ubiquitin-α-synuclein and, in the absence of any further treatment, inclusion formation was determined by immunocytochemistry using anti-HA antibody. We found that the ubiquitin-α-synuclein fusion protein formed significantly more inclusions when compared with α-synuclein alone (Fig. 8, B and C). Although this construct may not exactly correspond to the type of monoubiquitylation promoted by SIAH, it confirms that ubiquitylation is able to increase α-synuclein aggregation.

Characterization of α-Synuclein Inclusions Formed by SIAH—Lewy bodies were suggested to have amyloid properties because they were found to be partially stained with thioflavin S (36). We sought to determine whether the α-synuclein inclusions driven by SIAH display amyloid properties. For this, we carried out immunocytochemistry for α-synuclein and thioflavin S. We found that approximately one-third of α-synuclein inclusions were thioflavin S-positive (Fig. 9A), indicating that monoubiquitylated α-synuclein inclusions have amyloid-like features.
We also investigated whether α-synuclein inclusions can recruit other proteins present in Lewy bodies. We transfected SH-SY5Y cells with α-synuclein and SIAH-2, in the presence of ubiquitin, synphilin-1 or UCH-L1. We found the presence of ubiquitin and UCH-L1 in α-synuclein inclusions (Fig. 9B, upper and bottom panels). Synphilin-1 was recruited to α-synuclein inclusions as well (Fig. 9B, middle panels).

**Effect of Monoubiquitylation on α-Synuclein Disease Mutants—**
We next sought to investigate the effect of the disease mutations on α-synuclein monoubiquitylation and aggregation. We first carried out in vitro ubiquitylation assays where we incubated recombinant α-synuclein (wild-type, A53T, A30P, or E46K) with SIAH-2 and purified components of the ubiquitin system. We found no difference in the monoubiquitylation levels among the different α-synuclein disease mutants and wild-type protein in vitro (Fig. 10A). We also carried out in vivo ubiquitylation experiments. For this, SH-SY5Y cells were transfected with HA-α-synuclein (wild type, A53T, A30P, or E46K) and myc-SIAH-2, and monoubiquitylation of immunoprecipitated α-synuclein was determined by Western blot analysis. We found that SIAH-2 equally monoubiquitylated wild-type and mutant α-synuclein constructs (data not shown).

To investigate the effect of monoubiquitylation on the ability of α-synuclein disease mutants to aggregate, SH-SY5Y cells were transfected with HA-α-synuclein mutants and myc-SIAH-2, and monoubiquitylation of immunoprecipitated α-synuclein was determined by Western blot analysis. We found that SIAH-2 equally monoubiquitylated wild-type and mutant α-synuclein constructs (data not shown).
Monoubiquitylation of α-synuclein A53T mutant increases its tendency to form inclusions in human dopaminergic cells. A, His-α-synuclein mutants (wild-type (WT), A53T, A30P, or E46K) were incubated with recombinant SIAH-2, UbCH5b, ubiquitin K0, and the indicated components of the ubiquitin system. The levels of His-α-synuclein ubiquitylation were determined by Western blot using an antibody to α-synuclein (α-Syn). B, SH-SYSY cells were transfected with HA-α-synuclein (wild-type or A53T mutant) and incubated with 10 μM lactacystin, 10 mM NH₄Cl, and 10 mM 3-MA for 12 h. Immunocytochemistry was carried out using anti-HA (red) and anti-Myc (green) antibodies. Nuclei were stained with TOPRO-3. Scale bar, 25 μm. C, quantification of the percent of inclusion body formation in SH-SYSY cells transfected with HA-α-synuclein (wild-type, A53T, or A30P mutant) and incubated with Me₂SO as vehicle or 10 μM lactacystin, 10 mM NH₄Cl, and 10 mM 3-MA for 12 h. Error bars represent standard error of 3–4 independent experiments. **, significantly different from Me₂SO vehicle control at p < 0.01. α, significantly different from the values of wild-type at p < 0.05.

Next, we addressed the effect of monoubiquitylation to α-synuclein toxicity. For this, we co-transfected SH-SYSY cells with HA-α-synuclein and myc-SIAH-2, and we treated the cells with lactacystin, ammonium chloride, and 3-MA, which greatly increase the amount of monoubiquitylated α-synuclein and inclusion formation (Figs. 5B and 7A). We found that cells containing α-synuclein inclusions exhibited more cell death than those devoid of inclusions, suggesting that monoubiquitylated α-synuclein inclusions are toxic to cells (Fig. 11).

**DISCUSSION**

Mutations of the α-synuclein gene in familial PD and its presence in Lewy bodies of sporadic PD implicate α-synuclein as a central player in the formation of Lewy bodies and in the disease (4). In this study, we show that α-synuclein interacts in vivo with and is monoubiquitylated by endogenous SIAH. We also found that monoubiquitylation promotes α-synuclein aggregation into inclusion bodies, linking α-synuclein monoubiquitylation to Lewy body formation. In addition, we observed that monoubiquitylated α-synuclein inclusions are toxic to dopaminergic cells. Our results shed light on the mechanisms that regulate α-synuclein aggregation and toxicity, with implications to the pathogenesis of the disease.

Lewy bodies are known to be heavily ubiquitylated, and ~10% of α-synuclein purified from Lewy bodies was found to be monoubiquitylated (28–30, 54). Ubiquitylation was only observed in the aggregated forms present in Lewy bodies, being absent from soluble cytosolic α-synuclein (30). However, the role of ubiquitylation and Lewy body formation has been unclear. A key question in the field is whether the ubiquitylation process per se plays a role in α-synuclein aggregation in PD or is merely a secondary event caused by the accumulation of aggregated proteins that are eventually ubiquitylated over time. We now report for the first time that monoubiquitylation of α-synuclein is important to promote its aggregation, and we propose that monoubiquitylation of α-synuclein might represent a primary event for the formation of Lewy bodies.

Supporting an important role of α-synuclein monoubiquitylation for Lewy body formation is the finding that monoubiquitylation by SIAH robustly increased the in vitro aggregation of α-synuclein, as observed by Western blot and electron microscopy assays. Yet, when only 10% of α-synuclein was monoubiquitylated, we still observed a significant increase in its aggregation, suggesting that even small amounts of monoubiquitylation are enough to trigger α-synuclein aggregation, and this may be important for Lewy body formation.

In addition to increased aggregation, we found that monoubiquitylated α-synuclein promotes the formation of amorphous aggregates rather than fibrils (55). A similar amorphous aggregation of α-synuclein has been shown previously to be elicited by polyamines and low pH (56, 57). Moreover, Lewy bodies are known to contain amorphous aggregated proteins in their core (58, 59), implying that monoubiquitylated α-synuclein could perhaps work as a seed for the formation of inclusions in α-synucleinopathies.

We found that α-synuclein is monoubiquitylated by SIAH at lysines 10, 12, 21, 23, 34, 43, and 96 (Table 1), which include lysines known to be ubiquitylated in Lewy bodies (lysines 12, 21, and 23) (30). When ⅔ of α-synuclein lysines were mutated to arginines, including the ones we identified, monoubiquitylation was not abolished (Fig. 2). This indicates that adjacent lysines can become ubiquitylated when the preferred ones are mutated. This phenomenon has been reported previously for other ubiquitylated proteins (42, 43) and reflects the promiscuous nature of protein ubiquitylation.

There is no consensus on the identity of the lysines monoubiquitylated in aggregated α-synuclein from α-synucleinopathy brains. Anderson and co-workers (30) identified by mass spectrometry that α-synuclein purified from Lewy bodies of diffuse Lewy body disease brains is
ubiquitylated by lysines 12, 21, and 23. These lysines are ubiquitylated by SIAH (Table 1). On the other hand, Hasegawa et al. (28) identified the α-synuclein peptide-(6–13) (containing the lysine 10 we now identified) to be ubiquitylated in insoluble fractions of multiple system atrophy brains but did not determine which lysines were modified. Both studies processed α-synuclein by anion-exchange chromatography, and one included immunopurification with anti-phospho-α-synuclein antibodies (30). Such an approach might miss ubiquitylation of nonphosphorylated α-synuclein, and it is not possible to exclude the ubiquitylation of additional lysines in Lewy bodies. Therefore, the identification of α-synuclein lysines 10, 34, 43, and 96 that are also ubiquitylated by SIAH provide new information on additional possible sites of α-synuclein ubiquitylation.

Although polyubiquitylation is associated with protein degradation by the proteasome (60), monoubiquitylation has been shown to regulate different cellular functions, such as sorting of proteins in the late endosomal pathway, histone function, and DNA repair (51, 61). We showed in this study that α-synuclein, either monoubiquitylated by SIAH or directly fused to ubiquitin, aggregates within cells. The reason for the increase in α-synuclein aggregation by the addition of a few ubiquitin molecules is not clear. The hydrophobic region of α-synuclein is essential for its aggregation and filament assembly (62). Ubiquitin has a hydrophobic surface encompassed by its five-strand β-sheet (63, 64). This hydrophobic surface patch of ubiquitin interacts with hydrophobic patches of several ubiquitin-binding proteins and enzymes of the ubiquitin-proteasome pathway (63, 64). Thus, it is possible that addition of a few ubiquitin chains to α-synuclein renders α-synuclein more prone to aggregate by increasing the number of hydrophobic regions in the protein. This monoubiquitylated α-synuclein could serve as a seed for interacting with additional α-synuclein molecules and formation of aggregates. Our finding may also apply to other neurodegenerative diseases, because they are also characterized by the presence of inclusion bodies that are positive for ubiquitin (65). Our data imply that the accumulation of monoubiquitylated proteins may be a widespread mechanism involved in the formation of inclusions in different neurodegenerative diseases.

Compatible with the nondegradative role of monoubiquitylation, we found no evidence for the degradation of α-synuclein by SIAH. Thus, we raise the possibility that both the nonubiquitylated and the monoubiquitylated α-synuclein are degraded to similar extents by the proteosomal, lysosomal, and autophagic activities.

α-Synuclein was shown to be polyubiquitylated by Parkin and UCH-L1 (31, 32). However, the finding that α-synuclein purified from Lewy bodies is monoubiquitylated rather than polyubiquitylated suggests that other E3 ubiquitin ligases (s), such as SIAH, may be responsible for the ubiquitylation of α-synuclein. We have shown that SIAH is present in Lewy bodies of PD patients (22), suggesting that it may participate in Lewy body formation. Our present findings showing that SIAH co-immunoprecipitates with α-synuclein and that α-synuclein monoubiquitylation and aggregation are prevented by shRNA to SIAH suggest that SIAH may be the physiological E3 ubiquitin ligase of α-synuclein. Moreover, SIAH also interacts with synphilin-1, a protein that binds to both α-synuclein and Parkin, and is also present in Lewy bodies (22, 26, 27, 66–70).

It has been shown that the levels of monoubiquitylated proteins can be regulated by deubiquitinases (49). We found that the levels of α-synuclein monoubiquitylation are significantly increased when, instead of wild-type ubiquitin, α-synuclein is monoubiquitylated by a ubiquitin molecule that is less sensitive to deubiquitinases (G76A) (50). This suggests that a still unidentified deubiquitinase can reduce the levels of α-synuclein monoubiquitylation. Identification of the endogenous enzyme that deubiquitylates α-synuclein will clarify the mechanisms that regulate the levels of monoubiquitylated α-synuclein.

We also showed that the α-synuclein A53T mutant had an increased tendency to form inclusions when monoubiquitylated by SIAH, suggesting that monoubiquitylation could decrease the threshold for the α-synuclein A53T mutant to aggregate. Because multiplication of the α-synuclein gene was also shown to lead to disease (8–10), it is conceivable that increased steady-state levels of α-synuclein could promote a net increase in the amount of monoubiquitylated α-synuclein, leading to its aggregation. Therefore, we raise the possibility that the levels of monoubiquitylated α-synuclein may be relevant not only to sporadic disease but also to some forms of familial PD.

A variety of cell and animal models of different neurodegenerative diseases has been shown to develop inclusion bodies that inversely correlate with cell death (65). In PD, we and others have shown that synphilin-1 and synphilin-1A inclusion bodies can be cytoprotective (22, 68, 71–73). In this study, however, we found that inclusions formed by monoubiquitylated α-synuclein are not protective to cells but rather increase cell death. These findings are in accordance with recent data showing that inclusions of aggregated α-synuclein formed in Dro sophila are neurotoxic (74). In this framework, it is possible that α-synuclein inclusions may be toxic to cells and that co-aggregation of additional PD-related proteins, such as synphilin-1, may decrease this toxicity. Thus, although our present results do not support a protective role for monoubiquitylated α-synuclein in Lewy bodies, we still cannot predict how formation of Lewy bodies affect dopaminergic neuron viability in PD patients because of its complex protein composition.

In summary, we propose that the monoubiquitylation of α-synuclein by SIAH is important to promote its aggregation into inclusion bodies. It is conceivable that dysregulation of different proteolytic pathways may lead to the accumulation of monoubiquitylated α-synuclein and, as a consequence, accumulation of α-synuclein aggregates. Our findings shed light on the mechanisms involved in α-synuclein aggregation, with implications for Lewy body formation and possibly for dopaminergic cell survival.

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