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Aggregated and Monomeric α-Synuclein Bind to the S6′ Proteasomal Protein and Inhibit Proteasomal Function*

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The accumulation of aggregated α-synuclein is thought to contribute to the pathophysiology of Parkinson's disease, but the mechanism of toxicity is poorly understood. Recent studies suggest that aggregated proteins cause toxicity by inhibiting the ubiquitin-dependent proteasomal system. In the present study, we explore how α-synuclein interacts with the proteasome. The proteasome exists as a 26 S and a 20 S species. The 26 S proteasome is composed of the 19 S cap and the 20 S core. Aggregated α-synuclein strongly inhibited the function of the 26 S proteasome. The IC_{50} of aggregated α-synuclein for ubiquitin-independent 26 S proteasomal activity was 1 nM. Aggregated α-synuclein also inhibited 26 S ubiquitin-dependent proteasomal activity at a dose of 500 nM. In contrast, the IC_{50} of aggregated α-synuclein for 20 S proteasomal activity was > 1 μM. This suggests that aggregated α-synuclein selectively interacts with the 19 S cap. Monomeric α-synuclein also inhibited proteasomal activity but with lower affinity and less potency. Recombinant monomeric α-synuclein inhibited the activity of the 20 S proteasomal core with an IC_{50} > 10 μM, exhibited no inhibition of 26 S ubiquitin-dependent proteasomal activity at doses up to 5 μM, and exhibited only partial inhibition (50%) of the 26 S ubiquitin-independent proteasomal activity at doses up to 10 mM. Binding studies demonstrate that both aggregated and monomeric α-synuclein selectively bind to the proteasomal protein S6′, a subunit of the 19 S cap. These studies suggest that proteasomal inhibition by aggregated α-synuclein could be mediated by interaction with S6′.

A multisubunit complex, termed the proteasome, manages protein turnover in the body. Proteins can be either degraded directly by the proteasome, or they can be tagged with an 8-K_D protein, termed ubiquitin. Three different forms of the proteasome exist in a cell: the 20 S ubiquitin-independent proteasome, the 26 S ubiquitin-independent proteasome, and the 26 S ubiquitin-dependent proteasome. The 20 S particle forms the core of each form of proteasome (1, 2). Both the 26 S ubiquitin-dependent and -independent proteasomes contain the 20 S particle plus an additional smaller cap, which has a sedimentation coefficient of 19 S. Although smaller than the 20 S particle, the 19 S particle is also a multisubunit structure. The protein subunits that compose the 19 and 20 S particles are all known. The 19 S cap contains at least 18 different subunits, whereas the 20 S particle contains 28 subunits (1, 2). The protein S6′ (also known as tat binding protein 1 and Rpt5) is in the 19 S cap and is of particular interest because it was recently shown to directly bind ubiquitinated proteins, which suggests that it is required for ubiquitin-dependent proteasomal function (3).

Although the 26 S proteasome is responsible in both ubiquitin-dependent and -independent protein degradation (4, 5), the 20 S proteasome functions only in ubiquitin-independent protein degradation and is involved in 70–80% of the selective recognition and degradation of mildly oxidized proteins in the cytosol (4, 5). The 26 S proteasomal ubiquitin-dependent pathway degrades all ubiquitinated proteins within the cell and is the primary degradation pathway of the cell. The E1 ubiquitin-activating enzyme forms a thiodiester bond with mono-ubiquitin. The E2 ubiquitin-conjugating enzyme displaces the E1 enzyme and allows for conjugation of multiple ubiquitin moieties with one another. The E3 ubiquitin ligase enzyme binds both to the substrate targeted for degradation and to the E2 enzyme. The E2 and E3 enzymes are displaced, leaving a multichained ubiquitinated substrate protein that is targeted to the 26 S proteasome. This is both an ATP- and ubiquitin-dependent pathway (6–13).

Recent studies suggest that protein aggregates cause toxicity by inhibiting proteasomal function. Extended polyglutamine repeats, such as occur in mutant forms of huntingtin associated with Huntington's disease, aggregate readily (14–16). Polyglutamine aggregates inhibit ubiquitin-dependent proteasomal function (17). Aggregates of other proteins, such as the cystic fibrosis transmembrane receptor, also inhibit ubiquitin-dependent proteasomal function in cell culture (17). Many other proteins with hydrophobic domains also aggregate, and overexpressing the aggregation-prone domains of these proteins is toxic (18). The mechanism of toxicity for most aggregates is unknown.

Blockade of proteasome activity is toxic to many cell types and appears to be potentially important to many neurodegenerative diseases. Proteasomal inhibition causes apoptosis in many cell lines and is being tested as a potential chemotherapy (19). Although proteasomal inhibition causes rapid toxicity in cell culture, the slow accumulation of protein aggregates in neurodegenerative diseases might produce a correspondingly slow inhibition of the proteasome.

α-Synuclein is the major component of Lewy bodies, which are intracellular inclusions that form in Parkinson's disease

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(PD)[1] (20, 21). The association of α-synuclein with Lewy bodies suggests that protein aggregation represents an important aspect of the pathophysiology of α-synuclein and of PD. The link between α-synuclein and protein aggregation has been strengthened by the discovery of mutant forms of α-synuclein, A53T and A30P, that are associated with rare cases of familial PD (22, 23). Both mutations accelerate aggregation of α-synuclein (24–27). The link between α-synuclein and aggregation suggests that understanding the mechanism of toxicity induced by protein aggregates could provide important insights into the mechanism of cell death in PD.

Native α-synuclein has been shown to bind both fatty acids and many different proteins, including phospholipase D, G proteins, synphilin-1, protein kinase C, 14–3-3 protein, parkin, and the dopamine transporter (28–34). In addition, rat α-synuclein has been shown to bind to rat S6′ (35). Of these proteins, only synphilin-1 and parkin have been identified in Lewy bodies (33, 36, 37). Perhaps because of the pleiotropic binding properties of native α-synuclein, overexpressing it in cells produces multiple cellular effects. α-Synuclein inhibits protein kinase C activity, phospholipase D activity, and the activity of the dopamine transporter, and α-synuclein has chaperone activity (28, 29, 34, 38). Overexpressing α-synuclein also inhibits proteasomal function (39). The link between α-synuclein and the proteasome is intriguing but is not directly related to the pathophysiology of Parkinson's disease, because overexpressed α-synuclein retains a native structure until the cell is subjected to a stress, such as incubation with rotenone or ferrous chloride (40–44). Thus, whether aggregated α-synuclein inhibits proteasomal function is unknown, and the mechanism by which it might inhibit the proteasome is also unknown.

In this study we examine the interaction of α-synuclein with the three different types of proteasome and demonstrate that aggregated α-synuclein binds to S6′ and inhibits ubiquitin-dependent proteasomal function.

MATERIALS AND METHODS

Cell Lines, Transfections, Chemicals, and Antibodies—The human cell line HEK 293 and the human neuroblastoma cell line BE-M17 were grown in OPTIMEM (Cell Grow) plus 10% fetal bovine serum supplemented with 200 μg/ml G418 (Sigma), as needed. G418 was used for selection. Transfections utilized FuGENE at a 3:1 ratio to DNA, 4 μg per 10-cm dish. Recombinant α-synuclein was generated using wild-type α-synuclein inserted into a ProEX-His_6 plasmid (Invitrogen) as described previously (41). Antibodies used include monoclonal anti-α-synuclein (1:1000 IB, Transduction Labs); polyclonal anti-S6′ (1:1000, Affiniti); monoclonal anti-S6′ (1:1000, Affiniti); polyclonal anti-PA700 (1:1000, Affiniti); monoclonal anti-10b (1:1000, Affiniti); polyclonal anti-α subunit 20 S (1:1000, Affiniti); and polyclonal anti-α-synuclein (against amino acids 116–131, 1:1000).

Pull-down Assay—Brain samples were precleared with nickel-agarose for one hour at 4 °C to eliminate proteins that directly bind to nickel-agarose (Invitrogen). These samples were incubated overnight with 5 μg of recombinant α-synuclein (His-tagged), either aggregated or monomeric. Samples were incubated with nickel-agarose for one hour to allow binding of the His-tagged α-synuclein (monomeric or aggregated), and then they were centrifuged at 1000 rpm for 1 min. Samples were washed three times with immunoprecipitation buffer (50 mM Tris-HCl, 10 mM EGTA, 100 mM NaCl, 0.5% Triton-X, 1 mM dithiothreitol, 1 mM protease inhibitor mixture (Sigma), pH 7.4) and run on 8–16% SDS gradient polyacrylamide gels (BioWhitaker).

Immunoprecipitations—Protein concentration was determined using BCA protein assay (Pierce), and 500 μg of each sample was used per immunoprecipitation in immunoprecipitation buffer (50 mM Tris-HCl, 10 mM EGTA, 100 mM NaCl, 0.5% Triton-X, 1 mM dithiothreitol, 1 mM proteasome inhibitor mixture (Sigma), pH7.4). Samples were precleared using protein G-Sepharose beads (Seize X, Pierce) for 1 h at 4 °C and incubated with antibody overnight at 4 °C while rocking. Samples were washed three times with immunoprecipitation buffer, resuspended in 2× dithiothreitol protein loading buffer, boiled for 5 min at 90 °C, and run on 8–16% SDS gradient polyacrylamide gels (BioWhitaker).

Aggregation of α-Synuclein—Recombinant α-synuclein incubated for 2 months at 37 °C in phosphate-buffered saline while shaking at 800 rpm; aggregation was confirmed by performing immunoblot analysis.

Immunoblot Analysis—Transfers to polyvinylidene difluoride (Bio-Rad) were done overnight at 4 °C at 0.1 A/gel in transfer buffer. The immunoblot was blocked in 0.2% I-block (Tropix) in Tris-buffered saline with 0.1% Tween 20 for one hour at room temperature while shaking. We then incubated blots overnight at 4 °C in primary antibody at appropriate concentration in 5% bovine serum albumin in Tris-buffered saline/0.1% Triton X-100. Blots were washed three times, 10 min each, and incubated three hours in secondary antibody (1:5000, Jackson Laboratories) in I-block at room temperature. Blots were washed three times and developed using a chemiluminescent reaction (PerkinElmer Life Sciences).

Sucrose Gradients—10–30% linear sucrose gradients were prepared using Hoefer SG 15 gradient maker (Amersham Biosciences) following the manufacturer's recommendations. Approximately 10 mg of monomeric or aggregated α-synuclein, as determined by BCA protein assay (Pierce), was added to the top of the gradient; they were centrifuged using a SW41 rotor for 16 ½ hours at 40,000 rpm at 20 °C. After centrifugation, 0.5-ml fractions were collected; 20 μl of each fraction was run on an 8–16% gradient gel, and immunoblotted.

In Vitro 20 S Ubiquitin-independent Chymotryptic Proteasomal Activity Assay—We incubated aggregated or monomeric α-synuclein at various concentrations with purified 20 S proteasome (human erythrocytes, BioMol) for 30 min and then added a fluorogenic substrate (Suc-LLVY-AMC, BioMol). Ten minutes later, the samples were analyzed with a GeminiXS SpectraMax fluorescent spectrophotometer (Amersham Biosciences) using an excitation wavelength of 360 nm and an emission wavelength of 460 nm.

In Vitro 20/26 S Ubiquitin-independent Chymotryptic Proteasomal Activity Assay—Aggregated or monomeric α-synuclein at various concentrations was incubated with 250 μg of HEK 293 cell lysates, as determined by BCA protein assay (Pierce) in assay buffer (10 mM Tris-HCl, pH 7.8, 0.5 mM dithiothreitol, 5 mM $MgCl_2$, and 5 mM ATP) for 30 or 60 min at 37 °C while shaking at 800 rpm. We then added a fluorogenic substrate (Suc-LLVY-AMC, BioMol) and incubated samples an additional 30 min at 37 °C while shaking at 800 rpm. Solutions were analyzed using an excitation wavelength of 360 nm and an emission wavelength of 460 nm with the GeminiXS SpectraMax spectrophotometer (Amersham Biosciences).

In Vitro 26 S Ubiquitin-dependent Proteasomal Activity Assay—Substrates were generated with an *in vitro* transcription and translation of substrate proteins using a T7 promoter in *Escherichia coli* lysate (Promega), supplemented with [^{35}S]methionine, and then partially purified by high-speed centrifugation and ammonium sulfate precipitation as described (45). The protease substrate for ClP assays was derived from barnase, which is a ribonuclease from *Bacillus amyloliquefaciens*; the protease substrate for proteasomal assays was derived from *E. coli* dihydrofolate reductase (DHFR) (45, 46). A ubiquitin moiety was added to the N terminus of the substrate proteins via a 4-amino acid linker from the *E. coli* lac repressor (45). Substrate proteins were constructed in pGEM-3Zf (+) vectors (Promega) and were verified by sequencing. The reaction was resuspended in 40 μl of buffer (25% (v/v) glycerol, 25 mM $MgCl_2$, 0.25 mM Tris/HCl, pH7.4) to which 5 μl of the *in vitro* reaction containing the radiolabeled ubiquitinated substrate protein was added with 35 μl of rabbit reticulocyte lysate (Green Hectares, containing 1 mM dithiothreitol) that is ATP-depleted as described (45). We incubated the reactions with and without monomeric or aggregated α-synuclein. Concentration of α-synuclein was determined by BCA protein assay (Pierce). We incubated at 37 °C for 7 min to allow initial cleavage of substrate proteins. Ubiquitination and degradation was initiated by the addition of ATP and an ATP-regenerating system (0.5 mM ATP, 10 mM creatine phosphate, 0.1 mg/ml creatine phosphokinase, final concentrations). Reactions were incubated at 37 °C. At designated time points (15, 30, 45, 60, 90, 120, 150, and 180 min), small aliquots were removed and transferred to ice-cold 5% trichloroacetic acid. The trichloroacetic acid-insoluble fractions were analyzed by 10% SDS-PAGE and quantified by electronic autoradiography.

Statistics—All statistics were performed using a multifactorial analysis of variance analysis using the Statview statistical package.

[1] The abbreviations used are: PD, Parkinson's disease; DHFR, dihydrofolate reductase; DHFR-U, dihidrofolate reductase with a degradation tag; UPS, ubiquitin proteasomal system.

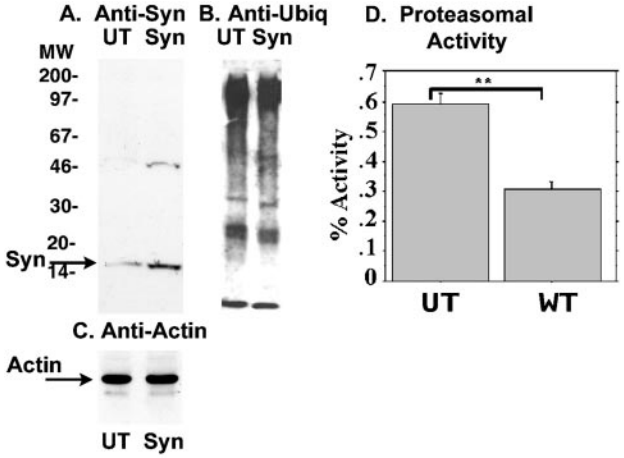

FIG. 1. **Effects of α-synuclein overexpression on the proteasomal system.** *A* and *B*, BE-M17 neuroblastoma cells were transfected with vector or wild-type α-synuclein and immunoblotted with antibodies to α-synuclein (*A*), ubiquitin (*B*), and actin (*C*). No differences in levels of ubiquitin-conjugated proteins were observed among cells transfected with vector or wild-type α-synuclein. This is a representative immunoblot from experiments that have been repeated at least three times. *D*, activity of the ubiquitin-independent proteasomal system in cell lines expressing wild-type α-synuclein compared with untransfected cells (**, $p < 0.0005$). These data represent the combined data from five experiments each containing 5 data points for each sample.

RESULTS

Overexpressing α-Synuclein Inhibits Proteasomal Degradation—To begin analyzing how α-synuclein might interact with the proteasome, wild-type α-synuclein was stably expressed in human neuroblastoma BE-M17 cells by transient transfection, and ubiquitin-dependent and -independent proteasomal activity was quantified. Because α-synuclein does not form aggregates spontaneously under these conditions, this experiment addresses whether increased concentration of cellular α-synuclein inhibits proteasomal activity. Immunoblotting of the cellular lysates demonstrated a significant increase in the α-synuclein levels in the transfected cells (Fig. 1*A*). Next, we investigated whether overexpressing α-synuclein affects the steady state levels of ubiquitin-conjugated proteins, which provides a measure of the ubiquitin-dependent proteasomal system. The BE-M17 cells expressing vector or wild-type α-synuclein were immunoblotted with anti-ubiquitin antibody. The amount of ubiquitin-conjugated proteins did not differ among the groups of transfected cells (Fig. 1, *B* and *C*).

We also investigated how overexpressing α-synuclein affects ubiquitin-independent proteasomal degradation. Previous studies report that cell lines overexpressing α-synuclein exhibit lower ubiquitin-independent proteasomal activity (39). To investigate ubiquitin-independent proteasomal activity, we measured hydrolysis rates of fluorogenic peptide analogues in cells transiently or stably overexpressing α-synuclein (Fig. 1*D*). No difference in activity was observed in cells transiently transfected with α-synuclein (data not shown). However, cell lines stably expressing wild-type α-synuclein showed an approximately 50% reduction in ubiquitin-independent proteasomal degradation, depending on the transgene (39) (Fig. 1*D*). These data suggest that α-synuclein does affect ubiquitin-independent proteasomal function.

α-Synuclein Inhibits the 20 S Proteasome—An increasing number of studies suggest that the state of α-synuclein aggregation plays a key role in the pathophysiology of PD. To better understand how α-synuclein affects the proteasome, we generated recombinant monomeric α-synuclein and aggregated α-synuclein. The aggregated α-synuclein was generated by aging recombinant α-synuclein at 37 °C for 2 months. Aggregation of α-synuclein was verified by immunoblot analysis (Fig. 2*A*). The aggregated protein ran as a smear with an average molecular weight of ~160,000 (Fig. 2*A*). The immunoblot of the aged, aggregated α-synuclein also exhibited some reactivity at 16,000. This could reflect either that the sample had some non-aggregated, monomeric α-synuclein remaining or that some of the α-synuclein could be dissociated from the aggregate by SDS. To examine this question, we fractionated monomeric or aged α-synuclein (37 °C for 2 months) by centrifugation in a sucrose gradient and immunoblotted each of the 25 fractions with anti-synuclein antibody. The initial sample used before the fractionation is shown in the first lane (Fig. 2*B*, labeled *In*). Monomeric α-synuclein had a low density and was most abundant in fractions 1–6 (Fig. 2*B*, *top*). In contrast, the aggregated α-synuclein sample showed nothing in the early fractions (corresponding to a low density) and migrated exclusively in the last fraction, suggesting a high density (Fig. 2*B*, *fraction 25*, *bottom*). Immunoblots of fraction 25 for the aggregated sample showed a small amount of SDS-sensitive α-synuclein that migrated as a monomer following exposure to SDS during the immunoblotting. This SDS-dissociable α-synuclein was particularly evident following longer exposures (Fig. 2*B*, *fraction 25B*, *bottom*). This suggests that aggregated α-synuclein contains SDS-sensitive and SDS-resistant aggregated protein.

Next, we examined the activity of purified 20 S proteasome particles in the presence of varying amounts of monomeric or aggregated α-synuclein using synthetic fluorescent peptides to monitor proteasomal activity. Increasing doses of monomeric α-synuclein progressively inhibited proteasomal activity (Fig. 3*A*). The IC_{50} for inhibition of the proteasome by monomeric α-synuclein was ~16 μM, assuming α-synuclein could achieve complete inhibition. Aggregated α-synuclein also inhibited the 20 S ubiquitin-independent proteasomal activity, exhibiting a maximal inhibition similar to that of monomeric α-synuclein (Fig. 3*B*).

To determine whether the inhibition was at the level of the proteasome or due to binding of the peptide substrate, we examined whether varying the level of substrate affected the α-synuclein-dependent proteasomal inhibition. Inhibition of the 20 S proteasome by monomeric α-synuclein increased with increasing substrate concentration (Fig. 3*C*). Increased proteasomal inhibition by α-synuclein might occur because larger effects are possible at higher rates of substrate degradation. These data indicate that the proteasomal inhibition that was observed did not result from substrate binding and substrate sequestration by α-synuclein. Thus, α-synuclein appears to inhibit the proteasomal activity via an interaction with the proteasome, rather than by binding substrate peptide.

Aggregated α-Synuclein Inhibits the 26 S Proteasome—Next, we examined the effects of monomeric and aggregated α-synuclein on a mixture of the 20 and 26 S proteasomes in HEK 293 cell lysates. Monomeric α-synuclein inhibited the 20 S/26 S proteasome mixture only partially, which could reflect greater inhibition of the 20 S proteasome complex and less inhibition of the 26 S proteasome complex (Fig. 4*B*). The concentration producing maximal inhibition of the 20 S/26 S proteasome complex was similar to that seen for the 20 S proteasome complex (> 10 μM), based on 50% maximal inhibition (Fig. 4*B*). Aggregated α-synuclein also inhibited the 20 S/26 S ubiquitin-independent proteasomal mixture. Based on an estimated molecular weight for aggregated α-synuclein of 160,000, we calculated that the IC_{50} of aggregated α-synuclein for the 20 S/26 S proteasome was 1 nM (Fig. 4*A*). The ability of aggregated α-synuclein to inhibit a mixture of the 26 S and 20 S proteasomes, but not the 20 S proteasome, suggests that aggregated

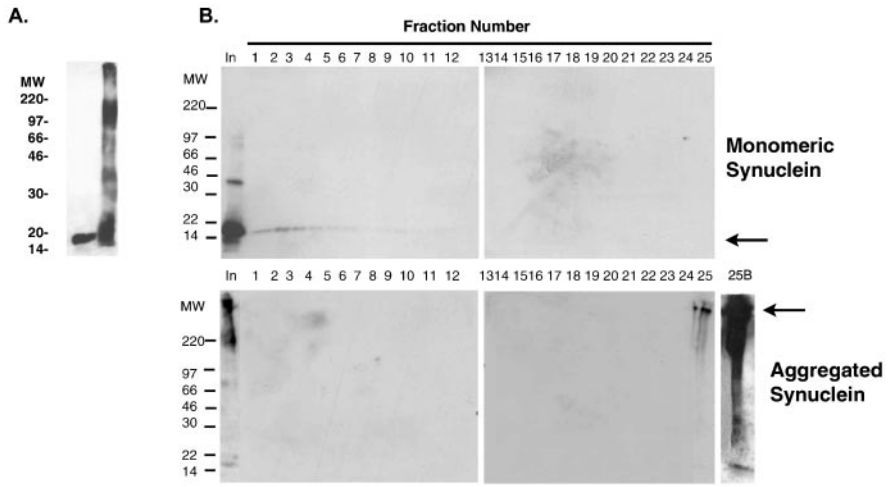

FIG. 2. **Analysis of monomeric and aggregated α-synuclein.** *A*, fresh α-synuclein (*lane 1*) migrated at about 16,000, which is consistent with a monomeric size. Aggregated α-synuclein (*lane 2*) exhibited both low and high molecular weight species following the process of immunoblotting, which involved heating in 2% SDS for 5 min, running on PAGE, and immunoblotting. This is a representative immunoblot from an experiment that had been repeated three times. *B*, samples of fresh (*top*) and aged (*bottom*) α-synuclein were fractionated on a 5–30% sucrose gradient, and each fraction was immunoblotted. An immunoblot of the non-fractionated starting material is shown in the first lane, *IN′*. The monomeric α-synuclein was present predominantly in the early fractions, suggesting a low molecular weight; the aged α-synuclein was present in the last fraction, suggesting a high molecular weight and little if any free monomeric α-synuclein. Because some SDS-sensitive aggregated α-synuclein appeared to be present in the aged Input sample, we overexposed the aged fractionated sample to determine whether it also contained any SDS-sensitive aged α-synuclein. A long exposure of fraction 25 (*lane 25B*) demonstrates the presence of 16,000 α-synuclein, suggesting that some of the aged, aggregated α-synuclein can be dissociated by SDS. This is a representative immunoblot from an experiment that had been repeated three times.

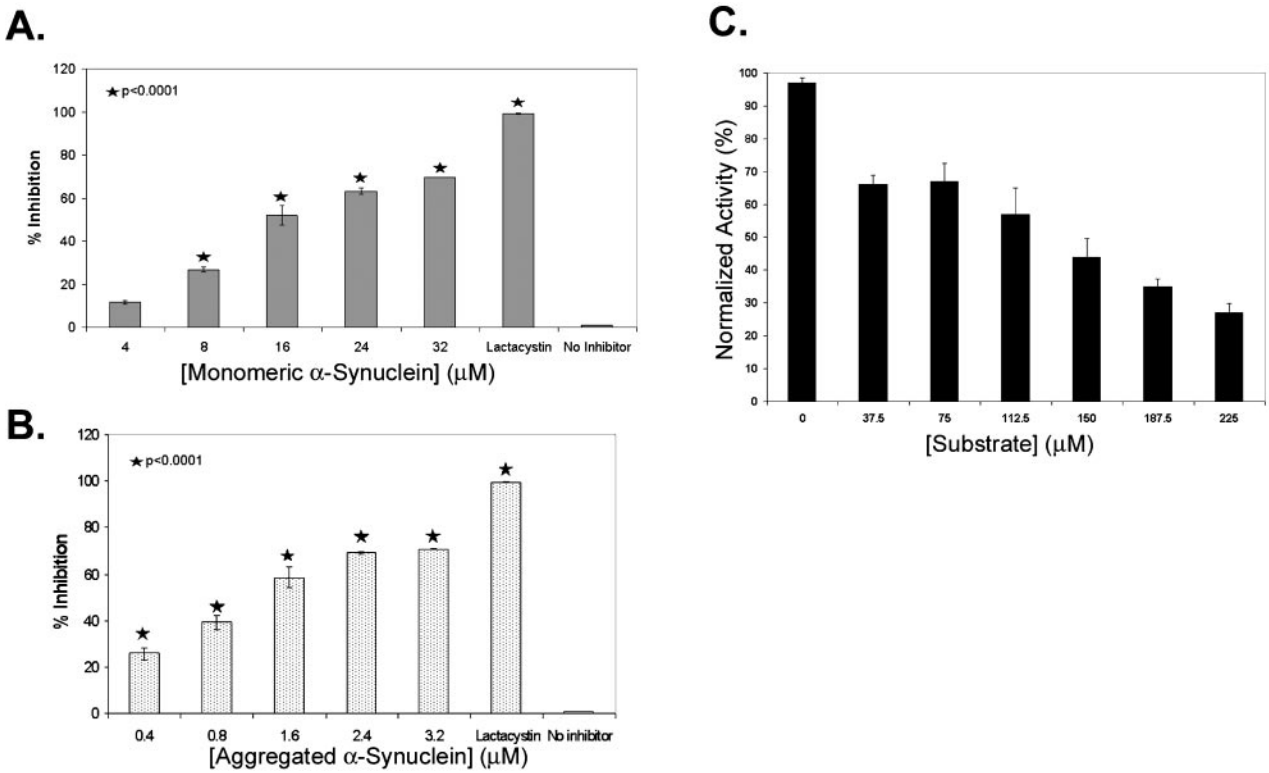

FIG. 3. **Effects of monomeric and aggregated α-synuclein on the 20 S proteasome.** *A*, inhibition of the 20 S proteasome by monomeric α-synuclein dose response. Five data points were used for each sample. *B*, inhibition of the 20 S proteasome by aggregated α-synuclein dose response. Five data points were used for each sample. *C*, substrate dependence of proteasomal inhibition by α-synuclein. Five data points were used for each sample.

α-synuclein selectively inhibits the 26 S proteasome.

Aggregated Synuclein, but Not Monomeric Synuclein, Inhibits Protein Degradation by the 26 S Proteasome—The greater ability of aggregated α-synuclein compared with monomeric α-synuclein in inhibiting 26 S ubiquitin-independent proteasomal activity raises the possibility that 26 S ubiquitin-dependent proteasomal function might also be selectively inhibited by aggregated α-synuclein. To investigate this, we examined ubiquitin-mediated degradation of a fusion protein made up of barnase and *E. coli* dihydrofolate reductase that had been fused with an N-terminal degradation tag (DHFR-U) (45). Prior studies show that degradation of ubiquitinated DHFR-U by reticulocyte lysates is mediated by the 26 S proteasome (45). We used this system to investigate how monomeric and aggregated α-synuclein affect ubiquitin-mediated proteasomal degradation. Degradation of ubiquitinated DHFR-U was examined

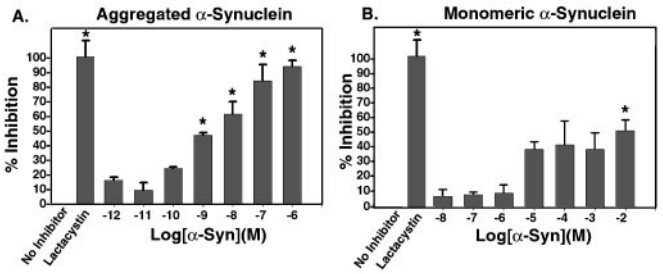

FIG. 4. **Inhibition of ubiquitin-independent proteasomal degradation by the 20 S/26 S proteasome with aggregated (*A*) and monomeric (*B*) α-synuclein using HEK 293 lysates.** The percent inhibition was normalized to the inhibition produced by the proteasomal inhibitor lactacystin (25 μM). *, $p < 0.002$ compared with no inhibitor.

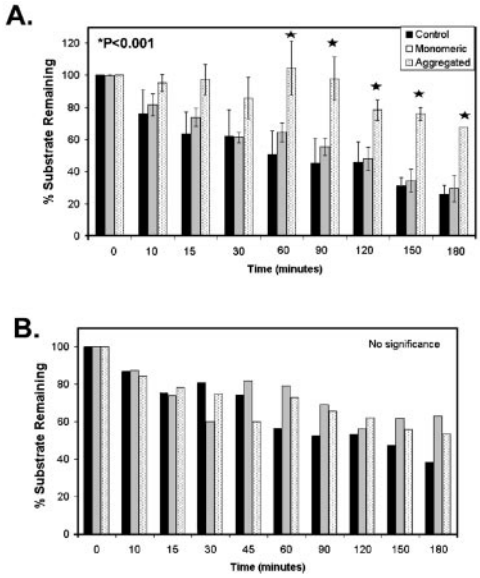

FIG. 5. **Inhibition of ubiquitin-dependent proteasomal degradation by aggregated α-synuclein.** *A*, aggregated α-synuclein (0.5 μM) inhibits ubiquitin-dependent degradation of DHFR-U by reticulocyte lysates by the 26 S proteasome, whereas monomeric α-synuclein (5 μM) does not inhibit degradation of DHFR-U by reticulocyte lysates by the 26 S proteasome. The overall analysis of variance was significant at $p < 0.001$. *Stars* show significance relative to DHFR-U in the absence of added α-synuclein. Each sample point was performed in triplicate. *B*, lack of inhibition of Clp1 by monomeric or aggregated α-synuclein. Degradation of DHFR-U was not significantly different between degradation of DHFR-U alone or in the presence of aggregated (0.5 μM) or monomeric (5 μM) α-synuclein. Each sample point was performed in triplicate.

in the presence of monomeric or aggregated α-synuclein (Fig. 5*A*). The half-life of DHFR-U was 125 min both under basal conditions and in the presence of 5 μM monomeric α-synuclein (Fig. 5*A*, *gray bars*). However, the half-life of DHFR-U greatly increased in the presence of 500 nM aggregated α-synuclein (Fig. 5*A*, *dotted bars*). No inhibition was seen with 50 nM aggregated α-synuclein (data not shown). These data indicate that 26 S ubiquitin-dependent proteasomal degradation is selectively inhibited by aggregated α-synuclein.

To determine whether inhibition of ubiquitin-dependent proteasomal degradation was specific to the 26 S proteasome, we also examined degradation of barnase that had been fused with a 65-amino acid N-terminal tag (DHFR-65) that allows the protein to be recognized and degraded by the bacterial proteasomal analog ClpAP (45). DHFR-65 was incubated with ClpAP alone or in the presence of 5 μM monomeric α-synuclein or in the presence of 500 nM aggregated α-synuclein, and the rate of degradation was monitored. Neither monomeric nor aggregated α-synuclein inhibited degradation of DHFR-65 by ClpAP (Fig. 5*B*). This indicates that proteasomal inhibition by aggregated α-synuclein is specific for the ubiquitin-dependent 26 S proteasomal system.

Native and Aggregated α-Synuclein Bind S6′—The ability of aggregated α-synuclein to inhibit degradation mediated by the 26 S proteasome could be explained by interaction between aggregated α-synuclein and a protein in the 19 S cap that is present in the 26 S proteasome but not the 20 S proteasome. Studies with rat α-synuclein suggest that α-synuclein binds the rodent 19 S proteasomal component S6′ (35). Based on this work, we investigated whether human α-synuclein interacts with S6′. His-tagged recombinant native or aggregated α-synuclein was incubated overnight with substantia nigra or cingulated cortex from normal human brain and then precipitated with nickel-agarose. The precipitates were immunoblotted with antibodies to S6′. A representative immunoblot with native α-synuclein is shown in Fig. 6*A*, and a pull-down with native or aggregated α-synuclein is shown in Fig. 6*B*. Both aggregated and monomeric α-synuclein associated with S6′. The term 'native' is used in this discussion because the overnight incubation of recombinant α-synuclein with the lysates appeared to promote formation of some recombinant α-synuclein dimer, in addition to the more abundant α-synuclein monomer (Fig. 6*B*, *lower panel*). Co-association of α-synuclein with S6′ was also observed by immunoprecipitating endogenous α-synuclein and immunoblotting for S6′ (Fig. 6*C*). To test the selectivity of the association, we examined whether α-synuclein binds other components of the 19 S proteasomal cap, such as Rpn12 and subunit 10b. Neither Rpn12 nor subunit 10b was observed to co-precipitate with α-synuclein (Fig. 6*D*, immunoblot for *10b* shown). It was not possible to test the association of S6′ with α-synuclein by immunoprecipitating S6′, because none of the antibodies to S6′ that we tested were successfully able to precipitate S6′ (data not shown). Together, these data suggest that both monomeric and aggregated α-synuclein bind S6′.

DISCUSSION

Proteasomal inhibition is known to be toxic to many cell types and is thought to contribute to the pathophysiology of neurodegenerative diseases (17, 18, 47). Our data demonstrate that overexpressing α-synuclein inhibits 20 and 26 S proteasomal activity. The relationship between overexpressed α-synuclein and the pathophysiology of PD, though, is unclear. Overexpressing α-synuclein in mammalian neurons does not lead to its spontaneous aggregation, except after delays of 6–12 months (48–51). Because protein aggregation is thought to play a critical role in the pathophysiology of neurodegenerative diseases and aggregation of α-synuclein appears to be important to the pathophysiology of PD, we sought to design experiments that would allow analysis of the actions of aggregated α-synuclein. To investigate whether aggregated α-synuclein interacts with the proteasome, we examined the behavior of α-synuclein that had been aggregated *in vitro*. We observed that aggregated α-synuclein inhibits both ubiquitin-dependent and -independent 26 S proteasomal activity. The IC_{50} of aggregated α-synuclein for ubiquitin-independent 26 S proteasomal activity was 1 nM, which was over 1000-fold higher than the IC_{50} for 20 S proteasomal activity. In contrast, monomeric α-synuclein inhibited 20 and 26 S proteasomal activity with an $IC_{50} > 10$ μM.

The high affinity of aggregated α-synuclein for inhibiting 26 S proteasomal activity could be explained by binding of aggregated α-synuclein to a protein in the 19 S cap, which is the proteasomal complex that binds to the 20 S proteasome and confers ubiquitin-dependence, as discussed below (3). Consist-

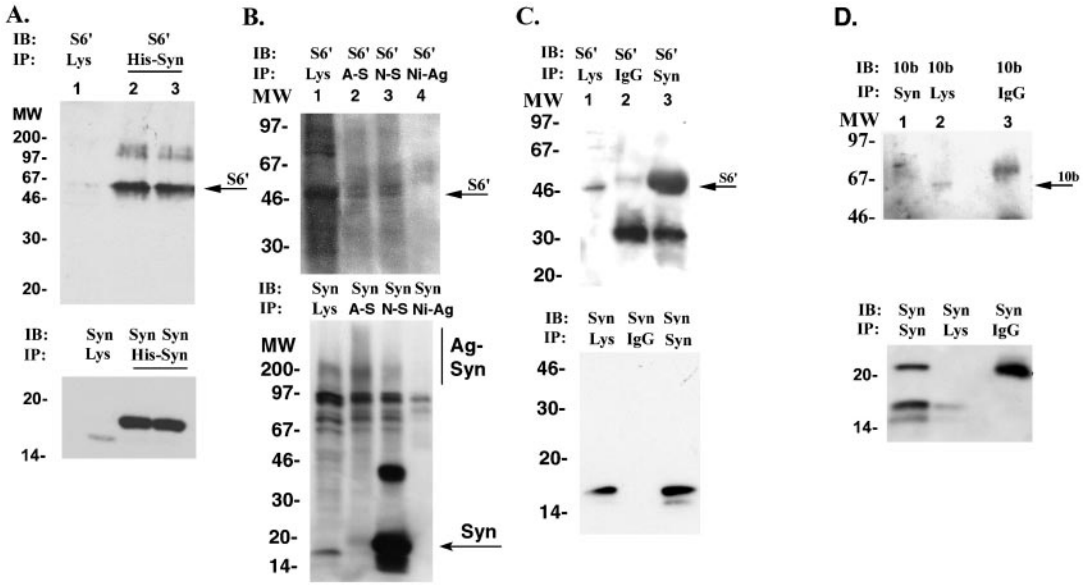

FIG. 6. **α-Synuclein binds S6′.** *A*, *upper panel*, immunoblot showing S6′ in brain lysate (*lane 1*, 10 μg) and precipitation of S6′ by monomeric His-tagged recombinant α-synuclein (*lanes 2* and *3*). *Lower panel*, reprobe of the same immunoblot with anti-α-synuclein antibody. The *arrow* points to the band corresponding to the S6′ protein. The α-synuclein band in lane 1 is lower than that in lanes 2 and 3 in the lower panel because the protein in lane 1 is endogenous α-synuclein, whereas the protein in lane 2 and 3 is His-tagged protein. *B*, *upper panel*, immunoblot showing S6′ in brain lysate (*lane 1*, 30 μg), precipitation of S6′ by aggregated (*lane 2*, *A-S*) or native His-tagged recombinant α-synuclein (*lane 3*, *N-S*), and lack of precipitation of S6′ using Ni-agarose pull-down without recombinant α-synuclein (*lane 4*). The *arrow* points to the band corresponding to the S6′ protein. *Lower panel*, reprobe of the same immunoblot with anti-α-synuclein antibody. The *arrow* points to monomeric α-synuclein, and the *bar* demonstrates the position of aggregated α-synuclein. The band at 36 kDa in lane 3 likely represents an α-synuclein dimer that might have been promoted by incubation of a large amount of recombinant α-synuclein with lysate but was not present in most other experiments (for example, see *panel C*). *C*, *upper panel*, immunoblot of S6′ showing the association of S6′ with α-synuclein following an immunoprecipitation of endogenous α-synuclein by anti-synuclein antibody. The *arrow* points to the band corresponding to the S6′ protein. *Lane 1* (*Lys*), crude brain lysates that had not been subject to immunoprecipitation. *Lane 2* (*IgG*), immunoprecipitation with nonspecific preimmune IgG antibody. *Lane 3* (*Syn*), immunoprecipitation with anti-α-synuclein antibody. *Lower panel*, reprobe with anti-α-synuclein antibody. *D*, no association was observed between α-synuclein and other proteasomal components, such as the 19 S subunit 10b. The *arrow* points to the band corresponding to the 10b protein, which is present in the lysates (*lane 2*) but not immunoprecipitated with α-synuclein (*lane 1*) or nonspecific IgG (*lane 3*). The abbreviations for this panel are the same as for panel B. All immunoblots in this figure are representative immunoblots from experiments that had been repeated at least three times.

ent with this hypothesis, we observed that α-synuclein binds to S6′, which is a subunit of the 19 S cap that was recently shown to bind polyubiquitinated proteins (3). Both aggregated and monomeric α-synuclein bind the S6′ protein. The interaction appears to be selective for S6′ because no association was observed with other 19 S proteasomal proteins, such as Rpn12 or subunit 10b.

Binding of α-synuclein to S6′ is consistent with prior publications. Ghee *et al*. (35) demonstrated that rat S6′ (also termed Tat binding protein-1, TBP1) binds α-synuclein using the yeast two-hybrid method. The association was confirmed by showing that an epitope-tagged S6′ could pull down α-synuclein following transfection of both proteins into HEK 293 cells. However, this study did not demonstrate interaction using the endogenous proteins and also did not investigate whether human α-synuclein binds to human S6′. In addition, Ii *et al.* (52) have documented the presence of proteasomal proteins in Lewy bodies, which supports our observation that aggregated α-synuclein binds S6′. The information presented in this study provides the functional relevance for these observations by showing that binding of α-synuclein to the proteasome inhibits proteasomal function.

The function of S6′ was recently identified and suggests a mechanism explaining why aggregated α-synuclein might inhibit the activity of the 26 S proteasome. The S6′ protein appears to function in the 19 S proteasomal cap as the docking protein for ubiquitin-conjugated proteins and is essential for binding of ubiquitin-conjugated proteins by the proteasome (3). Because aggregated α-synuclein is much larger than monomeric α-synuclein and often contains covalent cross-links, binding to S6′ might inhibit the function of the 19 S protein by competing with binding of other ubiquitin-conjugated proteins. Bound aggregated α-synuclein might occupy the unfolding proteins associated with proteasomal degradation, and the aggregate might also physically block the pore of the 19 S cap. This model provides an explanation for the ability of aggregated α-synuclein to interfere with both the ubiquitin-dependent and -independent 26 S proteasomal function.

Many other protein aggregates have been shown to be toxic to cells (18). Both aggregated cystic fibrosis transmembrane receptor and polyglutamine repeat exhibit toxicity that correlates with proteasomal inhibition (17, 18, 47). This study focuses attention on the interaction between S6′ and protein aggregates. Whether S6′ has a particular affinity for α-synuclein or is a general target for all protein aggregates remains to be determined. Inhibiting the ubiquitin-dependent proteasomal system (UPS) is known to be toxic, perhaps because it induces apoptosis (19). Inhibiting the UPS causes the accumulation of many toxic proteins, such as Pael-R, which was recently identified as a parkin substrate (53). Inhibiting the UPS is also known to cause the accumulation of protein aggregates in the endoplasmic reticulum (17, 47). Inhibiting the UPS could alter the regulation of cell cycle proteins (54). Reduced degradation of cell cycle proteins could account for the apparent abnormal activation of the cell cycle proteins observed in many neurodegenerative processes (55, 56).

Proteasomal inhibitors have recently been shown to induce degeneration of the dopaminergic neurons of the substantia nigra and induce α-synuclein aggregation (57). The tendency of α-synuclein to accumulate under conditions of proteasomal in-

hibition raises the possibility that the accumulation of aggregated α-synuclein adds to the proteasomal inhibition and increases the toxicity associated with proteasomal inhibition.

The discordance between the rapid kinetics of cell death associated with UPS inhibition in cell culture and the slow nature of degeneration in PD is notable. This discordance might be explained by the slow appearance of aggregated α-synuclein. α-Synuclein does not form aggregates under basal conditions when transiently overexpressed, but studies in transgenic mice show that overexpressing α-synuclein does lead to a delayed accumulation of aggregated α-synuclein (48–51). The slow rate of accumulation of aggregated α-synuclein could also lead to a correspondingly gradual inhibition of the UPS during the course of PD. Hence, progressive inhibition of the UPS by aggregated α-synuclein might be a gradual process in PD. Together these data suggest a model in which the gradual accumulation of aggregated α-synuclein progressively inhibits S6′ function, which leads to a gradual but progressive inhibition of the UPS and the progressive neurodegeneration that occurs in PD.

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REFERENCES

1. Glickman, M. H., Rubin, D. M., Fried, V. A., and Finley, D. (1998) *Mol. Cell. Biol.* **18,** 3149–3162
2. Voges, D., Zwickl, P., and Baumeister, W. (1999) *Annu. Rev. Biochem.* **68,** 1015–1068
3. Lam, Y. A., Lawson, T. G., Velayutham, M., Zweier, J. L., and Pickart, C. M. (2002) *Nature* **416,** 763–767
4. Davies, K. J. (2001) *Biochimie (Paris)* **83,** 301–310
5. Shringarpure, R., Grune, T., and Davies, K. J. (2001) *Cell. Mol. Life Sci.* **58,** 1442–1450
6. Rechsteiner, M. (1991) *Cell* **66,** 615–618
7. Rechsteiner, M., Hoffman, L., and Dubiel, W. (1993) *J. Biol. Chem.* **268,** 6065–6068
8. Goldberg, A. L. (1992) *Eur. J. Biochem.* **203,** 9–23
9. Hershko, A., and Ciechanover, A. (1998) *Annu. Rev. Biochem.* **67,** 425–479
10. Coux, O., Tanaka, K., and Goldberg, A. L. (1996) *Annu. Rev. Biochem.* **65,** 801–847
11. Baumeister, W., Walz, J., Zuhl, F., and Seemuller, E. (1998) *Cell* **92,** 367–380
12. Tanaka, K. (1998) *J. Biochem. (Tokyo)* **123,** 195–204
13. Tanaka, K. (1998) *Biochem. Biophys. Res. Commun.* **247,** 537–541
14. Satyal, S. H., Schmidt, E., Kitagawa, K., Sondheimer, N., Lindquist, S., Kramer, J. M., and Morimoto, R. I. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 5750–5755
15. Paulson, H. L., Perez, M. K., Trottier, Y., Trojanowski, J. Q., Subramony, S. H., Das, S. S., Vig, P., Mandel, J. L., Fischbeck, K. H., and Pittman, R. N. (1997) *Neuron* **19,** 333–344
16. Martindale, D., Hackam, A., Wieczorek, A., Ellerby, L., Wellington, C., McCutcheon, K., Singaraja, R., Kazemi-Esfarjani, P., Devon, R., Kim, S. U., Bredesen, D. E., Tufaro, F., and Hayden, M. R. (1998) *Nat. Genet.* **18,** 150–154
17. Bence, N. F., Sampat, R. M., and Kopito, R. R. (2001) *Science* **292,** 1552–1555
18. Bucciantini, M., Giannoni, E., Chiti, F., Baroni, F., Formigli, L., Zurdo, J., Taddei, N., Ramponi, G., Dobson, C. M., and Stefani, M. (2002) *Nature* **416,** 507–511
19. Jesenberger, V., and Jentsch, S. (2002) *Nat. Rev. Mol. Cell. Biol.* **3,** 112–121
20. Spillantini, M., Schmidt, M., Lee, VM-Y, Trojanowski, J., Jakes, R., and Goedert, M. (1997) *Nature* **388,** 839–840
21. Spillantini, M. G., Crowther, R. A., Jakes, R., Hasegawa, M., and Goedert, M. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 6469–6473
22. Polymeropoulos, M. H., Lavedan, C., Leroy, E., Ide, S. E., Dehejia, A., Dutra, A., Pike, B., Root, H., Rubenstein, J., Boyer, R., Stenroos, E. S., Chandrasekharappa, S., Athanassiadou, A., Papapetropoulos, T., Johnson, W. G., Lazzarini, A. M., Duvoisin, R. C., Di Iorio, G., Golbe, L. I., and Nussbaum, R. L. (1997) *Science* **276,** 2045–2047
23. Kruger, R., Kuhn, W., Muller, T., Woitalla, D., Graeber, M., Kosel, S., Przuntek, H., Epplen, J., Schols, L., and Riess, O. (1998) *Nature Gen.* **18,** 106–108
24. Conway, K., Harper, J., and Lansbury, P. (1998) *Nature Med.* **4,** 1318–1320
25. Conway, K. A., Lee, S. J., Rochet, J. C., Ding, T. T., Williamson, R. E., and Lansbury, P. T., Jr. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 571–576
26. Wood, S., Wypych, J., Steavenson, S., Louis, J., Citron, M., and Biere, A. (1999) *J. Biol. Chem.* **274,** 19509–19512
27. Biere, A. L., Wood, S. J., Wypych, J., Steavenson, S., Jiang, Y., Anafi, D., Jacobsen, F. W., Jarosinski, M. A., Wu, G. M., Louis, J. C., Martin, F., Narhi, L. O., and Citron, M. (2000) *J. Biol. Chem.* **275,** 34574–34579
28. Ostrerova, N., Petrucelli, L., Farrer, M., Mehta, N., Alexander, P., Choi, P., Palacino, J., Hardy, J., and Wolozin, B. (1999) *J. Neurosci.* **19,** 5782–5791
29. Jenco, J., Rawlingson, A., Daniels, B., and Morris, A. (1998) *Biochemistry* **37,** 4901–4909
30. Pronin, A. N., Morris, A. J., Surguchov, A., and Benovic, J. L. (2000) *J. Biol. Chem.*
31. Engelender, S., Kaminsky, Z., Guo, X., Sharp, A., Amaravi, R., Kleiderlein, J., Margolis, R., Troncoso, J., Lanahan, A., Worley, P., Dawson, V., Dawson, T., and Ross, C. (1999) *Nature Gen.* **22,** 110–114
32. Sharon, R., Goldberg, M. S., Bar-Josef, I., Betensky, R. A., Shen, J., and Selkoe, D. J. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98,** 9110–9115
33. Choi, P., Golts, N., Snyder, H., Petrucelli, L., Chong, M., Hardy, J., Sparkman, D., Cochran, E., Lee, J., and Wolozin, B. (2001) *NeuroReport* **12,** 2839–2844
34. Lee, F. J., Liu, F., Pristupa, Z. B., and Niznik, H. B. (2001) *FASEB J.* **15,** 916–926
35. Ghee, M., Fournier, A., and Mallet, J. (2000) *J. Neurochem.* **75,** 2221–2224
36. Wakabayashi, K., Engelender, S., Yoshimoto, M., Tsuji, S., Ross, C. A., and Takahashi, H. (2000) *Ann. Neurol.* **47,** 521–523
37. Schlossmacher, M. G., Frosch, M. P., Gai, W. P., Medina, M., Sharma, N., Forno, L., Ochiishi, T., Shimura, H., Sharon, R., Hattori, N., Langston, J. W., Mizuno, Y., Hyman, B. T., Selkoe, D. J., and Kosik, K. S. (2002) *Am. J. Pathol.* **160,** 1655–1667
38. Souza, J. M., Giasson, B. I., Lee, V. M., and Ischiropoulos, H. (2000) *FEBS Lett.* **474,** 116–119
39. Tanaka, Y., Engelender, S., Igarashi, S., Rao, R. K., Wanner, T., Tanzi, R. E., Sawa, A., Dawson, V. L., Dawson, T. M., and Ross, C. A. (2001) *Hum. Mol. Genet.* **10,** 919–926
40. Ostrerova-Golts, N., Petrucelli, L., Hardy, J., Lee, J., Farrer, M., and Wolozin, B. (2000) *J. Neurosci.* **20,** 6048–6054
41. Golts, N., Snyder, H., Frasier, M., Theisler, C., Choi, P., and Wolozin, B. (2002) *J. Biol. Chem.* **277,** 16116–16123
42. Lee, H. J., Choi, C., and Lee, S. J. (2001) *J. Biol. Chem.* **25,** 25
43. Betarbet, R., Sherer, T. B., MacKenzie, G., Garcia-Osuna, M., Panov, A. V., and Greenamyre, J. T. (2000) *Nat. Neurosci.* **3,** 1301–1306
44. Uversky, V. N., Li, J., and Fink, A. L. (2001) *FEBS Lett.* **500,** 105–108
45. Lee, C., Schwartz, M. P., Prakash, S., Iwakura, M., and Matouschek, A. (2001) *Mol. Cell* **7,** 627–637
46. Matouschek, A., Azem, A., Ratliff, K., Glick, B. S., Schmid, K., and Schatz, G. (1997) *EMBO J.* **16,** 6727–6736
47. Johnston, J., Ward, C., and Kopito, R. (1998) *J. Cell Biol.* **143,** 1883–1898
48. Lee, M. K., Stirling, W., Xu, Y., Xu, X., Qui, D., Mandir, A. S., Dawson, T. M., Copeland, N. G., Jenkins, N. A., and Price, D. L. (2002) *Proc. Natl. Acad. Sci. U. S. A.* **99**
49. Giasson, B. I., Duda, J. E., Quinn, S. M., Zhang, B., Trojanowski, J. Q., and Lee, V. M. (2002) *Neuron* **34,** 521–533
50. Kahle, P. J., Neumann, M., Ozmen, L., Muller, V., Odoy, S., Okamoto, N., Jacobsen, H., Iwatsubo, T., Trojanowski, J. Q., Takahashi, H., Wakabayashi, K., Bogdanovic, N., Riederer, P., Kretzschmar, H. A., and Haass, C. (2001) *Am. J. Pathol.* **159,** 2215–2225
51. Masliah, E., Rockenstein, E., Veinbergs, I., Mallory, M., Hashimoto, M., Takeda, A., Sagara, Y., Sisk, A., and Mucke, L. (2000) *Science* **287,** 1265–1269
52. Ii, K., Ito, H., Tanaka, K., and Hirano, A. (1997) *J. Neuropathol. Exp. Neurol.* **56,** 125–131
53. Imai, Y., Soda, M., Inoue, H., Hattori, N., Mizuno, Y., and Takahashi, Y. (2001) *Cell* **105,** 891–902
54. Yew, P. R. (2001) *J. Cell. Physiol.* **187,** 1–10
55. Husseman, J. W., Nochlin, D., and Vincent, I. (2000) *Neurobiol. Aging* **21,** 815–828
56. Chang, B. D., Watanabe, K., Broude, E. V., Fang, J., Poole, J. C., Kalinichenko, T. V., and Roninson, I. B. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 4291–4296
57. McNaught, K. S., Bjorklund, L. M., Belizaire, R., Isacson, O., Jenner, P., and Olanow, C. W. (2002) *Neuroreport* **13,** 1437–1441