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β-Synuclein Reduces Proteasomal Inhibition by α-Synuclein but Not γ-Synuclein*

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The accumulation of aggregated α-synuclein is thought to contribute to the pathogenesis of Parkinson's disease. Recent studies indicate that aggregated α-synuclein binds to S6′, a component of the 19 S subunit in the 26 S proteasome and inhibits 26 S proteasomal degradation, both ubiquitin-independent and ubiquitin-dependent. The IC_{50} of aggregated α-synuclein for inhibition of the 26 S ubiquitin-independent proteasomal activity is ~1 nM. α-Synuclein has two close homologues, termed β-synuclein and γ-synuclein. In the present study we compared the effects of the three synuclein homologues on proteasomal activity. The proteasome exists as a 26 S and a 20 S species, with the 26 S proteasome containing the 20 S core and 19 S cap. Monomeric α- and β-synucleins inhibited the 20 S and 26 S proteasomal activities only weakly, but monomeric γ-synuclein strongly inhibited ubiquitin-independent proteolysis. The IC_{50} of monomeric γ-synuclein for the 20 S proteolysis was 400 nM. In monomeric form, none of the three synuclein proteins inhibited 26 S ubiquitin-dependent proteasomal activity. Although β-synuclein had no direct effect on proteasomal activity, co-incubating monomeric β-synuclein with aggregated α-synuclein antagonized the inhibition of the 26 S ubiquitin-independent proteasome by aggregated α-synuclein when added before the aggregated α-synuclein. Co-incubating β-synuclein with γ-synuclein had no effect on the inhibition of the 20 S proteasome by monomeric γ-synuclein. Immunoprecipitation and pull-down experiments suggested that antagonism by β-synuclein resulted from binding to α-synuclein rather than binding to S6′. Pull-down experiments demonstrated that recombinant monomeric β-synuclein does not interact with the proteasomal subunit S6′, unlike α-synuclein, but β-synuclein does bind α-synuclein and competes with S6′ for binding to α-synuclein. Based on these data, we hypothesize that the α- and γ-synucleins regulate proteasomal function and that β-synuclein acts as a negative regulator of α-synuclein.

The pathology of Parkinson's disease (PD)[1] is characterized by a loss of dopaminergic neurons in the substantia nigra and the formation of intracellular inclusions, termed Lewy bodies. One of the primary components of Lewy bodies is α-synuclein, which is a small, abundant protein (1). α-Synuclein is implicated in the pathophysiology of PD because it is the primary component of Lewy bodies and because three mutations in the α-synuclein protein, A30P, E46K, and A53T, have been linked to the development of familial PD (2–4).

The pathophysiology of PD is incompletely understood, but increasing evidence suggests that proteasomal inhibition contributes significantly to the neurodegenerative process. The proteasome consists of a 20 S organelle that is a large proteolytic complex and is the core of each form of the proteasome core (5). The 20 S proteasome is thought to degrade oxidized proteins but has also been shown recently to internally cleave α-synuclein (6). Both the 26 S ubiquitin-independent and 26 S ubiquitin-dependent proteasomes contain the 20 S core plus smaller caps on each end of the 20 S core that have sedimentation coefficients of 19 S. One of the functions of the 19 S cap is to recognize ubiquitinated proteins, which is mediated by a protein in the 19 S cap, termed S6′ (6).

Aggregated proteins, such as those that accumulate as inclusion bodies in the brains of individuals with neurodegenerative disease, appear to potently inhibit proteasomal activity (7). Polyglutamine repeat proteins, such as those that occur with Huntington's disease, appear to be particularly potent inhibitors of ubiquitin-dependent proteasomal function in cell culture (7). Our own work indicates that aggregated α-synuclein also inhibits proteasomal activity potently (8). One mechanism through which α-synuclein binds the proteasome is via the proteasomal protein S6′ (8–10). Both monomeric and aggregated α-synuclein interact with S6′, but aggregated α-synuclein is a much more potent inhibitor of 26 S proteasomal activity than monomeric α-synuclein. Aggregated α-synuclein inhibits 26 S proteasomal activity with an IC_{50} (<5 nM) that is >1000-fold lower than that of monomeric α-synuclein (16 μM) (8). Other studies suggest that α-synuclein also inhibits the 20 S proteasome, although it also appears able to be cleaved by the 20 S proteasome (11, 12). The proteasomal hypothesis of neurodegeneration is supported by post-mortem studies showing that proteasomal activity is reduced in the

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[1] The abbreviations used are: PD, Parkinson's disease; DHFR-U, fusion protein of *E. coli* dihydrofolate reductase, barnase, and an N-terminal degradation tag.

substantia nigra from subjects who died with PD, although one study failed to detect changes in proteasomal activity in brain tissue from subjects who suffered from PD (13–15). Proteasomal inhibition in neurodegeneration might be enhanced by positive feedback, because drug-induced proteasomal inhibition increases the accumulation of aggregated α-synuclein (16–18). These studies highlight the potential importance of the proteasome in the pathophysiology of PD.

α-Synuclein is part of a family of proteins that also includes β and γ-synuclein (19). β-Synuclein is 78% homologous to α-synuclein, and γ-synuclein is 58% homologous to α-synuclein. Expression of α- and β-synuclein appears to be inversely regulated. β-Synuclein is the predominant RNA message expressed in the brain, although α-synuclein is the predominant protein expressed in the brain (20). A form of inverse regulation also occurs at the biochemical level, because β-synuclein inhibits the aggregation of α-synuclein *in vitro* and *in vivo* (19, 21). γ-Synuclein is also known as persyn and BCSG-1 (breast cancer-specific gene 1) (22). γ-Synuclein is significantly up-regulated in >70% of late stage breast and >85% of ovarian carcinomas (23). γ-Synuclein has multiple actions. It stimulates the ligand-dependent transcriptional activity of estrogen receptor-α in cultured breast cancer cells, stimulates cell proliferation, and up-regulates matrix metalloproteases, which are enzymes implicated in tumorigenesis and neurodegeneration (24, 25).

Because of the homology of the three-synuclein proteins and the link between α-synuclein and the proteasome, we set to understand how α-, β-, and γ-synucleins interact with the proteasomal system. In this study, we examine the interactions of α-synuclein, β-synuclein, and γ-synuclein with the three different proteasome pathways and demonstrate differential regulation by the three synuclein proteins. We demonstrate that β-synuclein does not inhibit proteasomal function but does prevent aggregated α-synuclein from inhibiting the 26 S proteasome. In addition, we observed that γ-synuclein potently inhibits the 20 S proteasome but is not affected by β-synuclein.

MATERIALS AND METHODS

Cell Lines, Transfections, Chemicals, and Antibodies—HEK-293 cells were also grown in OPTIMEM (Cell Grow) plus 10% fetal bovine serum. Recombinant α-synuclein and β-synuclein was generated using wild type α-synuclein inserted into a ProEX-His_6 plasmid (Invitrogen) as described previously (26, 27). γ-Synuclein was cloned into the XhoI and HindIII sites of the pTrcHisA vector (Invitrogen) and purified like α-synuclein and β-synuclein. Neurologically normal human cortical brain tissue (male, 72 years old, 8-h post mortem interval) was provided by the Loyola University Brain Tissue Bank directed by J. M. Lee. The antibodies used included monoclonal anti-α-synuclein (1:1000 immunoblot, 1:100 immunocytochemistry; Transduction Labs), monoclonal anti-S6′ (1:1000 immunoblot; Affiniti), and monoclonal anti-His (1:1000; Roche Applied Science).

Pull-down Assays from Brain—Brain samples were pre-cleared with nickel-agarose for 1 h at 4 °C to eliminate nonspecific proteins binding to the nickel-agarose (Invitrogen). Samples were incubated overnight with 10 μg of recombinant monomeric α-, β-, or γ-synuclein (His-tagged). Following this overnight incubation, samples were incubated with nickel-agarose for 1 h to allow binding of the His-tagged protein and then 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, and 1 mM proteasome inhibitor mixture (Sigma), pH 7.4) and run on 8–16% gradient SDS-polyacrylamide gels (Cambrex).

Pull-down Assay from Purified 20 S—Purified 20 S proteasome (Biomol) was incubated overnight with 10 μg of recombinant protein. Samples were incubated with nickel-agarose for 1 h to allow binding of the His-tagged protein and then centrifuged at 1000 rpm for 1 min. Samples were washed 3 times with immunoprecipitation buffer (50 mM Tris-HCl, 10 mM EGTA, 100 mM NaCl, 0.5% Triton X-100, 1 mM dithiothreitol, and 1 mM proteasome inhibitor mixture (Sigma), pH 7.4) and run on 8–16% gradient SDS-polyacrylamide gels (Cambrex).

Aggregation of α-Synuclein—Recombinant α-synuclein was aggregated by incubating samples for 1–2 months at 37 °C while rotating (5 mg/ml concentration in phosphate-buffered saline). Aggregation was confirmed by performing immunoblot analysis.

Immunoblot Analysis—Samples were re-suspended in 2× or 4× dithiothreitol loading buffer, heated for 5 min at 90 °C, and run on 8–16% gradient SDS-polyacrylamide gels (Cambrex). Transfers to polyvinylidene difluoride (Bio-Rad) were done overnight at 4 °C and 150 mA for 12 h. Blots were washed once with Tris-buffered saline plus 0.1% Tween 20 (TBS-T) and then blocked with 5% milk (Carnation) in TBS-T for 1 h at room temperature while shaking. The primary antibody was added overnight in 5% bovine serum albumin (Sigma) in TBS-T and incubated at 4 °C while shaking. Blots were then washed three times while shaking in TBS-T for 10 min per wash. A secondary antibody (Jackson Laboratories) was added (1:5000) in 5% milk in TBS-T. Blots were washed three times while shaking in TBS-T and developed using a chemiluminescent reaction (PerkinElmer Life Sciences).

In Vitro 20 S Ubiquitin-independent Proteasomal Activity Assay—Increasing concentrations of the recombinant protein of interest were incubated with purified 20 S proteasome (human erythrocytes; Biomol) in a 20 S proteasome assay buffer (Biomol) for 10 min at 30 °C, and then a 75 μM fluorogenic substrate (succinyl-Leu-Leu-Val-Tyr-7-amino-4-methylcoumarin; Biomol) was added. Lactacystin was used as an inhibitor control at 25 μM. Half-volume 96-well plates were used (Biomol). This substrate measures chymotryptic activity of the proteasome. Reactions were read immediately using a Gemini XS machine (Molecular Probes) to excite the solution at a wavelength of 380 nm, and the fluorescence emission was read at the wavelength of 460 nm.

In Vitro 20/26 S Ubiquitin-independent Proteasomal Activity Assay—At increasing concentrations, the recombinant protein of interest was incubated with 75 μg of HEK-293 whole cell lysates, as determined by BCA Protein Assay (Pierce) in buffer (50 mM Tris-HCl, pH 7.5, 1 mM EDTA) for 10 min at 30 °C. Half-volume 96-well plates were used (Biomol). We then added a fluorogenic substrate (succinyl-Leu-Leu-Val-Tyr-7-amino-4-methylcoumarin; Biomol). Samples were excited at a wavelength of 380 nm and the emission was read at a wavelength of 460 nm and fluorescence was read using the Gemini XS machine (Molecular Probes).

In Vitro 26 S Ubiquitin-dependent Proteasomal Activity Assay—These experiments were done by *in vitro* transcription and translation of substrate proteins using a T7 promoter in rabbit reticulocyte lysate (Promega) supplemented with [^{35}S]methionine and then partially purified using high speed centrifugation and ammonium sulfate precipitation as described (28). The protease substrate for C1P assays was derived from barnase, which is a ribonuclease from *Bacillus amyloliquefaciens*; the protease substrate for proteasomal assays was derived from *Escherichia coli* dihydrofolate reductase (DHFR) (28, 29). An ubiquitin moiety was added to the N terminus of the substrate proteins via a four-amino acid linker from the *E. coli lac* repressor (28). Substrate proteins were constructed in pGEM-3Zf (+) vectors (Promega) and verified by sequencing. The reaction was re-suspended in 40 μl of buffer (25% (v/v) glycerol, 25 mM $MgCl_2$, and 0.25 M Tris/Cl, pH 7.4) to which 5 μl of the *in vitro* reaction containing the radiolabeled ubiquitinated substrate protein was added with 35 μl of a rabbit reticulocyte lysate (Green Hectares) that was ATP-depleted as described (28). The reactions were incubated with the recombinant protein of interest or with no protein as the negative control. Protein concentrations were determined by BCA protein assay (Pierce). Initial cleavage of substrate proteins was enabled by a 7-min incubation at 37 °C. Ubiquitination and degradation were initiated by the addition of ATP and an ATP regenerating system (0.5 mM ATP, 10 mM creatine phosphate, and 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, and the trichloroacetic acid-insoluble fractions were analyzed by electrophoresis on 10% SDS-polyacrylamide gels and quantified by electronic autoradiography.

RESULTS

Monomeric α-Synuclein Mildly Inhibits the 20 S Proteasome, but γ-Synuclein Potently Inhibits the 20 S Proteasome—We began investigating how the different synucleins might modulate the activity of the 20 S proteasome. We examined the activity of 20 S proteasome in the presence of increasing concentrations of monomeric α-synuclein using synthetic fluorogenic peptides to monitor proteasomal activity. The purity of

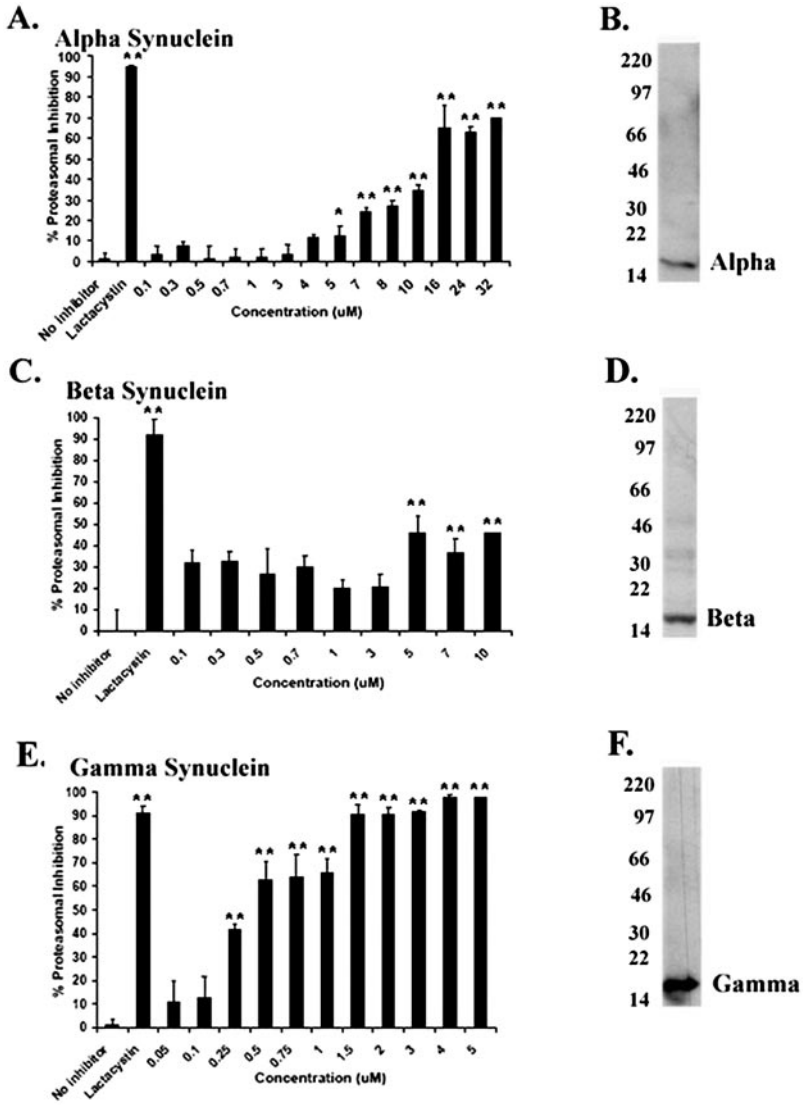

FIG. 1. **Modulation of 20 S chymotryptic proteasomal activity by monomeric α-, β-, and γ-synuclein.** *A*, ubiquitin-independent chymotryptic activity of the 20 S proteasome in the presence of increasing concentrations of α-synuclein. Monomeric α-synuclein inhibits the 20 S proteasome with an IC_{50} of 12 μM. Experiments were repeated a minimum of four times each. **, $p < 0.0001$ compared with no inhibitor. *B*, immunoblot of purified recombinant monomeric α-synuclein. *C*, ubiquitin-independent chymotryptic activity of the 20 S proteasome in the presence of increasing concentrations of β-synuclein. Experiments were repeated a minimum of four times each. **, $p < 0.0001$ compared with no inhibitor. *D*, immunoblot of purified recombinant monomeric β-synuclein. *E*, ubiquitin-independent chymotryptic activity of the 20 S proteasome in the presence of increasing concentrations of γ-synuclein. γ-Synuclein inhibits with an IC_{50} of 400 nM. Experiments were repeated a minimum of four times each. **, $p < 0.0001$ compared with no inhibitor. *F*, immunoblot of purified recombinant monomeric γ-synuclein.

the monomeric α-synuclein was confirmed by immunoblot (Fig. 1*B*). Monomeric α-synuclein mildly inhibited the chymotryptic, 20 S ubiquitin-independent proteasomal activity in a dose-dependent manner (Fig. 1*A*). The IC_{50} for inhibition of the proteasome was ~15 μM in these experiments, assuming that α-synuclein could achieve 100% maximal inhibition (Fig. 1*A*). This IC_{50} is similar to our previously published results demonstrating that monomeric α-synuclein inhibits the 20 S ubiquitin-independent proteasome with an IC_{50} of 16 μM. Our previous study also demonstrated that proteasomal inhibition by monomeric α-synuclein is not due to an interaction between α-synuclein and the fluorogenic substrate and is not sensitive to the concentration of a fluorogenic substrate (8).

Next we examined the effect of β-synuclein on the 20 S ubiquitin-independent proteasome. We confirmed the purity of the β-synuclein by SDS-PAGE (Fig. 1*D*). Increasing concentrations of recombinant β-synuclein were incubated with purified 20 S proteasome. A fluorogenic peptide was used that measures the chymotryptic proteasomal activity in an ubiquitin-independent manner. Increasing concentrations of β-synuclein had very little effect on the 20 S chymotryptic, ubiquitin-independent activity; we saw modest inhibition at all concentrations of β-synuclein (Fig. 1*C*). Two potential reasons might explain the low level inhibition of the 20 S proteasome in the presence of β-synuclein. 1) Modest inhibition might be due to an interaction of β-synuclein with the 20 S proteasome, although we detected no interaction with the 20 S α4, 20 S ζ, and 20 S α5 subunits of the 20 S proteasome. 2) β-Synuclein binds weakly to the 20 S proteasome as it cleaves β-synuclein (in addition to cleaving α-synuclein) (12).

Recombinant γ-synuclein behaved differently than either α- or β-synuclein. The purity of the recombinant γ-synuclein protein was confirmed by SDS-PAGE (Fig. 1*F*). The activity of γ-synuclein on the 20 S proteasome was tested in the presence of increasing amounts of monomeric γ-synuclein using a synthetic fluorescent peptide to monitor chymotryptic proteasomal activity. γ-Synuclein potently inhibited the 20 S chymotryptic activity with an IC_{50} for inhibition of 400 nM (Fig. 1*E*). We confirmed that the inhibition seen by γ-synuclein was not due to the binding of γ-synuclein to the substrate by showing that γ-synuclein did not alter the substrate dependence (data not shown). This suggests that γ-synuclein inhibits 20 S proteasomal activity *in vitro* by binding to a component of the 20 S proteasome.

Monomeric γ-Synuclein Potently Inhibits the 20/26 S Proteasome Mixture—Because the 26 S proteasome contains the 20 S organelle, we hypothesized that γ-synuclein would inhibit the 20/26 S proteasomal system present in HEK-293 cell lysates. Both β-synuclein and γ-synuclein were tested using the same methods described above. Recombinant β-synuclein protein

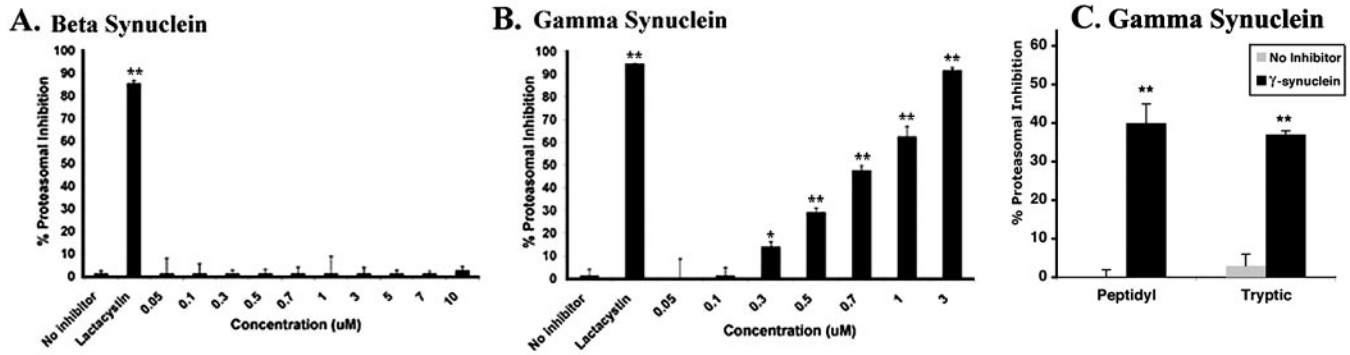

FIG. 2. **Modulation of 26 S chymotryptic ubiquitin-independent proteasomal activity by monomeric β- and γ-synuclein.** *A*, ubiquitin-independent chymotryptic activity of the 26 S proteasome in the presence of increasing concentrations of β-synuclein. Experiments repeated a minimum of 4 times each. **, $p < 0.0001$ compared with no inhibitor. *B*, ubiquitin-independent chymotryptic activity of the 26 S proteasome in the presence of increasing concentrations of γ-synuclein. γ-Synuclein inhibits with an IC_{50} of 600 nM. Experiments were repeated a minimum of three times. *C*, tryptic and peptidyl activity of the 26 S proteasome in the presence of 0.6 μM γ-synuclein. **, $p < 0.01$.

was incubated with whole HEK-293 lysates, and proteasomal activity was measured by the adding the chymotryptic fluorogenic peptide. Monomeric β-synuclein had no effect on the 20/26 S ubiquitin-independent, chymotryptic proteasomal activity (Fig. 2*A*). However, monomeric γ-synuclein potently inhibited the 20/26 S ubiquitin-independent chymotryptic proteasomal activity with an IC_{50} of 600 nM (Fig. 2*B*). γ-Synuclein also inhibited the tryptic and peptidyl activities of the proteasome (Fig. 2*C*). The similarities between the IC_{50} of monomeric γ-synuclein on the 20 S proteasome alone (IC_{50} of 400 nM) and that of the 20/26 S proteasomal mixture suggests that inhibition of the 20/26 S proteasomal activity by γ-synuclein is primarily due to a direct action of γ-synuclein on the 20 S proteasome alone.

Monomeric α-Synuclein, β-Synuclein, and γ-Synuclein Do Not Inhibit the 26 S Ubiquitin-dependent Pathway—We demonstrated previously that aggregated α-synuclein inhibits the 26 S ubiquitin-dependent pathway, unlike monomeric α-synuclein (8). Although β-synuclein and γ-synuclein do not aggregate as readily, we asked whether β-synuclein or γ-synuclein inhibit 26 S ubiquitin-dependent degradation. To investigate this question, we examined ubiquitin-mediated degradation of a fusion protein of *E. coli* dihydrofolate reductase, barnase, and an N-terminal degradation tag (DHFR-U) (28). Prior studies demonstrate that ubiquitinated DHFR-U is degraded by the 26 S proteasome in reticulocyte lysates (28). Previously, we demonstrated that aggregated α-synuclein inhibited 26 S ubiquitin-dependent proteolysis, with a half-life of DHFR-U greatly increased (>100-fold, from 15 min to ≫180 min) in the presence of 500 nM aggregated α-synuclein as compared with basal conditions (8). We used this same system to investigate whether monomeric β-synuclein and γ-synuclein inhibit ubiquitin-mediated proteasomal degradation (Fig. 3). Degradation of ubiquitinated DHFR-U was examined in the presence of monomeric α-synuclein, β-synuclein, or γ-synuclein. The half-life of DHFR-U was 125 min under basal conditions as well as in the presence of 5 μM monomeric α-synuclein, β-synuclein, or γ-synuclein (Fig. 3). The absence of inhibition indicates that monomeric α-synuclein, β-synuclein, and γ-synuclein have no effect on 26 S ubiquitin-dependent proteolysis. These data suggest that only aggregated α-synuclein can selectively inhibit the 26 S ubiquitin-dependent protein degradation pathway.

β-Synuclein Antagonizes the Inhibition of the 26 S Proteasome Caused by Aggregated α-Synuclein—Because of the high degree of homology between α- and β-synuclein, we hypothesized that β-synuclein might interfere with the binding of aggregated α-synuclein to S6′. To investigate this question, we incubated recombinant β-synuclein with whole HEK-293 cell lysates for 10 min and then incubated reactions with the recombinant aggregated α-synuclein for 10 min. Fluorogenic substrate was added, and the fluorescence was immediately determined and quantified. We observed that the addition of β-synuclein largely blocked inhibition of the 26 S ubiquitin-independent proteasome by aggregated α-synuclein when added before the aggregated α-synuclein (Fig. 4*A*). The β-synuclein was unable to antagonize the proteasomal inhibition induced by aggregated α-synuclein when it was added after the α-synuclein, possibly because the short duration of the assay (10 min) did not allow for a thermodynamic equilibrium to be established. The antagonism by β-synuclein was not due to disaggregation of the α-synuclein, because co-incubation of β-synuclein with aggregated α-synuclein did not change the size of the aggregate, as judged by immunoblot (Fig. 4*C*). This suggests that β-synuclein functions as a negative regulator of α-synuclein.

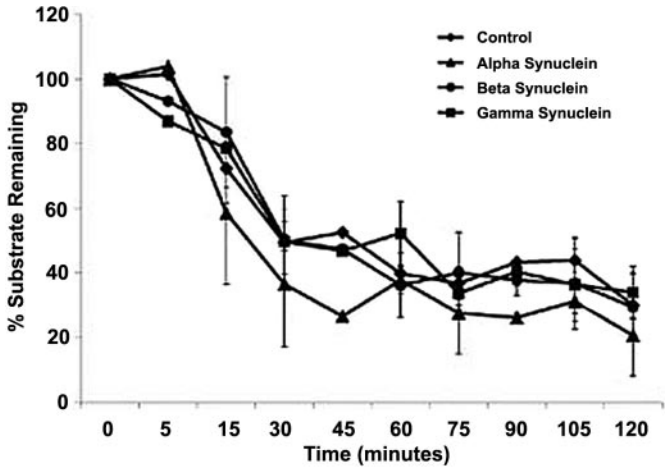

FIG. 3. **There is no effect of monomeric α-, β-, or γ-synuclein on the 26 S ubiquitin-dependent proteasomal activity.** Each sample point was performed in triplicate.

β-Synuclein Does Not Prevent the Inhibition of the 20 S Proteasome Caused by Monomeric γ-Synuclein—Because γ-synuclein is ~50% homologous to β-synuclein, we also asked whether β-synuclein prevented the inhibition of the 20 S ubiquitin-independent proteasomal system by γ-synuclein. Using the same experimental setup as above, we incubated β-synuclein with purified 20 S human proteasomes prior to the addition of γ-synuclein. β-Synuclein did not prevent the inhibition caused by γ-synuclein on the 20 S ubiquitin-independent proteasomal pathway (Fig. 4*B*). The positive control for inhibition was incubation with γ-synuclein alone (Fig. 4*B*). This

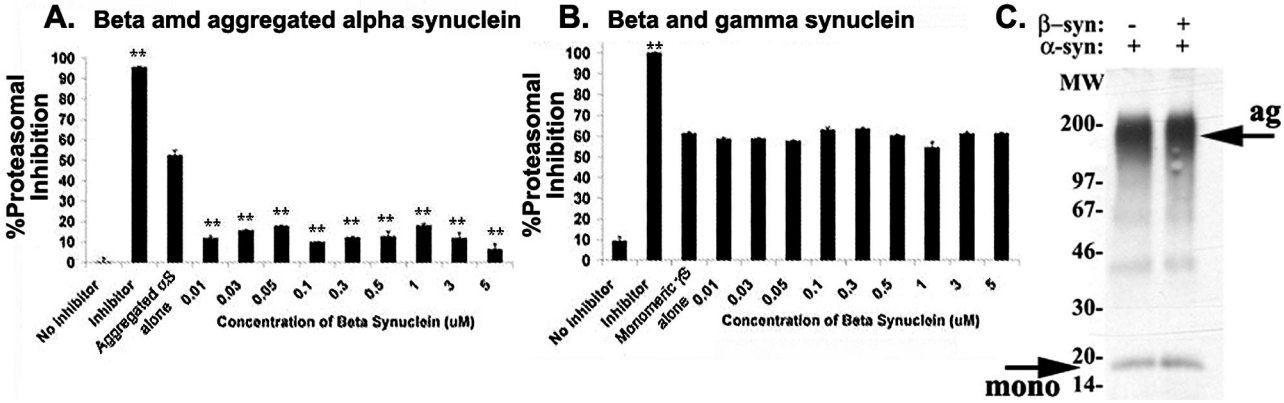

FIG. 4. **Co-incubation of α- and β-synucleins prevents proteasomal inhibition caused by aggregated α-synuclein.** *A*, co-incubating β-synuclein with 5 nM aggregated α-synuclein (α*S*) reduces the inhibition caused by aggregated α-synuclein on the 26 S ubiquitin-independent proteasome. **, $p < 0.0001$ compared with aggregated α-synuclein alone. *B*, co-incubating β-synuclein with 500 nM of monomeric γ-synuclein (γ*S*) has no effect on proteasomal inhibition caused by 500 nM monomeric γ-synuclein on the 20 S ubiquitin-independent proteasome. **, $p < 0.0001$ compared with monomeric γ-synuclein alone. *C*, co-incubating β-synuclein (5 μM) with aggregated α-synuclein (50 nM) for 30 min, which is longer than the typical duration of the proteasomal assays, did not change the aggregation state of α-synuclein as judged by an immunoblot with an anti-α-synuclein antibody (*lower arrow* labeled *mono* points to the size of monomeric α-synuclein, whereas the *upper arrow* labeled *ag* points to the size of aggregated α-synuclein).

suggests that β-synuclein selectively antagonizes the functions of α-synuclein.

α-Synuclein, but Not β-Synuclein, Binds to the 19 S Subunit S6′—We demonstrated previously that monomeric and aggregated α-synuclein interact selectively with the 19 S proteasomal subunit S6′. A sample immunoblot demonstrating this interaction is shown in Fig. 5*A*. To test the selectivity of the association of α-synuclein and S6′, we examined whether α-synuclein binds other components of the 19 S proteasomal cap, namely Rpn 1, Rpn 12, Rpt 1/subunit 7, Rpt 2, Rpt 3/subunit 6b, Rpt 4/subunit 10b, and Rpt 6 (data not shown). No interactions were observed, suggesting that α-synuclein selectively interacts with S6′ (data not shown and Fig. 5*C*) (8). We propose that aggregated α-synuclein mediates its inhibition of the 26 S proteasome via interaction between α-synuclein and S6′.

We have demonstrated that β-synuclein selectively prevents inhibition of the 26 S proteasome by aggregated α-synuclein and does not affect inhibition of the 20 S proteasome caused by γ-synuclein. The antagonism of β-synuclein could be explained by a competition of β-synuclein with α-synuclein for binding to S6′ or by direct binding of β-synuclein to α-synuclein. To test whether β-synuclein interacts with S6′, we incubated His-tagged recombinant β-synuclein overnight with human control cingulated cortex and then precipitated out the His-tagged protein with nickel-agarose, pulling down the His-tagged protein and other proteins attached to β-synuclein. The precipitates were immunoblotted for S6′ (Fig. 5*A*, *top*) and the histidine tag (Fig. 5*A*, *bottom*). S6′ was readily detected in the lysate but not in the pull-down analysis, which indicates that S6′ and β-synuclein do not interact.

β-Synuclein Interacts with α-Synuclein in the Human Brain—Next, we tested whether β-synuclein binds directly to α-synuclein and whether this binding prevents α-synuclein from interacting with S6′. A previous study by Hashimoto *et al.* demonstrated that α-synuclein and β-synuclein co-immunoprecipitate from brains of transgenic mice or transfected cell lines overexpressing both α- and β-synuclein (21). To test whether α-synuclein and β-synuclein interact in the human brain, we added recombinant His-tagged β-synuclein to neurologically normal (control) human brain lysates, pulled down β-synuclein from the human control brain, and immunoblotted for α-synuclein (Fig. 5*B*, *top*). We confirmed pull-down of the His-tagged β-synuclein by immunoblot analysis for the His tag (Fig. 5*B*, *bottom*). These data confirm that α-synuclein and β-synuclein interact in the human cingulated cortex. This interaction provides a mechanism by which β-synuclein might negatively regulate α-synuclein function.

β-Synuclein Competes with S6′ for Binding to α-Synuclein in the Human Brain—Because β-synuclein binds α-synuclein, we hypothesized that β-synuclein might compete with S6′ for binding to α-synuclein. To test this possibility, α-synuclein (His-tagged) was incubated with control human cortex with or without β-synuclein (no His tag) (Fig. 5*C*, *top*). Nickel-agarose was used to pull down His-tagged α-synuclein, and immunoblot analysis was performed. S6′ was detected in the pull-down of α-synuclein (Fig. 5*C*, *top*, *lane 2*) and in the lysate (Fig. 5*C*, *top*, *lane 4*). However, S6′ was not detected in the sample containing α-synuclein and β-synuclein together (Fig. 5*C*, Fig. 5*C*, *top*, *lane 1*) nor in the sample containing only nickel-agarose (Fig. 5*C*, *top*, *lane3*). Immunoblotting with an anti-His antibody confirmed that α-synuclein was successfully pulled down in each sample containing His-α-synuclein (Fig. 5*C*, *bottom*). These data suggest that β-synuclein negatively regulates the interaction of α-synuclein with the proteasome in the human brain.

DISCUSSION

The high degree of homology between α-, β-, and γ-synucleins suggests that their functions are also homologous, but a clear understanding of how the functions of these three proteins might relate is lacking. In this paper we use the proteasome as a target to investigate how the actions of the three synucleins impact on this particular organelle. We observed that α- and γ-synuclein directly inhibit ubiquitin-independent proteasomal function. α-Synuclein inhibits the 26 S proteasome, possibly by binding to S6′, a member of the 19 S subunit. γ-Synuclein inhibits that 20 S proteasome through a binding site that remains undefined. β-Synuclein does not appear to bind directly to the proteasome but does antagonize the actions of α-synuclein by binding α-synuclein and preventing its interaction with the 19 S protein S6′. These results identify a biological organelle, the proteasome, which is modulated by all three synucleins and allows study of how the actions of the synuclein homologues might integrate.

Proteasomal inhibition by γ-synuclein contrasts sharply with proteasomal inhibition by α-synuclein. Monomeric γ-synuclein potently inhibits proteasomal activity, but potent inhibition of

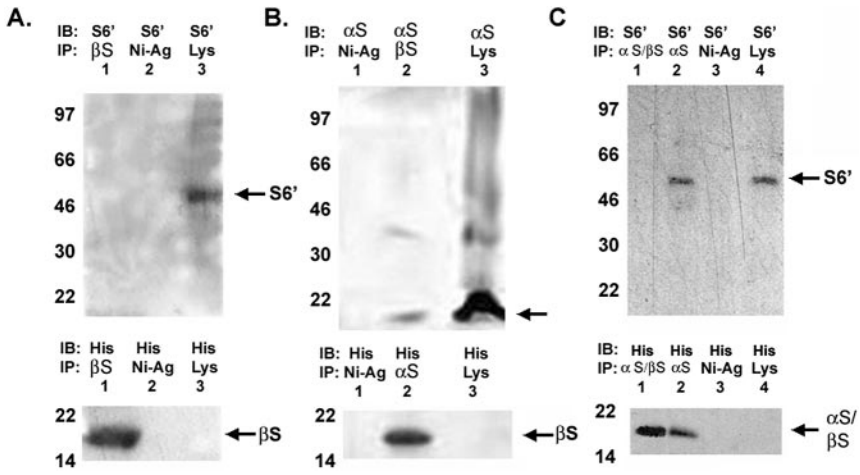

FIG. 5. **Co-incubation with β-synuclein inhibits association of α-synuclein and the proteasomal subunit S6′.** *A*, *top,* immunoblot of S6′ of a nickel-agarose pull-down of His-tagged β-synuclein (*βS*) failed to detect S6′ (*lane 1*), but S6′ was detected in the lysates (*Lys*) (*lane 3*). Nickel-agarose (*Ni-Ag*) was also blank. *Bottom*, reprobing of above immunoblot with anti-His antibody (*His*) detects the His-tagged β-synuclein (*lane 1*). *B*, *top*, nickel-agarose (*Ni-Ag*) pull-down of His-tagged β-synuclein (*βS*) detects binding to α-synuclein (*αS*) in the human control cortex (*lane 2*). α-Synuclein is also detected in the lysates (*Lys*) (*lane 3*). *Bottom*, reprobing of the above immunoblot with anti-His antibody (*His*) detects the His-tagged β-synuclein (*βS*) (*lane 2*). *C*, *top,* co-incubation of monomeric α- and β-synuclein (*αS/βS*) inhibits binding of α-synuclein to S6′ (*lane 1*) as compared with α-synuclein alone (*αS*) (*lane 2*). Immunoblot of S6′. S6′ is also detected in the lysates (*Lys*) (*lane 3*). *Bottom*, reprobing of above immunoblot with anti-His antibody (*His*) detects the His-tagged α-synuclein.

proteasomal activity by α-synuclein requires the protein to be aggregated. The importance of aggregation for proteasomal inhibition by α-synuclein raises the possibility that dimerization or oligomerization of α-synuclein contributes to its biological function. The two synucleins also differ in the site of proteasomal inhibition. α-Synuclein binds to S6′, a protein in the 19 S cap, and inhibits the 26 S proteasome, whereas γ-synuclein inhibits the 20 S proteasome through a binding site that remains to be defined. One characteristic that α- and γ-synucleins share in common is that both types of monomeric protein inhibit ubiquitin-independent proteasomal function but do not inhibit ubiquitin-dependent proteasomal function. The mechanism for restriction of the inhibition to the ubiquitin independent cascade remains to be determined and is surprising, because both the 20 S proteasome and the 19 S cap are required for ubiquitin-dependent proteasomal function.

The ubiquitin-dependent and ubiquitin-independent proteasomal activities have very different functions in the cell. The 20 S ubiquitin-independent proteasomal pathway is responsible for degradation of 70–80% of all mildly oxidized proteins and functions as a degradation mechanism with very broad specificity (30, 31). Little is known about the function of the 26 S ubiquitin-independent proteasomal system, because the only protein known to be degraded by this pathway is ornithine decarboxylase. The 26 S ubiquitin-dependent pathway is the best studied pathway and plays an important role in the regulated destruction of polyubiquitinated proteins. The levels of many proteins are regulated by polyubiquitination and the subsequent degradation by the 26 S ubiquitin-dependent proteasomal system. This ubiquitin-dependent degradation requires the S6′ protein, one of several proteins in the cap that sits over the 20 S organelle and enables degradation of ubiquitinated proteins. Two types of caps attach to the 20 S proteasome. The 19 S proteasomal cap combines with the 20 S proteasome to make the classic 26 S proteasome, whose role in degradation of ubiquitinated proteins is well documented. The 11 S proteasomal cap has been identified more recently (32). This smaller cap is termed the COP9 signalosome and uses proteins that are also present in the 19 S cap (33). COP9 participates in signal transduction mediated by the SCF and other cullin-based ubiquitin ligases (33).

β-Synuclein appears to modulate proteasomal function by indirect mechanisms. Monomeric β-synuclein has little or no affect on the 20 S ubiquitin-independent, 26 S ubiquitin-independent, and 26 S ubiquitin-dependent proteasomal activities *in vitro*. The ability of β-synuclein to bind α-synuclein raised the possibility that β-synuclein was able to prevent the inhibition of the 26 S ubiquitin-independent proteasome by aggregated α-synuclein *in vitro*. Consistent with this hypothesis, we observed that β-synuclein prevented the inhibition of aggregated α-synuclein on the 26 S ubiquitin-independent proteasome at very low concentrations (concentrations of >10 nM). When the addition of β-synuclein preceded that of aggregated α-synuclein, we did not observe inhibition. These results suggest that proteasomal inhibition by aggregated α-synuclein is not rapidly reversible by β-synuclein. However, we cannot rule out that the slow on-off kinetics of the proteins might preclude observing competition between α- and β-synuclein, because the 10-min incubation time might be insufficient for a thermodynamic equilibrium to be established. Further studies suggested that the biochemical basis for the antagonism was linked to direct binding of α-and β-synucleins. Hashimoto *et al.* demonstrated previously by immunoprecipitation assays that α-synuclein and β-synuclein associate with one another when both proteins are overexpressed in tissue culture or *in vivo* in doubly transgenic mice (21). Our studies confirmed this observation and demonstrated that β-synuclein interacts with α-synuclein in the human brain. These results suggest a hypothesis that β-synuclein prevents aggregated α-synuclein from inhibiting the 26 S proteasome by competing with S6′ for binding to α-synuclein. At this point in time we do not know whether β-synuclein acts simply by binding α-synuclein or by binding directly to S6′, although our initial studies failed to detect binding of β-synuclein to S6′. This suggests that β-synuclein may be a natural negative regulator of α-synuclein function. However, these studies cannot exclude the possibility that β-synuclein might also interact with a proteasomal subunit other than S6′.

β-Synuclein has 78% homology to α-synuclein, but the function of β-synuclein remains unclear (19). No physiological activity has yet been identified for the protein. Previous research has noted that β-synuclein might have utility as a therapeutic

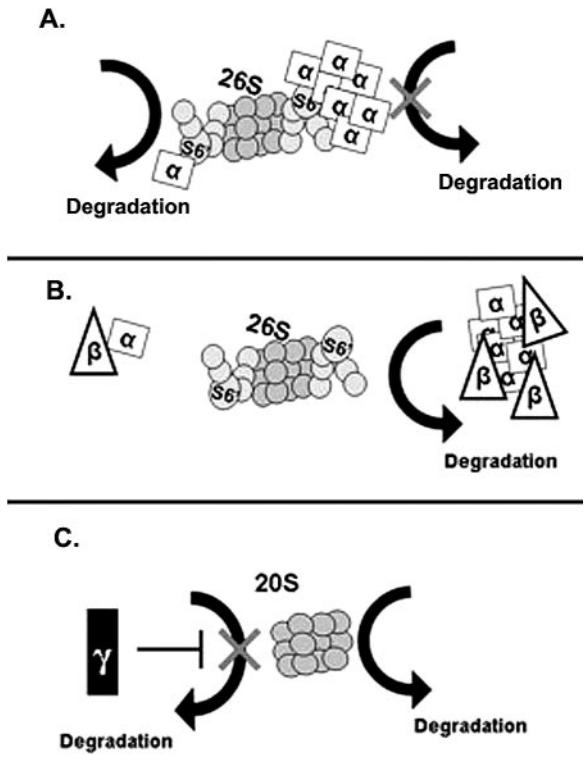

FIG. 6. **Hypothetical mechanism of action of the synucleins at the proteasome.** *A*, both monomeric and aggregated α-synuclein bind S6′, but binding of aggregated α-synuclein might inhibit activity by sterically inhibiting access of substrates to the proteasome. *B*, β-synuclein binds α-synuclein and prevents its interaction with S6′. β-Synuclein also prevents binding of the aggregated α-synuclein to the proteasome, thereby antagonizing the inhibition of aggregated α-synuclein on the 26 S proteasome. *C*, γ-synuclein binds and inhibits the 20 S proteasome.

strategy for inhibiting the aggregation of α-synuclein (19, 21). Our observations extend this concept by demonstrating that the binding of β-synuclein to aggregated α-synuclein can antagonize the actions of aggregated α-synuclein. The studies above demonstrate that β-synuclein prevents the potent proteasomal inhibition mediated by aggregated α-synuclein. β-Synuclein is unlikely to be acting on aggregated α-synuclein by disaggregating the large SDS-resistant α-synuclein aggregates, because the β-synuclein was added to the aggregated α-synuclein just 10 min prior to the assay. Gel electrophoresis performed on the α/β mixture up to 1 h after the addition of β-synuclein suggests that β-synuclein does not disaggregate aggregated α-synuclein (data not shown). The alternative explanation for the antagonism by β-synuclein is that the binding of β-synuclein prevents the binding of aggregated α-synuclein to the proteasome. Consistent with this hypothesis, we observed that β-synuclein was unable to antagonize proteasomal inhibition cause by aggregated α-synuclein under conditions where aggregated α-synuclein was pre-incubated with a proteasome mixture (data not shown); the prior addition of aggregated α-synuclein allows the α-synuclein to bind to S6′ before binding to β-synuclein.

Several lines of evidence support the idea that β-synuclein is a natural negative regulator of α-synuclein. α-Synuclein and β-synuclein have been shown to interact with one another *in vitro* and *in vivo* (21). β-Synuclein inhibits the aggregation of α-synuclein *in vitro*, and peptides derived from the sequence of β-synuclein inhibit the aggregation of α-synuclein (19, 21). We demonstrate in this paper that β-synuclein interacts with α-synuclein and can prevent α-synuclein from binding to the human proteasomal subunit, S6′. Studies using transgenic mice also show evidence that β-synuclein inhibits the aggregation of α-synuclein. Transgenic mice overexpressing α-synuclein display Lewy body-like inclusions and develop motor deficits; double α-synuclein and β-synuclein transgenic mice have a 40% reduction of Lewy body-like inclusion formation, and the functional deficits are prevented (34). Studies of the distribution of expression of α-synuclein and β-synuclein also suggest that regulation of the two proteins is linked. α-Synuclein and β-synuclein exhibit similar distributions (35). Although β-synuclein is the predominate RNA message, α-synuclein is the predominate protein in the brain (20). The high expression of β-synuclein RNA message suggests that translation of β-synuclein can be readily up-regulated when necessary for regulation of α-synuclein. However, proof that α and β-synuclein interact *in vivo* would require demonstration by a sensitive method such as fluorescence resonance energy transfer. The idea that α-synuclein and β- synuclein are antagonistic is strengthened by studies demonstrating that in cases of dementia with Lewy bodies, α-synuclein accumulates in locations where β-synuclein is undetectable (20, 36). The multiple cases of opposing actions or opposing expression suggest that β-synuclein might be a natural negative regulator of α-synuclein function.

Based on the studies presented in this manuscript and available in the literature, we suggest an integrated model describing how α-synuclein, β-synuclein, and γ-synuclein regulate proteasomal activity (Fig. 6). We propose that α-synuclein acts as a chaperone to regulate 26 S proteasome degradation via interactions with S6′. This proposal is based on the data showing that monomeric α-synuclein binds selectively to S6′ and that S6′ functions to tether polyubiquitinated proteins to the 26 S proteasome. Age, toxins, or other agents may stimulate α-synuclein aggregation. Aggregated α-synuclein inhibits the 26 S proteasome. Based on the data presented above, we hypothesize that β-synuclein functions as a negative regulator of α-synuclein at the 26 S proteasome (Fig. 6*A*). In this model, β-synuclein is able to modulate α-synuclein binding to S6′, which determines the degree of activation of 26 S ubiquitin-independent function (Fig. 6*B*). Based on our observations, we also hypothesize that γ-synuclein regulates 20 S degradation (Fig. 6*C*). Whether this regulation is by a direct binding of γ-synuclein to the 20 S core or an indirect mechanism remains to be determined. The ability of γ-synuclein to modulate 20 S function suggests a role for γ-synuclein in the cellular response to oxidative stress, because the 20 S ubiquitin-independent proteasomal pathway is responsible for degradation of 70–80% of all mildly oxidized proteins (30, 31). Further studies need to be done to confirm binding between γ-synuclein and the 20 S proteasome.

These results have several implications for PD. Results from several groups, including our own, suggest that aggregation of α-synuclein in PD causes proteasomal inhibition. High doses of proteasomal inhibition are known to be toxic. However, some recent papers have also questioned whether α-synuclein inhibits the proteasomal *in vivo*. Sawada *et al.* suggest that low level proteasomal inhibition could be protective (perhaps by enhancing the stress/heat shock response), and Martìn-Clemente *et al.* show that elevated levels of α-synuclein do not inhibit the proteasome *in vivo* (18, 37). However, the article by Martìn-Clemente *et al.* did not address whether aggregated α-synuclein might inhibit the proteasome (37). β-Synuclein might function to moderate the proteasomal inhibition caused by aggregated α-synuclein and thereby limit proteasomal inhibition. Antagonism of proteasomal inhibition by β-synuclein supports use of β-synuclein- or β-synuclein-derived peptides for treatments of synucleinopathies.

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