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13. ABSTRACT *(Maximum 200 Words)*

α-Synuclein is a protein that aggregates to form pathological structures, termed Lewy bodies, in the brains of patients with Parkinson's disease (PD). Aggregation of α-synuclein is thought to be harmful to neurons, and mutations that increase the tendency of α-synuclein to aggregate are associated with familial PD. The aim of this proposal is to understand factors that stimulate or inhibit α-synuclein aggregation, and the methods focus on metals, which our preliminary data suggests modulates α-synuclein aggregation. During the first year of this proposal we characterized the affects of eight metal ions on α-synuclein aggregation in vitro: iron (II and III), copper (II), Nickel (II), Manganese (II), Magnesium (II), Zinc (II), Calcium (II, not a metal, but tested) and Aluminum (III). We observed three classes of interaction based on conformational changes and aggregation. Iron and copper induced α-synuclein aggregation. Magnesium, Calcium and Zinc caused similar conformational changes; magnesium inhibited α-synuclein aggregation, zinc caused dimerization and calcium caused only conformational changes. The other metals had no effect. Iron (II), Copper and Magnesium exerted similar effects in neurons grown in cell culture, suggesting that magnesium might be useful in preventing the pathology of PD.

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Progress Report: DAMD17-01-1-0781
Mechanisms of α-synuclein aggregation and toxicity

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Introduction

The goal of this grant proposal is to investigate the interaction of α-synuclein with metals. Our preliminary studies suggested that metals, such as iron, might promote synuclein aggregation. In this proposal, we sought to understand which metals interact with α-synuclein, how these metals might modulate α-synuclein aggregation, and how they might affect the interaction with other proteins relevant to Parkinson's disease. The work aims for the first year were to investigate the biochemical interaction of iron with α-synuclein *in vitro*, examining the pH dependence, stoichiometry, sequence requirements and affects of iron on α-synuclein in neurons.

Body:

Work from Aim 1 that was Accomplished

The results of the work proposed in Aim 1 are described in articles 1, 2 and 5 of the appendix (1, 2). We first characterized α-synuclein by UV spectroscopy, and observed that binding of α-synuclein to metals could be monitored by UV spectroscopy. The UV spectrum of α-synuclein exhibits a peak at 310 and a second peak at 345.

In aim I we proposed to determine the pH dependence of iron binding with α-synuclein, the stoichiometry of iron binding to α-synuclein, the sequence requirements and the role of metal binding in neurons. Much of our progress is described in the article in appendix 1 and 5 (1, 3). We were able to use UV fluorescence spectroscopy to determine the affinity of iron for α-synuclein, which is described in reference 1. We also determined the pH dependence, and determined the sequence requirements. The sequence requirements were not included in the Golts paper (1), but reside largely at the C-terminus. Deletion of the C-terminal 37 residues reduces the affinity for iron over 4-fold.

We also had proposed to examine α-synuclein by electrospray ionization mass spectroscopy to examine the stoichiometry. We successfully performed electrospray, in collaboration with Sandy Markey, at NIH, but the results were erratic in that often no protein signal was detected (Appendix 7). In the interim, Loyola has purchased a SELDI mass spectrometer, and we have chosen to continue to pursue this work at Loyola because SELDI is better at analyzing large proteins than electrospray ionization mass spectroscopy. This work continues. We also had intended to measure the affinity by equilibrium dialysis. Although we attempted to do this, both in our laboratory and in collaboration with Ashley Bush (at Harvard, Boston), we were unable to do so. The reason we were unable to use equlibrium dialysis is that α-synuclein binds so avidly to dialysis tubing, that virtually all of the α-synuclein is lost during equilibrium dialysis experiments. Since these experiments require careful quantification, the extensive loss of α-synuclein renders the data meaningless. Given that we have determined affinity by UV spectroscopy, we have not pursued the dialysis experiments further.

We also successfully investigated how each metal affects α-synuclein aggregation. We observed that iron and copper both induce α-synuclein aggregation. Zinc, in contrast, induces dimerization of α-synuclein but not aggregation. None of the other metals,

magnesium, calcium, manganese, nickel or aluminum, affect α-synuclein aggregation on their own. This data is described in references 1 and 2.

Work relevant to Aims 2 and 3:

In aim 2, we proposed to investigate how different metals might interact to affect α-synuclein aggregation. We identified 3 classes of binding states for the interaction of metals with α-synuclein. Class I consists of the redox active metals, Fe(II) and Cu(II), which reduce fluorescence at both 310 and 345 nm. Class II consists of Mg(II), Zn(II) and Ca(II), which increase fluorescence at 345 nm but do not affect fluorescence at 310 nm. Class III consists of Ni(II), Mn(II) and Al(III), which do not affect the fluorescence of α-synuclein.

We studied the interaction of each of the metals in classes I and II. In the course of this work, we made the striking observation that Magnesium abolishes aggregation induced by either Fe(II) or Cu(III). In aim 2, we also proposed to examine aggregation in cell culture, and observed that magnesium prevents α-synuclein aggregation in neuronal cell lines following induction by iron (appendix 5) (3). We also have looked another model for α-synuclein aggregation, rotenone toxicity, and observed that magnesium also prevents rotenone-induced α-synuclein aggregation.

Work on Aim 3 will require that we develop a colony of transgenic mice over-expressing mutant α-synuclein. We have made significant progress towards this aim because we have acquired A30P α-synuclein mice from Philipp Kahle and Christian Haass at the University of Munich, in Germany. We currently have these mice at Loyola and are expanding the colony. Having this colony will allow us to examine α-synuclein aggregation in primary neurons grown in cell culture, as proposed in Aim 3.

Unanticipated Discoveries Relevant to this Grant Proposal:

During the past year we have made a number of discoveries and advances that are pertinent both to this grant proposal and towards understanding the pathophysiology of Parkinson's disease.

1. We have been able to over-express A53T α-synuclein in C. Elegans, and we have shown that these transgenic worms exhibit increased vulnerability to rotenone-induced toxicity, compared to non-transgenic worms or worms over-expressing β-synuclein, a close homologue of α-synuclein (appendix 9). Development of this worm is important because this represents a simple animal model for studying α-synuclein aggregation and toxicity. Use of this worm enables rapid analysis of putative therapeutic agents for PD, and also biochemical and genetic analysis of α-synuclein aggregation.
2. In years 2 and 3, we proposed to examine the interaction of α-synuclein with other proteins, such as protein kinase C, which binds α-synuclein. We have recently shown that α-synuclein binds to the proteasomal protein S6', and that aggregated α-synuclein inhibits both ubiquitin-dependent and ubiquitin independent proteasomal activity very potently (IC50 = 10 nM) (appendix 6).

This observation is very important because it provides a direct link between α-synuclein aggregation (such as occurs in PD) and toxicity. Proteasomal proteasomal inhibition is known to be toxic to cells, and is hypothesized to contribute to the mechanism of neurodegeneration in many diseases (4, 5). This work could explain why α-synuclein aggregation is toxic. In addition, we hypothesize that binding to S6' represents a general mechanism of toxicity of ubiquitinated protein aggregates.

3. We have discovered that another protein relevant to PD, termed parkin, also aggregates readily. This is important for several reasons. First, we and others have shown that parkin accumulates in Lewy bodies in PD. This suggests that parkin might act like α-synuclein to induced Lewy body formation and neurodegeneration. The link between parkin and PD is particularly compelling because there is a particular mutation in parkin, at R275W, that causes late onset Parkinson's disease (in contrast, other mutations cause a disease termed ARJP that exhibits loss of the same neurons as in PD, but none of the pathology seen in PD). Why does R275W cause PD? The answer is surprising. We have shown that the mutation causes parkin to aggregate spontaneously, much like the A53T mutation causes α-synuclein to aggregate. This suggests that transgenic animals over-expressing R275W parkin might provide a novel animal model for PD. To test this, we are generating transgenic mice over-expressing WT parkin or R275W parkin. We have also generated plasmids to study WT and R275W parkin in C. Elegans, and are in the process of creating transgenic worms containing these parkin constructs.

An interesting aspect of the parkin work relates to the interaction between parkin and α-synuclein. Both proteins co-exist in Lewy bodies. Our preliminary data provide the intriguing observation that parkin aggregation is reduced in cells over-expressing α-synuclein. This suggests that an endogenous function of α-synuclein might be to act as a protein chaperone, and prevent aggregation of proteins such as parkin. This hypothesis is consistent with our prior observation that α-synuclein is homologous to another protein chaperone, 14-3-3.

Key Research Accomplishments:

- Determined affinity and pH dependence of iron for α-synuclein
- Determined affinity of Cu(II), Mg(II), Zn(II) and Ca(II) for α-synuclein, and determined that Ni(II), Mn(II) and Al(III) do not bind α-synuclein.
- Performed electrospray ionization mass spectroscopy of α-synuclein.
- Demonstrated that iron causes α-synuclein aggregation in neurons, and that α-synuclein increases the vulnerability to iron of neurons.
- Demonstrated that magnesium inhibits α-synuclein aggregation and protects neurons against iron-mediated toxicity.
- Demonstrated that α-synuclein binds the proteasomal protein S6'.

- Developed an additional model for α-synuclein toxicity and aggregation using C. Elegans over-expressing α-synuclein.

Reportable Outcomes:
The research performed this year has resulted in publication of 4 articles relevant to α-synuclein. These articles are listed below, and are provided in the appendices.

Articles relevant to α-synuclein and the DAMD grant award that were published in last year:

1. Golts, N., Snyder, H., Frasier, M., Theisler, C., Choi, P., **Wolozin, B**. Magnesium inhibits spontaneous and iron-induced aggregation of alpha –synuclein. J. Biol. Chem. 277:16116-23 (2002).

2. **Wolozin, B**. and Golts, N., Synuclein, iron and Parkinson's disease, The Neuroscientist. 8(1): 22-32 (2002).

3. Ahn, B. H., Rhim, H., Kim, S. Y., Sung, Y. M., Lee, M. Y., Choi, J. Y., **Wolozin, B.**, Chang, J. S., Lee, Y. H., Kwon, T. K., Chung, K. C., Yoon, S. H., Hahn, S. J., Kim, M. S., Jo, Y. H., and Min, D. S. Alpha-synuclein interacts with phospholipase D isozymes and inhibits pervanadate induced phospholipase D activation in human embryonic kidney 293 cells. J Biol Chem. 277(14):12334-42 (2002).

4. Choi, P., Golts, N., Snyder, H., Petrucelli, L., Chong, M., Hardy, J.,Sparkman, D., Cochran, E., Lee, J.M., **Wolozin, B.,** Co-association of parkin and α-synuclein, NeuroReport. 12: 2839-45 (2001).

Conclusions:
Significance: Our results clearly demonstrate that redox active metals can induce α-synuclein aggregation both in vitro and in cell culture. This is important because it suggests that metals could play an important in stimulating the pathology in PD. Conversely, chelating metal could be protective against PD. A similar strategy has been shown to be effective in preventing formation of Alzheimer type pathology in an animal model of Alzheimer's disease, and is now in clinical trials for treatment of Alzheimer's disease (6).

Our results also show that magnesium inhibits α-synuclein aggregation, which suggests that magnesium might be useful in treating PD. Magnesium therapy poses a challenge for clinical application because it is unclear how much reaches the brain, and it can cause gastrointestinal distress. However, magnesium therapy is already used clinically to treat patients with preeclampsia during pregnancy.

Recommended changes: 1.) I would like permission to include S6' as one of the proteins that I study as part of this proposal (Aim 2, parts 2, 4 and 5 on the Statement of Work). I believe that the discovery that α-synuclein binds S6' represents an important discovery, and that it is worthwhile to add study of S6' to our protocol. The reason study

of the interaction of S6' with α-synuclein is important is that S6' appears to be directly related to the neuronal injury in PD, and development of inhibitors of synuclein binding to S6' could be a potential avenue of therapy. 2.) I would also like to investigate α-synuclein aggregation in C. Elegans, as a simple *in vivo* model. In Aim 4, studies in C. Elegans offer elegant methods for using genetics to determine the role of mitochondrial dysfunction in α-synuclein toxicity.

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1. Golts, N., Snyder, H., Frasier, M., Theisler, C., Choi, P., and Wolozin, B., Magnesium inhibits spontaneous and iron-induced aggregation of alpha-synuclein, *J Biol Chem, 277*, 16116 (2002).
2. Wolozin, B., and Golts, N., Iron and Parkinson's disease, *Neuroscientist, 8*, 22 (2002).
3. Golts, N., Snyder, H., Frasier, M., Theisler, C., Choi, P., and Wolozin, B., Magnesium inhibits the aggregation of α-synuclein in cultured cells, *J. Alz. Dis., (submitted)* (2002).
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5. Bence, N. F., Sampat, R. M., and Kopito, R. R., Impairment of the ubiquitin-proteasome system by protein aggregation, *Science, 292*, 1552 (2001).
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Appendices:

Articles relevant to α-synuclein and the DAMD grant award that were published in last year:

1. Golts, N., Snyder, H., Frasier, M., Theisler, C., Choi, P., **Wolozin, B**. Magnesium inhibits spontaneous and iron-induced aggregation of alpha –synuclein. J. Biol. Chem. 277:16116-23 (2002).

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Articles in Review:

5. Golts, N., Snyder, H., Frasier, M., Theisler, C., Choi, P., **Wolozin, B.**, Magnesium inhibits aggregation of α-synuclein in cultured cells.

6. Snyder, H., Mensah, K., Theisler, C., Matouschek, A. and **Wolozin, B.**, Aggregated and Monomeric α-Synuclein bind to the S6' Proteasomal Protein and Inhibit Proteasomal Function.

Work in progress:

7. Electrospray ionization mass spectroscopy of α-synuclein. A. pH 5.5, B. pH 3.5.
8. Spontaneous aggregation of Parkin R275W.
9. Development of a C. Elegans model of α-synuclein toxicity: rotenone and metal toxicity studies.

Appendix 1

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Magnesium Inhibits Spontaneous and Iron-induced Aggregation of α-Synuclein*

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Multiple studies implicate metals in the pathophysiology of neurodegenerative diseases. Disturbances in brain iron metabolism are linked with synucleinopathies. For example, in Parkinson's disease, iron levels are increased and magnesium levels are reduced in the brains of patients. To understand how changes in iron and magnesium might affect the pathophysiology of Parkinson's disease, we investigated binding of iron to α-synuclein, which accumulates in Lewy bodies. Using fluorescence of the four tyrosines in α-synuclein as indicators of metal-related conformational changes in α-synuclein, we show that iron and magnesium both interact with α-synuclein. α-Synuclein exhibits fluorescence peaks at 310 and 375 nm. Iron lowers both fluorescence peaks, while magnesium increases the fluorescence peak only at 375 nm, which suggests that magnesium affects the conformation of α-synuclein differently than iron. Consistent with this hypothesis, we also observe that magnesium inhibits α-synuclein aggregation, measured by immunoblot, cellulose acetate filtration, or thioflavine-T fluorescence. In each of these studies, iron increases α-synuclein aggregation, while magnesium at concentrations >0.75 mM inhibits the aggregation of α-synuclein induced either spontaneously or by incubation with iron. These data suggest that the conformation of α-synuclein can be modulated by metals, with iron promoting aggregation and magnesium inhibiting aggregation.

Parkinson's disease (PD)[1] is a common motor disorder that affects about 1% of population over the age of 65 (1). The disease is characterized by progressive neurodegeneration predominantly affecting dopaminergic neurons in the nigrostriatal system (2). The degenerating neurons develop intracellular inclusions, termed Lewy bodies, which are composed of a dense core of filamentous and granular material (3). Recent studies indicate that α-synuclein is a major filamentous component of Lewy bodies (3, 4). Genetic studies suggest that α-synuclein plays a key role in the pathophysiology of PD, because mutations in α-synuclein, at A53T or A30P, are associated with early-onset familial PD (5, 6).

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[1] The abbreviation used is: PD, Parkinson's disease.

The accumulation of aggregated protein underlies the pathophysiology of many neurodegenerative disorders, and increasing evidence suggests that aggregated α-synuclein plays a key role in the pathophysiology of PD. α-Synuclein has a strong tendency to aggregate and does so spontaneously *in vitro* at a slow rate (7–9). Both the A53T and the A30P mutations in PD increase the tendency of α-synuclein to aggregate. Many studies in cultured neurons, and some studies in transgenic animals, suggest that α-synuclein aggregation is linked to cellular toxicity and neurodegeneration (10–12). In cell culture, formation of α-synuclein aggregates correlates with cell injury (10). Overexpressing α-synuclein in *Drosophila* leads to an age-dependent accumulation of aggregated α-synuclein and associated neurodegeneration (12). Masliah and colleagues also observed that aggregated α-synuclein is associated with loss of markers in dopaminergic neurons, although other studies of α-synuclein overexpression in transgenic mice have been less conclusive (11, 13, 14). Thus, increasing lines of evidence suggest that aggregation of α-synuclein is associated with the degeneration of dopaminergic neurons and suggest that α-synuclein contributes to the neurodegenerative processes occurring in PD.

Recombinant α-synuclein aggregates spontaneously following prolonged incubation *in vitro*. Recently, we and others have shown that α-synuclein also aggregates rapidly following exposure to Fe(II) (10, 15). *In vitro*, Fe(II) accelerates the rate of α-synuclein aggregation. For example, similar amounts of aggregation are induced *in vitro* by incubating 23 μM α-synuclein alone for 30 days or with 50 μM $FeCl_2$ for only 3 days, suggesting that 50 μM Fe(II) increases the rate of α-synuclein aggregation about 10-fold (see discussion below). These observations suggest that interaction with iron could greatly accelerate α-synuclein aggregation.

The factors regulating α-synuclein aggregation in the brain are poorly understood. Some studies suggest that neurotoxins, such as the pesticide rotenone or paraquat, stimulate α-synuclein aggregation (16). The involvement of metals in PD suggests that metals might also play a role in the aggregation of α-synuclein and the pathophysiology of PD. Epidemiological studies have shown that exposure to metals is associated with PD. For instance, individuals with industrial exposure to iron, copper, and/or lead have high rates of PD (17). Neuropathological studies show that synucleinopathies are generally associated with iron accumulation, which is consistent with a pathological link between iron and α-synuclein (18). Brains from patients with PD, type I iron storage disease (Hallorvorden-Spatz disorder), and multiple systems atrophy all show increased iron content (19). In PD the levels of iron are increased over controls, and Fe(II) has been identified as a major component of Lewy bodies (20–26). How iron contributes to Lewy body formation and the pathophysiology of PD, though, is not

understood. In the experiments described below, we examine the regulation of α-synuclein aggregation using both spontaneous and iron-induced α-synuclein aggregation *in vitro* and show contrasting actions of iron and magnesium on α-synuclein aggregation. These studies have important implications for the pathophysiology of PD and other synucleionopathies.

EXPERIMENTAL PROCEDURES

Materials—α-Synuclein (wild-type, A53T, and A30P) was cloned into the *NcoI*/*NotI* site of the Pro-Ex His 6 vector (Invitrogen). To generate recombinant α-synuclein, BPer (Pierce) reagent was used to solubilize the recombinant α-synuclein from the isopropyl-1-thio-β-D-galactopyranoside-induced bacterial lysates, which were then passed over a nickel-agarose column for purification. All spectrophotometric analysis were repeated three to five times.

Immunoblotting—Cells were harvested with SDS lysis solution (2% SDS, 10 mM Tris, pH 7.4, 2 mM β-glycerol phosphate, 1 μM AEBSF). The amount of protein was determined using the BCA assay (Pierce), 5–30 μg per lane was run on 14% SDS-polyacrylamide gels and transferred to nitrocellulose (200 mA, 12 h). The nitrocellulose was then incubated 1 h in 5% I-block (Tropix)/phosphate-buffered saline, washed, incubated overnight in primary antibody, washed, then incubated 3 h in peroxidase-coupled secondary antibody and developed with chemiluminescent reagent (PerkinElmer Life Sciences).

Thioflavine-T Measurements—For analysis of aggregation using thioflavine-T, 23 nM α-synuclein was incubated in 10 μM thioflavine-T (in 50 mM glycine, pH 8.5) and measured by fluorescence (λ_{ex} = 440, λ_{em} = 450–600 nm).

Cellulose Acetate Assay—To analyze aggregation of α-synuclein by filtration, samples were diluted into 100 μl of water, filtered through cellulose acetate (0.2 μm pore size), washed with 200 μl phosphate-buffered saline, and then immunoblotted as described above.

RESULTS

Iron Quenches Tyrosine Fluorescence of α-Synuclein: Evidence of Association—To understand factors regulating α-synuclein aggregation, we investigated the interaction of different metals with α-synuclein using tyrosine fluorescence (27, 28). Tyrosine fluorescence has been used to monitor the association of various metals with a number of proteins, including Aβ, α-transducin and, more recently, α-synuclein (27–29). In these studies tyrosine fluorescence is used as an indicator of changes in protein conformation or binding of metals. Exciting tyrosine at 280 nm elicits fluorescence that peaks at 310 nm for monomeric tyrosine and at 350–400 nm for tyrosinate (30). The fluorescence spectrum of α-synuclein yielded fluorescence peaks at 310 and 375 nm (Fig. 1, *A* and *B*). Tyrosinate reactivity occurs when the phenolic hydroxyl group of tyrosine forms hydrogen bonds with carboxyl groups in nearby aspartates or glutamates. The fluorescence peak of α-synuclein at 375 nm showed a pH dependence similar to that of tyrosinate (Fig. 1*C*), which is consistent with the pH dependence of fluorescence due to tyrosinate. The peak at 375 nm had the highest intrinsic fluorescence at low pH and showed little change in fluorescence at pH > 7.0 (Fig. 1*C*). Further studies confirmed that the peak at 375 nm is not due to tyrosine dimerization, because both gel electrophoresis and mass spectrometry of the α-synuclein showed that the α-synuclein was monomeric (Fig. 1*D*, Coomassie gel of recombinant α-synuclein shown), and in addition, tyrosine dimerization of α-synuclein reduced its intrinsic fluorescence (Fig. 1, *E* and *F*, described further below). These results suggest that the peak at 375 nm is due to tyrosinate, which could result from proton transfer from the phenolic hydroxyl to aspartic or glutamic acid protein acceptors (30).

Metals Show Three Patterns of Interaction with α-Synuclein—Next we used the fluorescence to examine the interaction of α-synuclein with metals. We observed three classes of interaction with α-synuclein. Class I metals included iron (Fe(II) and Fe(III)) and copper (Cu(II)) and decreased the fluorescence at both 310 and 375 nm (Fig. 1*A*). Class II metals included magnesium, zinc, and calcium, and increased the fluorescence at 375 nm, but did not affect the fluorescence at 310 nm (Figs. 2*A* and 3*A*). Class III metals included nickel and manganese and did not affect α-synuclein fluorescence (data not shown). We proceeded to examine the fluorescence of α-synuclein in more detail to determine whether the metal induced changes α-synuclein fluorescence reflected interaction with metals or some other process, such as tyrosine dimerization. To examine whether the changes in fluorescence could be explained by tyrosine dimerization, we exposed monomeric α-synuclein to 312 nm light for 2 h (which is a process that induces tyrosine dimerization) and analyzed the emission fluorescence spectrum with excitation at either 280 or 315 nm. The emission spectrum derived using excitation at 280 nm showed that ultraviolet-irradiation reduced α-synuclein fluorescence strongly at 375 nm, but only weakly at 310 nm (Fig. 1*E*). The contrast between the changes in fluorescence induced by ultraviolet irradiation and by metals suggests that the changes in α-synuclein fluorescence induced by metals are not due to tyrosine dimerization. Analysis of the emission fluorescence spectrum of α-synuclein following excitation at 315 nm showed a reduced fluorescence at 380 nm for the ultraviolet-irradiated α-synuclein (Fig. 1*F*). In contrast, iron increased, rather than decreased, the fluorescence of α-synuclein as measured using the 315 nm excitation. These data indicate that the iron-induced quenching of α-synuclein fluorescence is not due to cross-linking of α-synuclein mediated by tyrosine dimerization.

Dose Dependence of Metal Binding—Plotting of the dose dependence of iron-induced fluorescence quenching showed a dose-dependent decrease in fluorescence, with an IC_{50} = 173 μM and a Hill coefficient of 1.0 (R2 = 1.0, $p < 0.0001$) (Fig. 1*B*), indicating one binding site or multiple binding sites with the same affinity and no cooperativity (Fig. 1, *A* and *B*). There is a small amount of binding of iron to α-synuclein between 1–10 μM Fe(II), which is a range that could be physiologically relevant (intracellular free iron is about 1.5 μM) (31).

The effect of magnesium on α-synuclein differed dramatically from that of iron. Magnesium increased the fluorescence at 375 nm but did not affect the fluorescence at 310 nm (Fig. 2*A*). Binding of magnesium to α-synuclein was also striking because the tyrosine fluorescence showed a sharp stepwise increase between 60 and 80 μM of magnesium indicating cooperative binding (Fig. 2*A*). The cooperative regulation of tyrosine fluorescence, specifically at 375 nm, suggests that magnesium causes a conformational change in synuclein differing from that induced by iron. Co-incubating 80 μM magnesium with iron did not prevent iron-induced quenching of α-synuclein tyrosine fluorescence and in fact increased affinity of α-synuclein for iron from 173 to 50 μM (Fig. 2*B*). These data suggest that iron and magnesium bind to different sites on α-synuclein.

Zinc and calcium also increased the fluorescence of α-synuclein at 375 nm, but showed a more graded pattern of interaction (Fig. 3*A*). Jensen and colleagues recently noted a similar pattern of binding of calcium to α-synuclein (29). Interestingly, immunoblots of recombinant α-synuclein following incubation with zinc showed that zinc induced formation of a prominent band at 32 kDa, consistent with formation of an SDS-resistant α-synuclein dimer (Fig. 3*B*). Neither magnesium nor calcium induced formation of SDS-resistant dimers identifiable by immunoblot (data not shown).

We also examined how the A53T mutation in human α-synuclein affected binding of iron and magnesium. The A53T mutation did not change the apparent affinity of iron for α-synuclein (data not shown), but did abolish the interaction between magnesium and α-synuclein (Fig. 2*C*). Previous stud-

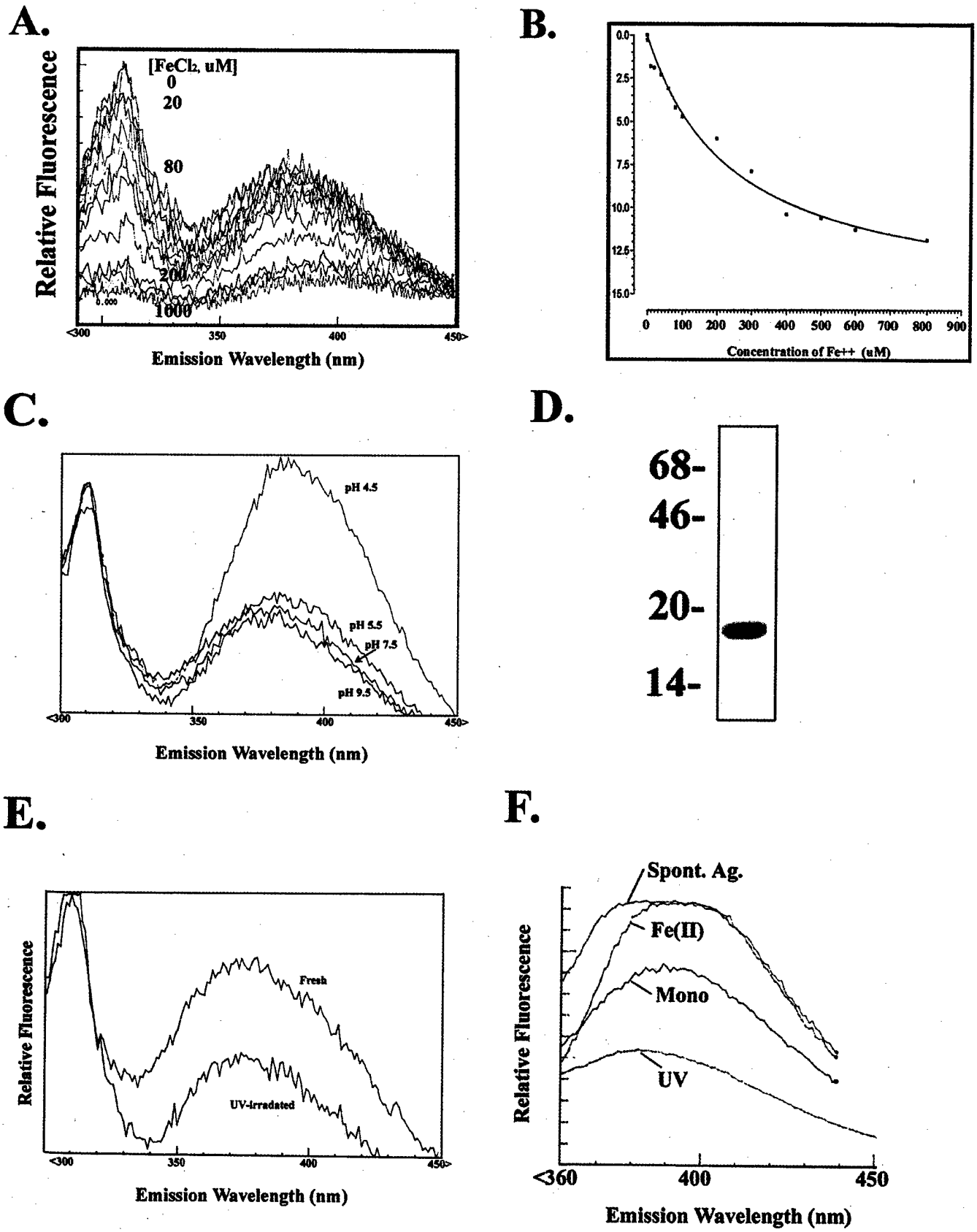


FIG. 1. **Interaction of iron with recombinant α-synuclein.** *A*, excitation of wild-type α-synuclein at λ_{ex} 280 nm produces a biphasic fluorescence spectrum with peaks at 310 and 375 nm. Incubating α-synuclein with increasing doses of $FeCl_2$ yields a progressive quenching of both peaks. *B*, quantification of the relative fluorescence from Fig. 1*A* of 1 μM α-synuclein at 310 nm during quenching by Fe(II). *C*, pH dependence of wild-type α-synuclein fluorescence using λ_{ex} 280 nm and λ_{em} 290–450 nm. The pH sensitivity of the fluorescence peak at 375 indicates that this fluorescence is due to the presence of tyrosinate. *D*, identification of recombinant α-synuclein following PAGE electrophoresis by staining with Coomassie Blue. The presence of a single α-synuclein band at 16 kDa shows that there is no dimerization. *E*, reduction in α-synuclein fluorescence following ultraviolet (UV) irradiation (2 h). The reduction in fluorescence of α-synuclein occurred mainly around the peak at 375 nm (λ_{ex} = 280 nm;

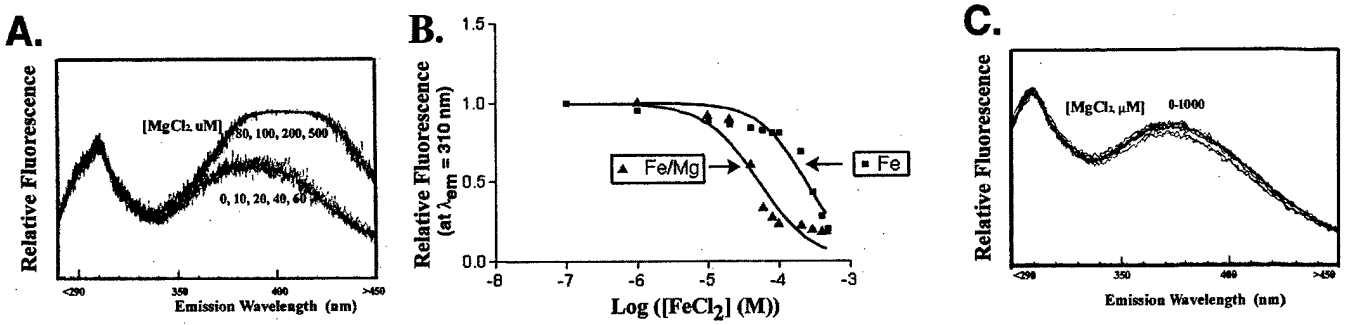
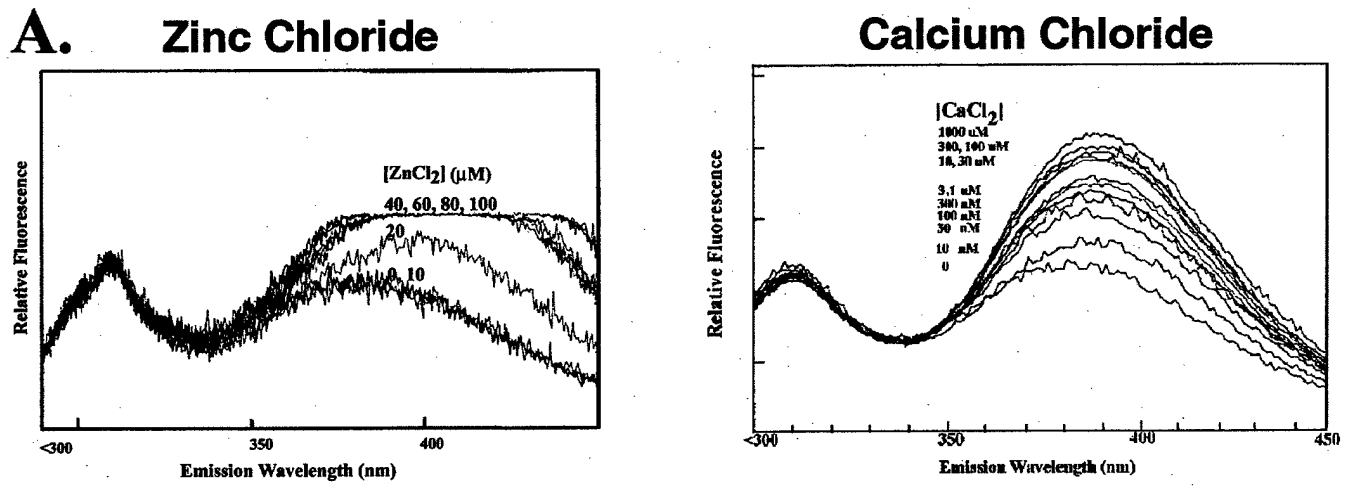


FIG. 2. A, fluorescence of wild-type α-synuclein in the presence of increasing doses of $MgCl_2$ shows increased fluorescence emission at 375 nm and no change at 310 nm, using an excitation wavelength of 280 nm (λex = 280 nm; λem 290–450 nm). *B*, a representative plot showing that magnesium increases the affinity of α-synuclein for iron. α-Synuclein shows an affinity for iron that is 5-fold lower when incubated in the presence of 100 μM $MgCl_2$. *C*, fluorescence emissions of A53T α-synuclein in the presence of increasing doses of $MgCl_2$ shows no change at 375 or 310 nm, using an excitation of 280 nm.



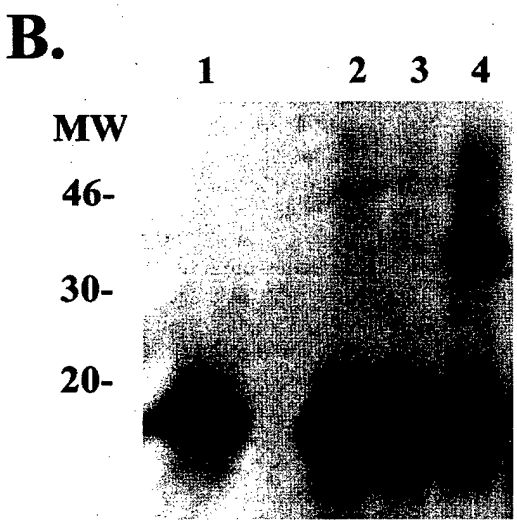


FIG. 3. **The effects of zinc on α-synuclein fluorescence and dimerization.** *A*, incubating zinc or calcium with recombinant α-synuclein increases the peak of α-synuclein emission fluorescence at 375 nm in a graded manner, using an excitation wavelength of 280 nm (λ_{ex} = 280 nm; λ_{em} 290–450 nm). *B*, immunoblot of recombinant α-synuclein after incubation with zinc shows formation of a 32-kDa band consistent with formation of an SDS-resistant α-synuclein dimer. *Lane 1*, 0 nM $ZnCl_2$; *lane 2*, 100 nM $ZnCl_2$; *lane 3*, 200 nM $ZnCl_2$; *lane 4*, 400 nM $ZnCl_2$.

ies have shown that the A53T mutation changes the conformation of α-synuclein by increasing its helical content (5). These conformational changes might either reduce binding of magnesium to α-synuclein or prevent the conformational change associated with binding of magnesium to α-synuclein.

Magnesium Inhibits α-Synuclein Aggregation—The differing effects of magnesium and iron on the fluorescence spectrum of α-synuclein suggested to us that magnesium and iron might

λ_{em} 290–450 nm), which contrasts with the changes in fluorescence induced by iron. *F*, UV irradiation (2 h) also reduces α-synuclein fluorescence following excitation at λ_{ex} = 315 nm; λ_{em} 350–450 nm. UV-induced inhibition of α-synuclein fluorescence contrasts with the increase in fluorescence induced by iron or spontaneous aggregation of α-synuclein.

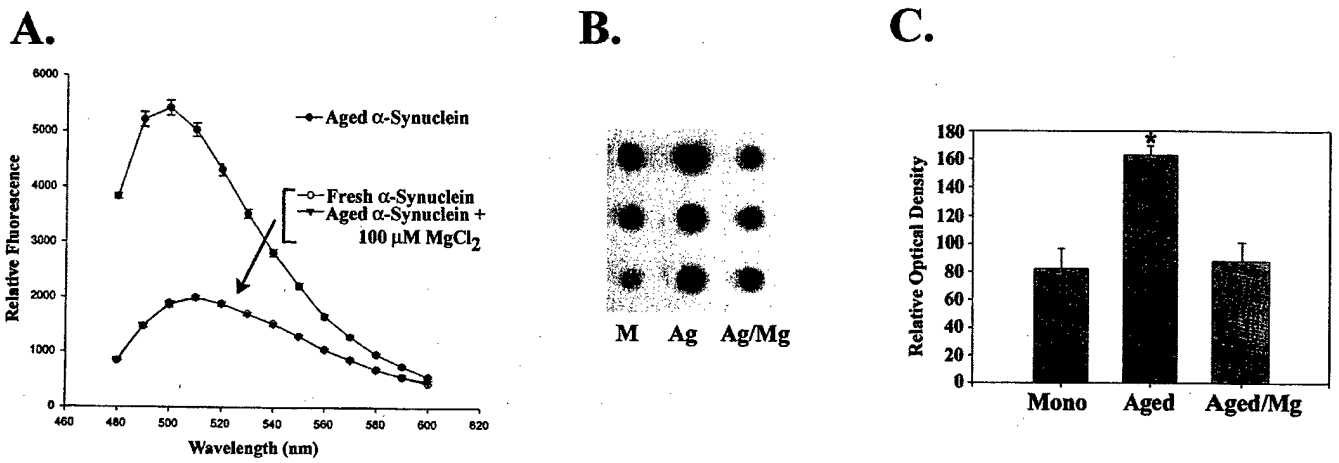


FIG. 4. **Magnesium inhibits the spontaneous aggregation of α-synuclein.** *A*, α-synuclein was prepared freshly (*Mono*) or aged 30 days at 37 °C to induce spontaneous aggregation (*Ag*) ± 0.8 mM Mg (*Ag/Mg*). Then the samples were then analyzed by Thioflavine-T fluorescence. The spontaneously aggregated α-synuclein gave strong fluorescence, while the sample aged in the presence of magnesium had a fluorescence curve identical to that of fresh α-synuclein. *B*, α-synuclein was prepared freshly (*M*, monomeric) or aged 30 days at 37 °C to induce spontaneous aggregation (*Ag*, aged) or aged 30 days at 37 °C in the presence of 0.8 mM $MgCl_2$ (*Ag/Mg*, aged plus Mg^{2+}). Then the samples were filtered through a cellulose acetate membrane and immunoblotted with rabbit anti-α-synuclein antibody. Incubating the α-synuclein with magnesium prevented formation of the large aggregates that are captured by the membrane. *C*, quantification of the optical density of the dot blots using the NIH Image program ($n = 6$, *, $p < 0.001$, analysis of variance factorial).

also induce different conformational states. We hypothesized that the conformational changes induced by binding of magnesium to α-synuclein might inhibit α-synuclein aggregation. To test this, we examined whether magnesium could inhibit the spontaneous aggregation of α-synuclein. α-Synuclein (23 μM) was incubated for 30 days at 37° ± $MgCl_2$ (500 μM). To measure the amount of aggregation, the α-synuclein was diluted to 23 nM in the presence of 10 μM thioflavine-T (in 50 mM glycine pH 8.5), and the fluorescence spectrum was measured. The solution of aged α-synuclein showed a strong fluorescence peak at 480, indicating the presence of abundant β-pleated sheet structures (Fig. 4*A*). Prior experiments have shown that the spontaneous aggregation of α-synuclein proceeds through a mechanism involving β-pleated sheet formation, and that thioflavine-T, which binds to proteins with β-pleated sheet structure, accurately measures α-synuclein aggregation (9). Using thioflavine-T we observed that samples incubated in the presence of magnesium showed only base-line levels of fluorescence, indicating that magnesium prevented the formation of β-pleated sheet structures and the aggregation of α-synuclein (Fig. 4*A*). To verify that the magnesium was inhibiting α-synuclein aggregation, we measured the amount of aggregated α-synuclein in each sample by capturing the aggregates with 0.2-μm cellulose acetate filters, measuring the amount of retained α-synuclein by dot blot, and quantitating the resulting optical density. The results of the cellulose acetate assay paralleled the thioflavine-T assay and showed that magnesium prevented the spontaneous aggregation of α-synuclein (Fig. 4, *B* and *C*). Thus, two independent methods show that magnesium inhibits the spontaneous aggregation of α-synuclein *in vitro*.

Magnesium was also able to prevent iron-induced α-synuclein aggregation. α-Synuclein (8 μM) was incubated with 50 μM $FeCl_2$ for 72 h and then analyzed by thioflavine-T fluorescence or cellulose acetate. In both cases, α-synuclein samples co-incubated with 500 μM magnesium chloride showed little aggregation (Fig. 5, *A* and *B*). The amount of thioflavine-T fluorescence induced by iron was less than that induced by spontaneously aggregated α-synuclein, which likely indicates that spontaneously induced α-synuclein contains more β-pleated sheet structure. Indeed analysis of iron-induced α-synuclein aggregates by circular dichroism did not show formation of β-pleated sheet structures, which suggests formation of a more amorphous aggregate (Fig. 5, *C* and *D*). These data suggest that magnesium inhibits the formation of α-synuclein aggregates containing either β-pleated sheet structure (via spontaneous aggregation) or amorphous structure (via iron-induced aggregation).

We also examined aggregation by immunoblot analysis, which has been successfully used to examine aggregation of α-synuclein, as well as aggregation of other proteins implicated in neurodegenerative disease, such as the huntingtin and PrP proteins (29, 32–35). In these assays, wild-type recombinant α-synuclein (8 μM) was incubated with 0–3 mM $FeCl_2$ and 0 or 100 μM $MgCl_2$ for 24 h at 37 °C. The samples were immunoblotted with anti-α-synuclein antibody, and the total amount of α-synuclein reactivity above 46 kDa (which includes structures larger than a dimers) was quantified by video densitometry (Fig. 6, *A* and *B*). We observed a reduction in formation of high molecular weight immunoreactivity of α-synuclein at all but the highest dose of iron ($n = 3$, $p < 0.0001$). These results support the hypothesis that magnesium inhibits aggregation of α-synuclein induced following treatment with iron.

Since we did not observe interaction between magnesium and A53T α-synuclein using tyrosine fluorescence, we tested whether aggregation of recombinant A53T α-synuclein was also insensitive to magnesium. We incubated recombinant A53T α-synuclein with 0–3 mM $FeCl_2$ and 0 or 100 μM $MgCl_2$ for 24 h, then immunoblotted the α-synuclein and quantified aggregation by video densitometry, as described above (Fig. 6, *C* and *D*). We did not observe consistent inhibition of iron-induced aggregation of A53T α-synuclein by magnesium. This suggests that magnesium cannot inhibit iron-induced aggregation of A53T α-synuclein.

DISCUSSION

These data demonstrate that iron (II), magnesium, zinc, and calcium all interact with α-synuclein. Our data primarily rely on tyrosine fluorescence as a measure of the interaction of α-synuclein with metals. The K_i of α-synuclein for iron is 173 μM, and the K_i of magnesium for α-synuclein is between 60 and 80 μM. These affinities are consistent with an affinity of α-synuclein for calcium, determined by Nielson and colleagues (29). Nielson and colleagues also confirmed their tyrosine flu-

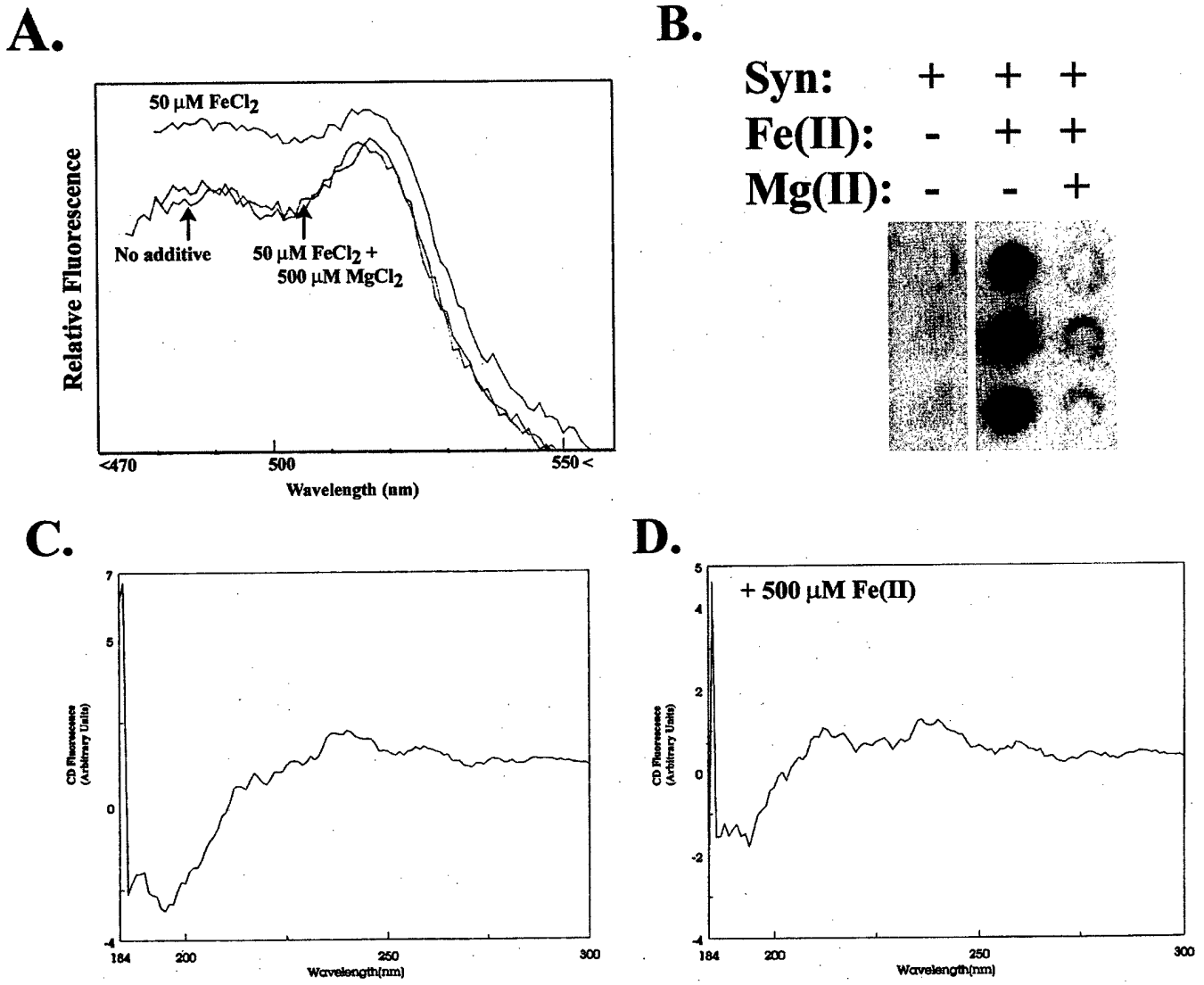


FIG. 5. **Magnesium inhibits iron-induced α-synuclein aggregation.** *A*, thioflavine-T fluorescence of α-synuclein (8 μM) following a 3-day incubation in the presence of 50 μM $FeCl_2$ ± 0.8 mM $MgCl_2$. *B*, analysis of the affects of magnesium on iron induced α-synuclein aggregation ± magnesium. α-Synuclein was prepared freshly (*Mono*) or aged 3 days at 37 °C in the presence of 50 μM $FeCl_2$ ± 0.8 mM $MgCl_2$. Then the samples were filtered through a cellulose acetate membrane and immunoblotted with rabbit anti-α-synuclein antibody. Cellulose acetate assay of spontaneously aggregated synuclein ± Mg^{2+}. *C*, circular dichroism spectrum of native α-synuclein (160 μg/ml). *D*, circular dichroism spectrum of α-synuclein (160 μg/ml) following incubation with 500 μM $FeSO_4$ for 5 days.

orescence studies using equilibrium dialysis; our attempts at using equilibrium dialysis were stymied by extensive binding of α-synuclein to dialysis membranes. However, the studies of Nielson and colleagues show that tyrosine fluorescence provides an accurate indication of metal-synuclein binding interactions. The apparent affinity of α-synuclein for magnesium is strong enough to allow interaction of α-synuclein with magnesium in living cells, where the average intracellular concentration of magnesium is about 0.5 mM. This suggests that this interaction could have physiological significance.

Although binding of magnesium to α-synuclein occurs at a concentration range that is physiologically significant, the concentration of free iron in the cell is much lower (<1.5 μM), which is far below the affinity of α-synuclein for iron that we observed (173 μM) (36). However, studies using cell culture and neuropathology both suggest that α-synuclein interacts with iron. Incubating cells with iron induces α-synuclein aggregation in viable cells, which suggests that the concentration of iron in a cell is sufficient to induce α-synuclein aggregation under some conditions. In addition, α-synuclein aggregates in iron type I storage disease, and iron co-localizes with α-synuclein in Lewy bodies (19). Although it is unclear how α-synuclein might interact with iron in the living cell, it is possible that cofactors increase the affinity of α-synuclein for iron sufficient to allow a physiological interaction. Many other factors might also affect the behavior of α-synuclein. For instance, binding to lipids and phosphorylation or binding to β-synuclein have all been shown to change the biochemistry of α-synuclein, and these agents might increase its affinity for iron (44). Our preliminary studies examining magnesium already provide a hint of modulation. The K_i of iron (II) drops to 50 μM in the presence of magnesium. Future studies might unravel the biochemistry of α-synuclein further.

Although binding of magnesium appears to introduce a conformation that promotes binding of iron, this same conformational change inhibits aggregation of α-synuclein. We hypothesize that magnesium either changes the conformation of α-synuclein to one that resists aggregation or induces dimerization to a structure that resists aggregation (Fig. 7). The ability of zinc to induce SDS-resistant α-synuclein dimers, cou-

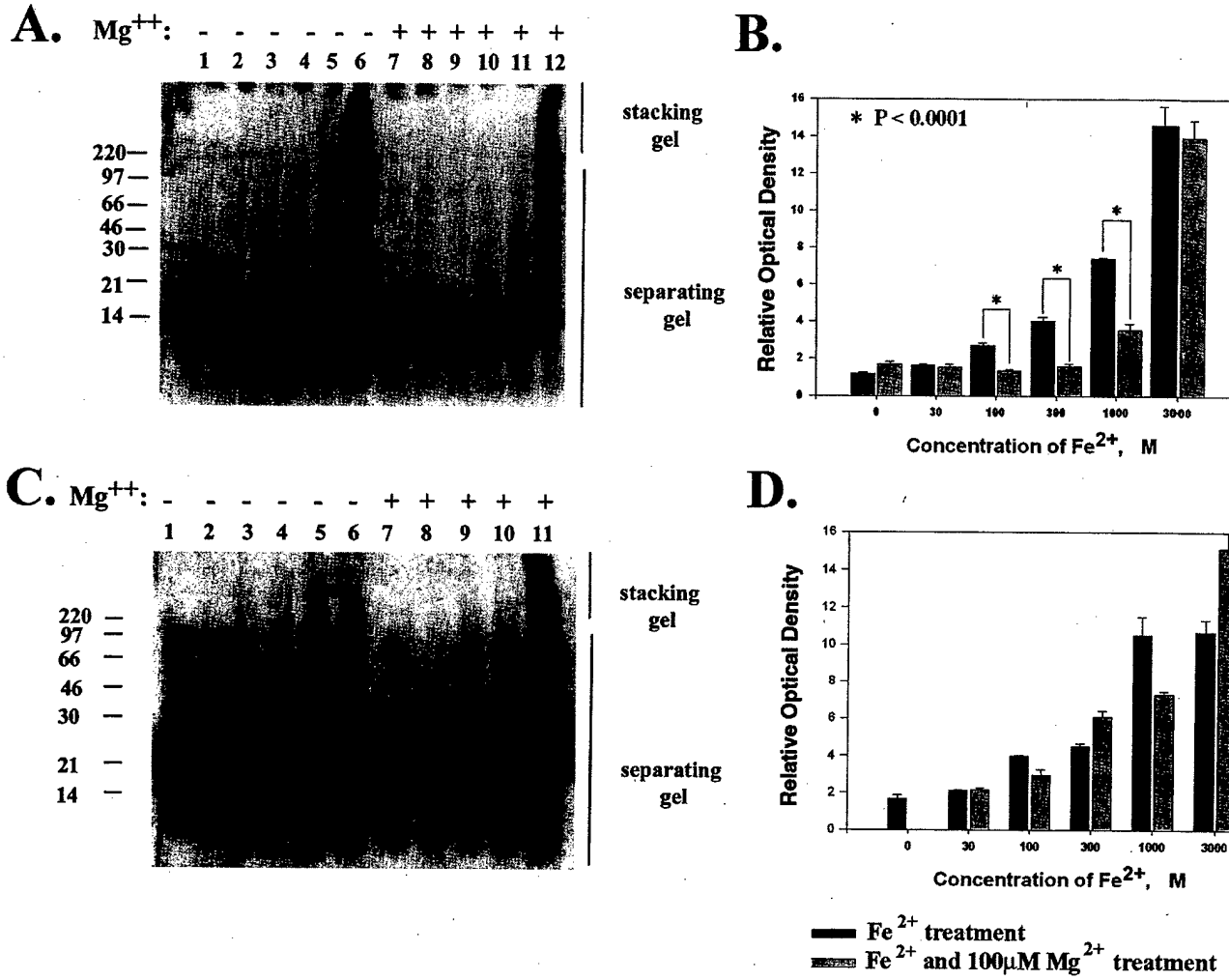


FIG. 6. **Immunoblotting of α-synuclein following incubation with varying doses of Fe(II) ± 100 μM $MgCl_2$.** *A*, immunoblotting of recombinant wild-type α-synuclein following treatment with 0–3 mM $FeCl_2$ plus 0.1 mM $MgCl_2$ for 1 day. The mean optical density above 46 kDa was quantified and used as an index of aggregation. Increasing doses of $MgCl_2$ reduced the formation of high molecular weight aggregates of α-synuclein. Concentrations (μM) of iron salts: 0 (*lanes 1* and *7*), 30 (*lanes 2* and *8*), 100 (*lanes 3* and *9*), 300 (*lanes 4* and *10*), 1000 (*lanes 5* and *11*); 3000 (*lanes 6* and *12*). Concentrations (μM) of $MgCl_2$: 0 (*lanes 1–6*), 100 (*lanes 7–12*). *B*, quantification of the aggregate formation by video densitometry showed a dose dependent decrease in aggregate formation that was statistically significant at 0.1 mM $MgCl_2$ ($n = 3$ for each point). *D*, immunoblotting of recombinant A53T α-synuclein following treatment with 0–3 mM $FeCl_2$ plus 0.1 mM $MgCl_2$ for 1 day. The mean optical density above 46 kDa was quantified and used as an index of aggregation. Increasing doses of $MgCl_2$ did not consistently inhibit formation of high molecular weight aggregates of α-synuclein. *E*, quantification of aggregate formation by video densitometry, showing that magnesium did not inhibit aggregation of A53T α-synuclein ($n = 3$ for each point).

pled with the similarity the changes in tyrosine fluorescence observed with magnesium and zinc, suggest that magnesium might induce dimerization of α-synuclein in a manner similar to that of zinc. Future studies using nuclear magnetic resonance spectroscopy will need to be performed to investigate further how magnesium affects the conformation of α-synuclein.

The most important observation made in this paper is that magnesium inhibits the aggregation of α-synuclein. This observation is supported by our use of four independent lines of investigation (immunoblot, cellulose acetate filtration, and thioflavine-T fluorescence). The type of aggregate measured by each assay likely differs slightly. Immunoblotting detects aggregates that are stable enough to resist both heating and SDS. Cellulose acetate filtration and thioflavine-T are more gentle methods that can detect both stable aggregates and also aggregates that might be re-dissolved by SDS. Thioflavine-T recognizes aggregate with a β-pleated sheet structure. Interestingly, spontaneously aggregated α-synuclein shows much more fluorescence by thioflavine-T than iron-induced aggregate, suggesting that the former has more β-pleated sheet structure. We have also taken care to examine two forms of α-synuclein aggregation: spontaneous and iron-induced aggregation. Many studies show that α-synuclein has a strong tendency to spontaneously aggregate, and this is the most widely accepted method for inducing α-synuclein aggregation (7–9). Metal-induced aggregation has only been investigated by a small number of groups, but is perhaps the only method currently available for inducing α-synuclein aggregation in cultured cells (10, 15, 37). The ability of magnesium to inhibit α-synuclein aggregation induced by both protocols (spontaneous and iron-induced) suggests that this is a robust phenomenon.

Increasing evidence suggests that metals play a pivotal role in the pathophysiology of neurodegenerative disorders. Zinc and copper greatly accelerate aggregation of β-amyloid and might play a critical role in neurotoxicity induced by β-amyloid (38, 39). Copper and manganese both bind to the prion protein and appear to influence the clinical course of prion-induced neurodegeneration (40, 41). Iron levels are increased in brains of patients with PD, and iron is present in Lewy bodies. Neu-

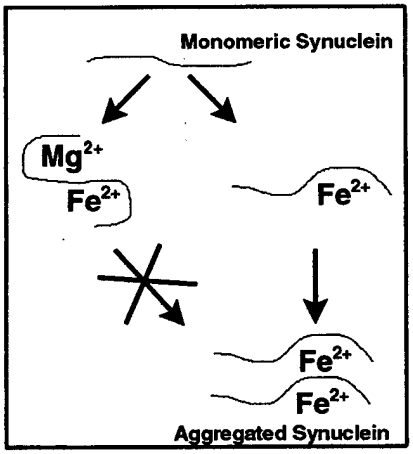


FIG. 7. **Model of interaction of iron and magnesium with α-synuclein.** In this model, iron binds to α-synuclein and promotes aggregation. Magnesium appears to bind to a different site than does iron and therefore does not inhibit binding of iron. We hypothesize that binding of magnesium to α-synuclein induces a conformational change that prevents formation of large α-synuclein aggregates.

romelanin selectively binds Fe(III) and might liberate Fe(II) as the Fe(III) is reduced to Fe(II) (via the Haber Weiss reaction) by free radicals produced in response to the oxidative stress associated with PD, providing a potential source of Fe(II) to accelerate α-synuclein aggregation (42, 43). On the other hand, magnesium levels are reduced in brains of patients with PD (21–26). If iron accelerates α-synuclein aggregation, then the abundance of iron in the substantia nigra could increase the tendency of Lewy bodies to accumulate in this region. In a companion paper,[2] we extend our studies from the test tube to neurons, and show that magnesium also inhibits aggregation of α-synuclein in neurons. Together, these data raise the possibility that iron and magnesium might also modulate α-synuclein aggregation in the brain.

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Appendix 2

■ REVIEW

Iron and Parkinson's Disease

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Multiple studies implicate iron in the pathophysiology of Parkinson's disease (PD). In the brains of patients with PD, iron levels are elevated and the levels of iron-binding proteins are abnormal. Iron has been suspected to contribute to PD because Fe(II) is known to promote oxidative damage. Recent studies suggest that an additional mechanism by which iron might contribute to PD is by inducing aggregation of the α-synuclein, which is a protein that accumulates in Lewy bodies in PD. NEUROSCIENTIST 8(1):22–32, 2002

KEY WORDS *Synuclein, Lewy body, Aggregation, Oxidation, Ferritin, Transferrin, Lactoferrin, Neuromelanin*

Parkinson's disease (PD) primarily results from loss of dopaminergic neurons in the substantia nigra, which leads to the bradykinesia, dyskinesia, rigidity, and tremor that characterize PD. Studies of the neuropathology, molecular genetics, and epidemiology of PD have each provided important clues about the pathophysiology of PD, but the work is not sufficiently advanced to provide a comprehensive picture explaining the etiology and progression of PD. The research presented in this review will explore two different lines of PD research that have recently converged. Studies of the neuropathology of PD have consistently implicated iron in the pathophysiology of PD. Iron is the most abundant metal in the human body, with levels of iron being particularly high in the brain and liver. Iron takes on added significance for the pathophysiology of PD because the levels of iron in the substantia nigra are higher in patients with PD than in patients with Alzheimer's disease or brain donors who were neurologically normal at the time of death. Increased iron could contribute to the pathophysiology of PD, because Fe(II), ferrous iron, promotes oxidative damage. Recent research has shown that iron also promotes the aggregation of α-synuclein. α-Synuclein is a protein that was identified by molecular genetic studies of familial PD. This abundant protein is one of the major components of Lewy bodies, which are intracellular proteinaceous inclusions that accumulate in PD and are a pathological hallmark of PD. The convergence of iron and α-synuclein research provides a model for integrating separate arms of the cascade of biochemical events that characterize the pathophysiology of PD.

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The Chemistry of Metals

Metals, such as iron and copper, play a critical role in the functioning of our bodies because of their labile chemistry. Metals can readily accept or donate electrons because their outer orbitals are not filled. The ability of metals to exchange outer orbital electrons allows them to catalyze oxidation and reduction (redox) reactions. Many proteins take advantage of the redox capability of metals by positioning metals near the active site of the enzyme. For instance, enzymes such as superoxide dismutase use metals such as copper, zinc, and manganese to catalyze the reduction of superoxide anions. The proteins of the electron transport chain, such as the cytochromes, have more than 20 iron atoms, which are used to transport electrons through the chain. The electron transport chain progressively reduces the energy of electrons released from oxygen in a process that drives oxidative phosphorylation and provides the energy that powers our cellular metabolism.

Metals can also form strong bonds with particular amino acids in proteins and with nucleic acids. Iron and copper form covalent bonds with cysteines, and most metals form ionic bonds with acidic amino acids, such as aspartate or glutamate. The spatial coordination provided by the metal-protein bonds plays a critical structural role in many proteins. Iron coordinates the structure of hemoglobin and binds oxygen. Similarly, in copper/zinc superoxide dismutase, zinc plays an important structural role whereas copper plays a catalytic role. Zinc and magnesium are also critical to the function of many DNA binding proteins, such as DNA polymerases. Thus, metals play a pivotal role in the biology of our body.

Despite being required for the functioning of many enzymes, the ability of metals to catalyze redox reactions and bind amino acids in proteins can have deleterious consequences for the cell. Nonspecific redox reactions and nonspecific chelation by proteins can wreak havoc in the cell. The best characterized redox reactions are the Fenton and Haber Weiss reactions, which are catalyzed by Fe (II) and Cu (II):

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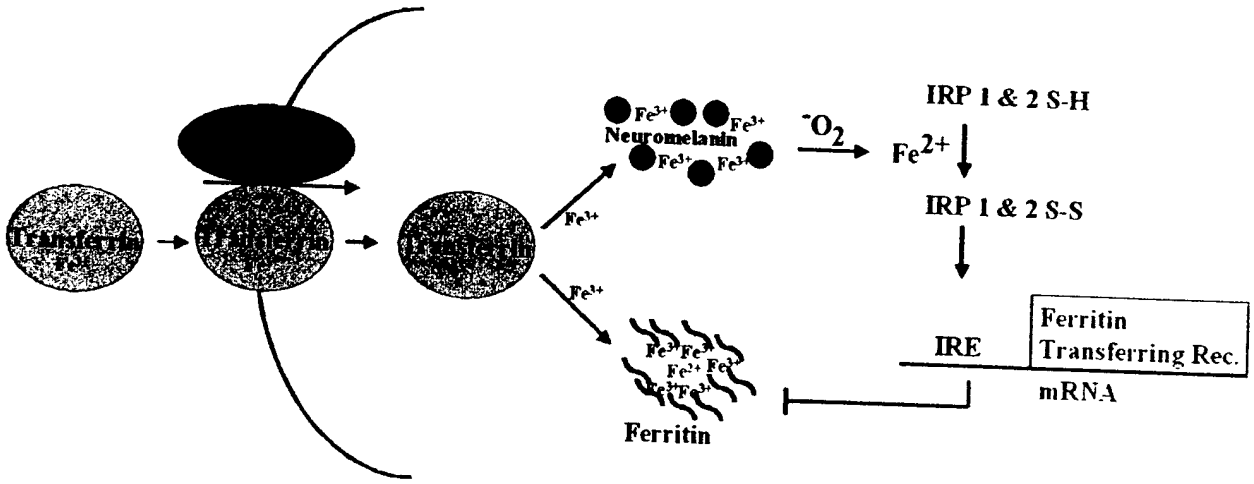


Fig. 1. Iron metabolism: Iron is transported by transferrin and lactoferrin (only transferrin is shown in the figure). The transferrin-iron complex is transported into the cell by the transferrin receptor. The iron is then released to an unidentified chaperone, which distributes the iron throughout the cell as needed. Ferritin serves as a store for excess iron. In dopaminergic neurons, neuromelanin also avidly binds Fe(III). Redox active Fe(II) might be generated from the store of Fe(III) in the neuromelanin after reaction with superoxide or other oxidants. The Fe(II) reacts with iron regulatory protein (IRP), which activates it and allows it to bind to iron response elements (IRE) in front of ferritin and transferrin receptor mRNA. Binding of IRP inhibits transcription of ferritin and transferrin receptor.

I. $O_2^{\cdot} + Fe^{3+} \rightarrow O_2 + Fe^{2+}$ Haber Weiss Reaction

II. $H_2O_2 + Fe^{2+} \rightarrow 2H_2O + OH^{\cdot} + OH^{-} + Fe^{3+}$ Fenton Reaction

These reactions lead to production of hydroxyl ions, which are highly reactive free radicals that rapidly oxidize proteins, lipids, and DNA. Nonspecific chelation of metals by amino acids present in proteins is also harmful because it stimulates protein aggregation. Protein aggregation is often toxic to cells for many different reasons, such as loss of activity or aggregate-mediated inhibition of organelle function. To avoid the deleterious side effects of metals, our bodies have developed an extensive machinery to transport, store, and utilize metals safely, thereby minimizing uncontrolled free radical production and uncontrolled protein aggregation.

Despite the presence of biochemical machinery to manage metals, nonspecific metal-induced oxidation and protein aggregation do occur, and metal-induced cellular damage accrues over our lifetime. This is particularly true in the brain, which contains a large number of nondividing cells that accumulate damaged protein, lipid, and DNA. The accumulation of oxidation products is particularly evident in the substantia nigra, where the amount of neuromelanin progressively increases throughout life. Metal-mediated oxidation and protein aggregation also play important roles in the progression of many neurodegenerative diseases that appear after decades of life. As will be explained below, metals appear to contribute to the process of protein aggregation or cellular toxicity in diseases such as PD, Alzheimer's disease, some forms of amyotrophic lateral sclerosis, Wilson's syndrome, and Hallervorden-Spatz syndrome. Each of these diseases is also characterized by the accumulation of markers of oxidative damage, which is consistent with a putative linkage between metals and free radical–mediated damage.

The Physiology of Iron Storage and Transport

To understand the factors that might regulate the accumulation of iron, it is helpful to examine normal iron metabolism. Iron in the form of Fe(III) is abundant but very insoluble under physiologic conditions. In the gut, Fe(III) is solubilized by reduction to Fe(II), whereupon it is taken up by the intestinal cells. After uptake, Fe(II) rapidly oxidizes to Fe(III), where it is bound by two iron transporters, transferrin and lactoferrin, and transported throughout the body. To enter cells, these two proteins are taken up by the transferrin and lactoferrin receptors, respectively. Transferrin and lactoferrin enter the cell and release the iron upon exposure to acidic pH to an unidentified carrier, whereupon the iron is taken up by enzymes in the cell or bound by the iron storage protein ferritin (Fig. 1). Ferritin plays a critical role in the cell because it stores excess iron by forming a cage composed of more than 20 ferritin subunits that is able to sequester up to 4500 atoms of iron. The cage contains Fe(III) in its periphery and the reactive Fe(II) at its core. The wall of Fe(III) effectively protects the cell from the reactive Fe(II) species by sequestering Fe(II) away from important biological molecules, such as proteins, DNA, and lipids. Ferritin exists as two isoforms, a heavy subunit (H, 21 kDa) and a light subunit (L, 19 kDa) (Ponka and others 1998). The heavy subunit contains ferroxidase activity that converts Fe(II) to Fe(III) and facilitates the sequestration of the ferric ion (Ponka and others 1998). The two ferritin isoforms are coded by two different genes, and the ratio between H and L subunits differs among different organs and among cell types. For instance, in the brain, H-rich ferritin is more abundant in

neurons whereas L-rich ferritin is more abundant in microglial cells (Ponka and others 1998).

Regulation of iron storage and transport is determined by the amount of transferrin and lactoferrin receptor and by the amount of ferritin produced (Ponka and others 1998). Increased transferrin or lactoferrin receptor leads to increased iron transport. Ferritin levels determine the amount of free iron in the cell. Increased ferritin increases iron sequestration and reduces free iron levels. Two homologous proteins, Iron Regulatory Protein 1 and 2 (IRP1 and 2), regulate levels of transferrin receptor, lactoferrin receptor, and ferritin in the cell (Thomson and others 1999). IRP2 appears to be the key regulatory species in the brain. Binding of free iron to IRP2 causes oxidation of IRP2, which stimulates its binding to regulatory sequences in the relevant mRNAs and in the genome, termed iron regulatory elements. Binding of IRP1 and 2 to RNA and DNA inhibits production of transferrin, lactoferrin, and ferritin. Thus, more iron causes more IRP activity and less production of transferrin, lactoferrin, and ferritin.

The substantia nigra also contains an additional mechanism for storing iron. Neuromelanin accumulates in the substantia nigra with age as a byproduct of the oxidation of dopamine. Neuromelanin is potentially important to our understanding of PD because it binds Fe(III) avidly under physiological conditions and thus sequesters iron (Zecca and others 2001). Multiple studies have documented that iron increases in the substantia nigra with age, and much of this increase could be due to the concomitant increase in neuromelanin (Zecca and others 2001). Binding of iron by neuromelanin might reduce free cytoplasmic iron and cause a compensatory increase in iron transport, with a corresponding increase in total neuronal iron content. The consequences of this gradual increase in iron content are currently unclear but could be somehow related to the selective vulnerability of pigmented neurons seen in the substantia nigra in PD (Hirsch and others 1989).

The Pathophysiology of Iron Storage and Transport in PD

The pathophysiology of PD appears to exert a strong effect on the transport and storage of iron. An increasing number of reports suggest that systemic iron metabolism is abnormal in PD. Markers of iron transport in the serum, such as transferrin or lactoferrin, appear to be reduced in PD, although the particular reports differ in the details of which transporter is altered (Logroscino and others 1997; Grau and others 2001). In the brain, iron accumulates more in the substantia nigra cases of PD than in control cases (Martin and others 1998). Because iron uptake is mediated by transferrin and lactoferrin, investigators have examined the expression of these proteins in PD. Lactoferrin immunoreactivity is increased in affected dopaminergic neurons in patients with PD compared with control patients, whereas transferrin levels are decreased (Faucheux, Herrero, and others 1995; Faucheux, Nillesse, and others 1995). The

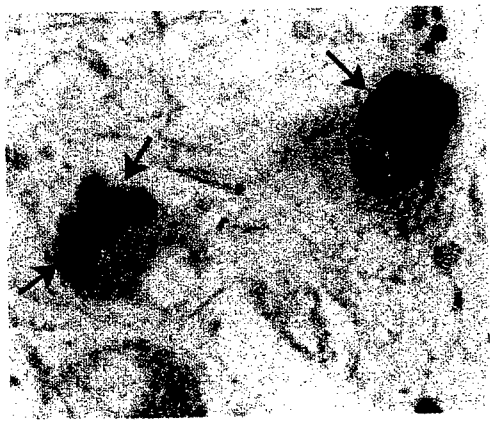


Fig. 2. Immunocytochemical staining with anti-α-synuclein antibody showing the accumulation of α-synuclein in Lewy bodies in the substantia nigra of a patient who died from Parkinson's disease (*arrows*).

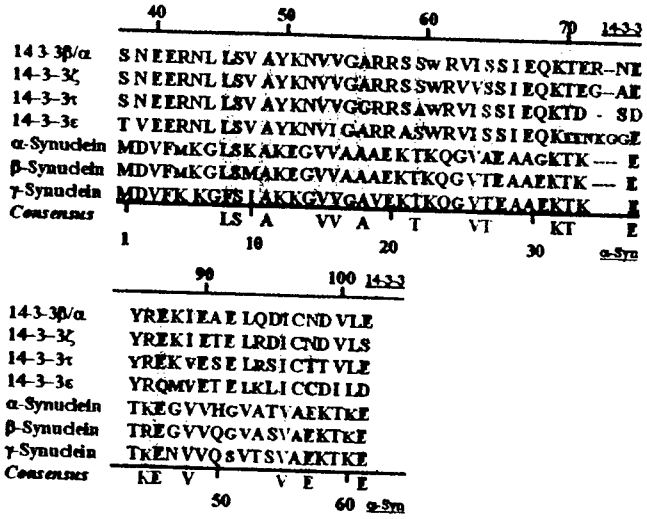
increased immunoreactivity is selective for areas showing neurodegeneration, and it is not observed in cholinergic neurons in patients with PD. The increase of lactoferrin in the dopaminergic neurons of the substantia nigra in patients with PD might be responsible for the excessive accumulation of iron in dopaminergic neurons in PD. The meaning of the changes in systemic iron transport is less clear, but systemic changes could easily be an indication of a fundamental problem with iron metabolism in PD.

Ferritin is also altered in PD. The level and H/L ratio of ferritin isoforms varies among pathological conditions. The changes in PD are striking because of the absence of correlation with changes in iron levels. Patients with PD show decreased levels of both ferritin subunits (compared with age-matched control patients) and no change in the H/L ratio in the substantia nigra, despite increased iron content in the substantia nigra in PD (Connor and others 1995). In contrast, in Alzheimer's disease there is a small increase in iron and an increase in the H/L ratio. The absence of homeostatic adjustments in ferritin levels in PD, in response to the changes in iron content, could simply reflect increased sequestration of iron by another species in the substantia nigra in PD (such as neuromelanin or α-synuclein, as described below), or might result from abnormal regulation of iron storage proteins as part of the pathophysiology of PD.

α-Synuclein Binds Iron

Recent studies into the molecular neuropathology of PD suggest an additional mechanism, involving the protein α-synuclein, that might also contribute to the dysregulation of iron metabolism in the substantia nigra in PD. α-synuclein is implicated in PD because two different mutations in α-synuclein, A53T and A30P, are associated with three different kindreds of familial PD, and because α-synuclein is one of the most abundant components in Lewy bodies (Fig. 2) (Polymeropoulos and others 1997; Spillantini and others 1997; Kruger and

A. Homology of α-Synuclein with 14-3-3



B. Proteins That Interact with α-Synuclein

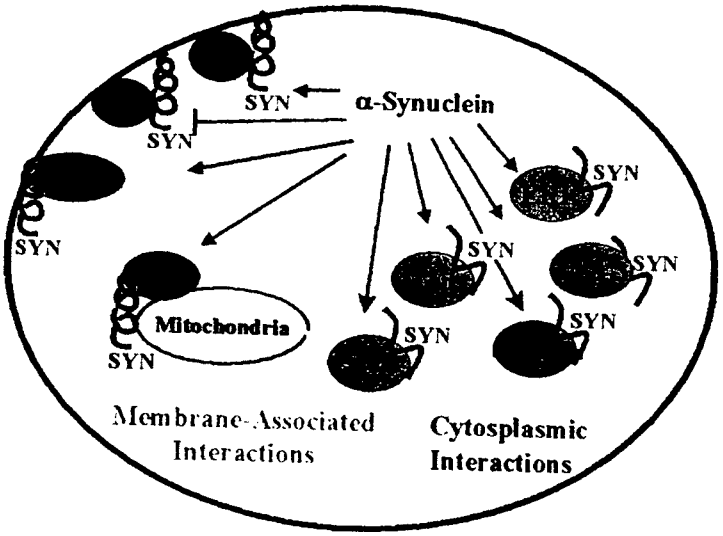
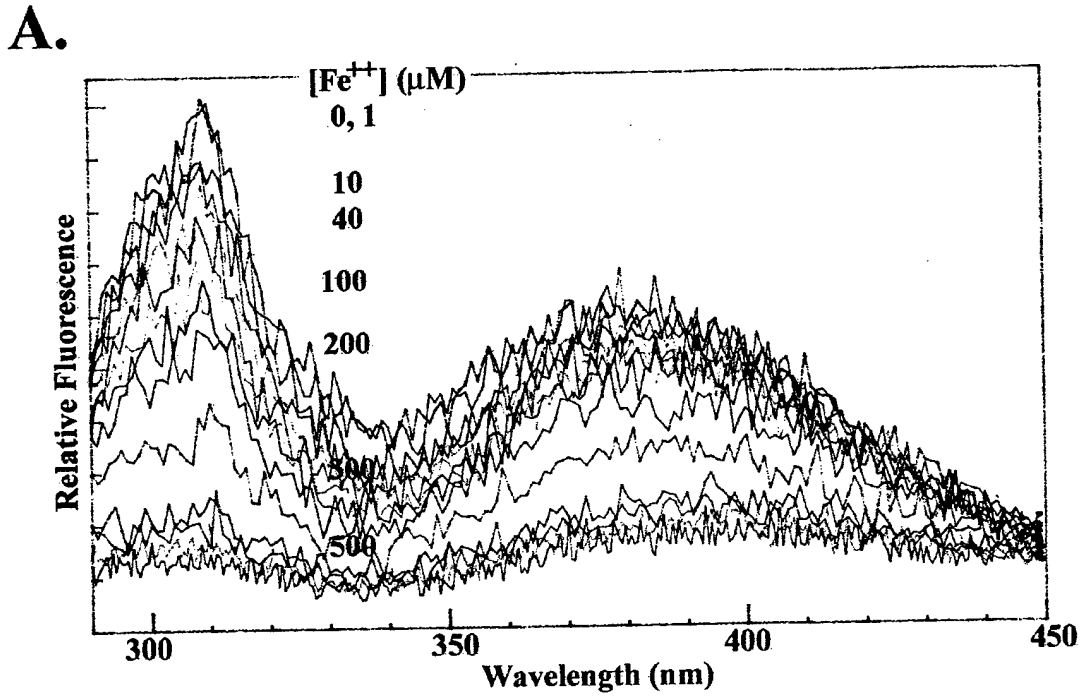


Fig. 3. α-synuclein biology. *A.* Homology between 14-3-3 isoforms and synuclein homologues. *B.* Soluble α-synuclein can be cytoplasmic or membrane bound. α-synuclein is thought to adopt an α-helical structure upon interaction with acidic phospholipids, whereas in solution α-synuclein has a random structure. Proteins that bind α-synuclein at the membrane are shown in green, whereas proteins that bind α-synuclein mainly in the cytoplasm are shown in blue. Protein kinase C binds synuclein both in the cytoplasm and at the membrane, but the biologically significant interaction appears to be at the membrane where binding to α-synuclein inhibits PKC activity. Abbreviations: Syn = α-synuclein; GP = G-Protein kinases; PLC-δ = phospholipase C δ; ERK = extracellular regulated kinase; PKC = protein kinase C.

others 1998). α-synuclein is part of a larger family of proteins that includes two other homologues, termed β and γ synuclein (Fig. 3). α-synuclein is a very acidic protein, particularly at its C-terminus. In the C-terminal domain, 16 of 42 amino acids (38%) are the acidic amino acids glutamate or aspartate. High percentages of acidic amino acids are also observed in other proteins that are designed to bind iron, such as ferritin, as well as in proteins that inadvertently bind iron, such as neurofilament. We have studied the interaction of α-synuclein with iron, and we observe ready association between the two proteins. Studies using tyrosine fluorescence as an indicator of synuclein-metal interactions suggest that α-synuclein can interact with micromolar levels of Fe(II), which is potentially significant because free levels of intracellular iron are about 1.5 μM in peripheral cells and could be higher in neurons of the substantia nigra (Golts and others 2001). Using recombinant α-synuclein incubated in saline, we have observed a small amount of binding at 1.5 μM Fe(II) (Fig. 4*A*). The amount of iron bound to α-synuclein in neurons could be much higher, though, because our preliminary evidence suggests that lipids and other cofactors (such as magnesium) can greatly increase the affinity (Golts and others 2001). Hence, a significant amount of neuronal α-synuclein might associate with iron.

Binding of iron to α-synuclein plays a significant role in the pathophysiology of PD, because iron accelerates α-synuclein aggregation greatly. Published reports show that exposure of 8 μM α-synuclein to 100 mM Fe(II)



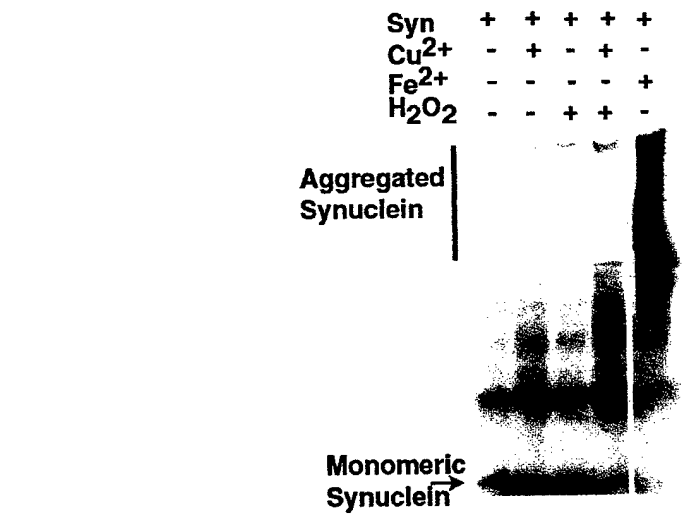


Fig. 4. *A.* Binding of iron to recombinant α-synuclein in phosphate-buffered saline, as measured by fluorescence at λex = 280, λem = 290–450. The peak at 310 is due to endogenous fluorescence of the four tyrosines in α-synuclein, and the peak at 375 nm is due to fluorescence from ionized tyrosines, termed tyrosinate. Some Fe(II) binding is apparent in the 1 to 10 μM range, although cofactors that increase the affinity of α-synuclein for iron at least tenfold will need to be identified before α-synuclein can be clearly said to interact with iron at physiological concentrations. *B.* Immunoblot of recombinant α-synuclein following incubation alone, with 0.5 mM Fe(II), or with 1 mM Cu(II) + 100 μM H_2O_2 for 3 days. Iron strongly induces α-synuclein aggregation and is a more potent inducer of α-synuclein aggregation at neutral pH than copper.

plus 100 mM hydrogen peroxide in vitro induces aggregation in 24 h, but our studies show that doses of iron as low as 100 μM induce aggregation of α-synuclein in 24 h, without additional oxidants (Fig. 4*B*) (Hashimoto, Hsu, and others 1999; Golts and others 2001). In contrast, to achieve spontaneous aggregation in vitro in absence of other agents, α-synuclein must be incubated at a concentration of 230 μM for 7 to 40 days (Conway and others 1998). Interestingly, iron-containing proteins, such as cytochrome C, also appear to accelerate α-synuclein

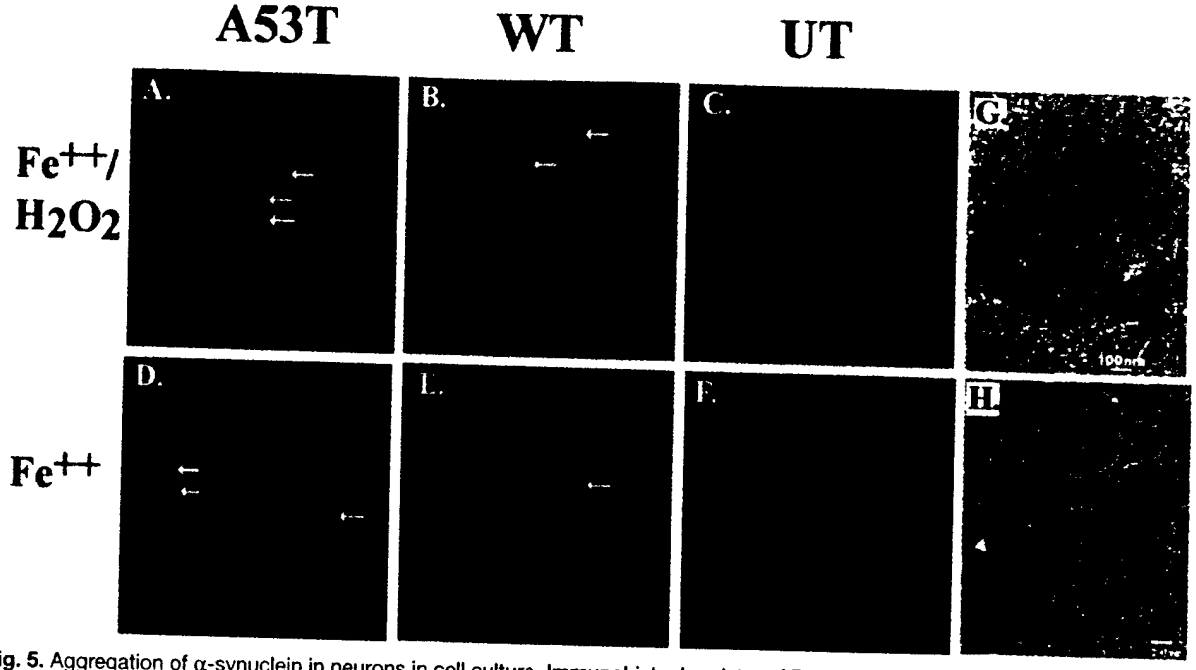


Fig. 5. Aggregation of α-synuclein in neurons in cell culture. Immunohistochemistry of BE-M17 cells overexpressing α-synuclein following exposure to 10 mM $FeCl_2$ + 100 μM H_2O_2 for 3 days. The cells show inclusions of both α-synuclein and ubiquitin. *G,H*, Electron microscopy shows that these aggregates contain long fibrils with a diameter of approximately 10 nm (G, mag. 35,000×). Some cells containing aggregates had both fibrillar deposits (triangle) and organelles that were intact (arrow), suggesting that aggregation can occur in living cells (*H*, mag. 20,000×). Copyright 2000 by the Society for Neuroscience.

aggregation (Hashimoto, Takeda, and others 1999). Whether this is due to transfer of iron from the carrier to α-synuclein or aggregation of α-synuclein independent of iron remains to be determined.

Exposing neuronal cells to Fe(II) also induces α-synuclein aggregation. Incubating neurons in medium containing 0.1 to 0.3 mM $FeCl_2$ for 4 days induces formation of ubiquitin-positive α-synuclein aggregates (Fig. 5) (Ostrerova-Golts and others 2000). Although not Lewy bodies, the aggregates resemble Lewy bodies. They contain abundant α-synuclein and ubiquitin, and the α-synuclein is aggregated into filaments with β-pleated sheet structure that bind Thioflavine-S (Figs. 5 & 6). However, unlike filaments in Lewy bodies, the experimentally induced filaments do not extend radially outward from the core of the aggregate. This suggests that iron is a useful agent to induce α-synuclein aggregation and model Lewy body formation but that the aggregates that form differ somewhat from the Lewy bodies present in PD.

Overlap between α-Synuclein Pathology and Iron Accumulation

Neuropathological studies also suggest a connection between α-synuclein aggregation and iron accumulation. Lewy bodies in PD contain iron [Fe(II)], which suggests that aggregated α-synuclein might bind iron in vivo. Other diseases showing α-synuclein accumulation also show accumulation of iron. For example, Hallervorden-Spatz disease is a neuroaxonal dystrophy that shows axonal swellings, occasional Lewy bodies, and accumulation of iron in the substantia nigra, red nucleus, and globus pallidus. Both the axonal swellings and Lewy bodies contain aggregated α-synuclein (Newell and others 1999; Galvin and others 2000). Iron also appears to accumulate along with α-synuclein in multiple systems atrophy. This disease shows widespread degeneration of neurons in the substantia nigra, putamen, olivary nucleus, pontine nuclei, and cerebellum; iron accumulation; and inclusions containing α-synuclein that are present in oligodendrocytes and neurons (Dickson and others 1999). The accumulation of both aggregated α-synuclein and iron in at least three different neurodegenerative diseases suggests that the two processes might be connected. Whether iron induces the accumulation of α-synuclein or aggregated α-synuclein sequesters free iron remains to be determined.

Are Protein Aggregates Harmful?

The research described above suggests that iron might promote α-synuclein aggregation, but is this bad? The significance of aggregates in neurodegenerative diseases is a source of strong debate. Inclusions of aggregated protein occur in most neurodegenerative diseases. α-synuclein accumulates in PD, Aβ accumulates in Alzheimer's disease, tau protein accumulates in frontotemporal dementias, and inclusions consisting of proteins with expanded polyglutamine repeats occur in diseases such as Huntington's disease and spinocerebellar ataxia. In each case, mutations in the proteins that accumulate are associated with familial forms of the illness. Each of these mutations promotes aggregation of the

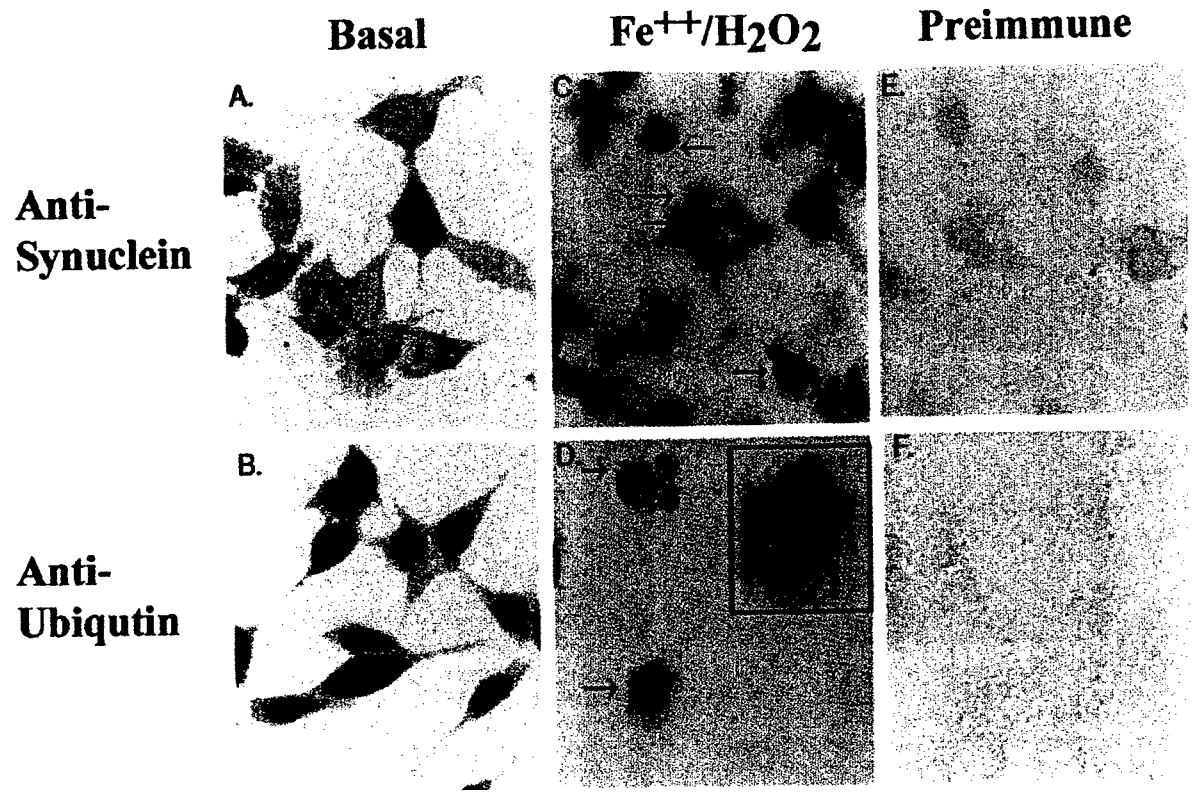


Fig. 6. Identification of α-synuclein aggregates by thioflavine-S staining and by electron microscopy. Thioflavine-S staining of cells expressing (*A* and *D*) A53T α-synuclein; (*B* and *E*) wildtype (WT) α-synuclein; or (*C* and *F*) untransfected (UT) cells with 10 mM $FeCl_2$ ± 100 μM H_2O_2 for 72 h. Bright green foci (*arrows*) are aggregates containing β-pleated sheet structure. Copyright 2000 by the Society for Neuroscience.

protein or a product of the protein (Hashimoto, Hsu, and others 1999; Jarrett and others 1993; Nacharaju and others 1999). In addition, each case overexpressing the mutant protein in transgenic animals causes a neurodegenerative disease that partially resembles the human disease, although there are often significant differences between the transgenic and human diseases. Overexpressing α-synuclein (A53T or wild type) in *Drosophila* leads to delayed degeneration of dopaminergic neuron, motor disturbances, and development of inclusions resembling Lewy bodies (Feany and Bender 2000). In mice, overexpressing amyloid precursor protein containing any of several familial mutations produces structures resembling neuritic plaques, tau pathology around the neuritic plaques, and some memory loss (Price and others 2000). Transgenic mice overexpressing a P301L mutation in tau protein develop neurofibrillary tangles and progressive motor impairments (Lewis and others 2000). Overexpressing huntingtin protein with an expanded polyglutamine repeat produces nuclear and cytoplasmic inclusions in the striatum and cortex, striatal neurodegeneration, and premature death (Davies and others 1997). There are often differences between human disease and the disease occurring in the animal models, but the differences do not negate a clear conclusion that mutant forms of these proteins can produce aggregation and neurodegeneration.

The question not addressed by these animal models is whether aggregation is the cause of the neurodegeneration. Increasing evidence suggests that the consolidated protein aggregate that makes up the inclusion body in each disease might not be the actual toxic agent producing neurodegeneration. In PD, Lewy bodies are present mainly in surviving neurons. In Alzheimer's disease, small oligomers of Aβ have been shown to be much more toxic than large aggregates of Aβ (Lambert and others 1998). In Huntington's disease, inhibiting nuclear aggregation of the huntingtin protein does not appear to inhibit cell death (Saudou and others 1998). The large, consolidated protein aggregate, such as a Lewy body, has a relatively circumscribed boundary. In contrast, micro-aggregates (perhaps bound to redox active metals) can diffuse throughout the neuron, interfere with the function of multiple target molecules, and thereby cause neurodegeneration. The Aβ aggregates bind to several proteins linked to cell death pathways, including the p75 NGF receptor and RAGE, and huntingtin might interfere with the function of cbp, a protein that binds the transcription factor CREB (Wolozin and Behl 2000; Nucifora and others 2001). Based on this logic, it is possible that the Lewy body might actually evolve from diffusible α-synuclein micro-aggregates. Consolidation of aggregated α-synuclein into Lewy bodies might protect the neuron by sequestering the micro-aggregates.

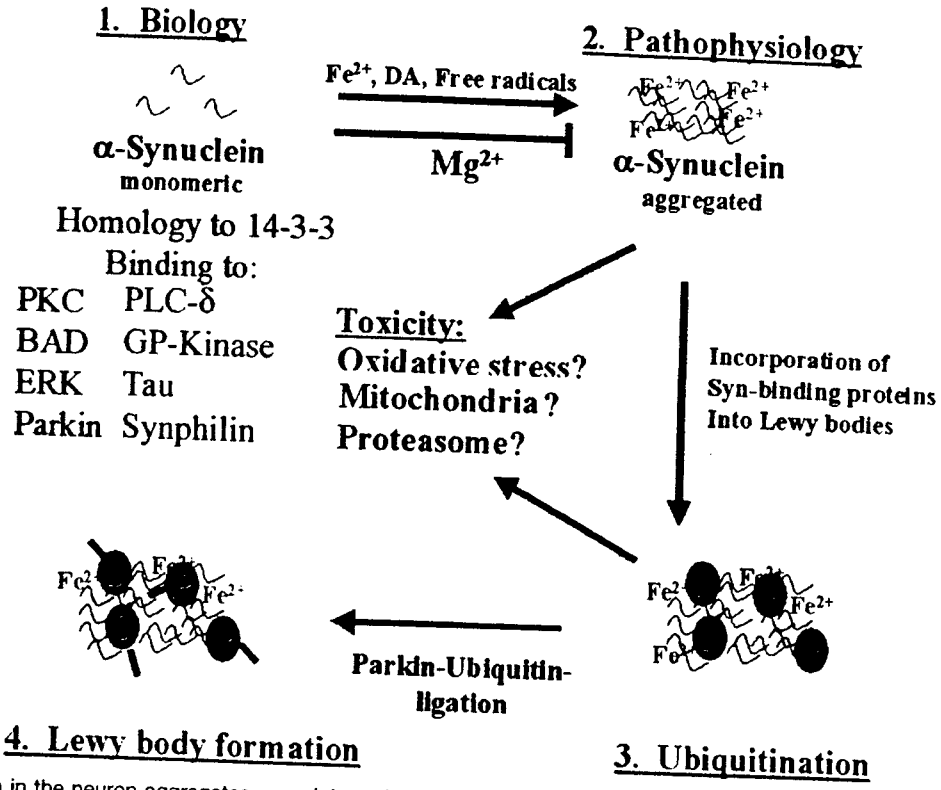


Fig. 7. α-synuclein in the neuron aggregates upon interaction with iron under oxidative conditions. The mechanism of toxicity of α-synuclein is not yet understood but could occur via oxidative stress or inhibition of mitochondrial or proteasomal function. The aggregated α-synuclein binds other proteins, such as parkin (P) and synphilin (S). These proteins, and possibly α-synuclein, become ubiquitinated. The entire complex then consolidates to form the Lewy body.

Knowledge of the molecules that interact with the α-synuclein aggregates could provide critical information about the mechanism of α-synuclein toxicity and strategies to interfere with α-synuclein toxicity.

Proteins That Interact with α-Synuclein

A simple model of α-synuclein biology divides it into two basic states: native and aggregated. Understanding the interactions of native (cytoplasmic or membrane bound) α-synuclein with different proteins provides important insights into the normal biology of α-synuclein, whereas understanding the interactions of aggregated α-synuclein is likely to provide critical insights into the pathophysiology of α-synuclein. Multiple proteins have been shown to interact with native α-synuclein, including phospholipase D, 14-3-3 proteins, protein kinase C isoforms, Bad, extracellular regulated kinase, G-proteins, and tau protein (Fig. 3*B*) (Jenco and others 1998; Jensen and others 1999; Ostrerova and others 1999; Pronin and others 2000). Any protein that binds aggregated α-synuclein will likely be present in Lewy bodies where there is an accumulation of α-synuclein. Our studies suggest that none of these proteins accumulates in Lewy bodies, and hence it is likely that none of these proteins interacts significantly with aggregated α-synuclein. On the other hand, the two proteins that do bind aggregated α-synuclein also accumulate in Lewy bodies (Fig. 7). Synphilin is a protein that was identified by virtue of its ability to bind α-synuclein (at the N-terminus) (Engelender and others 1999). Synphilin promotes the aggregation of α-synuclein and is present in Lewy bodies (Engelender and others 1999; Wakabayashi and others 2000). Parkin is a ubiquitin-ligase that is associated with autosomal recessive juvenile parkinsonism, as well as with occasional cases of familial PD. Parkin also accumulates in Lewy bodies and has the ability to bind aggregated α-synuclein (Choi, Ostrerova-Golts, and others 2000; Choi, Golts, and others 2001). These data suggest that proteins that bind to aggregated α-synuclein also accumulate in Lewy bodies.

Mechanisms of Toxicity Related to α-Synuclein

The connection between parkin and α-synuclein in PD is particularly interesting because it suggests that α-synuclein interacts with proteins in the ubiquitin-proteasomal cascade. TBP1 is a proteasomal protein that binds α-synuclein (Ghee and others 2000). Two of the three proteins that have been shown to be associated with parkinson syndromes when mutated are members of the ubiquitin-proteasomal cascade. Parkin, the most commonly mutated protein associated with parkinsonism, is a ubiquitin ligase that binds substrates for ubiquitination. UCH-L1 is a ubiquitin C-terminal hydrolase that cleaves polyubiquitin stretches to regenerate ubiquitin monomers that are required for the process of ubiquitination. In addition, the proteasomal cascade, which is known to

be very sensitive to oxidative damage, is impaired in PD (McNaught and Jenner 2001). These findings are part of a growing body of evidence suggesting that the ubiquitin-proteasomal cascade plays an important role in PD, and perhaps in other neurodegenerative diseases. Inhibiting proteasomal activity is known to induce rapid cell death in cells grown in culture. Loss of parkin activity impairs the ubiquitin-proteasomal cascade and causes neurodegeneration. It is unlikely that binding of α-synuclein to parkin inhibits parkin function, because Lewy bodies contain abundant ubiquitin. However, binding of aggregated α-synuclein to the proteasome could inhibit proteasomal function, either directly or by bringing Fe(II) into proximity with the proteasome and promoting oxidative damage to the proteasome.

The mechanisms of degeneration in PD are only beginning to be understood, and other models of degeneration are equally compelling. The leading model of neurodegeneration in PD focuses on oxidative damage and mitochondrial dysfunction. The simplest hypothesis is that aggregation of α-synuclein induces an accumulation of redox active iron within the cytosol and this iron catalyzes free radical production by Fenton and Haber-Weiss chemistry. Fe(III) is normally stored within ferritin clusters or in neuromelanin and does not cause oxidative damage. In this model, endogenous superoxide reduces Fe(III) associated with neuromelanin to Fe(II), by the Haber-Weiss reaction. Being soluble, the Fe(II) diffuses into the cytoplasm where it binds to α-synuclein and induces aggregation (Fig. 7). The iron-synuclein complex ineffectively sequesters Fe(II), which might stimulate oxidative damage in the cell (Fig. 7). This would increase oxidative stress in the dopaminergic neurons of the substantia nigra. Multiple neurochemical studies show strong evidence of oxidative stress in the substantia nigra in PD. α-synuclein isolated from brains of PD donors contains nitrotyrosine (Giasson, Duda, and others 2000). The antioxidants glutathione and tetrahydrobiopterin are strongly reduced, the amount of oxidized protein is greatly increased, and the neuroprotective protein NF-κB is translocated to the nucleus, which occurs during its activation (Perry and others 1982; Hunot and others 1997; Nakamura and others 1997).

Mitochondrial dysfunction enters into this model via environmental damage or genetic susceptibility. Genetic differences in our ability to metabolize toxins appears to impact on PD. Polymorphisms in the cytochrome P450 system are known to affect the metabolism of toxins, and some of these polymorphisms are associated with PD (Chen and others 1995). Multiple environmental toxins have been associated with PD. Individuals who work in steel mills or other jobs in which they are exposed to metals develop PD at a higher rate (Rybicki and others 1993). Individuals exposed to agricultural toxins, such as pesticides or herbicides, also develop PD at a higher rate (Semchuk and others 1991; Liou and others 1997). Heavy metals and many agricultural toxins are known to inhibit the electron transport chain in the mitochondria. A recent study also showed that rats exposed to low levels of the mitochondrial toxin rotenone also develop Lewy bodies and dopaminergic cell death (Betarbet and others 2000). This experiment provides a direct link between the mitochondrial impairment and PD because rotenone disrupts complex I of the electron transport chain. Disruption of the electron transport chain is hypothesized to lead to release of proteins of the electron transport chain into the cytoplasm. Such release might stimulate α-synuclein aggregation directly or might by synergism with iron cytoplasmic iron (Hashimoto, Takeda, and others 1999). Alternatively, because many of these proteins contain iron, their release could stimulate α-synuclein aggregation by releasing iron. The strength of the mitochondrial model of PD is that it provides a framework for integrating proteins implicated in PD with the environmental risk factors that are known to be associated with PD.

Therapeutic Approaches to PD

The evolving pathophysiology concepts focusing on the dual roles of α-synuclein and iron in PD suggest new therapeutic strategies. If α-synuclein aggregation contributes significantly to neurodegeneration in PD, then inhibiting α-synuclein aggregation might be one of the most straightforward therapeutic approaches. Aggregation might be inhibited by identifying chemicals that bind to α-synuclein and prevent aggregation, or by simply inhibiting α-synuclein expression, which slows the kinetics of aggregation. Pharmacological agents that can inhibit aggregation have been successfully developed for Aβ, which suggests that similar agents might be developed for α-synuclein. One approach that has been successfully used is to identify regions of α-synuclein that are required for aggregation, and then design peptides that inhibit aggregation by binding to the targeted sequence (Soto and others 1998). A 12-amino acid stretch in the middle of α-synuclein appears to be necessary for aggregation, and so agents that bind to this region might inhibit α-synuclein aggregation (Giasson, Murray, and others 2000). For instance, analogues of thioflavine-S and Congo Red, which are chemicals that bind to β-pleated sheet structures, might inhibit α-synuclein aggregation. Developing analogues that selectively target α-synuclein, however, is essential because nonspecific inhibition of β-pleated sheet formation would interfere with the functioning of other proteins that require β-pleated sheet structure for their function. Selective β-sheet breakers are in development for Aβ, and it is reasonable to presume that selective β-sheet breakers could also be developed for α-synuclein.

Reducing α-synuclein expression might also inhibit aggregation of α-synuclein because aggregation of α-synuclein is strongly concentration-dependent (Wood and others 1999; Ostrerova-Golts and others 2000). Studies with transgenic mice suggest that inhibiting α-synuclein expression might have few side effects because an α-synuclein knockout mouse has been created, and this mouse has only mild deficits in synaptic functioning, and no behavioral deficits (Abeliovich and others 2000). Anti-sense technology might be a feasible

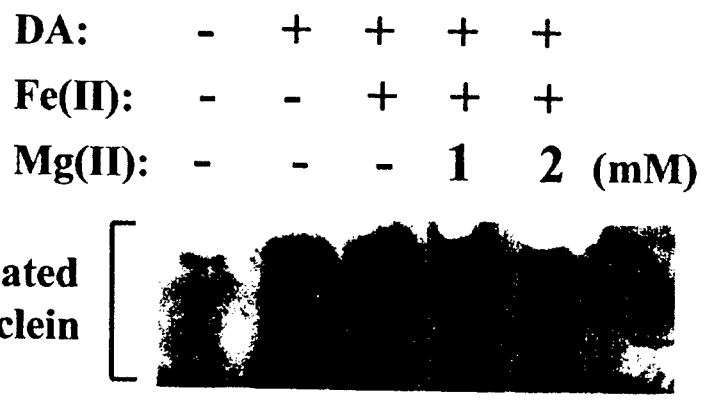


Fig. 8. Inhibition of α-synuclein aggregation by magnesium. BE-M17 cells overexpressing wildtype α-synuclein were incubated in growth medium ± 0.3 mM $FeCl_2$/50 μM dopamine ± 0–2 mM $MgCl_2$ for 4 days. The cell lysates were then centrifuged 1 h at 100,000 × g. The pellet (30 μg protein per sample) was immunoblotted with anti-α-synuclein antibody. Iron-induced formation of high molecular weight aggregates, whereas magnesium reduced the amount of aggregation.

therapeutic approach in PD because anti-sense DNA successfully reduces α-synuclein expression (Ostrerova and others 1999; Murphy and others 2000). Thus, the technologically sophisticated approach of using anti-sense technology is at least theoretically promising.

Targeting the interaction of α-synuclein with iron is another approach that might successfully inhibit α-synuclein aggregation. Chelators such as the iron-chelator deferoxamine might reduce the content of iron in the substantia nigra and thereby reduce α-synuclein aggregation. However, iron is required by many enzymes; hence, iron chelation therapy is unlikely to be tolerated by patients.

We have discovered another approach that focuses on metals and also inhibits α-synuclein aggregation. In the course of surveying how metals interact with α-synuclein, using tyrosine fluorescence as a marker of metal binding to α-synuclein, we observed two patterns of interaction. Iron and copper fall into one class, whereas zinc, magnesium, and calcium fall into a second class (Golts and others 2001). Although iron and copper induce α-synuclein aggregation, we have observed that magnesium inhibits α-synuclein aggregation. Incubating α-synuclein with magnesium (1–2 mM) greatly decreases both spontaneous and iron-induced α-synuclein aggregation in vitro (Golts and others 2001). Magnesium also inhibits iron-induced α-synuclein aggregation in cell lines overexpressing α-synuclein (Fig. 8). Pathological studies of PD support a connection between magnesium and PD because two reports show that patients with PD have lower levels of magnesium in the brain than age-matched controls (Barbiroli and others 1999). It is possible that the reduction in magnesium contributes to α-synuclein aggregation. This suggests that magnesium might be used by itself, or as an adjuvant to any future therapy to inhibit α-synuclein aggregation, and possibly delay the progression of PD. Use of magnesium as an adjuvant therapy in PD is also promising because the dose of magnesium that inhibits aggregation is similar to that already used in the clinics to treat pre-eclampsia. Thus, changes in the content of metals in the substantia nigra of patients with PD appears to exert a large impact on the tendency of α-synuclein to aggregate, and by manipulating these metals it might be possible to delay or stimulate the progression of PD.

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Appendix 3

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α-Synuclein Interacts with Phospholipase D Isozymes and Inhibits Pervanadate-induced Phospholipase D Activation in Human Embryonic Kidney-293 Cells*

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α-Synuclein has been implicated in the pathogenesis of many neurodegenerative diseases, including Parkinson's disease and Alzheimer's disease. Although the function of α-synuclein remains largely unknown, recent studies have demonstrated that this protein can interact with phospholipids. To address the role of α-synuclein in neurodegenerative disease, we have investigated whether it binds phospholipase D (PLD) and affects PLD activity in human embryonic kidney (HEK)-293 cells overexpressing wild type α-synuclein or the mutant forms of α-synuclein (A53T, A30P) associated with Parkinson's disease. Tyrosine phosphorylation of α-synuclein appears to play a modulatory role in the inhibition of PLD, because mutation of Tyr^{125} to Phe slightly increases inhibitory effect of α-synuclein on PLD activity. Treatment with pervanadate or phorbol myristate acetate inhibits PLD more in HEK 293 cells overexpressing α-synuclein than in control cells. Binding of α-synuclein to PLD requires phox and pleckstrin homology domain of PLD and the amphipathic repeat region and non-Aβ component of α-synuclein. Although biologically important, co-transfection studies indicate that the interaction of α-synuclein with PLD does not influence the tendency of α-synuclein to form pathological inclusions. These results suggest that the association of α-synuclein with PLD, and modulation of PLD activity, is biologically important, but PLD does not appear to play an essential role in the pathophysiology of α-synuclein.

α-Synuclein is a small, highly conserved presynaptic protein of unknown function that has been implicated in the development of neurodegenerative diseases, such as Alzheimer's (AD)[1] and Parkinson's disease (PD). α-Synuclein is known to be a structural component of the filaments in Lewy bodies of PD and dementia with Lewy bodies (1–5). α-Synuclein also contributes to the intracellular inclusions of multiple system atrophy (6, 7), and a fragment has been found in senile plaques in Alzheimer's disease. Two point mutations (A53T, A30P) in α-synuclein are genetically linked to familial PD (8, 9). However, the mechanism by which α-synuclein is involved in PD and in the accumulation in Lewy bodies remains elusive. The recent observation that both mice and flies expressing a human α-synuclein transgene recapitulate some characteristics of PD suggests that α-synuclein could be involved directly in the development of this disease (10, 11). Structurally, α-synuclein is a small acidic protein of 140 amino acid residues that contains three modular domain, including an amino-terminal lipid binding α-helix, an amyloid binding domain that encodes the non-Aβ component (NAC) of AD plaques, and a carboxyl-terminal acidic tail. The structure of α-synuclein allows the molecule to exist in either a random or a natively unfolded conformation or as an α-helix in the presence of phospholipids (12, 13), suggesting a highly dynamic regulation of α-synuclein function that depends on the local cellular milieu.

Reported binding targets for α-synuclein include Tau (14), 14-3-3 (15), protein kinase C (15), synphilin-1 (16), Elk (17), Tat-binding protein 1 (18). Recent studies have suggested various cellular roles for α-synuclein that include possible modifications of membrane and cell surface signaling events (19). Although normal cellular functions of α-synuclein are unknown, several observations suggest the synuclein may serve to integrate presynaptic signaling and membrane trafficking.

Recently, α-synuclein has been identified as a potent and selective inhibitor of phospholipase D2 (PLD2) *in vitro*. PLD hydrolyzes phosphatidylcholine to produce phosphatidic acid and diacylglycerol (20). This suggests that the level and modification of α-synuclein may affect phospholipase D2 activity *in vitro*, thereby modulating the cleavage of membrane lipids and membrane biogenesis (19). Thus far, two isoforms of PLD have

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[1] The abbreviations used are: AD, Alzheimer's disease; PD, Parkinson's disease; PLD, phospholipase D; PLC, phospholipase C; PMA, phorbol 12-myristate 13-acetate; PKCα, protein kinase C α; DMEM, Dulbecco's modified Eagle's medium; HEK cells, human embryonic kidney cells; NAC, non-Aβ component of Alzheimer's disease plaques; GST, glutathione *S*-transferase; PBS, phosphate-buffered saline; H & E, hematoxylin and eosin; wt, wild-type.

been described, PLD1 and PLD2 (21–24). Activity of 120-kDa PLD1 is regulated by multiple inputs, including phosphatidylinositol 4,5-bisphosphate, protein kinase C, the Rho family proteins, and ADP-ribosylation factor proteins. PLD2 is a 106-kDa protein that share 50–55% homology with PLD1. PLD2 is reported to have a much higher basal activity than PLD1 and appears to be insensitive to further stimulation by the known activators of PLD1. The primary lipid product of PLD, phosphatidic acid, exhibits a number of biological activities *in vitro* and may be an important mediator of processes controlling vesicular transport and changes in cell morphology (25). In neuronal cells, PLD activation has been linked to pathways involved in cell growth, differentiation, and neurotransmitter release (26, 27). Although many studies continue to focus on the functional relationships and the isozyme specificities of the PLD isozymes, the molecular mechanism of the regulation of the PLDs has not been fully elucidated.

In this regard, the identification of PLD-binding partners may provide clues toward the understanding of complex regulatory mechanism of PLD in different cells. Since direct cell cytotoxicity of α-synuclein is still controversial, α-synuclein might interact with other proteins to cause neurodegeneration. The investigation of regulation of PLD by α-synuclein is required to gain insight into the role of these proteins under normal and pathological condition. As a step in this effort, we now report for the first time that α-synuclein binds to PLD1 and PLD2 and inhibits pervanadate-induced PLD activation in human embryonic kidney 293 cells. Moreover, we show that the state of tyrosine phosphorylation of α-synuclein plays a modulatory role in pervanadate-induced PLD activity.

EXPERIMENTAL PROCEDURES

Materials—Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum, and LipofectAMINE were purchased from Invitrogen. Protein A-Sepharose and glutathione-Sepharose 4B were from Amersham Biosciences. Biotech. Hydrogen peroxide and sodium orthovanadate were from Sigma, and anti-phosphotyrosine antibody (Tyr(P)) (4G10) were from Upstate Biotechnology. The antibody to PKCα was purchased from Santa Cruz. Mouse monoclonal antibody to α-synuclein was generated using GST-α-synuclein as an antigen. Rabbit polyclonal antibody that recognizes both PLD1 and PLD2 was generated as described previously (28). Phosphatidylbutanol standard was from Avanti Polar Lipid. *myo*-[2-^{3}H]Inositol and [9,10-^{3}H]myristate were purchased from PerkinElmer Life Sciences. Silica gel 60 A thin layer chromatography plates were from Whatman. Horseradish peroxidase-conjugated anti-mouse IgG and anti-rabbit IgG were from Kirkegaard & Perry Laboratory (Gaithersburg, MD). The ECL Western blotting detection kit was from Amersham Biosciences.

Cell Culture and Transfection—HEK 293 cells were maintained in Hepes-buffered DMEM (Invitrogen) supplemented with 10% (v/v) fetal bovine serum under 5% CO_2. The cells were transfected with the indicated plasmid DNA and LipofectAMINE (Invitrogen) according to manufacturer's instructions. G418 (500 μg/ml) was used for selection.

Co-immunoprecipitation and Immunoblot—HEK 293 cells were lysed with lysis buffer (20 mM Hepes, pH 7.2, 1% Triton X-100, 1% sodium deoxycholate, 0.2% SDS, 150 mM NaCl, 1 mM Na_3VO_4, 1 mM NaF, 10% glycerol, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM phenymethylsulfonyl fluoride) and precleared with preimmune IgG and protein A-Sepharose for 30 min at 4 °C with rocking. Protein concentrations were determined using the Bio-Rad Protein Assay with bovine serum albumin as a standard. Equal protein aliquots of precleared cell lysates (1 mg) were incubated with the indicated antibodies and 30 μl of a 1:1 slurry of protein A-Sepharose beads for 4 h at 4 °C. The immune complexes were collected by centrifugation and washed five times with a buffer (20 mM Tris, pH 7.5, 1 mM EDTA, 1 mM EGTA, 150 mM NaCl, 2 mM Na_3VO_4, 10% glycerol, and 1% Nonidet P-40) and resuspended in sample buffer. Immune complexes were subjected to SDS-PAGE and Western blot analysis using the indicated antibody. The protein bands were visualized using ECL (Amersham Biosciences).

Construction and Preparation of GST Fusion Proteins—The full-length cDNA of human PLD1 or PLD2 was digested into fragments containing specific domains. These individual PLD1 or PLD2 fragments were then ligated into the *Eco*RI or *Sma*I site of the pGEX4T1 vector. Subcloning and the polymerase chain reaction (PCR) were used to produce the expression vectors encoding the respective GST fusion proteins. *Escherichia coli* BL21 cells were transformed with individual expression vectors encoding the GST fusion proteins, and after harvesting the cells, the GST fusion protein expressed were purified by standard methods (29) using glutathione-Sepharose 4B (Amersham Biosciences).

Preparation of Rat Brain Extract—Rat brain (2 g) was homogenized in lysis buffer using a polytron homogenizer. After centrifugation at 100,000 × *g* for 1 h at 4 °C, the resulting supernatant was used to investigate potential α-synuclein or PLD binding domains. Protein concentrations were determined using the methods developed by Bradford (30).

In Vitro Binding Experiment—Clarified lysates (1 mg) of rat brain were incubated with 3 μg of GST fusion proteins immobilized on glutathione-Sepharose beads in a final volume of 500 μl of lysis buffer for 1.5 h at 4 °C. Protein complexes were collected by centrifugation and washed four times with washing buffer (1% Triton X-100, 150 mM NaCl, 20 mM Tris-HCl, pH 8.0, 20 mM NaF, 2 mM sodium orthovanadate, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml aprotinin). Associated protein complexes were resolved by SDS-PAGE and transferred to a nitrocellulose membrane. Immunoreactivity was detected using the indicated antibodies, horseradish peroxidase-conjugated secondary antibodies, and ECL according to the manufacturer's instructions.

Measurement of Phosphoinositides Hydrolysis by PLC—Cells were plated into 60-mm dishes at 5 × 10^5 cells per dish and grown for 1 day. The cells were then labeled with *myo*-[2-^{3}H]inositol (2 μCi/ml) in inositol-free DMEM for 20 h. Subsequently, the labeled cells were washed and pretreated with 20 mM LiCl for 15 min in DMEM containing 20 mM Hepes, pH 7.2, and 1 mg/ml bovine serum albumin. Stimulation was initiated by the addition of pervanadate for 50 min and terminated by the addition of ice-cold 5% $HClO_4$. After 30 min in an ice bath, extracts were centrifuged, diluted with distilled water, and applied to Bio-Rad Dowex AG 1-X8 anion exchanger column. The column was then washed with 10 ml of distilled water followed by 10 ml of 60 mM ammonium formate containing 5 mM of sodium tetraborate. Total inositol phosphates were eluted with a solution containing 1 M ammonium formate and 0.1 M formic acid.

Phospholipase D Assay—PLD activity was assessed by measuring the formation of [^{3}H]phosphatidylbutanol, the product of transphosphatidylation in the presence of 1-butanol. HEK 293 cells were subcultured in six-well plates at 2 × 10^5 cells/well. The cells were serum-starved in DMEM for 24 h before the start of the assay. For the final 20 h of serum starvation, the cells were labeled with 1 μCi/ml [9,10-^{3}H]myristic acid. The cells were washed three times with 5 ml of phosphate-buffered saline (PBS) and pre-equilibrated in serum-free DMEM for 1 h. For the final 10 min of preincubation, 0.3% butan-1-ol was included. At the end of the preincubation, cells were treated with pervanadate or PMA for the indicated times. Incubations were terminated by removing the medium, washing with 5 ml of ice-cold PBS, and adding 1 ml of ice-cold methanol. Cells were scraped off the plates using a policeman, and the lipids were extracted and separated with methanol/chloroform/0.1 N HCl (1:1:1) according to the method of Bligh and Dyer (31). The lower phase was dried under N_2, resuspended in 30 μl of chloroform/methanol (2:1), and spotted onto silica gel 60A thin layer chromatography plate (Whatman). The plates were developed in the upper phase of the solvent system of ethyl acetate/iso-octane/H_2O/acetic acid (55:25:50:10) and stained with iodine. A phosphatidylbutanol standard (Avanti Polar Lipids) was used to locate the bands, which were scraped into scintillation mixture. Radioactivity incorporated into total phospholipids was measured, and the results were presented as percentage of total lipid counts/min incorporated into phosphatidylbutanol to normalize the results.

Immunofluorescent Staining—To examine the co-localization of α-synuclein with PLD1, a double-immunofluorescence technique was used. Three male Sprague-Dawley rats (3–6 months old) were deeply anesthetized with 4% chloral hydrate (1 ml/100 mg) and were sacrificed by transcardial perfusion with a fixative containing 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. Cryostat coronal sections (25 μm thick) were cut throughout the mesencephalon and were processed for double-immunofluorescence histochemistry. Free floating sections were blocked in 10% normal donkey serum and normal goat serum in 0.01 M PBS for 1 h. Incubation with primary antibodies was performed with a mouse monoclonal antibody against α-synuclein and an affinity-purified anti-PLD1 antibody overnight at 4 °C. After washing in PBS, the sections were incubated with a mixture of fluorescein isothiocyanate-conjugated goat anti-mouse IgG (Jackson ImmunoResearch; diluted at 1:100) and Cy3-conjugated goat anti-rabbit IgG (Jackson Im-

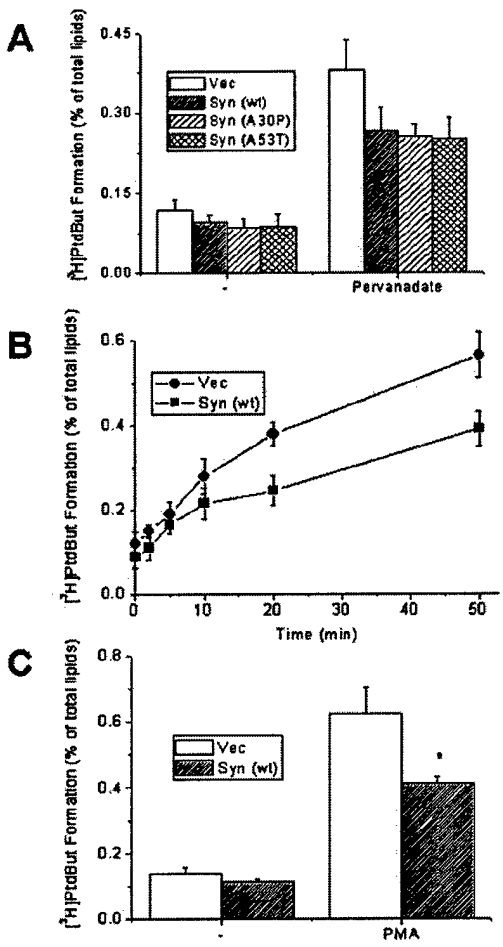

FIG. 1. **Overexpression of α-synuclein inhibits pervanadate-induced PLD activation in cells.** *A*, HEK 293 cells expressing wild-type (*wt*), A30P, and A53T α-synuclein as well as vector-transfected cells were cultured in six-well plates, labeled with [^{3}H]myristate, and treated with pervanadate (100 μM Na_3VO_4 and 0.5 mM H_2O_2) for 30 min. *B*, vector and α-synuclein HEK 293 cells were treated with pervanadate for different times. *C*, vector and α-synuclein HEK 293 cells were treated with PMA for 50 min. The radioactivity incorporated into phosphatidylbutanol was measured as described under "Experimental Procedures." Results are the means ± S.D of three independent experiments. *, $p < 0.05$ compared with PMA-treated control cells.

munoResearch; diluted at 1:100) for 2 h at room temperature. To test the specificity of immunostaining, control sections were processed in an identical manner but with the primary or secondary antibodies omitted. Slides were viewed using a confocal microscope (MRC-1024, Bio-Rad). Images were converted to TIFF format, and contrast levels of images were adjusted using Adobe Photoshop.

Formation of Eosinophilic Inclusions—We performed hematoxylin and eosin (H & E) staining according to standard cell biology techniques (16, 32). At 30 h post-transfection, the co-transfected HEK 293 cells were fixed with 3.7% formaldehyde-PBS buffer for 15 min at room temperature and hydrated with distilled water for 10 min. The hydrated cells were stained with Mayer's hematoxylin solution (Sigma) for 5 min and washed in distilled water. Cells were destained with 1% HCl in ethanol for 30 s and neutralized with 0.2% ammonia for 30 s. The processed cells were stained with eosin solution (Eosin Y, 0.5% aqueous solution, Sigma) for 1 min, followed by washing in distilled water. The cells were dehydrated by rinsing in the following concentration series of ethanol, 70, 80, and 100%, at room temperature and then mounted with 100% glycerol. All cells were counted in fields chosen at random from five different circles of the cell culture well (all cells were counted in five randomly selected fields under a light microscope at a magnification of ×400). Values are expressed as percentages of cells containing eosinophilic protein aggregates with pinkish color relative to total cells. Error bars shown in this study represent S.D. derived from means for three independent replicates.

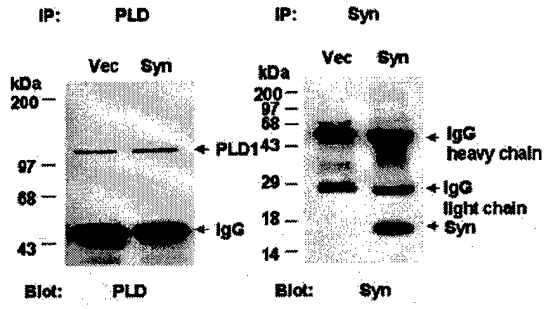

FIG. 2. **Expression of PLD1 in HEK 293 cells.** Vector or α-synuclein-expressing cells were lysed, and immunoprecipitates (*IP*) were prepared using anti-PLD or anti-α-synuclein (*Syn*) antibodies. Immunoprecipitants were analyzed by SDS-polyacrylamide gel electrophoresis, followed by transfer of proteins to nitrocellulose membrane and Western blotting (*Blot*) with anti-PLD or α-synuclein (*Syn*) antibodies. The results shown are representative of three separate experiments.

Statistical Analysis—Data are expressed as means ± S.D. The Student's *t* test was used where appropriate. A probability of $p < 0.05$ was considered statistically significant.

RESULTS

α-Synuclein Inhibits Pervanadate or PMA-induced PLD Activity in HEK 293 Cells—To investigate whether α-synuclein might affect PLD activity in human cell lines, we generated a line of 293 HEK cells overexpressing wild-type (wt), A30P, or A53T α-synuclein, as well as a vector transfected control cell line (Vec). Pervanadate, a complex of vanadate and hydrogen peroxide, is a competitive inhibitor of protein-tyrosine phosphatase that works by irreversible oxidation and functions on intact cells because of its cell permeability (33). Pervanadate is also known to stimulate PLD activity (34–36). Pervanadate stimulated PLD activity less in wild-type, A30P, or A53T α-synuclein cell lines than in the control cell line (Fig. 1*A*). Inhibition of pervanadate-stimulated PLD activity in α-synuclein HEK 293 cells occurred in a time dependent manner, compared with that of control cells (Fig. 1*B*). We also examined an effect of α-synuclein on another activator of PLD, PMA. PMA-induced PLD activation in cells expressing α-synuclein also was more reduced, compared with that of control cells (Fig. 1*C*). To examine the relative contribution of PLD protein to PLD activity in HEK cells, we investigated the expression level of PLD isozymes. By immunoprecipitation and Western blot analysis using anti-PLD antibody, cells overexpressing wild-type α-synuclein or vector-transfected cell line were found to express similar levels of PLD1 (Fig. 2). However, PLD2 was not detected in either cells (data not shown). Similar results were observed in HEK 293 cells stably transfected with A30P or A53T (data not shown). To further demonstrate the inhibitory effect of α-synuclein on the PLD activity, we transiently co-transfected α-synuclein and PLD1 or PLD2 into HEK 293 cells. Transfected cells were labeled with [^{3}H]myristic acid and then either left untreated or stimulated with pervanadate (100 μM sodium orthovanadate and 0.5 mM H_2O_2). Cells co-transfected with α-synuclein and PLD1 or PLD2 showed less pervanadate-induced PLD activation than cells transfected with PLD and vector (Fig. 4). These results demonstrate that α-synuclein inhibits both PLD1 and PLD2 activation induced by pervanadate in mammalian cells.

α-Synuclein Is Tyrosine-phosphorylated by Pervanadate in HEK 293 Cells—Protein-tyrosine phosphorylation plays a pivotal role for the functional properties of numerous proteins. α-Synuclein appears to contain four tyrosine residues, which are consensus sequences for tyrosine kinase-mediated phosphorylation. Recently, it was reported that α-synuclein is ty-

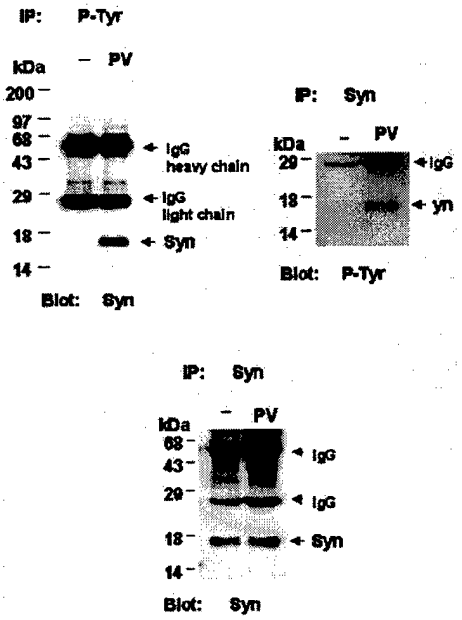

FIG. 3. **Pervanadate-induced tyrosine phosphorylation of α-synuclein in cells.** HEK cells overexpressing wild-type α-synuclein were treated with pervanadate (*PV*) (100 μM Na_3VO_4 and 0.5 mM H_2O_2) for 20 min. Cells were immunoprecipitated with anti-α-synuclein or anti-Tyr(P) antibody. Resulting immunoprecipitants were separated in duplicates by SDS-PAGE and transferred to nitrocellulose membranes using anti-Tyr(P) or α-synuclein (*Syn*) antibodies. The results shown are representative of three separate experiments.

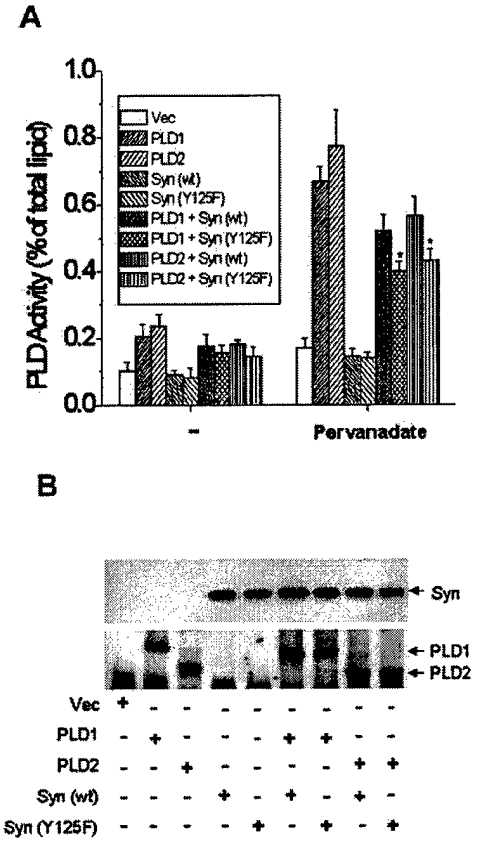

FIG. 4. **Effect of tyrosine phosphorylation state of α-synuclein on pervanadate-induced PLD activation.** *A*, HEK 293 cells were transiently transfected with various constructs and labeled with [^{3}H]myristic acid for 18 h. The cells were treated with or without pervanadate (100 μM Na_3VO_4 and 0.5 mM H_2O_2) for 30 min in the presence of 0.3% 1-butanol. The radioactivity incorporated into phosphatidylbutanol was measured as described under "Experimental Procedures." Results are the means ± S.D of three independent experiments. *, $p < 0.05$ compared with cells co-transfected with α-synuclein (*wt*) and PLD1 or PLD2 and treated with pervanadate. *B*, the lysates from the HEK 293 cells used in the experiments were analyzed by Western blotting as described under "Experimental Procedures."

rosine-phosphorylated by the Src family of protein-tyrosine kinases such as c-Src and Fyn (37). We investigated tyrosine phosphorylation of α-synuclein in transfected cells. Immunoprecipitated α-synuclein from either pervanadate-treated stably transfected HEK 293 cells expressing human α-synuclein was separated by SDS-PAGE in duplicate. Western blot analysis was then performed with phosphotyrosine specific antibody (4G10), or α-synuclein antibody, which specifically recognizes α-synuclein (Fig. 3). The cell lysates were also immunoprecipitated with anti-phosphotyrosine antibody and then analyzed by immunoblotting with anti-α-synuclein antibody. Pervanadate (100 μM sodium orthovanadate and 0.5 mM H_2O_2) induced tyrosine phosphorylation of α-synuclein.

Effect of Tyrosine Phosphorylation State of α-Synuclein on Pervanadate-induced PLD Activation—We investigated the effect of the tyrosine phosphorylation state of α-synuclein on pervanadate-induced PLD activity by mutating each of the tyrosines in α-synuclein. Mutation of Tyr^{125} to Phe was the only tyrosine mutation to alter the activity of α-synuclein. This reduced tyrosine phosphorylation to ~5% of the wild-type control, whereas other single amino acid changes do not change tyrosine phosphorylation significantly. Next, we examined the effect of tyrosine phosphorylation state on the ability of α-synuclein to inhibit PLD activity. The cells were co-transfected with PLD1 or PLD2 along with the Y125F mutant construct or wild-type control α-synuclein. After co-transfection, cells were trated with or without pervanadate (100 μM Na_3VO_4 and 500 μM H_2O_2). Mutation of Tyr^{125} to Phe slightly increased the inhibitory effect of α-synuclein on pervanadate-induced PLD activity. (Fig. 4*A*). The expression level of PLDs or α-synuclein was similar in these transfection experiment (Fig. 4*B*). Thus, the tyrosine phosphorylation state of α-synuclein appears to modulate PLD activity.

α-Synuclein Associates with PLD1 and PKCα—To explore the significance of this inhibition, we examined whether α-synuclein interacts with PLD isozymes. HEK 293 cells overexpressing α-synuclein were treated for 20 min with or without pervanadate. The lysates were immunoprecipitated with anti-PLD antibody, and the precipitates were probed with monoclonal anti-α-synuclein antibody (Fig. 5*A*). The presence of α-synuclein in the PLD immune complex was apparent. In a reciprocal experiment, α-synuclein was immunoprecipitated by antibody to α-synuclein. Subsequent immunoblotting with the anti-PLD1 revealed PLD1 expression (Fig. 5*A*). Interestingly, α-synuclein was constitutively associated with PLD1. Furthermore, both α-synuclein and PLD1 proteins were associated with PKCα in cells overexpressing α-synuclein (Fig. 5, *B* and *C*). α-Synuclein was also associated with PLD2 in co-transfection experiments (Fig. 5*D*). Interestingly, pervanadate did not alter the interaction of α-synuclein with PKCα or PLD. To examine whether the effect of α-synuclein on PLD is specific, we investigated the effect of α-synuclein on the activity of other lipid modifying enzyme and their interaction (Fig. 6). Pervanadate induced an increase in PLC activity in the HEK 293 cells, but α-synuclein did not affect PLC activity (Fig. 6*A*) and did not associate with PLC-β1 (Fig. 6*B*) in cells overexpressing α-synuclein, suggesting that the effect of α-synuclein on PLD is specific.

Amphipathic Repeat Region and NAC Domain of α-Synuclein Is Involved in the Interaction with PLD1—To map the region on

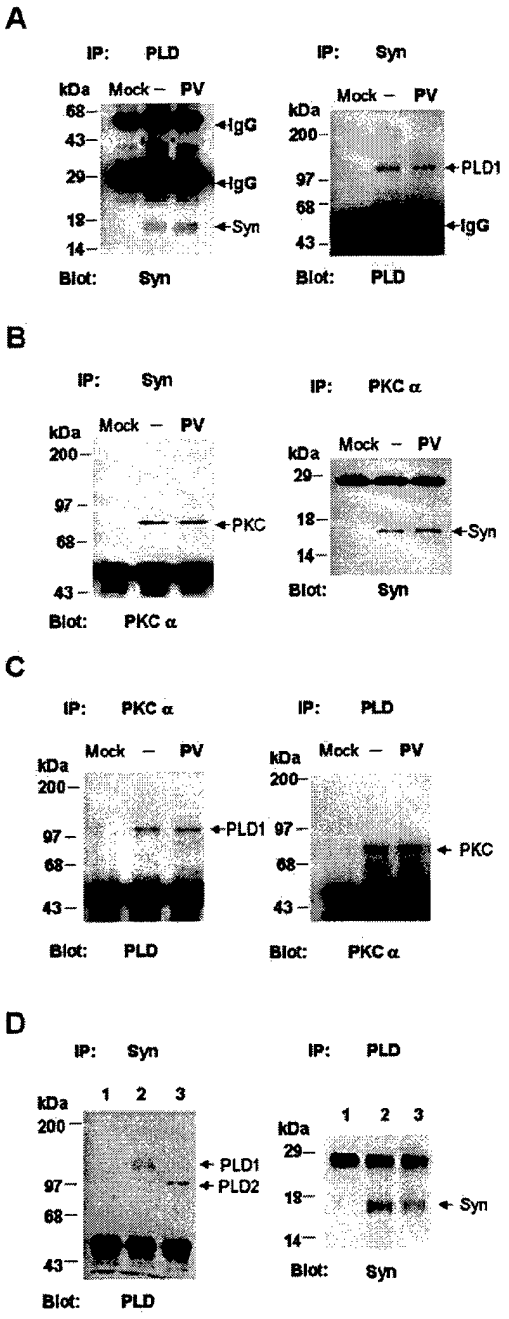

FIG. 5. **Interaction of α-synuclein with PLD and PKCα.** *A–C*, HEK 293 cells expressing α-synuclein were stimulated without or with pervanadate (*PV*) (100 μM Na_3VO_4 and 0.5 mM H_2O_2) for 20 min. The cells were then lysed, and immunoprecipitates (*IP*) were prepared using anti-PLD, anti-α-synuclein (*Syn*), or anti-PKCα antibody. The immunoprecipitates were analyzed by SDS-polyacrylamide gel electrophoresis, followed by transfer of proteins to nitrocellulose membranes and Western blotting (*Blot*) with anti-PLD, anti-α-synuclein (*Syn*), or anti-PKCα antibodies. *D*, HEK 293 cells were transiently transfected with the empty vector (*lane 1*), PLD1 and α-synuclein (*lane 2*), or PLD2 and α-synuclein (*lane 3*). After immunoprecipitation with the anti-PLD or anti-α-synuclein antibody, the immunoprecipitates were then immunoblotted with anti-α-synuclein or anti-PLD antibody. "*Mock*" represents a mock-precipitated control. The blots shown are representative of three independent experiments.

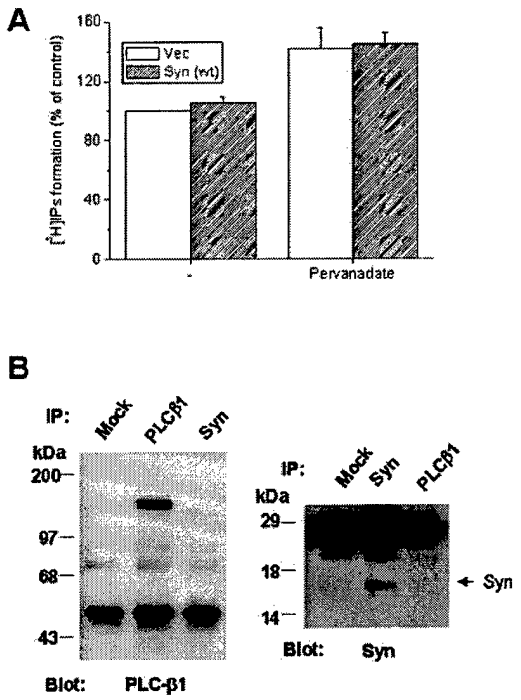

FIG. 6. **α-Synuclein does neither affect PLC activity nor interact with the enzyme.** *A*, subconfluent, quiescent cells were labeled with 2 μCi/ml *myo*-[2-^{3}H]inositol in inositol-free DMEM for 20 h and then stimulated with pervanadate (100 μM Na_3VO_4 and 0.5 mM H_2O_2) for 50 min in the presence of 20 mM LiCl, and total inositol phosphates were measured as described under "Experimental Procedures." Results are the means ± S.D of three independent experiments. *B*, the cells were then lysed, and immunoprecipitates (*IP*) were prepared using anti-PLCβ1 or anti-α-synuclein (*Syn*) antibody. The immunoprecipitates were then subjected to Western blotting (*Blot*) with anti-PLCβ1 or anti-α-synuclein (*Syn*). The blots shown are representative of three independent experiments.

the α-synuclein protein that is responsible for the interaction with PLD1, we prepared GST-fused human α-synuclein protein, GST-Syn-whole (amino acid residues 1–140), GST-Syn-N (amino acid residues 1–60), GST-Syn-NAC (amino acid residues 61–95), and GST-Syn-C (amino acid residues 96–140) and used them in *in vitro* binding experiments. As shown in Fig. 7, the GST-Syn-N and GST-Syn-NAC containing the amphipathic repeat region and the NAC domain, respectively, precipitated PLD1 from rat brain lysates. Thus, these data suggest that the amphipathic repeat region and the NAC domain might be involved in the interaction with PLD1.

α-Synuclein Associates with a Region (Amino Acids 1–331) of PLD1—Next, we analyzed the interaction site(s) of PLD1 with α-synuclein. To identify the PLD1 sequence involved in the α-synuclein binding, we constructed the GST fusion proteins shown in Fig. 8*A*. The lysates from rat brain were precipitated with GST-PLD1 fragments (Fig. 8*B*) and analyzed by Western blotting with anti-α-synuclein antibody. GST-PLD1 (amino acid 1–331) was found to be the region that most potently bound to α-synuclein. It appears, therefore, that the region between amino acids 1 and 331 containing the phox and pleckstrin homology domains may be important for the interaction with α-synuclein.

Interaction of PLD1 with the α-Synuclein in Rat Brain—As with HEK 293 cells overexpressing α-synuclein, we found that in rat brain tissues, PLD1 co-precipitates with endogeneous α-synuclein by using a co-immunoprecipitation method on rat brain (Fig. 9*A*). We tried to further check whether PLD1 co-localized with α-synuclein. Recently, we reported endogeneous PLD1 expression in neurons of the substantia nigra par compact (28), which are affected in PD. Expression of α-synuclein has been also reported in these neurons (38, 39). Therefore, rat brain sections were double-immunolabeled with anti-PLD1 and anti-α-synuclein antibodies to identify the co-localization of

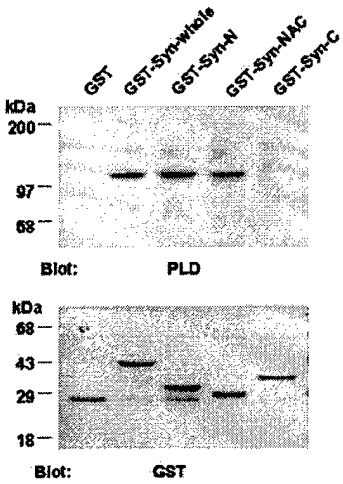

FIG. 7. **Amphipathic repeat region and NAC domain of α-synuclein interact with PLD1.** α-Synuclein was fragmented into individual domains consisting of NH_2 terminus (1–60), NAC (61–95), COOH terminus (96–140). The fragments and whole protein were cloned as GST fusion proteins, expressed in *E. coli*, and purified using glutathione-Sepharose beads. Equal amounts (1 mg) of GST or GST fusion proteins (GST-Syn fragment) were incubated with rat brain extract as described under "Experimental Procedures." The precipitated proteins were subjected to immunoblot analysis using antibody against PLD1 (*upper panel*). The amount of the GST fusion protein was visualized by Western blotting using anti-GST antibody (*lower panel*). The results shown are representative of three separate experiments.

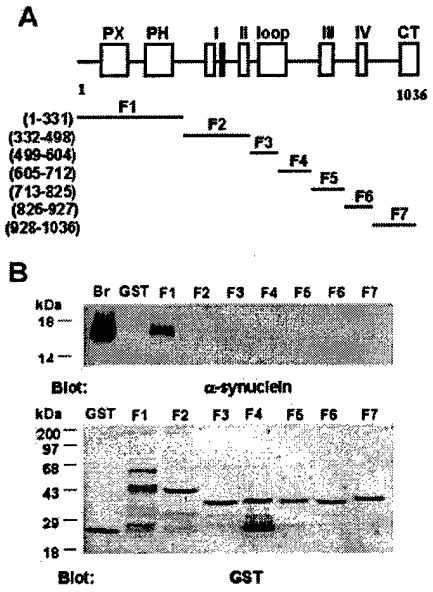

FIG. 8. **α-Synuclein associates with phox and pleckstrin homology domains of PLD1.** *A*, a schematic representation of the structure of PLD1 is shown on the *top*. The possible functions of each box have been proposed or demonstrated in Ref. 24. *Boxes* are the regions of highly conserved sequences in PLD. *PX*, phox domain; *PH*, pleckstrin homology-like domain; *I–IV*, conserved regions in the PLD family (24). *loop*, loop region, *CT*, COOH terminus. *B*, equal amounts (1 mg) of GST or GST fusion proteins (GST-PLD1 fragments, *F1–F7*) were incubated with rat brain extract as described under "Experimental Procedures." The precipitated proteins were subjected to immunoblot analysis using antibody against α-synuclein (*upper panel*). The amount of the GST fusion protein was visualized by Western blotting using anti-GST antibody (*lower panel*). The results shown are representative of three separate experiments.

α-synuclein with PLD1 in the same neuron. Examination with confocal microscopy revealed that all α-synuclein immunoreactive neurons in the substantia nigra pars compacta co-localized

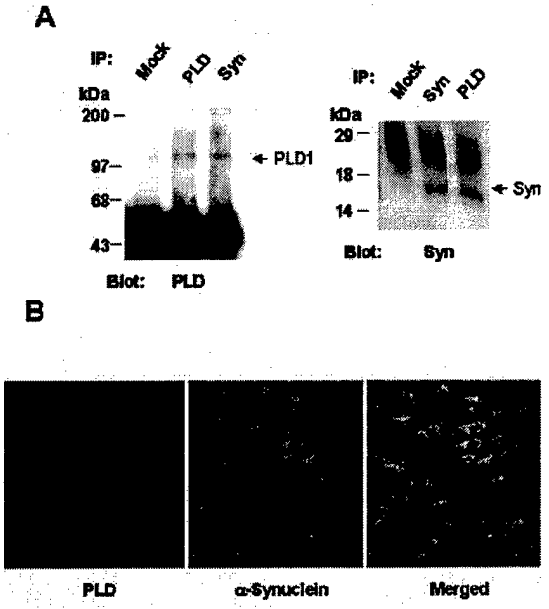

FIG. 9. **Interaction of PLD1 with α-synuclein in rat brain.** *A*, α-synuclein and PLD was co-immunoprecipitated from rat brain lysates using antibody to PLD or α-synuclein. The resulting immunoprecipitates were immunoblotted with antibodies to α-synuclein or PLD. *Mock* represents a mock-precipitated control. *B*, Cy3-labeled, PLD1 immunoreactive cells and fluorescein isothiocyanate-labeled, α-synuclein immunoreactive cells in the neuron of the substantia nigra pars compacta were visualized with confocal microscopy. Superimposed images display the co-localization of, respectively, PLD1-labeled and α-synuclein-labeled neurons in the substantia nigra pars compacta. *Scale bars*: 50 μm. The results shown are representative of two separative experiments.

with PLD1 immunoreactivity (Fig. 9*B*). Taken together, these results suggest that regulation of PLD by α-synuclein might occur through *in vivo* interaction.

Quantification of Eosinophilic Inclusions—Since α-synuclein was observed to interact with both PLD1 and PLD2, we assessed their functional significance in terms of the formation of intracellular cytoplasmic inclusions, which is pathological characteristics of PD (8, 9, 40, 41). The morphological composition of cells was determined by hematoxylin and eosin (H & E) staining to evaluate the formation of inclusion bodies in cells transfected with both PLD and α-synuclein proteins (Fig. 10*A*). Consistent with the previous result, we observed that ~6% cells out of total cell numbers had eosinophilic inclusion bodies in cytoplasm when HEK 293 cells were co-transfected with constructs encoding NAC and full-length synphilin-1, which were used as a positive control (Fig. 10*B*) (16). The percentage of cells that develop inclusion bodies is ~10–20%, out of cells expressing both NAC and synphilin-1 based on a transfection efficiency of 30–40%, which was calculated using a green fluorescent protein reporter plasmid. In contrast, cells co-transfected constructs encoding NAC and PLD1 or NAC and PLD2 exhibited only ~1–2% of eosinophilic inclusion bodies (Fig. 10*B*). In addition, cells co-expressing α-synuclein and PLD1 or PLD2 revealed same effect that was observed in cells co-exprexressing NAC and PLDs (data not shown). This is similar to the results that were obtained from cells expressing any protein alone, such as NAC, α-synuclein, PLD1, or PLD2 protein. These results suggest that the specific interaction between PLDs and α-synuclein might not be an essential feature in the formation of inclusion bodies in mammalian cells.

DISCUSSION

We demonstrate for the first time that α-synuclein binds to both PLD1 and PLD2 and inhibits its enzymatic activity in

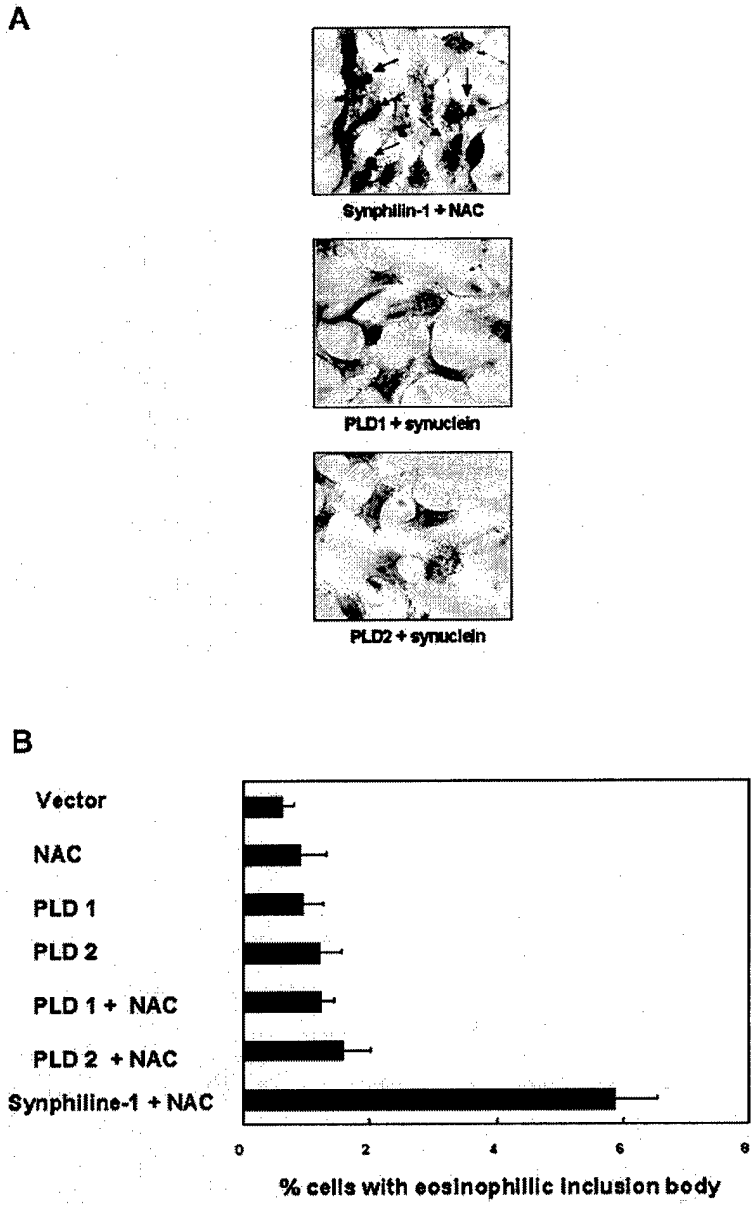

FIG. 10. **Formation of eosinophilic cytoplasmic inclusion bodies.** *A*, HEK 293 cells co-transfected with constructs encoding NAC (amino acids 61–95 of α-synuclein) and full-length synphilin-1 develop cytoplasmic eosinophilic inclusions when stained with H & E. The inclusions were hardly detected in cells co-transfected with constructs encoding NAC plus PLD1 or NAC plus PLD2. *B*, quantification of eosinophilic inclusion formation in HEK 293 cells transfected with various constructs. Approximately 6% of cells co-transfected with constructs encoding NAC and synphilin-1 co-stained eosinophilic inclusions, whereas co-transfection of constructs encoding NAC and PLD1 or PLD2 resulted in the formation of less than 1% of eosinophilic inclusion. Values are expressed as percentages of cells containing eosinophilic protein aggregates with *pinkish color* relative to total cells. Results are the means ± S.D of three independent experiments.

human cell lines. α-Synuclein has been implicated in Parkinson's and Alzheimer's disease. It is of great interest to determine the specific function(s) of α-synuclein because of its potential importance in the pathogenesis of these diseases. The link between α-synuclein and PLD appears to be particularly intriguing, because α-synuclein inhibits PLD2 activity *in vitro* (20), and PLD activity was significantly increased in in Alzheimer's disease brain tissues as compared with control tissues (42). Recently, we confirmed the increase in PLD1 protein in AD brain and also observed that PLD co-localized with β-amyloid (data not shown). Taken together, these results suggest that modulation of PLD by α-synuclein might play a role in some aspects of the pathophysiology of neurodegenerative diseases. Further investigations of the regulation of PLD by α-synuclein could provide valuable insights into the role of these proteins play in normal and pathological conditions.

To investigate this issue, we examined the regulation of PLD by α-synuclein in HEK 293 cells overexpressing α-synuclein. Pervanadate or PMA-stimulated PLD activation was decreased in cells overexpressing α-synuclein, compared with that of vector-transfected cells. Cells overexpressing $Ala^{53} \rightarrow Thr$ (A53T) and $Ala^{30} \rightarrow Pro$ (A30P) mutant α-synuclein, which have been associated with familial forms of PD, showed greater inhibition of PLD than vector-transfected cells. By immunoprecipitation and Western blot analysis using an anti-PLD antibody, the HEK 293 cells were found to express similar levels of ~120-kDa PLD1 protein. However, the 105-kDa PLD2 protein was not detected in any of the cell lines, indicating that the PLD activity shown in these cells is due to mainly to PLD1. Using *in vitro* studies, α-synuclein has been reported to inhibit PLD2 activity more potently than PLD1 activity (20). However, we found that in co-transfection experiments, α-synuclein inhibited pervanadate-stimulated PLD activity. This suggests that α-synuclein can inhibit both forms of PLD. The ability of α-synuclein to inhibit PLD1 in cells might reflect differences between *in vitro* and *in vivo* environments. Recently, α-synuclein was reported to inhibit PKC activity in HEK 293 cells (15), which could contribute to the inhibition of PLD activity. Inhibition of PKC by α-synuclein could contribute to the decrease in pervanadate-induced PLD activation in cells overexpressing α-synuclein. However, it is possible that this inhibition might also result from a direct interaction between

PLD and α-synuclein. Here we demonstrated that α-synuclein is constitutively associated with PLD1 in cells, and α-synuclein forms a triple complex with PLD and PKCα in a ligand-independent manner. The association between α-synuclein and PKC is consistent with prior results observed by Ostrerova and colleagues (15). We also demonstrated that α-synuclein associates with PLD2 in co-transfected cells. We found that α-synuclein co-immunoprecipitated with endogeneous PLD from rat brain tissue and is co-localized with PLD in neurons in the substantia nigra pars compacta of rat brain, indicating that these two proteins interact *in vivo*. The PLD1 binding site in α-synuclein resides in the amino acid residues 1–95 containing 6 or 7 conserved repeats with the consensus core sequence KTKEGV.

α-Synuclein has been shown to interact with phospholipids. This interaction is also facilitated mainly by a conserved NH_2-terminal 95 residues, which changes its structure from "unfolded" to α-helical upon binding to lipids (12). Because of α-synucleins ability to interact with lipids and their association with synaptic vesicles, it has been suggested that synucleins might be involved in intracellular vesicular trafficking (43). Tau interacts with the acidic COOH-terminal region (residues 89–140) of α-synuclein through its microtubule-binding domain. This opens the possibility that α-synuclein might have a bridging functions that might serve to bring different classes of ligands together. α-Synuclein binds to a region between amino acids 1 and 331 of PLD1. This region contains the NH_2-terminal pleckstrin homology and phox domains, which are known to be involved in protein-protein interaction as well as binding of phospholipids (44). The interaction sites of PLD2 with α-synuclein showed similar patterns as with PLD1 (data not shown). α-Synuclein did not affect the activity of PLC and other lipid-modifying enzymes and did not associate with PLC-β1 in cells overexpressing α-synuclein, suggesting that the effect of α-synuclein on PLD is specific.

Protein-tyrosine phosphorylation is thought to be important in regulating synaptic function and plasticity (45, 46). It was reported recently that α-synuclein can be tyrosine-phosphorylated by the Src family tyrosine kinase in a co-transfection experiment and *in vitro* using purified kinases (37). This tyrosine phosphorylation occurs primarily on tyrosine 125. It is difficult to speculate on the functional consequences of tyrosine phosphorylation of α-synuclein, because its normal function has not been elucidated definitively. The putative role of α-synuclein in regulating intracellular vesicular trafficking and signaling appears particularly interesting. Mice lacking α-synuclein show abnormal dopamine release (47). α-Synuclein exists in the cytoplasm in presynaptic neurons, but is also loosely associated with synaptic vesicles (48). Covalent modification, such as phosphorylation, is a likely candidate for regulation of α-synuclein at the synapse, and covalent modification could be important in modulating its function. Although the functional consequences of phosphorylation of the tyrosine 125 residue of α-synuclein remain to be elucidated, tyrosine phosphorylation could regulate the ability of α-synuclein to bind synaptic vesicles and thereby regulate protein-protein interactions. Here we demonstrate that the phosphorylation state of tyrosine 125 of α-synuclein modulates the activity of PLD. A mutation Tyr^{125} to Phe in α-synuclein (Y125F) that mimics dephosphorylation increases the ability of α-synuclein to inhibit pervanadate-induced PLD activation. This regulatory axis could affect exocytosis, because PLD is thought to be an important component of the exocytotic machinery (49).

The discovery of abnormal protein aggregates or accumulation has been described in a number of neurodegenerative diseases. We tried to investigate that the interaction between α-synuclein and PLD isozymes has a role in inclusion body formation in PD. Recently, it was reported that when constructs encoding portions of α-synuclein and synphilin-1 are co-transfected in mammalian cells, the cells formed eosinophilic cytosolic inclusions resembling the Lewy bodies of PD (16). Although α-synuclein aggregates by itself *in vitro*, it may be that aggregation *in vivo* is facilitated by an associated protein such as synphilin-1. When constructs encoding synphilin-1 and full-length α-synuclein or the non-Aβ component AD amyloid (NAC) portion of α-synuclein were co-transfected, we observed that ~6% of cells had cytosolic phase-dense inclusions. Cytosolic inclusions were eosinophilic when stained with H & E. In contrast, when constructs encoding PLD isozyme (PLD1 or PLD2) were co-transfected along with α-synuclein or NAC, only ~1–2% of cells had eosinophilic inclusions, which is similar to that present in control cells. Thus, our data suggest that PLD isozymes may not modulate α-synuclein aggregation.

In summary, our study suggests that α-synuclein modulates the activity of PLD by protein-protein interactions, but this interaction might not be involved in regulation of the formation of cytoplasmic inclusion bodies in mammalian cells. However, we cannot rule out the possibility that PLD does modulate α-synuclein aggregation in the brain, because the environment of HEK 293 cells differs from that of the aged human brain. Although the function of both α-synuclein and PLD remains largely unknown, identification of binding partners and examination of how and where the complexes form in the cell provide important tools for understanding the physiology of PD.

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Appendix 4

Co-association of parkin and α-synuclein

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Parkin and α-synuclein are two proteins that are associated with the pathophysiology of Parkinson's disease (PD). Parkin is present in Lewy bodies and axonal spheroids in brains affected by PD, and mutations in parkin cause hereditary forms of Parkinsonism. α-Synuclein is a major component of Lewy bodies and is associated with rare cases of PD. We now show that parkin binds to α-synuclein, including conditions associated with α-synuclein aggregation. Parkin and α-synuclein complexes were observed in BE-M17 cells under basal conditions, in BE-M17 cells under oxidative conditions and in brains from control or PD donors. Double staining of PD brains shows parkin and α-synuclein co-localize to the same pathological structures (both Lewy bodies and axonal spheroids). These results suggest that parkin interacts with α-synuclein and could contribute to the pathophysiology of PD more generally than was previously considered. *NeuroReport* 12:1–5 © 2001 Lippincott Williams & Wilkins.

Key words: Aggregation; Chaperone; Immunoprecipitation; Lewy body; Pathology; Ubiquitination

INTRODUCTION

Parkin and α-synuclein are two proteins that appear to play important roles in causing Parkinsonian syndromes. α-Synuclein is one of the major protein components of Lewy bodies, a pathological hallmark of Parkinson's disease (PD) [1,2]. The A53T and A30P mutations in α-synuclein have been linked to familial PD, which suggests that α-synuclein is capable of causing PD under some conditions [3,4]. The α-synuclein protein has a disordered structure in solution and a strong tendency to aggregate, particularly in the presence of a nidus (such as micro-aggregates of α-synuclein) or after exposure to divalent metals such as the ferrous or cupric ions [5–9]. The A53T and A30P mutations in α-synuclein both appear to increase the tendency of α-synuclein to aggregate. This increased propensity to aggregate is thought to precipitate PD in patients carrying these mutations.

Mutations in parkin are the most common cause of an early onset form of Parkinsonism, termed autosomal recessive juvenile Parkinsonism (ARJP), and mutations in parkin have also been associated with some cases of PD [10–12]. Parkin was recently shown to be a ubiquitin ligase [13]. The proteins that associate with parkin are largely unknown, as are the substrates of parkin, with the exception of the septin, hCDCrel-1, which is ubiquitinated by parkin [14]. In ARJP, loss of parkin expression and/or function leads to degeneration of the neurons of the substantia nigra. ARJP can present without Lewy bodies, which suggests that parkin does not induce α-synuclein aggregation, however in PD parkin co-localizes to some Lewy bodies and all axonal spheroids, which are pathological structures that contain aggregated α-synuclein [15,16]. The association between parkin and structures containing aggregated α-synuclein, Lewy bodies and axonal spheroids, suggested to us that parkin might bind to α-synuclein and contribute to the pathophysiology of PD.

MATERIALS AND METHODS

Cell lines, transfections, chemicals: BE-M17 cells were grown in OPTIMEM (Gibco/BRL) plus 10% FBS supplemented with 200 fg/ml G418, as needed. The α-synuclein and parkin cDNAs were cloned into pcDNA3.1 at the *Not*I site (α-synuclein), and the *Xba*I and *BamH*I site (sense parkin). The anti-sense parkin was cloned by cutting parkin a 411 bp cDNA segment complementary to a 411 nucleotide segment of parkin extending from the 5′ start site, blunt ending the fragment with mung bean nuclease and cloning into the *Eco*RV site of pcDNA3.1 (anti-sense parkin). The recombinant α-synuclein was cloned into the NcoI/NotI sites of the ProEx bacterial expression vector (GIBCO/BRL). The antibodies used for the analyses include: rabbit anti-parkin (1:5000, against amino acids 305-323 of human parkin, Chemicon), goat anti-parkin (1:1000, against amino acids 83-97 of human parkin, Southwest Immunology), rabbit anti-α-synuclein (1:2000) [9]; mouse anti-α-synuclein (1:1000, Transduction Laboratories), rabbit anti-ubiquitin (1:1000, Dako), mouse anti-actin (1:3000,

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ICN), FITC-anti-goat IgG (1:30, Jackson Immunoresearch) and rhodamine-anti-rabbit IgG (1:150, Jackson, Immunoresearch).

Human tissue: Substantia nigra were obtained from three cases of PD (mean age 68.0 ± 2.3 years, mean postmortem interval 5.2 ± 0.4 h) and three control cases (mean age 69 ± 13.8 years, mean postmortem interval 8.8 ± 1.9 h).

Immunoblotting: Cells are harvested with SDS lysis solution (2% SDS, 10 mM Tris, pH 7.4, 2 mM β-glycerol phosphate, 1 μM AEBSF). Protein was determined using the BCA assay (Pierce): 30 μg per lane was run on 12% polyacrylamide gels (with a 4% stacking gel) and transferred to nitrocellulose (200 mA, 6 h). The nitrocellulose was then incubated for 1 h in 5% milk/PBS, washed, incubated overnight in primary antibody, washed, then incubated for 3 h in peroxidase-coupled secondary antibody and developed with chemiluminescent reagent (DuPont).

Immunoprecipitations: Homogenates were extracted with 1% Triton X-100 in PBS plus protease and phosphatase inhibitors. The lysates were spun down (10 000 × *g*, 15 min) and the supernatants precleared with protein A or G sepharose (Pharmacia). Primary antibody (1 μg) was then added to 500 μg protein lysate and incubated at 4°C for 2 h. The immunocomplexes were precipitated by incubation with protein A sepharose (for rabbit primary antibodies) or protein G sepharose (for mouse and goat primary antibodies) at 4°C for 2 h. Following binding of the solid phase substrate, the samples were washed five times in TBS/1% Triton X-100 and immunoblotted.

Immunofluorescence: Following removal of paraffin, the samples were washed, permeabilized by incubation for 30 min with 0.2% Triton-X 100, blocked with 5% dry milk/1% goat serum/PBS, washed, incubated overnight in primary antibody in 2% BSA/0.1% Triton-X 100/PBS, washed, incubated for 3 h in secondary antibody in 2% BSA/0.1% Triton-X 100/PBS, washed and then coverslipped.

RESULTS

To investigate whether parkin associates with α-synuclein, we determined whether α-synuclein and parkin could be co-precipitated. We began by examining binding of parkin to α-synuclein in the BE-M17 cells under basal conditions. Parkin was immunoprecipitated from lysates of human neuroblastoma BE-M17 cells, and the resulting immuno-

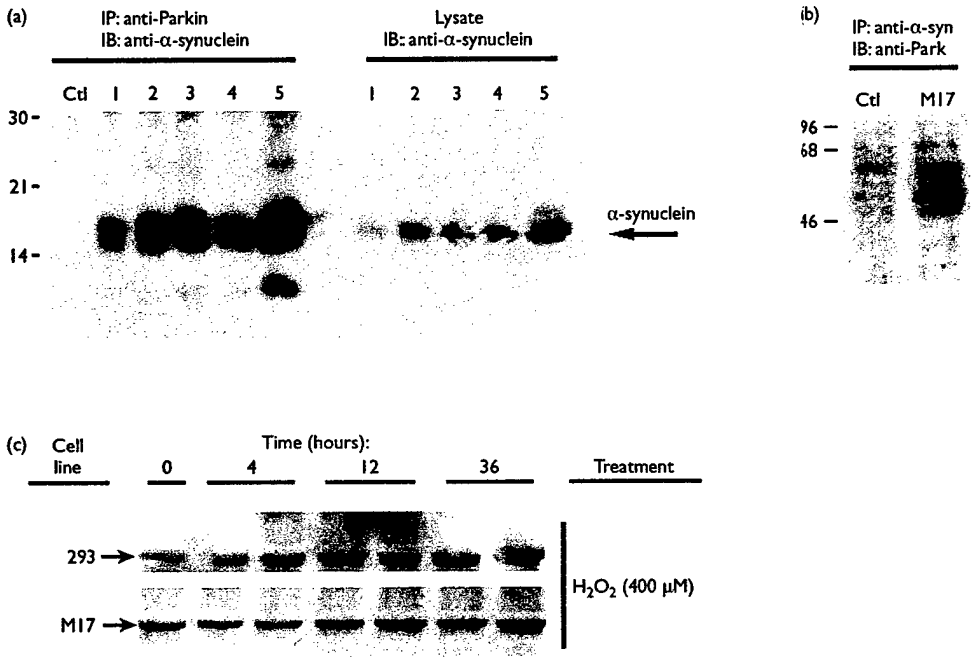

Fig. 1. Parkin expression and synuclein binding is increased under conditions that induce synuclein aggregation. **(a)** Immunoprecipitation of parkin from BE-M17 cells lines under basal conditions or after treatment with 0.3 mM $FeCl_2$ plus 50 μM dopamine, followed by immunoblotting with anti-α-synuclein antibody. Ctrl, immunoprecipitation performed with cell lysate and non-specific IgG. Lane 1, untransfected cells. Lane 2, cells overexpressing wildtype α-synuclein. Lane 3, cells over-expressing A53T α-synuclein. Lane 4, cells over-expressing A30P α-synuclein. Lane 5, A53T cells treated with iron plus dopamine. **(b)** Immunoprecipitation of α-synuclein followed by immunoblotting with anti-parkin antibody using BE-M17 cells. Ctrl, immunoprecipitation with cell lysate, performed with non-specific IgG in lieu of anti-α-synuclein antibody; M17, immunoprecipitation with cell lysate and anti-α-synuclein. **(c)** Changes in expression of parkin in 293 HEK and BE-M17 cells in response to oxidative stress over time. Each experiment was repeated 3–5 times.

blots were probed with α-synuclein antibody. α-Synuclein–parkin complexes were readily detectable in the human neuroblastoma BE-M17 cells (Fig. 1a). Binding was present in untransfected cells (Fig. 1a, lane 1), and binding was increased in cells over-expressing either wildtype (lane 2), A53T (lane 3), or A30P α-synuclein (lane 4). We also examined whether parkin can associate with α-synuclein under conditions of oxidative stress, such as those that might occur in the brains of patients with PD. We treated BE-M17 cells expressing A53T α-synuclein with 1 mM ferrous chloride and 50 μM dopamine for 3 days, which induces oxidative stress and α-synuclein aggregation [9]. We observed that α-synuclein bound to parkin, suggesting that parkin and α-synuclein can associate under conditions of oxidative stress (Fig. 1a, lane 5). Interestingly, the amount of parkin associated with α-synuclein under these conditions was greater than under basal conditions. α-Synuclein–parkin complexes were also detectable in BE-M17 cells after immunoprecipitating α-synuclein and immunoblotting with parkin (Fig. 1b). These results show that parkin binds to α-synuclein, including under oxidative conditions such as might be present in the substantia nigra in PD.

The increased association of α-synuclein with parkin appeared to be explained, in part, by the tendency of α-synuclein and parkin levels to increase in response to oxidative stress. Immunoblotting cellular lysates showed that α-synuclein levels were increased in cells treated with iron plus dopamine, which is consistent with our prior observation that α-synuclein levels increase during cell stress (Fig. 1a, lane 5) [17]. In separate experiments, we observed that subjecting cells to oxidative stress also increased the expression of parkin (Fig. 1c). Expression of endogenous parkin increased within 12 h of applying hydrogen peroxide to either HEK 293 cells (Fig. 1c, top panel) or BE-M17 cells (Fig. 1c, bottom panel). This suggests that the levels of both α-synuclein and parkin can increase during acute oxidative stress.

Next we sought to determine whether parkin associates with α-synuclein in brain tissue. First we investigated whether recombinant α-synuclein would associate with parkin (Fig. 2a). His-6 tagged α-synuclein (5 μg) was added to a lysate of human cingulate cortex (500 μg in PBS plus 1% Triton X-100, protease and phosphatase inhibitors), mixed and incubated for 1 h. Next, the recombinant α-synuclein was precipitated using nickel–agarose slurry (25 μl), washed three times in lysis buffer and immunoblotted. The resulting immunoblot showed a prominent band at 53 kDa, which is the expected mol. wt for parkin and co-migrated with a similar protein present in the lysate (Fig. 2a). Omission of recombinant α-synuclein from the procedure eliminated the 53 kDa band, although there was some immunoreactivity present in that region of the gel which probably represents other proteins non-specifically absorbed by the nickel–agarose resin. These data suggest that recombinant α-synuclein can associate with parkin.

Parkin–α-synuclein complexes could also be detected by direct immunoprecipitation of parkin from human brain. To examine brain tissue, we immunoprecipitated α-synuclein from substantia nigra of three PD and three control donors (using 500 μg, control and PD lysates, and rabbit anti-α-synuclein antibody). Immunoblots showed that parkin was associated with the precipitated α-synuclein, however there was no clear difference in the amount of parkin-α-synuclein complex (Fig. 2b). Parkin–α-synuclein complexes were also evident after immunoprecipitating the lysates with anti-parkin antibody and probing with anti-α-synuclein antibody (Fig. 2c, arrow). The amount of parkin in the lysates did not differ consistently among the samples. The additional band above the parkin band (Fig. 2c) is due to non-specific binding of the secondary antibody to the rabbit anti-α-synuclein antibody. Notably, no such band was seen in Fig. 1a because the blot was performed with a different secondary antibody that exhibited higher species specificity. These data indicate that α-synuclein and parkin associate, and that the association is present both in the normal brain and in brain tissue from patients with PD.

The observation of α-synuclein-parkin complexes in brains from cases with PD raised the possibility that parkin and α-synuclein might co-localize in pathological structures. Parkin and α-synuclein have both been noted to be present in Lewy bodies and axonal spheroids, but we have not determined whether parkin and α-synuclein co-exist together in the same pathological structures [2,15,16]. To determine whether parkin and α-synuclein co-localize to the same pathological structures in the brains of patients with Parkinson's disease, we performed immunocytochemistry using double staining with antibodies to parkin and α-synuclein. Substantia nigra from the brains of Parkinson donors was double-stained with anti-α-synuclein and anti-parkin antibodies. We identified Lewy bodies and axonal spheroids labeled with both anti-α-synuclein antibody (Fig. 3b,f) and anti-parkin antibody (Fig. 3c,g). Use of pre-immune serum gave no reactivity (Fig. 3e). Immunostaining for parkin or α-synuclein was also performed on substantia nigra sections from neurologically normal cases. Both parkin (Fig. 3j) and α-synuclein (Fig. 3k) antibodies clearly labeled neuronal cell bodies and processes. Omission of primary antibody precluded any immunoreactivity, although neuromelanin within dopaminergic neurons was evident (Fig. 3i). The presence of parkin and α-synuclein in the same pathological structures suggests that parkin can associate with aggregated α-synuclein.

DISCUSSION

We have shown that parkin and α-synuclein associate under both normal and pathological conditions, such as PD. The double staining studies support the proposal that parkin and α-synuclein associate by showing that both parkin and α-synuclein can be co-localized to the same pathological structures, both Lewy bodies and axonal spheroids, and together within neurons and neuronal processes.

Both parkin and α-synuclein are associated with Parkinsonian syndromes. α-Synuclein is a major component of Lewy bodies, and is present in axonal spheroids. These observations suggest that α-synuclein plays an important role in the pathophysiology of PD. Mutations in parkin are associated with autosomal recessive juvenile parkinsonism, as well as with some cases of PD [10,11]. Whether parkin plays a more general role in PD is unknown.

Increasing evidence suggests that parkin might be involved more generally in the pathophysiology of PD.

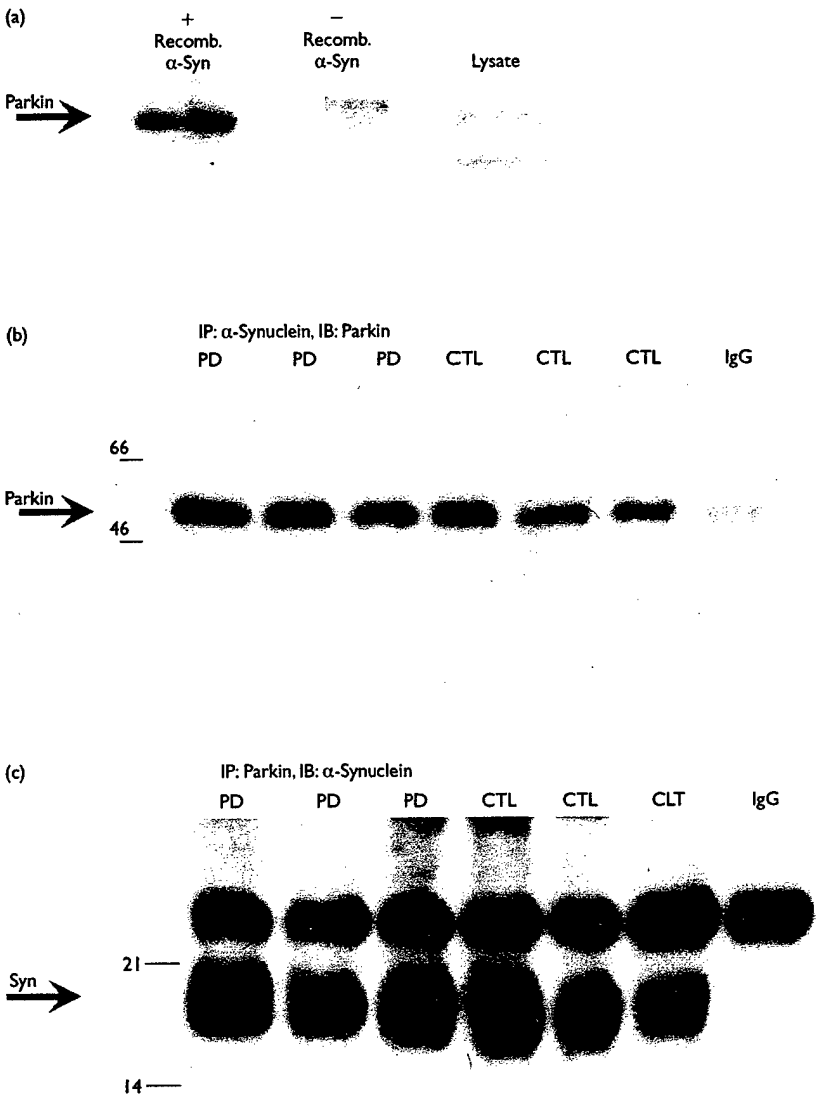

Fig. 2. Co-immunoprecipitation of parkin and α-synuclein. **(a)** Pulldown of parkin from human cingulate brain tissue by incubation with recombinant α-synuclein, and immunoblotting of the precipitate with anti-parkin antibody. **(b)** Immunoprecipitation of α-synuclein from human substantia nigra brain tissue, followed by immunoblotting with anti-parkin antibody (Ctrl, neurologically normal; PD, Parkinson's disease). The lane marked IgG refers to an immunoprecipitation of brain lysate performed with non-specific IgG. **(c)** Immunoprecipitation of parkin from human substantia nigra brain tissue, followed by immunoblotting with anti-α-synuclein antibody. Each experiment was repeated 3–5 times.

Both our laboratory and that of Shimura and colleagues have recently shown that parkin can be observed in Lewy bodies in cases of PD [15,16]. In addition, we have shown that parkin is also present in most, if not all, axonal spheroids in cases of PD [15]. Our observations presented in this manuscript show that parkin and α-synuclein associate and are present in the same pathological structures. This suggests that parkin might play a significant role in the pathophysiology of PD in general. The nature of this role remains unanswered. The data presented in this manuscript indicate that parkin normally associates with α-synuclein, even under conditions of oxidative stress, and might also be capable of associating with aggregated α-synuclein. As aggregated α-synuclein accumulates in the brains of patients with PD, binding of parkin to aggregated α-synuclein could lead to the accumulation of parkin in Lewy bodies and axonal spheroids. Because parkin is an ubiquitin ligase, it is possible that the association of α-synuclein with parkin contributes to the regulation of the ubiquitin-dependent proteasomal cascade under normal conditions [13,14]. Moreover, the association of parkin with aggregated α-synuclein could contribute to the ubiquitination of proteins in Lewy bodies and axonal spheroids.

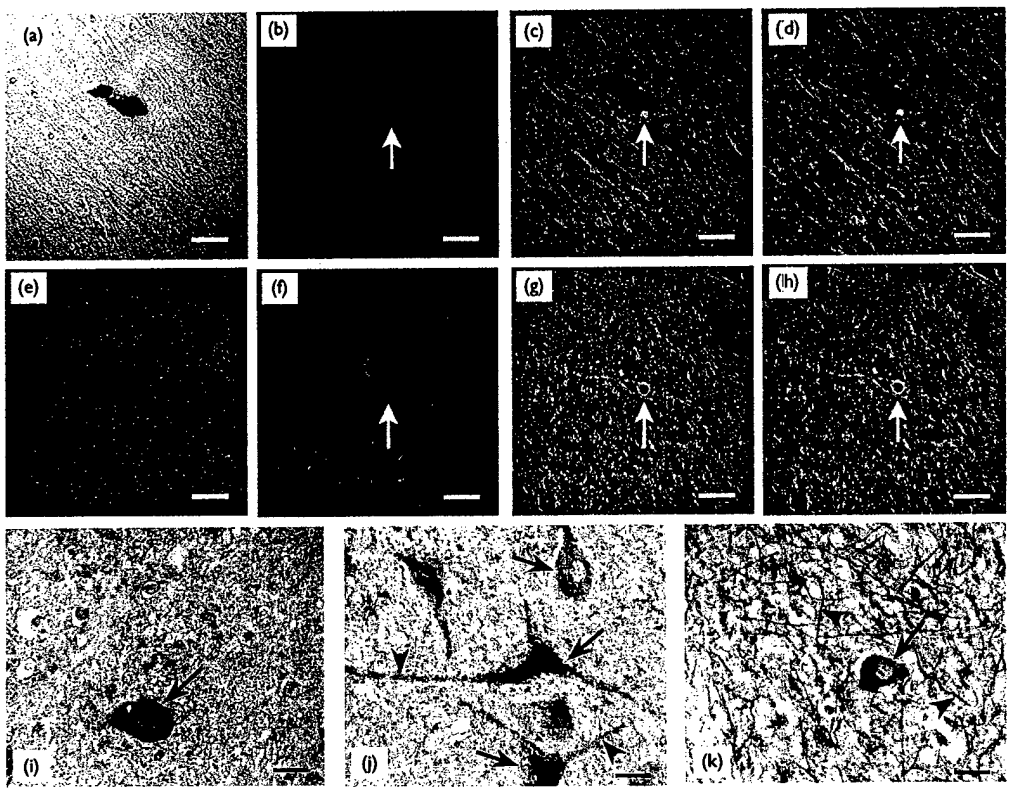

Fig. 3. Immunocytochemistry of substantia nigra from PD substantia nigra. **(a)** Staining of a brightfield photograph of a dopaminergic neuron containing neuromelanin. **(b)** Staining of the same section with anti-α-synuclein antibody reveals an immunoreactive Lewy body (arrow). **(c)** The same Lewy body is also stained with anti-parkin antibody (arrow). **(d)** Overlaying of (b) and (c) demonstrates co-localization of α-synuclein and parkin staining (arrow). **(e)** Staining with pre-immune serum yielded no reactivity. **(f)** An axonal spheroid stained with anti-α-synuclein antibody (arrow). **(g)** The same axonal spheroid is also stained with anti-parkin antibody (arrow). **(h)** Overlaying (f) and (g) demonstrates co-localization of α-synuclein and parkin staining (arrow). **(i)** Omission of primary antibody revealed no staining in control substantia nigra sections. However, neuromelanin was still clearly visible (arrow). **(j)** Immunostaining of control substantia nigra sections with anti-parkin antibody revealed clear staining of neuromelanin-containing neuronal cell bodies (arrows) and neuronal processes (arrowheads). **(k)** Immunostaining of control substantia nigra sections with anti-synuclein antibody revealed immunoreactivity resembling parkin staining, with cell bodies (arrow) and cell processes (arrowheads). Bar = 20 μm.

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Appendix 5

MAGNESIUM INHIBITS THE AGGREGATION OF α-SYNUCLEIN IN CULTURED CELLS

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Abbreviations: Parkinson's disease (PD); Lewy bodies (LB).

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Abstract

α-Synuclein aggregation contributes to the pathophysiology of Parkinson's disease. We have recently shown that magnesium inhibits aggregation of recombinant α-synuclein *in vitro,* but whether magnesium inhibits α-synuclein aggregation in neurons is unknown. To investigate this, we examined the effects of exogenously applied magnesium on the aggregation of α-synuclein in neurons. First we compared α-synuclein aggregation induced by either rotenone or iron (FeII) in neuroblastoma cells. Iron and rotenone both induced α-synuclein aggregation and the accumulation of high molecular weight ubiquitin adducts, although iron induced more α-synuclein aggregation than rotenone. The high molecular weight ubiquitin conjugates also occurred in cells exhibiting α-synuclein aggregation, and the ubiquitin conjugates co-precipitated with α-synuclein. Magnesium (1 mM or greater) inhibited both α-synuclein aggregation and the accumulation of high molecular weight ubiquitin adducts induced by either iron or rotenone. Magnesium was effective both in cell lines and primary neurons. The reduction of α-synuclein aggregation was apparent using either immunoblot and immunocytochemical analysis using anti-α-synuclein antibody. We also examined iron-mediated toxicity, which is increased in neuroblastoma cells over-expressing α-synuclein. Magnesium (1.2 mM) inhibited iron-mediated neurotoxicity in neuroblastoma cells over-expressing α-synuclein. These data show that magnesium inhibits α-synuclein aggregation and toxicity in cell culture, which suggests that magnesium might impact on α-synuclein aggregation in Parkinson's disease.

Introduction

Parkinson's disease (PD) is a common neurodegenerative disorder characterized by loss of dopaminergic neurons of the substantia nigra, producing symptoms such as bradykinesia and tremor. The pathological hallmark of PD are Lewy bodies (LB), which are proteinaceous intracellular inclusions composed primarily of α-synuclein and ubiquitin, as well as smaller amounts of numerous other proteins [2, 7, 12, 51, 52]. α-Synuclein is thought to play a central role in the neurodegeneration occurring in PD because mutations in α-synuclein, at A53T or A30P, cause familial PD, which indicates that changes in α-synuclein biology are sufficient to cause disease [32, 48].

Increasing evidence suggests that aggregation of α-synuclein is a central process that underlies the pathophysiology of PD. α-Synuclein has a strong tendency to aggregate, and does so spontaneously at a slow rate *in vitro* [14, 27, 59]. The mutations in PD that are associated with familial PD, at A53T and the A30P, both increase the tendency of α-synuclein to aggregate *in vitro* and *in vivo* [20, 43]. Aggregation of α-synuclein appears to be associated with neurotoxicity. Studies both in cultured neurons, and in transgenic animals, show that the accumulation of α-synuclein aggregates is linked to cellular toxicity and neurodegeneration [20, 38, 43]. Formation of α-synuclein aggregates in neuroblastoma correlates with iron mediated toxicity [43], and although not directly related to α-synuclein aggregation, oer-expressing α-synuclein causes acute toxicity in dopaminergic neurons grown in cell culture [60]. Over-expressing α-synuclein leads to an age-dependent accumulation of aggregated α-synuclein, and associated neurodegeneration in *Drosophila* [2, 20], and in also to varying degrees in some transgenic mice [38]. For instance, Masliah and colleagues also observed that

aggregated α-synuclein is associated with loss of markers in dopaminergic neurons [38]. On the other hand, other studies show that some mice can tolerate high levels of α-synuclein without developing neurodegenerative changes [31, 56]. Together, these data suggest that aggregation of á-synuclein could contribute to neurodegeneration in PD.

Recently, two methods have been developed for inducing α-synuclein aggregation in cell culture. The redox active metals, Fe(II) or Cu(II), induce α-synuclein aggregation *in vitro* [28, 43, 45]. The amount of iron-induced aggregation increases during co-incubating with an oxidant, such as dopamine or hydrogen peroxide. Iron also induces α-synuclein aggregation cell culture [43]. Neuroblastoma cells over-expressing A53T develop aggregated α-synuclein upon incubation with 1 mM Fe(II) for 3 days. Wild type α-synuclein aggregates somewhat less readily, and iron needs to be combined with an oxidant, such as dopamine or hydrogen peroxide, to induce α-synuclein aggregation. Incubating neuroblastoma cells over-expressing wild-type α-synuclein or primary cultures of rat cortical neurons develop α-synuclein aggregates after incubation with 50 μM dopamine plus 1 mM Fe(II) for 3-5 days [43]. Rotenone is a mitochondrial inhibitor that has also been shown to induce aggregation of α-synuclein both in cell culture and *in vivo* [6, 55].

Both methods have strengths and weaknesses in modeling α-synuclein aggregation. Based on results in the published literature, iron appears to produce more α-synuclein aggregation than rotenone [34, 43]. High doses of iron, though, are toxic and can aggregate many proteins, although in neuroblastoma cells α-synuclein is selectively aggregated by Fe(II) in the range of 1 – 3 mM [43]. Rotenone is of interest because it can induce structures resembling Lewy bodies and cause selective degeneration of

dopaminergic neurons of the substantia nigra when administered to the rat [6]. Rotenone, though, is also quite toxic. Both iron and rotenone are clinically relevant to synucleinopathies. Epidemiological studies link PD to both pesticides and metal exposure [24, 25, 35, 49]. Rotenone has an additional connection to PD because it acts in a manner analogous to MPTP, which is a known human dopaminergic neurotoxin [53]. Iron has an additional connection to PD because it is toxic to neurons and accumulates in neurodegenerative diseases, such as Hallervorden-Spatz disease, Multiple Systems Atrophy and PD [16, 21, 26, 37]. These neuropathological studies suggest a link between iron and α-synuclein aggregation because each of these diseases also accumulates α-synuclein aggregates [16, 21, 52]. Thus, both types of treatment recapitulate particular aspects of PD.

The presence of α-synuclein in Lewy bodies and associations of α-synuclein mutations with PD suggests that α-synuclein is directly involved in the process of neurodegeneration in PD. The putative involvement of α-synuclein aggregation in PD has lead to extensive interest in developing methods to inhibit α-synuclein aggregation because such methods could provide insight into potential therapies for PD. We recently demonstrated that magnesium inhibits α-synuclein aggregation *in vitro* [23]. In the current study, we show that magnesium also inhibits aggregation of α-synuclein aggregation in primary rat cortical neurons and in neuroblastoma cells. In addition, we show that magnesium protects against toxicity associated with α-synuclein aggregation.

Methods

Materials: α-Synuclein (Wildtype, A53T and A30P) was cloned into the NotI site of pcDNA3. The sequence of each construct was confirmed by DNA sequencing. For

production of recombinant protein, α-synuclein was inserted into the NcoI/NotI site of the Pro-Ex His 6 vector (GIBCO/BRL). Antibodies used include: polyclonal anti-α-synuclein (SC1, 1:2000 for immunoblotting and 1:500 for immunocytochemistry against human α-synuclein, residues 116-131), monoclonal anti-α-synuclein-1 (1:1000, Transduction Labs), polyclonal rabbit anti-ubiquitin (1:1000 for immunoblotting and 1:500 for immunocytochemistry, Dako).

Cell Culture: Cells were grown in OPTIMEM (Gibco/BRL) supplemented with 10% FBS, non-essential amino acids, sodium pyruvate and 500 μg/ml G418, as needed. G418 was used for selection. Primary cortical neurons were harvested from E18 rat fetuses and grown on poly-D-lysine coated dishes in neurobasal medium (GIBCO). Following treatment the cells were harvested in buffer containing 20 mM Tris, pH 7.4, 2 mM EDTA, 0.25 M sucrose, and 20 μg/mL protease inhibitor cocktail (Sigma). The cell lysate was sonicated and centrifuged at 100,000 x g at 4°C for 1 hr, the protein content determined by BCA assay (Pierce) and 30 μg of each sample immunoblotted as described above.

Immunoblotting: Cells were harvested with SDS lysis solution (2% SDS, 10 mM Tris, pH 7.4, 2 mM β-glycerol phosphate, 1 μM AEBSF). The amount of protein was determined using the BCA assay (Pierce), 5-30 μg per lane was run on 14% SDS polyacrylamide gels and transferred to nitrocellulose (200 mAmp, 12 hrs). The nitrocellulose was then incubated 1 hr in 5% milk/PBS, washed, incubated overnight in 1° antibody, washed, then incubated 3 hrs in peroxidase coupled 2° antibody and developed with chemiluminescent reagent (NEN).

Cell fractionation: For cell fractionation the cells were harvested in buffer containing 20 mM Tris, pH 7.4, 2 mM EDTA, 0.25 M sucrose, and 20 μg/mL protease inhibitor cocktail (Sigma). The cell lysate was sonicated and centrifuged at 100,000 x g at 4°C for 1 hr.

Immunohistochemistry: For light microscopy, cells are fixed with 4% paraformaldehyde, washed, permeabilized by incubation for 30 min with 0.2% Triton-X 100, blocked with 5% dry milk/1% goat serum/PBS, washed and then incubated overnight in 1° antibody (1:500). Development is with an ABC kit and 3', 3'-diaminobenzidine as per manufacturer's directions (Vector).

Immunoprecipitation of α-synuclein aggregates: Cell lysates were harvested as insoluble material collected by centrifugation at 100,000 x g for 1 hr, as described in above. The sample (0.5 mg) was pre-cleared by incubating with 25 μg protein G-agarose for 1 hr, centrifuging 10,000 rpm 15 min (which is a speed that does not precipitate α-synuclein aggregates unless they are coupled via antibody to agarose beads), then 2 μl rabbit anti-α-synuclein antibody was added to the supernatant and incubated overnight at 4°C and then precipitated by incubating with 25 μl protein G-agarose for 1 hr and spun down. Laemli sample buffer was added, and then analyzed by immunoblot using 12% PAGE using polyclonal anti-ubiquitin antibody.

Propidium iodide staining: To measure viability using vital dye staining, the cells were incubated in growth medium containing 5 μM propidium iodide for 5 min, and then each well was photographed at two different sites chosen randomly and the number of positive cells were quantitated. Bright field photographs were also analyzed to insure that each well contained similar numbers of cells.

Results

The ability of magnesium to inhibit aggregation of wild-type α-synuclein *in vitro* suggests that it might also be able to inhibit aggregation in living neurons. To test this we examined the ability of magnesium to inhibit α-synuclein aggregation in primary cortical neurons grown in cell culture. Primary cortical neurons were treated with 1 mM $FeCl_2$ plus 0 – 1.5 mM $MgCl_2$ for 3 days, the α-synuclein was immunoblotted and the amount of aggregate determined by video densitometry (fig. 1A and B). Treatment with increasing doses of magnesium from 0.2 to 1.5 mM produced dose-dependent reduction in formation of high molecular weight α-synuclein aggregates (fig. 1A and B). Doses as low as 0.5mM of magnesium produced statistically significant reduction in formation of α-synuclein positive aggregates. Magnesium was also able to inhibit α-synuclein aggregation induced by 1mM $FeCl_2$ and 50 μM dopamine for 4 days in BE-M17 neuroblastoma cells over-expressing wild type α-synuclein (fig. 1C). Dopamine needs to be added because iron induces α-synuclein aggregation less effectively in BE-M17 than in primary neurons.

Our previous studies showed that neurons treated with iron and dopamine developed α-synuclein-positive inclusions in parallel with the formation of SDS-resistant aggregates [43]. We proceeded to investigate whether magnesium also prevents formation of α-synuclein-positive inclusions in BE-M17 neurons stably over-expressing wild-type α-synuclein. The neurons were treated with 1 mM $FeCl_2$ and 50 μM dopamine ± 1 mM $MgCl_2$ for 3 days, and then examined by immunocytochemistry using anti-α-synuclein antibodies. Neurons treated with iron showed abundant α-synuclein-positive

inclusions, and looked shrunken and injured. Neurons treated with iron and magnesium looked healthier and showed little accumulation of α-synuclein–positive inclusions (fig. 2). Some of the cells treated with iron plus magnesium showed some vacuoles, which suggests that the magnesium did not completely protect the cells from the toxicity of iron.

We proceeded to investigate whether magnesium could inhibit aggregation of α-synuclein in cells induced by reagents other than iron. Rotenone has also been shown to induce α-synuclein aggregation both *in vivo* and in neurons grown in cell culture [6, 55]. We observed that treating BE-M17 cells with rotenone (40 nM) for 12 days induced formation of high molecular weight ubiquitinated-proteins (fig. 3A). Aggregated α-synuclein also accumulated after 12 days of treatment with rotenone (fig. 3B). The accumulation of ubiquitin adducts was more apparent than the accumulation of aggregated α-synuclein (fig. 3B). The cells continued to divide during the treatment period, indicating that they were still viable. The amount of ubiquitin-adduct that accumulated was similar to the amount that occurred in cells treated with iron (1mM $FeCl_2$) for 3 days (fig. 3A). Treating the cells with magnesium strongly inhibited the rotenone-induced accumulation of ubiquitin-conjugate (fig. 3A). However, magnesium only weakly inhibited the accumulation of aggregated α-synuclein (fig. 3B, note arrow).

Ubiquitinated proteins co-localize with α-synuclein in Lewy bodies in PD brain tissue. To examine whether α-synuclein associates with the ubiquitinated aggregates that accumulate we induced α-synuclein aggregation in primary cortical neurons, immunoprecipitated the α-synuclein, and then immunoblotted with anti-ubiquitin antibody to pull down ubiquitinated α-synuclein binding proteins. The primary cortical neurons were treated with 0.3 mM iron and 50 μM dopamine for 3 days, α-synuclein was

immunoprecipitated with anti-α-synuclein antibody, and the immunoprecipitate was immunoblotted with anti-ubiquitin antibody. We observed abundant high molecular weight ubiquitinated products that co-immunoprecipitated with α-synuclein after treatment with 0.3 mM iron and 50 μM dopamine for 3 days (fig. 4A). Moreover, treating the cells with magnesium produced a strong decrease in ubiquitin immunoreactivity (fig 3A and C, $n=3$, $P<0.0001$). We performed a number of controls to verify that the ubiquitin reactivity was associated with α-synuclein (fig. 4B). Experiments showed that the high molecular weight products are part of a complex containing α-synuclein because omission of the primary antibody, use of non-specific IgG antibody, or use of anti-raf antibody (which we had previously shown does not co-immunoprecipitate with α-synuclein) in place of anti-α-synuclein antibody failed to precipitate a significant amount of ubiquitinated aggregates (fig. 4B) [42]. The ubiquitinated products that co-precipitate with α-synuclein probably represent proteins other than α-synuclein, because recent results suggest that most α-synuclein is not ubiquitinated [50].

Magnesium-induced inhibition of α-synuclein aggregation also correlated with neuroprotection, as measured by a propidium iodide assay, which is a vital dye assay that monitors membrane integrity. For the propidium iodide viability assay, cells are incubated with 5 μM propidium iodide for 5 min, and the numbers of cells with ruptured membranes that allow leakage of propidium iodide into the cell are scored. The untransfected and wild type α-synuclein transfected neurons were incubated with 1.5 mM $FeCl_2$ plus 50 μM dopamine ± 0.8 $MgCl_2$ for 3 days, then incubated with 5 μM propidium iodide for 5 min and the positive nuclei were scored. Cells over-expressing α-

synuclein showed a large increase in toxicity (fig. 6A & B), while untransfected cells showed little toxicity. Magnesium largely reversed the toxicity in the wild type α-synuclein over-expressing neurons (fig. 5A & B). These results suggest that magnesium can protect against toxicity associated with the aggregation of α-synuclein.

Discussion

The data in this manuscript show that increasing extracellular magnesium by 0.8 – 1.5 mM significantly inhibits aggregation of α-synuclein and an associated accumulation of ubiquitinated proteins both in neuroblastoma cells over-expressing α-synuclein and in primary cultures of rat cortical neurons. The ability to inhibit α-synuclein aggregation and the accumulation of ubiquitinated proteins covers aggregation induced by either iron ($FeCl_2$) or rotenone. Inhibition of α-synuclein aggregation is evident using any of multiple methods, including detecting aggregates by immunoblotting α-synuclein, detecting α-synuclein inclusions by immunocytochemistry, and by detecting high molecular weight ubiquitinated proteins associated with α-synuclein aggregates. Previously we have shown that cells over-expressing α-synuclein show increased vulnerability to iron-mediated toxicity that parallels formation of α-synuclein aggregates. In this manuscript, we repeat this observation, and also show that doses of magnesium that inhibit α-synuclein aggregation also inhibit iron-mediated toxicity. Together, these data suggest that magnesium can inhibit the aggregation of α-synuclein in cells grown in culture.

We have recently shown that magnesium inhibits α-synuclein aggregation *in vitro* [23]. However, the biology of α-synuclein aggregation *in vitro* might differ significantly

from than in living cells because the cellular milieu contains many factors that are not present *in vitro*. For instance, α-synuclein is partially membrane bound in the cell, and increasing evidence suggests that membrane bound α-synuclein has a greater tendency to aggregate than free α-synuclein [34, 47]. In addition, α-synuclein might also interact with other proteins in the cell, such as 14-3-3, protein kinase C, synphilin-1 (in neurons), β-synuclein and phospholipase D, all of which could affect the tendency of α-synuclein to aggregate [19, 29, 30, 42]. The interaction of Hsp-70 with α-synuclein, for instance, appears to inhibit aggregation of α-synuclein, as well as the associated cellular toxicity [2]. Cellular α-synuclein can also be post-translationally modified, such as by phosphorylation, nitration, glycosylation, or other as yet unidentified changes [22, 41, 50]. α-Synuclein might also interact with small molecules, such as calcium, or other yet undefined cofactors [23, 40, 44]. Given the complexity of the cellular milieu, the biology of α-synuclein might differ in the cell from *in vitro*. For instance, recent data show that dopamine inhibits α-synuclein aggregation *in vitro*, but our studies in living cells show that dopamine accelerates α-synuclein aggregation [13, 43]. This dopamine-mediated acceleration of α-synuclein aggregation is consistent with the pathology of PD, which shows an accumulation of α-synuclein aggregates in dopaminergic neurons [13]. The differences in biology of α-synuclein in the test tube and in the cell emphasize the importance of investigating whether magnesium also inhibits α-synuclein in living cells.

The accumulation of ubiquitinated proteins in cells exhibiting α-synuclein aggregation represents a significant difference between the process of α-synuclein aggregation in cells and *in vitro*. Increasing evidence indicates that intracellular protein

aggregates, termed aggresomes, inhibit the proteasome [4]. The accumulation of ubiquitin-conjugated proteins in cells treated with rotenone or iron suggests that the ubiquitin-dependent proteasomal inhibition system is inhibited under these conditions. Proteasomal inhibition could result from the direct action of rotenone or iron, and also from inhibition by aggregated α-synuclein. The sensitivity of ubiquitin immunoblots might reflect inhibition of the proteasome by both large and small α-synuclein aggregates. In contrast, immunoblot analyses with anti-α-synuclein antibodies detect large, highly stable, SDS-resistant protein aggregates, which might not include less stable α-synuclein proto-fibrils. Increasing data suggests that small proto-fibrils are the toxic species in neurodegenerative diseases, and these toxic aggregates can inhibit the proteasome [4, 33, 58]. Our observation that ubiquitinated proteins accumulate in cells exhibiting α-synuclein is consistent with this model.

Magnesium might also have direct protective effects on the proteasome that are independent of anti-aggregating effects of magnesium on α-synuclein. Magnesium has been shown to increase proteasomal activity using *in vitro* assays [46]. This suggests that increasing cellular magnesium could increase the activity of these magnesium-dependent proteins. Direct action on the proteasome might explain the greater efficacy of magnesium for inhibiting the accumulation of ubiquitinated proteins than inhibiting the accumulation of α-synuclein aggregates during rotenone treatment.

The kinetics of α-synuclein aggregation appears to be relatively slow, compared to other proteins that aggregate, such as Aβ or long stretches of poly-glutamine. Perhaps because of this, α-synuclein does not spontaneously aggregates in cells grown in culture. However, factors such as mutation, toxins and metals, all of which are associated with

PD, stimulate α-synuclein aggregation sufficiently to observe the process in cell culture [6, 43, 55]. Rotenone is a mitochondrial inhibitor that induces α-synuclein aggregation both in the brain and in cells grown in culture [6, 55]. Iron accumulation is also associated with α-synuclein aggregation in the brain, and exposure of cells grown in culture to iron induces formation of filamentous α-synuclein aggregates [21]. The ability of similar stimuli to promote α-synuclein aggregation both in the brain and in cell culture suggests that the mechanisms of aggregation in the brain and in cell culture share features in common. The ability of magnesium to inhibit aggregation induced by either rotenone or iron, and either *in vitro* [23] or in cell culture, suggests a broad ability of magnesium to inhibit α-synuclein aggregation, and that magnesium might also inhibit α-synuclein aggregation induced by rotenone in the brain, although this hypothesis remains to be tested.

The observation of modulation of α-synuclein aggregation by magnesium or iron adds to a growing body of evidence implicating metals in the pathophysiology of neurodegenerative disorders. Zinc and copper greatly accelerate aggregation of β-amyloid and might play a critical role in neurotoxicity induced by β-amyloid [1, 10]. Copper and manganese both bind to the prion protein, and appear to influence the clinical course of prion-induced neurodegeneration [8, 57]. Iron and copper induce α-synuclein aggregation [44, Ostrerova-Golts, 2000 #1305, 45]. Iron levels are increased in substantia nigra of patients with PD, and iron is present in Lewy bodies. Conversely, magnesium levels are reduced in brains of patients with PD [3, 15, 18, 26, 36, 54].

The reciprocal regulation of α-synuclein aggregation by magnesium and iron raises the possibility that the abundance of iron in the substantia nigra could contribute to

the tendency of Lewy bodies to accumulate. Study of α-synuclein pathology suggests that α-synuclein interacts with iron, at least under pathological conditions. Neurodegenerative diseases exhibiting iron accumulation, such as multiple systems atrophy and Hallervorden-Spatz disease, accumulate aggregates of α-synuclein. In addition, iron is present in Lewy bodies [11]. However, most iron that accumulates in neurons in the substantia nigra is bound up as a ferric ion chelate with neuromelanin, while the iron that accumulates in glia and microglia is caged in ferritin complexes [5, 17]. This suggests that there is little free ferrous iron present in the substantia nigra, which is the type of iron that would contribute to oxidative stress in PD.

Whether α-synuclein aggregation is caused by pesticides, such as rotenone, or redox active metals, the ability of magnesium to modulate α-synuclein aggregation and toxicity raises the possibility that magnesium levels might be able to reduce α-synuclein aggregation in PD. If aggregation of α-synuclein plays a fundamental role in the neurodegenerative processes occurring in AD, then increasing neuronal magnesium levels could inhibit neurodegeneration mediated by α-synuclein. The amount of magnesium required to inhibit α-synuclein aggregation is within the range commonly employed in clinical protocols using magnesium (a serum concentration of 1-1.5 mM Mg^{++}). Clinical studies on humans indicate that it is possible to raise magnesium levels in the brain by administering magnesium intraveneously or ad libidum [9, 39]. Together, these studies suggest that magnesium supplementation might be able to ameliorate the deleterious affects of aggregated α-synuclein both in transgenic models of synucleinopathies, and in patients suffering from PD and related synucleinopathies.

Figure 1: Inhibition of aggregation of α-synuclein by magnesium in primary cortical neurons.

A. Immunoblotting of α-synuclein in lysates from primary cortical neurons following treatment with 1 mM $FeCl_2$ and 0 – 1.5 mM $MgCl_2$ for 3 days. Increasing doses of $MgCl_2$ reduced the formation of high molecular weight aggregates of α-synuclein.

B. Quantitation of the aggregate formation by video densitometry showed a dose dependent decrease in aggregate formation that was statistically significant at 0.5 mM $MgCl_2$.

C. Immunoblotting of α-synuclein in lysates from human BE-M17 neuroblastoma cells stably over-expressing wild type α-synuclein following treatment with 1mM $FeCl_2$ and 50 μM dopamine for 4 days. Increasing doses of 0.5 – 1.5 mM $MgCl_2$ inhibited formation of high molecular weight aggregates of α-synuclein. Lane1: Basal, Lanes 2-5: 1mM $FeCl_2$ and 50 μM dopamine Lanes 3-5: 0.5, 1 and 1.5 mM $MgCl_2$.

Figure 2: Magnesium inhibits formation of α-synuclein positive inclusions in human neuroblastoma cells.

Human BE-M17 neuroblastoma cells stably over-expressing wild-type α-synuclein were treated with 1 mM $FeCl_2$ and 50 μM dopamine plus 0 or 1 mM $MgCl_2$ for 4 days and then stained with anti-α-synuclein antibody using a peroxidase-ABC kit and 3', 3'-diaminobenzidine for color development.

A. Untreated cells.

B. Cells treated with iron, which showed inclusions, shrinkage and degeneration.

C. Cells treated with magnesium plus iron, which showed reduced inclusion formation and reduced toxicity. Magnification=40X for all panels.

Figure 3: Magnesium inhibits rotenone induced α-synuclein aggregation.

BE-M17 cells over-expressing wild type α-synuclein were incubated with 1 mM $FeCl_2$ for 3 days or 40 nM rotenone ± 1.2 mM $MgCl_2$ for 12 days. The lysates were then immunoblotted with either rabbit anti-ubiquitin antibody (panel A) or mouse anti-α-synuclein antibody (right B). The rotenone treatment induced strong accumulation of high molecular weight ubiquitin-adducts and some of accumulation of aggregated α-synuclein (the vertical line denotes the region of the gel defined as containing high molecular weight ubiquitinated proteins). Magnesium was able to inhibit the rotenone-induced accumulation of high molecular weight ubiquitin-adducts. Magnesium also partially reduced the accumulation of α-synuclein aggregation, evident in the region highlighted by the arrow.

Figure 4: Inhibition of α-synuclein aggregation also reduces levels of ubiquitinated proteins associated with α-synuclein aggregates.

A. Primary cortical neurons were treated with 0.3 mM $FeCl_2$ plus 50 μM dopamine and 0 – 2 mM $MgCl_2$ for 3 days. The lysates were then immunoprecipitated with anti-α-synuclein antibody and immunoblotted with anti-ubiquitin antibody. Increasing doses of $MgCl_2$ reduced the formation of high molecular weight ubiquitinated aggregates.

B. Controls for the immunoprecipitations in panel A. As a negative control, lysate was subjected to immunoprecipitation using protein G (lane 1). As additional negative controls, lysates ± $FeCl_2$ (1 mM) and dopamine (50 μM) were also subjected to immunoprecipitation using non-specific IgG (lanes 2 & 3) or an antibody against an antigen that is not α-synuclein (anti-raf, lanes 4 & 6). For a positive control, lysate + $FeCl_2$ (1 mM) and dopamine (50 μM) were immunoprecipitated with α-synuclein antibody. The immunoblot was then probed with a combination of anti-ubiquitin and α-synuclein antibody (the anti-α-synuclein antibody was included to demonstrate the presence of α-synuclein in immunoprecipitate in lane 5, note arrow). The arrow points to monomeric α-synuclein and the bar highlights high molecular weight α-synuclein/ubiquitin aggregates. With the exception of the positive control (lane 5), the immunoprecipitates showed little high molecular weight aggregate. The positive control for this immunoblot, shown in lane 5, has abundant high molecular weight aggregate, although this lane lacks reactivity in the stacking gel (such as is observed in panel A, lane 2 & 3) due to experimental variability.

C. Quantification of the aggregate formation by video densitometry showed a dose dependent decrease in formation of ubiquitinated aggregates.

Figure 5: Magnesium protects against α-synuclein-mediated toxicity.

A. Propidium iodide analysis untransfected or wild-type transfected BE-M17 cells following treatment with 1.5 mM $FeCl_2$ and 50 μM dopamine ± 0.8 mM $MgCl_2$

for 2 days. Treating with magnesium increased survival of the α- synuclein transfected cell line. White cells represent injured cells that allow entry of the propidium iodide (5 μM). Bright field pictures of the wells containing wildtype cells demonstrate that there are similar numbers of cells in each well. Magnification=10X

B. Quantitative analysis of propidium iodide from separate dishes of cells (n=4).

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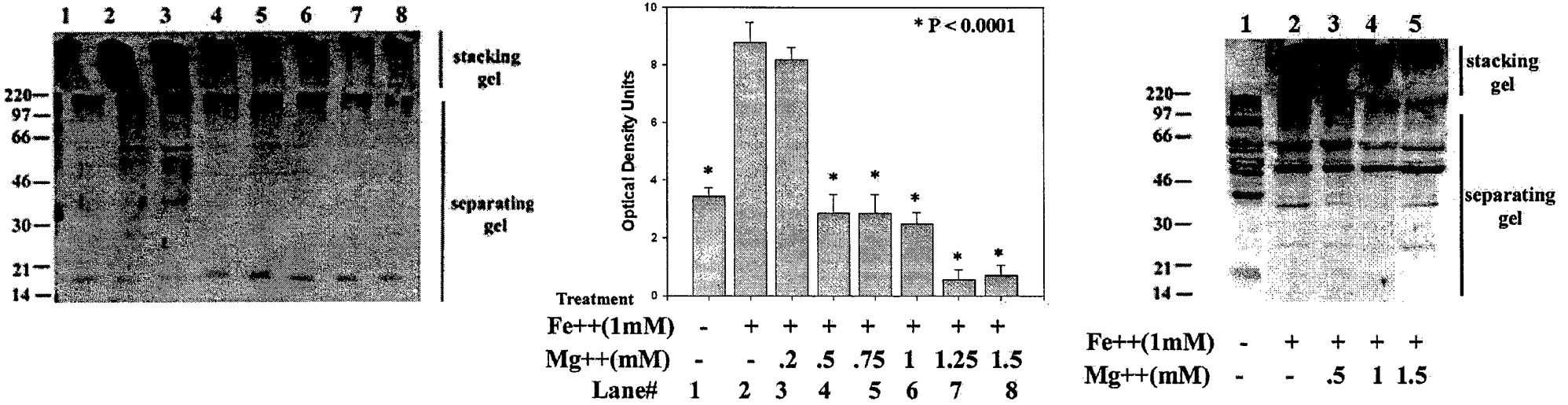
Figure 1


A.
1 2 3 4 5 6 7 8
220
97
66
46
30
21
14
stacking gel
separating gel
B.
* P < 0.0001
Optical Density Units
10
8
6
4
2
0
Treatment
Fe++(1mM) - + + + + + + +
Mg++(mM) - - .2 .5 .75 1 1.25 1.5
Lane# 1 2 3 4 5 6 7 8
C.
1 2 3 4 5
220
97
66
46
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21
14
stacking gel
separating gel
Fe++(1mM) - + + + +
Mg++(mM) - - .5 1 1.5


A.
B.
C.

Figure 3

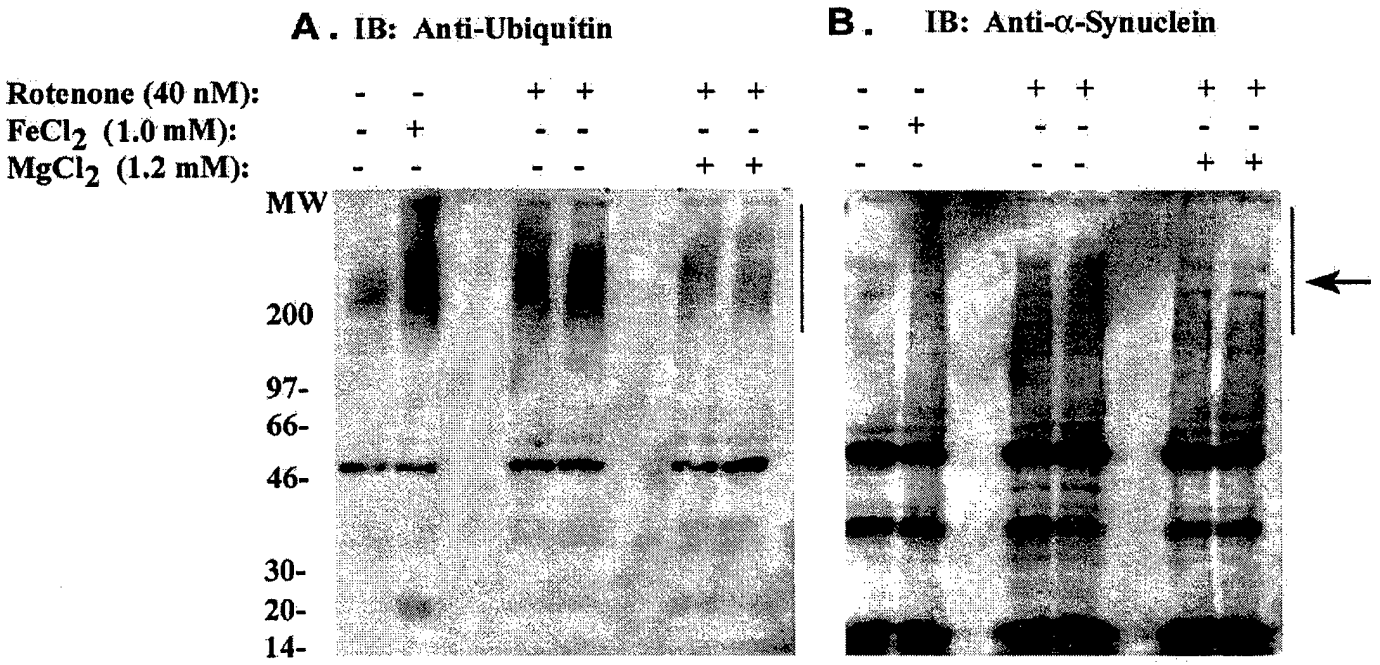

A. IB: Anti-Ubiquitin
B. IB: Anti-α-Synuclein
Rotenone (40 nM): - - + + + + - - + + + +
FeCl2 (1.0 mM): - + - - - - - + - - - -
MgCl2 (1.2 mM): - - - - + + - - - - + +
MW
200
97-
66-
46-
30-
20-
14-

Figure 4

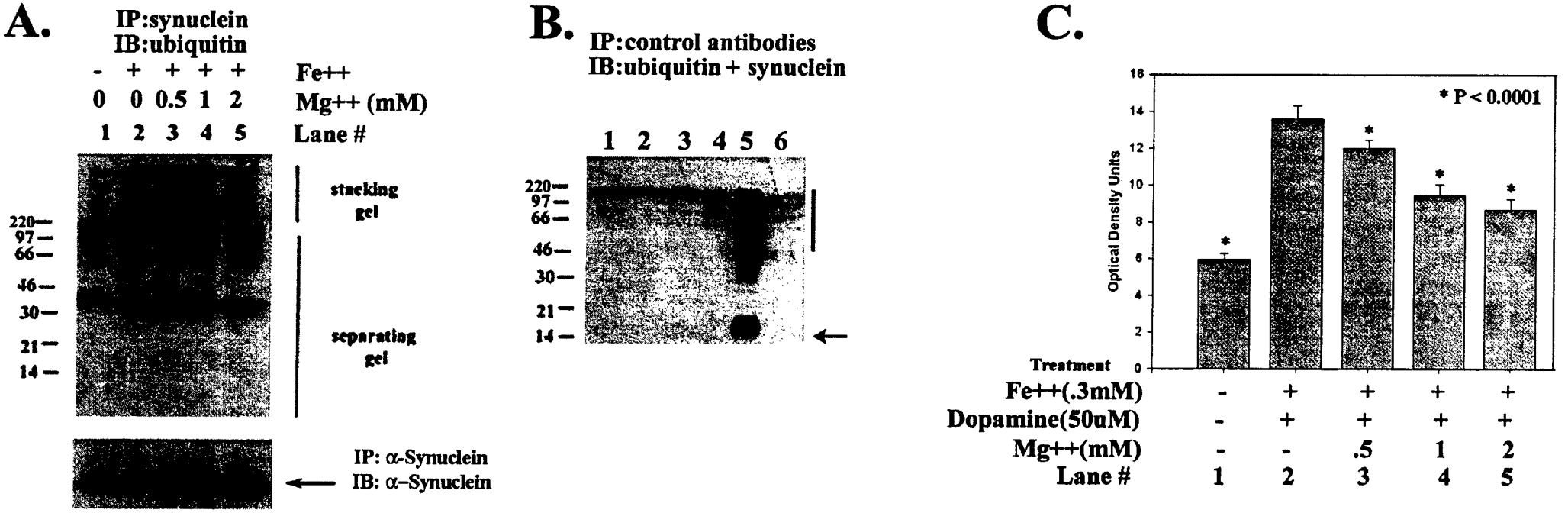
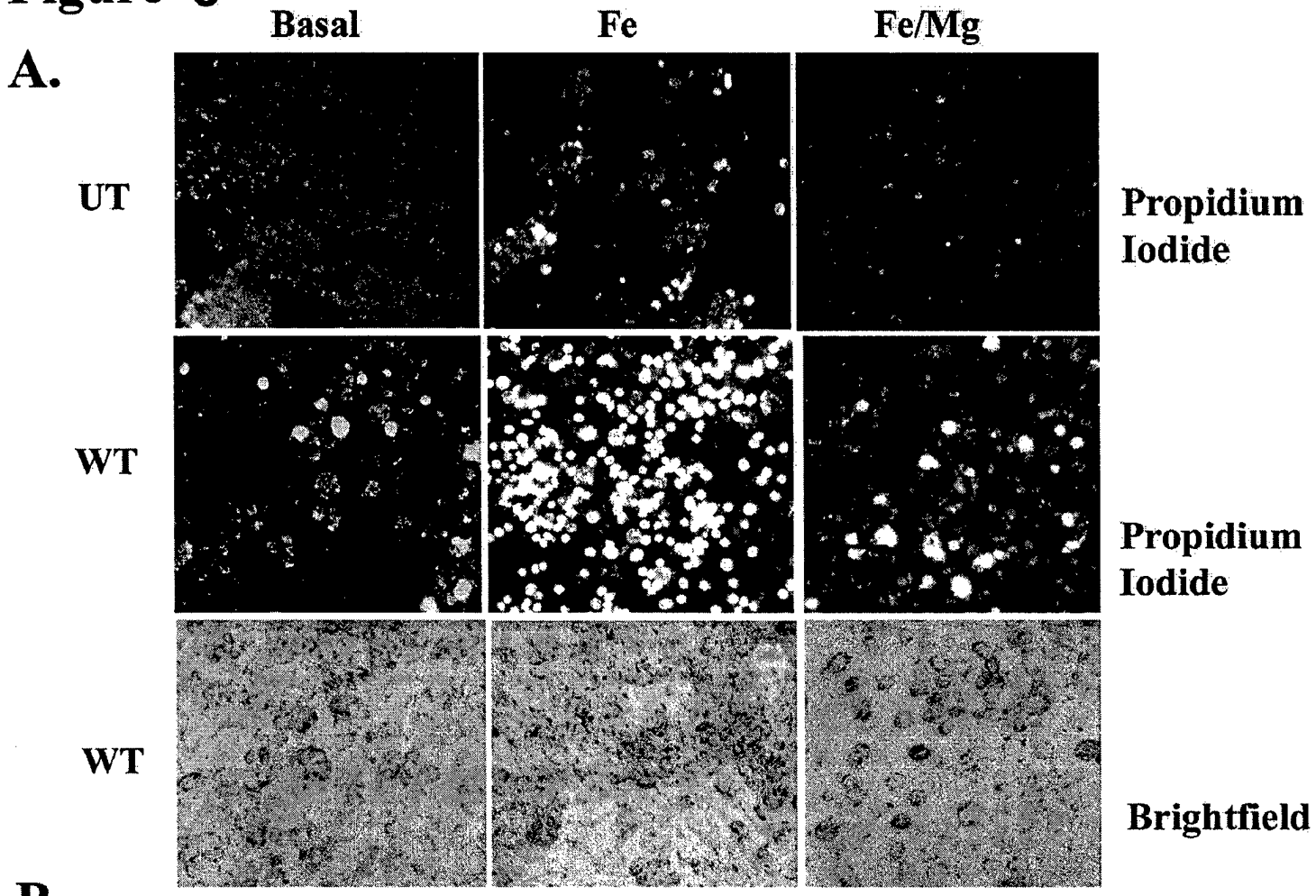

A.
IP:synuclein
IB:ubiquitin
- + + + +
0 0 0.5 1 2
1 2 3 4 5
Fe++
Mg++ (mM)
Lane #
220
97
66
46
30
21
14
stacking gel
separating gel
IP: α-Synuclein
IB: α-Synuclein
B.
IP:control antibodies
IB:ubiquitin + synuclein
1 2 3 4 5 6
220
97
66
46
30
21
14
C.
* P < 0.0001
Optical Density Units
16
14
12
10
8
6
4
2
0
Treatment
Fe++(.3mM) - + + + +
Dopamine(50uM) - + + + +
Mg++(mM) - - .5 1 2
Lane # 1 2 3 4 5

Figure 5


A.
Basal
Fe
Fe/Mg
UT
WT
WT
Propidium Iodide
Propidium Iodide
Brightfield
B.
PI Positive Nuclei
360
300
250
200
150
100
50
0
Basal
FeCl2
FeCl2/MgCl2
*
UT
WT

Appendix 6

Aggregated and Monomeric α-Synuclein bind to the S6' Proteasomal Protein and Inhibit Proteasomal Function

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Abbreviations: DHFR, dihydrofolate reductase; PD, Parkinson's disease; UPS, ubiquitin proteasomal system

Keywords: Parkinson's disease, Lewy Body, ubiquitin, neurodegeneration, aggresome, ubiquitin proteasomal system, RPT5, tat binding protein 1

Abstract:

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 26S and a 20S species. The 26S proteasome is composed of the 19S cap and the 20S core. Aggregated α-synuclein strongly inhibited the function of the 26S proteasome. The IC50 of aggregated α-synuclein for ubiquitin-independent 26S proteasomal activity was 1 nM. Aggregated α-synuclein also inhibited 26S ubiquitin-dependent proteasomal activity at a dose of 500 nM. In contrast, the IC50 of aggregated α-synuclein for 20S proteasomal activity was >1 μM. This suggests that aggregated α-synuclein selectively interacts with the 19S cap. Monomeric α-synuclein also inhibited proteasomal activity, but with lower affinity and less potency. Recombinant monomeric α-synuclein inhibited the activity of the 20S proteasomal core with an IC50 >10 μM, exhibited no inhibition of 26S ubiquitin-dependent proteasomal activity at doses up to 5 μM, and exhibited only partial inhibition (50%) of the 26S ubiquitin-independent proteasomal activity at doses up to 10 mM. Binding studies demonstrate that both aggregated and monomeric α-synuclein selectively binds to the proteasomal protein S6', a subunit of the 19S cap. These studies suggest that proteasomal inhibition by aggregated α-synuclein could be mediated by interaction with S6'.

Introduction:

A multi-subunit 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 KD protein, termed ubiquitin. Three different forms of the proteasome exist in a cell: the 20S ubiquitin-independent proteasome, the 26S ubiquitin-independent proteasome and the 26S ubiquitin-dependent proteasome. The 20S particle forms the core of each form of proteasome (1,2). Both the 26S ubiquitin-dependent and ubiquitin-independent proteasomes contain the 20S particle plus an additional smaller cap, which has a sedimentation coefficient of 19S. Although smaller than the 20S particle, the 19S particle is also a multi-subunit structure. The protein subunits that compose the 19S and 20S particles are all known. The 19S cap contains at least 18 different subunits, while the 20S particle contains 28 subunits (1,2). The protein S6' (also known as tat binding protein 1 and Rpt5) is in the 19S 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).

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 (4-6). Polyglutamine aggregates inhibit ubiquitin-dependent proteasomal function (7). Aggregates of other proteins, such as the cystic fibrosis transmembrane receptor, also inhibit ubiquitin-dependent proteasomal function in cell culture (7). Many other proteins with hydrophobic domains also aggregate, and over-expressing the aggregation-prone domains of these proteins are toxic (8). 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 (9). 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 (PD) (10,11). 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 (12,13). Both mutations accelerate aggregation of α-synuclein (14-17). 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 (18-24). In addition, rat α-synuclein has been shown to bind to rat S6' (25). Of these proteins, only synphilin-1 and parkin have been identified in Lewy bodies (23,26,27). Perhaps because of the pleiotropic binding properties of native α-synuclein, over-expressing it in cells produces multiple cellular effects. α-Synuclein inhibits protein kinase C activity, phospholipase D activity, the activity of the dopamine transporter, and α-synuclein also has chaperone activity (18,19,24,28). Over-expressing α-synuclein also inhibits proteasomal function (29). The link between α-synuclein and the proteasome is intriguing, but is not directly related to the pathophysiology of Parkinson's disease because over-expressed α-synuclein retains a native structure until the cell is subjected to a stress, such as incubation with rotenone or ferrous chloride (30-34). 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 manuscript 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.

Methods:

Cell lines, transfections, chemicals and antibodies: cells were grown in OPTIMEM plus 10% FBS supplemented with 200 ug/mL of G418, as needed. The human cell line HEK 293 and the human neuroblastoma cell line BE-M17 were grown in OPTIMEM (Cell Grow) plus 10% FBS supplemented with 200 ug/mL of G418 (Sigma), as needed. G418 was used for selection. Transfections utilized FuGene at a 3:1 ratio to DNA, 4ug per 10cm dish. Recombinant α-synuclein was generated using wild type α-synuclein inserted into a ProEX-his$_6$ plasmid (Gibco/Invitrogen) as described previously (31). Antibodies used include: monoclonal anti-α-Synuclein (1:1000 IB, 1:100 ICC, Transduction labs); polyclonal anti-S6' (1:1000, Affiniti); monoclonal anti-S6' (1:1000, Affiniti); polyclonal anti-PA700 (1:1000, Affiniti); polyclonal anti-α subunit 20S (1:1000, Affiniti); and polyclonal anti- α-Synuclein (against amino acids 116-131, 1:1000).

Pull down assay: Brain samples were pre-cleared with nickel agarose for one hour at 4°C to eliminate proteins that directly bind to nickel agarose (Invitrogen/ Gibco). 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 minute. Samples were washed three times with immunoprecipitation buffer (50mM Tris-HCl, 10mM EGTA, 100mM NaCl, 0.5% Triton-X, 1mM DTT, 1mM Protease inhibitor cocktail (Sigma), pH7.4) and run on 8-16% SDS gradient polyacrylamide gels (Biowhitaker).

Immunoprecipitations (IP): Protein concentration was determined using BCA protein assay (Pierce) and 500ug of each sample was used per IP in immunoprecipitation buffer (50mM Tris-HCl, 10mM EGTA, 100mM NaCl, 0.5% Triton-X, 1mM DTT, 1mM Proteasome inhibitor cocktail (Sigma), pH7.4). Samples were pre-cleared using Protein G sepharose beads (Seize X, Pierce) for 1 hour at 4°C and incubated with antibody overnight at 4°C while rocking. Samples were washed three times with immunoprecipitation buffer, resuspended in 2X DTT protein loading buffer, boiled for 5 minutes 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 PBS while shaking at 800 rpm and aggregation was confirmed by performing immunoblot analysis.

Immunoblot analysis: Transfers to PVDF (BioRad) were done overnight at 4°C at 0.1Amps/ gel in transfer buffer. The immunoblot was blocked in 0.2% I-block (Tropix) in TBS with 0.1% Tween-20 for one hour at room temperature while shaking. We then incubate blots overnight at 4°C in primary antibody at appropriate concentration in 5% BSA in TBST. Blots were washed three times, ten minutes 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 (NEN).

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

In Vitro 20/26S Ubiquitin-Independent Chymotryptic Proteasomal Activity Assay: Aggregated or monomeric α-synuclein at various concentrations was incubated with 250ug 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 DTT, 5 mM $MgCl_2$, and 5 mM ATP) for 30 or 60 minutes at 37°C while shaking at 800 rpm. We then added a fluorogenic substrate (Suc-LLVY-AMC, BioMol) and incubated samples an additional 30 minutes at 37°C while shaking at 800 rpm. Solutions were analyzed using an excitation wavelength of 360 nm, and an emission wavelength of 460nm with the GeminiXS SpectraMax spectrophotometer (Molecular Dynamics).

In Vitro 26S Ubiquitin-Dependent Proteasomal Activity Assay: Substrates were generated with an *in vitro* transcription and translation of substrate proteins using a T7 promoter in E. Coli lysate (Promega Corp), supplemented with [^{35}S] methionine, and then partially purified by high-speed centrifugation and ammonium sulfate precipitation as

described (35). The protease substrate for ClP assays was derived from barnase, which is a ribonuclease from Bacillus amyloliquefaciens, while the protease substrate for proteasomal assays was derived from E. coli dihydrofolate reductase (DHFR) (35,36). An ubiquitin moiety was added to the N-terminus of the substrate proteins via a 4-amino acid linker from the E. coli lac repressor (35). Substrate proteins were constructed in pGEM-3Zf (+) vectors (Promega Corp) and were verified by sequencing. The reaction was resuspended in 40 µl buffer (25% [v/v] glycerol, 25mM MgCl2, 0.25M Tris/Cl [pH7.4]) to which 5µL of the *in vitro* reaction containing the radio labeled ubiquitinated substrate protein was added with 35 µl rabbit reticulocyte lysate (Green Hectares, containing 1 mM DTT) that is ATP depleted as described (35). We incubated the reactions with and without monomeric or aggregated α-synuclein. Concentration of α-synuclein determined by BCA Protein Assay (Pierce). We incubated at 37°C for seven minutes to allow initial cleavage of substrate proteins. Ubiquitination and degradation was initiated by the addition of ATP and an ATP-regenerating system (0.5mM ATP, 10mM creatine phosphate, 0.1 mg/ml creatine phosphokinase, final concentrations). Reactions were incubated at 37°C and at designated time points (15, 30, 45, 60, 90, 120, 150, and 180 minutes), small aliquots were removed and transferred to ice-cold 5% trichloroacetic acid (TCA), and the TCA-insoluble fractions were analyzed by 10% SDS-PAGE and quantified by electronic autoradiography.

Results:

Over-expressing α-synuclein inhibits proteasomal degradation

To begin analyzing how α-synuclein might interact with the proteasome, wild type and A53T α-synuclein were expressed in human neuroblastoma HEK 293 cells by transient transfection, and ubiquitin-dependent and ubiquitin-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. 1A). We investigated whether over-expressing α-synuclein affects the steady state levels

of ubiquitin-conjugated proteins, which provides a measure of the ubiquitin-dependent proteasomal system. To investigate the ubiquitin-dependent proteasomal system, HEK 293 cells were transfected cells with vector, wild type or A53T α-synuclein. The lysates were collected 48 hrs after transfection with the α-synuclein constructs, and then immunoblotted with anti-ubiquitin or anti-α-synuclein antibody. The amount of ubiquitin-conjugated proteins did not differ among the groups of transfected cells (fig. 1B).

We also investigated how over-expressing α-synuclein affects ubiquitin-independent proteasomal degradation. Previous studies report that cell lines over-expressing α-synuclein exhibit lower ubiquitin-independent proteasomal activity (29). To investigate ubiquitin-independent proteasomal activity, we measured hydrolysis rates of fluoregenic peptide analogues in cells transiently or stably over-expressing α-synuclein (fig. 1C). No difference in activity was observed in cells transiently transfected with α-synuclein (data not shown), however cell lines stably expressing either wild type or A53T α-synuclein showed approximately a 50% reduction in ubiquitin-independent proteasomal degradation depending on the transgene (29) (fig. 1C). These data suggest that α-synuclein does affect proteasomal function.

α-Synuclein inhibits the 20S 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 of 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. 2A). The aggregated protein ran as a smear with an average molecular weight of approximately 160 kDa (fig. 2A). Next, we examined the activity of purified 20S 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. 2B). The IC50 for inhibition of the proteasome by monomeric α-synuclein

was approximately 16 μM, assuming α-synuclein could achieve complete inhibition. Aggregated α-synuclein also inhibited the 20S ubiquitin-independent proteasomal activity, exhibiting a maximal inhibition similar to that of monomeric α-synuclein (fig. 2B).

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 20S proteasomal by monomeric α-synuclein increased with increasing substrate concentration (fig. 2C). Increased proteasomal inhibition by α-synuclein might occur because larger effects are possible at higher rates of substrate degradation. These data indicates 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 26S proteasome:

Next, we examined the effects of monomeric and aggregated α-synuclein on a mixture of the 20S and 26S proteasome in HEK 293 cell lysates. Monomeric α-synuclein inhibited the 20S/26S proteasome mixture only partially, which could reflect greater inhibition of the 20S proteasome complex and less inhibition of the 26S proteasome complex (fig. 3). The concentration producing maximal inhibition of the 20S/26S proteasome complex was similar to that seen for the 20S proteasome complex (>10 μM), based on 50% maximal inhibition (fig. 3). Aggregated α-synuclein also inhibited the 20S/26S ubiquitin-independent proteasomal mixture. Based on an estimated molecular weight for aggregated α-synuclein of 160 kDa, we calculated that the IC50 of aggregated α-synuclein for the 20S/26S proteasome was 1 nM (fig. 3). The ability of aggregated α-synuclein to inhibit a mixture of the 26S and 20S proteasomes, but not the 20S proteasome suggests that aggregated α-synuclein selectively inhibits the 26S proteasome.

Aggregated synuclein, but not monomeric synuclein, inhibits protein degradation by the 26S proteasome.

The greater ability of aggregated α-synuclein compared to monomeric α-synuclein in inhibiting 26S ubiquitin-independent proteasomal activity, raises the possibility that 26S 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) (35). Prior studies show that degradation of ubiquitinated DHFR-U by reticulocyte lysates is mediated by the 26S proteasome (35). We used this system to investigate how monomeric and aggregated α-synuclein affect ubiquitin-mediated proteasomal degradation. Degradation of ubiquitinated DHFR-U was examined in the presence of monomeric or aggregated α-synuclein (fig. 4A). The half-life of DHFR-U was 125 min under basal conditions, and also in the presence of 5 μM monomeric α-synuclein (fig. 4A, gray bars). However, the half-life of DHFR-U greatly increased in the presence of 500 nM aggregated α-synuclein (fig. 4A, dotted bars). No inhibition was seen with 50 nM aggregated α-synuclein (data not shown). These data indicate that 26S ubiquitin-dependent proteasomal degradation is selectively inhibited by aggregated α-synuclein.

To determine whether inhibition of ubiquitin-dependent proteasomal degradation was specific to the 26S 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 (35). 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. 4C). This indicates that proteasomal inhibition by aggregated α-synuclein is specific for the ubiquitin-dependent 26S proteasomal system.

Native and Aggregated α-synuclein bind S6'

The ability of aggregated α-synuclein to inhibit degradation mediated by the 26S proteasome, could be explained by interaction between aggregated α-synuclein and a

protein in the 19S cap, which is present in the 26S proteasome but not the 20S proteasome. Studies with rat α-synuclein suggest that α-synuclein binds the rodent 19S proteasomal component S6' (25). Based on this work, we investigated whether human α-synuclein interacts with S6'. His-tagged recombinant native or aggregated α-synuclein was incubated over night with substantia nigra or cingulated cortex from normal human brain, and then precipitated with nickel agarose. The precipitates were then immunoblotted with antibodies to S6'. A representative immunoblot with native α-synuclein is shown in figure 5A, and a pull down with native or aggregated α-synuclein is shown in figure 5B. 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 (figure 5B, lower panel). Co-association of α-synuclein with S6' was also observed by immunoprecipitating endogenous α-synuclein, and immunoblotting for S6' (fig. 5C). To test the selectivity of the association, we examined whether α-synuclein binds other components of the 19S proteasomal cap, such as Rpn12 and subunit 10b. Neither Rpn12 nor subunit 10b were observed to co-precipitate with α-synuclein (fig. 5D, 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 binds S6'.

Discussion:

Proteasomal inhibition is known to be toxic to many cell types, and is thought to contribute to the pathophysiology of neurodegenerative diseases (7,8,37). Our data demonstrate that over-expressing α-synuclein inhibits 20S and 26S proteasomal activity. The relationship between over-expressed α-synuclein and the pathophysiology of PD, though, is unclear. Over-expressing α-synuclein in mammalian neurons does not lead to its spontaneous aggregation, except after delays of 6-12 months (38-41). 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 ubiquitin-independent 26S proteasomal activity. The IC50 of aggregated α-synuclein for ubiquitin-independent 26S proteasomal activity was 1 nM, which was over 1000-fold higher than the IC50 for 20S proteasomal activity. In contrast, monomeric α-synuclein inhibited 20S and 26S proteasomal activity with an IC50 > 10 μM.

The high affinity of aggregated α-synuclein for inhibiting 26S proteasomal activity could be explained by binding of aggregated α-synuclein to a protein in the 19S cap, which is the proteasomal complex that binds to the 20S proteasome and confers ubiquitin-dependence, as discussed below (3). Consistent with this hypothesis, we observed that α-synuclein binds to S6', which is a subunit of the 19S cap that was recently shown 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 19S proteasomal proteins, such as Rpn12 or subunit 10b.

Binding of α-synuclein to S6' is consistent with prior publications. Ghee and colleagues demonstrated that rat S6' (also termed Tat binding protein-1, TBP1) binds α-synuclein using the yeast two-hybrid method (25). 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, Mori and colleagues, have documented the presence of proteasomal proteins in Lewy bodies (42), which supports our observation that aggregated α-synuclein binds S6'. The information presented in this manuscript 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 26S proteasome. The S6' protein appears to function in the 19S 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 19S 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 19S cap. This model provides an explanation for the ability of aggregated α-synuclein to interfere with both the ubiquitin-dependent and independent 26S proteasomal function.

Many other protein aggregates have been shown to be toxic to cells (8). Both aggregated cystic fibrosis transmembrane receptor and polyglutamine repeat exhibit toxicity that correlates with proteasomal inhibition (7,8,37). The work presented in this manuscript 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 (9). Inhibiting the UPS causes the accumulation of many toxic proteins, such as Pael-R, which was recently identified as a parkin substrate (43). Inhibiting the UPS is also known to cause the accumulation of protein aggregates in the endoplasmic reticulum (7,37). Inhibiting the UPS could alter the regulation of cell cycle proteins (44). Reduced degradation of cell-cycle proteins could account for the apparent abnormal activation of the cell cycle proteins observed in many neurodegenerative processes (45,46).

Proteasomal inhibitors have recently been shown to induce degeneration of the dopaminergic neurons of the substantia nigra and induce α-synuclein aggregation (47). The tendency of α-synuclein to accumulate under conditions of proteasomal inhibition 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 over-expressed, but studies in transgenic mice show that over-expressing α-synuclein does lead to a delayed accumulation of aggregated α-synuclein (38-41). The slow rate of accumulation of aggregated α-synuclein could also lead to a correspondingly gradual inhibition 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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FIGURE LEGENDS

Figure 1: Effects of α-synuclein over-expression on the proteasomal system.

A, B. HEK 293 cells were transfected with vector, wild type or A53T α-synuclein, and immunoblotted with antibodies to α-synuclein (A) or ubiquitin (B). No differences in levels of ubiquitin-conjugated proteins were observed among cells transfected with vector, wild type or A53T α-synuclein.

C. Activity of the ubiquitin-independent proteasomal system in cell lines expressing wild type or A53T α-synuclein compared to untransfected cells (**$p<0.0005$).

Figure 2: Effects of monomeric and aggregated α-synuclein on the 20S proteasome.

A. Immunoblotting of monomeric and aggregated α-synuclein.
B. Inhibition of the 20S proteasome by monomeric and aggregated α-synuclein – dose response.
C. Substrate dependence of proteasomal inhibition by α-synuclein

Figure 3: Inhibition of ubiquitin-independent proteasomal degradation by the 20S/26S 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 to no inhibitor.

Figure 4.: 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 26S proteasome, while monomeric α-synuclein (5 μM) does not inhibit degradation of DHFR-U by reticulocyte lysates by the 26S proteasome. The overall ANOVA was significant at $P<0.001$. Stars show significance relative to DHFR-U in absence of added α-synuclein.
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.

Figure 5: 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, while 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 α-synuclein dimer that might have been promoted by incubation of 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') shows crude brain lysates that had not been subject to immunoprecipitation (IP). Lane 2 (IgG) shows immunoprecipitation with non-specific pre-immune IgG antibody. Lane 3 (Syn) shows 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 19S 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 non specific IgG (lane 3). The abbreviations for this panel are the same as for panel B.

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Figure 1

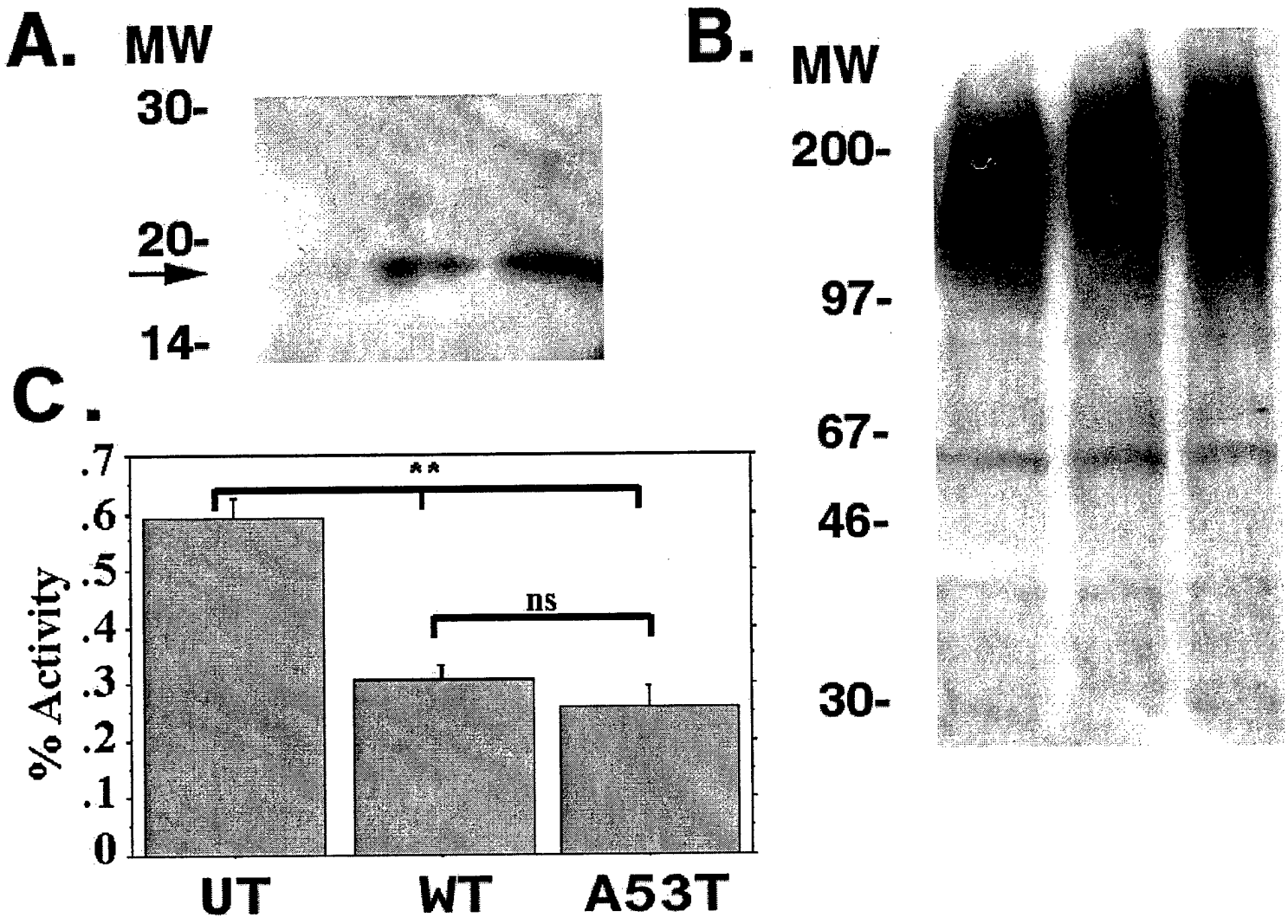

A.
MW
30-
20-
14-
B.
MW
200-
97-
67-
46-
30-
C.
% Activity
.7
.6
.5
.4
.3
.2
.1
0
**
ns
UT
WT
A53T

Figure 2

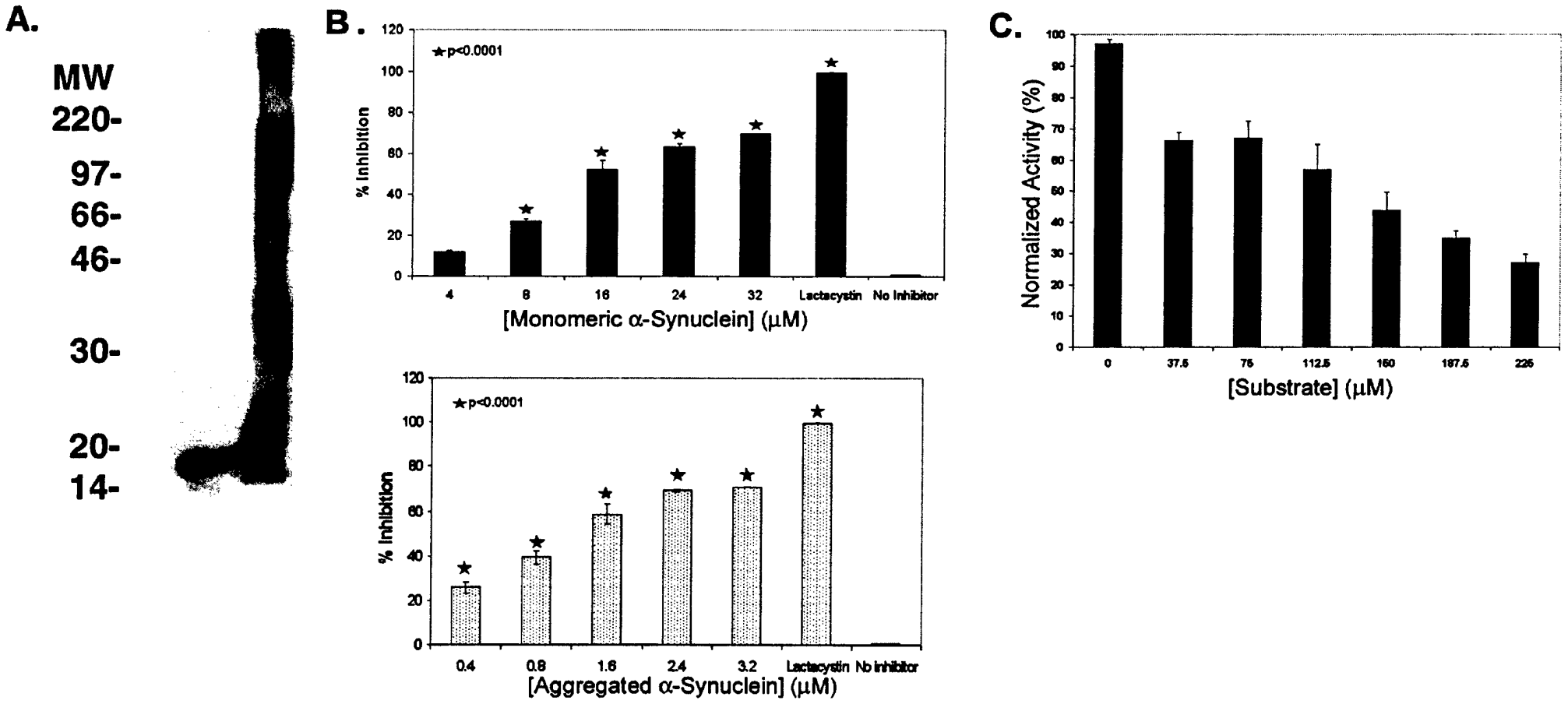

A.
MW
220-
97-
66-
46-
30-
20-
14-
B.
★p<0.0001
% Inhibition
120
100
80
60
40
20
0
4
8
16
24
32
Lactacystin
No Inhibitor
[Monomeric α-Synuclein] (μM)
★p<0.0001
% Inhibition
0.4
0.8
1.6
2.4
3.2
Lactacystin
No Inhibitor
[Aggregated α-Synuclein] (μM)
C.
Normalized Activity (%)
100
90
80
70
60
50
40
30
20
10
0
0
37.5
75
112.5
150
187.5
225
[Substrate] (μM)

Figure 3

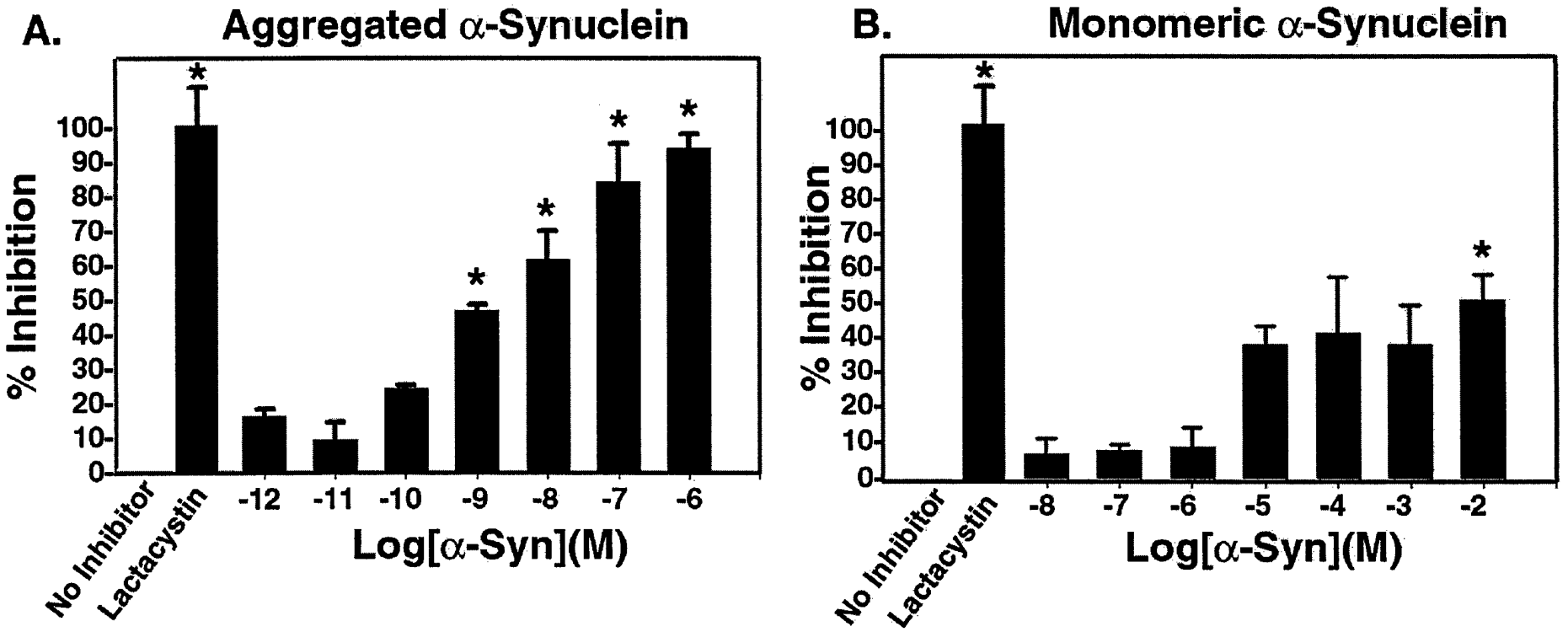
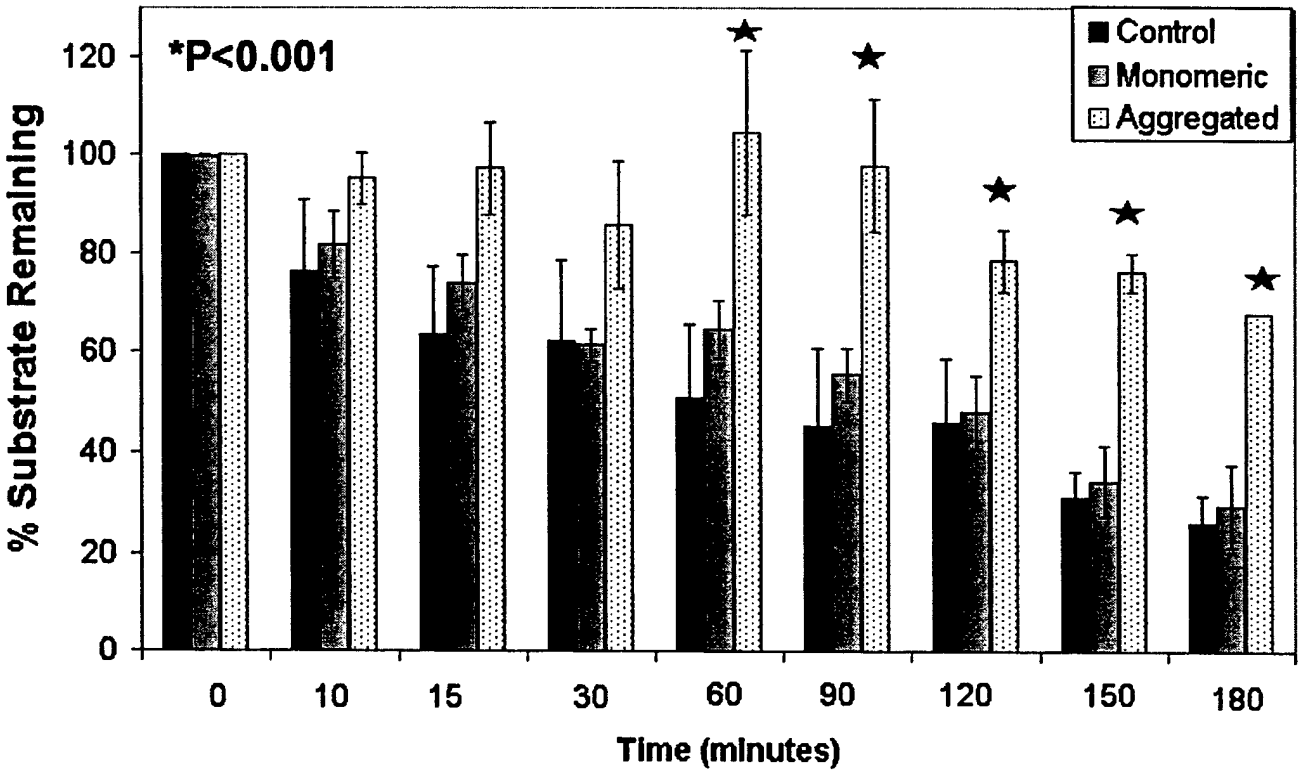

A.
Aggregated α-Synuclein
% Inhibition
100
90
80
70
60
50
40
30
20
10
0
No Inhibitor
Lactacystin
-12
-11
-10
-9
-8
-7
-6
Log[α-Syn](M)
B.
Monomeric α-Synuclein
% Inhibition
100
90
80
70
60
50
40
30
20
10
0
No Inhibitor
Lactacystin
-8
-7
-6
-5
-4
-3
-2
Log[α-Syn](M)

Figure 4

A.


*P<0.001
Control
Monomeric
Aggregated
% Substrate Remaining
120
100
80
60
40
20
0
0
10
15
30
60
90
120
150
180
Time (minutes)

B.

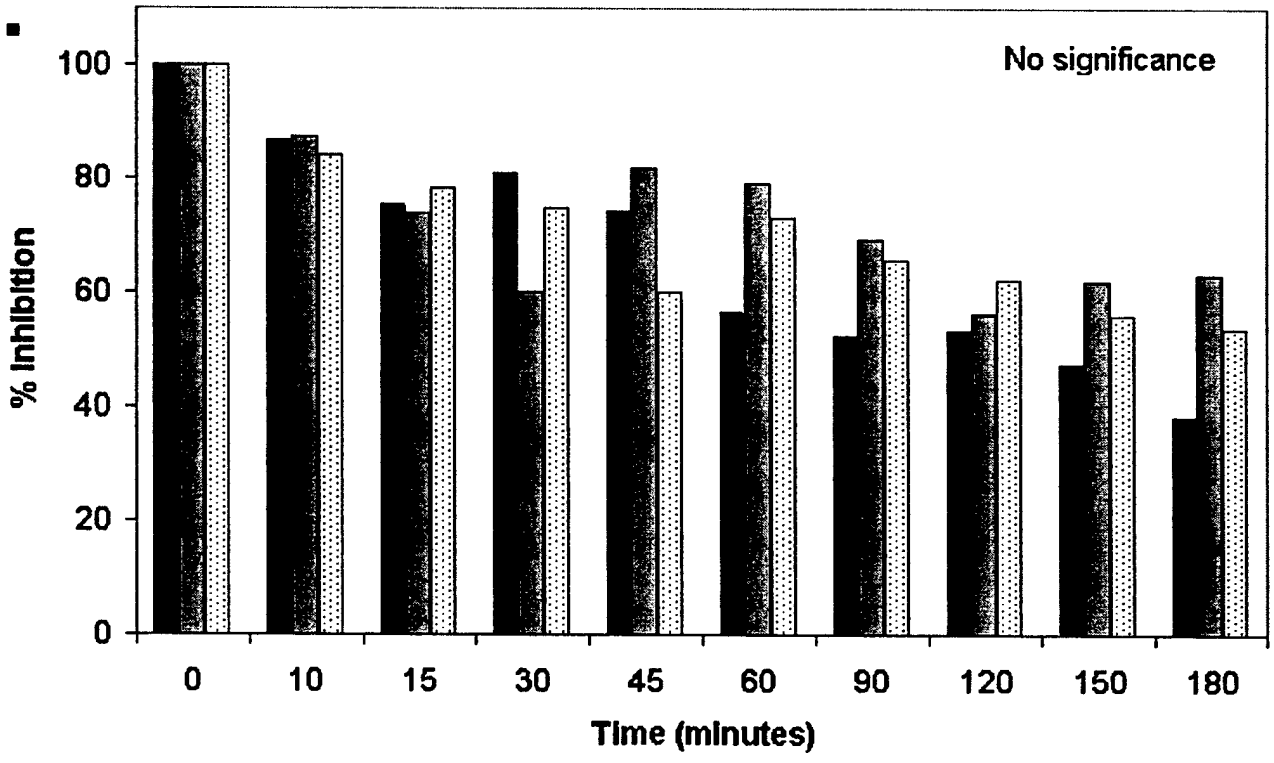
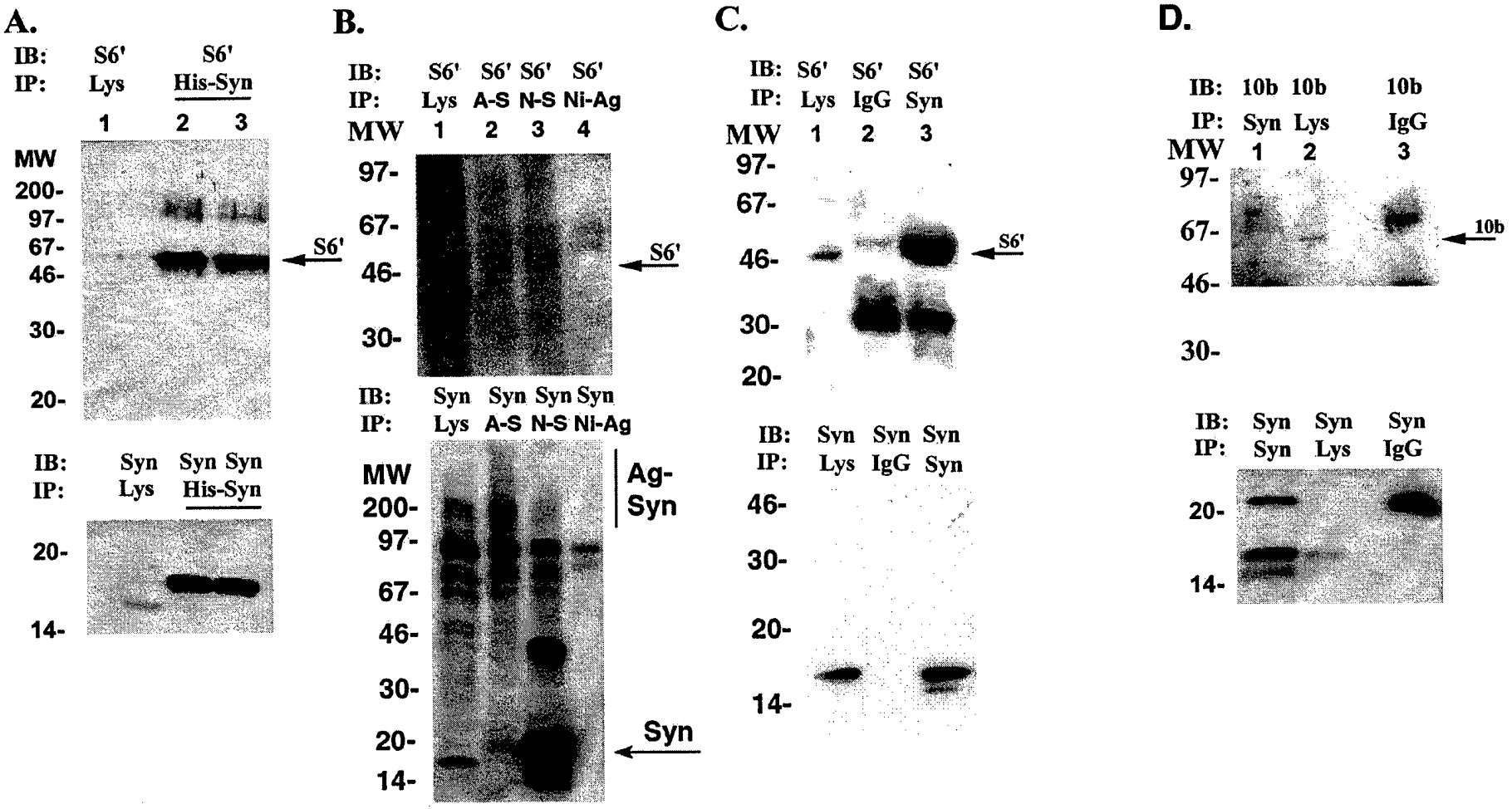

No significance
% Inhibition
100
80
60
40
20
0
0
10
15
30
45
60
90
120
150
180
Time (minutes)

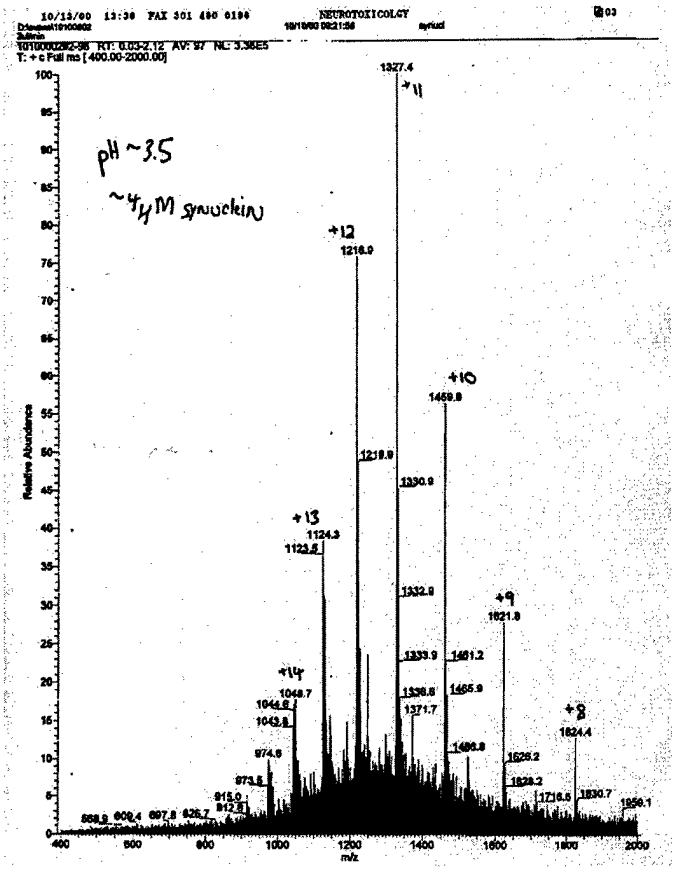
Figure 5


A.
IB: S6' S6'
IP: Lys His-Syn
1 2 3
MW
200-
97-
67-
46-
30-
20-
S6'
IB: Syn Syn Syn
IP: Lys His-Syn
20-
14-
B.
IB: S6' S6' S6' S6'
IP: Lys A-S N-S Ni-Ag
MW 1 2 3 4
97-
67-
46-
30-
S6'
IB: Syn Syn Syn Syn
IP: Lys A-S N-S Ni-Ag
MW
200-
97-
67-
46-
30-
20-
14-
Ag-Syn
Syn
C.
IB: S6' S6' S6'
IP: Lys IgG Syn
MW 1 2 3
97-
67-
46-
30-
20-
S6'
IB: Syn Syn Syn
IP: Lys IgG Syn
46-
30-
20-
14-
D.
IB: 10b 10b 10b
IP: Syn Lys IgG
MW 1 2 3
97-
67-
46-
30-
10b
IB: Syn Syn Syn
IP: Syn Lys IgG
20-
14-

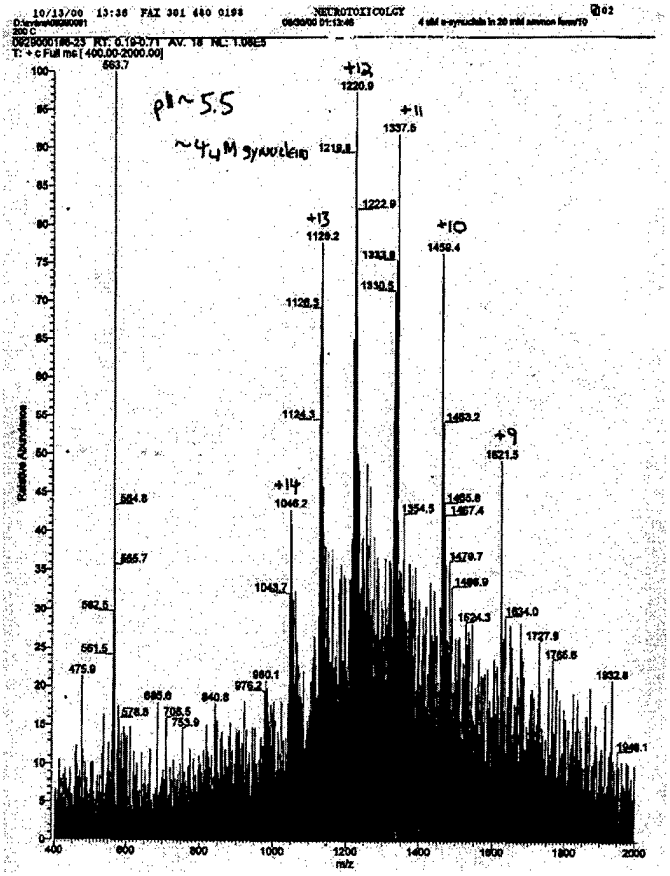
Appendix 7

Appendix 7

A.



B.



Appendix 8

Supplemental findings that are relevant to α-synuclein aggregation, but not included in the grant proposal for DAMD17-01-1-0781.

Benjamin Wolozin, Loyola University Medical Center

We have developed several models relevant to α-synuclein aggregation and Parkinson's disease. While none of the models are ready yet for publication, each is quite promising and being actively pursued.

Appendix 8.

We have made the intriguing observation that the R275W parkin mutation, which is the only parkin mutation associated with late onset Parkinson's disease (PD), demonstrating that this mutation causes spontaneous aggregation of parkin (that is reduced in cells over-expressing α-synuclein). We have generated vectors for transgenic expression of WT and R275W parkin in C. Elegans, to test in the worm whether R275W parkin spontaneously aggregates (fig. 1). (We are also generating transgenic mice over-expressing R275W parkin). We anticipate that this work will provide a novel model for studying Lewy body formation.

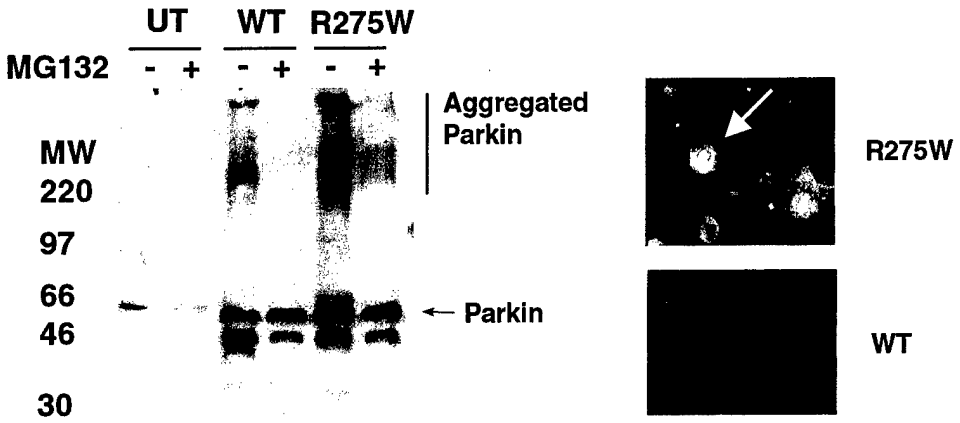


Figure 1: R275W parkin has an increased tendency to aggregate compared to WT parkin. Note the increased high molecular weight parkin on the immunoblot, corresponding to aggregated (or poly-ubiquitinated) parkin, and the increased large and small accumulation of parkin seen by anti-parkin immunohistochemistry in transfected cells (arrow).

Appendix 9.

In collaboration with Marius Hoener, we have been able to over-express A53T α-synuclein in C. Elegans, and we have shown that these transgenic worms exhibit increased vulnerability to rotenone-induced toxicity, compared to non-transgenic worms or worms over-expressing β-synuclein, a close homologue of α-synuclein (fig. 2). Development of this worm is important because this represents a simple animal model for studying α-synuclein aggregation and toxicity. Use of this

worm enables rapid analysis of putative therapeutic agents for PD, and also biochemical and genetic analysis of α-synuclein aggregation.

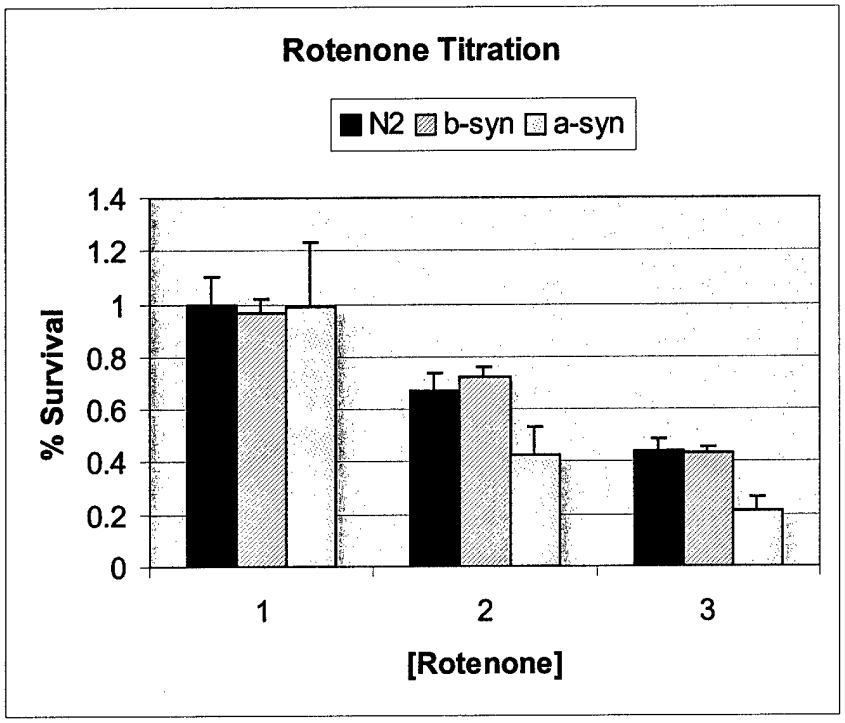


Figure: 2 α-Synuclein worms show reduced survival in the presence of 0, 0.1 and 0.2 mM rotenone (groups 1, 2, 3, respectively) after two days incubation.