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Newly Discovered α-Synuclein Familial Mutant E46K and Key Phosphorylation and Nitrosylation-Deficient Mutants are Toxic to Yeast

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Summary

Parkinson's disease is a neurodegenerative disorder that is caused by the loss of dopaminergic neurons in the substantia nigra. Misfolding of α-synuclein is thought to cause this selective cell death. In our α-synuclein yeast overexpression model, we previously demonstrated that α-synuclein, is non-toxic to yeast, runs 8-10 kDa higher on protein gels and aggregates minimally *in vivo*. Recently, a new familial mutant of a-synuclein E46K was discovered. α-Synuclein is a diversely post-translationally modified protein and we hypothesized that one of these modifications may underlie its aberrant size. Here, we show that E46K is more toxic to yeast than other familial α-synuclein mutants. α-Synuclein mutants lacking key phosphorylation and nitrosylation sites are surprisingly toxic as well, but do not account for α-synuclein's aberrant size. Interestingly, all these mutants localize to the periphery of yeast cells and show variable levels of *in vivo* aggregation.

Introduction

Accumulation of misfolded proteins as insoluble aggregates occurs in several neurodegenerative diseases including Parkinson's disease (PD) (Dawson, 2003). PD is the second most common and fatal neurodegenerative disorder in the aging population. Symptoms of PD include resting tremor, rigidity, slowness of movement, gait disturbances, and postural instability (Dawson, 2003). These symptoms are believed to be due to the loss of dopaminergic neurons in the substantia nigra, which is accompanied by the formation of insoluble inclusions known as Lewy bodies (LBs). Lewy bodies contain the misfolded protein α-synuclein (Sharon et al., 2003). It has been shown that the formation of these α-synuclein aggregates coincides with disease onset and the symptoms observed (Giasson et al., 2002). α-Synuclein was first associated with PD when a missense mutation (A53T) in the gene encoding this cytosolic protein was linked to a rare, familial dominant form of PD (Polymeropoulos et al., 1997). A second missense mutation (A30P) was then found in another PD family (Kruger, et al., 1998). Just recently, a new familial mutation, E46K was discovered, however, it has yet to be characterized (Zarranz, 2004). Despite the rarity of these α-synuclein mutations, which account for only 5% of the all cases observed in PD, their recognition has opened up the field of PD research (Sharon et al., 2003) However, sporadic PD accounts for 95% of all cases. In sporadic PD the misfolding of normal wild-type α-synuclein is a spontaneous event.

α-Synuclein is a small 140 amino acid (sizing to 14 kDa), natively unfolded, heat stable, soluble protein. It is abundant in the brain and is estimated to constitute approximately 1% of total brain proteins, and is localized primarily near presynaptic terminals of neurons (Clayton and George et al., 1998). Even though α-synuclein is a heat stable protein and natively unfolded, it can aggregate in cells to form proteinaceous inclusions under pathological conditions (Duda et al., 2000). The A53T familial mutant of α-synuclein appears to induce disease onset by increasing fibril formation; *in vitro* studies have shown that recombinant mutant A53T α-synuclein protein fibrillizes faster than wild-type α-synuclein (Conway et al., 2000; Giasson et al., 1999). These and other α-synuclein inclusions have been shown to contain high molecular weight insoluble α-synuclein proteins that are resistant to strong denaturing conditions; this suggests that α-synuclein proteins within these inclusions are covalently cross-linked (Tu et al., 1998; Norris et al., 2003). The biochemical factors that induce aberrant α-synuclein aggregation and the precise molecular alterations of α-synuclein that lead to the formation of pathological inclusions remains poorly understood (Paxinou et al., 2001).

Post-translational modifications of α-synuclein may be responsible for the formation of the proteinaceous inclusions seen in PD patients. Recent studies support a role for oxidative and/or nitrative stress in α-synuclein post-translational modifications. Previous studies have shown that α-synuclein was nitrated in pathological lesions such as LBs (Giasson et al., 2000). Furthermore, other studies have indicated that nitrating agents such as peroxynitrite/CO_2 or myeloperoxidase/H_2O_2/nitrite can nitrate and oxidize α-synuclein at tyrosine (Tyr) residues, resulting in the formation of highly stable dityrosine oligomers (Souza et al., 2000). Cross-linking or the formation of covalent bonds between amino acids on the same or different molecules of α-synuclein was observed when human recombinant α-synuclein was exposed to nitrating agents aw well (Souza et al., 2002).The formation α-synuclein filaments by nitrating agents, provides evidence for a role for nitrating–agent induced modifications in the stabilization of α-synuclein inclusions (Souza et al., 2000). Other studies have also shown that oxidation by dopamine, inhibited the conversion of protofibrils to fibrils, leading to the accumulation of α-synuclein protofibrils (Conway et al., 2001). Additionally, it was investigated if nitration and oxidization induced the aggregation of a-synuclein. Human embryonic cells (HEK 293) transfected with wild-type and mutant α-synuclein exposed to nitrating and oxidizing agents had extensive formation of α-synuclein intracellular aggregates (Paxinou et al., 2001). The role of oxidative and /or nitrative species

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on the conversion of monomeric, soluble α-synuclein to soluble oligomers and finally to insoluble α-synuclein inclusions remains unclear (Norris et al., 2003). It is known that Tyr residues and serine residues in α-synuclein are targets for nitration (Souza et al., 2000).

A recently published yeast model of α-synuclein expression in yeast (Outerio and Lindquist, 2003) showed that α-synuclein localized to the plasma membrane, formed intracellular inclusions, and influenced vesicle trafficking in wild-type cells. Our lab has recently developed a *Saccharomyces cerevisiae* (*S. cerevisiae)* model to study protein misfolding associated with PD (Johnson 2003; Sharma 2004). In this model wild-type, and the two-disease-associated forms of α-synuclein, A30P and A53T, and the artificial double mutant (A30P/A53T) were expressed in yeast. Multiple sizes for all four of α-synuclein were observed, with a majority of proteins appearing to be ~10 kDa higher in size than the predicted 14 kDa size (Johnson, 2003). This may be due to *in vivo* post-translational modifications of α-synuclein (i.e. by addition of nitro, oxy, or phosphoryl groups). Our yeast model showed that wild-type (WT) and mutant forms of α-synuclein do not cause toxicity to yeast cells. Furthermore, GFP-tagged WT, A53T, and double mutant (A30P/A53T) α-synuclein localize to the membrane of yeast cells, while A30P protein remained diffused in the cytoplasm. WT, A53T and A30P/A53T α-synuclein also showed formation of foci in the cytoplasm.

In this study, we investigated the role of the familial mutant E46K in α-synuclein aggregation, toxicity, and expression. Additionally, we investigated the role of Tyr residues at amino positions 39, 125 and the serine residue at position 129 in α-synuclein post-translational modification, aggregation formation, toxicity, and localization.

Results

Expression of S129A, Y39F, and Y125F mutants and the Familial Mutant E46K

Western analysis was conducted with lysates made for cells expressing the four mutant (S129A, Y39F, Y125F, and E46K) forms of α-synuclein in order to compare the expression of α-synuclein. Wild-type and mutant α-synucleins were subcloned into yeast pYES2.1 expression vector under the control of a galactose-inducible promoter GAL1. Growing cells in the presence of galactose induced optimal expression by 24 hours. α-Synuclein expression of these mutants has not been previously shown. Lysates were run on denaturing gels; all forms of GFP-tagged α-synuclein migrated at ~62 kDa. This expression has been previously described among other mutant forms of α-synuclein (Sharma, 2004). Anti-V5 antibody was used for protein detection (Figure 1). α-Synuclein was visualized by staining with Coomassie Blue. The Coomassie stain showed that equal amounts of protein were loaded into each lane (data not shown). All forms of GFP-tagged α-synuclein showed similar levels of α-synuclein expression and gels showed a lack of SDS insoluble aggregates of the mutant forms α-synuclein because proteins were not observed at high molecular weights. However, it was expected that E46K mutant α-synuclein would display protein bands at higher molecular weight because of the possible presence of aggregated α-synuclein. Additionally, it was expected that S129A, Y39F, and Y125F mutant α-synuclein would migrated at a lower molecular weight of ~52 kDa because they lack the sites necessary for post-translational modification.

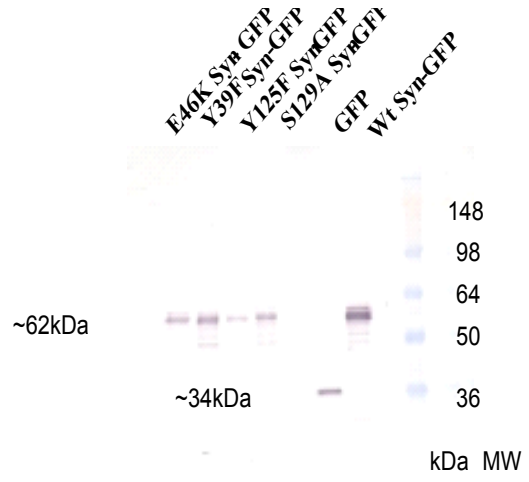

Figure 1. α-Synuclein Expression In Newly Created Mutants
GFP alone migrated at ~34kDa, and the major band for the wild-type α-synuclein-GFP(CT) fusion protein was observed at ~62kDa. When expressed the fusion protein in the newly created mutants a similar expression pattern was observed. A prominent band at ~62kDa was observed for the various mutant α-synuclein-GFP(CT) fusion proteins. The Anti-V5 antibody was used for protein detection. MW = Molecular Weight Marker.

S129A, Y39F, and Y125F is Toxic to Yeast

To assess if α-synuclein expression was toxic to yeast cells, optical density and spotting analyses were performed. Optical density readings were taken during logarithmic growth phase. α-Synuclein toxicity of these mutants has not been previously shown, but surprisingly cells expressing S129A, Y39F, and Y125F mutant forms of α-synuclein showed major growth deficiencies compared to cells expressing wild-type α-synuclein (Figure 2A). It was expected that these mutants would show a reduction in α-synuclein toxicity because these mutants lack key sites for post-translational modification. For a more sensitive toxicity assay, we spotted five-fold, serially diluted yeast cells onto plates containing inducing (galactose) and non-inducing (glucose) media (Figure 2C). Cells expressing wild-type α-synuclein grew similarly to cells carrying the parent vector. There was an observed mild growth defect for cells expressing Y39F, Y125F, and S129A mutant α-synuclein (Figure 2C). These results are consistent with the toxicity observed in the optical density assay, however, a smaller lag growth was observed. The controlled cells expressing GFP showed no lag in growth compared to the cells expressing parent plasmid alone. The glucose plates served as controls and showed that cell density within each dilution was equal in that each column showed similar cell growth (Figure 2C).

S129A, Y39F, and Y125F Localize Near Yeast Plasma Membranes

In order to investigate the localization of α-synuclein in live yeast cells, we tagged wild-type and the mutant forms of α-synuclein with GFP. GFP served as a visual marker for the protein α-synuclein. GFP was tagged to

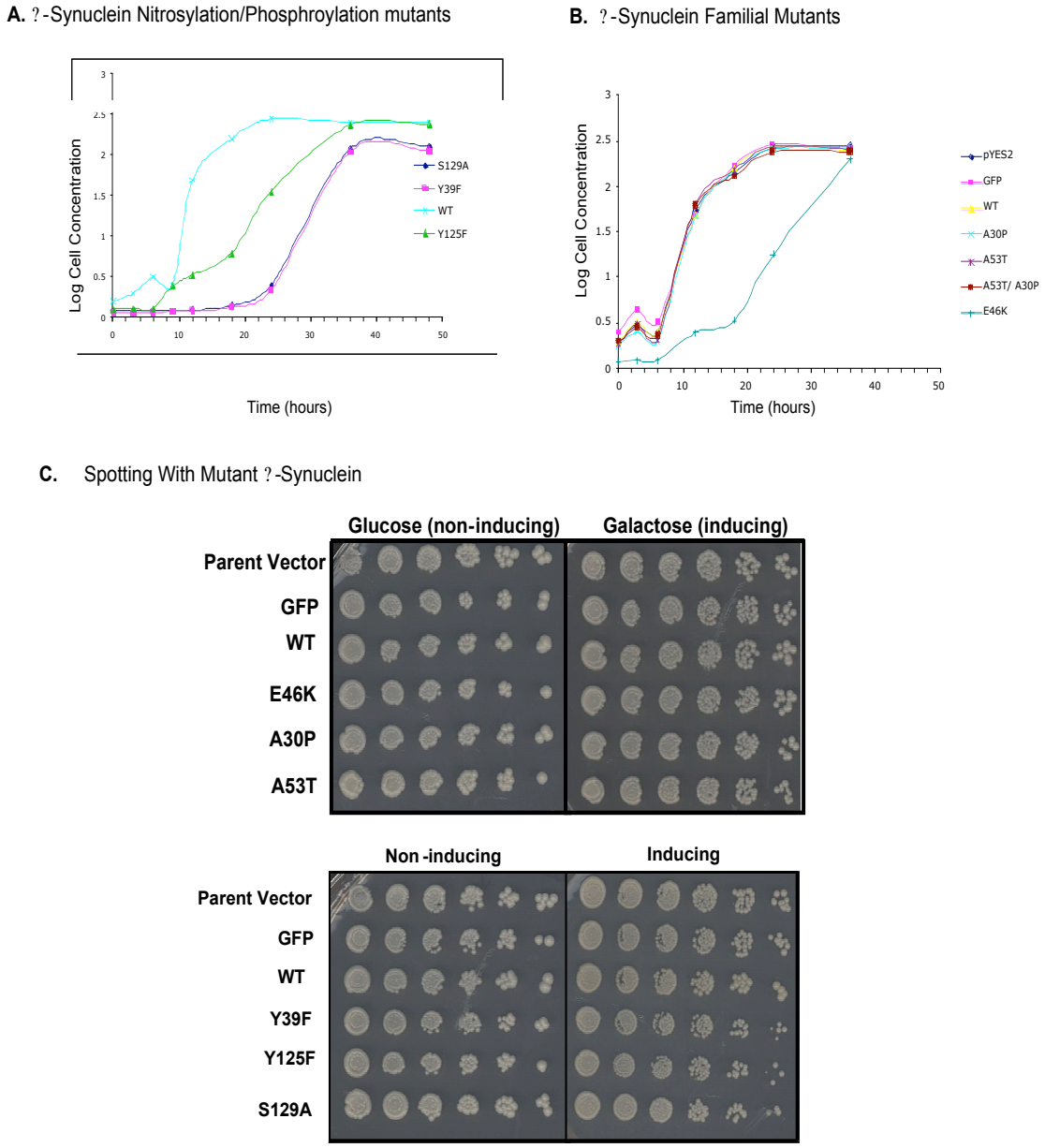

Figure 2. α-Synuclein Inhibits Growth Selectively
OD600 readings were taken at several time points for yeast cells induced for α-synuclein expression. When compared to cells containing just the parent plasmid (pYES2), α-synuclein expression did not have a drastic effect on growth for A30P, A53T and A53T/A30P mutants. The E46K mutant showed a large lag growth compared to wild-type a-synuclein and other mutant α-synucleins. **(B)** All mutants showed a lag in growth rate when compared to the wild-type a-synuclein expressing cells. **(C)** Growth was also analyzed by spotting five-fold-serially diluted yeast cells on plates containing non-inducing (glucose) or inducing (galactose) medium. All yeast cells grew to the same extent on non-inducing plates. On inducing plates, cells expressing the Y39F, Y125F, and S129A mutant a-synuclein displayed modest growth defects, but not wild-type a-synuclein or cells expressing parent plasmid alone.

the C-terminus of the α-synuclein gene because the α-synuclein protein was more stable and it maintained the folding and activity of α-synuclein to a better extent. Due to the toxicity of S129A, Y39F, and Y125F in yeast cells, we conducted live cell fluorescence microscopy to determine if α-synuclein localized differently in the dying yeast cells compared to the parent strain. α-Synuclein was expressed for 24 hours in galactose media. C-terminal GFP-tagged wild-type and Y39F, Y125F, S129A, mutant α-synuclein localized near the cell membranes of yeast (Figure 3A). When the GFP control protein was expressed it remained primarily distributed throughout the cells cytoplasm as expected (Rines et al., 2002). These findings are consistent with previous α-synuclein localization studies observed in our lab (Sharma, 2004). However, it was unexpected

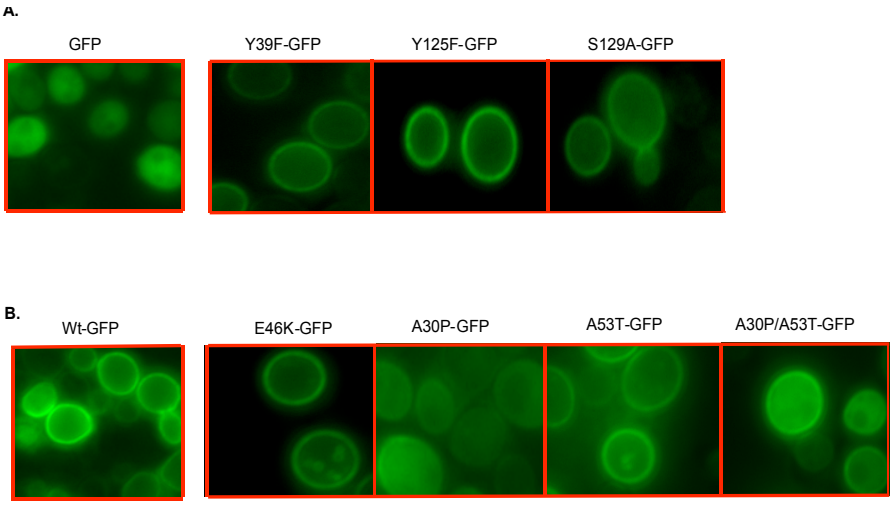

Figure 3. Fluorescence Patterns in Yeast
(A) With GFP fluorescence, (top panel), Y39F. Y125F, and S129A mutants displayed bright halos, indicating α-synuclein's localization near yeast plasma membranes. Control cells (with vectors containing the GFP gene alone) showed diffuse fluorescent staining, and GFP was distributed throughout the cell. **(B)** Wild-type, E46K, A53T, and double mutant α-synuclein (tagged with CT-GFP) displayed bright halos. A small number of cells expressing the mutant forms A53T and E46K displayed foci that could be indicative of α-synuclein's aggregation. The A30P mutant α-synuclein, however, remained distributed within the cytosol.

that the S129A, Y39F, and Y125F mutant showed toxicity when expressed in yeast cells but there was no observed change in its localization.

E46K is Toxic to Yeast
We further questioned whether the expression of E46K mutant α-synuclein affected growth of yeast cells. Previous studies have shown that α-synuclein is toxic to neurons (Masliah, E., et al., 2000). Again, optical density readings and spotting assays were performed during logarithmic growth phase. Minor differences were observed between the growth rates of the control parent vector and GFP cells and cells expressing wild-type α-synuclein and A30P, A53T, and A53T/A30P mutant α-synuclein (Figure 2B). However, cells expressing E46K mutant α-synuclein showed a major lag in growth compared to cells carrying the parent vector or those expressing wild-type α-synuclein. Spotting assays showed different results compared to the optical density assays. Cells expressing wild-type and the various familial mutant forms of α-synuclein grew to the same extent as cells with the parent vector and control GFP protein. The glucose plates served as controls and showed that cell density within each dilution was equal in that each column showed similar cell growth (Figure 2C).

E46K Localizes Near Plasma Membrane and Forms Cytoplasmic Foci
C-terminal GFP-tagged E46K mutant α-synuclein showed similar α-synuclein localization as S129A, Y39F, and Y125F mutants. Again, α-synuclein was expressed in the parent strain and cells containing the E46K mutant for 24 hours in galactose media. E46K, A53T, and A30P/A53T α-synuclein localized near the periphery of the cell (Figure 3B). Additionally, a small population of cells expressing E46K and A53T mutant α-synuclein formed foci in the cytoplasm (Figure 3B). The A30P mutant did not localize to the periphery and it remained distributed throughout the cytoplasm (Figure 3B). The A30P/A53T double mutant showed an intermediary phenotype (Figure 3B). The protein mainly remained distributed throughout the cytoplasm and to a lesser extent it localized to the cell membrane. Furthermore, the GFP control was primarily distributed throughout the cytoplasm.

Discussion

The main goal of Parkinson's disease research is to find an effective way to reverse or avoid the protein aggregation of α-synuclein and the neuronal death associated with this disease. Many animal models have been developed to understand the pathology of Parkinson's disease; models have focused on evaluating the potential toxicity of α-synuclein and the effects it has on the advancement of PD. However, many of the pathological aspects of PD still remain unclear. *S. cerevisiae* has served as an excellent model for studying many facets of Parkinson's. Through this model we have been able to examine the protein folding and misfolding of α-synuclein.

α-Synuclein increased size is not due to phosphorylation at Serine 129 and nitrosylation at Tryosines 39 or 125
Previous Western analysis conducted in our lab has shown that a-synuclein from yeast lysates migrates to ~ 62 kDa in denaturing protein gels in contrast to the predicted mass of 53 kDa (14 kDa for α-synuclein protein, 34 kDa for GFP protein, and 5 kDa epitope tags). Thus, α-synuclein migrates ~8-10 kDa higher than predicted. In both human patients and *E.coli* models, α-synuclein runs at its predicted size rarely (Conway et al., 2000). Our observed 62 kDa α-synuclein band is consistent with these findings. We believed that α-synuclein migrated to a higher predicted molecular weight due to post-translational modifications located on Serine 129, Tyrosine 39 and 125. The serine 129 residue of α-synuclein has been linked to phosphorylation modifications and tyrosine 39 and 125 has been linked to nitrosylation modifications. Thus, in

this study we used site-directed mutagenesis to alter these residues to examine if they contribute to specific post-translational modifications in α-synuclein. However, the western analysis showed that there was no change in the migration pattern of these altered α-synucleins (S129A, Y39F, and Y125F). These findings suggest that α-synuclein's increased size is not due to phosphorylation at serine 129 and nitrosylation at tyrosines 39 and 125.

Other studies have shown that α-synuclein may run higher than expected due to a glycosylation modification (Shimura et al., 2001). Additionally, other studies have shown that α-synuclein may run at a higher molecular weight than predicted because of lipidation (Sharon et al., 2001). Finally, α-synuclein has is misfolded in neurons. When proteins become misfolded they are ubiquitinated by a cascade of ubiquitin enzymes in the ubiquitin proteasome pathway (Ciechanover and Brundin, 2003). Thus, α-synuclein may be running higher due to ubiquitin post-translational modifications at lysine residues. We are unsure why α-synuclein migrates to 62 kDa in our study, but it may be due to one or more of these three forms of post-translational modifications.

S129A, Y39F, and Y125F Showed Unexpected Toxic properties

The optical density and spotting results indicate that when S129A, Y39F, and Y125F α-synuclein is expressed in yeast cells; they are less viable than cells expressing wild-type α-synuclein. These results were unexpected because post-translational modifications of α-synuclein, such as nitrosylation and phosphorylation may be responsible for the formation of the proteinaceous inclusions seen in PD patients (Giasson et al., 2000). It has been shown that the formation of these α-synuclein aggregates coincides with disease onset and the symptoms observed (Giasson et al., 2002). Thus, by altering the sites in α-synuclein that are responsible for nitrosylation and phosphorylation we would see less toxicity, however, the exact opposite was observed. In fact, these results suggest that these post-translational modifications are beneficial not detrimental to cells expressing α-synuclein. When cells are expressing α-synuclein with unaltered 39, 125, 129 residues no toxicity is observed in both spotting and optical density assays.

***In Vivo* Membrane Association of S129A, Y39F, and Y125F α-Synuclein**

S129A, Y39F, and Y125F α-synuclein has extensive peripheral localization in live yeast cells. This is consistent with the live yeast cell microscopy experiments conducted by Sharma, 2004. However, initially it was expected that α-synuclein would be distributed throughout the yeast's cytosol, with foci to indicate aggregation formation of the protein. α-Synuclein's association to yeast plasma membranes agrees with the research findings pertaining to α-synuclein's interaction with lipids. In α-synuclein's amino acid sequence there is a 11-residue repeat, which is common among apolopoproteins; this repeat sequence is believed to be responsible for its ability to bind synthetic phospholipid vesicles (Davidson et al., 1998). α-Synuclein has motifs that resemble those of fatty-acid binding proteins, and α-synuclein binds to fatty-acids *in vitro* (Sharon et al., 2001). Current research has implicated that α-synuclein interacts with dopamine transporters on the neuronal membrane and it regulates the amount of dopamine that enters the cell (Wersinger et al., 2003). This finding suggests a possible functional role of α-synuclein. In our research α-synuclein's association to the yeast plasma membrane demonstrates its ability to bind to phospholipids (Sharma, 2004). The cytoplasm of yeast cells is much smaller that that of neurons, thus α-synuclein may have an easier ability to bind phospholipids in yeast membranes (Sharma, 2004).

E46K does not form SDS-insoluble aggregates

Western analysis showed that the familial mutant E46K migrates at ~62 kDa. Previous Western analysis conducted in our lab has shown that α-synuclein from yeast lysates expressing wild-type α-synuclein and the other familial mutants A53T and A30P migrates to ~62 kDa in denaturing protein gels (Sharma, 2004). However, it was expected that E46K α-synuclein expression would show multiple high running bands that correspond to dimers and aggregates because a case study in 2003 stated that E46K mutant form of α-synuclein is more prone to aggregation compared to the other familial mutants (Zarranz, et al, 2003). Hence in our studies, E46K α-synuclein may not be aggregating to dimers and higher order structures to significant amounts in yeast cells, or aggregates do form but are separated during denaturation steps before loading proteins on gels for electrophoresis analysis.

E46K Toxicity May Be Due To Increased Misfolding

In PD, one hypothesis for the death of substantia nigra neurons is that misfolded/aggregated α-synuclein creates toxic conditions. In mice and fruit fly models overexpression of α-synuclein results in cell death (Masliah et al. 2000; Feany and Bender, 2000). Thus it was expected that α-synuclein expression of E46K would decrease cell viability to some extent. The optical density results show that cells expressing mutant E46K α-synuclein had a large lag in growth compared to cells expressing wild-type and A53T, A30P, and A53T/A30P mutant forms of α-synuclein. Previous studies conducted in our lab have shown that the mutant forms A53T, A30P, and A53T/A30P only exhibit a slight lag in growth in both optical density and spotting assays (Sharma, 2004). However, spotting assays show that the E46K mutant shows no difference in growth compared to cells carrying parent vector or other forms of α-synuclein. Thus, toxicity assays should be repeated again to make any significant conclusions about E46K toxicity.

GFP microscopy results show that at 24 hours of expression, when toxicity is occurring, E46K α-synuclein has extensive peripheral localization in live yeast cells, again suggesting α-synuclein's affinity for lipids. In addition, our GFP microscopy studies show that the E46K mutant is more prone to aggregation compared to the A53T mutant. This observed increase in aggregation of E46K α-synuclein may relate to the increase in toxicity observed in yeast cells expressing this protein, because it is proposed that neurons die due to the toxic conditions α-synuclein creates when it is misfolded (see proposed Model in Figure 4). Furthermore, we found that the A30P mutant does localize to plasma membranes, thus this mutation may reduce α-synuclein's affinity for lipids. Some studies show that the A30P familial mutation greatly reduces α-synuclein's ability to bind to lipids (Jensen et al., 1998; Cole et al., 2002). However, other studies find that the A30P mutations have little effect on lipid binding (Perrin et al., 2000). Our studies suggest that this mutation causes α-synuclein to lose lipid affinity, and this may

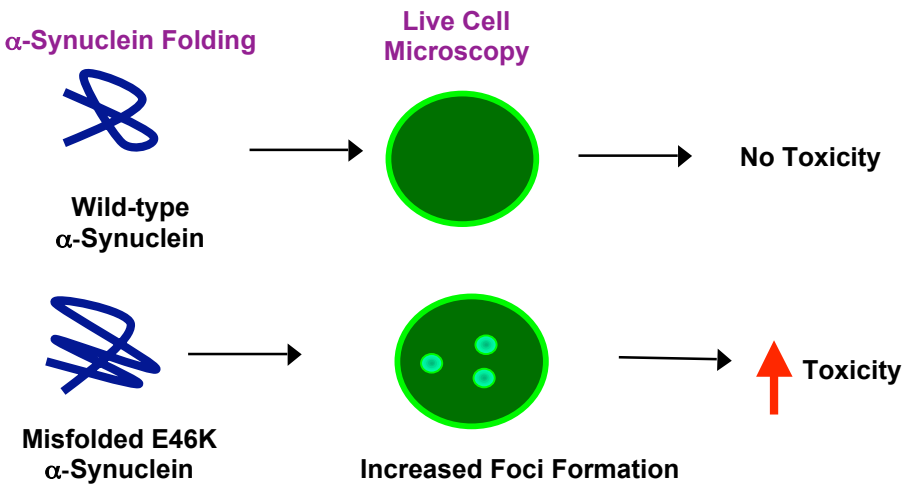

Figure 4. Hypothetical Model Characterizing α-Synuclein's Misfolding and Aggregation *In Vivo*
Live cell microscopy displayed different fluorescence patterns for yeast cells expressing wild-type and mutant α-synuclein tagged with GFP. Abnormal formation of α-synuclein aggregates are highlighted by distinct fluorescent foci. Mutant E46K α-synuclein displayed foci formation that is indicative to aggregation. Whereas wild-type α-synuclein localized to the plasma membrane of cells. It was predicted more foci would be seen in E46K-mutant cells as compared to their wild-type expressing cells. This increase in foci formation for E46K expressing cells may account for its observed toxicity. It has been described that misfolded or aggregated α-synuclein may be toxic to neurons in the substantia nigra of Parkinson's disease patients.

prevent it from proceeding with its normal function (Sharma, 2004). These results agree with *in vitro* studies showing that α-synuclein aggregation is accelerated by the A53T mutation (Conway et al., 1998; Narhi et al., 1999).

The first yeast model published in 2003, describes similar results to our GFP fluorescence studies (Outerio and Lindquist, 2003). In this yeast model, the wild-type and A53T mutant α-synuclein forms showed peripheral localization. The A30P mutant form did not localize to the periphery and lead to lipid accumulation. However, they reported that wild-type α-synuclein formed inclusions similar to those seen in the A53T yeast model. These results differ from our lab's findings in that we do not see inclusion structures in our wild-type yeast model.

Conclusion

Our studies have enhanced our understanding of the misfolding, aggregation, and post-translational modifications of α-synuclein. Western and GFP studies imply that α-synuclein may not be nitrosylated or phosphorylated. Identification of other key post-translational modifications may elucidate α-synuclein misfolding in PD. Additionally, evaluation of the newly discovered E46K mutant recapitulates many properties seen in α-synuclein behavior in PD patients including increase toxicity with mutant α-synuclein. Further examination of this major toxicity observed in cells expressing S129A, Y39F, Y125F, and E46K mutants may aid in the discovery of factors that can inhibit or correct α-synuclein misfolding.

Experimental Procedures

Preparation of Familial Mutant E46K and Tyr—Phe Mutant

α-Synuclein Proteins E46K, Y39F, Y125F, S129A, mutant α-synucleins cDNAs were created using site-directed mutagenesis (Invitrogen). 1.6 μL methylation buffer, 1.6 μL of 10X SAM buffer, 1 μL DNA methylase, and 10.8 μL of water were added to 1 μL of purified plasmid DNA containing α-synuclein. The reactions were incubated at 37°C for one hour. A PCR reaction was used to amplify vector DNA with a new missense mutation. Primers were designed to make the particular mutations

S129A:5'GCTTATGAAATGCCT**G**CTGAGGAAGGGTATCAA3';
Y125F: 5'CCTGACAATGAGGCTT**T**TGAAATGCCTTCTGAG3'
Y39F:5'AAAGAGGGTGTTCTCT**T**TGTAGGCTCCAAAACC3';
E46K:5'ATGTAGGCTCCAAAACCAAG**A**AGGGAGTGGTGC3'.

These mutant cDNAs were expressed in *Escherichia coli* DH5a-T1. α-Synuclein expression plasmids were transformed as described (Burke, 2000) into URA-3-deficient *S. cerevisiae* yeast strains. For selection, yeast cells were grown on synthetic-complete media lacking uracil (SC-URA). Presence of α-synuclein constructs was confirmed by polymerase-chain reaction. The pYES2.1 vector, containing a galactose inducible promoter (GAL1), allowed for regulated α-synuclein expression. Table 1 lists all α-synuclein-GFP constructs in yeast expression vectors that were investigated.

Table 1. List of α-synuclein-GFP constructs in Yeast Expression Vectors

Construct	Vectors
WT α-Synuclein-GFP(CT)	TOPO PYES 2.1
A30P α-Synuclein-GFP(CT)	"
A53T α-Synuclein-GFP(CT)	"
A30P/A53T α-Synuclein-GFP(CT)	"
E46K α-Synuclein-GFP(CT)	"
Y39F α-Synuclein-GFP(CT)	"
Y125F α-Synuclein-GFP(CT)	"
S129A α-Synuclein-GFP(CT)	"
GFP	"

GFP Microscopy and Quantification

Yeast cells were viewed under the Zeiss Axiovert-100 fluorescent microscope and images were acquired under blue-filter settings and deconvoluted using the Metamorph 4.0 imaging software. Quantification was carried out by counting

cells in several areas of the slide and calculating the ratio of cells displaying certain patterns: general fluorescence (cytoplasmic localization), bright halos (peripheral localization), and foci (possible aggregation).

Western Analyses
Yeast cells ($2.5x10^7$ cells/ml) were washed in 50mM Tris (pH 7.5), 10mM NaN_3 and solubilized in Electrophoresis Sample Buffer (ESB; Burke, 2000) containing 2% SDS, 80mMTris (ph 6.8), 10% glycerol, 1.5% DTT, 1mg/ml bromophenol blue, and a cocktail of protease inhibitors and solubilizing agents (1% Triton-X 100, 1mM PMSF, 1mM benzamidine, 1mM sodium orthovanadate, 0.7 μg/ml pepstatin A, 0.5 μg/ml leupeptin, 10μg/ml E64, 2μg/ml aprotinin, and 2μg/ml chymostatin). Samples were run on pre-cast 10-20% Tris-Glycine SDS gels (Invitrogen), using SeeBlue as the molecular standard (Invitrogen). Gels were transferred to PVDF membranes and probed with the antibody: anti-V5 (Invitrogen).

Growth Analyses
For OD_{600} analysis, absorbance readings of yeast cells expressing α-synuclein were taken at 600nm using the Hitachi U-200 spectrophotometer. For spotting, cells were grown to mid-log phase in SC-URA raffinose (2%), normalized to equal densities, serially diluted (five-fold), and spotted on SC-URA glucose (2%) or galactose (2%) plates. Photographs were scanned after 2-3 days of growth.

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References

Ciechanover, A. and Brundin, P. 2003. The ubiquitin-proteasome system in neurodegenerative diseases: Sometimes the chicken, sometimes the egg. *Neuron* 40: 427-446.

Clayton, D. & George, J. 1998. The synucleins: a family of proteins involved in synaptic function, plasticity, neurodegeneration and disease. *Trends. Neurosci.* 21: 249-254.

Cole, N.B., Murphy, D.D., Grider, T., Rueter, S., Brasaemle, D., and Nusbaum, R.L. 2002. Lipid droplet binding and oligomerization properties of the Parkinson's disease protein α-synuclein. *J. Biol. Chem.* 277: 6344-6352.

Conway, K., Harper, J., Lansbury, P. 1998. Accelerated *in vitro* fibril formation by a mutant α-synuclein linked to early-onset Parkinson's disease. *Nat. Med.* 4: 1318-1320.

Conway, K., Harper, J., Lansbury, P. 2000 b. Fibrils formed *in vitro* from α-synuclein and two mutant forms linked to Parkinson's disease are typical amyloid. *Biochemistry* 39: 2552-2563.

Davidson, W.S., Jonas, A., Clayton, D.F., George, J.M. 1998. Stabilization of α-synuclein secondary structure upon binding to synthetic membranes. *J. Biol. Chem.* 273: 9443-9449.

Dawson, T.M. and Valina Dawson. (2003). Molecular Pathways of Neurodegeneration in Parkinson's Disease. *Science.* 302: 819-822.

Duda,, J.E., Murray, I.V., Chen, O., Souza, J.M., Hurtig, H.I., Ischiropoulos, H., Trojanowski, J.Q., and Lee, V.M. 2000. Oxidative damage linked to neurodegeneration by selective alpha-synuclein nitration in synucleinopathy lesions. *Science* 290: 985-989.

Feany, M. and Bender, W. 2000. A *Drosophila* model of Parkinson's disease. *Nature* 23: 294-298.

Giasson, B., Uryu, K., Trojanowski, J., Lee, V. 1999. Mutant and wild type human α-synucleins assemble into elongated filaments with distinct morphologies *in vitro*. *J. Biol. Chem.* 274: 7619-7622.

Giasson, B., Duda, J.E., Murray, I.V., Chen, O., Souza, J.M., Hurtig, H.I., Ischiropoulos, H., Trojanowski, J.Q., and Lee, V.M. 2000. Oxidative damage linked to neurodegeneration by selective alpha-synuclein nitration in synucleinopathy lesions. *Science* 290: 985-989.

Giasson, B., Duda, J., Quinn, S, Zhang, B., Trojanowski, J., Lee, V. 2002. Neuronal α-synucleinopathy with severe movement disorder in mice expressing A53T human α-synuclein. *Neuron* 34: 521-533.

Jensen, P.H., Nielsen, M., Jakes, R., Dottis, C.G., and Goedert, M. 1998. Binding of α-synuclein to brain vesicles is abolished by familial Parkinson's disease mutation. *J. Biol. Chem.* 273: 26292-26294.

Johnson, B.E. 2003. Expression and characterization studies of α-synuclein to model Parkinson's disease in yeast. *Undergraduate Senior Thesis – Lake Forest College, Biology.* 2 May 2003.

Krüger, R., Kuhn, W., Müller, T., Woitalla, D., Graeber, M., Kösel, S., Przuntek, H., Epplen, J., Schöls, L., Riess O. 1998. Ala30Pro mutations in the gene encoding α-synuclein in Parkinson's disease. *Nat. Genet.* 18: 106-108.

Masliah, E. Rockenstein, E., Veinbergs, I., Mallory, M., Hashimoto, M., Takeda, A., Mucke, L. 2000. Dopaminergic loss and inclusion body formation in α-Synuclein mice: implications for Neurodegenerative disorders. *Science* 287:1265-1269.

Narhi, L., Wood, S.J., Steavenson, S., Jiang, Y., Wu, G.M., Anafi, D., Kaufman, S.A., Martin, F., Sitney, K., Denis, P., Louis, J.C., Wypych, J., Biere, A.L., and Citrons, M. 1999. Both familial Parkinson's disease mutations accelerate α-synuclein aggregation. *J. Biol. Chem.* 274: 9843-9846.

Norris, E.H., et al. 2003. Effects of oxidative and nitrative challenges on a-synuclein fibrillogenesis involve distinct mechanisms of protein modifications. *The Journal of Biological Chemistry*. 278: 27230-27240.

Outeiro, T.F. and Lindquist, S. 2003. Yeast cells provide insight into alpha-synuclein biology and pathobiology. *Science* 203: 1772-1775.

Paxinou, E., et al. (2001). Induction of a-synuclein aggregation by intracellular nitrative. *The Journal of Neuroscience*. 21:8053-8061.

Polymeropoulos, M., and 20 others. 1997. Mutations in the α-synuclein gene identified in families with Parkinson's disease. *Science* 276: 2045-2047.

Rines, D.R., He, X., and Sorger, P.K. 2002. Quantitative Microscopy of Green Fluorescent Protein-Labeled Yeast Cells. Guthrie, C. and Fink, G.R. (Ed.) Guide to Yeast Genetics and Molecular and Cell Biology. Vol. 351: 16-34. New York: Academic Press.

Sharma, N. Baker's yeast promotes understanding of α-synuclein's functionaland
pathogenic roles. *Undergraduate Senior Thesis-Lake Forest College, Biology*. Lake Forest College. 30 Apr 2004.

Sharon, R., Goldberg, M., Bar, I., Betensky, R., Shen, J., Selkoe, D. 2001. α-Synuclein occurs in lipid-rich high molecular weight complexes, binds fatty acids, and shows homology to the fatty-acid binding proteins. *Proc. Natl. Acad. Sci. USA* 98: 9110-9115.

Sharon, R., Bar-Joseph, E., Frosch, M.P., Walsh, D.M., Hamilton, J., and Selkoe, D. 2003. The formation of highly soluble oligomers of α-synuclein is regulated by fatty acids and enhanced in Parkinson's disease. *Neuron* 37: 583-595.

Shimura, H., Schlossmacher, M., Hattori, N., Frosch, M., Trockenbacher, A., Schneider, R., Mizuno, Y., Kosik, F., Selkoe, D. 2001. Ubiquitination of a new form of α-synuclein by parkin from human brain: Implications for Parkinson's disease. *Science* 293: 263-269.

Souza, J.M., Giasson, B., Chen, O., Lee, V.M., and Ischiropoulos, H. 2000. Dityrosine cross-linking promotes formation of stable alpha-synuclein polymers. Implication of nitrative and oxidative stress in the pathogenesis of neurodegenerative synucleinopathies. *J. Biol. Chem.* 275: 18344-18349.

Tu, P.H., Galvin, J.E., Baba, M., Giasson, b., Tomita, t., Leight, s., Nakajo, S., Iwatsubo, T., Trojanowski, J.Q., and Lee, V.M. 1998. *Ann. Neurol.* 44: 415-422.

Wersinger, C., Prou, D., Vernier, P., Niznik, H.B., and Sidhu, A. 2003. Mutations in the lipid-binding domain of alpha-synuclein confer overlapping yet distinct, functional properties in the regulation of dopamine transporter activity. *Mol. Cel.. Neurosci.* 24: 91-105.

Zarranz JJ, Alegre J, Gomez-Esteban JC, Lezcano E. Ros R, Ampuero I, Vidal, L., Hoenicka J, Rodriguez O, Atares B, Llorens V, Gomez Tortosa E, del Ser T, Munoz DG,de Yebenes JG.The new mutation, E46K, of alpha-synuclein causes Parkinson and Lewy body dementia. *Annals of Neurology*. 2004 Feb;55(2):164-73.