Phosphorylation and Alanine-76 Contribute to α-Synuclein's Plasma Membrane Binding and Aggregation

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Abstract

Parkinson's disease (PD) is an incurable neurodegenerative disease, which afflicts nearly 4 million people worldwide. The hallmark symptom of PD is the formation of Lewy bodies containing aggregated, phosphorylated, and membrane phospholipid associated α-synuclein. The molecular determinants for α-synuclein aggregation and membrane association are still unknown. Past studies suggest that alanine-76 and phosphorylation at serines 87 and 129 may contribute to aggregation and membrane-association, which we tested here in two yeast models. By studying phosphorylation mutants (S87D and S129D), we found evidence that phosphorylation increases α-synuclein plasma membrane association in budding yeast and intracellular vesicular aggregation in fission yeast. Study of an A76R and A76E mutant demonstrated that this site promotes α-synuclein membrane association in budding yeast and aggregation in fission yeast, but the extent of aggregation is sensitive to the charge of the side chain. Thus, both yeast models help illuminate the molecular basis of α-synuclein pathology.

Introduction

Parkinson's disease (PD) was first described by James Parkinson almost 200 years ago (Lee et al., 2006). Today, it is estimated that PD affects nearly 4 million people worldwide (Rochet et al., 2004). Resting tremors, rigidity, and difficulty in initiating movements are key symptoms of PD and manifest due to the death of specific dopaminergic neurons found in the substantia nigra (Lozano et al., 2005; Rochet et al., 2004). There are two forms of PD: familial and sporadic (Polymeropoulous et al., 2007). Sporadic PD has been linked to environmental toxins, pesticides, and head trauma. However, the molecular mechanisms behind the onset of sporadic PD are still unclear. Familial PD is better understood, and at least seven genes have been identified that, when mutant, cause familial Parkinson's disease. These genes are α-synuclein (Polymeropoulous et al., 1997), Parkin (Kitada et al., 1998), UCH-L1 (Valente et al., 2004), DJ-1 (Bonifati et al., 2003), PINK 1 (Valente et al., 2004), LRRK2 (Paisan-Ruiz et al., 2004; Zimprich et al., 2004), and ATP13A2 (Fonzo et al., 2007). Aggregated α-synuclein is found in both forms of the disease, suggesting that aggregation is important in both sporadic and familial PD onset (Spillantini et al., 1998). These formations are called Lewy bodies and are composed of several other proteins in addition to α-synuclein (Spillantini et al., 1998; Yamada et al., 2004).

The α-synuclein present in the Lewy bodies of human PD patients is heavily modified. In human patients, α-synuclein is heavily phosphorylated at serine-129 and to a lesser extent at serine-87 (Fujiwara et al., 2002). Phosphorylation at ser-129 increases aggregation of α-synuclein in vitro and in vivo (Fujiwara et al., 2002; Gorbatyuk et al., 2007). Although the role of phosphorylation in promoting aggregation is well understood, the role of phosphorylation in cell death is less clear. Chen et al., (2004) observed phosphorylation dependent toxicity in a Drosophila model. However, in a rat model, ser-129 phosphorylation had a protective effect (Gorbatyuk et al., 2007, McFarland et al., 2009). Due to this conflict, the impact of phosphorylation on cellular toxicity needs to be investigated further.

The tendency of α-synuclein to aggregate is an important pathological feature of PD. The amino acids 71-82 make up the non-beta amyloid component region (NAC) of α-synuclein (Giasson et al., 2001). This region contains numerous hydrophobic amino acids and is essential for protein aggregation (Giasson et al., 2001). The importance of the NAC domain in α-synuclein's ability to aggregate is illustrated by the structure of β-synuclein. β-synuclein differs structurally from α-synuclein in that it lacks the NAC region and does not aggregate (Biere et al., 2000). Also, when the NAC domain is deleted from α-synuclein, aggregation does not occur in vitro or in vivo (Giasson et al., 2001, Periquet et al., 2007). In 2004, alanine-76 within the NAC region was mathematically predicted to directly influence α-synuclein aggregation (Chiti et al., 2004). Giasson et al., (2001) showed that when alanine-76 was mutated to a charged amino acid, α-synuclein's ability to aggregate was affected. While the NAC region has been studied extensively in model organisms, the specific amino acid alanine-76 has not been well studied beyond cell culture. Thus, the role of alanine-76 in aggregation and membrane association is not well understood in vivo.

In this study, we used a yeast model of Parkinson's disease to evaluate the contribution of phosphorylation and alanine-76 on α-synuclein's properties. Yeast models are cost effective, and experiments can be finished quickly. Additionally, the genome has been sequenced, and yeast genes are highly homologous to humans (Bostein et al., 1997). Finally, yeast have a history of successfully modeling neurodegenerative diseases (Outeiro and Lindquist, 2003; Dixon et al., 2005, 2005; Zabrocki et al., 2005; Cooper et al., 2006). In our lab, wild-type α-synuclein generally localizes to the plasma membrane in our budding yeast model (Sharma et al., 2006) and aggregates in our fission yeast model (Brandis et al., 2006). This allows us to study the roles of serine phosphorylation and alanine-76 to the two PD pathology linked α-synuclein properties: phospholipid binding and aggregation.

We first tested the hypothesis that phosphorylation at serine-129 and serine-87 contributes to the aggregation and protective properties of α-synuclein by studying α-synuclein phosphorylation mimic mutants S87D and S129D. We asked if these phosphorylation mutants would alter their localization compared to wild-type, impact toxicity, or accumulate in our yeast models. Phosphorylation deficient mutants were studied by a collaborator and used as controls (Fiske et al., 2009). We found that phosphorylation increased membrane association in budding yeast and intracellular vesicular aggregation in fission yeast.

Next, we tested the hypothesis that alanine-76 contributes to α-synuclein membrane localization and aggregation. Our approach was to mutate the hydrophobic

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alanine to a hydrophilic arginine. We showed that alanine-76 promoted membrane association and aggregation in budding and fission yeast, respectively. However, the extent of aggregation was sensitive to the charge of the mutated *amino acid.*

Materials and Methods

Materials and Methods listed below are adapted from Sharma et al. (2006), and described below briefly again.

α-Synuclein Constructs
Human wild-type (WT) α-synuclein cDNAs were provided by Christopher Ross of Johns Hopkins University. Using Invitrogen site directed mutagenesis, WT α-synuclein was mutated into A76E, A76R, S87A, S129A, S87D, and S129D. In order to confirm the mutations, the mutants were sent to the University of Chicago for sequencing. WT and mutant α-synuclein cDNAs were subcloned into the pYES2.1/V5-His-TOPO yeast expression vector for budding yeast and the pNMT1 TOPO-TA expression vector for fission yeast. In order to tag GFP to the C-terminus of α-synuclein, α-Synuclein cDNAs were subcloned into the pcDNA3.1/C-terminal GFP and pcDNA3.1/N-terminal GFP mammalian expression vectors using Invitrogen. The GFP tagged α-synuclein gene was then PCR-amplified and subcloned using the pYES2.1/V5-His-TOPO yeast expression vector and the pNMT1 TOPO-TA expression vector. The α-synuclein-GFP pYES2.1/V5-His-TOPO and pNMT1 TOPO-TA vectors were separately transformed into DH5α *E. coli* cells. The parent pYES2 vector, parent pNMT1 vector (provided by Judy Potashkin, Rosalind Franklin University of Medicine and Science), and GFP in pYES2.1/V5-His- TOPO vector or pNMT1 vector served as controls. Mutant A76E, S87A, and S129A were created using the following primers:

S87D:
Forward:
5' GTGGAGGGAGCAGGGGACATTGCAGCAGCC 3'
Reverse: 5'CCCTGCTCCCTCCACTGTCTTCTGGGCTAC 3'

S129D:
Forward:
5' GCTTATGAAATGCCTGACGAGGAAGGGTATC 3'
Reverse: 5' AGGCATTTCATAAGCCTCATTGTCAGGATC 3'

A76R:
Forward:
5'-CAGTG GTGACG GGT GTGACACGAGTAGCCCAG A-3'
Reverse:
5'- CAACCTCCTCGTCACCACTGCCCACACTGT-3'

Yeast Strains
Budding yeast strain Y4741 is from Open Biosystems and fission yeast strain TCP1 (h- leu1-32) is from Invitrogen.

Yeast Expression
For budding yeast, α-synuclein vectors were transformed into BY4741 as described in Burke et al., 2000. PCR amplification confirmed the presence of the vector in our budding yeast. pYES2 vector has a galactose inducing promoter. Thus, in order to produce α-synuclein, cells were grown on SC-Uracil galactose media. To grow cells without α-synuclein induction, SC-uracil glucose media was used. TCP1 was transformed with the pNMT vectors using a lithium-acetate transformation method described by Alfa et al.1993. To produce α-synuclein, cells were grown in the inducing media Edinburgh Minimal Medium (EMM)-Thiamine. To grow without producing α-synuclein, cells were grown in EMM+Thiamine. Then, 500µl of cells were inoculated in 25 mL EMM - thiamine (to express α-synuclein).

Western Analyses
At 24 and 48 hour time points, yeast cells were washed with 10 mM NaN3 and solubilized in Electrophoresis Sample Buffer (ESB; Burke, 2000). Lysates were run on Invitrogen 10-20% acrylamide gels in a running buffer containing 10% SDS. SeeBlue molecular ladder was used as the standard. The gels were then transferred to PVDF membranes. Once washed with blocking buffer, the membranes were probed with antibodies. For both yeasts, α-synuclein was detected with an anti-V5 antibody. Anti-PGK was used for a loading control in budding yeast and anti-beta actin was used as a loading control in fission yeast. Bands were visualized by detecting alkaline phosphatase activity.

Growth Curve Analysis
Budding yeast were grown overnight in SC-Ura glucose and centrifuged. The cells were then washed with water two times and diluted to a final volume of 10 mL. Cells were then counted using a hemocytometer. The specific amounts of cells were then pipetted into 25 mL of SC-Ura galactose. At the time periods of 0, 3, 6,12, 18, 24, 36, and 48, 1mL of cells was pipetted into a cuvet and absorbance was read by a spectrophotometer. The absorbance numbers were then plotted versus time in hours. For fission yeast, the experiment was completed similarly to budding yeast only the cells were grown in EMM+Thiamine overnight and placed into EMM-Thiamine for the 48 hour time period.

GFP Microscopy
Yeast cells were grown overnight in 10ml SC-URA glucose (or EMM + thiamine for fission yeast) at 30°C and 200 rpm. Yeast were grown in SC-URA galactose media (or EMM-thiamine for fission yeast) to induce protein expression. 4 µL of cells were placed on a slide and viewed using a Nikon TE2000-U fluorescence microscope. The cells were viewed using a Nikon TE2000-U fluorescent microscope. Pictures of yeast cells were acquired and analyzed using the program Metamorph.

Dilution Spotting
Yeast cells were grown overnight in 10 mL of SC-URA glucose (EMM +thiamine for fission yeast) for a day. Cells were washed and counted. Cells were then diluted five times by pipetting 20 µL of cells in 80 µL of water five times. These six different dilutions were then pipetted onto SC-URA galactose (EMM-thiamine for fission yeast) solid media dishes and onto non-inducing solid media dishes for a control.

Statistical Analysis
Optical density: Student t-test performed on the collective readings for the three Optical Density analyses performed for budding yeast and fission yeast. Calculations were made at 18 and 24 hours. For each student t-test, the mutants were being compared in growth to the parent plasmid.
Microscopy: Using DIC images, 750 cells were counted for the mutants and parent plasmid at 24 and 48 hours. Fluorescence images were then evaluated and labeled as cytoplasmically diffused, aggregated, halo, or halo and diffused. The final counts for each cell type, mutant or parent plasmid were then compared.

Results

In this study, two types of yeast models were used to study the properties of α-synuclein: budding yeast strain BY4741 and fission yeast strain TCP1. The pYES2 galactose-

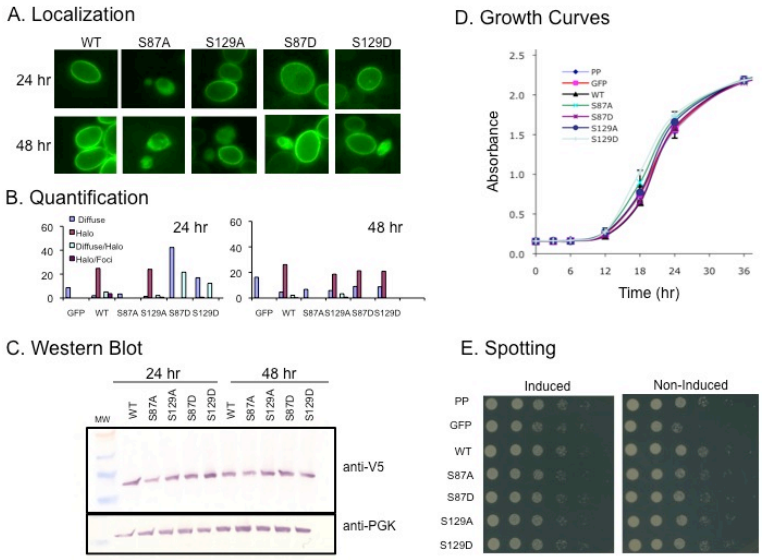

Figure 1: Phosphorylation mimic Alpha-synuclein is membrane bound and non-toxic in BY4741 budding yeast
A. Alpha-Synuclein localization: Alpha-synuclein WT, S87A, S129A, S87D, and S129D localization at 24 and 48 hours post expression. WT, S87D, and S129D localized to the plasma membrane. Likewise, S87A and S129A localized to the cytoplasm (n=2).
B. Localization quantification: 750 cells of each transformed alpha-synuclein construct in BY4741 were counted and scored for five different florescence localization patterns: diffuse, halo, foci, weak halo and diffuse, and weak halo and foci (n=2).
C. Protein expression: Western blotting was used to assess the amount of WT and phosphorylation mutant alpha-synuclein constructs present in BY4741 yeast at 24 and 48 hours. WT, S129A, S87D, and S129D showed similar expression. Expression of S87A decreases slightly compared to WT (n=2).
D. Growth curve: Optical density 600 nm was used to evaluate growth in BY4741 budding yeast expressing WT, S87A (18 hrs- P=0.409 and 24 hrs- P=0.265), S129A (18 hrs P=0.792 and 24hrs- P=0.489). S87D (18 hrs- P=0.516 24 hrs- P=0.665), and S129D (18 hrs P=0.203 and 24 hrs P=0.057). Phosphorylation mimic and deficient mutants and WT alpha-synuclein were statistically compared to PP. (n=3).
E. Spotting: Five-fold serial dilutions on glucose (non-inducing media) and galactose (inducing media). WT, S87A, S129A, S87D, and S129D showed no toxicity. (n=3).

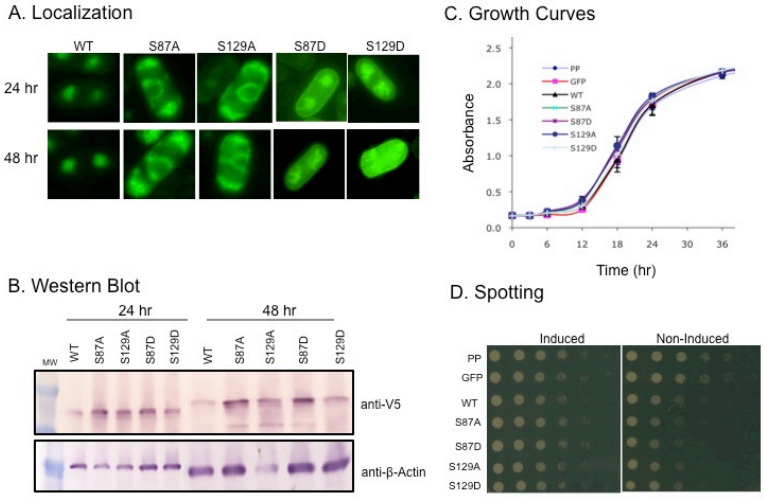

Figure 2: Phosphoryalation mimic Alpha-synuclein aggregates and localizes to the cytoplasm in fission yeast
A. Alpha-Synuclein localization: Fluorescence microscopy was used to visualize GFP-tagged alpha-synuclein at 24 and 48 hrs. WT aggregates at 24 and 48 hrs. At 24 hrs, S87D aggregates while S129D is cytoplasmically diffuse by 48 hrs. Likewise, S87A and S129A localized to intracellular organelles and to the cytoplasm (n=1).
B. Protein expression: Western blotting was used to assess the amount of WT, phosphorylation mimic, and deficient mutant alpha-synuclein present in TCP1 yeast at 24 and 48 hrs. Expression of S87A, S129A, and S87D showed similar expression. S129D showed a slight decrease in expression (n=2).
C. Growth curve: Optical density 600 nm was used to evaluate growth in TCP1 fission yeast expressing WT, S87A (18 hr- P=0.320 and 24 hr- P=0.492), S129A (18 hr P=0.250 and 24hr- P= 0.304), S87D (18 hr- P=0.433 24 hr- P=0.447), and S129D (18 hr P=0.570 and 24 hr P=0.533)
Phosphorylation mimic mutants and wild-type alpha-synuclein were statistically compared to PP (n=3).
D. Spotting: Five-fold serial dilutions on Emm+T (non-inducing media) and Emm-T (inducing media). WT, S87A, S129A, S87D, and S129D are shown. WT, phosphorylation mimic and deficient mutants showed slight toxicity when compared to PP and GFP-alone in TCP1. However, no phosphorylation dependent toxicity was observed (n=3).

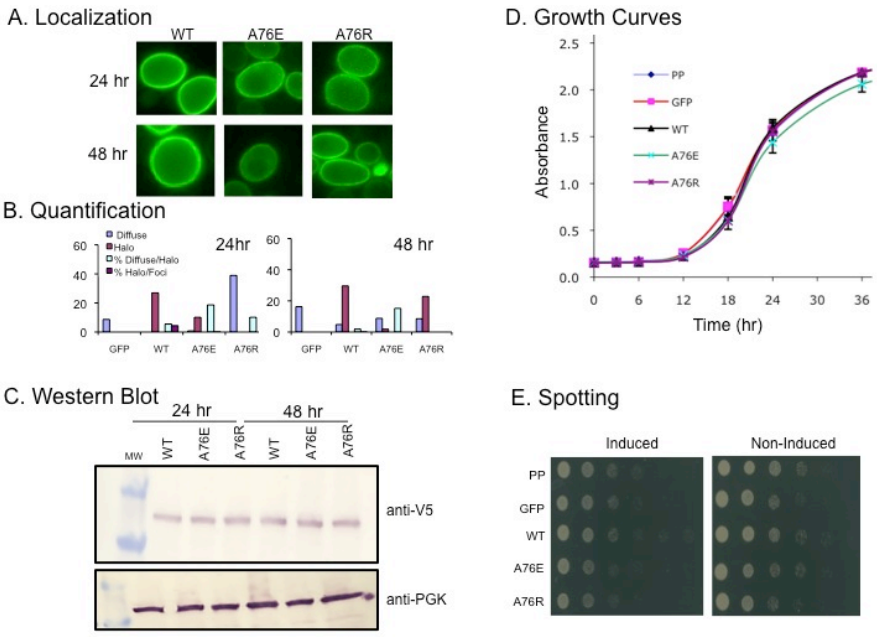

Figure 3: Alanine-76 is important to membrane localization.
A. Alpha-Synuclein localization: Alpha-synuclein WT, A76R, and A76E localization at 24 and 48 hours post expression. WT localized to the plasma membrane, A76R, and A76E localized to the cytoplasm. However, by 48 hrs A76R localizes to the plasma membrane (n=2).
B. Localization quantification: 750 cells of each transformed a-synuclein construct in BY4741 were counted and scored for five different florescence localization patterns: diffuse, halo, foci, weak halo and diffuse, and weak halo and foci (n=2).
C. Protein expression: Western blotting was used to assess the amount of WT and alanine-76 mutant a-synuclein constructs present in BY4741 yeast at 24 and 48 hrs. WT, A76E, and A76R showed similar expression in by4741 yeast. (n=2).
D. Growth curve: Optical density 600 nm was used to evaluate growth in BY4741 budding yeast expressing WT, A76R (18hrs-P=0.248 and 24hrs P=0.959), and A76E (18hrs- P=0.384 and 24hrs- P=0.477). No significant toxicity was observed with WT, A76R or A76E when compared to PP (n=3).
E. Spotting: Five-fold serial dilutions on glucose (non-inducing media) and galactose (inducing media). No alpha-synuclein dependent toxicity was observed (n=3).

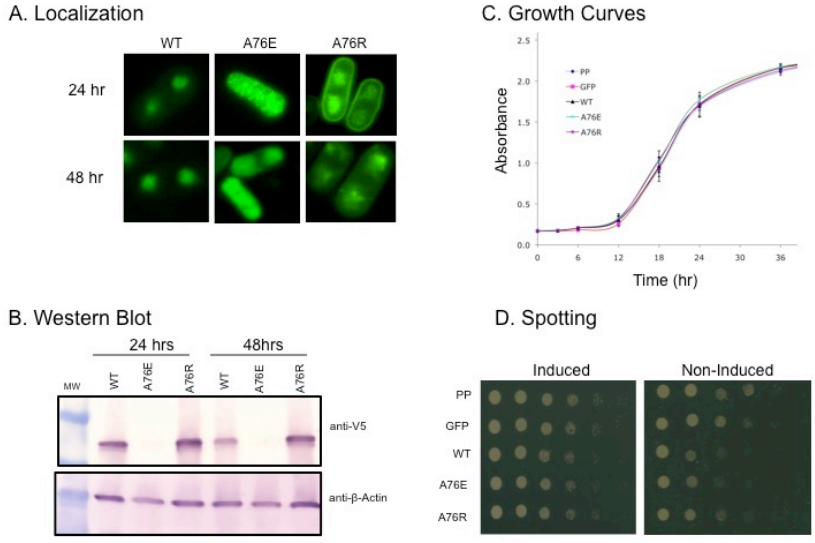

Figure 4: Alanine-76 is important to aggregation if fission yeast
A. Alpha-Synuclein localization: Alpha-synuclein WT, A76R, and A76E localization at 24 and 48 hours post expression. WT and A76R aggregated in fission yeast. However, A76R localized to the cytoplasm. (n=2).
B. Protein expression: Western blotting was used to assess the amount of WT and alanine-76 mutant alpha-synuclein present in TCP1 yeast at 24 and 48 hrs. WT and A76R showed similar expression. A76E showed a dramatic decrease in expression when compared to WT (n=2).
C. Growth curve: Optical density 600 nm was used to evaluate growth in TCP1 fission yeast expressing WT, A76R (18hrs P= 0.576 and 24hrs P=0.908), and A76E (18hrs P=0.622 and 24hrs P=0.530). Alanine-76 mutants and wild-type alpha-synuclein were compared to PP and GFP-alone. No toxicity was observed. (n=3).
D. Spotting: Five-fold serial dilutions on Emm+T (non-inducing media) and Emm-T (inducing media). WT, A76R, and A76E showed alpha-synuclein dependent toxicity when compared to PP and GFP-alone, however, no additional toxicity was observed in the alanine-76 mutants. (n=3).

promoting vector expressed α-synuclein in budding yeast and the PNMT1 vector in fission yeast. We utilized four assays to assess the properties of α-synuclein. Live cell GFP microscopy evaluated where α-synuclein localized. Western blotting analyzed the expression of α-synuclein in the cells. Finally, two well established toxicity assays, OD-600 growth curve and five fold serial dilution spotting, analyzed growth of the cells.

Phosphorylation is important to α-synuclein's membrane binding

We first evaluated the effect of phosphorylation on α-synuclein in the BY4741 budding yeast strain. We analyzed localization and showed that wild-type binds to the membrane. In support of our hypothesis, S87D and S129D α-synuclein phosphorylation mimic mutants localized to the plasma membrane similarly to wild-type by 48 hours (Figure 1A). Likewise, the α-synuclein phosphorylation deficient mutants, S87A and S129A, did the opposite and localized to the cytoplasm and less to the plasma membrane (Figure 1A). However, the phosphorylation mimics were expressed at levels similar to WT (Figure 1B). Furthermore, cells expressing α-synuclein, whether wild-type or phosphorylation mutants, all grew similar to cells expressing the parent vector and GFP (Figure 1C & D).

α-Synuclein aggregation is affected by phosphorylation

Secondly, we asked if α-synuclein aggregation would be affected by phosphorylation in TCP1 fission yeast. We first showed that wild-type α-synuclein tends to form intracellular aggregates in fission yeast. In support of our hypothesis, S87D α-synuclein mutant localized to intracellular vesicles similar to wild-type (Figure 2B). Likewise, S87A and S129A localized to the cytoplasm with less aggregation. Interestingly, S129D formed aggregates, but to a lesser extent than S87D and wild-type and also localized heavily to the cytoplasm (Figure 2A). Again, the phosphorylation mimic conformation did not correlate with a significant change in expression. However, a slight decrease in expression was seen in S129D when compared to S87D (Figure 2B). Lastly, no significant decrease in growth was observed among fission yeast expressing any of the α-synuclein constructs (Figure 2C). The five fold spotting assay, however, did show a slight α-synuclein dependent toxicity (Figure 2D). Phosphorylation did not contribute any additional protection to the cells (Figure 2D).

Alanine-76 contributes to α-synuclein's plasma membrane association

Our next goal was to evaluate the affects of the hydrophobic amino acid alanine -76 within the NAC domain. We first analyzed localization and showed that wild-type α-synuclein in BY4741 budding yeast strain localized to the plasma membrane (Figure 3A). In support of our hypothesis, A76E and A76R α-synuclein localized to the cytoplasm and less to the plasma membrane (Figure 3A). Over time, however, A76R became primarily membrane bound while A76E continued to localize to the cytoplasm (Figure 3B). Neither alanine-76 mutation altered expression (Figure 3B). Lastly, cells expressing α-synuclein, whether wild-type or alanine-76 mutants, all grew similar to GFP and parent plasmid (Figure 3C & D).

Alanine-76 is important to the aggregation of α-synuclein

Finally, we examined the role of alanine-76 in fission yeast. First we showed that wild-type α-synuclein aggregates in fission yeast (Figure 4A). In support of our hypothesis, A76E localized to the cytoplasm and less aggregation was seen (Figure 4A). The A76R mutant phenotype was complex and seemed to aggregate and localize to the cytoplasm. Interestingly, A76R did not cause a change in expression while A76E decreased significantly in expression (Figure 4B). Finally, α-synuclein dependent toxicity was observed in the spotting assay but no significant growth decrease in growth curve analysis (Figures 4C & D). However, no additional toxicity due to A76R or A76E when compared to wild-type α-synuclein was observed (Figure 4D).

Discussion

The tendency for α-synuclein to aggregate and associate with phospholipids is important in Parkinson's disease pathology since Lewy bodies are composed of aggregated and lipid-associated α-synuclein. The goal of this study was to understand the importance of phosphorylation in α-synuclein's ability to bind membranes, aggregate, and induce toxicity. Secondly, we sought to understand the role of alanine-76 in α-synuclein's ability to bind membranes and aggregate. We first found that phosphorylation increases α-synuclein plasma membrane association in budding yeast and intracellular vesicular aggregation in fission yeast. Secondly, we found that alanine-76 promotes α-synuclein membrane association in budding yeast and aggregation in fission yeast. Lastly, we found that neither of the phosphorylation mimics enhanced toxicity in budding or fission yeast.

Phosphorylation is important to α-synuclein membrane binding and aggregation

Our first major finding that S87D and S129D increases plasma membrane localization in budding yeast and S87D increased aggregation in fission yeast supports our hypothesis that phosphorylation contributes to α-synuclein's ability to bind to phospholipids and aggregate. Likewise, S87A and S129A did the opposite and localized to the cytoplasm in both fission and budding yeast. Also, while S129D localized to the cytoplasm, some fraction was membrane bound as well. These findings further demonstrate that phosphorylation at serine 129 and also serine 87 contributes to PD pathology linked properties of α-synuclein as shown in previous studies in vitro (Fujiwara et al., 2002; Smith et al., 2005). Studies have shown that phosphorylation at serine-129 enhances aggregation in vivo (Gorbatyuk et al., 2007). However, Chen et al., (2005) showed that ser-129 phosphorylation did not cause aggregation. The finding that phosphorylation at ser-129 in fission yeast became more cytoplasmically diffuse further demonstrates the role of phosphorylation in large aggregates but not smaller oligomeric α-synuclein aggregates (Silveira et al., 2008). Some aggregates that were not bound to vesicles were seen but α-synuclein was mainly cytoplasmically diffuse. Small oligomeric species, which have been linked to toxicity, may form when α-synuclein is not able to be phosphorylated. These small species may be too small to see under live cell GFP microscopy, but could be forming in both the budding and fission yeast.

Also, the contribution of phosphorylation to α-synuclein's ability to bind phospholipids is not well documented. Paleologou et al., (2008) showed phosphorylated α-synuclein did not alter membrane interactions when compared to phosphorylation deficient mutants. This contradicts our results; however, Sharon et al., (2003) showed that aggregated α-synuclein in Lewy bodies, which are mainly composed of phosphorylated α-synuclein, bound to the phospholipids of vesicles. The findings in our budding yeast model help demonstrate that phosphorylated α-synuclein does help binds phospholipids. Studies showed that α-synuclein changes conformation in order to bind to membranes. Thus, when phosphorylated in budding and fission yeast, α-synuclein may adopt a shape that allows

binding to phospholipids. The extra expression in fission yeast may allow α-synuclein, while bound to phospholipids, to aggregate as well and even bind to the plasma membrane (Brandis et al., 2006). Thus, these findings suggest that phosphorylation of α-synuclein is not only important in aggregation but also membrane binding as well.

Alanine-76 contributes to α-synuclein's aggregation and membrane binding

Our second major finding is that alanine-76 is important to α-synuclein's ability to bind lipids and aggregate. These findings supported the hypothesis that alanine-76 contributes to α-synuclein's ability to localize to the plasma membrane and aggregate. A76E localized to the plasma membrane in both budding and fission yeast while A76R localized to the cytoplasm in budding yeast and seemed to aggregate in fission yeast. A76Rs inability to cytoplasmically localize in fission yeast and extensively in budding yeast is supported by Giasson et al., (2001). Glutamic acid was shown to be a stronger hydrophilic mutation than arginine, possibly contributing to the different properties of the two mutants. Changing this hydrophobic amino acid to a hydrophilic one may completely change the properties of the NAC region. The NAC region is important to α-synuclein aggregation; changing its hyrdrophobic property may be similar to deleting the region or at least an important part of the region (Waxman et al., 2009). Soper et al., (2008) showed that when the NAC region was intact, α-synuclein formed aggregates bound to vesiclzes. Without the NAC region, however, these aggregates did not formed. Hydrophobicity plays a significant role in the tertiary structure of a protein. Thus, without the key property of a hydrophobic region, α-synuclein may not have been able to take on the structure that allows it to aggregate.

The field lacks data on the NAC region of α-synuclein and its role in membrane binding. Our data further develops the role of alanine-76 in phospholipid binding. Soper et al., (2008) showed that, without the NAC domain, aggregates could not form and these aggregates are bound to vesicles. This may mean that, to some extent, the NAC region is important to membrane binding. This may be a small role because the same experiment showed that α-synuclein could still bind to the plasma membrane without the NAC region. Suggested earlier, as with phosphorylation, α-synuclein may need a certain tertiary structure in order to bind phospholipids. Since phosphorylation can still occur in these mutants, but the NAC region's property has changed, the certain tertiary structure that α-synuclein forms may be impacted by both factors. Thus without one or the other we have shown a decrease of membrane binding in our budding yeast (Sharma et al., 2006).

No toxicity due to phosphorylation

Our final finding is that phosphorylation did not cause additional toxicity in our yeast model. Our hypothesis was not supported, and additionally the lack of phosphorylation did not cause toxicity in our yeast model either. In fact we showed α-synuclein toxicity in our fission yeast only. Interestingly, other labs have shown α-synuclein toxicity in their budding yeast models. (Outeiro and Lindquist, 2003). The lack of toxicity in our budding yeast model may be due to several key reasons. First, the vector system we use may not express enough protein to cause misfolding and aggregation. We used a 2μ expression system instead of having the protein implemented into the genome. Thus, we may not have been able to over express α-synuclein sufficiently to cause misfolding and aggregation. If this occurs, certain proteins such as Hsp70 may help α-synuclein fold correctly preventing aggregation (Hartl et al., 2002). Secondly, the strain of yeast we used may affect the properties of α-synuclein. A-synuclein may have a slightly different role in BY4741 or TCP1 when compared to other strains. Finally, yeasts, although they are simple organisms, they are advanced in protecting themselves. Yeast may be able to excrete the plasmid when the protein is starting to cause toxicity. Thus, the plasmid may be able to produce enough α-synuclein, but the yeast does not allow it to.

We expected phosphorylation would to be a toxic agent to the cells. At first, these results conflict with two major studies: in a fly and rat model it has been shown that phosphorylation cause toxicity and protection respectively (Chen et al., 2004; Gorbatyuk et al., 2007). This difference in toxicity may be due to the organism or the type of vector expressing α-synuclein. However, our finding may further develop the role of phosphorylation and toxicity that has been shown in previous studies (Silveira et al., 2008; McFarland et al., 2009). Both studies showed that α-synuclein toxicity was independent of phosphorylation. These interesting conflicts in the field could be due to the fact that each lab uses a different expressing vector or over-express α-synuclein to a larger extent. However, recently McFarland et al., (2009) showed that phosphorylation at tyrosine 125 protected against the toxicity due to serine-129 phosphorylation. If ser-129 phosphorylation is additionally toxic to our yeast cells, there may be enough tyrosine-125 phosphorylation to protect against this toxicity. This is supported by the results showing that phosphorylation deficient mutants do not show additional toxicity either.

There are important studies that still need to be completed. First, additional research will complete analysis on phosphorylation and the role of alanine-76 in membrane binding and aggregation in different strains of yeasts. Different strains may help lead to better understandings of the roles of phosphorylation and alanine-76 in aggregation and membrane binding. Secondly, truncation mutants of α-synuclein will be evaluated. Evidence shows that the c-terminus is truncated in 15% of α-synuclein aggregated in Lewy bodies. Periquet et al., (2007) showed that truncating the c-terminus in vivo enhances the ability for α-synuclein to aggregate and causes toxicity. Thus, truncation of the three regions of α-synuclein may lead to additional insights into properties of α-synuclein. Finally, properties of α-synuclein due to phosphorylation at tyrosine-125 should be evaluated.

Conclusion

The mechanism behind α-synuclein's ability to aggregate is still a question that remains unanswered. Answering the question whether phosphorylation speeds up aggregation could lead to new drug targets for Parkinson's disease patients. If aggregation is toxic to cells and phosphorylation enhances α-synuclein's ability to aggregate, scientists could target certain phosphatases that dephosphorylate α-synuclein which would hopefully decrease the amount of aggregated α-synuclein. Understanding phosphorylation and alanine-76's role in aggregation is still necessary and could help lead to possible drug treatments.

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