Assessing alpha-Synuclein Degradation through the MVB/Endocytosis Pathway

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Abstract

Parkinson's disease (PD) is a neurodegenerative disorder linked to the accumulation, misfolding, and aggregation of the α-synuclein protein in dopaminergic neurons of the midbrain, which might ultimately lead to the death of these neurons. Until recently, the proteasome was believed to be the main pathway for α-synuclein degradation; however, strong evidence emerged that supports the lysosome as another mechanism that effectively degrades the protein. The two pathways to the lysosome are autophagy and endocytosis. Here I propose the autophagy as a mechanism for α-synuclein degradation is available, even though some studies point to the MVB/endocytosis pathway a route for membrane bound and extra cellular protein degradation. I propose the hypothesis that the MVB/endocytosis pathway is a route through which α-synuclein is targeted to the lysosome. Using S. cerevisiae as a model system, I will assess whether α-synuclein accumulates, changes localization, and induces cell toxicity in yeasts genetically compromised for endocytosis at the pre-ESCRT step: vps27 and vps34. The pre-ESCRT gene knockouts will be compared with intact endocytosis intact yeast strains, while also comparing α-synuclein containing vectors to α-synuclein absent vectors. With around four million people affected worldwide, finding a cure for PD is a worldwide concern and priority.

Background

Our society is affected with a variety of human disorders; amongst them are a group of disorders classified as the neurodegenerative disease disorders. These disorders affect the brain and are characterized by the death of specific neurons. Parkinson’s disease (PD) is a neurodegenerative disorder first described in 1817 by James Parkinson. The disorder is incurable, and classic symptoms include resting tremors, muscular rigidity, bradykinesia, and postural instability (1). The importance of studying this disease is apparent when looking at the statistics: around four million people worldwide are afflicted, and it is expected that by 2040, neurodegenerative disorders such as Alzheimer’s disease and Huntington disease will surpass cancer as the leading cause of death (2). These diseases are caused by the accumulation of different proteins. In PD specifically, α-synuclein is the protein thought to be involved (3). Lewy bodies, which are inclusions of α-synuclein, are found in the substantia nigra region of the midbrain in PD patients. These individuals experience a loss of dopamine neurons in this area of the brain (2, 4, 5, 6). The loss of these dopamine-secreting cells leads to the motor deficiencies seen in the patients. PD can occur in two forms: sporadic or familial. Initially, the update of 1-Methyl-4-phenyl-4-propinopyridine (MPPP), a byproduct of synthetic heroin, caused PD-like symptoms in patients (7). Now it is known that the misfolding and accumulation of wild-type (WT) α-synuclein leads to the sporadic case of PD. Point mutations in α-synuclein, on the other hand, cause familial forms of the disease. Six genes have been identified in the development of familial PD: α-synuclein (1), parkin (8), UCH-L1 (9), DJ-1 (10), PINK1 (11), and LRRK2 (12). Currently, no cure for PD has been found, but some medications to alleviate the symptoms are available. Unfortunately, some patients eventually become resistant to the medication (2).

α-Synuclein is a neuronal protein that is found in the brain. It is a protein that has been implicated in a variety of Neurodegenerative diseases, including PD and AD. The presence of a weak transient or residual secondary structure in the protein is what may be playing a role in amyloid fibril formation, therefore playing a role in its aggregation. Its plastic characteristic is mainly due to its C-terminal region, which is acidic and always remains unstructured (13). A large element of the protein is in its localization in presynaptic nerve terminals (14) and its ability to bind to phospholipids membranes (15). Recently, data implicates that α-synuclein is delivered to the plasma membrane through its interaction with the secretory pathway (16). In yeast models, WT and the other familial mutant, E46K, localize in the plasma membrane. The familial mutant A30P localizes in the cytoplasm (17). To further support α-synuclein’s gain of function characteristics in PD, experiments in animal models have shown PD-like symptoms with α-synuclein over expression. This was demonstrated with wild-type α-synuclein and its familial mutants (A30P and A53T) in mice (18), flies (19), and worms (20). The past decade has given tremendous insight into the PD field, but some questions on the molecular basis of PD remain unanswered. Some questions include: if α-synuclein is toxic to yeast, what is the causative agent? What is the role of oxidative stress in PD pathogenesis? Another major question in the field is if α-synuclein accumulation is what causes PD. If so, a better understanding of the different routes α-synuclein takes to become degraded is essential for developing treatments. The lysosome as a degradation pathway has been less studied than the proteasome. Studies have shown that proteasome inhibition leads to α-synuclein inclusion formation (21, 22). A knockout mouse model for the essential subunit of the 26S proteasome resulted in mice with intra-neuronal Lewy-like inclusions and neurodegeneration in the nigrostrital pathway (23). These results highlight the proteasome as a key organelle in α-synuclein degradation. Recently, accumulating evidence implicated the lysosome as a means for α-synuclein degradation (24). Two pathways to the lysosome exist: autophagy and endocytosis. Focus on the lysosome has built up due to mounting contradicting evidence that demonstrated no changes in levels of α-synuclein with proteasome inhibition (21, 25). Cuervo et al. (2004) conducted experiments that showed WT α-synuclein is internalized and degraded by the lysosome through chaperone-mediated autophagy (CMA). The A30P and A53T mutants, on the other hand, were not degraded using this mechanism (26). Results were confirmed in similar experiments with inhibited CMA and macroautophagy that resulted in WT α-synuclein accumulation (27). Other experiments with autophagy found evidence of the presence of α-synuclein in autophagic-like vesicles. Data also showed that autophagy inducers, like rapamycin, increased the clearance of α-synuclein in PC12 cell lines (28).

Although some evidence links α-synuclein degradation to the lysosome, like the studies mentioned above, these mostly focus on autophagy, one of the two
pathways to the lysosome. Little evidence exists that focuses on the other pathway to the lysosome for α-synuclein degradation: the endocytosis pathway. The endocytosis pathway brings in proteins from outside or from the plasma membrane into the cell and to the lysosome. Proteins are degraded using multivesicular bodies (MVBs), which are vesicles that form inside the late endosome. MVBs are formed when the endosome membrane invaginates into the lumen. The invagination is made possible by the endosomal-sorting complex required for transport (ESCRT). The ESCRT complex helps form MVBs, which then make it possible for those membrane-bound proteins to be degraded once the endosome and lysosome fuse (29, 30, 31). Few studies focus on the endocytosis/MVB pathway as a mechanism for α-synuclein degradation. This is relevant because studies in yeast knockouts of key endocytosis genes show α-synuclein dependent toxicity (32). Also, data shows that in C. elegans endocytosis gene knockouts, there was an accumulation of phosphorylated α-synuclein (33). Even though α-synuclein is mostly a cytoplasmic protein, small amounts of its aggregated form are found outside the cells in PD patients, in particular in the blood plasma and cerebrospinal fluid. Lee, H. J. et al. (2008) showed that this extra cellular accumulation of α-synuclein was internalized using the endocytosis pathway and eventually degraded by the lysosome (34). Therefore, my hypothesis is that the MVB/endosome pathway is a route through which α-synuclein is targeted to the lysosome.

S. cerevisiae (budding yeast) will be used as a model organism for the role of the endocytosis/MVB pathway in α-synuclein degradation. This eukaryotic model system is effective in the study of different neurodegenerative diseases; budding yeast are used in the study of Huntington’s disease and PD, among others (16, 17, 32, 35, 36). The MVB pathway to the lysosome has been conserved from yeasts to humans because of its importance (30). It is also easy to use these organisms, because their genetic code has been decoded, and the entire gene knockout library is available. Eleven proteins are involved in the ESCRT complex. Five of these eleven yeast genes were studied under Alex Ayala’s senior thesis work in our lab (37) and the remaining five are currently being studied. The two pre-ESCRT proteins, vps34 and vps27, have not been fully analyzed, and their role in α-synuclein degradation is unknown. vps34 has been found to be required for protein and membrane trafficking events such as MVB formation (38), vps27 also mediates endosomal protein sorting (39). I propose that vps34 and vps27 are required for the functioning of this pathway and that the absence of them would lead to disruptions in the MVB/endosome pathway and accumulation of proteins, including α-synuclein.

Significance

Intellectual Merit: No treatment for the inconvenience and hardships caused by neurodegenerative disorders exists today. Around four million individuals are affected with PD, and out of those, 500,000 to one million are afflicted in North America alone. On top of the high number of afflicted individuals, the costs of treatment are unapproachable for many. Patients nowadays spend more than $200 a month for a typical drug treatment regimen (40). The research being done will help in understanding the mechanism by which α-synuclein is removed from PD patients and eventually lead to the development of a cure or information that will help in the cure of similar diseases.

Broader Relevance: The broader relevance to this research as an undergraduate professor at a liberal arts college can be seen through the accomplishments of the undergraduate researchers working in my lab. Through lab work, these students receive an in-depth, hands on, and focused biological learning experience. These students also learn key laboratory techniques that can be applied in any field. We are training well-prepared undergraduates for diverse biological careers in research, medicine and allied health.

Specific Aims

1. Assess if α-synuclein accumulates in yeasts genetically compromised for endocytosis at the pre-ESCRT step.

2. Assess α-synuclein localization in yeasts genetically compromised for endocytosis at the pre-ESCRT step.

3. Assess if α-synuclein induces cellular toxicity in yeasts genetically compromised for endocytosis at the pre-ESCRT step.

Design and Methods

1. Assess if α-synuclein accumulates in yeasts genetically compromised for endocytosis at the pre-ESCRT step.

Rationale: To evaluate if α-synuclein is accumulating in the cells due to the endocytosis pathway being compromised, we will evaluate α-synuclein expression in specific gene knockouts for proteins at the pre-ESCRT step in yeast. In past papers from our lab, Western blots and loss of induction assays have been an effective way to show accumulation. Western blots will be performed because of their effectiveness in codon for α-synuclein using the anti-V5 antibody. Loss of induction assays will confirm Western blot results and give α-synuclein degradation comparisons over time (36, 37).

Method: Three groups of cells will be examined: the two knockout strains for pre-endocytosis/MVB pathway genes (vps34 and vps27) and the control with intact endocytosis genes (BY4741). Using Western blot and loss of induction assays, accumulation of α-synuclein will be examined in yeasts genetically compromised for endocytosis and intact endocytosis pathway yeast cells.

1. Yeast Expression: α-synuclein expression plasmid vectors will be inserted into the above budding yeast strains using the transformation technique described in Botelho et al. (2000). For selection, yeast cells will be grown on media lacking uracil (SC-URA). Plasmid polymerase chain reaction (PCR) will confirm α-synuclein expression. The pYES2.1 vector will allow for regulation of α-synuclein expression because of the presence of the galactose inducible promoter (GAL1). V5 and Green fluorescent protein (GFP) are also present in vectors.

2. Development of 5 plasmid constructs: Five different plasmids will be inserted to create the five different plasmid constructs: Parent plasmid alone (PP), plasmid with GFP, wild-type α-synuclein with GFP (WT), A30P mutant α-synuclein with GFP, and E46K mutant α-synuclein with GFP will be the five plasmid constructs for the three different groups. Therefore, the parent plasmids, cells with GFP (non-toxic proteins), and cells with α-synuclein vectors under non-inducing conditions will serve as controls.

3. Testing for α-synuclein in Western Blots: The cells will be grown in galactose for 24 hours, equal numbers of yeast cells expressing GFP, WT, A30P, and E46K for both vps knockout strains and BY4741 will be made into cell lysates as described in Sharma et al. (2006). Using SeeBlue as a molecular standard, these proteins will be run in Tris-glycerine gels, transferred to a PVDF membrane, washed as described by Sharma et al. (2006), and blotted for both anti-V5 and anti-PGK antibodies. The anti-V5 antibody is
detecting for the α-synuclein protein. The results seen in the anti-V5 blot will give the accumulation results for α-synuclein. The anti-PGK blot will be the loading control. Two Western blots for each knockout strain and BY4741 will be performed to confirm results.

4. Testing for α-synuclein in Loss of Induction: The cells will be grown in galactose for 24 hours as described above, and then they will be transferred to glucose media and grown for 24 hours. Cell lysates will be prepared as described above at 24-hour galactose, 0, 6, 12, and 24 hour glucose for each knockout strain and BY4741. The cells will be washed and run on the gels as described above. Results will demonstrate how well the cells are degrading the α-synuclein left after the induction media. Two loss of induction blots for each knockout strain and BY4741 will be performed to confirm results.

Prediction: In the knockout strains, WT and E46K will accumulate and thus show a stronger/thicker band in the western blot compared to the mutant strains and GFP control. In the loss of induction assay, WT will take longer to degrade in the knockout strain compared to the BY4741 parent control. The familial PD mutant, A30P, will show no difference.

2. Assess α-synuclein localization in yeasts genetically compromised for endocytosis at the pre-ESCRT step.

Rationale: Cell fluorescent microscopy is a valuable way to observe protein localization within a cell. Tagging GFP to α-synuclein has been an effective method in past studies (17, 36). Although some labs use immunofluorescence to tag for α-synuclein, GFP has been characterized as a better method (17). Using GFP, WT α-synuclein localizes intensely at the plasma membrane (17). The valuable use of GFP is seen by its discovery leading to the acquisition of the Nobel Prize in Chemistry in 2008. When key endocytosis genes are compromised, it will be expected that the localization of α-synuclein will change.

Method: Both knockout strain and BY4741 cells will be grown in galactose media for 24 hours, and the localization of the different cell lines described above will be determined using a Nikon TE2000-U florescent microscope, and the images will be acquired and quantified using Metamorph 4.9 Imaging Software. The localization will again be visualized at 48 hours after induction. Detailed methods are described in Sharma et al. (2006). A total of 750 cells will be counted with each plasmid construct for each time point. The ratio of cells displaying specific fluorescence patterns will be calculated and graphed for each plasmid construct. Two picture acquisition trials for each knockout strain and BY4741 will be performed at both time points to confirm results.

Prediction: In yeast models, WT and the other familial mutant, E46K localize in the plasma membrane. The familial mutant A30P localizes in the cytoplasm (17). I predict that the WT and E46K α-synuclein localization phenotype will change. It will differ from the usual BY4741 WT and E46K membrane localization as seen in past studies with our lab (36). I expect the α-synuclein to form small aggregates within the yeast cell as is seen in human PD brains. A30P localization will not change.

3. Assess if α-synuclein induces cellular toxicity in α-synuclein genetically compromised for endocytosis at the pre-ESCRT step.

Rationale: α-Synuclein is linked to cell death (17). Past labs have used growth curves and spotting assays as good measures of cellular toxicity in α-synuclein inducing media (17, 36). Vps28, an important gene in the ESCRT-I complex has shown toxicity in WT α-synuclein with growth curves and spotting assays (32). Spotting assays will confirm growth curve results and together be better analysis of cell toxicity.

Method: The different plasmid constructs and three groups described above will be used. Growth curves and spotting assays will be performed.

1. Growth Curves: Growth curves will be created with the five plasmid constructs and with a BY4741 PP control. Detailed methods described in Sharma et al. (2004). Cells will be grown overnight in glucose media and will have equal starting concentrations before being placed into galactose. The absorbance of the five different cultures will be measured and recorded at 0, 3, 6, 12, 18, 24, 36 and 48 hours using Hitachi U-2000 Spectrophotometer. The absorbance readings will be plotted against time to produce a growth curve. Two absorbance readings will be averaged for each plasmid construct for each time trial. Finally, three different growth curves for each knockout strain and BY4741 will be performed to confirm results. All three trials will be placed into one cumulative growth curve with averaged readings. T-tests will be used to measure significant differences in the curves.

2. Spotting: Cells will be grown in glucose and counted as described above. Equal concentrations of cells for each of the six plasmid constructs will be serially diluted (5-fold) into microwell plates. 3ml will be spotted in glucose and galactose plates using multi-channel pipettes and grown for either 24 hrs or 48 hours. Three glucose and galactose plates will be spotted for each trial. There will be three total trials done for each plasmid construct.

Prediction: I predict that vps27 and vps34 WT and E46K plasmid constructs will grow, overall, slower than the BY4741 PP control because α-synuclein is causing toxicity to the yeast, and endocytosis is compromised. A30P will not show any toxicity in the cells.

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