Creating a plasmid for the study of A30P and A53E mutations and the effects of decreased sumoylation in alpha-Synuclein in Parkinson's

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Abstract  
Parkinson’s disease (PD) is a neurodegenerative disease that can be characterized by its negative affect on motor function. In PD there is aggregation of α-synuclein caused by either familial or sporadic mutations of the gene in the dopaminergic neurons of the substantia nigra. There are several familial mutations that are known to cause this aggregation in the cells, for example A30P, and several mutations that are newly discovered, such as A53E. One other factor thought to affect the solubility of α-synuclein is sumoylation. While these familial mutations and sumoylation have been studied separately, it is not known how they interact on the same gene. We hypothesize that when sumo is blocked through a mutation, K96R and K102R, α-Synuclein will increase aggregation. This study will be focused on transforming a plasmid into yeast that will be able test both the familial mutations A30P and A53E coupled with a mutation that blocks sumoylation. Two gene fragments containing these mutations were successfully subcloned into the pYES2.1 TOPO E.coli vector and checked for correct orientation. Orientation was confirmed through whole cell and plasmid based PCR and a gene sequencing analysis. The plasmid vector for the A30P mutation was not successfully transformed into yeast but A53E plasmid vector was successfully transformed. These mutations, now expressed in yeast, will allow for future studies to be completed that may increase knowledge of Parkinson’s disease as a whole and how SUMO mutations in these two familial α-synuclein mutations affect aggregation and disease pathology.

Introduction  
Parkinson’s disease (PD) is a neurodegenerative disease that affects motor function. More common in older adults, around the age of 60, PD usually presents itself first in the form of tremors on one side of the body. These tremors gradually worsen into other symptoms that spread to both the right and left side of the body. Other common symptoms include a combination of rigidity, bradykinesia, and loss of postural reflexes (Fahn, 2011). PD comes in two varieties, sporadic and familial. Sporadic is more common and characterized by general occurrences in the population. Familial is less common and is inherited from a family member.

In the brain, PD affects the substantia nigra in which there is a striatal dopamine deficiency due to loss of dopaminergic neurons in the substantia nigra. In the early stages of the disease, the medulla oblongata is affected first then the basal ganglia. A post-mortem examination of a patient’s brain shows that there is the presence of Lewy bodies, or cytoplasmic inclusions, consisting of a protein called α-Synuclein in the damaged cells.

α-Synuclein is a 140 amino acid soluble protein. It has 3 distinct regions, N, M, and C. The N-terminal region has repeating sequences that relate to a lipid-binding motif, which helps protein binding to phospholipid vesicles. The central region, the M region, is extremely hydrophobic, and has been implicated in forming amyloid fibrils. The C-terminal region is hydrophilic and rich in proline, glutamate, and aspartate. It helps with chaperone activity to α-Synuclein. α-Synuclein does not have a secondary or tertiary structure; it is an unfolded protein because it mainly acts as a chaperone. (Irvine, El-Agnaf, Shankar, & Walsh, 2008). In PD mutant α-Synuclein forms aggregates and increases PD pathology. However, mutations in α-Synuclein only account for a small percentage of PD cases.

There are several known α-Synuclein mutations that cause Familial PD such as A30P (Kruger, et al. 1998), E46K (Zarranz, et al. 2003), H50Q (Ghosh, 2013), G51D (Lesage, 2013), A53E (Pasanen, 2014), and A53T (Polymeropoulos, et al. 1997). The familial mutants focused on in this study are A30P and A53E. A30P is a better-known mutation, in which the Arginine at the thirtieth amino acid is mutated to Praline. A53E is more newly discovered and has not been studied in depth; the Arginine at the fifty-third amino acid is mutated to Glutamic Acid (Ghost, 2014).

SUMO is a protein that binds to α-Synuclein to aid in solubility and prevent aggregation. Sumoylation, the process that is initiated by the binding of SUMO to a protein, helps to regulate aggregation in a cell by increasing protein solubility. SUMO proteins are a family of small proteins that are covalently attached to and detached from other proteins in cells to modify their function (Hay, 2005). SUMO proteins are similar to ubiquitin, and are directly by an enzymatic cascade analogous to that involved in ubiquitination. However, instead of tagging a protein, SUMO matures, then cleaves its last four amino acids to bind with a lysine (Dorval & Fraser, 2006).

In this study, there is a mutation in the C terminus of both α-Synuclein familial mutations that affects SUMO binding and prevents sumoylation. The lysines at the 96th and 102nd amino acid, that is normally where SUMO binds, are replaced with arginines. This mutation does not allow sumoylation to occur, potentially causing aggregates of α-Synuclein to form. These aggregates are characteristic of Parkinson’s (Krumova, et al., 2011).

It is known that sumoylation improves solubility, and that the mutated form of the gene causes aggregation. It is not known how the sumo mutation and the familial mutation interact and affect Parkinson’s pathology. We hypothesize that when SUMO is blocked through the mutation, α-Synuclein will increase aggregation.

Yeast will be used to test this hypothesis. Yeast or Saccharomyces cerevisiae is a good model organism because it has a short generation time and can grow on a variety of media. This makes it readily available and can provide an invaluable starting point for later analysis in more complex organisms. This organism can be easily transformed with extra-chromosomal plasmids or by genomic insertion.

In the context of studying Parkinson’s disease, yeast can express α-Synuclein and any mutants of α-Synuclein in the same way as humans do as they make and fold proteins in the same way. If a mutated plasmid vector is inserted into yeast and then expressed as a protein, the yeast can be studied for the affects of the mutant protein. In this study, the yeast with mutated forms of α-Synuclein and the sumoylation mutations will be looked at for any effects. These results are compared to yeast with the wild type form of α-Synuclein, which are normally functioning. From this experiment, we can see how yeast can control their cells when their cellular environment becomes toxic (Outeiro, & Lindquist, 2003). The aim of this study is to...
take two plasmids containing the mutated α-synuclein gene fragments with SUMO modifications, and tagged with GFP, and to transform them into E.coli, purify and analyze them, and then simulate how the fragments would act. We amplify the familiar mutant α30P and A53E α-synuclein gene fragments with the additional K62R and K102R modifications, respectively (Figure 1A). The two genes were then fused C-terminally with GFP and a 2-5' His tag from a mammalian cell culture plasmid. We then inserted the plasmids into a plasmid vector and transformed into E. coli. The subcloned vector will then be checked for correct orientation using whole cell PCR and plasmid purification. The plasmid PCR is also used for expression vector and amplified in bacteria. After selecting the correct orientation of DNA in the vectors and purifying them from bacteria. These vectors will then be transformed into yeast and grown on plates to be used in later experiments.

**Results**

**Gene Fragment Amplification by PCR**

A protocol for a procedure used to develop a yeast model (Figure 1C) with α30P and A53E modifications was to amplify the genes from a plasmid created earlier. In the plasmid the mutated gene was linked with GFP and a V5 a His tag is used to aid in the later study of Parkinson’s (Figure 1A). We used PCR (polymerase chain reaction) and the appropriate forward and reverse primers (Figure 1B) in order to amplify the gene. To ensure that some product was successfully amplified different amounts of plasmid were used for a total of 7 PCR reactions including positive and negative controls (Table 1). See methods section for complete details.

**Gene Fragment Purification by Gel Electrophoresis**

In order to tell if there was PCR product, gel electrophoresis was run with each of the 7 PCR reactions. Two gels were run, one specifically checking for purification of the two specific gene fragments, Gel 1, and one checking for PCR product against the positive and negative controls, Gel 2. We predicted the bands containing the fragments (Figure 2A) to be near 1185 base pairs because of calculations made by looking at the length of the DNA in the vectors plus the length of GFP attached. The actual gel showed that the bands were in the predicted position, meaning that the forward and the reverse controls (lanes 4, 5, 6, and 7) worked (Figure 2B). In Gel 1 the bands were extremely faint under UV light which was unclear whether there was PCR product of α30P and A53E.

To purify the DNA from the gels, the bands were cut out, indicating that the bands were faint. It was more difficult to separate the product from the gel and be sure that the bands were fully cut. We imaged the gel after the bands were cut out. The cleaved DNA from the gels was then used, whether there was PCR product of α30P and A53E.

**Subcloning into Plasmid**

In this step, the PCR product from the previous whole cell and plasmid PCR was analyzed in order to see if there was a vector in which the fragment is in the correct orientation. Again, it was necessary to confirm as is shown in a gel where all of the colonies chosen for isolation to put into yeast.

**Discussion**

The purpose of this study was to look at two α-synuclein fragments each with a familiar Parkinson’s mutation, α30P and A53E, and two SUMO modifications, K62R and K102R, and transform them into a yeast vector: A30E transformed successfully and A30P did not transform successfully.

We successfully purified and extracted the A30P and A53E gene fragments from the original plasmid through PCR amplification and gel electrophoresis purification. The purification was confirmed as is shown in a gel where all of the colonies chosen for isolation to put into yeast.

The forward primer used was designed to bind to the gene promoter and the reverse primer used binds to the 3’-end of the gene fragments. If there will be no PCR product if the gene is in the incorrect orientation, and product if the orientation is correct.

For plasmid based PCR, the plasmid first needs to be extracted from the E. coli. In order to be able to transform yeast to other organisms. We used an alkaline treatment to gently lyse the E. coli cells that had grown in the liquid LB+AMP liquid overnight culture. To extract the DNA in the vectors and purify them from bacteria. These vectors will then be transformed into yeast and grown on plates to be used in later experiments.
To separate and purify amplified gene fragments, gel electrophoresis was run with each PCR product. The appropriate fragment was excised from the gel, eluted in water, and used for subsequent cloning into a plasmid vector. The vector used by this lab was the pYES2/V5-His-TOPO vector, which is available from Invitrogen.

In conclusion, the transformation of the A53E plasmid into yeast was effective, and the transformed cells were able to grow on selective media containing uracil. This result indicates that the A53E plasmid was successfully integrated into the yeast genome and that the yeast cells were able to synthesize the α-synuclein protein.

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References


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Figure 1. Overall Project Design. (A) Alpha-synuclein cartoon Wild type vs. A30P fragment and A53E fragment being studied. (B) PCR primers used for gene amplification. (C) Overall schematic of experimental design. Step 1 completed by Alex Roman beforehand, step 2-7 to be done in this study.
Figure 2. Gene Fragment Purification by Gel Electrophoresis (A) Computerized ideal gel of gene fragment Purification. Gel 1 should have bands at about 1100 bp for wells labeled Tube 2, 1, 5, & 6. Gel 2 should have bands at 1100 for wells labeled tube 1, 2, 5, 6, & 7, and bands near the well for tubes 3 & 4, the negative controls. (B) PCR gel photograph before removal of DNA. There were bands near 1100 bp as expected in Gel 1. Gel 2 did not run as expected, only tube 7 had the expected result. (C) PCR photograph after the removal of DNA. The bands previously at ~1100 bp were cut out successfully. (D) Gel of Purified PCR products, bands are faint but visible in lanes 6 and 7. Lanes 2-5 are not relevant to this paper.

Figure 3. Picture of E. coli transformation plates. (A) E.coli, LB + AMP, 10/3/14, PYES2, A30P, 200 μL shows minimal growth. Selected colonies circled for future testing. (B) E.coli, LB + AMP, 10/3/14, PYES2, A53E, 200 μL shows minimal growth. Selected colonies circled for future testing. (C) E.coli, LB + AMP, 10/3/14, PYES2, A30P, 30 μL and E.coli, LB + AMP, 10/3/14, PYES2, A53W, 30 μL show no bacterial growth. (D) Positive transformation control shows growth.
Figure 4. Orientation Check II: Fragment A30P. (A) Computerized ideal gel with in which all tested colonies (see fig 3A) have correct orientation and plasmid based and whole cell PCR are successful. (B) Actual gel photograph of the A30P fragment gel. Whole cell PCR was unsuccessful, there are no bands. Plasmid based PCR was successful and shows two colonies with the correct orientation (shown with red circles). These two colonies, 2 and 4, are selected for further study. (C) Gel showing that the plasmid based PCR preparation was correct for both fragments, there are bands in all the wells.

Figure 5. Orientation Check II: Fragment A53E. (A) Computerized ideal gel with in which all tested colonies (see fig 3B) have correct orientation and plasmid based and whole cell PCR are successful. (B) Actual gel photograph of the A53E fragment gel. Whole cell PCR was unsuccessful, there are no bands. Plasmid based PCR was successful and shows two colonies with the correct orientation (shown with red circles). These two colonies, 1 and 4, are selected for further study.
Figure 5. Orientation Check II: Fragment A53E. (A) Computerized ideal gel with in which all tested colonies (see fig 3B) have correct orientation and plasmid based and whole cell PCR are successful. (B) Actual gel photograph of the A53E fragment gel. Whole cell PCR was unsuccessful, there are no bands. Plasmid based PCR was successful and shows two colonies with the correct orientation (shown with red circles). These two colonies, 1 and 4, are selected for further study.

Figure 6. Picture of Yeast Transformation Plates. (A) SC-URA plated with yeast transformed with fragment A53E. Growth on the plates indicates that the transformation was successful. (B) SC-URA plated with yeast transformed with fragment A30P. No growth on the plates indicates that the transformation was not successful, though the positive and negative control were successful. (C) YPD control plates show that the s. cerevisiae FYK471 yeast were growing efficiently.
Table S1. PCR primer design table. Each PCR tube for gene fragment purification was prepared with the specified amount of master mix, forward primer, reverse primer, plasmid, and water, then put through PCR. Reference this table when looking at Figure 2.