Mutating the α-Synuclein gene to examine the relationship between acetylation and glycation and familial mutations for future studies in *S. cerevisiae*

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**Summary**

Parkinson’s disease (PD), a motor neurodegenerative disease, exhibits a cellular pathology of α-synuclein aggregates called Lewy bodies in dopaminergic neurons of the substantia nigra. Although several genes are implicated in PD, six familial mutations (A30P, E46K, A53T, G51D, H50Q, and A53E) on the α-synuclein gene, SNCA, are known to cause Early-Onset Parkinson’s. However, a model of pathology has not been identified. The hypothesis that post-translational modifications on the protein α-synuclein mediate or moderate familial mutant pathology will be tested and mutations in the gene responsible for α-synuclein, SNCA, will be developed that mimic and block two post-translational modifications responsible for altering α-synuclein’s ability to aggregate: acetylation and glycation. Primary studies show that acetylation reduces α-synuclein’s toxicity, whereas glycation exacerbates it. While the K6Q/K10Q familial mutants were unsuccessful, we were able to create a K10R A53T mutant. In addition, we were able to create a K10Q A53T mutant. Now that these tools are available in yeast (*S. cerevisiae*), we can study their functional properties. We hypothesize that the K10R A53T mutant will be more prone to aggregation than A53T α-synuclein as it mimics glycation and blocks acetylation. We also predict that the K10Q A53T mutant will be less prone to aggregation than A53T α-synuclein as the new mutant mimics acetylation and blocks glycation. The results of functional assays will test our predictions and elucidate potential mechanisms and components involved in the cellular pathology of Parkinson’s disease.

**Introduction**

Parkinson’s disease (PD) is a fatal and incurable neurodegenerative disease characterized by motor symptoms, such as bradykinnesia (the slowing of movement), involuntary movements, resting tremors, and unstable posture (Obeso et al., 2010). PD can be sporadic, as 95% of PD cases are, or familial (Dauer & Przedborski, 2003). Both types of PD are caused by the death of dopaminergic neurons in the substantia nigra (Panigrahi et al., 2015) which contain abnormal cytoplasmic inclusions called Lewy bodies (Spillantini et al., 1998). Lewy bodies are composed of clumps of aggregated protein, the main component being α-synuclein (Klein & Westenberger, 2012; Spillantini et al., 1998).

Irregularities in certain key genes, including *PINK1*, *LRRK2*, *Parkin*, *DJ1*, *ATP13*, and the *SNCA* gene are strongly linked to heritable, monogenic PD (Bonifati, 2002; Martinez et al., 2017; Ramirez et al., 2006; Rassu et al. 2017; Villeneuve et al. 2014). Six mutations on the *SNCA* gene have been identified that cause PD: A30P, E46K, A53T, G51D, H50Q, and A53E.

The protein α-synuclein is highly abundant presynaptically and is essential for the wellbeing of dopaminergic neurons (Abeliovich et al., 2000). Although the exact function of α-synuclein remains unknown, it is believed to be involved in vesicle transport and neurotransmission, lipid-binding, and enzyme regulation (Dev et al., 2003). The protein is 140 amino acids long and contains three functional domains, each possessing distinct properties (Fiske & DebBurman, 2010). The N-domain is responsible for α-synuclein’s membrane-binding function, the M-domain gives the protein its aggregation properties, and the C-domain is responsible for the protein’s solubility (Fiske & DebBurman, 2010).

All six familial mutations are located in the N-terminus of the protein. Yeast studies have informed much of what we know about the familial mutants of α-synuclein. Wild-type α-synuclein has been shown to be toxic in yeast, causing yeast cells expressing the protein to grow less in serial dilution spotting assays (Ciaccioli et al., 2013). Although the other familial mutants show similar or aggravated toxicity (measured through serial dilution spotting assays), A30P and G51D seem to reverse that toxicity; cells containing these mutants seem to grow better than those expressing wild-type α-synuclein (Ong et al., 2017).

The familial mutants of α-synuclein also localize in live yeast cells in distinct ways. The mechanism through which the protein’s localization affects cell growth and survival is unknown; however, some interesting patterns arise. While H50Q, E46K, and A53T exhibit primarily membrane-binding characteristics 24 hours after α-synuclein expression is activated, A53E forms some intracellular foci (Sharma et al., 2006; Slovang et al., 2011; Ong et al., 2017). A30P and G51D tend to remain diffuse throughout the cell (Pooreva et al., 2015). This non-accumulation of A30P and G51D in yeast could provide an explanation for why these mutants seem to have better growth than their counterparts which either bind to the membrane or form aggregates.

This protein can be post-translationally modified and these post-translational modifications can affect its aggregation and toxicity. Some examples of the modifications it can undergo include nitration, phosphorylation, and sumoylation (Giasson, 2000; Krumova et al., 2011). Two other modifications α-synuclein can undergo are acetylation and glycation. Acetylation is the process whereby acetyl groups attach to select lysines on the N-terminus of α-synuclein (de Oliveira et al., 2017). Preliminary studies showed that blocking α-synuclein acetylation exacerbated toxicity in rats (de Oliveira et al., 2017). On the other hand, glycation, an age-associated covalent modification wherein sugars attach to select lysines in the N-terminal of α-synuclein, worsens the protein’s toxicity and oligomerization (Vicente, 2017). Although the effects of these covalent modifications have been studied on wild-type α-synuclein, little is known about whether there is an interaction between these post-translational modifications and α-synuclein’s six familial mutations.

Therefore, we decided to focus on the interaction between acetylation and glycation and the familial mutants of α-synuclein using the budding yeast model of α-synuclein established by the DebBurman Lab at Lake Forest College (Sharma et al., 2006). Yeast is an excellent model organism as its genome is fully sequenced, its genes are easily manipulated, and several of our protein-folding mechanisms are fundamentally conserved in that organism.

In order to manipulate our chosen covalent modifications, we created mutations on the main glycation and acetylation sites that artificially block or mimic either of these modifications. While our colleagues created mutations that manipulated glycation and blocked acetylation, our goal was to mimic acetylation and block glycation through a double mutation: K6Q/K10Q. We aimed to create six versions of α-synuclein that contained both the K6Q/K10Q mutations and one familial mutation to be able to determine whether acetylation and glycation moderate α-synuclein familial mutant toxicity.

We used a site-directed mutagenesis design to create these mutants and transform them into yeast (see Results section, Figure 1B). Our first main aim was to create the K6Q/K10Q double mutation on a pre-existing familial mutant template using mutagenesis polymerase chain reaction (Mutagenesis PCR, see Method section, Step 3: Plasmid Based PCR). The next aim was to check if the PCR was successful by running a DNA gel to see whether the mutant was successfully created and amplified (see Method section, Step 3: Plasmid Based PCR). Our third aim was to transform the plasmid into DH5α E. coli to destroy the unmutagenized template DNA and further amplify the mutant (see Method section, Step 2: Template DNA, Vectors, and Bacterial and Yeast Cells Used). The fourth aim of our project was to purify the plasmid from the bacterium and confirm the DNA of the correct size was present. It was imperative to then check the sequence of the plasmid to ascertain that it had the desired mutations (see Method, Step 5: DNA Sequencing). Once that was done, the plasmid would be ready to be transformed into yeast (see Method, Step 6: Yeast Transformation) for functional studies that would tell us about the possible interaction between the familial mutants and acetylation.

We hypothesized that the K6Q/K10Q mutant would lessen the toxicity of the familial mutant but toxicity would not be fully rescued. This novel tool will later help us study the effects of these modifications on the six familial mutants to ascertain the presence or absence of an interaction.
Results

Overall Experimental Design and Creation of Primers for K6Q/K10Q

Our goal was to mutagenize the wild-type α-synuclein (SNCA) gene in a pYES2 plasmid to make six versions of the plasmid, each containing the two acetylation-mimicking and glycation-blocking mutations, K6Q and K10Q, as well as one of the six familial mutations (Figure 1A). To do this, we used a six-step experimental design (Figure 1B). Our first step was to design the PCR primers to create the mutant (step 1) (Figure 1C). We then checked for the success of our specific primers by running a DNA gel (step 2). The third step was to transform the plasmid into DH5α E.coli to destroy the template and amplify the mutant. We then purified our mutant plasmid (step 4) and sent the pure plasmid to the University of Chicago for DNA sequencing (step 5). Finally, if the sequence came back correctly, we transformed the mutant into yeast to perform functional studies (step 6).

The familial mutants of α-synuclein were created by our peer teacher, Rosemary Thomas (data not shown). Agarose gel electrophoresis was used to check the primers bound correctly to the template DNA (Figure 1D). The thick bands present on the gel in lanes 2-7 around the 1kb mark showed that the forward and reverse primers bound the DNA and amplified it. The DNA synthesized by the forward primer (lanes 2, 4, and 6) was slightly heavier than the reverse primer-synthesized DNA (lanes 3, 5, and 7).

Mutagenesis and Gel Electrophoresis of K6Q/K10Q with H50Q, G51D,
and A53E and K6Q with H50Q, G51D, and A53E

We performed mutagenesis Polymerase Chain Reaction (PCR) to create the double mutation K6Q/K10Q on H50Q, G51D, and A53E and to amplify the products (see Methods, Step 3: Plasmid-Based PCR). We used agarose gel electrophoresis to determine the success of the PCR mutagenesis by qualitatively comparing the band for the template DNA (if there was one at all) to the band for the DNA that had undergone mutagenesis (see Methods, Step 3: Plasmid-Based PCR). At the 10kb mark, we expected to see thick bands in the lanes containing the positive control (lane 2) and the familial mutations (lanes 4, 6, and 8) and thinner bands from the template in the lanes containing the negative controls (lanes 3, 5, and 7) (Figure 2A). However, no DNA was observed at the 10kb mark in lanes 2-8, showing that neither the template nor the PCR products were visible (Figure 2B).

The PCR mutagenesis reaction was rerun in a second attempt to add the acetylation-mimicking and glycation-blocking double mutation K6Q/K10Q of α-synuclein onto the familial mutant templates. We ran a second gel electrophoresis to determine the success of the PCR. We expected to see the thinnest bands showing the template at the 10kb mark in lanes 2, 5, and 8, where we put the negative controls. We expected to see thicker bands in lanes 3, 4, 6, 7, 9, and 10, with the thickest bands in lanes 4, 7, and 10, where we put the fully concentrated familial mutants (Figure 2C). Again, no DNA was visible at the 10kb mark in the expected lanes, indicating that the reaction was unsuccessful (Figure 2D). Due to this, a new mutagenesis product for a different mutant, K6Q A53E, was obtained from Niam Abyesirirwadena and Isaac Ordonez. In lanes 7 and 8, their gel shows that they successfully mutated and amplified K6Q A53E of the correct size: approximately 10 kb (Figure 3A, B).

![Figure 1. Overall Experimental Design and Creation of Primers for K6Q/K10Q](image1)

(A) Comparison of WT α-synuclein plasmid to mutant plasmids (K6Q/K10Q A30P, K6Q/K10Q E46K, K6Q/K10Q A53T, K6Q/K10Q H50Q, K6Q/K10Q G51D, K6Q/K10Q A53E).

![Figure 2. Mutagenesis and Gel Electrophoresis of K6Q/K10Q on H50Q, G51D, and A53E](image2)

(A) Computerized ideal gel of PCR products for the mutagenesis of K6Q/K10Q on H50Q, G51D, and A53E α-synuclein. Lane 1 contains High Mass DNA Molecular Weight Ladder. Lane 2 contains mutagenesis positive control. Lane 3 contains mutagenesis negative control (K6Q/K10Q -H50Q). Lane 4 contains mutagenesis familial mutant (K6Q/K10Q +H50Q). Lane 5 contains K6Q/K10Q -G51D. Lane 6 contains K6Q/K10Q +G51D. Lane 7 contains K6Q/K10Q -A53E. Lane 8 contains...
Bacterial Transformation, Plasmid Purification, and Sequencing of K6Q A53E

To further amplify K6Q A53E and destroy the template used to create the mutant (see Methods Step 2: Template DNA, Vectors, and Bacterial and Yeast Cells Used and Methods Step 4: Plasmid Purification), we transformed the PCR product into DH5α E. coli and observed significant growth of the E. coli over one day (Figure 4A, B). We observed no growth in the positive and negative control plates (Figure 4C). After transforming the PCR product into DH5α E. coli, we purified plasmid from four distinct colonies (see Methods, Step 4: Plasmid Purification). We then performed gel electrophoresis to check whether the plasmid was both present in the colony and the correct size. We expected to see four thick bands at the 10kb mark in lanes 2-5 (Figure 5A). The gel showed plasmid of the correct size was isolated from all four colonies and had been amplified by the bacterium (Figure 5B).

We then checked the sequence of our mutated α-synuclein by sending the plasmids purified from colony 1 and colony 2 of E. coli containing K6Q A53E for sequencing to the University of Chicago’s DNA sequencing facility. The sequences for both purified plasmids were incorrect (see Appendix A).

Figure 4. Bacterial Transformation of K6Q A53E
(A) Picture of 20µL and 80µL of K6Q A53E LB+Amp plates displaying no growth of E.coli on day zero (B) Picture of 20µL and 80µL of K6Q A53E LB+Amp plates displaying growth of E.coli on day one, (C) Picture of positive control, negative control, and mutagenesis negative control 20µL LB+Amp plates all displaying no growth after one day.

Figure 5. Plasmid purification of K6Q A53E and Mutagenesis of K10R A53T and K10Q A53T
(A) Computerized ideal gel of K6Q A53E purified plasmids (lanes 1-5) and of PCR products for K10R A53T and K10Q A53T (lanes 6-10). Lane 1 contains High Mass DNA Molecular Weight Ladder. Lane 2 contains K6Q A53E plasmid 1. Lane 3 contains K6Q A53E plasmid 2. Lane 4 contains K6Q A53E plasmid 3. Lane 5 contains K6Q A53E plasmid 4. Lane 6 contains high mass DNA ladder. Lane 7 contains mutagenesis negative control (K10R -A53T). Lane 8 contains mutagenesis positive control (K10R +A53T). Lane 9 contains K10Q -A53T. Lane 10 contains K10Q +A53T. (B) Gel image of K6Q A53E purified plasmids (lanes 1-5) and of PCR products for K10R A53T and K6Q A53T (lanes 6-10). Lane 1 contains High Mass DNA Molecular Weight Ladder. Lane 2 contains K6Q A53E plasmid 1. Lane 3 contains K6Q A53E plasmid 2. Lane 4 contains K6Q A53E plasmid 3. Lane 5 contains K6Q A53E plasmid 4. Lane 6 contains high mass DNA ladder. Lane 7 contains mutagenesis negative control (K10R -A53T). Lane 8 contains mutagenesis positive control (K10R +A53T). Lane 9 contains K10Q -A53T. Lane 10 contains K10Q +A53T.

Yeast Transformation of K6Q A53E
Since the sequences for the plasmids from colonies 1 and 2 were incorrect, we chose to transform the plasmids from colonies 3 and 4 into S. cerevisiae. We observed no growth on day zero (Figure 6A). However, by day one and day three, we observed significant growth of S. cerevisiae in each of the plates (Figure 6B, C). Furthermore, we observed growth in theYPD and SC-Ura positive control plates and theYPD negative control plate; however, we observed no growth in the SC-Ura negative control plate (Figure 6D). After transforming plasmids 3 and 4 into S. cerevisiae, we discovered that the sequences for plasmids 3 and 4 were also incorrect (see Appendix B).

Figure 6. Yeast Transformation of K6Q A53E
(A) Representation of SC-Ura and YPD plates used to obtain K6Q A53E plasmids and sc mutants from yeast colonies (B) Photograph of SC-Ura and YPD plates used to obtain K6Q A53E plasmids and sc mutants from yeast colonies.
Mutagenesis and Gel Electrophoresis of K10R A53T and K10Q A53T
We also performed mutagenesis PCR to create the mutations of K10R A53T and K10Q A53T. Once the mutants were created, we used agarose gel electrophoresis to determine if the mutagenesis was successful. We expected to see thick bands in lanes 8 and 10 at the 10 kb mark, where the respective positive controls for K10R A53T and K10Q A53T were located. Conversely, we expected to see thinner bands at the 10kb mark in lanes 7 and 9, where the respective negative controls for K10R A53T and K10Q A53T were placed (Figure 5A). The gel indicated that our PCR reactions were successful, since bands were visible in the lanes containing PCR product (Figure 5B).

Bacterial Transformation of K10R A53T and K10Q A53T and Plasmid Purification of K10R A53T
We then attempted to transform both mutants into DH5α E. coli. After one day, the K10R A53T LB+Amp plates displayed growth of the E. coli, while the K10Q A53T LB+Amp plates displayed no growth (Figure 7A, B). Consequently, we only purified plasmid from the colonies of the K10R A53T mutant, and four distinct colonies were used. We used agarose gel electrophoresis to determine if the plasmid was present in the colony and was the correct size. We expected to see four thick bands on the gel in lanes 3-6 at the 10kb mark (Figure 8A). Plasmid of the correct size was observed in each lane of the gel (Figure 8B).

Sequencing and Yeast Transformation of K10R A53T
The purified plasmids from colonies 1 and 2 were sent to the University of Chicago for DNA sequencing, and the sequences were correct (data not shown). Therefore, we attempted to transform plasmids 1 and 2 into S. cerevisiae. We observed significant growth of S. cerevisiae in all the K10R A53T plates for both plasmids 1 and 2 (Figure 9A). We observed noticeable growth in the YPD and SC-Ura positive control plates and the YPD negative control plate after one day; however, we observed no growth in the SC-Ura negative control plate (Figure 9B).
The efficacy of the forward and reverse primers for K6Q/K10Q are shown in lanes 6 and 7, where thick bands indicate significant amplification and, in turn, correct adherence of the primer to the template.

K6Q/K10Q mutagenesis

K6Q/K10Q mutagenesis of the familial mutants was attempted twice. Both attempts yielded negative results with a lack of amplification (Figure 2). A possible reason for the unsuccessful PCR mutagenesis is the inherent difficulty of a double mutation mutagenesis. A double mutation mutagenesis requires the adherence of the primer to the template with two mismatched base pairs instead of the one involved in a single mutation mutagenesis. In addition, an innate difficulty of the mutated region is that the primer has a 5-base pair tail on the 5' end instead of the desired 6-base pair length. This increases the difficulty of primer-to-template adherence. Finally, in Figure 2B, lane 10, which contains the forward primer check positive control, showed no band in the gel. The lack of the band shows an ineffective adhering primer was used and may be the reason that the mutagenesis of K6Q/K10Q onto the familial mutant templates failed.

K6Q A53E mutagenesis

As the delegation of work shifted over the course of the project, we began working with a K6Q A53E mutant that was created by our colleagues, Niam Abeysiriwardena and Isaac Ordonez. Evidence for the success of their K6Q A53E mutagenesis can be found in Figure 3B; a distinct band is present in lane 8 containing the PCR mutagenesis product K6Q A53E.

K6Q A53E bacterial transformation

With the verified K6Q A53E product, the next step was to transform the product into DH5-α E. coli for two reasons. Firstly, the bacteria would act as a vector for later yeast transformation by amplifying K6Q A53E product via rapid division. Secondly, the bacteria would degrade the original template so that only the PCR mutagenesis product would be transformed into E. coli and then yeast. Ampicillin, an antibiotic, was used to selectively kill bacteria without the transformed plasmid while those with the plasmid and its constitutive gene for ampicillin resistance survived. These data are shown in Figure 4A and Figure 4B where growth of select and disparate colonies over the course of 24 hours is indicative of successful bacterial transformation. A query with this portion of the project is why the positive control showed no growth after 24 hours. While this is unexpected, the growth of bacterial samples containing our experimental PCR mutagenesis product makes this query insignificant due to experimental success.

K6Q A53E plasmid purification

After transforming the K6Q A53E into E. coli, the sequence of the plasmid had to be confirmed for the certified validity of genotype and post-translational condition. In order to confirm the PCR mutagenesis product, the plasmid was amplified in E. coli, and select colonies viable on the LB+ Amp medium were selected for sequencing. To isolate the plasmid, lysis and a sequence of precipitations were used. The plasmid product was then run on a gel as shown in Figure 5B. Bands of high intensity and similar length in lanes 2 through 5 show the amplification of DNA of roughly the correct size to be sent for sequencing.

K6Q A53E yeast transformation

Plasmids 1 and 2 returned with incorrect sequences, suggesting an incorrect mutagenesis; therefore, we transformed plasmids 3 and 4. However, those plasmids also came back with an incorrect sequence. The plasmids were then grown on the SC-Ura plates and colonies from the SC-Ura plates were selected for sequencing. To isolate the plasmid, lysis and a sequence of precipitations were used. The plasmid product was then run on a gel as shown in Figure 5B. Bands of high intensity and similar length in lanes 2 through 5 show the amplification of DNA of roughly the correct size to be sent for sequencing.

Discussion

Project Overview

The overall aim of the project was to successfully create glycation and acetylation mutants of the six familial mutants (A30P, E46K, A53T, G51D, H50Q and A53E) that block or mimic acetylation and glycation at different lysine-coding regions of the SNCA gene. In addition, our peer teachers, Rosemary Thomas ’18 and Chisomo Mwale ’19, created K6Q A53E. The overall aim of the project was to successfully create a53T from Plasmid 2 on LB+Amp plates displaying growth of S. cerevisiae with no growth on the SC-Ura negative control.

Experimental Overview

K6Q/K10Q primer check

Given that our project involved the PCR mutagenesis of an SNCA gene template, the primers involved in mutagenesis had to be confirmed. With the assistance of our lab peer teacher, Rosemary Thomas, the forward and reverse primers involved in our K6Q/K10Q double mutation were confirmed to bind correctly to the template DNA (Figure 1D). The efficacy of the forward and reverse primers for K6Q/K10Q are shown in lanes 6 and 7, where thick bands indicate significant amplification and, in turn, correct adherence of the primer to the template.
K10R A53T and K10Q A53T mutagenesis

In addition to working on the K6Q A53E mutant, we worked to mutagenize a K10R and a K10Q mutant of A53T in order to assist our colleagues. The same method of PCR mutagenesis was used to perform the desired mutation. The success of the mutagenesis is shown by distinct bands in lanes 8 and 10 in Figure 5B.

K10R A53T and K10Q A53T bacterial transformation

Next, we verified the K10R A53T and K10Q A53T PCR mutagenesis products. We then transformed the plasmids into yeast using the same method and for the same reasons discussed above for the K6Q A53E bacterial transformation: to amplify and isolate the intended plasmid. Bacterial transformation was deemed a success due to the colonies observed in Figure 7B after one day compared to Figure 7A.

K10R A53T plasmid purification

After successfully amplifying discrete colonies of the K10R and K10Q mutants of A53T, we purified the K10R A53T plasmid while our colleagues worked on purifying the K10Q A53T plasmid. We selected four colonies from the K10R A53T plates and used the plasmid purification method mentioned above to isolate the transformed plasmid. Plasmid purification was confirmed by the presence of four large bands of similar weight in lanes 3 through 6 of Figure 8B.

K10R A53T yeast transformation

After Dr. Wilcox informed us that plasmids 1 and 2 of K10R A53T had the correct sequence, we transformed said plasmids into yeast. The same SC-Ura medium was used as in the K6Q A53E yeast transformation. Proliferation after three days of incubation was observed on the SC-Ura Figure 9A, indicating a successful yeast transformation.

Future Studies

With the tools created by our group and groups like ours in BIOL 221X, the next step is to test the phenotypes of the modified familial mutants. Our hypothesis is that the Parkinson’s phenotype of α-synuclein aggregation is mediated by post-translational modifications. In turn, we would expect to see fewer aggregates in wild-type α-synuclein with modifications that mimic acetylation and block glycation but higher aggregation in wild type α-synuclein with modifications that block acetylation and mimic glycation. In terms of the familial mutants that are predisposed to form foci, we would expect to see a decrease in aggregation in familial mutants with modifications that mimic acetylation and block glycation and increased aggregation in familial mutants with modifications that block acetylation and mimic glycation. In addition to the variances in phenotypes, any abnormal interactions between modifications at specific lysine sites could indicate whether K6 or K10 is implicated to a greater extent in the Parkinson’s cellular pathology.

In addition to these tools, others can be made due to other amino acids implicated in α-synuclein aggregation. Research found a suite of amino acid locations that are implicated to have a high propensity to be acetylated in rat brains; Lysines 6, 34, 45, 96, and serine 42 of α-synuclein are prime sites for acetylation (Lundby et al., 2012). The researchers made note that a reason for the implication of serine 42 is how it flanks lysine 43. In addition to acetylation, lysine sites are highly relevant in glycation. Due to the shared affinity of both acetylation and glycation modifications to lysine groups, there is a potential interaction between the two. Since acetylation is predicted to rescue a pankinsonian phenotype and glycation is predicted to amplify the phenotype, a complete assay of acetylation and glycation at all possible sites could implicate the specific modifications that make α-synuclein.

With the proposed hypothesis of post-translational modifications implicated in Parkinson’s cellular pathology, the connection between them and the six familial mutants must be defined. For example, the A53T familial mutant substitutes an arginine for a tyrosine, neither of which are implicated in the lysine-dependent post-translational modifications. If our hypothesized model is correct, we would expect all of the familial mutants to harbor significantly more post-translational modifications that are associated with aggregation than wild-type. To measure this, we would propose an ELISA (enzyme-linked immunosorbent assay) to determine which post-translational modifications are present and to what magnitude (O’Connor 2001). A potential experiment that would disprove the proposed mechanism of familial mutant aggregation would entail isolating the familial mutant α-synuclein via Western blot and analyzing whether the isolated protein has a propensity to aggregate to a significantly greater extent than wild-type α-synuclein. In addition to familial mutants, other genes are implicated in α-synuclein aggregation, leading to Parkinson’s. For our model to be correct, we would run the same ELISA procedure in wild-type α-synuclein and familial mutants that mimic acetylation and glycation at varying locations.

To confirm how post-translational modifications affect α-synuclein toxicity, we propose a Co-immunoprecipitation assay that quantifies the interaction or lack of interaction between α-synuclein and chaperone proteins. If the post-translational modifications decrease the ability of α-synuclein to interact with chaperone proteins, this may be a mechanism that confers the toxic phenotype. To identify the level of toxicity the post-translational modifications confer on wild-type and familial mutants, we proposed a cell viability/death assay with the now-transformed, post-transitionally modified familial mutants. This can also be performed with serial dilution spotting (DebBurman 2013). In addition, we propose fluorescent microscopy assays to identify the aggregation or solubility of α-synuclein (DebBurman 2013). This will also allow the identification of α-synuclein localization with the three-way interaction of wild-type vs familial mutant vs post-translational modification.

In summary, we and our collaborators in BIOL 221X created tools to test a hypothesis of Parkinson’s cellular pathology. This work explores the possible links between familial and sporadic Parkinson’s through post-translational modifications, a powerful tool that can provide insight into the molecular basis of Parkinson’s disease. Functional assays in yeast will test our hypothesis that glycation will create or exacerbate a parkinsonian α-synuclein phenotype while acetylation will rescue or maintain a wild-type α-synuclein phenotype. We hope to expand these future projects to tie in genes other than SNCA such as PINK1, LRRK2, Parkin, DJ1, ATP13. We predict these mutants use the same mediating post-translational modifications to confer their parkinsonian pathology.

Method

We used a site-directed mutagenesis protocol developed by Invitrogen to make our mutations and insert them into yeast.

Step 1: Primer Design and Synthesis

The forward and reverse primers used to create the mutations were made according to the directions specified in (DebBurman, 2013, p. 20-23). Primers were designed by referring to the α-synuclein sequence provided in (DebBurman, 2013, p. 22). The forward primer contained up to six codons identical to the six codons on the α-synuclein gene that precede the location of the mutation, followed by the codon coding for the mutant amino acid, flanked by the six codons that follow the location of the mutation. For example, the first five codons in the forward primer for the K6Q mutation would be the same as those present on positions 1-5 on WT α-synuclein. Following this, CAA (coding for glutamine, Q) would replace AAA (the original codon, which codes for lysine, K, which is normally present on position 6 of the α-synuclein gene). The CAA would be flanked by the same codons present from positions 7-11 on WT α-synuclein. The reverse primer contained a sequence complementary to the forward primer. The primer sequences used are listed in Figure 1C.

Step 2: Template DNA, Vectors, and Bacterial and Yeast Cells Used

The α-synuclein gene to be mutated was supplied by Thermofisher in the plasmid vector pYES2. Apart from the α-synuclein gene SNCA, this vector also contains the Ura3 and AmpR genes, making it advantageous in certain environments to the survival of yeast and bacteria that lack these genes. We used this system to drive the expression of this plasmid in our organism of choice.

We used DH5α E.coli cells to rapidly multiply the plasmid overnight. These bacteria do not have a gene for ampicillin resistance, which means that they need the pYES2 plasmid in order to survive in a medium containing ampicillin, an antibiotic that kills the bacteria. DH5α E.coli also cleaves methylated DNA that enters its cells.

We used BY4741 cells as our strain of yeast as they lack the ability to make uracil, an essential nucleotide used in RNA. This means that they need the pYES2 plasmid to survive in media that lack uracil.

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Step 3: Plasmid-based PCR

Plasmid-based mutagenesis Polymerase Chain Reaction (PCR) is a procedure that amplifies DNA to create several thousand copies of the new mutant. The reaction works by running a specific ratio of DNA polymerase, forward and reverse primers, template DNA and a master mix (mixture of dNTPs, buffer solutions, and ions) in a thermal cycle. We also added the enzyme methylese. The amounts added are specified in (DebBurman, 2013, p. 32). The reaction functions by separating the two complementary strands of DNA, thereby allowing the respective primers to bind to the strands, and, consequently, allowing the polymerase to synthesize new copies of DNA using the dNTPs present in the mixture. Before mutagenesis, the DNA template was methylated so that when the plasmids were transformed in DH5α E.coli, only the new mutagenized, unmethylated DNA would remain after the methylated template was destroyed by the bacterium.

Methylation occurs for 20 minutes at 37°C; PCR then continues at 94°C for two minutes. After the two minutes the temperature remains at 94°C for 20 s, drops to 57°C for 30 s and finally rises again, remaining at 68°C for five minutes. This cycle occurs three times. The reaction is incubated at 68°C for an additional five minutes and is finally stored at 4°C in the machine until it is removed.

Once the mutagenesis PCR reaction was complete, the products underwent gel electrophoresis in order to check whether DNA amplification occurred. In addition, a positive control was run by our peer teacher Rosemary to ensure that the PCR machine and the reagents were working correctly. Primer checks were also run on the gel to ensure that our primers worked correctly. Once we confirmed that our PCR reaction had amplified the DNA, we proceeded to transform the plasmid into DH5α E.coli.

Step 4: Plasmid Purification

Following our transformation of the plasmid into bacterial cells according to guidelines specified in (DebBurman, 2013, p. 36-38), we grew the bacteria on LB+Amp plates to ensure that only the E.coli that integrated the plasmid in their cells survived, due to the AmpR gene present in the plasmid. After 18 hours, we picked four distinct colonies and grew them overnight in separate test tubes containing liquid LB+Amp. These liquid cultures were then used to purify plasmid.

Plasmid purification is a set of reactions involving cell lysis, separation of plasmid DNA from genomic DNA by precipitation, and a series of filtrations that result in the extraction of the pure plasmid from cells. We used the MiniPrep Plasmid Purification Kit developed by Qiagen to perform this reaction. The full protocol we used is described in (DebBurman, 2013).

Once the reaction was complete, the results were run on a gel to confirm that plasmid of the right size was present in the cells. If a thick band of the correct size (measured qualitatively by looking at the DNA ladder) was present for the mutant, some plasmid DNA was present.

Step 5: DNA Sequencing

The plasmids that showed appropriate bands were sent to the University of Chicago’s sequencing facility to check if we were successful in creating the specific mutations. The sequence should mimic the sequence of WT α-synuclein apart from the two/three sites we mutated using PCR. For example, the K60A53E α-synuclein would have the same codon sequence as WT α-synuclein apart from positions 6 and 53. A correct sequence also meant that there were no random mutations on other sites of the gene.

Step 6: Yeast Transformation

After confirming that the sequence of the plasmid was the desired one, we transformed our plasmid into BY4741 yeast. This strain of budding yeast lacks the ability to create uracil, an important nucleotide in RNA. The plasmid containing the mutated α-synuclein gene also contains Ura3. Therefore, BY4741 cells need the plasmid to survive in a medium that lacks uracil. Hence, after having transformed our plasmid into BY4741, we plated the yeast onto SC-URA glucose media to ensure that the surviving yeast contained the plasmid.

Note: Eukaryon is published by students at Lake Forest College, who are solely responsible for its content. The views expressed in Eukaryon do not necessarily reflect those of the College.

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