UNDERSTANDING THE STRUCTURE AND FUNCTION OF PROTEIN KINASE C-EPSILON USING SITE DIRECTED MUTAGENESIS

by

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UNDERSTANDING THE STRUCTURE AND FUNCTION OF PROTEIN KINASE C-EPSILON USING SITE DIRECTED MUTAGENESIS

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ABSTRACT

This research is focused on gaining a better understanding of PKC-epsilon a calcium-dependent protein kinase involved in a wide range of cellular functions including cell proliferation, survival, and apoptosis. The interest in PKC-epsilon derives from the discovery of a *de novo* mutation in the PKC-epsilon gene in patients suffering from SHORT syndrome. This syndrome is a debilitating disorder characterized by short stature, hyperextensibility, ocular depression, Rieger anomaly, and teething decay. This project involved recapitulating the naturally occurring *de novo* mutations *in vitro* as well as determining if other mutations in PKC-epsilon could cause similar disease-state phenotypes. Using a technique known as Site Directed Mutagenesis mutations were introduced into the PKC-epsilon gene and the effects of these mutations on the protein expression were assessed. This mutational analysis will help identify the regions of PKC-epsilon that are vital for its function. This will help elucidate the effect of the same mutations in patients and could help correlate with the severity of disease. Obtaining a clearer picture of the different regions of the PKC-epsilon protein allows for future studies to focus on successfully fixing these regions when they become damaged and could therefore be used to help patients with SHORT syndrome.

Introduction

The Protein Kinase C (PKC) family of protein enzymes are calcium dependent kinases that are activated via G-protein coupled receptor pathways and are influential in a wide variety of cellular functions. Prime examples of these functions include cell proliferation, survival, apoptosis, and migration. The PKC family has a wide variety of different isoforms totaling fifteen in humans, and all PKCs are made up of a catalytic and a regulatory domain tied together via a hinge region. The catalytic domain is highly conserved between the different isoforms whereas the regulatory domain is what primarily differs. PKC isozymes are found throughout the body systems and have been found to have display unique functional properties occasionally acting as tumor suppressor or tumor inducing kinases. PKCε in particular is known to be an oncogenic member of the PKC family as it contributes to cancer initiation and progression via overexpression in certain epithelial tumors. PKCε is primarily associated with cardiomyocytes where it regulates muscle cell contraction and metabolism but in the scope of this project PKCε is analyzed due to its role in the presentation of SHORT syndrome.

The “SHORT” in SHORT syndrome stands for Short stature, Hyperextensibility, Ocular depression, Rieger anomaly, and Teething delay describing some of the characteristic physiological presentations of the disease. Patients who are thought to have SHORT syndrome will typically only exhibit a maximum of three of the mentioned traits although insulin resistance, low BMI, and cardiac malformities can also be a part of the disease. Although SHORT syndrome is rare, scientific interest stems from the discovery of *de novo* mutations found to cause the SHORT syndrome physiology. When SHORT syndrome was first discovered it was believed to only be caused by a mutation in the regulatory domain of the

Phosphoinositide-3-Kinase Regulatory Subunit 1 (PIK3R1) gene, specifically a substitution of arginine to tryptophan at position 649. This mutation impacts the regulatory subunit of the phosphoinositide-3-kinase (PI3K) protein and caused impaired PI3K-dependent AKT activation decreasing signaling along the PI3K-AKT-mTOR network, and diminishing the phosphorylation of targets that promote proper cell growth.

However, in recent years patients with SHORT syndrome physiology have been found to contain a wide variety of mutations in the PRKCE gene that codes for PKCε. These *de novo* mutations are believed to reduce mTORC2-dependent priming of AKT due to decreased activation by PKCε. More specifically, when a mutation is present mTORC2, a protein complex known to

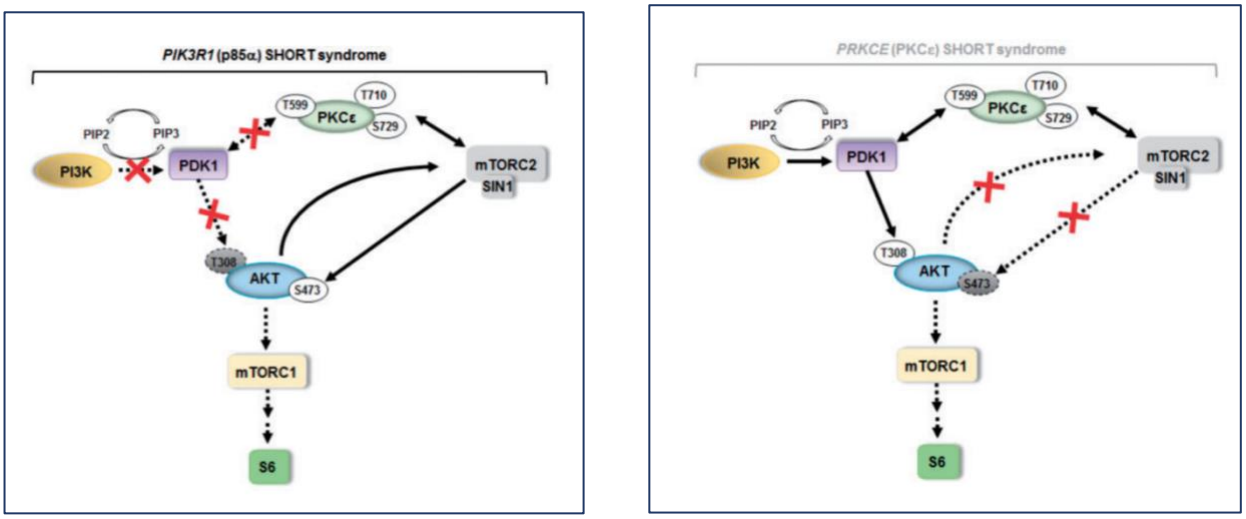


Figure 1: PI3K and PKCe SHORT Syndrome Signaling Pathway
SHORT Syndrome was originally believed to only be caused by a mutation in the PI3K gene. Discovery of a mutation in PKC-epsilon altered the mutated protein in the pathway. Mutated PKC-epsilon causes decreased activation of mTORC2, which can then no longer activate AKT and causes diminished signaling that leads to decreased protein synthesis and can hinder regular cellular functions. (1)

mediate phosphorylation of specific cellular targets and promote their activation, is no longer properly activated by PKCε. Without proper activation mTORC2 can no longer phosphorylate AKT, a key intermediate that is responsible for phosphorylating further targets such as the

ribosomal subunit S6. Without phosphorylation S6 is not activated and cannot carry out its function as a ribosomal protein and assist in the translation of mRNA to protein. In a cell with a nonfunctioning S6 there is decreased gene expression as the genetic material can no longer be translated to protein and therefore the cell loses the functionality associated with the protein that can no longer be produced. PKCε mutations can cause an overall decrease of phosphorylation along the pathway resulting in diminished cell proliferation due to a decrease in protein production. The first of these mutations to be found was E599K (a glutamic acid to lysine change at amino acid 599) which altered a highly conserved residue in the kinase domain of PKCε. After this initial *de novo* mutation was discovered, many other mutations in the PRKCE gene were found in patients with SHORT syndrome physiology leading the focus of this project to be understanding the function of PKCε in this bodily context and determining which parts of the PKCε protein are the most critical in terms of function. Via the *in vitro* recapitulation of naturally occurring mutations, we are able to determine what mutations would result in similar SHORT syndrome phenotypes. The project hypothesis centers on determining which amino acids are critical to protein function and we predict that if amino acids are critical, mutating and compromising them will result in a change to protein function.

Methods

Site Directed Mutagenesis of PKCε: To generate the different PKCε mutants, site directed mutagenesis kits from New England Biolabs (NEB) were used. Bacterial plasmid PCDNA3 template containing the PKCε coding sequence (Addgene, Cat# 10795) was used in combination with primers designed in-lab to generate mutations that had been observed in patients with SHORT syndrome physiology. The primers used were designed by obtaining the

patient sequence from Gen Bank and generating a primer approximately 20 nucleotides long that was complementary to the region of the gene where the mutation was to be generated (Table 1). A special thanks to my fellow colleague Leah Marut for designing the primers used throughout the course of this project.

Table 1: List of Primers used for Site Directed Mutagenesis (SDM) in this study.

A159S		
Q5SDM_2/9/2022_F	GCGGCAGGGGTCCGTCAGGCGCA	Tm=74 Ta (annealing temp) =72
Q5SDM_2/9/2022_R	TTCCTCGGCCGCATGCGTTC	Tm=73 Ta(anneal temp)=72
R268W		
Q5SDM_7/25/2021_F	GGGACTCTTGTGGCAGGGTTTGC	Tm=71 Ta (annealing temp) =69
Q5SDM_7/25/2021_R	CAGAGCAGGGACCCACAG	Tm=68 Ta. (Anneal temp) =69
V288M		
Q5SDM_4/13/2021_F	TGAGACCAACATGGCTCCCAACT	Tm=67 Ta (annealing temp) =65
Q5SDM_4/13/2021_R	CATCGACGGTGAACATTCATTTTG	Tm=64 Ta (anneal temp) =65
R372Q (CAG)		
Q5SDM_7/15/2021_F	ATTTGACAACCAGGGAGAGGAGC	Tm=57 Ta(annealing temp)=58
Q5SDM_7/15/2021_R	GACAAGGCTTTCCGAATG	Tm=60 Ta(anneal temp)=58
R372Q (CAA)		
Q5SDM_5/6/2022_F	ATTTGACAACCAAGGAGAGGAGC	Tm=62 Ta(annealing temp)=61
Q5SDM_5/6/2022_R	GACAAGGCTTTCCGAATG	Tm=60 Ta(anneal temp)=61
R372G		
Q5SDM_7/25/2021_F	ATTTGACAACGGCGGAGAGGAGC	Tm=58 Ta(annealing temp)=59
Q5SDM_7/25/2021_R	GACAAGGCTTTCCGAATG	Tm=60 Ta(anneal temp)=59
R396W		
Q5SDM_4/13/2021_F	TGGCGAAGTCTGGCAAGGCCAGG	Tm=75 Ta (annealing temp) =72
Q5SDM_4/13/2021_R	TTCTCACCGGGGCTCATCAGC	Tm=72 Ta (anneal temp) =72
T453R		
Q5SDM_4/28/2021_F	CGTGGACTGCCGGATGACAGAGAAG	Tm=60 Ta (annealing temp) =58
Q5SDM_4/28/2021_R	TCATCATCCTGAAGGATG	Tm=57 Ta (anneal temp) =58
G491S		
Q5SDM_2/16/2022_F	ATATGTAAATAGTGGAGACCTCATG	Tm=58 Ta (annealing temp) =59
Q5SDM_2/16/2022_R	TCCATGACGAAAAAGAGG	Tm=58 Ta (anneal temp) =59

G492A

Q5SDM_2/2/2023_F TGTAAATGGTGCAGACCTCATGTTTC Tm=62 Ta (annealing temp) =60
Q5SDM_2/2/2023_R TATTCCATGACGAAAAAGAGG Tm=59 Ta(anneal temp)=60

G492E (GAA)

Q5SDM_5/6/2022_F TGTAAATGGTGAAGACCTCATGTTTC Tm=62 Ta (annealing temp) =65
Q5SDM_5/6/2022_R TATTCCATGACGAAAAAGAGG Tm=59 Ta (anneal temp) =60

E599K

Q5SDM_4/13/2021_F GCTGATGTACAAGATGATGGCTGGACAGC Tm=72 Ta (annealing temp) =72
Q5SDM_4/13/2021_R ACCCCCAGGGCCCACCAG Tm=76 Ta (anneal temp) =72

A609T

Q5SDM_4/28/2021_F TCCCTTTGAGACCGACAATGAGG Tm=64 Ta (annealing temp) =65
Q5SDM_4/28/2021_R GGCTGTCCAGCCATCATC Tm=66 Ta (anneal temp) =65

By looking at the patient sequence the exact nucleotide change was determined, and the primer was modified with the change so that it would generate a mutant plasmid.

Once the mutant plasmid is generated, amplification occurs via PCR with annealing temperatures varying based on the individual primers (Table 2).

Table 2: Annealing temperature used for each primer.

Mutant	Sequence	Annealing Temperature (°C)
A159S	GCGGCAGGGGTCCGTCAGGCGCA	67
T453R	CGTGGACTGCCGGATGACAGAGAAG	55
G491S	ATATGTAAATAGTGGAGACCTCATG	55
E599K	GCTGATGTACAAGATGATGGCTGGACA	62
A609T	TCCCTTTGAGACCGACAATGAGG	59
V288M	TGAGACCAACATGGCTCCCAACT	59
G492A	TGTAAATGGTGCAGACCTCATGTTTC	55
R268W	GGGACTCTTGTGGCAGGGTTTGC	66
G492E	TGTAAATGGTGAAGACCTCATGTTTC	55
R372Q	ATTTGACAACCAAGGAGAGGAGC	55

Once amplification is complete the DNA undergoes the KLD (Kinase, Ligase, and Dpn1) reaction where different chemical compounds assist in preparing the DNA sample for

transformation. Dpn1 digests the methylated DNA template that does not have the mutation in order to minimize the possibility of non-mutated DNA transferring into the bacteria during transformation. Kinase adds phosphate groups that stabilize the new mutated plasmids, and ligase re-circularizes those same mutated plasmids.

Bacterial Transformation: Following KLD, competent bacteria from the NEB kits are used to perform bacterial transformation with the mutated plasmid. This transformation involves heat shock of the bacteria which opens the bacterial cell wall and allows for the uptake of exterior plasmids. Once the bacteria have undergone heat shock, they are allowed to grow in nutrient rich SOC while in a shaker set to 37°C for one hour. The bacteria are then plated onto nutrient-rich agar plates containing ampicillin and allowed to grow overnight in an incubator set to 37°C. The plasmid template used to perform the site directed mutagenesis contains a gene that provides ampicillin resistance so, idealistically only the bacteria that took up the mutated plasmid during heat shock will be able to survive on the plate due to the added functionality of ampicillin resistance.

Bacterial Mini-preps: Following the plating and incubation of the bacteria, colonies were selected at random and grown out in LB broth with ampicillin to increase the amount of potentially mutated DNA. The colonies were allowed to grow for 16 hours after which the DNA was extracted from selected cultures using a mini-prep protocol. The bacterial cultures are spun down with a centrifuge to remove the LB broth supernatant and the remaining bacterial pellet is treated with lysozyme, an enzyme that lyses the bacteria and TEG buffer (Tris pH 8, EDTA, Glucose) which help stabilize molecules like DNA and RNA. Once the pellet is dissolved, SDS detergent and 0.2M NaOH are added to help neutralize the pH of the bacterial lysate while ammonium acetate (7.5M) is added to help in the precipitation of proteins. The

contents of the bacteria are then centrifuged causing heavier components like the cell wall and proteins to form a pellet and lighter components like the plasmid DNA to remain suspended in the supernatant. This supernatant is then treated with isopropanol and ethanol that help stabilize the DNA and protect it from degradation enzymes. The samples are centrifuged again to separate the ethanol and the DNA, the ethanol is removed, and the DNA is resuspended in TE (Tris pH 8, EDTA). These mini-preps allow for the plasmid DNA to be isolated and purified preparing it to be used to perform sequencing reactions where one can check for the presence of the desired mutation.

Sequencing: Once the DNA was isolated a sequencing protocol was used to determine if the mutation of interest was located within the plasmid. The primers bracketed the region being sequenced to ensure that only the region where the mutation would appear would be sequenced. The gene was amplified using PCR and the resulting sample was exposed to BET, small metal spheres that bound to the DNA and to a presented magnet to isolate the DNA from the supernatant. The supernatant was removed, and the DNA was washed with 70% ethanol to remove any impurities and then resuspended in a resuspension buffer. Subsequent sequencing using a genetic analyzer was then performed over the course of 2 hours. Once the genetic analyzer sequenced the samples, analysis was performed with the Sequencher software which allowed for the comparison of the sample sequences and the WT PKCε sequence. This alignment allowed for the confirmation of the presence of the desired mutation if SDM had been successful.

Bacterial Midi-preps: Once the presence of the mutation was confirmed, the original culture was grown out in an exponential manner until a 150 mL culture was obtained. This culture then underwent a similar protocol to the miniprep instead following the manufacturer instructions on

a Qiagen midi-prep kit. These midi-preps generated a greater amount of mutated DNA that could then be used for subsequent studies including protein analysis by western blot and kinase assays.

Western Blot: The mutant DNA samples from the midi-prep were transfected into HEK-293 cells that were allowed to grow for 48 hours in order to produce mutated copies of the PKCε protein. These cells were then lysed with lysis buffer and centrifuged to separate the cellular components from the proteins. The mutated proteins isolated from the cells underwent a Bradford assay to measure the protein concentrations. Once protein concentration was determined the samples were loaded onto a previously poured SDS-page gel. Once the gel finished running the samples were transferred onto a membrane in order to increase the durability of the medium and the samples were probed with anti-PKCε antibody (Cell Signaling Cat# 9253). This western blot study helped determine the amount of protein that was produced by each of the cells transfected with different mutant DNA.

Results

Optimizing SDM Conditions:

After an initial round of mutagenesis, the following modifications were made to the protocols to increase their efficiency.

1. All the annealing temperatures were decreased by 3-5°C in order to increase the likelihood of the engineered primers to bind to the template DNA and therefore increase the likelihood of mutant generation.
2. The KLD step of the protocol was also altered increasing the duration from 10 minutes to 30 minutes in a water bath and 30 minutes on the benchtop. The use of a water bath generated a more ideal environment for Kinase and Dpn1 which need a warm environment to better degrade the template DNA. The increased time simply gave Kinase

3. and Dpn1 a greater amount of time to degrade the template. Greater template degradation ensures that transformed plasmids are more likely to contain the mutated version of the plasmid and generate bacterial colonies that contain the mutation.

Analyzing Mutants: Using the methods outlined above eight different mutants were successfully created over the course of a year and a half (Table 3).

Table 3: Results of Site Directed Mutagenesis of PKCε

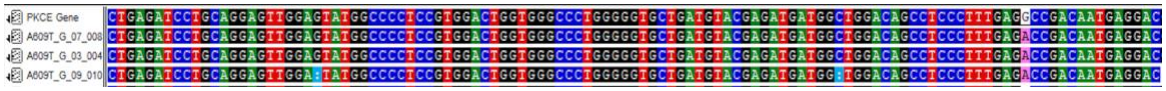
Mutant	Sequence	Sequencing Result	Findings
T453R	CGTGGACTGCCGGATGACAGAGAAG	☑	Only desired mutation
G491S	ATATGTAAATAGTGGAGACCTCATG	☑	Only desired mutation
A609T	TCCCTTTGAGACCGACAATGAGG	☑	Only desired mutation
A159S	GCGGCAGGGGTCCGTCAGGCGCA	☑	Only desired mutation
V288M	TGAGACCAACATGGCTCCCAACT	☑	Only desired mutation
R268W	GGGACTCTTGTGGCAGGGTTTGC	☑	Only desired mutation
G492A	TGTAAATGGTGCAGACCTCATGTTTC	☑	Only desired mutation
G492E	TGTAAATGGTGAAGACCTCATGTTTC	☑	Only desired mutation
E599K	GCTGATGTACAAGATGATGGCTGGACA	☑	Truncated due to undesired mutation

Sequencing: Successful generation of a mutant was confirmed via analysis of the gene sequence using Sequencher software to compare the wild type (WT) sequence to that of the mutant sequence.

Figure 2: Mutant Sequencing Analysis
Analysis of sequencing reactions was performed with Sequencher software which aligned the different sequences with wild type (WT) and highlighted any differences. In the enlarged portion shown the top line is WT sequence which shows a thymine (T) while all the sequencing reactions show a mutated version with cytosine (C) which was the desired change.

As seen inf figure 2, the software highlights any discrepancies between the two sequences making identification of the desired mutation straightforward. Each of the mutants was analyzed using this software and is listed below with the desired mutation highlighted.

- A609T



- V288M

- A159S

- R268W

- G491S

- G492A

- E599K

- T453R

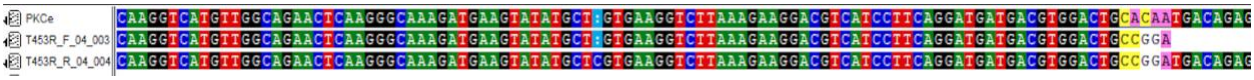


Figure 3: Sequencher analysis of PKCε mutants.

Expression of Mutants in Mammalian Cells: After confirming the mutation in the DNA sequence, DNA samples were transfected into HEK-293 cells in order to determine the impact of the mutation on mammalian cell expression of the PKCε protein.

After transfection the cells were allowed to grow and the PKCε protein was isolated and purified. Protein concentration per sample was determined via Bradford assay and samples were then run on a western blot with an anti-PKCε antibody. Mammalian cell expression and western blot showed that the levels of protein expression were equivalent across the different mutants and compared to the WT.

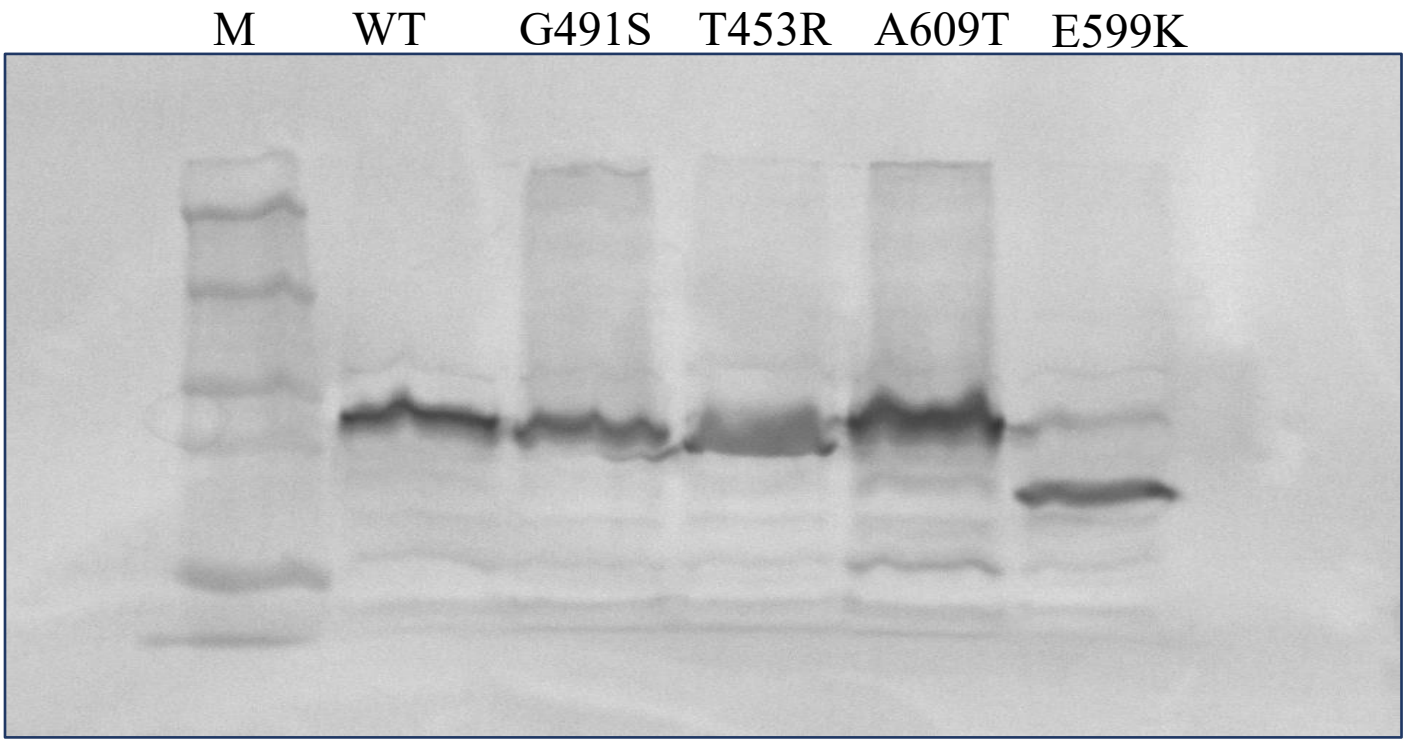


Figure 4: Expression of Mutants
Western Blot with four PKC-epsilon mutants and wild type (WT). All mutants show similar levels of expression as WT. The E599K mutant ran faster on the gel indicating that it was smaller in size than the other mutant proteins.

When analyzing protein size on the western blot all mutants were the same size in KD as wild type (WT). The only exception was E599K, where the band was seen to run faster indicating that

it is of a smaller size. Due to E599K running faster on the gel and being shorter in length sequencing of the entire gene was performed to determine if any undesired mutations had inadvertently been inserted into the sequence.

Analysis of E599K: When E599K was analyzed via western blot it was determined to express a form of PKCε protein that ran faster on the gel in comparison to both WT and other mutants. This piqued interest since it potentially showed that the E599K mutant contained another undesired mutation that was causing the protein to become truncated. In order to determine if this was the case, full gene sequencing was done on the E599K mutant DNA by using existing SDM primers as well as newly generated primers to cover the entire gene sequence. The results of this full gene sequencing showed the desired mutation as well as three substitution mutations. On their own these mutations did not raise any particular concern, but when the mutant sequence was translated to the amino acid sequence and compared to wild type the change in the amino

acid sequence in subsequent truncation was quite evident.

PKCe Amino Acid Sequence

MDYKDDDDKVVFNGLLKIKICEAVSLKPTAWSLRHAVGPRPHTFLLDPYIALNVDDS
RIGQTATKQKTNSPAWHDEFVTDVCNGRKIELAVFHDAPIGYDDFVANCTIQFEELL
QNGSRHFEDWIDLEPEGRVYVIIDLSGSSGEAPKDNEERVFRERMRPRKRQGAVRRR
VHQVNGHKFMATYLRQPTYCSHCRDFIWGVIGKQGYQCQVCTCVVHKRCHELIITKC
AGLKKQETPDQVGSQRFSVNMPHKFGIHNYKVPTFCDHCGSLLWGLLRQGLQCKVCK
MNVHRRCETNVAPNCGVDARGIAKVLADLGVTPDKITNSGQRRKKLIAGAESPQPAS
GSSPSEEDRSKSAPTSPCDQEIKELENNIRKALSFDNRGEEHRAASSPDGQLMSPGE
NGEVRQGQAKRLGLDEFNFIKVLGKGSFGKVMLAELKGKDEVYAVKVLKKDVILQDD
DVDCTMTEKRILALARKHPYLTQLYCCFQTKDRLFFVMEYVNGGDLMFQIQRSRKFD
EPRSRFYAAEVTSALMFLHQHGVIYRDLKLDNILLDAEGHCKLADFGMCKEGILNGV
TTTTFCGTPDYIAPEILQELEYGPSVDWWALGVLMYEMMAGQPPFEADNEDDLFESI
LHDDVLYPVWLSKEAVSILKAFMTKNPHKRLGCVASQNGEDAIKQHPFFKEIDWVLL
EQKKIKPPFKPRIKTKRDVNNFDQDFTREEPVLTLVDEAIVKQINQEEFKGFSYFGE
DLMP-S

E599K Amino Acid Sequence

MDYKDDDDKVVFNGLLKIKICEAVSLKPTAWSLRHAVGPRPHTFLLDPYIALNVDDS
RIGQTATKQKTNSPAWHDEFVTDVCNGRKIELAVFHDAPIGYDDFVANCTIQFEELL
QNGSRHFEDWIDLEPEGRVYVIIDLSGSSGEAPKDNEERVFRERMRPRKRQGAVRRR
VHQVNGHKFMATYLRQPTYCSHCRDFIWGVIGKQGYQCQVCTCVVHKRXHELIITKC
AGLKKQETPDQVGSQRFSVNMPHKFGIHNYKVPTFCDHCGSLLWGLLRQGLQCKVCK
MNVHRRCETNVAPNCGVDARGIAKVLADLGVTPDKITNSGQRRKKLIAGAESPQPAS
GSSPSEEDRSKSAPTSPCDQEIKELENNIRKALSFDNRGEEHRAASSPDGQLMSPGE
NGEVRQGQAKRLGLDEFNFIKVLGKGSFGKVMLAELKGKDEVYAVKVLKKDVILQDD
DVDCTMTEKRILALARKHPYLTQLYCCFQTKDRLFFVMEYVNGGDLMFQIQRSRKFD
EPRSRFYAAEVTSALMFLHQHGVIYRDLKLDNILLDAEGHCKLADFGMCKEGILNGV
TTTTFCGTPDYIAPEILQELEYGPSVDWWALGXADVXDDGWDSLPLRPTMRTTYLSP
SSMTTCCTQSGSARRLSAS

Figure 5: Comparison of Amino Acid Sequences between wild type (WT) PKCε and mutant E599K.

As seen in figure 5, the E599K protein codon sequence was not only shorter but also displayed evidence of a frameshift mutation that greatly altered the end portion of the protein with all amino acids past position 602 being mutated. This mutation is likely a result of error during the site directed mutagenesis stage and therefore further attempts to recapitulate the mutant must occur.

Discussion

Over the course of this research project the overall goal was to successfully recapitulate PKCε mutations that were observed in patients experiencing SHORT Syndrome. Below is a visual representation of the mutants which were attempted.

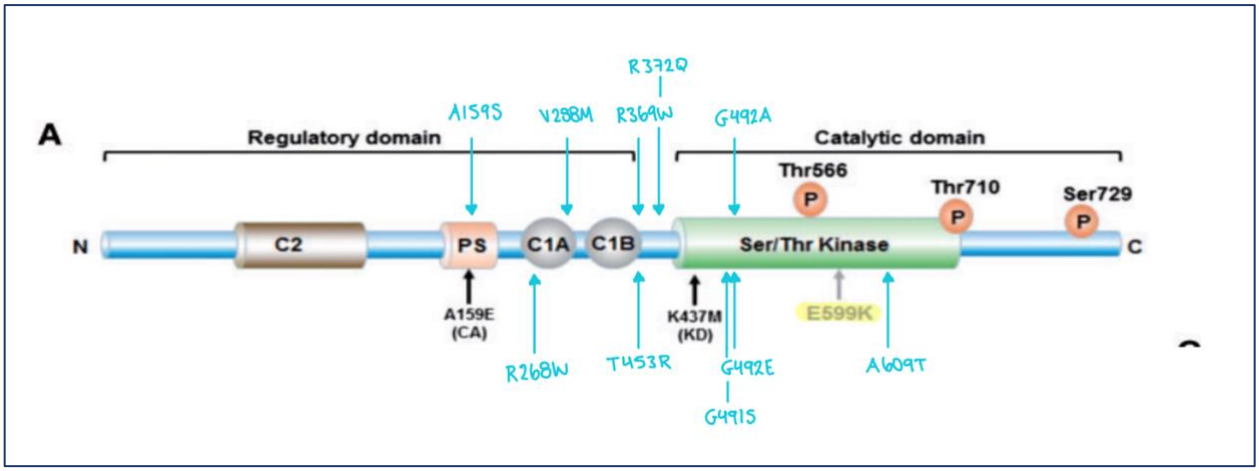


Figure 6: SHORT Syndrome PKC-epsilon Mutations
The mutations listed in blue were found in patients with SHORT Syndrome. The location of the mutations gives us an idea of how the mutation would affect the function of the protein. These mutations were recapitulated to test the effect on the PKC-epsilon protein in cell culture.

These mutants are located throughout the PKCε gene and the severity of the presentation of SHORT Syndrome may be associated with the region of the gene in which the mutation occurs. E599K, A609T, and G492E/A are all located in the highly conserved catalytic domain of the gene and could have more on an impact as they would have a direct effect on the kinase activity

of the protein and result in the greatest loss of function. The mutations located in the regulatory domain like A159S or V288M could result in a change in the activity level of the protein, e.g., constitutively active enzyme. The mutations that lie between the two domains like R369W and T453R could still generate noticeable impacts as they could affect the tertiary and quaternary structure of the protein making it more challenging to interact with substrates. *In vitro* generation of these mutations was key in understanding the ways in which different mutations affected the structure and functionality of the protein. Of the twelve potential mutations that were identified in patients, eight were successfully recreated *in vitro* and four of them were analyzed via western blot. The reasoning behind the limitations on mutation generation was due to complications with the site directed mutagenesis protocol that did not always yield the desired mutation. Western blot analysis was limited by the DNA concentrations of the remaining mutants which were not high enough for effective transfection into mammalian cells. From the western blot analysis, it was determined that the mutations likely did not impact the overall structure of the protein as all the proteins but one were the same size as the WT. This most likely means that the mutations impact the functionality of the protein, and this is what causes the loss of function that results in SHORT syndrome presentation. Moving forward with the research further western blot analysis on abundance and size will be needed to determine the potential impacts of the mutations of the PKCε protein. Further analysis pertaining to the functionality of the PKCε protein will also be needed in order to determine the impact of the patient mutations. Functionality of PKCε can be assessed using *in vitro* kinase assays that determine the amount of phosphorylation of a known substrate of the enzyme. Since PKCε is a known kinase that has distinct molecular targets in its cellular pathway performing a kinase assay would allow for further understanding as to how the mutations impact the protein. A potential target for the kinase assay would be AKT as it would

allow for the demonstration of PKCε phosphorylation of substrates and if the mutations do in fact affect functionality. Overall, gaining a better understanding of how the mutations impact the PKCε protein will allow for further research that determines if these impacts can be reversed. This greater understanding may also help future research regarding potential treatments or relief for patients experiencing SHORT syndrome.

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