ASSOCIATION OF C3PO & PLCβ

A Major Qualifying Project Report

Submitted to the Faculty of the

WORCESTER POLYTECHNIC INSTITUTE

in partial fulfillment of the requirements for the

Degrees of Bachelor of Science

in

Chemistry &

Biology/Biotechnology

by

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CDR Deadline: April 26, 2018

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ABSTRACT

While Phospholipase-Cβ (PLCβ) plays an important role on the plasma membrane by generating calcium signals, it also has a significant population in the cytosol where it shows affinity for Component 3 Promoter of RISC (C3PO), which is an enhancer of RNA silencing. This project focused on PLCβ's inhibition of RNA silencing *in vitro* and in PC12 cells by evaluating the hydrolysis of fluorescent oligonucleotides. We find that PLCβ binds and inhibits C3PO, allowing the expression of genes whose sequence would otherwise be degraded.

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ACKNOWLEDGEMENTS

BACKGROUND

This chapter first explores the role of the Phospholipase C family of proteins and their role within the cell, including the structure and function they share. It then pivots to the isozyme PLCβ, and what makes this particular type of PLC unique, including its activator and presence within the cytosol. Of particular interest is its relationship with the C3PO protein, which is also covered in greater detail. Ultimately, it concludes by discussing recent findings regarding what is hypothesized to be the two proteins role in RNA silencing, a type of post-translational gene regulation, and its impact on cell differentiation.

Phospholipase C

Phospholipase C (PLC) proteins are a family of enzymes known to selectively catalyze the hydrolysis of the phospholipid, phosphatidylinositol 4,5-biphosophate (PIP_2) along the cell membrane. The group is generally targeted for research purposes because the reaction generates two important regulatory molecules, 1,2-diacylglycerol (DAG) and 1,4,5-triphosphate (IP_3), which influence Ca^{2+} concentration within the cell (Kadamur and Ross, 2013).

Although each family member has this similar phospholipid hydrolytic function, the members are not the same. Each isozyme maintains 40-50% of their structure, including a TIM barrel that serves as the activity site, an X-Y linker that may block the activity site in unactivated specimen, and several other structures (**Figure-1**) below. Mammalian PLC isozymes are then categorized into 4 groups based upon structural differences further away the activity site (Kadamur and Ross, 2013). This project focuses on one group in particular – PLCβ.
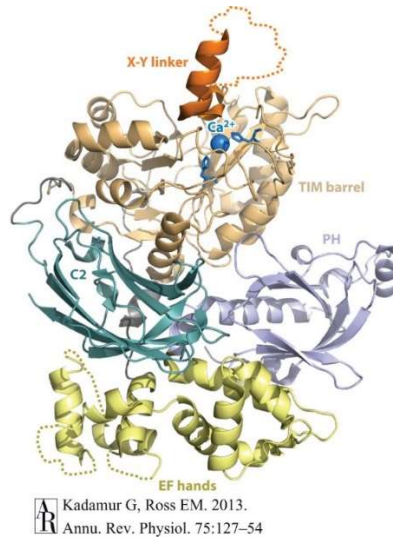
Figure 1- The Conserved Structure Found in all PLC Family Members. Shown is a TIM barrel serving as the active region surrounded by the X-Y Linker, PH and EF Hands, this particular model is from PLCβ2 (Kadamur and Ross, 2013)

PLCβ

PLCβ distinguishes itself by having an extension of roughly 400 amino acids at the C-terminus that form a three stranded coil. PLCβ's activation along this site by G_q proteins is unique amongst the PLC family. The activation by G_q allows it to respond to stimuli outside the cell (**Figure-2**) (Kadamur and Ross, 2013). Previous studies have demonstrated that the membrane bound G proteins are capable of anchoring and orienting PLCβ, allowing the phosphorylation of PIP_3 to progress without the X-Y linker blocking the TIM barrel (Fukami et al., 2010).
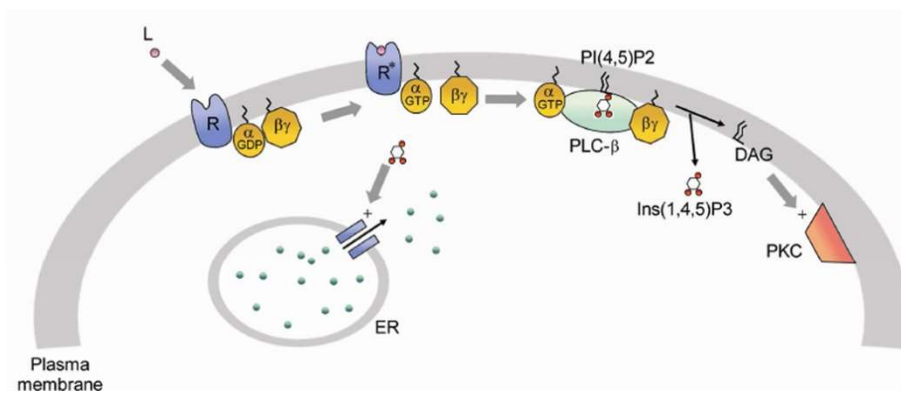

Figure 2 - A model for PLCβ function on the cell membrane - note the activation by G protein, unique to this isozyme

PLCβ has been shown to regulate calcium, and a variety of effects result from its inactivity in PLCβ KO mice. The absence of PLCβ1 induces epilepsy, with mice typically dying at post-natal day three. PLCβ2 KO also alters the ability for mice to taste. Removing PLCβ3 induces myeloproliferative disease and atherosclerosis while PLCβ4 KO causes depression. (Fukami et al., 2010)

However, to say this is all due to calcium regulation paints an incomplete picture of PLCβ's role within the cell. Despite the lack of G-protein, there remains a significant population of PLCβ in the cytosol (Aisku, Runnels and Scarlata, 2010). This suggests PLCβ is performing a function independent of its activator, in other words it could be a moonlighting protein, performing a secondary task entirely independent of its known function.

PLCβ1 Affiliation to TRAX

The Scarlata lab previously identified translin-associated factor X (TRAX) as a novel binding partner to PLCβ, using PLCβ1 (Aisiku, Runnels and Scarlata, 2010). Like $G_{\alpha q}$ TRAX binding also occurs on the C-terminus, although with a lower affinity. Through immunofluorescent imaging it was shown that despite its preference, TRAX and PLCβ1 are colocalized in HEK293 cells. Further experiments concluded that only $G_{\alpha q}$ or TRAX can interact with PLCβ1, but even with forced activation of G protein to generate an excess concentration of activators, colocalization in the cytosol persisted (Aisiku, Runnels and Scarlata, 2010).

TRAX, C3PO, and RNA Silencing

Isolated TRAX is unstable and thus it is only found associated with the nucleotide binding protein translin, where the two naturally form the octamer called component 3

promoter (C3PO) of RNA induced silencing complexes(RISCs)(**Figure-3)**. Duplex microRNAs (miRNAs) bind to C3PO which degrades one of the strands leaving the other to bind to its target mRNA on RISC and become degraded halting that gene's expression (Philip et al., 2013). In models for C3PO activity, it was designated to be a purely a promoter of RISCs where Argonaut 2 (Ago2) was the catalytic component of the structure (**Figure-4**). However, recent experiments in our lab have cast a shadow of doubt upon this idea, as there has been little association between C3PO and Ago2, which suggests independent hydrolysis.

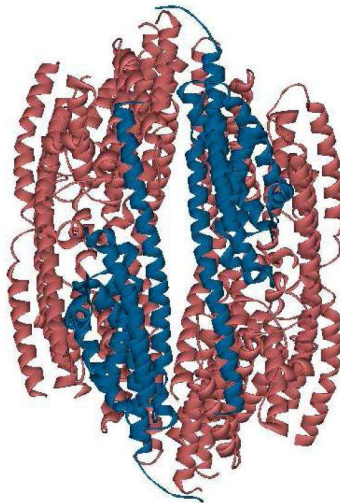*Figure 3- Three dimensional structure of the octamer C3PO – TRAX, the protein known to bind with PLCβ, is seen in blue, Translin, the nucleotide binding protein, is in red (Sahu, Philip and Scarlata., 2013)*
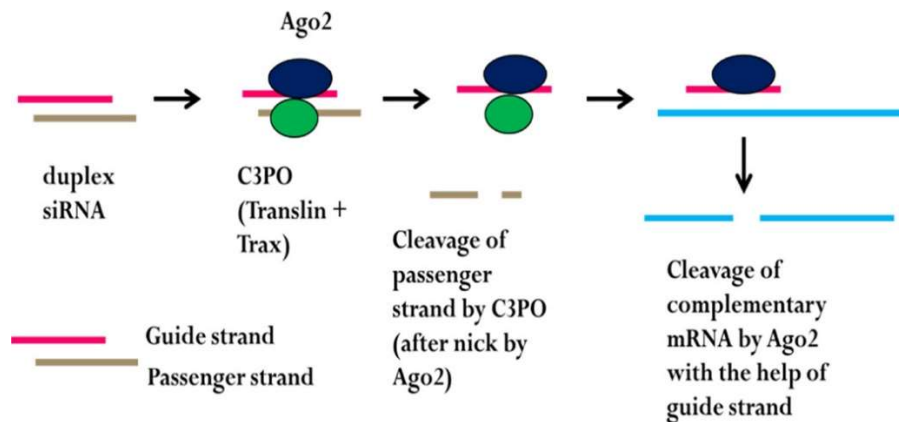

Figure 4- A early model of the role C3PO plays in RNA silencing. Note: the model considers Ago2 the catalytic component of RNA silencing (Phillip et al., 2013).

A recent study asserts that this RNA silencing has significant impact on the learning process in mammalian brains. Knock out TRAX/Translin mice struggled to perform in long term memory tests, showing impaired results in novel object tests when compared to wild type mice, and significantly reduced synapse activity (Park et al., 2017).

A typical indicator for C3PO activity is the number of miRNAs within the cell. Looking at the hippocampal region of wild type and TRAX/Translin knock-down mice in learned and homecaged populations, it was seen that there is a substantial change in several miRNAs. Learned KO mice had a far higher number of targeted miRNAs than their wt counterparts, while homecaged mice did not have a significant difference (Park et al, 2017). This shows that the genes C3PO regulate play a role in learning, with the difference in gene expression demonstrating the necessity of this post transcriptional gene regulation to occur.

PLCβ1's Interaction with RNA Silencing

Our lab's hypothesis is that PLCβ1 is a significant factor in this gene regulation. Binding to C3PO occurs in a 1 to 1 ratio and inhibits the hydrolysis of nucleotides (Aisikku, Runnels and Scarlata, 2010). This function likely plays a role in neural differentiation in KO mice that were largely issues related to the synapse development in neuronal cells. On a cellular level, the Scarlata lab was also able to demonstrate that without PLCβ1, PC12 cells are unable to differentiate, furthering this idea that both PLCβ1 and C3PO are necessary for proper differentiation and development to occur in the brain (Garwain and Scarlata, 2016).

Co-localization of PLCb1 and C3PO has been demonstrated in previous studies, and binding has been shown to occur, but several questions remain with regards to how this inhibition of hydrolysis occurs (Scarlata et al., 2016). It has not been conclusively determined whether C3PO is capable of hydrolyzing independently of Ago2 in cells, nor whether PLCβ1'sregulatory mechanism is universal.

The current model for how PLCβ1 interacts with C3PO (**Figure-5**) is that it binds to the two exposed areas of TRAX in C3PO and halts hydrolysis. Whether the structure needs to be in its active state or not remains to be seen, although it has been observed that the greatest inhibition of hydrolysis are genes most targeted by C3PO, which could imply a better attachment to TRAX when C3PO is most active.
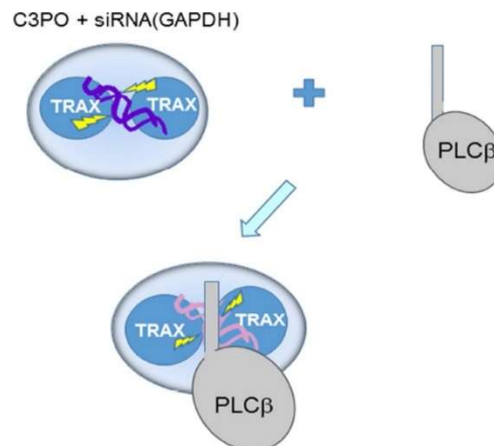

Figure 5- A model for how PLCβ binds to C3PO, with the 400 amino acid extended coil interfering with the two TRAX proteins ability to hydrolyze the nucleotide sequence

Connection to G-protein

One of the experiments that has been conducted by the Scarlata lab has been to excite G protein coupled receptors (GPCR) using carbachol – a synthetic agonist to acetylcholine. What has been found is that when G_q proteins are excited, cytosolic PLCβ migrates towards the membrane and away from C3PO.

The M_1 muscarinic acetylcholine receptor is both the GPCR most commonly associates with G_q proteins and most localized in the hippocampus region of the brain. In homozygous M_1 knock out mice there is a significant change in behavior, with mice performing significantly worse on several of the cognitive tests that involve hippocampal learning – specifically when memories of tasks have to be transferred to other parts of the brain (Anagnostaras et al., 2002).

The importance M_1 has with regards to the transfer of information through the brain and C3PO's impact on learning in general are too similar to ignore. The association of PLCβ and C3PO ultimately will serve as a bridge that connects the GPCR and cellular differentiation to create a better model for learning in mammals.

PROJECT PURPOSE

While it is known that C3PO is inhibited by PLCβ, the mechanism for its influence is less established. This project first aimed to observe the impact of the two proteins with regards to hydrolysis of oligonucleotides *in vivo*. It then pivots into the manner in which this inhibition occurs, looking at how altering the order in which oligonucleotides are exposed to both PLCβ and C3PO *in vitro* impacts hydrolysis rates. Ultimately, we try to illuminate how PLCβ is used as a tool to regulate C3PO's post-transcriptional gene regulation and implement it into the larger narrative surrounding how learning occurs on a cellular level.

METHODS

Throughout this project specific protocols were followed to ensure the integrity of the results.

Protein Gels

The purified proteins were analyzed on SDS gels followed by Coomassie Blue staining. Separating gels were composed of 2.3mL of dH$_2$O, 5.0mL of 30% acrylamide, 2.5mL pH 8.8 1.5M tris, 100μL of 10% SDS, 100 μL of 10% APS and 6μL TEMED. The stacking gel was made by mixing together 2.2mL of dH$_2$O, 680μL of 30% acrylamide, 1mL of 0.5M Tris (pH=6.8), 40μL of 10% SDS, 40 μL of 10% APS and 4μL of TEMED.

Bradford Assay

Concentrations of proteins were determined by running Bradford assays. Bovine serum albumin was used to create standards in a series of compared to a 10x diluted sample of each protein elution. 0.5 μl of Bradford solution was then added to each well, and the absorbance measurements taken at 280 nm.

Fluorescent Oligonucleotides

The rate of hydrolysis was observed using a fluorescent oligonucleotide designed to emit a photon of a different wavelength as it was broken down. Having already seen miR103-3p impacted by PLCβ's presence in the cell, it was chosen as the template for a double stranded DNA that had 6-Carbofluorescein (FAM) tagged on the 3' end and 5-Carboxytetramethylrhodamine (TAMRA) on tagged on the 5' end. Due to the small length of the oligonucleotide, upon excitation of FAM, Forster Resonance Energy Transfer (FRET) would occur causing a greater signal from TAMRA at 590 nm, and far less from FAM's 520 nm (**Figures-6 and 7**). As the oligonucleotide was degraded and

the distance between the two fluorophores increased, FRET's impact lessened and the signal at 520 nm gradually would increase, while the 590 would decrease.
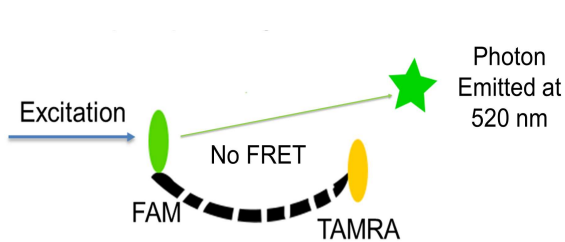

Figure 7 - Hydrolyzed oligonucleotide behavior, emission at 520 nm is an indicator that hydrolysis has occured
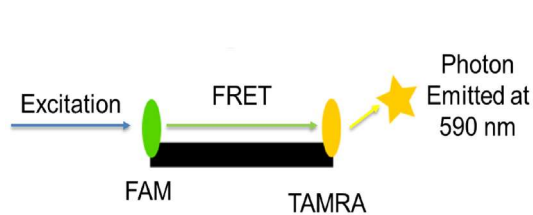
Figure 6 - Intact oligonucleotide behavior, emission at 590 nm is an indicator that hydrolysis has slowed.

Fluorometry and FRET

A fluorometer was used in order to observe FRET in vitro. 2.5 µl of 100 µM of the oligonucleotide was pipette into a microcuvette under variable conditions, maintaining a constant volume of 120 µl to ensure the same concentration of fluorescent molecules. Solutions were excited using a single photon xenon laser at 490 nm. Using a steady state emission scanning, the emitted photons were observed from 500-650 nm with step sizes of 1 nm, over three averages after a 103 µsec delay and 50 µsec delay time. 25 shots were fired per reading with a frequency of 150 Hz. Fluorometer experiments were performed in triplicate and averaged in order to perform a T-test for significant difference.

Cell Type

PC12 cells are derived form a pheochromocytoma of the rat adrenal medulla that have embryonic origin stemming from the neural crest. They are capable of differentiating into neuron-like cells developing physical features such as dendrites.

Cell culture

PC12 cells were maintained in Dulbecco's modified Eagle's medium which contained DMEM (Dulbecco's Modified Eagle Medium), GIBCO, 320-355mosM supplemented

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with 50 mL Fobium Fetal Bovine Serum (FBS), 5mL P/S and 5mL of 100mm Sodium

Pyruvate. When the cells were split, 2ml of trypsin were added (in a 100mm dish) and the

plate was incubated at room temperature for 2 minutes in order for the cells to detach

from the plate.

Transfection

Expression of both TRAX and PLCβ were knocked down in PC12 cells by transfecting

complementary siRNA by electroporation. This protocol adapted from Molecular

Cloning: A Laboratory Manual. Cells were grown to near 100% confluence and washed

with sterile PBS. The cells were then trypsinized, centrifuged 5 minutes at 1500× g and

resuspended in 10mL of fresh growth medium. 800 µl of cells were pipeted into a 0.4cm

BioRad cuvette and placed in an electroporator (BioRad Gene Pulser Xcell). Cells were

then plated and covered with fresh medium.

Microinjection and FLIM

Cells were fixed by washing with Hanks' Balanced Salt Solution (HBSS) media before

imaging with ISS vista vision software attached to a two photon excitation confocal

microscope. Fluorescence lifetime was imaged from 500-540 via channel three, with a

threshold set to 60 and data smoothed twice. Excitation was performed at 780 nm.

Microinjections were performed with 100 µM solution of oligo's injected with an

injection pressure of 40 hPa over 0.65 seconds, with an Eppendorf produced Femtotips.

RESULTS

PLCβ1 is necessary for the differentiation of PC12 cells, but the mechanism for its influence is less established. Prior to the beginning of this project, RNAseq data were collected showing the number of miRNAs present in knock-down PC12 cells in undifferentiated and differentiated states, but this data was never evaluated. From almost 400 sequences, 49 were shown to have a magnitude of 10,000 copies, only 14 of which were shown to have a change in expression of greater than 15% in KO cells. Of these, miR-103-3p was chosen for further testing.

This project began by first observing the change in rate of hydrolysis in differing cell types. Due to interference in intensity that could arise in cells Fluorescence Lifetime (the duration of time a molecule fluoresces) at 520 nm was used as an indicator for how the oligonucleotides were interacting with C3PO. In this experiment FRET would cause the lifetime to decreases, so as hydrolysis occurs the lifetime at 520 increases.

The phasor plot below (**Figure-8**) shows the first trial where wild type PC12 cells were microinjected with the fluorescent miR. Single fluorescence lifetime is ideally plotted along the arc moving across the diagram, however interference due to FRET moves the overall fluorescence below the arc. As time goes on, the fluorescence increases and behaves more similarly to if there was only a single fluorophore. The pixels that were plotted appear in purple in the cell image – their distribution throughout the cell matches C3PO's presence throughout the cytosol.

The average lifetime in the phasor plots were then taken and made into a distribution graph, where the actual change in fluorescence lifetime could be compared from the first 3 minutes to the last data point taken at 20 minutes. This increase is due to

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the photons emitted from FAM unable to excite TAMRA as the distance between the two
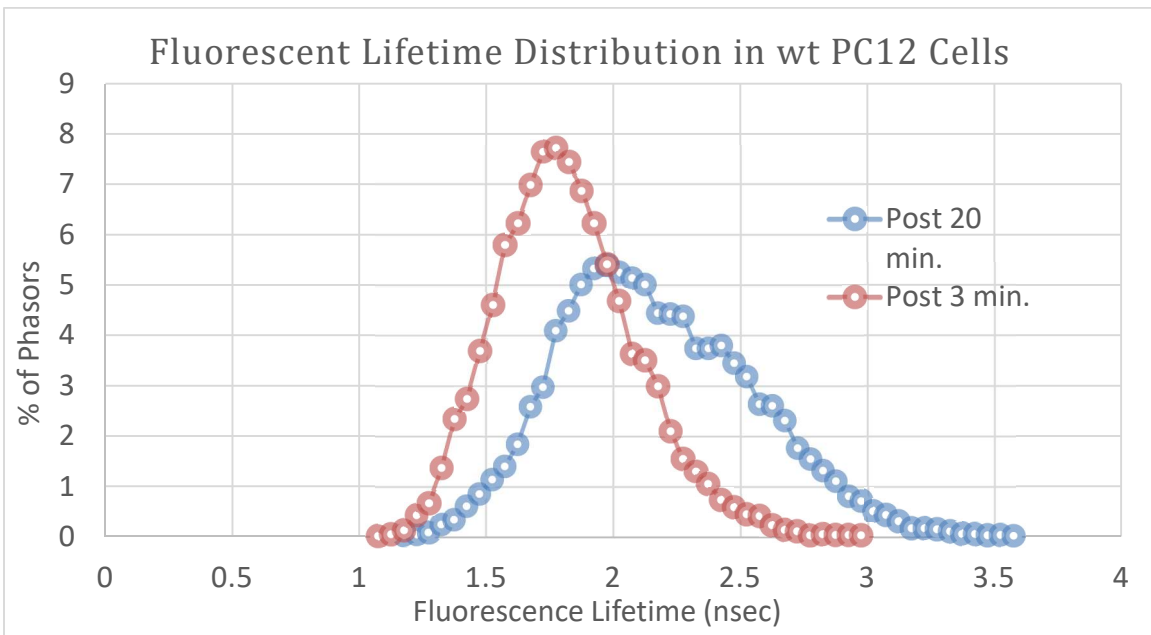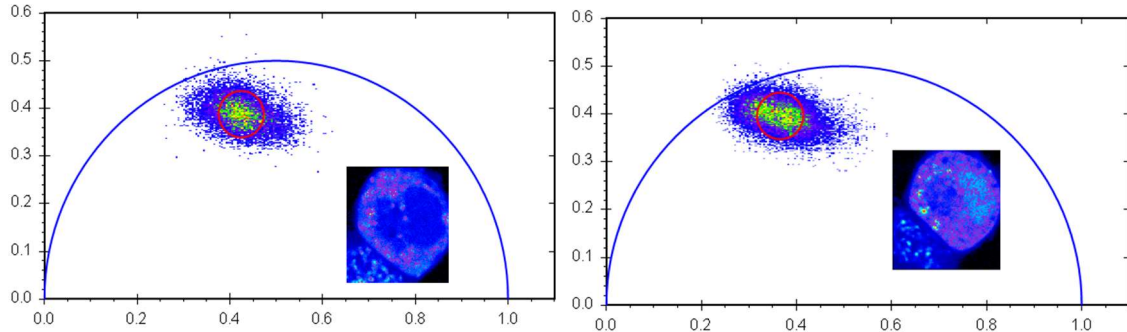fluorophores increases with hydrolysis.

Figure 8 - Top Left shows the phasor plot of wild type PC12 Cell injected with the fluorescent nucleotide after three minutes, the top right panel shows the same cell after 20 minutes. Below is a lifetime distribution graph showing the most frequent lifetimes

Looking at the phasor diagram from PLCβ1 knock down cells, it's clear that the

shift towards the central arc is more drastic. This fits the model of PLCβ inhibiting the

hydrolysis of microRNAs. With C3PO left unchecked, the degradation of

oligonucleotides is very rapid and FRET is rapidly eliminated. The oligonucleotides are

also seen fluorescing evenly throughout the cell, again consistent with C3PO's location.

Consistent with this data is the Lifetime Distribution graph (**Figure-9**), which shows a

much more drastic shift over the course of 20 minutes – FAM's lifetime extends to a 2.5 nsec, vs the almost 2.0 shown in wt lifetime after 20 minutes.
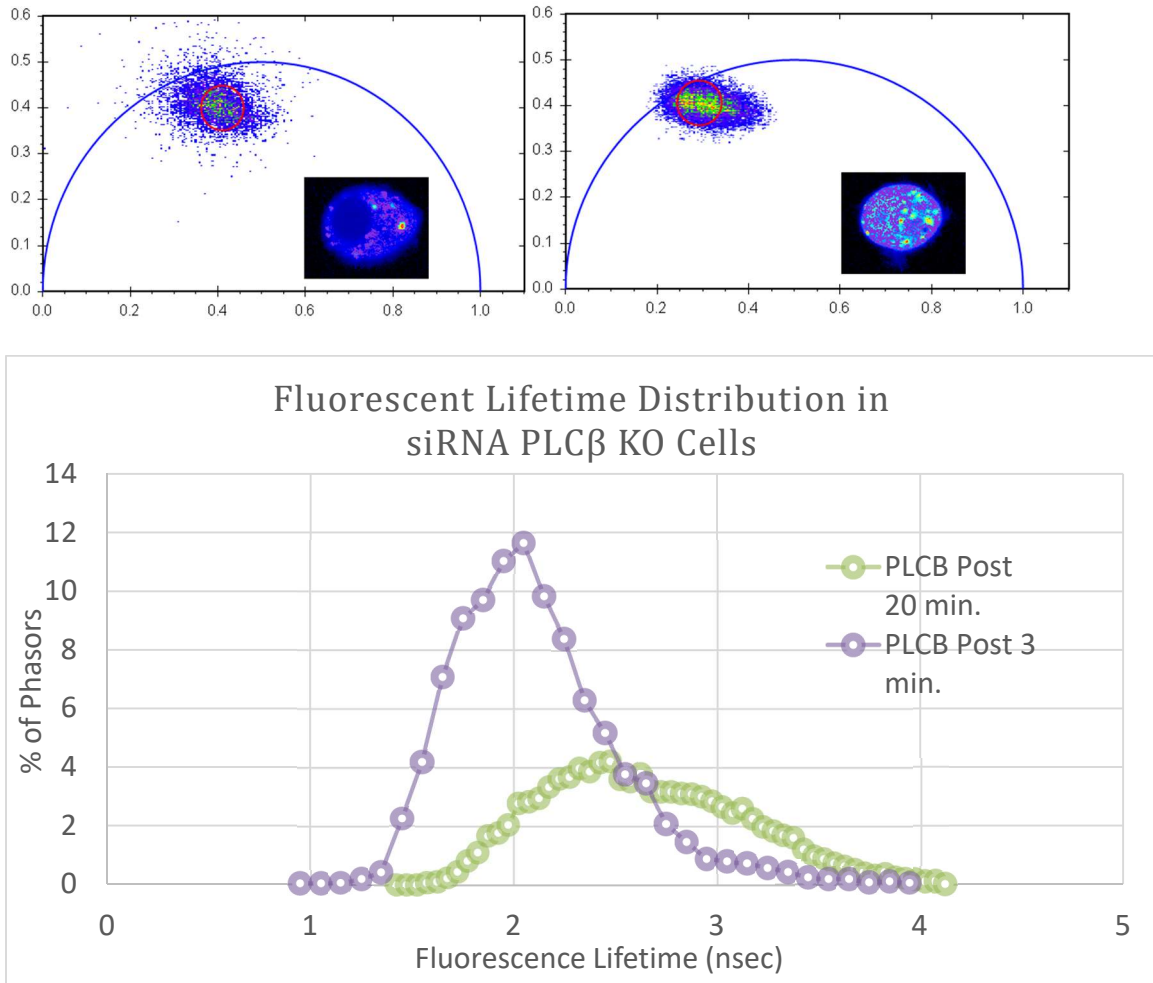

Figure 9Top Left shows the phasor plot of PLCβ Knock Down PC12 Cell injected with the fluorescent nucleotide after three minutes, the right shows the same cell after 0 minutes. Below is a lifetime distribution graph showing the most frequent lifetimes

In cells where TRAX was knocked–down (**Figure-10**), the fluorescence lifetime remained beneath the central arc on the phase diagram, meaning FRET was maintained and the oligo was not hydrolyzed as effectively. Interestingly, the limited number of points in the phasor plot that did fluoresce at 520 nm were all found in isolated areas in the cell, suggesting high levels of localization inconsistent with C3PO's behavior. Furthermore, in the lifetime distribution graph, lifetime was not shown to increase, but

remained below 1.5 nsec; a stark difference from the increasing trends viewed in previous trials.
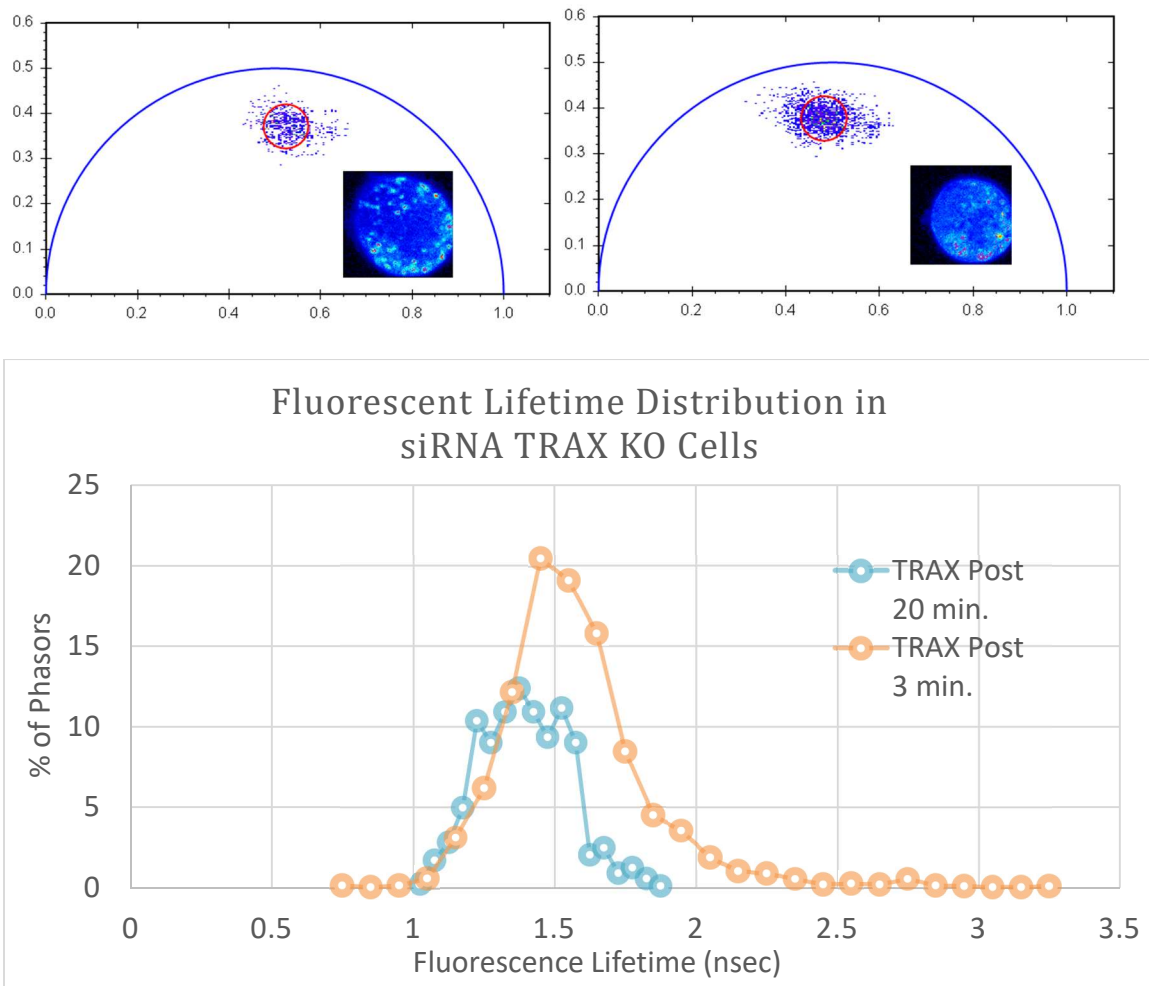

Figure 10 - Top Left shows the phasor plot of TRAX Knock Down PC12 Cell injected with the fluorescent nucleotide after three minutes, the right shows the same cell after 20 minutes. Below is a lifetime distribution graph showing the most frequent lifetimes

The interaction between PLCβ1 and C3PO is given further clarity by experiments testing their interactions in vitro. By mixing the two proteins and oligonucleotide in a cuvette, the observed reaction cannot rely on external factors such as Ago2. In order to ensure the validity of the results, we first tested the purity of our protein samples. The result of a protein gel stained with coomassie blue for PLCβ1 (**Figure-11**). Single bands appear in the appropriate location, indicating the purification was successful.
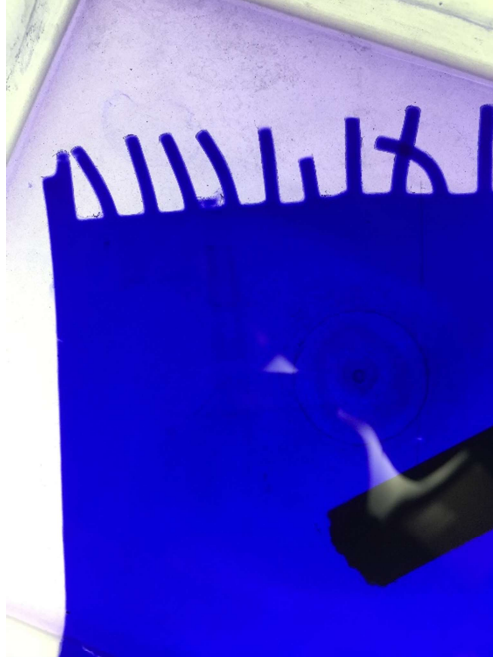
Figure 11 – Protein Gel results from the purification of PLCβ1, the dark colour was a result of overstaining, but the two bands to the left of the ladder are still visible.

A similar protein gel was performed to determine the purity of C3PO (**Figure-12**), however this gel was performed in SLS buffer, which denatures C3PO into its components translin and TRAX. The results are consistent with this, as we see two similarly sized bands in the appropriate location next to the ladder.
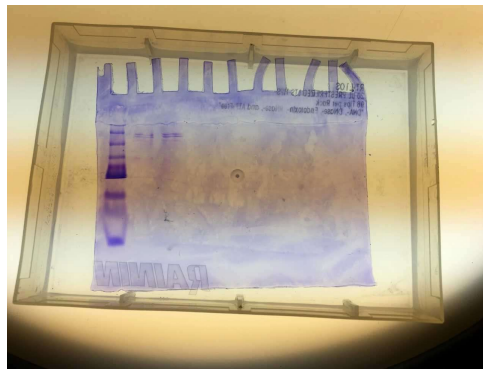

Figure 12 - The results from the C3PO protein gel - note the two bands consistent with the tertiary structure - 6 Translin and 2 TRAX

Evaluating the fluorescence over time in terms of the rate at which the ratio between fluorescence intensity at 520 and 590 increased was used as a barometer for how

fast C3PO hydrolyzed the oligonucleotide when C3PO was allowed to interact with the

oligonucleotides for three minutes before the addition of PLCβ1, or when PLCβ1 was

added three minutes before C3PO. Raw data for a trial of each condition can be seen in

Figure 13 below. The increase in 520 expression demonstrates C3PO's ability to

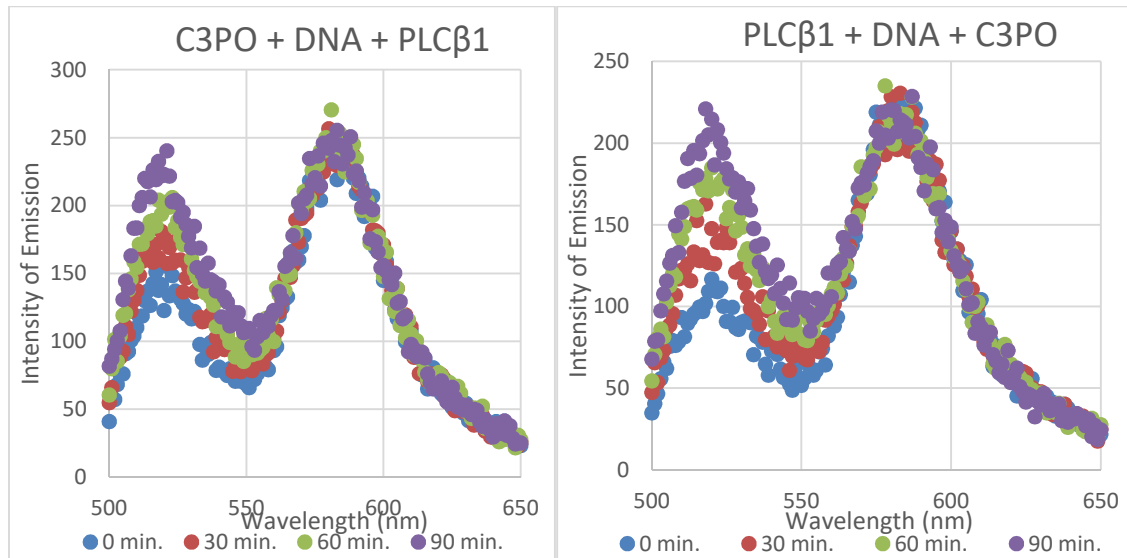hydrolyze nucleotides without external factors.

Figure 13 – Left: Raw data from trial one of the fluorometer when C3PO and DNA were allowed three minutes to react before PLCβ1 was added. Right: Raw data from trial one of the fluorometer when PLCβ1 and DNA were allowed three minutes to react before C3PO was added.

While the intensity is highly variable, the rate of change in intensity is a more

consistent indicator of hydrolysis and was found to be consistent between multiple trials.

After trial one, rather than compiling the 150 points from every reading, a 10 point

moving average was taken around both 520 and 590 nm. The ratio of these two averages

was plotted over time, producing a linear trend from which we could better compare the

rate of hydrolysis between the different conditions, and gauge similarity between trials
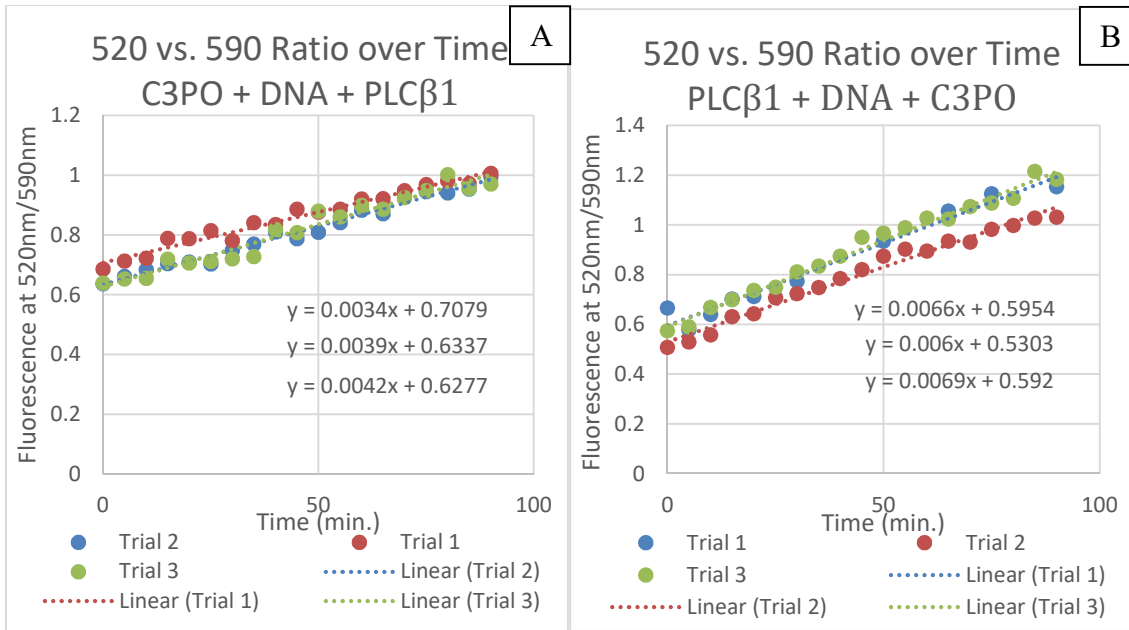
(**Figure 14**).

Figure 14 - Left: The rate the ratio of fluorescence intensity at 520 nm/590 nm when C3PO was exposed to DNA first. Right: The same ratio except when PLCβ1 is added first

The average rate of change is shown in **Figure 15** below, where it can be clearly visualized that PLCβ1 has a greater impact after C3PO binds to the nucleotides. This difference passed the 95% confidence interval producing a P-value of 0.0026, suggesting PLCβ1 inhibits the most effectively when C3PO is active.
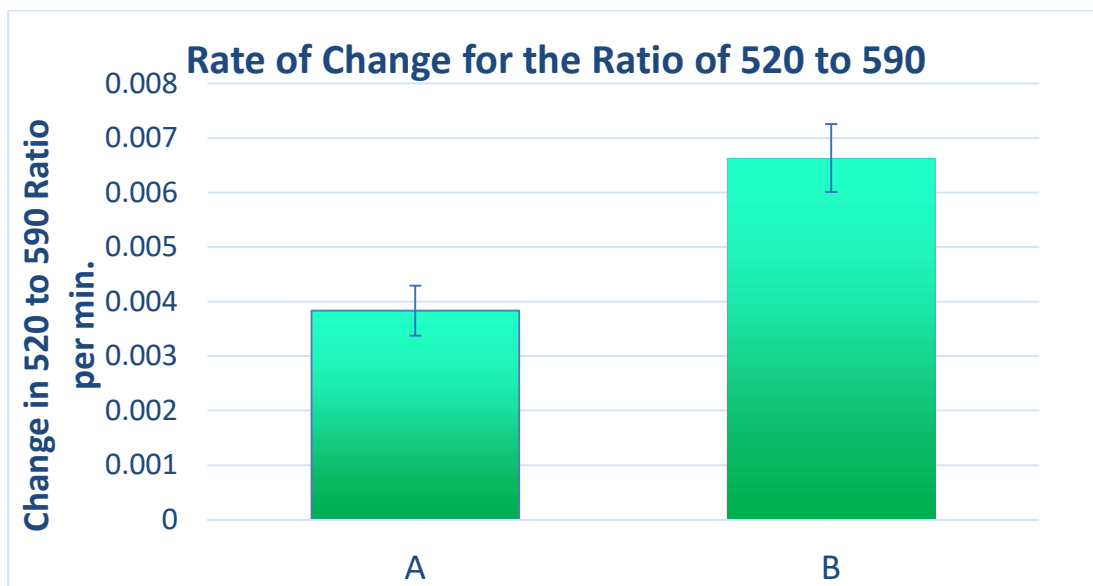

Figure 15 - A comparison of the average change in fluorescence intensity emitted at 520/590 nm, an indicator for how quickly the oligonucleotides are being hydrolyzed by C3PO

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DISCUSSION

Demonstrating a definitive association of PLCβ1 and C3PO in this project is a bridge that connects G protein coupled receptors, RNA silencing and cell differentiation and will develop a better map for how learning occurs in mammals. Through our experiments, we were able to develop an even clearer idea of how the two proteins work together.

In line with our lab's previous findings, we were able to observe C3PO hydrolyzing oligonucleotides without Ago2, allowing the protein to have functions outside the stress granules and RICS where Ago2 resides. The project's further discovery that C3PO is better inhibited by PLCβ1 when oligonucleotide is already bound grants us a more detailed model for how inhibition occurs. This also opens up the possibility that PLCβ1 selectively inhibits C3PO based upon the sequence of nucleotides it has bound. However, we did not test the variety of miRNAs that they both interact with, or whether nor did we test a perfect pairing of nucleotides is required.

We were able to demonstrate that the hydrolysis rates of oligonucleotide in untreated cells are between values observed when PLCβ1 was knocked down and C3PO was knocked down. This result shows they both are required to maintain normal gene regulation. This in conjunction with the previous studies that demonstrated that learning is dependent on G_q , C3PO components as well as the observation that C3PO and PLCβ1 are required for PC12 differentiation (Anagnostaras et al., 2002, Park et al., 2017, Garwain and Scarlata 2016). These results help build the framework for how all these parts are involved in neuronal differentiation in mammals

Unfortunately, we were unable to test whether or not activating GPCRs would increase the rates of hydrolysis as it draws PLCβ1 out of the cytosol. If this was the case, it would directly link the neurotransmitter's external GPCR-mediated signal to the post transcriptional gene regulation that C3PO conducts. Until this is investigated, we cannot say conclusively that this relationship exists, although we remain confident it serves as the bridge between neurotransmitters and gene regulation, completing this pathway.

The only experiment that did not behave as expected was the solution FRET of miR hydrolysis study looking at the intensity of the 520nm and 590nm peaks. Based upon our model, we believed that 590nm should decrease as 520nm increased correlating to a reduction in FRET, but instead the signal at 590nm remained constant. This is likely due to quenching that occurred as a result of cutting of the oligonucleotides, although this remains uncertain. As lifetime tends to give more consistent results with less interference, further trials should be done with a modified procedure that utilizes the confocal microscope to perform in vitro trials.

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