Purification and Association of PLCβ1, C3PO and Ago2

A Major Qualifying Project Report Submitted to the faculty of WORCESTER POLYTECHNIC INSTITUTE in partial fulfillments for the requirements for the degree of Bachelor of Science in Biochemistry by

Sara Stillings

This report represents the work of one or more WPI undergraduate students submitted to the faculty as evidence of completion of a degree requirement. WPI routinely publishes these reports on the web without editorial or peer review.

Date:
4/28/2022
Project Advisor:
Professor Suzanne Scarlata

Table of Contents

Abstract 2

Acknowledgements 3

Introduction 4

Materials and Methods 6

Cell Culturing 6

Fluorescence Lifetime Imaging Measurements (FLIM) 6

Ago2 Transfection 6

Ago2 Purification 6

Western Blot 7

Results 8

Ago2, PLCβ1 and TRAX interactions 8

Overexpression in Loren cells 12

Ago2 Overexpression and Purification 13

Conclusions and Future Directions 16

References 17

Abstract

Phospholipase C β1 (PLCβ1) is a protein involved in a signal transduction pathway that causes a cell to release Ca^{2+} into the cytoplasm as a result of G protein activation. Argonaute 2 (Ago2) is a multifunctional protein involved in RNA silencing leading to mRNA degradation and is incorporated into protein-mRNA aggregates called stress granules (SGs). Stress granules help cells survive stressful environmental conditions by protecting mRNA and proteins from degradation. Component 3 promoter of RISC (C3PO) is a member of the RNA induced silencing complex (RISC) that is composed of TRAX and translin subunits. PLCβ1 binds to both C3PO and Ago2 in the cytosol to inhibit RNAi activity and mediate stress granule aggregation, respectively. Here we show evidence of interaction between these proteins and we have implemented a procedure in which proteins could be purified to investigate the molecular details of their association.

Acknowledgements

I would like to thank Professor Suzanne Scarlata for giving me the opportunity to do this project in her lab and for all her support and feedback throughout this year. I would also like to thank Lela Jackson for her advice and patience and for teaching me the skills I needed to complete this project throughout the year. I would like to thank the past and present members of Scarlata and Gericke's lab for their welcoming and kind energy. I would like to give thanks to Worcester Polytechnic Institute for providing me the chance to work in Scarlata's lab and for the experiences I have had and the topics I have learned throughout the past four years. Lastly, I would like to thank my friends and family for supporting me and especially for helping me out with any lab mishaps that might've happened during the course of the year.

Introduction

G-protein coupled receptors (GPCRs) are a group of transmembrane receptors.[1] These proteins respond to many different signals or neurotransmitters, causing a signal transduction. When a ligand binds to a GPCR, G proteins can be activated with the exchange between GDP to GTP. When one specific G protein, Gαq, becomes activated, it increases its affinity to phospholipase C β1 (PLCβ1). PLCβ1 then hydrolyzes phosphatidylinositol 4,5 bisphosphate (PIP_2) into diacylglycerol (DAG) and 1,4,5 inositol trisphosphate (IP_3). IP_3 then diffuses to the endoplasmic reticulum where it binds to a receptor to release Ca^{2+} into cytoplasm. This calcium release can lead to numerous activities and responses.[3] PLCβ1 plays an important role on the plasma membrane, however, it should be noted that PLCβ1 is also present in the cytosol. However, its presence in the cytosol is not well understood as its only regulator is Gαq, which only appears at the plasma membrane. This is why looking into the possible other roles of PLCβ1 is of high interest.

Argonaute 2 (Ago2) is a protein that is a component of the RNA-induced silencing complex (RISC).[5] When RISC has bonded to a double stranded RNA, siRNA is degraded, leaving the guide strand to find the matching mRNA. Once this match is found, the mRNA is then cleaved by Ago2 to begin its degradation by C3PO. The component 3 promoter of RISC (C3PO) is an activator of RISC and is made of two subunits: translin and TRAX. This activator has been observed to bind Ago2 when mRNA is being cleaved, showing it is an essential component for this process.[6]

Stress granules (SGs) are membraneless pockets of stalled mRNA. These aggregates are present from many environmental stresses, such as heat or osmotic stress, until stressors have been alleviated. It is believed that these SGs help cells survive environmental conditions. It has been observed that Ago2 has been present in these stress granules and it has been concluded that these stress granules are formed when there is not a match between the mRNA and the guiding strand in the RISC complex.

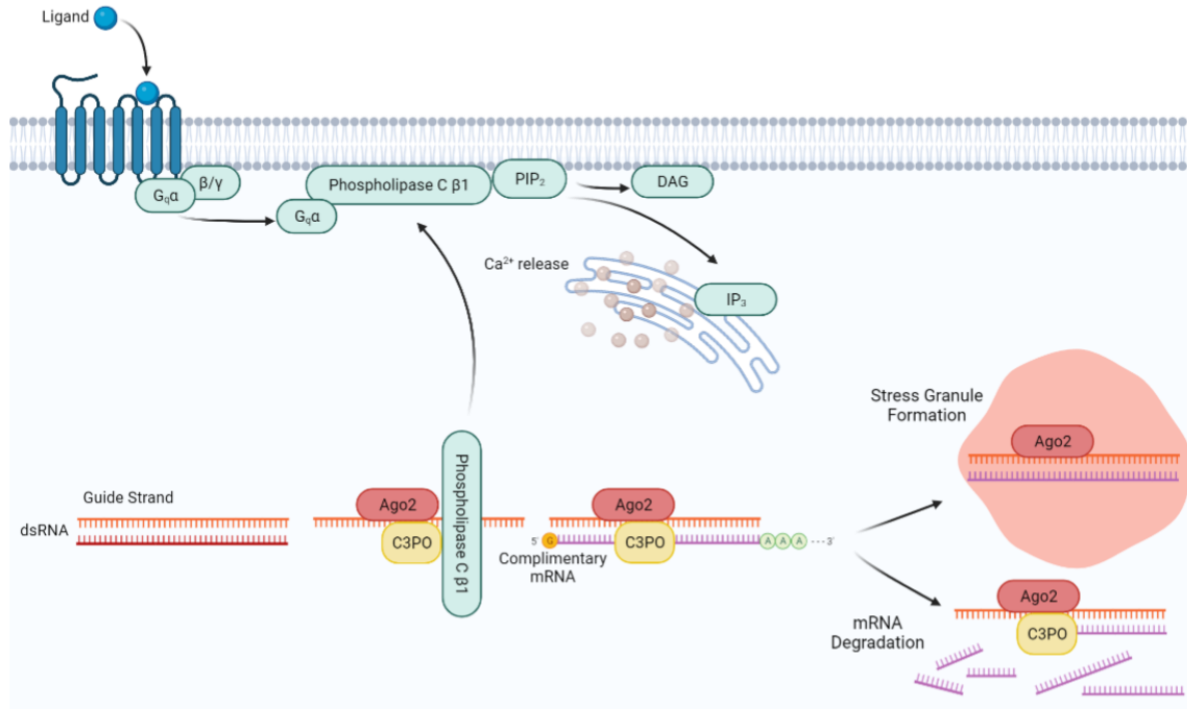

Figure XX. *An image of the pathways that both involve PLCβ1. The top blue and green pathway follows the GPCR pathway that leads to the release of* Ca^{2+}*. The orange, red and purple pathway follows the RNA silencing pathway that leads to the degradation of mRNA. It also presents Ago2 encapsulating SGs formation. In the RNA silencing pathway, a portion of the RISC complex bound to PLCβ1 can be observed.*

Preliminary data from our group suggests that PLCβ1, Ago2 and C3PO are associated together in the cytosol. PLCβ1 seems to have a high affinity for TRAX and could help with mediating stress granule formation.[3] However, the reason for PLCβ1 to be binding to Ago2 and C3PO is not obvious and it would be best to observe these proteins in better detail. This points to the importance of making a procedure to purify both PLCβ1 and Ago2 proteins. This would help to better understand their interactions with C3PO and to observe a cell's behavior or response environment stressors and what these proteins' roles are to those stressors.

Materials and Methods

Cell Culturing

The cell lines used were PC12 cells and HEK-293 cells obtained from American Tissue Cell Culture and HEK-293-TAP-PLCβ1 cells ("Loren's cells") were gifted from Dr. Loren Runnels, which express PLCβ1 using a Flp-In system (Invitrogen). To culture the PC12 cells, the media was made up of Dulbecco's Modified Eagle Medium (DMEM) with 10% horse serum HI, 5% fetal bovine serum (FBS) and 1% pen strep. To differentiate, Dulbecco's Modified Eagle Medium (DMEM) with 1% horse serum HI and 1% pen strep with the addition of nerve growth factor (1 μg/mL) from Bon Opus Biosciences was used. For Loren's cells, regular HEK293 cell media was used by combining Dulbecco's Modified Eagle Medium (DMEM) with 10% FBS and 1% pen strep. To induce Loren's cells into overexpress PLCβ1, the medium would be supplemented with 1μg/mL tetracycline for at least 60 hours before being collected.

Fluorescence Lifetime Imaging Measurements (FLIM)

To perform FLIM, PC12 cells were split into 2 mL glass bottom dishes, transfected with fluorescent tagged proteins and differentiated. Two days later, FLIM measurements were performed on the dual-channel confocal fast FLIM (Alba version 5; ISS Inc.) equipped with photomultipliers and a Nikon Eclipse Ti-U inverted microscope. A 60× Plan Apo (1.2 numerical aperture, water immersion) objective and a mode-locked, two-photon titanium-sapphire laser (Tsunami; Spectra-Physics) were used in this study. The lifetime of the laser was calibrated each time before experiments by measuring the lifetime of Atto 435 in water with a lifetime of 3.61 ns (reference) at 80, 160, and 240 MHz. The samples were excited at 800/850 nm, and emission spectra were collected through a 525/50 band-pass filter. For each measurement, the data were acquired until the photon count was >300. Fluorescence lifetimes were calculated by allowing ω to be equal to 80 MH.

$$\tau = \frac{S}{G * 2\pi * \omega}$$

For FLIM, plasmids used were:

eGFP-PLCβ1

eGFP-hAgo2

mCherry-TRAX

Ago2 Transfection

The pCIneo-HA-Ago2 was purchased from Addgene, and made in the lab of Witold Filipowicz & Ramesh Pillai (Addgene plasmid # 115362 ; http://n2t.net/addgene:115362 ; RRID:Addgene_115362). PC12 cells were transiently transfected with the hAgo2 plasmid with Lipofectamine 3000 (Invitrogen, Inc.) as instructed by the manufacturer in antibiotic free medium.

Ago2 Purification

To purify the Ago-2 protein, Pierce Anti-HA Magnetic beads (Thermo Scientific 88838) were used. To start, PC12 cells would be transfected and then incubated overnight. The medium was

changed to one containing antibiotics and cells were incubated for an additional 24 to 48 hours. The following procedure is following the instructions for the Pierce Anti-HA Magnetic Beads. Magnetic beads were washed with Lysis/Wash buffer (0.025M Tris, pH 7.4, 0.15M NaCl, 0.001M EDTA, 1% NP40, 5% glycerol, protease inhibitor cocktail) two times. Lysate is then added and incubated with beads for 30 minutes. Beads are collected to the side of the tube with a magnetic stand and supernatant is removed. Beads are then washed with Lysis/Wash buffer two more times, with supernatant discarded both times. To elute the protein, beads are washed with an elution buffer (0.1M glycine, pH 2.0) and then neutralized with a neutralizing buffer (1M Tris, pH 8.5).

Western Blot

For the western blot, the percent of SDS gel made depended on the molecular weight of the protein. For Ago2, the SDS percentage was 10% while for PLCβ1, the SDS percentage was made to be 8%. The protein samples were mixed with a sample buffer and then boiled at 70°C for 10 minutes. The samples were then loaded into the SDS gel, as well as a ladder. The protein on the gel was then transferred onto a nitrocellulose membrane (Bio-Rad, Inc.). The membrane was then blocked with 5% milk in TBST for an hour, and then incubated overnight with the protein tagged primary antibody. The primary antibodies used were Anti-Ago2 antibody (Abcam, ab32381) and Anti-PLC β1 Antibody (Santa-Cruz Biotechnology Inc., sc5291). After three 10 minute washes in TBST, the membrane was incubated with a secondary antibody and then washed again in TBST before imaging with ECL solution. The secondary antibodies used were mouse anti-rabbit IgG-HRP (Santa-Cruz Biotechnology Inc., sc2357) and an anti-mouse secondary antibody. To perform a control, actin would be tagged, and the antibody used was Anti-β-Actin antibody (Santa-Cruz Biotechnology Inc., sc-47778).

Results

Ago2, PLCβ1 and TRAX interactions

It has been seen that Ago2, PLCβ1 and TRAX (a component of C3PO) all interact with each other, but this was needed to be confirmed. To observe protein-protein interaction, fluorescent lifetime imaging measurements (FLIM) can be used. For FLIM, interactions between Ago2 and TRAX were observed as well as PLCβ1 and TRAX.

To observe Ago2 and TRAX interactions, Ago2 had a GFP tag and TRAX had a mCherry tag. Because mCherry has a lower excitation wavelength, the lifetime should decrease if there is an interaction between the two proteins.

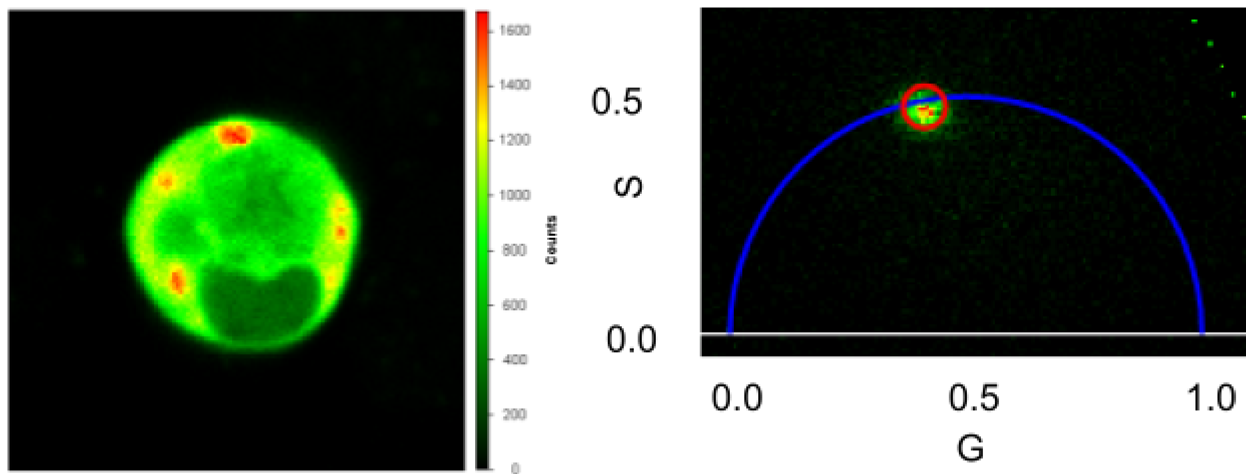

Figure XX. *Live cell image with Ago2 tagged with GFP in a differentiated PC12 cell and the accompanied phasor plot. The average lifetime was around 2.22 ns for this specific cell.*

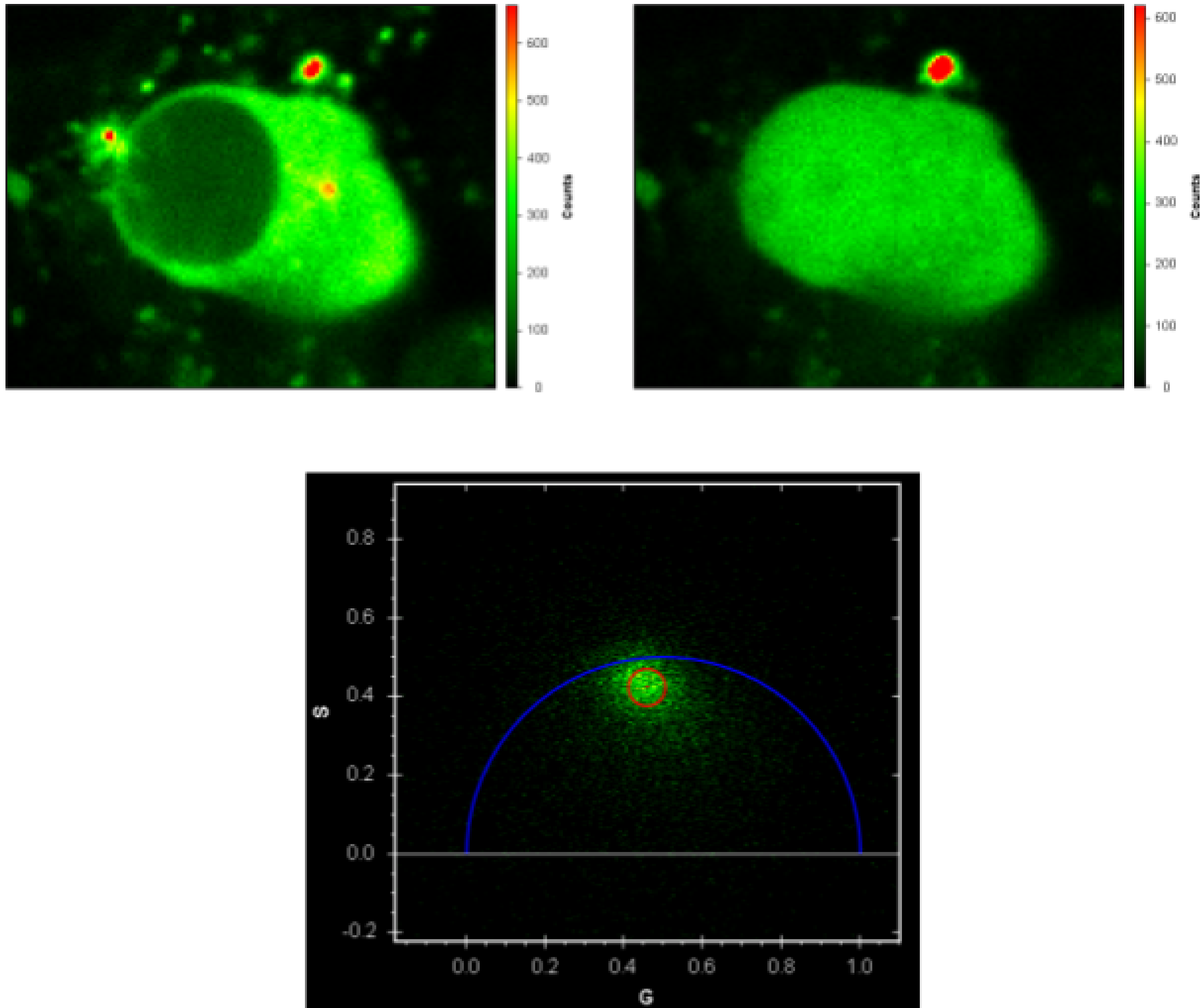

Figure XX. *Live cell image with Ago2 tagged with GFP (left) and TRAX tagged with mCherry (right) in a differentiated PC12 cell and accompanied with its phasor plot. The average lifetime was around 1.87 ns for this specific cell. It can also be noticed that in the Ago2 image, there is a clear Ago2 in the cytosol, yet this high concentration cannot be seen with TRAX. This may point to an Ago2 stress granule being present.*

Within the images of cells with both tagged Ago2 and TRAX, there were some with high concentrations of Ago2 in specific spots, but the TRAX image did not match these spots (such as in the figure above). This suggests possible stress granules being formed in some of the cells. This also confirms that C3PO is not present in these Ago2 stress granules when they form.

To observe PLCβ1 and TRAX interactions, PLCβ1 had a GFP tag and TRAX had a mCherry tag. And so, as before, the lifetime should decrease if there is an interaction between the two proteins.

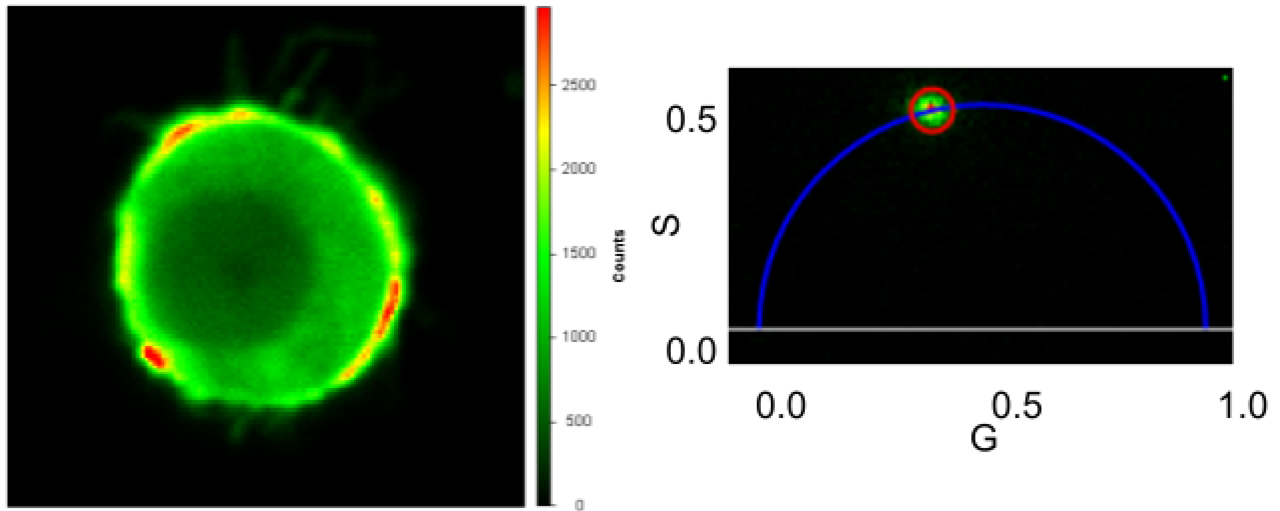

Figure XX. *Live cell image with PLCβ1 tagged with GFP in a differentiated PC12 cell and the accompanied phasor plot. The average lifetime was around 2.46 ns for this specific cell. The concentration of PLCβ1 is very high on the plasma membrane, as seen with color coordinating to the higher count values on the outline of the cell, however, it can be seen there is a cytosolic population of PLCβ1.*

Within the images of cells with PLCβ1 tagged, it can be noticed that these cells have a high concentration of the protein on the plasma membrane (as seen with the bright ring on the edge of the cell), however with there still being a brightness within the cell, this confirms a protein population within the cytosol in the cell.

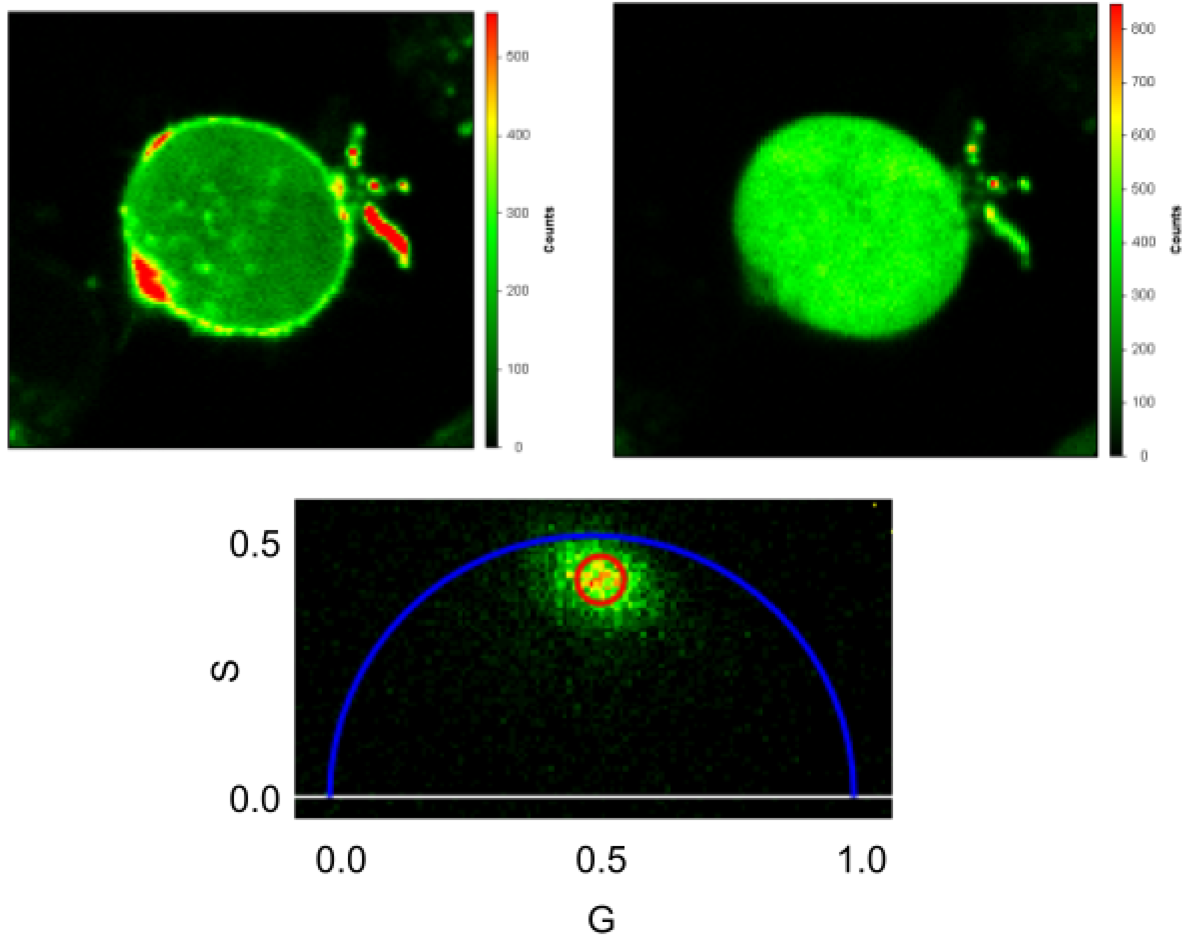

Figure XX. *Live cell image with PLCβ1 tagged with GFP (left) and TRAX tagged with mCherry (right) in a differentiated PC12 cell and accompanied with its phasor plot. The average lifetime was around 1.57 ns for this specific cell.*

Overall, the average lifetime in cells with just Ago2 was 2.26 ns and the average lifetime in cells with Ago2 and TRAX was 1.98 ns. This is quite a significant drop and even in the figure below, the error bars do not cross, showing it is significantly different. A similar trend can also be observed with the PLCβ1 and TRAX FLIM results. The average lifetime in cells with just PLCβ1 was 2.46 ns while, when TRAX is also present, the lifetime drops to 1.95 ns. These drops in lifetime confirm there is protein interaction between Ago2 and TRAX as well PLCβ1 and TRAX.

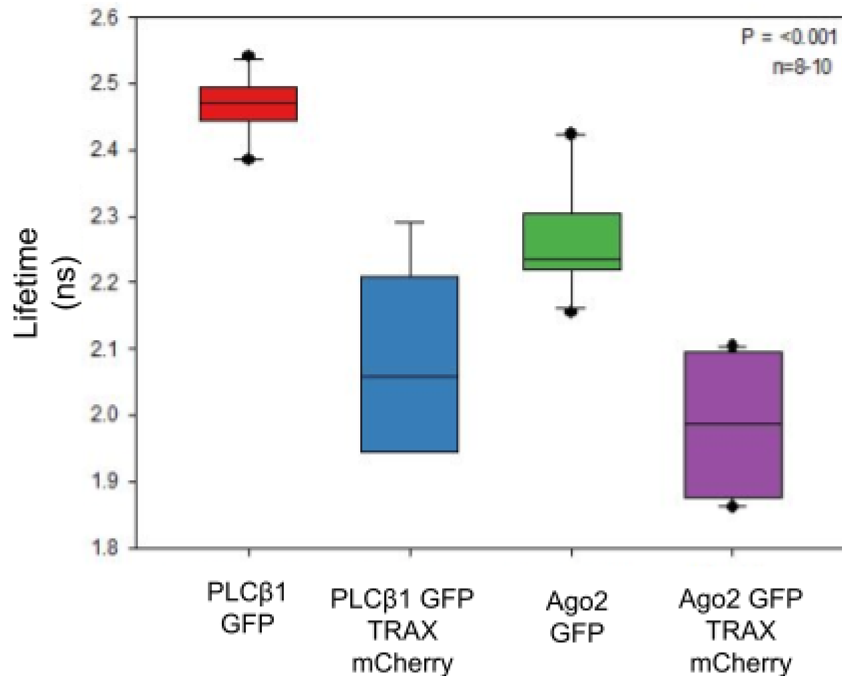

Figure XX. *Graph of the fluorescence lifetime measurements with PLCβ1 and Ago2 and both interacting with TRAX in differentiated PC12 cells. Both PLCβ1 and Ago2 were tagged with a GFP protein, while TRAX was tagged with a mCherry protein. It can be observed that there is a significant drop in fluorescence lifetime between PLCβ1 and PLCβ1 with TRAX, showing that there is an interaction occurring between PLCβ1 and TRAX. Similarly, there is a significant drop in fluorescence lifetime between Ago2 and Ago2 with TRAX, showing that there is an interaction also occurring between Ago2 and TRAX.*

Overexpression in Loren cells

To ensure that Dr. Loren Runnels's cells did in fact overexpress PLCβ1, a western blot was performed. Quantitatively, there is a significant increase in the boldness of the line between with and without tetracycline present, while the actin images have less of a difference.

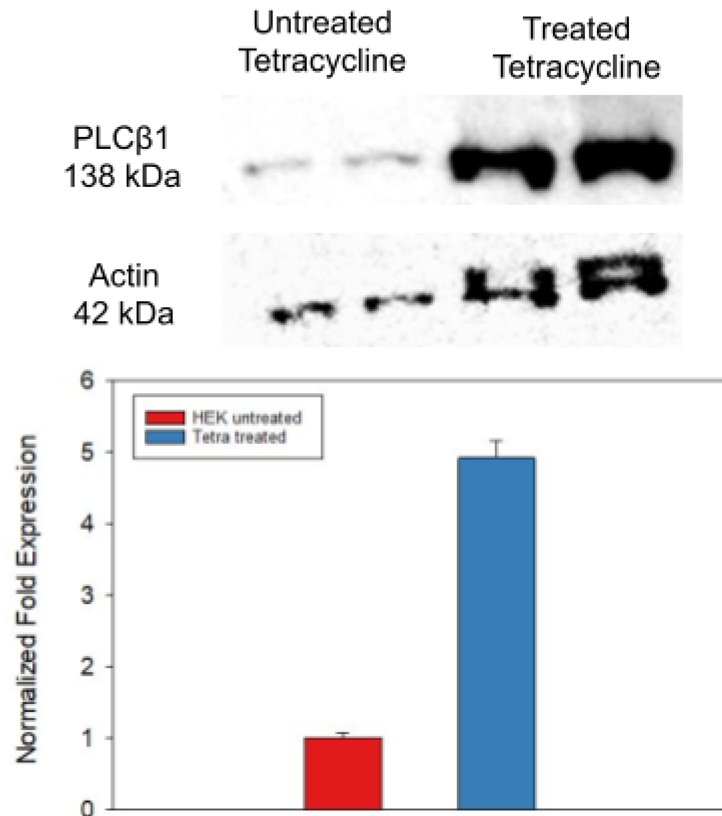

Figure XX. *Graph of the amount of PLCβ1 in Loren cells treated with and without tetracycline for 60 hours and western blot images of PLCβ1 and Actin tagged. A western blot was used to measure the protein amounts. The expression was normalized with the amount of β-actin present.*

After normalizing the expression of PLCβ1 with the actin bands, it can be observed that there is about a five fold increase of expression of PLCβ1 in the Loren cells in the presence of tetracycline.

Ago2 Overexpression and Purification

Before purifying Ago2, it was best to determine which cell line would be best for production of Ago2. It is well known that HEK293 are a suitable cell line for protein expression, but PC12 cells have been in use in the lab, and so a comparison of their protein production was needed.

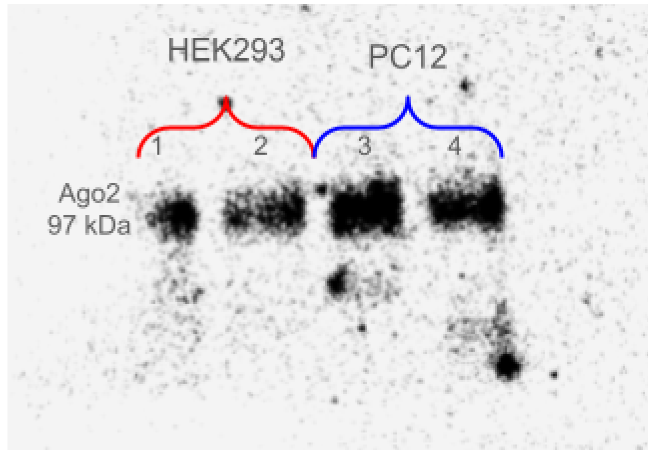

Figure XX. *Expression of Ago2 in HEK293 and PC12. Fractions 1 and 3 were not transiently transfected with the Ago2 plasmid.*

When performing the blot, the control used was β-actin, however, it did not seem to appear very well from each blot. However, a Bradford Assay was run before running the western, which ensured the protein concentrations for each sample would be similar. This allows a side by side comparison of the protein expression of Ago2 in HEK293 and PC12 to be done. It can be seen in the western blot image that there seems to be slightly better expression of Ago2 in the PC12 cells over the HEK293 cells, which made us pick to use PC12 cells to purify Ago2.

The purification of Ago2 was then implemented in PC12 cells. Using Pierce's Anti-HA magnetic beads allowed the purification to be done very quickly. After purification, a western blot was run to ensure Ago2 had been successfully purified.

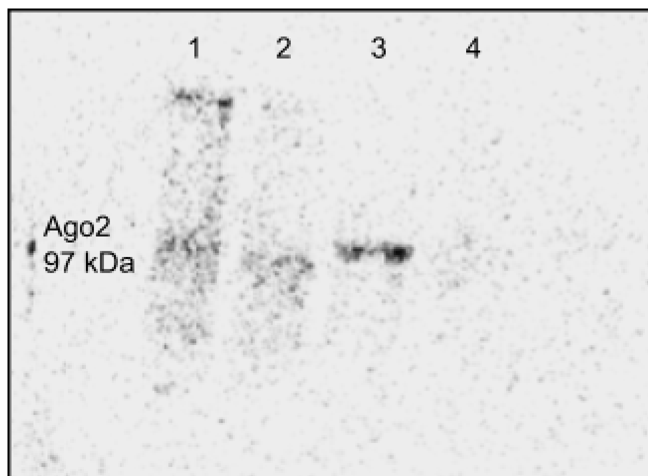

Figure XX. *Western blot image of the fractions of Ago2 purification. It can be observed that both fractions 2 and 3 appear to have a large amount of Ago2. Fraction 1 seems to have a protein streak and it is unclear why this would be the case.*

Within the western blot, it can be seen that fractions two and three have strong bands, which indicates purified protein. However, within the first band, there seems to be a long streak, or at least two bands. What this streak means is unknown but could be looked into another time. But, based on the western blot, it can be concluded that Ago2 has been successfully purified from PC12 cells.

Conclusions and Future Directions

The cytosolic roles of PLCβ1 still need to be explored. With the current knowledge that it does interact with the RISC complex, more specifically Ago2 and C3PO, but why it does is still a question to look into. This study into purifying these proteins was made to support future endeavors of looking into the interactions of them.

With the confirmation of the FLIM results, not only do Ago2 and C3PO interact, and the same can be said with PLCβ1 and C3PO, but that C3PO also does not appear to be present in the Ago2 stress granules. It can also be assumed that PLCβ1 is also not present in these stress granules, however, in order to confirm this, it would be best to purify these proteins to observe their bonding behaviors or to perform more FLIM experiments with PLCβ1 and Ago2 having different fluorescent tags.

It has also been found that with the presence of Gαq, that TRAX seems to have an increase in concentration in the nucleus.[4] It would be of interest to observe the movement or concentrations of other proteins involved in the RISC complex in the presence of different signals, whether that be Gαq or possibly other ones. Another would be to look at the FLIM results of these proteins also with higher concentrations of other signals from the GPCR pathway as well.

By purifying Ago2, studies into its interactions can now be delved into more so. With the ease of the magnetic beads, the purification process for Ago2 is relatively simple, allowing for accessibility of receiving high volumes of Ago2. This then allows observers to use purified Ago2 to see changes in behavior of PLCβ1 or C3PO in the presence of high concentrations of Ago2. This can also allow a better look into the crystal structure of Ago2 and its interaction with mRNA.

There still needs to be a successful procedure for the PLCβ1 purification. With the knowledge that the Loren cells do in fact overexpress PLCβ1 by a five fold amount, it can now allow a more successful purification process with the use of them. It has been proven that PLCβ1 can be purified from Loren cells.[4] By using that procedure, one can hopefully successfully purify PLCβ1, allowing for better observations of the protein.

Through this research, this lays the support for future endeavors into PLCβ1 with Ago2 and C3PO. There is much to learn about the interactions between the RISC complex and PLCβ1, and with the steps to have purified protein laid out allows for ease to study these.

References

1. Adams, J. U. In *Essentials of Cell Biology*; O'Connor, C., Ed.; NPG Education: Cambridge, MA, 2010.
2. Garwain, O.; Pearce, K. M.; Jackson, L.; Carley, S.; Rosati, B.; Scarlata, S. Stimulation of the Gαq/Phospholipase CB1 Signaling Pathway Returns Differentiated Cells to a Stem‐like State. *The FASEB Journal* **2020**, *34* (9), 12663–12676.
3. Jackson, L.; Qifti, A.; Pearce, K. M.; Scarlata, S. Regulation of Bifunctional Proteins in Cells: Lessons from the Phospholipase CB/G Protein Pathway. *Protein Science* **2019**, *29* (6), 1258–1268.
4. Philip, F.; Guo, Y.; Aisiku, O.; Scarlata, S. Phospholipase CB1 Is Linked to RNA Interference of Specific Genes through Translin‐Associated Factor X. *The FASEB Journal* **2012**, *26* (12), 4903–4913.
5. Pillai, R. S.; Artus, C. G.; Filipowicz, W. Tethering of Human Ago Proteins to Mrna Mimics the MIRNA-Mediated Repression of Protein Synthesis. *RNA* **2004**, *10* (10), 1518–1525.
6. Qifti, A.; Jackson, L.; Singla, A.; Garwain, O.; Scarlata, S. Stimulation of Phospholipase CB1 by GAq Promotes the Assembly of Stress Granule Proteins. *Science Signaling* **2021**, *14* (705).
7. Sahu, S.; Philip, F.; Scarlata, S. Hydrolysis Rates of Different Small Interfering RNAS (Sirnas) by the RNA Silencing Promoter Complex, C3PO, Determines Their Regulation by Phospholipase CB. *Journal of Biological Chemistry* **2013**, *289* (8), 5134–5144.
8. Thermo Fisher Scientific. (2014). Pierce Anti-HA Magnetic Beads: User Guide. Retrieved from Document Connect (thermofisher.com)