ISSN: 0975 -8542

Journal of Global Pharma Technology

Available Online at: www.jgpt.co.in

RESEARCH ARTICLE

Determine The PH Domaine of PLC-gamma in the Egg of Starfish during Fertilization

Rana Hussein Naser, Alyaa Maan Abdelhameed, Aalhan Mohammed Alwan

University of Diyala\ College of Science\ Department of Biotechnology-Biological Research/Iraq.

Abstract

The PLC gamma function in fertilization has been indispensable over the years of researches. The fertilization and the insight communications with the human beings, plants and animals started from the beginning of human life. In this study, the PH-domain of PLCγ will be fused with GFP and this PH-GFP fusion protein will be used for localization studies in the starfish Patiria miniate.

Keywords: *Domaine; PLC-gamma; egg; Fertilization*

Introduction

Fertilization

Fertilization can be defined as the process in which the two gametes, eggs and sperms interact to start the progression [1, 2]. The mammalian eggs and sperms need to reach the female genitals to further enhance the development of series which will result in the fertilization and eventually in the creation of a new human being [3].

Many researches and medical experiments have identified numerous mammalian eggs and sperms gene which are the contributors for the development of eggs and process of fertilization [4, 5]. Amusingly, the gametes are the products which have molecules in the somatic cells. Runft et al [6].Reported egg activation as a marked process due to which egg returns to the cell-cycle and initiates embryonic development process and consequent studies reported rise in level of free cytoplasmic Ca^{2+} helped egg to return to the cell-cycle [7,10]. The mechanism, signal molecules and techniques involved in this important process are being studied by scientific community from past era to till date [11] and thus providing the opportunity for more future inventions.

Role of PLC γ in Fertilization

Phospholipase C known as PLC is a form of enzymes that slashes phospholipids. This enzyme has considerable role in a variety of pathways for example signaling, metabolic etc and by the hydrolysis of membrane lipid phosphatidylinositol 4, 5-bisphosphate to produce inositol phosphate 3 (IP_3) and diacylglycerol (DAG) (12). DAG and IP_3 are second messengers, which may begin the process of signal transduction by activation of intracellular Ca^{+2} release and protein kinase C and supplementary take part in different signaling pathways [13, 14].

It is most generally taken to be one and the same with the human structures of enzymes, which have an imperative role in eukaryotic cell physiology, in meticulously signaling the transduction pathways. There are thirteen kind of PLC which is classified into six categories of isotypes, including β, γ, δ, ε, ζ, η this is according to the structure [12, 14]. PLC has a vital role in responses to the cellular signals and also to extracellular signals [15].

PH Domains

Pleckstrin homology (PH) domains are small protein units which be found in numerous proteins implicated in cellular development in which membrane fundamentally participates in signaling pathways, vesicular trafficking, and cytoskeletal reorganization [16]. There are numerous PH domains which have been involved in membrane aiming via explicit communications with meticulous phosphoinositides, however in most of examples, the structural source for these interface has not been clarified.

©2009-2019, JGPT. All Rights Reserved

Undeniably, the role for the preponderance of PH domains remains to be recognized. Lemmon et al [17].Studied that PH domain ligands which helps in membrane binding also have interesting roles in interactions of protein with protein and protein with lipid.

Materials and Methods

Bioinformatics

Bioinformatics was used to design primers with BsrG1 restriction site to amplify the PH domain of starfish PLCγ and insert it in pRSETA-PAGFP plasmid to make a PH-GFP fusion protein. First, AmPLCγ full-length cDNA sequence and PH domain were obtained from the NCBI database. Next, Primer1 analysis in Biology Workbench was used to design forward primer and reverse primer to amplify PH domain.

PCR

PCR was used to amplify the PH domain from full-length Am PLCγ plasmid. The table describes sample (PH domain, zero DNA control, forward primer control, and reverse primer control) used for PCR reaction.

	PH domain	Zero DNA control	Forward Primer control	Reverse Primer control
DNA	1 µl	-	1 µl	1 µl
PCR master mix	12.5 µl	12.5 µl	12.5 µl	12.5 µl
Forward primer	3 µl	3 µl	3 µl	-
Reverse primer	3 µl	3 µl	-	3 µl
DI water	5.5 µl	6.5 µl	8.5 µl	8.5 µl

The PCR was run for 40 cycles with annealing temperature at 58°C. When the PCR was finished, the samples were run on a 1% agarose gel

Agarose Gel Electrophoresis

Agarose gel electrophoresis was used to analyze plasmids after PCR, restriction digestion, and dephosphorylation 1% agarose gel was made in 1X TAE buffer (1mM EDTA, 40mM Tris). The contents were heated in microwave until boiling. Next, the contents cooled, then 1µl ethidium bromide was added, and contents were poured in electrophoresis gel mold. A comb was inserted into the gel and the gel was solid ~30 minutes. When the gel was solid, comb was removed, 1X TAE buffer was added to electrophoresis chamber and samples were loaded. The samples were run until the dye migrated ¼ length of the gel, and then electrophoresis was stopped.

DNA Extraction

Plasmid DNA was extracted using a QIAGEN plasmid purification kit. First, 2 ml liquid bacterial culture was grown in LB-media containing ampicillin overnight in 37°C shaking water bath. The next day, 1.5 ml was removed from the culture and was centrifuged for 10 minutes at 13000 rpm at room temperature.

Then, the supernatant was removed and the cell pellet was re-suspended in 100 µl of a 50 mM glucose, 25 mM Tris-HCl, and 10 mM EDTA solution. Next, 200 µl of a basic solution (0.2N NaOH and 1% SDS) was added, the sample was vortexed, and the contents incubated at room temperature for 1 to 2 minutes.

Next, 150 µl of acid solution (3M Potassium Acetate and 11.5% Acetic Acid) and 150 µl of Chloroform/Isoamyl Alcohol (24:1, v/v) were added. The supernatant was removed and placed into a new tube. Next, 95% Ethanol was added at 3X the volume of the sample. The contents were mixed and microfuged. The supernatant was discarded and 500 µl of 70% Ethanol was added to the pellet to wash it. Finally, the pellet was dried, and then re-suspended in 30 µl of EB buffer and 1µl RNAse.

Restriction Digestion and Dephosphorylation

The PH domain and pRSETA-PAGFP plasmid were treated with the BsrG1 restriction enzyme for 1 hour at 37°C, then the PH domain and plasmid were run on a 1% agarose gel. Then the pRSETA-PAGFP plasmid was incubated in 3 µl phosphate buffer and 1 µl phosphatase (CIAP) at 37°C for 1 hour, then 65°C for 15 minutes. Next the dephosphorylated plasmid was run on a 1% agarose gel electrophoresis.

Gel Purification

PH domain treated with BsrG1 and PAGFP treated with BsrG1 and dephosphorylated, were run on a 1% agarose gel. The PH domain bands and PAGFP bands were cut out of the gel with a razor and put in microfuge tubes. The QIAGEN gel purification kit was used to melt the plasmids out of gel.

©2009-2019, JGPT. All Rights Reserved

First, QG buffer three times weight of the gel was added, the samples were vortexed, then heated until the gel dissolved. After checking gel and saw it is yellow, added isopropanol the same weight as the gel and centrifuged for 1 minute at 10000rpm, then discarded the flow-through. Then PE buffer used to wash it and centrifuged for 1 minute at 10000rpm again then discard the flow-through. Add buffer EB to elute DNA into the new tube.

Cloning

PAGFP and PH domain melted from the gel were ligated by incubating them together with T4 DNA ligase at 4°C overnight. Next, *E. coli* DH5α was transformed with PH-PAGFP plasmid.

The transformation procedure: 1) A 2 ml overnight culture of *E. coli* DH5α was mixed with PH-PAGFP plasmid and incubated on ice for 30 minutes 2) Bacteria were heat-shocked at 42°C for 30 seconds, then incubate on ice for 2 minutes 3) Then, S.O.C medium was added to the tube and incubate at 37°C shaking water bath for 30 minutes 4) Bacteria were put on LB-agar plates containing ampicillin and grown at 37°C overnight.

Fusion Protein Expression

First, 2 ml overnight cultures of the *E. coli* DH5α with PH-PAGFP plasmid were grown overnight at 37°C. Next, 0.5 ml of the culture was transferred to new tube with LB-media with ampicillin and the other culture stored at 4°C. The culture was grown in a shaking water bath at 37°C for 30 minutes. Next, 200 μl sample of the culture was saved as "Uninduced" sample at -80°C. To remaining culture, 20 μl of 0.1M IPTG (Isopropyl β-D-1-thiogalactopyranoside) was added and it was grown for 2 hours in the 37°C shaker. After 2 hours, 100 μl sample was saved as the "Induced" sample at freeze.

Confocal Microscopy

Confocal microscope was used to see GFP-PH fusion protein in bacteria DH5α *E. coli*. The GFP was activated with UV light.

Results and Discussion

To evaluate the detected proteins, PCR and gel electrophoresis of PH domain of PLC γ, DNA extraction, restriction digestion and purification of PH-PAGFP clone gel, fusion protein gel for expression, and confocal microscope were used .For data analyses, NCBI datasets and restriction mapping were used. Results of all these experiments are as follows:

A)

ACAAGAAGAAGCTGACGCCCCAGGAGGTGGCCAGCGTCACCAAGATGCTGAAAATGGGCA
CCGTCCTGACGCGCTTCTACGGCAAACGACGACCGGAAAGGAGGTCGTTCGAAATCTGCA
TGGAGACGCGGCAGATACTGTGGAGGCGACAGACTGGGCGGACAGACGGAGCAGTTAAAA
TTCGTGAGATAAAAGAGATTCGTCCCGGTAAGAACTCACGAGACTTCGAGAGGTGGCCGG
ATGAAGCCAAGAAGTATGATACCTCGCTCTGTCTTGTCATATGCTACGGTGCCGAGTTCAG
ACTCAAGAGCTTGTCCGTCGTTGCCGGCAATGCCGATGAACGACACAAGTGGATCGTCGG
CCTCAACTGGCTAGTGGAA.

B)

ACAtGtAcAAGCTGACGCCCCAGGAGGTGGCCAGCGTCACCAAGATGCTGAAAATGGGCAC
CGTCCTGACGCGCTTCTACGGCAAACGACGACCGGAAAGGAGGTCGTTCGAAATCTGCAT
GGAGACGCGGCAGATACTGTGGAGGCGACAGACTGGGCGGACAGACGGAGCAGTTAAAAT
TCGTGAGATAAAAGAGATTCGTCCCGGTAAGAACTCACGAGACTTCGAGAGGTGGCCGGA
TGAAGCCAAGAAGTATGATACCTCGCTCTGTCTTGTCATATGCTACGGTGCCGAGTTCAGA
CTCAAGAGCTTGTCCGTCGTTGCCGGCAATGCCGATGAACGACACAAGTGGATCGTCGGC
CTCAACTGGCTtGTacAA

C)

GtAcAAGCTGACGCCCAGGAGGTGGCCAGCGTCACCAAGATGCTGAAAATGGGCACCGTCC
TGACGCGCTTCTACGGCAAACGACGACCGGAAAGGAGGTCGTTCGAAATCTGCATGGAGA
CGCGGCAGATACTGTGGAGGCGACAGACTGGGCGGACAGACGGAGCAGTTAAAATTCGTG
AGATAAAAGAGATTCGTCCCGGTAAGAACTCACGAGACTTCGAGAGGTGGCCGGATGAAG
CCAAGAAGTATGATACCTCGCTCTGTCTTGTCATATGCTACGGTGCCGAGTTCAGACTCAA
GAGCTTGTCCGTCGTTGCCGGCAATGCCGATGAACGACACAAGTGGATCGTCGGCCTCAA
CTGGCTt

©2009-2019, JGPT. All Rights Reserved

D)

GCTGtAcAAGCTGACGCCCCAGGAGGTGGCCAGCGTCACCAAGATGCTGAAAATGGGCACC
GTCCTGACGCGCTTCTACGGCAAACGACGACCGGAAAGGAGGTCGTTCGAAATCTGCATG
GAGACGCGGCAGATACTGTGGAGGCGACAGACTGGGCGGACAGACGGAGCAGTTAAAATT
CGTGAGATAAAAGAGATTCGTCCCGGTAAGAACTCACGAGACTTCGAGAGGTGGCCGGAT
GAAGCCAAGAAGTATGATACCTCGCTCTGTCTTGTCATATGCTACGGTGCCGAGTTCAGAC
TCAAGAGCTTGTCCGTCGTTGCCGGCAATGCCGATGAACGACACAAGTGGATCGTCGGCC
TCAACTGGCTtGTACAAGTAAGAATTCGAAGCTTGATCCGGCTG.

Figure 2: PH domain primers with BsrG1 restriction sites A) PH domain of starfish PLCγ with the primers (blue). B) PH domain with BsrG1 sites in primers (lower letters – blue) C) PH domain after cut with BsrG1 enzyme (blue). D) The sequence of pRSETA – PAGFP with BsrG1 restriction site. Everything green is GFP domain from pRSETA plasmid after digest with BsrG1

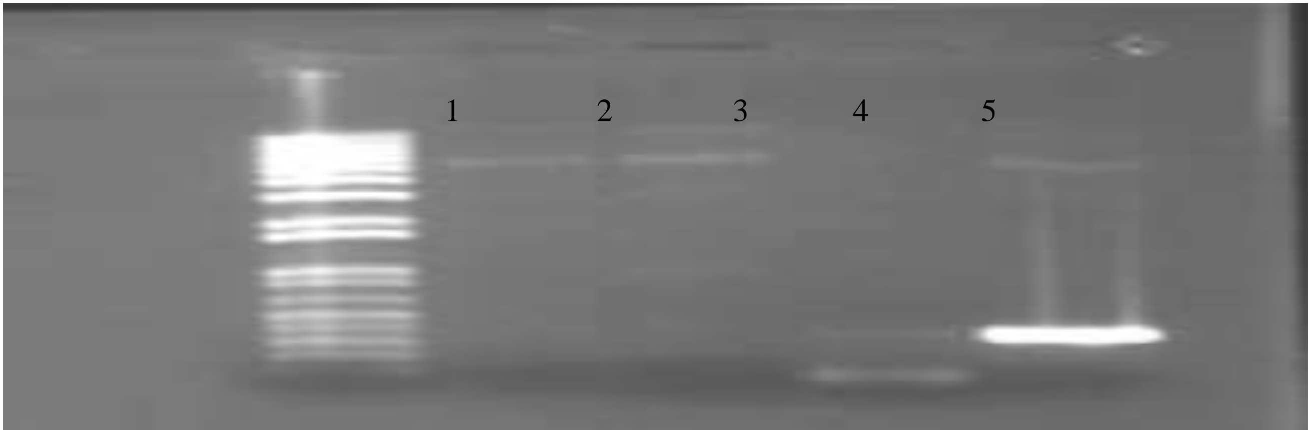


Figure 3: PH domain amplified from full-length AmPLCγ DNA by PCR. The PH domains amplified from the full length PLCγ plasmid using the primers in Figure 1. Lane 1 is the 1Kb Plus ladder, lane 2 is zero DNA control, lane 3 is the reverse primer control, lane 4 is the forward primer control, and lane 5 is template DNA and primers.

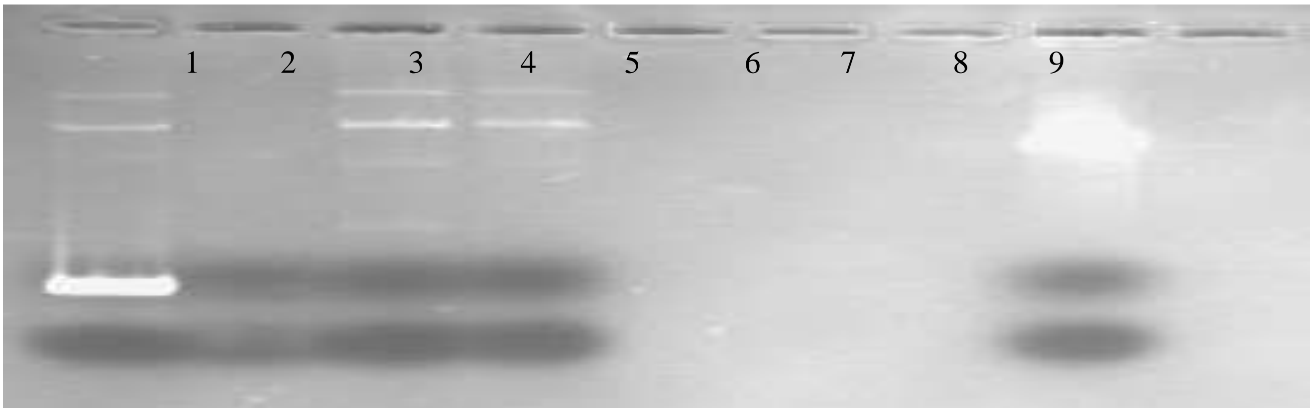
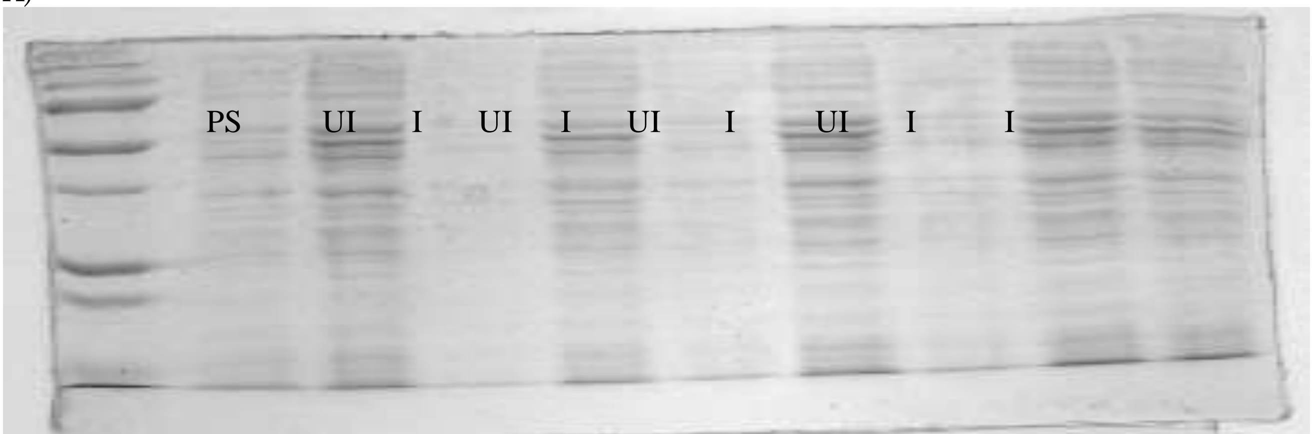
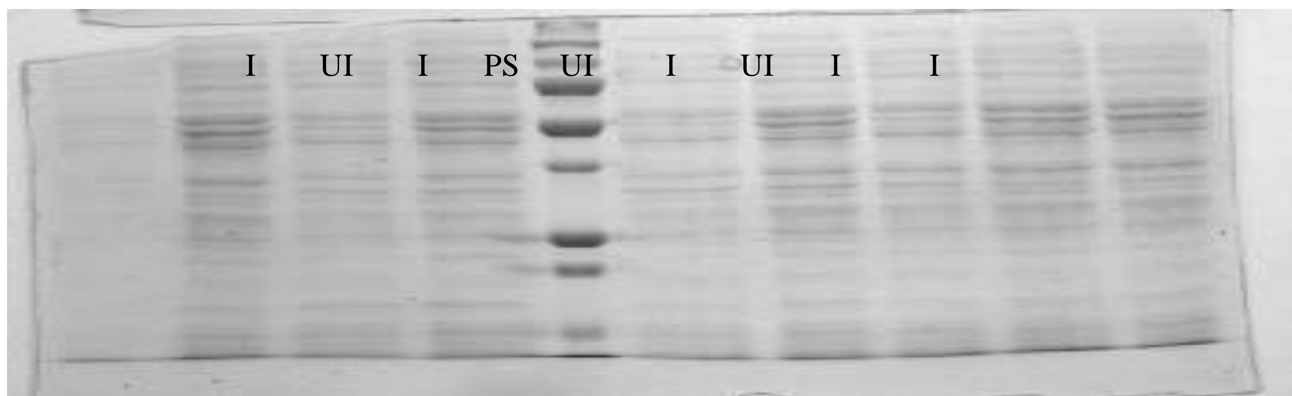


Figure 4: PH domain was cloned into the PAGFP plasmid. After PH domain was amplified and melted from gel, it cut with BsrG1. The pRSETA-PAGFP also cut with BsrG1, then dephosphorylated. The digested PH domain and PAGFP fragments were ligated together to make PH-PAGFP cloned plasmid. Lane 1 is the PH domain after cut with BsrG1, lane 2 is zero DNA control treatment with restriction BsrG1, lane 3 is reverse primer control treat with BsrG1, lane 4 is the forward primer control treat with BsrG1, lane 8 is pRSETA-PAGFP after being cut with BsrG1 and dephosphorylated

A)



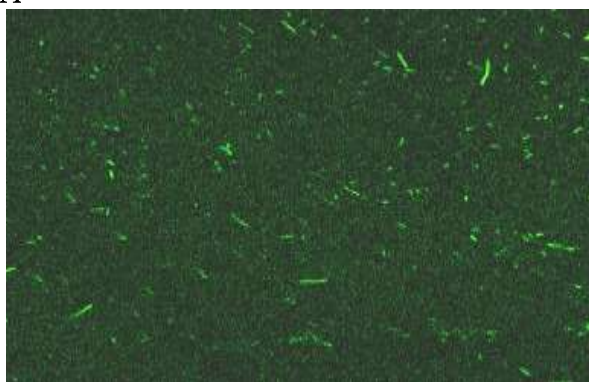
©2009-2019, JGPT. All Rights Reserved



B)

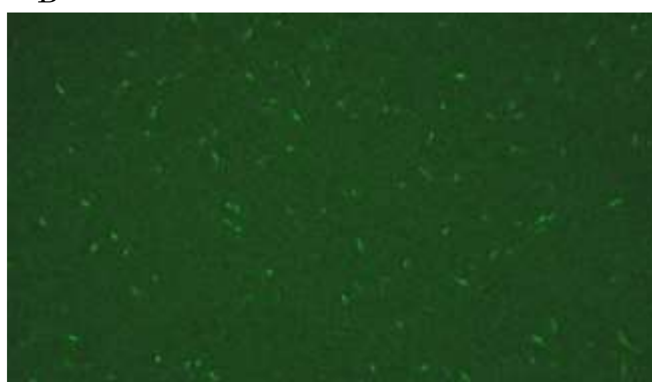
Figure 5: PH-GFP protein isolated from *E. coli.* DH5α. The PH-PAGFP clone was transformed into *E. coli* DH5α, the bacteria was induced to express the fusion protein with IPTG, and the sample was run on 10% SDS-PAGE gel. A) Lane 1 precision plus protein standards (PS) = 250,150,100,75,50,37,25,20,15 kD and other lanes are uninduced (UI) or induced (I) sample. B) Uninduced (UI), induced (I), or protein standard (PS). The fusion protein size (PH domain + GFP) is 41 kD (PH domain is 380 bp/3 = 126 amino acids, 126 x 110 = 13,860 Da or 13 kD, GFP = 28 kD, GFP + PH = 41 kD), so we can notice in both A&B at the protein standard size 50 Kd, fusion protein (PH domain and GFP plasmid) is found

A



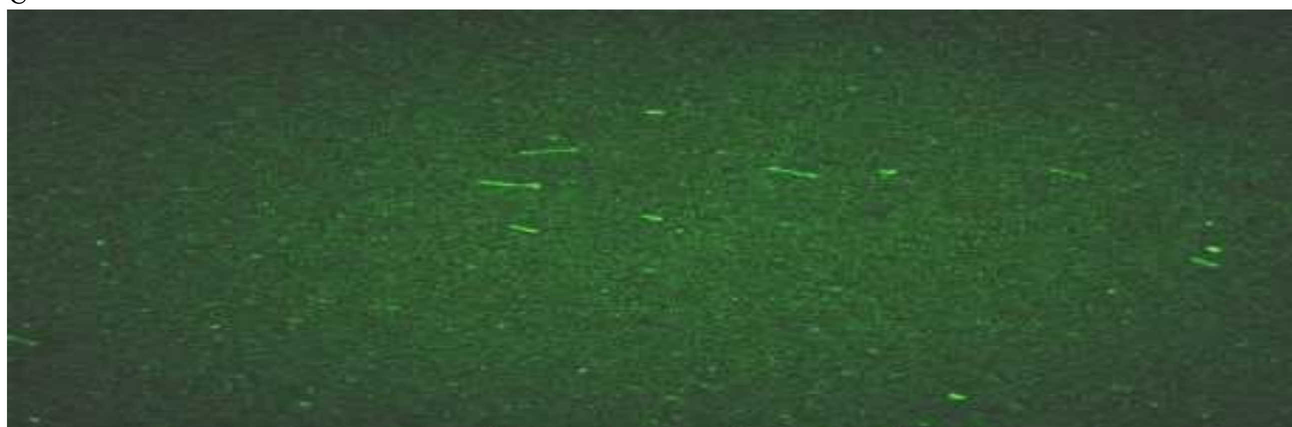
PA-GFP CONTROL AFTER UV

B



PH-PAGFP BEFORE UV

C



PH-PAGFP AFTER UV

Figure 6: Confocal microscopy. The induced bacteria were put under UV light to see GFP (green) in the bacteria. A) PAGFP used as control after UV. B) Bacteria with PH-PAGFP before UV C) Bacteria with PH-PAGFP after UV

We aimed to observe the localization dynamics of PLC γ in starfish *Asteria miniata*, in the final step, the cloning for synthesis of fusion protein to check the expression of PLC γ PH domain with a florescent tag to observe its localization was performed. The next, aim was to check whether the fusion product in the transformants obtained was in a frame and is expressed properly. Hence, the DHF5α containing the fusion product were induced with IPTG. As shown in the Figure 4, A&B, we observed a strong induction of protein around 50kD indicating the expression of 41kD fusion product. To recheck the validity of PH-PAGFP we exposed these DHF5α clones to UV to observe the fluorescence by confocal microscopy. As shown in the figure 5, we can observe that the bacteria containing PH-PAGFP or PA-GFP alone showed green fluorescent after UV exposure as against the one without exposure to UV.

©2009-2019, JGPT. All Rights Reserved

The next step is to observe the expression of this fusion protein PH-PAGFP in starfish eggs and to observe its localization kinetics pre- fertilization. Alternatively, we can induce the bacteria to express the fusion protein Am PH-GFP and perform microinjection experiments in starfish eggs. The fusion protein would be injected in the starfish eggs at varying concentrations and time points before or after sperm interaction and the subsequent Ca^{++} will be monitored with the standard techniques.

We hope that this fusion will interfere with the endogenous Am PLCγ activity and thus would give us an insight into the precise mechanisms and localization events before or after sperm-oocyte activation. Further, this fusion protein might interact with and localize to the other cytosolic compartments (Non-membrane) in response to the sperm interaction and might act to Ca^{++} by hitherto unknown means as shown previously for the other members of PLC family [18, 19].

References

1. Sillers PJ, Forer A (1985) Ca^{++} in fertilization and mitosis: the phosphatidylinositol cycle in sea urchin gametes and zygotes is involved in control of fertilization and mitosis. Cell Biol. Int. Rep., 9(3): 275-82.
2. Wilson EB (1925) The Cell in Development and Heredity (New York: Macmillan.
3. Ikawa M, Inoue N, Benham MA, Okabe M (2010) Fertilization: a sperm's journey to and interaction with the oocyte, 984-994.
4. Krawetz SA (2005) Paternal contribution: new insights and future challenges. Nat Rev Genet 6(8): 633-42.
5. Whitaker M (2006) Calcium at fertilization and in early development. Physiol. Rev., 86(1): 25-88.
6. Runft LL, Carroll DJ, Gillett J, O'Neill FJ, Giusti AF, Foltz KR (2004) Identification of a starfish egg PLC-γ that regulates Ca2+ release at fertilization. Dev. Biol., 269: 220-236.
7. Whitaker M, Steinhardt, RA (1985) Ionic signaling in the sea urchin egg at fertilization. In: Metz, C.B., Monroy, A. (Eds.), Biology of Fertilization vol. 3. Academic Press, San Diego, CA, 167-221.
8. Carroll DJ, Albay DT, Hoang KM, O'Neill FJ, Kumano M and Foltz KR (2000) The relationship between calcium, MAP kinase, and DNA synthesis in the sea urchin egg at fertilization. Dev. Biol., 217: 179-191.
9. Stricker SA (1999) Comparative biology of calcium signaling during fertilization and egg activation in animals. Dev. Biol., 211: 711-718.
10. Zucker RS, Steinhardt RA (1978) Prevention of the cortical reaction in fertilized sea urchin eggs by injection of calcium chelating ligands. Biochim. Biophys. Acta, 541: 459-466.
11. Florman HM and Ducibella T (2006) Fertilization in Mammals. In: Neill JD, editor. Physiology of Reproduction Elsevier; San Diego CA: 55–112.
12. Rhee SG, Bae YS (1997) Regulation of phosphoinositide-specific phospholipase C isozymes. J. Biol. Chem., 272: 15045-15048.
13. Rhee SG (2001) Regulation of phosphoinositide-specific phospholipase C. Annu. Rev. Biochem., 70: 281-312.
14. Singer AG, Beauchamp GK and Yamazaki K (1997) Volatile signals of the major histocompatibility complex in male mouse urine. Proc. Natl. Acad Sci., USA 94: 2210-2214.
15. Putney WJJr (2002) PLC-[gamma]: an old player has a new role (phospholipase C). nature cell biology, 4: 280-281.
16. Singh SM, Murray D (2003) Molecular modeling of the membrane targeting of phospholipasC pleckstrin homology domains, NCBI, 1934-1953.
17. Lemmon MA, Falasca M, Ferguson KM and Schlessinger J (1997) Regulatory recruitment of signaling molecules to the cell membrane by pleckstrin-homology domains. Trends Cell Biol., 7: 237-242.
18. Watt SA, Kular G, Fleming IN, Downes CP, Lucocq JM (2002) Subcellular localization of phosphatidylinositol 4,5-bisphosphate using the pleckstrin homology domain of phospholipase C δ1. Biochem. J., 363: 657-666.
19. Yu Y, Nomikos M, Theodoridou M, Nounesis G, Lai FA, Swann K (2012) PLCζ causes Ca2+ oscillations in mouse eggs by targeting intracellular and not plasma membrane PI(4,5)P2. Mol. Biol. Cell, 23: 15.

©2009-2019, JGPT. All Rights Reserved