DETECTION THE PROTEIN INITIATES STARFISH EGG ACTIVATION DURING FERTILIZATION

Rana Hussein Naser Al- Qaysi¹, Alyaa Abdelhameed², Zainab Abd Mohammed³, Aalhan Mohammed Alwan⁴, Nada Khalid Alharbi⁵ & Wanisa Abdussalam Mohammed⁶ ¹Biotechnology Dep., College of Science, University of Diyala, Iraq. ²Al-Muqdad College of Education, University of Diyala, Iraq.

Abstract:

The role of an interaction between PLC γ and the proteins during fertilization is still unclear. To recognize whether fertilization includes PLC β - Ca2+ and PLC γ release mechanisms, GST-SH2 fusion protein was designed and injected into the egg of *Asterina miniata* sp. In this study, the mixture of SH2-pGEMT-Easy vector was combined with the component cell of DH5 α , the results proved the success of SH2 domains insertion into SH2-pGEMT-Easy vector using AmPLC γ plasmid. AmPLC γ was treated with EcoR1 to make a GST-SH2 fusion protein. The designed new SH2 primer including EcoR1 and Xho 1 digestion site and clone them into pGEX vector system, could be directly detect the interaction protein with SH2 domain with an easy purification.

Keywords: Fertilization, Starfish, PLCy, Asterina miniata sp

INTRODUCTION

When fertilized, the eggs experience a free cytoplasty of Ca2 +, which is required to stimulate embryogenesis. In the eggs of starfish, studies consumed inhibitors prepared versus vertebrate proteins have described that this Ca²⁺ increase demands in egg Src family kinase (SFK) that indirectly or directly initializes phospholipase C-gamma (gamma-PLC) to yield IP3, that leads the release of Ca²⁺ from the reticulum of egg's endoplasmic (ER) [reviewed in Semin. Cell Dev. Biol. 12 (2001) 45]. To test in more features the parameters of endogenous in eggs of starfish that are demanded for the release of Ca²⁺ at fertilization, an oocyte cDNA encoding gamma-PLC was separated from the starfish Asterina miniata.

This cDNA, specified Am-gamma PLC, encodes a protein with 49% identity to mammalian gamma-PLC1. A 58-kDa Src family kinase reacted with recombinant Am-gamma PLC Src homology 2 (SH2) scopes in a particular, the responsive manner for fertilization. Sea urchin egg gamma-PLC immunosuppressively directed to an antimonopoly directed toward am-gamma PLC discovered gamma-PLC fertilisator-dependent gamma-PLC phosphorylation. Injecting the eggs of starfish with the tandem SH2 scopes of Am- gamma PLC (which prevents of the activation of gamma-PLC) specifically prevented release of Ca^{2+} at fertilization. These results refers that an endogenous the egg of starfish gamma-PLC scope-intercede technique.

The gamma-PLC in fertilization has been indispensable over the years of researches. The fertilization and the insight interactions of the human beings, plants and animals started from the beginning of human life. Cell signal transduction refers to cells during intracellular receptors or the cell membrane feelings informational molecules stimulation by intracellular signal transduction system conversion, thereby affecting cell biological learning function of the process. (Ramadan et al., 2012; Shimohama et al., 1995; Park et al., 1994).

During fertilization the sperm triggers a chain of activities in the egg to cause mitosis and development. Besides the sperm delivers its gene, the egg also needs a signal to start the development (K Swann et al. 2004). The significant observed signal in the egg during fertilization is the increased concentration of calcium in egg cytoplasm (Stricker 1999). The activation of egg keeps still if no changed concentration of calcium in the egg while the sperm fusing with the egg (Kline JT & Kline D1992). Hence, the increased calcium signal in the egg is the key element during the beginning of fertilization. The initial calcium rise from egg's endoplasmic reticulum is caused by inositol 1,4,5- trisphosphate (IP3) that is the construction of phospholipase C γ (Ian K. Towny et al. 2009).

Phospholipase C γ is the enzyme that hydrolyzes the membrane phospholipid, phosphatidylinositol 4,5- bisphosphate (PIP₂), to produce inositol 1,4,5- trisphosphate (IP3) and diacylglycerol (DAG). Phospholipase C γ contains many domains from C terminal to N terminal - PH domain, two SH2 domain, domain of X catalytic, SH3 domain, domain of Y catalytic and domain of C2. When growth factor binds to the receptor, it triggers autophosphorylation of the receptor on tyrosine residue, which has interaction with NH₂⁺- terminal SH2 domain. This action leads COOH⁻-terminal SH2 domain and PH domain to companion with the membrane and start to hydrolyze PIP₂ [1].

Aspect of PLC gamma in fertilization

During the fertilization, the sperm initializes an egg to reenter the cycle of cell and forming a new zygote. An intracellular calcium that release from the endoplasmic reticulum can induce egg activation. PLC molecules able to detect the fertilization-calcium association in the new generation via IP3 [2]. The Ca²⁺ important in egg activation was released between 1920s and 1930s. Whereas, the study about molecular signaling pathway that involved in sperm and egg fusion is continuous until today[3] Study the regulation of IP3 receptor and calcium signaling has important role on cell function such as concentration of smooth muscles, fertilization, immune response and gene expression[4]. The interaction of molecular mechanisms help to create different calcium signaling that mediated by IP3[5].

The levels of intracellular calcium help to regulate IP3R by store the depletion, conformational changes in IP3 receptor. Also, this interaction can regulate some various signals molecules that effect many physiological processes[6].

METHODS AND MATERIALS

Bioinformatics

To design the SH2 primer, Am PLC γ full line cDNA sequence and SH2 domian were obtained from NCBI. To design SH2 forward primer-5'-ACCCGGAACTCTACATGGAC-3' and Am PLC γ SH2 reverse primer-5'-CTAGTAG CGTTGACTCGGGGG-3'.

pGEMT-Easy vector sequence that contain EcoR1 restriction site SH2 domain and the primer aattcgatt acccggaac tctacatgga cccaaaccag tttgtaccaa aggtgacagt gaaagccctg tatgactaca gagc ccaacg agacgatgag ttgacattct gcaagcacgc catcatcacc aatgtagata agcaagacct tggctggtgg agoga gact acggcggcaa gaagaacatg tggttcccct ccaactacgt ggaagagacc caaccaaatg acaacagccc cgagtcaacg ctactag aatcactagtg were downloaded. To choose pGEX6p-1 as the subclone vector, pGEX6p-1, pGEX6p-2, and pGEX6p-3 cDAN sequence were downloaded from NCBI using ORF Finder [7].

Cell culture and Plasmid Extraction

In this research, two ways of plasmid extraction were used: QIAGEN kit (Qiagen,MD,USA and traditional method . The quality of DNA was assessed via agarose gel electrophoresis. While the yield and purity of extracted gDNA was assessed using a NanoDrop spectrophotometer (NanoDropND-1000) recording nucleic acid content at 260 nm, protein contamination at 280 nm and various contaminants at 230 nm. The ratios (260:280 and 260:230) give an indication of purity with values as close to 2.0 as possible.

Plasmid DNA was resumed from overnight cultures using a plasmid miniprep extraction kit (Qiagen, UK) according to the manufacturer's guidelines . Plasmid PLC γ was purified using QIAGEN plasmid miniprep kit. A single colony of *E.coli* was grown with AMP on LB broth (10 ml) at 37°C while being shaken 200 rpm overnight. The culture was centrifuged (8000 rpm; 3 min; 25°C) [7].

PCR and Gel electrophoresis

The full length of Am PLCy cDNA was used as a template and the bioinformatics method was used to design SH2 primers. Am PLCy SH2 forward primer is 5'-ACCCGG AACTCTACATGGAC-3' and Am PLCγ SH2 reverse primer is 5'-CTAGTAGCGTTGACTCG GGG-3'. The DNA template was amplified using the full length of Am PLCy. PCR Mastermix, two primers and ddH₂O in the PCR tube. Run 25 cycles with annealing temperate 58°C and then run 1% gel to confirm the PCR product .The DNA sample was loaded on 0.1 agarose with ethidium bromide and ran for 1 hour at 125 volt [7].

Transformation

The mixture of SH2-pGEMT-Easy vector was combined with the component cell of DH5 α (1:50) and incubated in ice for 45 min and then heat shock at 35 °C bath water for 45 seconds. Then it incubated on the ice for 2 min and then shake with S.O.C medium for 1 hour [7].

X-gal blue-white selection

Spread 100ul 20mg/ml X-gal and 40ul 100mM IPTG on each pre-made agar plate and then wait 30 minutes until the plates dry. Pour each transformation tube on two plates for 400ul and 600ul. Incubate these plates at 37°C overnight [7].

Restriction Digestion and Ligation

The plasmid was treated with EcoR1 at 37 °C for 1 hour. For ligation SH2 fragment, pGEX6p-1, and pGEMT-Easy vector were mixed (3:1) with T4 DNA ligase at 4 °C overnight. Then the plasmid was extracted and digested using the selected enzyme and analysed by agarose gel (0.7%) electrophoresis to confirm its integrity [7].

Gel Purification

pGEX6p-1 plasmid and SH2 domain were run in 0.7 % gel electrophoresis; pGEMT-Easy plasmid treated with EcoR1. pGEX6p-1 and SH2 domains gels were cut into two individuals tubes. QIAGEN kit was used for purification. Two tubes were weighted and filled with QG buffer (3:1). Vortex and heat were used until the gel dissolved. Isopropanol was used and

centrifuge for 1 minute at 10000rpm. PE buffer was used to wash the sample and recentrifuge. EB buffer was added to elute the DNA into new tube [7].

Dephosphorylation

pGEX6p-1 was incubated with 5 μ l of 0.01u/ μ l CIAP at 37°C for half hour. The CIAP was re-added and incubated at 37 °C for 30 minutes. To stop the reaction, stop buffer (200mM NaCl, 1mM EDTA, 10mM Tris-HCL ,and 0.5% SDS) and CIAP were added. To remove the supernatant into the new tube, phenol:chloroform:isoamyl alcohol were added at ratios 25:24:1, respectively. Ammonium acetate and 100% ethanol were used to wash it and then dry the pellet. Add EB buffer to save the sample[7].

RESULTS AND DISCUSSION

The Sperm at fertilization starts a spreading increase of Ca2 + in the cell, that is focal to the production of the Cell [8]. In echinoderm as well as the eggs of vertebrate, the increase in outcomes of Ca^{2+} , at minimal in a huge part for the release of Ca^{2+} from the response of endoplasmic reticulum to an increase in inositol trisphosphate IP3 [9]. However, the description of how the IP3 is produced at fertilization is not mentioned.

The phosphatidylinositol bisphosphate (PIP2) generate IP3, by the work of a phospholipase C [10]. This enzyme's family contains δ , γ and β isoforms. The initialization of PLC β is activated by G proteins, while the initialization of PLC γ is activated by tyrosine kinases. PLC δ regulation is badly grasp, although the initialization for enzymatic of all 3 PLC isoforms can be stimulated by a raise in Ca²⁺ [11]. Very probably the production of IP3 at the findings of fertilization from the initialization of one of these isoforms of phospholipase C.

Both PLCy and PLCB path ways are offer in eggs. Exogenous receptors known to release Ca2+ by a G protein/PLCβ pathway like Expression in mammalian, frog and starfish eggs, and allows the release of Ca^{2+} in eggs when the corresponding agonists are used like muscarinic m1or serotonin 2c receptors [11]. This refers that corresponding G proteins and functional PLCB are offer. Likewise, expression in frog eggs and starfish of exogenous receptors recognized to release Ca²⁺ by a PLC γ / tyrosine kinase pathway, like receptors for PDGF or EGF, allows release of Ca²⁺ to these agonists response [12]. The receptors of pointmutated that do not activate PLC γ do not cause release of Ca²⁺ [12]. These results refers that a PLC γ functional is offer. In such experiments the existence of PLC γ has been described by immunoblotting but have not been implemented in mammalian eggs [13]. In this research, SH2 domain of phospholipase $C\gamma$ showed interaction with a specific protein (unidentified) during the early stage of fertilization and calcium release. SH2 domain localization was detected through cloning SH2 into cloning pGEX6p-1 vector to make a GST-SH2 fusion protein. The fusion protein has ability to improve the expression and the solubility of the target protein. GST fusion protein is a high-quality purification protein with few protease cleavage sites [14].

Furthermore, maltose binding protein (MBP)-SH2 fusion protein could be used to promote the solubility of SH2 domain or GFP-SH2 fusion protein to detect the position of SH2 domain in the cell [15].

Design SH2 of PLCy PCR primer

SH2 primer was used to amplify SH2 domain of AmPLC γ . Figure 1- presented the full-length of AmPLC γ cDNA sequences using the NCBI primer design, the AmPLC γ sequence

was used to design forward and reverse SH2 primers using primer design program: 5'-ACCCGGAACTCTACATGGAC-3'F and 5'-CTAGTAG CGTTGACTCGGGG-3'R.

ORIGIN tttcagaatg gccaccaaca gccttacaa gaagaagctg acgcccag aggtggc cgtcaccaag atgctgaaa tgggcaccgt cctgacggc tttacaggca aacgacg ggaaggagg tcgttcgaaa tcggtgga gacgeggcag atactgtgga ggcgaca tgggcggaca gacggacgag ttaaaattcg tgggataaa gggatcgtc ccggtaa tccacgagac ttcgaaggt ggccggatga agccagagag tatgatact cgctcg gtcatatgc tacggtgccg agtcagaccgt catgaggg tcgtcggaca atagatgg aggtagacga cacaagtggg tcgtcgcct cactggcg ggtaggagaca atagatggt cgatgaacga cacaagtgga tcgtcggcct cactggcg ggtaggaggt cggagaca ataaatt aggtacaca aggggcat cgggcgca atgaggtgt acgcgggggg ttctacgcca tggggga aagtatacca aggggctat taagatgggt acgcggggg ttctacgcca tggggagac aatggattg gacggtta acggtgcat caagaggg tccacagg ggtcggtgg acaagca fgatggatga caggagcata aggacatta caagagggg tccacagtggg gacagggg gacggggg aatggattg gacggcttg tccagtca caaattgg gatcggtgg acaaggag ttctcctagc caagagga accggcg accggacaat ccatact attccag gggaggt fol tgacgagtt gaaggacctt gggcacac ggcaacaat cctatgcag gagtcggg caaaga gatggaatcg tttctgacgg acctggccg accggacag gagtcggac caaatt gatggaatg tgggacgact tggacacac gctctgaat gagtcggg caagaa taatgagatt gtggacgact tggacacac gctctgaat gagtcggg caagat taatgagatt gtggacgact tggacacac gctctgaat gagtcggg caagat ttgttacg atggcgcc gatgttcga acttgacgt tcgtaggg cgacgt ttgtttacg atggcgcc gatgttcga acttgacgt tcgtgagg cgacgt ttgttacga gggcggg tggacgacat gagtcgga cgacgt tgaaggcc tagaggc cgaagt ttgttacga ggggcg tgacgacat tggacgacat caatata cattgagg gacgtgggg cgacgg gatgaaag gagggcc tgggcgacaacac gctctgaat actgtgagg cgacgtaggacgacaat ttggacggag gacgt gaaggacgacaacac gacgtggagac caaattgggg gacgggggg cgacgacgagagaga gatggagat gtggacgact gggcgacaacaacac gctctgaat actggaggacgacgagacga	
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Amplify SH2 domain of AmPLCy

DH5 α cells were grown overnight on LB broth, and the full length of AmPLC γ plasmid was used as PCR template. The result shows that the size of AmPLC γ plasmid is 3816 bp (Figure 2).

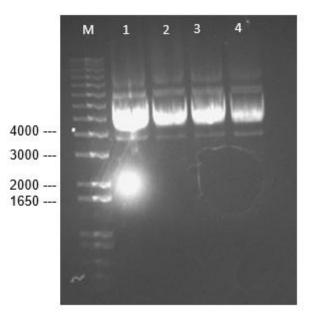


Figure 2: Agarose gel (0.7%) electrophoresis of the PCR product of AmPLCγ plasmid (3816 bp) on 125 volt for 1 hour. Line M: DNA marker (1kb); lines 1- 4 PCR- positive results. Continuously, in figure 3 the size of SH2 domain product was 199bp.

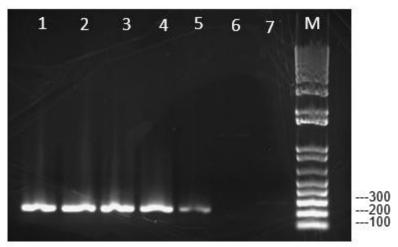


Figure 3: Agarose gel (0.1%) electrophoresis of the PCR product of SH2 product by AmPLCγ plasmid (199 bp) on 125 volt for 1 hour. Line M: DNA marker (1kb); lines 1-4 are samples . line 5 without template DNA(control1); line 6 without forward SH2 primer (control 2); line 7 without reverse SH2 primer (control 3). 2 µl of DNA template, 12.5 µl PCR master Mix, and 2 µl if each primer with dH₂O to total 25 volume. The annealing temperature was set to 58 °C and run 25 cycles. 20 µl of DNA and 4 µl loading dye were loaded per each well.

Clone PCR product to pGEMT -Easy vector

To confirm the digestion of EcoR1 at GAATTC two sites of SH2 domain using pGEMT-Easy vector, T4 DNA ligase kit (Promega,UK) was used. SH2 domain was inserted into SH2pGEMT-Easy vector at 4°C. SH2- pGEMT-Easy vector was transformed to DH5α cell (1:50). pGEMT-Easy contains *lacZ* that use in X-gal blue-white selection to detect bacterial plasmid and SH2 domain. Overnight incubation (white clones), and plasmid DNA extraction using EcoR1 and run on 1% agar gel. Figure 4 presented only line2 and line9 contain SH2 domain. Hence, save No.2 and No.9 tubes to do sub-clone.

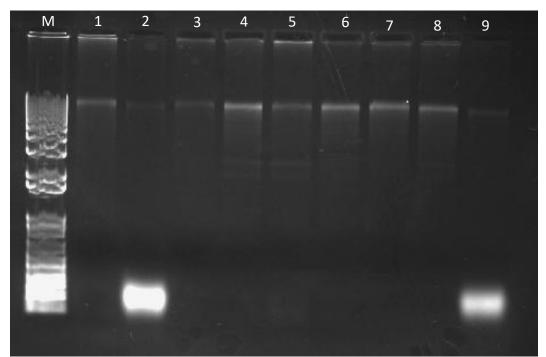


Figure 4: Agarose gel (1%) electrophoresis of gel of SH2-pGEMT-Easy plasmid DNA digestion. Mix 3 μl of plasmid DNA, 2 μl of 10X buffer, 1 μl EcoR1 and 14 μl dH₂O at 37 °C for 1 hour. Load 20 μl DNA and 4 μl dye per well .

Sub-clone SH2 domain in pGEMT-Easy into pGEX6p-1 vector

pGEX6p-1 plasmid and SH2-pGEMT-Easy plasmid (tube No. 2 and 9)were treated with EcoR1. Then 0.7% agar gel was used to run SH2 domain and pGEX6p-1.; after that pGEX6p-1 was treated with phosphatase. T4 DNA ligase was used to insert SH2 domain into pGEX6p-1 at 4 °C overnight. To confirm the transformation, the plasmid DNA was extracted and digested using these enzymes and analysed by agarose gel electrophoresis to confirm its integrity. Figure 5 does not show no SH2 domain in pGEX6p-1 vector.

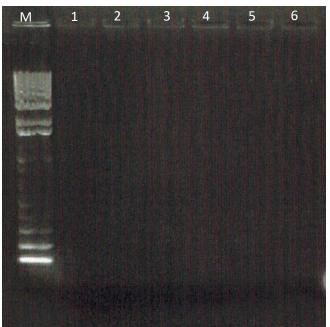


Figure 5: Agarose gel (1%) electrophoresis of gel of SH2-pGEX6p-1 plasmid DNA digestion. Mix 3 µl of plasmid DNA, 2 µl of 10X buffer, 1 µl EcoR1 and 14 µl dH₂O at 37 °C for 1 hour. Load 20 µl DNA and 4 µl dye per well.

Re-pick clone from X-gal blue-white choice plate

Due to the negative results of the earlier experiment, 12 clones were picked from X-gal bluewhite selection plate and grown in LB broth overnight. The SH2-pGEMT-Easy plasmid DNA was extracted and treated with EcoR1. Gel electrophoresis of purified plasmid gave many bands without pointed to SH2 domain (Fig.6). Due to the low efficiency of EcoR1, PCR was used to examine the presence of SH2 domain in the SH2-pGEMT-Easy plasmid. SH3pGEMT-Easy plasmid was used as a template for SH2-pGEMT-Easy plasmid to run PCR. The results proved the success insertion of SH2 domains into The SH2-pGEMT-Easy vectors (Fig. 7).

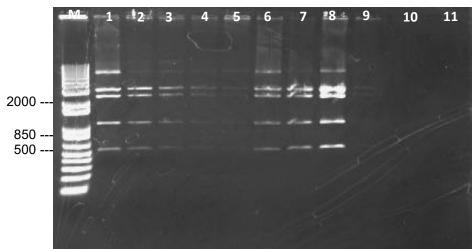


Figure. 6: Agarose gel (1%) electrophoresis of gel of of SH2-pGEMT-Easy plasmid DNA digestion. The result showed many bands without pointed to SH2 domain. Mix plasmid 3µl plasmid DNA, 2µl 10X buffer, 1µl EcoR1 and 14µl dH₂O and incubate 37°C for 1 hour.

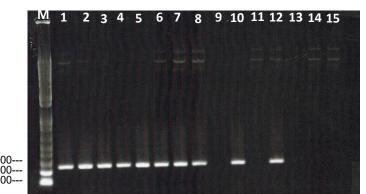


Figure. 7: Agarose gel (1%) electrophoresis of gel of SH2 domain product by PCR using SH2-pGEMT-Easy template.

The forward SH2 primer is 5'-ACCCGGA ACTCTACATG GAC-3' and the reverse SH2 primer is 5'-CTAGTAGCGTTGACTC GGGG-3'. 1 μ l of DNA template, 12.5 μ l PCR Master Mix, and 2 μ l if each primer with dH₂O to total 25 volume. The annealing temperature was set to 58 °C and run 25 cycles. 20 μ l of DNA and 4 μ l loading dye were loaded per each well. The gelelectrophpresis was run at 125 volt for 1 hour. Lines 1-12 are samples; line 13 without DNA template(control 1); line 14 without forward SH2 primer (control 2); and line 15 without reverse SH2 primer (control 3).

The insertion of SH2 domain into pGEMT-Easy vector was amplified using SH2-pGEMT-Easy plasmid DNA (Fig.7). This positive result proved the frustration of SH2 digestion with EcoR1. The main goal of this process is to create GST-SH2 fusion protein and design a new SH2 primer including EcoR1 and Xho 1 digestion site and clone them into pGEX vector system. Furthermore, the GST-SH2 fusion protein could be directly detect the interaction protein with SH2 domain with an easy purification.

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