The Role of FAK (Focal Adhesion Kinase) in Fertilization of Starfish Asterina Miniata PLCγ

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ABSTRACT

Fertilization need increased the concentration of Ca^{+} for egg activation. Inositol 1,4,5-trisphosphate (IP3), the second messenger of phospholipase Cγ, regulates the initial calcium rise from egg's endoplasmic reticulum. However, FAK (focal adhesion kinase) is a non-receptor tyrosine kinase, which is known to create docking sites for signaling molecules that contain SH2 domains, including PLCγ. PLCγ participates in other signaling pathways besides fertilization and the role FAK may play in fertilization has not been determined. The goal of these experiments is to determine is the FAK is the link between Src and PLCγ, as well as, confirming or not confirming if Src directly or indirectly activates PLC.

دور FAK في اخصاب نجم البحر

الخلاصة

عند الاخصاب مطلوب زيادة تركيز الكالسيوم لتنشيط البويضة, الاينوزيتول Inositol 1,4,5-trisphosphate (IP3) وهو عبارة عن الرسول الثاني من PLCγ والذي ينظم ارتفاع الكالسيوم الأولي في الشبكة الإندوبلازمية للبويضة بينما FAK وهو عبارة عن تيروزين كيناز غير مستقل والذي يعرف كمواقع لارساء جزيئات نقل الإشارة التي تحتوي على المجالات SH2 بما في ذلك PLCγ والتي تشارك في مسارات إشارات أخرى إلى جانب الاخصاب كما ان FAK قد يلعب دور في الاخصاب لكن لم يتم تحديده, والهدف من هذه التجارب هي تحديد فيما اذا FAK هو الرابط بين src و PLCγ ، وكذلك للتأكد او عدمه فيما اذا src ينشط او لاينشط PLCγ

INTRODUCTION

Fertilization can be defined as sperm signaling the egg to reenter the cell cycle and begin embryonic development. At the molecular level, fertilization can be identified as a factor(s) from the sperm activates a signal transduction pathway in the egg that leads to Ca^{2+} release from the endoplasmic reticulum. The two best-studied pathways of fertilization are a heterotrimeric G-protein that activates PLC-beta in frog, mammalian, and starfish eggs, and a receptor tyrosine kinase that activates PLC gamma in frog and starfish (Carroll et al., 1997); however, recent studies, in which inhibitors of PLC-β were injected into mice and did not inhibit fertilization-induced Ca^{2+} release, suggest that this pathway does not contribute to Ca^{2+} release (Malcuit et al., 2006) .

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Because fertilization occurs internally in mammals, a different, more accessible model needed to be used to study fertilization. Starfish - a member of the deuterostome superphylum, which includes animals who develop the anus as the first opening during development and echinoderm subphylum - are frequently used as a model to study fertilization. Starfish fertilize externally, meaning they release their sperm or oocytes into the surrounding environment in which the sperm fertilizes the egg. The sperm or oocytes are stored in the arms of the starfish. Retrieval, which is simple and does not hurt the starfish, consists of poking a hole in the dorsal surface of the arm with a 3-mm sample corer and using tweezers to pull out oocytes or sperm.

Not only do starfish provide easy access to oocytes and sperm, many of the signal transduction pathways and protein compositions are very close in identity to that of mammals. *Asterina miniata* PLC-γ (a protein of interest in this experiment) is 49% identical to mammalian PLC-γ (Runtt et al ., 2004). Also, studies have indicated that starfish MAP kinase (a receptor tyrosine kinase involved in many signaling pathways, especially mitosis and meiosis, in mammals) functions in maturation and early development events, such as metaphase I arrest, meiosis I to meiosis II transition, apoptosis, and initiation of development, confirming starfish oocytes and eggs are a useful model system (Kishimoto, 2004).

The receptor tyrosine kinase bound in the plasma membrane of the oocyte responsible for directly or indirectly (Runft et al ., 2004) activating PLCγ is a Src family kinase. If directly activating, it binds specifically to the SH2 (Src homology 2) domains on PLCγ (Guisti et al., 1999). The pathway by which these proteins signal Ca^{2+} release starts with a factor(s) from the sperm (Malcuit and colleagues, 2006, suggest it is PLCζ from sperm that binds to Src). The sperm factor (SF) binds to Src, which exists as two monomers until activated, when it dimerizes triggering the intrinsic tyrosine kinases to cross-phosphorylate. The phophorylated tyrosines become a landing pad for the SH2 domains of PLCγ or another factor, which binds to the SH2 domains of PLCγ. Next, PLCγ cleaves the lipid phosphoinositide bisphosphate (PIP_2) via hydrolysis, creating inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 travels to the endoplasmic reticulum causing release of intracellular Ca^{2+} by binding to the IP_3 receptor in the egg's ER (Runft et al., 2004).

SF
↓
Activation of Src
↓
Activation of PLCγ
↓
$PIP_2 \rightarrow IP_3$ + DAG
↓
Ca^{2+} release from the ER

Materials and Methods

In order to study the hypothesized action of FAK binding to PLCγ, affinity interactions were performed using PLCγ - GST fusion proteins, along with SDS-PAGE and western-blotting techniques to analyze results.

Making of PLCγ-GST Fusion proteins

LB-amp plates were made and E. coli strains, which included an insert of DNA that coded for a protein(s) of choice, were streaked on them and grown in a 37°C incubator overnight. 2 mL LB-amp liquid cultures of the bacteria were grown overnight in a 37°C shaking water bath. The whole 2 ml overnight culture was transferred to 50 ml of freshly made LB-amp. The cultures were placed back into the 37°C shaking water bath for 30 minutes so they would be in log phase, in which the artificial inducer IPTG was added (100 µl of 0.1M stock). Upon addition of IPTG, the cultures were left in the 37°C shaking water bath for 3 hours, allowing time for the lac operon to be induced so the bacteria produce their natural and synthetic proteins. Samples were taken every hour and after three hours, the cultures were centrifuged and the bacterial pellets were stored in a -70°C freezer ((Runft et al., 2004).

The day the affinity interactions were performed, the pellets were removed from the freezer and the pellets were resuspended in 5 ml of PBS/0.2% Triton X-100. Next, the resuspended pellets were sonicated at 10 microns for 3 minutes. After sonification, the tubes were centrifuged for 15 minutes in the Sorvall RT600B Refrigerated Centrifuge at 6500 rev/min at 5°C. After centrifugation, the supernatants were transferred to a new tube. Next, the glutathione-S-transferase (GST) beads were prepared:

A) Glut-sepharose were removed from the refrigerator

B) 0.5 ml of beads were transferred to a 15 ml tube

C) 5 ml of PBS/0.2% Triton X-100 were added the beads in the 15 ml tube and the contents were centrifuged for 2 min on the Sorvall RT600B Refrigerated Centrifuge at 6500 rev/min at 5°C.

D) The rinsing of the beads with PBS/0.2% Triton X-100 was performed 3 times

Once the GST beads were prepared, the supernatants from the bacterial pellets were transferred to the 15 ml tube of GST beads and then the beads and supernatant were placed on a rocker at 4°C for 1.5 hours. After rocking, the contents of the tube were centrifuged in the Sorvall RT600B Refrigerated Centrifuge at 6500 rev/min at 5°C for 1 minute. The supernatant was discarded and the beads were washed as per steps A through D above. After completing the third wash, the beads were stored in the refrigerator. A BioRad® SDS-PAGE and BioRad® protein assay were run on the beads to determine whether or not there was successful isolation of our protein of interest and the concentration of the protein (Runft et al., 2004).

Affinity Interactions

First, an oocyte lysis buffer was prepared on ice. The lysis buffer was made of the following (Gish et al.,1995) :

Chemical	Stock	Amount for 10 mls
HEPES, pH 7.0	1 M	200 µl
NaCl	5M	300 µl
Na_3VO_4	1M	100 µl
NaF	1M	100 µl
EDTA	0.5 M	20 µl
Na-β-glycerophosphate	0.5 M	1.2 mls
NP-40	100%	100 µl
Protease Inhibitor Cocktail (PIC III)	1000X	10 µl
DI H_2O		q.s to 10 mls

Next, starfish oocytes were obtained from the freezer. There were different types of ooctyes, each taken at different time's intervals during fertilization, such as: unfertilized, fertilization at 1 minute, fertilization at 5 minutes, etc. While kept on ice, 1ml of the lysis buffer was added to each sample using a hypodermic needle and syringe (pipetting up and down several times to break open the oocytes). The samples were allowed to sit on ice for 20 minutes and then they were centrifuged at 4°C for 20 minutes at 12,000 g. After centrifugation, the supernatants were transferred to pre-chilled microfuge tubes and 20 µl of PLCγ-GST beads were added (Runft et al., 2004). The sample was placed on a rocker at 4°C for 1.5 hours and then centrifuged for 2 minutes at 12,000 g at 4°C. After centrifuging, the sample was washed 4 times with 1 ml of PBS/0.2% Triton X-100. Upon completion of washing, the sample was put through an SDS-PAGE (as per BioRad® protocol).

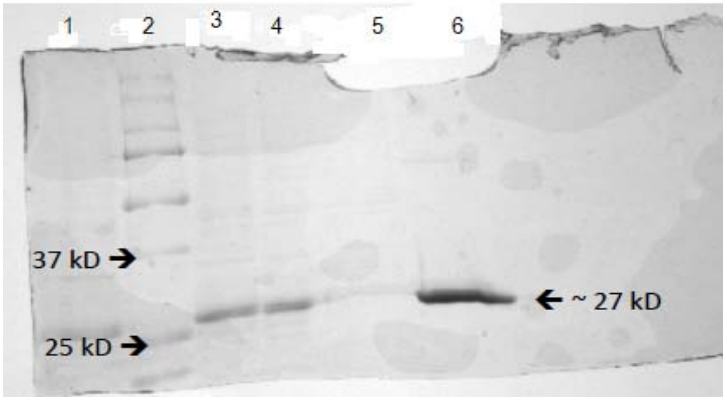
Results and Discussion

Databases have indicated that the phosphorylation of PLC, the activity of SFKs and activity profile of PLC gamma are a confirmation of the hypothesis that the interaction between the sperm and egg triggers an SFK (Rongish and Kinsey, 1999). Consequently, the phosphorylation and activation of PLC gamma release IP3 and finally Ca^{2+} (Xu and Eck, 1997). It is important to note that the release of Ca^{2+} occurs in tens of seconds following sperm-egg interaction, but the maximal SFK activity as well as PLC gamma phosphorylation and activation takes some time (Moarefi, & Kuriyan, 1997). This occurs after insemination. Studies have shown that elevations in SFK activity can be detected within 15s after insemination. Furthermore, interaction between PLC gamma and SFK can be is detectable within 15 s (Xu, and Eck, 1997; Sicheri, and Kuriyan, 1997). Current data show that the activation of SFKs takes center stage upstream of the Ca^{2+} release (Rongish and Kinsey, 1999). The current model explaining the role of PLC gamma in fertilization posits that the rapid response manifested in the egg is anchored on a threshold (Runft et al., 2004). Switch like signaling protocol shave also been described in other systems such as the immune system (Runft et al., 2004; Xu and Eck, 1997).

However, the mode of interaction between SFK and PLC-gamma has not been elucidated. It is not clear whether the SFK phosphorylates and activates PLC gamma or it

phosphorylates tyrosine residues at the cell membrane in order to provide binding sites for PLC gamma (Rongish and Kinsey, 1999). In starfish for example, three SFKs have been identified: *Am*SFK3, *Am*SFK2, and *Am*SFK1 (O'Neill and Foltz, 2004). It has been found that both *Am*SFK3 and *Am*SFK1 are essential in cell activation. The activity of *Am*SFK1 kinase activity increases transiently during fertilization (O'Neill and Foltz, 2004). In addition, *Am*SFK3 has been found to possess a very transient kinase activity in response to fertilization (O'Neill and Foltz, 2004). Understanding the genetical features of *Am*PLC gamma, SpSFK1A, AmSFK3, *Am*SFK2, and *Am*SFK1 will be essential in shading more light on the genetical features that influence fertilization

The results consisted of pictures of SDS-PAGE gels from the affinity interaction experiments performed; the first round of affinity interaction experiments did not give the expected results. The isolation of GST only gave a strong signal on the gel, but the isolation of PLCγSH2SH2-GST produced a weak signal. A protein assay was conducted on the PLCγSH2SH2-GST and the concentration of protein was 2.7082 µg/µl. These results suggested that the concentration was not represented well by the gel, so affinity interactions were continued. The results of the affinity interactions showed a band around the 50 kD range. When this gel was transferred to nitrocellulose and incubated with a mouse antiphosphotyrosine antibody as a control, but nothing showed up on the blot. This result was odd because antiphophotyrosine should have produced a signal by, at least, binding to PLCγSH2SH2-GST; however, PLCγSH2SH2-GST only produced a faint signal in the gel, so it may not have bound to the antibody or even transferred to the nitrocellulose. After this result, it was concluded that the bacteria did not express the PLCγSH2SH2-GST insert well and the protein needed to be re-expressed using a different strain of bacteria.



Figure(1):10%SDS-PAGA gel of PGEX-GST isolation from bacteria

Figure 1 showed the Lane 1 was a 10 µl sample of the bacteria before IPTG was added and denoted as uniduced (UI). Lane 2 was a 3 µl sample of a BioRad® Precision Plus Protein Standard. Lanes 3 and 4 were10 µl samples of the bacteria 2 and 3 hours after IPTG was added and a denoted as induced (I). Lane 5 was a 20 µl sample of the bacterial lysate after the GST beads were added and represented any proteins that did not bind to

the beads. Lane 6 was a 20 µl sample of the bacterial proteins that bound to the beads.

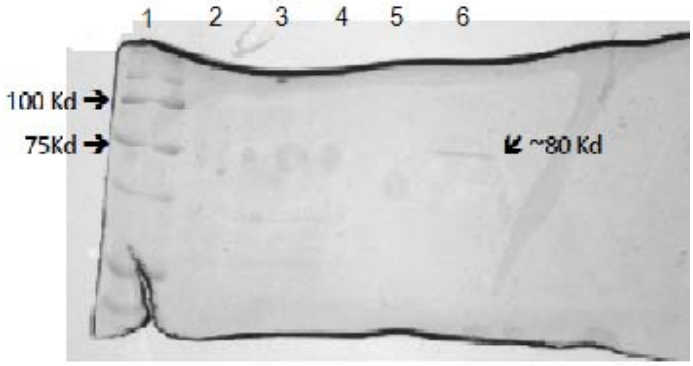


Figure (2). 10% SDS-PAGE gel of the pGEX with the PLCγSH2SH2-GST insert isolation from bacteria

Figure 2 showed the Lane 1 was a 3 µl sample of a BioRad® Precision Plus Protein Standard. Lane 2 was a 10 µl sample of the bacteria before IPTG was added and denoted as uniduced (UI), others lanes as figure 1.

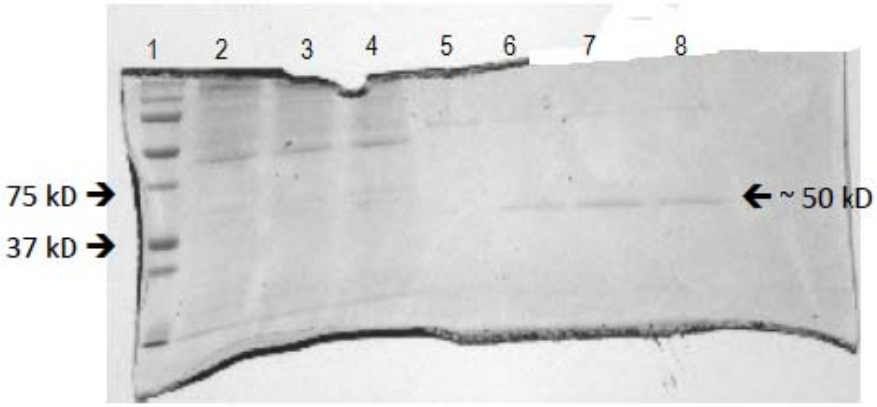


Figure (3). 10% SDS-PAGE gel of the affinity interactions performed with the PLCγSH2SH2-GST beads

Figure 3 showed the Lane 1 was a 3 µl sample of a BioRad® Precision Plus Protein Standard. The beads were inserted into Pisaster oocyte/egg samples that were unfertilized (UF), taken 10 minutes after fertilization (F10), or taken 20 minutes after fertilization (F20). A 20 µl sample of the proteins that did not bind to the beads in the UF,

F10, or F20 oocytes were in lanes 2,3 and 4. A 20 µl sample of the proteins that did bind to the beads in the UF, F10, or F20 oocytes were in lanes 6,7, and 8. Lane 5 was a 20 µl sample of the PLCγSH2SH2-GST beads.

For the second round of affinity interactions, Bov PLCγ SH2(N+C)-GST in Top 10 F cells produced a very strong band in the gel. This band corresponds with the band produced by the SH2(N+C)-mut and SH2(N+C)-wt by Carroll and colleagues(in 1997), which confirms that protein was successfully isolated with the beads. The affinity interactions have not been performed with this protein, but this gel suggests a good starting point for determining the role of FAK.

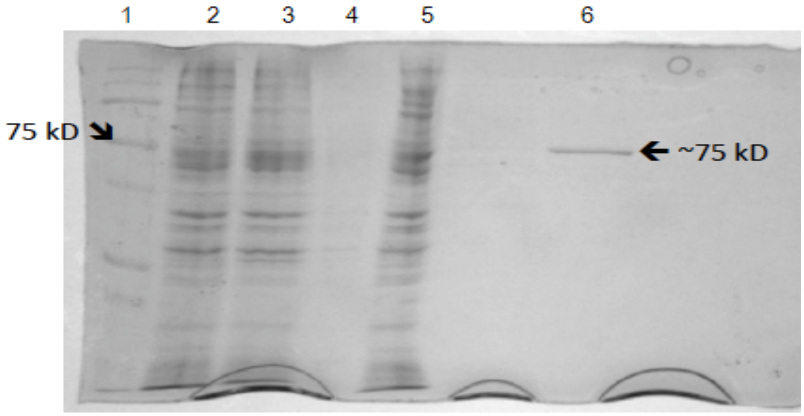


Figure (4). 10% SDS-PAGE gel E. coli with the Bovine PLCγSH2(N+C)-GST isolation.

Figure 4 showed the Lane 1 was a 3 µl sample of a BioRad® Precision Plus Protein Standard. Lane 2 was a 20 µl sample of the bacteria before IPTG was added and denoted as uniduced (UI). Lanes 3 and 4 were 20 µl samples of the bacteria 1 and 2 hours after IPTG was added and a denoted as induced (I). Lane 5 was a 20 µl sample of the bacterial lysate after the GST beads were added and represented any proteins that did not bind to the beads. Lane 6 was a 20 µl sample of the bacterial proteins that bound to the beads.

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