Chapter 4

C. elegans phospholipase C (Ce-PLC-210) mediates ovulation

Introduction

In reponse to extracellular signals, the phosphoinositol specific phospholipase C (PLC) hydrolyzes phosphatidylinositol 4,5 bisphosphate (PIP_2) into diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP_3). These second messengers initiate an intracellular signal transduction pathway by activating either protein kinase C or mobilizing calcium from internal stores, both of which control diverse biological processes (rev. by Berridge and Irvine, 1989). IP_3 levels have been shown to modulate ovulation in *C. elegans* hermaphrodites. (Clandinin et al., 1998; Bui and Sternberg, 2002, Chapter 3). Regulating PIP_2 concentration likely has consequences for downstream signaling events activated by its breakdown products DAG or IP_3. *C. elegans* ovulation defective mutants have proved to be a tractable system with which to study the regulation of proteins, which proposedly alter IP_3 levels thereby affecting ovulation. For example, phenotypic examination of *ipp-5* mutants defective in the 5-phosphatase, indicate that the 5-phosphatase plays a more critical role than the IP_3 3-kinase in down reguatling signaling.

Three established families of phosphoinositol specific PLC have been identified that catalyze the breakdown of PIP_2: PLC γ (145kDa), PLC β (150kDa), PLC δ (85kDa) (rev. by Majerus et al., 1990). For all the three PLC isoforms, the catalytic X and Y domains and C2 regulatory region as well as a plectin homology (PH) domain and EF hand domain are conserved. The modular architecture of the PLC γ isoform differs by number of PH domains and presence of SH2 and SH3 domains. Figure 1 shows a summary of the structural architecture of the PLC isoforms. Each of these family members are regulated by independent mechanisms. PLC β is regulated by heterotrimeric G proteins (rev. by Berridge, 1993). Recent

work has shown that PLC δ is regulated by capacitive calcium entry (Kim et al., 1999). The PLC γ isoform is regulated upon tyrosine phosphorylation following binding to receptor (RTK) and nonreceptor tyrosine kinases via its SH2 and SH3 domains (rev. by Majerus et al., 1990). LET-23 RTK activation stimulates IP_3 production required for the ovulation pathway in *C. elegans* (Clandinin et al., 1998). A *C. elegans* PLCγ has been identified in the genome (*C. elegans* Sequencing Consortium, 1998) and likely acts in this conserved pathway; although, a mutant has not yet been isolated a mutant in suppressor screens of *let-23* or *lin-3* sterility (Lesa, G., and Clandinin, T. personal comm.). A PLC β homolog, *egl-8* (Trent et al., 1983; Thomas, J.,1990; Miller K., et al., 1999), has been identified. However, this mutant has no observable affect on ovulation (Bastiani, C. personal comm.). The phospholipase C enzyme that contributes to IP_3 production required for ovulation has been elusive. A new class of PLC isoform, PLC-210, has been first identified and biochemically characterized in *C. elegans*(Shibatohge et al., 1998). Recently, the closest related human isoform , PLC ε, was found (Lopez et al., 2001; Song et al., 2001), although the cellular function of this new isoform still remains unclear. By studying the function of PLC-210 in *C. elegans* in the context of ovulation, I hope to gain a better understanding of its in vivo role.

The *C. elegans* phospholipase C, Ce-PLC-210, was isolated in a yeast two hybrid screen for LET-60/Ras binding proteins (Shibatohge et al., 1998). In addition to the X, Y, and C2 domain conserved among phospholipase C proteins, the unique presence of two additional domains in Ce-PLC-210 defines a novel class (Figure 1). In the C terminal domain, is a Ras-associating (RA) domain structurally homologous to those on the RalGDS and AF-6 proteins. In the N-terminal domain, is the CDC25-like domain, structurally homologous to guanine nucleotide exhange proteins for Ras,

such as *Drosophila* SOS. PLC-210 is an 1898 amino acid protein of 210 kDa that exhibited hydrolyzing activity towards phosphatidylinositol 4,5- bisphosphate into inositol 1,4,5-triphosphate *in vitro* (Shibatohge et al., 1998). To investigate its functional role in an intact organism, Ken-ichi Kariya isolated two deletion mutants in *C. elegans plc-210.* Based on his initial observations that these mutants were sterile, PLC-210 is required for fertility. As part of a collaboration, I am further investigating the basis for sterility defect and present the genetic characterization.

Results

plc-210 affects fertility and ovulation

To better understand the functional role of PLC-210 in an *in vivo* context, two deletion mutants were generated: *plc-210(cu1),* where one fourth of the Y domain, the entire C2 and RA1 domains are deleted; and *plc-210(cu2),* which lacks half of the Y domain, the entire regulatory C2 and RA1 domains (Figure 2). PLC PIP_2 hydrolyzing activity requires the catalytic X and Y domains which are conserved among phosphoinositide-specific PLC proteins (Fleischman, et al., 1986). Although, the function of the RA1 domain is unknown, this domain is essential for Ras-interaction (Shibatohge et al., 1998). These deletion mutants are likely loss-of-function mutations for any function dependent on Ras-interactions and for PLC activity, leading to decreased IP_3 production and response.

I examined the brood size of both *plc-210* deletion mutants and confirmed that they are semi-sterile: *plc-210(cu1)* has an average brood of 5.4 ± 3.6 (n=20) and *plc-210(cu2)* has an average brood of 8.8 ± 5.6 (n=48). In comparison, a wild type produces an average brood of 281.2 ± 21.5, n=17 (Table 1). Low fertility often stems from ovulation defects (Clandinin et al., 1998; Bui and Sternberg, 2002, Chapter 3).

I examined the gonads of mutant *plc-210* animals under Nomarski optics and noted that multiple eggs are stuck in the spermatheca, a bi-valved smooth muscle-like compartment containing the sperm. From my initial observations, it appeared that multiple oocytes in the spermatheca may result from the spermatheca enveloping multiple oocytes at once, similar to what occurs in *ipp-5(sy605)* mutants which are defective for the inositol 5-phosphatase. In *ipp-5(sy605)* mutants, two or sometimes three oocytes are ovulated at one time; however, only one is fertilized and undergoes embryogensis (Bui and Sternberg, 2002). To determine whether the sterility of *plc-210* mutants results from a similar mechanical defect in ovulation, I conducted Nomarski video microscopy analysis on young adult *plc-210* mutant worms. Nomarski video recordings of ovulation in these mutants reveal a novel mechanical ovulation exit defect, as well as an entry defect.

In *plc-210* mutants, the oocytes that remain in the spermatheca are fertilized and undergo cellular divisions. This phenotype is easily distinguishable by mounting worms and viewing them under Nomarski optics; I saw multiple eggs jam the spermatheca because the spermatheca uterine valve fails to dilate in a timely fashion to release the fertilized egg into the uterus. Each oocyte was at a different point of embryogenesis, indicating they were ovulated at different times (Figure 3). I have never observed ovulation of more than one oocyte per cycle (n=56). Thus, this phenotype is different than the *ipp-5* mutant phenotype. I examine it further below.

In wild-type animals, the oocytes align the proximal gonad and mature in an assembly line fashion as they proceed proximally towards the spermatheca. At ovulation, the epithelial ovarian sheath contracts and the spermatheca valve dilates to envelop an egg and fertilize it. Figure 4 shows a series of Nomarski photomicrographs of a wild type and a *plc210* mutant animal taken during ovulation.

(video 1). In a wild-type worm, the spermatheca-uterine (sp-ut) valve dilates within five minutes of being ovulated to allow passage of the fertilized egg into the uterus, where it is eventually laid through the vulva (McCarter et al. 1997; Clandinin et al. 1998). In *plc-210* mutants, I observe an entry defect, where the spermatheca dilates to ovulate the oocyte, but then the spermatheca does not contract properly so that oocyte is refluxed back through the open spermatheca valve into the oviduct, tearing the oocyte (see video 2, Figure 4B).

In addition to the entry defect, I also observe a novel exit defect. In *plc-210* deletion mutant animals, the sp-ut valve fails to dilate and release a fertilized oocyte into the uterus within the normal time frame. Sometimes, I see several eggs jammed in the spermatheca (Figure 4B, video 1). To determine whether the entry defect where the spermatheca fails to contract properly allowing an oocyte to reflux back into the oviduct is independent and not a secondary affect of the spermatheca jammed with multiple fertilized eggs, I observed if there were any entry defective first ovulations in young adult mutants. For *plc-210(cu2)* mutants, 50%(n=8) had entry defects, while 33% (n= 3) had entry defects in *plc-210(cu1)* mutants. This indicates the entry defect is separable from the exit defect and is likely not a result of the presence of multiple eggs stuck inside the spermatheca. Furthermore, in young adult animals, I observed the second ovulation in which there was one egg stuck inside the spermatheca and no eggs in the uterus. In *plc-210(cu2)* mutants, 67% (n=6) of second ovulations were entry defective, while for *plc-210(cu1)* mutants, 75% (n=4) were entry defective. I also observed third ovulations in young adult animals where one egg was in the uterus and one egg was inside the spermatheca. In *plc-210(cu2)* mutants, 57% (n=14) of third ovulations were entry defective, while for *plc-210(cu1)* mutants, 60% (n=5) were entry defective (see Table

2). This suggests the entry defect does not appear to get progressively worse with successive ovulations.

In summary, for *plc-210(cu2)* mutants, 57% (n=28) of the ovulations observed were entry defective in that the distal spermatheca relaxed/dilated inappropriately. Similarly, 58% (n=12) of ovulations observed in *plc-210(cu1)* mutant animals had entry defects. Every ovulation event is not entry defective, and this defect is separable from the exit defect.

I next determined whether the presence of multiple eggs jammed in the spermatheca somehow inhibits the dilation of the sp-ut valve and prevents timely release of the egg into the uterus. To answer this, I recorded and observed whether the exit defect occurs as early as in the very first ovulation of a young adult, when there would be no eggs inside the spermatheca. I observed in 100% of the first ovulations in *cu1*(n=23) and *cu2* (n=29), the sp-ut valve failed to dilate on time even after the subsequent ovulation occurred, thereby trapping multiple fertilized eggs in the spermatheca. This suggests that the delayed dilation of the sp-ut valve is not due to the physical presence of eggs jammed in the spermatheca. I interpret this phenotype resulting from defects in timing of dilation of the sp-ut valve. I also observed the second ovulation in mutant young adults, and 100% are exit defective in *plc-210(cu1),* n=35, and *plc-210(cu2),* n=40. For the third ovulation, where one egg was in the uterus and one egg was stuck inside the spermatheca, in 100% of the mutant animals the next ovulated oocyte also failed to exit the spermatheca on time (*cu1,* n=19; *cu2,* n=14). Upon successive ovulations, an egg is released into the uterus. This exit defect phenotype is 100% penetrant on a per animal basis (see Table 2).

In mutant *plc-210* animals, I also note that the oocytes in the proximal gonad subsequently goes **e**ndo**m**i**t**o**o**tic (Emo). The Emo sterile phenotype is similar to that observed in *lin-3* and *let-23* reduction-of-function mutants, where the spermatheca fails to dilate sufficiently (McCarter et al., 1999; Clandinin et al., 1998). In Emo mutants, the proximal oocyte fails to be ovulated and gets trapped in the oviduct; subsequently, the DNA undergoes multiple rounds of **e**ndo**m**i**t**o**o**tic replication. The Emo phenotype occurs either when the oocyte refluxes back into the gonad and is not subsequently ovulated, or from the failure of later onset ovulations.

To test whether the fertility/ovulation defect is recessive or dominant, I examined both mutants as heterozygotes. *plc-210(cu1)*/+ animals have an average brood of 295.8 ± 42.8 (n=20; Table 1), while *plc-210(cu2)*/+ animals have an average brood of 273.9 ± 64 (n=14; Table 1). I next examined the gonads and scored the heterozygotes for an exit defect. Like the sterility phenotype, the ovulation exit defect is recessive as 100% of the animals have wild-type ovulations (*plc-210(cu1)*/+, n=40; *plc-210(cu2)*/+, n=39). Together, the data indicate the ovulation defect and sterility phenotypes are recessive for both deletion mutants.

To confirm the phenotype I observed is attributed to the deleted region in the *plc-210* locus, I examined *plc-210* mutant transgenic animals carrying a wild-type copy of *plc-210* on a transgene and *sur-5::GFP* as a co-marker of transformation and assayed for rescue of the ovulation defect. As a control, 100% of nontransgenic animals that did not carry the GFP marker had the exit defect (n=12), recognized by the presence of multiple eggs in the spermatheca. Mutant transgenic animals carrying the wild-type *plc-210* as an extrachromasomal array showed rescue. In n=49/52 gonad arms, I observed rescue for the exit defect. Thus, the defects I observed are

likely a result of the deleted regions in the *plc-210* locus, because adding wild-type copies of the gene rescues the phenotype.

PLC-210 is expressed in the adult spermatheca

To see where PLC-210 might function, I examined the expression of a *plc-210* ::EGFP reporter that includes the N terminal 87 amino acids of PLC-210 fused to EGFP. Transgenic animals carrying an integrated transgene with a 3.9 kb region upstream of the 5' ATG, including the first exon, first intron, and second exon fused to EGFP cDNA show expression in the adult spermatheca (Figure 5) . Additionally, I see expression in the vulva and neurons. Similarly, ITR-1 (Gower et al., 2000; Dal Santo et al., 2000), LFE-2, LET-23 (Clandinin et al., 1998) and IPP-5 (Bui and Sternberg 2002 ,Chapter 3) which act in the adult spermatheca to modulate its contraction behavior are expressed in the adult spermatheca. Spermathecal expression of PLC-210 is consistent with it acting in the adult spermatheca to modulate its dilation/contraction behavior.

Mosaic analysis can indicate what tissue a gene's function is required. Genes affecting fertility likely function in the gonad. The gonadal lineage can be separated into the somatic gonad and the germline. The spermathecal cell lineage is part of the somatic gonad while oocytes are part of the germline (Kimble and Hirsh, 1979). Mosaic analysis indicates that *plc-210* is not required in the germline, which is what I expect if it is acting in the spermatheca (Kariya, K. personal comm.). This suggests PLC-210 likely functions in the somatic gonad.

plc-210* is not suppressed by mutations in *lfe-1(gf)* or *lfe-2(lf)

I examined genetic interactions of *plc-210* with genes that are known to regulate ovulation. Ovulation is dependent on a conserved IP_3 signaling pathway (Clandinin et al., 1998) in which LIN-3/EGF signals through LET-23 RTK and likely stimulates phospholipase mediated hydrolysis of PIP_2 into IP_3, which presumably releases internal calcium through LFE-1, the IP_3R homolog (Figure 5). LFE-2, an IP_3 3-kinase and IPP-5, a 5-phosphatase homolog, also negatively regulate IP_3 signaling, which is crucial for proper ovulation. (Clandinin et al., 1998; Bui and Sternberg 2002, Chapter 3). Either too low or too high amounts of signaling cause ovulation defects, and the Emo sterile phenotype in the most severe cases observed (Bui and Sternberg, 2002, Chapter 3). For example, animals with reduction-of-function (rf) mutations in *lin-3* or *let-23* are presumed to have inadequate signaling resulting in the Emo sterile phenotype. A gain-of-function mutation in *lfe-1* or loss-of-function mutation in *lfe-2* can suppress the sterile phenotype of *lin-3(rf)* and *let-23(rf)* (Clandinin et al., 1998). I tested whether *lfe-1* or *lfe-2* mutations can suppress the sterility associated with *plc-210* deletion mutants. If PLC-210 acts upstream in the pathway, then I predict either *lfe-1* or *lfe-2* mutations will suppress the sterility defect. The average brood of *lfe-1 unc24; plc-210(cu1)* is 9.4 ± 6.7 (n=37) in comparison to the single mutant, *plc-210(cu1*), which has an average brood of 7.8 ± 5.0 (n=47, Fisher's exact test p=0.2297; Table 1). The average brood of *unc-57 lfe-2; plc-210(cu1)* is 9.5 ± 6.9 (n=51, p=0.1626; Table 1). The single mutant, *plc-210(cu2),* has an average brood of 8.4 ± 7.1 (n=41,Table 1). In comparison, *lfe-1 unc24; plc-210(cu2)* has a brood of 9.9 ± 6.0 (n=21, p=0.4605; Table 1) while *unc-57 lfe-2; plc-210(cu2)* has a brood of 8.4 ± 7.1 (n=41, p=0.8051; Table 1). The genetic data indicate *lfe-1* and *lfe-2* fail to suppress the sterility of both *plc-210* mutants. Additionally, I do not observe

suppression of the sp-ut valve defect, as multiple eggs are jammed in the spermatheca (n=many; Figure 3). This suggests that *plc-210* may not be upstream of *lfe-1* or *lfe-2*. Alernatively, *plc-210* may be acting in some parallel pathway or downstream.

Conclusions

In this study, I have characterized a novel type of phosphatidyl inositol phospholipase C, Ce-PLC-210, which has two additional domains not present in other family members: a CDC25 -like domain and a RAS binding (RA) domains). Mutants with deletions in both the catalytic PIP_2 hydrolyzing domain and RA domain result in an ovulation defect indicating Ce-PLC210 has a critical function in ovulation. It is unclear which of these domains plays a regulatory role in ovulation. Ovulation in *C. elegans* is dependent on an IP_3 signaling pathway (Clandinin et al., 1998). In *lin-3* or *let-23* mutants which have insuffficient levels of IP_3 signaling, ovulation defects result because of inappropriate/failed dilation of the spermatheca distal valve. Mutations in *lfe-1* and *lfe-2,* which encode IP_3R and IP_3 3-kinase, respectively, can suppress the ovulation defect in *lin-3* or *let-23* mutants. Levels of IP_3 signaling modulate ovulation and proteins that control levels of IP_3 are important for proper ovulation (Bui and Sternberg, 2002). However, both *lfe* mutations do not suppress the delayed dilation defect of the spermatheca-uterine valve associated with *plc-210* mutants. This is unexpected, as *plc-210* mutants with catatytic domain deletions are loss-of-function and proposedly have lower levels of signaling. Thus, I would expect the *lfe* mutations acting downstream, which increase IP_3 levels, would compensate for the loss of phospholipase hydrolyzing activity in *plc-210* mutants). It would be important to determine whether the effects on ovulation are independent of PIP_2 hydrolysis. Alternatively, ovulation effects of PLC-210 may still be dependent on

PIP_2 hydrolysis, but independent of IP_3. Epistasis genetic data suggest it is possible that *plc-210* functions in an IP_3 independent pathway to control dilation of the spermatheca to regulate ovulation. DAG, the other breakdown product of receptor mediated PIP_2 hydrolysis, may have a signaling function in ovulation. The closest related human isoform, PLCε, is activated by the G protein $G_{\alpha 12}$ (Lopez et al., 2001), suggesting it is possible that the PLC-210 may also be activated by other receptors.

Future experiments should be (1) to determine whether the function of PLC-210 in ovulation is mediated through the RAS GEF activity (through the CDC25 -like domain or the RAS binding (RA) domains) or PIP_2 hydrolysis (2) to test the functional conservation of Ce-PLC210 and the most similar human isoform, PLCε, as its function is unknown. I can examine the functional conservation, by assaying for rescue of the ovulation defect in transgenic *plc-210* mutant worms carrying a transgene of the human *plc* ε gene. I can test which functional domain regulates ovulation, by assaying for rescue of the ovulation defect in transgenic *plc-210* mutant animals carrying a transgene of a mutated copy of *plc-210* which is catalytically inactive for PIP_2 hydrolysis. If there is functional conservation betweenn Ce PLC-210 and hPLCε, I can use the mutant hPLCε H1144L cDNA (Lopez et al., 2001), which is incapable of PIP_2 hydrolysis. Any effects on ovulation would be independent of the second messenger IP_3, suggesting that DAG, the other breakdown product of PIP_2 hydrolysis, may have a signaling function. DAG is a known second messenger (rev. by Berridge, M.J.). We have not yet investigated the role of DAG in ovulation. It would be interesting to observe interactions with *C. elegans* mutants in the DAG signaling pathway: the DAG kinasa, *dgk-1 (*Hajdu-Cronin, Y.M, 1999; Nurrish, S. et al., 1999), which converts DAG into phosphatidic acid, and a PKC homolog, *tpa-1* (Sano et al., 1995), which is an effector of DAG signaling.

Similarly, I can test for the functionality of the other motifs in PLC-210 by mutating each domain separately and assaying for rescue of the ovulation defect in transgenic mutant *plc-210* animals. The ability to assay for protein function by inducing mutations to test for functional domain requirements in transgenic nematodes will be a useful analytic tool for functional analysis.

The function of the RA domain is unknown. In vitro studies show that mutations within the effector region of Ha-Ras disrupt binding to PLC-210 (Shibatohge et al., 1998). Thus, the authors conclude that PLC-210 is a ras effector. It is possible that since neither *lfe-2* or *lfe-1* can rescue the ovulation defect, that function of PLC-210 may not be mediated through IP_3, but through ras signaling. Prior genetic studies show that a *let-60* ras gain-of-function mutation failed to rescue the ovulation defect of *lin-3* or *let-23* reduction-of-function mutants, suggesting ras activaton does not affect spermathecal dilation in ovulation (Clandinin et al., 1998). It would be important to resolve the issue of whether ras activation is important for the function of PLC-210 in ovulation. The caveat of analyzing the *let-60* reduction-of-function mutants that have been isolated thus far in screens for regulators in vulva induction, is that they most likley have been selected for having healthy brood sizes and minimal fertility problems. Loss-of-function mutations in *let-60* are dead, and so we cannot assay for its contribution in ovulation.

The RA domain may target *plc-210* to the membrane closer to its substrate, PIP_2; however, for hPLCε, which is most similar to *C. elegans plc-210*, the RA domain seems not be that important for membrane targeting, as a construct containing only the N terminal guanine nucleotide exchange factor (GEF) domain is highly targeted to membranes (Lopez et al., 2001). The unique presence of the CDC25-like domain may play some functional role in ovulation. This domain contains the

conserved RasGEF catalytic motif signature suggesting that ras activation by promoting exchange of GTP for GDP may be important for regulating dilation of the spermatheca. It would be most interesting to explore further if the function of RA domain and ras signaling in ovulation.

Ce- PLC-210 represents a novel class of phopholipase C. Currently, the cellular function of the closest related human isoform PLCε is unknown. Our studies in *C. elegans* may give us some clues about its physiologic role. Genetic analysis of *plc-210* deletion mutants indicate it functions in ovulation by modulating spermathecal contraction/dilation. Future studies should elucidate the requirements of the bifunctional protein domains in ovulation, which may play a vital role linking multiple pathways to regulate spermatheca contraction behavior in ovulation.

Materials and methods

Methods for maintaining strains at 20°C were according to Brenner (1974.)

The deletion mutants, *plc-210(cu1)* and *plc-210(cu2)* were isolated by Ken-ichi Kariya using the method of G. Moulder and R. Barstead, (personal comm. http://pcmc41.ouhsc.edu/Knockout/).

The following alleles were used: LG1, *unc-57(ad592), lfe-2(sy326)*; LGIV, *unc24(e138), lfe-1/itr-1(sy290), dpy-20(e1282)*; LGX, *lon-2(e678)*. (Strains are from Brenner 1974; Clandinin et al., 1998).

Strain construction

recessive/dominance test

N2 males were mated to *plc-210* hermaphrodites. Cross male progeny which are hemizygous for *plc-210* were mated to *dpy-20(e1282)*. Cross progeny which are

distinguished as non-Dpy hermaphrodites are heterozygous for *plc-210* were subcloned. I assayed these animals for brood size and ovulation exit defects. This was performed for *plc-210(cu1)* and *plc-210(cu2)*.

PS 4194 and PS4197 *lfe-1 unc-24; plc-210*

N2 males were mated to *plc-210(cu1)* hermaphrodites. Cross males, *plc-210*/+ were mated to *lfe-1 unc-24; lon-2*. F1 non-Unc, non-Lon worms of the genotype *lfe-1 unc-24*/++*; lon-2*/ *plc-210* were picked to individual plates .

F2 *unc-24*, non-Lon animals were cloned and of the putative genotype *lfe-1 unc-24; plc-210*. I looked in the next generation to confirm homozygosity of *plc-210* by ensuring there were no *lon-2* segregants. The same genetic scheme was followed for *plc-210(cu2)*.

PS 4195 and PS 4196 *unc-57 lfe-2; plc-210*

N2 males were mated to *plc-210(cu1)* hermaphrodites. Cross males, *plc-210*/+ were mated to *unc-57 lfe-2; lon-2*. F1 non-Unc, non-Lon worms of the genotype *unc-57 lfe-2*/++*; lon-2*/ *plc-210* were cloned.

F2 *unc-57*, non-Lon animals were cloned. Of the putative *unc-57 lfe-2; plc-210*, I looked in the next generation to confirm homozygosity of *plc-210* by ensuring there were no *lon-2* segregants. The same genetic scheme was followed for *plc-210(cu2)*.

Microscopy and image processing

Worms were anesthetized for 30 min in M9 with 0.1% tricaine and 0.01% tetramisole (Sigma Inc.) before mounting and recording (McCarter et al., 1999). Animals were mounted on 5% agarose pads with 20 μl of M9/anesthetic, covered with an 18 mm glass cover slip, and the edges of the cover slip were sealed with

Vaseline. Observations were made at 20°-23° C. Animals were mounted on a Zeiss Axioscope and recorded under Nomarski optics (63X or 100X objective) for no more than 6 hours. The microscope was connected to an MTI 72 CCD video camera module and VCR. Images were recorded on VHS tape in real time. While under anesthetic, oocyte maturation, sheath and spermathecal activity at ovulation proceed while pharyngeal pumping and egg laying cease (McCarter et al., 1999). For ovulation movies, VHS video recordings were dubbed onto DVCAM digital tapes, captured as computer DV stream files via fire wire, and then the frame speed increased by 20000X for *plc-210 (c1)*, or 10000X for *plc-210(c2),* using the software program Final Cut Pro. Video compression for web playback on Quicktime Player 5, was performed using the Sorensen Squeeze Application Program, Sorensen Video 3 compressor. Still frame images (720X480 pixels) were grabbed from the ovulation computer captured DV stream file (Digital Media Center, Caltech).

For fluorescent microscopy, animals were viewed with 100X or 63X objective under a GFP filter. Images were collected using a Hamamatsu C4742-95 digital camera, transferred to a G3 Macintosh running Open Lab Imaging 1.7.8r3 Software, and assembled in Photoshop. I examined animals from two strains *plc-210(c1) Is[plc-210::gfp],* and *N2 Is[plc-210::gfp* (K. Kariya), both of which have the *plc-210:: gfp* reporter transgene integrated into the chromosome. The reporter construct contains 3.85 kb of 5' upstream sequence, the first exon, the first intron (2.26 kb), and the second exon fused to EGFP cDNA. This construct expresses a fusion protein of PLC-210 N-terminal 87 amino acids fused to EGFP (personal comm. K. Kariya).

Brood Assay

L4 larvae hermaphrodites were serially transferred to fresh plates every day for 4 days at 20°C and progeny counted two days after eggs hatched.

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Table 1 Genetic characterization of *plc-210*

genotype	**brood**	**(n)**	**p value**
A)			
+/+	281.2 ± 21.4	17	
plc-210(cu1)	7.8 ± 5.0	47	
plc-210(cu1)/+	295.8 ± 42.8	20	
lfe-1; plc-210(cu1)	9.4 ± 6.7	37	0.2297
lfe-2; plc-210(cu1)	9.5 ± 6.9	51	0.1626
B)			
+/+	281.2 ± 21.4	17	
plc-210(cu2)	8.8± 5.6	48	
pl-c-210(cu2)/+	273.9 ± 64	14	
lfe-1; plc-210(cu2)	9.9 ± 6.0	21	0.4605
lfe-2; plc-210(cu2)	8.4 ± 7.1	41	0.8051

Legend.

Both deletion mutations in *plc-210* cause fertility defects. A) Brood sizes in background of *plc-210(cu1)*. B). Brood sizes in background of *plc-210(cu2)*. All *lfe-1(sy290)* chromosomes are marked with *unc-24*. All *lfe-2* chromosomes are marked with *unc-57*.

Table 2. Ovulation phenotype of *plc-210* deletion mutants

	plc-210 genotype			
	cu1	(n)	*cu2*	(n)
First ovulation:				
Entry defect	33%	(3)	50%	(8)
Exit defect	100%	(23)	100%	(29)
Second ovulation:				
Entry defect	75%	(4)	67%	(6)
Exit defect	100%	(35)	100%	(40)
Third ovulation:				
Entry defect	60%	(5)	57%	(14)
Exit defect	100%	(19)	100%	(14)

Legend. *plc-210* mutant young adult animals were mounted and scored for ovulation defects via Nomarski video microscopy. (n) represents the number of gonad arms. In most cases, we scored both gonad arms in an animal if they were visable in the plane of focus. Two different defects were observed. In some cases, an entry defect occurs where the spermatheca distal valve does not contract/dilate appropriately, such that an ovulated oocyte is refluxed back into the oviduct. An exit defect occurs when the spermatheca uterine valve fails to dilate in a timely fashion to release a fertilized egg into the uterus. Consequently multiple eggs are trapped in the spermatheca. A fertilized egg is eventually released upon successive ovulations.

Figure 1. Schematic structure of PLC family functional domains. X and Y domains, catalytic motifs; PH, plectin homology domains; C2 calcium regulatory domain; SH, src homology domain; RA, ras- associating domain. Adapted from Lopez et al. 2001.

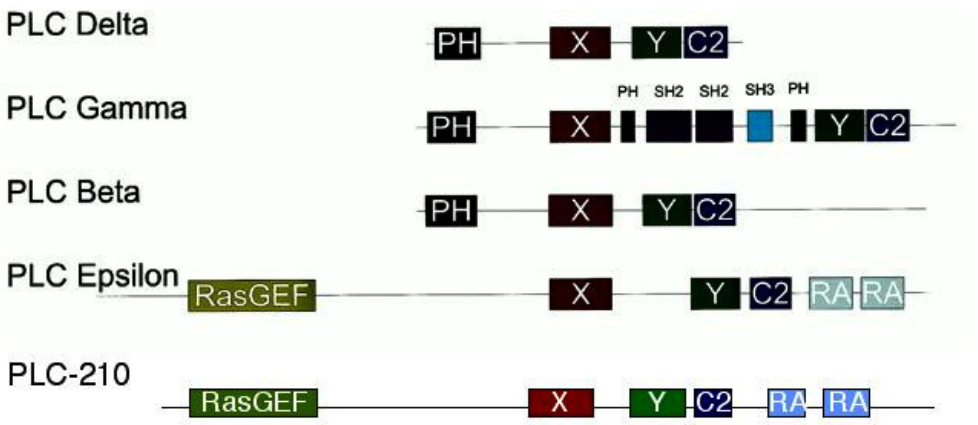

PLC Delta
PH
X
Y
C2
PLC Gamma
PH
SH2
SH2
SH3
PH
PH
X
Y
C2
PLC Beta
PH
X
Y
C2
PLC Epsilon
RasGEF
X
Y
C2
RA
RA
PLC-210
RasGEF
X
Y
C2
RA
RA

Figure 2. Schematic representation of Ce PLC-210. The arrows represent the regions deleted in *plc-210(cu1)* and *plc-210(cu2)* deletion mutants. *cu1* lacks one fourth of the catalytic Y domain, the entire regulatory C2 and RA1 ras-associating domains. *cu2* lacks half of the catalytic Y domain, the entire regulatory C2 and RA1 domains.

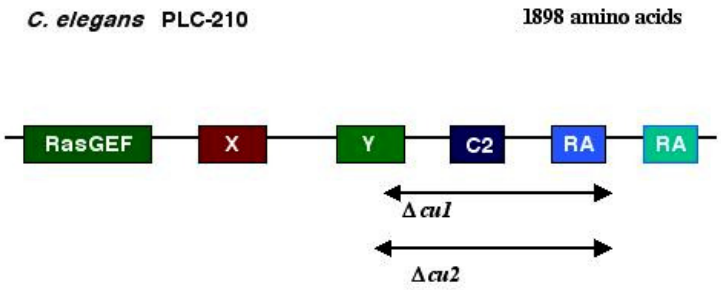

C. elegans PLC-210
1898 amino acids
RasGEF
X
Y
C2
RA
RA
Δcu1
Δcu2

Figure 3. Nomarski photomicrographs of *plc-210* mutant hermaphrodite gonads. The animal is oriented with the ventral side up, the oviduct with oocytes (Oo), aligned to the left and the uterus to the right. (A) In *plc-210* mutants animals, the spermatheca-uterine valve fails to dilate in a timely fashion to release a fertilized egg into the uterus. Consequently, multiple eggs remain trapped in the spermatheca and will be eventually released upon successive ovulations. Note two eggs at different points of embryogenesis inside the spermatheca. The arrowhead points to the proximal most recently fertilized egg which has not undergone as many cellular divisions as the distal egg (arrow). (B) *lfe-1 unc-24; plc-210(cu2)* (C) *unc-57 lfe-2; plc-210(cu2)*. 63X objective. Scale bar, 20 μm.

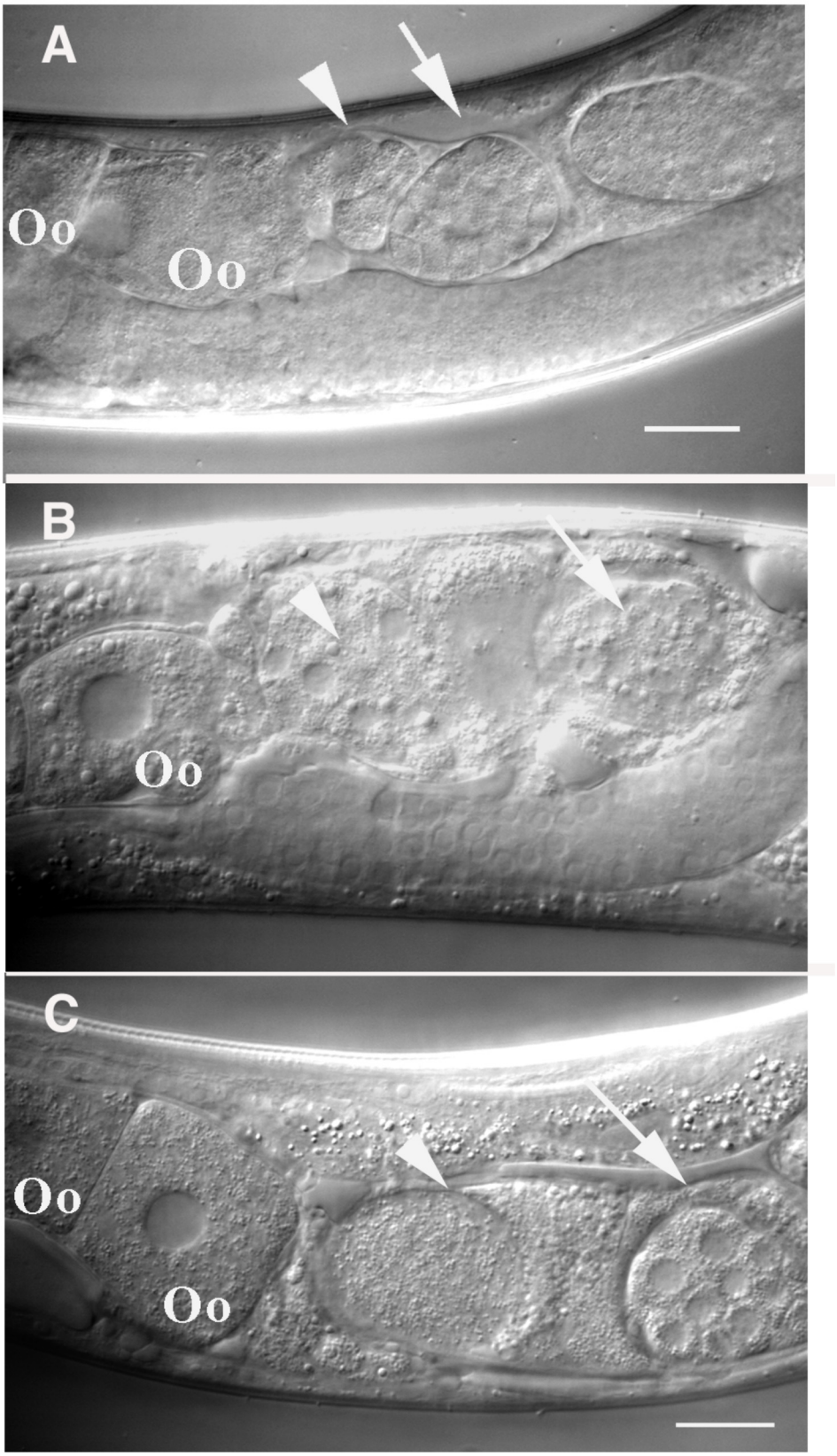
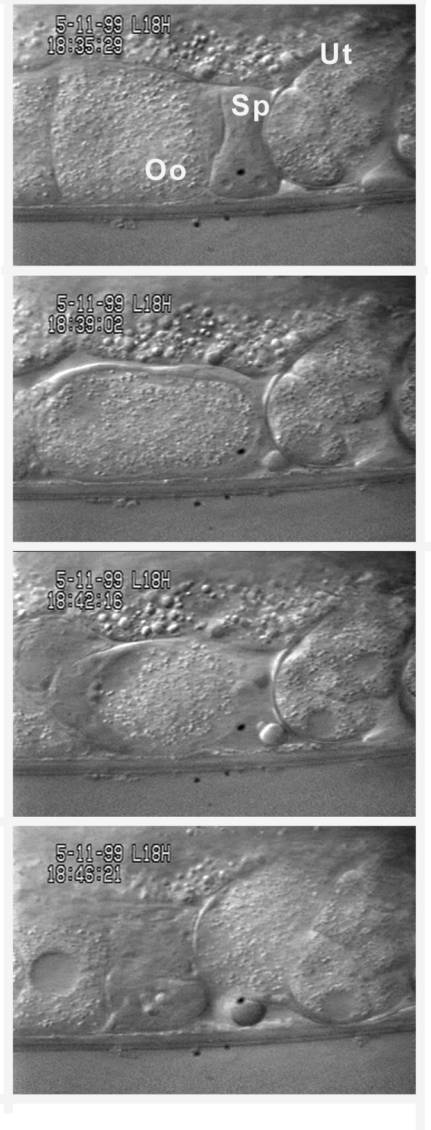

A
Oo
Oo
B
Oo
C
Oo
Oo

Figure 4. Nomarski photomicrograph ovulation sequence

The animal is oriented ventral side down, with oocytes (Oo) aligning the oviduct to the left and the uterus (ut) to the right. Sp denotes spermatheca. (A) wild-type gonad taken during ovulation. (B) *plc-210* mutant gonad taken during ovulation. Two eggs are trapped inside the spermatheca (Sp), demarked by the white double headed arrow. The white arrowhead points to the distal most fertilized egg that is eventually released into the uterus.


5-11-99 L18H
18:35:29
Ut
Sp
Oo
5-11-99 L18H
18:39:02
5-11-99 L18H
18:42:16
5-11-99 L18H
18:43:21

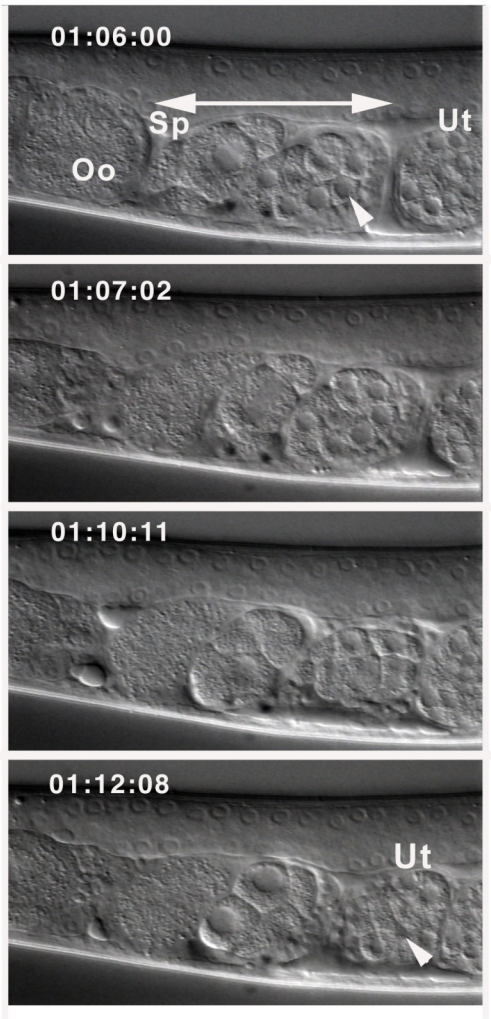
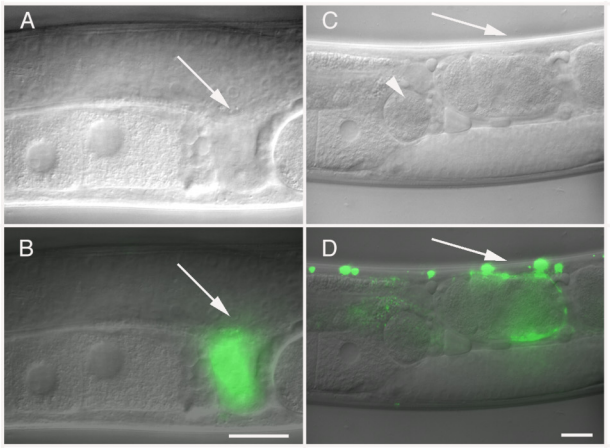

01:06:00
Sp
Ut
Oo
01:07:02
01:10:11
01:12:08
Ut

Figure 5. Expression of *plc-210*

Nomarski and fluoresence photomicrographs of transgenic worms carrying enhanced green fluorescent protein under the control of *plc-210* promoter. A) Nomarski photomicrograph of a wild-type transgenic worm. B). Fluorescence photomicrograph of the same animal. 100X objective. Scale bar, 20 μm. C) Nomarski photomicrograph of a *plc-210(cu1)* deletion mutant transgenic worm. D). Fluorescence photomicrograph of the same animal. 63X objective. Scale bar, 20 μm. Animals are positioned ventral side down. Oocytes aligning the oviduct are to the left. Note the torn oocyte left in the proximal oviduct and multiple fertilized eggs trapped in the spermatheca. The white arrows indicate the position of the spermatheca.


A
C
B
D