Chapter 5

Summary

This body of work combines molecular and genetic techniques to analyze IP_3 signaling downstream of the *Caenorhabditis elegans* LET-23 epidermal growth factor receptor homolog.

Extensive pathway analysis has been conducted for the LET-23 RTK (Aroian,1992; Clandinin, 1998; Lesa, 1998; Chang, 2000) and has greatly increased our understanding of the mechanisms of RTK-mediated signal transduction.
A crucial insight provided by Clandinin's work was to demonstrate that signal specificity can be achieved by activation of distinct signaling pathways downstream of the LET-23 RTK. While vulva induction and viability is mediated by LET-23 activation of a RAS signaling cassette, fertility is mediated by a ras-independent pathway, requiring the inositol triphosphate receptor (Clandinin et al., 1998).

Inositol phosphates are important for intracellular signaling (Berridge, 1993). Most of what is known about inositol phosphate signaling comes from biochemical and molecular cloning studies. The elegant genetic study by Clandinin uncovered the IP_3 receptor (IP3R) and IP_3 3-kinase (IP_3 3K) worm homologs, ITR-1 and LFE-2, in a screen for suppressors of the *lin-3* ovulation defect. It is one of the few in vivo analyses to date implicating a physiologic function that is dependent on IP_3 signaling. Much is unknown about in vivo regulation of IP_3 signaling, thus I collaborated with Minquin Wang in a screen to identify new components that regulate IP_3 signaling. In Chapter II, I describe a screen for suppressors of the double mutant defective in the IP_3 3K and IP3R, *lfe-2; itr-1,* which causes an ovulation defect and sterility similar to the *lin-3* reduction of function mutation. I report initial characterization of four candidates isolated: one of which is a wild-type revertant of the gain-of-function ITR-1/IP3R. Genetic analysis of the other suppressors indicate these mutations decrease the gain-of function activity of *itr-1(sy290)*. Two suppressor mutations are

tightly linked to *itr-1* and may be second-site *itr-1* intrageneic mutations. Further DNA sequence analysis will be needed to confirm this. The sequence of the IP_3R suggests there are many factors involved in the regulation of IP_3R and cloning of these mutant suppressors may elucidate critical factors which down regulate the receptor.

In an attempt to further dissect regulation of the inositol signaling pathway, I took a reverse genetics approach. Nomarksi video microscopy shows that the *lfe-2* mutant, which is defective for IP_3 3-kinase activity, has no observable defects in ovulation. Since IP_3 3K phosphorylates IP_3 into IP_4 thereby controlling IP_3 levels and signaling, we proposed an alternate pathway in worms must exist to down regulate signaling for ovulation. Mammalian studies have identified a 5-phosphatase that also metabolizes IP_3, thereby down regulating signaling (Drayer et al., 1996). Making use of the knock out technology, we screened for a targeted deletion in the *C. elegans* type I 5-phosphatase gene, *ipp-5*. In Chapter III, the genetic characterization of *ipp-5* is described. In contrast to *lfe-2* mutants, *ipp-5* mutants have a novel ovulation defect. Normally during ovulation, the myoepitheleal sheath lining the oviduct contracts and the distal spermatheca dilates and extends to envelop an oocyte (McCarter et al. 1999). Phenotypic analyses of *ipp-5* using video microscopy analysis demonstrate an important effect of negatively regulating IP_3 signaling is to prevent overextension and dilation of the distal spermatheca. Prior to this, it was unclear whether the IP_3 3K or the 5-phosphatase was more important for attenuating IP_3 signaling. My work established that the 5-phosphatase plays a predominant role in negatively regulating IP_3 signaling, in the context of *C. elegans* ovulation.

Differences in signaling levels can have important biological consequences, indicating signal thresholds are important for precision. Additional genetic analysis of

other mutants and mutant combinations presented in Chapter III shows that varying IP_3 signaling levels can translate into qualitatively distinct responses with respect to spermathecal contraction/dilation behaviour. Mutations in signaling proteins, which lead to decreased or elevated levels of IP_3 signaling result in appropriate dilation/ contraction of the spermatheca causing ovulation/fertlity defects.

The genetic data thus far delineate a pathway where LET-23 RTK signaling results in PIP2 hydrolysis and IP_3 production, which activates IP_3 signaling required for ovulation. Genetic screens have not recovered the PLCγ, which is encoded in the genome (*C. elegans* Sequencing Consortium, 1998) and is predicted to mediate PIP2 hydrolysis. However, a Japanese group has discovered a novel phospholipase C, Ce PLC210 in *C. elegans* (Shibatohge et al., 1998). Essentially nothing was known about the function of the *C. elegans* PLC-210. Although the most similar human isoform, PLCε, was recently isolated, its physiological function, largely uncharacterized, remains a mystery. I was primarily intersted in this Ce PLC210 upon recently learning from Ken-ichi Kariya that these deletion mutants were sterile and exhibited an ovulation defect, and thus began a colloborative project to study its function. Chapter IV describes the genetic characterization of *C. elegans* PLC-210. My work indicates it plays a critical regulatory role in modulating dilation of the spermatheca-uterine valve in ovulation. My genetic epistasis data show that mutations in *lfe-2* and *itr-1,* which can suppress *lin-3* and *let-23* reduction of function mutations, fail to suppress the ovulation defects in *plc-210* deletion mutants. Thus, its exact mechanisms of regulation in signaling are unclear. Unlike other phospholipase C isoforms, it uniquely contains a CDC25- like ras guanine nucleotide exchange factor domain and two ras-associating domains along with the conserved catalytic

phospholipase domains. Future functional studies will implicate which domain is important for its regulatory role in ovulation.

Future challenges

Molecular genetic techniques have allowed us to dissect function of metabolites and signaling pathways. Work presented in the thesis body provides the most complete pathway analysis of IP_3 signaling in a model organism to date and implicates a physiological function dependent on IP_3 signaling. My systematic genetic analysis of various mutants which alter IP_3 signaling show that an optimal level of IP_3 signaling is required for proper ovulation in *C. elegans*. IP_3 signaling in other systems has an established role in mobilizing intracellular calcium which activates various programs (rev by Berridge, 1993). Methods for detection that allow fine temporal and spatial resolution of signaling events will provide more detailed analysis of signaling mutants to understand how changes in signaling levels affect physiology.

Tsien and colleagues have developed a fluorescent in vivo calcium detector, the cameleon (1997). Structurally, the cameleon consists of two different green fluorescent protein variants that have different excitation wavelengths, which flank the calcium binding domain of calmodulin. Upon binding calcium, conformational changes bring one GFP variant in close contact with the other GFP variant to allow fluorescence resonance energy transfer, thereby using the emitted wavelength of one to excite the other. This type of sensor is advantageous over standard dyes, because it does not involve timely injection of each animal assayed. Furthermore, we can use promoters to target specific expression of the cameleon in a tissue or cell of interest.

The use of optical indicators is well suited to the nematode, which is transparent and easily amenable to transgenic technology. Kerr et al. (2000) have shown that the cameleon can be used to measure calcium transient changes in the pharynx and neurons in *C. elegans*. In an attempt to study calcium dynamics in ovulation, I have constructed spermathecal promoter cameleon fusions and examined transgenic animals carrying this GFP calcium sensor. Unfortunately, in all the different transgenic lines generated, expression of the cameleon appears to be under some unknown regulatory mechanism. The same promoters driving GFP expression express strongly; however, the promoter cameleon fusions show weaker to nonexistent expression, coexpression in the pharynx, and its expression levels are not affected. Overcoming these problems will enable us to provide a more quantitative means of analyzing how various mutations that perturb IP_3 signaling translate into differences in calcium signaling, which ultimately affect physiologic function.

The definitive role of IP_4 as a second messenger remains unknown. Cullen et al. isolated an IP_4 binding protein, $GAP1^{IP4BP}$, as a putative IP_4 receptor and identified it as a member of the GTPase activating protein family (Cullen et al., 1991; 1995). Molecular modeling and site-directed mutagenesis identified residues within the C terminal PH domain that confer specificity for IP_4 binding (Cozier et al., 2000). The *C. elegans* closest homolog, GAP-1, which was isolated as a regulator of ras signaling in vulva induction (Hajnal et al, 1997), also has the conserved IP_4 binding domain. We can perform genetic analysis of *C. elegans* mutants to uncover a function for IP_4 signaling. Several stop mutations in *C. elegans* GAP-1 that truncate the protein before the PH domain were described (Hajnal et al., 1997).

In Chapter III, I described a genetic deletion mutant in the 5-phosphatase, *ipp-5,* which has a known role dephosphorylating IP(1,3,5)3 into IP(1,4)2 in other systems.

In these *ipp-5* mutants, we expect to have increased levels of IP_3 levels available for conversion to IP(1,3,4,5)4 by LFE-2/ IP_3 3-kinase. It is possible that in *ipp-5* mutants, the ovulation defect could in part be attributed to increased levels of IP_3 and hence IP_4, which could have signaling capacity. We can assay for a function of IP_4 by building a double mutant with *ipp-5* and one of the *gap-1* mutants, *n1683,* which has a stop codon right before the PH IP_4 binding domain. If GAP-1 acts as an IP_4 receptor, these double mutants should not be able to signal through GAP-1, where the IP_4 binding domain is missing. No effects on fertility were reported for the single mutant *gap-1*(Hajnal, et al. 1997). In a sensitized background of the *ipp-5 gap-1* double mutant, if we observe suppression or improvements of the *ipp-5* ovulation phenotype, then we can conclude that the phenotype, in part, resulted from IP_4 signaling through GAP-1. If this were the case, then GAP-1 appears to be a bifunctional protein that acts as a RAS GAP that mediates Ras signaling, but additionally acts as a receptor for IP_4 to mediate IP_4 signaling in ovulation.

That the putative IP_4 receptor, $GAP1^{IP4BP}$, is a member of the GTPase activating protein family that has IP_4 stimulated activity on RAS, suggests a link between phopholipase derived and RAS signaling (Cullen et al., 1995). This has not been examined for the *C. elegans* GAP-1. In Chapter IV, I describe the involvement of CE PLC210 in ovulation, which also suggests a potential link. The CE PLC-210 and its closest human homolog, PLC ε, has CDC25-like RAS guanine nucleotide exchange factor domain, and RA associating domain in addition to the PIP2 hydrolysis catalytic domains (Shibatohge et al., 1998; Lopez et al., 2001; Kelley et al., 2001; Song et al., 2001). In vitro studies show that the effector domain of Ha-RAS must be intact for its binding to the RA domain of PLC-210 suggesting PLC210 is an effector of RAS (Shibatohge et al., 1998). We do not know what the functional

consequences of RAS binding are and furthermore, we do not know what functional domains in PLC-210 are important for ovulation. This bifunctional protein suggests there may be some like between inositol and ras signaling. RAS signaling was not implicated in ovulation as *ras* gain of function mutations did not suppress the sterility of *lin-3* and *let-23* (Clandinin et al., 1998). The challenge will be to understand if and how these pathways integrate to control an appropriate response.

As more studies reveal integration of signaling pathways and defects in signaling contribute to cancer and other genetic diseases, it will be of high interest to work towards increasing our understanding of regulatory mechansims of signaling processes. Progress has been made from biochemical and genetic analyses, showing that signaling pathways are conserved across species from nematodes to vertebrates. Thus, it is likely that future studies using model organisms will continue to provide insights to mechanisms of signal transduction. Our accumulating knowledge of signaling proteins can perhaps one day be applied to development of therapeutic pharmacological agents that modulate signaling.

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