The Caenorhabditis elegans CPI-2a cystatin-like inhibitor has an essential regulatory role during oogenesis and fertilization*

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Running title: C. elegans CPI-2a inhibitor is essential for oogenesis

* This work was supported by The National Institute of Health Grant # AI48057 to (SL)
Summary

In the present study we characterized a sterile cpi-2a(ok1256) deletion mutant in *C. elegans* and showed that CPI-2a has an essential regulatory role during oogenesis and fertilization. We have also shown that the CPI2a inhibitor and both Ce-CPL-1 and Ce-CPZ-1 enzymes are present in the myoepithelial sheath surrounding germ cells, oocytes and embryos as well as in the yolk granules within normal oocytes. Staining of mutant worms with anti-yolk protein antibodies have indicated that the proteins are not present in the mature oocytes. Moreover, GFP expression was absence or reduced in cpi-2a/yp170:gfp mutant oocytes although it was expressed in one of the successfully developed embryo. Based on these results we hypothesize that the sterility in cpi-2a(ok1256) mutant worms is potentially caused by two possible mechanisms: 1) defects in the uptake and/or processing of yolk proteins by the growing oocytes; 2) indirect induction of defects in cell-cell signaling that is critical for promoting germ line development, oocyte maturation, ovulation and fertilization. Defect in any of these processes would have detrimental effects on the development of normal embryos and consequently normal production of progenies as we observed in cpi-2a mutant worms. This is the first study that demonstrates the expression of cysteine proteases and their endogenous inhibitor in the gonadal sheath cells surrounding germ cells and oocytes, which indirectly have established their potential involvement in proteolytic processing of molecules within the gonadal sheath cells such as components of the ECM or the cytoskeletal proteins, which are essential for proper cell-cell signaling activities of the gonadal sheath cells during normal maturation and ovulation processes.

Introduction

Cystatins are endogenous cysteine protease inhibitors that have received a good deal of attention because of their potential regulatory functions. Cysteine proteases are important group of proteolytic enzymes that have traditionally been viewed as lysosomal mediators of terminal protein degradation but more recently have been found to have a more expanded role in cellular physiology. These roles appear to include apoptosis, MHC class II immune responses, prohormone processing, and extracellular matrix remodeling important to bone development (1-6). The cysteine proteases, cathepsin B, L and H, as well as the aspartic protease cathepsin D have been shown to be essential enzymes required for the proteolytic processing of the vitellogenin into yolk proteins during oogenesis (7) and embryonic development in many organisms (8-10). In a mouse model cathepsin B and L have been shown to be required for normal embryo development and uterine decidualization, and that the decidua, which is a transient tissue that develops in the uterine chamber of the mouse and known to secret enzyme inhibitors, contributes to the control of the cysteine proteases by a coordinated expression of cystatin C during mouse implantation (11); the cystatin C message and protein levels are regulated in a specific spatial and temporal pattern that correlates with cathepsin B and L production. In addition, the perturbation of cysteine protease activity during early implantation caused abnormal embryo development and uterine decidualization. The human cystatin C is highly abundant in body fluids and has a role in many important physiological functions (12,13).

The physiological activities of the cathepsin-like cysteine proteases are controlled by their specific endogenous protein inhibitors. Any imbalance between the enzymes and their cystatin-like inhibitors can create in mammalian systems uncontrolled proteolysis seen in inflammatory disorders and during tumor growth [reviewed in (14)]. For example, the persistent phenotype of many metastatic cell types was shown to be associated with increased production of cathepsin-like enzymes and their abnormal regulation by cystatin C (15-17). The importance of cysteine proteases and their inhibitors to the normal development of embryos was demonstrated by treatment of pregnant female animals with the cysteine protease inhibitor, E-64, resulting in stunted embryos (18). In rats, the in vitro perturbation of the yolk sac with E-64 or leupeptin, another specific cysteine protease inhibitor, resulted in decreased
protein processing and embryo growth retardation (19), suggesting that enzyme activity is required for normal breakdown of yolk proteins during embryogenesis (20).

Based on size, location and homology the members of the cystatin superfamily are subdivided into stefin family (type 1), cystatin family (type 2) and kininogen family (type 3) (21), all composed of at least one 100-120 amino acid domain containing a highly conserved reactive site consisting of 5 amino acids, QXVXG (22-24). The type 2 cystatin; cystatin C, D, S, SN and SA are tight-binding inhibitors of papain-like cysteine proteases such as cathepsin B, H, L, and S (12,24-26). They are secreted proteins of approximately 120 amino acid residues containing a single inhibitory domain. The best studied representatives of the cystatin family type 2 are the human cystatin C and the chicken cystatin (12,14,22,27). Type 2 cystatin has been also identified and characterized in many parasites such as Trypanosoma cruzi, T. congolense and T. brucei (28-30) Brugia malayi (31), Litomosoides sigmodontis (32) Onchocerca volvulus (33), Haemonchus contortus (34), Acanthocheilonema viteae (35) and Schistosoma mansoni (36). The precise function of the cystatins in the parasites is mostly unknown although Bm-CPI-2, a cystatin from B. malayi, was shown to suppress the host immune response by interfering in the processing of MHC class II-restricted antigen processing via direct inhibition of the human cathepsins S, L and B (31,37).

In the human parasitic O. volvulus nematode two cystatin-like protease inhibitors encoded by Ov-cpi-1 and Ov-cpi-2 were identified. Based on its localization in the parasites during development in the host, the Ov-CPI-2 was proposed to play a role in the regulation of the endogenous parasite cysteine proteases during molting, cuticle and eggshell remodeling and embryogenesis (38). The Ov-CPI-1 was identified in the O. volvulus EST database; however, its specific role during filarial development is still unknown. Recent studies have demonstrated that the O. volvulus cathepsin L (Ov-cpl) (39) and cathepsin Z (Ovcpz) (40) are localized in the same regions as Ov-CPI-2, implying that Ov-CPI-2 may regulate both enzymes during O. volvulus development. Moreover, using RNAi we have shown that both enzymes are essential for third- to fourth-stage larva molting (41). Using the C. elegans model we have confirmed that the homologues of the filarial enzymes in C. elegans, Ce-cpz-1 and Ce-cpl-1, have essential roles not only during molting but also during embryogenesis (39,42). In both O. volvulus and C. elegans these enzymes may act as proteolytic enzymes processing and/or degrading cuticular proteins during molting and other proteins involved in embryogenesis (41,42). The C. elegans CPL-1 enzyme was also shown to have a specific role in yolk protein processing (43).

The free living nematode, C. elegans, also expresses only two cystatin molecules, Ce-cpi-2a (R01B10.1) and Ce-cpi-1 (K08B4.6). The two cystatins have considerable level of sequence homology to B. malayi, L. sigmodontis, A. viteae, and O. volvulus cystatin inhibitors (44). Biochemical analysis of the inhibitory activity of Ce-CPI-2a and Ce-CPI-1 have shown that although both inhibit the human cathepsin B, L and S with Ki values ranging from 0.0143 to 33.88, both inhibitors have distinct interactions with each enzyme based on the differing Ki values with each of the enzymes (37). Our preliminary data indicated that the cpi2a and cpi-1 genes are expressed differentially during C. elegans development, which suggests that they have distinctive regulatory roles through their interaction with their individual target enzymes, and potentially in discrete processes. In this study we have used the C. elegans model to provide direct evidence of the functional role(s) of the C. elegans cpi-2a during worm’s development and show that its role during C. elegans reproduction is essential. The function of Ce-CPI-2a during development is potentially due to its putative interaction with both the Ce-CPL-1 and Ce-CPZ-1 cathepsin-like enzymes.

**Experimental Procedures**

**Nematode strains and culture conditions**

All Caenorhabditis elegans strains used in this study were maintained and propagated at 20°C on small petri plates containing nematode growth medium (NGM) and seeded with the
Escherichia coli strain OP50 (Brenner 1974). The wild-type strain N2 (Bristol) was received from the C. elegans Genetics Center, Minneapolis, MN, USA and was used to create the various transgenic strains. The C. elegans homozygous mutant cpi-2a allele (ok1256)V and cpi-1 allele (ok1213)IV strains as well as the DH1033 strain were also provided by C. elegans Genetics Center.

Stage-specific profile of the Ce-cpi-2a mRNA transcript using Real-Time PCR
A synchronous population of all developmental stages was prepared as described previously (42). First strand cDNA was generated from 1µg of total RNA using the Omniscript RT kit (Qiagen, Valencia, CA) and priming with random hexamers. The specific cDNA fragment of Ce-cpi-2a was amplified using the forward 5’-CGTCTTCGCTCTCATTGCCATTTC-3’ and the reverse 5’-GTAGTAGTAAAGTCGGTTGTGGGTCG-3’ primers. The copy number of each transcript was quantified by Real-Time PCR using QuantiTech™ SYBR Green PCR kit (Qiagen, Valencia, CA). The following PCR conditions were used: 50°C for 2 min, 95°C for 15 min, followed by 40 cycles of 94°C for 15 sec, 64°C for 30 sec, and 72°C for 45 sec. The copy number of the transcript within each stage-specific cDNA preparations, and the standard curves for cpi-2a and ama-1 were drawn as described previously (42). The ama-1 gene, which encodes the large subunit of RNA polymerase II, was used as the control gene. The levels of ama-1 (45) are relatively constant during development and thus suitable for comparison of transcript levels between stages of the worm.

Analysis of the temporal and spatial expression patterns of the cpi-2a transgene
In order to investigate the cell specific and the temporally regulated expression of cpi-2a (Figure 1A) in vivo we created C. elegans transgenes using a cpi-2a: lacZ fusion reporter construct. The translational fusion construct for cpi-2a, designated (pSL112A) (Figure 1B), contained a 900 bp promoter region upstream to the ATG and the 1.4 kb coding sequence consisting of all three exons of the Ce-cpi-2a and was generated by PCR from C. elegans genomic DNA. This PCR fragment of 2.3 kb was then cloned into the PCR 2.1 cloning vector (TOPO cloning kit, Invitrogen, Carlsbad, CA) before excision and sub-cloning into a C. elegans β-galactosidase (lacZ) reporter vector pPD90.23, which contains the nuclear localization signal (NLS) motif. The C. elegans transgenes were created as described previously using the pRF4 as the selection marker (46,47). Third (F3) and subsequent generations of four transgenic lines exhibiting the roller phenotype were stained for β-galactosidase activity as previously described (42).

Analyses of cpi-2a mutant
To further our understanding of the role of cpi2a in C. elegans development we obtained a deletion mutant, the cpi-2a(OK1256) allele, which has a deletion of 1801 bp that covers all three cpi-2a exons and the 3’ UTR resulting in a complete removal of the coding sequences within the R01B10.1a gene. The mutant was backcrossed three times using wild type N2 (Bristol) strain males according to a standard protocol (48) and maintained as homozygous worms. The homozygous mutant allele was sequenced to confirm the deletion site using inner right sequence primer CCACAATTCCAATCCCAATC corresponding to position 1833 to1813 bp in relation to ATG start codon and inner left sequence ATTTTCTGGCAATTTCGTG corresponding to position –265 to -245 bp in relation to ATG start codon in cpi-2a gene. Individual homozygous mutant hermaphrodites were grown on plates at 20°C and their self-progeny were used in subsequent experiments. For morphological comparison between mutant and wild type strains, living animals were observed using Nomarski differential interference contrast (DIC) microscopy.

Fertility and brood analyses
To measure fertility of the homozygous cpi-2a(OK1256) worms, individual hermaphrodites were placed onto NGM plates seeded with E. coli OP50 and the generation of viable progeny was observed. The worms were transferred to fresh NGM plates every 18-20 hours followed by counting the eggs and larvae for four
consecutive days. If a hermaphrodite worm did not produce during a 5-day period any viable embryo or it produced very low (5-20) number of viable embryos the worm was then classified as sterile.

Rescue of the sterile phenotype due to cpi-2a mutation using C. elegans transgenic strains

To rescue the cpi-2a mutation by complementation, several transgenic rescue constructs were created using a C. elegans expression vector (pPD95.75) without the GFP (Figure 1): 1) The cpi-2a cDNA was placed under the control of its endogenous 900 bp promoter (Figure 1C; pSL112C ); 2) The cpi-1 cDNA was placed under the control of the Ce-cpi-2a 900 bp promoter (Figure 1D; pSL151A); and 3) The genomic sequence encoding for the full length cDNA of the R01B10.3 gene under its own 900 bp promoter region (Figure 1E; pSL149A). The rescue of the cpi-2a(ok1256) mutant strain with the full genomic sequence of R01B10.3 was designed to confirm that the phenotype due to deletion was only due to the knockdown of the cpi-2a transcript. The opposite strand of the genomic sequence of cpi2a (R01B10.1) encodes another gene named R01B10.3 (alpha-amylase) which is 396 bp long and composed of 3 exons (Figure 1F) and corresponds to the second intron of cpi-2a on the other strand.

Transgenic lines for each rescue construct were created by the injection of each rescue plasmid DNA along with pRF4 into the gonad of C. elegans N2 strain as described above. The procedure to rescue deletion mutant worms using transgenic strains followed the procedures described by Janke et al (49). In brief: heterozygous mutant males were created by crossing hermaphrodite cpi-2a(ok1256) mutant worms with wild type males; 15-20 individual transgenic hermaphrodites expressing the rescue gene were then crossed, each with 10-12 heterozygous mutant males. After 36 h of mating, the hermaphrodites were transferred individually to fresh plates and allowed to produce progeny. The F1 progeny was screened for males exhibiting the roller phenotype (rol-6). The presence of a roller phenotype in the progeny of the crossed worms was an indicator of the presence of the transgene within the worms and thus successful crossings. In addition, single worm PCR was performed on the roller worm to ensure that these worms contained the rescue gene. Twenty five L4 roller hermaphrodites from a successful mating plate were individually picked and transferred to fresh plates to allow self-fertilization. For each transgene rescue experiment the individual worms were of two possible genotypes: 1) cpi-2a(ok1256)/+; cpi-2a (Ex) or +/-; cpi-2a (Ex): 2) cpi-2a(ok1256)/+; cpi-1(Ex) or +/-; cpi-1 (Ex): 3) cpi-2a(ok1256)/+; R01B10.3 (Ex) or +/-; R01B10.3 (Ex). The Ex designates extrachromosomal array for each rescue gene. The worms of both genotypes were screened for the presence of sterile or non-sterile animals over 4 days. If the worms produced <20 progenies in 4 days they were considered sterile or non-rescued. If the worms produced >150 worms in 4 days they were considered rescued successfully by the transgenic strain. The rescue was correlated with the presence or absence of the rescue genes as well as their genotype (the presence and absence of cpi-2a deletion) using PCR on single worms (fertile and non fertile animals) with the corresponding gene specific primers. The progenies of the heterozygous fertile or non fertile roller worms were self-fertilized to obtain homozygous. We used single worm PCR on roller mother to confirm their genotype and then the progenies of homozygous worms were tested for fertility by growing them individually on separate plate. The presence of fertility in cpi2a/cpi2a; cpi-2a (Ex) worms indicated that these worms were rescued. The continued presence of sterility in cpi2a/cpi2a; R01B10.3 (Ex) or cpi2a/cpi2a; cpi-1(Ex) indicated that both genes did not rescue the sterile phenotype of cpi-2a(ok1256).

Co-localization by immunofluorescence of the CPI-2a cystatin inhibitor with two C. elegans endogenous cysteine proteases; Ce-CPZ-1 and Ce-CPL-1

Rabbit anti-CPI-2a antibodies and mouse anti-CPL-1 or anti-CPZ-1 antibodies were used for the co-localization of their corresponding inhibitor and enzymes in C. elegans embryos, mixed larvae and adult worms by immunofluorescence assays (IFA). The rabbit
anti-Ce-CPI-2a antibodies were kindly provided by Susanne Hartman (Molecular Parasitology, Humboldt University, Berlin, Germany). Because the anti-CPI-2a antibodies cross-reacted with the Ce-CPI-1 recombinant protein (data not shown), we used the cpi-1(ok1213) mutant strain for staining with the anti-CPI-2a antibodies, which allowed the determination of the specific locations of the CPI-2a native protein. The Ce-cpi-1(ok1213) allele has a deletion of 1244 bp that covers 460 bp of the promoter region as well as the gene coding for exon 1, exon 2 and part of exon 3 within K08B4.6; thus not expressing any fragment of CPI-1 that could cross react with the anti-CPI-2a antibodies. The Ce-cpi-1(ok1213) mutant strain does not have any obvious phenotypes and its reproduction is normal. The procedures for sample preparation and staining were essentially the same as described previously for Ce-CPZ-1 and Ce-CPL-1 (39,42). In brief, C. elegans cpi-1 mutant gravid hermaphrodites were washed with PBS and cut open to release the embryos. The embryos were then fixed in methanol/acetone using the freeze-cracking protocol (50). Whole-mount fixation of the mutant animals was performed according to a modification of the method of Finney and Ruvkun (51). The fixed embryos or the permeabilized whole worms were treated with 1% BSA for 1 h before reaction with antibodies. The embryos or mixed stage larvae and adult worms were incubated with a mixture of rabbit anti-CPI-2a and mouse anti-CPL-1 or CPZ-1 antibodies at a dilution of 1:200 each. The samples were incubated with the antibodies at 4°C overnight. After washing, the samples were incubated with a mix of FITC-conjugated goat anti-rabbit (1:250) and Texas Red-conjugated goat anti mouse (1:250) secondary antibodies. Embryos were incubated with secondary antibodies for 2h at 37°C, while the larvae and adult worms were incubated at 4°C overnight. Specimens were mounted on slides with 15 µl of mounting medium, VECTASHIELD containing DAPI, and viewed under Axioskop 2 plus fluorescence microscope (Zeiss, Germany) using appropriate filter sets.

Ultrastructural localization of the native Ce-CPI-2a protein in C. elegans
In order to study the subcellular localization of the native CPI-2a protein during C. elegans development a mixed population of cpi-1 mutant worms was collected and fixed for 60 min using 4% parafomaldehyde/0.1% glutaraldehyde in 0.1M phosphate buffer, pH 7.4 containing 1% sucrose. The fixed worms were then processed for immunoelectron microscopy as previously described (33,40). Thin sections of embedded C. elegans Ce-cpi-1(ok1213) mutant worms were probed with rabbit anti-Ce-CPI-2a antibody before incubation with 15 nm gold particles coated with anti-rabbit IgG (Amersham Pharmacia Biotech). Rabbit pre-immune serum was used as the control. In addition, we probed thin sections in similar regions of the Ce-cpi-1(ok1213) mutant worms with mouse antibodies raised against Ce-CPL-1 and Ce-CPZ-1 according to published protocols (39,42).

Localization of the C. elegans yolk proteins by immunofluorescence
Mouse monoclonal antibodies to C. elegans yolk proteins YP170 (PIIA3) and YP88 (OIC1) (52,53) were kindly provided by Susan Strome (University of Indiana). For detection of the yolk proteins in C. elegans wild type and cpi-2a mutant worms, fixed embryos and permeabilized whole worms were processed as described above before reaction with monoclonal antibodies to Ce-YP170 and YP88 yolk proteins at a 1:100 dilution for embryos and 1:200 dilution for permeabilized whole worms. Texas Red-conjugated goat anti-mouse secondary antibodies were used at a 1:250 dilution. Specimens were then mounted on slides and analyzed as described above.

To explore whether the YP170 yolk protein is transported and taken up normally by cpi-2a mutant oocytes and embryos homozygous cpi-2a mutants carrying the YP170::GFP transgene were generated by standard genetic crosses and GFP levels and distribution were compared to that in wild-type (strain-DH1033). In wild type worms the YP170::GFP protein is transported from the hermaphrodite intestine via the pseudocoelom to the gonad for endocytosis by oocytes (Grant and Hirsh, 1999).
Results

The cystatin-like inhibitors of *C. elegans*

Two predicted *C. elegans* cystatin-like inhibitors were identified in the Wormbase database (http://www.wormbase.org). They are encoded by cosmids R01B10.1a and K08B4.6 and were named *Ce-cpi-2a* and *Ce-cpi-1*, respectively. The *Ce-cpi-2a* gene maps to chromosome V (accession number AF068718) and *Ce-cpi-1* maps to chromosome IV (accession number AF100663). The predicted *Ce-CPI-2a* protein contains 143 amino acids (15.7 kDa), while the *Ce-CPI-1* protein contains 139 amino acids (15.0 kDa). Both amino acid sequences of *Ce-CPI-2a* and *Ce-CPI-1* contains sequence features characteristics of the *O. volvulus* onchocystatin, *Ov*-CPI-2 (M37105), and contain the conserved glycine residue in the 5' end, a stretch of five amino acids QVVAG and a PW dipeptide in the C-terminal part of a molecule, all of which are a characteristic feature of type 2 cystatins (23). These motifs are believed to form the inhibitory site loop that is directed against the papain binding motif (MatInspector/Gene regulation: hormone receptor family), T3alpha1 (zinc dependent DNA-binding) and CRE-BP1 (DNA binding motif) (MatInspector/Gene regulation: downstream). Both amino acid sequences of *Ce-cpi-2a* and *Ce-cpi-1* contain the conserved region and PW motifs, which are essential for its activity as an inhibitor. Therefore, this truncated protein is not predicted to be bioactive.

Temporal expression of *cpi-2a* transcript during development

Quantitative real time PCR was performed on total RNA prepared from synchronized populations of *C. elegans* at two-hour intervals. The *C. elegans ama-1* gene was used as an internal control transcript to allow the relative quantification of *cpi-2a* expression in each stage. The *cpi-2a* transcript was present throughout development; however its expression varied between stages. Notably, the *cpi-2a* transcript increased approximately 2.5-3.5 hours before each molt: the L1/L2 molt (11.5 h after hatching), the L2/L3 molt (18.5 h); the L3/L4 (26 h) and the L4/adult molt (36h) (Figure 2A & B). Subsequently to each molt the transcript levels decreased. Because in our previous studies we reported a role of cysteine proteases during molting in *C. elegans* (39,42) it was of interest to compare the temporal expression levels of the *Ce-cpl-1* and *Ce-cpz-1* transcripts in relation to that established for *cpi-2a*. Interestingly, the transcription levels of *Ce-cpl-1* were also elevated before each molt and at the same times as those of *Ce-cpi-2a* (Figure 2a). The decrease in the transcription levels after the L2/L3, L3/L4 and L4/adult was also similar, except that after the L1/L2 molt the *cpi-2a* transcript went up but the *cpi-1* remained low. The elevated *cpi-2a* transcription profile before each molt also coincided with the *C. elegans* *Ce-cpz-1* transcript levels but only before the L2/L3, L3/L4 and L4/adult molt (Figure 2b), and the timing before the molt was 2 hours. The reason for the significant increase in the relative expression levels of *cpi-2a* and the enzyme encoded genes during the L2/L3 molting stage is presently unknown. These results implied that CPI-2a may play an important role during molting perhaps through regulating the functions of its putative target CPL-1 and/or CPZ-1 enzymes. During adult stage development the levels of *cpi-2a* were moderately elevated and stable similarly as those of *Ce-cpl-1* and *Ce-cpz-1* (data not shown).

The spatial pattern of *Ce-cpi-2a* expression

The *cpi-2a* gene is 1.436 kb long and composed of 3 exons and 2 introns (Figure 1A). The spatial expression pattern of *Ce-cpi-2a* was examined after transformation of *C. elegans* N2 (Bristol) strain with a *cpi-2a: lacZ* fusion construct (pSL112A) containing NLS. The translational construct was created by fusion of 900 bp of the putative promoter region, and the 1.4 bp of *cpi2a* coding sequence consisting of all three exons (Figure 1B). The putative promoter region contains regulatory elements common in different organisms such as: GATA, AP-1, SP1, Oct-1, PAR-alpha 1 (member of steroid hormone receptor family), T3alpha1 (zinc dependent DNA-binding) and CRE-BP1 (DNA binding motif) (MatInspector/Gene regulation: downstream).
Interestingly, the cpi-2a promoter does not contain CAAT or TATAA boxes. The importance of these regulatory elements in the cpi-2a promoter remains to be analyzed.

The cpi-2a:lacZ transgenic strains expressed cpi-2a during worm’s development (Figure 3). The expression was first observed in few embryonic cells within the very early stages of embryos (Figure 3a). During embryonic progression many more cells were stained, specifically the embryonic intestinal, pharyngeal and hypodermal cells and some other unidentified cells (Figure 3b). As the embryos progressed to pretzel stage, the expression in several of the hypodermal and intestinal cells was maintained (Figure 3c). During the L1 to L4 development cpi-2a was highly expressed in the intestinal and hypodermal cells, as well as in the pharyngeal cells including pharyngeal gland cells (Figure 3d). In adult stages, in addition to embryos in the uterus, the lacZ staining was mostly confined to the pharyngeal gland and pharyngeal muscle cells (Figure 3e) and few hypodermal cells in the tail of the worm (Figure 3f).

**Analysis of the cpi-2a(ok1256) mutant strain**

To establish the specific role(s) of the *C. elegans* CPI-2a cystatin during development, we analyzed a *C. elegans* homozygous cpi-2a(ok1256) deletion allele mutant strain obtained from the *C. elegans* gene knockout project at Oklahoma Medical Research Foundation. Before analysis, the mutant strain was backcrossed 2-3 times. Although the resulting homozygous cpi-2a(ok1256) mutant adult worms appeared normal the majority of them (90%) developed into sterile adults producing at the most 16 fertile embryos over a 4 day period. Most of the embryos (98%) produced by those worms were laid in the first 24 hours of the egg laying. After that the gonad of these egg laying mutant worms contained abnormal oocytes. The laid embryos went through normal larval development to adult hermaphrodites but those adult hermaphrodites were still sterile. About 10% of the mutant hermaphrodites were completely sterile and no eggs were laid. In these worms although one or two of the germ cells were transformed into developed oocytes, the oocytes never matured, ovulated and produced a fertilized embryo. Wild type worm usually produce ~300 fertile embryos during the same period. Notably, the somatic gonad as well as the germ cells and the sperm cells appeared normal in the cpi-2a(ok1256) mutant worms during early meiosis I and later developmental stages as evidenced by DAPI staining (Figure 4a-d). However, continue development of the oocytes in the proximal gonad did not occur probably because of defects in the timely maturation and ovulation of oocyte that resulted accumulation of several disorganized germ cells nuclei (arrow) near the bend region in the cpi-2a mutant gonad (Figure 4d). Moreover, providing cpi2a mutant hermaphrodites with wild type sperms by mating with wild type males did not restore their fertility indicating that the sterility in cpi-2a(ok1256) is oocyte specific.

The production of fertilized embryos depends on the normal development of oocytes, their timely maturation, ovulation and fertilization. Theoretically any one of these steps could be rate limiting for the production of fertile embryos. It appears that in the cpi-2a(ok1256) mutant hermaphrodites all the three processes were affected as significant defects in oocyte maturation, ovulation and/or the progression of the fertilized zygote through the spermatheca were observed. The phenotypes observed (Figure 4) include: the few newly developed oocytes were not properly shaped and clustered in the normal assembly-line-like fashion although some of the previously matured oocytes developed into normal embryos (Figure 4f); production of the few normal embryos was restricted to only one gonad arm of the mutant hermaphrodites (Figure 4g); only one oocyte with an intact nuclear envelope developed; however, it did not mature and thus ovulate. The other oocytes in the proximal gonad arm were mostly undeveloped (Figure 4h); endomitotic oocytes were present in the most proximal end of gonad, undeveloped oocytes were present close to the bend region and no embryos were present in the uterus (Figure 4i).
To confirm that the sterile phenotype in these mutant worms is due to the deletion of the cpi2a transcript and not the transcript encoded by the R01b10.3 gene on the opposite strand, we performed rescue experiments with two transgenic lines containing cpi-2a (Ex) or R01b10.3 (Ex). The wild type copy of cpi-2a gene rescued the sterile phenotype; progenies of all crossed worms were fertile and produced on average 150 healthy embryos over a 4 day period and they were positive for cpi-2a (Ex) as indicated by PCR and contained two genotypes: cpi-2a(Ok1256)/+; cpi-2a (Ex) (n=10) and +/-; cpi2-a (Ex) (n=15). Progenies from the heterozygous cpi-2a(Ok1256)/+; cpi-2a (Ex) were self-fertilized to obtain homozygous cpi-2a(Ok1256)/+; cpi-2a (Ex), all of which were fertile and thus rescued. Out of the 25 worms expressing the R01b10.3 (Ex) gene 7 were of the genotype cpi-2a(Ok1256)/+; R01b10.3 (Ex) and they produced on average only 17 healthy embryos over a 4 day period, similarly to the cpi-2a(Ok1256) mutant worms. Progenies from these heterozygous worms were then self-fertilized to obtain homozygous cpi-2a(Ok1256)/+; cpi-2a (Ok1256)/+; R01b10.3 (Ex), all of which were still fertile indicating that R01b10.3 (Ex) gene was unable to rescue the sterile phenotype of cpi-2a(Ok1256). The other 18 worms had the genotype +/-; R01b10.3 (Ex) and produced on average 143 normal healthy progenies on the same period. These results indicate that the sterile phenotype was the result of disruption in the normal function of the CPI2a inhibitor. Interestingly, the cpi-2a mutant sterile phenotype could not be rescued using its homologous Ce-cpi-1 cDNA (K08B4.6) under the cpi-2a promoter (Figure 1D), confirming our hypothesis that both inhibitors have distinctive regulatory roles through interaction with their individual target enzymes.

**Localization of the native CPI-2a protein in C. elegans by immunofluorescence and immunoelectron microscopy and its co-localization with the Ce-CPL-1 and CPZ-1 enzymes**

Using IFA we found that the CPI-2a protein is expressed in the sheath cell cytoplasm surrounding the germ cells and oocytes (Figure 5a), in many embryonic cells within the embryos (Figure 5d), and in eggshells (Figure 5g). The CPI-2a protein was also present in the cuticle of all larval stages (data not shown). To establish whether the C. elegans CPI-2a cystatin is spatially co-expressed with its potential target cysteine proteases, we performed co-localization analyses of CPI-2a with two of the well characterized cysteine proteases of C. elegans; Ce-CPL-1 and Ce-CPZ-1, which were shown to function in embryogenesis and larval development (39,42). The CPI-2a protein was co-localized with CPL-1 during oogenesis and embryonic development; they were both present in the same locations in the sheath cell cytoplasm which is concentrated between the germ cells (Figure 5c, a honeycomb pattern), in embryonic cells within the developing embryos (Figure 5f), and in the eggshells (Figure 5i). They also co-localized in the cuticle of the embryos (data not shown). CPI-2a also co-localized with CPZ-1 in the developing oocytes (Figure 6c) as well as the sheath cell cytoplasm around the germ cells. They also co-localized in the cuticular regions of all larval stages (data not shown), however, while CPI-2a was present along the ecdysed cuticle of a molting worm, the CPZ-1 enzyme was only expressed in part of the ecdysed cuticle (Figure 6g). These results suggest that CPI-2a may regulate the functions of both CPL-1 and CPZ-1 but distinctly, in different regions and at differing times during the worm’s development.

To identify more precisely the subcellular regions within the worms where the native CPI-2a protein is localized, thin sections from various developmental stages of Ce-cpi-1(Ok1213) mutant worms were stained with anti-CPI-2a antibodies for immunoelectron microscopy analysis. The CPI-2a native protein was localized in the cuticle of all stages (Figures 7a) as demonstrated also by IFA. During oogenesis and embryogenesis, the CPI-2a was expressed in the gonadal sheath surrounding the germ cells, oocytes and embryos (Figures 7b, 7c and 7f), the yolk granules within developing oocytes (Figure 7c insert), yolk protein platelets within the body cavity (Figure 7d), in embryos (Figure 7f) and in the eggshells surrounding the embryos (Figures 7f & 7g). Notably, CPI-2a was also highly expressed in the developing sperms.
within the spermatheca (Figure 7e).

To further investigate whether CPI-2a may regulate the functions of its putative two target enzymes Ce-CPL-1 and Ce-CPZ-1 during reproduction, we extended our previous localization studies of the Ce-CPL-1 and Ce-CPZ-1 native enzymes (39,42) and examined more closely their presence within the gonad. It appeared that both enzymes are highly expressed in germ cells, the yolk granules within the developing oocytes, in embryos and the eggshells as well as the gonadal sheath surrounding these cells (Figure 8). Distinctively, both enzymes are not expressed in the sperm cells or the yolk protein platelets (data not shown).

Detection of yolk proteins in cpi-2a mutant worms
Cysteine proteases and cystatins have been shown to have essential roles in the proteolytic processing of vitellogenin into yolk proteins during oogenesis (7,11,55-57) and embryogenesis (8-10,19,20,58). In C. elegans cpl-1(vc322) mutant worms the loss of CPL-1 activity was shown to lead to an aberrant processing and/or conformational changes in yolk proteins, resulting in embryonic lethality phenotype. In addition, in the cpl-1 mutant embryos the recycling and/or degradation of the RME-2 yolk receptor was significantly delayed, but not completely blocked (43). Because loss of CPI-2a activity caused developmental defects in oocytes, we examined whether the processing of yolk proteins in these mutant worms was also affected. Antibodies to the C. elegans YP170 and YP88 proteins were used to localize the yolk proteins in homozygous cpi-2a mutant and wild type N2 worms (Figure 9a-c). In wild type worms, the development of oocytes occurs in the proximal end of the C. elegans U-shaped gonad, which has a distal-to proximal polarity with respect to the development of germ cells that go through three stages of development and differentiate into developing oocytes. Once matured the oocytes are fertilized and produce embryos (61). The development of late stage oocytes and embryos depends on nutrients in particularly the yolk proteins. The C. elegans yolk is secreted from its site of synthesis, the

YP170::GFP fusion protein in wild type DH1033 strain and in homozygous cpi-2a/yp170::gfp worms, we found that in the mutant worms the GFP expression was either absent or only present at very low intensity in the oocyte located most proximately to spermatheca, although the embryo next to the spermatheca that has developed in this mutant worm was positive for YP170::GFP expression (Figure 9g). The negative GFP expression coincided with the presence of abnormal oocyte in the mutant worm (Figure 9f). In the wild type (strain DH1033) GFP expression was observed in the two late-stage oocytes proximal to the spermatheca and in the embryos (Figure 9e). These results suggest that the imbalance between cysteine proteases expressed in these cells and their potential cystatin inhibitor in these mutant worms might have affected the normal uptake and/or processing of the yolk proteins.

Discussion
The C. elegans cystatin, CPI-2a has an essential regulatory role during oogenesis and fertilization. The cli-2a (ok1256) mutant is sterile. We hypothesize that the sterility is caused by two possible mechanisms: 1) defects in the uptake and/or processing of yolk proteins by the growing oocytes; 2) indirect induction of defects in cell-cell signaling that is critical for promoting germ line development, oocyte maturation, ovulation and fertilization. Defect in any of these processes would have detrimental effects on the development of normal embryos and consequently normal production of progenies as we observed in cpi-2a mutant worms.

In wild type worms, the development of oocytes occurs in the proximal end of the C. elegans U-shaped gonad, which has a distal-to proximal polarity with respect to the development of germ cells that go through three stages of development and differentiate into developing oocytes. Once matured the oocytes are fertilized and produce embryos (61). The development of late stage oocytes and embryos depends on nutrients in particularly the yolk proteins. The C. elegans yolk is secreted from its site of synthesis, the
intestine, into the pseudoceolomic space (body cavity), and is ultimately taken up into vesicles (yolk granules) within the growing oocytes (62) by a receptor-mediated endocytosis pathway (60). The primary translation products are precursors, or vitellogenins, which are cleaved and modified to yield the mature yolk proteins (63). Proteases have been implicated in yolk processing during oogenesis and embryogenesis but their identity, location, regulation and precise role(s) have not been studied in detail. In the chicken, cathepsin D has been identified as the major enzyme involved in yolk processing (64) whereas in *Xenopus*, amphibians and fish, in addition to cathepsin D the cysteine proteases cathepsin L and cathepsin B have been also implicated (65-68). The studies in *Xenopus* have suggested that aspartic protease activity is also required for yolk-receptor dissociation (68). In insects, however, cathepsin L and B, rather than cathepsin D, appeared to be the major enzymes involved in yolk degradation (8,58,69). A potential role of cathepsin L and cathepsin F cysteine proteases in the yolk protein processing events during oocyte maturation and/or early embryogenesis was also described in *Fundulus heteroclitus* (57). The importance of regulating cysteine proteinase activities during development of oocytes and embryos by their endogenous cystatin inhibitors is not as yet fully understood. However, in rats the influence of cysteine protease inhibitors on embryogenesis was demonstrated by the treatment of pregnant female animals with the cathepsin inhibitor, E-64, resulting in stunted embryos (18). In addition, in vitro perturbation of the rat yolk sac with the E-64 or leupeptin inhibitors resulted in decreased protein processing and embryo growth retardation (19) confirming that controlled enzyme activity is required for normal breakdown of yolk proteins during embryogenesis (20). The regulation of yolk degradation by cysteine proteases and their endogenous inhibitor has also been inferred from biochemical studies on *Ornithodoros* eggs (70,71).

In *C. elegans*, our studies (39) and those by Britton and Murray (43) have shown that Ce-CPL-1 and the yolk proteins were both expressed in intestinal cells and probably transported together from the intestine to the gonad where they are taken up into the same compartments within developing oocytes and embryos. Moreover, we have shown that the Ce-CPL-1 cathepsin L-like enzyme is essential for embryogenesis (39); in cpl-1(vc322) mutant worms the embryos were developmentally arrested with approximately 100-150 cells and failed to undergo morphogenesis. The loss of *C. elegans* CPL-1 activity lead to an aberrant processing and/or conformational changes in yolk proteins resulting in abnormal yolk granules fusion. In addition, in the cpl-1 mutant embryos the recycling and/or degradation of the RME-2 yolk receptor is significantly delayed, but not completely blocked (43). The Ce-CPZ-1 cathepsin Z was also reported to function in embryogenesis although its role was only partially essential (10-20% embryonic lethality in the ok497 mutant worms) (42). Our present studies extended the possible roles of these cysteine proteases and their putative inhibitor during oogenesis. Both Ce-CPL-1 and Ce-CPZ-1 enzymes as well as the CPI-2a inhibitor are present in the myoepithelial sheath surrounding germ cells, oocytes and embryos, as well as in yolk granules within normal oocytes pointing to the possibility that the CPI-2a inhibitor may regulate both enzymes during normal uptake and/or processing of yolk proteins by the mature oocytes. When the inhibitor is absent, its target enzymes are possibly not controlled and proceed into untimely and/or abnormal yolk protein uptake and/or processing. Consequently, the normal maturation of oocytes and their subsequent timely ovulation and fertilization is affected. Our hypothesis is supported by the absence of staining of oocytes within the cpi-2a mutant worms with anti-yolk protein antibodies and the absence or reduced GFP::YP170 expression in cpi-2a/yp170:gfp mutant oocytes although it was expressed in one of the successfully developed embryo. Our present study suggest that cathepsin L and Z may have a direct role in yolk protein uptake and processing, although we cannot rule out an indirect role for both enzymes, activating other proteases, such as cathepsin D or other cathepsin L and cathepsin B that have not as yet been characterized, which might be involved in these processes. Defining the precise role of cysteine
proteases and their putative inhibitor during oogenesis will require further studies.

In *C. elegans*, cell-cell signaling is also critical for promoting germ line development, oocyte maturation, and ovulation. During ovulation, the mature oocyte is expelled from the gonad arm by contraction of the proximal myoepithelial sheath and dilation of the distal spermatheca, however, not much is known about the regulatory mechanism that govern this process (72). Both the proximal sheath and distal spermatheca cells were shown to be required for oocyte meiotic maturation and ovulation. Ablation of sheath cells resulted in delays in oocytes meiotic maturation (72). Ablation of sheath and distal spermatheca cells can also trap mature oocytes in the gonad arm where they endomitotically replicate their DNA (72,73). Mature oocytes that are not ovulated on schedule also become endomitotic within the gonad arm (74-76). Moreover, aberrant signaling between the oocytes and the surrounding gonadal sheath cells can lead to abnormal sheath motility or contractions necessary for ovulation (59).

We suggest that *Ce*-CPI-2a regulate the proteolytic processing of molecules within the gonadal sheath cells, directly or indirectly, which are essential for proper cell-cell signaling activities of the gonadal sheath cells during normal maturation and ovulation processes. Our electron microscopy data demonstrated the presence of the CPI-2a inhibitor and its putative target enzymes, CPL-1 and CPZ-1, in the gonadal sheath cells surrounding germ cells, oocytes and embryos. This is the first study that actually demonstrated the expression of cysteine proteases and their endogenous inhibitor in these cells, which indirectly shows the involvement of cathepsin-like enzymes and their potential regulation by CPI-2a in cell-cell signaling processes and/or contraction of the proximal myoepithelial sheath required for oocyte maturation/ovulation in *C. elegans* and perhaps in other nematodes.

The myoepithelial sheath cells of the proximal ovary are morphologically smooth muscle-like cells with distinct thick and thin filaments that are organized into a nonstriated manner (59) and are required for ovulation of mature oocytes (72). Several cytoskeletal proteins are implicated in sheath contraction. The contraction of the ovarian muscle requires tropomyosin and troponin C, which are associated with actin filaments in the myoepithelial sheath. Suppression of both proteins by RNAi inhibited gonadal contraction and resulted in the accumulation of endomitotic oocytes in the gonad (77). Disruption of the MUP-2 troponin T (75) and PAT-3 β-integrin (78) functions also resulted in ovulation defects, however, their localization in the sheath cells at subcellular level has not been examined. In addition, perturbation of β-integrin or talin causes defects in the gonadal morphogenesis and also disrupted oocyte maturation and gonad sheath cell structure (79,80). Contractile muscle cells showed disorganization of the actin cytoskeleton leading to complete paralysis, a phenotype that was also observed with depletion of *pat-2* and *pat-3* integrins, suggesting that the ovulation defects observed might be partly due to structural defects rather than defects in the regulation of contraction. Recent studies have confirmed the role of integrin and integrin-associated proteins in gonad function; suppression of *pat-4*/integrin-linked kinase (ILK) and *unc-112*/Mig-2 transcripts by RNAi caused oocyte accumulation in the proximal gonad and distal tip cells migration defects. It was further determined that failed ovulation was due to defective contraction and dilation of somatic gonad structures, including spermatheca and gonad sheath. Actin cytoskeleton in the proximal gonad of the RNAi animals appeared disorganized, indicating that RNAi of *pat-4* or *unc-112* inhibited the overall assembly of actin cytoskeleton in somatic gonad (81). Taking into consideration that in vertebrates degradation of ECM by cathepsins B, H, K, L and S is believed to play an important role in ovarian function (56,82-84), we predict that also in *C. elegans* CPL-1 and CPZ-1 and their putative CPI-2a inhibitor that are localized to the myoepithelial sheath cells may also have a role in the remodeling of components of the ECM or the cytoskeletal proteins in these cells. More studies are required to validate this hypothesis.

Based on our immunoelectron localization data,
the C. elegans CPI-2a cystatin is highly expressed in sperm cells and therefore it is tempting to speculate that it has a role in regulating target enzyme(s) within the sperm cells. Several cystatin related genes are expressed in the male reproductive tract of mouse and human (85). The mouse cystatin and cathepsin L are thought to promote sperm maturation through modification of sperm cell surface proteins and soluble proteins in the surrounding fluid (86). The identity of the CPI-2a target enzymes in these processes in C. elegans is unknown; our present studies show that the CPL-1 and CPZ-1 enzymes are not expressed in sperm cells. There are several uncharacterized cathepsin B-like enzymes in the C. elegans database (www.wormbase.org) for which RNAi screens have suggested that they are required for embryogenesis; F57F5.1 (87) and T10H4.12 (88). In addition, there are few other cathepsin L enzymes that are distantly related to Ce-CPL-1 and a cathepsin F in the C. elegans genome (89) that are not as yet characterized and might have a role in sperm maturation. Regardless, it appears that in cpi-2a mutant hermaphrodites the sperm cells are intact and that by providing cpi-2a mutant worms with sperm from wild type worms by mating did not restore fertility. There results indicate that cpi-2a has no essential function in sperm cells. As CPI-2a is co-localized with CPL-1 and CPZ-1 also in the eggshells, both the enzymes and their inhibitor might also have a role during eggshell formation/morphogenesis. Based on our observation of the cpi-2a mutant worms it is hard to predict if their roles are essential as the majority of the oocytes never developed into embryos.

Although the regulatory role of CPI-2a appears to be essential only during oogenesis, our present studies have also suggested that it may interact with CPL-1 and CPZ-1 during other developmental processes as well, in particular during molting. Its regulatory roles in this process, however, are not essential and thus detrimental to molting. The putative interaction between O. volvulus cathepsin L and Z and their endogenous inhibitor cystatin during O. volvulus molting was predicted many years ago (33) and was the basis for the study of cysteine proteases and their cystatin inhibitors in C. elegans during molting. Expression of the cpi-2a transcript and accumulation of the CPI-2a protein are tightly coordinated in cell/tissue specific manner during the post-embryonic development. The cpi-2a reporter construct is expressed in many hypodermal and pharyngeal cells similarly as observed for cpl-1 and cpz-1 (39,42). The expression of cpi-2a transcript along with those of cpl-1 and cpz-1 is elevated prior to molting. The transcript levels of both cpi-2a and cpl-1 were approximately 2.5-3.5 hours before the L1/L2 molt, L2/L3 molt; L3/L4 and the L4/adult molt. In comparison, the elevated cpi-2a transcription profile coincided with the Ce-cpz-1 transcript levels only before the L2/L3, L3/L4 and L4/adult molt, and the timing before each molt was 2 hours. Moreover, the enzymes and their inhibitor are co-localized in the cuticle of molting larvae. These analyses suggest that the role of CPI-2a during molting is potentially through its putative interaction with both enzymes. In our previous studies we have shown that Ce-cpl-1 may have a direct role in degradation of the old cuticle, in processing of the new cuticle and/or in digesting cuticular anchoring proteins (39). Alternatively, we suggested that CPL-1 may act indirectly to process and activate other enzymes or hormones involved in molting. The function of CPZ-1 during molting is more dominant than that of CPL-1; 20-80% of F1 had molting defects after RNAi and in the mutant worms (42).

Although the role of cystatins in the regulation of cysteine proteases during oogenesis and embryogenesis have been established in many organisms, our studies are the first to demonstrate that also in C. elegans cathepsin L and cathepsin Z as well as their putative endogenous cystatin inhibitor, Ce-CPI-2a, play a role during oogenesis and fertilization. Future studies will focus on identifying the proteins that are targeted by these enzymes during oogenesis and embryogenesis in order to elucidate their distinctive functions.
Acknowledgements

*C. elegans* homozygous mutants *cpi-2a* allele (ok1256)V and *cpi-1* allele (ok1213)IV were produced by *C. elegans* gene knockout project at Oklahoma Medical research Foundation and was provided by *C. elegans* Genetics Center, University of Minnesota, Minneapolis, MN. We thank Susan Stromme for providing anti-YP170 and anti-YP88 monoclonal antibodies. We also thank Andy Fire for providing the *gfp* and *LacZ* expression vectors. The *C. elegans* strain DH1033 expressing *yp170:gfp* was also provided by *C. elegans* Genetics Center, Minneapolis, MN. The rabbit anti-*Ce*-CPI-2a antibodies were kindly provided by Susanne Hartman (Molecular Parasitology, Humboldt University, Berlin, Germany). We thank Core Sciences lab, NYBC for DNA sequencing.

The abbreviations used are: *ama-1*, α-amanitin-resistant gene; bp, base pair(s); BLAST, Basic local alignment search tool; *cpi*, cysteine protease inhibitor; DAPI, 4’6-diamidino-2-phenylindole; kb, kilobase(s); L1, first-stage larvae; L2, second-stage larvae; L3, third-stage larvae; L4, fourth-stage larvae; *lacZ*, β-galactosidase gene; RT-PCR, reverse transcriptase polymerase chain reaction; *YP*, yolk protein.

References


**FIGURE LEGENDS**

FIG. 1. Genomic organization of the *C. elegans* genes. (A) Genomic organization of Ce-cpi-2a. (B) Translational fusion construct was generated by fusion of a 0.9 kb promoter region upstream of the cpi-2a ATG and its all three exons in frame with lacZ (pSL112A). NLS, nuclear localization signal within the...
lac Z reporter constructs. ATG, the codon for the first methionine within the expression constructs. To rescue cpi-2a(ok1256) mutant worms by complementation three rescue constructs in the expression vector pPD95.75 were generated: 1) cpi-2a cDNA (C), which contained the 0.9 kb cpi-2a promoter fused to cpi-2a cDNA (pSL112C) followed by unc-54 3’ UTR; 2) cpi-1 cDNA (D), which contained the 0.9 kb of cpi-2a promoter fused to cpi-1 cDNA (pSL151A); and 3) R01B10.3 gene, which included the 0.9 kb of R01B10.3 promoter and its complete genomic sequences (pSL149A). The genomic organization of R01B10.3 is present in F. Exons are indicated as shaded boxes and the numbers under the boxes indicate the amino acid residues within that exon. Promoters and the introns between the exons are indicated by a solid line.

FIG. 2. Temporal pattern of cpi-2a gene expression as determined by Real-Time PCR. The levels of the cpi-2a transcript in each stage of development were measured and compared with the previously determined transcript levels of C. elegans Ce-cpl-1 and cpz-1. The graph shows the ratio (+ SD) between cpi-2a or cpl-1 (A) and cpi-2a or cpz-1 (B) levels and those of the constitutively expressed control gene ama-1 (y-axis). The ama-1 gene was used as an internal control transcript to allow the relative quantification of cpi-2a, cpl-1 and cpz-1 expression in distinct developmental stages (x-axis). The RNA from mixed-stage embryos and from synchronized larval and adult populations collected at 2-h intervals was isolated for the preparation of stage-specific cDNA. Stage-specific molting within the life cycle is indicated by vertical arrow. Each experimental point was repeated at least twice.

FIG. 3. Cell-specific expression of Ce-cpi-2a: lacZ during C. elegans development. Lines of transgenic C. elegans carrying the cpi-2a: lacZ reporter gene were created as described under “Experimental procedures.” The histochemical stain for β-galactosidase produces insoluble products in nuclei transcribing the cpi-2a gene. In all transgenic lines, the expression of cpi-2a was observed in hypodermal, intestinal and pharyngeal cells of: (a) few cells of early stage embryo (arrows); (b) many cells in embryo of later stage of development (arrow); (c) pretzel stage embryo (arrow); (d) pharyngeal (arrow) and intestinal cells (arrowhead) in larvae; (e) the pharyngeal muscle cell (arrowhead) in the anterior portion of an adult worm. Note that expression is also observed in the pharyngeal gland cells (arrow); and (f) the hypodermal cells in the posterior region of an adult worm (arrow). Transgenic C. elegans worms were photographed using Nomarski optics (magnification X400).

FIG. 4. Nomarski (DIC) and fluorescent images of N2 wild-type worms and cpi-2a(ok1256) mutant worms having defects during oogenesis. Homozygous cpi-2a(ok1256) mutant worms were analyzed as described in “Experimental procedures”. (a) DIC photograph of a wild type gonad; (b) Normal DNA organization in wild type germ cells (solid line), oocytes (arrows) and spermatheca (sp) was visualized by DAPI staining; (c) DIC photograph of a cpi-2a mutant gonad for comparison; (d) Normal DNA organization in germ cells (solid line) in the distal gonad arm as visualized by DAPI staining. However, several disorganized germ cells nuclei (arrow) near the bend region in the cpi-2a mutant gonad were observed. These cells did not differentiate into oocytes. Only a single oocyte (arrowhead) was present in the proximal gonad arm. The internal view of an N2 wild-type adult gonad arm is presented in “e”; the line along the proximal arm indicates regions where the oocytes are arranged in a single row. Small arrow indicates normal spermatheca and a long arrow indicates fertilized embryos. Representative of cpi-2a mutant phenotypes are presented in: (f) cpi-2a(ok1256) gonad showing few fertilized embryos (big arrow), normal spermatheca (small arrow) and disorganized, loosely arranged and enlarged oocytes (line); (g) two gonad arms of cpi-2a(ok1256): one proximal gonad arm is filled with fertilized embryos (arrows) while the other arm is devoid of either oocytes or embryos (line); (h) mutant hermaphrodite proximal gonad displaying a single oocyte with an intact nuclear envelope (big arrow) that did not mature and ovulate. Arrow indicates a normal spermatheca. The other oocytes in the proximal gonad arm were mostly undeveloped (arrowhead); (i) endomitotic oocytes were present in the most proximal end of the mutant gonad (line) while undeveloped oocytes were present close to the bend region (arrowhead). Germ cells are indicated by “gc”, vulva by “v” and spermatheca by ‘sp’. Fluorescent images (b and d) were taken using Zeiss fluorescent microscope and fluorescein/isothiocyanate filter sets (magnification X400).
All the other images were taken using Nomarski optics (magnification X400).

**FIG. 5. Co-localization of the native CPI-2a and Ce-CPL-1 proteins.** Embryo and whole mount fixation were performed as described under “Experimental procedure.” CPI-2a was labeled with FITC-conjugated goat anti-rabbit, while CPL-1 was labeled with Texas Red-conjugated goat anti mouse secondary antibodies. The CPI-2a inhibitor (a) and the CPL-1 enzyme (b) are both localized in the same regions (c) of germ cells (arrow) and in developing oocytes (arrowhead); CPI-2a (d) and CPL-1 (e) are both localized in the embryonic cells (arrows) and to the same cells (f); CPI-2a (g) as well as CPL-1 (e) are expressed in the eggshells of empty embryo (arrows) at the same regions (i). Localization of CPI-2a and CPL-1 proteins was observed under Zeiss fluorescent microscope using fluorescein/isothiocyanate filter sets (magnification X400).

**FIG. 6. Co-localization of native CPI-2a and Ce-CPZ-1 proteins.** Embryo and whole mount fixation were performed as described under “Experimental procedure.” CPI-2a was labeled with FITC-conjugated goat anti-rabbit, while CPZ-1 was labeled with Texas Red-conjugated goat anti mouse secondary antibodies. CPI-2a (a) was localized in both germ cells (arrow) and oocytes (lines) while CPZ-1 (b) was found in developed oocytes (lines), however, CPZ-1 staining was very faint in germ cells (arrow). CPI-2a and CPZ-1 co-localized (c) in developing oocytes (line), and to some extent also to the germ cells. During ecdysis (d) of the old cuticle both CPI-2a (e) and CPZ-1 (f) are present at the same regions in the cuticles (g) of the molting worm (arrow). However, while CPI-2a was present along the ecdysed cuticle, the CPZ1 enzyme was expressed in only a part of the ecdysed cuticle (g). Localization of CPI-2a and CPZ-1 proteins was observed under Zeiss fluorescent microscope using fluorescein/isothiocyanate filter sets (magnification X400).

**FIG. 7. Ultrastructural localization of the native CPI-2a protein in *C. elegans* at different stages of development.** Antibodies raised against the *C. elegans* recombinant inhibitor was used to study the subcellular localization of the CPI-2a native protein as described under “Experimental Procedures.” The top panel is a DIC photograph of a semi-thin section of *C. elegans cpi-1* mutant hermaphrodite stained with toluidine blue and showing the regions (a-c; e-g) where the immunoelectron analyses were performed. Specific labeling of the native CPI-2a protein is observed in the cuticle of the worm (a, cu); sheath cell (b, sh) surrounding the germ cells (gc); sheath cell surrounding oocytes (c, oo) as well as within yolk granules (yg) [see insert in c]; yolk platelets (yp) within the body cavity (d); developing sperms within the spermaphthea (e, arrowhead); sheath and eggshell (es, arrowhead) surrounding the embryos (f and g). Each bar is 250 nm.

**FIG. 8. Ultrastructural localization of the native CPL-1 and CPZ-1 proteins in *C. elegans.* Antibodies raised against the *C. elegans* CPL-1 and CPZ-1 enzymes were used to study the subcellular localization of CPL-1 and CPZ-1 proteins as described under “Experimental Procedures.” (a-c) CPL-1 is localized to the sheath cells (sh), germ cells (gc), oocytes (oo), yolk granules (yg), and embryo (emb); (d-f) CPZ-1 is also present in sheath cell (sh), germ cells (gc), oocytes (oo), yolk granules (yg) and embryo (emb). Each bar is 250 nm.

**FIG. 9. The expression of yolk proteins in wild type and cpi-2a mutant worms:** Antibodies to the *C. elegans* YP170 and YP88 proteins were used to localize the yolk proteins in homozygous *cpi-2a* mutant and in wild type N2 worms. Both antibodies gave similar patterns of staining. Figures a-c represent images obtained with monoclonal antibodies to YP170. The yolk protein is localized to the three most proximal oocytes in the gonad (a, arrow) and inside the embryonic cells (b, arrows) of wild type hermaphrodites. The yolk protein, however, is absent from the developing oocytes of the *cpi-2a* mutant hermaphrodite (c, thick line). Figures e and g compare the expression of YP170::GFP fusion protein in wild type DH1033 strain (e) and in homozygous *cpi-2a/yp170::gfp* mutant worms (g). The Nomarski images of the worms are presented in d and f, respectively. In DH1033 strain the GFP expression is observed in the two late-stage oocytes proximal to the spermaphthea and in the embryos (e). In the mutant
worm (g) the GFP expression was either absent or only present at very low intensity in the oocyte located most proximately to spermatheca, although the embryo next to the spermatheca that has developed in this mutant worm was positive for YP170::GFP expression. Note that the negative GFP expression coincided with the presence of an abnormal oocyte in the mutant worm (f). Oocyte (oo); embryo (emb). DIC and fluorescent images were taken using Zeiss fluorescent microscope using fluorescein/isothiocyanate filter sets (magnification X400).
Figure 1

A  
Promoter (0.9 kb)  ATG  Coding seq (1433bp)  
1 37 38 63 64 143 aa  

Ce-cpi-2a

B  
cpi-2a promoter  ATG  cpi-2a coding seq (1419bp)  

pSL112A

C  
cpi-2a promoter  cpi-2a cDNA (432bp)  
unc-54 3’ UTR  

pSL112C

D  
cpi-2a promoter  cpi-1 cDNA (420bp)  

pSL151A

E  
R01B10.3 coding seq (397bp)  Promoter (0.9 kb)  

pSL149A

F  
Coding seq (397bp)  ATG  Promoter (0.9 kb)  
98 55 54 28 27 1 aa  

R01B10.3

250 bp
Figure 2

A

Relative expression of cpl-1

B

Relative expression of cpz-1

Relative expression of cpl-2a

Hours post L1 arrest

L1 L2 L3 L4 Adult
molt 1 molt 2 molt 3 molt 4
Figure 3
Figure 5

CPI-2    CPL-1    Merge

a

b

C

d

e

f


g

h

i
Figure 6

DIC  CPI-2a  CPZ-1  MERGE

![Image of figure 6 with DIC, CPI-2a, CPZ-1, and MERGE columns]
Figure 7
Figure 8

CPL-1

CPZ-1

(a) sh

(b) sh

(c) emb

(d) sh

(e) sh oo yg

(f) emb

Figure 8
Figure 9
The Caenorhabditis elegans CPI-2a cystatin-like inhibitor has an essential regulatory role during oogenesis and fertilization
Sarwar Hashmi, Jun Zhang, Yelena Oksov, Qiongmei Ji and Sara Lustigman

*J. Biol. Chem. published online July 20, 2006*

Access the most updated version of this article at doi: 10.1074/jbc.M600254200

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