We have analyzed the expression and function of Ce-cpz-1, a Caenorhabditis elegans cathepsin Z-like cysteine protease gene, during development of the worm. The cpz-1 gene is expressed in various hypodermal cells of all developmental stages and is specifically expressed in the gonads and the pharynx of adult worms. Disruption of cpz-1 function by RNAi is specifically expressed in all developmental stages and caused morphological defects in the head or tail region of the animal. The related Onchocerca volvulus cathepsin Z protein suggests that the function of CPZ-1 during molting might be conserved in other nematodes. Based on the cpz-1 RNA interference and cpz-1 deletion mutant phenotypes, it appears that cpz-1 have additional roles during morphogenesis. Deletion of cpz-1 coding sequence or inhibition of cpz-1 function by RNAi also caused morphological defects in the head or tail region of living nematodes (5, 6). The only two published nematode cathepsin Z-like enzymes were postulated to function during molting (6), which is an obligatory process for nematode development and potentially in other fundamental biochemical processes that are still undefined (5). The role of the Onchocerca volvulus CPZ during molting was evidenced by its distinct localization, as determined by using monospecific antibodies raised against the O. volvulus LOVCP enzyme (remnamed Ov-cpz) and thin sections of the parasitic nematode. The native enzyme was localized in the region where the separation between the cuticles of the third-stage larvae (L3) and the fourth-stage larvae (L4) during molting takes place (6). The homologous enzyme in Toxocara canis (Tc-cpz), however, was also highly expressed in adult stages, which precluded its exclusive role during T. canis molting. Unfortunately, it is still difficult to

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The Caenorhabditis elegans Cathepsin Z-like Cysteine Protease, Ce-Cpz-1, Has a Multifunctional Role during the Worms’ Development*

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‡ The abbreviations used are: CPZ, cysteine protease cathepsin Z-like protein; ama-1, α-amanitin-resistant gene; BLAST, basic local alignment search tool; cpz-1, cysteine protease cathepsin Z-like gene; DAPI, 4′,6-diamidino-2-phenylindole; dsRNA, double-stranded RNA; GFP, green fluorescence protein; kb, kilobase(s); L1, first-stage larvae; L2, second-stage larvae; L3, third-stage larvae; L4, fourth-stage larvae; lacZ, β-galactosidase gene; RNAi, RNA interference; RT, reverse transcriptase; PBS, phosphate-buffered saline; NLS, nuclear localization signal; NGM, nematode growth medium; ECM, extracellular matrix; GST, glutathione S-transferase.

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elucidate gene functions directly in parasitic nematodes because of the lack of molecular genetic approaches to directly investigate the role of a desired gene in the biology/biochemistry of these organisms. To conclusively uncover the broad range of the cathepsin Z enzyme function in the parasitic nematodes, we took advantage of the presence of CPZ-encoding sequences in the free-living nematode, *Caenorhabditis elegans*, and the availability of molecular tools that provide for high throughput characterization of genes in this powerful model system. The *C. elegans* has only two genes that encode cathepsin Z-like cysteine proteases, which we named Ce-cpz-1 (F32B5.8) and Ce-cpz-2 (M04G12.2); this makes it an ideal in vivo system for studying the putative functions of the homologous genes in the parasitic nematodes for which the most important biochemical interactions are likely to be conserved (7, 8). The use of the *C. elegans* model system for the study of homologous genes found in many other organisms including humans has already revolutionized the progress toward the confirmation of genes that can become targets for new drug development (7, 8). The amino acid sequence of Ce-CPZ-1 is 82 and 73% identical to the *O. volvulus* and *T. canis* CPZ enzymes, whereas Ce-CPZ-2 shows only 57 and 53% identity, respectively. Because of the greater identity of Ce-CPZ-1 to the parasitic nematode sequences, we chose to study the functional role of Ce-CPZ-1 during *C. elegans* development as the model system for the parasitic CPZ enzymes, and consequently validate whether these enzymes in the parasitic nematodes could become potential targets for the development of therapeutic intervention. Cysteine proteases in many other parasitic systems have been identified as potential targets for drug or vaccine development (9, 10).

**EXPERIMENTAL PROCEDURES**

The *Cathepsin* Z-like Cysteine Proteases of *C. elegans*—A BLAST search (11) of the *C. elegans* database (www.sanger.ac.uk/projects/C_elegans/wormpep1) using the *O. volvulus* *Ov*-CPZ amino acid sequence (U71150) identified two predicted *C. elegans* cathepsin Z-like genes (F32B5.8 and M04G12.2), which we named Ce-cpz-1 and Ce-cpz-2, respectively. Alignment of the CPZ protein sequences within the GenBank database was made using ClusterW multiple sequence alignment. The Ce-CPZ-1 protein encoded by the F32B5.8 gene had the highest similarity with the *Ov*-CPZ protein (82%) in comparison to 57% identity of Ce-CPZ-2 with *Ov*-CPZ, and is therefore the focus of the studies outlined below. Few cDNA clones corresponding to the F32B5.8 gene were expressed in the Expressed Sequence Tag Data Base, one of which contained the full-length Ce-CPZ-1 cDNA sequence (clone yk94b1, accession no. D66235). The pBluescript phagemid of the *xZAP II* phage clone yk94b1 (obtained from Yuji Kohara, *C. elegans* Consortium, National Institute of Genetics, Mishima, Japan) was excised and the DNA sequenced in both directions to confirm the predicted amino acid sequence of the F32B5.8 gene. Based on this cDNA sequence and trans-splicing patterns, there appear to be 5 exons in the F32B5.8 gene. Based on the cDNA sequence and trans-splicing patterns, there appear to be 5 exons in the F32B5.8 gene. Based on the cDNA sequence and trans-splicing patterns, there appear to be 5 exons in the F32B5.8 gene. Based on the cDNA sequence and trans-splicing patterns, there appear to be 5 exons in the F32B5.8 gene. Based on the cDNA sequence and trans-splicing patterns, there appear to be 5 exons in the F32B5.8 gene.

**Constitution of cpz-1 Transgenic *C. elegans* Strains—Two types of reporter fusion construct were generated. The transcriptional fusion construct designated pSL108A contained a 2.3-kb promoter region of the Ce-cpz-1 and was generated by PCR from *C. elegans* genomic DNA (Fig. 1C). It was cloned into the *lacZ* reporter vector pPD90.23, which contains the nuclear localization signal (NLS) motif. The translational fusion constructs designated pSL103 and pSL109 contained a genomic fragment of Ce-cpz-1, which included a 1.4-kb promoter region and the first 4 exons (Fig. 1C). The fragment gene for these constructs was amplified from *C. elegans* genomic DNA and subsequently cloned into the PCR 2.1 cloning vector (TOPO cloning kit, Invitrogen) before excision and subcloning into two *C. elegans* expression vectors: 1) pPD57.75 (pSL103), which expressed the gene in frame with *gfp* and 2) pPD90.23, which expressed the gene in frame with β-galactosidase (pSL107A) and in the nuclei of the worms. Sequencing of the final constructs was performed to confirm that the coding sequence of cpz-1 is expressed in both translational constructs was in frame with *gfp* or the *lacZ* sequences.

* C. elegans adult hermaphrodites were transformed by microinjection around 80 mg/μl recombinant plasmid DNA, prepared using the Concert™ rapid plasmid miniprep system (Invitrogen), into their gonadal syantum as described previously (14, 15). A plasmid DNA (pRf4) containing the dominant selectable marker gene rol-6 (su1006), which encodes a mutant collagen, was co-injected (~80 mg/μl) with the Ce-cpz-1:loxZ or Ce-cpz-1:gfp fusion constructs. *C. elegans* expressing the rol-6 gene continuously roll over, thereby providing a visible phenotype for the selection of transgenic worms (16). F2 rollers were picked and transformed into individual Escherichia coli plates to establish a number of independent lines. Lines in which F3 and subsequent generations showed the rol phenotype were stained for *lacZ* (Fig. 2) and subsequently transferred to *E. coli* and subcloned into two 2-phenylindole, final concentration 0.1% (w/v) as a co-stain to aid in the identification of cell types (17). cpz-1:gfp transgenics were visualized by mounting live transgenic worms on a 2% agarose pad in 0.01% sodium azide as described (18).

**Double-stranded RNA (dsRNA) Preparation and RNA Interference (RNAi)—** RNAi procedure was carried out using dsRNA as described by Fire et al. (19) and Tabara et al. (20). The full-length Ce-cpz-1 cDNA clone (yk94b1) in pBluescript was used as the template for RNA synthesis. The cDNA was first amplified with commercially available T3 and/or T7 primers with random hexamers. The specific cDNA fragment of cpz-1 was then amplified using the forward 5'-GCTTCTTCGCTTAT-3' and the reverse 5'-AAGCTGACACCATGCTGTTGG-3' primers and the copy number quantified by real-time PCR using QuantiTech™ SYBR Green PCR kit (Qiagen). Another set of primers, forward 5'-GCTTCTTCGCTTAT-3' and reverse 5'-TTCTTTTCTCTCGCTTC-3', was used for the specific amplification of an internal control, the *ama-1* transcript (13). Both sets of primers were designed to span an intron to distinguish cDNA from contaminating gDNA products. The following PCR conditions, which allowed reactants to remain in excess, were used: 50 °C for 2 min, 95 °C for 15 min, followed by 45 cycles of 94 °C for 15 s, 62 °C for 30 s, and 72 °C for 45 s. To determine the copy number of each construct within the transgenic dsRNA preparations, standard curves for *cpz-1* and *ama-1* were drawn based on quantitative PCR using known amounts of template DNA in a range of 10–10⁶ copies. The relative content of the transcript corresponding to *cpz-1* is expressed in each developmental stage as the ratio between its copy numbers relative to that of *ama-1*.

**CPZ Is Essential for *C. elegans* Development**
were identified by Western blot using antibodies against GST or anti-
manufacturer. Specific fractions containing the purified recombinant protein
The soluble fraction was then subjected to preparative separation using
37°C. Each mouse received a total of 10 μg of protein using the repetitive immunizations/multiple sites strategy (23, 24). Each mouse anti-serum was raised against the purified CPZ-1 recombinant

buffer one more time. After centrifugation, the pellet was suspended in
500-ml culture were harvested and lysed by sonication in 40 ml of 50

coli
Biosciences). The recombinant GST-CPZ-1 protein was expressed in
enzyme (amino acids 23–2633 in relation to ATG start codon. Indi-

and adults were collected and washed in PBS and Ruvkun Fixation
Buffer before being fixed and permeabilized using 1% paraformalde-
hyde in Ruvkun Fixation Buffer for 30 min and two freeze-thaw cycles
in dry/ethanol bath. The fixed embryos or the permeabilized whole
worms were treated with a blocking solution (60 mg/ml normal goat
serum in PBS; Jackson Immunoresearch Laboratories, Inc., Westgrove,
PA) for 1 h before reaction with antibodies. Primary anti-Cpz-1 anti-

bodies were used at 1:20 and 1:40 dilution. Fluorescein isothiocyanate-
conjugated anti-mouse secondary antibodies were used at a 1:50
dilution. Specimens were mounted on slides with 15 μl of mounting
medium, Vectashield containing DAPI (Vector Laboratories, Inc., Bur-
lingsame, CA). The edges of the cover slips were sealed with nail polish
and viewed under fluorescence microscope using appropriate filter sets.

ultrastructural Localization of the Native Ce-Cpz-1 Protein in C. elegans—to study the subcellular localization of the native CPZ protein
during C. elegans development, samples were collected from
synchonized cultures during the molting processes throughout C. elegans
development. Worms were fixed for 60 min in 4% paraformaldehyde, 0.1% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, containing 1%
sucrose. The fixed worms were then processed for immunoelectron
microscopy as previously described (6, 26, 27). Thin sections of C. elegans
embedded worms were probed with mouse antiserum raised against the recombinant C. elegans CPZ-1 fusion polypeptide before
incubation with 15 nm gold particles coated with anti-mouse IgG (Am-
ershaw Biosciences). Mouse preimmune serum was used as the control.

RESULTS

The C. elegans Cathepsin Z-like Enzyme Ce-cpz-1—BLASTP search with the O. volvulus cathepsin Z-like protease Oc-Cpz
(accession no. U71150), identified only two closely related protein
sequences in the C. elegans data base (ACeDB); one in cosmid F32B5, which maps to chromosome 1 (accession no. AF003148) and the second in cosmid M04G12, which maps to chromosome 5 (accession no. Z81103). We have designated the two putative cathepsin Z-like sequences Ce-cpz-1 (F32B5.8) and

Ce-cpz-2 (M04G12.2). A comparison of the F32B5.8 genomic sequence and the yk94b1 cDNA clone encoding the full-length amino acid sequence of the corresponding protein revealed that the first Met residue in the N terminus sequence of F32B5.8 predicted by Genfinder was incorrect. This was also
confirmed by cloning the 5′-noncoding region of the cDNA
using the 22-nucleotide splice leader sequence, SL1, of C. el-

gens. In C. elegans, SL1 tends to be spliced very close, and
often immediately adjacent, to the initiating methionine codon,
and may possibly play a role in translation initiation (28). The putative ATG start codon of Ce-cpz-1 is at bp 25,362 instead of
bp 27,752 of cosmid F32B5. The corresponding Ce-cpz-1 cDNA
therefore encodes a protein of 306 amino acids, containing a signal peptide of 23 residues, a propeptide of 42 amino acid,
and a mature enzyme of 261 amino acid residues (Fig. 1A). By
analogy with other cysteine proteases, the putative processing
site of the zymogen to the mature form of the enzyme had been
assigned to the Asp266-Leu bond. The Ce-cpz-2 sequence comprises 467 amino acid residues with a putative zymogen-
processing site at the Asp266-Leu bond. Although the CPZ subfamily
is characterized by a short propeptide (41–42 amino acids)
relative to those present in other cysteine proteases (1), Ce-
cpz-2 is an exception. It has a very long propeptide of 208
amino acid residues.

The amino acid sequences encoding the mature Ce-cpz-1
and Ce-cpz-2 enzymes have 82 and 57% identity with the
Oc-cpz-1 amino acid sequence, and 73 and 53% identity with the
T. canis (Tc-Cpz) cathepsin Z-like sequence, respectively. All
the nematode cathepsin Z-like sequences, Ce-cpz-1, Ce-cpz-2,
Oc-cpz-1, and Tc-Cpz (Fig. 1B), contain upstream to the cyste-
eine active site the conserved HIP amino acid sequence and an
additional two other peptide insertions within the protein se-
quence characteristic of this new subfamily within the papain
family of cysteine proteases (1, 5). Although the O. volvulus
sequence of the CPZ proteins was the first one cloned (6), it was
only subsequently classified as cathepsin Z once a homologous
CPZ sequence was cloned from the human brain (Hs-Cpz) (1). The human cathepsin Z is 62, 50, 60, and 57% identical to Ce-cpz-1, Ce-cpz-2, Ov-cpz, and Tc-cpz, respectively. Interestingly, only the nematode cathepsin Z-like sequences have downstream of the cysteine active site a stretch of 5 amino acids, CGSCW, that is identical to the amino acid sequence within the conserved cysteine active site, CGSCWAF (Fig. 1, A and B) (5, 6). The significance or the function of the CGSCW repeat is unknown.

The following is the gene structure for Ce-cpz-1. The gene is composed of 5 exons and 4 introns, spanning 2.8 kb. The second and the fourth introns of Ce-cpz-1 are unusually large (570 and 1110 bp); the first and third introns are 55 and 56 bp long (Fig. 1C). Interestingly, the gene structure is very similar to that of the Ov-cpz genomic sequence (accession no. U71150), which contains 8 exons (Fig. 1D). Regardless, both contain a very short third exon that encodes only the conserved sequence of the cysteine active site (CGSCWAF). This gene structure is different from that of the cpz-2 gene (data not shown). Because of the close structural similarity among cpz-1, Ov-cpz, and Tc-cpz, we have chosen to study cpz-1 in great length and thus utilized the C. elegans model system to elucidate its function(s).
CPZ Is Essential for C. elegans Development

Fig. 2. Temporal pattern of Ce-cpz-1 gene expression as determined by real-time PCR. The levels of the cpz-1 transcript in each stage of development were compared. The graph shows the ratio (± S.D.) between cpz-1 levels and those of the constitutively expressed control gene ama-1 (y axis). We used the ama-1 gene as an internal control transcript to allow the relative quantification of cpz-1 expression in each stage. The mRNA was isolated from mixed-stage embryos and synchronized larval and adult populations collected at 2-h intervals and used for the preparation of stage-specific cDNA as indicated on x axis. Stage-specific molting within the life cycle is indicated by vertical and horizontal arrows. Note that cpz-1 transcripts increased in the intermolt period of each larval stage and at 40 h when the fertilized eggs go through the process of embryogenesis. Each experimental point was repeated at least twice.

during nematode development in comparison to those postulated in the parasitic nematodes (5, 6).

The Transcript Corresponding to Ce-cpz-1 Gene Is Constitutively Expressed in All Developmental Stages of C. elegans and Is Elevated Prior to each Molt—To determine the temporal expression profile of the Ce-cpz-1 mRNA transcript during C. elegans development, quantitative real-time PCR was performed on total RNA prepared from synchronized populations of C. elegans at 2-h intervals. We used the ama-1 gene as an internal control transcript to allow the relative quantification of cpz-1 expression in each stage. It has previously been shown that the levels of ama-1, which encodes the large subunit of RNA polymerase II (29), are relatively constant during development, and it is therefore a suitable control gene for comparison of transcript levels between different stages of the worm (13, 30). As shown in Fig. 2, although the cpz-1 transcript is present throughout development, it is least expressed in embryos and during L1 to L2 growth. Interestingly, the level of the cpz-1 transcript increased significantly prior to the L2/L3 molt at 16 h and decreased after the L2/L3 molt (~20 h), and then periodically increased before the L3/L4 molt (~24 h) and the L4/adult molt (~34 h). In addition, a significant increase in its level occurred during the period when the early part of embryogenesis takes place (~40 h after L1). During adulthood there is a 2–3-h period when the fertilized eggs go through the process of embryogenesis (31).

Temporal and Spatial Expression of Ce-cpz-1 in Transgenic Worms—The spatial expression pattern of Ce-cpz-1 was examined after transformation of C. elegans with a gfp reporter fusion cpz-1:gfp construct (pSL103) or with cpz-1:lacZ fusion constructs (pSL107A and pSL108A) containing a NLS cassette (Fig. 1F). Three independent transgenic lines carrying an extrachromosomal array of the reporter gene and the pRF4 transformation marker were established for each construct. The three lines for each transgene showed similar expression patterns. For a more detailed analysis of the expression patterns and to identify the individual cells showing promoter activity, reporter gene constructs containing the NLS cassette were used. Transgenic lines created after injection of either transcriptional (pSL108A) or translational (pSL107A) cpz-1:lacZ constructs showed identical expression patterns indicating that the 1.4-kb promoter region within pSL103 and pSL107A contains all the required regulatory elements. A typical cpz-1: lacZ expression pattern is shown in Fig. 3. β-Galactosidase expression was observed in many cells, including gut, hypodermal, and other cells within the developing embryos (Fig. 3, a and b). However, because of the intense staining in some parts of the developing embryos, the identification of the many stained cells was not possible. In post-embryonic stages, β-galactosidase staining was present in the hypodermal cells of all larval stages (Fig. 3c) and young adults (Fig. 3, e and f) until they developed to fully mature gravid hermaphrodites (7–10 days after L4 adult hermaphrodite). β-Galactosidase staining was also detected in intestinal cells. The cpz-1:lacZ expression in all stages was specifically detected in the large hypodermal syncytium covering most of the worm (hypo7), and in the hypodermal cells of the head (hypo4 and hypo6) and occasionally in hypodermal cells in the tail. β-Galactosidase staining was also present in few pharyngeal cells of the adult worms but not in the pharyngeal cells of larval stages (Fig. 3, d and e). Expression of cpz-1:gfp translational construct was detected in all developmental stages except embryos. The cpz-1:gfp expression was restricted to the hypodermis, with additional expression in the pharynx and the gonad only in the L4 and adult worms stages (Fig. 4). The specific expression in the pharynx of adult worms was similar to that observed in cpz-1:lacZ transgenic worms. Interestingly, there was no embryonic expression of the gfp construct. The reason for the absence of embryonic expression is unknown, but could be because of some intrinsic effects of the gfp construct on such temporal expression.

Ce-cpz-1 Is Critical for Molting and Normal Development of C. elegans—RNAi was used to determine the function of cpz-1 during development and specifically its potential role(s) during molting. RNAi on stage-specific C. elegans worms was employed using both the soaking and the feeding methods (20, 21). RNAi with the Ce-cpz-1 activity resulted mostly in severe molting and/or morphological defects and some embryonic developmental arrest (Fig. 5). Inactivation of Ce-cpz-1 in L4 larvae by soaking or by injection in young hermaphrodites resulted in embryonic arrest at >50-cell stage of development (Fig. 5b). Moreover, the development of a small percentage (12%) of the laid embryos that escaped the embryonic arrest and progressed to the L2 stage was arrested at the L2 to L3 molt. In these worms the old cuticle remained attached to the mid-portion of the body resulting in a constriction in the mid body (Fig. 5c). A similar percentage of embryonic lethality was obtained when L4 larvae were fed on cpz-1 dsRNA.

For a finer analysis of the temporal effects of cpz-1 gene suppression, RNAi assays were performed on various stage-specific worms using soaking and feeding methodology. When L1 worms were soaked in cpz-1 dsRNA, 20% of the treated L1 had severe structural defects in anterior or posterior regions of the L2 body. The head of the affected larvae was strikingly swollen and curved (Fig. 5d), and the tail was bifurcated (Fig. 5f). Soaking of L2 in cpz-1 dsRNA resulted in L3 to L4 molting defects (23%) and morphological defects (35%) in L4. The treated L2 arrested at the L3/L4 molt as a result of incomplete shedding of old cuticle (Fig. 5h). The morphological defects were confined to the gonads and included abnormal gonad extension and improper vulva opening (Fig. 5i). When L2 larvae were fed on cpz-1 dsRNA, 80% of the larvae arrested at the L3/L4 molt; these worms under Nomarski optics had major morphological defects in the anterior region of the worms (Fig. 5k). With the exception of feeding L2 on cpz-1 dsRNA, the
percentage of phenotypes resulting from RNAi was relatively low and ranged between 20 and 80%. Regardless, we obtained similar results using the different treatment methods in repeated experiments, except that the feeding of L2 with cpz-1 dsRNA has increased the percentage of the arrested molting phenotype from 23% to 80%. The soaking of L2 larvae in dsRNA had more severe effects, possibly related to a high level of cpz-1 transcripts in this stage of the worm (Fig. 2).

**Mutation in cpz-1 Gene Affects C. elegans Development**—Because of the low penetrance and variability of the stage-specific RNAi phenotypes, it was of importance to analyze a cpz-1 deletion mutant. Sequence analysis of cpz-1 (ok497) homozygous mutant revealed a 1,925-bp deletion in the coding sequence of the gene, which includes a deletion of a portion of exon 1 (amino acids 39–49) and complete removal of exons 2–4, which also resulted in the loss of an open reading frame between exon 1 and exon 5. Homozygous cpz-1 (ok497) mutant animals were examined using Nomarski microscopy to study the effects of disrupting the normal function of cpz-1. Individual cpz-1 mutant worms were raised at 15, 20, or 25 °C and examined for phenotypic differences. At all these temperatures, cpz-1 homozygous mutant worm were both viable and fertile. However, at 20 and at 25 °C, ~20% of the cpz-1(ok497) mutant embryos showed embryonic lethality (Fig. 6a). Some embryos that escaped embryonic lethality although they completed the L1 molt have shown morphological defects in the head (Fig. 6b) or the tail of the L2 worms (Fig. 6c). These phenotypes were similar to that obtained after cpz-1 RNAi (Fig. 5, d and f). Interestingly, at 15 °C higher and broader phenotypic defects were observed. Approximately 60% of the mutant embryos showed embryonic lethality, and ~6% of the F1 generation larvae had molting defects; some larvae hatched to L1 but they could not separate themselves from the eggshell completely (Fig. 6d), and few larvae arrested at the L2/L3 molt (Fig. 6e).

These larvae often could not shed the L2 cuticle during L2/L3 molt, resulting in the formation of severe constriction of unshed cuticle in L3 animals. In addition, few cpz-1 mutant animals showed partially detached cuticle at the head during L2/L3 molt (Fig. 6f); these animals were stuck within the old cuticle and were thus unable to proceed with ecdysis. Similar to cpz-1 RNAi, ~5% of the cpz-1 mutant worms developed gonad defect in L4 (Fig. 6g).

**Localization of the Native CPZ-1 Protein in C. elegans by Immunofluorescence and Immunoelectron Microscopy**—Antibodies raised against the recombinant CPZ-1 protein (amino acid residues 22–306) were first confirmed to not cross-react with the Ce-CPZ-2 and Ce-CPL recombinant proteins by Western blot (data not shown) before they were used to localize the CPZ-1 native protein on whole worms and in thin sections. Using immunofluorescence staining of fixed embryos and permeabilized whole mounted worms, the native Ce-CPZ-1 protein was localized in many cells in early embryonic stages (Fig. 7a). DAPI was added to the mounting media to identify stained nuclei. Although few hypodermal cells were identified, the exact identification of many other cells was not possible because of the extensive staining. During morphogenesis of the embryos, the native CPZ-1 protein was localized along the hypodermal region, whereas the embryo was at the lima bean stage (at 6 h within the embryogenesis process) (Fig. 7b). In all larval stages (L1–L4), a restricted pattern of CPZ-1 localization became apparent, with the most prominent staining detectable throughout the hypodermis (Fig. 7c). In L4 worms the native CPZ-1 protein was also localized in the hypodermis, but in addition, the CPZ-1 protein was also detected in few gonad cells (Fig. 7c) and along the whole length of the pharynx of adult worm (Fig. 7d).

To identify more precisely the hypodermal regions within the worms where the native CPZ-1 protein is localized, thin sections from various developmental stages of *C. elegans* were stained for immunoelectron microscopy analysis. In larval and adult stages, the CPZ-1 native protein was localized in the cuticular regions of the worms (Fig. 8, a and e). In molting larvae (Fig. 8, b–d), the protein was localized in both the new and old cuticles, in particular in the interface where the old cuticle is being degraded before ecdysis (Fig. 8c).
ing cycles and are dependent upon precise control of a complex
components by the hypodermis are tightly coupled to the molt-
cuticle synthesized by the underlying layer of the hypodermal
tissue, a large syncytium that extends throughout the length of
entire cuticle is shed at each molt and replaced with the new
growth temperature (20 °C). The temperature sensitivity of
nts strain showed defects in embryogenesis and morphogene-
synthesis during molting was thus established by the phenotypes
renewal was pointed tail (arrow); c, late L4 worm showing defects in the gonad extension (arrow); d, L2 worm with morphological defect in the head (arrow); e, wild type worm with a normal head (arrow); f, structural defect in the tail of L2 (arrow); g, wild type worm with a normal pointed tail (arrow); h, L3 worm with a partially detached cuticle at the head while the buccal plug between mouth and the cuticle is still attached (arrow); i, late L4 worm showing defects in the gonad extension (arrow); j, late L4 with a normal gonad (arrow); k, a worm arrested at the L3/L4 molt. All photographs were taken using Nomarski optics (original magnification, ×400).

**DISCUSSION**

In the present study, we have shown that cathepsin Z plays several critical roles in the biology of nematodes. Suppression of gene expression by RNAi or by cpz-1 (ok497) deletion in mutant strain has resulted in varying degree of functional loss including molting defect, morphological defect, and embryonic lethality. Similar to cpz-1 RNAi worms, the cpz-1 (ok497) mutant strain showed defects in embryogenesis and morphogene-
sis at the normal growth temperature (20 °C). Only at lower temperature (15 °C) did the cpz-1 mutant also show molting defects, similar to those seen with RNAi. The importance of Ce-cpz-1 in molting was thus established by the phenotypes obtained after cpz-1 RNAi and from initial analysis of homozy-
gous cpz-1 (ok497) mutant worms and is supported by the distinct expression and localization of the gene product in the hypodermis and the pharynx of **C. elegans** worms.

Molting in nematodes has three major steps: 1) separation of the old cuticle from the hypodermis (apoplistis), 2) formation of new cuticle arising from the outermost surface of the hypodermis, and 3) the shedding of the old cuticle (ecdysis) (32). In **C. elegans**, components of the old cuticle are degraded before ecdysis in mechanisms that are not yet established and that are believed to be dependent in part on the function of proteases and other components within the cuticle (33–36). The entire cuticle is shed at each molt and replaced with the new cuticle synthesized by the underlying layer of the hypodermal tissue, a large syncytium that extends throughout the length of the nematode. Both the synthesis and secretion of cuticular components by the hypodermis are tightly coupled to the molting cycles and are dependent upon precise control of a complex series of cellular events (35). The cuticle is mostly composed of collagen proteins, however, in each larval stage a unique set of collagen genes are produced by the hypodermal cell and exported to the surface (37). Notably, successful molting depends on the complete expulsion of the pharyngeal lining (32). About 30 min before ecdysis, the posterior bulb of the pharynx begins to twitch spasmodically, the cuticular lining breaks, and the posterior piece passes back into the intestine. Then the old cuticle of the body wall becomes inflated around the tip of the head, and the nematode pulls back from it repeatedly until the remainder of the cuticular lining of the pharynx is detached and then expelled through the mouth. The nematode then escapes from the old cuticle by pushing against the softened old cuticle with its head until a hole is made (32). Failing to expel the anterior piece can cause a cuticular plug in its mouth, which is not easily dislodged and can result in death by starvation.

It appears that the **C. elegans** CPZ-1 is similar to several other proteases of nematodes that have been reported to participate in this crucial process of molting during the development of nematodes. The role of these proteases was suggested to be in the digestion of old cuticle, degradation of the cuticular anchoring proteins, and/or the activation of peptide molting hormones or other molting enzymes by processing their proen-
zymes (38). Proteases such as serine, cathepsin L-like cysteine proteases, and/or aminopeptidases that are active during molting have been described in **Phocanema decipiens** (39), **Ancylostoma** (40), **Homonchus contortus** (41, 42), and filariae (43, 44). In the filarial parasites, **Dirofilaria immitis** and **Brugia pahangi**, metalloproteases have been suggested to be ini-
CPZ Is Essential for *C. elegans* Development

CPZ enzyme has been suggested to have an essential role during molting (6). In our previous studies, we have shown that the *C. elegans* CPL-1 and the filarial cathepsin L play a potentially important role in molting (18).

We propose that *cpz-1* may be involved indirectly in the process of degradation of anchoring proteins that are present in the old cuticle, which needs to be digested before shedding of the old cuticle occurs. The immunoelectron microscopy data clearly indicate the presence of CPZ-1 native protein in both the new and the old cuticles during molting, specifically at the time when the separation between the old and the new cuticles occurs. At the end of this process, a thinner old cuticle with remaining struts of the median cuticular zone is observed. In addition, CPZ-1 may acts as a proteolytic enzyme that degrades cuticular and/or other proteins that are part of the pharyngeal lining. Consequently, CPZ-1 may advance the complete separation process of the cuticles along the length of the worm body as well as in the pharynx even before complete ecdysis occurs.

Further support for its digestive activity is based on previous studies on its homologous protein, *Ov-CPZ*, in *O. volvulus*. In *O. volvulus*, the native *Ov-CPZ* enzyme was localized specifically in the region where the separation between the cuticle of L3 and L4 during molting occurs, and culturing of *O. volvulus* L3 in the presence of synthetic irreversible inhibitors resulted in an incomplete separation between the two cuticles (6). In *O. volvulus*, the old cuticle is not degraded before ecdysis; however, the anchoring protein between the cuticles needs to be digested before complete ecdysis. Alternatively or additionally, *CPZ-1* may participate in the processing of other nematode proteins that are critical for completion of the molting process, such as collagenases, peptide molting hormones, and/or other enzymes secreted by the worms during molting (38, 39, 41).

Similar indirect function during *C. elegans* molting have been attributed to the *C. elegans* LR-P-1 protein (35, 36). Mutation in the *lrp-1* gene conferred an inability to shed and degrade all of the old cuticle at each of the larval molts. The extracellular part of LR-P-1 was postulated to be required for the activation of collagenases or other proteases secreted during molts that partially degrade the old cuticle. Interestingly, both LR-P-1 and CPZ-1 are expressed on the apical surface of the hyp7 cells that cover 90% of the nematode’s surface area (36).

The periodic molting in *C. elegans* as well as in other nematodes requires strict temporal and spatial regulation of the expression of multiple genes that jointly regulate this process. Control mechanisms that regulate such a complicated process are poorly understood, but recent studies have implicated two *C. elegans* nuclear hormone receptors (NHR-23 and NHR-25) in the regulation of molting (45–47). Molting defects were observed after interference with *C. elegans nhr-25* gene expression by RNAi. The arrested L1-L2 worms were unable to shed their cuticle properly during molting as whole cuticles or parts of cuticle remained attached to the rectum (38, 39). Soaking or feeding of all four larval stages of worms in nhr-23 dsRNA also resulted in molting defects, however, in the later developmental stages of the worms suggesting that *nhr-23* may also regulate molting at each of the *C. elegans* larval developmental stages (47, 48). So far, it is not clear whether both proteins have a direct function or their role is through downstream gene(s) that are not activated because of the nhr-23 or nhr-25 RNAi inactivation. It is possible that a cascade of proteins take part in the molting process of *C. elegans*, some of which may or may not regulate the expression of others. The penetration of both *cpz-1* RNAi and the mutant phenotypes was low, suggesting that there are other genes might contribute additively to *cpz-1* function during development. For example, the *C. elegans* cathepsin L, Ce-cpl-1, was shown to be involved in molting (18). Interestingly, during larval development, the mRNA transcript levels of *cpl-1* and *nhr-23*, similar to that of the *cpz-1* gene, increase before the molts. Future studies on *cpz-1* mutant involving other genes that function during molting will shed light on the interaction between *cpz-1* and other molting genes.

A comprehensive functional analysis of all *C. elegans* genes using RNAi has been initiated (49, 50) and, in conjunction with genome-wide functional genomic approaches (51–53), including assays characterizing protein-protein interactions, will contribute to uncover the molecular basis and the components that are essential for molting.

Besides molting, the *C. elegans* *cpz-1* appears to have additional regulatory function(s). Disruption of *cpz-1* resulted in...
abnormal head or tail morphology of L2 worms when L1 were fed or soaked on \(cpz-1\) dsRNA. Similar phenotypes were observed in homozygous \(cpz-1\) (ok497) deletion mutant. The expression of \(cpz-1\) in hypodermal cells may suggest that \(cpz-1\) is also involved in morphological processes. Two forms of collagenous extracellular matrix (ECM) are present in nematodes: the cuticle that forms the exoskeleton and the basement membrane that surrounds the tissues. The ECM, both the cuticular and the basement membrane, is essential for the viability of all stages, as it helps maintain the worm’s post-embryonic body shape (37). The cuticular ECM is predominantly composed of small highly cross-linked collagens that are assembled through complex post-translational modifications to form the cuticle in each of the newly developed larval stages. Previous studies have shown that mutations or inappropriate expressions of any of the temporarily expressed collagen genes during development can cause a variety of gross morphological defects (37, 54–56). We suggest that \(cpz-1\), which is expressed in many hypodermal cells, may have an upstream regulatory role of the hypodermal morphogenetic pathway and, as such, be responsible for the proper proteolytic processing of some of the procollagens expressed during larval development. Insufficient maturation of these precursors might produce abnormal cuticle and thus a deformed body. Notably, these abnormalities were only found in L2 stages. Another protease that belongs to the subtilisin-like proteinase family has been shown to be part of a similar process. It was shown to regulate the processing of the SQT-1 cuticle collagen (37, 57). The \(sqt-1\) mutants exhibit the roller phenotype. Interestingly, inhibition of \(nhr-23\), \(nhr-25\), and \(lrp-1\) gene expression also resulted in some morphological abnormalities. All these genes, including \(cpz-1\), are highly expressed in the major hypodermal cells in all larval stages of nematodes, which supports their involvement in epidermal differentiation during or after molting (45, 46, 48).

In addition, \(cpz-1\) RNAi in L2 worms as well as \(cpz-1\) deletion mutant resulted in improperly developed gonad, suggesting another role for \(cpz-1\) in the somatic gonad development. This is supported by \(cpz-1\)-\(gfp\) expression in the gonadal tissues of transgenic young hermaphrodite and the presence of the native \(CPZ-1\) protein in some portion of the vulva. There are examples of \(C.\ elegans\) collagen-modifying enzyme, which affect morphology and development. For example, GON-1 is an ECM-modifying enzyme affecting organ morphogenesis (58); \(gon-1\) mutant phenotype shows severe abnormality in the gonad. \(CPZ-1\) may have a role similar to that of the GON-1 metalloprotease, which was suggested to direct the expansion of gonad by remodeling.
Moreover, CPZ-1 has an additional function during embryonic development. Both cpz-1 mutant and cpz-1 RNAi caused embryonic lethality in the developing embryos. During normal development the cpz-1 transcript (reporter construct) and the protein are both expressed in many embryonic cells, including hypodermal cells, and in the hypodermis of the later stage embryos. Interestingly, both cpz-1 mutant and the cpz-1 RNAi-affected embryos failed to progress beyond the cell proliferation stage arresting before morphogenesis, may suggest that cpz-1 directly or indirectly target those proteins that are essential in cell fate specification and organization of tissues and organs during development. The hypodermis plays a critical role in altering the shape of the embryo during C. elegans morphogenesis (59). The hypodermal cells originate on the dorsal surface of the embryo and migrate ventrally to enclose the embryo at the beginning of morphogenesis (59, 60). Subsequently, the hypodermal actin cytoskeleton reorganizes, forming a parallel of actin fiber, and the contraction of these actin fibers within the hypodermal cells determines the shape of the embryo during elongation (59). Future experiments aimed at the identification of the protein(s) that are targeted by CPZ-1 activity when it controls these morphological processes would clarify the distinct function(s) of CPZ-1.

In summary, we suggest that CPZ-1 has a multifunctional role during C. elegans development. Although there are parallels between localization of the O. volvulus and C. elegans CPZ-1 enzymes during molting in both nematodes, the precise function(s) of the cpz-1 gene in other processes in both the free living and the parasitic nematodes will need further confirmation. These observations are encouraging for future investigation in the discovery of selective inhibitors unique to CPZ and thus will aid in the development of potential chemotherapeutic agents that will interfere with normal development of parasitic nematodes. The unique structure of cathepsin Z among other members of the papain family of cysteine proteases will be of great value in designing specific inhibitor useful as a research tools to investigate the physiological roles of this enzyme.

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