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Calreticulin, a Calcium-binding Molecular Chaperone, Is Required for Stress Response and Fertility in *Caenorhabditis elegans*

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Calreticulin (CRT), a Ca^{2+}-binding protein known to have many cellular functions, including regulation of Ca^{2+} homoeostasis and chaperone activity, is essential for heart and brain development during embryogenesis in mice. Here, we report the functional characterization of *Caenorhabditis elegans* calreticulin (*crt-1*). A *crt-1* null mutant does not result in embryonic lethality but shows temperature-dependent reproduction defects. In *C. elegans* CRT-1 is expressed in the intestine, pharynx, body-wall muscles, head neurons, coelomocytes, and in sperm. *crt-1* males exhibit reduced mating efficiency and defects late in sperm development in addition to defects in oocyte development and/or somatic gonad function in hermaphrodites. Furthermore, *crt-1* and *itr-1* (inositol triphosphate receptor) together are required for normal behavioral rhythms. *crt-1* transcript level is elevated under stress conditions, suggesting that CRT-1 may be important for stress-induced chaperoning function in *C. elegans.*

INTRODUCTION

Calreticulin (CRT) is a 46-kDa protein ubiquitously expressed in all cells of higher organisms (Michalak *et al.*, 1999). Encoded by a single gene, CRT was first purified and identified as a Ca^{2+}-binding protein of the skeletal muscle sarcoplasmic reticulum (Ostwald and MacLennan, 1974) and was later genetically identified in mouse (Smith and Koch, 1989) and in other mammals (Fliegel *et al.*, 1989). It is a highly conserved protein with >90% amino acid sequence identity among the mammalian forms (Michalak *et al.*, 1992).

Calreticulin is divided into three structural and functional domains: a highly conserved N-domain, a proline-rich P-domain, and a highly acidic C-domain that binds Ca^{2+} with high capacity and low affinity (K_d = 2 mM; B_{max} = >25 mol of Ca^{2+}/mol of protein) (Nash *et al.*, 1994; Corbett and Michalak, 2000). The N terminus has a cleavable signal sequence that is processed cotranslationally, and there is a C-terminal KDEL endoplasmic reticulum (ER) retention signal. However, calreticulin has also been shown to localize to the nucleus and/or the cytoplasm of certain cells (Krause and Michalak, 1997).

Calreticulin has been implicated in diverse cellular functions, both in the ER and outside the ER environment. Within the ER lumen, CRT has been shown to play an important role as a lectin-like chaperone similar to calnexin, an integral ER membrane protein (Bergeron *et al.*, 1994; Helenius *et al.*, 1997). Due to its Ca^{2+}-buffering property in the ER, CRT is also known to regulate intracellular Ca^{2+} homeostasis (Fliegel *et al.*, 1989; Treves *et al.*, 1990; Michalak *et al.*, 1992; Krause and Michalak, 1997). CRT is also known to act as a modulator of nuclear-hormone receptor-mediated gene transcription (Burns *et al.*, 1994; Dedhar *et al.*, 1994) and integrin-mediated calcium signaling and cell adhesion (Coppolino *et al.*, 1997; Goicoechea *et al.*, 2000; Kwon *et al.*, 2000). More importantly, calreticulin-deficient mice show embryonic lethality along with heart defects such as decreased ventricular wall thickness, indicating that CRT is essential for heart and brain development in mice (Mesaeli *et al.*, 1999; Rauch *et al.*, 2000).

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Due to its relatively short life cycle, *C. elegans* has always been an excellent model for developmental and genetic studies. Because it is also a convenient model for Ca^{2+} homeostasis studies (Baylis *et al*., 1999; Dal Santo *et al*., 1999; Kraev *et al*., 1999), we undertook a functional study of the *C. elegans* calreticulin, a Ca^{2+}-binding protein. The calreticulin gene (*crt-1*) of the free-living nematode *C. elegans* has also been cloned and sequenced (Smith, 1992). *crt-1* is physically mapped to the center arm of chromosome V (LGV) corresponding to the yeast artificial chromosome Y38A10A. *C. elegans* CRT-1, like in vertebrates, is encoded by a single gene (Smith, 1992). The amino acid sequence was thereafter deduced and alignments with mouse calreticulin show an overall 61% identity. In this study, we report the expression pattern of CRT-1, and demonstrate that the deletion mutant shows temperature-dependent reproductive defects, suggesting CRT-1 may be important for the survival of *C. elegans* in a stress-induced environment. Recently, *crt-1* alleles have been identified as suppressors of necrotic cell death in *C. elegans* (Xu *et al*., 2001). We further show that *crt-1* mutants exhibit reduced fertility, suggesting that CRT-1 is important for proper development of *C. elegans* sperm and oocyte.

MATERIALS AND METHODS

C. elegans Strains

The nematode *C. elegans* Bristol type (N2), CB1282 *dpy-20(e1282) IV*, CB224 *dpy-11(e224) V*, DH245 *fem-2(b245) III*, JT73 *itr-1(sa73) IV*, and DR190 *dpy-13(e184),unc-24(e138) IV* were obtained from the *Caenorhabditis* Genetics Center at the University of Minnesota (St. Paul, MN). The *crt-1* mutant KJ216 *crt-1(jh101)* was isolated by reverse genetics method (this study), 4 *crt-1* alleles (*bz29*, *bz30*, *bz31*, and *bz50*) were obtained from the laboratory of M. Driscoll and three alleles (*sy328, sy331, sa73*) of *itr-1* were from the laboratories of P. Sternberg, J. Thomas, and E. Jorgenson. Worm breeding and handling were conducted as described (Brenner, 1974).

Isolation of C. elegans Calreticulin cDNA, Northern Analysis, and DNA Injection

To obtain full-length *crt-1* cDNA, two primers were designed based on the *crt-1* sequence reported previously (Smith, 1992): 5′-ATGCGAATTCATGAAATCACTCTGCC-3′ upstream primer and 5′-ATGCGTCGACTTAGAGCTCATCGTGTGTCC-3′ downstream primer. These primers were used to amplify a mixed stage *C. elegans* cDNA library (kindly provided by P. Okkema and A. Fire) by polymerase chain reaction (PCR). Amplification was carried out for 30 cycles: 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min. A full-length *crt-1* cDNA of size 1188 bp was obtained, subcloned into pGEM-T vector (Promega, Madison, WI), and confirmed by sequencing. For Northern blotting, total RNA was prepared from staged animals as described (Krause, 1995; Cho *et al*., 2000). To examine the change in the *crt-1* gene transcription level under stress, we treated wild-type animals with 7% ethanol. Mixed stage animals grown on regular growth plates were harvested and incubated in complete S medium in the absence or presence of 7% ethanol. The animals were aerated by shaking at 250 rpm. After 6 h of incubation, total RNA was purified from each sample and Northern blotting was performed as described previously. Wild-type and *crt-1(jh101)* worms were grown at 16, 20, and 25°C, and total RNA was extracted to conduct Northern analysis as before. Signals were detected by exposing the blots to an x-ray film or to an imaging plate of an image analyzer (BAS-1500, Fujifilm; Fuji Photo Film, Tokyo, Japan). For the rescue experiment, a genomic *crt*-1 clone was constructed from a 3-kb *Hind*III-*BamH*I fragment obtained by the genomic library (λgt11) screening and a downstream region clone from PCR reaction. The resulting 6-kb fragment containing a *crt*-1 genomic DNA (pRC101) was injected at a 2-, 10-, and 50-ng/μl concentration mixed with 100 ng/μl pRF4 plasmid.

Preparation of Polyclonal Antibodies and Western Analysis

The full-length *crt-1* cDNA was subcloned into pGEX-4T1 (Amersham Pharmacia Biotech) and overexpressed as glutathione *S*-transferase (GST) fusion protein. The fusion protein was purified on Glutathione-Sepharose (Amersham Pharmacia Biotech) and used to immunize rabbits. Total proteins obtained from wild-type worms and *crt-1* mutants were prepared and used for Western blotting as described previously (Cho *et al*., 2000).

Ca^{2+}-binding Assay and Aggregation Assays

Ca^{2+}-binding assay for CRT-1 was performed as described previously (Maruyama *et al*., 1984). The full-length *crt-1* cDNA fused to pGEX-4T1 (see above) was overexpressed in the presence of isopropyl β-D-thiogalactoside, resolved by SDS-PAGE, transferred to polyvinylidene difluoride membrane, and probed with $^{45}Ca^{2+}$ (Cho *et al*., 2000). After washing with 50% ethanol, the membrane was dried and exposed to an imaging plate of an image analyzer (BAS-1500, Fujifilm).

For aggregation assay, purified GST-CRT-1 (see above) was released from GST by thrombin cleavage and further purified with the use of Superdex-75 column in 50 mM HEPES buffer, pH 7.5, containing 10% glycerol, and dialyzed in TSC buffer (10 mM Tris-HCl, 0.15 M NaCl, 5 mM $CaCl_2$, pH 7.2). Protein aggregation assay was carried out with the nonglycosylated protein, citrate synthase (CS) (Roche Biomedical) according to the methods described (Saito *et al*., 1999). Rabbit IgG and bovine serum albumin (BSA) was used as a control.

Isolation of crt-1 Deletion Mutants from a Mutagenized DNA Library

A synchronous population of wild-type N2 worms (P0) was collected and mutagenized in M9 buffer containing 0.5 μg/ml 4,5,8′ trimethylpsoralen (T6137; Sigma, St. Louis, MO). Mutagenesis and worm harvest was carried out as described (Liu *et al*., 1999). Screening of mutants from the mutagenized DNA library was carried out by a nested PCR-based method and subsequent sib selections as described (Barstead, 1999). A homozygous line of animals with a 1.1-kb deletion relative to the wild-type was isolated. This animal was outcrossed six times to wild-type animals to establish the strain KJ216 *crt-1(jh101)* and was used in subsequent analysis. To obtain a homozygous line of *crt-1(jh101)* males, *crt-1(jh101)* homozygous hermaphrodites were crossed to wild-type males, and the heterozygous males thus obtained were crossed to homozygous parents. A line of homozygous *crt-1(jh101)* males was obtained and the deletion region for both the hermaphrodites and the males was determined by nested PCR followed by sequencing the PCR products.

Immunostaining and Whole-Mount In Situ mRNA Hybridization

Wild-type *C. elegans* was immunostained as described (Ahnn and Fire, 1994; Miller and Shakes, 1995; Cho *et al*., 2000). Animals were freeze-cracked and stained with anti-CRT-1 primary antibody and goat anti-rabbit secondary antibody (rhodamine conjugated). Slides were mounted and observed under a fluorescence microscope (Olympus BX50; Olympus, Tokyo, Japan). Immunofluorescence localization of CRT-1 in sperm cells of wild-type and *crt-1(jh101)* males was performed as described (Arduengo *et al*., 1998).

Whole-mount in situ experiments were carried out as described (Seydoux and Fire, 1994, 1995). Digoxigenin-labeled *crt-1* DNA probes were made by PCR amplification, and alkaline phosphatase-labeled anti-digoxigenin Fab fragment (Roche Molecular Biochemicals) was used to detect signals. Samples were viewed under a light microscope for color reactions

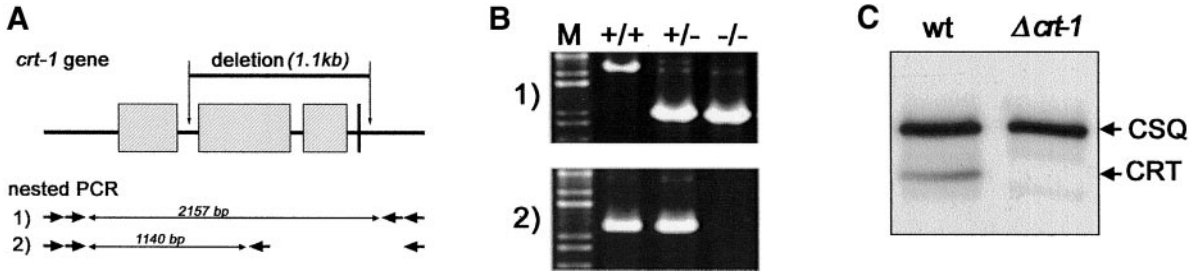

Figure 1. Genomic organization of *crt-1(jh101)* deletion mutant and protein analysis. (A) A 1.1-kb deletion, which removes a portion of intron 1 through 3′UTR′, is shown by a horizontal bar. The 1 and 2 indicate the primer sets for nested PCR (shown by arrows) used during initial sib selection and homozygosity checking, respectively. (B) PCR bands obtained from single worm PCR, wild-type (+/+), heterozygote (+/−), and homozygote, with the use of the primer sets described in A. Absence of band in 2 (−/−) confirms that the worm is a homozygote. (C) Western blotting shows CRT-1 (55 kDa) and CSQ-1 (calsequestrin as a control) proteins in N2 worms (lane 1) and *crt-1(jh101)* (lane 2).

and under a fluorescence microscope for 4,6-diamidino-2-phenylindole (DAPI) staining.

Immunogold Staining

Immunogold staining with adult N2 worms and *crt-1 (jh101)* was carried out as described (Yu and Chai, 1995). Specimens were incubated for 2 h at room temperature with primary antibody (rabbit polyclonal anti-CRT-1). After a thorough wash in 1% bovine serum albumin and 0.01% Tween 20 in phosphate-buffered saline, the specimens were reincubated overnight at 4°C with 5-nm gold-conjugated goat anti-rabbit IgG (British BioCell Research, Cardiff, United Kingdom). For silver enhancement, a commercial kit was used (Amersham Pharmacia Biotech) and the background was stained with uranyl acetate and lead citrate. Samples were air-dried and examined under a transmission electron microscope (Jeol 1200 EXII).

Phenotype Analysis

crt-1(jh101) deletion mutant was characterized microscopically for phenotypic defects. The brood size of the wild-type and mutant worms at 16, 20, and 25°C was estimated. Sperm fertility and mating efficiency of *crt-1(jh101)* males were studied by estimating the outcross progeny. Three *crt-1(jh101)* males were allowed to cross to CB224 *dpy-11(e224)V* on fresh plates. Each cross was then transferred to a fresh plate for each of the next 4 d. Total progeny on the plates was counted and the number of dumpy phenotype worms and wild-type worms were scored as self-progeny and outcross progeny, respectively. The percentage of outcross progeny versus total progeny was determined and scored as mating efficiency. To examine sperm, *crt-1* and control N2 mate plates were shifted to 25°C after cross. L4 males then moved to plates without hermaphrodites to accumulate sperm overnight, and then males were dissected and sperm activated with pronase treatment as described (L'Hernault and Roberts, 1995).

To examine the role of *crt-1* in receptor-mediated endocytosis of yolk proteins, the green fluorescent protein (GFP) expression of YP170::GFP in *crt-1(jh101)* mutant worms was visualized. *crt-1(jh101)* homozygous males were crossed to an integrated line DH1033 *(bIs1[vit-2::GFP, rol-6; sqt-1(sc103)]* (kindly provided by B. Grant), and the progeny thus obtained were visualized for changes in the expression pattern of YP170::GFP. This fluorescent *YP170::GFP* integrated line is useful to study the yolk transportation from the intestine to the oocytes by fluorescence microscopy, thereby allowing an in vivo analysis of secretion and endocytosis.

To study the defecation cycle in *crt-1* null mutant, *crt-1(jh101*) homozygous males were crossed to JT73 *itr-1(sa73) IV* mutant strain to obtain the *crt-1; itr-1* double mutants. Trans-heterozygous animals thus obtained were cloned and individual progeny was scored for defecation cycle under dissecting microscope as described (Thomas, 1990). The *crt-1* mutation was confirmed by nested PCR (see above) and the *itr-1* mutation was confirmed by sequencing the *itr-1* genomic DNA.

RESULTS

Isolation of a crt-1 Deletion Mutant, crt-1(jh101), in C. elegans

The calreticulin gene *(crt-1*) of *C. elegans* has been previously identified, physically mapped toward the center of chromosome V, and sequenced (Smith, 1992). A full-length *crt-1* cDNA was obtained by PCR from a mixed stage worm cDNA library and contained an open reading frame of 1188 bp. *C. elegans* calreticulin (CRT-1) is composed of 395 amino acid residues and shows 61% identity with mouse CRT (Smith, 1992), and 58% identity with human calreticulin.

To examine *crt-1* function in vivo, we isolated a *crt-1* deletion mutant, *crt-1(jh101),* by a PCR-based screening of a chemically mutagenized library. The deletion removed ~1.1 kb of the *crt-1* gene (Figure 1, A and B), including two-thirds of the *crt-1* coding regions. The deletion region was further confirmed by nested PCR with the use of an inner downstream primer that produced no PCR band in the *crt-1 (jh101)* homozygous mutant but showed a 1.1-kb band with a wild-type or *crt-1* heterozygous mutant (Figure 1B, 2). To analyze the CRT-1 protein, we performed Western blotting experiments with anti-CRT-1 antibodies. Anti-CRT-1 antibody detected a single band of 55 kDa from protein extracts of a mixed stage population of wild-type animals (Figure 1C). However, no protein band was detected from *crt-1(jh101)* mutants, indicating that these deletion mutants are functionally null. We also failed to detect protein in two nonsense alleles (*bz29* and *bz30*) reported by Xu *et al.* (2001).

C. elegans CRT-1 Expresses in Intestine, Pharynx, and Body-Wall Muscles

Before characterizing mutant animals we examined the expression of *crt-1* in wild-type worms. First, we performed Northern blot experiments with RNA extracted from staged wild-type (N2) animals with the use of a 1040-bp *Pvu*II-*Sac*I *crt-1* cDNA fragment as a probe. A single 1.4-kb transcript was detectable at all stages of development (Figure 2A). To further determine the temporal and spatial expression of

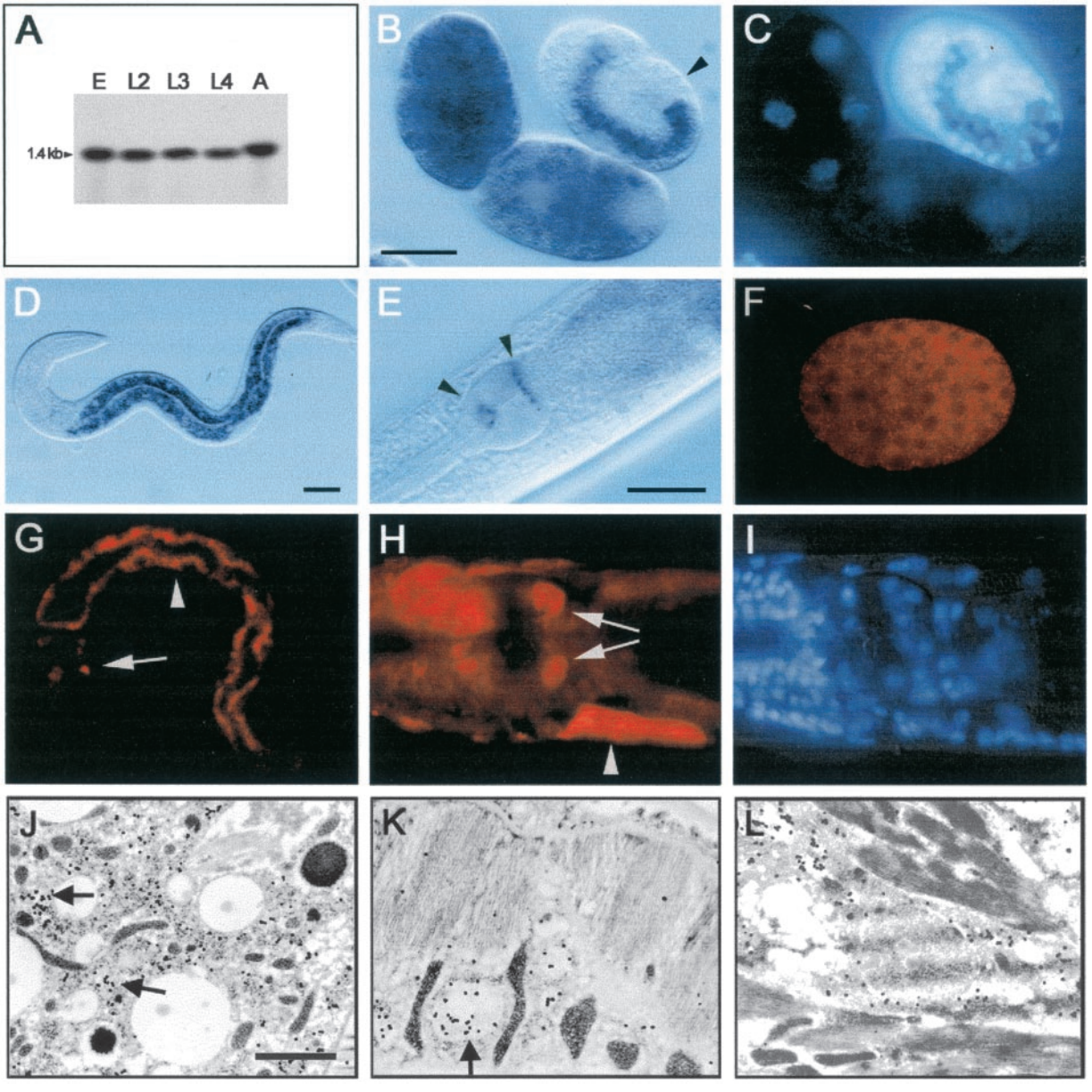

Figure 2. Expression pattern of *crt-1* in *C. elegans*. (A) Northern blot analysis with ^{32}P-labeled *crt-1* probe detects a 1.4-kb band at all developmental stages. Eggs (E); larval stages (L2–L4); adults (A). (B–E) In situ localization of *crt-1* RNA at different developmental stages. With the use of an antisense probe, robust signals are observed in a two-cell stage embryo and in a four-cell stage embryo. Restricted signals along the midline are observed in intestinal precursor cells, arrowhead indicates anterior part of the embryo. (C) DAPI-stained embryos shown in B to visualize the nuclei. (D) Strong signal is observed along the intestine of a larva and in the pharyngeal bulb (E, arrowheads). (F–I) Immunofluorescence localization of CRT-1 in wild-type worms with the use of anti-CRT-1 antibodies. Staining is observed in the cytoplasm of early embryo stage (F), along the intestinal wall of a larva (G), and in the terminal bulb region of the pharynx (arrows) and some excretory cells (arrowhead) in the head (H). (I) DAPI-stained worm shown in H. Bar, 20 μm. (J–L) Immunogold electron micrograph showing the subcellular localization of CRT-1. (J) Transverse section of the intestine showing clustered signals of CRT-1 as gold particles around the intestinal vacuoles (arrows) in wild-type worms. (K) Transverse section of the body-wall muscle structure showing CRT-1 signals (arrow) at the cytoplasmic face of the muscle cells in wild-type animals. (L) Section through the pharynx shows signals in the cytoplasmic regions between muscular structure. Bar, 0.5 μm.

crt-1, whole-mount in-situ hybridization experiments were conducted with an antisense probe (Figure 2, B–E). The *crt-1* transcripts, both maternal and zygotic, were detected during early embryonic stages and the expression was maintained through adult stages (Figure 2, B–E). The signals from the maternally transcribed messages were detected as early as two-cell stage embryo and continued through four-cell stage. As the embryo went through morphogenesis, a strong

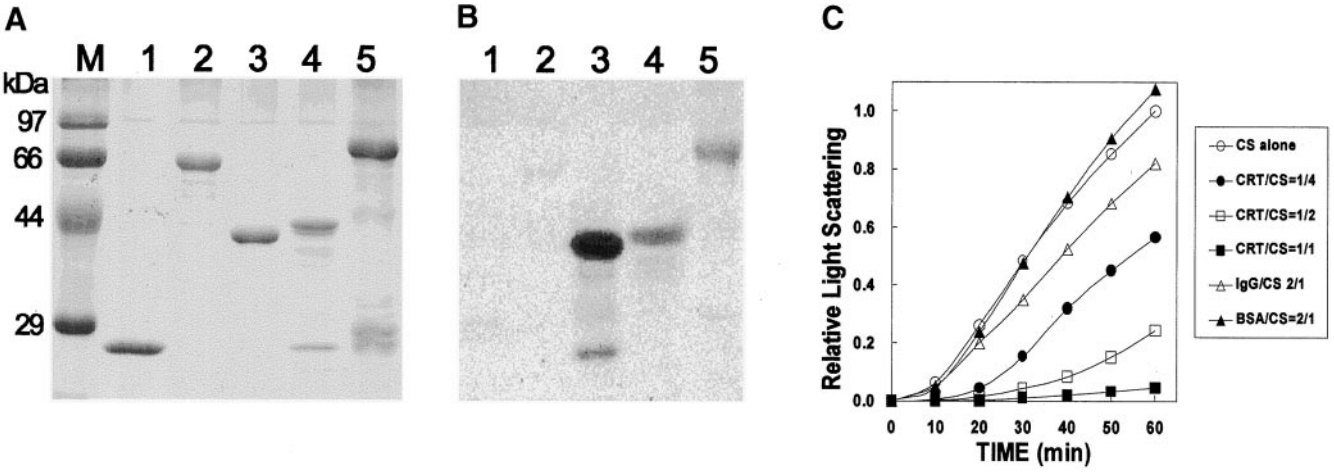

Figure 3. CRT-1 is a Ca^{2+}-binding molecular chaperone. (A) An SDS-PAGE gel stained with Coomassie blue showing GST (lane 1), BSA (lane 2), GST-calcineurin B (lane 3), thrombin-digested GST-CRT-1 (lane 4), GST-CRT-1 (lane 5), and molecular weight markers (lane M). (B) Ca^{2+}-binding assay. A corresponding gel shown in A with positive bands, indicating the $^{45}Ca^{2+}$-labeled proteins (GST-calcineurin lane 3; GST-CRT-1 lane 4; CRT-1 lane 5). GST (lane 1) and BSA (lane 2) are used as negative controls. (C) Suppression of thermal aggregation by CRT-1. CS was incubated at 45°C in the absence and presence of CRT-1 (0.25–1 μM) or IgG (2 μM). Aggregation of CS was monitored by measuring light scattering at 360 nm.

but restricted signal was detected along the midplane of the embryo indicative of intestinal precursor cell expression (Figure 2, B and C). During larval and adult stages, CRT-1 was shown to express strongly along the intestine (Figure 2D), which was in conformity with the embryonic expression patterns. In addition, distinct signals were observed in the pharyngeal bulb region (Figure 2E). However, no signals were detected in control experiments with a sense strand probe.

To further localize CRT-1 in situ, we performed whole-mount immunostaining with anti-CRT-1 antibodies in a mixed stage wild-type population. CRT-1 was detected in the cytoplasm, presumably from the endoplasmic reticulum network, of the early embryo (~100-cell stage) (Figure 2F) and a strong intestinal staining was observed in both larval and adult worms (Figure 2G). Signals were also observed in the terminal bulb region of the pharynx and in excretory cells in the head (Figure 2, H and I, arrows and arrowhead, respectively). Faint but specific staining was also observed in head neurons and body-wall muscle cells (see below).

Next, we localized CRT-1 at the subcellular level in specific tissues of wild-type worms by immunogold electron microscopy with the use of anti-CRT-1 antibodies (see MATERIALS AND METHODS). In intestinal cells, clustered signals of CRT-1 were observed around the intestinal vacuoles and intestinal granules of wild-type worms (Figure 2J). The intestinal cells of *C. elegans* are characterized by the appearance of such granules and vacuoles alongside the intestinal lumen. In body-wall muscles, distinct enclosed signals of CRT-1 in the cytoplasmic regions of the muscle cells were observed (Figure 2K). Interestingly, this localization of CRT-1 is somewhat different from that of calsequestrin, which appeared to localize at sarcoplasmic reticulum in body-wall muscle (Cho *et al.*, 2000). In the pharyngeal muscle cells, signals were detected in the cytoplasmic regions between muscular structures (Figure 2L).

CRT-1 Binds Ca^{2+} and Functions as Molecular Chaperone

Calreticulin is well known to bind Ca^{2+} and contains two distinct types of Ca^{2+}-binding sites located at different regions of the protein, one high affinity/low capacity and the other low affinity/high capacity (Baksh and Michalak, 1991). To examine whether CRT-1 can bind Ca^{2+}, we overexpressed full-length recombinant GST-CRT-1 in *Escherichia coli* and performed Ca^{2+} overlay experiments with $^{45}Ca^{2+}$. Purified GST-CRT-1 and purified CRT-1 after thrombin cleavage showed Ca^{2+}-binding activities (Figure 3B, lanes 4 and 5), whereas GST alone showed no Ca^{2+}-binding activity (Figure 3B, lane 1). Thus, Ca^{2+} overlay experiments confirmed that CRT-1, like other Ca^{2+}-binding proteins in *C. elegans* such as calcineurin B (Figure 3B, lane 3), has binding activity for Ca^{2+}.

To examine whether CRT-1 functions as a molecular chaperone, we tested its ability to suppress thermal aggregation of a nonglycosylated protein CS as shown by Saito *et al.* (1999). On heating at 45°C, CS denatures and forms large aggregates and CRT-1 effectively suppressed this thermal aggregation at 1:4 and 1:2 M ratio to CS and completely suppressed at 1:1 M ratio (Figure 3C). On the other hand, purified twofold molar excess of either rabbit IgG or BSA had little or no effect. *C. elegans* CRT's ability to suppress aggregation of nonglycoproteins in vitro is as effective as that of purified rabbit calreticulin (Figure 3C; Saito *et al.*, 1999). Therefore, *C. elegans* CRT-1, like other calreticulins and other molecular chaperones, is capable of suppressing thermal aggregation of a nonglycosylated protein.

crt-1 Mutant Is Viable and Shows Temperature-dependent Reproductive Defects

crt-1(jh101) mutant worms were examined for phenotypic defects. To our surprise, mutant animals did not show dras-

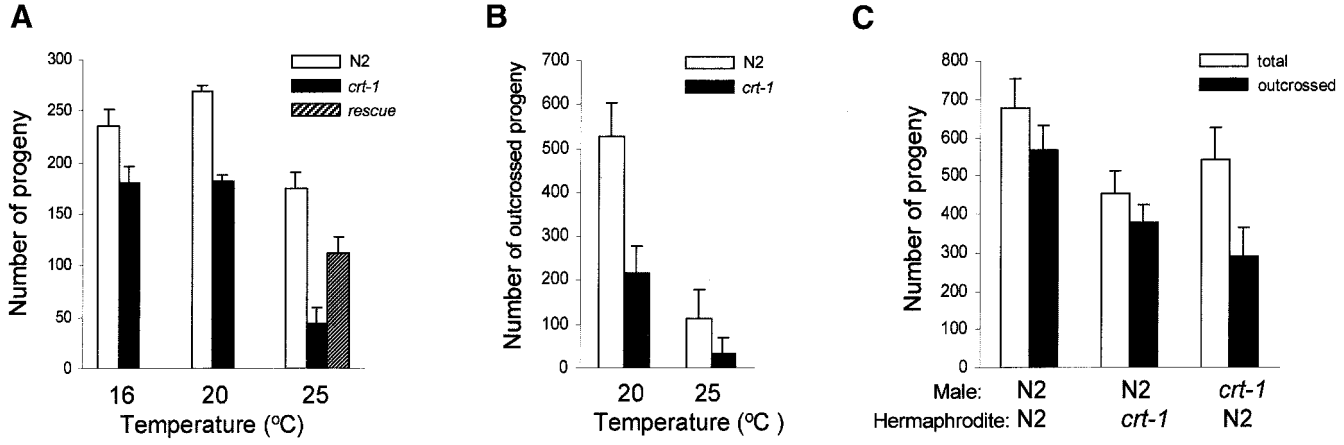

Figure 4. Reproductive defects in *crt-1* mutants. (A) Brood sizes of *crt-1 (jh101)* or wild type worms were examined at 16°C, 20°C, or 25°C. $n > 50$ in each case. The defect is rescued by injection of the genomic *crt-1* gene. (B) Mating efficiency of males as determined by the number of outcross progeny was examined for *crt-1* and wild-type animals. $n > 30$ (n: individual cross) at 20°C and 25°C. (C) Reciprocal out crosses between wild type (N2) and *crt-1*. In each case, cross-progeny was measured by counting male progeny and multiply by 2.

tic phenotypes except that they grow more slowly and were slightly shorter in body length than wild type. On careful examination, the *crt-1* mutants showed somewhat low brood size distinguishable from wild-type animals at 16 and 20°C. However, at 25°C, brood size was significantly reduced compared with wild-type animals (Figure 4A). Similar defects were observed with *crt-1(bz29)* and *crt-1(bz30)* alleles (Xu *et al.*, 2001). Thus, mutants lacking functional CRT-1 show temperature-dependent reproduction defects. We further tested whether this defect was indeed caused by the *crt-1(jh101)* deletion mutation through a rescue experiment (see MATERIALS AND METHODS). Reproduction defects were rescued substantially although not completely when the *crt-1* genomic DNA was reintroduced into mutants by microinjection (Figure 4A).

We compared the mating efficiency of the *crt-1(jh101)* and wild-type males. Although the *crt-1(jh101)* males exhibited slightly sluggish motility, normal copulatory behavior was observed compared with wild type. However, the mating efficiency, as determined from the number of outcross progeny (see MATERIALS AND METHODS), was shown to be significantly lower in *crt-1(jh101)* at both 20°C and 25°C (Figure 4B). We further tested the possibility that somatic gonad or/and oocyte development of hermaphrodite was also impaired in the *crt-1(jh101)* mutants. As shown by the numbers of out-crossed progeny in reciprocal crosses between wild type and *crt-1* male versus hermaphrodite, oocytes produced by *crt-1(jh101)* hermaphrodites were much less fertile than those of wild type (Figure 4C). Based on this observation we speculate that *crt-1* might function not only in oocyte development (and/or somatic gonad) but also in the sperm and that the sperm and oocyte derived from the mutant animals during mating were not as competent as wild type. First, to address this possibility of sperm defect, wild-type and *crt-1(jh101)* male sperm were carefully examined and immunostained with anti-CRT-1 antibodies (see MATERIALS AND MTETHODS; Figure 5). As expected, robust staining was observed in the cytoplasm of wild-type sperm as well as in residual bodies (Figure 5, A1–C3), whereas staining was completely abolished in the *crt-1* mutants (Figure 5, D3). This observation confirms that *crt-1* is expressed in the sperm and probably plays an important role in sperm development. We also stained *crt-1* mutant sperm with monoclonal antibody 1CB4, which specifically visualizes the fibrous body-membranous organelle (FB-MO) complexes known to be important for spermatogenesis (Okamoto and Thomson, 1985). The 1CB4 staining pattern of *crt-1* mutant appeared to be normal (Figure 5 E3), suggesting that morphogenesis and/or proper partitioning of FB-MO is not defective in *crt-1* mutant sperm. However, when the activated *crt-1* sperm were examined, they appeared to have slightly shorter pseudopods, and nuclei that were often off center, and were generally smaller than wild-type sperm (Figure 5,F and G). These defects are similar to those seen in a group of genes required for sperm development, which includes *spe-17* (Shakes and Ward, 1989; L'Hernault *et al.*, 1993). Taken together, our data suggest that *crt-1* is critical in the fertility of the worms and/or in late sperm development.

Second, we reasoned that CRT-1 could function in synthesis and/or endocytosis of the yolk protein in the developing oocytes. To address this question, we crossed *crt-1(jh101)* homozygous males to an integrated line DH1033 (*bIs1[vit-2::GFP, rol-6; sqt-1(sc103)]* (kindly provided by B. Grant), and visualized the GFP expression of the yolk protein (YP170:: GFP) in the intestine and during yolk uptake in the oocytes (see MATERIALS AND METHODS). Contrary to our prediction, there were no visible differences in the GFP expression patterns of YP170::GFP in the *crt-1* mutant line (*vit-2:: gfp;crt-1*) when compared to the DH1033 transgenic line, *vit-2::gfp*. Green fluorescence was observed in developing oocytes and in different-staged embryos in both the fluorescent lines (data not shown). These data indicate that although CRT-1 may function in oocyte development (and/or in somatic gonad), but is not essential in the receptor-mediated endocytosis of oocytes.

crt-1 May Function in Stress Response

We have observed temperature-dependent brood-size defects for the *crt-1(jh101)* allele where brood size was shown

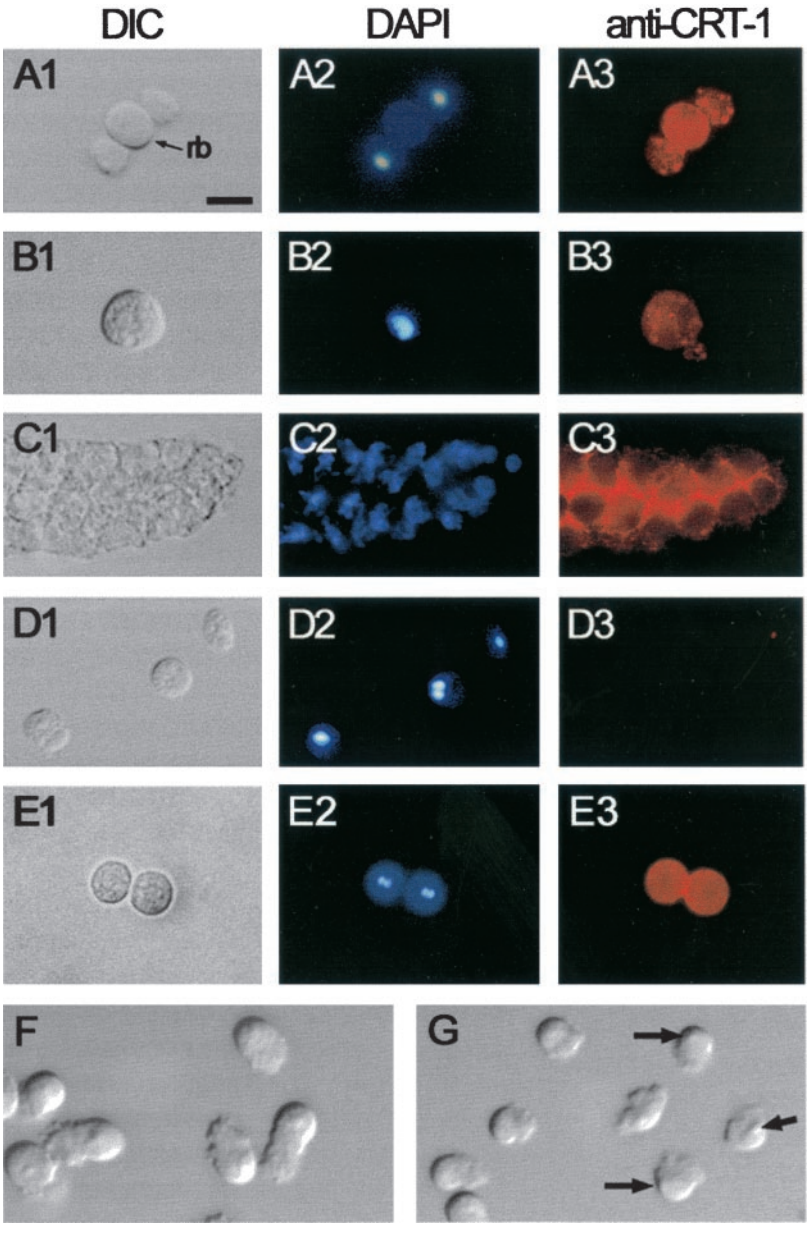

Figure 5. Immunolocalization of CRT-1 in sperm cells. (A–C) Wild-type sperm show normal staining with anti-CRT-1. (A) Wild-type spermatids budding from the residual body (rb) are stained with anti-CRT-1, whose nuclei are shown by DAPI staining. (B) Wild-type spermatid. (C) Male gonad containing spermatids and are stained with anti-CRT-1 antibodies. (D) *crt-1(jh101)* mutant spermatids that fail to be stained by anti-CRT-1 antibodies, indicating the absence of functional CRT-1 in the mutant worms. (E) mAb 1CB4 stains FB-MO complexes of the *crt-1* sperm. Nomarski images of wild-type (F) and *crt-1* (G) sperm, arrows point to the examples of nuclei that are off center in the cell.

to be significantly decreased at a higher temperature compared with wild type (Figure 4A). It is likely that the mutant, in the absence of CRT-1 protein, is defective in coping with elevated temperature stress due to lack of chaperoning activity. We checked the level of *crt-1* message in wild-type worms grown at different temperatures (see MATERIALS AND METHODS). In fact, the transcript level was increased with temperature approximately twofold (Figure 6A). Even though there was no CRT-1 protein produced in the *crt-1(jh101)* deletion mutants, the truncated transcript of a 600-bp band was detected. Interestingly, the level of the truncated message was also increased twofold at 25°C (Figure 6B).

We also reasoned that CRT-1 could function as a molecular chaperone in other stress responses, like ethanol stress. To test this, we conducted Northern analysis for *crt-1* in wild-type worms before and after stress-induced treatment with 7% ethanol. The transcript level of *crt-1* increased approximately threefold, compared with the untreated control worms (Figure 6C). We confirmed the quantities of the loaded RNA were identical by means of hybridization with actin or tubulin probes, whose transcription level is not altered by elevated temperature and ethanol (Figure 6). These data further indicate that worms responded to certain stress conditions, such as ethanol stress, by elevating gene transcription of stress-responsive genes such as *crt-1*. Calreticulin has been known to possess chaperoning function (Figure 2C), and hence, we infer that *crt-1* gene transcription may be elevated to meet the necessary chaperoning function during stress conditions.

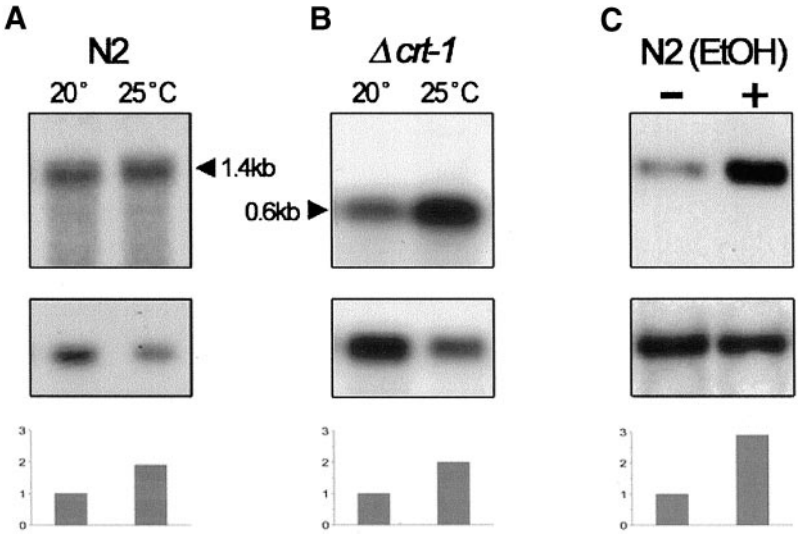

Figure 6. Stress signals affect the level of *crt-1* transcription. (A and B) Northern blot analysis of *crt-1* for wild-type and *crt-1(jh101)* worms grown at different temperatures as indicated. The *crt-1* transcripts (1.4 kb) for the wild-type (A) or the *jh101* allele (0.6 kb) (B) are shown with temperature (top), loading control *act-1* transcripts (middle), and normalized levels (bottom). (C) Northern blot analysis of *crt-1* worms after ethanol stress condition. Lane 1 shows the *crt-1* transcript under the normal condition and lane 2, the *crt-1* transcript 6 h after treatment of 7% ethanol. Tubulin transcripts are shown as loading control.

CRT-1 in Association with Inositol Triphosphate (IP_3) Receptor Is Important for Defecation Cycle and Fertility in C. elegans

Inositol triphosphate (IP_3) receptors are Ca^{2+}-release channels localized to the ER and allow Ca^{2+} release from the intracellular stores into the cytoplasm (Berridge, 1993; 1997). The IP_3 receptor in *C. elegans (itr-1)*, like *crt-1*, is expressed predominantly in the intestine, and mutations in the *itr-1* gene show abnormal behavioral rhythms during defecation (Dal Santo *et al*., 1999) and decreased fertility (Clandinin *et al*., 1998). In particular, defecation cycles are lengthened in the *itr-1(sa73)* mutants (Dal Santo et al., 1999), and brood sizes are significantly reduced in *itr-1(sy328)*, and *itr-1(sy331)* mutants (Clandinin *et al*., 1998). Because CRT-1 and IP_3R are both known to regulate Ca^{2+} homeostasis within the cells, and because both are expressed in intestine, we sought to investigate whether *crt-1* may interact genetically with these mutant alleles. First, we measured brood sizes and defecation cycles for *crt-1(jh101)* and for *itr-1(sa73, sy331, sy328)* mutants and wild type. We found that *crt-1* mutants showed reduced brood size, as already shown in Figure 4A, but showed defecation cycles that were almost identical to those of wild-type worms (Table 1). As previously reported, all three alleles of *itr-1(sa73, sy331, sy328)* mutants showed significantly reduced brood sizes, and only one allele of *sa73* showed significantly prolonged defecation cycles (Table 1 and Dal Santo et al., 1999). Interestingly, we found that the two alleles of *itr-1(sy328, sy331)* mutant showed slightly shortened defecation cycles, which could be due to the fact that these alleles are gains of function mutants (Clandinin *et al*., 1998). All double mutants of *crt-1;itr-1(sa73)*, *crt-1;itr-1(sy328)*, and *crt-1;itr-1(sy331)* grew very slowly even at 20°C and produced much smaller number of progeny compared to either *crt-1(jh101)* or *itr-1(sa73, sy328, sy331)* single mutant animals (Table 1). In particular, the double homozygote animal of *crt-1;itr-1(sa73)* exhibited very small numbers of progeny showing high fractions of arrested embryos and much longer defecation cycles or no defecation at all. In summary, *crt-1* genetically interacts with *itr-1* and shows synergistic effects both on brood sizes and defecation cycles. Hence, these data suggest that *crt-1* in association with *itr-1* is important during Ca^{2+} signaling for normal reproduction and defecation cycles.

DISCUSSION

CRT-1 and Ca^{2+} Homeostasis

We obtained a deletion mutant of *crt-1* by PCR-based screening of TMP/UV-mutagenized library. This mutant, *crt-1(jh101)*, produced no CRT-1 protein as evidenced from our Western analysis. To our surprise, mutant animals were viable and displayed no drastic defects other than reduced brood size. The decreased brood size at a higher temperature suggested that the homozygote mutant was sensitive to the elevated temperature and hence was unable to grow normally. Because we showed *crt-1* is expressed in pharyngeal and body-wall muscle cells, it is likely that the mutant homozygote may have impaired function in these tissues in absence of CRT-1. Hence, an argument could be made that the low brood size and slow growth in *crt-1(jh101)* may be attributed to reduced feeding behavior and/or defective muscle function. Reduced brood size together with several

Table 1. Genetic characterization of the *crt-1(jh101)*

Genotype	Brood size	Defecation cycle (s)	n
N2 (WT)	287 ± 6	53.5 ± 2.6	30
crt-1(jh101)	180 ± 23	53.6 ± 2.9	30
itr-1(sa73)	106 ± 13	111 ± 22	10
itr-1(sy328)	121 ± 18	43.7 ± 5.7	12
itr-1(sy331)	193 ± 26	45.7 ± 5.7	13
crt-1;itr-1(sa73)	43 ± 13*	>10 min**	20
crt-1;itr-1(sy328)	41 ± 9	60.0 ± 2.8	10
crt-1;itr-1(sy331)	106 ± 62	60.6 ± 7.4	10

* Low brood size plus embryonic lethality (~30%).

** Longer than 10 min, or no defecation observed.

other phenotypes related to muscle defects were also previously observed in the *unc-68(e540)* (ryanodine receptor) null mutants (Sakube *et al.*, 1997; Maryon *et al.*, 1998). *unc-68* encodes the ryanodine receptor, which is a Ca^{2+} release channel localized at sarcoplasmic reticulum and one of the important components in the excitation-contraction coupling of muscle cells. Recently, we have identified and characterized *C. elegans* calsequestrin, *csq-1*, a Ca^{2+}-sequestering protein in body-wall and pharyngeal muscles, and showed that CSQ-1 is not essential for body-wall muscle functions in *C. elegans* (Cho *et al.*, 2000). Because both CSQ-1 and CRT-1 may play important roles in regulating Ca^{2+} homeostasis as a Ca^{2+} buffer, it is possible that CRT-1 may be dispensable in muscle cells of *C. elegans*. In fact, pharyngeal pumping of *crt-1(jh101)* mutants was comparable to wild-type animals and other muscle functions, including enteric muscle contraction, appeared to be normal (Table 1). Hence, our data on reduced brood size may not be reflected in the feeding defects of *crt-1* knockout worms but rather may be reflected in the defects in fertilization.

CRT-1 and Germ Cell Development

Mating efficiency by the *crt-1* males was also significantly low, which may account for the reduced brood size. Despite normal copulatory behavior, *crt-1* males were much less efficient in producing cross progeny, suggesting that the male-derived sperm were not competent to fertilize the oocytes in vivo. Furthermore, immunohistochemical analyses of *crt-1* sperm and wild-type sperm (Figure 5) strongly suggest that CRT-1 plays a crucial role in sperm development in the nematode. Calreticulin and other chaperone proteins, such as calmegin and calnexin, have also been implicated in sperm development and fertilization in mammals (Nakamura *et al.*, 1993; Nash *et al.*, 1994; Ohsako *et al.*, 1994; Ikawa *et al.*, 1997).

When the morphology of *crt-1* spermatids at early stages of spermatogenesis was compared with that of wild type no difference was observed (Figure 5, A–C). Budding from residual bodies also appeared to be normal, and the morphogenesis and/or partitioning of fibrous body-membranous organelle was unaffected as shown by staining with 1CB4 monoclonal antibody, suggesting that early stage spermatogenesis in *crt-1* mutants progresses normally. However, when spermatids activate into spermatozoa with a single motile pseudopod several defects were noticed. The mutant sperm were generally smaller than wild type, and their pseudopods were slightly shorter. It was also noticed that the nuclei of mutant sperm were often off-center (Figure 5, F and G). Taken together, our data indicate that *crt-1* mutant sperm may have defects in the late stages of spermatogenesis. Similar phenotypes were previously observed in mutants *spe-10(hc104)* and *spe-17(hcDf1),* and these genes encode a protein with a zinc finger motif and a novel protein with unknown function, respectively (Shakes and Ward,1989; L'Hernault *et al.*, 1993; Lindsey and L'Hernault, unpublished data).

The decreased ferility in *crt-1* mutants also appeared to be due to defective oocytes as evidenced by out-cross experiments (Figure 4C). Defective oocytes may have been resulted from abnormal oocyte development or/and deficient somatic gonad in *crt-1* mutants, suggesting CRT-1 may have a role either in somatic gonad or oocyte development. Since our in situ hybridization and immunostaining data show that *crt-1* is expressed predominantly in the intestine of the worms. *vit-2* (vitellogenin) is another Ca^{2+}-binding yolk protein known to be expressed in the intestines and important for oocyte development (MacMorris *et al.*, 1994; Speith *et al.*, 1985; Speith and Blumenthal, 1985). Based on these facts, we reasoned that CRT-1 could function in synthesis and/or endocytosis of the yolk protein in the developing oocytes. Contrary to our prediction, there were no visible differences in the GFP expression patterns of YP170::GFP in the *crt-1* mutant line (*vit-2::gfp;crt-1*) when compared to the DH1033 transgenic line, *vit-2::gfp*, suggesting that receptor mediated endocytosis occurs normally in *crt-1* mutant lines. These data indicate that CRT-1 is not essential in the receptor-mediated endocytosis of oocytes.

CRT-1 Functions as Molecular Chaperone

Chaperone function of mammalian calreticulin for both glycosylated and nonglycosylated proteins has been directly demonstrated in vitro (Saito *et al.*, 1999). Our results also showed *C. elegans* CRT-1 effectively suppresses thermal aggregation of citrate synthase, one of the nonglycosylated proteins previously used for chaperone activity assay. These data confirm that CRT-1 has a conserved function as a molecular chaperone in vitro, and thus we may postulate that CRT-1 functions in vivo in response to thermal stresses such as elevated temperatures. To test the possibility that temperature-sensitive defects seen in *crt-1* mutants could result from the absence of CRT-1 chaperoning function, we examined the transcript level for the *crt-1* gene in wild-type and in deletion mutant *crt-1(jh101)* worms. As shown in Figure 6, A and B, there was a significant increase in message level in response to temperature increase. Additionally, the ethanol treatment experiment, which was independently designed and performed to identify genes responding to ethanol stress, revealed that *crt-1* is one of the genes whose transcripts was elevated significantly (Figure 6C; Kwon and Lee, unpublished observation). These data again suggest that the CRT-1 functioning as a molecular chaperone may be important for *C. elegans* to cope with environmental stresses such as ethanol treatment. Similarly, enhanced expression of calreticulin upon stressed by heat shock and ionizing radiation has been reported in retinal epithelium and human squamous sarcoma cells, respectively (Ramsamooj *et al.*, 1995; Szewczenko-Pawlikowski *et al.*, 1997). Therefore, like in mammals, *C. elegans* CRT-1 may function as a molecular chaperone in response to stress conditions. However, it appears that *crt-1* may be one of the mid- or late-response genes in stress-inducing environments in *C. elegans*. For example, when we performed a microarray analysis with RNAs from ethanol-stressed worms the *crt-1* gene was one of the genes induced only after several hours of treatment (Kwon and Lee, unpublished observation). In this respect, it is also intriguing that the reproduction defects seen at elevated temperatures were somewhat progressive with generations of worms continuously grown at elevated temperatures. This suggests that accumulated damages from stressful environments may slowly affect normal growth of *C. elegans* in the absence of CRT-1 function. The rescue experiment, which was conducted with *crt-1(jh101)* mutant worms grown at 20°C due to technical difficulties and then subsequently maintained at 25°C, did show a significant recovery of the defects (Figure 4A). However, the recovery was not complete and there are several possible reasons for the incomplete rescue. First, we selected and examined progeny of the F1 or F2 transgenic animals from the injection

(n = 30). Because they are not stable integrated lines there may be mosaic expression of the introduced *crt-1* gene, which could not fully rescue CRT-1 function in every tissue, including germ cells. Second, the standard concentration of *crt-1* DNA for microinjection was somehow toxic even to the wild-type worms, suggesting that there might be a tight regulation of endogenous gene expression of calreticulin in *C. elegans*. Therefore, the reintroduced *crt-1* gene could not be faithfully expressed to rescue the defects. Nevertheless, introduction of *crt-1* gene substantially rescued reproduction defects of mutants, indicating that phenotypes characterized in this study are indeed resulted from the deleted *crt-1* gene.

CRT-1 and Inositol Triphosphate (IP_3) Receptor in C. elegans

Inositol triphosphate (IP_3) receptors are Ca^{2+} release channels localized to the ER and allow Ca^{2+} release from the intracellular stores into the cytoplasm (Berridge,1993; 1997). The IP_3 receptor in *C. elegans* (*itr-1)* is encoded by *dec-4* (defecation cycle period abnormal) (Dal Santo *et al*., 1999), and, like *crt-1*, is shown to be expressed predominantly in the intestine. Mutations in the *itr-1* gene, *lfe/itr-1* (*sy328, sy290, sy331*), *itr-1(sa73)* and *itr-1(cj5)*, show significantly reduced brood size, abnormal behavioral rhythms during defecation, and defects in ventral enclosure during embryogenesis, respectively (Clandinin *et al*., 1998; Dal Santo *et al*., 1999; Thomas *et al*., personal communication). In order to test genetic interaction between *crt-1* and *itr-1*, we have generated double mutants using two alleles of gain of function mutants *lfe-1/itr-1(sy328, sy331*) (Clandinin *et al*., 1998) and one allele of loss of function mutant *itr-1(sa73)* mutants (Dal Santo *et al*., 1999). As shown in Table 1, all the double mutants exhibited drastically reduced brood sizes, suggesting that *crt-1*, in association with *itr-1*, is important for fertility in *C. elegans*. It has been shown that *itr-1* is involved in ovulation (Clandinin *et al*., 1998), which may require coordinated contraction triggered by calcium release. Therefore, the reduction of fertility in *crt-1* mutant could be explained in part by defects in oocyte development, including ovulation and/or gonadal sheath.

For defecation cycles, we found two alleles of *lfe-1/itr-1* mutants show slightly shortened cycles compared to wild type. It is conceivable that gain of function mutation in IP_3 receptor could accelerate defecation rhythm. In fact, both of these mutants have been mapped to IP_3 binding sites and have been characterized as gain of function alleles (Clandinin *et al*., 1998). The other allele, *sa73*, which has been mapped to the central domain of IP_3 receptor, showed prolonged defecation cycle as shown previously (Dal Santo *et al*., 1999). The *crt-1* mutant alone exhibits normal defecation cycles but significantly prolonged cycles in association with all three alleles of *itr-1* mutants (Table 1). In particular, *sa73* allele in double with *crt-1*, showed the most drastic additive effect on defecation cycle. The *crt-1;itr-1(sa73)* double mutants were able to lay fertilized embryos, but very high fractions (up to 30%) of embryonic lethality were observed. Nevertheless, the double homozygote animal itself was viable and exhibited much longer defecation cycles (longer than 10 min, n> 20) or no defecation at all. Taken together, we suggest that *crt-1* in association with *itr-1* is important during Ca^{2+} signaling for normal defecation cycles and fertility in *C. elegans*.

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