RESEARCH ARTICLE

Alternative chaperone machinery may compensate for calreticulin/calnexin deficiency in *Caenorhabditis elegans*

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Received: May 12, 2005
Revised: September 21, 2005
Accepted: September 21, 2005

Proper folding and maintenance of the native structure are central to protein function and are assisted by a family of proteins called chaperones. Calreticulin and calnexin are ER resident chaperones well conserved from worm to human. Calreticulin/calnexin knock-out mice exhibit a severe phenotype, whereas in *Caenorhabditis elegans*, calreticulin [*crt-1(jh101)*]- and calnexin [*cnx-1(nr2009)*]-null mutant worms exhibit only a mild phenotype, suggesting the possible existence of alternative chaperone machinery that can compensate for the deficiency of calreticulin and/or calnexin. In order to rapidly identify the compensatory chaperone components involved in this process, we analyzed the proteome of *crt-1*(*jh101*) mutants and [*crt-1(jh101);cnx-1(nr2009)*] double mutants. When grown at 20°C, we found that five proteins were up-regulated and two proteins were down-regulated in *crt-1(jh101)* mutants; nine proteins were up-regulated and five proteins were down-regulated in [*crt-1(jh101);cnx-1(nr2009)*] double mutants. In addition, elevation of the cultivation temperature to 25°C, which is still permissive to growth but causes specific defects in mutants, led to the identification of several additional proteins. Interestingly, the consistent increment of heat shock protein-70 family members (hsp70) together with protein disulfide isomerase (PDI) at all the examined conditions suggests the possible compensatory function imparted by hsp70 and PDI family members in the absence of calreticulin and/or calnexin.

Keywords:
Caenorhabditis elegans / Calnexin / Calreticulin / Chaperone

1 Introduction

Protein folding and assembly are important post-translational steps that ensure the formation of biologically active proteins. These processes *in vivo* are assisted by a number of ER molecular chaperones such as calreticulin (CRT), calnexin (CNX), immunoglobulin-binding protein (Bip/GRP78), ER resident protein (ERp72), and glucose-regulated protein (GRP94) [1, 2].

Calreticulin is a major Ca^{2+}-binding molecular chaperone found in a number of diverse species [3]. Calreticulin participates in the folding and maturation of a large number of newly synthesized proteins and glycoproteins [3, 4]. The nematode *Caenorhabditis elegans* has single calreticulin, which is 58% identical and 71% similar to its human counterpart at the amino acid level. Calreticulin knock-out mice show embryonic lethality along with heart development defects, indicating that calreticulin is essential for embryogenesis in

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Abbreviations: CNX, calnexin; **CRT,** calreticulin; **HSP,** heat shock protein; **PDI,** protein disulfide isomerase; **UPR,** unfolded protein response

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mammals [5, 6]. In marked contrast, *C. elegans* calreticulin [*crt-1(jh101)*]-null mutants are viable and showed only a mild phenotypic defect: temperature-dependent reproduction defect [7]. Calreticulin is functionally coupled with another ER chaperone molecule, calnexin, which is a Ca^{2+}-binding transmembrane protein. Calreticulin and calnexin establish the so-called "calreticulin/calnexin cycle" [4]. Both bind with nascent polypeptide or glycoprotein and assist in proper folding. The physical association of calreticulin/calnexin with another folding enzyme, ERp57, which is a thiol oxidoreductase, enables the simultaneous exertion of disulfide bond formation while folding. Calnexin knock-out mice survived only up to 4 wk after birth [8]. However, *C. elegans* calnexin mutants, like calreticulin mutants, are viable and showed only a mild phenotypic defect (Lee *et al.*, unpublished results). Besides calreticulin and calnexin, other chaperones have been found to act in the ER. For example, immunoglobulin-binding protein Bip/GRP78 and GRP94 assist in the folding process; protein disulfide isomerase (PDI) catalyzes disulfide bond formation and isomerization. *C. elegans* homologues of Bip/GRP78, GRP94, PDI and ERp57 have been reported, which are *hsp-3/4*, endoplasmin, *pdi-2* and *pdi-3,* respectively. The existence of conserved chaperone components and the availability of viable calreticulin and calnexin mutants provide an exciting opportunity to study the chaperone system in *C. elegans*. The latter property is particularly noteworthy because *C. elegans* is the only multicellular organism identified to date in which the knock-out of either calreticulin (*crt-1*) or calnexin (*cnx-1*) or a combination of both is viable. Since the *crt-1(jh101)* and *cnx-1(nr2009)* mutants showed only a mild phenotype, we hypothesized that perhaps the other chaperone system could potentially be up-regulated to compensate for calreticulin/calnexin deficiency. To identify the other chaperone system(s) rapidly, we performed comparative proteome analysis of differentially expressed proteins in the *crt-1(jh101)* and [*crt-1(jh101);cnx-1(nr2009)*] double mutant worms at two different temperatures. We identified a number of differentially expressed proteins by comparative proteome analysis of mutant *C. elegans*. Among them, we confirmed the expression levels of HSP-3 (Bip/GRP78) and PDI-2 mRNA. Calreticulin-null mutants showed increased expression of HSP-3 and PDI-2. Our results describing the relationship between the calreticulin/calnexin chaperone system and the Bip chaperone system indicate that the latter system may compensate for the deficiency of the calreticulin/calnexin chaperone system in *C. elegans*.

2 Materials and methods

2.1 Nematode culture

C. elegans strains (wild-type N2, *crt-1(jh101)*, *cnx-1(nr2009)*, [*crt-1(jh101);cnx-1(nr2009)*]) were grown at 20° and 25°C in S medium (50 mM phosphate buffer, pH 6.0, 0.1 M NaCl, 10 mM potassium citrate, 3 mM $CaCl_2$, 3 mM $MgSO_4$, 0.05 mM EDTA, 0.025 mM $FeSO_4$, 0.01 mM $MnCl_2$, 0.01 mM $ZnSO_4$, and 0.001 mM $CuSO_4$) with 5 μg/mL β-sitosterol (Steraloids, Newport, RI, USA) using the OP50 strain of *Escherichia coli* as a food source. The worms were grown for 8 days, during which they were supplied with both 25-azacoprostane hydrochloride and β-sitosterol at a concentration of 5 μg/mL. Following this period, worms were harvested by centrifugation and separated from remaining bacteria by flotation on 35% sucrose. After washing with M9 buffer (44 mM KH_2PO_4, 21 mM Na_2HPO_4, 86 mM NaCl, pH 7.0, and 1 mM $MgSO_4$), mixed stages of worms were stored at −70°C until use.

2.2 Sample preparation for 2-DE

Worms were washed with distilled water and suspended with worm lysis buffer containing 1% DTT, 0.5% carrier ampholyte, 5 mM EDTA, 7 M urea, 2 M thiourea, 4% CHAPS, and protease inhibitor. Suspensions were sonicated for about 30 s on ice, and the soluble fractions were collected by centrifugation at 36 000 × *g* for 40 min at 4°C. The protein concentration of the soluble fraction was determined by the Bradford method using BSA as a standard. Aliquots were stored at −70°C until use.

2.3 2-D PAGE

Protein samples (500 μg) were applied onto an IPG strip (Immobiline Dry Strip, pH 3–10 nonlinear, 18 cm; Amersham Biosciences) by in-gel redhydration using an IPGphore unit under constant voltage (10 V) for 14 h. IEF was performed at 500 V for 1 h, followed by 1000 V for 1 h, and the voltage was gradually increased to 8000 V for 1 h. After IEF for another 4 h, each strip was incubated for 15 min in 50 mM Tris, pH 8.8, 6 M urea, 20% glucose, 2% SDS, and 1% DTT followed by an additional incubation for 15 min in the same solution containing 2.5% iodoacetamide instead of DTT. The equilibrated strip was placed onto a 9∼16% gradient polyacrylamide gel, and the gel was electrophoresed at 120 V in running buffer (25 mM Tris (pH 8.8), 192 mM glycine, 0.1% SDS). After fixing the proteins in 40% methanol and 5% phosphoric acid for at least 1 h, the gel was stained with CBB R-250 overnight. After destaining with 10% methanol and 10% acetic acid, the gel image was obtained using a GS-710 densitometer (Bio-Rad, CA, USA). The digitalized gel images were normalized and comparatively analyzed using the PDQuest program (V. 6.1).

2.4 Protein identification

Proteins were identified by PMF as described by Jensen *et al.* [9] with minor modifications. The selected protein spots were excised from gels, and washed with deionized water and ACN. Then, proteins were reduced with 10 mM DTT in 0.1 M NH_4HCO_3 at 56°C for 1 h, and alkylated with 55 mM IAA at room temperature for 30 min. The gel pieces were dried and soaked in 100 ng of sequencing grade trypsin on ice for 1 h.

After the removal of residual trypsin, the gel was incubated in 50 mM NH_4HCO_3 containing 5 mM $CaCl_2$ at 37°C for 12 h. Digested peptides were extracted in 0.1% TFA and 60% ACN three times, and the pooled samples were dried using a Speed-Vac. Recovered peptides were mixed with 0.5% TFA and diluted with CHCA. The sample (1 μL) was loaded on a plate and dried for crystallization. Masses of peptides were determined using a Voyager-DE STR MALDI-TOF mass spectrometer (PE Biosystems, Framingham, MA, USA). Calibration was performed using the internal mass of trypsin. Database searching was carried out with Peptident (www.expasy.ch/tools/peptident.html), MS-Fit (prosector.ucsf.edu/ ucsfhtml3.4/msfit.htm), or ProFound (prowl.rockerfeller.edu/cgi-bin/ ProFound).

2.5 SDS-PAGE and immunoblot analysis

Protein samples were incubated in SDS sample buffer (60 mM Tris, pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.1% bromophenol blue) at 95°C for 5 min. Proteins were separated using SDS-PAGE and transferred onto an NC membrane using a Bio-Rad wet transfer unit. The blots were then blocked with 5% w/v nonfat dry milk in TBS-T solution (25 mM Tris, pH 7.5, 150 mM NaCl, 0.05% w/v Tween 20). After washing in TBS-T, the blots were incubated for 1 h with polyclonal anti-CRT, anti-CNX, or anti-GRP78/Bip antibody at a 1:1000 dilution, followed by horseradish peroxidase conjugated anti-rabbit secondary antibodies. Immunoreactive bands were detected by ECL reagents (Pierce).

2.6 RT-PCR

Total RNA was extracted from 0.2 g of frozen worms using TRI reagent (Molecular Research Center) after crushing with a mortar and pestle in liquid nitrogen. The RNAs were treated with DNase I (TAKARA) and extracted with phenol/chloroform. Absence of genomic DNA was confirmed by PCR with a sample of total RNA using the primers described below. The first strand of cDNA was synthesized using 50 ng of total RNA, Moloney murine leukemia virus reverse transcriptase (Invitrogen), 1 mM dNTPs, 0.5 μg of oligo(dT) primers (Invitrogen), and 20 U RNasin (Promega) in a 20-μL volume. The mixture was incubated at 37°C for 1 h, and the reactions were terminated by heating at 95°C for 5 min. PCR was performed using 5 ng of the cDNA on a DNA thermal cycler (PerkinElmer, Boston, MA, USA). To ensure the reliability of mRNA extraction and reverse transcription, all samples were assayed for the constitutively expressed gene actin-1 (*act-1*). RT-PCR was performed using gene-specific primers: *act-1* (forward, 5'-gctatgttccagccatccttcttg-3'; reverse, 5'-tagaagcacttgcggtgaacgatg-3'), *hsp-3* (forward, 5'-tggccgtaacactgttatcccaac-3'; reverse, 5'-accttcttgtcatcctcagcgaac-3'), *pdi-2* (forward, 5'-tcttgaactcttgctcgagcttgg-3'; reverse, 5'-ctcggtggtgatctcttcgaagtc-3'). After RT-PCR, 10-μL aliquots of the products were subjected to 2% agarose gel electrophoresis and stained with ethidium bromide.

3 Results

3.1 Calreticulin and calnexin do not compensate each other by elevating the protein level

Knock-out of either calreticulin or calnexin renders severe phenotypes in mice. To our surprise, neither calreticulin-null mutants nor calnexin-null mutants exhibited any severe phenotype in *C. elegans*. Based on this observation, and the fact that calreticulin and calnexin have been shown to have similar molecular function [10, 11], we tested the hypothesis that perhaps in the absence of one of these proteins the level of the other protein would be elevated in compensation in *C. elegans*. We tested this possibility by analyzing the level of each protein in the genetic background where the other protein is completely absent. However, as shown in Fig. 1, there is no significant change in the level of calreticulin in calnexin mutants and *vice versa*, suggesting that calreticulin and calnexin might not compensate each other by elevating the protein level.

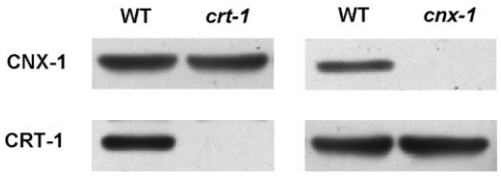

Figure 1. Expression of calreticulin (CRT-1) and calnexin (CNX-1) in the calreticulin mutant [*crt-1(jh101)*] or calnexin mutant [*cnx-1(nr2009)*]. Western blot shows that each mutant is a molecular null (no protein was detected) and the level of CRT-1 is the same in wild-type and *cnx-1(nr2009)* worms. Similarly, the level of CNX-1 is the same in wild-type and *crt-1(jh101)* worms.

3.2 Identification of differentially expressed proteins in *crt-1(jh101)* and [*crt-1(jh101);cnx-1(nr2009)*] mutants grown at 20°C

To systematically determine the molecular mechanisms associated with the calreticulin/calnexin cycle in the ER, we attempted to identify and characterize the proteins differentially expressed in the *crt-1(jh101)*-null mutant worms grown at conventional cultivation temperature (20°C) by 2-DE. Proteins were extracted from each strain with urea lysis solution and separated by 2-D PAGE (see Section 2.3). Approximately 350 protein spots were visualized with CBB R-250. Comparison of these relatively abundant proteins on 2-D gels using PDQuest revealed that seven spots were reproducibly changed, with their expression levels altered more than twofold in six independent experiments. These proteins were identified by PMF using MALDI-TOF MS and database searching. We found that seven proteins were differentially expressed in *crt-1(jh101)* mutants when grown at 20°C compared to the wild-type worms (Table 1 and Fig. 2). Among them, five proteins

Table 1. List of differentially expressed proteins in *crt-1*-deletion mutants compared to wild-type worms at 20°C

Spot no.	Accession no.	M_r (kDa)	p*I*	Protein name	Accuracy	Sequence coverage	Expression level
11	Q17813	61.1	6.3	UDP-glucuronyl and UDP-glucosyl transferase	50 ppm	7%	decreased
12	Q01291	30.7	5.5	40S ribosomal protein S0 (RPS-0)	20 ppm	17%	decreased
13	P27420	73	5.1	Heat shock protein 70 (HSP-3)	20 ppm	19%	increased
14	Q81G53	49	4.76	Protein disulfide isomerase (PDI-2)	20 ppm	23%	increased
15	O61396	64.1	8.66	*N*-acetylgalactosaminyl transferase 6 (GLY-6)	40 ppm	5%	increased
16	P27604	48	5.85	*S*-adenosylhomocysteine hydrolase	10 ppm	17%	increased
17	O62107	38	5.83	UDP-glucose 4-epimerase	10 ppm	22%	increased

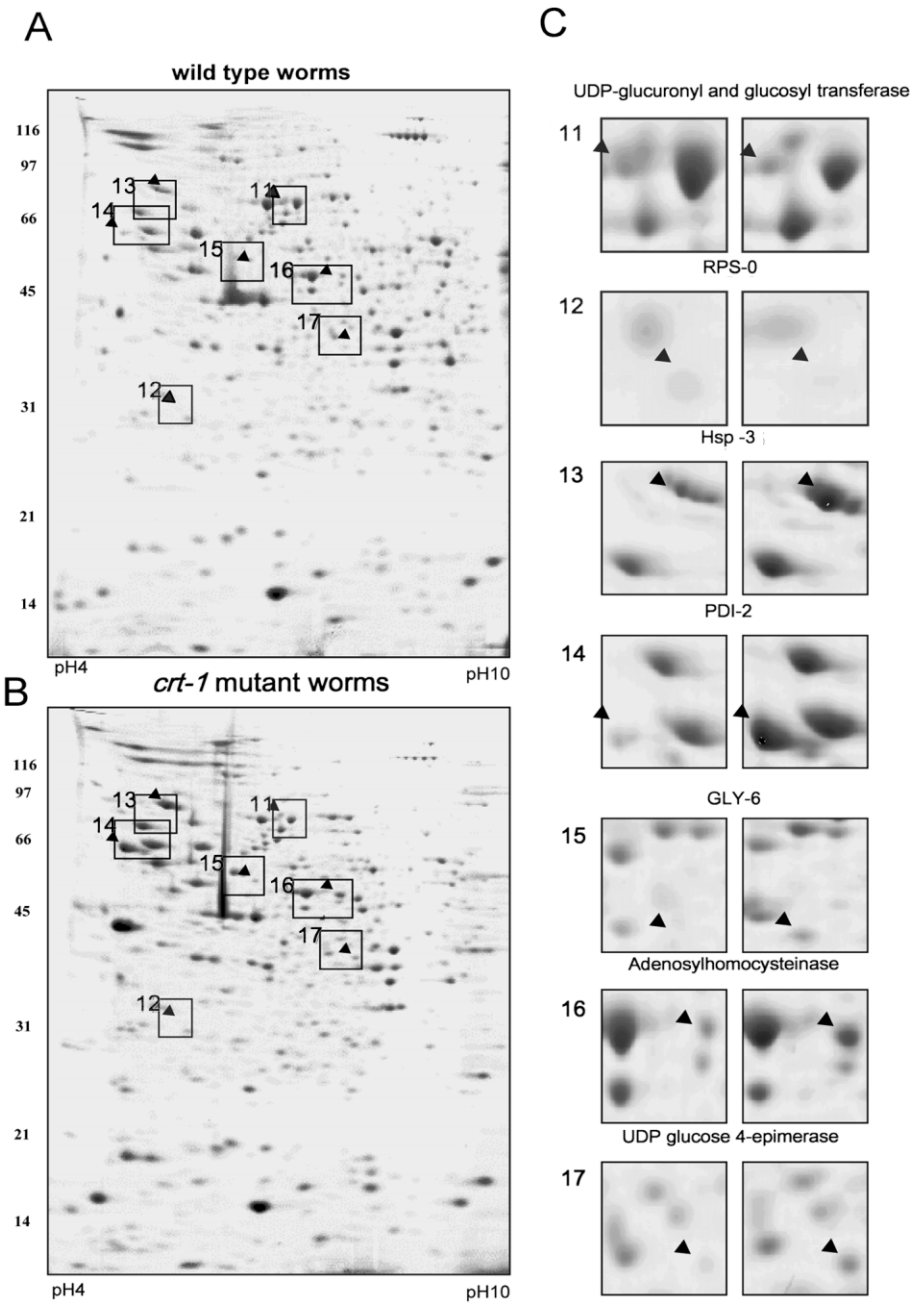

Figure 2. Comparison of 2-DE gel maps of proteins isolated from either wild-type (WT) or calreticulin-null mutant worms grown at 20°C. Total proteins extracted from wild-type (A) or calreticulin-deletion mutant worms (B) were separated by 2-D PAGE and stained with silver. Each protein spot was numbered for subsequent MS analysis. (C) The regions where protein spots changed in abundance were enlarged to show differential expression of each protein spot. The numbers indicate the corresponding regions in 2-D gels.

were increased in *crt-1(jh101)*-deletion mutants and two proteins were decreased more than twofold. The proteins found to be increased were heat shock protein Bip/GRP78 (HSP-3), PDI-2, glycosyl transferase, adenosylhomocysteinase, and UDP-glucose 4-epimerase. The two proteins found to be decreased in *crt-1(jh101)*-deletion mutants were UDP-glucuronosyltransferase family member and 40S ribosomal protein S0 (RPS-0).

Also, we identified 14 differentially expressed protein spots in wild-type and [*crt-1(jh101);cnx-1(nr2009)*] double mutant worms grown at 20°C by 2-D PAGE (Table 2 and Fig. 3). Five proteins that were down-regulated in [*crt-1(jh101);cnx-1(nr2009)*] double mutants were outer membrane protein F (OMP-F), alcohol dehydrogenase, membrane-associated acyl-coA-binding protein (MAA-1), and troponin T (TNT-2). Nine proteins that were up-regulated were heat shock protein Bip/GRP78 (HSP-3), heat shock protein Bip/GRP78 (HSP-4), vacuolar H^+-ATPase, PDI-2, vitellogenin (VIT-2), *S*-adenosylhomocysteine dehydrolase, glucose 4-epimerase, actin (ACT-4), and oxygen evolving enhancer protein.

3.3 Identification of differentially expressed proteins in *crt-1(jh101)* and [*crt-1(jh101);cnx-1(nr2009)*] mutants grown at 25°C

Since *crt-1(jh101)* and *cnx-1(nr2009)* mutants showed retarded growth and a pronounced decrease in brood size at 25°C, the temperature which is still permissible for the wild-type worm, we were interested in investigating the effect of temperature change on the alteration of protein profiles in the mutants. Comparison of the relative abundance of protein spots in each pair (wild-type and mutants) of gels revealed that many proteins were differentially expressed. These protein spots were identified using MALDI-TOF MS. We found that nine proteins were differentially expressed in wild-type and *crt-1*-deletion mutant worms when grown at 25°C (Table 3 and Fig. 4). Up-regulated proteins in calreticulin-deletion mutant worms were HSP-4 (heat shock protein Bip/GRP78), PDI-3, UDP-glucose 4-epimerase, and LBP-2 (lipid-binding protein); down-regulated proteins in calreticulin-deletion mutant worms were DNJ-20, OMP-F, ACT-4, hypothetical protein, and DHS-22 (dehydrogenase). Twenty-one protein spots were differentially expressed in [*crt-1(jh101);cnx-1(nr2009)*] double mutant worms grown at 25°C (Table 4 and Fig. 5). Up-regulated proteins were HSP-3 (heat shock protein Bip/GRP78), PDI-2, INT-1 (initiation factor 4A), VIT-2 (vitellogenin), *S*-adenosylhomocysteine hydrolase, UDP-glucose 4-epimerase, and LEC-1 (galectin). Down-regulated proteins in [*crt-1(jh101);cnx-1(nr2009)*] double mutant worms were ACT-4, acyl co-A dehydrogenase, GPD-2 (glyceraldehydes 3-phosphate dehydrogenase), DNJ-20, EFF-2 (elongation factor-2), a hypothetical protein, and DHS-22 (dehydrogenase).

3.4 Transcriptional up-regulation of *hsp-3* and *pdi-2* in *crt-1(jh101)* and [*crt-1(jh101);cnx-1(nr2009)*]

Our comparative proteome analysis indicates that the expression of HSP-3 and PDI-2 protein increases in the absence of CRT-1/CNX-1 in *C. elegans*. The observed increment in HSP-3 and PDI-2 levels could be either due to transcriptional up-regulation or due to post-transcriptional events. In order to distinguish between these two possibilities, we performed RT-PCR with two mutants grown at different temperatures using two gene-specific (*hsp-3* and *pdi-2*) primer sets. As shown in Fig. 6A, a distinctly increased expression of *hsp-3* and *pdi-2* mRNA was observed in both mutants. Expression of the *hsp-3* and *pdi-2* genes was normalized to that of *act-1*. Furthermore, the HSP-3 protein level was analyzed by Western blot using a polyclonal antibody

Table 2. List of differentially expressed proteins in *crt-1;cnx-1* double mutants compared to wild-type worms at 20°C

Spot no.	Accession no.	M_r (kDa)	p*I*	Protein name	Accuracy	Sequence coverage	Expression level
51	P02931	36.4	4.69	Outer membrane binding proteins F (OMP-F)	20 ppm	24%	decreased
52	Q17334	37.7	6.1	Alcohol dehydrogenase	30 ppm	24%	decreased
53	Q9XVX9	31	7.0	Membrane-associated-acyl CoA binding protein (MAA-1)	50 ppm	12%	decreased
54	Q9XTZ5	37.2	8.95	Dehydrogenase (DHS-22)	50 ppm	10%	decreased
55	Q72071	53.8	5.0	Troponin T (TNT-2)	20 ppm	9%	decreased
56	P27420	73	5.1	Heat shock protein 70 (HSP-3)	20 ppm	19%	increased
57	P20163	72.2	5.03	Heat shock protein 70 (HSP-4)	10 ppm	15%	increased
58	Q9XW92	66.5	5.1	Vacuolar H+-ATPase (VHA-13)	50 ppm	44%	increased
59	Q8IG53	49	4.76	Protein disulfide isomerase (PDI-2)	20 ppm	23%	increased
510	P05690	188	6.42	Vitellogenin 2 precursor (VIT-2)	50 ppm	5%	increased
511	P27604¡¡	48	5.85	*S*-adenosylhomocysteine hydrolase	10 ppm	17%	increased
512	O62107	38	5.83	UDP-glucose 4-epimerase	10 ppm	22%	increased
513	P10986	37	5.4	Actin (ACT-4)	30 ppm	16%	increased
514	O62277	24.4	5.7	Oxygen-evolving enhancer protein	20 ppm	46%	increased

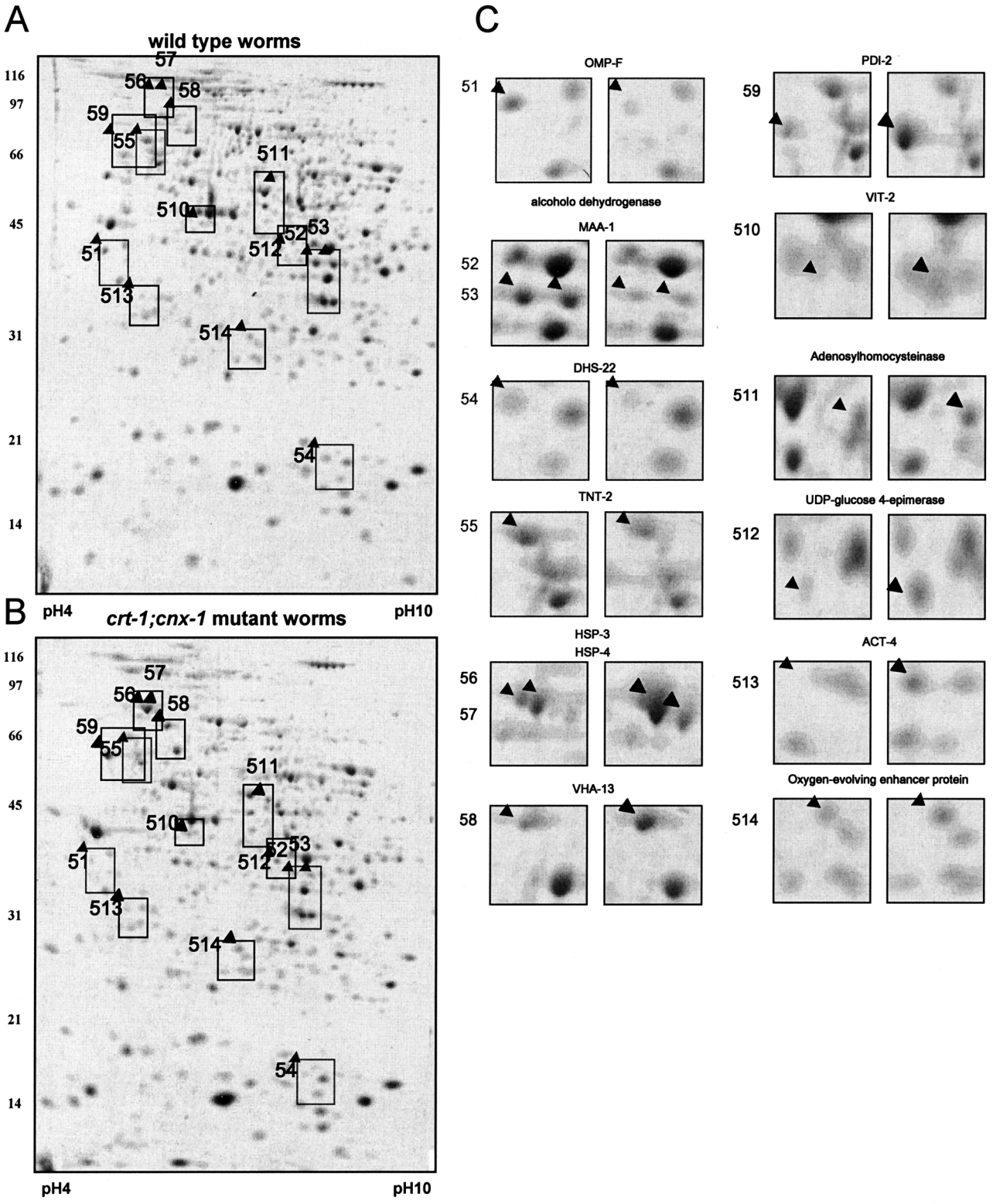

Figure 3. Comparison of 2-DE gel maps of proteins isolated from either wild-type (A) or calreticulin/calnexin double mutant [*crt-1(jh101);cnx-1(nr2009)*] worms grown at 20°C. Total proteins extracted from wild-type or calreticulin/calnexin double mutants were separated by 2-D PAGE and stained with silver. Each protein spot was numbered for subsequent MS analysis. (C) The regions where protein spots changed in abundance were enlarged to show differential expression of each protein spot. The numbers indicate the corresponding regions in 2-D gels.

Table 3. List of differentially expressed proteins in *crt-1*-deletion mutants compared to wild type-worms at 25°C

Spot no.	Accession no.	M_r (kDa)	p*I*	Protein name	Accuracy	Sequence coverage	Expression level
21	Q8MPX3	55.9	6.12	DNaJ domain (DNJ-20)	50 ppm	6%	decreased
22	P02931	36.4	4.69	Outer membrane protein F (OMP-F)	20 ppm	24%	decreased
23	P10986	41.7	5.3	ACTIN (ACT-4)	50 ppm	30%	decreased
24	O44604	17.4	6.0	Hypothetical protein	50 ppm	41%	decreased
25	Q9XVS9	37.2	8.95	Dehydrogenase (DHS-22)	50 ppm	10%	decreased
26	P20163	72.2	5.03	Heat shock protein 70 (HSP-4)	10 ppm	15%	increased
27	O17908	54.9	6.0	ERp57 precursor (PDI-3)	10 ppm	23%	increased
28	O62107	38	5.83	UDP-glucose 4-epimerase	10 ppm	22%	increased
29	Q20224	18.8	6.22	Lipid binding protein (LBP-2)	10 ppm	49%	increased

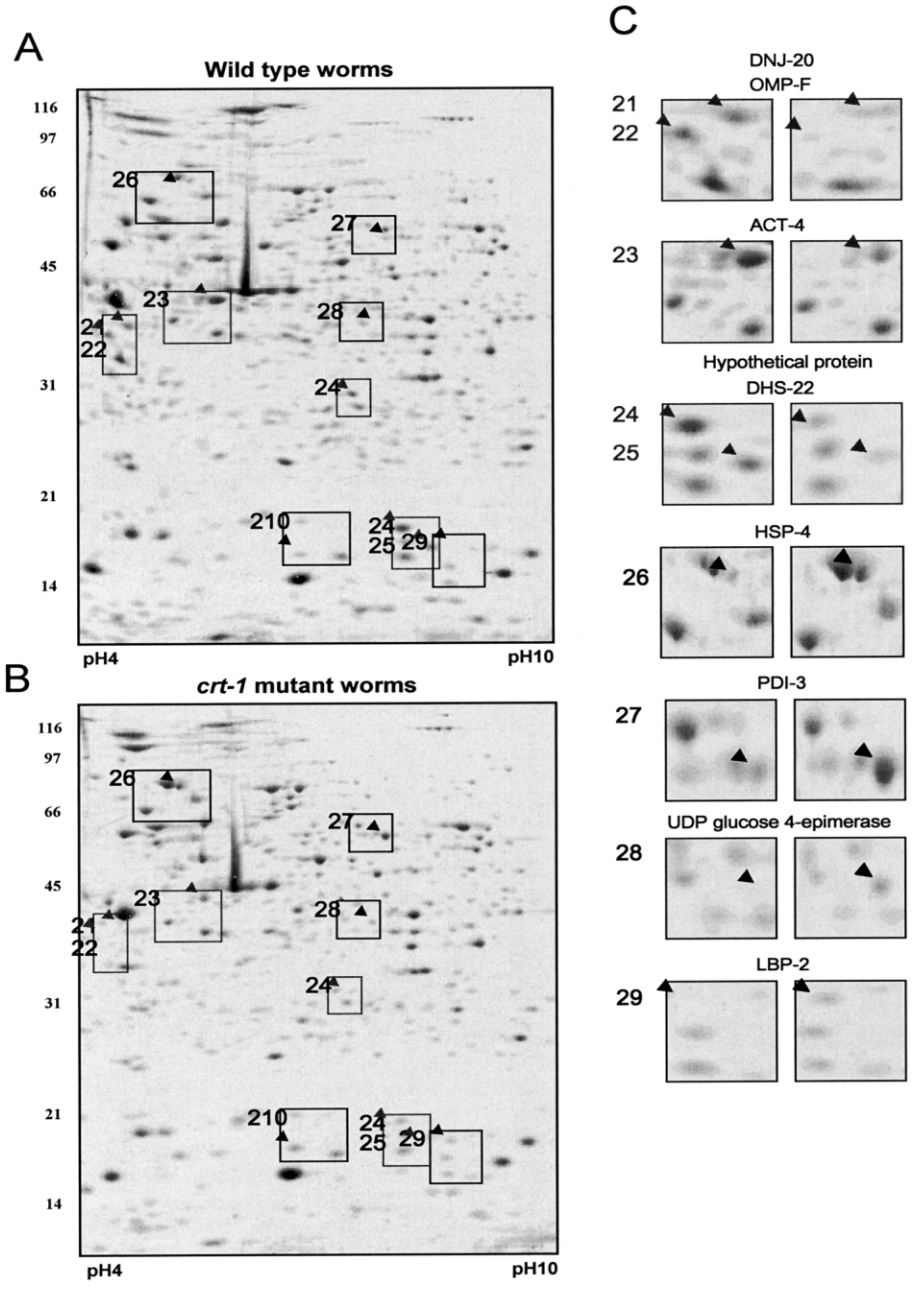

Figure 4. Comparison of 2-DE gel maps of proteins isolated from either wild-type (A) or calreticulin null mutants [*crt-1(jh101)*] (B) grown at 25°C. Total proteins extracted from wild-type and calreticulin-deletion mutants were separated by 2-D PAGE and stained with silver. Each protein spot was numbered for subsequent MS analysis. (C) The regions where protein spots changed in abundance were enlarged to show differential expression of each protein spot. The numbers indicate the corresponding regions in 2-D gels.

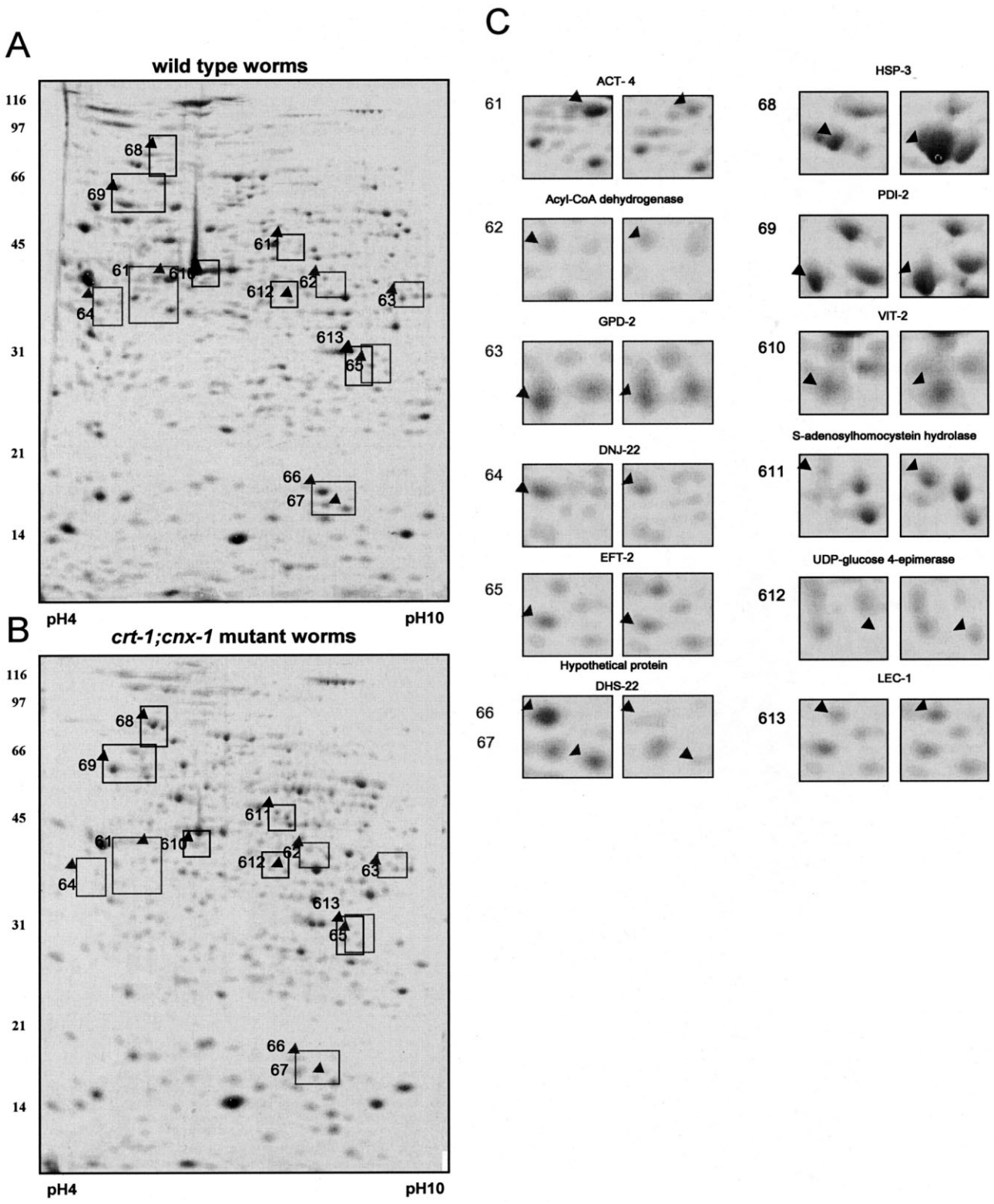

Figure 5. Comparison of 2-DE gel maps of proteins isolated from either wild-type (A) or calreticulin/calnexin double mutant [*crt-1(jh101);cnx-1(nr2009)*] (B) worms grown at 25°C. Total proteins extracted from wild-type (A) and calreticulin/calnexin double mutants (B) were separated by 2-D PAGE and stained with silver. Each protein spot was numbered for subsequent MS analysis. (C) The regions where protein spots changed in abundance were enlarged to show differential expression of each protein spot. The numbers indicate the corresponding regions in 2-D gels.

Table 4. List of differentially expressed proteins in *crt-1;cnx-1* double mutants compared to wild-type worms at 25°C

Spot no.	Accession no.	M_r (kDa)	p*I*	Protein name	Accuracy	Sequence coverage	Expression level
61	P10986	41.7	5.3	ACTIN 4 (ACT-4)	50 ppm	30%	decreased
62	Q966M3	47	6.4	Acyl-CoA dehydrogenase (ACDH-1)	30 ppm	13%	decreased
63	P17329¡¡	36.5	6.5	Glyceraldehyde 3-phosphate dehydrogenase 2 (GPD-2)	20 ppm	14%	decreased
64	Q8MPX3	55.9	6.12	DNaJ domain (DNJ-20)	50 ppm	6%	decreased
65	P29691	94.6	6.1	Elongation factor 2 (EFT-2)	50 ppm	8%	decreased
66	O44604	17.4	6.0	Hypothetical protein	50 ppm	41%	decreased
67	Q9XVS9	37.2	8.95	Dehydrogenase (DHS-22)	50 ppm	10%	decreased
68	P27420	73	5.1	Heat shock protein 70 (HSP-3)	20 ppm	19%	increased
69	Q8IG53	49	4.76	Protein disulfide isomerase (PDI-2)	20 ppm	23%	increased
610	P05690	188	6.42	Vitellogenin 2 precursor (VIT-2)	50 ppm	5%	increased
611	P27604¡¡	48	5.85	*S*-adenosylhomocysteine hydrolase	10 ppm	17%	increased
612	O62107	38	5.83	UDP-glucose 4-epimerase	10 ppm	22%	increased
613	O45904	32.7	6.4	Galectin (LEC-1)	20 ppm	37%	increased

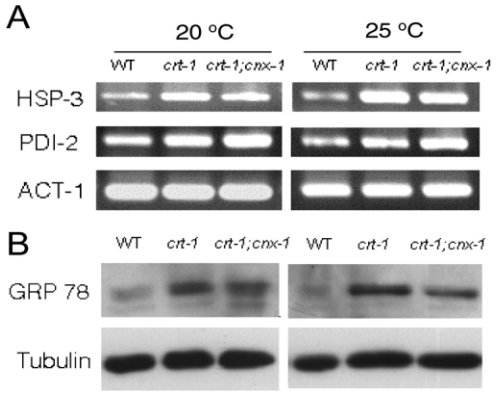

Figure 6. (A) RT-PCR analysis of *hsp-3* and *pdi-2* genes identified from 2-DE gels and Western blot analysis of HSP-3 protein. Both (A) the transcriptional level of *hsp-3* and *pdi-2* and the (B) protein level of HSP-3 were increased in *crt-1(jh101)* and [*crt-1(jh101);cnx-1(nr2009)*] mutant worms grown at indicated temperatures (20°C and 25°C).

raised against Bip/GRP78. Expression of HSP-3 protein was normalized to that of tubulin. As shown in Fig. 6B, enhanced expression of HSP-3 protein was observed in *crt-1(jh101)* and [*crt-1(jh101);cnx-1(nr2009)*] mutants grown at different temperatures relative to wild-type worms. Taken together, these results show that *hsp-3* and *pdi-2* are transcriptionally up-regulated and the protein level of HSP-3 is also up-regulated.

4 Discussion

4.1 Calreticulin and calnexin cannot compensate each other by elevating the protein level in *C. elegans*

During folding, proteins with N-linked oligosaccharides interact transiently and specifically with two homologous lectin-like ER chaperones, calreticulin and calnexin. Both proteins are chaperones that share several functions, including Ca^{2+} binding, lectin-like activity, and recognition of misfolded proteins. However, a major distinction between these two proteins is that calreticulin is a luminal protein, which is able to travel freely within the ER lumen, whereas calnexin is a transmembrane protein [12]. Some proteins serve as a substrate for both calreticulin and calnexin, whereas other proteins are specifically recognized by either calreticulin or calnexin, suggesting overlapping as well as non-overlapping substrate specificity between calreticulin and calnexin [13–18]. Our previous study showed that *crt-1(jh101)* and *cnx-1(nr2009)* mutants displayed only a mild phenotype and [*crt-1(jh101);cnx-1(nr2009)*] double mutants showed an additive but not a synergistic phenotype (Lee *et al.*, unpublished results). As shown in Fig. 1, there is no significant change in calreticulin/calnexin expression in the absence of the other molecule. Taken together, our results indicate that calreticulin and calnexin might not compensate each other by elevating the protein level, which further suggests that in the absence of calreticulin/calnexin, *C. elegans* may have other chaperones to cope with the deficiency of calreticulin/calnexin. Our observation is consistent with a similar study carried out in mammalian cells [19].

4.2 Functions of Bip and PDI may compensate for calreticulin/calnexin deficiency in *C. elegans*

In the absence of calreticulin/calnexin, *C. elegans* was viable and showed only mild phenotypes such as smaller brood size and slower growth rate compared to wild-type worms [7]. This result suggested that *C. elegans* may have other chaperone systems to cope with the deficiency of calreticulin/calnexin chaperone machinery. Comparative proteome analysis of *crt-1(jh101)* mutants and [*crt-1(jh101);cnx-1(nr2009)*] double mutants revealed that Bip/GRP78 homologues (HSP-3/HSP-4) and PDI family members (PDI-2/PDI-3) increased more than twofold, suggesting the possibility that Bip ho-

mologues and PDI family members may compensate for the lack of function(s) of the calreticulin/calnexin chaperone system in *C. elegans*. Indeed, it has been proposed that during the translocation of glycoproteins into the ER, a choice is made between chaperone systems; one of the systems comprises Bip/GRP94 and PDI and the other consists of calreticulin/calnexin and ERp57 [20].

Furthermore, a kinetic protein folding model has been proposed where Bip functions as a chaperone and PDI as a catalyst, and this model might also apply to other pairs of ER folding chaperones and catalysts [21]. The independent actions as well as the cooperation of chaperones are necessary for correct folding. Some chaperones bind immature proteins thereby forming a scaffold, allowing other chaperones to access the immature protein. The cooperation of Bip and PDI has been shown for the folding of antibodies *in vitro* [22], and with calreticulin for the folding of coagulation factor VII [18].

A role of Bip in protein folding was initially inferred from its binding properties and from the fact that its expression is induced by the accumulation of unfolded proteins in the ER [23]. *C. elegans* has two homologues of mammalian Bip, HSP-3 and HSP-4, and three PDI family members (PDI-1, 2, and 3). Expression of HSP-3 and PDI-2 was increased both in *crt-1(jh101)* mutants and in [*crt-1(jh101);cnx-1(nr2009)*] double mutants, suggesting that HSP-3 and PDI-2 may be required to partially compensate for the lack of calreticulin.; Moreover, expression of HSP-4 (Bip homologue) is increased in [*crt-1(jh101);cnx-1(nr2009)*] double mutants, suggesting that additionally enhanced expression of HSP-4 may be required for to compensate for the lack of calnexin.

4.3 Deficiency of the CRT/CNX chaperone system may trigger unfolded protein response

The unfolded protein response (UPR) is a transcriptional and translational intracellular signaling pathway activated by the accumulation of unfolded proteins in the lumen of the ER. It has been shown that accumulation of unfolded proteins induced by ER stress activates UPR target genes [24, 25]. The studies utilizing *C. elegans* as a genetic model system showed that *C. elegans* undertakes UPR to maintain ER homeostasis so as to cope with ER stress [25]. The most well characterized transcriptional target of the UPR is the gene encoding Bip (GRP78). *C. elegans* has two homologues of mammalian Bip: HSP-3 and HSP-4.

To elucidate the mechanism for *hsp-3* and *pdi-2* induction, their promoter regions were analyzed. The *hsp-3* promoter has three ERSE-1-like sequences (ER stress-response element) [26] and three transcriptional activator (ATF/CREB) recognition sites [27, 28], suggesting the potential importance of *hsp-3* in UPR (Fig. 7). Interestingly, the promoter of *pdi-2* also has an ERSE-1-like sequence (Fig. 7). Under compromised chaperone functioning, as in the absence of CRT-1/CNX-1, the numbers of unfolded proteins are likely to increase, which seems to trigger the UPR. Up-regulation of UPR target genes, notably *hsp-3* and *pdi-2*, may eventually provide alternative chaperone machinery for circumventing the CRT-1/CNX-1 deficiency.

4.4 *C. elegans* provides a genetic model to study the ER chaperone system

One of the outstanding questions regarding the requirement of the calreticulin/calnexin cycle in animals is whether HSP-3 and PDI-2 are the only redundant ER chaperone systems compensating for the loss of calreticulin/calnexin function. The *crt-1(jh101)* mutant and *cnx-1(nr2009)* mutant worms are very similar. For example, a temperature-sensitive reproduction defect and slower growth were observed for both mutants. In future studies, we should be able to determine whether over-expression of *hsp-3* and *pdi-2* could compensate for the phenotypic defects observed in *crt-1* mutants. Elucidating the role of HSP-3 and PDI-2 in worms could provide insight into the basis of the ER chaperone system. Furthermore, the simplified genetic system described here should provide insight into the roles and interactions of ER chaperone molecules and folding enzymes. The nematode may be ideal for elucidating the ER chaperone system, because its pathways are similar but simpler than those in mammals, and worms with mutations in these pathways are viable and convenient for analysis. Finally, the growth

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I. ERSE-1 like sequence: CCAAT-N9-CCACG

 hsp-3   -409   CCAAT-CGGCGAGCG-CCGCG  -427
         -343   CCTAT-CGTCCTAGG-CCACG  -325
         -193   CGAAT-GCTCAAGGT-CCACT  -211
 Pdi-2   -88    CCAAT-CGACGCGTT-CCACG  -70

II. Transcrptional activator CREB/ATF recognition sites: TGACGT

 hsp-3   -1433  CAGTT-TGACGT-TTAGA  -1438
         -493   GTAAC-TGACGT-GTCCC  -488
         -357   TCTGC-TGACGT-TGCCT  -352
```

Figure 7. Potential UPR regulatory elements in the promoters of *hsp-3* and *pdi-2*. Numbers are relative to the ATG start codon.

defect in *C. elegans* harboring defects in the *crt-1*/*cnx-1* and *hsp-3*/*pdi-2* pathways may provide an ideal model with which to identify and evaluate molecules that influence these pathways.

This work was supported by grants M10503010001 (D. H. Kim) from the System Biology Program, M103KV010019–05K2201–01920 (J. Ahnn) from the BRC Frontier program and FRP05C2–360 (Y. J. Yoo) from the 21C Frontier Functional Proteomics Projects from Korea Ministry of Science and Technology.

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