Requirement of V-ATPase for Ovulation and Embryogenesis in Caenorhabditis elegans*

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Vacuolar-type proton translocating ATPase (V-ATPase)1 is a ubiquitous enzyme responsible for the acidification of cellular compartments in eukaryotic cells (for reviews, see Refs. 1–3). The enzyme is a multi-subunit complex formed from a peripheral V1 sector with catalytic sites and a membrane-bound V0 sector with a proton pathway. At least 13 subunits are required for V-ATPase activity in yeast (1). Acidification generated by V-ATPase is required for intracellular processes such as receptor-mediated endocytosis, protein sorting, and zymogen activation (2). The proton electrochemical gradient established by V-ATPase energizes transporters for neurotransmitter uptake into synaptic vesicles and neurosecretory granules (4). Furthermore, the same enzyme localized in the plasma membranes has critical functions in renal intercalated cells for regulation of transepithelial acid-base transport (5), in osteoclasts for bone remodeling (6, 7), and in seminal ducts for spermatogenesis (8).

In Caenorhabditis elegans, five vha genes (vha-1, vha-2, and vha-3 for c subunit; vha-4 for c; vha-11 for C) encoding V-ATPase subunits have been found (9, 10). They are predominantly expressed in H-shaped excretory cells contributing to the excretory system in adult worms. Although all vha genes are highly expressed in embryonic stages, their functions are unknown.

During C. elegans embryogenesis, embryos undergo a series of four unequal divisions to produce five somatic founders (AB, E, MS, C, and D) (11). At the beginning of gastrulation, two E daughter cells move to the interior of an embryo to eventually generate intestinal cells. We observed that VHA-11, the C subunit of V-ATPase, was predominantly localized in the intracellular organelles of intestinal cells during late embryogenesis and that the generation of acidic compartments in the intestine was dependent on vha-11 expression. In addition, double strand RNA interference was used to silence V-ATPase gene-specific expression. Worms injected with vha-11 double strand RNA became sterile, due to the failure of ovulation. The oocytes underwent multiple rounds of DNA replication without cytokinesis and became polyplid, probably because ovulation was not coupled with oocyte maturation in the injected worms, suggesting that V-ATPase activity or inside-acidic organelles are essential for oocyte maturation.

EXPERIMENTAL PROCEDURES

Preparation of Antibodies against VHA-11—A cDNA fragment encoding VHA-11 was cloned into the pET-32 expression vector and introduced in Escherichia coli BL21(DE3) cells (Novagen). After induction, a recombinant protein (corresponding to 99–384 residues of VHA-11) was purified using an Ni-NTA column (Qiagen) and then injected into albino rabbits. The polyclonal antibodies were affinity-purified using the same recombinant protein.

Immunoblot Analysis—Wild type worms grown on plates were harvested, washed with M9 buffer (85 mM NaCl, 1 mM MgSO4, 16.8 mM Na2HPO4, and 22 mM KH2PO4), and then suspended in lysing buffer (62.5 mM Tris-HCl (pH 6.8), 10% glycerol, and 2% sodium dodecyl sulfate). They were disrupted by rapid mixing with acid-washed glass beads (0.2–0.3 mm ø), followed by heating at 95 °C for 5 min. After centrifugation at 20,000 × g for 5 min, the resulting supernatant was subjected to protein concentration determination using BCA protein assay reagent (Pierce). Samples were separated by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate and then transferred electrochemically onto nitrocellulose membranes. Immunodetection was carried out using alkaline phosphatase-conjugated antibodies, 5-bromo-4-chloro-3-indolyl phosphate, and 4-nitroblue tetrazolium chloride.

Construction of Plasmids for Double Strand RNA Preparation—The full-length cDNA clone coding for either VHA-4 or VHA-11 (9, 10) was used for preparation of double strand RNA (dsRNA). A 1.9-kb EcoRV EcoRI fragment of the E. coli lacZ gene from pPD21.28 (12) was subcloned into pBluescript II KS (Stratagene) as a control. A 0.6-kb Ddel/EcoRV segment of PCV10 (9) was used as the vha-2 open reading frame. The 3'–untranslated regions of the vha-1, vha-2, and vha-3 cDNAs were amplified by PCR using the following primers: vha-1, 5'-ggagctcattttgcttcaattgca-3' and 5'-caatgttaatgcttacattttgcttcaattgca-3'; vha-2, 5'-aggatctttgcttacattttgcttcaattgca-3' and 5'-aagaatttcgcttacattttgcttcaattgca-3'; vha-3, 5'-ggagctcattaattttgcttcaattgca-3' and 5'-aagaatttcgcttacattttgcttcaattgca-3'.
Immunohistochemical analysis of VHA-11. A, detection of VHA-11 in C. elegans total lysate. A total lysate of yeast cells carrying either a vector (Vector) or the pVHA-11 expression plasmid (pVHA-11), and a C. elegans lysate (C. elegans) were subjected to polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate, blotted onto nitrocellulose membranes, and then incubated with affinity-purified anti-VHA-11 antibodies. The amounts of the yeast and C. elegans lysates used were 30 and 10 μg, respectively. The solid arrow indicates the position of the VHA-11 protein (40 kDa). The open arrowhead indicates a nonspecific band for the yeast lysates. B–D, immunohistochemical localization of VHA-11 in C. elegans. An adult worm (B), embryos (~200 cells) at the cleavage stage (C), and embryos at the comma stage (D) were fixed and then incubated with the affinity-purified anti-VHA-11 antibodies. VHA-11 was significantly observed in H-shaped excretory cells in the anterior part of an adult worm (B), consistent with a previous report involving the GFP reporter gene (9). The arrow indicates the position of the nucleus of an H-shaped excretory cell. In C and D, VHA-11 was localized in dot-like structures around the nuclei and also detected in the cytoplasm. Arrowheads indicate the dot-like structures with which VHA-11 was densely associated. Scale bars indicate 50 μm (B) or 10 μm (C, D).

RESULTS
Localisation of V-ATPase in Embryonic Intestine and Adult H-shaped Excretory Cells—We have identified five vha genes (vha-1, vha-2, vha-3, vha-4, and vha-11) encoding V-ATPase subunits in C. elegans (9, 10). Their expression was determined using the corresponding promoter sequences connected upstream of GFP or lacZ reporter gene. These promoters were highly active in H-shaped excretory cells and the rectum of adult worms (9, 10).

To identify the cells and organelles in which V-ATPase is localized, polyclonal antibodies against VHA-11 were raised. VHA-11 is a functional V-ATPase subunit and has no isoforms in nematodes (10). As shown in Fig. 1A, the affinity-purified antibodies recognized a single protein band (40 kDa) on an immunoblot of a wild type nematode lysate. A band corresponding to the same size was also detected for the lysate of yeast cells harboring the vha-11 expression plasmid but not for that of yeast cells with a vector. These results indicated that the antibodies specifically recognized a 40-kDa vha-11 gene product.

Using the anti-VHA-11 antibodies, immunohistochemical analysis was carried out. In the adult stage, VHA-11 was expressed mainly in an H-shaped excretory cell (Fig. 1B), and also in intestinal cells (data not shown), consistent with expression of the vha-11::GFP reporter gene (10). In embryonic stages, VHA-11 was detected as dot-like intracellular compartments around the nuclei (Fig. 1C, arrowheads). Diffuse staining in the cytoplasm could be seen at all embryonic stages. Beginning at the comma stage, dense dot-like staining became visible in the cytoplasmic stream of GFP or lacZ reporter gene. These results indicate that V-ATPase is localized in intracellular compartments.
of embryos, especially those of intestinal cells.

**Generation of Acidic Compartments in the Embryonic Intestine by V-ATPase**—The predominant expression of V-ATPase in the embryonic intestine suggests that acidic organelles are present in intestinal cells. Consistent with this suggestion, acridine orange, a weak basic dye, was highly accumulated in embryonic intestinal cells (Fig. 2, A and B). The accumulation was detected predominantly in the cytoplasmic perinuclear regions of intestinal cells in confocal sections (Fig. 3), indicating that inside-acidic compartments are present in the embryonic intestine. The acridine-orange staining disappeared upon either treatment with bafilomycin A1, a specific inhibitor of V-ATPase (Fig. 2 C), or the addition of 5 mM ammonium chloride (data not shown). Inactivation of *vha-11* expression by RNA interference prevented the accumulation of acridine orange in the intestine (Fig. 2 D and also see below). These results indicate that V-ATPase mediates the acidification of intracellular compartments in the embryonic intestine.

**Embryonic Lethality Caused by Silencing of *vha-11* Expression**—RNA interference is effective for abolishing expression of a specific gene in *C. elegans* (13). We injected *vha-11* dsRNA into adult worms to disrupt the gene expression in progenies. At 24 h after injection of the *vha-11* dsRNA, the VHA-11 protein in embryos became undetectable, whereas introduction of the control *lacZ* dsRNA was ineffective (Fig. 4 A). Silencing of *vha-11* expression was observed not only in embryos retained in the uterus but also in eggs harvested from plates (Fig. 4 A, lanes 2 and 4). These results suggest that introduction of the *vha-11* dsRNA completely turned the gene expression off during embryogenesis.

All embryos lacking *vha-11* expression were essentially lethal (Table I), and their development was arrested at various stages. 15–30% of the embryos arrested at the one-cell stage. More than 30% of the embryos became lethal after gastrulation. The development of the other embryos was arrested between two-cell stage and gastrulation. The disappearance of VHA-11 in the embryonic intestine of *vha-11* dsRNA-injected worms was confirmed immunochemically: no dot-like staining was detected in the intestine of comma-stage embryos (Fig. 4 D). On the other hand, significant staining was observed in embryos from *lacZ* dsRNA-injected worms as well as those

![Fig. 2. Embryonic acidic organelles accumulating acridine orange.](image)

**Fig. 2.** Embryonic acidic organelles accumulating acridine orange. Embryos from wild type (A, B, C) and *vha-11* dsRNA-injected (D) worms were vitally stained with acridine orange for 30 min. Lateral (A, C) and ventral (B, D) views of embryos at the comma stage are shown. Arrowheads indicate acidic compartments stained with acridine orange. Acridine orange staining was not observed in embryos treated with bafilomycin A1 (C) or in embryos from a *vha-11* dsRNA-injected worm (D); arrows indicate the positions of intestinal cells. Scale bar indicates 10 μm.

![Fig. 3. Acridine-orange staining of embryonic intestines.](image)

**Fig. 3.** Acridine-orange staining of embryonic intestines. Embryos at the comma (A–D) and 2-fold (E, F) stages were stained with acridine orange. Fluorescent (A, C, E) and corresponding differential interference contrast (B, D, F) images were acquired by confocal microscopy. Lateral (A, B, F) and ventral (C, D) views are shown. Scale bar indicates 10 μm.
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without injection (Fig. 4, B and C). It was noteworthy that diffuse staining in the cytoplasm was also decreased in embryos from vha-11 dsRNA-injected worms. Consistent with the loss of VHA-11, acridine-orange staining was not observed in intestinal cells of embryos from the vha-11 dsRNA-injected worms (Fig. 2D). These results indicate that vha-11 is an essential gene for embryogenesis, particularly the formation of acidic compartments in the intestine.

Vo Subunit Genes vha-1 and vha-4 Are Required for Embryogenesis but Not vha-2 and vha-3—Because worms became lethal upon silencing of gene expression of vha-11 for the C subunit in the peripheral V1 sector, it was of interest to examine the genes for Vo subunits. A series of dsRNAs for proteolipid subunits was used to examine the effects on embryogenesis. C. elegans V-ATPase has two proteolipids, i.e., proteins of 23 kDa (VHA-4) and 16 kDa (VHA-1, VHA-2, and VHA-3) (9, 10). Introduction of vha-11 dsRNA led to embryonic lethality, similar to the case of vha-11 dsRNA (Table I), indicating that 23-kDa proteolipid is required for embryonic development.

Embryonic lethality was also observed with the vha-1 or vha-2 dsRNA coding for a 16-kDa proteolipid. However, introduction of the vha-1 or vha-2 dsRNA may abolish the expression of other 16-kDa proteolipid genes, because they exhibit high identity: 60% amino acid identity between VHA-1 and VHA-2; 100% between VHA-2 and VHA-3 (9, 10). Expected from the high identity of VHA-1, VHA-2, and VHA-3, no specific antibodies could be produced (data not shown). Thus, non-identical 3′-untranslated region (UTR) of each vha gene (vha-1, vha-2, or vha-3) was examined for embryonic development together with the presence of the corresponding transcript. The dsRNA for vha-1 UTR caused embryonic lethality, but those for vha-2 UTR and vha-3 UTR did not (Table I).

Consistent with the results, the vha-1 transcript was essentially undetectable after injection of the vha-1 UTR dsRNA, although the vha-2 and vha-3 transcripts were present (Fig. 5). In contrast, injection of the dsRNA for vha-2 UTR caused the vha-2 and vha-3 expressions to silence, but significant amounts of vha-1 were expressed. The dsRNA for vha-3 UTR lowered only the corresponding transcript. Upon injections of vha-2 UTR + vha-3 UTR dsRNA, no embryonic lethality was observed (Table I), and the vha-1 and vha-3 transcripts were detectable in embryos from the injected worms. These results indicate that the vha-1 expression is essential for embryogenesis, whereas vha-2 and vha-3 are not required.

Defective Ovulation in Worms Injected with the dsRNAs for V-ATPase Subunits—Because the acidic organelles generated by V-ATPase are important for embryogenesis, we were also interested in their roles in oogenesis. Nematode gonads display distal-proximal polarity in hermaphrodites (17), germ cells proliferate in the distal gonads, and oogenesis occurs in the loop and proximal regions (Fig. 6A). Condensation of chromosomes in diakinesis is observed in the proximal gonad (Fig. 6B, arrowheads).

We observed that vha-11 dsRNA-injected worms were able to produce eggs. However, the egg numbers gradually decreased, and the worms finally became sterile after 24 h. This sterility continued for about 4 days, and then the worms started pro-

TABLE I
Injection of dsRNAs for V-ATPase subunits leads to embryonic lethality

| dsRNA      | Injected worms | Progeny  | Embryonic lethality |
|------------|----------------|---------|---------------------|
| Mock       | 17             | 15 /808 | 4.6                 |
| lacZ       | 15             | 2 /301  | 0.7                 |
| vha-1      | 14             | 115 /10 | 92.4                |
| vha-2      | 14             | 117 /5  | 95.9                |
| vha-4      | 12             | 177 /8  | 95.7                |
| vha-11     | 15             | 207 /10 | 95.4                |
| vha-1 UTR  | 15             | 226 /11 | 95.4                |
| vha-2 UTR  | 14             | 45 /905 | 12.9                |
| vha-3 UTR  | 11             | 4 /372  | 1.1                 |
| vha-2 UTR + vha-3 UTR | 14 | 52 /229 | 18.5 |

[2] Immunofluorescence analysis of embryos from vha-11 dsRNA-injected worms. A, VHA-11 became undetectable after injection of vha-11 dsRNA. Embryos (1-μg proteins) from worms injected with either lacZ (lanes 1, 3) or vha-11 dsRNA (lanes 2, 4) were subjected to gel electrophoresis and immunoblotting. Proteins were extracted from embryos either prepared from gravid worms (lanes 1, 2) or harvested from agar plates (lanes 3, 4). The arrowhead indicates the position of VHA-11. B–D, comma stage embryos from control (B), lacZ dsRNA- (C), and vha-11 dsRNA-injected (D) worms were immunochimically stained with antibodies against VHA-11: arrowheads indicate dot-like structures associated with VHA-11 in the intestine. Scale bar indicates 10 μm.
dsRNA injected

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vha-1
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vha-2
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vha-3
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rp21

FIG. 5. Detection of transcripts for vha-1 and vha-2 in embryos. Transcripts for vha-1, vha-2, and vha-3 were detected in embryos from worms injected with dsRNA for vha-1 UTR, vha-2 UTR, vha-3 UTR, or vha-2 UTR + vha-3 UTR by RT-PCR using the corresponding primers. As a control, transcript for ribosomal protein (rp21) was amplified. The PCR products were separated by polyacrylamide gel electrophoresis, and the gel was stained with SYBR Green I (Molecular Probes).

Producing viable eggs again. To visualize the germ cell nuclei in the sterile worms, they were fixed at 48 h after injection of the vha-11 dsRNA and then stained with DAPI. Oocytes with condensed chromosomes at the diakinesis stage of meiotic prophase were also detected (Fig. 6C, arrowheads). They were found in the distal gonads near the loop region, but in the proximal gonads of non-injected worms (Fig. 6B). The location was different in the injected worms, because abnormal oocytes with strong DAPI staining filled the proximal gonad (Fig. 6C). The chromosomes became loose and dispersed in the cytoplasm, showing H-shaped staining (Fig. 6D). Spermathecae were observed near the vulva due to the accumulation of oocytes, and no fertilized embryos were found in the uterus (Fig. 6, E and F). These findings indicate that oocytes are trapped in the gonads and then undergo endomitotic DNA replication, because ovulation fails. Endomitotic oocytes contain polyploid nuclei possibly due to the multiple rounds of DNA replication without cytokinesis (18–20). Essentially the same results were obtained on injection of the vha-1, vha-2, or vha-4 dsRNA. These results indicate that V-ATPase, and thus acidic organelles, are essential for oocyte ovulation.

DISCUSSION

V-ATPase was localized immunochemically in dot-like structures of embryonic cells using antibodies against VHA-11. It may be associated with intracellular organelles such as lysosomes and endosomes, similar to in other eukaryotic cells (2). In comma stage embryos, V-ATPase was highly expressed in intestinal cells being localized in their intracellular compartments. The distribution in embryos was similar to that of the acidic compartments stained with acridine orange. The C. elegans intestine is known to contain numerous storage granules (21). Furthermore, labeled probes are taken through endocytosis and accumulated within granules in which acridine orange is stored (22), suggesting that the acidic pH generated by V-ATPase is required for nutrient intake by intestinal cells.

We used RNA interference (13) as a powerful tool for silencing V-ATPase gene-specific expressions. The progenies exhibited defects dependent on the time after injection of dsRNA. C. elegans germ cells in the gonads exhibit distal-proximal polarity during proliferation and meiotic prophase progression (17). Thus, each germ cell may show a defect, depending on the progress of oogenesis. vha-11 dsRNA-injected worms produced eggs up to 24 h, but the eggs did not hatch. The development of the eggs was randomly arrested at different stages. The amount of vha tran-
scripts is high at the embryo rather than the larval and adult stages (10). Thus, the difference in the stage at the time of arrest may depend on the amount of maternal mRNA present in each embryo. 15–30% of the embryos arrested as one-cell stage, suggesting that the cell division cycle may require acidic compartments generated by V-ATPase. Inactivation of proteolipid subunit genes also caused embryonic lethality. Furthermore, similar results were obtained on injection of the dsRNA for F55H2.2, which is homologous to genes for the D subunit in the V1 sector.2 These results suggest that V-ATPase activity itself is essential for the progress of embryonic development.

Two isoforms (VHA-1 and VHA-2/VHA-3) for 16-kDa proteolipids are found in C. elegans: vha-2 and vha-3 encode an identical protein (10). RNA interference analysis of these proteolipids indicated that embryonic development requires VHA-1 but not VHA-2/VHA-3. So far, 16-kDa proteolipid isoforms have been identified in S. cerevisiae (23, 24), S. pombe (25), and D. melanogaster (26). In S. cerevisiae, two proteolipids (Vma3p and Vma11p) are essential for growth at neutral pH (24) and are components of each V0 sector of yeast V-ATPase (27). However, only single genes for 16-kDa proteolipide have been found in mouse (28) and human (29). Unlike yeast, only VHA-1 is required for embryonic development in C. elegans. It became of interest to study roles of the VHA-2/VHA-3 proteolipid in adult worms.

Yolk granules are embryonic organelles and store essential nutrients that support embryonic development (30). Their contents, yolk proteins, are derived from vitellogenins and have been shown to be taken up into acidic compartments of C. elegans oocytes through endocytosis (31). YP170, one of the C. elegans vitellogenins, is also taken up by oocytes through an endocytotic pathway mediated by the RME-2 receptor protein (32). Thus, it is reasonable to assume that disruption of the genes required for endocytosis affected oogenesis. As expected, worms injected with the dsRNA for clathrin or adaptin gene produced inhibition of yolk protein uptake by oocytes.

The accumulation of endomitotic oocytes was observed in the gonads of worms injected with the dsRNAs for V-ATPase subunits. So far, only mutants defective in ovulation have been reported (18–20). One of them, the emo-1 mutant, has a zygotic defect in ovulation and eventually produces oocytes with polyploid nuclei (18). Because the emo-1 gene encodes a homologue of Sce61 gamma, which is a component of the protein translocation pore in the endoplasmic reticulum in eukaryotic cells, it is proposed that the emo-1 mutation disrupts the transport of oocyte proteins required for ovulation (18). In addition to emo-1, the introduction of the dsRNAs for small GTPase (ATP-ribosylation factor) and coatamer genes necessary for clathrin-mediated endocytosis also leads to the accumulation of endomitotic oocytes (32), supporting the hypothesis that the inhibition of protein transport causes ovulation failure and the production of polyploid nuclei. A defect in oocyte ovulation caused by the dsRNAs for V-ATPase subunits is consistent with reports that acidic compartments are essential for protein trafficking such as endocytosis (3, 33).

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