Multiple Genes for Vacuolar-type ATPase Proteolipids in Caenorhabditis elegans

A NEW GENE, vha-3, HAS A DISTINCT CELL-SPECIFIC DISTRIBUTION

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In the vacuolar-type H+-ATPase (V-ATPase), highly hydrophobic subunits known as the proteolipids are components of the integral membrane V₀ sector. Previously, we described the identification of three different proteolipid genes in Caenorhabditis elegans (Oka, T., Yamamoto, R., and Futai, M. (1997) J. Biol. Chem. 272, 24387–24392): vha-1 and vha-2 encoded 16-kDa subunits, and vha-4, a 23-kDa isoform. We report here that a third 16-kDa gene, vha-3, has been identified on chromosome IV. This is the first example in which four proteolipid genes have been found in a single organism. vha-2 and vha-3 exhibited 85% nucleotide identity within the open reading frames which encoded the identical amino acid sequence. Northern blot analysis indicated that all four genes were expressed in a similar pattern during the worm life cycle; however, studies with transgenic worms indicated that the vha-3 gene was expressed differently from other proteolipid genes in a cell-specific manner. These results implied that the isofoms of the proteolipids may be related to functional differences of V-ATPases in various cell types.

Another new gene, vha-11, contained seven exons and was found to be located immediately downstream of vha-3. The two genes constitute a single transcriptional unit. The VHA-11 protein had 384 amino acids and shared strong sequence similarities with the C subunit, a component of the peripheral V₁ sector of the V-ATPase, from yeast, bovine, and human. Expression of the vha-11 cDNA complemented a null mutation of VMA5, the yeast C subunit gene, thus demonstrating that vha-11 was the functional C subunit of C. elegans.

The vacuolar-type H⁺-ATPase (V-ATPase) is a ubiquitous proton pump found in a variety of intracellular organelles including the endosome, lysosome, Golgi apparatus, and clathrin-coated vesicle (1–3). The V-ATPase-mediated acidification of organellar lumens is critical for protein sorting, lysosomal activation, and receptor-mediated endocytosis (4–7). In addition, V-ATPase is found in the plasma membranes of kidney and bladder where the enzyme has a key role in pH regulation (8, 9) of osteoclasts for bone resorption (10) and of seminal duct for spermatogenesis (11).

V-ATPase has two distinct sectors called V₁ and V₀ which are analogous to F₁ and F₀ of F-type ATPases (12), respectively. V₁ is the peripheral membrane sector and is composed of eight subunits. A and B subunits form the catalytic complex, and six other subunits (C-H) link the A-B hexamer complex to V₀ (13). V₀ is the integral membrane sector making up the proton pathway and consists of the proteolipid (16 and 23 kDa) and 100- and 36-kDa subunits (13).

A hydrophobic 16-kDa protein called the proteolipid is a major component of the V₀ sector and has a N,N′-dicyclohexylcarbodiimide-reactive glutamate that is essential for proton transport (14, 15). The cDNA clones for proteolipids have been identified from a number of species. Three proteolipid genes are independently required for yeast V-ATPase function (14–17): VMA3 and VMA11, both of which code for 16-kDa proteolipids, and VMA16, which codes for a 23-kDa isoform. Higher eukaryotic cells also have multiple proteolipid genes. Previously, we reported that Caenorhabditis elegans has three genes (vha-1, vha-2, and vha-4; Ref. 18). At this time, the number of proteolipid genes in mammalian cells is unclear. Although four genomic clones were isolated from a human library, only one corresponded to the cDNA and the other three clones appeared to be of pseudogenes (19).

In this study, we demonstrate that a third 16-kDa proteolipid gene (vha-3) is present in the C. elegans genome. The vha-3 and vha-2 genes exhibit strong similarity and encode identical polypeptides but are expressed differently in a cell-specific manner. Another newly described gene, vha-11, is located just downstream of vha-3 and codes for a hydrophilic polypeptide very similar to the V-ATPase C subunits of yeast (20, 21), bovine (22), and human (23). Expression of the vha-11 cDNA restores growth of the yeast vma5 mutant that lacks the C subunit gene (20). The results demonstrate that the vha-11 gene product is the C subunit.

EXPERIMENTAL PROCEDURES

Culture and Transformation of Worms—Wild-type strain Bristol N2 was cultured as described (24). Transformation was carried out using the selectable marker plasmid pRF4 (25).

Sequencing cDNA Clones—The C. elegans expressed sequence tag (EST) clones, yk413h1 (vha-3) and yk434g8 (vha-11), were kindly provided by Y. Kohara and sequenced using the Dye Terminator DNA sequencing kit (Applied Biosystems). The 5' regions were amplified from total RNA taken from a mixed population of worms by RT-PCR using spliced leader primers and gene-specific primers (vha-3, 5'-gacctgcactcttcac-3' and vha-11, 5'-caacctcctgctcttgatac-3') as described (18). The resulting PCR products were sequenced and ligated with the EST cDNAs to construct full-length cDNA clones. The nucleotide sequence data reported in this paper will appear in the DDBJ, EBI, and
GenBank™ nucleotide sequence data bases with the following accession numbers: vha-3, AB009566; and vha-11, AB009567.

Northern Blot Analysis—Synchronously growing worms were prepared using the alkaline hypochlorite method (26). Total RNA was prepared from C. elegans of mixed growth stages using TriZOL™ LS Reagent (Life Technologies, Inc.) according to the recommendations of the manufacturer. 15 μg of total RNA was electrophoresed on a 1.5% agarose, 6% formaldehyde gel and transferred to a Hybond-N+ membrane (Amersham Pharmacia Biotech). Hybridizations were carried out using probes labeled by the random primed DNA labeling kit (Boehringer Mannheim) and QuikHyb solution (Stratagene) according to the recommendations of the manufacturer. The blot was also hybridized with a probe from the ribosomal protein gene rp21 as a loading control (27). The filter was scanned by the Image-Analyzer BAS-1000 (Fuji Film) for semi-quantitative estimation of transcript levels.

Genomic Southern Blots—Genomic DNA was prepared from a mixed population of worms and subjected to Southern blot analysis. For low stringency conditions, the filters were washed at room temperature for 10 min with 5× SSC containing 0.1% SDS, followed by a further wash at 60 °C for 10 min (27).

Constructions of lacZ Reporter Plasmids—The upstream region of vha-3 was amplified directly from C. elegans genomic DNA by PCR using primers (5′-gcgacaggtactgcaggatattg-3′ and 5′-gcggtttccaagtcgtcgacattt-3′). The maximum amount of upstream region was used but without introducing the regulatory regions of neighboring genes. The 2.2-kb product was digested with PstI and SalI and inserted into pPD89.03, a lacZ reporter plasmid, to create pCV3–03. pCV02 was constructed by inserting the 1.3-kb BamHI to SalI fragment, which contained the regulatory region of vha-1 and vha-2 (18), into pPD89.03. Transgenic worms were fixed and stained for β-galactosidase using 5-bromo-4-chloro-3-indolyl-β-D-galactoside (28). Identification of cell types has been previously described using Nomarski optics (18, 29).

FIG. 1. Nucleotide alignment of vha-2 and vha-3 and gene structure of vha-3 and vha-11. A, nucleotide alignment of vha-2 and vha-3, which code for identical 16-kDa proteolipids. Identical residues are indicated by boxes. Amino acid residues are shown by one-letter symbols. B, gene structure of vha-3 and vha-11. Closed and shaded boxes represent coding and untranslated regions, respectively. The identified spliced leaders (SL1 or SL2) are indicated upstream of the first exon of each gene. The 2.2-kb fragment containing the 5′ upstream regulatory region was fused with a lacZ reporter gene in pCV3–03 for testing expression pattern.
Rescue of Yeast vma5 Null Mutant with C. elegans vha-11—cDNA was ligated downstream of the TDH3 promoter of pKT10, a yeast expression vector (30). The resulting plasmid was introduced into the yeast Dvma5 mutant SF838–1D (MAT a leu2–3,112 ura3–52 his4–519 ade6 gal2 pep4–3 Dvma5::LEU2, Ref. 20). Transformants were streaked on YPD plates (2% peptone, 1% yeast extract, and 2% glucose), including either 50 mM sodium succinate, pH 5.0, or 50 mM potassium phosphate, pH 7.5, and were incubated at 30 °C for 3 days to score the growth phenotype.

RESULTS

A Third V-ATPase 16-kDa Proteolipid Gene, vha-3, in C. elegans—Previously, we identified two proteolipid genes, vha-1 and vha-2, in C. elegans which had 60% amino acid identity (18). We searched for additional genes using RT-PCR. One PCR product was highly similar to the vha-2 gene, and analysis of the C. elegans genome data base indicated that the PCR product was a third V-ATPase proteolipid gene, vha-3 (19).

FIG. 2. Alignment of V-ATPase C subunit amino acid sequences and rescue of yeast Dvma5 by C. elegans vha-11. A, alignment of the V-ATPase C subunits from nematode, yeast (Vma5p) (20, 21), bovine (22), and human (23). The deduced amino acid sequences were aligned for maximal homology. Conserved residues among at least three species are indicated by boxes. B, rescue of the yeast Dvma5 mutation by vha-11. Yeast cells harboring vector with no insert (Vector), the vha-11 expression plasmid (vha-11), or the VMA5 gene on a single-copy plasmid (VMA5) were streaked on either YPD, pH 5.0, or YPD, pH 7.5, and incubated at 30 °C for 3 days before phenotypic scoring.
uct matched the Y38F2 YAC genomic clone from chromosome IV. We named the corresponding gene *vha-3* and searched the EST data base for a full-length clone. We found only one clone (yk413h1) that contained a part of the sequence of the PCR product.

A full-length clone of *vha-3* was obtained by RT-PCR using primers specific for *vha-3* and the spliced leaders that are attached to the 5’ end of almost all processed transcripts in *C. elegans* (31). The entire *vha-3* cDNA was 1421 bp (not including polyadenylation) with an open reading frame of 486 bp. The coding region of *vha-3* was 85% identical to that of *vha-2* (Fig. 1A), whereas the 3’-untranslational region (935 bp) of *vha-3* was 461 bp longer and had no similarity with that of *vha-2*. *vha-3* encoded a 161-amino acid polypeptide completely identical to the VHA-2 protein. 38% of *vha-3* codons had silent substitutions when compared with *vha-2* (Fig. 1A); almost all changes (90%) were the wobble position. Comparison of genomic and cDNA sequences revealed that *vha-3* is an intronless gene (Fig. 1B), whereas *vha-2* has two introns (18).

The V-ATPase C Subunit Gene, *vha-11*, of *C. elegans* Is Linked to *vha-3*—Sequence comparison with the Y38F2 YAC genomic clone revealed that clone yk434g8 mapped immediate downstream of *vha-3*. An open reading frame was found in yk434g8 that shared high sequence identity with the V-ATPase C subunits of other species. We named the gene *vha-11* and obtained the entire *vha-11* cDNA by RT-PCR. Comparison with genomic DNA and cDNA sequences indicated that the *vha-11* gene consisted of seven exons (Fig. 1A). The 1639-bp cDNA contained a coding region for a 384-amino acid polypeptide without any putative transmembrane regions. The *vha-11* protein exhibits 37%, 56%, and 56% sequence identity with the C subunits of yeast (Vma5p) (20, 21), bovine (22), and human (23), respectively (Fig. 2A).

To address whether the *vha-11* protein is the V-ATPase C subunit, the *vha-11* cDNA was placed under control of the yeast *TDH3* constitutive promoter. The resulting plasmid was introduced into a yeast *vma5* null mutant that grows very slowly at neutral pH (20). Expression of *vha-11* completely restored growth of the *vma5* mutant (Fig. 2B), thus demonstrating that the *vha-11* protein can substitute as a C subunit in yeast.

Identification of *vha-3* and *vha-11* Transcripts—To confirm that *vha-3* and *vha-11* genes are expressed, Northern blot analysis was performed using RNA from a mixed-stage culture. The *vha-3* probe hybridized with three transcripts (0.9, 1.0, and 1.4 kb; see Fig. 3A). The 1.4-krb transcript corresponded to *vha-3* because it was consistent with the length of the cDNA. Two weaker bands (0.9 and 1.0 kb) showed the same mobility as *vha-2* transcripts and are likely because of cross-hybridization. The amount of the *vha-3* transcript was roughly five percent of *vha-1* or *vha-2*. A single band (1.5 kb) was detected for the *vha-11* transcript whose size was compatible with that of the cDNA (Fig. 3B).

Sequence comparison of genomic and cDNA clones showed that only five base pairs were present between *vha-3* and *vha-11* (Fig. 1B), suggesting that they constitute a polycistronic unit similar to *vha-1* and *vha-2*. To confirm whether *vha-3* and *vha-11* are in a single gene cluster and whether their transcripts are trans-spliced, RT-PCR analysis was carried out using primers specific for the spliced leaders (SL1 and SL2). The *vha-3* mRNA exclusively received SL1, and *vha-11* was trans-spliced with SL1 or SL2, suggesting that the two genes are transcribed as a polycistronic unit and that *vha-3* is located immediate downstream of the promoter.

Presence of Four *vha* Proteolipid Genes in *C. elegans*—To

**Fig. 3. Northern blot analysis of *vha-3* and *vha-11* transcripts.** Total RNA from a mixed stage population was electrophoresed, blotted, and hybridized to probes from the coding regions of each gene. Transcript sizes were estimated using RNA standards run on the same gel. A, arrowheads show positions of the *vha-3* (closed) and *vha-2* (open) transcripts. B, arrowhead represents the *vha-11* transcript.

**Fig. 4. Genomic Southern blot analysis of the four *vha* genes.** *C. elegans* genomic DNA (15 µg) was digested with *EcoRI* (lane 1), *EcoRV* (lane 2), *HincII* (lane 3), or *PvuII* (lane 4), subjected to electrophoresis, blotted to a filter, and hybridized with probes from the coding regions of each gene. Blots were washed under the low stringency condition as detailed under “Experimental Procedures.” Arrowheads represent hybridization with indicated genes. DNA standards were stained with ethidium bromide, and their sizes are shown in kilobase pairs.
search for other C. elegans proteolipid genes, Southern blot analysis was carried out under low stringency conditions. The vha-1 probe hybridized with single bands, the sizes of which were consistent with the length predicted from the genomic sequence (Fig. 4). No other bands were detected even under low stringency conditions, thus indicating that vha-1 is a single gene and no other homologue exists in this organism.

In the case of vha-2 and vha-3, two bands were always observed when genomic DNA was treated with different enzymes. As shown for vha-3 in Fig. 4, the strong signals represented vha-3, and the weak bands were because of cross-hybridization with vha-2. Similar hybridization patterns were observed with vha-2. These results strongly suggest that no additional vha-2 or vha-3 homologues are present. vha-4, encoding the 23-kDa V-ATPase proteolipid (18), was also in single copy in the worm genome. Taken together, these results indicate that only four vha proteolipid genes are present in C. elegans.

Expression of Proteolipid Genes during the Worm Life Cycle—The obvious but interesting question is what distinguishes vha-2 and vha-3 since they code for the identical protein. We first assessed the expression of each gene as a function of life cycle. Northern blot analysis was carried out using RNA from different developmental stages. All four proteolipid genes were highly expressed in the embryo and much lower during larval stages (Fig. 5). The results suggest that the V-ATPase activities may be important during embryogenesis and cell proliferation (32). The expression of all genes gradually increased again leading up to the adult stage. When in the dauer stage, an obligate stage of diapause, all four transcripts were present but at approximately 2-fold higher levels than the L2 or L3 stage as judged from quantitation of the blot scans.

No significant differences were observed on the expression patterns between vha-2 and vha-3 through all stages. We note that the vha-2 gene was expressed at higher levels than vha-1 in the embryo, L1, and L4 stages (Fig. 5). On the other hand, the amount of vha-1 and vha-2 transcripts were similar at other stages. This observation points out that transcription of the two genes, even though in a polycistronic unit, may be differentially regulated at least during the larval stages by an unknown mechanism.

Preferential Expression of vha-3 in Gastric and Hypodermal Cells—Although V-ATPase genes are ubiquitously expressed, vha-1, vha-2, and vha-4 were expressed at much higher levels in the H-shaped excretory cell (18). To determine the expression pattern of the vha-3 gene, a fusion gene was constructed by inserting a genomic fragment containing the vha-3 5’ transcriptional regulatory sequences through to the first two codons of the reading frame into a lacZ expression vector (Fig. 1B). Surprisingly, expression of the vha-3 fusion gene was significantly different in the adult worm from the other 16-kDa proteolipid genes. vha-1 and vha-2 were predominantly expressed in the H-shaped excretory cell and rectum (Fig. 6C), which confirmed previous results obtained with GFP (green

![Fig. 5. Stage-specific Northern blot analysis of the four vha genes.](http://www.jbc.org/)

![Fig. 6. Expression of vha-3 in gastric and intestinal cells.](http://www.jbc.org/)
fluorescent protein) fusion genes (18). In contrast, vha-3 was mainly expressed in gastrointestinal and hypodermal cells, in addition to the H-shaped excretory cell (Fig. 6, A and B). After prolonged staining, the canals of the H-shaped excretory cell also became visible (arrowheads). Similar results were obtained in transgenic worms carrying the reporter gene fused to the 5′ upstream region of vha-11 (data not shown), supporting that vha-3 and vha-11 are transcribed as a polycistronic unit. These results demonstrate that the transcriptional regulation of vha-3 is different from that for vha-1 and vha-2.

**DISCUSSION**

We have identified two genes (vha-3 and vha-11) coding for V-ATPase subunits of *C. elegans*. The 1421-bp vha-3 cDNA encodes the 16-kDa proteolipid. This is the fourth gene coding for isoforms of the proteolipid, vha-11 consists of seven exons and codes for a 384-amino acid hydrophilic polypeptide. The VHA-11 protein exhibited significant similarities to the V1 sector C subunits of yeast (20, 21), bovine (22), and human (23), and the vha-11 cDNA complemented a yeast vma5 (C subunit) mutation, thus indicating that vha-11 is a functional counterpart of VMA5.

The vha-3 and vha-11 genes are tandemly located on chromosome IV as a single polycistronic unit. Transgenics analysis showed that vha-3 was most strongly expressed in intestine, hypodermis, and the H-shaped excretory cell (Fig. 6). Parallel expression patterns of a reporter gene fused to the 5′ upstream region of vha-11 support that vha-3 and vha-11 are transcribed as a polycistronic unit. As shown previously, vha-1 and vha-2, which are two other genes that code for the 16-kDa proteolipid, are also transcribed as a unit (18). Furthermore, the first gene of both units (vha-1 and vha-3) were found to have unusually long 3′-untranslated regions and no introns. In contrast to the vha-1/vha-2 unit, the vha-3/vha-11 consists of genes for one V0 and one peripheral V1 subunit. Genes for other V-ATPase subunits, B, D, E, F, G, H, a and d, have been identified from the *C. elegans* genome project data base (ACeDB WS2 4–16) based on sequence similarities to known yeast subunits (13); however, none of these are in a polycistronic unit. Thus the transcriptional units of vha-3/vha-11 and vha-1/vha-2 are unique among V-ATPase genes in *C. elegans*. Finally, we note that vha-3/vha-11 and vha-1/vha-2 gene pairs are functionally related. Most *C. elegans* gene clusters consist of unrelated genes, although lin-15/lin-15B and fbp-1/fbp-16 pairs are clearly functionally related (31, 33).

Southern blot analysis of the *C. elegans* genome showed that additional homologues of the proteolipid genes are not present, therefore suggesting that only four proteolipid genes (vha-1, vha-2, and vha-3, 16-kDa subunit; vha-4, 23 kDa) are present. This is the first report of four proteolipid genes in the same genome. Among these genes, vha-3 and vha-2 share very high nucleotide similarity within the coding region and encode identical polypeptides. Although 38% of the codons used for vha-3 and vha-2 are different, the codon usage is not significantly different from other *C. elegans* open reading frames (34). Two cDNAs for very similar proteolipids (16 kDa) were also isolated from cotton and shared 95% identity within the coding region (35). The functions of the two similar genes now found in plants from cotton and shared 95% identity within the coding region (18). Furthermore, the first gene which are two other genes that code for the 16-kDa proteolipid, VMA5.

In addition to the V0 sectors in different cell types. Similarly, cell-specific expression of mammalian V1 subunits is also known. There are brain and kidney forms of the B subunit that have been identified in human and bovine. These B subunits share 84% amino acid identity (37, 38). The possibility that different isoforms define functional differences of the V-ATPases is under investigation.

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