

## LUMINAL ACIDIFICATION OF DIVERSE ORGANELLES BY V-ATPase IN ANIMAL CELLS

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### Summary

Eukaryotic cells contain organelles bounded by a single membrane in the cytoplasm. These organelles have differentiated to carry out various functions in the pathways of endocytosis and exocytosis. Their lumina are acidic, with pH ranging from 4.5 to 6.5. This article describes recent studies on these animal cell organelles focusing on (1) the primary proton pump (vacuolar-type

H<sup>+</sup>-ATPase) and (2) the functions of the organelle luminal acidity. We also discuss similarities and differences between vacuolar-type H<sup>+</sup>-ATPase and F-type ATPase. Our own studies and interests are emphasized.

Key words: ATPase, V-ATPase, organelle, endomembrane organelle, proton pump, vacuolar-type ATPase.

### Introduction

Highly differentiated endomembrane (single-membrane-bounded) organelles are involved in the dynamic membrane trafficking processes essential for protein sorting and targeting, receptor-mediated endocytosis, neural or hormonal signal transduction and other processes (Mellman et al., 1986; Goldstein et al., 1985). These organelles include the Golgi apparatus, secretory vesicles, coated vesicles, endosomes, lysosomes and synaptic vesicles. Their lumina are acidic, ranging in pH from 4.5 to 6.5, thus generating a transmembrane electrochemical proton gradient with respect to the near-neutral cytoplasm. The acidic luminal pH is established by a proton-pumping vacuolar-type ATPase (V-ATPase) in combination with ion channels and transporters, whose varying distribution contributes to the organelle-specific luminal contents. The acidic pH is required for organelle functions such as hydrolysis of macromolecules, release of ligands from receptors and processing of preproteins. The proton electrochemical gradient, consisting of the pH gradient ( $\Delta\text{pH}$ ) and/or membrane potential ( $\Delta\psi$ ), provides a driving force for the accumulation of hormones or transmitters into secretory vesicles, synaptic vesicles or synaptic-like microvesicles (microvesicles). A diversity of organelles is ensured by the accurate sorting and transport of the specific components into the lumina or membranes of the corresponding organelles. In this regard, it has become apparent recently that certain organelle-specific membrane protein(s) function in docking/fusion with transport vesicles (Ferro-Novick and Jahn, 1994).

In this article, we briefly summarize recent studies on animal acidic organelles that possess V-ATPase. We include results from our own laboratories and emphasize our interests. Similarities between V-ATPase and F-ATPase (ATP synthase) are also discussed. Information about related fields not mentioned here can be found in reviews (Forgac, 1989; Anraku, 1996; Stevens and Forgac, 1997; Futai et al., 1998).

### Endomembrane organelles with an acidic lumen

#### *Visualization of the acidic luminal pH of endomembrane organelles*

The acidic luminal pH of the endomembrane organelles can be visualized using pH-sensitive fluorescent dyes, such as Acridine Orange and fluorescein isothiocyanate (FITC)-dextran, or by immuno-gold electron microscopy using 3(2,4-dinitroanilino)-3'-amino-*N*-methyl dipropylamine (DAMP) (Anderson and Orci, 1988; Yoshimori et al., 1991). Acridine Orange and DAMP are lipophilic amines, and their non-protonated forms permeate membranes. They become non-permeant once protonated in the acidic lumina of the organelles, yielding pH-dependent distributions. The fluorescence of accumulated Acridine Orange is qualitatively proportional to the pH of the organelle (dark yellow to orange) in mouse or human cell lines (Yoshimori et al., 1991). The organelle pH can be estimated by counting gold particles in immuno-gold electron micrographs using DAMP (Yoshimori

et al., 1991). Lysosomal pH was estimated to be approximately 5 using this method (Yoshimori et al., 1991).

The fluorescence of FITC-dextran incorporated into endosomes or lysosomes can be correlated with the intra-organellar pH by monitoring the excitation wavelength. That the acidic pH is generated by V-ATPase can be confirmed using inhibitors such as bafilomycin and concanamycin (Bowman et al., 1988; Dröse et al., 1993; Ito et al., 1995). The lysosomal pH of a cultured human carcinoma cell line was approximately 4.8, as estimated by FITC-dextran incorporation, and increased to approximately 6 upon treatment with bafilomycin (Yoshimori et al., 1991). The advantage of inhibitors is that they are effective at low concentration and do not inhibit F-ATPase or P-type ATPase (Bowman et al., 1988). Inhibition was prevented by adding the  $V_o$  sector (Hanada et al., 1990) or the  $a$  subunit (Zhang et al., 1994), indicating that bafilomycin inhibits the enzyme by binding to the  $V_o$  sector  $a$  subunit. In contrast to ionophores, bafilomycin does not alter organelle morphology even after prolonged administration (Yoshimori et al., 1991; Umata et al., 1990). However, ionophores such as nigericin can be used for a short-term assay to disrupt  $\Delta$ pH in cultured cells. Analysis using inhibitors supported the hypothesis that an acidic luminal pH is required at the later stage of receptor-mediated endocytosis of epidermal growth factor (Yoshimori et al., 1991).

#### *Organelles with an acidic luminal pH*

At what developmental stage do mammals establish acidic compartments? We studied this question using preimplantation mouse embryos cultured *in vitro* (G.-H. Sun-Wada, Y. Wada and M. Futai, in preparation). Fertilized eggs to eight-cell embryos showed a weak diffuse granular staining with Acridine Orange throughout the cytoplasm. However, in compacted eight-cell embryos and morulae stages, staining became apparent. The staining patterns disappeared immediately when embryos were exposed to a weak base such as ammonium chloride. In the blastocyst, acidic compartments were concentrated exclusively in the perinuclear region of trophectoderm cells, while staining in the inner cell mass was still diffuse. These results suggest that acidic compartments are formed and that their morphologies change during an early developmental stage. The polarized distribution of the compartments may be essential for early mammalian development. Immunofluorescence microscopic analysis using an antibody to subunit  $B$  confirmed that the V-ATPase is localized to these acidic compartments.

Small regions of the plasma membrane, called coated pits, form clathrin-coated vesicles during receptor-mediated endocytosis (Mellman et al., 1986). The regions containing receptors with bound ligand are incorporated into the vesicles. After shedding the clathrin coat, these vesicles form early endosomes and fuse with lysosomes. During exocytosis, vesicles derived from endosomes or from the Golgi network fuse with plasma membranes and secrete neurotransmitters or hormones. We have been interested in the accumulation of

transmitters into neural synaptic vesicles and endocrine cell microvesicles. The electrochemical proton gradient established by the V-ATPase in combination with ion channels or transporters provides the driving force for transporting transmitters into vesicles. Glutamate is accumulated into synaptic vesicles in a manner dependent on membrane potential (Moriyama and Futai, 1990; Moriyama et al., 1990), whereas the transport of monoamines and  $\gamma$ -aminobutyric acid (GABA) is dependent on  $\Delta$ pH (Schuldiner et al., 1995). The acidic interior of these vesicles also resulted in the accumulation of neuron blockers (lipophilic cations), which penetrate the vesicles in a non-protonated lipophilic form and accumulate inside as protonated forms (Moriyama et al., 1993b). This accumulation dissipates the  $\Delta$ pH and inhibits  $\Delta$ pH-coupled transport. Thus, the lipophilic cations inhibit the accumulation of monoamines or GABA, but not glutamate transport driven by membrane potential. Compounds such as 1-methyl-4-phenylpyridinium ( $MPP^+$ ), which is known to cause selective degradation of dopaminergic neurons, are transported specifically by a  $\Delta$ pH-coupled monoamine transporter (Moriyama et al., 1993a). These findings indicate that the acidic compartments are important pharmacologically for considering the mechanisms of action of hydrophobic drugs.

Microvesicles are small organelles (approximately 50 nm in diameter) with a morphology similar to the synaptic vesicles and membrane components that are necessary for docking/fusion with plasma membranes and transmitter accumulation (Moriyama et al., 1996). They have specific transporters coupled to V-ATPase. Examples include pancreatic  $\beta$  cells (GABA; Thomas-Reetz et al., 1993), PC12 cells (acetylcholine; Bauerfeind et al., 1993) posterior pituitary cells (norepinephrine; Moriyama et al., 1995) and pinealocytes (glutamate; Moriyama and Yamamoto, 1995). Microvesicles and synaptic vesicles are similar to each other in protein composition, having V-ATPase, transmitter transporters and factors required for docking with plasma membranes. However, they are not exactly the same. Pinealocyte microvesicles are devoid of synapsin and contain synaptophysin with a slightly different electrophoretic mobility from that of synaptic vesicles (Moriyama and Yamamoto, 1995). Synaptic vesicle protein 2 (SV2) has two known isoforms: SV2A has been shown to be present in neuronal and endocrine cells, whereas SV2B is neuron-specific (Bajjalieh et al., 1994). However, SV2B has been found in pinealocyte and clonal pancreatic  $\alpha$  cells (Hayashi et al., 1998). Thus, the distribution of SV2B does not always define a vesicle as being of neuronal origin. The rates of release of transmitters from the two vesicles are different: the rates are of the order of seconds for microvesicles but only milliseconds for synaptic vesicles (Yamada et al., 1996). The mechanism by which transmitters accumulate into microvesicles and synaptic vesicles are similar: membrane-potential-dependent transport of glutamate into pinealocyte microvesicles (Moriyama and Yamamoto, 1995) or  $\Delta$ pH-driven transport of GABA into pancreatic  $\beta$  cell vesicles (Thomas-Reetz et al., 1993) and of monoamine into

posterior pituitary cells (Moriyama et al., 1995). Studies on the roles of microvesicles in pinealocytes have contributed to our understanding of the roles of glutamate in endocrine cells (Yamada et al., 1996; Moriyama et al., 1996).

V-ATPase has also been localized to the plasma membrane of epithelial cells, including those in the seminal duct (Breton et al., 1996), in osteoclasts (Chatterjee et al., 1992), in kidney proximal tubules (Gluck, 1992) and in bladder (Tomochika et al., 1997). These localizations are consistent with the role of acidic pH in these tissues or cells. Plasma membrane V-ATPase is also present in insect midgut, Malpighian tubules and sensory sensilla (Wieczorek et al., 1999) as well as frog skin (Harvey, 1992). It is of interest to study the biochemical differences between V-ATPase localized to plasma membranes and to endomembrane organelles.

### V-ATPase: catalysis and structure

#### V-ATPase catalysis

The V-ATPase is a primary proton pump with a structure and mechanism similar to those of F-ATPase (Nelson, 1992; Anraku, 1996; Stevens and Forgac, 1997; Futai et al., 1998; Nelson and Harvey, 1999). The V-ATPase functions only as an ATP hydrolase, pumping protons away from the cytoplasmic compartment, whereas the F-ATPase is an ATP synthase driven by an electrochemical proton gradient and functions as an ATPase only in exceptional cases (Futai et al., 1989). ATP hydrolysis by V-ATPase does not follow simple Michaelis–Menten kinetics: the chromaffin granule V-ATPase has three  $K_m$  values (Hanada et al., 1990) and, as for F-ATPase, single-site/multisite catalysis has been suggested from studies of the yeast enzyme (Uchida et al., 1988; Hirata et al., 1989).

It is of interest to know whether V-ATPase is a reversible enzyme. Synthesis of ATP by V-ATPase is dependent on an electrochemical proton gradient, as has been shown in isolated plant vacuoles (tonoplast-enriched vesicles) by measuring the exchange reaction (Façanha and de Meis, 1998) that leads to the incorporation of radioactive phosphate into ATP. ATP synthesis has also been assayed using a luciferin/luciferase

assay (Dupaix et al., 1989; Schmidt and Briskin, 1993). However, detailed studies with inhibitors for related enzymes including V-ATPase, F-ATPase and adenylate kinase are still required. The plant pyrophosphatase has been expressed in yeast vacuoles (Kim et al., 1994). Both V-ATPase and pyrophosphatase are functional in the isolated vacuoles and form electrochemical proton gradients depending on the presence of ATP and pyrophosphate, respectively. We were interested in the pyrophosphatase system to test the reversibility and regulation of V-ATPase. The yeast vacuole could synthesize ATP, depending on the electrochemical proton gradient established by the pyrophosphatase (Hirata et al., 2000). These results indicate that the V-ATPase is a reversible enzyme. The vacuole was a good system for studying the regulation of V-ATPase by  $\Delta pH$  or membrane potential: analysis of  $\Delta pH$  formation with ATP and/or pyrophosphate suggests that V-ATPase is not strictly regulated by proton gradient.

#### Catalytic $V_1$ sector

Like the  $F_1$  and  $F_0$  sectors of F-ATPase, V-ATPase has a ball-and-stalk structure (see, for example, Moriyama et al., 1991; for a review, see Forgac, 1989) and membrane extrinsic and intrinsic sectors, termed  $V_1$  and  $V_0$ , respectively, from the analogy (Fig. 1). The  $V_1$  sector contains catalytic domains, and the  $V_0$  sector constitutes a proton pathway. The  $V_1$  subunits for the catalytic domains are *A* (67–73 kDa), *B* (55–60 kDa), *C* (41 kDa), *D* (34 kDa), the stalk subunits are *E* (33 kDa), *F* (14 kDa), *G* (15 kDa) and *H* (50–57 kDa) and the  $V_0$  subunits are *a* (100–116 kDa), *c''* (19–23 kDa), *d* (38–39 kDa) and *c* and *c'* (14–17 kDa) (Stevens and Forgac, 1997; Arai et al., 1988). The 9.7 kDa  $V_0$  subunit was found recently in insect, human, murine and bovine sources (Merzendorfer et al., 1999). The molecular mass of the  $V_1$  sector is approximately 500 kDa with a stoichiometry of  $A_3B_3C_1D_1E_1F_1G_3H_1$ , whereas the molecular mass of the  $V_0$  sector is approximately 250 kDa with a stoichiometry of  $a_1d_1c''_1(c \text{ and } c')$ . Phenotypes of yeast lacking V-ATPase have been useful for defining the subunits of the enzyme (Anraku, 1996; Stevens and Forgac, 1997):

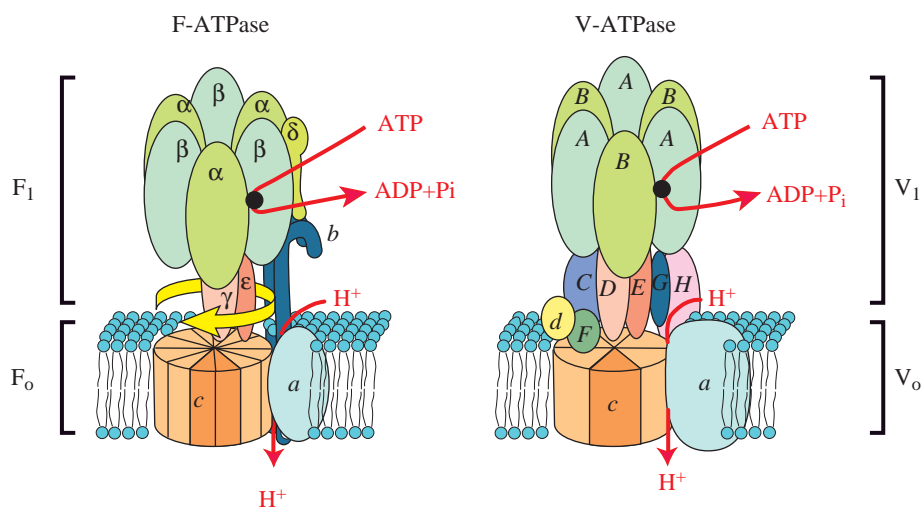
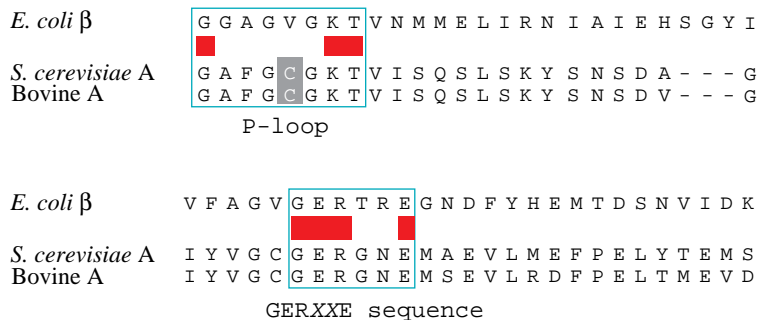


Fig. 1. Structural models of F-ATPase and V-ATPase. Subunit structures and ATP-hydrolysis-dependent  $H^+$  transport by V-ATPase and F-ATPase are shown schematically. Homologous subunits, such as subunits *A* and  $\beta$  of V-ATPase and F-ATPase, respectively, are indicated by the same colours.

Fig. 2. Alignment of the sequence of the F-ATPase  $\beta$  subunit of *Escherichia coli* and the V-ATPase A subunits of *Saccharomyces cerevisiae* and ox. Parts of the sequence of the F-ATPase  $\beta$  subunit and V-ATPase A subunits are aligned. The P-loop (Gly-X-X-X-Gly-Lys-Thr) and the GERXXE sequence (Gly-Glu-Arg-X-X-Glu) are boxed, and conserved residues in both V- and F-ATPase are emphasized with a red bar. The roles of the cysteine residue in the V-ATPase P-loop are discussed in the text; both motifs contain catalytic residues.



mutants show no growth at pH 7.5, failure to accumulate pigment in an *ade2* cell and sensitivity to  $\text{Ca}^{2+}$ . Mutants lacking a V-ATPase can grow at pH 5.5, indicating that the loss of V-ATPase causes conditional lethality (Nelson and Nelson, 1990). Utilizing these phenotypes, yeast subunits and assembly factors have been identified genetically (Stevens and Forgac, 1997; Anraku, 1996). Certain V-ATPase subunits from other eukaryotes, such as *Caenorhabditis elegans* subunit C, have been identified functionally by complementation with yeast mutants (Oka et al., 1998). Most of the  $V_1$  subunits were identified biochemically in mammals (Stevens and Forgac, 1997) and insects (Wieczorek et al., 1999).

Like the  $\alpha$  and  $\beta$  subunits of  $F_1$ , V-ATPase has three pairs of A and B subunits. The catalytic subunit A of V-ATPase shares approximately 25% identity with the  $\beta$  subunit of F-ATPase (Bowman et al., 1992). Subunit A contains an insertion of approximately 100 amino acid residues not found in the  $\beta$  subunit. This insertion reduces the identity between the two subunits and also suggests that structural and functional differences may exist between the two ATPases. Catalytic residues identified in the P-loop (Gly-X-X-X-Gly-Lys-Thr) and the GERXXE sequence (Gly-Glu-Arg-X-X-Glu) of the  $\beta$  subunit are conserved in subunit A (Futai et al., 1989; Futai and Omote, 1996; Omote and Futai, 1998) (Fig. 2). A cysteine residue is conserved in the P-loop (Gly-X-X-X-Cys-Gly-Lys-Thr) of V-ATPase but not in that of F-ATPase. *N*-ethylmaleimide (NEM) modifies this cysteine and inhibits ATPase activity (Feng and Forgac, 1992). The regulatory role of the cysteine was suggested by Feng and Forgac (1994): the P-loop cysteine (Cys261) of the bovine coated-vesicle ATPase forms a disulphide bond with Cys539 of the same subunit and inactivates the enzyme. The two cysteines are predicted to be approximately 1.3 nm apart, suggesting that the distortion of the nucleotide binding site may occur upon disulphide bond formation. F-ATPase lacks the cysteine in the P-loop, consistent with its insensitivity to NEM. However, the introduction of a cysteine into the corresponding position makes the F-ATPase sensitive to NEM (Iwamoto et al., 1994).

Yeast mutagenesis experiments confirmed that the lysine (Lys263) in the P-loop and the glutamate (Glu286) in the GERXXE sequence are catalytically essential (Liu et al., 1997). In the proposed mechanism of F-ATPase catalysis, the lysine binds the  $\beta/\gamma$  phosphate of ATP, and the glutamate activates a water molecule (Abrahams et al., 1994; Futai and Omote, 1996). The positive charge of the arginine in GERXXE is also

required for catalysis, and the last glutamate of the GERXXE sequence is essential for catalytic cooperativity in F-ATPase (Futai and Omote, 1996). The conservation of these residues suggests that V-ATPase may have similar catalytic mechanism. The  $V_1$  subunit B is a counterpart of the  $F_1$   $\alpha$  subunit, and they share approximately 25% identity (Bowman et al., 1992; Inatomi et al., 1989); the  $\alpha$  subunit residues required for the catalytic cooperativity of the  $F_1$  sector are conserved in the B subunit of the  $V_1$  sector. The  $V_1$  subunit corresponding to the  $\gamma$  subunit of  $F_1$  is not known, although  $V_1$  subunit D (yeast Vma8p) may be the counterpart, as suggested by their predicted structural similarity (Nelson et al., 1995; Graham et al., 1995).

#### Proton channel $V_o$ sector

The  $V_o$  sector consists of at least four different subunits. The extremely hydrophobic  $V_o c$  subunit (16 kDa), also called the proteolipid because of its lipophilicity, has been cloned from yeast *Saccharomyces cerevisiae* (Nelson and Nelson, 1989; Umemoto et al., 1991), *Caenorhabditis elegans* (Oka et al., 1997, 1998), *Manduca sexta* (Dow et al., 1992), *Drosophila melanogaster* (Finbow et al., 1994), ox (Mandel et al., 1988), mouse (Hanada et al., 1991) and human (Hasebe et al., 1992). It is the counterpart of the  $F_o c$  subunit (8 kDa). The amino and carboxyl halves of the  $V_o c$  subunit are homologous, suggesting that the subunit may have resulted from a duplication of an ancestral gene (Nelson, 1992; Mandel et al., 1988). The  $c$  subunit has four hydrophobic transmembrane  $\alpha$  helices (I, II, III, IV), and the amino (I, II) and carboxyl (III, IV) domains are individually homologous to the  $F_o c$  subunit. However, unlike the  $F_o c$  subunit, the  $V_o$  amino-terminal domain does not have glutamate or aspartate in the middle of domain II. The glutamate is only present in domain IV, where it is critical for proton translocation, because it could not be replaced by other amino acid residues including glutamine (Noumi et al., 1991). Domain IV has the highest degree of sequence conservation of any domain in the  $c$  subunits so far sequenced, supporting its essential role in proton pumping. Six copies of the  $c$  subunit are present in the  $V_o$  complex (Stevens and Forgac, 1997); thus, 24 transmembrane  $\alpha$  helices from the six  $c$  subunits are present in  $V_o$ , whereas the same number of  $\alpha$  helices in  $F_o$  requires 12 copies of the  $c$  subunit, as clearly confirmed recently (Jones and Fillingame, 1998). The V-ATPase proteolipid (subunit  $c$ ) has also been suggested to be a component of the insect gap junction (Finbow et al., 1994).

$V_o$  has another proteolipid (subunit  $c''$ , 23 kDa) found in yeast (Hirata et al., 1997), *C. elegans* (Oka et al., 1997), mouse (G. H. Sun-Wada, Y. Wada and M. Futai, in preparation) and human (Nishigori et al., 1998). The sequence of this proteolipid can be aligned with the  $V_o c$  subunit except that an additional 50 amino-terminal residues are present. Like the  $c$  subunit, the amino- and carboxyl-terminal halves of the 23 kDa subunit share homology. This protein has five putative transmembrane domains. *C. elegans* subunit  $c''$  (*vha-4*) exhibited 52% identity with yeast *Vma16p* and 64% with a human homologue (ATP6F). A mouse homologue is 96% identical with ATP6F. A glutamate residue in the middle of the third transmembrane segment is conserved in all proteolipids so far sequenced. Despite the homology with the  $c$  subunit, the *VMA16* gene coding for the 23 kDa proteolipid is also essential for the activity of yeast V-ATPase (Hirata et al., 1997). No isoform for the  $c''$  subunit is found in yeast and *C. elegans*, in which the entire genomes have already been sequenced.

The  $V_o$  subunit  $a$  is a large protein (116 kDa in mammals and 100 kDa in yeast) (Stevens and Forgac, 1997). This subunit may be a counterpart of  $F_1$  subunit  $a$ , which is proposed to function as a part of the stator in  $F_1F_o$  (Junge et al., 1997). This subunit can be divided into two domains; a hydrophilic amino-terminal domain and a carboxyl half forming 6–7 transmembrane domains. V-ATPase subunit  $G$  shows significant homology with the  $b$  subunit of  $F_o$  (Hunt and Bowman, 1997). Subunit  $b$  has a single membrane-spanning domain and an extra-membrane domain, both possibly  $\alpha$

helices, and is suggested to serve as a stator that holds the  $\alpha_3\beta_3$  complex in place while the  $\gamma\epsilon c$  complex rotates. However, V-ATPase subunit  $G$  apparently lacks a membrane-spanning domain. Thus, if the  $G$  and  $b$  subunits play the same role, the  $G$  subunit should form a complex with a hydrophilic domain of the  $a$  subunits of  $V_o$  and also  $V_1$  subunit(s). From the sequence identities, it is not easy to identify the counterparts of  $F_1 \delta$  and  $\epsilon$  subunits that may interact with  $G$ .

Overall structural similarities (Fig. 1) suggest that V- and F-ATPase utilize the same basic mechanisms for ATP hydrolysis, proton translocation and energy coupling. Thus, the mechanism of action of V-ATPase may include the rotation of the subunit complex during ATP-dependent proton translocation, as shown for the F-ATPase (Noji et al., 1997; Junge et al., 1997; Omote et al., 1999).

### Heterogeneity of V-ATPase

As described above, V-ATPase pumps protons into diverse endomembrane organelles to generate their unique acidic luminal pH, and the same enzyme in the plasma membranes of epithelial cells acidifies external compartments, such as those in the urinary bladder (Tomochika et al., 1997), and alkalizes other compartments, such as those in caterpillar midgut (Wieczorek et al., 1999). It is tempting to speculate that different V-ATPase subunit isoforms contribute to the organelle-specific luminal pH, and are localized to the plasma membrane to acidify or alkalize the external cytosol. Since V-

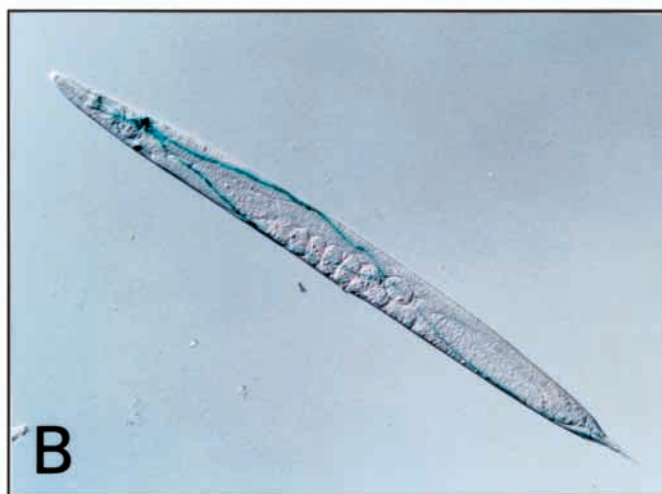
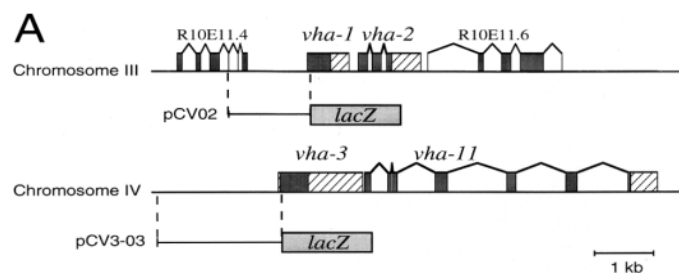


Fig. 3. The *Caenorhabditis elegans vha-1*, *vha-2* and *vha-3* genes coding for the  $c$  subunits (16 kDa proteolipid). (A) Gene organization of *vha-1*, *vha-2* and *vha-3* genes. Filled and hatched boxes represent coding and untranslated regions, respectively. (B) Expression of the *vha-1* gene in the H-shaped excretory cell. Expression of the *vha-1::lacZ* fusion gene (pCV02) in a young adult transgenic animal is shown. *lacZ* expression is seen in the H-shaped excretory cell. (C) Expression of the *vha-3* gene in gastric and intestinal cells. The *lacZ* reporter gene (pCV3-03) is under the control of a 2.2 kilobase (kb) upstream regulatory region of *vha-3*. *vha-11* is the gene for subunit  $C$ . See text and Oka et al. (1997, 1998) for more details.

ATPase is a complicated multisubunit enzyme, changing a single subunit may affect the properties of the entire enzyme. Thus, the roles of V-ATPase subunit isoforms are of interest and should be studied carefully.

#### *Isoforms of the c subunit*

Two genes (*VMA3*, *VMA11*) code for the yeast *c* and *c'* subunits, respectively, and their products are 56% identical in amino acid sequence (Anraku, 1996). They are not redundant genes, and Vma3p and Vma11p are both essential for the activity of V-ATPase, indicating that they are subunits of the same enzyme (Umemoto et al., 1991). We have detected three *C. elegans* genes coding for the *c* subunit (Fig. 3). Two of them (*vha-1*, *vha-2*) form an operon on chromosome III (Oka et al., 1997), and the third gene (*vha-3*) and the subunit *C* gene (*vha-11*) form a cluster on chromosome IV (Oka et al., 1998). The *vha-1* gene product is 66% identical to that of *vha-2* or *vha-3* and 61% identical to that of yeast Vma3p. Since the *vha-1* and *vha-2* gene products share equal similarities with the Vma3p and Vma11p proteins, we cannot conclude that the two yeast genes correspond to the *C. elegans* counterparts. *vha-2* and *vha-3* produce an identical protein, although the nucleotide sequences of their coding regions differ by 15%. In this regard, two cDNAs for closely similar proteolipids have been isolated from cotton, and their coding region shared 95% identity (Hasenfratz et al., 1995).

Northern blot analysis indicated that three proteolipid genes are expressed in a similar pattern during the life cycle of *C. elegans* (Oka et al., 1998). However, studies with transgenic worms indicated that the genes are expressed differently in a cell-specific manner. The *vha-1* and *vha-2* genes were expressed in most of the larval cells, but predominantly in an H-shaped excretory cell in the adult (Oka et al., 1997). The H-shaped cell is a large mononuclear cell with bilateral excretory canals extending along the length of the worm's body. The cell body forming a bridge between the two canals is positioned on the ventral epidermal ridge slightly posterior to the nerve ring. The expression pattern of the *c* subunit was similar to that of the subunit *c''* proteolipid coded by the *vha-4* gene and of the  $V_1$  sector *B* subunit, indicating that an entire V-ATPase is highly expressed in an H-shaped cell. Immunohistochemical localization of the subunit *C* protein confirmed the high level of expression of V-ATPase in the H-shaped cell (T. Oka and M. Futai, in preparation). A P-glycoprotein (PGP-3) is also predominantly expressed in the H-shaped cell, and the *pgp-3* deletion mutant is sensitive to colchicine and chloroquine, suggesting that the P-glycoprotein exports toxic compounds or metabolic waste from the H-shaped cell to the exterior (Broeks et al., 1995). Thus, the V-ATPase that is highly expressed in the H-shaped cell may be supporting such a transport function.

The *vha-3* gene was mainly expressed in gastrointestinal and hypodermal cells in addition to the H-shaped cell (Oka et al., 1998). In contrast, *vha-2* was highly expressed in the H-shaped cell and was not detectable in gastrointestinal and hypodermal cells even after prolonged staining with the reporter gene

product. These results strongly suggest that expression of the two *vha* genes is differentially regulated according cell type.

The presence of cell-specific isoforms in *C. elegans* prompted us to survey the *c* subunit isoforms in mammals. However, only one gene was found for the human *c* subunit, although three pseudogenes were identified (Hasebe et al., 1992). Similarly, two pseudogenes were identified for mouse, and Southern blot analysis suggested that no closely related homologue was present (T. Noumi, H. Inoue and H. Kanazawa, in preparation). It should be noted that negative results in the blotting analysis do not always indicate absence of a gene. In this regard, the presence of the *C. elegans* *vha-3* isoform could be deduced from Southern blotting with the reading frame of *vha-2* as a probe, but not with that of *vha-1* (Oka et al., 1998).

#### *Kidney-type and ubiquitous isoforms of the B subunit*

Two cDNAs encoding homologous but distinct *B* subunits ( $B_1$ ,  $B_2$ ) have been cloned from ox, human and mouse (Nelson et al., 1992). Their amino acid sequences share approximately 80% identity: the  $B_1$  subunit is known to be a kidney-type subunit, whereas  $B_2$  is ubiquitous. The V-ATPase in kidney is responsible for acid secretion by distal nephrons, and the  $B_1$  subunit is found at the apical surface of acid-secreting cells. Karet et al. (1999) identified 15 different mutations (premature termination, frameshift, missense substitutions and splice site mutations) in the human *ATP6B1* gene coding for the  $B_1$  subunit. All the altered residues in the missense mutations are conserved in the *B* subunit homologues from human to *Neurospora crassa* (Bowman et al., 1992). Patients with *ATP6B1* mutations had distal renal tubular acidosis, a condition characterized by impaired renal acid secretion and resulting in metabolic acidosis (Karet et al., 1999). Patients also had sensorineural hearing loss: consistent with this finding, expression of *ATP6B1* in the cochlea and endolymphatic sac has been demonstrated in mouse. These results suggest that expression of *ATP6B1* is required for endolymph pH homeostasis and normal auditory function.

#### *Isoforms of subunit a*

Yeast has two genes, *VPH1* and *STV1*, coding for isoforms of  $V_0$  subunit *a* (Manolson et al., 1992, 1994). Their gene products (Vph1p and Stv1p) are found in distinct compartments, probably vacuoles and endosomes/Golgi network, respectively. On the basis of the sequence similarity, four candidate genes for *C. elegans* subunit *a* could be found in its genome (T. Oka, unpublished results). These subunit *a* isoforms may be responsible for the organelle- or cell-specific localization.

Three subunit *a* isoforms seem to be present in mammals: the subunit for coated-vesicles from bovine brain (Peng et al., 1994), the protein for which cDNA was originally cloned as coding for a putative immune regulator from murine T cells (Lee et al., 1990) and a putative subunit from human osteoclast (Li et al., 1996). Sequence divergence between the three subunits is more than that expected from species differences.

We have cloned mouse cDNAs coding for three subunit *a* isoforms, termed *a1*, *a2* and *a3*, coding for polypeptides of 832, 856 and 834 amino acid residues, respectively (T. Toyomura, T. Oka, C. Yamaguchi, Y. Wada and M. Futai, in preparation). Their amino acid sequences are approximately 50% identical: the homology is higher in the hydrophobic region of the carboxyl-terminal half and weak in the amino-terminal half between residues 70 and 300. It is also noteworthy that the regions between residues 660 and 720 have essentially no homology. These regions may contribute to the tissue- or organelle-specific distributions. All three proteins seem to have similar topologies, giving 6–9 transmembrane domains (Stevens and Forgac, 1997; Leng et al., 1999). Immunoblots have indicated different tissue distributions of the three isoforms. It should be noted that the subunit *a* isoforms have been found in organisms ranging from yeast to mammals.

The results of immunoreactivity and northern blotting suggest that the distribution of mammalian V-ATPase subunit isoforms is different among cells and among organelles. We may further speculate that the subunit isoform determines the specific location of V-ATPase. Tissue-specific isoforms of the bovine subunit *a1*, formed by alternative RNA splicing, have also been identified (Peng et al., 1994).

#### V-ATPase as a physiologically essential enzyme

Is V-ATPase essential for the growth of higher eukaryotes? Yeast cells lacking V-ATPase cannot grow at neutral pH but can grow at acidic pH. Growth at acidic pH may be explained because an acidic luminal pH in the organelles can be generated by fusion with pinocytotic vesicles containing acidic fluid (Nelson and Nelson, 1990). These results suggest that V-ATPase is not essential for yeast growth as long as the medium is acidic.

Higher animals, including mammals, are not likely to survive without V-ATPase because it is ubiquitously expressed in tissues and physiologically important for various functions. Thus, deletions of only a tissue-specific protein such as the *B1* subunit are likely to result in a viable phenotype (Karet et al., 1999). However, it is not known at what stage of animal development the defective V-ATPase has its lethal effect.

The obvious test to answer this question is to create an animal lacking an essential V-ATPase subunit. We were interested in the *vha-11* gene coding for V<sub>1</sub> subunit *C* of *C. elegans*. The VHA-11 protein exhibits 37, 56 and 56% sequence identity with the *C* subunit of yeast (*Vma5p*), ox and human, respectively (Oka et al., 1998). The yeast *Vma5p* protein is essential for growth at neutral pH, and only one corresponding gene is found in the *C. elegans* genome. Expression of *vha-11* completely restored the growth of the yeast *vma5* mutant, confirming that *vha-11* codes for a functional subunit *C*. The transgene with the *vha-11* promoter region was expressed in an adult H-shaped cell, as for other V-ATPase subunits. Thus, it should be obvious that the disruption of this gene would affect the animal seriously. Gene function in *C. elegans* is conveniently disrupted using RNA interference

(RNAi) by double-stranded RNA (Montgomery et al., 1998). The double-stranded RNA has been shown to target and disrupt the corresponding gene activity. When we introduced double-stranded RNA corresponding to the *vha-11* transcript into the adult gonad, no live offspring were obtained from the animal (T. Oka and M. Futai, in preparation). These results suggest that V-ATPase, and therefore an acidic organelle lumen, is essential for the hatching of the worm.

The first knock-out of V-ATPase in an animal was obtained from *Drosophila melanogaster*. A fly mutant with lethal P-element insertions in the *vha55* gene for the *B* subunit was identified by Davies et al. (1996). Deletion of the *B* subunit locus was shown to be lethal, whereas point mutations gave varying phenotypes that ranged from lethal to surviving flies. These results suggest that V-ATPase in organelles or plasma membranes is essential for the development from larva to fly.

The same question can be asked regarding mammalian V-ATPase. The mouse subunit *c* gene (PL16) is an appropriate target to knock out because no isoforms have been found. Chromosomal PL16 was replaced by the neomycin-resistance gene using target-directed mutagenesis (Inoue et al., 2000). No PL16<sup>-/-</sup> mouse has been identified at birth, while PL16<sup>+/-</sup> embryos were viable and showed no noticeable abnormality. These results indicate that the homozygous loss of the *c* subunit gene caused embryonic lethality. However, the PL16<sup>-/-</sup> embryo could develop into the blastocyst stage *in vitro* (G. H. Sun-Wada, Y. Wada and M. Futai, in preparation). Furthermore, the mutant blastocysts formed acidic compartments similar to those of the wild type, suggesting that the maternal mRNA is sufficient for establishing an acidic compartment up to the blastocyst stage. Embryos of the same stage were also detected *in vivo*, but none was attached to the uterine epithelium after 7 days *post coitus*. These results suggest that V-ATPase or acidic organelles are essential immediately before gastrulation in mammals.

It is also of great interest to disrupt V-ATPase in specific cells or tissues using a conditional knock-out system. An initial attempt has been made to rescue a PL16<sup>-/-</sup> mouse by introducing a transgene for subunit *c*, and to knock out the transgene in defined cells at specific developmental stage. These experiments are currently in progress (T. Yoshimizu, G. H. Sun-Wada, Y. Wada and M. Futai, in preparation).

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