Journal of Bioenergetics and Biomembranes, Vol. 35, No. 4, August 2003 (© 2003)

Vacuolar H^+ Pumping ATPases in Luminal Acidic Organelles and Extracellular Compartments: Common Rotational Mechanism and Diverse Physiological Roles

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Cytoplasmic organelles with an acidic luminal pH include vacuoles, coated vesicles, lysosomes, the Golgi apparatus, and synaptic vesicles. Acidic compartments are also known outside specialized cells such as osteoclasts. The unique acidic pH is formed by V-ATPase (Vacuolar type ATPase), other ion transporters, and the buffering action of proteins inside the organelles. V-ATPase hydrolyzes ATP and transports protons inside an organelle or extracellular compartment. We have summarized recent progress on mouse V-ATPases and their varying localizations together with their mechanism emphasizing similarities with F-type ATPases.

KEY WORDS: V-ATPase; ATP synthase; proton pump; osteoclast; subunit isoform; rotational catalysis; synaptic vesicle.

INTRODUCTION

Various organelles with a luminal acidic pH are found in the cytoplasm of eukaryotic cells (for reviews, see Anraku, 1996; Futai *et al.*, 2000; Nelson and Harvey, 1999; Nishi and Forgac, 2002; Stevens and Forgac, 1997). They include vacuoles, coated vesicles, endosomes, lysosomes, the Golgi apparatus, and chromaffin granules. Acidic compartments are also formed outside specialized cells, an example being the resorption lacuna formed between the osteoclast plasma membrane and the bone surface. The unique acidic pH of lumens of organelles or extracellular compartments are formed by vacuolar type H^+ ATPases (V-ATPases), which translocate protons across membranes coupled with ATP hydrolysis. Similar to F-ATPase (ATP synthase, F_1F_0), V-ATPase comprises a membrane extrinsic domain (V_1) and a transmembrane proton pathway (V_0) (Fig. 1). Yeast V_1 is formed from *A, B, C, D, E, F, G*, and *H* subunits, and V_0 from *a*, *c*, *c′*, *c″*, and *d* (Anraku, 1996). In other criteria, both ATPases are formed from a catalytic hexamer ($\alpha_3\beta_3$ or A_3B_3), stalks, and a proton pathway.

A mutant lacking each subunit gene cannot grow at neutral pH (*vma* phenotype), possibly because of defective organelle acidification. The subunit structure of mammalian V-ATPases are the same as that of the yeast one except that *c′* is not present. V-ATPases are functionally conserved during evolution, as evidenced by the complementation of yeast *Vma* mutants with mammalian (Imai-Senga *et al.*, 2002; Murata *et al.*, 2002; Sun-Wada *et al.*, 2002, 2003) or *Caenorhabditis elegans* (Oka *et al.*, 1998) cDNA coding for the corresponding subunits.

Yeast V-ATPase is an assembly of 13 different subunits (Anraku, 1996), whereas minimal F-ATPases such as those from bacteria and chloroplasts are formed from eight ($\alpha_3\beta_3\gamma\delta\varepsilon ab_2c_{10}$) and nine subunits (Futai *et al.*, 1989), respectively. Despite the differences in physiological roles between F and V-ATPase, corresponding subunits have been assigned based on their limited amino

Key to abbreviations: V-ATPase, vacuolar H^+ ATPases (V_0V_1); F-ATPase, ATP synthase (F_0F_1); dpc, days post-coitus.

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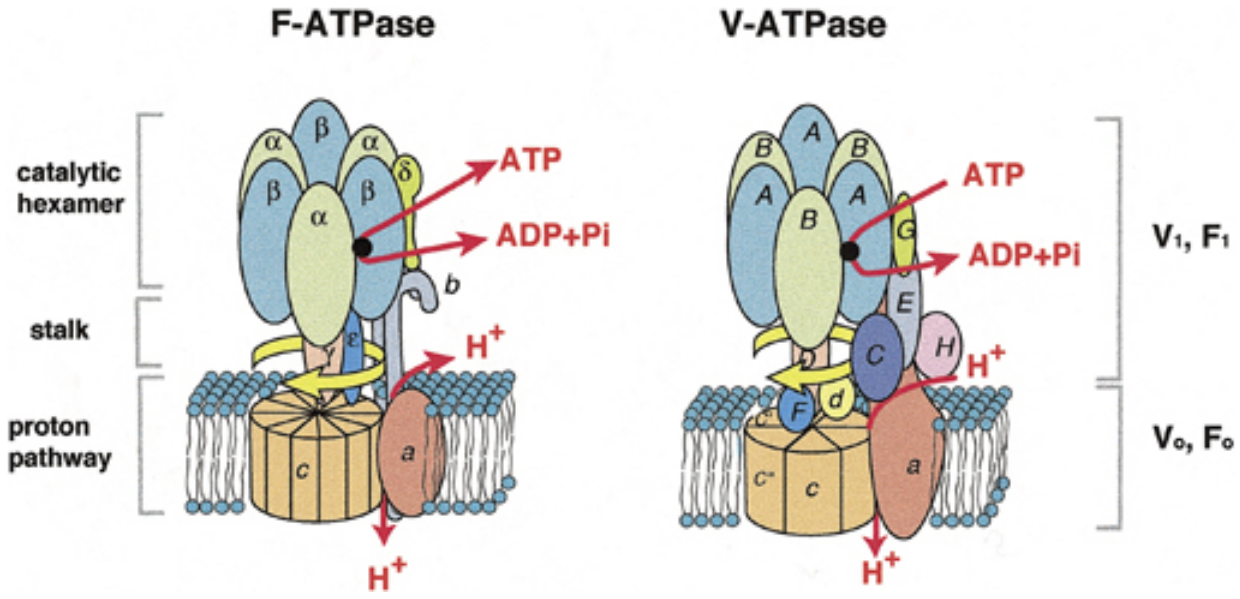

Fig. 1. Subunit organization of V-ATPase and F-ATPase. The structures of V-ATPase and F-ATPase are schematically shown together with catalysis and proton transport. Membrane intrinsic (V_0, F_0), and peripheral (V_1, F_1) sectors, the catalytic hexamer, stalk regions, and proton pathway are indicated. Corresponding subunits between F- and V-ATPase are shown in the same colors.

acid sequence homology: *A* and *B* of the V_1 catalytic hexamer correspond to β and α of F_1, respectively, and V_0 *c* is a duplicated form of F_0 *c*. Thus, it is of interest to determine whether or not the catalysis and proton translocation mechanisms of V-ATPase are different from those of F-ATPase.

Although V-ATPase is obviously a ubiquitous proton pump found in almost all mammalian cells, it shows unique physiological roles depending on its localization in organelles or plasma membranes. Thus, V-ATPase may have structural information for its localization to a specific membrane to play its roles. Furthermore, the luminal pH of organelles or compartments vary between 4.5 and 6.5, indicating that they may have different mechanisms for maintaining their unique acidic environments. The mechanisms may include biochemical differences of V-ATPases, their densities in membranes, or coupling with ion or solute transporters localized in the same membrane. An interesting question is whether or not the V-ATPases in a variety of membranes differs in catalysis, proton transport, and subunit structure. This question can be asked more specifically as: "Is the osteoclast plasma membrane V-ATPase the same as those in endomembrane organelles including lysosomes and the Golgi apparatus?", or "Is V-ATPase localized in the kidney epithelial cell plasma membrane the same as in osteoclast?" It may be reasonable to assume that the V-ATPase localizations are determined by V_0 sector subunits through interactions with other membrane proteins. The transmembrane V_0 subunits may also sense and establish the unique luminal pH of specific organelles. Thus, studies on V_0 subunits or possibly their isoforms, or those interacting with V_0 should be fruitful for answering these pertinent questions. Consistent with this notion, isoforms have been found recently for mouse and *C. elegans* subunits of the V_0 sector and the stalk region of V_1 (Imai-Senga *et al.*, 2002; Murata *et al.*, 2002; Sun-Wada *et al.*, 2002, 2003).

In this article, we discuss the common mechanisms and the physiological roles of V-ATPases, with special reference to subunit isoforms and F-ATPase. Our discussion is also closely related to the basic questions in cell biology including those regarding the biogenesis of organelles or plasma membranes. We intend to emphasize studies in our own laboratory, and other aspects of V-ATPase not discussed in this article can be found in the reviews previously published (Anraku, 1996; Futai *et al.*, 2000; Nelson and Harvey, 1999; Nishi and Forgac, 2002; Stevens and Forgac, 1997) and elsewhere in this mini-review series.

COMMON MECHANISM FOR CATALYSIS AND PROTON TRANSLOCATION BY V-ATPase

Catalytic Site and Proton Translocation

The high resolution X-ray structure of bovine F_1 revealed that three copies of the α and β subunits

are located alternately around the amino and carboxyl terminal α helices of the γ subunit (Abrahams *et al.*, 1994). The three β subunits observed in the structure may correspond to different catalytic stages of the enzyme: βT, ATP-bound form; and βD, ADP-bound; βE, no nucleotide. The ATP hydrolysis by F_1 exhibits three different K_m values for ATP, i.e. 10^{-9}, 10^{-6}, and 10^{-4}M, respectively (Futai *et al.*, 1989). The rate for single site (unisite) catalysis is very low ($10^{-3}s^{-1}$), but it is enhanced $\sim 10^5$-fold upon ATP binding to two other sites (multisite catalysis). Paul Boyer proposed a binding change mechanism from extensive kinetic studies (Boyer, 1997). In the mechanism, the product release from one catalytic site is stimulated upon substrate binding to the other sites. The F-ATPase catalytic site is formed from the β subunit glutamates, arginine and threonine (Fig. 2(a)) (Futai and Omote, 1996). It is striking that these β subunit catalytic residues are

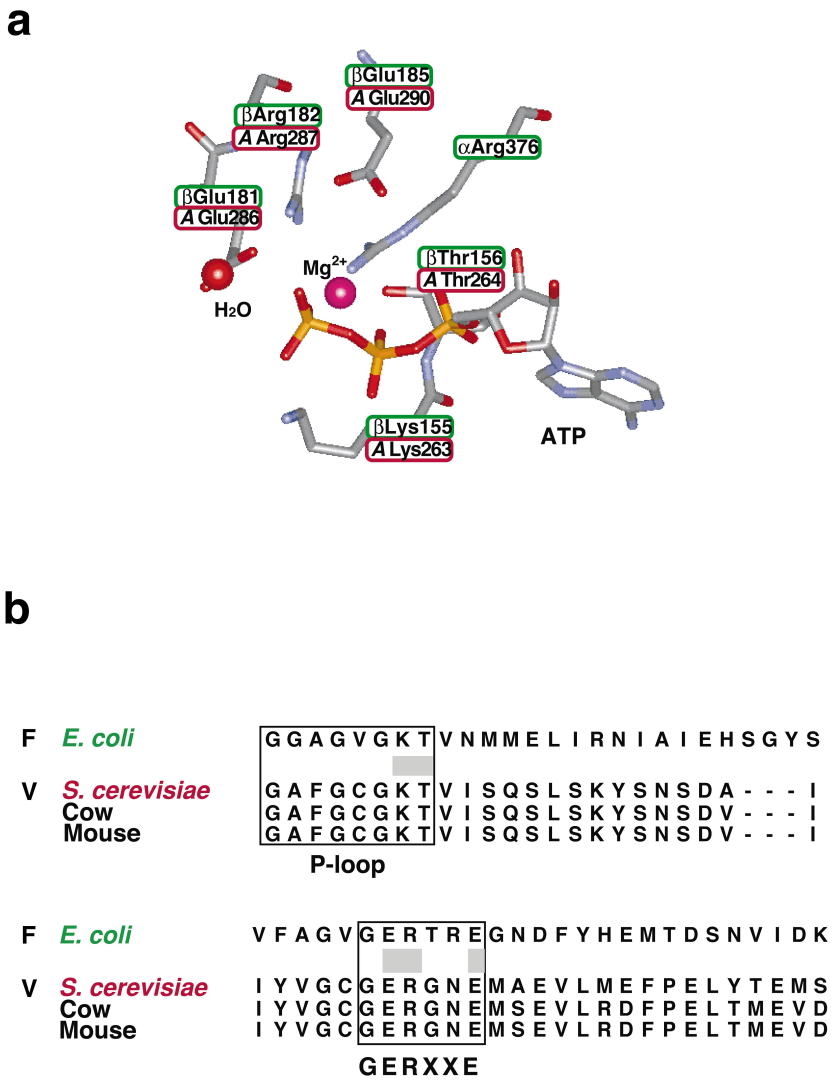

Fig. 2. Catalytic site of F-ATPase and its conservation in V-ATPase. (a) Catalytic site residues of F-ATPase are shown with bound ATP. The structure is from bovine X-ray analysis (Abrahams *et al.*, 1994) and catalytic residues (in green boxes) are numbered following the *E. coli* enzyme (Omote and Futai, 1996). The corresponding yeast V-ATPase residues are also shown (in red boxes) (Liu *et al.*, 1997; MacLead *et al.*, 1998). (b) Conservation of catalytic residues in V- and F-ATPase. Alignment of the *E. coli* β subunit of F-ATPase, and the *A* subunit of V-ATPase from yeast, cow, and mouse is shown. The P-loop (glycine-rich sequence) and conserved GERXXE motif are shown.

conserved in the V-ATPase *A* subunit of the A_3B_3 hexamer (Fig. 2 (a) and (b)). The results of mutational studies on the *A* subunit residues are consistent with their catalytic roles (Liu *et al.*, 1997; MacLead *et al.*, 1998). Kinetic studies indicated that V-ATPase exhibits three K_m values (5 μM, 30 μM, and 300 μM), but its catalytic cooperativity is much lower than that of F-ATPase (Hanada *et al.*, 1990).

Mutation studies have also indicated that the proton pathway for *E. coli* F-ATPase is formed from Asp-61 and Arg-210 of the *c* and *a* subunit, respectively (Cain and Simoni, 1989; Eya *et al.*, 1991; Fillingame *et al.*, 2000). The *c* subunit, also called the 8 kDa proteolipid, is formed from two transmembrane helical domains connected by a cytoplasmic loop. The Asp-61 residue is located in the middle of the carboxyl terminal α helix, and 10–14 copies (c_{10-14}) of the *c* subunit form a ring structure (Girvin and Fillingame, 1994; Jiang *et al.*, 2001). More complicated proton pathway of yeast V_0 is formed from copies of the *c* (Umemoto *et al.*, 1991), *c*′ and *c*″ (Hirata *et al.*, 1997) subunits together with the *a* and *d* subunits. Subunits *c* and *c*′, encoded by the yeast *VMA3* and *VMA11* genes, respectively (Hirata *et al.*, 1997; Umemoto *et al.*, 1991), are a duplicated form (16 kDa) of the ancestral *c* subunit, and show 56% identity with each other in amino acid sequence. V_0 may form a ring structure from *c*, *c*′, and *c*″(possibly $c_4c'c''$) similar to that of F-type ATPase, and the Glu residue for proton transport is located on the fourth helix of the *c* and *c*′ subunits (Glu-137 and Glu-145, respectively), and in the third helix of the *c*″ (Glu-188) subunit, indicating that V_0 and F0 have 6 and 10–14 proton translocating residues, respectively. Although V_0 and F_0 subunit *a* do not exhibit significant sequence homology, mutations of the Arg-210 and Arg-735 residues, respectively, abolished proton translocation (Cain and Simoni, 1989; Eya *et al.*, 1991; Fillingame *et al.*, 2000; Kawasaki-Nishi et al., 2001). Thus, the glutamate of the *c*, *c*′ and *c*″ subunits, and arginine of the *a* subunit participate in proton transport, similar to F-ATPase having functional aspartate and arginine residues in the *c* and *a* subunits, respectively.

Only a single gene has been found for the human (Hanada *et al.*, 1991) and mouse (Hayami *et al.*, 2001) *c* subunits (also called the proteolipid or 16 kDa subunit), although several homologous pseudogenes have been found in both organisms. The mouse protein exhibites 83% and 84% sequence similarity to those of the yeast (Umemoto *et al.*, 1991) and *C. elegans* (Oka *et al.*, 1997) proteins, respectively, and the conserved essential glutamate is implicated in proton transport (Noumi *et al.*, 1991). These results suggest that mammals have only two proteolipid subunits corresponding to *c* and *c*″ of yeast. Thus, a knockout mouse blastocyst with a disrupted *c* subunit gene (*Atp6V*$_0$*c*) dies before its implantation into the uterine membrane (Sun-Wada *et al.*, 2000). Naturally, the blastocysts lack organelles with an acidic luminal pH, resulting in an altered organellar morphology.

Rotational Catalysis

Consistent with the binding-change mechanism, the γ subunit occupying the central space of the $\alpha_3\beta_3$ hexamer of F-ATPase rotates, interacting alternately with the three β subunits (Noji *et al.*, 1997; Omote *et al.*, 1999). Furthermore, we (Sambongi *et al.*, 1999), and Junge and coworkers (Pänke *et al.*, 2000) have shown continuous rotation of a complex of the γ and *c* ring of purified F-ATPase, observing rotation of an actin filament connected to the *c* subunit, when the $\alpha_3\beta_3$ hexamer was immobilized on a glass surface. Similarly, rotation of the $\alpha_3\beta_3$ hexamer was observed when the *c* ring was immobilized (Tanabe *et al.*, 2001). Finally, the rotation of the *a* subunit or $\alpha_3\beta_3$ hexamer relative to the *c* ring has been shown in membranes (Nishio *et al.*, 2002). These results clearly indicate that F-ATPase is a motor enzyme of which the rotor and stator are interchangeable.

Although V-ATPase is significantly different from F-ATPase in structure, kinetics and physiological roles, it has been speculated that V-ATPase has a rotary mechanism (Futai *et al.*, 2000; Nishi and Forgac, 2002). We were prompted to examine this possibility, by introducing a histidine-tag and a biotin-tag to the *c* and *G* subunits, respectively, to immobilize V-ATPase on a glass surface (Hirata *et al.*, in press) (Fig. 3). Upon the addition of ATP, we observed continuous counter clockwise rotation of an actin filament connected to the *G* subunit of V-ATPase. Although a stoichiometry of two has been determined for subunit *G* (Xu *et al.*, 1999), rotation was observed in case that the enzyme contained only single actin filament. It is possible that physical perturbation may occur if the enzyme connects with two actin filaments simultaneously. This rotation is inhibited by concanamycin, a specific V-ATPase inhibitor, but not by azide, an inhibitor of F-ATPase. Since bafilomycin, a similar antibiotic to concanamycin, has been shown to bind to V_0 (Hanada *et al.*, 1990), possibly its subunit *a* (Zhang *et al.*, 1994), the rotation of the *a* subunit was obviously blocked by the tightly bound antibiotic. These results suggest that V-ATPase and F-ATPase carry out similar rotational catalysis. However, the different structures of the membrane sectors (ab_2c_{10-14} and $ac_4c'c''d$ for *E. coli* F_0 and yeast V_0, respectively) and stators of the two enzymes (Fig. 1) suggest that their individual rotation mechanisms may be different.

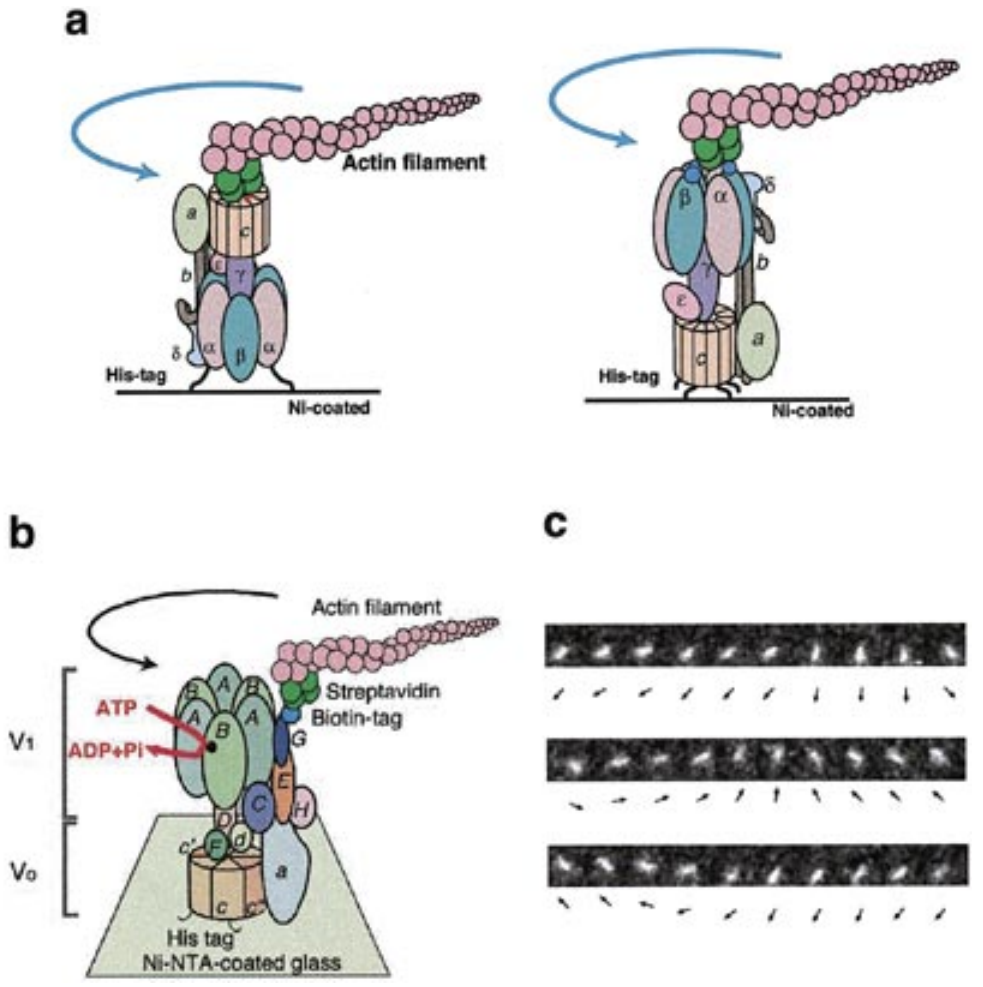

Fig. 3. Rotational catalysis of F-ATPase and V-ATPase. (a) Two experimental systems used for observing rotational catalysis of F-ATPase. F-ATPase was immobilized through a His-tag attached to the α subunit or the *c* subunit, and an actin filament was connected to the *c* or α subunit for the rotation assay. The directions of rotation are indicated. (b) Experimental system used for observing rotational catalysis of V-ATPase. V-ATPase was immobilized on a glass surface through a His-tag introduced into the *c* subunit, and an actin filament was attached to the biotin-tag connected to the *G* subunit. (c) Rotation of an actin filament, connected to the *G* subunit. Upon addition of ATP, counter clockwise rotation of the filaments was observed. Typical sequential video images are shown (10 ms interval), the direction of the actin filaments being shown schematically by arrows below the video images.

SUBUNIT ISOFORMS IN MEMBRANE SECTOR V_0

Diverse Proteolipid Subunits

The *c* subunit of F_0, and the *c*, *c*′, and *c*″ of V_0 are also called proteolipids. As discussed above, two yeast genes, *VMA3* and *VMA11*, encode *c* and *c*′ subunits respectively, and the *VMA16* gene encodes another 23 kDa proteolipid *c*″ in *Saccharomyces cerevisiae* (Umemoto *et al.*, 1991). *C. elegans* has an operon consisting of the two genes, *vha-1* and *vha-2*, encoding 169 and 161 residue *c* subunits, respectively (Oka *et al.*, 1997). The two proteins exhibit ~60% identity, and 55–67% similarity with the *c* subunit of yeast, mouse and cow. We could not conclude the correspondence of *C. elegans vha-1* and *vha*-2 to the yeast counterpart because they share essentially equal similarities with Vma3p and Vma11p. *C. elegans* has a third *c* subunit gene (*vha-3*), which encodes an identical polypeptide to that encoded by *vha-2* (Oka *et al.*, 1998). However, *vha-2* was expressed predominantly in H-shaped excretory cells, whereas *vha-3* was mainly expressed in gastrointestinal and hypodermal cells in addition to H-cells. Thus, the two genes are differently regulated depending on the cell type.

The organism has the *vha-4* gene coding for the 23 kDa protein corresponding to c'' proteolipid (Oka *et al.*, 1997). The Vha-4 protein exhibited 52% identity with yeast Vma16p. The reporter gene experiments suggest that the three isoforms may be used in the V_0 sectors of different organelles. In contrast to yeast and *C. elegans*, mammals only have one gene for the *c* subunit (16 kDa proteolipid) (Hanada *et al.*, 1991; Hayami *et al.*, 2001), thus having no c' subunit.

Diverse *a* Subunits and Their Roles

The yeast *a* subunit is encoded by two genes, *VPH1* and *STV1*, exhibiting 54% identity in amino acid sequence, and Vph1p (Manolson *et al.*, 1992) and Stv1p (Manolson *et al.*, 1994) are localized to vacuoles and prevacuolar compartments (possibly the Golgi or endosomes), respectively. Excess expression of Stv1p in the Δ*vph1* and Δ*stv1* strains result in mislocation of *Stv1p* to vacuoles (Manolson *et al.*, 1994). Thus, V-ATPase with *Stv1p* and that with *Vph1p* localized in the same membranes could be compared enzymologically (Kawasaki-Nishi *et al.*, 2001; Nishi and Forgac, 2000). V-ATPase with Stv1p showed an about fivefold lower ratio of proton transport to ATP hydrolysis than that with Vph1p. These results suggest that the *a* subunit has roles for determining efficiency for proton translocation, assuming that reduced expression of the V_1 part (in the Δ*vph1* / Δ*stv1* strain with Stv1p) does not affect the measurement of proton translocation. Studies on a chimeric *a* subunit constructed from Stv1p and Vph1p suggested that the amino terminal region of the *a* subunit regulates the targeting of V-ATPase (Kawasaki-Nishi *et al.*, 2000). The same approach also suggests that the carboxyl-terminal domain affects energy coupling between proton transport and ATP hydrolysis.

It was reasonable to assume that higher eukaryotes or mammalians have more complicated isoform patterns than yeast, because they have unique cells with specific organelles or ones forming extracellular acidic compartments. As expected, even a simple model animal like *C. elegans* has four *a* subunit isoforms. Their genes, *vha-5*, *vha-6*, *vha-7*, and *unc-32*, code for proteins of 873, 865, 966, and 894 amino acid residues, respectively, with nine highly conserved transmembrane segments, and exhibit 34–49% overall sequence identity with each other (Oka *et al.*, 2001). They are expressed in a cell-specific manner during development: *vha-5*, excretory H cells; *vha*-5, intestine; *vha-7*, hypodermal cells; and *unc-32*, nerve cells. Consistent with neural expression, the *unc-32* mutation was originally isolated on the basis of defective movement, and mutant alleles including four recently isolated ones correspond to the *unc-32* gene (Pujol *et al.*, 2001). Disruption of gene expression by RNA interference experiments (RNAi) clearly showed that three of them are essential genes; *unc-32* is essential for embryogenesis, and *vha-5* and *vha-6* are required for larva development, whereas *vha-7* is dispensable. These results suggest that V-ATPases with different isoforms are expressed in specific cells, and that their acidic organelles or compartments are essential for nematode development.

The cDNAs for three isoforms, *a*1, *a*2 and *a*3, were isolated from mouse, and found to exhibit ∼50% amino acid sequence identity with each other (Nishi and Forgac, 2000; Toyomura *et al.*, 2000;). It was hard to match *C. elegans* isoforms to those of mouse. Northern analysis showed their expression in various mouse tissues: *a*1, mainly in brain and liver; *a*2, heart, kidney, in brain and liver; and *a*3, strongly in heart and liver. The *a*1 isoform is 95 and 97% identical to the subunit *a* from cow (Peng *et al.*, 1994) and rat (Perin *et al.*, 1991) clathrine-coated vesicles, respectively. *a*2 is identical to a putative immunoregulatory protein from mouse except for two amino acid replacements (Lee *et al.*, 1990). Mouse *a*2 is 91% identical to cow subunit *a*. The *a*3 isoform shows 84% identity with 116 kDa polypeptide from human osteoclastoma cells (Li *et al.*, 1996).

The cDNA for a fourth isoform, *a*4, was isolated from mouse recently (Oka *et al.*, 2001; Smith *et al.*, 2001). The *a*4 is 63, 54, and 48% identical with mouse *a*1, *a*2, and *a*3, respectively. Unlike other isoforms, the *a*4 gene is transcribed after the 14 dpc embryo stage when primitive glomerulli are observed, and differentiation of the kidney has started. The *a*4 transcript is only found in the kidneys of adult mice (Oka *et al.*, 2001). The V-ATPase with the *a*4 isoform is localized immunochemically to the apical and basolateral membranes of the α- and β-intercalated cells in the collecting duct, respectively. These results indicate that V-ATPase with the *a*4 isoform is required for apical proton secretion from α cells, and bicarbonate secretion from the apical membrane of β cells (Fig. 4). These results are consistent with the notion that V-ATPase with the *a*4 isoform is involved in mammalian acid/base homeostasis and closely related to the differentiation of collecting tubules.

Mutations in the human *ATP6N1B* gene have been shown to cause recessive renal tubular acidosis (Smith *et al.*, 2000). The gene product exhibits ∼86% identity with the mouse *a*4 isoform, suggesting that it is the human *a*4 isoform. Human *a*4 is highly expressed in kidney intercalated cells of the collecting duct. The incidence of this disease also suggests that *a*4 cannot be replaced by the other three isoforms.

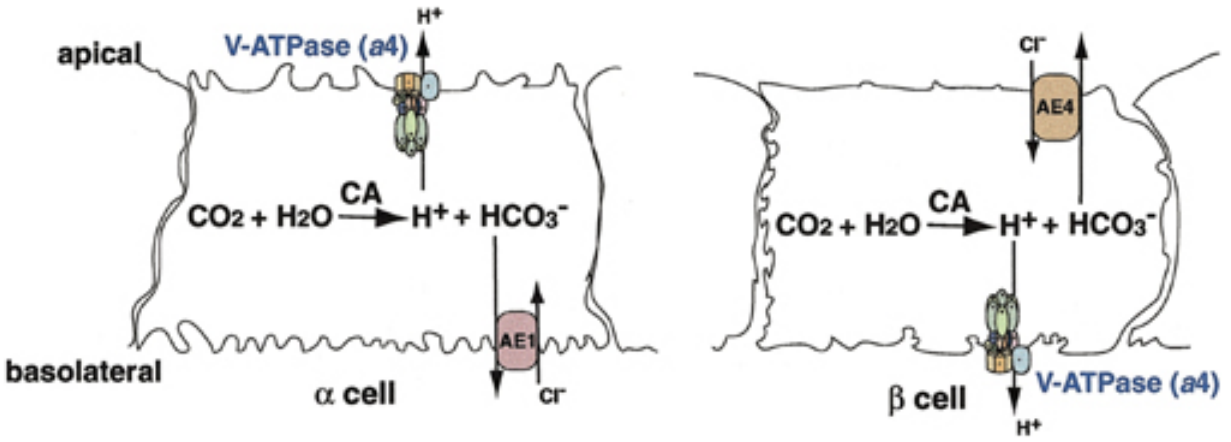

Fig. 4. Schematic representation of the two major subtypes of intercalated cells in the cortical collecting duct. Both cell types contained cytosolic carbonic anhydrase II (CA), which is responsible for intracellular production of protons and bicarbonate. α-cells, proton-secreting cells have an apical V-ATPase with an *a*4 isoform and a basolaterally located chloride/bicarbonate exchanger (AE1). β-cells have *a*4 containing V-ATPase of the opposite polarity. The apically located anion exchanger is AE4 (for detail see Oka *et al.*, 2001).

Localization of a Unique V-ATPase in Osteoclasts

V-ATPase is a proton pump localized in the plasma membranes of osteoclasts, and acidifies the resorption lacuna formed between the bone and the osteoclasts (Fig. 5(a)) (Toyomura *et al.*, 2003). Osteoclasts could be induced from bone marrow cells in the presence of 1.25 $(OH)_2D_3$ (Toyomura *et al.*, 2000). Upon induction, the amount of the *a*3 isoform increased together with V_1 sector subunit. *a*3 was detected immunochemically in the osteoclast plasma membrane and its vicinity, whereas *a*1 was found in the dot-like structure of the cytoplasm, and *a*2 was not detectable at all. These results indicate that V-ATPase with the *a*3 isoform is the proton pump in the osteoclast plasma membrane. The *a*3 isoform together with V_1 was localized with microtubules, suggesting that V-ATPase with the *a*3 isoform (in the transport vesicles or organelles) interacts with microtubules and is carried to the plasma membrane. Disruption of the *Atp6i* gene for mouse *a*3 causes severe osteopetrosis due to the loss of osteoclast-mediated acidification of resorption lacunae (Li *et al.*, 1999). A spontaneous mouse osteopetrotic mutation (Nakamura *et al.*, 1997) has been mapped to the same gene (Scimeca *et al.*, 2000). These results establish that the *a*3 isoform is the essential subunit for the osteoclast plasma membrane V-ATPase.

Is V-ATPase with *a*3 localized to the plasma membrane of an osteoclast progenitor? To address this question, RAW 264.7, a murine macrophage line, could be a good model cell. When cultured with RANKL (receptor activator of nuclear factor κB ligand), RAW 264.7 can form multinuclear cells expressing osteoclast markers such as tartrate resistant acid phosphatase (TRAP), calcitonine receptor and cathepsin K (Huang *et al.*, 2000). Thus, we can follow the formation of the osteoclast-specific plasma membrane during differentiation (Toyomura *et al.*, in press). We found that the V-ATPase with the *a*3 isoform was localized in lysosomes before induction. Similar to the case of osteoclasts derived from bone marrow cells, the *a*3 isoform is localized to the plasma membranes of the multinuclear cells derived from RAW 264.7. Markers of lysosomes such as lamp2 (lysosome associated membrane protein 2) also showed the plasma membrane localization in multinuclear cells. These results suggest that the osteoclast plasma membrane, at least partly, is derived from lysosomes. Thus, the biochemical question regarding V-ATPase localization leads to unveiling a novel mechanism underlying the formation of the osteoclast plasma membrane from those of lysosomes.

SUBUNIT ISOFORMS IN V_1 AND THE STALK REGION OF V-ATPase

Isoforms of the mammalian *B* subunit of the V_1 sector were found earlier: B_1 is specifically found in kidney and cochlea, and *B*2 is expressed ubiquitously (Karet *et al.*, 1999). However, isoforms for other subunits became known only very recently. Extensive analysis of a cDNA library revealed the presence of isoforms of the *d*, *C*, *E*, and *G* subunits mainly located in the stalk region between the A_3B_3 hexamer and the V_0 sector (Fig. 1 and Table I) (Imai-Senga *et al.*, 2002; Murata *et al.*, 2002; Sun-Wada

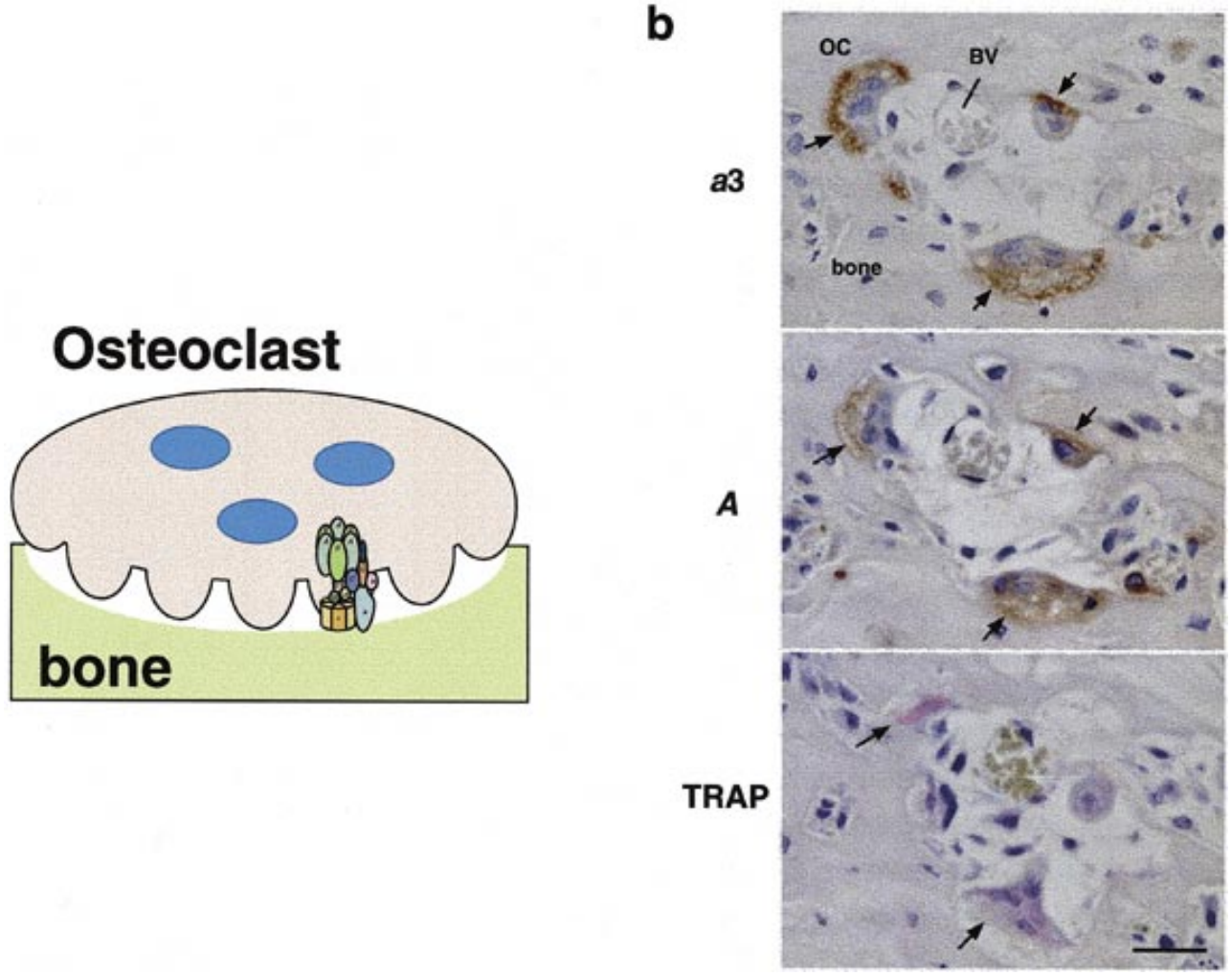

Fig. 5. Localization of a unique V-ATPase in the osteoclast plasma membrane. (a) Schematic model of an osteoclast. A model of an osteoclast is shown together with a resorption lacuna formed between the plasma membrane and the bone surface. The presence of V-ATPase in the plasma membrane is schematically shown. (b) Histochemistry of areas around the bone surface. A mouse femora was fixed, decalcified, and embedded. Sections were stained for tartrate-resistant acid phosphatase (TRAP) (a marker for osteoclasts) and, the *a*3 isoform (*a*3) and subunit *A*(*A*) of V-ATPase. Plasma membranes of multinuclear osteoclasts (OC) are indicated by arrows. A blood vessel (BV) is also indicated. Bar, 25 μm.

et al., 2003). Similar to in the case of the *B* subunit, they include isoforms (*C*1, *E*1, *G*2, and *G*3) uniquely expressed in a tissue- or cell-specific manner, and ones (*C*2, *E*2, and *G*1) expressed ubiquitously.

V-ATPases With *E*1 and *E*2 Isoforms

We have identified the mouse *Atp6e1* and *Atp6e2* genes encoding testis-specific (*E*1) and ubiquitous (*E*2) isoforms of subunit *E*, respectively (Sun-Wada *et al.*, 2002 and Table I). They comprise 226 amino acid residues, and exhibit ~70 % identity with each other and ~33% identity to yeast Vma4p. Expression of mouse *E*1 and *E*2 complementes the yeast Δ*vma4* mutation, indicating that they are functional V-ATPase subunits. Yeast V-ATPase with the mouse *E*1 and *E*2 isoforms showed similar ATPase activity with apparent K_m^{ATP} of ~150 μM, whereas that with Vma4p showed an apparent K_m^{ATP} of ~300 μM. The kinetic difference between V-ATPase with the yeast and mouse *E* subunits suggests that this subunit interacts with the catalytic hexamer.

Yeast cells expressing V-ATPase with mouse *E*1 showed a temperature-sensitive growth phenotype. However, the *E*1 enzyme retained ATPase activity at 37°C: the activity of the enzyme with *E*1, *E*2, or Vma4p increased

Table I. V-ATPase Subunits and Their Coding Genes

Domain		Subunit	Yeast gene	Mouse isoform (mammalian)	Mouse gene
	Catalytic hexamer	*A*	*VMA1*		*Atp6A1*
	(A_3 B_3)	B	*VMA2*	*B*1	*Atp6V1B1*
				*B*2	*Atp6V1B2*
		C	*VMA5*	*C*1	*Atp6V1C1*
				*C*2-a	*Atp6V1C2a*
				*C*2-b	*AtpV1C2b*
V_1		*D*	*VMA8*		*Atp6V1D1*
		E	*VMA4*	*E*1	*Atp6V1e1*
				*E*2	*Atp6V1e2*
	Stalks	F	*VMA7*		*Atp6V1F1*
		G	*VMA10*	*G*1	*Atp6V1g1*
				*G*2	*Atp6V1g2*
				*G*3	*Atp6V1g3*
		H	*VMA13*		*Atp6V1H1*
		d	*VMA6*	*d*1	*Atp6V$_0$d1*
				*d*2	*Atp6V$_0$d2*
	Proton pathway	*a*	*STV1*	*a*1	*Atp6V$_0$a1*
			VPH1	*a*2	*Atp6V$_0$a2*
				*a*3	*Atp6V$_0$a3*
V_0				*a*4	*Atp6V$_0$a4*
		c	*VMA3*		*Atp6V$_0$c*
		c′[a]	*VMA11*		
		c″	*VMA16*		*Atp6V$_0$f*

Note. Subunits and their coding genes are summarized for yeast and mouse. Mouse isoforms found are shown.
[a] Subunit *c*′ is not found in mammals.

about twofold when the assay temperature was shifted from 25°C to 37°C. The *E*1 enzyme was completely inactive in proton transport at 37°C, whereas the *E*2- or Vma4p-containing enzyme retained the transport activity. The *E*1 V-ATPase could not couple ATP hydrolysis and proton transport, thus being defective in energy coupling. These results also suggest the close interaction of the *E* subunit with the proton pathway.

Atp6e1 is only transcribed in testis, and the *E*1 isoform is localized specifically in the developing acrosomes of spermatids and those of mature sperm (Sun-Wada *et al.*, 2002). On the other hand, *E*2 was expressed in all tissues examined and in the perinuclear organelles of spermatocytes. Although three *a* isoforms (*a*1, *a*2, and *a*3) were found in testis, only *a*2 was expressed in acrosome. Presence of *a*2 and *E*1 in testis V-ATPase was confirmed by immunoprecipitation. The *a*1 was expressed in almost all testis cells and *a*3 specifically in Sertoli cells, but *a*1 and *a*3 were not detectable in mature acrosomes. We have also identified human genes, *ATP6E*1 and *ATP6E*2, encoding *E*1 and *E*2, respectively (Imai-Senga *et al.*, 2002). Both isoforms complement the yeast Δ*vma4* mutation, *E*1 being specifically expressed in testis similar to that of mouse. The two plant *E* subunit isoforms (Kawamura *et al.*, 2000) did not show highly restricted expression similar to mammalian *E*1 (Sun-Wada *et al.*, 2002).

V-ATPase With Isoforms for the *G* and *d* Subunits

Crider *et al.* (1997) found two bovine *G* subunit isoforms in biochemical studies, although they did not report tissue expression or organellar localization. We have identified mouse *Atp6g1* and *Atp6g2* (Table I), encoding the *G*1 and *G*2 isoforms of the *G* subunit, respectively (Murata *et al.*, 2002). Both isoforms comprise 118 amino acid residues, and show 62% identity with each other and high similarity (76%) to yeast Vma10p. *G*1 was found in all tissues examined, whereas *G*2 was found exclusively in brain. *G*2 could complement the yeast deletion mutant Δ*vma10* lacking the *G* subunit, whereas *G*1 did not show this complementation, possibly due to the weakly conserved carboxyl terminal region. *G*2 was localized in synaptic vesicles where *G*1 was undetectable, suggesting that V-ATPase with *G*2 is involved in the vesicle acidification. Consistent with the brain expression, *G*2 was induced at 10 dpc in mice and maintained at all later stages. Furthermore, a third isoform, *G*3, was found more recently

(Smith *et al.*, 2002; Sun-Wada *et al.*, 2003). *G*3 exhibits ~53% identity with *G*1 or *G*2, and ~75% similarity to yeast Vma10p, and is only found in kidney.

We identified *Atp6v1c1* and *Atp6v1c2* (Table I) coding for the *C*1 and *C*2 isoforms of the subunit *C*, respectively (Sun-Wada *et al.*, 2003). *C*1, consisting of 384 amino acid residues, is 76% similar to yeast Vma5p, and expressed ubiquitously. *Atp6v1c*2 codes for the*C*2-a and *C*2-b isoforms. *C*2-a contains 46 additional amino acid residues compared with *C*1, and *C*2-b is a splicing variant of *C*2-a. *C*1 and *C*2-b are 57.2% identical with each other, and the *C*2 transcript is found predominantly in lung and kidney, and observed after 17 dpc. However, *C*2-b lacking the additional residues from *C*2-a is only found in lung alveolar type II cells which produce pulmonary surfactant. *C*1, *C*2-a, and *C*2-b could complement yeast Δ*vma*5. Thus, V-ATPase with *C*2-a and *C*2-b is responsible for acidification of specific organelles, lamellar bodies, in alveolar type II cells (Sun-Wada *et al.*, manuscript in preparation).

It is of interest to note that V-ATPase with the *C*1 gene showed a similar K_m to that with Vma5p, whereas those with *C*2-a and *C*2-b exhibited lower values. V-ATPase with mouse *C* subunit showed lower proton translocation. These results suggest that the *C* subunit has a close functional interaction with catalytic hexamer A_3B_3 and the V_0 proton pathway. In contrast to mammalian counterparts, *C. elegans* has a single gene *VHA-11* coding for a *C* subunit (Oka and Futai, 2000). The experiment involving disruption of *VHA-11* gene expression indicated that V-ATPase and inside-acidic organelles are required for ovulation and embryogenesis (Oka and Futai, 2000).

Two subunit *d* isoforms, *d*1 and *d*2, exhibit 68% identity with each other, and 45 and 42% identity with yeast Vma6p, respectively (Sun-Wada *et al.*, 2003). *d*1 is only expressed in kidney, whereas *d*2 is expressed ubiquitously in various tissues.

DIVERSE MAMMALIAN V-ATPases

In contrast to the role of F-ATPase in ATP synthesis in mitochondria, V-ATPase is localized in many organelle membranes and plasma membranes forming extracellular compartments with an inside acidic pH, and carries out diverse physiological roles. Isoforms have been found for subunits forming a membrane sector or a stalk between V_0 and the catalytic A_3B_3 hexamer. The isoforms in the stalk region are: *C* subunit, *C*1, *C*1 and *C*1-a and *C*2-b; *E*, *E*1 and *E*2; *G*, *G*1, *G*2 and *G*3; *d*, *d*1 and *d*2. It is striking that so many isoforms have been found recently, although only isoforms *B*1 and *B*2 for the *B* subunit have been identified so far (Nelson, 1992). In all cases, one of the isoforms is expressed in specific tissues or cells, whereas the other one is ubiquitously expressed. We examined isoform localization in specific cells in the tissues exhibiting high expression. However, it is still possible that the isoforms are expressed in specific cells in tissues

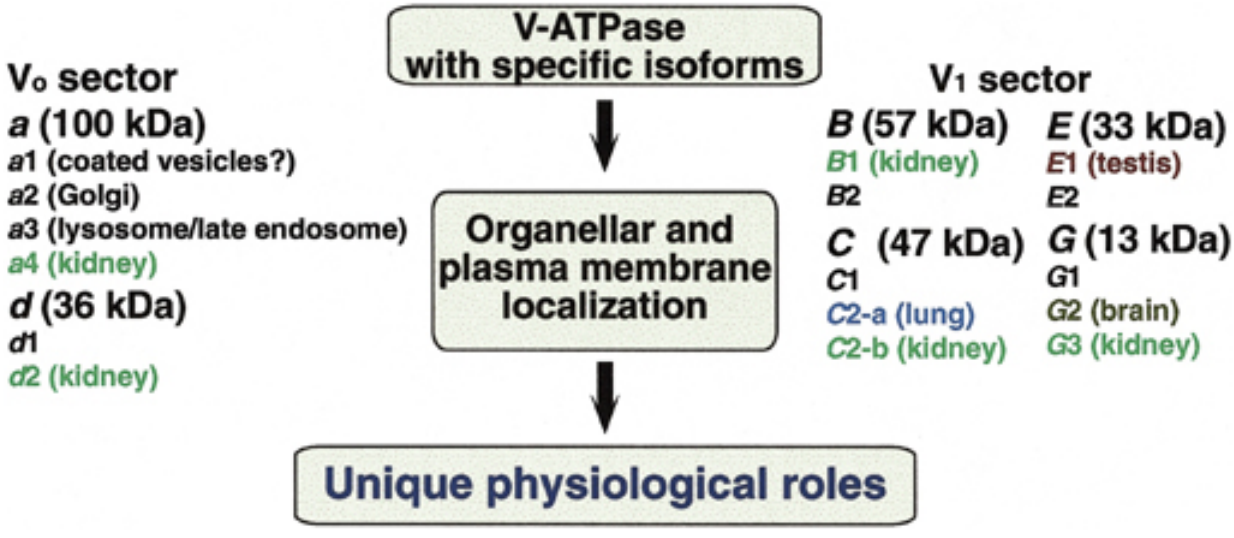

Fig. 6. Roles of subunit isoforms of V-ATPase. Subunit isoforms are summarized: isoforms expressed specifically in unique cells or tissues are indicated. Isoforms expressed in the same tissues are shown by the colored letters, and those expressed ubiquitously are indicated without highlighting. A conceptual model of the roles of subunit isoforms is also shown.

not apparently showing expression on Northern analysis. The expression of the *B*1 isoform in the inner ear is the example of such an isoform (Karet *et al.*, 1999).

The stalk subunits interact with the V_0 sector and/or V_1 catalytic hexamer A_3B_3, as shown by cross-linking and two-hybrid analysis (Arata *et al.*, 2002; Puopolo *et al.*, 1992). Thus, they may affect V_0 H^+ transport, as suggested above for the *C* and *E* subunits. The interaction with the V_0 sector may also be important for targeting V-ATPase to specific membranes of organelles or plasma membranes. Of the four subunit *a* isoforms, *a*4 is expressed specifically in the kidney collecting duct, and other isoforms are expressed more widely in various tissues. However, it can be assumed that the stalk subunit interacts with the subunit *a* isoform, affecting the V-ATPase for targeting to specific membranes. Examples may be the V-ATPase with the *a*2/*E*1 and *a*3/*G*2 isoforms found in acrosomes (Sun-Wada *et al.*, 2002) and synaptic vesicles (Murata *et al.*, 2002), respectively.

In summary, as also shown in Fig. 6, V-ATPase plays various physiological roles in unique cells or organelle membranes utilizing specific isoforms. The isoforms may respond to different signals for targeting to the specific organelles or plasma membrane of the specialized cells.

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