Mouse Proton Pump ATPase C Subunit Isoforms (C2-a and C2-b) Specifically Expressed in Kidney and Lung∗

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The vacuolar-type H+-ATPases (V-ATPases) are multimeric proton pumps involved in a wide variety of physiological processes. We have identified two alternative splicing variants of C2 subunit isoforms: C2-a, a lung-specific isoform containing a 46-amino acid insertion, and C2-b, a kidney-specific isoform without the insert. Immunohistochemistry with isoform-specific antibodies revealed that V-ATPase with C2-a is localized specifically in lamellar bodies of type II alveolar cells, whereas the C2-b isoform is found in the plasma membranes of renal α and β intercalated cells. Immunoprecipitation combined with immunohistological analysis revealed that C2-b together with other kidney-specific isoforms was selectively assembled to form a unique proton pump in intercalated cells. Furthermore, a chimeric yeast V-ATPase with mouse the C2-a or C2-b isoform showed a lower \( K_{\text{m}} \), proton, and lower proton transport activity than that with C1 or Vma5p (yeast C subunit). These results suggest that V-ATPases with the C2-a and C2-b isoform are involved in luminal acidification of lamellar bodies and regulation of the renal acid-base balance, respectively.

Highly differentiated endomembrane organelles, including the Golgi apparatus, lysosomes, endosomes, and secretory vesicles, have a luminal acidic pH, which is required for various cellular functions. The acidic pH is established by a ubiquitously expressed multisubunit proton pump, vacuolar type H+-ATPase (V-ATPase)1 (for reviews, see Refs. 1–5). In addition to the intracellular organelles, the V-ATPase is localized in the plasma membranes of highly differentiated cells, including osteoclasts (6), kidney intercalated cells (7), and male tract epithelial cells (8), where it is required for bone metabolism, urine acidification, and spermatogenesis, respectively. Furthermore, the same enzyme is required for the acidification of specialized organelles, including synaptic vesicles (9, 10) and acrosomes (11). Thus, assembly of V-ATPase, its targeting to final destinations, and its proper regulation are essential for the diverse physiological functions.

V-ATPase has a membrane peripheral V1 sector for ATP hydrolysis and an integral VO sector for proton translocation and exhibits similarity to F-ATPase (ATP synthase) in both structure and catalytic mechanism. The ATP-dependent conformational changes are transmitted between the peripheral complex (V1 or F1) and the proton pore (VO or FO) through a number of subunits forming a stalk (1, 4, 5). We have demonstrated that the catalytic mechanism involving subunit rotation is conserved in V- and F-ATPases (12–16). Deletion of mammalian VO subunit c, which is encoded by a single gene (17, 18), has been shown to cause an embryonic lethal phenotype (19), indicating that the enzyme is essential in early development. The subunits in the stalk region are required for activity and assembly in yeast (20) and mammals (11, 21).

Recent studies suggested that the diverse physiological functions of V-ATPase are established through the utilization of specific subunit isoforms(s) (21–25), the basic functional enzyme structure being maintained. Multiple isoforms have been found for the largest subunit, \( a \), of the VO sector in nematode (26), chicken (27), mouse (6, 28–30), and humans (31). The mammalian \( a4 \) is specifically expressed in renal intercalated cells (29, 32). Consistently, \( a4 \) mutations cause renal acidosis (32), whereas a defect of \( a3 \), a component of the osteoclast plasma membrane V-ATPase (6, 25), results in osteopetrosis (33). The \( a1, a2, \) and \( a3 \) subunit isoforms exhibit different subcellular localizations (25). V-ATPases with kidney- (\( d2, G3, \) and \( C2 \)) (22, 34), testis- (E1) (11, 21), and brain- (G2) (24) specific VO subunit isoforms are involved in renal acidification, fertilization, and neurotransmitter accumulation, respectively.

We have reported that the mouse V-ATPase C subunit has two isoforms, C1 being expressed ubiquitously, and C2 specifically in kidney and lung (22). In this study, we found that C2 shows further diversity (C2-a and C2-b) due to alternative mRNA splicing. They exhibited different expression in kidney and lung. C2-a was specifically expressed in type II alveolar epithelial cells, and localized to the lamellar bodies specialized for the storage and secretion of surfactant phospholipids (35), whereas C2-b was found predominantly in the plasma membrane of renal intercalated cells. Immunoprecipitation revealed that the kidney-specific isoforms, including B1, G3, d2, a4, and C2-b, were present in the same complex, whereas the ubiquitously expressed B2, C1, G1, and other \( a \) isoforms were found in different complex. These results indicated a selective assembly of V-ATPase subunit isoforms in vivo. In addition, a chimeric yeast V-ATPase with the mouse C2-a or C2-b isoform was functional in yeast vacuoles but showed a lower \( K_{\text{m}} \) value and lower proton transport activity than that with C1 or yeast C subunit (Vma5p).

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EXPERIMENTAL PROCEDURES

Isolation of Splice Variants of C2 Isoform—Full-length cDNA from kidney or lung (Marathon-Ready cDNA, Clontech) was used as a template in RT-PCR using primers GH175/6 (5'-ATCCAGCTCTAGGTTTCCCGTTAATT-TCCG-GC-3') and GH88 (5'-CTAGTCAAGAAGGGCTAGGTCGATA-3'), amplified two open reading frames of C2 isoforms (designated as C2-a and C2-b) with different sizes. The amplified PCR fragments were subcloned and sequenced. Primers GH176 (5'-ATCCAGCTCTAGGTTTCCCGTTAATT-TCCG-GC-3') and GH176 (5'-TACACCGCTAGGTTTCCCGTTAATT-TCCG-GC-3') were used to examine tissue-specific expression. After amplification, the products were analyzed on 1% SeaKem GT agarose gel (BMA).

Northern Blot Analysis and In Situ Hybridization—Mouse multiple-tissue blots were purchased from Clontech. The PCR products amplified with primers GH176/GH88 and the kidney cDNA as the template, or with primers GH175/GH176 and lung cDNA as the template were used as probes.

(Digoxigenin)-11-UTP-labeled single-stranded RNAs were prepared with a digoxigenin RNA labeling mixture and the corresponding T3 or T7 RNA polymerase (Roche Applied Science). The 712- and 1192-bp T7 RNA polymerase (Roche Applied Science). The 712- and 1192-bp products were analyzed on 1% SeaKem GT agarose gel (BMA).

RESULTS

Identification of Alternative Splicing Variants for C2 Isoform—We found that the mouse V-ATPase C subunit has two isoforms, C1 and C2 (22). C1 is expressed ubiquitously, whereas C2 is detected specifically in kidney and lung (22). Using the full-length cDNA prepared from lung or kidney, we amplified two open reading frames (C2-a and C2-b) (Fig. 1, A and B). C2-a, which corresponds to C2 cDNA isolated previously (22), contained 46 additional amino acid residues (Pro976 to Glu1312) compared with C2-b. A search of a mouse genomic data bank (www.ensembl.org/Mus_musculus/) with the sequence of C2-a cDNA revealed that the 138-bp insertion was exactly encoded by exon 12 of the C2 isoform gene Atp6v1c2 (total 15 exons) (Fig. 1C). Human cDNAs corresponding to C2-a and C2-b were also found (78.3% identity to mouse cDNA) in human EST clones (e.g. GenBank accession number BE549333) encoding the human counterparts.

Tissue Variations in Expression of Alternatively Spliced Forms of C2—The tissue distributions of alternatively spliced forms of the C2 messenger RNA were examined by RT-PCR with primers (GH175 and GH176) corresponding to sequences within the insertion region. The C2-b transcript was found mainly in kidney, whereas C2-a was found predominantly in lung (Fig. 1B). This was confirmed by Northern analysis. A C2-a-specific probe (probe 1, Fig. 1C) recognized a single band for lung (Fig. 1D, left panel), whereas the probe corresponding to the entire open reading frame of C2-a (probe 2, Fig. 1C) detected signals for both kidney and lung (Fig. 1D, right panel).

Antibodies for C1 and C2 were generated against isoform-specific synthetic peptides Glu12 to Lys28 and Asp12 to Ser20.
respectively, and affinity-purified with columns conjugated with the corresponding recombinant proteins. The epitope regions are highly conserved in various mammals (Fig. 2A). Immunoblotting with the purified antibody against C2a revealed 38,000 and 40,000 molecular weight proteins in kidney and lung, respectively (Fig. 2B, lower panel), the molecular weights of both bands corresponding to those predicted from the corresponding cDNAs. We did not detect a protein band for lung with a molecular weight corresponding to C2-b. These results indicated that the C2-a isoform is the major form expressed in lung and that C2-b is kidney-specific. On the other hand, the C1 antibody recognized a 38,000 molecular weight band for all tissues examined (Fig. 2B, upper panel), confirming the previous Northern analysis results (22).

Localization of C2-a in Lung—In situ hybridization with the C2-a antisense probe clearly demonstrated the localization of C2-a mRNA in mouse alveolar corner cells usually located at the branching region of an alveolar wall (Fig. 3A). Type I alveolar cells with the typical flattened shape were not stained. The sense probe did not reveal any significant signals (Fig. 3B).

Immunohistochemical staining with the C2-specific antibody revealed that the C2-a isoform is localized specifically to type II alveolar epithelial cells having large and rounded nuclei with a prominent nucleolus and vacuolated cytoplasm (Fig. 4A). Type II cells secrete surfactants to reduce the surface tension within the alveoli to prevent alveolar collapse during expiration (35), and their cytoplasm is filled with vesicles containing phospholipids in the form of lamellar bodies. Staining with antibodies against C2 and ABCA3 transporter, a 180-kDa lamellar body

![Table I](image)

**Table I**

| Subunit isoform | Peptide sequence | Refs. |
|-----------------|------------------|-------|
| B1              | QGAAQDPASDTAL (C-terminal) | This study |
| B2              | LSEEYPRDSAKH (C-terminal) | This study |
| C1              | [EKTCQQTWEKLHATK](#) | This study |
| C2              | [DKENLQALERMNVT](#) | This study |
| d1              | [VIDDLKEMVKEF](#) | This study |
| d2              | [REFDYFRHNSLEPLSTFL](#) | This study |
| E1              | [MYKIAKDKVQI](#) | 24 |
| G1              | [QETREKMTVLQ](#) | 16 |
| G2              | [GATRRQYQG](#) | 25 |
| G3              | [UETLKV](#) | 25 |
| a1              | [RCCEMDKLF](#) | This study |
| a3              | [HHRHRNTQQR](#) | 18 |
| a4              | [KIHMQSQA](#) | 20 |

* The polyclonal antibodies against the a2 isoform were generated with bacterially expressed recombinant protein containing the C-terminal region (position, 615–742) (18).
membrane protein, clearly showed that C2-a was expressed specifically in type II cells (Fig. 4A, arrows). The A subunit of V-ATPase was also detected at a high level in type II alveolar cells. In contrast, the C1 isoform was expressed at a similar level in airway epithelial cells, alveolar macrophages, pulmonary vessels, airway smooth muscle cells, and connective tissue cells (Fig. 4A, C1). We also examined the intracellular localization of C2-a by confocal microscopy and found that the staining signals of C2-a were superimposed on those of ABCA3 (Fig. 4B).

Localization of C2-a in type II cells was also confirmed by immunoelectron microscopy. The gold particles for anti-C2 antibodies labeled the membranes of lamellar bodies, whereas the C1 antibodies mainly stained the membranes of other organelles (Fig. 5, arrows). These results indicated that a V-ATPase with the C2-a isoform is localized in the membranes of lamellar bodies, suggesting a unique V-ATPase is involved in the acidification of lamellar bodies in type II alveolar cells.

Localization of C2-b and Other Kidney-specific Subunit Isoforms—Immunohistochemical analysis was carried out to identify the renal cells expressing the C2-b isoform. No significant signal was observed for glomeruli or proximal and distal convoluted tubules (Fig. 6A). The C2-b isoform was strongly expressed in the cortical and medulla collecting ducts (Fig. 6, A and B). In the cortical collecting ducts, α and β intercalated cells are responsible for proton and bicarbonate secretion, respectively (7, 42). The α and β cells have a V-ATPase localized on their apical and basolateral plasma membranes, respectively (7). Peanut lectin agglutinin is associated with the apical membranes of β cells but not with those of α cells (43). Double immunostaining indicated that all intercalated cells expressing the C2-b isoform on the basolateral surface were stained with the peanut lectin at the apical region, indicating that β cells have the basolateral C2-b isoform (Fig. 6C). The α cells have Cl⁻/HCO₃⁻ exchanger (anion exchanger 1, AE1) on their basolateral membranes (44–46). All intercalated cells having the

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**Fig. 2. Western blotting analysis of C1 and C2 expression in mouse tissues.** A, the mouse epitopes and corresponding regions in human and rat. The unconserved residues are shown in red. B, immunoblotting using affinity-purified antibodies against each isoform. Lysates of murine liver, brain, kidney, lung, spleen, testis, and thymus together with the lysates of yeasts expressing C1 and C2 were subjected to 12% polyacrylamide gel electrophoresis in the presence of SDS (30 μg of proteins/lane).

**Fig. 3. In situ localization of C2-a in lung.** RNA in situ hybridization in sections with C2-specific antisense (A) and sense (B) riboprobes is shown. The sections were counterstained with methylene green. C2 expression was detected in alveolar type II cells. The probe region used for hybridization is also shown. Bar, 10 μm.
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lized kidney membrane fraction and found that both anti-
forms prompted us to examine whether they were assembled
The intercalated cell-specific localization of kidney-specific iso-
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specific antibodies (Table I) revealed that the other kidney-
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localization at intercalated cells and brush border (29,
30). The subunit c proteolipid, which is encoded by a single
gene in mouse (18, 19), was co-immunoprecipitated with either
B1 or B2 (Fig. 8G). These results indicated that the kidney-
specific isoforms of Vo sector subunits, together with those of
V1 sector, were present in the same V-ATPase.

Selective Interactions of Subunit Isoforms in Mouse Kidney—
The intercalated cell-specific localization of kidney-specific iso-
forms prompted us to examine whether they were assembled
selectively to form a unique V-ATPase. We tested each anti-
body to immunoprecipitate the enzyme complex from solubi-
lized kidney membrane fraction and found that both anti-B1
and anti-B2 antibodies could efficiently precipitate the entire
family V-ATPase complexes. We have used the biotinylated
antibodies followed by horseradish peroxidase (HRP)-conju-
gated streptavidin to detect the corresponding isoform in im-
umnoprecipitated complex. No proteins in kidney lysate re-
acted with HRP-streptavidin (data not shown), indicating that
no endogenous kidney proteins could be recognized by HRP-
streptavidin. The specificities of all the antibodies (Table I)
were confirmed and used below (data not shown).

As described above, the V1 sector contained three kidney-
specific isoforms, B1, C2-b, and G3. Immunoprecipitation with
anti-B1 or anti-B2 antibody revealed that C2-b and G3 were
co-precipitated with the kidney isoform B1 (Fig. 7, A and B),
whereas the ubiquitous counterparts C1 and G1 were mostly
detected in the precipitates of the ubiquitous B2 (Fig. 7, C and
D). These results indicated that kidney-specific subunits B1,
C2-b, and G3 were present in the same V1 sector of the
V-ATPase complex. The G1 isoform was also co-precipitated
with the B1, although the amount was significantly less than that
with B2 (Fig. 7D). It should be mentioned that the G2 isoform
is not expressed in kidney (24). The E subunit contained two
isoforms, and the E2 is the only isoform expressed in kidney.
Immunoprecipitation revealed that the E2 is co-precipitated
with both B subunit isoforms (Fig. 7E).

The ubiquitously expressed d1 isoform was co-immunopre-
cipitated with both B1 and B2 (Fig. 8A), whereas, the kidney-
specific d2 was associated mainly with the B1 containing
complex (Fig. 8B). Meanwhile, the ubiquitously expressed a1, a2,
and a3 were co-immunoprecipitated predominantly with B2,
but not with B1 (Fig. 8, C–E). The kidney-specific a4 was
co-precipitated with both the B1 and B2 (Fig. 5F), consistent
with its localization at intercalated cells and brush border (29,
30). The subunit c proteolipid, which is encoded by a single
gene in mouse (18, 19), was co-immunoprecipitated with either
B1 or B2 (Fig. 8G). These results indicated that the kidney-
specific isoforms of Vo sector subunits, together with those of
V1 sector, were present in the same V-ATPase.

Kinetic Analyses of V-ATPase Containing C Subunit Iso-
forms—C1 and C2-a could functionally replace the yeast coun-
terpart, Vma5p (22). When expressed under the control of the
yeast TDH3 constitutive promoter, C2-b was also able to com-
plement a deletion mutation (vam5Δ) similar to C1 and C2-a,
indicating that the chimeric yeast V-ATPases with C1, C2-a,
and C2-b can be compared functionally. Vacular membranes
were isolated from Δvma5 cells expressing each mouse isoform
or Vma5p. Western blot analysis revealed that the amounts of
the A subunit in the cells expressing the chimeric V-ATPases
with C1, C2-a, and C2-b were about 50%, 35%, and 30% of that
observed in vacuolar membranes with Vma5p (Table II, rela-
tive expression), indicating that the expression level of the
chimeric V-ATPase was lower than that of the yeast enzyme.
Corresponding to the expression levels, the specific activities of the V-ATPases with C1, C2-a, and C2-b were also lower than that for Vma5p (Table II). The \( K_m \) (ATP) values of the V-ATPases with C1 and Vma5p were similar, whereas those with C2-a and C2-b were significantly lower.

We found that the ATP-dependent proton transport activity in vacuolar membranes expressing the V-ATPases with C1, C2-a, and C2-b were lower than that observed for the enzyme with yeast Vma5p. The apparent proton transport activity of the V-ATPase with C2-b was about 10% of that with Vma5p. Furthermore, the efficiency of energy coupling for C2-b was significantly low, when compared with the chimeric V-ATPases with C1 and C2-a (Table II). These results suggest that the C subunit is related to the energy coupling of V-ATPase.
TABLE II

| Subunit isoform | Relative expression level of V-ATPase | ATPase activity | Proton pumping | Estimated energy coupling |
|-----------------|--------------------------------------|-----------------|---------------|--------------------------|
|                 | % relative to Vma5p | $K_{\text{m,ATP}}$ (µM) | Specific activity (µmol/min·mg) | % $\mu F$ relative to Vma5p | $\mu F_{\text{ATPase}}$ |
| Vma5p           | 100 | 192 | 0.49 | 100 | 204 |
| C1              | 50 | 194 | 0.28 | 39.2 | 141 |
| C2-a            | 35 | 126 | 0.11 | 24.2 | 220 |
| C2-b            | 30 | 110 | 0.14 | 9.7 | 69 |

a ATPase activities were assayed on isolated vacuolar membranes containing Vma5p, C1, C2-a, or C2-b (10 µg of membrane protein) over the ATP concentrations range of 50 µM to 1.5 mM. Activities were measured in the absence and presence of 1 µM concanamycin A, and the results shown represent the concanamycin A-sensitive fraction of the activity. $K_{\text{m,ATP}}$ and specific activities were calculated from Lineweaver-Burk plots of reciprocal of initial ATP concentration versus reciprocal of initial rate of ATPase activity (µmol/min·mg).

b ATP-dependent proton transport activities were estimated from the initial rate of ATP-dependent fluorescence (microfarads ($\mu F$)) quenching in the presence of 0.5 mM ATP using the fluorescence dye AMCA in the absence or presence of 1 µM concanamycin A. The transport assay buffer was the same as that used for measurement of ATPase. The level of quenching was calculated from the difference between the maximum level of fluorescence quenching after addition of ATP and the level after addition of 5 µM NH₄Cl. Values are expressed as relative values.

**Fig. 8.** V₉ subunit isoforms co-immunoprecipitate from solubilized kidney membranes. Mouse kidney membrane fractions were incubated with n-octyl-β-glucopyranoside, and the solubilized fraction was subjected to immunoprecipitation with anti-B1 and B2 antibodies and Western blot analysis. Each panel shows a Western blot using the indicated biotinylated anti-isoform antibodies. Lane 1, proteins from membrane fraction; lanes 2 and 3, proteins immunoprecipitated with anti-B1 (α-B1), B2 (α-B2) antibodies, respectively; and lane 4, preimmune IgG (IgG). The arrows indicated the positions of corresponding isoforms.
We have identified two isoforms of V-ATPase C subunit: C1, expressed ubiquitously; C2, found predominantly in kidney and lung (22). Comparison of the amino acid sequences of the isoforms revealed that C2 contained an insertion of 46 residues not found in C1. Here, we showed that the C2 isoform has two splicing variants: C2-a, previously C2, a lung-specific isoform containing a 46-amino acid insertion; C2-b, a kidney-specific isoform without the insert.

Subunit C is believed to be a peripheral stalk component that may not be essential for enzyme activity in a reconstituted system (31) but is important for assembly of the V1 complex (20). When VMA5, however, both a fully assembled VO complex and a core V1 sub-complex were formed (48). These results indicated that the lamellar organelles and endocytic vesicles in proximal tubules.

selectively assembled into the same complex to form a unique renal collecting tubules, similar to other kidney-specific sub-complex with ubiquitous subunit isoforms. On the other hand, have several features distinguishable from those of intracellular membranes (54); (C2-a)

under the control of the

spliced by expression of Vma5p or mouse

in lamellar bodies contains a unique lipid aggregation (50). Our results indicated that the V-ATPase ing of surfactant proteins, or surfactant protein-dependent acidification revealed that the V-ATPase with isoforms may be involved in regulation of the V-ATPase activity required in

be involved in regulation of the V-ATPase activity required in

acidic internal pH of

that allows vectorial proton secretion (for a review, see Ref. 57).

DISCUSSION

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