α4, a Unique Kidney-specific Isoform of Mouse Vacuolar H\(^+\)-ATPase Subunit α*

Toshihiko Oka‡, Yoshiko Murata‡, Miwako Namba‡, Takao Yoshimizu‡, Takao Toyomura‡, Akitsugu Yamamoto§, Ge-Hong Sun-Wada‡, Naotaka Hamasaki†, Yoh Wada‡, and Masamitsu Futai‡

From the 2Division of Biological Sciences, Institute of Scientific and Industrial Research, Osaka University, Core Research for Evolutionary Science and Technology (CREST) of the Japan Science and Technology Corporation, Osaka 567-0047, Japan, the 2Department of Physiology, Kansai Medical University, Moriguchi, Osaka 570-8506, Japan, and the 2Department of Clinical Chemistry and Laboratory Medicine, Kyushu University, Fukuoka 812-8582, Japan

The vacuolar-type H\(^+\)-ATPase (V-ATPase) translocates protons across membranes utilizing the energy of ATP hydrolysis (for reviews, see Refs. 1–5). The organellar acidic pH is important for renal acid/base homeostasis.

In this study, we have isolated a mouse cDNA coding for a fourth isoform (α4) of the membrane subunit α of V-ATPase. This isoform was specifically expressed in kidney, but not in the heart, brain, spleen, lung, liver, muscle, or testis. Immunoprecipitation experiments, together with sequence similarities for other isoforms (α1, α2, and α3), indicate that the α4 isoform is a component of V-ATPase. Moreover, histochemical studies show that α4 is localized in the apical and basolateral plasma membranes of cortical α- and β-intercalated cells, respectively. These results suggest that the V-ATPase with the α4 isoform, is important for renal acid/base homeostasis.

A ubiquitous vacuolar-type H\(^+\)-ATPase (V-ATPase)\(^1\) translocates protons across membranes utilizing the energy of ATP hydrolysis (for reviews, see Refs. 1–5). The organellar acidic pH generated by V-ATPase is responsible for various processes including zymogen activation, receptor-mediated endocytosis, macromolecule degradation, and protein sorting. The enzyme is also found in plasma membranes, where it transports protons outside cells such as osteoclasts (6, 7), renal-intercalated cells (8), and epithelial cells of the seminal duct and bladder (9, 10).

V-ATPase has a membrane peripheral V\(_1\) sector for ATP hydrolysis and an integral V\(_\text{h}\) sector for proton translocation (1–5). The V\(_\text{h}\) sector consists of at least five subunits (α, c, c\(^\prime\), c\(^\prime\prime\), and d) (11). Subunit α is the largest (116 kDa) of the V-ATPase subunits, and its isoforms have been found in yeast (12), chicken (13), mouse (7, 14, 15), cow (16, 17), and human (18). These isoforms exhibit different distributions in organelles and tissues. Yeast isoforms (Vph1p and Stv1p) are localized in vacuoles and Golgi/endsomes, respectively (12). Three isoforms (α1, α2, and α3) have been found previously in mammals (7, 14–18). These isoforms may be important for the localization of V-ATPase in various organelles or plasma membranes.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBankTM/EBI Data Bank with accession number(s) AB050903.

Experimental Procedures

CDNA Cloning and Nucleic Acid Blotting—A mouse EST (expressed sequence tag) clone, 2099716 (19), coding for a part of the α4 isoform, was sequenced. To obtain the 5′-region of the α4 cDNA, total RNA was prepared from C57BL/6J male mice kidneys (age, 8 weeks; Japan SLC). Reverse transcriptase-polymerase chain reaction was carried out using gene-specific primers, and the product was ligated with the EST clone to create a full-length cDNA for the α4 isoform. The nucleotide sequence reported in this study will appear in the DDBJ, EMBL, and GenBankTM\(^*\) data bases with the accession number AB050903.

Northern blot analysis was carried out with multiple tissue blots (CLONTECH) as described by Toyomura et al. (7). A probe was prepared from the cDNA clone (between +1660 and +2167 bp, numbering from the first letter of the initiation codon), and labeled with [α-\(^{32}\)P]dCTP using rediprimeTM II random prime labeling system (Amersham Pharmacia Biotech). Each filter was hybridized with the probe using ExpressHyb Hybridization Solution (CLONTECH) at 68 °C for 60 min.

Genomic DNA (10 μg) from C57BL/6J mice was digested with restriction enzymes, subjected to agarose gel electrophoresis, and then blotted onto a filter. A DNA fragment (between +792 and +1073 bp) of the α4-coding region was used to prepare a labeled probe. Hybridization was performed as described above, and radioactivity was analyzed with a BAS-1000 (Fuji Film).

Preparation of Kidney Cortex Membrane Fraction—All operations were carried out at 4 °C. Kidney cortex (about 2.7 g) was obtained from ten ICR mice (age, 8 weeks), and suspended in 14 ml of 10 mM HEPES-KOH pH 7.4 containing 0.25 mM sucrose, 10 mM KCl, 5 mM MgCl\(_2\), 1 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and complete protease inhibitor mixture (amount recommended by the Company) (Roche Diagnostics). The suspension was homogenized in a Wheaton homogenizer. The supernatant fraction, obtained by centrifugation at 500 × g for 5 min, was centrifuged at 10,000 × g for 15 min. The supernatant was centrifuged at 100,000 × g for 30 min. The precipitate was suspended in 2.4 ml of PBS containing 1 mM EDTA and 10% glycerol and stored at −80 °C until use.

Immunohistochemistry—The synthetic peptide KHQKSQLQSFTIHE-DAVEGDH (positions 665–685 of the α4 isoform) was used to immunize albino rabbits for antibodies against the α4 isoform. The resulting serum was applied to a peptide-conjugated column, and antibodies were further purified using a recombinant protein-conjugated column. Antibodies against human Cl\(^-\)/HCO\(_3\)^− exchanger (AE1) were obtained by injecting a human polypeptide (His\(^{16}\)-Val\(^{21}\)) into a rabbit (20). Immunoblot was carried out.
Kidney-specific V-ATPase Subunit α Isoform

Identification of the α4 Isoform of V-ATPase Subunit α—Three subunit α isoforms have been identified in mouse and chicken (7, 13, 15). This finding prompted us to search for the fourth mammalian isofrom. Analysis of the mouse kidney EST library (19) led us to identify a clone 2099716, coding for a protein highly homologous to mouse α1, α2, or α3 isoforms. The clone encoded an open reading frame of 280 amino acid residues, but apparently lacked a 5′-terminal region. The 5′-region was obtained by reverse transcriptase-polymerase chain reaction from mouse kidney RNA.

The entire cDNA was 3014 bp (not including polyadenylation) and contained a coding region for 833 amino acid residues with two potential N-linked glycosylation sites (Asn402 and Asn493). The predicted protein exhibited 63, 54, and 48% similarities to the mouse α2, α3, and α4 isoforms, respectively. A hydrophathy plot of the protein suggested a structure with nine transmembrane regions (Fig. 1, I–IX), i.e. similar to other isoforms. The amino-terminal domain (1–74) of the protein were highly similar to other isoforms (α1, 74.2%; α2, 65.6%; α3, 64.6%).

Mutational analysis of the yeast VPH1 gene showed 11 amino acid residues that are essential for activity, assembly, or intracellular sorting of subunit α (22). All of them except His729 (yeast numbering) were completely conserved in all mouse isoforms (Fig. 1, asterisk). Based on the structure and sequence similarities, the protein encoded by the cDNA was named the a4 isofrom. Southern blot analysis of mouse genomic DNA gave out as described previously (7). For immunoprecipitation experiments, kidney cortex membranes (2 mg/ml) were incubated at 4 °C for 2 h in 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 2% octyl glucoside, and 200 mM NaCl. The supernatant fraction, obtained by centrifugation at 100,000 × g for 30 min, contained solubilized V-ATPase.

Histology—ICR mice (age, 8–10 weeks; Japan SLC) were anesthetized and perfused briefly with PBS, pH 7.4 and then fixed with 4% paraformaldehyde in PBS for 15 min. The kidneys were removed, incubated in the same solution overnight at 4 °C, and cut transversely into 5-mm thick blocks. They were successively infiltrated with 30% sucrose in PBS, embedded in OCT compound (Miles), and stored frozen. Frozen blocks were sectioned at 4 μm thickness and mounted on MASC-coated slides (Matsunami Glass). The sections were rinsed with PBS containing 0.05% Tween 20 (PBST), and incubated for 30 min with 0.3% H2O2 in methanol. They were blocked with PBS containing 1.5% normal goat serum, and then developed using VectaStain Elite ABC reagent (VECTOR Laboratories). After washing with PBST, the sections were further incubated with a biotinylated goat anti-rabbit IgG (VECTOR Laboratories) in PBS containing 1.5% normal goat serum, and then developed using VectorStain ABC reagent (VECTOR Laboratories). After washing with PBST, the sections were counterstained with hematoxylin and mounted with Eukitt (Kindler).

Electron Microscopy—The pre-embedding silver enhancement immunogold method was used, as described previously (21). Mice were anesthetized with ether and perfusion-fixed for 10 min with 4% paraformaldehyde and 0.2% picric acid in 0.1 M sodium phosphate (pH 7.4). The fixed kidneys were removed and incubated for another 50 min in the same solution. Cryo sections (6 μm in thickness) were reacted with 5 μg/ml anti-α4 antibodies overnight, followed by incubation with colloidal gold (1.4-nm diameter)-conjugated secondary antibodies. The gold labeling was intensified using a silver enhancement kit (Nano Probes).

RESULTS

Mouse α4 isoform.
Kidney-specific V-ATPase Subunit a Isoform

Kidney-specific Expression of the a4 Gene—It was of interest to determine which tissue(s) expresses the a4 gene. A 3.3-kb single transcript was detectable exclusively in kidney (Fig. 2B), i.e. no signals were observed in heart, brain, spleen, lung, liver, skeletal muscle, or testis even after longer exposure of the filter (data not shown). These results were consistent with the EST clone 2099716 (19) as isolated from the kidney library. These findings suggest that the a4 isoform is a kidney-specific subunit a. During embryonic development, the a4 gene was transcribed from 15-dpc embryos, whereas no significant signal was detectable in 7- and 11-dpc embryos (Fig. 2B).

Detection of the a4 Isoform in Kidney—Antibodies were generated using a synthetic peptide for immunohistochemical studies on the localization of the a4 isoform. The affinity-purified antibodies specifically recognized a single 94-kDa protein in a lystate of yeast cells expressing an a4 expression plasmid (Fig. 2C), i.e. they did not react with other isoforms (data not shown). The position of the band matched the molecular weight (95,603) calculated from the deduced amino acid sequence. Bands corresponding to 95–106 kDa were observed for kidney membranes (Fig. 2C), whereas no significant bands were detectable for other tissues. The multiple bands may correspond to glycosylated forms because bovine subunit a is known to acquire N-linked oligosaccharides (23). The a4 isoform was detected mainly in the cortical membrane fraction of kidney (Fig. 2D). Other isoforms and subunits including subunit A that have a catalytic site were distributed equally in cortical and medullar fractions (Fig. 2D and data not shown).

Immunoprecipitation of V-ATPase with the a4 Isoform—Membranes were obtained from kidney cortex and treated with octylglucoside. The soluble fraction was incubated with antibodies against a specific band (Fig. 2A), indicating that only one gene for a4 is present in the mouse genome.

Kidney-specific expression of the a4 isoform. A, genomic Southern blot analysis of the a4 isoform. Mouse genomic DNA (10 μg) was digested with various endonucleases and electrophoresed on an agarose gel. After blotting, the filter was hybridized with radioactive probe. B, Northern blot analysis of the a4 isoform. Poly(A)+ RNAs (2 μg) of various adult tissues and whole embryos were hybridized with radioactive probe. The blot was also hybridized with a control probe of β-actin. Arrowhead indicates the position of the transcripts. C, kidney-specific presence of the a4 isoform. Total proteins (20 μg) from various mouse tissues and yeast cells expressing a4 (a4 in yeast!) were separated by the gel electrophoresis in the presence of SDS and then incubated with antibodies against a4. The arrowhead indicates the position of the a4 subunit that was not glycosylated. D, presence of the a4 isoform in the renal cortex and medulla. Total proteins (30 μg) of whole kidney, medulla, or cortex were separated by gel electrophoresis, and then incubated with antibodies against the a3, a4, or A subunit.

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bodies against the a4 isoform, and the immunoprecipitate was subjected to polyacrylamide gel electrophoresis in the presence of SDS. As shown by immunoblotting (Fig. 3), the precipitate contained subunit A of membrane extrinsic V_1 sector and the c subunit of V_O, suggesting that the a4 isoform is a component of kidney cortical V-ATPase.

Localization of the a4 Isoform in Intercalated Cells of the Cortical Collecting Ducts—Immunohistochemical analysis was carried out to identify the renal cells expressing the a4 isoform. No significant signal was observed in glomeruli or proximal and distal convoluted tubules (Fig. 4A). The a4 isoform was strongly expressed in the cortical collecting ducts (Fig. 4A) and found specifically on the apical and basolateral surfaces of certain epithelial cells (Fig. 4B, arrowheads). The signal diffused through the cytoplasm was often observed in the cortical collecting ducts (Fig. 4B, arrow). Two types of epithelial cells (principal and intercalated cells) are present in cortical collecting ducts (24). Immunoelectron microscopic analysis revealed that the a4 isoform was concentrated on the apical (Fig. 5A) and basolateral (Fig. 5B) surfaces of intercalated cells detected as mitochondria-rich epithelial cells (24), whereas no signal was found in principal cells (Fig. 5C).

In the cortical collecting ducts, two kinds (α and β) of intercalated cells are responsible for proton and bicarbonate secretion, respectively (8). The α and β cells have V-ATPase localized on their apical and basolateral plasma membranes, respectively (25). Peanut lectin agglutinin (PLA) is associated with the apical membranes of rabbit β cells but not with those of α cells (26). Although it is not a specific marker in rodent, double immunostaining indicated that all intercalated cells expressing the a4 isoform at the basolateral surface were apical PLA-positive (Fig. 4C). Electron micrographs (Fig. 5) showed that the cells expressing the a4 isoforms in the basolateral surface exhibit extensive invagination in the basolateral membranes and a few microvilli in the apical surface (Fig. 5B). This morphology is reported to be characteristic of rat β-intercalated cells (24, 27). Considering both the immunofluorescence and immunoelectron microscopy evidence, we suggest that the mouse β cells have basolateral a4 isoforms.

The α cells have Cl⁻/HCO₃⁻ exchanger (AE1) on their basolateral membranes (28–30). All intercalated cells having the
apical a4 isoform were basolateral AE1-positive, indicating that α cells have apical a4 (Fig. 4D). These results indicate that the V-ATPase with the a4 isoform is localized specifically on the apical and basolateral surfaces of α- and β-intercalated cells, respectively.

DISCUSSION

The subunit a is forming the intrinsic membrane Vₐₐ sector of V-ATPase. Three α isoforms (α1, α2, and α3) were found previously in chicken, mouse, cow, and human (7, 13–18). We have identified a fourth subunit α isoform (a4) of mouse V-ATPase in this study. Transcripts of the a4 isoform were found in 15- and 17-dpc embryos, but not in 7- and 11-dpc embryos. The differentiation of mouse kidney proceeds after 14-dpc when primitive glomeruli are observed. At the same time, the number of collecting tubules also increases in association with the development of glomeruli (31). Considered with the finding that the a4 isoform was expressed in collecting ducts, these observations suggest that a4 gene expression is closely related to the differentiation of renal collecting tubules.

Renal intercalated cells in cortical collecting ducts have been classified into at least two cell types (α and β) (8, 24, 32). For acid/base homeostasis, α- and β-intercalated cells are thought to be involved in proton and bicarbonate secretion, respectively (8, 32). The α cells express V-ATPase on their apical membranes and AE1 on their basolateral membranes (8, 28–30), suggesting that the V-ATPase with the a4 isoform is required for apical proton secretion in α cells.

On the other hand, β cells are thought to be mirror images of α-intercalated cells, with apical Cl⁻/HCO₃⁻ exchanger and basolateral V-ATPase (8, 32). As described above, a4 was clearly detectable on the β cell basolateral membrane. A new Cl⁻/HCO₃⁻ exchanger (AE4) was recently found in the apical surface of β cells (33), suggesting that a combination of AE4 and V-ATPase with a4 is important for bicarbonate secretion from apical membranes of β cells.

Smith et al. (34) have reported recently that the mutations in the human ATP6N1B gene cause recessive distal renal tubular acidosis. The ATP6N1B gene product is highly expressed in kidney and exhibits 85.8% identity with the mouse a4 isoform, suggesting that the ATP6N1B gene codes for a human counterpart of the mouse a4 isoform (Fig. 1). The ATP6N1B product has been suggested to be present on the apical surface of kidney-specific V-ATPase Subunit a Isoform.
intercalated cells in cortical collecting ducts. In contrast, the mouse α4 isoform was clearly detectable not only on the apical surface of α-intercalated cells but also on the basolateral surface of β cells, implying that human α4 may also be present on the basolateral surface of β cells.

Two isoforms (B1 and B2) of subunit B have been identified in the mammalian V-ATPases V1 sector (35, 36). The B1 isoform is expressed specifically in kidney (35–37), whereas B2 is ubiquitously observed. Mutations in the human B1 gene also cause distal renal tubular acidosis (37), similar to that in the ubiquitously observed. Mutations in the human B3 gene also cause renal tubular acidosis (37), whereas B2 form is expressed specifically in kidney (35, 36). The B1 isoform was clearly detectable not only on the apical cells, implying that human α4 may also be present on the basolateral surface of β cells. Two isoforms (B1 and B2) of subunit B have been identified in the mammalian V-ATPases V1 sector (35, 36). The B1 isoform is expressed specifically in kidney (35–37), whereas B2 is ubiquitously observed. Mutations in the human B1 gene also cause distal renal tubular acidosis (37), similar to that in the ubiquitously observed. Mutations in the human B3 gene also cause renal tubular acidosis (37), whereas B2 form is expressed specifically in kidney (35, 36). The B1 isoform was clearly detectable not only on the apical cells, implying that human α4 may also be present on the basolateral surface of β cells.

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