Localization and Regulation of the ATP6V0A4 (a4) Vacuolar H\(^+\)-ATPase Subunit Defective in an Inherited Form of Distal Renal Tubular Acidosis

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Abstract. Vacuolar-type H\(^+\)-ATPases (V-H\(^+\)-ATPases) are the major H\(^+\)-secreting protein in the distal portion of the nephron and are involved in net H\(^+\) secretion (bicarbonate generation) or H\(^+\) reabsorption (net bicarbonate secretion). In addition, V-H\(^+\)-ATPases are involved in HCO\(_3\)- reabsorption in the proximal tubule and distal tubule. V-H\(^+\)-ATPases consist of at least 13 subunits, the functions of which have not all been elucidated. Mutations in the accessory ATP6V0A4 (a4 isoform) subunit have recently been shown to cause an inherited form of distal renal tubular acidosis in humans. Here, the localization of this subunit in human and mouse kidney was studied and the regulation of expression and localization of this subunit in mouse kidney in response to acid-base and electrolyte intake was investigated. Reverse transcription–PCR on dissected mouse nephron segments amplified a4-specific transcripts in proximal tubule, loop of Henle, distal convoluted tubule, and cortical and medullary collecting duct. a4 protein was localized by immunohistochemistry to the apical compartment of the proximal tubule (S1/S2 segment), the loop of Henle, the intercalated cells of the distal convoluted tubule, the connecting segment, and all intercalated cells of the entire collecting duct in human and mouse kidney. All types of intercalated cells expressed a4. NH\(_4\)Cl or NaHCO\(_3\) loading for 24 h, 48 h, or 7 d as well as K\(^+\) depletion for 7 and 14 d had no influence on a4 protein expression levels in either cortex or medulla as determined by Western blotting. Immunohistochemistry, however, demonstrated a subcellular redistribution of a4 in response to the different stimuli. NH\(_4\)Cl and K\(^+\) depletion led to a pronounced apical staining in the connecting segment, cortical collecting duct, and outer medullary collecting duct, whereas NaHCO\(_3\) loading caused a stronger bipolar staining in the cortical collecting duct. Taken together, these results demonstrate a4 expression in the proximal tubule, loop of Henle, distal tubule, and collecting duct and suggest that under conditions in which increased V-H\(^+\)-ATPase activity is required, a4 is regulated by trafficking but not protein expression. This may allow for the rapid adaptation of V-H\(^+\)-ATPase activity to altered acid-base intake to achieve systemic pH homeostasis. The significance of a4 expression in the proximal tubule in the context of distal renal tubular acidosis will require further clarification.

The kidneys play a major role in the regulation and maintenance of body acid-base homeostasis. This is achieved by two mechanisms, the reabsorption of filtered bicarbonate in the proximal segments and the additional secretion of acid or base equivalents in the distal segments (1). All processes require the function of a V-type (vacuolar) H\(^+\)-ATPase expressed in the plasma membrane (1,2). Approximately 70 to 80% of the total filtered bicarbonate is reabsorbed in the proximal tubule, an additional 15% is reabsorbed along the thick ascending loop (TAL) of Henle and the distal tubule, and only 5% is reabsorbed in the connecting segment (CNT) and cortical collecting duct (CCD) (1). In the proximal tubule, protons are secreted across the apical brush border membrane into urine, where they react with HCO\(_3\)\(^-\) to form CO\(_2\), which then diffuses into the cells, is hydrated, and regenerates H\(^+\) and HCO\(_3\)\(^-\). HCO\(_3\)\(^-\) is released into blood, whereas the H\(^+\) reenters the cycle across the apical membrane. The majority of H\(^+\) in the proximal tubule is secreted by several Na\(^+\)/H\(^+\) exchanger isoforms, including NHE-3 (1). However, it has also been demonstrated that approximately 40% of the overall bicarbonate reabsorption in the proximal tubule is independent of luminal Na\(^+\) and thus may reflect H\(^+\)-ATPase–mediated H\(^+\) secretion (3). Reabsorption of bicarbonate in the TAL is also thought to depend on
both Na\(^+\)/H\(^+\) exchange and V-type H\(^+\)-ATPase activity (4,5). In the CNT and collecting duct, bicarbonate reabsorption is driven and mediated mainly by the V-type H\(^+\)-ATPase expressed in type A intercalated cells. V-type H\(^+\)-ATPases also play an important role in the secretion of bicarbonate/base equivalents by type B intercalated cells of the CCD. There, V-H\(^+\)-ATPases are expressed at the basolateral surface and mediate H\(^+\) secretion into blood; this H\(^+\) arises from the intracellular generation of HCO\(_3\)-, which is released into urine through Cl\(^-\)/HCO\(_3\)- exchange across the apical membrane. In addition, an H\(^+\)/K\(^+\)-ATPase is found in the collecting duct and may contribute to H\(^+\) excretion under certain circumstances, such as dietary K\(^+\) depletion (6).

The significance of V-H\(^+\)-ATPases in renal H\(^+\) secretion is highlighted by two forms of inherited distal renal tubular acidosis (dRTA) (7,8). Mutations in the ATP6V1B1 gene cause dRTA combined with sensorineural deafness as a result of expression of the B1 V-H\(^+\)-ATPase subunit in the intercalated cells of the collecting duct and some specialized cells of the inner ear (9,10). Recently, a second form of dRTA was identified, caused by mutations in the ATP6V0A4 gene coding for the a4 V-H\(^+\)-ATPase subunit (11). This form of dRTA may also be associated with deafness in some cases (12).

V-type H\(^+\)-ATPases consist of at least 13 subunits arranged in a peripheral V\(_{p}\) and membrane-bound V\(_{o}\) domain (for review, see (13,14)). For many of these subunits, organ- or cell-specific isoforms that may be involved in the cell-specific regulation or targeting of the pump complex exist as suggested by studies in yeast (14,15). Little is known about the function of the mammalian homologs of the yeast isoforms and the specific roles of cell-specific isoforms. For the a subunit, four isoforms have been identified in C. elegans, chicken, cow, mouse, and human, which display distinct organ expression patterns (11,16–23). On the basis of Northern blotting, the a1, a2, and a3 subunits are mainly found in extrarenal tissues but also show some expression in the kidney (16,17,20,21). In contrast, the a4 subunit has the most restricted expression and has been detected only in kidney, epididymis, and inner ear in human and mouse (11,12,18,22). However, differences in the localization of the a4 subunit in kidney were reported, with the initial report describing localization in only one subset of intercalated cells in human kidney (11). In mouse, Oka et al. (18) localized the a4 subunit to both type A and type B intercalated cells, whereas we recently demonstrated that the a4 subunit could be found in all types of intercalated cells and also with strong expression in the initial segment of the proximal tubule in mouse kidney (22). These discrepancies could reflect technical differences (e.g., use of antigen retrieval, fixation of the tissue) or species-specific expression patterns and need to be resolved to elucidate further the role of tissue-specific V-H\(^+\)-ATPase isoforms as well as the particular role of this disease causing a4 V-H\(^+\)-ATPase subunit. Therefore, we examined the localization of the a4 subunit in mouse and human kidney and, using antigen retrieval techniques, demonstrated expression in most nephron segments. In addition, we show that although the protein expression levels of the a4 subunit are not influenced by acid-base status, there is a marked subcellular redistribution of the a4 subunit in intercalated cells in response to acid-base changes and dietary potassium intake.

### Materials and Methods

**Animals**

C57BL/6J mice (male, 25 to 30 g; Jackson Laboratories, Bar Harbor, ME) were maintained on a standard diet and had access to drinking water *ad libitum*. Mice were given either 2% sucrose/0.28 M NH\(_4\)Cl or 2% sucrose/0.28 M NaHCO\(_3\) in the drinking water for 24 h, 48 h, or 7 d as described previously (24–26). These treatments have been shown to induce mild metabolic acidosis or alkalosis in rodents (26,27). The control group received only 2% sucrose in their drinking water. An additional group of mice was provided a K\(^+\)-depleted diet for 7 or 14 d (Bio-serv, Frenchtown, NJ) as described previously (25,28). Each group consisted of 8 to 10 animals for each time point and treatment, respectively. Four animals of each group were used for Western blotting, and the remaining 4 to 6 were used for immuno-histochemistry. For blood analysis, mice were anesthetized and heparinized venous blood was collected and analyzed immediately for blood gases and electrolytes on a Radiometer ABL 505 (Radiometer, Copenhagen, Denmark) blood gas analyzer. Urine was collected as spot urine, and pH was measured immediately using a pH microelectrode (Lazar Research Laboratories, CA) connected to Thermo Orion 290 pH meter. All studies were approved by the Yale Animal Care and Use Committee or the Swiss Veterinäramt (Zurich, Switzerland).

**Western Blotting**

Animals were killed, and kidneys were rapidly harvested. After homogenization in an ice-cold K-HEPES buffer (200 mM mannitol, 80 mM K-HEPES, 41 mM KOH [pH 7.5]) with pepstatin, leupeptin, K-EDTA, and PMSF as protease inhibitors, the samples were centrifuged at 1000 × g for 10 min at 4°C and the supernatant was saved. Subsequently, the supernatant was centrifuged at 100,000 × g for 1 h at 4°C, and the pellet was resuspended in K-HEPES buffer containing protease inhibitors. After measurement of the total protein concentration (Bio-Rad, Hercules, CA), 50 μg of crude membrane protein was solubilized in Laemmli sample buffer, and SDS-PAGE was performed on a 10% polyacrylamide gel. An initial gel was stained with Coomassie blue to confirm equal loading among samples. For immuno-blotting, the proteins were transferred electrophoretically from unstained gels to polyvinylidene fluoride membranes (Immobilon-P, Millipore, Bedford, MA). After blocking with 5% milk powder for 60 min, the blots were incubated with the primary antibodies (rabbit anti-a4 raw serum 1:5000 [the anti-a4 serum had been produced in rabbit using a C-terminal peptide of the human sequence that had a N-terminally introduced cysteine for linkage to keyhole limpet hemocyanin (KLH; NH\(_2\)-CKFSPPFSFKHLGTAEE-COOH) (11)]) and mouse monoclonal anti-actin (42 kD; Sigma, St. Louis, MO) 1:500 either for 2 h at room temperature or overnight at 4°C. After washing off the primary antibody and subsequent blocking, blots were incubated with the secondary antibodies (donkey anti-rabbit 1:10,000 and sheep anti-mouse 1:5000 IgG-conjugated with horse radish peroxidase [Amersham Life Sciences]) for 1 h at room temperature. Antibody binding was detected with the enhanced chemiluminescence ECL kit (Amersham Pharmacia Biotech, UK) before exposure to x-ray film (Kodak). All films were scanned and analyzed using SCION Imaging software.
loops of Henle. Magnification, (red) and AQP-2 (green). Note also apical staining in two adjacent cells. (A) No staining was observed in cortical sections incubated with the anti-a4 serum plus the immunizing peptide. (B) Phase contrast photo of the same region as in A. G, glomerulus; PT, proximal tubule; C, cortical collecting duct. (C) Immunolocalization of a4 in the brush border membrane of proximal tubules. (D) Triple labeling for a4 (red), the calcium-binding protein calbindin (green), and the AQP-2 water channel (blue; which is specifically expressed in the principal cells of the collecting duct). Co-localization of calbindin (green) and a4 (red) in the same cells are seen, whereas no blue staining was observed, indicating expression of a4 in the distal tubule. (E) Double labeling for a4 (red) and the water channel AQP-2 (green) in the cortical collecting duct (CCD), demonstrating expression of a4 in intercalated cells but not principal cells. Note that all cells are stained either for AQP-2 or for a4, suggesting a4 expression in all types of intercalated cells. (F) Double labeling of an outer medullary collecting duct (OMCD) for a4 (red) and AQP-2 (green). Note also apical staining in two adjacent loops of Henle. Magnification, ×400.

**Immunohistochemistry**

**Human Kidney.** Three different human kidney specimens were obtained from Dr. K. Klingel (Department of Pathology, University of Tübingen, Tübingen, Germany), which originated from renal tumor resections but showed no tumor infiltration. The use of the tissue for research was approved by the local ethics commission. Sections were fixed with paraformaldehyde by immersion and embedded in paraffin. Five-micrometer sections were cut, deparaffinized with xylol, washed with decreasing ethanol concentrations, and subsequently rehydrated with PBS before immunohistochemistry was performed as described below.

**Mouse Kidney.** C57BL/6J mice were anesthetized with ketamine and perfused through the left ventricle with PBS followed by paraformaldehyde-lysine-periodate fixative (29). Kidneys were removed and fixed overnight at 4°C by immersion in paraformaldehyde-lysine-periodate. Kidneys were washed three times with PBS, and 5-μm cryosections were cut after cryoprotection with 2.3 M sucrose in PBS for at least 12 h. Immunostaining was carried out as described previously (28,30). Sections were incubated with 1% SDS for 5 min, washed three times with PBS, and incubated with PBS containing 1% BSA for 15 min before the primary antibody. The primary antibodies (rabbit anti-ATP6V0A4 [a4] serum 1:500, rabbit anti-NCC serum 1:500; gift of J. Loffing, Institute of Anatomy, University of Zurich, Zurich, Switzerland), and goat anti-human AQP-2 [Santa Cruz Biotechnology, Santa Cruz, CA] 1:100, mouse monoclonal anti-calbindin-D 28k [Swant, Bellinzona, Switzerland] 1:10,000) were both diluted in PBS and applied either for 75 min at room temperature or overnight at 4°C. Sections were then washed twice for 5 min with high NaCl PBS (PBS + 2.7% NaCl), once with PBS, and incubated with dilutions of the secondary antibodies (donkey anti-rabbit S86 [1:1000], donkey anti-mouse 488 [1:400], donkey anti-goat 488 [1:200; Molecular Probes, Eugene, OR] or donkey anti-goat Cy5 [Jackson Immunolab]) at the given dilutions for 1 h at room temperature. Sections were again washed twice with high-NaCl PBS and once with PBS before mounting with VectaMount (Vector Laboratories, Burlingame, CA). Sections were viewed either with a Zeiss LSM 410 confocal microscope or with a Leica CLSM confocal microscope. Images were processed (overlays) using Adobe Photoshop. For cell counts, kidneys from four animals for each treatment were used and 5 to 10 pictures from at least two sections from each kidney were taken for each nephron segment analyzed. Cells were counted as being positive either for a4 (intercalated cells) or for AQP-2 (principal cells). Intercalated cells were further classified on the basis of the predominant subcellular distribution of a4 immunostaining as described previously (24).

**Tubule Isolation and Reverse Transcription–PCR**

C57BL/6J mice (male, 25 to 30 g; Jackson Laboratory) were anesthetized with ketamine and perfused through the left ventricle with PBS containing 250 μg/ml collagenase (Sigma C-9891). The kidneys were rapidly removed, and coronal slices 2 to 3 mm in thickness were cut and incubated in PBS/collagenase at 37°C for 15 min. After rinsing with ice-cold PBS several times, the different nephron segments were hand-dissected and transferred into Trizol. Total RNA was extracted, purified following the manufacturer’s protocol, and reverse-transcribed using oligo(dT)12–18 as primer according to standard methods. Aliquots of the resulting DNA were amplified by PCR using three different pairs of specific primers, each spanning at least one intron. The primer sequences used for Figure 3 were gcagtgcatcatcgccgagatc (forward) and gaacataggctggacactccaag (reverse). Reactions (in a 20-μl volume) contained 1× buffer, 0.2 mM dNTPs, 1.5 mM MgCl2, 1 μM primers, and 5 U TaqDNA polymerase (Roche). Cycling conditions were [95°C, 64°C, 72°C] × 38 cycles, preceded by denaturing at 95°C for 3 min and followed by final extension at 72°C for 10 min. Whole mouse cortex and water provided positive and negative controls, respectively. Products were resolved on a 2% agarose gel stained with ethidium bromide and photographed under ultraviolet light.
Localization of a4 in Human Kidney

Differences in a4 expression patterns were previously reported in human and mouse kidney (11,18,22). Thus, we first sought to examine whether these discrepancies were explained by differences in tissue preparation such as the use of antigen retrieval techniques. An antibody raised specifically against the human a4 subunit was used to localize the vacuolar H^+-ATPase a4 subunit in human kidney sections (11,22). In the absence of any antigen retrieval techniques on human kidney sections, only a weak but specific signal could be found in the intercalated cells along the collecting duct (data not shown). However, antigen retrieval using either SDS or microwaving unmasked specific staining also in other nephron segments, suggesting that the failure of previous studies to detect a4 expression in the human proximal tubule (11) was indeed related to technical differences in antigen retrieval techniques.

The specificity of the staining was demonstrated by preincubation of the antibody with the immunizing peptide, which abolished all staining (Figure 1, A and B). Also, no staining was observed when the preimmune serum or the secondary antibody alone was used (data not shown). a4-specific staining was observed in the brush border and in the subapical compartment of the proximal tubule, with more intense staining seen in the early segments (S1 and S2; Figure 1C). Furthermore, apical staining was seen in the thick ascending limb and the distal convoluted tubule (DCT; Figure 1D). Intercalated cells in the CNT, CCD and medullary collecting duct, particularly the outer medullary collecting duct (OMCD), showed strong labeling. The staining was restricted to intercalated cells as demonstrated by double labeling of the principal cell-specific water channel AQP-2. Triple labeling of the calcium-binding protein calbindin-D28k and the AQP-2 water channel was performed to confirm localization of a4 in the DCT. In human kidney, calbindin-D28k is expressed both in the DCT and in the CCD, whereas AQP-2 is only found in the principal cells of the CNT and collecting duct (31). As shown in Figure 1D, co-localization of calbindin-D28k with a4 was observed (green, calbindin-D28k; red, a4; blue, AQP-2) in nephron segments not expressing AQP-2, consistent with localization in the DCT. In the intercalated cells of the CNT and CCD, a4 expression was restricted to cells negative for AQP-2, thus indicating a4 expression in intercalated cells. Notably, all AQP-2–negative cells were positive for a4, consistent with a4 expression in all types of intercalated cells. Most intercalated cells in the CNT and CCD showed an apical localization of a4 (Table 1); cells with a bipolar, basolateral, or diffuse staining were only rarely observed. In the medullary collecting duct, a4 expression was confirmed by reverse transcription–PCR (RT-PCR). Mouse kidney was hand-dissected, and total RNA was extracted from specific nephron segments and total mouse kidney. RT-PCR was performed with intron-spanning primers specific for mouse a4 (Atp6n1b) as described in Materials and Methods. A band of the expected size of approximately 500 bp was found on an ethidium bromide–stained gel for all segments and total kidney. Omitting RNA from the RT-PCR reaction yielded no bands. The lanes were loaded with RT-PCR products from the following samples: 1, proximal tubule; 2, loop of Henle; 3, distal tubule; 4, CCD; 5, medullary collecting duct; 6, no RNA (water); 7, total kidney.
expression was confined to the apical side of all intercalated cells.

**Localization of a4 in Mouse Kidney**

To compare the localization of a4 in another species and to examine a4 expression and localization in response to acid-base status and electrolyte intake, we also studied a4 in mouse kidney. The anti-a4 serum recognized only one band of approximately 115 kD in membrane fractions obtained separately from mouse kidney cortex or medulla. Preincubation with the immunizing peptide abolished this band (Figure 2A). Furthermore, Western blotting with membrane fractions obtained from several mouse tissues showed a specific signal only in kidney and epididymis, not in other major organs expressing high levels of V-H⁺-ATPase, such as brain (Figure 2B). To investigate the segmental expression of ATP6V0A4 mRNA along the mouse nephron, we isolated RNA from hand-dissection nephron segments and performed reverse transcription–PCR using three different sets of nested primers specific for the murine a4 isoform, each pair of which spanned at least one intron. Expression was found in the proximal tubule, the loop of Henle, distal tubule, CCD, and medullary collecting duct (Figure 3).

Similar to our findings in human kidney, a4 was localized by immunohistochemistry to both the cortex and the medulla (Figure 4C). All staining could be abolished by preincubation of the serum with the immunizing peptide, and no staining was observed with the preimmune serum alone (Figure 4, A and B). a4 staining was seen in the brush border membrane and apical compartment of the proximal tubule with stronger staining in the early segments (S1 > S2 > S3; Figure 5A). Thick ascending limb and the early distal tubule were stained weakly on the apical side. In the late distal tubule, a4 staining was stronger and co-localized with calbindin-D28k but in a different subset of cells (Figure 5B). The localization of a4 in the distal tubule was further confirmed by staining for the thiazide-sensitive Na/Cl co-transporter NCC, which in mouse is expressed in the distal tubule but not elsewhere (32). a4 immunoreactivity was seen in consecutive sections in the same segment as NCC staining (Figure 5F and G). In the CNT and CCD, a4 staining was found only in cells negative for the water channel AQP-2, indicating that a4 is expressed in intercalated cells but not in principal cells (Figure 5, B and C). All cells negative for AQP-2 were positive for a4, with a mixed pattern of staining (apical, bipolar, or diffuse intracellular) in the CCD (Figure 5C) and only apical staining in the OMCD and initial inner medullary collecting duct (Figure 5D).
Regulation of a4 Expression and Localization in Mouse Kidney by Acid-Base and Electrolyte Status

To gain further insight into the potential physiologic role of the a4 subunit, we provided mice different dietary challenges that have been shown to induce metabolic acidosis (NH4Cl loading), metabolic alkalosis (NaHCO3 loading), or mild hypokalemia (K+ depletion). The effect of the diets on systemic blood pH, bicarbonate, and electrolytes as well as on urinary pH was controlled and is summarized in Table 2. Loading with NaHCO3 did not result in a significant elevation of venous bicarbonate levels but led to a pronounced urine alkalization. Similarly, chronic K+ depletion caused a significant fall of serum K+ levels without increasing venous bicarbonate levels. The normal bicarbonate levels found under NaHCO3 loading and chronic K+ depletion may be due to the analysis of venous blood samples. NH4Cl loading of the drinking water for 24 h, 48 h, or 7 d caused metabolic acidosis but did not alter a4 protein expression levels in total kidney membrane fractions (Figure 6) or separate fractions obtained from cortex or medulla (data not shown; Figure 6). Also, NaHCO3 loading for 24 h, 48 h, or 7 d did not influence a4 protein expression levels (Figure 6). Similarly, induction of K+ depletion for 1 or 2 wk with dietary potassium restriction, a condition known to induce metabolic alkalosis and stimulate vacuolar H+-ATPase activity in the distal tubule and CCD (33,34), did not alter a4 protein expression levels in total kidney (Figure 7) or separate cortical and medullary preparations (data not shown).

Immunohistochemistry, however, demonstrated marked differences in the subcellular localization of a4 in response to the different electrolyte challenges as summarized in Table 3. None of the treatments affected the ratio of intercalated to principal cells. NH4Cl loading led to a redistribution of a4 staining in intercalated cells from a diffuse or bipolar pattern to a strong rim-like apical staining that could be seen as soon as after 24 h and remained up to 1 wk (Figure 8, A and B). The pronounced apical staining was seen in the DCT, CCT, and medullary collecting duct. Cells with basolateral staining were only rarely observed in CCD. In the proximal tubule, no alteration of staining could be detected at the level of light microscopy immunohistochemistry after acid loading. In contrast, after NaHCO3 loading, a predominantly bipolar a4 staining was found in many intercalated cells of the CCD and a more cytosolic a4 distribution was observed in the CNT and medullary collecting duct (Figure 8, C and D).

Chronic dietary potassium depletion is often associated with metabolic alkalosis. The increased activity of V-H+-ATPases in the distal tubule and CCD has been implicated in the development of metabolic alkalosis in this setting, and a more pronounced apical staining of the V-H+-ATPase 70 kD (A) and 31 kD (E) subunits was described in intercalated cells of these nephron segments in response to K+ depletion (1,33,34). In agreement with these findings, we also found strong apical labeling of intercalated cells in the DCT, CNT, CCD, and medullary collecting duct after K+ depletion (Figure 8, E and F, Table 3). In the DCT, CNT, and CCD, staining was primarily apical, with almost no intracellular or basolateral staining observed.

Discussion

Vacuolar H+-ATPases are involved in a variety of cellular functions in the kidney, such as protein endocytosis from the lumen in the proximal tubule (35), recycling of transport proteins such as the water channel AQP-2 (36), insertion of vesicles into the membrane, and lysosomal degradation of proteins (2,37). Inborn defects of the ATP6V1B1 or ATP6V0A4 V-type H+-ATPase subunits have been shown to affect the kidney and to manifest themselves as syndromes of dRTA often associated with sensorineural deafness (8,9,11,12). Thus, for understanding the role of these subunits and the development of the syndromes caused by the loss of their activity, the tissue and cellular and subcellular distribution need to be determined. a-subunit homologs have recently been cloned from several species, including human (11), mouse (18,22), and C. elegans (19). Different patterns of a4 subunit expression in human and mouse kidney, however, have been reported (11,18,22). To resolve these discrepancies, we studied a4 expression in these two species and examined a4 regulation in response to acid-base status and electrolyte intake in mouse kidney.

Expression of the a4 subunit was found along the human and mouse nephron in all segments known to express V-H+-ATPases: the proximal tubule, loop of Henle, the CNT, CCD,

Table 2. Urine and blood data from mice treated with different diets

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<th>Control</th>
<th>NH4Cl 1 Day</th>
<th>NH4Cl 2 Days</th>
<th>NH4Cl 7 Days</th>
<th>NaHCO3 1 Day</th>
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<td>pH</td>
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<td>5.64 ± 0.04*</td>
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<td>Blood pH</td>
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<td>n.t.</td>
<td>7.13 ± 0.04*</td>
<td>7.26 ± 0.03</td>
<td>n.t.</td>
<td>7.22 ± 0.04</td>
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<tr>
<td>HCO3⁻</td>
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<td>9.2 ± 0.9*</td>
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<td>18.5 ± 1.4</td>
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<td>n.t.</td>
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* Significantly different from control group (P ≤ 0.05).

n.t., not tested.
Figure 5. Expression of α4 along the mouse nephron. (A) Labeling of mouse kidneys with anti-α4 serum (red) and the principal cell-specific water channel AQP-2 (green). Strong staining for α4 of the brush border membrane of the early proximal tubule segments (S1, S2) and of some distinct cells in an adjacent CCD was seen. (B) Connecting tubule with α4 (red) localized to the apical side of intercalated cells, principal cells were marked with the AQP-2 water channel (blue), the calcium-binding protein calbindin is marked in green. (C) Double labeling for α4 (red) and AQP-2 (green) showing α4 staining in a subset of cortical collecting cells negative for AQP-2 (principal cells). All cells negative for AQP-2 were positive for α4, demonstrating expression in all types of intercalated cells. Note that several patterns of subcellular α4 staining are visible as described previously for other H^+^-ATPase subunits in the CCD with apical (arrow head), bipolar (arrow), and basolateral (*) localization (2). (D) Expression of α4 (red) in the OMCD was seen only in cells devoid of the AQP-2 (green) water channel. (E) Localization of α4 in the early and late distal convoluted tubule (DCT1, DCT2) and CCD. Mouse kidneys were stained against α4 (red), the calcium-binding protein calbindin (green) expressed in both DCT and CCD and the AQP-2 water channel specifically expressed in the principal cells of the connecting segment (CNT) and collecting duct. α4 staining (red) is seen in proximal tubules, early and late DCT, and intercalated cells of the CNT and CCD. DCT1 and DCT2 are identified by calbindin staining (green) but the absence of AQP-2 (blue). Note the transition of the early DCT (DCT1) and late DCT (DCT2) marked by the appearance of calbindin and the α4 staining in both segments (arrowheads). (F and G) Localization of α4 in the distal tubule. Consecutive sections were stained against the thiazide-sensitive Na/Cl co-transporter NCC (red), specifically expressed in mouse distal tubule, and the AQP-2 water channel (green; F) or against the α4 vacuolar H^+^-ATPase subunit (red), calbindin (green), and AQP-2 (blue; G). Co-localization of NCC and α4 demonstrate expression of α4 also in distal tubule. Magnifications: ×600 in B and C, ×550 in D, ×400 in E, ×500 in G.

OMCD, and initial inner medullary collecting duct (2). In the proximal tubule, expression was seen in the brush border and apical compartment, suggesting a role in bicarbonate reabsorp-
Materials and Methods. The induction of K+actin (42 kD), and the ratio of α4:actin was calculated as described in Methods. For controlling for protein expression levels were assessed by Western blotting of a crude membrane fraction prepared from total kidney. For controlling for protein loading, all Western blot membranes were stripped and reprobed for a4 protein. Similar results were obtained after only 1 wk of a low-potassium diet (data not shown).

Subcellular localization of the α4 vacuolar H+-ATPase subunit in intercalated cells in mouse kidney in response to different diets. Indeed, a1, a2, and a3 transporters participate not only in proton extrusion/bicarbonate reabsorption but also in more complex cellular processes such as receptor-mediated endocytosis and lysosomal degradation of proteins and also may be involved in exocytotic processes (1,2,35,38). That dRTA patients do not show evidence of generalized proximal tubular dysfunction may reflect that these other V-H+-ATPase functions may be performed by other a isoforms under normal conditions. α4 was found not only in the intercalated cells of the CCD and medullary collecting duct but in additional nephron segments and that mutations in the a4 coding gene ATP6VOA4 are associated with only distal renal tubular acidosis but not a more generalized tubular dysfunction raises several questions as to the function of the α4 subunit in the proximal tubule and loop of Henle. In the proximal tubule, V-H+-ATPases participate not only in proton extrusion/bicarbonate reabsorption but also in more complex cellular processes such as receptor-mediated endocytosis and lysosomal degradation of proteins and also may be involved in exocytotic processes (1,2,35,38).

Table 3. Subcellular localization of the α4 vacuolar H+-ATPase subunit in intercalated cells in mouse kidney in response to different diets

<table>
<thead>
<tr>
<th>Diet</th>
<th>IC (%)</th>
<th>PC (%)</th>
<th>Apical (%)</th>
<th>Subapical (%)</th>
<th>Diffuse (%)</th>
<th>Bipolar (%)</th>
<th>Basolateral (%)</th>
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<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>CNT</td>
<td>40.7 ± 1.8</td>
<td>59.3 ± 1.8</td>
<td>73.3 ± 7.5</td>
<td>18.3 ± 6.0</td>
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<td>39.4 ± 1.5</td>
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<td>27.3 ± 3.9</td>
<td>20.1 ± 3.9</td>
<td>17.8 ± 3.1</td>
<td>26.9 ± 4.0</td>
<td>8.0 ± 2.4</td>
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<td>42.4 ± 10.3</td>
<td>52.6 ± 10.8</td>
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<tr>
<td>IMCDi</td>
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<td>71.2 ± 9.3</td>
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<tr>
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<td>79.8 ± 5.6</td>
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<td>16.5 ± 6.9*</td>
<td>72.4 ± 7.9*</td>
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<td>23.0 ± 2.8</td>
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<tr>
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<td>17.5 ± 11.6*</td>
<td>80.1 ± 11.2*</td>
<td>2.4 ± 2.4</td>
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<td>IMCDi</td>
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<tr>
<td>CNT</td>
<td>40.9 ± 3.7</td>
<td>59.1 ± 3.7</td>
<td>77.2 ± 8.0</td>
<td>18.3 ± 6.2</td>
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</tr>
<tr>
<td>CCD</td>
<td>38.1 ± 1.2</td>
<td>61.9 ± 1.2</td>
<td>55.9 ± 2.7*</td>
<td>17.7 ± 2.6</td>
<td>9.2 ± 2.1*</td>
<td>15.7 ± 2.4*</td>
<td>1.4 ± 0.9*</td>
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<tr>
<td>OMCD</td>
<td>36.2 ± 2.0</td>
<td>63.8 ± 2.1</td>
<td>82.8 ± 2.1*</td>
<td>17.2 ± 2.1*</td>
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<td>IMCDi</td>
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<td>85.1 ± 4.8*</td>
<td>13.8 ± 4.2*</td>
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* Significantly different from control condition.
Figure 8. Alterations in acid-base status and chronic K\textsuperscript{+} depletion led to changes in subcellular a4 localization in mouse kidney. Mouse kidneys were stained for a4 (red) and AQP-2 (green). (A) DCT and CCD after 2 d of NH\textsubscript{4}Cl loading showing in most intercalated cells a rim-like apical staining. Intercalated cells in the DCT2 were bulging into the lumen and appeared hypertrophic as described before for the intercalated cells of the medullary collecting duct under chronic potassium depletion (50). Only a few intercalated cells with diffuse or basolateral staining were visible (data not shown). (B) Medullary collecting duct from the transition of outer to inner medulla after 2 d of acid loading with prominent apical a4 staining in intercalated cells. (C) CCD after 1 wk of bicarbonate loading with most intercalated cells showing mainly cytosolic or bipolar staining typical for type B or non–type A/B bicarbonate secreting intercalated cells. (D) In the OMCD after 1 wk of bicarbonate loading, a4 protein was diffusely localized in the cytosol and on the apical side of intercalated cells, suggesting downregulation of proton secretion. (E) Chronic K\textsuperscript{+} depletion for 2 wk led to a strong apical a4 staining of intercalated cells in the CCD. Only very few intercalated cells with diffuse or basolateral staining could be found (data not shown). (F) In the same kidney, apical staining of intercalated cells in the convoluted (late) distal tubule seemed to be more enhanced. Magnifications: \( \times 400 \) in A through C, E, and F; \( \times 520 \) in D.
scripts have been detected by Northern blotting and reverse transcription–PCR of total kidney RNA preparations (16,20,21,23). However, the cellular and subcellular localizations of the α1, α2, and α3 proteins in kidney are unknown at present.

That α4 was previously not detected in human and mouse proximal tubule may reflect technical differences in immunostaining methods, as we were unable to detect significant and specific α4 immunostaining in the proximal tubule or loop of Henle without antigen retrieval. Similarly, other proteins such as the Na+/K+-ATPase have been detected only in intercalated cells after antigen retrieval (40).

Induction of metabolic acidosis or alkalosis as well as changes in the dietary intake of electrolytes are known to alter the activity and subcellular localization of the vacuolar H+-ATPase in different nephron segments (2,33,34,41–44). In the distal tubule and CCD, induction of metabolic acidosis leads to the activation of mainly type A acid-secretory intercalated cells, whereas metabolic alkalosis stimulates type B intercalated cells (2,24,45,46). Consistent with this change in intercalated cell appearance and function, the α4 subunit was found in the apical membrane of most intercalated cells in the late DCT, CNT, and CCD after NH4Cl loading. Even though we did not correlate the subcellular localization of the α4 subunit with markers of type A and type B intercalated cells (e.g., AE1, pendrin), it is very likely that the redistribution of the α4 subunit to different subcellular localizations in the CCD is associated with the activation of acid and/or inactivation of bicarbonate secretion in this segment under the different conditions as previously shown (28,46–48). Similarly in chronic K+ depletion, a condition previously shown to induce metabolic alkalosis partly through increased reabsorption of bicarbonate and secretion of protons (1), prominent apical staining of intercalated cells in all segments was seen as described previously for the E (31 kD) subunit (34). Only a few intercalated cells with diffuse or basolateral staining could be observed under both conditions, acid loading or chronic K+ depletion (data not shown). In contrast, after alkali loading, most intercalated cells in the CCD showed a strong bipolar or basolateral labeling. These findings are in good agreement with earlier reports showing trafficking of other H+-ATPase subunits in response to metabolic acidosis or alkalosis or dietary electrolyte intake (2). In addition, Western blotting for the α4 subunit in separate corticomedullary fractions under the different treatments showed no changes in the protein expression levels. In contrast, Fejes-Toth and Naray-Fejes-Toth (49) suggested changes in mRNA levels of the E (31 kD) subunit in response to metabolic acidosis or alkalosis. However, Bastani et al. (24) showed no changes in the E (31 kD) H+-ATPase subunit mRNA and protein levels under these conditions, consistent with observations obtained in our own laboratory (C. Wagner, unpublished results). Thus, the majority of observations suggest that regulation of H+-ATPase activity in response to acid-base status is mediated by trafficking of pumps or pump subunits, rather than changes in subunit expression levels.

In summary, we demonstrate expression of the α4 H+-ATPase subunit in different nephron segments of human and mouse kidney, namely proximal tubule, loop of Henle, DCT, CNT, CCD, and medullary collecting duct. α4 protein levels are not affected by changes in acid-base status or electrolyte intake. However, on a subcellular level, α4 subunits are redistributed to the apical side of intercalated cells after acid-loading or chronic K+ depletion or to the basolateral side in the CNT and CCD after alkali loading. That loss of α4 subunit function in a syndrome of inborn dRTA does not affect proximal tubular function will need further clarification but may suggest a compensatory role for other α subunit isoforms.

Acknowledgments

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References


