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The H^+-ATPase B1 subunit localizes to the thick ascending limb and distal convoluted tubule of rodent and human kidney

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Abstract

The vacuolar-type H⁺-ATPase B1 subunit is heavily expressed in the intercalated cells of the collecting system, where it contributes to H⁺ transport, but has also been described in other segments of the renal tubule. This study aims to determine the localization of the B1 subunit of the vacuolar-type H⁺-ATPase in the early distal nephron, encompassing thick ascending limbs (TAL) and distal convoluted tubules (DCT) in human kidney and determine if the localization differs between rodents and humans. Antibodies directed against the H⁺-ATPase B1 subunit were used to determine its localization in paraffin embedded formalin fixed mouse, rat, and human kidneys by light microscopy and in sections of lowicryl embedded rat kidneys by electron microscopy. Abundant H⁺-ATPase B1 subunit immunoreactivity was observed in the human kidney. As expected, intercalated cells showed the strongest signal, but significant signal was also observed in apical membrane domains of the distal nephron, including TAL, macula densa and DCT. In mouse and rat, H⁺-ATPase B1 subunit expression could also be detected in apical membrane domains of these segments. In rat, electron microscopy revealed that the H⁺-ATPase B1 subunit was located in the apical membrane. Furthermore, the H⁺-ATPase B1 subunit colocalized with other H⁺-ATPase subunits in the TAL and DCT. In conclusion, the B1 subunit is expressed in the early distal nephron. The physiological importance of H⁺-ATPase expression in these segments remains to be delineated in detail. The phenotype of disease causing mutations in the B1-subunit may also relate to its presence in the TAL and DCT.

Keywords

Proton pump, Acidosis, Acid-base balance, ATP6V1B1, distal tubule.
Introduction

The kidney plays a critical role in maintaining acid-base homeostasis. Several mechanisms exist whereby the kidney can alter the tubular transport capacity for $\text{H}^+$ and $\text{HCO}_3^-$ in response to changes in systemic pH. The roles of the proximal tubule and intercalated cells (IC) of the connecting tubule (CNT) and collecting ducts (CD) in acid-base balance have been well defined. The early portion of the distal nephron, namely the distal tubule, encompassing the thick ascending limb (TAL) and distal convoluted tubule (DCT) (22) also contributes significantly to acid-base transport in kidney. In fact, detailed physiological measurements suggest that up to 15% of the filtered $\text{HCO}_3^-$ can be reclaimed by the loop of Henle (7) and that 5-10% of $\text{HCO}_3^-$ may be reabsorbed in the superficial distal convolution accessible by micropuncture (5, 6, 9). The contribution by the DCT remains to be clearly delineated, but significant differences have been observed in $\text{HCO}_3^-$ delivery between early and late puncture sites (5, 6, 9, 38). Dissection of the underlying cellular mechanisms responsible for $\text{HCO}_3^-$ reabsorption in the distal convolution (encompassing the DCT, CNT and initial cortical collecting duct (CCD)) is complicated by the gradual appearance of IC along the length of it. The molecular pathway of IC mediated $\text{HCO}_3^-$ reabsorption is well established and is ascribed to the primary activity of $\text{H}^+$-ATPases. The molecular mechanism responsible for the secretion of $\text{H}^+$ across the apical membrane of the distal tubule has been ascribed to both $\text{Na}^+/\text{H}^+$ exchangers and $\text{H}^+$-ATPases (2, 7, 14, 38). $\text{H}^+$-ATPases are likely involved due to the presence of IC along the length of the distal convolution. However, a role for $\text{H}^+$-ATPases in non-IC is supported by the $\text{H}^+$-ATPase inhibitor Bafilomycin A1, blocking $\text{HCO}_3^-$ absorption at lower concentrations in the early distal convolution, as opposed to the late part of the distal convolution (38) where IC are more plentiful.

Most studies on the role and regulation of the $\text{H}^+$-ATPase in the kidney have focused predominantly on the IC. Numerous $\text{H}^+$-ATPase subunits have been found expressed in the IC and mutation or deletion of the $\text{H}^+$-ATPase B1 subunit in humans or mice, lead to distal renal tubular acidosis (dRTA) (12, 18). Loss of function mutations in the B1 subunit of the $\text{H}^+$-ATPase are associated with more severe forms of distal renal tubular acidosis, as compared to dominant mutations in AE1 (17-19). These forms often present with hypokalemia and a salt losing phenotype that are not exclusively explained by dysfunction of the type A IC. This could indicate a role for the B1-subunit outside the type A cells (15). In the vast majority of studies
conducted on the localization or regulation of the H\textsuperscript{+}-ATPase B1 subunit, the only reported localization has been to the IC. The *ATPV1B1* gene encodes the human H\textsuperscript{+}-ATPase B1 subunit and transgenic insertion of eGFP or Cre driven by a 6.5 kb *ATPV1B1* gene promoter fragment in transgenic mice, has shown that expression of these transgenes was limited to IC throughout the nephron as well as principal-like cells and IC within the CNT of mouse kidney. No expression has been reported elsewhere (24, 25). Furthermore, the Cre recombinase driven by the *ATPV1B1* promoter is now frequently used to delete transgenes in IC and CNT cells (21, 31, 32).

Early immunolabeling studies documented expression of the 31 kD H\textsuperscript{+}-ATPase E subunit in the early distal nephron, including DCT, and CNT of rat (4), and subsequent studies additionally reported expression in the TAL (26, 29). Furthermore, Brown *et al* found the 56 kD subunit (unspecified B1/B2) expressed in the DCT (4). Later studies using B1 specific antibodies directed against the bovine epitope of B1 localized the subunit to the DCT of rat kidney, but not the TAL (26), while an antibody directed against the same epitope revealed expression in the outer medulla of TAL as well as the DCT (29). In these studies, immunolocalization of the B1 subunit to the DCT was listed as data not shown, and thus DCT labelling of the B1 subunit has apparently only been shown by Paunescu and colleagues in mouse kidney (30). Although these previous studies were carefully performed, the localization of the B1 subunit and antibody specify was not verified using *Atp6v1b1*-deficient (KO) mice. This is desirable, since a risk of immuno-cross-reactivity exists with the highly homologous B2-subunit, which has also been found expressed in the TAL, DCT, CNT in addition to the IC of rat and mouse kidney in early distal nephron (29). In conclusion, the localization of the H\textsuperscript{+}-ATPase B1 subunit in the early distal nephron relies on few studies and has not been determined in humans (26, 29).

The current study aimed to answer the following queries: *i)* Is the H\textsuperscript{+}-ATPase B1 subunit expressed in the early distal tubule in the human kidney as well as mouse and rat? *ii)* Is the localization of the H\textsuperscript{+}-ATPase B1 subunit within the early distal tubular segments similar between rodents and human? *iii)* Is the H\textsuperscript{+}-ATPase B1 subunit localized in the apical plasma membrane in the early distal tubule? *iv)* Does the H\textsuperscript{+}-ATPase B1 subunit colocalize with other H\textsuperscript{+}-ATPase subunits in these segments?
Materials and Methods

Animal experiments.

Mice with a targeted deletion of the Atp6v1b1 gene have been generated and described previously (12). Tissue from this mouse strain was obtained from previously published studies (15, 16). Mice overexpressing eGFP after a 6.5 kb fragment of the ATP6V1B1 promoter immediately upstream of the coding region of the gene have also been described in detail previously (25). Tissue from this mouse strain was obtained from previously published studies ((15, 16). Monoclonal antibodies against SLC12A3 were produced in mice for this study in accordance with Danish Law under the animal experimental permits #2014-15-0201-00043.

Isolation and processing of tissue from human kidney

Tissue was obtained from kidneys removed by nephrectomy due to renal carcinoma. Kidney tissue was retrieved from parts of the nephrectomised kidneys that were not affected by tumour growth. After isolation of the tissue, specimens were immersion fixed in 10% formalin for 3 hours and subsequently placed in phosphate buffered saline (PBS) until they were dehydrated and subsequently embedded in paraffin. The samples were obtained after each patient involved in the study had given informed written consent (n=5). The Biomedical Research Ethics Committee of Southern Denmark approved all listed experiments under the licence S-20140159.

Primary antibodies.

The following antibodies were used to evaluate the expression of the H+ -ATPase B1 subunit in human and murine kidney: ATP6V1B1H7659: Rabbit polyclonal anti-ATP6V1B1 antibody directed against most C-terminal portion of the rat H+ -ATPase B1 subunit and described previously (10). ATP6V1B1S: Rabbit polyclonal antibody directed against the N-terminal portion of the human H+ -ATPase B1 subunit (HPA031847, Sigma Aldrich, St Louis, MO, USA). ATP6V1B1SC: Goat polyclonal antibody directed against the N-terminus of the human H+ -ATPase B1 (N-20, sc-21206, Santa Cruz Biotechnology, Dallas, TX, USA). The specificity of the antibodies against ATP6V1B1 was confirmed by staining tissue from ATP6V1B1-KO mice (Figure 1A-F). Furthermore, the following antibodies were used to determine...
localization of other acid-base transporters and specific markers for individual nephron segments throughout the renal tubule. i) Goat polyclonal antibodies against AQP2 (C-17, Santa Cruz Biotechnology). ii) Rabbit polyclonal antibodies against NKCC2 (SLC12A1, HPA014967, Sigma-Aldrich). iii) Rabbit polyclonal antibodies against NCC (SLC12A3 HPA028748, Sigma-Aldrich). iv) Mouse monoclonal antibody against plasma membrane Ca\(^{2+}\)-ATPase 4 (PMCA4) (JA9, ab2783, Abcam, Cambridge, UK). v) Goat polyclonal antibodies against eGFP (Abcam). vi) Rabbit polyclonal antibodies against ATP6V1E1 (PA5-29899, ThermoFischer Scientific, Slangerup, Denmark). vii) Rabbit polyclonal antibodies against ATP6V1B2 (HPA008147, Sigma-Aldrich). viii) Rabbit polyclonal antibodies against ATP6V1G1 (PA00135A0Rb, Cusabio Technologies, Houston TX, USA). ix) Mouse monoclonal antibodies directed against the human thiazide-sensitive NaCl co-transporter encoded by the SLC12A3 gene were generated as described in detail previously (39). In brief, 30 µg of peptide (GEPRKVRPTLADLHSFLQEG corresponding to amino acids 77-97 of the human SLC12A3) coupled onto diphtheria toxoid was mixed with GERBU adjuvant, injected into NMRI mice twice over a 14 day interval. Mice received an intravenous booster injection of the conjugated peptide with adrenaline, 3 days prior to fusion of spleen cells. Fusion was done as described previously using SP2 myeloma cell line as fusion partner (20). Clones were screened by ELISA and cloned by limiting dilution. Resulting clones were characterized for their ability to stain mouse and human kidney. Subsequent evaluation of the clones was done by immunohistochemistry on kidneys from Slc12a3 deficient mice (34), to evaluate specificity of the antibodies. Clone 9 (SLC12A3\(_C9\)), which showed labelling in the DCT in WT mice and no labelling in Slc12a3-deficient mice was used for the current manuscript (Fig 1O and P).

**Immunohistochemical labelling using HRP-conjugated secondary antibodies.**

Immunostaining was performed as previously described on paraffin embedded formalin fixed kidneys from mice (3) and rats (28). Briefly, kidney tissue was rehydrated in Tissue-Tek Tissue-clear (Sakura Finetek, Brøndby, Denmark) and graded ethanol and boiled in Tris-EGTA buffer (TEG, 10 mM Tris, 0.5 mM EGTA, pH = 9.0). Endogenous peroxidases and free aldehyde groups were blocked by addition of 0.6% H\(_2\)O\(_2\) and 50 mM NH\(_4\)Cl in PBS. Sections were incubated overnight at 4°C with primary antibody added to 0.1% Triton
X-100 in PBS, washed and incubated with secondary horseradish peroxidase conjugated antibodies (HRP, DakoCytomation, Glostrup, Denmark) or using the ImmPRESS™ Polymer HRP Reagent. The DAB⁺ Substrate Chromogen System was used to visualize HRP activity (K3467, DakoCytomation). Sections were counterstained with hematoxylin. Pictures were obtained using an Olympus BX51 microscope (Olympus, Ballerup, Denmark).

**Immunofluorescence labelling of tissue.**

Staining was done essentially as described previously (3) and according to the same protocol as outlined above for immunohistochemical labelling, with modifications. After secondary horseradish peroxidase conjugated antibodies, a fluorescent Cy3-coupled TSA substrate (TSA Cyanine 3, Perkin Elmer, Waltham, MA, USA) was used. Sections were then reboiled in TEG buffer to remove bound antibodies and followed by a new round of immunolabeling, using primary antibodies and subsequent Alexa-labeled 488 secondary antibodies. For eGFP localization with SLC12A3 and ATP6V1B1\textsubscript{H7659} in ATP6V1B1-eGFP mice, kidney sections were incubated with primary antibodies and subsequently co-incubated with secondary antibodies conjugated to Alexa fluorophores (Molecular Probes, Eugene, OR, USA) (1). Images were obtained using an Olympus BX51 microscope (Olympus, Ballerup, Denmark). Brightness was adjusted on the entire image for preparation of figures.

**Processing of tissue for transmission electron microscopy.**

Rat kidney tissue from control rats was fixed and processed as previously described (11, 13). Lowicryl sections were labeled with the ATP6V1B1\textsubscript{H7659} antibody at 1:500 dilution and imaged in a Philips Morgagni transmission electron microscope operating at 80 kV. Images were taken with a MegaView III CCD camera (1376 x 1036 pixels, 16 bit). Multiple image alignment was used to combine 25 original images taken at 2.2k magnification to create overview images. High magnification images, to document subcellular localization of immunogold, were obtained at 44k magnification. Images were converted to 8 bit and intensity/contrast-adjusted using ImageJ software for preparation of the figure.
Results

Characterization of antibodies against the H\(^+\)-ATPase B1-subunit (ATP6V1B1) and the thiazide sensitive NaCl cotransporter (SLC12A3) in mouse, rat and human kidney.

We tested three different antibodies against ATP6V1B1. Two polyclonal antibodies directed against the N-terminus of the human ATP6V1B1 protein, namely the ATP6V1B1\(_S\) from Sigma and the ATP6V1B1\(_SC\) from Santa Cruz as well as a previously characterized polyclonal antibody directed against most C-terminal portion of the rat ATP6V1B1\(_H7659\) (10). All antibodies were tested on kidney tissue from human and rat as well as wildtype (WT) and Atp6v1b1-deficient (KO) mice (12). All three antibodies recognize epitopes that are very abundant in the IC, but also present in the early distal nephron, although the antibodies display varying affinities across the species investigated (Figure 1). In human kidney, the ATP6V1B1\(_S\) antibody strongly recognized epitopes in the early distal tubule and IC (Figure 1A), while no staining could be found in rat (Figure 1B). In mouse kidney, ATP6V1B1\(_S\) only recognized IC in WT mice, while no staining was seen in kidney from Atp6v1b1-KO mice (Figure 1C-D). In human kidney, ATP6V1B1\(_H7659\) staining appeared much weaker, but still recognized IC and cells in the early distal nephron (Figure 1E). The ATP6V1B1\(_H7659\) antibody stained early distal nephron segments in kidney tissue from rat (Figure 1F) and WT-mice (Figure 1G), while staining was completely absent in Atp6v1b1-deficient mice (Figure 1H). The ATP6V1B1\(_SC\) antibody also stained the early distal tubule and the IC in human (Figure 1I), rat (Figure 1J) and WT mouse (Figure 1K), however, in the Atp6v1b1 KO, residual staining was observed in IC and other cells of the distal nephron (Figure 1L). In conclusion, the ATP6V1B1\(_H7659\) antibody seems to give the most complete and specific description of the localization of the B1 subunit in mouse kidney, since all staining disappears in the KO and both IC and distal tubules are stained. The ATP6V1B1\(_SC\) antibody, which is raised to recognize the human ATP6V1B1, cross-reacts with another protein in the distal tubule of mouse kidney. The absence of staining in the distal tubule of mice using the ATP6V1B1\(_S\) antibody indicates this antibody has lower affinity for the mouse ATP6V1B1 than ATP6V1B1\(_H7659\), however the ATP6V1B1\(_S\) showed much higher affinity for the human ATP6V1B1 than ATP6V1B1\(_H7659\). In this study, the ATP6V1B1\(_H7659\) was used at a 1:100 dilution, whereas previous studies applying the antibody used >10x more diluted solutions (10, 11). This explains why staining of the distal tubule is not consistently described in previous studies using ATP6V1B1\(_H7659\) and...
highlights that ATP6V1B1 is less abundant in distal tubule cells than in IC in mice. For the remainder of the study, the ATP6V1B1_{H7659} antibody was used to evaluate ultrastructural and immunohistochemical localization and regulation of the ATP6V1B1 subunit in the distal nephron of mouse, rat and human, while the ATP6V1B1_{S} antibody was used alongside ATP6V1B1_{H7659} for clarity in the human kidney only. The ATP6V1B1_{SC} antibody was not used for the remainder of this study. An antibody was also generated against the human thiazide-sensitive NaCl cotransporter (NCC/SLC12A3) encoded by the SLC12A3 gene, to aid in the colocalization of the H^+-ATPase B1 subunit to the DCT, where SLC12A3 is expressed. This monoclonal antibody denoted SLC12A3_{C9} showed the expected pattern of localization by immunohistochemistry on kidneys from human (Figure 1M), rat (Figure 1N) and WT mouse (Figure 1O), while no detectable staining was seen in Slc12a3 KO mice (34) (Figure 1P).

In order to anticipate whether the antibodies employed would react across species an alignment was made between parts of the amino acid sequence of H^+-ATPase B1 and B2 subunits from mouse, rat and human used to generate antibodies against the H^+-ATPase B1 subunit. Multiple amino acid sequence alignments was accomplished with CLUSTALW (https://www.genome.jp/tools-bin/clustalw) in order to align the N-terminal (upper) and C-terminal (lower) domains of the H^+-ATPase B1 and B2 subunits from mouse, rat and human (Figure 1Q). The boxed sequence in the N-terminal alignment is the epitope used to produce antibodies against ATP6V1B1_{S}. The boxed sequence in the C-terminal alignment indicates the epitope used to generate the ATP6V1B1_{H7659} antibody. Santa Cruz Biotechnology does not provide information on the specific epitope used for their ATP6V1B1_{SC} antibody.

H^+-ATPase B1 subunit localizes to the apical membrane domains in distal nephron cells of mouse kidney.

Immunolabelling with the ATP6V1B1_{H7659} antibody revealed ATP6V1B1 protein to be abundantly expressed in the IC in mouse kidney as well as distal tubular segments (Figure 2A). In the cortex, expression of the H^+-ATPase B1 subunit was evident in distal nephron segments including the DCT and the cortical TAL (Figure 2A). In the medulla, H^+-ATPase B1 staining was observable in the outer stripe, similar to the cortex, and gradually declined in the inner stripe of the outer medullary portion of the mouse TAL (Figure 2B). Expression of the H^+-ATPase B1 subunit was detectable in IC all the way to the inner medullary collecting...
ducts (CD) (Figure 2B). To confirm the finding of the ATP6V1B1 subunit in specific segments of the distal nephron, double labelling was performed with known cellular markers. Colocalization was performed with SLC12A1 (Figure 2C), which localizes to the TAL and SLC12A3 that localizes to the DCT (Figure 2D). Using both markers, colocalization could be confirmed. Colocalization was also performed with the plasma membrane Ca\(^{2+}\)-ATPase 4 (PMCA4). In mouse and rat, PMCA4 is expressed in the cortical TAL, macula densa and the early part of the distal convoluted tubule (DCT1), with comparatively higher expression in the late part of the distal convoluted tubule (DCT2) and CNT segments, and a gradual decline towards the cortical collecting duct (1). Using this marker, it was clear that distal nephron cells with low as well as high intensity staining of PMCA4 were also positive for ATP6V1B1 in their apical membrane domains (Figure 2E). Double labelling with ATP6V1B1\(_{H7659}\) and Aquaporin 2 (AQP2) documented expression of the H\(^+\)-ATPase B1 subunit outside the collecting duct (Figure 2F).

Unlike the staining pattern we found for the H\(^+\)-ATPase B1 subunit in kidney, previous studies using transgenic mice driving protein expression after a 6.5 kb fragment of the human ATP6V1B1 promoter have shown expression of both Cre recombinase and eGFP in both cell types of the CNT and in IC of the CD (24, 25), but no expression of Cre or eGFP in the early portion of the distal nephron. Using ATP6V1B1-eGFP transgenic mice we compared the expression of eGFP with the expression of the H\(^+\)-ATPase B1 subunit. Immunodetectable expression of eGFP was limited to the ICs of the collecting duct and the CNT segment as well as non-ICs in the CNT as reported previously (24, 25), while no colocalization with SLC12A3 in the DCT could be found (Figure 2G). Co-labelling with antibodies directed against eGFP and the ATP6V1B1\(_{H7659}\) antibody highlighted the expression of the H\(^+\)-ATPase B1 subunit in tubule segments devoid of immunodetectable eGFP expression driven by the 6.5 kb human ATP6V1B1 promoter fragment (Figure 2H). Within the CNT, weak but significant immunoreactivity for the H\(^+\)-ATPase B1 subunit was observed in the very apical region of the non-ICs, which were immunoreactive for eGFP (Figure 2H). This small signal in CNT-cells was weaker than the signal in DCT-cells.

Ultrastructural and immunohistochemical localization of the H\(^+\)-ATPase B1 subunit in early distal tubule of rat kidney.
In rat, a similar pattern of expression was evident as seen in mouse, with localization of the B1-subunit to apical membrane domains of the cells in the early distal nephron and IC (Figure 3A-B). In comparison to the mouse, the H+-ATPase B1 subunit in the medullary TAL appeared restricted towards cortex and reduced in expression as the TAL descended through the outer stripe of the medulla. Similar to mouse, colocalization was evident with SLC12A1 in the TAL (Figure 3C) and SLC12A3 in the DCT (Figure 3D) segments, confirming expression in the early distal nephron. Similarly, distal nephron cells with low and high intensity staining of PMCA4 stained for ATP6V1B1H7659 in their apical membrane domains (Figure 3E). Double labelling with ATP6V1B1H7659 and AQP2 confirmed localization of the H+-ATPase B1 subunit to IC in the collecting ducts as well as in cells outside the collecting duct (Figure 3F).

To evaluate whether the H+-ATPase B1 subunit localized to the apical plasma membrane, immunoelectron microscopy was performed on rat sections using the ATP6V1B1H7659 antibody. Representative images of cell membranes of distal tubule cells showed immunogold labeling for the H+-ATPase B1 subunit (Figure 4). Labeling was found in apical aspects of the plasma membrane (marked by arrows) and in intracellular sites, including submembranous vesicles (marked by arrowheads).

The H+-ATPase B1 subunit is abundantly expressed in the early portion of the distal nephron in human kidney.

In human kidney, the H+-ATPase B1 subunit was abundant in the IC of the cortical collecting system (Figure 5A), but immunoreactivity was also comparatively substantial in the cortical early distal nephron segments, which was especially evident using the ATP6V1B1s antibody (Figure 5A). In the outer medulla, staining was seen in collecting duct IC and at a lower intensity in the TAL cells (Figure 5B). At long development times, a weak apical DAB-staining could also be detected in CNT and principal cells of the collecting system, however the signal was definitively weaker than the signal observed in the DCT and TAL. A similar pattern of expression, albeit with an overall lower intensity, was observed in human kidney using the ATP6V1B1H7659 antibody (Figure 5C-D). Furthermore, complete colocalization was observed between the ATP6V1B1H7659 and ATP6V1B1s antibodies in human kidney (Figure 5E). Colocalization was seen with ATP6V1B1 and SLC12A1 in the TAL (Figure 5F). Not all TAL cells showed positive staining for the H+-
ATPase B1 subunit. Furthermore, colocalization was detected with SLC12A3 in the DCT (Figure 5G). ATP6V1B1 also localized to ICs, next to AQP2-positive principal cells in the collecting ducts, as well as cells to cells outside the collecting duct (Figure 5H). Colocalization of PMCA4 was not performed in human as the transporter is not expressed in different amounts along the entire DCT (1).

In mice and humans, the immunoreactivity for the H⁺-ATPase B1 subunit in the macula densa appeared intense compared to the segments adjacent to the macula densa. In rats however, the macula densa did not show immunoreactivity for the H⁺-ATPase B1 subunit (Figure 6). Apart from the macula densa, the H⁺-ATPase B1 subunit seemed generally similarly localized in human, rat and mouse kidney, but the relative immunoreactivity for of the H⁺-ATPase B1 in the early portion of the distal nephron compared to the IC of the CD appeared more intense in humans than in rodents. However, we did not attempt to make a quantitative estimate of this impression, as a direct comparison between species is not possible based on the available data.

The B2, E1, and G1 subunits of the H⁺-ATPase localize to the TAL and DCT in mouse and human kidney.

To investigate whether other subunits of the H⁺-ATPase colocalized with the B1 subunit in the TAL and DCT enabling H⁺-ATPase activity, the localization of the H⁺-ATPase B2 subunit (ATP6V1B2), the H⁺-ATPase E1 subunit (ATP6V1E1), and the H⁺-ATPase G1 subunit (ATP6V1G1) was examined in mouse and human kidney by immunohistochemistry. Immunostaining for H⁺-ATPase B2 subunit was most intense in the cortical proximal tubules and distal nephron segments in mouse kidney (Figure 7A-B). Here colocalization was found in apical membrane domains with SLC12A1 in the TAL (Figure 7C) and with SLC12A3 in the DCT (Figure 7D). Furthermore, immunostaining for the H⁺-ATPase B2 subunit in human kidney showed a similar pattern of localization (Figure 7E-F). The distribution of the H⁺-ATPase E1 subunit was also assessed in mouse and human kidney by immunohistochemistry. In mouse kidney, abundant immunoreactivity was observed in cortical proximal tubules and distal nephron segments, while IC’s stained most strongly throughout the nephron (Figure 8A-B). Staining for ATP6V1E1 was found in apical membrane domains of TAL and DCT, where it colocalized with SLC12A1 and SLC12A3, respectively (Figure 8C-D). In human kidney, similar staining was observed, with predominating IC staining throughout.
the kidney and robust staining in proximal and distal tubules (Figure 8E-F). \( H^+ \)-ATPase G1 subunit expression was also investigated along the mouse and human distal nephron by immunohistochemistry. In mouse kidney, staining was observed in both proximal and distal tubules. Further the IC stained in both the cortex and medulla (Figure 9A-B). The ATP6V1G1 subunit colocalized with SLC12A1 and SLC12A3 in the TAL and DCT, respectively (Figure 9C-D). \( H^+ \)-ATPase G1 subunit expression was likewise detected in proximal and distal tubular segments of human kidney, with abundance in IC’s as well (Figure E-F).
Discussion

The main aim of this study was to investigate if the H\(^+\)-ATPase B1 subunit is expressed in the early portion of the distal nephron in human kidneys and if this is different from rat or mouse kidneys. Our data strongly suggest that the H\(^+\)-ATPase B1 subunit is expressed along the TAL and DCT nephron segments in all three species investigated. This is based on the following observations i) H\(^+\)-ATPase B1 subunit is expressed in apical membrane domains in human kidney, where it colocalizes with SLC12A1 and SLC12A3, respectively. ii) Similarly, H\(^+\)-ATPase B1 subunit expression can be detected in early distal tubular segments identified by TAL and DCT markers in rat and mouse kidneys as described previously (26, 29, 30). iii) Ultrastructurally, the H\(^+\)-ATPase B1 subunit localizes to the plasma membrane and is not restricted to intracellular sites. iv) The specificity of the antibodies (ATP6V1B1\(^{H7659}\) and ATP6V1B1\(^{SC}\)) used to detect the B1 subunit in the early portion of the distal nephron was confirmed in tissue from Atp6v1b1 deficient mice.

As in previous studies of the B1 subunit in the early distal nephron, these conclusions are based on immunodetection and we thus performed extensive tests of the antibodies employed. Both of the antibodies generated against a human sequence (ATP6V1B1\(^{S}\) and ATP6V1B1\(^{SC}\)) showed similar staining patterns in the human kidney, with prominent staining of the IC and robust staining of the early distal nephron encompassing the TAL and DCT. Since the N-terminal localization of the epitopes used to generate the polyclonal ATP6V1B1\(^{S}\) and ATP6V1B1\(^{SC}\) antibodies against the human ATP6V1B1 sequence shows overlapping homology with the ATP6V1B2 subunit, we tested the specificity of these antibodies in Atp6v1b1-deficient mice, generated previously (12). Using these mice, Finberg et al has documented that B1 subunit expression in the IC is present in wild-type and heterozygotes, while completely absent in the Atp6v1b1-deficient mice (12). In the present study, the ATP6V1B1\(^{SC}\) antibody showed residual staining in Atp6v1b1-deficient mice, and we concluded this antibody not to be specific for the B1-subunit. Using the ATP6V1B1\(^{S}\) antibody, no immunostaining could be observed in Atp6v1b1-deficient mice, although it should be noted that the signal obtained by the ATP6V1B1\(^{S}\) antibody in mouse kidney was significantly weaker than seen with the other antibodies (ATP6V1B1\(^{S}\) and ATP6V1B1\(^{H7659}\)). The ATP6V1B1\(^{S}\) antibody only detected expression of the H\(^+\)-ATPase B1 subunit in the IC of wild-type mice, suggesting that the antibody has less affinity for the murine than for the human ATP6V1B1 epitope. The ATP6V1B1\(^{H7659}\) antibody
directed against the unique C-terminal of the rat ATP6V1B1 showed prominent staining in IC and similar staining in the early portion of the distal nephron in human kidney as obtained with the other antibodies, albeit with lower overall intensity in human, than in mouse and rat. This can be explained by differences in the C-terminal sequence between the rodent and human B1-subunits. We found the signal from the ATP6V1B1H7659 to be completely absent in Atp6v1b1-deficient mice, documenting the specificity of this antibody and its usefulness to investigate the expression of the H^+-ATPase B1 subunit in the early distal nephron.

In all three species, the intensity of immunolabelling for the H^+-ATPase B1 subunit was much stronger in IC than in cells of the DCT and TAL segments. This is in line with previous findings of H^+-ATPase B1 subunit expression in mice (30). Thus, there appear to be no major difference between the species investigated in terms of localization and it is therefore reasonable to assume that functional studies of the H^+-ATPase in the early portion of the distal nephron of rodents will translate to human physiology. We did not attempt to make a direct quantitative comparison of the expression levels in the DCT and TAL segments between species. However, the difference between the labelling intensity of IC and the early portion of the distal nephron appeared less pronounced in sections of human kidney than in both rat and mouse kidney. Furthermore, immunolocalization of the H^+-ATPase B1 subunit to the macula densa differed between species, with lower relative abundance of the subunit in rat as compared to the surrounding tubular epithelium (Table 1).

The localization of the B1 subunit to the apical membrane of DCT cells, suggests a role in trans-epithelial transport processes in the segment and not limited to a function in intracellular vesicles. Yet, as opposed to the IC and TAL, basolateral extrusion mechanisms for HCO_3^- reabsorption have not been described for the DCT. Given that Brown and colleagues have reported expression of the B1 subunit in apical endosomes from papillary collecting duct principal cells without other H^+-ATPase subunits (33), it is important to clarify whether the H^+-ATPase B1 subunit localizes with other H^+-ATPase subunits in the distal tubule. Several lines of evidence suggest that it may do so. First, Brown and coworkers have identified the H^+-ATPase 31 KDa (H+-ATPase E) subunit and the 56 KDa (includes both H^+-ATPase B1 and H+-ATPase B2) subunits by electron microscopy at the apical plasma membrane of the distal convoluted tubule (4).
Furthermore, deep sequencing data from isolated rat nephron segments generated and analyzed by Lee, Chou, and Knepper shows abundant mRNA expression of the H\(^+\)-ATPase B1 subunit in both the TAL and DCT (23). Here they also found that multiple other H\(^+\)-ATPase subunits are expressed along the length of the early distal nephron, such as the H\(^+\)-ATPase a4 subunit in which disease causing mutations are described (35). Furthermore, the study also uncovered H\(^+\)-ATPase E1, G1, and B2 subunit mRNA expressed throughout the nephron (23), with notable expression in the TAL and DCT. However, since the DCT and CNT segments in rat kidney contain ICs, expression levels obtained from these segments may reflect a mixture of cell types. In contrast, TAL segments are completely devoid of ICs and still display expression of several of H\(^+\)-ATPase subunits (23). A recent study based on single cell transcriptomics of mouse kidney also detected a number of the H\(^+\)-ATPase subunits, including B1, E1, G1 and B2 in the cell cluster classified as DCT cells (27). Finally, immunohistochemistry in this study shows expression of the H\(^+\)-ATPase E1, G1, and B2 subunits in both the TAL and DCT. Thus it is likely that the H\(^+\)-ATPase B1 subunit will be associated with other subunits and participates in multi-subunit complexes that drive H\(^+\) transport in TAL and DCT as well.

Studies using transgenic mice to drive expression of Cre recombinase or eGFP after a 6.5 kb fragment of the human ATP6V1B1 promoter showed expression in both cell types of the CNT (ICs and CNT cells) as well as in ICs of the CD (24, 25). No expression of Cre or eGFP was observed in the early portion of the distal nephron (DCT and TAL). In the light of the data summarized above, it is evident that this transgenic mouse line only expresses Cre or eGFP in a subset of the cell types expressing the B1 subunit. However, the transgenic mouse raises the intriguing question if the marked expression of eGFP in CNT-cells reflects endogenous expression of the B1 subunit. The eGFP expression was originally hypothesized to be due to "some intrinsic and unique property of cells in the CNT" (24), but no studies have been able to clarify this further. A recent study shows that distinct cell types in distal nephron epithelia considered to perform unique functions, share common embryological precursors and that several differentiation pathways may lead to the same functional cell type (37). Perhaps the mechanism responsible for Cre and eGFP expression in the transgenic mouse line relates to these cellular differentiation patterns, which lead to the complex cellular architecture of the distal nephron, including at least 7 functionally differentiated cell types.
Although it should be kept in mind, that the observed immunoreactivity in the CNT-cells is markedly weaker than in the DCT-cells, the immunohistochemical results presented in this study support the presence of small amounts of H⁺-ATPase B1 subunit protein in CNT cells. Moreover, cluster analysis of single cell transcriptomics of mouse kidney resulted in identification of a transitionary cell type in the distal nephron, which express genetic markers of both ICs (including Atp6v1b1) and collecting duct principal cells (including Aqp2), and thus possibly may be the CNT-cells (27). Surprisingly, Atp6v1b1 was also found in 28% of the cells assigned to the cluster of collecting duct principal cells and the traditional view, that expression of the H⁺-ATPase B1-subunit is the identifying hallmark of ICs, was further questioned by the fact, that the Atp6v1b1 gene was not found to be among the core group of genes identifying the IC's (27).

The results of this study are not consistent with substantial expression of the B1 subunit in CD principal cells. However, we cannot exclude that low levels of B1 protein are present in some principal cells. Our results are in general in accordance with the findings based on transcriptomics, as they indicate the presence of B1 subunit protein in a wider range of cell types than ICs, in particular the TAL and DCT.

As mentioned, loss of function mutations in the Atp6v1b1 gene encoding the B1 subunit of the H⁺-ATPase associate with more severe forms of distal renal tubular acidosis than dominant mutations in AE1 (17-19). Patients suffering loss of function mutations in the Atp6v1b1 gene often present with a salt losing phenotype and hypokalemia, which seems not to be explained alone by dysfunction of type A IC (15), but points towards important functions of the H⁺-ATPase B1 subunit in other cell types, including type B and non-A-nonB IC and/or cells on the early distal nephron. In line with this, a recent report describes a mouse model with renal ablation of the Atp6ap2 gene encoding a H⁺-ATPase accessory protein (36). In this model, dRTA was observed in addition to a severe urinary concentrating defect caused by a dysfunction of the TAL.

Since AE1 is exclusively expressed in type A IC, the phenotype of the dominant mutations of AE1 can be ascribed to a lack of acid excretion mediated by type A IC. The more severe effect of mutations in the H⁺-ATPase B1 subunit may thus reflect the broader expression of the H⁺-ATPase B1 subunit along the early distal nephron as documented in this study. However, the different phenotypes may also reflect a difference in the severity of recessive versus dominant mutations in AE1, with some degree of residual function of type A IC being retained in patients with dominant mutations. The H⁺-ATPase could also be involved in more
basal cellular functions, including contribution to maintenance of the electrochemical gradient across the plasma membrane as has been proposed in IC (8). Finally, as the B1 subunit is part of the V1 complex of the H^+-ATPase, forming “the rotor” unit responsible for the hydrolysis of ATP, we cannot exclude that its localization to the apical membrane of the TAL and the DCT is associated to other ATPase equipped proteins.

In summary, this study documents by immunohistochemistry the H^+-ATPase B1 subunit to be expressed in the apical membrane domains of TAL, macula densa and DCT in human and rodent kidney. Furthermore, by immunoelectron microscopy the subunit is localized to the apical plasma membrane in the DCT. The immunoreactivity for H^+-ATPase B1 subunit was less intense in the TAL and DCT as compared to IC of the same species. The documented expression of the B1 subunit in renal tubular cells other than IC may nevertheless contribute to the more severe dRTA seen in patients, with loss of function mutations in the B1 subunit as compared to dominant mutations in intercalated cell specific AE1.

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Conflict of Interest

None
References


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Immunohistochemical staining of the H⁺-ATPase B1 subunit (ATP6V1B1) along the distal nephron identified in mouse, rat and human kidney in the current study. Comparison has only been made between different nephron segments within each species investigated. No assessment of staining intensity has been made between species, due to possible species differences in antigen binding. isomTAL: inner stripe of outer medullary thick ascending limb; osomTAL: outer stripe of outer medullary thick ascending limb; cTAL: cortical thick ascending limb; DCT: distal convoluted tubule; CNT: connecting tubule; CCD: cortical collecting duct; OMCD: outer medullary collecting duct; IMCD: inner medullary collecting duct. +, ++, and +++: expression with increasing intensity; -: expression absent or below limit of detection; IC: IC cells are responsible for the detected immunoreactivity.
Figure Legends

**Figure 1. Validation of antibodies in human, rat, WT mice and gene specific knockout mice.**

A-D) Immunostaining for ATP6V1B1 in kidney tissue from human (A), rat (B) wildtype mouse (WT) (C) and Atp6v1b1-deficient (KO) (D) mice using the ATP6V1B1S antibody. E-H) Immunostaining of the H⁺-ATPase B1 subunit in kidney from human (E), rat (F), WT mouse (G) and Atp6v1b1-KO mouse (H) using the ATP6V1B1H7659 antibody. I-L) Immunostaining for ATP6V1B1 in kidney tissue from human (I), rat (J), WT mouse (K) and Atp6v1b1-KO mouse (L) using the ATP6V1B1SC antibody. M-P) Immunostaining for SLC12A3 in kidney tissue from human (M), rat (N), WT mouse (O) and Slc12a3-KO mice (P) using the SLC12A3c9 antibody. Q) Alignment of the parts of the amino acid sequence of the H⁺-ATPase B1 and H⁺-ATPase B2 subunits from mouse, rat and human used to generate antibodies against the H⁺-ATPase B1 subunit. Upper alignment shows the sequence used to generate the ATP6V1B1S antibody, lower alignment shows the amino acid sequence used to generate the ATP6V1B1H7659 antibody.

**Figure 2. The H⁺-ATPase B1 subunit is expressed in apical membrane domains along the early portion of the mouse distal nephron**

A-B) Immunostaining for H⁺-ATPase B1 subunit (ATP6V1B1) in mouse kidney cross-section using the ATP6V1B1H7659 antibody in the cortex (A) and the medulla (B). In the cortex, expression was evident in the ICs as well as in early distal tubular segments. In the outer medulla, both TAL segments and ICs in collecting ducts were positive for ATP6V1B1 using the ATP6V1B1H7659 antibody. Towards the inner medullary portion of the kidney, staining was visible only in the ICs of the collecting duct system. Grey stippled lines demarcate the zones of the medulla. C) Representative confocal images showing detection of the ATP6V1B1 using the TAL marker SLC12A1 (green) and the ATP6V1B1H7659 antibody (red). Image from the cortical TAL. Staining is visible in IC as illustrated by arrowhead and in TAL as exemplified by asterisks. D) Confocal images showing localization of the ATP6V1B1 using the ATP6V1B1H7659 antibody (red) with the DCT marker, SLC12A3 (green). Again, an arrowhead indicates one IC and a DCT tubule is exemplified by an asterisk. E) Immunofluorescent staining with antibodies directed against the H⁺-ATPase B1 subunit using the ATP6V1B1H7659 antibody (red) with PMCA4 (green). Staining is present across the cortical TAL, the distal convoluted tubule (DCT1 and DCT2), in addition to the IC of the collecting...
system (arrowhead). F) Representative confocal images showing detection of the ATP6V1B1 using the ATP6V1B1\textsubscript{H7659} antibody (red) with AQP2 (green). IC are indicated by an arrowhead and early distal nephron tubules marked by asterisk. G) Confocal images showing immunofluorescence labeling of eGFP (green) with SLC12A3 in transgenic mice expressing eGFP after a 6.5 kb fragment of the ATP6V1B1 promoter. eGFP expression is visible in the collecting system as (including both CNT cells and IC, the latter indicated by arrowhead), but absent from DCT defined by SLC12A3 expression as exemplified by asterisk. H) Representative confocal images showing detection of eGFP (green) and ATP6V1B1 (red) using the ATP6V1B1\textsubscript{H7659} antibody in \textit{ATP6V1B1-eGFP} mice. IC are indicated by arrowheads and early distal tubular segments are indicated by asterisks. Weak immunoreactivity for ATP6V1B1 is visible in apical domains of CNT-cells that express eGFP (indicated by arrows).

**Figure 3.** The H\textsuperscript{+}-ATPase B1 subunit is expressed along the distal nephron of rat kidney. A-B) Immunostaining for H\textsuperscript{+}-ATPase B1 subunit (ATP6V1B1) in rat kidney tissue using the ATP6V1B1\textsubscript{H7659} antibody in the cortex (A) and the medulla (B). In the cortex, strong immunoreactivity was seen in the ICs, but also in apical domains in the early distal tubules. Similar to mouse, TAL segments and ICs in collecting ducts were positive for ATP6V1B1 in the outer medulla. C-F) Representative confocal images showing detection of the ATP6V1B1 using the ATP6V1B1\textsubscript{H7659} antibody (red) and colocalized with SLC12A1 (C, green), SLC12A3 (D, green), PMCA4 and (E, green) and AQP2 (H, green). Staining was similar to mouse, with IC illustrated by arrowheads and early distal tubules exemplified by asterisks.

**Figure 4.** Ultrastructural localization of the H\textsuperscript{+}-ATPase B1 subunit to the apical plasma membranes in rat. A-H) Representative high magnification electron microscopy images from the area of the distal tubule in the overview image. Immunoreactivity for the H\textsuperscript{+}-ATPase B1 subunit was found localized to the apical plasma membrane as indicated by arrows and apical intracellular compartments marked by arrowheads. Asterisks denote the tubular lumen.

**Figure 5.** The H\textsuperscript{+}-ATPase B1 subunit is expressed along the human distal nephron.
A-B) Immunostaining for ATP6V1B1 in human kidney using the ATP6V1B1<sub>S</sub> antibody. Abundant expression was evident in the IC, but significant strong immunostaining was also detected in distal tubular segments (A). As in mouse, both TAL segments and IC in collecting ducts were positive for ATP6V1B1 in the outer medulla (B), while only the IC of the collecting duct system were stained in the inner medullary portion of the kidney. C-D) Immunostaining of the H<sup>+</sup>-ATPase B1 subunit in kidneys using the ATP6V1B1<sub>H7659</sub> antibody in cortex (C) and outer medulla (D). Similar staining pattern to that of ATP6V1B1<sub>S</sub> was found in human kidney, albeit less intense. E) Representative confocal images showing detection of the ATP6V1B1 using the ATP6V1B1<sub>S</sub> antibody (green) and the ATP6V1B1<sub>H7659</sub> antibody (red). Staining is visible in IC as indicated by arrowheads and in early distal tubular structures marked by asterisks. Early distal tubules are stained by both antibodies in the human kidney. F) Confocal images showing localization of the ATP6V1B1 using the ATP6V1B1<sub>S</sub> antibody (red) with the SLC12A1 (green). Note clear colocalization between the two epitopes to apical domains in the TAL. G) Confocal images showing cellular colocalization between H<sup>+</sup>-ATPase B1 subunit staining from the ATP6V1B1<sub>S</sub> antibody (red) with the SLC12A3 (green). IC are indicated by arrowhead and colocalization of the epitopes to apical domains in the DCT is indicated by an asterisk. H) Confocal images showing colocalization of the AQP2 protein (green) in the collecting system and the ATP6V1B1 using the ATP6V1B1<sub>S</sub> antibody (red). Arrowhead denotes an IC in a collecting duct, which contain AQP2 positive principal cells and asterisk indicate ATP6V1B1 in tubule form the early distal nephron.

**Figure 6.** *Species differences in macula densa expression of the H<sup>+</sup>-ATPase B1 subunit.*

A-B) Immunostaining for ATP6V1B1 using the ATP6V1B1<sub>H7659</sub> antibody in mouse (A) and rat (B) kidney cortex. C) Immunostaining for ATP6V1B1 using the ATP6V1B1<sub>S</sub> antibody in human kidney. Note an apparent lower expression of the H<sup>+</sup>-ATPase B1 subunit in macula densa of rat. Asterisks denotes tubular segment containing the specialized macula densa cells.

**Figure 7.** *The H<sup>+</sup>-ATPase B2 subunit is expressed in the early distal nephron in mouse and human kidney.*
A) Immunostaining for H^+-ATPase B2 subunit (ATP6V1B2) in mouse kidney showed most abundant expression in the cortical portion of the kidney. Note strong staining of the proximal tubules and distal nephron segments. B) Less intense staining was observed in the outer and inner medullary portion of the kidney (B). Grey stippled lines demarcate the zones of the medulla. C) Confocal images showing localization of the ATP6V1B2 (red) with the SLC12A1 (green). D) Confocal images showing cellular colocalization between H^+-ATPase B2 subunit staining (red) with SLC12A3 (green). Note localization of ATP6V1B2 to apical domains in the TAL and DCT as marked by asterisks. E-F) Immunostaining for H^+-ATPase B2 subunit in human kidney in the cortex (E) and the medulla (F). Staining is visible in proximal tubules and distal nephron segments in the cortical labyrinth and medullary rays, and in the medulla.

**Figure 8. Distribution of the H^+-ATPase E1 subunit in mouse and human kidney.**

A) Immunostaining for H^+-ATPase E1 subunit (ATP6V1E1) in mouse kidney revealed abundant expression in cortical nephron segments, including the proximal tubules and distal nephron segments. B) Staining appeared less intense in the outer and inner medullary portion of the kidney. IC stained most strongly. Grey stippled lines demarcate the cortex from the medulla. C-D) Confocal images showing localization of the ATP6V1E1 (red) with the SLC12A1 (green, C) and with SLC12A3 (green, D) to apical domains in the TAL and DCT. Asterisks mark early distal tubules. E-F) Antibodies against the H^+-ATPase E1 subunit were used to stain human kidney. Akin to the mouse, staining was most abundant in cortical tubule segments, including proximal tubules and distal tubules. IC staining was strong in both cortex and the medulla (F).

**Figure 9. The H^+-ATPase G1 subunit is expressed along the mouse and human distal nephron.**

A-B) Immunostaining for H^+-ATPase G1 subunit (ATP6V1G1) in mouse kidney showed expression in the cortical and medullary portion of the kidney. Staining was found in both the proximal tubules and distal nephron segments, with pronounced IC staining in both cortex and medulla. Grey stippled lines demarcate the zone of the inner medulla. C-D) Confocal images showing localization of the ATP6V1G1 (red) with SLC12A1 (green, C) or SLC12A3 (green, D). E-F) Immunostaining for H^+-ATPase G1 subunit in human kidney in the cortex (E) and the medulla (F).
Figure 3

(A) ATP6V1B1

(B) ATP6V1B1

(C) SLC12A1

(D) SLC12A3

(E) PMCA4

(F) AQP2
Figure 7