Expression of the 56-kDa B2 subunit isoform of the vacuolar H\(^+\)-ATPase in proton-secreting cells of the kidney and epididymis

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The relative distribution of the B1 and B2 isoforms in proton-secreting urogenital epithelial cells has not been examined in depth. In particular, it is unclear whether both isoforms are coexpressed in IC and epididymal clear cells and whether they have similar or distinct intracellular localizations. It has been proposed that the particular isoform composition of the V-ATPase within a cell or tissue might be an important factor in determining the intracellular location and/or function of the V-ATPase (22, 35). Furthermore, recent studies on a knockout mouse that lacks the B1 isoform of the V-ATPase have shown not only that the mouse is viable but also that it does not develop detectable systemic acidosis when allowed unrestricted access to food and water (19). Furthermore, the mice appear to be fertile (K. E. Finberg, Dept. of Pathology, Massachusetts General Hospital, personal communication). This suggests that normal V-ATPase function might be maintained by the presence of the B2 isoform in proton-secreting cells of the urogenital tract.

To investigate whether the 56-kDa B2 subunit of the V-ATPase could potentially play a role in the regulation of proton transport in kidney and epididymis epithelial cells, we raised isoform-specific affinity-purified rabbit polyclonal antibodies and used them to study the distribution of the two B subunits isoforms in these organs. We found the 56-kDa B2 subunit of the V-ATPase to be expressed not only in the kidney proximal convoluted tubule and TAL, but also in the DCT, CS, and both A- and B-type IC of the CD in both species. The B2 isoform was also detected in all proton-secreting cells of the epididymis in both rat and mouse. Under baseline conditions, the B2 isoform was found predominantly on intracellular vesicles, but under some conditions, such as chronic carbonic anhydrase inhibition, it was also expressed on the apical plasma membrane of A-IC cells in the kidney. These data indicate that the B2 isoform can be integrated into the V-ATPase holoenzyme in specialized proton-secreting cells. It could, therefore, play a role not only in the acidification of intracellular organelles but also in transepithelial proton secretion and the maintenance of acid-base homeostasis.

**MATERIALS AND METHODS**

**Antibodies.** To identify H\(^+\)-ATPase-containing cells in both kidney and epididymis, we used an affinity-purified polyclonal antibody raised in chicken against a synthetic peptide corresponding to the 10 carboxy-terminal amino acids of the E (31 kDa) subunit of the V-ATPase, as described previously (11, 23). The rabbit polyclonal anti-V-ATPase (56-kDa B1 subunit) antibody was raised against a 10-amino acid peptide (PQDTEADTAL), corresponding to the COOH-terminal sequence of bovine V-ATPase, coupled to keyhole limpet hemocyanin (KLH) via a cysteine residue. This antibody was also characterized previously (11). A new rabbit polyclonal anti-V-ATPase (56-kDa B2 subunit) antibody was also generated as described above against a KLH-coupled peptide (EFYPRDSAKH) corresponding to the last 10 amino acids of the COOH-terminal tail of human V-ATPase B2 subunit.

For the peptide competition assay, the peptides against which the B1 and B2 antibodies were raised were dissolved in phosphate-buffered saline (PBS) containing 0.02% (wt/vol) sodium azide. Each antibody was preincubated in the presence of a 10-fold (wt/wt) excess of the respective immunizing peptide for 1 h at room temperature before the immunofluorescence staining protocol described in Immuno-fluorescence and confocal microscopy. Protein concentration in the peptide and antibody samples was determined using a SmartSpec 3000 spectrophotometer (Bio-Rad Laboratories, Hercules, CA).

For all anti-V-ATPase antibodies described, the immunizing peptides were synthesized by the Massachusetts General Hospital Peptide/Protein Core Facility and the respective antibodies were raised commercially (Cocalico Biologicals, Reamstown, PA). Each antibody was affinity purified from whole serum with the respective immunizing peptide by using the SulfoLink kit (Pierce, Rockford, IL) according to the manufacturer’s instructions.

A monoclonal antibody against the 28-kDa calcium-binding protein calbindin (mouse IgG1 isotype; Sigma-Aldrich, St. Louis, MO) was used at a final concentration of 14 μg/ml as a kidney connecting segment marker.

The following affinity-purified secondary antibodies were used: indocarbocyanine (Cy3)-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) at a final concentration of 1.5 μg/ml, fluorescein isothiocyanate (FITC)-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories) at 25 μg/ml, FITC-conjugated donkey anti-chicken IgY (Jackson ImmunoResearch Laboratories) at 25 μg/ml, and FITC-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories) at 8.3 μg/ml.

**Tissue preparation.** Adult male Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) and adult mice (C57BL6 × CBA F1 strain; Jackson Laboratory, Bar Harbor, ME) were anesthetized with pentobarbital sodium (Nembutal, 65 mg/kg body wt ip; Abbot Laboratories, North Chicago, IL) and perfused through the left cardiac ventricle with PBS (0.9% NaCl in 10 mM phosphate buffer, pH 7.4) followed by paraformaldehyde-lysine-periodate fixative (PLP: 4% paraformaldehyde, 75 mM lysine-HCl, 10 mM sodium periodate, and 0.15 M sucrose in 37.5 mM sodium phosphate). Both kidneys were removed and sliced, and both epididymides were harvested from each animal. The organs were further fixed by immersion in PLP for 4 h at room temperature and overnight at 4°C, extensively rinsed in PBS, and stored at 4°C in PBS containing 0.02% sodium azide until use. Where appropriate, one epididymis and one kidney were harvested and frozen in liquid nitrogen for Western blotting (see Protein extraction and Western blotting).

**Immunofluorescence and confocal microscopy.** Tissues prepared as described in Tissue preparation were cryoprotected in PBS containing 0.9 M sucrose overnight at 4°C and then embedded in Tissue-Tek OCT compound 4583 (Sakura Finetek USA, Torrance, CA), mounted on a cutting block, and frozen at −27°C. Sections (4 μm) were cut on a Reichert-Jung 2800 Frigocut cryostat (Leica Microsystems, Bannockburn, IL), collected onto Superfrost Plus precleaned, charged microscope slides (Fisher Scientific, Pittsburgh, PA), air-dried, and stored at 4°C until use.

Sections were rehydrated in PBS for 10 min and treated with 1% (wt/vol) SDS for 4 min for retrieval of antigenic sites, as previously described (18). Sections were subsequently washed three times for 5 min in PBS and incubated for 10 min in 1% (wt/vol) bovine serum albumin (BSA) in PBS with 0.02% sodium azide to prevent nonspecific staining, followed by a 90-min incubation in the primary antibody at room temperature. After three 5-min PBS washes, the secondary antibody was applied for 1 h at room temperature, and the slides were then rinsed again in PBS three times for 5 min. Slides were mounted in a 1:1 mixture of Vectashield medium (Vector Laboratories, Burlingame, CA) and 1.5 M Tris solution (pH 8.9). For dual staining with antibodies raised in different species, the primary antibodies were applied sequentially at the appropriate concentrations as described in Antibodies, with each primary antibody being followed by the corresponding secondary antibody.

Digital images were acquired using a Nikon Eclipse 800 epifluorescence microscope (Nikon Instruments, Melville, NY) with an Orca 100 charge-coupled device camera (Hamamatsu, Bridgewater, NJ). Images were then analyzed by using IPLab version 3.2.4 scientific image processing software (Scanalytics, Fairfax, VA) and...
Fig. 1. Detection of vacuolar H⁺-ATPase (V-ATPase) B2 subunit in kidney and epididymis by Western blotting. Thirty micrograms of mouse (lane 1) and rat (lane 2) kidney homogenates were subjected to SDS-PAGE, and blots were probed with an antibody raised against the V-ATPase B2 subunit. The 56-kDa band was abolished when the antibody was preincubated with the peptide antigen (lanes 3 and 4). Thirty micrograms of mouse (lane 5) and rat (lane 6) epididymis homogenates were subjected to SDS-PAGE, and blots were probed with the anti-B2 subunit antibody. Concentration of the V-ATPase enzyme by coimmunoprecipitation with an anti-V-ATPase E subunit antibody resulted in an increased B2 signal in mouse and rat epididymis (lanes 7 and 8, respectively).

Fig. 2. Peptide competition assays in rat kidney cortex using the B1 and B2 V-ATPase antibodies and the peptides against which the antibodies were raised (see Antibodies in MATERIALS AND METHODS). The V-ATPase subunit B1 antibody was applied alone (A) or after preincubation in the presence of the B1 (B) or B2 immunizing peptide (C). The V-ATPase B2 antibody was applied alone (D) or after preincubation in the presence of the B1 (E) or B2 immunizing peptide (F). The antibody against the B1 subunit strongly stained intercalated cells (IC) of the cortical collecting duct (CD) (A), whereas the B2 antibody stained proximal convoluted tubules (D). For each of the 2 antibodies, staining was completely abolished by preincubation with the respective immunizing peptide but not when preincubated with the peptide against which the other antibody was raised. Bars = 30 μm.
were imported into and printed from Adobe Photoshop version 6.0 image-editing software (Adobe Systems, San Jose, CA).

For confocal laser scanning microscopy, tissue sections were prepared as described. Confocal imaging was performed on a Radiance 2000 confocal microscopy system (Bio-Rad Laboratories) using LaserSharp 2000 version 4.1 software, and images were edited as described above.

Immunogold electron microscopy. Small pieces of rat kidney medulla prepared as described were cryoprotected in PBS containing 2.3 M sucrose. Ultrathin cryosections were cut on a Leica EM FCS at −80°C and collected onto Formvar-coated nickel grids. Sections were blocked on drops of 1% BSA in PBS for 10 min at room temperature and then incubated on drops of primary anti-B2 V-ATPase antibody or DAKO diluent alone (DAKO, Carpinteria, CA) for 2 h at room temperature. After being rinsed on drops of PBS, the grids were incubated on drops of goat anti-rabbit IgG secondary antibody coupled to 10-nm gold particles (Ted Pella, Redding, CA) for 1 h at room temperature. After being rinsed on drops of distilled water, the grids were stained on drops of uranyl acetate-tylose mixture for 10 min on ice and then collected on loops and allowed to dry. Sections were examined in a Philips CM 10 transmission electron microscope at 80 kV.

Protein extraction and Western blotting. Kidney and epididymis tissues from rats and mice were cut into smaller pieces and disrupted with a Tenbroeck tissue grinder in 3 ml of homogenization buffer [320 mM sucrose, 10 mM HEPES-KOH, pH 7.2, 1 mM EGTA, 0.1 mM EDTA, 1 mM DTT, and Complete protease inhibitors from Roche Applied Science (Indianapolis, IN)]. Homogenates were centrifuged for 10 min at 1,000 g at 4°C. Triton X-100 was added to the supernatant to a final concentration of 1%. After a second homogenization and centrifugation for 30 min at 16,000 g at 4°C, the supernatant was collected and the protein concentration was determined with the bicinchoninic acid protein assay (Pierce Biotechnology, Rockford, IL) using albumin as standard. Protein extracts were aliquoted and stored at −80°C. Protein (20–30 μg) was diluted in Laemmli reducing sample buffer, boiled for 5 min, and loaded onto Tris-glycine polyacrylamide 4–20% gradient gels (Cambrex, Rockland, ME). After SDS-PAGE separation, proteins were transferred onto an Immun-Blot polyvinylidene difluoride membrane (Bio-Rad Laboratories). Membranes were blocked in Tris-buffered saline (TBS) with 5% nonfat dry milk and then incubated overnight at 4°C with the primary antibody diluted in TBS with 2.5% milk. For competition experiments, the primary antibody was incubated for 1 h with a 10-fold excess of the corresponding peptide before the overnight incubation with the membrane. After four washes in TBS with 0.1% Tween 20 (TBST), membranes were incubated with a donkey anti-rabbit IgG antibody conjugated to horseradish peroxidase for 1 h at room temperature. After four further washes, antibody binding was detected with the Western Lightning chemiluminescence reagent (Perkin Elmer Life Sciences, Boston, MA).

Immunoprecipitation. Epididymis homogenate (800–1,000 μg) was incubated overnight at 4°C with a chicken antibody raised against the E subunit of the V-ATPase (see Antibodies) in a buffer containing 20 mM HEPES-KOH, pH 7.2, 100 mM KCl, 2 mM MgCl2, 2 mM CaCl2, and protease inhibitors. Anti-chicken IgY (50 μl) immobilized on agarose beads (Promega, Madison, WI) was added. After another hour of incubation at 4°C, proteins bound to the beads were recovered by centrifugation for 30 s at 500 g. Beads were washed four times in

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Fig. 3. Immunofluorescence labeling for the B1 and B2 subunits of the V-ATPase in the rat cauda epididymis. Strong staining is shown in the apical pole of clear cells of the rat cauda epididymis with anti-B1 (A) and anti-B2 (D) affinity-purified antibodies. Staining was completely abolished by preincubation of the anti-B1 and anti-B2 antibodies with their corresponding immunizing peptide (B and F, respectively). Preincubation of the anti-B1 antibody with the B2 immunizing peptide, and of the B2 antibody with the B1 peptide, did not inhibit the staining (C and E, respectively). Bars = 20 μm.

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the immunoprecipitation buffer containing 0.5% Triton X-100, resuspended in 30 μl of 2X Laemmli reducing sample buffer, and boiled for 5 min. After a brief centrifugation, supernatants were loaded on a polyacrylamide gel and analyzed as described Protein extraction and Western blotting.

RESULTS

Characterization of B1 and B2 V-ATPase antibodies. Western blotting using the affinity-purified antibody against the B2 subunit of the V-ATPase showed a single band at an apparent molecular weight of 56 kDa in mouse and rat kidney samples (Fig. 1, lanes 1 and 2, respectively). No bands were present when blotting was performed using antibody that had been preincubated with a 10-fold excess of the immunizing peptide (Fig. 1, lanes 3 and 4). The anti-B2 antibody detected a weak 56-kDa band in mouse and rat epididymis homogenates (Fig. 1, lanes 5 and 6, respectively). To enhance this signal, we coimmunoprecipitated the B2 subunit using the anti-V-ATPase E subunit (31 kDa) antibody. After this coimmunoprecipitation, a stronger B2 signal was detected in mouse and rat epididymis samples (Fig. 1, lanes 7 and 8, respectively). The specificity of the anti-B1 subunit antibody was shown previously (11).

Specificity of the B1 and B2 V-ATPase antibodies for immunofluorescence staining was further assessed by peptide competition assays in both kidney and epididymis. For these experiments, the B1 and B2 antibodies were preincubated in the presence of the immunizing peptides as described above, and the antibody-peptide mixture was used alongside the antibody alone in the regular immunostaining protocol. In rat kidney cortex, the V-ATPase B1 antibody alone revealed high levels of expression of this subunit in the proton-translocating IC of the CD (Fig. 2A). Conversely, the B2 subunit of the V-ATPase was shown to be highly expressed in cortical PT (Fig. 2D). Both these findings are in agreement with previous observations (29) and with our own results for mouse kidney cortex (data not shown). The staining was completely abolished by preincubation of antibodies with their respective immunizing peptide for both B1 (Fig. 2B) and B2 subunits (Fig. 2F). To check for cross-reactivity of the two V-ATPase 56-kDa antibodies, we preincubated the B1-specific antibody with the B2 immunizing peptide and the B2 antibody with the B1 peptide. No inhibition of staining was seen under these conditions, indicating the specificity of the antibodies for their corresponding B subunit peptide sequences (Fig. 2, C and E).

The immunocytochemical competition assays in rat epididymis yielded results similar to those for the kidney. As previously described (17), a strong staining for the B1 subunit was observed in clear cells (Fig. 3A). These cells were also brightly stained using the B2 subunit antibody (Fig. 3D). Adjacent principal cells (PC) were poorly labeled by the B1 and B2 antibodies. No staining was detected when anti-B1 and -B2

Fig. 4. Confocal microscopy showing double-immunofluorescence staining of rat kidney cortex for calbindin (A, green) and V-ATPase B2 subunit (B, red). Calbindin stained connecting segment (CS) and distal convoluted tubule (DCT) cells (A). Proximal tubules (PT) do not contain the 28-kDa calbindin protein. The B2 subunit of the V-ATPase is present in the CS and in the apical pole of PT and DCT cells (B). C: merged image in which colocalization of calbindin and V-ATPase B2 appears as yellow staining in CS and DCT cells. Bar = 30 μm.
Antibodies were preabsorbed with their respective immunizing peptides (Fig. 3, B and F, respectively), showing specificity of the antibodies. The staining was not affected when anti-B1 antibody was preincubated with the B2 immunizing peptide or when anti-B2 antibody was preincubated with the B1 peptide (Fig. 3, C and E, respectively), demonstrating no cross-reactivity of the antibodies.

Detection of V-ATPase B2 subunit in the kidney cortex. As shown in Fig. 2D, the B2 subunit of the V-ATPase exhibited a characteristic high level of expression in cortical PT of both rat and mouse kidney, with B2 being localized at the apical pole of the PT epithelial cells (Fig. 4). A monoclonal calbindin antibody was used to identify CS in the cortex. Calbindin was also detected in DCT cells as previously described (Fig. 4A) (32). Calbindin-positive CS cells were found to contain the B2 subunit of the V-ATPase in both rat (Fig. 4B) and mouse (mostly localized to the apical pole of the cells) (data not shown). Calbindin-negative cells, or IC, of the CS also expressed the B2 isoform, but the level of expression was variable among different cells and different tubules. Whereas
Fig. 6. Immunocytochemical localization of the V-ATPase in the renal collecting duct. The 56-kDa subunit B2 (A, red) was present in all IC of the mouse inner medullary (IM) CD, characterized by the presence of the 31-kDa E subunit of the V-ATPase (B, green). Some cells showed a marked colocalization of these V-ATPase subunits at their apical membrane (C). In other segments of the CD, in both the inner stripe (IS) of the outer medulla (D–F) and the cortex (CO) (G–I), the B2 subunit was expressed in IC but showed a less obvious apical staining pattern. Low levels of B2 expression were also seen in principal cells throughout the CD. G–I show B2 and E V-ATPase expression in the rat kidney cortical CD. Whereas A-IC (arrow) exhibit a pattern of diffuse/apical staining similarly to the medullary CD from both species, the B2 subunit showed a diffuse distribution in B-IC (arrowheads) and did not strongly colocalize with the E subunit at the basolateral plasma membrane. Bars = 10 μm.
many IC in the CS expressed higher B2 levels than calbindin-positive CS cells (Fig. 4, B and C), some IC exhibited lower B2 levels compared with those in CS cells.

B2 was expressed in most DCT cells and was concentrated at the apical membrane and in the subapical cytoplasmic domain in both rat (Fig. 4, B and C) and mouse kidney (not shown). A similar pattern was seen in the DCT of both species for the B1 subunit of the V-ATPase (not shown), as observed previously (29).

To further characterize V-ATPase expression in the CS and DCT, we performed a dual immunostaining experiment by using antibodies against the B2 and E subunits of the V-ATPase. In both CS (Fig. 5, A–C) and DCT (Fig. 5, D–F), most cells stained for both subunits (B2, red, and E, green). DCT cells expressed E V-ATPase localized mostly apically and subapically (Fig. 5, E), where it colocalized with B2 (Fig. 5, D). Unlike DCT cells, rat kidney CS cells exhibited a more diverse, mosaic-like pattern of staining for both B2 (Fig. 5A) and E subunits (Fig. 5B). Whereas most B-IC with distinct basolateral E subunit staining in the CS showed weak basolateral B2 staining, in the CD (see below), cells with a more intense basolateral B2 staining were sometimes encountered (Fig. 5, A–C, insets). In contrast, apical B2 staining was detectable in most cells in the CS.

B2 V-ATPase immunostaining in the kidney collecting duct. The B2 isoform of the 56-kDa subunit of the V-ATPase was expressed in all A-IC and B-IC of the CD in both mouse and rat kidney. The pattern of B2 V-ATPase immunostaining was in general vesicular or diffuse cytoplasmic and was often concentrated in the subapical cytoplasmic domain. Some IC, however, had a pattern of apical expression that appeared as a tighter apical band, indicative of apical membrane staining. Figure 6 shows typical examples of B2 and E subunit double staining in the CD of the mouse kidney inner medulla (A–C), inner stripe (IS) of the mouse outer medulla (D–F), and cortex of rat kidney (G–I). The two subunits are coexpressed in the same cells, and they mostly assume a dispersed cytosolic localization in cortical and outer medullary CD of control tissues (Fig. 6, D–I). The distribution of both B2 and E subunits was frequently more polarized in inner medullary CD cells (Fig. 6, A–C), with the sharp band of yellow staining in the merged image (Fig. 6C) revealing their colocalization at the level of the apical membrane. PC showed a weak expression of B2, usually detectable in a diffuse cytosolic pattern in the cortex (Fig. 6G). The B2 expression pattern in A-IC (Fig. 6G, arrow) closely resembles that in the A-IC of the medullary CD. Similarly to PC, the B2 distribution in B-IC assumed a diffused pattern. Even in those B-IC in which the basolateral pole of the
cell was more strongly stained for the 31-kDa subunit (Fig. 6, G and H, arrowheads), the 56-kDa B2 subunit did not show significantly higher levels of staining at the basolateral membrane than in the cytoplasmic domain.

To more clearly determine whether the B2 isoform could be located on the apical membrane of CD A-IC, we performed immunogold electron microscopy on rat kidney outer medulla sections of the CD (Fig. 7). In good agreement with the immunofluorescence results, immunoelectron microscopy revealed that B2 V-ATPase can localize either mostly to the apical membrane of A-IC (Fig. 7A) or mainly in the subapical cytoplasmic domain and to a lesser extent on the apical membrane (Fig. 7B).

Detection of V-ATPase B subunits in the thick ascending limb. Figure 8 shows the results of an immunolabeling experiment in which we used the anti-B1 and anti-B2 V-ATPase antibodies in consecutive cryostat sections of rat kidney. As previously reported (26, 29), the V-ATPase B2 subunit was expressed in all regions of the TAL in both rat and mouse kidney. Higher levels of B2 expression were seen toward the apical pole of the TAL cells, as shown in Fig. 8B (rat, IS of the outer medulla). This apical and subapical staining pattern was also seen in mouse kidney (not shown). However, both B1 and B2 isoforms were expressed in TAL cells in the cortex and outer stripe (OS) of both rat and mouse kidney. In contrast, the B1 subunit was not detectable in the IS of the outer medulla in either species. Figure 8A shows no B1 expression in the TAL of the IS, whereas the IC of the CD exhibit a high level of B1 expression. As shown in Fig. 8B, the B2 subunit is highly expressed in the IS TAL in the absence of the B1 subunit. In the OS, however, TAL do stain for the B1 isoform, and its localization is apical and subapical (Fig. 8C). OS TAL also stain for the B2 subunit in a similar pattern, whereas the PT staining for B2 is characteristic for both the OS (S3 segments) and cortex (Fig. 8D).

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**Fig. 8.** Immunofluorescence labeling of serial sections showing distribution of the 56-kDa B subunits of the V-ATPase in thick ascending limbs (TAL) in the inner stripe (IS) (A and B) and outer stripe (OS) (C and D) of rat kidney outer medulla. In the IS of the outer medulla, IC of the CD stained for the B1 subunit (A), whereas the TAL were negative. In contrast, in a consecutive section of the same IS region, a perinuclear and subapical punctate staining pattern for the B2 subunit was present in TAL (B). Conversely, TAL in the OS contained the B1 subunit of the V-ATPase in an apical location (C). In a consecutive section of the same OS region, the same TAL shown in C also stained positively for the B2 subunit (D). PT in the OS showed subapical B2 (D) but not B1 (C) staining, similar to the pattern previously seen in the cortex (see Fig. 2). Bars = 30 μm.
Localization of the B2 subunit of the V-ATPase in the collecting duct of acetazolamide-treated rats. We next determined whether the cellular localization of the 56-kDa B2 and 31-kDa E subunits of the V-ATPase would change in parallel in response to chronic carbonic anhydrase inhibition by acetazolamide, a condition in which A-IC are “activated” by membrane insertion of the V-ATPase (2). In agreement with previous reports, the E subunit staining pattern shifted in most A-IC of the CD in all kidney regions from a generally diffuse cytoplasmic or subapical and/or apical staining to a tight apical membrane localization, and this is shown for the OS of the outer medulla in Fig. 9B (see also arrows in E, IS). The 56-kDa B2 subunit staining pattern was also altered in response to acetazolamide in most A-IC, from a mainly cytoplasmic localization (see Fig. 6) to an apical and subapical distribution. The apical colocalization of the E and B2 subunits was demonstrated by a sharp band of yellow apical membrane staining (Fig. 9, C and F) and also in the subapical domain in the IC of the CD in all kidney regions.

As previously shown (2), we also noticed a significant reduction in the number of B-IC in acetazolamide-treated animals. With respect to B2 localization, we detected many B-IC that exhibited a diffuse cytosolic staining for this isoform, as described above for B-IC in control tissues. However, we also found some B-IC in acetazolamide-treated tissues that, unlike those in control animals, revealed a weak basolateral B2 staining (not shown).

Detection of V-ATPase B1 and B2 subunits in the epididymis by indirect immunofluorescence. Cryostat sections of PLP-fixed rat cauda epididymis were stained using antibodies raised against the B1 and B2 subunits of the V-ATPase. Double labeling for B2 and E subunits of the V-ATPase in rat cauda epididymis confirmed that B2 expression is strongest in the previously described (8–10) subunit B1- and E-positive clear cells (Fig. 10).

Higher resolution confocal microscopy confirmed that both B1 (Fig. 11A) and B2 subunits (Fig. 11E) are present in the apical pole of clear cells of mouse cauda epididymis. Double
V-ATPase insertion (2). Apical insertion of the B1 isoform is previously shown to enhance in rats treated with acetazolamide, which we have determined by immunofluorescence and confocal microscopy. The extent of this apical localization was greatly increased in rats treated with acetazolamide, which we have previously shown to “activate” A-IC by increasing apical V-ATPase insertion (2). Apical insertion of the B1 isoform is also greatly increased by acetazolamide treatment (2). We conclude that though the V-ATPase holoenzyme containing the B2 isoform is mainly found in the cytoplasm, where it plays a role in the acidification of intracellular organelles, it could also contribute to apical proton secretion in renal epithelia under some conditions. A proton-secretory role for the B2-containing V-ATPase has already been shown in osteoclasts, which do not express the B1 isoform (26).

In contrast to the strong apical membrane localization of B2 in some A-IC, basolateral plasma membrane expression of B2 in B-IC of the cortical CD was weak or absent, even in those cells in which the E subunit of the V-ATPase stained the basolateral pole of the cells more strongly than other cellular domains. However, in CS, distinct basolateral coexpression of the B2 and E subunits was found in a few cells. Furthermore, after acetazolamide treatment of rats, some B-IC in CD had a weak but distinct basolateral B2 staining. These results imply that the basolateral targeting and/or retention of the B2 subunit in B-IC may be less efficient than that of the B1 subunit. Interestingly, we previously reported that NHE-RF1 (Na\(^+\)/H\(^+\) exchanger regulatory factor), a member of the PDZ family of proteins, was expressed in B-IC but not A-IC (11) and proposed that NHE-RF1 might be involved in the bipolar targeting of the B1 V-ATPase subunit in B-IC. However, NHE-RF1 is probably not required for apical V-ATPase targeting, given that it was not detectable in A-IC, in which the plasma membrane V-ATPase is uniquely apical. The weak B2 staining of basolateral membranes of B-IC might, therefore, be related to the absence of the COOH-terminal PDZ-binding sequence from this isoform. In contrast, the COOH terminus of the B1 isoform contains a well-defined DTAL motif that allows it to interact with NHE-RF1 (11), which may permit basolateral accumulation of B1. It is also likely that the level of expression of the B2 subunit in any given IC might also determine our ability to detect low levels of basolateral staining. As previously shown for the E subunit (2), acetazolamide treatment might also upregulate B2 subunit expression, allowing its detection on basolateral membranes under these conditions.

In the epididymis, B2 isoform expression was readily detected in proton-secreting clear cells of rat and mouse tissues. The clear cells are, thus, similar to renal IC with respect to B1 and B2 coexpression in cells specialized for proton secretion. However, convincing colocalization of B2 with the 31-kDa E subunit in the extensive apical microvilli of these cells was not found under control conditions or after acetazolamide treatment of rats. Instead, B2 was always concentrated in a population of subapical vesicles in clear cells in both rat and mouse epididymis. Unlike the B1 subunit, B2 did not colocalize extensively with the E subunit in the apical plasma membrane microvilli. It is conceivable that the B2 isoform might not play the same role in transmembrane proton transport in epididymal clear cells as in kidney IC. Alternatively, it is possible that the acetazolamide treatment to which the animals were subjected in this report could fully activate renal IC but not epididymal clear cells. This is supported by the fact that localization of the B1 isoform was not modified in epididymal clear cells after acetazolamide treatment (data not shown). Further studies on activation of proton secretion in the epididymis are necessary to address these possibilities.

The idea that the B2 isoform can serve as a possible backup or alternative mechanism for the active role played by the B1 isoform in proton secretion is suggested by recent studies on...
Fig. 11. Confocal microscopy showing double-immunofluorescence labeling for B1 or B2 subunits with the E subunit of the V-ATPase in rat and mouse cauda epididymis clear cells. In all panels, arrows indicate the frontier between the apical membrane brush border and the subapical endosomal compartment of the clear cell. This frontier is also visible as a dark line that appears to separate these 2 cellular domains. In mouse epididymis, the B1 subunit was distributed between subapical vesicles and apical microvilli (A, red). Some vesicles are also seen deeper inside the cell. The same localization was observed for the E subunit (B, green). Merged image C shows an almost complete colocalization of the B1 and E subunits (yellow). The B2 subunit was also present in subapical vesicles but was absent from apical microvilli (E, red), whereas the E subunit was located both in subapical vesicles and the microvilli of the same cell (F, green). Merged image G shows partial colocalization of the B2 and E subunits in subapical vesicles (yellow) and little or no apparent colocalization in apical microvilli (green). A similar distribution was observed in clear cells of rat cauda epididymis for the B1 and E subunits (D) and for the B2 and E subunits (H). Bars = 10 μm.
knockout mice lacking the B1 isoform of the 56-kDa subunit. These animals are viable and do not develop detectable systemic acidosis when allowed unrestricted access to food and water. Their urine has, however, a higher pH than that of normal mice (3, 19). These findings suggest that the V-ATPase necessary for distal acidification by IC is at least partially functional, despite the lack of the B1 subunit. Furthermore, male knockout mice lacking the B1 isoform appear to be fertile (K. E. Finberg, personal communication), implying that luminal acidification in the epididymis, which is necessary for sperm maturation and storage, is not critically impaired by the lack of the B1 V-ATPase subunit. Direct examination of the V-ATPase subunit composition of IC and clear cells from these knockout mice is needed to provide more insight into these issues.

The cellular role of V-ATPase holoenzymes with distinct subunit compositions is coming under closer scrutiny with the discovery of increasing numbers of subunits that have different cell- and tissue-specific isoforms. It has been proposed that various V-ATPase subunit aggregations are responsible for differential intracellular localization and different targeting mechanisms and functions of the proton pump (22, 35). Each V1 sector of the V-ATPase contains three B subunits. However, it is not known whether a single V-ATPase holoenzyme complex contains only one type of B subunit isoform or whether “hybrid” complexes containing both B1 and B2 subunits are present in those cells in which the two isoforms are coexpressed. Coimmunoprecipitations with other V-ATPase subunits have not yet offered a definitive answer to this question. Subunits a4, c, d1, E2, and G1 were shown to coprecipitate with both B isoforms, whereas C2-b, d2, and G3 coimmunoprecipitate with B1 alone, and a1, a2, a3, and C1 coprecipitate with B2 alone (35). It is clear, therefore, that B1 and B2 subunits can occur in unique and separate V-ATPase complexes, but whether B1/B2 interaction occurs in yet other manifestations of the holoenzyme has not been specifically addressed.

Besides the A- and B-IC of the CD, the 56-kDa B2 subunit of the V-ATPase is also expressed in the kidney in the proximal convoluted tubule, TAL, DCT, and CS in both rat and mouse. In the TAL, a difference in B isoform expression was seen between the IS of the outer medulla and the rest of this tubule segment. IS TAL cells express only the B2 isoform, whereas both B1 and B2 are clearly detectable in the OS and the cortical TAL regions. A striking apical colocalization of B1 and B2 was also found in distal tubule and CS epithelial cells. The functional relevance of these patterns of B subunit expression remains to be determined.

In summary, our present data show that the B2 subunit of the V-ATPase is expressed in most, if not all, proton-secreting cells in the epithelium lining the nephron and the CD, and it is also present in proton-secreting clear cells of the epididymis. This subunit has a mainly cytoplasmic distribution in most cells under baseline conditions, but apical plasma membrane localization can be observed in a few kidney IC in the renal medulla. This membrane staining is increased in CD IC after chronic acetazolamide treatment, which results in an increased “activity” of these cells. These data indicate that in addition to its role in the acidification of intracellular organelles, the V-ATPase holoenzyme containing the B2 subunit is also poised to play a role in transepithelial proton secretion under some conditions.

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