Relocalization of the V-ATPase B2 subunit to the apical membrane of epididymal clear cells of mice deficient in the B1 subunit

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Submitted 30 November 2006; accepted in final form 20 March 2007

Da Silva N, Shum WW, El-Annan J, Păunescu TG, McKee M, Smith PJ, Brown D, Breton S. Relocalization of the V-ATPase B2 subunit to the apical membrane of epididymal clear cells of mice deficient in the B1 subunit. Am J Physiol Cell Physiol 293: C199–C210, 2007. First published March 28, 2007; doi:10.1152/ajpcell.00596.2006.—An acidic luminal pH in the epididymis contributes to maintaining sperm quiescent during their maturation and storage. The vacuolar H^+ ATPase (V-ATPase), located in narrow and clear cells, is a major contributor to luminal acidification. Mutations in one of the V-ATPase subunits, ATP6v1B1 (B1), cause distal renal tubular acidosis in humans but surprisingly, B1^-/- mice do not develop metabolic acidosis and are fertile. While B1 is located in the apical membrane of narrow and clear cells, the B2 subunit localizes to subapical vesicles in wild-type mouse, rat and human epididymis. However, a marked increase (84%) in the mean pixel intensity of B2 staining was observed in the B1 subunit-deficient in B1^-/- mice compared with B1^+/+. Immunogold electron microscopy showed abundant B2 in the apical microvilli of clear cells in B1^-/- mice. B2 mRNA expression, determined by real time RT-PCR using laser-microdissected epithelial cells, was identical in both groups. Semiquantitative Western blots from whole epididymis and cauda epididymidis showed no variation in the B1 subunit.

In mammals, the epididymis is composed of one to three highly coiled tubules divided into several morphologically distinct regions, including the initial segments, caput, corpus, and cauda epididymidis. These regions are further segmented into intraregional segments, which all contribute to the establishment of a succession of microenvironments in which spermatozoa mature and are concentrated, transported, and stored (19, 31, 49). The composition of the luminal microenvironment is controlled by distinct epithelial cell types: principal cells, narrow, clear cells, and basal cells sit at the base of the epithelium (25). Narrow, clear and principal cells play a major role in transepithelial acid/base transport. Like cells of the kidney proximal tubule, principal cells in the proximal epididymis express a set of enzymes and transporters that generate net bicarbonate reabsorption, with no net proton secretion (45). The luminal bicarbonate concentration is dramatically reduced during the passage of fluid through the proximal epididymis, and is maintained low in the distal epididymal regions and the vas deferens (37). In addition to bicarbonate reabsorption, active proton secretion takes place in the epididymal lumen, as reflected by the acidic pH of 6.8 measured in the distal portions of the tubule (15, 36, 37, 63). Net proton secretion is achieved by narrow and clear cells, in which large amounts of plasma membrane vacular H^+ ATPase (V-ATPase) and cytosolic carbonic anhydrase II (CAII) are expressed (8, 11, 14, 23, 24, 46, 47). Narrow cells are located in the initial segments of the epididymis, and clear cells are present in the remaining portions (caput, corpus, and cauda) of the epididymis.

Spermatozoa develop their ability to become motile and to fertilize an egg as they pass through the male excurrent ducts, which are composed of efferent ducts, epididymis, and vas deferens. Considerable changes in the composition of luminal fluid occur in the epididymis and include net water, Na^+, Cl^-, and HCO_3^- reabsorption, K^+ secretion, in addition to secretion and resorption of numerous proteins (2, 17, 18, 30, 36, 37, 58, 59). In addition, the lumen of the epididymis and vas deferens is maintained acidic compared with blood, and has a low bicarbonate concentration (36, 37). Low pH and bicarbonate are required for sperm maturation, for the prevention of premature activation of acrosomal enzymes, and for maintaining sperm in a quiescent, immotile state during their maturation and storage in the epididymis and vas deferens (1, 2, 16, 26, 32). Thus a defect in the acidification capacity of epididymis and vas deferens might have important consequences on the maturation and storage of spermatozoa, and, consequently, on male fertility (45).

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is catalytic, but the noncatalytic binding site of the B subunit may be involved in the regulation of V-ATPase activity. Two highly homologous 56-kDa isoforms of the B subunits (ATP6V1B1 and ATP6V1B2), coded by two distinct genes, have been identified. These two isoforms share a highly conserved central core of 469 amino acids, whereas the amino and carboxy termini are extremely divergent (39, 48). ATP6V1B1 (B1) was initially described as a kidney-specific isoform but was later identified in several organs including the eye, inner ear, and epididymis (8, 13, 34, 39, 60), whereas the so-called “brain” isoform, ATP6V1B2 (B2), is now considered to be ubiquitous (5, 60). We have shown previously that both B1 and B2 isoforms are enriched in V-ATPase-rich cells in the rat epididymis, as well as in renal intercalated cells (46). However, unlike the B1 subunit, most of the B2 immunostaining was concentrated in a population of subapical vesicles and was absent from the apical membrane. Therefore, we hypothesized that B1 is the functionally predominant subunit, whereas B2 could serve as an alternative subunit in situations where B1 function is impaired. This hypothesis was supported by the absence of any severe phenotype in mice lacking the B1 isoform, which were originally engineered to create a mouse model of distal renal tubular acidosis (dRTA) (22). dRTA is a disease in which the regulation of systemic pH is dramatically impaired and mutations of Atp6v1B1 coding for the B1 subunit have been shown to cause dRTA in humans (34, 35, 51). However, B1 null mice did not develop systemic acidosis under normal dietary conditions. In the kidney, the absence of the B1 subunit is partially compensated for by a modulation of B2 subcellular localization: the incorporation of B2-containing complexes is significantly increased in the apical membrane of type A intercalated cells from the inner medulla (22). Furthermore, B1−/− mice are fertile, suggesting that there is also a compensatory response to replace the loss of B1 function in the male excurrent duct. To determine whether a B2 subunit-based compensatory mechanism may be involved in the maintenance of an acidic luminal pH compatible with fertility, we undertook a quantitative analysis of B2 expression and localization in the epididymis of B1 knockout mice.

MATERIALS AND METHODS

Nomenclature. ATPase, H+ transporting, V1 subunit B, isoform 1 (ATP6V1B1) is coded by the gene Atp6v1B1. ATPase, H+ transporting, V1 subunit B, isoform 2 (ATP6V1B2) is coded by the gene Atp6v1B2. ATPase, H+ transporting, V1 subunit E-like 2 isoform 2 (ATP6V1E2) is coded by the gene Atp6v1E2. For simplicity, these proteins are referred to throughout the text as the B1, B2, and E2 subunits of the V-ATPase, respectively.

Experimental animals. B1−/− mice have been described previously (22). Animals were genotyped by PCR using primer sets specific for the wild-type (intron/exon 9) and deleted alleles (Neo cassette), using genomic DNA from tail clips as a template. Animal experiments were approved by the Massachusetts General Hospital Subcommittee on Research Animal Care, in accordance with the National Institutes of Health, Department of Agriculture, and Association for Assessment and Accreditation of Laboratory Animal Care requirements.

Primary antibodies. V-ATPase was identified in epididymal epithelial cells by using affinity-purified anti-B1, -B2, and -E2 antibodies raised against synthetic peptides and described previously (10, 23, 46). An additional anti-B2 antibody was raised in chicken against the peptide previously used to generate the rabbit antibody (EFYPRDZSAK). A cysite was added to the NH2-terminal portion of the peptide for coupling to keyhole limpet hemocyanin for antibody production and binding to an affinity column for purification of the antibody. Immunization of two chickens was performed by Cocalico Biologicals (Beamstown, PA). Immune sera were tested in our laboratory by immunofluorescence and Western blotting, and positive sera were affinity purified against the B2 peptide using the SulfoLink Kit according to the manufacturer’s recommendations (Pierce; Rockford, IL). The anti-AQP9 antibody was raised in rabbit against a COOH-terminal peptide of rat AQP9 and was affinity purified (42).

Total RNA extraction, reverse transcription, and polymerase chain reaction. Total RNA was isolated from epididymides using the RNeasy Mini kit (Qiagen, Valencia, CA). Genomic DNA contamination was removed by on-column incubation with 25 units of DNase I for 30 min at room temperature. DNA-free RNA was reverse transcribed (RT) and polymerase chain reaction (PCR) was performed with RT products as templates following a previously described procedure (29). PCR products were analyzed by electrophoresis on a 2.5% agarose gel containing GelStar stain (Cambrex BioScience, Rockland, ME). No template controls were performed by omitting cDNA template from the PCR amplification. The sequences of the oligonucleotide primers were the following: B1-FOR: C(C/T)AGCATGAGCGGGATCAT; B1-REV: TATAATCGAGAAGACCACGCC; B2-FOR: CGAACCTTGGATGAGCTTTGGACATT; B2-REV: GGTGCCTGAGGATCTCTTCTC; GAPD-FOR: TGACCAAGAGGCCCCTATCC; GAPD-REV: CCCTAGGCCCCCTCTGTTAT. Primers were synthesized by Sigma-Genosys (The Woodlands, TX). The amplicon sizes are 182 bp (B1), 87 bp (B2), and 98 bp (GAPD).

For epithelial cell-specific RT-PCR analysis, epithelial cells from wild type and B1−/− mouse cauda epididymides were laser microdissected, and total RNA was extracted and amplified in vitro. Amplified RNA samples were used as templates for real-time quantitative PCR (qRT-PCR). Laser capture microdissection, RNA amplification, and qRT-PCR were performed following previously described procedures (21). qRT-PCR analysis was performed using the B2 and GAPD primer sets described above, the iTaq SYBR Green Supermix with ROX reagent (Bio-Rad, Hercules, CA), and the 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA). A dissociation curve was generated after each SYBR Green PCR run to monitor the specificity of the amplification.

Tissue preparation. Adult wild-type and B1−/− mice were anesthetized with pentobarbital sodium and perfuned through the left ventricle with phosphate-buffered saline (PBS), followed by paraformaldehyde-lysine-periodate (PLP) fixative (4% paraformaldehyde, 75 mM lysine HCl, 10 mM sodium periodate, and 0.15 M sucrose in 37.5 mM phosphate). Epididymides were removed and further fixed by immersion in PLP overnight at 4°C, washed in PBS, and stored at 4°C in PBS containing 0.02% sodium azide.

Human epididymis sections were kindly provided by Dr. Ivan Sabolic (Institute for Medical Research and Occupational Health, Zagreb, Croatia). Normal regions of human epididymis were obtained during surgery from three adults (age 50–65 yr) with testicular or bladder neck carcinoma, or benign prostatic hypertrophy. Tissues were fixed in PLP by immersion and stored in PBS containing 0.02% sodium azide. The sections used in the present study are from the same tissue samples that were used previously (23, 42). The research was approved by the Zagreb Hospital Ethics Committee, and a written voluntary agreement was obtained from each patient before surgery was performed.

Immunofluorescence. Fixed tissues were cryoprotected in PBS with 30% sucrose for at least 2 h at room temperature, embedded in Tissue-Tek OCT compound (Sakura Finetek, Torrance, CA), and mounted and frozen on a cutting block. Tissues were cut in a Reichert Fricog cut microtome at 5 μm thickness and sections were placed onto Fisherbrand Superfrost Plus microscope slides (Fisher Scientific, Pittsburgh, PA). Sections were rehydrated in PBS for 15 min and pretreated with 1% (wt/vol) sodium dodecyl sulfate (SDS), an antigen retrieval technique that we have previously described (12). After three
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washes in PBS, slides were preincubated in 1% (wt/vol) bovine serum albumin in PBS with 0.02% Na-azide for 30 min to prevent non-specific staining, after which they were incubated with the different primary antibodies in antibody diluent (Dako North America, Carpenteria, CA) for 90 min at room temperature or overnight at 4°C. After two washes in high-salt PBS (containing 2.7% NaCl) and one wash in PBS, slides were incubated for 1 h at room temperature with Cy3- or FITC-conjugated secondary antibodies and washed again. For B2/ AQP9 and B1/B2 double staining, the two primary antibodies were applied simultaneously at the appropriate concentration, followed by the two secondary antibodies. Washed slides were mounted in Vectashield with 4,6-diamidino-2-phenylindole (Vector Labs, Burlingame, CA) and examined using a Nikon E800 epifluorescence microscope. Sections were digitally imaged using a Hamamatsu Orca charge-coupled device camera and IPLab software (Scanalytics, Fairfax, VA).

Quantification of mean pixel intensity of V-ATPase B2-associated fluorescence in the apical pole of clear cells of the cauda epididymis. Immunofluorescence slides were prepared as described above from three wild-type and three B1 knockout animals. Each experiment (incubation) included two sections from each animal. All slides were immunostained under identical conditions, and for a given incubation all the digital images were acquired using the same exposure parameters. Each image was corrected for its own unstained background value. The segmentation function of IPLab was used to select the regions corresponding to the B2-associated fluorescence in the apical pole of clear cells. The mean pixel intensity of each segment was measured, and the raw data were imported into Microsoft Excel for further statistical analysis. The final result was calculated from segments extracted from a total of 48 images (23 B1 wild-type and 25 B1−/−). Data were expressed for each group as means ± SE. This technique has been used previously in our laboratory to quantify the expression of aquaporin 2 in the kidney and of aquaporin 9 and V-ATPase in the epididymis (4, 7, 44).

Quantification of the distribution of clear cells. The relative number of clear cells was evaluated by comparing the length occupied by the apical pole of clear cells with the luminal perimeter of the tubules. V-ATPase B2 immunofluorescence slides were prepared as described above, and digital images were captured. Thirty tubules (15 for the wild-type group, 15 for the B1 knockout group) were randomly selected in cauda epididymidis images. The perimeter of each tubule and the length occupied by the clear cells, including rows of clear cells that are characteristic of mouse epididymis and individual cells, were measured using the IPLab software, from images that were calibrated for each microscope objective. Raw data were imported into Microsoft Excel for statistical analysis, and the final results were expressed as the percentage of tubule perimeter occupied by the apical pole of clear cells (means ± SE).

Immunoblot analysis. Epididymides and kidneys were dissected, frozen in liquid nitrogen and stored at −80°C. Tissues were disrupted with a Tenbroeck tissue grinder in homogenization buffer containing 10 mM HEPES, pH 7.5, 0.32 M sucrose, 1 mM EDTA, and Complete protease inhibitors (Roche Applied Science, Indianapolis, IN). The homogenate was centrifuged at 4°C for 10 min at 1,000 g, Triton X-100 (1%), Igepal CA-630 (0.05%), and dithiothreitol (1 mM) were added to the supernatant. After a second homogenization and centrifugation for 30 min at 16,000 g (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. Fifty micrometers (20 μg for kidney control) protein were digested in Laemmli-reducing sample buffer, heated for 5 min at 99°C and loaded onto a Tris-glycine polyacrylamide 4–20% gradient gel (Cambrex Bio Science, Rockland, ME). After SDS-page separation, proteins were transferred onto an Immob-Blot PVDF membrane (Bio-Rad, Hercules, CA). 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 and 0.02% sodium azide. After four washes in TBS with 0.1% Tween 20, membranes were incubated with an IgG conjugated to horseradish peroxidase (Jackson ImmunoResearch Laboratories, West Grove, PA) for 1 h at room temperature. After four further washes, antibody binding was detected with the Western Lightning chemiluminescence reagent (Perkin Elmer LAS, Boston, MA) or SuperSignal West Dura reagent (Pierce). To determine the specificity of the novel chicken anti-B2 antibody, preincubation of the antibody was performed with a 10-fold excess (wt/wt) of immunizing peptide for 1 h at room temperature before Western blot incubation. For quantitative analysis of Western blots, digital images were acquired with the EpiChem3 Darkroom and analyzed with the LabWork 4.6 software (UVP, Upland, CA). Membranes were blocked in 5% nonfat dry milk and then incubated overnight at 4°C with the primary antibody diluted in TBS with 2.5% milk and 0.02% sodium azide. After four additional washes, antibody binding was detected with the Western Lightning chemiluminescence reagent (Perkin Elmer LAS, Boston, MA) or SuperSignal West Dura reagent (Pierce).

Immunogold electron microscopy. Small pieces of fixed cauda epididymidis were embedded at low temperature (−45°C) with HM20 resin (Electron Microscopy Sciences, Hatfield, PA) in a Leica EM AFS, as we have described previously (43). Ultrathin sections were

Fig. 1. Double immunofluorescence localization of B1 and B2 subunits in mouse and rat epididymis. Confocal microscopy projection series (i.e., multiple confocal layers flattened into a single image) of epididymal sections double stained for B1 (green) and B2 (red). B1 subunit is mainly located in apical microvilli and subapical vesicles of clear cells from mouse (A and rat (D) epididymis. Longer microvilli are routinely detected in clear cells from rat epididymis compared with mouse epididymis. In both species, a weaker intracellular B1 staining is also detected in these cells. The B2 subunit is mainly located in subapical vesicles and is virtually absent from apical microvilli in clear cells from mouse (B) and rat (E) epididymis. The merged images in C and F show that the co-localization of B1 and B2 is restricted to subapical vesicles of clear cells (arrowheads in C; yellow) and is absent from apical microvilli (arrows in C; green). Bars = 5 μm.
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Detection of V-ATPase B1 and B2 subunits in clear cells of mouse and rat cauda epididymidis. Double-labeling confocal microscopy for B1 and B2 subunits in the mouse (Fig. 1, A–C) and rat (Fig. 1, D–F) cauda epididymidis showed that B1 (Fig. 1, A and D) is present in apical microvilli (arrows in Fig. 1C) as well as in the cytoplasm and subapical vesicles (arrowheads in Fig. 1C), as we have previously described (46). In contrast, the B2 subunit (Fig. 1, B and E) is barely detectable in apical microvilli but is present in subapical vesicles and cytoplasm throughout the cell, where it co-localizes with the B1 subunit (Fig. 1, C and F).

Western blot and immunofluorescence analysis was performed to characterize our novel chicken anti-B2 antibody (Fig. 2). By Western blot analysis, the antibody showed a single band at an apparent molecular mass of 56 kDa in mouse epididymis, mouse kidney, rat epididymis, and rat kidney samples (Fig. 2A: lanes 1–4, respectively). The signal was completely abolished when the antibody was preincubated with a 10-fold excess of the immunizing peptide (Fig. 2B). By immunofluorescence, clear cells (arrows) show the brightest staining for B2. A weaker staining was also detected in the apical membrane of principal cells (arrowheads) and in spermatozoa (Fig. 2C). The staining in all of these locations was completely abolished after preincubation of the antibody with the immunizing peptide (Fig. 2D).

**RESULTS**

**Detection of V-ATPase B1 and B2 subunits in clear cells of mouse and rat cauda epididymidis.** Double-labeling confocal microscopy for B1 and B2 subunits in the mouse (Fig. 1, A–C) and rat (Fig. 1, D–F) cauda epididymidis showed that B1 (Fig. 1, A and D) is present in apical microvilli (arrows in Fig. 1C) as well as in the cytoplasm and subapical vesicles (arrowheads in Fig. 1C), as we have previously described (46). In contrast, the B2 subunit (Fig. 1, B and E) is barely detectable in apical microvilli but is present in subapical vesicles and cytoplasm throughout the cell, where it co-localizes with the B1 subunit (Fig. 1, C and F).

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**Measurement of epididymal luminal pH.** Wild-type and B1 knockout mice were anesthetized as described above, and the vas deferens and epididymis were exposed. Retrograde perfusion through the vas deferens lumen was performed at a rate of 60 μl/h with the use of a syringe pump (model 100; KD Scientific, Holliston, MA). A small incision was made in the cauda region of the epididymis (between the proximal and distal cauda) to allow the fluid to flow out of the tubule. The pH of fluid was measured in vivo immediately as it was flowing out of the epididymis, using a proton-selective electrode positioned at the incision site. The proton-selective electrode was composed of a glass microelectrode, pulled from 1.5-mm diameter borosilicate glass (model TW150-4; World Precision Instruments, New Haven, CT) to obtain a final tip diameter of 2 to 4 μm, as described previously (8). The electrodes were silanized, front filled with a proton-selective ionophore (30 μm column, Hydrogen ionophore cocktail B, Fluka) and back filled with a solution of 100 mM KCl. Selective electrode potentials were measured with a high input impedance preamplifier with a unity gain (model AD515, Analog Devices), and the signal was digitized by an analog-to-digital board (DR 2800 series, Data Translations). An additional amplifier with a gain of 1,000-fold was placed between the head stage and the computer, and the circuit was completed with a 3 M KCl agar bridge positioned inside the scrotum cavity. The Nernstian slope of the electrode was determined before and after each experiment by measuring the potentials in commercial calibration solutions at pH 6.0, 7.0, and 8.0. This method allowed for an accurate measurement of the pH of the luminal content by avoiding any loss of CO2, which would lead to a rapid alkalinization of the fluid.

**Quantification of gold particle labeling.** The relative amount of B2-subunit present in the apical microvilli of clear cells was determined from the number of gold particles per unit length of apical membrane using IPLab software. Gold particles present in the apical membrane including microvilli were counted for each cell, and the number was divided by the total length of apical membrane and microvilli for that cell. A total of 33 cells were examined from 3 wild-type mice and a total of 63 cells were examined from 3 B1−/− mice. Data were expressed as mean gold particles per unit length of apical membrane ± SE for each group. This method has been used by our laboratory previously to quantify the level of V-ATPase present in the apical membrane of clear cells (43).
Localization of B1 and B2 subunits in human epididymis. In agreement with our previously published data (23), clear cells of human epididymis express abundant B1 in their apical microvilli, and lower amounts in intracellular vesicles (Fig. 3A, A': green). Double-labeling showed that the B2 subunit is enriched in clear cells compared with adjacent principal cells and that it is predominantly located in intracellular structures but not in apical microvilli of clear cells (Fig. 3B, B': red). A weak B2 staining was also detected in principal cells. The merged panels shown in Fig. 3C, C' demonstrate little or no co-localization between the B1 and B2 isoforms in the apical microvilli of clear cells (green).

B1 and B2 mRNA expression during rat epididymis postnatal development. A quantitative RT-PCR analysis of B1 and B2 isoform expression was performed with rat epididymal epithelial cells harvested by laser capture microdissection during postnatal development and in the adult rat (Fig. 4). The expression of B1 subunit mRNA increases considerably during the first 3 wk of postnatal development (200-fold increase) and remains strong in the adult (although it decreases significantly from its 2- to 4-wk levels), whereas B2 mRNA expression is very stable (maximum of 5-fold increase) from birth to adulthood. The marked increase in B1 expression correlates with the progressive appearance of clear cells during the first 3 wk of postnatal development (9). The subsequent decrease probably represents a “dilution” of B1-expressing clear cells within the epithelium as they are less tightly packed in the mature epithelium, as we have previously described (9). This result, therefore, further indicates that B1 is the predominant B subunit in clear cells of the epididymis.

Distribution of clear cells in the epididymis of wild-type and B1 knockout mice. The distribution of clear cells was examined in wild-type and B1-/- mice. Clear cells were identified by their absence of staining for aquaporin 9, a principal cell marker (42), and by their positive staining for B2 (Fig. 5A). Immunofluorescence images of B2 staining were acquired with different parameters (longer exposure time for the “red” B2 channel in the wild-type mice) to obtain images of comparable intensity for qualitative examination of the staining patterns. Narrow and clear cells are interspersed between principal cells in most segments of the epididymis. However, in mice, the proximal region of the cauda epididymidis contains a high proportion of clear cells aligned in long rows, in addition to individual clear cells (Fig. 5, A and B, arrows). Quantification of the length occupied by the apical region of B2-positive clear cells relative to the total luminal perimeter of each tubule revealed no significant difference between the two groups (40% ± 11 vs. 36% ± 10; Fig. 5C), indicating a similar distribution of clear cells in wild-type and B1-/- mice. In addition to epithelial staining, our antibody revealed a signif-

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Fig. 3. Localization of B1 and B2 in human epididymis. Double-labeling showed abundant B1 expression in clear cells of human epididymis, where it is located mainly in apical microvilli (A, A': green). Some B1-positive intracellular vesicles are also detected. In contrast, while B2 is also enriched in clear cells, its localization is mainly in intracellular structure, and it is not detected in apical microvilli (B, B': red). The merged images shown in C, C' confirmed the lack of colocalization of B1 and B2 in apical microvilli. In these images, no sperm are visible in the lumen as indicated by the absence of 4,6-diamidino-2-phenylindole (DAPI) staining (blue). A–C: Bars = 20 μm. A’–C’: Bars = 5 μm.
significant B2 expression in spermatozoa, in both B1 knockout mice and wild-type controls.

Quantitative immunofluorescence of subunit B2 in B1 knockout mouse cauda epididymidis. When the same parameters were used for image capture, the B2-associated immunostaining was noticeably more intense in the apical pole of clear cells from B1−/− mouse cauda epididymidis compared with wild-type animals (Fig. 6, A and C). A quantitative analysis of this variation was performed using IPLab software (Fig. 6, B and D). The mean pixel intensity of B2-associated staining in the apical region of clear cells (Fig. 6, A and C) was quantified using the IPLab segmentation function (highlighted in yellow in Fig. 6, B and D), as we have described previously (4, 44). A significant increase in the intensity of B2 apical staining was observed in B1 knockout mice compared with wild-type animals (Fig. 6E).

Subcellular localization of B2 in B1 knockout mouse cauda epididymidis. First, the subcellular localization of B2 was characterized by spinning disk confocal microscopy. Double immunostaining was performed for the B2 and E2 subunits, and images were acquired with identical parameters (laser power, gain, exposure time) for each of the subunits in wild-type and B1−/− mice. The E2 subunit is part of the V1 domain and is the only E isoform identified in epithelial cells (28, 54). It, therefore, allows for the visualization of all V-ATPase complexes, whether or not they contain B2. In wild-type mouse epididymis, a weak staining for B2 was observed in subapical vesicles and the cytoplasm of clear cells (Fig. 7A). The E2 subunit (Fig. 7B) was abundant in the apical microvilli and subapical vesicles, and was also detected in the cytoplasm. The green color in the merged image in Fig. 7C shows that relatively little B2 co-localizes with E2 in the apical microvilli of clear cells. In B1 knockout mice, an intense B2 staining was observed in the apical microvilli of clear cells (Fig. 7D), and the subapical and cytosolic staining was diminished, compared with the wild-type control. The E2-associated fluorescence was also shifted from the subapical region and cytosol, to the microvilli (Fig. 7E). The orange-yellow staining in Fig. 7F shows that B2 co-localizes with E2 in the apical membrane of clear cells in B1 knockout mice. Figure 7G is a schematic view of the results shown in Fig. 7, A–F, and includes higher magnification images of the apical region of clear cells.

The subcellular localization of B2 in the apical microvilli of clear cells was further examined by immunogold electron microscopy in wild-type (Fig. 8, A and C) and B1 knockout mouse epididymis (Fig. 8, B and D). More B2-associated gold...
particles were consistently detected in the microvilli of clear cells of B1−/− animals vs. wild-type mice. Quantification analysis revealed a significant increase in the amount of apical membrane-associated B2 subunit labeling in clear cells of B1−/− mice compared with wild-type mice (Fig. 8; see bar graph).

B1 mRNA expression in the epididymis of wild-type and B1 knockout mice. Conventional and quantitative RT-PCR analysis was performed to examine B1 mRNA expression in B1−/− vs. wild-type mice. As expected, conventional RT-PCR from RNA extracted from whole epididymides showed a strong B1 mRNA signal in the B1+/+ and B1+/− mice but no signal in the B1−/− mice (Fig. 9A top). In contrast, strong signals for B2 mRNA were obtained in all samples (Fig. 9A, middle), and no difference was detected between the samples, indicating no significant modulation of B2 mRNA expression in B1−/− mice, compared with B1+/− mice and B1+/+ controls. This result was confirmed by real-time quantitative PCR analysis performed with the same samples (not shown). Thus, overall B2 mRNA expression is not upregulated in the whole epidid-
ymis of B1 knockout mice. To more clearly determine whether the expression of the B2 isoform could be locally modulated in the distal portion of the epididymis rather than in the whole organ, we performed a quantitative analysis of B2 mRNA with samples representing exclusively epithelial cells of the cauda epididymidis. Real-time quantitative PCR performed with RNA extracted from laser-microdissected epithelial cells revealed that B2 mRNA expression is identical in B1/+ mice compared with wild-type controls (Fig. 9B).

B1 and B2 protein expression in the epididymis of B1 knockout mice. As expected, Western blots performed with whole epididymis protein extracts showed abundant B1 expression in B1/+ and B1/+ mice and no expression in B1/- mice (Fig. 10A). In contrast, similar levels of B2 protein were detected in B1/- mice, compared with B1/+ and B1/+ animals. A quantitative Western blot analysis restricted to the cauda region of the epididymis also showed that B2 protein expression is not significantly different in knockout animals compared with wild types (Fig. 10B). Together, these results confirm that the absence of functional B1 is not compensated by an upregulation of B2 expression in B1 knockout animals.

Measurement of epididymal luminal pH. The pH of the luminal fluid from cauda epididymidis was measured in wild-type and B1 knockout animals with a proton-selective electrode. Similar pH values of 6.7 were detected in both groups (Fig. 11). These results show that the acidification process is not impaired in mice lacking functional B1 subunit.

DISCUSSION

Genetic studies have shown that mutations in Atp6v1B1, the gene encoding the B1 subunit of V-ATPase, are associated with a loss of B1 function and trigger recessive distal renal tubular acidosis (dRTA) and irreversible hearing impairments.
(34, 53). In the kidney, B1 is expressed in the apical membrane of the proton-secreting type A intercalated cells of the collecting duct (3, 38, 39, 46). The pH of arterial blood must be maintained at 7.4, and impairment of acid-base homeostasis has dramatic consequences on cellular function, growth, and development. Atp6v1B1 mutations identified in dRTA patients result in the expression of abnormal V-ATPase complexes, and concomitant failure of the urinary acidification process is accompanied by severe symptoms. Interestingly, most of the dRTA patients with an Atp6v1B1 mutation also suffer from deafness (34), indicating that the kidney is not the only organ affected by the loss of B1 function. In the inner ear, proton secretion is required to maintain the pH of endolymph between 6.6 and 7.4 (20, 34, 52). Therefore, it is predicted that the acidification process could also be impaired in the male reproductive tract, which also expresses the B1 isoform, but it has not been possible to examine this so far in the B1 null human population because most of them are juveniles.

To characterize the role of V-ATPase in the kidney and create an animal model of dRTA, Finberg et al. (22) engineered B1-deficient mice. Unlike dRTA patients harboring homozygous Atp6v1B1 mutations, B1 null mice raised on a normal diet did not develop significant acidemia. However, an imposed acid load induced an impairment of urinary acidification, which led to significant systemic acidosis in knockout animals compared with wild-type controls, demonstrating an important role of B1 in renal proton secretion.

We have shown that both B1 and B2 isoforms of the V-ATPase are enriched in the apical pole of proton-secreting epididymal narrow and clear cells (8, 11), as well as kidney intercalated cells (46). An acidic luminal pH in the epididymis is necessary to keep spermatozoa in a quiescent state as they mature and are stored in the male excurrent duct, and impairment of the acidification process may affect male fertility. Contrary to B1, which is clearly the predominant B isoform in clear cells, B2 is less abundant and is mainly located in subapical vesicles (46). Our results showing a marked increase in B1 mRNA that correlates with the appearance of clear cells during the first 3 wk of postnatal development (9), whereas B2 mRNA levels remained stable, further confirmed that the B1 subunit is the predominant isoform in clear cells. The critical role of luminal acidification in male reproductive physiology was demonstrated by a recent study showing that mice lacking the transcription factor Foxi1 are infertile due to the inability of sperm to move up the female reproductive tract and fertilize an egg (6). This study established that Foxi1 is a major transcriptional regulator of several genes involved in proton secretion in epididymal narrow and clear cells, including Atp6v1B1 and CAII. Severe depletion of both B1 and CAII in Foxi1 knockout mouse epididymis resulted in a more alkaline luminal environment and severe impairment of sperm maturation, leading to infertility.

In contrast, the absence of significant fertility impairment in B1 null mice, which continue to express CAII, indicates that a compensatory mechanism, possibly involving the B2 subunit, is triggered in the male excurrent duct to maintain a luminal acidic pH. The present study shows that the loss of B1 function is neither compensated for by an increase in the relative number of V-ATPase-rich cells nor by an increase of total B2

Fig. 11. Luminal pH measurement in WT and B1 KO mouse cauda epididymis. Luminal pH was measured with a proton-selective electrode in the distal region of 6 WT and 7 B1 KO mouse epididymides. The luminal pH was not significantly different in the WT and in the B1 KO group (pH 6.71 and 6.76, respectively).
expression. However, quantitative immunofluorescence and immunogold analysis revealed that the B2 subunit is translocated from the subapical compartment to the apical membrane of clear cells in B1 null mice. Co-localization of B2 and E2 subunits indicated that B2-containing V-ATPase complexes, which are predominantly located in the subapical pole of clear cells in wild-type animals, are exported into the apical membrane and may, therefore, contribute to apical proton secretion. This was confirmed by the normal luminal acidic pH of their cauda epididymidis. Similarly, we have previously shown that medullary intercalated cells lacking the B1 subunit display an intense apical B2 staining, whereas B2 is predominantly intracellular in control animals (46).

This relocalization of the B2 subunit, which is the ubiquitous isoform responsible for the acidification of intracellular organelles, does not appear to impair the survival functions of clear cells, because their number and morphology remain intact in the B1−/− mice. Although expressed at lower levels compared with the B1 subunit, the B2 subunit is enriched in clear cells compared with other cell types. Therefore, it is most likely present in sufficient amounts in the subapical pool of vesicles and cytosol to compensate for the absence of B1, without altering the vital functions associated with B2 expression on acidifying organelles of clear cells in B1−/− mice.

The role of a particular isoform composition in the intracellular trafficking, location, and/or function of the V-ATPase is still poorly understood and is currently under investigation. Recent studies, performed in transfected kidney inner medullary collecting duct cells, showed that B1 constructs carrying the single amino acid mutations described in dRTA patients fail to assemble into functional V-ATPase complexes (62). It was suggested that mutated B1 subunits might impair the trafficking of V-ATPase by competing with wild-type subunit-containing complexes for as yet unidentified components of the protein/vesicle targeting machinery. Interestingly, the PDZ-binding motif that terminates B1 and that is absent in the B1 knockout model, which lacks the COOH terminus of the protein, is retained in the human B1 subunits carrying dRTA point mutations, thus preserving potentially important interactions with proteins involved in trafficking regulation. Alternatively, in humans, B1 subunits harboring single point mutations might assemble normally with the holoenzyme, thus preventing a compensatory association of the B2 subunit. Importantly, the B1 knockout mouse, which was generated by suppressing exons 7–11 of the Atp6v1B1 locus, does not express an abnormal B1 protein capable of interfering with the trafficking of wild-type B1-containing V-ATPase complexes or with the assembly of B2 into the holoenzyme. Therefore, we hypothesize that the absence of B1 in the knockout mouse model allows B2 to associate more freely with the holoenzyme that will be targeted to the apical membrane of epididymal clear cells. In accordance with this hypothesis, we and others have shown previously that the V0 sector a4 subunit is the predominant “a” isoform of the V-ATPase in the apical pole of narrow and clear cells (47, 50), and a4 has been shown to coimmuno-precipitate with both B1 and B2 subunits (55). Thus, one intriguing possibility is that V1 sectors bearing the B2 isoform are able to associate more readily with a4-containing V0 transmembrane sectors when B1 is absent and that targeting signals on the a4 subunit are responsible for the apical plasma membrane targeting of the holoenzyme. It has previously been suggested that the large transmembrane “a” subunits play a role in V-ATPase targeting (41, 56, 57). Indeed, recent work (27, 47) has shown that the a2 isoform has a predominantly intracellular localization in kidney proximal tubule cells and epididymal epithelial cells, whereas a4 is on the apical brush border membrane of proximal tubule cells and in the plasma membrane of intercalated cells and clear cells (27, 41, 47). The mechanisms underlying the insertion of the B2 subunit in V-ATPase complexes at the plasma membrane in the absence of B1 (compared with its intracellular localization in the presence of B1) are under current investigation in our laboratory and will provide new insights into how the V-ATPase-mediated acidification is regulated in the male excurrent duct.

**ACKNOWLEDGMENTS**

We thank Dr. Ivan Sabolic from the Institute for Medical Research and Occupational Health, Zagreb, Croatia, for providing the human epididymis sections used in this study.

**GRANTS**

This study was supported by National Institutes of Health Grants HD-40793 (to S. Breton), DK-38452 (to S. Breton and D. Brown), DK-42956 (to D. Brown), and DK-73266 (to T. G. Paunescu). The Microscopy Core of the Massachusetts General Hospital Program in Membrane Biology received additional support from National Institute of Diabetes and Digestive and Kidney Diseases Grant DK-38452, from the Center for the Study of Inflammatory Bowel Disease Grant DK-43351 and Boston Area Diabetics and Endocrinology Research Center Grant DK-57521.

**REFERENCES**


