Vacuolar H\textsuperscript{+}-ATPase B1 Subunit Mutations that Cause Inherited Distal Renal Tubular Acidosis Affect Proton Pump Assembly and Trafficking in Inner Medullary Collecting Duct Cells

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Point mutations in the B1 subunit of vacuolar H\textsuperscript{+}-ATPase are associated with impaired ability of the distal nephron to secrete acid (distal renal tubular acidosis). For testing of the hypothesis that these mutations interfere with assembly and trafficking of the H\textsuperscript{+}-ATPase, constructs that mimic seven known point mutations in inherited distal renal tubular acidosis (M) or wild-type (WT) B1 were transfected into a rat inner medullary collecting duct cell line to express green fluorescence protein (GFP)-B1WT or GFP-B1M fusion proteins. In co-immunoprecipitation studies, GFP-B1WT formed complexes with other H\textsuperscript{+}-ATPase subunits (c, H, and E), whereas GFP-B1M did not. Proteins that were immunoprecipitated with anti-GFP antibody from GFP-B1WT cells had ATPase activity, whereas proteins from GFP-B1M cells did not. Proton pump–mediated intracellular pH transport was inhibited in GFP-B1M–transfected cells but not in GFP-B1WT cells. GFP-B1WT and GFP-B1M are present in the apical membrane and increased with cellular acidification. In GFP-B1WT cells, the apical membrane fraction of GFP-B1, endogenous B1, and the 31-kD subunits of the H\textsuperscript{+}-ATPase increased with cell acidification. In GFP-B1M cells, the endogenous B1 and 31-kD subunits did not increase with acidification. B1 point mutations prevent normal assembly of the H\textsuperscript{+}-ATPase and also may act as an inhibitor of H\textsuperscript{+}-ATPase function by competing with endogenous intact H\textsuperscript{+}-ATPase for trafficking in inner medullary collecting duct cells.

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acuolar H\textsuperscript{+}-ATPases are ubiquitous multisubunit complexes that mediate the ATP-dependent transport of protons. In general, vacuolar H\textsuperscript{+}-ATPases consist of two domains: A cytoplasmic catalytic V\textsubscript{1} domain (640 kD) and a membrane-bound V\textsubscript{0} domain (260 kD) (1). This hetero-oligomer catalyzes the hydrolysis of ATP and the translocation of proton (H\textsuperscript{+}) across biologic membrane (1,2). Vacular H\textsuperscript{+}-ATPases acidify many intracellular compartments, such as the lysosomes, Golgi apparatus, secretory vesicles, and endosomes. In other specialized cells, such as the renal \alpha-intercalated cell, endolymph-forming inner ear cells, osteoclasts, and cells of the epididymis, the vacuolar H\textsuperscript{+}-ATPase mediates the transport of H\textsuperscript{+} across the plasma membrane (3).

In the kidney, intercalated cells of the late distal tubule, connecting segment, and cortical and medullary collecting ducts mediate the transport of H\textsuperscript{+} by an apical membrane H\textsuperscript{+}-ATPase. These cells express several renal-specific subunits, including the B1 and a4 subunit of the H\textsuperscript{+}-ATPase (4–6). It has been reported that mutations in the B1 and a4 subunit of the vacuolar H\textsuperscript{+}-ATPase cause inherited type I (or distal) renal tubular acidosis (dRTA) combined with or without sensorineural deafness (7,8). dRTA is a disorder with the following characteristics: Metabolic acidosis, hyperchloremia, and hypokalemia. Fifteen different ATP6V1B1 (B1 subunit) mutations in 19 different kindreds with recessive dRTA have been reported (7). These mutations include premature termination codons, frameshift mutations, splice site mutations, and some missense and other miscellaneous mutations. The exact mechanism(s) by which identified ATP6V1B1 mutations lead to impaired renal H\textsuperscript{+} secretion and, therefore, dRTA remains unknown.

To elucidate the molecular basis by which ATP6V1B1 mutations result in dRTA, we made seven constructs that mimic ATP6V1B1 point mutations that have been identified in inherited dRTA (L81P, R124W, M174R, T275P, G316E, P346R, and G364S) and transfected them into an inner medullary collecting duct (IMCD) cell line to produce stable cell lines that expressed either green fluorescence protein B1 wild-type (GFP-B1WT) or GFP-B1 mutant (GFP-B1M) fusion proteins. In this report, we demonstrate that ATP6V1B1 subunits with point mutations are not assembled into the multisubunit vacuolar H\textsuperscript{+}-ATPase, whereas WT are. However, the unassembled B1 mutant sub-
units were targeted to the apical membrane, and targeting was enhanced in response to acidic stimulation. In addition, targeting of the mutants interfered with apical membrane accumulation of the native H⁺-ATPase. Therefore, B1 mutants may act as an inhibitor of H⁺-ATPase function by competing with the endogenous intact H⁺-ATPase for the same trafficking process that directs H⁺-ATPase to the apical membrane in IMCD cells. In addition, the B1 subunit contains specific targeting information.

**Materials and Methods**

**Establishment of Stably Transfected Cell Lines that Express GFP-B1WT and GFP-B1M Proteins**

A PCR-based method was used to introduce point mutations into the kidney-specific isoform B1 subunit template. Primers (Figure 1) that were designed to amplify the human B1 template fragments to produce ATP6V1B1 point mutations that have been identified in inherited dRTA (L81P, R124W, M174R, T275P, G316E, P346R, and G364S) are depicted in Figure 1. Restriction endonucleases (Figure 1) and T4 DNA ligase were used to manipulate the construction of the template to achieve the targeted mutations. The ligased cDNA was inserted into pcDNA3.1/NT-GFP-TOPO (Invitrogen, Carlsbad, CA) vectors, and the constructed vectors for expression of GFP-B1WT and GFP-B1M were transfected into IMCD cells as described previously (9). All clones of recombinant mutant B1 vectors were sequenced to ensure that the correct insertions and intended mutations were present. IMCD cells were obtained from rat papillae as described previously (10). All clones of recombinant mutant B1 vectors were sequenced to ensure that the correct insertions and intended mutations were present. IMCD cells were obtained from rat papillae as described previously (10). Purified recombinant plasmids were used to transfect the IMCD cell using Lipofectamine 2000 (Invitrogen) (9). Transfected IMCD cells were selected in medium that contained 800 μg/ml G418. Stable cell lines (clonal) that express GFP-B1WT and GFP-B1M were transfected into IMCD cells as described previously (9). All clones of recombinant mutant B1 vectors were sequenced to ensure that the correct insertions and intended mutations were present. IMCD cells were obtained from rat papillae as described previously (10). Purified recombinant plasmids were used to transfect the IMCD cell using Lipofectamine 2000 (Invitrogen) (9). Transfected IMCD cells were selected in medium that contained 800 μg/ml G418. Stable cell lines (clonal) that express GFP-B1WT and GFP-B1M were transfected into IMCD cells as described previously (9).

**Reverse Transcription–PCR**

Reverse transcription for B1 and B2 subunit of the H⁺-ATPase was performed by using 0.1 μg of mRNA as template as described previously by us (9). The following primers were used for amplification of B subunits: Isoform 1 (homologous with human and mouse B1 isoform), upstream 5-ttg ttc agg tgt aga gga-3, downstream 5-tcg cgg aat gcc ctt gag cat-3; and isoform 2 (Rattus norvegicus ATPase, H⁺-transporting, V1 subunit, B isoform, 2, Atp6vlb2), upstream 5-caaagttggcttgccgagggatgag-3, downstream 5-cta gtg ctc aga gca tgc-3.

**H⁺-ATPase–Mediated Proton Transport**

The rate of H⁺-ATPase–mediated H⁺ transport was determined in monolayers of WT IMCD cells and transfected IMCD cells that express GFP-B1WT and GFP-B1M fusion proteins by a fluorospectrophotometric method (10). Briefly, quiescent IMCD cells that were grown on glass coverslips were incubated for 1 h at 37°C in NHB (in mM: 110 NaCl, 50 HEPES acid, 5 KCl, 1 MgCl₂, 5 K₂HPO₄, 1 CaCl₂, and 5 glucose [pH 7.2]) that contained 10 μM of the acetoxyethyl ester of 2′,7′-bis(carboxyethyl)-5(6)-carboxyfluorescein. The coverslip then was placed in a cuvette that contained 1 ml of NHB and secured at a 35-degree angle to the excitation beam. The monolayer was washed three times with NHB and then incubated in 1 ml of NHB. Fluorescence intensity was measured in a PerkinElmer (Boston, MA) model LS 650-10 fluorospectrophotometer equipped with a thermostatically controlled (37°C) cuvette holder at excitation wavelengths of 505 and 455 nm with a slit width of 5 nm and an emission wavelength of 560 nm with a slit width of 10 nm. Once intracellular pH (pHᵢ) was stable in NHB, Na⁺-independent pHᵢ recovery after a 20-mM NH₄Cl-induced acid load was determined when cells were incubated in CHB (similar to NHB, except 110 mM choline chloride replaced 110 mM NaCl) as described previously. The H⁺-ATPase–mediated pHᵢ recovery after acute acid load also was evaluated in the presence of the H⁺-ATPase inhibitor 1 μM bafilomycin (11). At the end of each experiment, the fluorescence intensity ratio was calibrated to pH, with potassium HEPES buffer that contained 10 mg/ml nigericin.

**Immunoprecipitation**

GFP-B1WT- and GFP-B1M–transfected IMCD monolayers that were scraped from the plastic plate were homogenized using Teflon-coated Dounce homogenizer in an IP buffer (in mM: 10 Tris HCl, 150 NaCl, 1 EDTA, and 1 EGTA with 1% Triton-100 and 0.5% NP-40, titrated to a pH of 7.4). Just before use, 1X protease inhibitor cocktail set I (cat. no. 539131; Calbiochem, La Jolla, CA) was added. The homogenate was centrifuged at 1000 × g at 4°C for 20 min to obtain a postnuclear supernatant. The supernatant was centrifuged further at 10,000 × g for 20 min at 4°C. A total of 800 μg of protein from the final supernatant was mixed with IP buffer to a total volume of 1 ml and subjected to immunoprecipitation with primary rabbit polyclonal anti-GFP antibody (1:250; BD Biosciences) or normal rabbit serum as negative control. The samples were processed as described previously (9).

**ATP/NADH Coupled Assay for ATPase**

The assay is based on a reaction in which the regeneration of hydrolyzed ATP is coupled to the oxidation of NADH (12,13). The ATPase hydrolysis rates were determined as the change in NADH absorbance at 340 nm in a Na⁺-K⁺-free reaction mixture that consisted of the immunoprecipitated samples supplemented with ATPase reaction buffer (25 mM triethanolamine hydrochloride [pH 7.5], 13 mM magne-
Acid Loading of Cells and Apical Membrane Preparation

IMCD monolayers were subjected to a reduction in cytosolic pH (pH) from 7.2 to 6.5 by methods described previously (14). Briefly, IMCD monolayers were washed with PBS to remove any DMEM and washed once with CHB-AC (CHB to which 10 mM potassium acetate was added). The monolayers then were incubated in CHB at 37°C for 15 min. As a control, IMCD monolayers were treated similarly but the CHB was replaced by NHB. During this time, the pH rapidly declined in the presence of CHB-AC to 6.5 as a result of reversal of the Na+/H+ exchanger and diffusion of acetic acid into the cell (14).

Apical membranes were isolated from the IMCD cells by a vesiculation method adapted by our laboratory for polarized epithelial cells (15). This method enriches the apical marker GP-135 nearly 10-fold compared with whole-cell homogenate, and the basolateral marker E-cadherin is barely detectable (15). Control (pH, approximately 7.2) or acid-loaded cells, in which the pH falls to 6.5, were incubated for 90 min at 37°C in a vesication medium (CHB or NHB) that contained 50 mM paraformaldehyde, 2 mM DTT, and protease inhibitors. Formaldehyde and DTT induced the formation of apical membrane vesicles that are released into the incubation medium. At the end of 90 min, the medium that contained the released apical membrane vesicles were collected, filtered through 37-mm nylon mesh to remove whole cells, and centrifuged at 20,000 rpm at 4°C in a Sorvall RCB for 1 h to pellet the vesicles. The pellet was aliquoted for protein determination and dissolved in SDS sample buffer for SDS-PAGE immunoblotting.

Immunoblot

Whole-cell homogenate and samples from the above studies were loaded onto a 10% polyacrylamide-SDS gel, run under reducing conditions, transferred to nitrocellulose filters, and reacted with antibody as described previously (9). The primary antibodies used were a rabbit polyclonal anti-31-kD or 56-kD B1 subunit of H⁺-ATPase (a gift from Dr. Dennis Brown, Harvard Medical School, Boston, MA), a rat monoclonal anti-GFP (Santa Cruz), a rabbit polyclonal antibody to v-ATPase c (ductin; Oncogene, San Jose, CA), and a mouse monoclonal anti-GP-135 (G.K. Ojakian, Downstate Medical Center, Brooklyn, NY). The secondary antibodies were coupled to horseradish peroxidase, and a rabbit polyclonal anti–31-kD or 56-kD B1 subunit at the mRNA and protein levels. Brain expresses the specific B subunit of the H⁺-ATPase that is inserted into the apical membrane of intercalated cells, we determined by reverse transcription–PCR and Western blot the B subunit isoforms expressed by these cells compared with intact kidney and brain. Figure 2 we demonstrates that both kidney and IMCD cells express both B1 and B2 isoforms of the 56-kD subunit at the mRNA and protein levels. Brain expresses only the B2 isoform of this subunit.

Expression of GFP-B1WT and GFP-B1M Constructs in Stably Transfected IMCD Cells

To evaluate the expression of GFP-B1 constructs in the stably transfected IMCD cells, we subjected lysates from these cell lines to immunoblot analysis for GFP protein levels using an anti-GFP antibody. Figure 3 shows that similar levels of GFP-B1 fusion proteins are expressed in all the mutants as well as in the WT B1-transfected cell lines. The similarity of expression level was permeabilized with 1% Triton-100 in PBS for 5 min and subsequently washed (three times, 5 min each) in PBS. For blocking of nonspecific background staining, cells were incubated with PBS-1% BSA for 30 min. Primary antibody (rabbit polyclonal anti-GFP antibody at a dilution of 1:250) was applied at 4°C overnight. After washing in PBS (three times, 5 min each), secondary anti-rabbit antibody (diluted 1:200) coupled to FITC (Jackson Immunologicals, West Grove, PA) or streptavidin-CY3 (1 μg/ml in PBS; Jackson Immunologicals) was applied for 1 h at room temperature. After further washing as above, slides were mounted in Vectashield (Vector Laboratories, Burlingame, CA) and diluted 1:1 in 0.1 M Tris-HCl (pH 8.0). Digital images were acquired with a confocal microscope (UltraView LCI, PerkinElmer). The images stacks were analyzed using Velocity 3.6 software, and apical XY and Z images were generated.

Results

Expression of the B Subunits in Cultured IMCD Cells

The IMCD cell that was used in this study functionally resembles collecting duct intercalated cells with regard to the characteristics of proton transport (10). To document that these cells express the specific B subunit of the H⁺-ATPase that is inserted into the apical membrane of intercalated cells, we determined by reverse transcription–PCR and Western blot the B subunit isoforms expressed by these cells compared with intact kidney and brain. Figure 2 we demonstrates that both kidney and IMCD cells express both B1 and B2 isoforms of the 56-kD subunit at the mRNA and protein levels. Brain expresses only the B2 isoform of this subunit.

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of GFP-B1 fusion protein ensures the comparability of analysis
of these cell lines in the following experiments.

Interaction of GFP-B1WT and GFP-B1M with the Other V₁
and a V₀ Subunit of the H⁺-ATPase. If the GFP-B1 fusion
protein can assemble with other H⁺-ATPase subunits, then it
should be possible, using an anti-GFP antibody, to co-immu-
noprecipitate additional H⁺-ATPase subunits with the GFP-B1
fusion protein from cell homogenates of stably transfected
cells. For this purpose, we evaluated the co-immunoprecipitability
of two V₁ subunits, the E (31 kD) and H (51 kD) subunits and one
V₀ subunit c (ductin consists of two 8-kD helices that form a
four-helix SDS-resistant bundle and therefore is detected as a
32-kD dimer [16]). Figure 4 demonstrates that all three subunits
(c, E, and H) of the H⁺ ATPase can be co-immunoprecipitated
with GFP-B1WT. Therefore, it seems that the WT GFP fusion
protein can interact with other subunits to form a V₁ sector and
that this sector can assemble with the V₀ sector to form an intact
H⁺-ATPase. In contrast, none of the three native subunits (c, E,
and H) was co-immunoprecipitated with any of the seven
mutant fusion proteins that were expressed by transfected
IMCD cells. It follows, then, that these point mutations produce
a protein that cannot assemble with the other subunits to form
a complete H⁺-ATPase.

ATPase Hydrolysis Activity. On the basis of the co-immu-
noprecipitation studies, we predicted that WT constructs as-
semble into complete molecules that have ATPase activity,
whereas mutant constructs do not. We determined the ATPase
activity of anti-GFP antibody (normal rabbit serum as negative
control) immunoprecipitates that were obtained from cells that
were transfected with B1WT and B1M constructs. The results in
Figure 5 show that the NADH absorbance at an optical density
of 340 decreased (ATP hydrolysis) with time in the B1WT
sample, whereas all the mutant samples remained unchanged.

Figure 3. Expressions of GFP-B1WT and GFP-B1M fusion pro-
tein in stably transfected IMCD cells. (A) Homogenates of
transfected IMCD cells (60 μg/lane) were immunoblotted with
an anti-GFP antibody to identify the fusion protein and with
anti-actin antibody as a loading control. (B) Densitometric anal-
ysis of the expression level of the GFP constructs in four stud-
ies. Similar levels of GFP-B1 fusion protein (81 kD) are ex-
pressed in all mutants as well as in the B1WT-transfected cell
lines.

Figure 4. Ability of GFP-B1WT or GFP-B1M to assemble with the E, H, and c subunits of H⁺-ATPase. (A) Immunoprecipitates of
800 μg of whole-cell homogenates from the stably transfected IMCD cell lines were prepared with a rabbit polyclonal anti-GFP
antibody. The immunoprecipitates were subjected to immunoblot analysis. The top panel was probed with an antibody to GFP
to identify the GFP-B1 fusion protein, and the bottom panel was probed with a rabbit antibody to the 31-kD H⁺-ATPase (E
subunit). (B) This study was similar to that depicted in A except that the last lane was immunoprecipitated with nonimmune rabbit
serum (NRS). The top panel was probed with an antibody to GFP, the middle panel was probed with an antibody to subunit H
(51 kD), and the bottom panel was probed with an antibody to subunit c (ductin) that recognizes c as a dimer (32 kD). (C)
Immunoblot for ductin in a membrane fraction of homogenates from untransfected IMCD cells and IMCD cells that were
transfected with B1WT or the point mutation M174R. These studies were repeated at least three times.
The ATPase activity was calculated to be 92 ± 0.06 μM/min per mg protein. Therefore, these point mutations do not assemble with other V1 or V0 subunits into a complex that has ATPase activity, whereas WT GFP constructs form a complex that has enzyme activity.

**Mutations of V-ATPase B1 Subunit Affect H+·ATPase–Mediated Proton Secretion.** To determine the proton translocating activity of the H+·ATPase, we measured the rate of Na+-dependent pH recovery after an acute acid load in the wild IMCD cells and GFP-B1–transfected cells (Figure 6). After acute cellular acidification, pH in both untransfected, wild IMCD cells and B1WT-transfected cells increased at the same rate of 0.040 ± 0.006 pH U/min and in both was nearly abolished by 1 μM bafilomycin a specific H+·ATPase inhibitor (pH recovery rate was 0.007 ± 0.002). However, pH recovery was significantly (P < 0.05; n = 5) reduced to a rate of 0.007 ± 0.002, 0.004 ± 0.002, 0.002 ± 0.002, 0.003 ± 0.002, 0.006 ± 0.004, 0.009 ± 0.004, and 0.015 ± 0.006 in L81P, R124W, M174R, T275P, G316E, P346R, and G364S B1M-transfected IMCD cells, respectively. Figure 7 demonstrates that whereas B1M-transfected cells demonstrate little or no pH recovery in the absence of extracellular Na+, addition of Na+ to one mutant cell line stimulates rapid pH recovery back to baseline in the one representative mutant studied. These data indicate that whereas the mutations inhibit H+·ATPase–mediated pH recovery, the Na+-dependent mechanism (Na+:H+ exchange) for pH recovery is unaffected.

**Mutations of B1 Subunit Affect Proton Pump Trafficking to Apical Membrane.** The previous results document that the B1M subunits do not assemble, have no ATP hydrolytic activity, and block H+·ATPase–mediated proton secretion in transfected IMCD cells. However, because native B1 subunits are expressed in transfected IMCD cells, one might expect these native subunits to form a completely normal H+·ATPase and participate in proton secretion, but this does not occur. Therefore, in some manner, each B1M must act as an agent that inhibits the function of the native H+·ATPase. One possible inhibitory mechanism may involve an effect on regulated exocytic insertion of the pump into the apical membrane.

To address this question, we first determined the effect of cell acidification on the distribution of GFP-B1 fusion proteins to the apical membrane. Apical membranes were isolated by a vesiculation method from cells that were incubated either in normal (NHB) or cell-acidifying medium (CHB-AC) and were assessed for GFP-B1 subunit content by immunoblot analysis (Figure 8). It is known that the vacuolar H+·ATPase is present in the apical membrane and that the amount is enhanced by acute cellular acidification (15). As expected the GFP-B1WT was present in the apical membrane when incubated in NHB and increased with acute cellular acidification. To our surprise, although the GFP-B1M did not assemble into the whole enzyme, B1M was present in the apical membrane–associated fraction. Furthermore, the apical membrane content of B1M increased in response to cell acidification to a comparable degree as that of WT. Immunohistochemistry studies (Figure 9) also showed that in both WT and one mutant (L81P), GFP-B1 translocates to the apical membrane upon cellular acidification. Unlike α-intercalated cells in the intact collecting duct, these transfected cultured IMCD cells after acidification retain considerable GFP-B1WT or L81P in cytosolic vesicles. This retention probably is the consequence of overexpression of this subunit. Similar morphologic observations were made with the other mutant cell lines. Therefore, unassembled B1M subunit can traffic to the apical membrane, and trafficking to the apical membrane increases in response to cell acidification.

**Mutant B1 Subunits Act as an Inhibitor of H+·ATPase Trafficking**

Because unassembled B1M traffic to the apical membrane and respond to the same signal for trafficking as the intact H+·ATPase, it is possible that this unassembled B1 competes with endogenous assembled B1 for the mechanism that mediates this process. Therefore, GFP-B1M may act as an agent that interferes with the normal regulation of the native apical membrane H+·ATPase. To test this, we evaluated the effect of expression of GFP-B1WT or GFP-B1M on the apical membrane translocation of endogenous B1 subunits in transfected IMCD cells (Figure 10). In GFP-B1WT–transfected cells, both GFP-B1 and endogenous B1 were present in the apical membrane fraction, and both increased with acute cellular acidification. However, in GFP-B1M–transfected cells, cell acidification resulted in an increase in the apical content of the mutant construct, but either no change or a decrease in apical content of endogenous B1 was noted. Enhanced insertion of B1M but not B1WT with cell acidification prevented the increase in the apical content of another H+·ATPase subunit, the 31-kd E subunit (Figure 11). The lack of an increase of both endogenous B1 and E subunit content of the apical membrane with enhanced trafficking of B1M after acute cellular acidification but not with enhanced

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*Figure 5. ATPase activity of GFP-B1WT or GFP-B1M. Immunoprecipitates from whole-cell homogenates (800 μg) from the stably transfected IMCD cell lines were prepared with anti-GFP antibody or NRS. The ATPase activity of immunoprecipitates was determined by an ATP/NADH-coupled assay and blanked against the NRS samples. Only GFP-B1WT had measurable ATPase activity (0.12 ± 0.02 OD units/min or 74 ± 06 μM/min). Values are means ± SEM; n = 3.*
trafficking of B1WT is consistent with the hypothesis that these point mutations inhibit the normal trafficking of assembled B1 to the apical membrane.

Discussion

Primary dRTA can be inherited as either an autosomal dominant or recessive trait (17,18). Patients with recessive dRTA are severely affected and often present with systemic metabolic acidosis, growth retardation, and hearing loss. Karet et al. (7,19) reported mutations that cause dRTA in ATP6V1B1, encoding the B1 subunit of the apical proton pump that mediates distal nephron acid secretion. The exact mechanisms by which the identified ATP6V1B1 mutations leads to impaired renal proton secretion and thus dRTA have not been determined. Whether these B1M subunits assemble with other components of the vacuolar H⁺-ATPase complex and whether the vacuolar H⁺-ATPase complex retains the ability to traffic appropriately to the apical surface of α-intercalated cells in the absence of a normal B1 subunit are not yet known. Several ATP6V1B1 mu-
tations that produce highly disruptive changes that are likely to result in loss of function of the encoded ATP6V1B1 protein were identified. These mutations introduce premature termination codons, frameshift mutations, missense mutations, and

Figure 8. Change in apical membrane distribution of B1 construct in response to cell acidification. (A) Preparation of apical membrane from control (NHB) and acutely acidified cells (CHB) was assessed for GFP-B1 subunits by Western blot with anti-GFP antibody. (B) The relative amount present in control and acidified cells was quantified by densitometric analysis of the Western blots. Values are means ± SEM (n = 4).

Figure 9. Immunolocalization of GFP-B1 in B1WT- and B1M-transfected IMCD cells before and after acidification. Control cells and cells that were subjected to an acute acid load were evaluated by immunohistochemistry for the distribution of GFP-B1WT or GFP-B1M (L81P). The apical membrane was biotinylated at 4°C. Then the monolayers were fixed and stained for the GFP fusion protein (FITC) and apical biotin (avidin-CY3). The stained specimens were evaluated by confocal microscopy. Optical sections (XY) at the apical level are depicted for the CY3 channel (red), FITC channel (green), and a merged channel. Z sections were generated from the merged image stacks (Velocity 3.6). In these Z sections, the apical surface is stained red in control and yellow after acidification. The yellow color in both the XY and Z sections indicate that GFP-B constructs co-localize with biotinylated proteins in the apical membrane.

Figure 10. Expression of B1M subunit blocks the trafficking of normal B1 to the apical membrane. Apical membrane fractions were isolated from IMCD cells that were stably transfected with either GFP-B1WT or GFP-B1 with a point mutation before (NHB) and after (CHB) intracellular acid loading. Equal amounts of protein were loaded for electrophoresis and subjected to immunoblotting (IB) with anti-GFP antibody (GFP), which detects only the GFP-B1 construct, with anti-B1 antibody (B1), which will detect both the GFP-B1 and the endogenous B1, or with anti-GP135, which detects a resident apical membrane protein.

Figure 11. Mutation of B1 subunit blocks the trafficking of normal H+-ATPase to the apical membrane. (A) Apical membrane fractions were isolated from IMCD cells that were transfected with either GFP-B1WT (WTB1) or mutant B1 (L81P-G364S) constructs before (NHB) or after an acute acid load (CHB). The isolated apical membrane was immunoblotted for GP135 (top; an apical membrane resident protein) and for the 31-kD subunit of the H+-ATPase (bottom). (B) Densitometric analysis of the GFP immunoblots shown in A. Values are means ± SEM; n = 3.
splice site mutations. Some kindreds who have dRTA with mutations of ATP6V1B1 that result in only a single amino acid substitution have been identified. We selected these single–amino acid substitution mutations to evaluate mechanisms by which B1 mutations disrupt H⁺ transport in a tissue culture model of the intercalated cell.

The constructs that mimic known point mutations, identified in inherited dRTA B1M or B1WT constructs were transfected into IMCD cells to express either GFP-B1M or GFP-B1WT proteins. To determine whether these constructs can be assembled with all the other subunits into an intact H⁺-ATPase, we tested whether subunit E (31 kD), H (51 kD), or c (dimer 32 kD) could be co-immunoprecipitated with the expressed GFP fusion B1 subunit. We selected these subunits as an index for assembly because the E subunit is critical for assembling the V1 portion of the H⁺-ATPase onto the V0 sector (20) and the c subunit to document that assembly of the V0 to V1 sectors to one another was complete. From our results, we conclude that GFP-B1M subunits are not assembled into an intact H⁺-ATPase because they did not form immunoprecipitable complexes with any of the three subunits examined (Figure 4). It is possible that the GFP moiety of the mutant fusion protein and not the mutation per se interfered with the assembly of this fusion protein with other H⁺-ATPase subunits. However, this is highly unlikely because GFP-B1WT formed an immunoprecipitable complex with the E, H, and c subunits. Consistent with the co-immunoprecipitation studies are the experiments in which the ATPase activity of immunoprecipitates was tested. ATPase activity requires not only formation of the V1 sector but also assembly of the V1 sector with the V0 sector (21). ATPase activity was detected only in immunoprecipitates from cells that were transfected with GFP-B1WT, whereas none of the mutants had activity. The lack of ATPase activity of immunoprecipitates from cells that were transfected with mutant constructs is additional evidence that these mutant constructs do not assemble into a functional H⁺-ATPase.

In this study, we also demonstrated that the B1M subunit has the ability to traffic to the apical membrane and that the amount of B1M present in the apical membrane is enhanced by acute cell acidification. In this regard, B1M trafficking resembles that of the intact nonmutant H⁺-ATPase (15). If the unassembled B1M utilizes the same mechanisms for trafficking to the apical membrane as the intact H⁺-ATPase, then the mutant could act as an inhibitor and compete with trafficking of the intact H⁺-ATPase assembled from native nonmutant B1 subunits. Consistent with this proposal are the following observations: (1) When trafficking of the B1M to the apical membrane is upregulated by acute cellular acidification, there is either no increase or a reduction in the intact H⁺-ATPase in the apical membrane; (2) cell lines that are transfected to express the B1M but not B1WT cannot respond to an acute acid load with enhanced proton transport. Therefore, in IMCD cells, the unassembled B1M subunits act as competitive inhibitors of trafficking of the endogenous, assembled H⁺-ATPase to the apical membrane and thereby reduce the capacity of these cells to transport H⁺.

Is it solely the failure of incorporating B1M subunits into functional plasma membrane proton pumps that causes the acidification defect that is observed in patients with recessive dRTA? If this is the case, then one would predict that Atp6v1b1 −/− animals should have a similar phenotype. Surprising, Atp6v1b1 −/− mice seem healthy, grow normally, do not have hearing defects, and are without systemic metabolic acidosis when on a normal diet and develop acidosis only when challenged (22,23). It is interesting that a recent study indicated that the absence of B1 expression induces a compensatory increase of the B2 isoform that potentially may act as a substitute isoform for B1 to increase H⁺-ATPase activity in these Atp6v1b1 −/− mice (23). These observations could be interpreted as suggestive evidence that the lack of functional B1 subunits is not the sole cause of the severe form of dRTA observed in these patients with this genetic disorder. However, the lack of B2 compensatory increases in human recessive forms of dRTA may account for the phenotype differences in mouse versus human disease. Alternatively, we speculate that B1M may contribute to the severity of the acidification defects that are observed in humans who harbor these ATP6V1B1 point mutations as compared with the phenotype observed in Atp6v1b1 −/− mice by effects that are in addition to their inability to assemble into H⁺-ATPase. If there is a compensatory increase of B2 isoform expression in humans who have recessive dRTA and harbor ATP6V1B1 point mutations as observed in Atp6v1b1 −/− animals, then the unassembled B1M may behave in the same manner as they do in the transfected IMCD cells, as inhibitors, competing with the compensatory B2 type H⁺-ATPase for trafficking to the plasma membrane. In heterozygotes, it may be that insufficient B1M accumulate to achieve effective competition with normal assembled H⁺-ATPase. These assumptions concerning the mechanism of human dRTA, although consistent with our observations in cultured cells, will remain speculative until direct determinations are made in human intercalated cells regarding the level of expression of B2 and mutant subunits (13). Because the B1 subunit does not have a transmembrane domain, its localization to the apical membrane must be mediated by interacting with some other molecules. The carboxy terminus of the B1 subunit terminates in a DTAL amino acid sequence (D, aspartic acid; T, threonine; A, alanine; L, leucine) that is typical of a PDZ-binding motif (24). It may be through this PDZ-binding motif that protein–protein interactions that are required not only for membrane localization but also for trafficking are formed (24). The PDZ protein that interacts with B1 to mediate apical membrane trafficking has not been identified. However, it has been suggested that sodium-hydrogen exchange regulatory factor, in β-intercalated cells, functions as the basolateral targeting protein for B1 (24). Sodium-hydrogen exchange regulatory factor was not detected in α-intercalated cells (24). None of the ATP6V1B1 nonsense mutations that have been identified to date in dRTA kindreds is located in the carboxy-terminal region of this protein (7,8,19). These B1 constructs with point mutations still retain their trafficking ability, although they are not assembled. Therefore, we propose that the B1 subunit probably contains the specific targeting information for both the isolated mutant subunit and the assembled H⁺-ATPase. The B1 point mutations act as inhibitors in cultured IMCD cells because they compete for the
same sorting-targeting processes as the fully assembled H\(^+\)-ATPase does in IMCD cells.

**Conclusion**

We believe that the point mutations of B1 that produce dRTA are unable to be assembled into a complete H\(^+\)-ATPase and that the unassembled B1M subunits interfere with the trafficking of native H\(^+\)-ATPase in the transfectected IMCD cells.

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**References**