Incomplete distal renal tubular acidosis from a heterozygous mutation of the V-ATPase B1 subunit

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HCO₃⁻ into the plasma occurs via anion exchanger 1 (AE1) (68). It is not mere coincidence that congenital forms of distal RTA (dRTA) in humans result from inactivating mutations of carbonic anhydrase II enzyme (23, 53, 55), the B1 or a4 subunits of V-ATPase (60, 67), or the AE1 Cl⁻-base exchanger (6, 7, 11).

These candidate genes were identified using classical genetics on kindreds derived from probands initially identified by clinicians. In general, phenotypic information is limited to clinical diagnosis of RTA based on hypobicarbonatemia without elevation of plasma anion gap or creatinine and concomitant unduly alkaline urine pH. The disorder may be brought to clinical attention by complications such as growth retardation, nephrolithiasis, and nephrocalcinosis (31). Most clinical data are limited to plasma HCO₃⁻ concentration and spot urinary pH with no further phenotypic characterizations.

Congenital dRTA usually follow autosomal recessive inheritance with the exception of some mutations of AE1, which are autosomal dominant. The basis for the recessive nature is usually attributed to adequacy of the normal functioning transporters provided by the single wild-type allele, or haposufficiency. In the case of AE1-related autosomal dominant dRTA (6, 11, 25, 29, 57, 69), in vitro data have suggested that mutant AE1 may prevent wild-type AE1 from reaching the membrane via heterodimerization or by mistargeting of the mutant to the apical membrane, thereby nullifying apical H⁺ secretion (13, 49, 50, 64). This dominant model by mutant AE1 remains to be proven in vivo. Other than these AE1 mutations, the accepted paradigm is that one needs two mutant alleles to produce dRTA. However, the foundation of this paradigm is based on the absence of overt clinical diagnoses in heterozygotes.

The clinical diagnosis of dRTA depends on the stringency of the definition and how one sets the threshold for defining renal acid underexcretion. Since metabolic acidosis is not a dichotomous trait, a forme fruste of dRTA can conceivably eclipse detection by conventional clinical tests, and indepth metabolic studies are not usually performed by practitioners. Wrong and coworkers (16, 71) recognized that defective distal renal acidification can be associated with a normal plasma HCO₃⁻ concentration and set the precedence for using acute acid loading to uncover renal acidification defects. Interestingly, a considerable fraction of the literature on this type of incomplete dRTA actually stems from patients with kidney stones. While most patients with calcium oxalate stones can maximally acidify their urine, up to 30% of patients with calcium phosphate stones have reduced ability to lower their urine pH (2, 22,
Distal apical H⁺ secretion is mediated by V-ATPase (5, 27), which belongs to a family of ATP-energized H⁺-translocating ion pumps (18). V-ATPases acidify intracellular organelles, which is essential for receptor-mediated endocytosis, lysosomal hydrolysis, intracellular protein targeting, processing of hormones, and uptake and storage of neurotransmitters. In specialized cells, plasma membrane-situated V-ATPases are instrumental in the regulation of bone resorption, synaptic transmission, endolymph pH regulation, and renal acid excretion. V-ATPases are complex proteins composed of at least 13 different subunits organized into 2 distinct functional domains: a peripheral catalytic sector (650-kDa V₁ domain (A₁B₁C₁D₁E₁F₁G₁H₁) and an intramembranous 260-kDa proteolipid V₀ domain [a₄d₁c₃/(c’c₆)] (24). In mammals, V₁ is restricted to the epithelia of the inner ear, epididymis, and distal renal tubule. Mutations in the B₁ subunit gene ATP6V1B1 are responsible for autosomal recessive dRTA with deafness (19, 28, 56, 60). The mechanisms by which some B₁ subunit mutations lead to V-ATPase dysfunction were unknown until recently, when the majority of mutant B₁ subunits were shown to fail to incorporate into the V₁ subunit (20, 73).

We posed the simple but fundamental question of whether heterozygous carriers of mutant B₁ have any detectable renal acidification abnormalities. In addition, if they do, is there evidence for negative dominance? We took a family known to harbor an inactivating truncation mutation of B₁ (21) and studied the heterozygous members for abnormalities in renal acidification, prompted by the fact that one of the heterozygotes developed severe kidney stones. Despite the fact that none of the heterozygotes carry the clinical diagnosis of RTA, we obtained evidence that the heterozygotes have abnormal baseline steady-state urinary chemistry, and two heterozygotes showed abnormal urinary acidification upon provocation. Because of the previous postulation of negative dominance of the mutant B₁ subunit over the wild-type subunit (73), we used in vitro biochemical, mammalian cell expression, and yeast complementation approaches to examine for this possibility. We conclude that the affected heterozygotes in our kindred are in a state of haploinsufficiency where the pump from the normal allele can sustain normal clinical chemistry but the burden of heterozygosity in this family is incomplete dRTA. Individuals with incomplete dRTA or calcareous nephrolithiasis with distinct chemical abnormalities should be considered as potential heterozygous carriers of mutant B₁ alleles.

METHODS

Patient experiments. Subjects were recruited from University of Texas Southwestern Medical Center clinics with approval by the Institutional Review Board, and all participants gave informed consent. Normal subjects comprised staff and students on campus. A systemic multichannel analysis was used for serum analytes (Beckman CX9ALX, Beckman, Fullerton, CA). Serum pH and Pco₂ were measured using a Radiometer ABL 5 (Radiometer America, Westlake, OH). Urine creatinine was measured via the picric acid method, sulfate was measured by ion chromatography, ammonium was assessed by the glutamate dehydrogenase method, and citrate was assessed enzymatically (Boehringer-Mannheim Biochemicals, Indianapolis, IN).

The ammonium chloride load test was conducted as previously described (33). Fasted subjects had urine collected hourly from 0700 to 1200 hours under mineral oil. At 0800 hours, 50-nmol ammonium chloride gelatin capsules were administered orally in two to four divided doses over 30 min. Water (250 ml) was given at 0700, 0800, 0900, 1000, and 1100 hours. Arterialized venous blood was obtained for chemistry and pH and blood gases at 0700, 1000, and 1200 hours. Urine was collected for 4–6 h depending on the response to the acid load. To perform urine minus blood Pco₂, which yields a surrogate for H⁺ flux in the presence of bicarbonaturia (17, 62), two forearm intravenous catheters were inserted: one for NaHCO₃ infusion and the other for blood sampling. Sodium bicarbonate (250 meq) in 750 ml was infused, and urine was collected every 30 min until the urine pH was >7.0, and the urine was then collected every 15 min for three times and analyzed for Pco₂, pH, total volume, and creatinine. Venous blood was drawn before the infusion and at the time of each of the last three urine collections for Pco₂ and pH.

For immunohistochemistry of the human kidney, intraoperative medullary biopsy samples (approved by the Institution Review Board of the University of Texas Southwestern Medical Center) were immersion fixed in 4% paraformaldehyde (4°C for 4 h). After being washed with PBS (4°C overnight), tissues were frozen in OCT, and 4-μm sections were cut and washed with PBS (for 15 min) followed by 0.1% Triton X-100 incubation (10 min). Sections were incubated with a blocking solution (PBS, 3% BSA, and 10% goat serum for 60 min) and then rabbit polyclonal anti-B₁ antibody (1:100; 4°C overnight) (47). After being washed with PBS, sections were incubated with Alexa fluor 488-conjugated goat anti-rabbit IgG (Invitrogen, Carlsbad, CA) and then with 1:80 rhodamine phalloidin (Invitrogen). After additional PBS washes, sections were mounted and then visualized with a Zeiss LSM510 microscope.

Mammalian cell expression experiments. PCR-generated cDNA fragments were inserted in frame into COOH-terminal single hemagglutinin (HA)-tagged PMH (Roche) or NH₂-terminal FLAG-tagged p3xFLAG-CMV-10 (Sigma-Aldrich, St. Louis, MO) vectors for mammalian expression. Synonymous mutations in B₁ were created by a QuickChange Multi Site-Directed Mutagenesis Kit according to the manufacturer’s instructions (Agilent Technologies). All constructs were verified by sequencing. Human embryonic kidney (HEK)-293 cells (American Type Culture Collection (ATCC) were cultured (37°C, 5% CO₂) in high-glucose (450 mg/dl) DMEM (GIBCO-BRL) supplemented with 10% (vol/vol) FBS, penicillin (100 units/ml), and streptomycin (100 μg/ml). Transfections were performed with Lipofectamine 2000 (Invitrogen), and cells were used 48 h later. The design of the small interfering (si)RNAs is shown in Fig. 3a, and siRNAs were custom synthesized by Invitrogen and added to cells at a final concentration of 200 μM.

Immunoblots and immunoprecipitation were performed as previously described for these cells (20). The primary antibodies used were as follows: anti-B₁ antibody, rabbit polyclonal (Xie Lab) and mouse monoclonal (SC-55544, Santa Cruz Biotechnology); anti-FLAG antibody, rabbit polyclonal antibody (F7425, Sigma) and mouse monoclonal (F3165, Sigma); and anti-HA antibody, rabbit polyclonal (SA5430064, Sigma) and mouse monoclonal (H3663, Sigma). Secondary horseradish peroxidase-conjugated sheep anti-rabbit IgG (Invitrogen), and mouse monoclonal (H3663, Sigma). Secondary immunoprecipitation, cells were lysed in buffer [containing 150 mM NaCl, 50 mM Tris-HCl (pH 8.5), 5 mM EDTA, 1 mM EGTA, 1% Triton X-100, 100 μg/ml PMSF, 2 μg/ml leupeptin, 2 μg/ml aprotinin, and 2 μg/ml pepstatin A], and lysates were cleared by centrifugation (14,000 g for 30 min). Precipitating antibodies (1:200 dilution) followed by prewashed protein G-agarose (50% slurry) were added (4 h at 4°C). After being washed with lysis buffer (3 times),
proteins were eluted from the beads with 2.5× loading buffer [2.5 mM Tris·Cl (pH 6.8), 2.5% SDS, 2.5% β-mercaptoethanol, and 25% glycerol], heated (95°C for 2 min), resolved by SDS-PAGE, transferred to polyvinylidene difluoride membranes, and probed with the stated primary antibodies.

For immunocytochemistry, HEK-293 cells were fixed 48 h after transfection with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, and blocked by 3% BSA and 10% goat serum for 1 h. Specimens were incubated with anti-FLAG mouse monoclonal antibody. Fluorescence images were acquired through a Zeiss ×100 objective lens using a Zeiss LSM-510 laser scanning confocal microscope.

Microsomal vesicles were isolated from HEK-293 cells (two 10-cm dishes) as previously described (32). The cell pellet was resuspended in ice-cold hypotonic lysis buffer [10 mM HEPES (pH 7.2), 1 mM EDTA, 5 μM diisopropyl fluorophosphate, 10 μg/ml pepstatin A, 10 μg/ml benzamidine, and 10 mg/ml benzamidine] and then homogenized by 10 strokes in a tight fitting Dounce followed by 15 strokes after the addition of an equal volume of sucrose buffer (500 mM sucrose and 10 mM HEPES, pH 7.2). The lysate was centrifuged (600 g for 15 min), the secondary supernatant was centrifuged again (100,000 g for 45 min), and the pellet was resuspended in buffer (250 mM sucrose, 10 mM HEPES, pH 7.2) at 2–6 mg/ml protein. Vesicular H+ pumping was assayed by measurements of acridine orange absorbance quenching in a SLM-Amino DW2-C dual wavelength spectrophotometer (change in absorbance at 492–540 nm) (14, 72). Vesicles (300 μg) were diluted into 1.6 ml assay buffer [200 mM NaCl, 30 mM Na-tricine (pH 7.5), 3 mM MgCl2, 0.5 mM EDTA, and 6 μM acridine orange]. Reactions were initiated by the addition of ATP (final concentration: 1.3 mM) and valinomycin (final concentration: 1 μM) and were terminated by the addition of 1.6 μM of H+ ionophore 1799.

Yeast transformation and growth selection. COOH-terminally HA-tagged expression constructs in p426TF or p425TF vectors (35) were transformed into Saccharomyces cerevisiae strain YBR127C (genotype MATa/MATalpha his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 lys2Δ0/+ met15Δ0/+ ura3Δ0/ura3Δ0 ∆VMA2/∆VMA2, ATCC) and grown at 30°C in YEPD medium (10 g/l yeast extract, 20 g/l peptone, 20 g/l d-glucose, and 100 mg/l adenine hemisulfate) or synthetic minimal (300 mM Na-tricine, 3 mM MgCl, 0.5 mM EDTA, and 6 μM acridine orange). Reactions were initiated by the addition of ATP (final concentration: 1.3 mM) and valinomycin (final concentration: 1 μM) and were terminated by the addition of 1.6 μM of H+ ionophore 1799.

RESULTS

Pedigree, clinical phenotype, and 24-h urine chemistry. Figure 1 shows the pedigree previously partially described (3, 21). The family members have advanced in age since the original publication (3), but the clinical diagnoses have not changed, with six family members carrying the diagnosis of dRTA. One heterozygote carrier presented with urolithiasis with a heavy stone load, which required surgical intervention (asterisk in Fig. 1). Interestingly, she also had unduly alkaline urine and unexplained hypocitraturia, which cannot be explained by unusual acid intake (urinary SO4 excretion: 31 meq/day) or alkali intake (urinary K+ excretion: 67 meq/day, estimated net gastrointestinal alkali absorption: 27 meq/day) (65). The combination of high urine pH and hypocitraturia suggest defective urinary acidification. This prompted us to screen the rest of the family. Stone risk profiles from 24-h or spot urines are shown in Fig. 1. Although none of the heterozygotes carriers were previously recognized by their physicians as having dRTA, all of them showed some degree of urinary abnormalities, such as hypocitraturia and hypercalciuria, that cannot be attributable to secondary causes. Some of them also have urine pHs that are slightly alkaline, but since these were obtained on outpatient random diets, one cannot definitely conclude on abnormality. We could not identify asymptomatic stones in the rest of the family members because the subjects declined imaging experiments.

### Table 1. 24-hour and spot urine values.

<table>
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<th>Parameter</th>
<th>Wildtype</th>
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<td>5.87</td>
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<tr>
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<td>102</td>
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</tr>
<tr>
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<td>0.339</td>
</tr>
<tr>
<td>K+</td>
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<td>3.0</td>
<td>0.003</td>
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**Fig. 1. Top:** genotype and phenotype of the pedigree. A GC insertion leads to loss of a phenylalanine and a frame shift, resulting and premature termination (p.Phe468fsX487). The arrow indicates the proband. *The patient without frank renal tubular acidosis (RTA) who presented with kidney stones. dRTA, distal RTA. Bottom: 24-hour and spot urine values. NA, not available.
H⁺ gradient and H⁺ flux measurements in heterozygotes. Some but not all family members consented to participate in more detailed metabolic experiments. To test whether the collecting duct can generate and sustain a high H⁺ gradient, we loaded the subjects with ammonium chloride to induce maximal H⁺ pumping. This maneuver has been shown to maximally lower urine pH in normal individuals (16, 51, 71). The two heterozygotes lowered their urine pH and increased ammonium excretion, but their urine pHs were not as low as the normal sibling (Fig. 2, A and B). The urinary ammonium excretion was numerically lower than but probably not that different from the wild type. The small numbers did not permit meaningful statistical analysis. The response in the homozygous patient was basically flat.

Acidic urine implies adequate pump and epithelial integrity to generate and maintain pH gradients. Since secretion of a small amount of H⁺ into the urine across a tight epithelium can theoretically maintain a high H⁺ gradient, this does not guarantee adequate flux of H⁺ to excrete the acid load. To examine that, we use the urine minus blood Pco₂ test to estimate H⁺ flux (62). Under high luminal HCO₃⁻ concentration, the generation of luminal CO₂ can only result from titration of luminal HCO₃⁻ from high rates of apical H⁺ secretion. Of the heterozygous subjects tested, none of them mounted a normal PCO₂ gradient (Fig. 2C).

The combination of abnormal steady-state baseline 24-h urine parameters and abnormal H⁺ gradient and H⁺ flux upon challenge beget the conclusion that these heterozygous individuals do not have normal urinary acidification and actually fit the criteria for incomplete dRTA.

Calcareous kidney stone and B1 expression in situ in a heterozygote. This investigation was initiated by one family member who presented with calcareous stones of significant load that eventually required surgical intervention. Somewhat unexpected was that the stone composition was not the calcium phosphate typically expected of dRTA patients. Instead, the subject had 80% calcium oxalate and 20% calcium phosphate stones. There was no evidence of nephrocalcinosis by a preoperative noncontrast computerized tomography scan (not shown), intraoperative inspection, and tissue histology. An intraoperative biopsy of the papillary tip in this patient revealed an abnormal cellular pattern of the B1 subunit. While the distribution of B1 in an unrelated normal individual showed mostly apical staining, the affected heterozygote showed a mixture of both apical and rather prominent diffuse intracellular staining (Fig. 2D). Our anti-B1 antibody does not recognize truncated B1; thus the signal likely represents wild-type B1 in V₁ domains that did not assemble into V₀V₁. The rest of the family members had no documented history of stones, but they did not consent to imaging experiments in the absence of a clinical diagnosis.

Examination for negative dominance in mammalian cells. The metabolic phenotypes indicated that the only truly normal individual in this family was the homozygous wild-type male member. In this kindred, most heterozygotes seemed to exhibit some abnormal (albeit mild) renal acidification. This can be due to either a dominant negative effect of mutant truncated B1 or haploinsufficiency of wild-type B1.

We previously characterized the p.Phe468fsX487 truncation mutation as one that prevents proper assembly of B1 into V₁, and the defect is not due to the loss of the PDZ binding motif at the COOH terminus (21). HEK-293 cells express both B1 and B2 subunits (RT-PCR; data not shown), which do not furnish a clean system to study transfected proteins. We knocked down the endogenous B subunits with siRNAs and engineered synonymous nucleotide mutations in transfected human B1 to protect it from the siRNA (Fig. 3A), siRNA reduced endogenous V-ATPase activity to ~25–30% of normal without affecting the expression of the transfected FLAG-tagged B1 (Fig. 3B). This knockdown is necessary to examine the function of heterologously expressed B1 as the endogenous B activity is strong and precludes functional readout of the transfected B1 (Fig. 4A).

The transfected wild-type B1 conferred robust V-ATPase activity, whereas mutant B1 was inactive (Fig. 4A). Cotransfection of mutant B1 appeared to reduce the H⁺ pump activity of wild-type B1 (Fig. 4A). At face value, this appears to support a dominant negative effect of the mutant over wild-type B1. However, one needs to exercise caution in this finding. The reduction of wild-type activity can be explained by the fact that cotransfection of mutant B1 decreases the expression of wild-type B1 (Fig. 4, B–D). When normalized
for the wild-type B1 antigen, the V-ATPase activity of cotransfected cells was actually normal.

To further confirm this point, we kept the amount of wild-type B1 constant and gradually increased the truncated mutant and searched for the presence of interference. When transfected mutant B1 expression was increased, the transfected wild-type B1 expression appeared to be decreased (Fig. 4C). However, a similar effect was seen in the converse experiment, where increasing wild-type B1 expression decreased mutant B1 expression (Fig. 4C). When both B1 subunits are driven to extremely high levels by strong promoters in these transient expression experiments, the two subunits may be competing for the same limited translational machinery. We fathom that this is in fact the more likely explanation than a dominant negative effect of the mutant on translation of wild-type B1.

To further test this hypothesis, we examined whether transfection of exogenous mutant B1 affects the expression of native B1 and its ability to assemble. As shown in Fig. 5, exogenous mutant B1 did not affect the level of native B1 expression, assembly, or function. This speaks strongly against a dominant negative effect of mutant B1 on native wild-type B1.

Examination for evidence of negative dominance in yeast.

To thoroughly refute the dominant negative hypothesis, we used a second system to test for dominance. Yeast provides an ideal system for studying transporters in general because of the

Fig. 3. Mammalian cell expression system. Endogenous B1 and B2 were knocked down in human embryonic kidney (HEK)-293 cells, and V-ATPase function was measured in isolated endosomes. A: design of the small interfering (si)RNAs targeting B1 and B2 and protection of the heterologously transfected B1. B: original tracings of the acridine orange assay. HEK-293 cells were transfected with control siRNA (left), combined B1 + B2 siRNA (middle), or B1 + B2 siRNA along with FLAG-tagged WT human B1 with synonymous mutations as shown in A (right). Tracings show ATP-induced endosomal acidification followed by the collapse of the pH gradient by H+ ionophore 1799 (*). C: experimental conditions were similar to those in B. Cell lysates were immunoblotted with anti-B1 or anti-FLAG antibody.

Fig. 4. Heterologous expression of WT and truncation mutant (TM) B1 in HEK-293 cells. A: V-ATPase function in endosomes was measured as ATP-induced acidification and expressed as a percentage of untransfected control cells. Top, WT and TM B1 were transfected into HEK-293 cells with scrambled control siRNA. Bottom, WT and TM B1 were transfected into HEK-293 cells where endogenous B1 and B2 were knocked down with siRNA. Each bar represents mean ± SE from three independent transfections with triplicate plates per transfection. Statistically significant differences were assessed by ANOVA. *P < 0.05. B: immunoblot of transfected FLAG epitope-tagged WT and TM B1 in conditions identical to those in A. Bottom. C: titration of transfected WT (FLAG tagged) and TM B1 [hemagglutinin (HA) tagged] where the amount of cDNA of one was kept constant and the other varied. D: HEK-293 cells were transfected with 4 µg FLAG-tagged WT B1 (top) or 4 µg FLAG-tagged WT B1 with 8 µg HA-tagged TM B1 (bottom) and stained with anti-FLAG antibody. Left, fluorescent images only; right, results superimposed on DIC images.
ease in obtaining definitive genetic null cells and the ability to use growth as a functional screen; thus, this host has been used extensively for studying V-ATPase subunits (26, 48). Therefore, an independent test of negative dominance by the mutant B1 subunit is H\textsuperscript+-pumping function in yeast, which is critical to confer the ability to grow in pH 7.5. As previously shown, human B1 complements the yeast homolog well in a growth assay (21). In null yeast, the presence of mutant B1 did not affect the expression level of B1 or the ability of wild-type B1 to support growth in pH 7.5 (Fig. 6A). This provides additional evidence showing that the truncated mutant protein exerts no significant effects on wild-type B1. We further examined whether there was any evidence of negative dominance in 12 other known human mutations to complete an exhaustive search using the yeast model. None of these mutations affected the ability of wild-type B1 to complement pump function in null yeast, and none of these mutations affected the level of B1 expression (Fig. 6B).

**DISCUSSION**

Inactivating mutations of the V\(_1\) subunit B1 and the V\(_0\) subunit a4 are associated with congenital autosomal recessive dRTA with sensorineural hearing loss (28, 56, 58, 60, 67). The mechanism of dysregulated acidification from B1 mutations was unclear until recently, when disrupted V-ATPase assembly in vitro was described as one mechanism for seven known ATP6V1B1 missense mutations (20, 73). The finding of abnormal acidification in the heterozygous carriers in this kindred was unexpected but unequivocal. The question is why these subjects should be abnormal given that they have one normal copy of B1 and, as far as we know, B1 expression is biallelic.

The current stoichiometric model of V\(_1\) (A\(_3\)B\(_3\)C\(_1\)D\(_1\)E\(_3\)F\(_1\)G\(_3\)H\(_1\)) confers a 0.875 (1–0.5\(^3\)) probability of incorporating at least one abnormal B1 subunit. A heterogeneous wild-type/mutant B1 can conceivably affect ATP hydrolysis by A\(_3\)B\(_3\) and impair pump function. However, we cannot find evidence showing that the truncated B1 is capable at all of incorporation into V\(_1\). However, mutant B1 can theoretically affect the synthetic pathway of wild-type B1 if both alleles are translated together. Based on the observation that overexpressed mutant B1 subunits can lower Na\textsuperscript+-independent cell pH recovery after acid load in a rat IMCD cell line, Yang and coworkers (73) proposed a dominant negative mechanism of transfected mutant B1 subunits over native wild-type B1 subunits. However, in the background of native B1, the effect of the transfected
protein is not easy to assay and certainly difficult to interpret. We reduced endogenous B1 to levels where we could see the activity of the transfected protein, and the initial results hinted at the possibility of negative dominance (Fig. 4A). However, any reduction in wild-type B1 activity was in fact seen only in the situation where both transfected proteins were driven to very high levels. Mutant B1 did not exert any dominant effect over wild-type B1 expression, assembly, or function (Figs. 5 and 6). To complete our search, we studied 12 naturally occurring human B1 mutations in null yeast and failed to find any dominant negative action for any of them. Although this was a single sample, the heterozygous carrier exhibited an abnormal pattern of staining of B1 in the renal medulla, which we interpret as unassembled mutant B1. The higher than expected intracellular staining likely represents the failure of mutant B1 to incorporate into V1, as we have observed in cell culture (20). At the moment, the situation where both transfected proteins were driven to very high levels. Mutant B1 did not exert any dominant effect over wild-type B1 expression, assembly, or function (Figs. 5 and 6). To complete our search, we studied 12 naturally occurring human B1 mutations in null yeast and failed to find any dominant negative action for any of them. Although this was a single sample, the heterozygous carrier exhibited an abnormal pattern of staining of B1 in the renal medulla, which we interpret as unassembled mutant B1. The higher than expected intracellular staining likely represents the failure of mutant B1 to incorporate into V1, as we have observed in cell culture (20). At the moment, the data in concert support the de facto conclusion that the clinical burden of heterozygosity in this family is likely due to haploinsufficiency.

We are cognizant of the fact that not all families with dRTA are linked to either ATP6V1B1 or ATP6V0A4 (56), suggesting possible additional loci and mechanisms for congenital dRTA. We cannot rule out the possibility that our clinical and metabolic findings can be related to other putative loci that modify urinary acidification. Nephropathia or nephrocalcinosis are common in subjects with dRTA (4, 8, 9, 66) due to the combination of alkaline urine and hypocitraturia and, to some extent, hypercalciuria (8, 37, 39, 40), although abnormalities in stone inhibitors, such as glycosaminoglycans and nephrocalcin, have also been proposed (36). These urinary abnormalities promote all calcium stones but, in particular, calcium phosphate stones, which is compatible with human population data that show increased prevalence of renal acidification defect in calcium phosphate stone formers (22, 45, 46).

Although there is universal agreement that calcium stones are common in dRTA, the consensus is that dRTA is a rare cause of kidney stones. However, decades of patient data have provided unequivocal evidence for impaired renal acidification in the general stone-forming population without frank Mendelian dRTA. In 7 studies (12, 38, 41, 42, 59, 61, 70) amounting to >1,000 stone formers, abnormalities in urinary acidification have been found in an average of 17% (range: 6–26%) of all-comers. Whereas the first stone suffer may have a <10% chance of abnormal renal acidification (43), in recurrent stone formers, the frequency has been estimated from 7% to 100% in 7 studies totaling >200 patients (1, 2, 10, 34, 44, 54, 63). Abnormalities in acidification are also more common in bilateral stones, ranging in prevalence from 36% to 64% in a total of 228 patients (15, 44).

The clinical data suggest prevalent defective renal acidification even in the general stone-forming population and is staggering high in specific subgroups. In a small number of patients with the triple feature of bilateral recurrent calcium phosphate stones, Konnak and coworkers (30) found that all of them had defective renal acidification. Although these individuals may have acquired incomplete dRTA, they can also be heterozygous carriers of candidate genes. However, no genotypic data were presented in any of these studies mentioned above. In fact, based on the supposition, albeit unverified, that heterozygous carriers of the dRTA candidate genes are completely normal individuals, genetic causes for subtle defects in acidification in stone formers were likely never examined. If heterozygous careers of mutant candidate genes of dRTA harbor subclinical defects that elude routine clinical chemistry and confer a propensity for disease rather causing disease, then one will not succeed in recognizing inheritance patterns at all. There is a need for database of sequence variance in candidate genes for dRTA in the general stone-forming population.

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DISCLOSURES

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AUTHOR CONTRIBUTIONS


REFERENCES

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