A novel missense mutation in SLC34A3 that causes hereditary hypophosphatemic rickets with hypercalciuria in humans identifies threonine 137 as an important determinant of sodium-phosphate cotransporter in NaPi-IIc

Graciana Jaureguiberry,1 Thomas O. Carpenter,3 Stuart Forman,2 Harald Jüppner,1 and Clemens Bergwitz1

1Endocrine Unit and 2Department of Anesthesiology, Massachusetts General Hospital, Boston, Massachusetts; and 3Department of Pediatrics and Endocrine Unit, Yale School of Medicine, New Haven, Connecticut

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Jaureguiberry G, Carpenter TO, Forman S, Jüppner H, Bergwitz C. A novel missense mutation in SLC34A3 that causes hereditary hypophosphatemic rickets with hypercalciuria in NaPi-IIc. Am J Physiol Renal Physiol 295: F371–F379, 2008. First published May 14, 2008; doi:10.1152/ajprenal.00900.2008.—The present study describes two novel compound heterozygous mutations, c.410C>T(p.T137M) (T137M) on the maternal and g.4225_50del on the paternal allele of SLC34A3, in a previously reported male with hereditary hypophosphatemic rickets with hypercalciuria (HHRH) and recurrent kidney stones (Chen C, Carpenter T, Steg N, Baron R, Anast C. Pediatrics 84: 276–280, 1989). For functional analysis in vitro, we generated expression plasmids encoding enhanced green fluorescence protein (EGFP) concatenated to the NH2 terminus of wild-type or mutant human type IIc Na-Pi cotransporter (NaPi-IIc), i.e., EGFP-hNaPi-IIc; EGFP-[M137]hNaPi-IIc; or EGFP-[Stop446]hNaPi-IIc. The V446Stop mutant showed complete loss of expression and function when assayed for apical patch expression in Xenopus laevis oocytes. Conversely, EGFP-[M137]hNaPi-IIc was inserted into apical patches of OK cells and into oocyte membranes. However, when quantified by confocal microscopy, surface fluorescence was reduced to 40% compared with wild-type. After correction for surface expression, the rate of 33P uptake by oocytes mediated by EGFP-[M137]hNaPi-IIc was decreased by an additional 60%. The resulting overall reduction of function of this NaPi-IIc mutant to 16%, taken together with complete loss of expression and function of g.4225_50del(V446Stop), thus appears to be sufficient to explain the phenotype in our patient. Furthermore, the stoichiometric ratio of 22Na and 33P uptake was increased to 7.1 ± 3.65 for EGFP-[M137]hNaPi-IIc compared with wild-type. Two-electrode studies indicate that EGFP-[M137]hNaPi-IIc is nonelectrogenic but displayed a significant phosphate-independent inward-rectified sodium current, which appears to be insensitive to phosphonoformic acid. M137 thus may uncouple sodium-phosphate cotransport, suggesting that this amino acid residue has an important functional role in human NaPi-IIc.

SLC34A3; rickets; hypercalciuria; nephrolithiasis

Address for reprints and other correspondence: C. Bergwitz, 1038 Thier Bldg., Massachusetts General Hospital, 50 Blossom St., Boston, MA 02114 (e-mail: cbergwitz@partners.org).

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the disappearance of NaPi-IIa (minutes) (29). Furthermore, NaPi-IIc protein appears to get reinserted into the membrane (8, 28), whereas NaPi-IIa is internalized via the clathrin-coated pits within minutes and degraded via the endosomal pathway (2). These differences in regulation may explain why NaPi-IIa can only partially compensate for the lack of NaPi-IIc in individuals affected by HHRH and why Npt-2c appears to compensate only partially for the lack of Npt-2a in the mouse (4, 31).

This inability to fully compensate for the lack of one transporter by expressing the respective other may also be related, at least partially, to the finding that Npt-2c is most prominently expressed during development (26). Consequently, adult Npt-2c null mice show no hypophosphatemia (27). Their residual moderate hypercalcuria and elevation of their serum calcium and 1,25(OH)2D levels further indicate that the observed small increase in Npt-2a mRNA and protein is not sufficient to fully normalize phosphate homeostasis (27). Similarly, Npt-2a null animals show renal phosphate wasting, hypercalcuria, nephrocalcinosis, and skeletal abnormalities until weaning. However, older mice lacking Npt-2a have serum phosphate levels that are only slightly lower then those of wild-type littermates, which may be related to the upregulation of Npt-2c expression in these mice (4, 31). Mice that are null for both sodium-phosphate cotransporters appear to have a more severe phenotype than those lacking either cotransporter alone (24), which further supports the hypothesis that each transporter has a partially nonredundant role in the regulation of phosphate homeostasis. The hypophosphatemia in individuals with HHRH persists life-long, which is in contrast to the mild phenotype observed in Npt-2c null mice. It is therefore conceivable that some human NaPi-IIc mutations have a dominant phenotype and thus impair renal phosphate reabsorption more severely than predicted by the findings in the Npt-2c null mouse (24).

Because of these considerations, we sought to explore how two novel human NaPi-IIc mutations affect sodium-phosphate cotransporter function. Besides describing two SLC34A3 mutations, c.410C>T(p.T137M) and g.4225_50del, which provide a molecular explanation for clinical and laboratory findings in a previously reported male who is affected by HHRH (11), our analyses indicated that the g.4225_50del mutation in SLC34A3 leads to poor mRNA expression, whereas the T137M mutation, which affects a conserved amino acid residue, appears to impair coupled sodium-phosphate cotransport.

**MATERIALS AND METHODS**

**Experimental subjects.** All clinical investigations described in this study was conducted after informed consent in accordance with the ethical standards of the Helsinki Declaration of 1975 (and as revised in 1983) and approved by the institutional review committees of Yale University and Massachusetts General Hospital (Boston, MA). The index case, a now 38-year-old Turkish man, presented at age 2 yr with knee and hip pain, fatigue, and difficulty walking. He developed genu valga and radiological rickets at age 5 yr and received vitamin D (5,000 U/day) for 3 wk, after which he was lost to follow-up. At age 10 yr, he was seen again with severe rickets, but treatment with vitamin D (40,000 U/day) and phosphate (1.1 g/day) again was stopped after 3 wk due to development of flank pain. At age 14 yr, he was admitted for a metabolic ward study, which established the clinical diagnosis of hypophosphatemic rickets with hypercalcemia (11). His height at the time was 148 cm (5th percentile), and his weight was 43 kg (10th percentile). His biochemical analyses showed mild hypophosphatemia (3.0–3.8 mg/dl, normal range 3.5–5.5); elevated 1,25(OH)2D (133–148 pg/ml, normal range 20–80); normal PTH (1.9–2.0 μg/ml, normal range <25); elevated alkaline phosphatase (23.6–37.9 IU/ml, normal range 5–19); normal calcium (9.8–10.4 mg/dl, normal range 8.5–10.5); normal 1,25(OH)2D (12.6–16.1 ng/ml, normal range 10–40); and normal renal function. His urinary calcium ranged from 5.8 to 6.4 mg·kg·1·day−1 (normal range <4), his Tmp/GFR was 2.6–3.2 mg/dl (normal range 2.7–5.6); his radiographical evaluation showed epiphyseal flaring; and the histomorphometric analysis of a tetracyclin-labeled iliac crest bone biopsy was consistent with osteomalacia. On a controlled diet containing 850 mg/day phosphate, 500 mg calcium, and 385 mg magnesium, his phosphate balance was negative. On 1.2–1.4 g elemental phosphate/day, his hypophosphatemia resolved and his urinary calcium excretion dropped to 0.09 mg·kg·1·day−1. He developed flank pain on day 11 of his admission and was found to have a left urethral stone. He remained off phosphate and vitamin D supplements for most of his adult life, during which time he suffered three additional episodes of renal colic due to stones. His biochemical analysis off therapy at age 31 yr continued to show hypophosphatemia due to renal phosphate losses (see Fig. 2B). He is the son of nonconsanguineous parents; his mother had a renal stone at age 4 yr, requiring surgical removal and a second stone during pregnancy, but had normal serum and urine biochemical findings. His paternal grandmother reportedly had recurrent renal stones, but biochemical analyses were not available; his father, sister, and daughter are clinically and biochemically unaffected (see Fig. 2B).

**Clinical studies.** Current fasting blood samples were taken after informed consent at Yale University or in the Duzen Laboratuvralari (Ankara, Turkey) and assayed for calcium, phosphate, creatinine, unfractionated alkaline phosphatase, 1,25(OH)2D, and intact PTH using commercially available kits.

**Genetic studies.** The entire SLC34A3 gene of the index case (III-2), spanning 5 kb including 800 bp of the 5′-promoter region and all 13 exons and intronic sequences, was amplified by PCR and subjected to nucleotide sequence analysis at the Massachusetts General Hospital DNA Sequencing Core Facility. PCR assays for the confirmation in III-2 and analysis of g.4225_50del and c.410C>T(p.T137M) in the available family members were designed as described (7). In brief, 20 μM of forward primer 50 and reverse primer 53 (see Table 1) were used with reagents from Qiagen (Valencia, CA), 250 μM dNTP at 94°C × 5 min initial denaturation followed by 40 cycles of 94°C × 1 min, 65°C × 1 min, 72°C × 1 min, and 72°C × 10 min-final extension to obtain a normal 397-bp and mutant 370-bp PCR product for g.4225_50del. To amplify c.410C>T(p.T137M), primers 32 and 33 were used at 94°C × 5 min for the initial denaturation followed by 40 cycles of 94°C × 1 min, 70°C × 1 min, 72°C × 2 min, and 72°C × 10 min final extension, followed by restriction enzymatic digest with NalIII to obtain fragments of 377 and 71 bp (wild-type) or 352, 71, and 24 bp (mutant) (Table 2).

Haplotype analysis was performed using the Genotyping Resource Center for Human Genetic Research (Massachusetts General Hospital, Boston, MA) by using microsatellite markers from deCODE Genetics (18), D9S1826 and D9S1838, and the markers CB-9 and CB-11 as described (7). Briefly, all fragments (CB-9, CB-11, D9S1826, and D9S1838) were made in a 7.5-μl PCR reaction that contained 3 ng of DNA, 2.5 mM MgCl2 (Applied Biosystems), 1 × PCR Buffer II (Applied Biosystems), 0.25 mM dNTPs (Amersham), 0.5 pmol of forward (labeled) and reverse primer, and 0.5 U AmpliTag Gold (Applied Biosystems). The reactions were cycled as follows: 95°C × 12 min for the initial denaturation and then 10 cycles of 94°C × 30 s, 58°C × 30 s, 72°C × 1 min, followed by 20 cycles of 89°C × 30 s, 55°C × 30 s, 72°C × 1 min, and 72°C × 40 min final extension. The products were amplified and run on an Applied Biosystems 3730XL DNA Analyzer along with GeneScan 500Liz as an internal standard.
The data were analyzed using Applied Biosystems GeneMapper v3.7 software package. GenBank accession numbers for SLC34A3 are as follows: genomic contig NT_024000.15; cDNA, NM_080877.1; and protein, NP_543153.1.

RT-PCR assay for ectopic expression of mutant NaPi-IIc mRNA in peripheral lymphocytes. To permit allele assignment of the identified mRNA species, we first performed segregation analysis for the marker SNP c.1140C>T (Table 2) at 94°C for 5 min for the initial denaturation, followed by 16 cycles at 94°C for 30 s, 55°C for 1 min, and 72°C for 10 min final extension. The PCR product was subjected to restriction endonuclease digestion with BstUI to obtain the fragments indicated in Table 2. RNA was extracted from peripheral lymphocytes of the index case III-2 using the Pax-Purification system (Qiagen, Valencia, CA) and subjected to RNase-free DNase treatment to remove residual genomic DNA followed by cDNA synthesis using a Qiagen Omniscript kit. Using primers 105 and 276 (Table 1) at 94°C for 5 min for the initial denaturation, followed by 40 cycles at 94°C × 1 min, 65°C × 1 min, 72°C × 1 min, and 72°C × 10 min final extension. The nested-PCR product was purified using spin columns (Qiagen, Qiagen PCR Purification Kit) and subjected to nucleotide sequence analysis with primers 275 and 104 at the Massachusetts General Hospital DNA Sequencing Core Facility.

Generation of pEGFP-[M137]hNaPi-IIc and pEGFP-[446Stop] hNaPi-IIc. NaPi-IIc tagged with enhanced green fluorescence protein (EGFP) at the NH2 terminus (pEGFP-NaPi-IIc) was generated using BP111/HindIII restriction sites and standard cloning techniques. A QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) was then used to introduce into pEGFP-NaPi-IIc the T137M mutation and the V446Stop mutation. The latter mutation truncates NaPi-IIc after amino acid 466, which represents one possible reading frame for g.4225_50del. The mutagenesis primers were designed with the silent site selector DNA mutagenesis program by David Nix, http://rana.lbl.gov/SSS/ (see Table 1). Fifty nanograms pEGFP-NaPi-IIc were then amplified using Pfu-turbo DNA polymerase (Stratagene), Qiaquick Q-reagent (5 μl) then amplified using Pfu-turbo DNA polymerase (Stratagene), Qiagen Qicheck Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) followed by addition of a poly-A tail (Ambion). cRNA was synthesized using the Message mMachine (Ambion, Austin, TX) followed by addition of a poly-A tail (Ambion) according to the manufacturer’s instructions and purified with NucAway spin columns (Ambion). cRNA integrity was verified on 1.3% agarose/TAE electrophoresis gels, and yield was determined by optical density at 260 nm.

Table 1. List of primers

<table>
<thead>
<tr>
<th>Primer</th>
<th>Orientation</th>
<th>Location</th>
<th>Sequence</th>
<th>AT</th>
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</thead>
<tbody>
<tr>
<td>32</td>
<td>Forward</td>
<td>Intron 4</td>
<td>GGAGGCCAAGCAGGAGAAC</td>
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<td>33</td>
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<td>Exon 13</td>
<td>TCCAGAAGATGAGGCGAACAC</td>
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<td>Exon 12</td>
<td>GAGGCCTGAGCCACGATCGT</td>
<td>63°C</td>
</tr>
<tr>
<td>50</td>
<td>Reverse</td>
<td>Exon 13</td>
<td>TGGTGACCCACCAGCTGTT</td>
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</tr>
<tr>
<td>53</td>
<td>Reverse</td>
<td>Exon 13</td>
<td>TACAGAGAATGAGGCGAACAC</td>
<td>65°C</td>
</tr>
<tr>
<td>104</td>
<td>Reverse</td>
<td>Exon 13</td>
<td>GTATACACCACAGAGGATGGC</td>
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<td>Exon 11</td>
<td>GTGGTCGCGCCGCGCTTCT</td>
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<td>190</td>
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<td>Exon 5</td>
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<tr>
<td>191</td>
<td>Reverse</td>
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<td>GAATGAGGAGGAGACATGCGTGTCCTGCAAGGCAGG</td>
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<tr>
<td>238</td>
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<td>pEGFP-C1</td>
<td>ATTGAGTGAAGAGAATGGCCAGAC</td>
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<tr>
<td>239</td>
<td>Reverse</td>
<td>Exon 13</td>
<td>ACAGAAGGGGAGAGCTGCTG</td>
<td>60°C</td>
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<tr>
<td>274</td>
<td>Reverse</td>
<td>Exon 12</td>
<td>GGGCTGTAGACTCTGCTCT</td>
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<tr>
<td>275</td>
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<td>Exon 12</td>
<td>CTGGCTGCGCGCTACTC</td>
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<tr>
<td>276</td>
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<td>Exon 13</td>
<td>CCAGCGCGTGGAGGACGATTAAC</td>
<td>65°C</td>
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<tr>
<td>280</td>
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<td>55°C</td>
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<tr>
<td>281</td>
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<td>CAAGTTGAGAAGGAGACATGCGTGTCCTGCAAGGCAGG</td>
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<tr>
<td>282</td>
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<td>Exon 10</td>
<td>CAGTACATATAAGGCGAACAC</td>
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AT, annealing temperature; EGFP, enhanced green fluorescence protein; NA, not applicable.

Table 2. PCR assays for SNP/mutations

<table>
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<tr>
<th>SNP/Mutation</th>
<th>Length</th>
<th>Exon</th>
<th>Primers</th>
<th>Enzyme</th>
<th>Bands After Restriction Digest</th>
</tr>
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<tbody>
<tr>
<td>c.1140C&gt;T</td>
<td>419 bp</td>
<td>12</td>
<td>46–274</td>
<td>BstUI</td>
<td>CC: 261,158 CT: 419, 261,158 TT: 419</td>
</tr>
<tr>
<td>g.4225_50del</td>
<td>397 bp</td>
<td>13</td>
<td>50–33</td>
<td>NA</td>
<td>wt: 377, 71; mut: 352, 71, 24 bp</td>
</tr>
<tr>
<td>wt, Wild-type; mut, mutation.</td>
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<td></td>
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</table>
Transient expression of mutant and wild-type NaPi-IIc in opossum kidney cells. Opossum kidney cells were obtained (12) and maintained in DMEM/F12 (1:1; Invitrogen, Carlsbad, CA), supplemented with 10% FBS (Hyclone, Logan, UT), 100 U of penicillin (base), 100 μg of streptomycin (base), and 0.25 μg of amphotericin B/ml (Invitrogen) at 37°C in 95% humidified air-5% CO2. Ten thousand cells per well were plated into a eight-well slide flask (Nalge Nunc, Naperville, IL), transfected with 0.1 μg plasmid DNA/well using Transfectamine (Qiagen) according to the manufacturer’s instructions, and cultured for an additional 3 days before fixation with 4% paraformaldehyde in phosphate-buffered saline containing 0.5 mM CaCl2 and 1 mM MgCl2 for 20 min at room temperature. Epifluorescent images were obtained using a Nikon Eclipse E800 fluorescent microscope (Nikon, Melville, NY) after nuclear counterstaining with propidium iodide.

X. laevis oocyte preparation and injection. X. laevis maintenance and oocyte harvest procedures were approved by the Subcommittee on Research and Animal Care of the Massachusetts General Hospital. X. laevis oocytes (Nasco, Fort Atkinson, WI) were harvested by a minilaparotomy using sterile techniques, defolliculated in OR2 (170 mM NaCl, 5 mM KCl, 2 mM MgCl2, 10 mM HEPES, pH 7.5) with 1 mg/ml collagenase I from Clostridium histolyticum (C-9891, Sigma, St. Louis, MO, or CLS1, no. 4197, Worthington, Lakewood, NJ) rocking for 2 × 90 min at room temperature, rinsed several times with OR2, and then cultured in ND96 (+ + +) [96 mM NaCl, 2 mM KCl, 1.8 mM CaCl2, 1 mM MgCl2, 10 mM HEPES (pH 7.5), 25 μCi/ml sodium 22Na] for a total volume of 200 μl. To determine 22Na efflux, the medium was changed to ND0, ND100 (+ + +) [96 mM NaCl, 2 mM KCl, 1.8 mM CaCl2, 1 mM MgCl2, 10 mM HEPES, and 0.01 mCi33P/ml in a total volume of 200 μl] for 20 min at room temperature. Delivery of superfusion solutions from reservoirs to the flow chamber was controlled by computer-activated solenoid valves. Currents were digitized at 200 Hz (Digidata 1200, Axon Instruments, Foster City, CA) and recorded on a personal computer running commercial software (Clampex8.2, Axon Instruments) as previously described (25).

Baseline current was determined at −50 mV with ND100. Oocyte viability was confirmed by resting membrane potentials below −18 mV in ND100 and by epifluorescence microscopy to confirm expression of EGFP-hNaPi-IIc. For intervals of 25 s, the solution then was changed to ND0, ND100 + 0.3 mM P, or ND100 + 0.3 mM Pi + 3 mM phosphonoformic acid (PFA). Current-voltage (I-V) plots of sodium-dependent currents were obtained either by sequential runs in ND100 and ND0 for 20 s in 20-mV steps from −120 to +40 mV, following 5-s equilibration at the new membrane potential, or using a ramp from −120 to +40 mV over 1,600 ms following 15-s equilibration in either ND100 or ND0 and 5-s adjustment to 120 mV. Sodium-dependent currents were calculated by subtracting the currents obtained in ND0 from those obtained in ND100.

Statistical and data analysis. Radioactive isotope uptake and electrophysiological experiments were repeated with oocytes from at least three donor frogs, four to five oocytes/experimental condition. The electrophysiological data were recorded with Clampex8.2 (Axon Instruments) and analyzed with Campfit8.2 (Axon Instruments) using a personal computer. Data are expressed as means ± SE.

RESULTS

Sequence and haplotype analysis. Lymphocyte DNA of the index case III-2 was used to generate four overlapping PCR products spanning 5 kb of genomic DNA, including 800 bp of the 5′-promoter region and all 13 exons and intronic sequences of SLC34A3, followed by nucleotide sequence analysis as described previously (7). Two novel heterozygous mutations, c.410C>T(p.T137M) and g.4225_50del, were identified (Fig. 1), which were absent in 94 and 124 control alleles, respectively. Furthermore, several known or novel polymorphisms were detected: hom. c.200G>A(R67H), het. c.496G>T(G166C), het. c.558G>A(Q186Q), hom. g.2704T>A, het. c.1140C>T(L385L), hom. g.3296G>C, hom. g.3710G>C, hom. g.3736C>T, hom. g.4107T>C, and hom. c.1538A>T(V513E) (7, 14, 19). Haplotype analysis indicated that c.410C>T(p.T137M) was inherited from the mother II-1, while g.4225_50del was inherited from the father II-2 (Fig. 2C),
RT-PCR analysis of SLC34A3 transcripts in lymphoblastoid cells. g.4225_50del deletes the last 25 nucleotides of intron 12 and the first nucleotide of exon 13 on the paternal allele. To determine whether this allele is transcribed, first-strand cDNA was synthesized from total RNA of peripheral lymphocytes of the index case III-2, followed by RT-PCR to amplify the region flanking the splice junction between exons 12 and 13, which is altered by g.4225_50del. The sequence obtained was assigned to one of the parental alleles using the single nucleotide polymorphism c.1140C>T(L385L) in exon 12, which is homozygous for cytidine in the mother and heterozygous for cytidine and thymidine in III-2 and the father (Fig. 2). A single 499-bp PCR fragment was obtained which was homozygous for cytidine at position c.1140, indicating that only the maternal allele had been amplified. The mutant transcript was likewise absent from lymphoblastoid cells of an unrelated individual affected by HHRH carrying g.4225_50del (5, 6). These findings suggest that transcripts containing g.4225_50del are either unstable (nonsense-mediated decay) or lack the annealing sites for primers 105 and 276 due to alternative splicing and deletion of exon 13.

Functional analysis of V446Stop. Because it cannot be definitively concluded from the absence of ectopic transcripts in peripheral lymphocytes or lymphoblastoid cells that truncated transcripts from the paternal allele are missing in the patient’s kidney, we next functionally evaluated this mutation in opossum kidney cells and in X. laevis oocytes. For this purpose, we generated an expression plasmid containing the cDNA encoding for EGFP concatenated to the NH2 terminus of wild-type and mutant forms of human NaPi-Ile (pEGFP-NaPi-Ile). This NH2-terminal EGFP tag has been successfully used for the functional evaluation of NaPi-IIa in the past (15) and does not affect the function of unmodified NaPi-Ile when identical amounts of cRNA are injected into oocytes and assayed for sodium-dependent phosphate uptake (data not shown). The EGFP tag permits the observation of membrane fluorescence as a measure of surface expression of the cotransporters using confocal fluorescent microscopy (Fig. 3E) (10). Images taken with constant laser settings were analyzed to obtain the relative membrane fluorescence, which was linear over a wide range of cRNA injected into the oocyte and permitted detection of as little as 20% difference in surface expression with a coefficient of variation of <10%, as shown in Fig. 3B. Using the Stratagene Quickchange method, a stop codon was introduced into pEGFP-NaPi-Ile in place of the first codon of exon 13 (V446Stop). This mutation truncates the NaPi-Ile protein after the fifth membrane-spanning domain, the site predicted from an alternatively spliced hypothetical paternal transcript containing g.4225_50del. X. laevis oocytes injected with 25 ng/oocyte polyadenylated cRNA encoding for EGFP-[446Stop]NaPi-Ile failed to show sodium-dependent 33P uptake (Fig. 3A) or measurable surface expression (Fig. 3B) compared with oocytes injected with 25 ng polyadenylated cRNA encoding for EGFP-NaPi-Ile. Furthermore, EGFP-[446Stop]NaPi-Ile accumulated intracellularly and failed to insert into apical patches of opossum kidney cells, the brush border equivalent of this cell line (10), when transiently transfected with pEGFP-[446Stop]NaPi-Ile (Fig. 3D).
surface expression, all subsequent experiments were performed with X. laevis oocytes injected with 50 ng of polyadenylated cRNA encoding for EGFP-[M137]hNaPi-IIc or 6.25 ng of polyadenylated cRNA encoding for EGFP-hNaPi-IIc, followed by $^{33}$P and $^{22}$Na isotope uptake experiments (13). $^{33}$P uptake by oocytes expressing mutant and wild-type transporters was, as expected, sodium dependent (data not shown). Furthermore, the half-maximal inhibitory concentrations for phosphate were similar for EGFP-[M137]hNaPi-IIc (0.14 ± 0.07 mM) and wild-type EGFP-hNaPi-IIc (0.43 ± 0.23 mM), and thus neither assay could explain the observed reduction in the rate of phosphate uptake. However, we observed a significantly increased Na conductance for EGFP-[M137]hNaPi-IIc, which resulted in an increased $^{22}$Na/$^{33}$P ratio of 7.1 ± 3.65 for the mutant cotransporter (compared with 2.3 ± 0.4 for wild-type) (Fig. 4C). $^{22}$Na uptake was independent of extracellular phosphate and resistant to treatment with PFA, a known inhibitor of type II sodium-phosphate cotransporters (15) (data not shown). After the oocytes were loaded with $^{22}$Na in ND0, no $^{22}$Na efflux was observed after incubation of the oocytes in nonradioactive ND0 for 60 min at room temperature, which argues against recycling of sodium and makes nonspecific toxic effects caused by the mutant cotransporter unlikely (data not shown).

To further characterize the nature of the increased sodium conductance, we performed dual electrode voltage-clamp studies, which indicated that EGFP-[M137]hNaPi-IIc was nonelectrogentic (Fig. 4A). The baseline leak current was reversed when sodium was removed from the superfuse but was unaffected by removal of potassium, calcium, or magnesium from the superfuse, or by replacement of the superfuse by LD100 (containing 100 mM LiCl in place of NaCl) (data not shown), indicating that it was selective for sodium ions. The hyperbolic shape of the I-V plot for EGFP-[M137]hNaPi-IIc furthermore indicates that this sodium-dependent conductance is inward rectified (Fig. 4B). Also, in this experimental setup no effect on sodium intake was observed with PFA and phosphate (Fig. 4A), indicating that the increase in the $^{22}$Na/$^{33}$P ratio is independent and different from coupled sodium-phosphate cotransport.

**DISCUSSION**

**Compound heterozygous mutations in SLC34A3.** Analysis of genomic DNA from the affected male III-2 revealed a compound heterozygous mutation in SLC34A3, which was absent in DNA from healthy controls. The paternal allele carries a missense mutation affecting a highly conserved amino acid residue (T137M), which, as shown here, may be an important determinant of sodium-phosphate cotransport. The paternal allele carries a deletion mutation (g.4225_50del), which abrogates ectopic transcription in peripheral lymphocytes of the index case and predicts truncation of the NaPi-IIc cotransporter after the fifth membrane-spanning domain. One possible transcript for g.4225_50del, [446Stop]hNaPi-IIc, furthermore, does not appear to be expressed in X. laevis oocytes. Thus the combined sequence alterations of SLC34A3 likely represent disease-causing mutations and lead to the development of HHRH in III-2.

Compound heterozygous SLC34A3 mutations are relatively frequent in HHRH, and in 10 of the 14 independent kindreds

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**Fig. 2.** Pedigree and haplotype analysis. A: pedigree. Solid circles or squares indicate individuals who developed rickets during childhood along with renal phosphate-wasting, hypophosphatemia, and hypercalciuria. Open symbols indicate healthy individuals. Samples with dashed lines are from individuals who were unavailable for genotyping.

**Fig. 3.** A: bar graph showing the rate of $^{33}$P uptake by oocytes in the presence of polyadenylated cRNA encoding for EGFP-hNaPi-IIc, followed by $^{33}$P and $^{22}$Na isotope uptake experiments (13). $^{33}$P uptake was reduced to 40% compared with cells expressing wild-type NaPi-IIc (Fig. 3C). To allow for similar levels of...
described to date, the affected patients carry such mutations (7, 16, 19). This may indicate a high mutational rate of SLC34A3 and/or a relatively high frequency of heterozygous SLC34A3 mutations in the general population. Carriers of SLC34A3 mutations on one allele often present with hypercalciuria; however, no biochemical abnormalities were observed in the heterozygous parents II-1 and II-2 investigated in this study.

Despite the presence of hypercalciuria in homozygous or heterozygous carriers of SLC34A3 mutations, renal calcifications have been underappreciated in the original clinical descriptions of patients with HHRH and were generally attributed to the inappropriate treatment with vitamin D analogs (11, 32). Recently, however, stone formation was noticed as part of the initial presentation in two kindreds, in which carriers were heterozygous for c.586G>A (G196R), g.2259_359del, or c.1402C>T (R468W) (7). Furthermore, recurrent nephrolithiasis was described in heterozygous and homozygous carriers of g.2259_2359del (14) and in homozygous carriers of c.586G>A (G196R) (5, 6). Thus renal stones may be more common in HHRH but may have previously been missed, if patients were asymptomatic and appropriate imaging studies were not done.

Renal stones were also observed in the present case and in some of his first-degree relatives. g.4225_50del was found in an unrelated case with HHRH, and multiple heterozygous carriers of the deletion in this kindred also suffer from recurrent renal stones (5). However, given the small number of kindreds and since stone disease is a common condition in the general population, it cannot be excluded at the present time that the observed association of stones with SLC34A3 mutations occurred by chance alone.

Loss of function of both SLC34A3 alleles explains renal phosphate wasting. Opossum kidney cells and X. laevis oocytes are well-established cell systems used to study sodium-phosphate cotransporters (23, 26). When visualized in vivo with EGFP, we found that membrane expression in X. laevis oocytes expressing EGFP-[M137]hNaPi-IIC and wild-type EGFP-hNaPi-IIC. X. laevis oocytes were injected with 50 ng of polyadenylated cRNA encoding for wild-type (c) and EGFP-[M137]hNaPi-IIC (n), followed after culture for 3 days by 33P uptake in ND100/2m MPi corrected for surface expression using confocal microscopy. Values are means ± SE of 3 independent batches of 4 oocytes each. D: expression of EGFP-[446Stop], EGFP-[M137]hNaPi-IIC, wild-type hNaPi-IIC, and hNaPi-IIC in opossum kidney cells and X. laevis oocytes. Opossum kidney cells were transfected with plasmid DNA of pEGFP-C1 encoding for EGFP (a), wild-type EGFP-hNaPi-IIC (b), EGFP-[M137]hNaPi-IIC (c), or EGFP-[446Stop]-hNaPi-IIC (d), incubated for 3 days at 37°C, followed by fixation with 4% paraformaldehyde/PBS (+Ca+Mg) and propidium iodide as the counterstain. For the following panels, 6.25 (e and f) or 50 ng (g and h) polyadenylated cRNA were injected into X. laevis oocytes. After culture at 18°C for 3 days, live-cell epifluorescent microscopy (×63) was used to qualitatively demonstrate membrane (bright rim) and intracellular expression of wild-type EGFP-hNaPi-IIC (e), wild-type EGFP-hNaPi-Ila (f), and EGFP-[M137]hNaPi-IIC (g), while EGFP-[446Stop]-hNaPi-IIC (h) was only expressed intracellularly as indicated by absence of the bright rim. E: confocal images of EGFP-[M137]hNaPi-IIC surface expression in X. laevis oocytes. For better quantification of surface expression, relative membrane fluorescence was assessed using an Axioplan Radiance 2000 confocal microscope system. Images from tangential and/or across membrane sections (white arrows) were saved as TIF files and imported into IPLab 3.9.5 r2 for quantification using a personal computer.

Fig. 3. A: 33P uptake into Xenopus laevis oocytes expressing enhanced green fluorescent protein (EGFP)-[446Stop]- and EGFP-[M137]hNaPi-Iic. Twenty-five nanograms of polyadenylated cRNA transcribed from the appropriate plasmids encoding for wild-type human type IIc Na-Pi cotransporter (NaPi-Iic), human NaPi-Iia, and the mutants T137M and V446Stop were injected into X. laevis oocytes, followed by incubation at 18°C until day 3, when 33P uptake was measured over 60 min in ND100+1 mM P. Values are means ± SE of 3 independent batches of 4 oocytes each. B: surface expression of EGFP-[446Stop], EGFP-[M137]hNaPi-Iic, and wild-type EGFP-hNaPi-Iic. Polyadenylated cRNA encoding for EGFP-[446Stop], EGFP-[M137]hNaPi-Iic, or wild-type EGFP-hNaPi-Iic (125–50 ng/oocyte) were injected into X. laevis oocytes, followed by incubation at 18°C for 3 days. Surface expression was determined by confocal microscopy as described in MATERIALS AND METHODS and in E. C: time course of 33P uptake into X. laevis oocytes expressing EGFP-[M137]hNaPi-Iic and wild-type EGFP-hNaPi-Iic. X. laevis oocytes were injected with 50 ng of polyadenylated cRNA encoding for wild-type (c) and EGFP-[M137]hNaPi-Iic (n), followed after culture for 3 days by 33P uptake in ND100+2 mM P, corrected for surface expression using confocal microscopy. Values are means ± SE of 4 independent batches of 4 oocytes each. D: expression of EGFP-[446Stop], EGFP-[M137]hNaPi-Iic, wild-type hNaPi-Iic, and hNaPi-Iic in opossum kidney cells and X. laevis oocytes. Opossum kidney cells were transfected with plasmid DNA of pEGFP-C1 encoding for EGFP (a), wild-type EGFP-hNaPi-Iic (b), EGFP-[M137]hNaPi-Iic (c), or EGFP-[446Stop]-hNaPi-Iic (d), incubated for 3 days at 37°C, followed by fixation with 4% paraformaldehyde/PBS (+Ca+Mg) and microphotography (×63) with propidium iodide as the counterstain. For the following panels, 6.25 (e and f) or 50 ng (g and h) polyadenylated cRNA were injected into X. laevis oocytes. After culture at 18°C for 3 days, live-cell epifluorescent microscopy (×10) was used to qualitatively demonstrate membrane (bright rim) and intracellular expression of wild-type EGFP-hNaPi-Iic (e), wild-type EGFP-hNaPi-Iia (f), and EGFP-[M137]hNaPi-Iic (g), while EGFP-[446Stop]-hNaPi-Iic (h) was only expressed intracellularly as indicated by absence of the bright rim. E: confocal images of EGFP-[M137]hNaPi-Iic surface expression in X. laevis oocytes. For better quantification of surface expression, relative membrane fluorescence was assessed using an Axioplan Radiance 2000 confocal microscope system. Images from tangential and/or across membrane sections (white arrows) were saved as TIF files and imported into IPLab 3.9.5 r2 for quantification using a personal computer.
changes a polar residue to a hydrophobic residue, which may extend further the first membrane-spanning region and thus lead to abnormal folding or membrane insertion, thereby explaining the observed 40% reduction in surface expression with this mutation. Truncation of the NaPi-IIc protein after the fifth membrane-spanning domain by V446Stop deletes determinants of surface expression in the third intracellular loop of NaPi-IIc (17) and the COOH-terminal intracellular tail, which binds to NHERF-1 and -3 (35). Since ectopic transcripts of the paternal SLC34A3 allele were absent from lymphoblastoid cells of the affected individual III-2, nonsense-mediated decay may further impair expression of the paternal allele.

Interestingly, the father, II-2, who carries g.4225_50del on one of his SLC34A3 alleles, is biochemically normal and has no history of childhood rickets, hypercalcitriuria, or renal stones despite a predicted 50% reduction of NaPi-IIc function. By comparison, 70% of the heterozygous carriers of another previously reported null-allele, c.228del, displayed hypercalcitriuria (7). Alimentary modifiers including the intake of vitamin D (5, 6) and genetic factors may explain the reduced penetrance of mutations of the affected individual III-2, nonsense-mediated decay may further impair expression of the paternal allele.

The relatively low abundance of NaPi-IIc in the proximal tubules, at least in adult rodents (26), and the lack of a hypophosphatemic phenotype in the murine knockout of Npt-2c (27) raise the possibility that human NaPi-IIc mutations result in dominant effects. However, the presence of deletion and missense mutations throughout the cotransporter molecule rather than a few mutational hot spots is generally considered more suggestive for loss-of-function mutations (30), and our finding that both mutations significantly reduce surface expression continue to support the previous proposal that HHRH is an autosomal recessive disorder caused by loss-of-function mutations in NaPi-IIc.

T137 is a key determinant of sodium-phosphate cotransport in NaPi-IIc. After correction for surface expression, the rate of phosphate uptake in X. laevis oocytes injected with cRNA encoding for EGFP-[M137]-hNaPi-IIc was 40% of wild-type. Thus, in addition to affecting membrane expression as discussed above, T137 appears to be an important determinant of cotransport in NaPi-IIc. Because the apparent affinity constant for phosphate remained unchanged, it appears unlikely that T137 is involved in substrate binding. T137 is located in the first intracellular loop, which contains residues that appear to be important determinants of sodium-phosphate cotransport and stoichiometry in the related cotransporter NaPi-Ila (1). Therefore, we explored the possibility that this mutation affects Na:P stoichiometry. Compared with wild-type NaPi-Ic, EGFP-[M137]-hNaPi-IIc permitted uptake of 7.1 ± 3.65 sodium ions for each phosphate ion (Fig. 4C). Under the assumption that divalent phosphate, in agreement with previous reports for NaPi-IIa, is the preferred substrate also for NaPi-IIc (13), this may be due to cotransport of five Na⁺ ions in the presence of phosphate. Electrophysiological evaluation of EGFP-[M137]-hNaPi-IIc by dual-electrode voltage-clamp studies, however, suggested that the Na:P cotransport stoichiometry is unaltered since EGFP-[M137]-hNaPi-IIc remains nonelectrogenic (Fig. 4A). Instead, we noticed that the phosphate-independent rectified sodium conductance of EGFP-[M137]-hNaPi-IIc is significantly increased and unaffected by other cations (Fig. 4B). Mutation of T137 to methionine may thus lead to an uncoupling of cotransport in NaPi-IIc, and this uncoupling may render cotransport and thus the rate of phosphate uptake less efficient. This finding is consistent with the observed unchanged apparent Kd, but decreased Vmax of the mutant transporter for phosphate. Increased sodium uptake may furthermore reduce the sodium gradient across the cell membrane, which could lead to a certain degree of toxicity in the proximal tubular cell and could conceivably cause internalization and reduction of surface expression of the mutant cotransporter.

In summary, we here describe two novel SLC34A3 mutations, c.410C>T(p.T137M) on the maternal allele and...
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