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Fanconi-Bickel Syndrome and Autosomal Recessive Proximal Tubulopathy with Hypercalciuria (ARPTH) Are Allelic Variants Caused by GLUT2 Mutations

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Context: Many inherited disorders of calcium and phosphate homeostasis are unexplained at the molecular level.

Objective: The objective of the study was to identify the molecular basis of phosphate and calcium abnormalities in two unrelated, consanguineous families.

Patients: The affected members in family 1 presented with rickets due to profound urinary phosphate-wasting and hypophosphatemic rickets. In the previously reported family 2, patients presented with proximal renal tubulopathy and hypercalciuria yet normal or only mildly increased urinary phosphate excretion.

Methods: Genome-wide linkage scans and direct nucleotide sequence analyses of candidate genes were performed. Transport of glucose and phosphate by glucose transporter 2 (GLUT2) was assessed using *Xenopus* oocytes. Renal sodium-phosphate cotransporter 2a and 2c (Npt2a and Npt2c) expressions were evaluated in transgenically rescued Glut2-null mice (tgGlut2$^{-/-}$).

Results: In both families, genetic mapping and sequence analysis of candidate genes led to the identification of two novel homozygous mutations (IVS4-2A>G and R124S, respectively) in *GLUT2*, the gene mutated in Fanconi-Bickel syndrome, a rare disease usually characterized by renal tubulopathy, impaired glucose homeostasis, and hepatomegaly. *Xenopus* oocytes expressing the [R124S]GLUT2 mutant showed a significant reduction in glucose transport, but neither wild-type nor mutant GLUT2 facilitated phosphate import or export; tgGlut2$^{-/-}$ mice demonstrated a profound reduction of Npt2c expression in the proximal renal tubules.

Conclusions: Homozygous mutations in the facilitative glucose transporter GLUT2, which cause Fanconi-Bickel syndrome, can lead to very different clinical and biochemical findings that are not limited to mild proximal renal tubulopathy but can include significant hypercalciuria and highly variable degrees of urinary phosphate-wasting and hypophosphatemia, possibly because of the impaired proximal tubular expression of Npt2c. **(*J Clin Endocrinol Metab* 97: 0000–0000, 2012)**

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Abbreviations: ARPTH, Autosomal recessive proximal tubulopathy and hypercalciuria; FBS, Fanconi-Bickel syndrome; GLUT2, glucose transporter 2; NPT2, sodium-phosphate cotransporter 2; WT, wild-type.

Several inherited disorders with impaired renal tubular reabsorption of phosphate due to proximal tubular dysfunction have been defined at the molecular level. A decreased renal threshold for phosphate reabsorption can occur as an isolated defect, for example, in patients with mutations in sodium-phosphate cotransporter 2c (NPT2c) (*SLC34A3*), who present with renal phosphate losses leading to rickets/osteomalacia but without evidence for additional tubular defects. In contrast, mutations in the main renal sodium-dependent phosphate cotransporter, NPT2a (*SLC34A1*), were recently shown to cause not only impaired renal phosphate reabsorption and hypophosphatemic rickets but also hypercalciuria and additional proximal tubular defects (1). Likewise, mutations in the ClC5 (chloride hydrogen exchanger) are the cause of Dent's disease (2), which is characterized by proteinuria and hypercalciuria and sometimes hyperphosphaturia. Furthermore, patients with the recently described syndrome of autosomal recessive proximal tubulopathy and hypercalciuria (ARPTH) can show variable defects in renal phosphate reabsorption together with hypercalciuria, proteinuria, and other proximal tubular defects (3). In addition, patients with Fanconi-Bickel syndrome (FBS), a rare recessive disorder caused by homozygous or compound heterozygous mutations in the glucose transporter 2 (GLUT2) (*SLC2A2*), can develop, besides an abnormal regulation of blood glucose level, generalized proximal tubulopathy, including a variable increase in urinary phosphate excretion (4).

Here we describe the identification of the genetic defect in two unrelated consanguineous families. The affected individuals in family 1 presented with profound early-onset urinary phosphate-wasting leading to severe hypophosphatemic rickets; family 2 was previously described as being affected by ARPTH (3). Genome-wide homozygosity mapping established linkage for both families to an overlapping region on chromosome 3q26 and nucleotide sequence analysis of candidate genes led to the identification of two different, novel homozygous *GLUT2* mutations. Subsequent investigations in tgGlut2$^{-/-}$ mice revealed a mild increase in urinary phosphate excretion due to much reduced proximal tubular expression of Npt2c, whereas Npt2a expression was only slightly impaired. Discovery of different homozygous GLUT2 mutations in patients affected by either early-onset severe rickets due to profound urinary phosphate-wasting or ARPTH establishes that mutations in this glucose transporter can lead to vastly different clinical and biochemical findings.

Materials and Methods

Description of the investigated families

Family 1

Two brothers, the products of distantly related, healthy parents from the Dominican Republic, are affected by severe hypophosphatemic rickets; an older sister is healthy (Fig. 1A). There was no family history of rickets, skeletal disorders, or abnormalities in mineral metabolism. The index case (1031C) presented at 5 months of age with impaired growth and signs of rickets (craniotabes, rachitic rosary, and flaring of the wrists and knees bilaterally) and was found to have severe hypophosphatemia [as low as 1.2 mg/dl (normal for age: 4.5–6.7)], elevated alkaline phosphatase, and mild metabolic acidosis, but normal calcium (Table 1). The 1,25$(OH)_2$ vitamin D levels were low-normal in subject 1031C and elevated in subject 1031E before treatment was initiated. Fibroblast growth factor 23 levels, measured when this assay became available while the patients were on treatment with oral phosphate and calcitriol, were within the normal range. Aminoaciduria and glycosuria as signs of a generalized defect in proximal tubular function were noted. Ophthalmological examination showed no evidence for cystinosis. Despite therapy with oral phosphate, bicarbonate, ergocalciferol, and activated vitamin D (calcitriol), hypophosphatemia and rickets persisted and he developed progressive genu valgum (knock-knees) and suffered a right femoral fracture at age 5 yr and a midshaft fracture of the right femur after minor trauma at age 13 yr. He furthermore developed nephrocalcinosis.

1031E (see Fig. 1A) presented also with impaired growth and rickets at the age of 7 months, which was, however, less severe than that of his older brother 1031C; laboratory testing revealed hypophosphatemia [2.3 mg/dl (normal for age: 4.5–6.7)], mild acidosis, and proximal tubular defects (Table 1). Therapy with oral phosphate and activated vitamin D was initiated. He also developed bilateral nephrocalcinosis.

A standard oral glucose tolerance test was performed after an overnight fast when patient 1031C was 12 yr 8 months old and his brother, patient 1031E, was 8 yr 11 months old. Two baseline samples were obtained for the measurements of plasma glucose, insulin, and C peptide; the results were averaged. A glucose drink (Glucola) was then given orally at a dose of 1.75 g/kg of body weight (maximum of 75 g) and blood samples were obtained every 30 min for 120 min for the measurement of plasma glucose, insulin, and C peptide. The fasting glucose levels were normal, but the 2-h plasma glucose levels were between 140 and 200 mg/dl as evidence for impaired glucose tolerance, according to the American Diabetes Association guidelines (Supplemental Data, published on The Endocrine Society's Journals Online web site at http://jcem.endojournals.org).

A younger sister (1031B, see Fig. 1A) is healthy and did not show any evidence for laboratory abnormalities when investigated at the age of 12 yr (data not shown).

Family 2

In the previously reported family 2 with ARPTH, a highly consanguineous Druze family from Israel, three individuals presented with hypercalciuria and were found to have generalized nonacidotic proximal tubulopathy (3) (Fig. 1D). Urinary calcium-creatinine ratios (milligrams per milligram) were 900, 500, and 350, respectively (normal for age: <220) in the affected family members (3). All three children had normal serum levels for calcium and PTH as well as normal 1,25$(OH)_2$ vitamin D levels, but glycosuria and generalized aminoaciduria. Two of the three affected individuals showed increased urinary phosphate excretion, which was associated in one patient with significant hypophosphatemia and radiographic evidence for rickets. The healthy parents are first-degree cousins, thus suggesting an au-

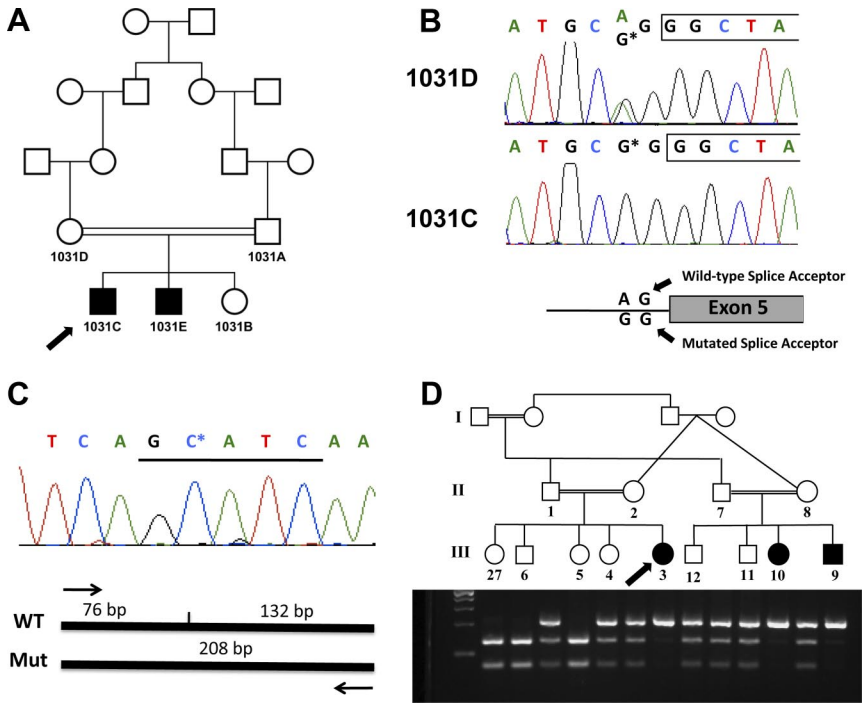

FIG. 1. GLUT2 mutations in family 1 and family 2. A, Pedigree of family 1. *Solid symbols*, Affected individuals; *arrow*, index case. B, Sequence chromatograms covering the splice acceptor site mutation IVS4–2A>G of *SCL2A2* (encoding GLUT2) using DNA from the unaffected mother 1031D (heterozygous for the mutation) and one of her affected sons, 1031C (homozygous for the mutation). The *star* indicates the mutated nucleotide. The first nucleotides of exon 5 are *boxed*. *Bottom picture* depicts the location of the mutation, which is predicted to cause skipping of exon 5. C, Sequence chromatogram of *SCL2A2*, the gene encoding GLUT2, using DNA from the index case in family 2. The *star* indicates the homozygous c.372 A>C missense mutation in exon 4 leading to the substitution of the conserved arginine at position 124 to serine (R124S). The loss of the recognition site GANTC (N is any nucleotide) for the endonuclease *HinfI* (*underlined*) was used to confirm the presence of the mutation. *Bottom*, PCR strategy for restriction enzyme digest of the WT, and mutant (Mut) allele to confirm the mutation. D, Pedigree of family 2. *Filled symbols*, Affected individuals; *arrow*, index case. *Bottom*, Confirmation of *SCL2A2* mutation c.372 A>C through restriction enzyme digest with *HinfI*. The genomic fragment was amplified by PCR, digested with *HinfI*, resolved on a 3% agarose gel and visualized by ethidium bromide staining. WT DNA revealed two fragments, homozygous mutant DNA from affected family members one fragment and heterozygous DNA from carriers three fragments (two from the WT and one from the mutated allele).

tosomal recessive mode of inheritance, rather than an X-linked disorder, such as Dent's disease, which can present with similar abnormalities; in addition, analysis of several X-chromosomal microsatellite markers excluded linkage to the gene encoding the ClC5 chloride channel. Because the clinical and laboratory findings could not readily be assigned to any previously described syndromes, the disorder in family 2 was referred to as autosomal recessive proximal tubulopathy with hypercalciuria (ARPTH) (3).

Genetic analysis

Family 1

The Institutional Review Board of the Massachusetts General Hospital (Boston, MA) approved the study, and informed consent was obtained from all participating subjects. Peripheral blood was obtained from both parents (1031D, 1031A) and all three children (1031C, 1031B, 1031E), and genomic DNA was isolated using standard methods. Genotyping was performed using the Affymetrix 10k single-nucleotide polymorphism platform, and homozygosity mapping was carried out using the software program easyLINKAGE (5) and Allegro (6).

Family 2

The study protocol was approved by the Institutional Ethics Review Committee and by the National Committee for Genetic Studies of the Israeli Ministry of Health, and informed consent was obtained from all participants or their legal guardians. Genomic DNA of three affected children and 10 healthy, first-degree relatives was extracted from peripheral blood leukocytes, and a genome-wide scan was performed with 378 fluorescently labeled microsatellite marker pairs using standard methods (1). Linkage to the locus on chromosome 3q26.1 was confirmed by genotyping of additional microsatellite markers derived from Marshfield maps (Mammalian Genotyping Service database of the Marshfield Medical Research Foundation, Marshfield, WI) in all study participants.

Intronic primers were used to amplify all coding exons and intron-exon junctions for candidate genes, which included *GLUT2*; primer sequences are available upon request.

The presence of the detected mutation in GLUT2 from family 2 was tested in 100 healthy, ethnically matched Druze control samples through Sanger sequencing.

Xenopus laevis oocyte experiments

Introduction of the identified mutations into the plasmid encoding human GLUT2

The c.372A>C mutation resulting in the replacement of arginine at position 124 to serine (R124S), to generate [R124S]GLUT2, was introduced by QuikChange (Stratagene, La Jolla, CA) into plasmid DNA encoding wild-type (WT) human GLUT2 (vector pSP64T, kindly provided by Dr. Graeme Bell, University of Chicago, Chicago, IL). The presence of the nucleotide change and the absence of additional mutations were confirmed by nucleotide sequence analysis.

Generation of cRNA for injection into oocytes

Linearized plasmid DNA encoding WT human GLUT2 (pSP64T) or the human glucose transporter GLUT3 (phGLUT3/XP) (both gifts from Dr. Graeme Bell, University of Chicago) as well as the plasmid encoding human NPT2a were used as template for cRNA synthesis as described (7) using Message mMachine (Ambion, Austin, TX).

Oocyte injections

Oocytes were harvested from female *X. laevis*, defolliculated, and injected as described (7). Oocyte injections were performed

TABLE 1. Laboratory values

Variable	Patient 1031C	Patient 1031E	Reference values
Age (yr)	5 months	7 months	
Sex	Male	Male	
Height (cm)	57.3 cm (less than third percentile)	63.8 cm (less than third percentile)	
Weight (kg)	5.34 kg (less than third percentile)	6.53 kg (less than third percentile)	
Bone pain	+++	++	
Leg deformities	+++	++	
Rickets	+++	++	
Blood tests			
Creatinine (mg/dl)	0.4	0.3	0.3–1.0
Glucose (mg/dl)	107[a]	82	70–110
Calcium (mg/dl)	8.6	9.4	8.5–10.5
Phosphate (mg/dl)	2.4	2.3	4.5–6.7
HCO_3 (mmol/liter)	19	17.3	22.0–27.0
Alk Phos (U/liter)	4290	1526	15–350
PTH (pg/ml)	94	64	10–60
25OHD (ng/ml)	34	43	9–46
$1,25(OH)_2D$ (pg/ml)	14	81	6–62
FGF23 (RU/ml)[b]	<50	68	<230
Urine tests			
Glycosuria	+++	++	Negative
Amino aciduria	+++	++	Negative
calcium/creatinine (mg/g)	2367	1730	<860
TRP[c]	0.24	0.48	>0.85

Laboratory values were obtained at the indicated age, before treatment was initiated, except for a and b.To convert the values for creatinine to micromoles per liter, multiply by 88.4. To convert the values for calcium to millimoles per liter, multiply by 0.25. To convert the values for phosphate to millimoles per liter, multiply by 0.323. To convert the values for glucose to milligrams per deciliter, divide by 0.05551. To convert the values for 25OHD (25-hydroxyvitamin D) to nanomoles per liter, multiply by 2.496. To convert the values for $1,25(OH)_2D$ (1,25-dihydroxyvitamin D) to picomoles per liter, multiply by 2.6. Alk Phos, Alkaline phosphatase; FGF23, fibroblast growth factor 23.

[a] Performed at 7 months of age.

[b] Performed at ages 13.5 and 9.75 yr, respectively, when both patients were treated with oral phosphate and ergocalciferol.

[c] The fractional tubular reabsorption of phosphate (TRP) was calculated according to the following formula: TRP = 1 − (renal phosphate clearance: creatinine clearance).

with 100 nl of ribonuclease-free water containing 50 ng cRNA. Microinjected oocytes were incubated at 18 C for 72 h before transport studies were performed; medium was changed every 24 h.

Glucose transport

Groups of five oocytes in six-well dishes were incubated with 200 μl of uptake buffer containing Barth's medium at pH 7.4 with 100 μM glucose and 0.1 μM 2-deoxy-D-[2,6-^{3}H]glucose (GE Healthcare Bio-Sciences Corp., Piscataway, NJ). After 30 min, glucose transport was terminated by aspirating the medium and rinsing the oocytes three times with ice-cold PBS containing 0.1 mM phloretin (MP Biomedicals, Solon, OH), a potent inhibitor of glucose transport. Oocytes were individually lysed in 0.5 ml of 10% sodium dodecyl sulfate, and radioactivity was determined by liquid scintillation counting. Five oocytes were used for each construct and each assay was performed in triplicate.

Phosphate uptake

^{33}P uptake studies were performed as described (7) using ND100 or ND0 supplemented with 1 and 10 mM potassium phosphate buffer (pH 7.4) containing 100 μCi/ml ^{33}P-orthophosphoric acid (PerkinElmer, Boston MA). To assess efflux of radiolabeled ^{33}P, cells were injected with cRNA encoding NPT2a (to allow preloading of cells with radioactive phosphate) and WT or mutant GLUT2, or vehicle; after loading the cells with ^{33}P, appearance of radioactivity in the medium was measured after 0, 30, and 60 min.

Evaluation of Npt2a and Npt2c expression in Glut2-null mice rescued through transgenic expression of Glut1 in the pancreas (tgGlut2$^{-/-}$ mice)

Glut2-null mice (Glut2$^{-/-}$) were rescued from the abnormal regulation of glucose homeostasis and early lethality through the transgenic expression of rat Glut1 (tg) in pancreas under the rat insulin promoter, as described (8). Six-week-old transgenic male mice were investigated that were null (tgGlut2$^{-/-}$), heterozygous (tgGlut2$^{-/+}$), or WT for Glut2 (tgGlut2$^{+/+}$); all animals were maintained on a standard chow containing 1.05% calcium and 0.8% phosphate. Concentrations of plasma and urinary inorganic phosphate were determined by the Phospha-C test (Wako, Osaka, Japan) and urine creatinine by the Stanbio Creatinine LiquiColor Test (Stanbio Laboratory, Boerne, TX). Fractional excretion index of phosphate (9) was calculated as follows: urine phosphate (milligrams per deciliter)/[urine creatinine (milligrams per deciliter) × serum phosphate (milligrams per deciliter)]. Western blot analysis was performed using single kidneys from three different animals per group that were frozen

immediately after removal from the animal. For immunohistochemical analyses, the contralateral kidneys were perfused *in vivo* with PBS and fixated with 4% paraformaldehyde before removal from the animal. Expression of Npt2a and Npt2c was evaluated in the kidneys of mice that were null, heterozygous, or WT for Glut2 using previously described antibodies (10). Brush border membrane vesicles, which were used for Western blot analysis, were prepared from whole kidneys using the Ca^{2+} precipitation method, as described previously (11).

Statistical analysis

For biochemical parameters of Glut2 knockout mice, comparisons of means were performed using the Student's *t* test. A $P < 0.05$ was considered indicative of statistical significance.

Results

Genetic analysis

To identify the molecular defects in both investigated consanguineous families, genome-wide homozygosity mapping was performed using DNA from all affected family members and from available healthy parents and siblings. For family 1 (see Fig. 1A), two large regions of homozygosity by descent were identified on chromosome 3q26.1 and 6q13–15 (parametric logarithm of the odds score in multipoint analysis: 3.4 for each of the two regions). Nucleotide sequence analysis of candidate genes in this region performed using genomic DNA from the index case (1031C) revealed a homozygous splice site mutation IVS4-2A>G (Fig. 1B) in *GLUT2* (*SLC2A2*), the gene encoding the facilitated glucose transporter. The healthy parents and the healthy sister are heterozygote for the identified mutation. This mutation, which has not been reported previously, is predicted to result in skipping of exon 5, followed by four amino acids that are unrelated to GLUT2 followed by a termination codon (Gln166AspfsX4), thereby eliminating more than half of the transporter protein (Fig. 2A).

Homozygosity mapping in family 2 (see Fig. 1D) also revealed homozygosity by descent for the region 3q26.1, *i.e.* the same chromosomal region that was identified in family 1. Direct nucleotide sequence analysis of DNA from the three affected family members revealed the novel homozygous missense mutation c.372A>C in GLUT2 (Fig. 1C), replacing an evolutionary conserved arginine in the first intracellular loop to a serine (R124S) (Fig. 2, A and B). This A>C nucleotide change removes the recognition site for the endonuclease *Hinf*I, thereby allowing confirmation of the identified nucleotide change in the PCR-amplified portion of *GLUT2* gene by restriction enzyme digest. After digesting the PCR products with *Hinf*I, gel electrophoresis revealed three DNA fragments in the unaffected, heterozygous family members, and, as expected, two bands in the healthy controls. In contrast, PCR-amplified DNA from the affected individuals resulted in the generation of one single band representing the mutant allele, thus confirming removal of the *Hinf*I recognition site (Fig. 1D, *lower panel*). These results confirmed homozygosity for the c.372A>C nucleotide change for the three affected children, heterozygosity for the parents, and only WT alleles or heterozygosity for the mutant allele for the unaffected children. The presence of this mutation was excluded in 100 healthy, ethnically matched Druze control samples.

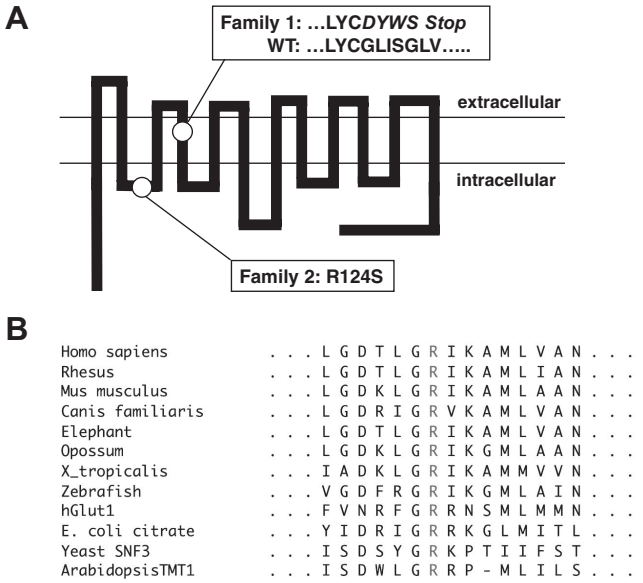

FIG. 2. Structure of GLUT2 transporter and conservation of R124. A, The IVS4-2A>G splice site mutation in family 1 is predicted to lead to skipping of exon 5 followed by four unrelated amino acid residues and a stop codon resulting in the truncation of most of the transporter protein. Predicted protein sequence caused by the absence (WT) or presence of the mutation is indicated. The R124S mutation resulting from the nucleotide change identified in family 2 is also indicated. B, Arg124 (highlighted in *red*) is changed to Ser (S) in family 2. Note that this arginine is conserved among different GLUT2 proteins and also among other members of the glucose transporter family (human GLUT1 depicted here) and of bacterial, yeast, and plant transporters.

X. laevis oocyte experiments

To investigate the effect of the R124S mutation on glucose transport, *Xenopus* oocytes were injected with cRNA encoding WT GLUT2, the [R124S]GLUT2 mutant, or GLUT3. GLUT2- as well as GLUT3-injected oocytes showed an approximately 50-fold increase in glucose uptake from a baseline of 55 ± 45 counts/min to 3037 ± 321 counts/min for GLUT2 and to 2661 ± 449 counts/min for GLUT3, thus confirming that both transporters were efficiently expressed and functional (Fig. 3A); the activity of both transporters was almost completely inhibited by the GLUT-specific inhibitor phloretin. Oocytes expressing [R124S]GLUT2 showed only an increase to 1505 ± 276

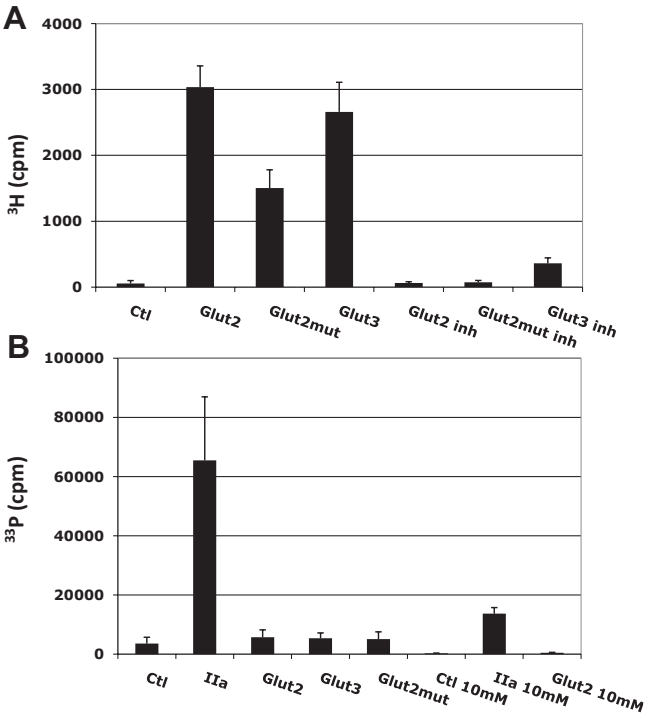

FIG. 3. Glucose and phosphate uptake studies. A, Glucose uptake by *X. laevis* oocytes expressing WT GLUT transporters or mutants as indicated. Twenty-five nanograms of polyadenylated cRNA transcribed from plasmids encoding WT human GLUT2, human GLUT3, or the R124S mutant identified in family 2 (Glut2mut) were injected into oocytes, followed by incubation at 18 C for 3 d, when uptake of 2-deoxy-d-[2,6-^{3}H]glucose was measured over 30 min in the absence or presence of the GLUT inhibitor phloritin (0.1 mM). ^{3}H was measured after lysis of the oocytes in scintillation fluid. Values are means ± SE of three independent experiments, each with five oocytes. B, Phosphate uptake by *X. laevis* oocytes expressing NPT2a or WT GLUT transporters or mutants thereof as indicated. Twenty-five nanograms of polyadenylated cRNA transcribed from the appropriate plasmids encoding NPT2a, GLUT2, GLUT3, or the R124S mutant identified in family 2 (Glut2mut) were injected into *X. laevis* oocytes, followed by incubation at 18 C for 3 d, when uptake of ^{33}P was measured over 60 min in either 1 or 10 mM potassium phosphate buffer. ^{33}P was measured after lysis of the oocytes in scintillation fluid. Values are means ± SE of three independent experiments, each with five oocytes. Ctl, Control.

counts/min, which represents a reduction of glucose transport by about 50% compared with the WT transporter. To test the hypothesis that GLUT2 directly facilitates phosphate transport, oocytes injected with cRNA encoding WT GLUT2, [R124S]GLUT2, NPT2a (positive control), or GLUT3 (negative control) were tested for phosphate uptake using uptake buffers containing 1 or 10 mM potassium-phosphate and 100 μCi/ml ^{33}P-orthophosphoric acid. Unlike NPT2a, expression of GLUT2 in oocytes failed to facilitate phosphate transport into the oocyte in the absence or presence of sodium (Fig. 3B). Furthermore, coexpression of GLUT2 WT or [R124S]GLUT2 together with NPT2a failed to enhance efflux of ^{33}P out of the oocyte (data not shown).

Evaluation of Glut2-null mice

To further explore how a lack of the GLUT2 transporter, which is expressed at the basolateral membrane of the proximal renal tubules, can lead to urinary phosphate-wasting and hypercalciuria, we investigated Glut2-null mice that are rescued from abnormal regulation of glucose homeostasis and early lethality by the transgenic expression of rat Glut1 under the control of a pancreas-specific promoter (tgGlut2$^{-/-}$) (8). Mice WT (tgGlut2$^{+/+}$), heterozygous (tgGlut2$^{-/+}$), or homozygous (tgGlut2$^{-/-}$) for Glut2-ablation had statistically indistinguishable plasma phosphate levels, but urinary phosphate excretion tended to be higher in tgGlut2$^{-/-}$ animals (fractional excretion index of phosphate: 43.1 ± 10.4 mg^{-1}/dl^{-1} for tgGlut2$^{-/-}$ *vs.* 23.7 ± 5.1 mg^{-1}/dl^{-1} for tgGlut2$^{+/+}$, mean ± SEM; $P < 0.07$).

Western blot analyses of the brush border membrane fractions from tgGlut2$^{-/-}$ mice revealed, in comparison with kidneys from tgGlut2$^{+/+}$ and tgGlut2$^{+/-}$ animals, a reduction of Npt2a protein by 17.6 ± 2% ($P < 0.05$), whereas expression of Npt2c was reduced by 69.1 ± 12% (Fig. 4A). These findings were confirmed by the immunohistochemical evaluation of kidney sections from tgGlut2$^{+/+}$ and tgGlut2$^{-/-}$ animals using antibodies directed against murine Npt2a and Npt2c, respectively (Fig. 4B). In single-tubule sections, Npt2a expression appeared unchanged and Npt2c expression significantly reduced in tgGlut2-null animals.

Discussion

Using genome-wide homozygosity mapping with 10k single-nucleotide polymorphism chips and microsatellite markers, we searched for the genetic defect in two unrelated consanguineous families with varying defects in proximal tubular phosphate reabsorption. In one family, affected members showed early onset urinary phosphate-wasting, resulting in severe hypophosphatemic rickets in addition to other defects in proximal tubular function. In the other, unrelated family, affected members had been diagnosed with ARPTH (autosomal recessive proximal tubulopathy and hypercalciuria) characterized by tubular dysfunction leading to significant hypercalciuria but no or only mild urinary phosphate losses (in two of three affected subjects, leading to rickets in only one) (3). Despite these phenotypic differences, linkage to an overlapping region of chromosome 3q26.1 was obtained for both families, and two novel, homozygous *GLUT2* mutations were identified in the affected individuals, thus establishing the diagnosis of FBS in both families.

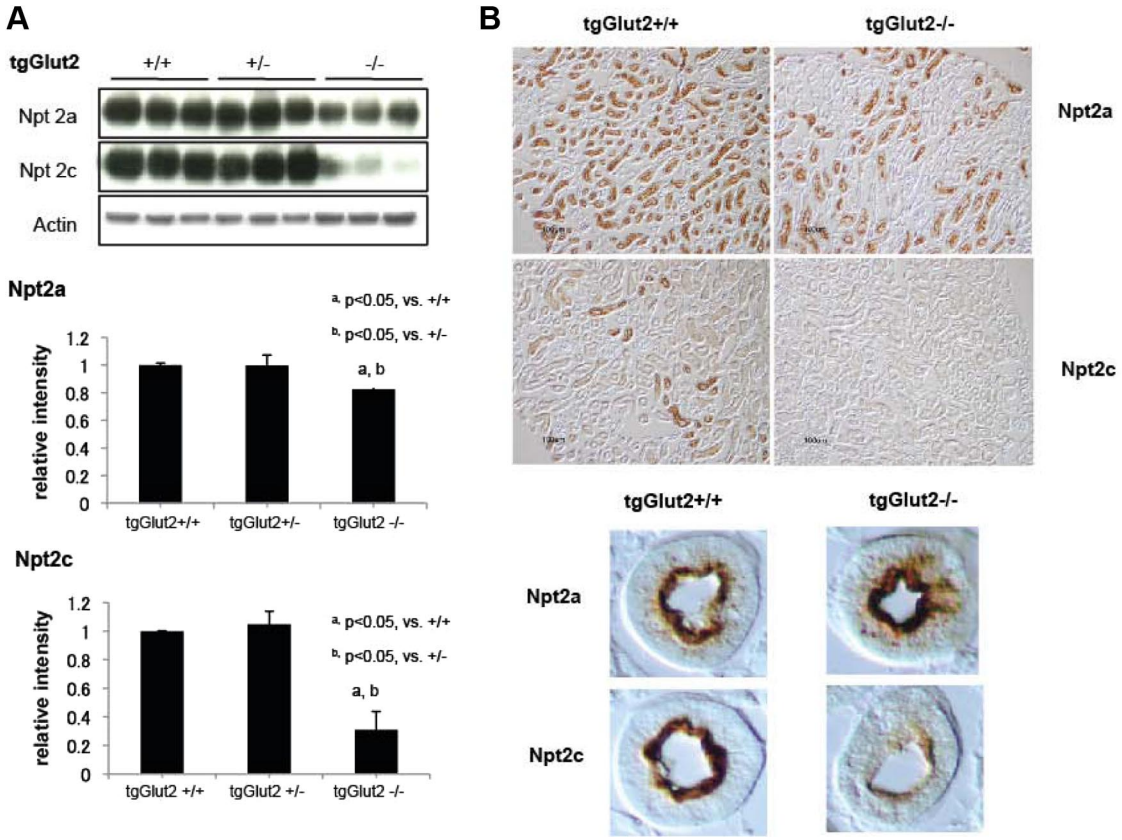

FIG. 4. Regulation of sodium phosphate transporters in rescued Glut2-null animals. Glut2-null mice were rescued from abnormal regulation of glucose homeostasis through the transgenic expression of rat Glut1 (tg) in pancreas under the rat insulin promoter. Six-week-old transgenic male mice that were null (tgGlut2$^{-/-}$), heterozygous (tgGlut2$^{-/+}$), or WT for Glut2 (tgGlut2$^{+/+}$) were investigated. A, *upper panel*, Western blot analysis of the brush border membrane fractions from kidneys demonstrates a mild reduction in Npt2a protein and a dramatic reduction in Npt2c protein; *lower panels*, quantification of the Western blot normalized for actin expression. B, Immunohistochemical evaluation of kidney sections (*upper panels*) and individual tubules (*lower panels*) from tgGlut2$^{-/-}$ and tgGlut2$^{+/+}$ animals confirmed the dramatic reduction of Npt2c protein.

In family 1, a novel splice-site mutation (IVS4-2A>G) was identified that is predicted to result in skipping of exon 5 followed by a premature termination codon and truncation of the resulting GLUT2 protein, which is presumably nonfunctional. In family 2, a novel missense mutation was identified that changes arginine at position 124 to serine (R124S). Arginine 124, located within the first intracellular loop of GLUT2, is strictly conserved not only in all members of the large family of glucose transporters but also in all members of the transport facilitator family, including plant and bacterial carrier proteins (12). Consistent with the constraint-based prediction of functional importance of this residue, previously reported *in vitro* mutagenesis experiments revealed an essential role of this arginine for the appropriate conformation of GLUT4 (12). Replacing the corresponding arginine in Glut4 with a lysine (R92L) resulted in 60% reduction of glucose transporter activity (12), which is similar to our *in vitro* findings with the R124S mutant of GLUT2.

Different homozygous GLUT2 mutations, often predicted to lead to a loss-of-function, have been reported as the cause of FBS, a rare autosomal recessive disease that was first described in 1949 (13–16). Affected children exhibit fasting hypoglycemia, presumably due to reduced glucose transport out of liver cells and postprandial hyperglycemia due to reduced flux of glucose into the pancreatic β-cell and therefore inappropriate insulin release (4). Children with FBS additionally develop enlargement of the liver and kidney, presumably because of increased glycogen accumulation, which may lead to proximal tubular dysfunction and could explain the renal phosphate and calcium losses that have been reported in some cases.

The affected members in our kindreds, however, showed no hepatomegaly when investigated, possibly due to the reported transient nature of hepatomegaly in FBS, and no apparent abnormalities based on random glucose levels. However, a formal oral glucose tolerance test, performed in the two affected members of family 1 after the genetic diagnosis of GLUT2 mutation has been established, did show impaired glucose tolerance (Supplemental Data). The profound, early-onset renal phosphate-wasting in the two affected individuals in family 1 and the degree of hypercalciuria observed in the affected members of family 2 appeared to be so unusual that a diagnosis of

TABLE 2. Comparison of the phenotypic features of the affected members in families 1 and 2

	Family 1	Family 2
Blood		
Hypophosphatemia	++	+/−
Metabolic acidosis	+	−
Hyperparathyroidism (mild)	+	−
Elevated $1,25(OH)_2D$ level	+/−	−
Urine		
Glycosuria	+	+
Generalized aminoaciduria	+	+
Hypercalciuria	++	++
Phosphaturia	++	+/−
Bone		
Rickets	++	+/−

+, Present; ++, pronounced; −, absent; +/−, present in some, but not all members. $1,25(OH)_2D$, 1,25-Dihydroxyvitamin D.

FBS was initially not entertained in either family. However, our molecular studies identified novel GLUT2 mutations in both kindreds, establishing the diagnosis of FBS and thus revealing a rather broad spectrum of clinical and laboratory findings in this disease (Table 2). Differences between the phenotypes in our families and that of classic FBS could be caused by additional genetic or nongenetic factors, such as modifier genes or environmental effects. Mutations in GLUT2 should therefore be considered in the differential diagnosis of hypophosphatemic rickets and in patients with hypercalciuria of unknown etiology.

The frame-shift mutation in family 1 is predicted to lead to a complete loss-of-function of GLUT2, and this mutation was therefore not investigated further in our *in vitro* studies; for the mutation in family 2 (R124S), we demonstrated a major reduction in glucose transport *in vitro*. In the S1 segment of the proximal tubule of the kidney, glucose is reabsorbed apically by the sodium-dependent glucose transporter SGLT2 and is transported out of the cell on the basolateral side by GLUT2. Because of this localization within the tubular cells, it is difficult to reconcile the mechanism through which loss-of-function mutations of the glucose transporter GLUT2 cause hypophosphatemia and hypercalciuria. We considered two possibilities: 1) that functional loss of GLUT2 at the basolateral membrane impairs glucose efflux from the proximal tubule leading to accumulation of glucose and/or glycogen in the cell and glucose toxicity, resulting in an impairment of phosphate transporters at the apical membrane; or 2) that GLUT2 itself, in addition to transporting glucose, also acts as a phosphate transporter, analogous to GLUT9 that was recently found to be a transporter of uric acid (17). Loss-of-function of GLUT2 in the latter scenario would therefore lead to disturbance of phosphate transport across the proximal tubular cell. However, our experiments using *X. laevis* oocytes expressing WT GLUT2 or the R124S mutant revealed no evidence that GLUT2 can transport phosphate into the cells. Likewise, GLUT2 failed to facilitate export of phosphate out of cells since oocytes, which had been injected with cRNA encoding both NPT2a (to allow preloading with radioactive phosphate) and GLUT2 showed no efflux of radioactivity into the medium. The results of these *in vitro* studies suggest, at least under our experimental conditions, that GLUT2 does not function as a phosphate transporter.

To further explore the question how the lack of GLUT2 transporter at the basolateral membrane of the proximal renal tubules can lead to urinary phosphate-wasting, we investigated mice that are null for Glut2 yet rescued from early lethality by the expression of rat Glut1 under the control of a pancreas-specific promoter (18). In the kidneys of these animals, a striking reduction of Npt2c protein was observed both by immunohistochemistry in the proximal tubules and by Western blots analysis using whole kidneys. Contrary to what would be expected if glucose toxicity were responsible for a general impairment of proximal tubular function, expression of Npt2a was only slightly reduced. The rescued Glut2-null animals (tg-$Glut2^{-/-}$) revealed a mild increase in urinary phosphate excretion yet no evidence for hypophosphatemia. This difference between phenotypes in human *vs.* mouse is similar to the laboratory findings in Npt2c-null animals that show no hypophosphatemia and only mild transient hypercalciuria (11). In contrast to this mild phenotype in mice, inactivating NPT2c mutations in humans lead to hereditary hypophosphatemic rickets with hypercalciuria (11), a disorder characterized by increased urinary phosphate excretion leading to hypophosphatemia and rickets as well as elevated $1,25(OH)_2$ vitamin D levels, hypercalciuria, and nephrocalcinosis/kidney stones in some affected individuals (19), *i.e.* findings similar to those observed in patient 1031E of family 1. However, hereditary hypophosphatemic rickets with hypercalciuria is quite variable, and some carriers of homozygous or compound heterozygous NPT2c mutations show little or no biochemical changes (20).

It remains to be determined what mechanisms lead to the significant reduction of Npt2c and the rather small reduction in Npt2a expression in Glut2-ablated mice. It is conceivable that disruption of Glut2 leads, through an unknown mechanism, to selective impairment of some other transporters. This may be similar to the findings in recently described patients with homozygous NPT2a mutations, who develop hypercalciuria and other features of Fanconi syndrome in addition to the expected renal phosphate-wasting (1). Our findings in patients with GLUT2 mutations and in patients with NPT2a mutations raise the possibility that mutations in certain specific transporters

in the proximal tubule can lead to defects in a subset of other transporters in the proximal tubule cell, which in turn can lead to unexpected phenotypes.

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