Differential Regulation of the Renal Sodium/Phosphate Co-Transporters NaPi-Ila, NaPi-Ilc and PiT-2 in Dietary Potassium Deficiency

Sophia Y. Breusegem1,#, Hideaki Takahashi1, Hector Giral-Arnal1, Xiaoxin Wang1, Tao Jiang1, Jill W. Verlander3, Paul Wilson1, Shinobu Miyazaki-Anzai4, Eileen Sutherland1, Yupanqui Caldas1, Judith T Blaine1, Hiroko Segawa4, Ken-ichi Miyamoto4, Nicholas P. Barry1,2 and Moshe Levi1,2

1Department of Medicine, Division of Renal Diseases and Hypertension, and
2Department of Physiology and Biophysics, University of Colorado Denver, 12700 E. 19th Avenue, Aurora, CO 80045
3Department of Medicine, Division of Nephrology, Hypertension, and Transplantation, University of Florida, Gainesville, FL 32610
4Department of Molecular Nutrition, Institute of Health Biosciences, University of Tokushima Graduate School, Tokushima, Japan

Running Head: NaPi-Ilc and PiT-2 in dietary potassium-deficiency

#Corresponding Author: Dr. Sophia Breusegem

Cambridge Institute for Medical Research
Wellcome Trust/MRC building
Hills Road
Cambridge
CB2 0XY
U.K.
Email: syab2@cam.ac.uk

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Abstract

Dietary potassium (K)-deficiency is accompanied by phosphaturia, and decreased renal brush border membrane (BBM) vesicle sodium (Na)-dependent phosphate (P_i) transport activity. We previously showed that K-deficiency in rats leads to increased abundance in the proximal tubule BBM of the apical Na/Pi co-transporter NaPi-IIa, but that the activity, diffusion and clustering of NaPi-IIa could be modulated by the altered lipid composition of the K-deficient BBM (Zajicek et al., Kidney Int. 60, 694-704, 2001; Inoue et al., J. Biol. Chem. 279, 49160-49171, 2004). Here we investigated the role of the renal Na/Pi co-transporters NaPi-IIc and PiT-2 in K-deficiency. Using Western blotting, immunofluorescence and quantitative real-time PCR we found that in rats and in mice K-deficiency is associated with a dramatic decrease in the NaPi-IIc protein abundance in proximal tubular BBM and in NaPi-IIc mRNA. In addition, we documented the presence of a third Na-coupled P_i transporter in the renal BBM, PiT-2, whose abundance is also decreased by dietary K-deficiency in rats and in mice. Finally, electron microscopy showed subcellular redistribution of NaPi-IIc in K-deficiency: in control rats, NaPi-IIc immunolabel was primarily in BBM microvilli whereas in K-deficient rats, NaPi-IIc BBM label was reduced and immunolabel was prevalent in cytoplasmic vesicles. In summary, our results demonstrate that decreases in BBM abundance of the phosphate transporter NaPi-IIc and also PiT-2 might contribute to the phosphaturia of dietary K-deficiency, and that the three renal BBM phosphate transporters characterized so far can be differentially regulated by dietary perturbations.

Keywords: hypokalemia, phosphaturia, SLC34A1, SLC34A3, SLC20A2
**Introduction**

The kidney plays a critical role in the maintenance of phosphate ($P_i$) homeostasis. Plasma phosphate levels are maintained through regulated re-absorption of filtered $P_i$ along the proximal tubule. Apical entry of $P_i$ into the proximal tubule epithelial cells is mediated by at least two brush border membrane (BBM) sodium-coupled phosphate ($P_i$) transporters: NaPi-IIa (SLC34A1) and NaPi-IIc (SLC34A3) (6, 9, 19, 20).

NaPi-IIa and NaPi-IIc respond to variations in dietary $P_i$, parathyroid hormone (PTH), FGF23 and other factors by changing their abundance in the proximal tubule BBM. However, the time scales of their regulation by dietary $P_i$ and PTH are quite different, with rat and mouse NaPi-IIa down-regulation by high dietary $P_i$ concentrations or PTH and up-regulation under low dietary $P_i$ occurring within one hour, while NaPi-IIc regulation takes up to 4 times longer (23, 24). And while their overall molecular structure is predicted to be very similar, important differences between these two $Na^+/P_i$ co-transporters exist. For example, NaPi-IIa is electrogenic, coupling $P_i$ transport (at physiological pH mainly $HPO_4^{2-}$) with the transport of 3 $Na^+$ ions. In contrast, NaPi-IIc is electroneutral, only transporting 2 $Na^+$ for every $P_i$ (2). In addition, we recently determined that NaPi-IIc interacts with some of the same proteins as NaPi-IIa (NHERF1 and NHERF3/PDZK1), but not others (Shank2E, PIST) (27).

To date, NaPi-IIa has been considered the main apical proximal tubule $P_i$ transporter, as deduced from studies with NaPi-IIa knock-out mice. The NaPi-IIa knock-out mice exhibit a 70% increase in phosphaturia, indicating that at least in mice NaPi-IIa might be responsible for up to 70% of the renal $P_i$ re-absorption, with NaPi-IIc or other
apical \( P_i \) transporters accounting for the remaining 30% (3). However, recent research suggests that in humans NaPi-IIc might be an important regulator of \( P_i \) balance. Indeed, mutations in Npt2c, the gene encoding NaPi-IIc, have been identified as the cause of hereditary hypophosphatemic rickets with hypercalciuria, a life-long phosphate wasting disease (4, 10, 12, 15). These findings, in addition to the studies indicating important differences between NaPi-IIa and NaPi-IIc physiology, have stimulated great interest in further studies of NaPi-IIc regulation.

Here we determined whether NaPi-IIc is also regulated by dietary potassium (K)-deficiency. We previously showed regulation of NaPi-IIa, NaPi-1 (a type I \( Na^+/P_i \) co-transporter (5)) and PiT-1 (a type III \( Na^+/Pi \) co-transporter (8, 29)) in rats fed a K-deficient diet. In particular we found that K-deficiency leads to increased abundance of these \( P_i \) transporters in the proximal tubule BBM; however, at the same time \( Na^+ \)-coupled \( P_i \) transport in BBM vesicles derived from K-deficient rats was reduced compared to \( Na^+/P_i \) coupled transport in BBM from control rats (32).

We next sought to determine if an altered lipid environment could explain the decreased \( Na^+/P_i \) cotransport activity in K-deficient BBM despite the increased NaPi-IIa abundance. We found that K-deficiency is associated with an increase in BBM sphingomyelin, glucosylceramide and ganglioside GM3 and a decrease in BBM lipid fluidity (32). Using scanning fluctuation correlation spectroscopy and molecular brightness analysis we further found that NaPi-IIa diffusion in K-deficient BBM was slowed and NaPi-IIa cluster size increased compared to the transporter’s diffusion and clustering in control BBM (11). Decreased diffusion and increased cluster size could both reduce the \( P_i \) transport activity of NaPi-IIa and thus contribute to the reduced Na+-
coupled Pi transport activity in K-deficient BBM despite the increased NaPi-IIa BBM abundance.

In this study we find that dietary K-deficiency is associated with a sharp decrease in NaPi-IIc BBM abundance, both in rats and mice. We suggest that this decrease contributes to the observed hypophosphatemia and increased urinary Pi excretion in K-deficiency. In addition, electron microscopy indicates redistribution of NaPi-IIc from the BBM microvilli to intracellular vesicles in dietary K-deficiency. Finally, we also find that PiT-2, another type III Na/Pi co-transporter, is localized in the apical BBM of the proximal tubule, is regulated by dietary Pi and is down-regulated in K-deficient rats and mice.
Materials and Methods

Animals
Male Sprague-Dawley rats weighing between 175 and 200 g were obtained at 6 weeks of age from Charles River Laboratories (Wilmington, MA). C57BL/6 mice weighing between 20 and 25 g were obtained at 6 weeks of age from Jackson Laboratory (Bangor, Maine). All animals were housed at the Center for Laboratory Animal Care at the University of Colorado Health Sciences Center and put on a 12-h light/dark cycle. At 8 weeks of age the animals were fed a control diet (Teklad Diet #TD88238, Harlan Laboratories, Madison, WI) or a potassium-deficient diet (Teklad Diet #TD95006) for 14 days. The diets were otherwise matched for their phosphorus, calcium, magnesium, sodium, protein, carbohydrate and fat content. We used 6 rats or 12 mice in each group per experiment, and the experiments were repeated three times. For the dietary Pi studies, animals were fed for 14 days a low Pi diet (0.1% Pi, Teklad diet #85010), a control Pi diet (0.6% Pi, Teklad diet #84122) or a high Pi diet (1.2% Pi, Teklad diet #85349). The animal studies were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Colorado. Urine potassium, phosphorus and creatinine were measured using commercially available kits from Stanbio Laboratory (Boerne, TX). Plasma creatinine was measured using a 3200 Q trap LC-MS/MS (Applied Biosystems Inc., Foster City, CA) according to (25).

Materials
All chemicals were obtained from Sigma except when noted. Two polyclonal rabbit anti-NaPi-IIa antibodies were generated, one by Affinity Bioreagents (Golden, CO) and one
Colorado State University (Fort Collins, CO), and used at 1:10,000 for Western blotting and at 1:250 for immunofluorescence microscopy. The rabbit polyclonal anti-NaPi-IIc antibody was used as described (22). A chicken anti-NaPi-IIc antibody was custom-made by Davids Biotechnologie (Regensburg, Germany) as described (27) and used at 1:1,000 for Western blotting and at 1:100 for immunofluorescence. A rabbit anti-PiT-2 antibody was also from Davids Biotechnologie and generated by injection of the peptide HCKVGSVVAVGWIRSRKA. The PiT-2 antibody was used at 1:1,000 for Western blotting and 1:50 for immunofluorescence. The NHERF1 antibody was a generous gift from Dr. E. Weinman (University of Maryland, Baltimore, MD) and used at 1:2500 for Western blotting. The PDZK1 (CLAMP1) antibody was from BD Biosciences (San Jose, CA) and used at 1:5000 for Western blotting. The EEA1 and Rab5 antibodies were also from BD Biosciences and used at 1:2000 and 1:250 respectively for Western blotting.

Isolation of rat BBM

Rats were anaesthetized via an intraperitoneal injection of 50 mg/kg Pentobarbital sodium (Pentothal, Abbott Laboratories, Chicago, IL). The renal vessels were clamped before removal of the kidneys. Thin slices from the superficial cortex (SC) were dissected on ice-cooled glass. The SC and juxtamedullary cortex (JMC) kidney slices were homogenized in 15 mL ice-cold isolation buffer consisting of 300 mM mannitol, 5 mM EGTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 16 mM HEPES and 10 mM Tris, pH 7.5, and 1 tablet Roche Mini-Complete per 200 mL buffer using a Polytron homogenizer (90 seconds at 40% power). The BBM were isolated from the homogenate
by Mg$^{2+}$ precipitation followed by differential centrifugation as described (7). Briefly, 0.54 ml of 1 M MgCl$_2$ and 21 ml of water were added to each 15 ml of kidney homogenate. After 20 minutes of shaking, the homogenate was centrifuged at 2,790 x g for 15 minutes. The supernatant was subjected to another round of Mg$^{2+}$ precipitation and the resulting supernatant was centrifuged at 40,000 x g for 30 minutes. The resulting BBM pellets were resuspended in a buffer containing 300 mM mannitol, 16 mM HEPES, 10 mM Tris, pH 7.5 and one Mini-Complete tablet (Roche, Indianapolis, IN) per 50 mL buffer. BBM total protein concentration was determined using the BCA assay (Pierce, Rockford, IL).

**Isolation of mouse BBM**

Mice were anaesthetized via an intraperitoneal injection of 50 mg/kg Pentobarbital sodium (Pentothal, Abbott Laboratories). After clamping of the renal vessels the kidneys were removed and thinly sliced. Kidney slices from 2 mice were combined in 7.5 mL isolation buffer consisting of 15 mM Tris/HCl, pH 7.4, 300 mM mannitol, 5 mM EGTA and 1 Roche Complete inhibitor tablet per 250 mL buffer. The kidney slices were homogenized using a Potter-Elvejham homogenizer with 8 to 10 rapid strokes and transferred to a chilled capable tube. Kidney residues remaining on the homogenizer were rinsed off with 10 mL water that was then added to the kidney homogenate. BBM were prepared by a double Mg$^{2+}$ precipitation analogous to the preparation of rat BBM. For the first Mg$^{2+}$ precipitation, 300 μL 1M MgCl$_2$ was added to the homogenate, the solution was vortexed and shaken every 5 minutes for 20 minutes before centrifugation at 2,500 x g for 15 minutes. The supernatant was subjected to a second Mg$^{2+}$
precipitation, and from the resulting supernatant the BBM was recovered by centrifugation at 38,000 x g for 40 minutes. The BBM was resuspended and its protein content quantified as above for the rat BBM.

**P_i transport assays**

Phosphate transport was measured by radioactive $^{32}$P_i uptake in freshly isolated BBM vesicles as described (32).

**Western Blotting**

BBM proteins (10 or 20 μg total protein) were separated by 7.5% or 10% SDS-PAGE (Criterion, Bio-Rad, Hercules, CA) and transferred onto nitrocellulose membranes (Bio-Rad). Membranes were blocked for 30 minutes at room temperature with 5% milk in TBST buffer (20 mM Tris/HCl, 150 mM NaCl, 0.5% Tween, pH 7.4) before incubation with primary antibodies diluted in TBTS/milk overnight at 4ºC. After 4 washes with TBST, membranes were incubated with HRP-conjugated goat secondary antibodies (Pierce) diluted 1:10,000 for 1 hr at room temperature. HRP was detected following 2 minutes of incubation in Supersignal West Pico Chemiluminescent Substrate or Supersignal West Dura Extended Duration Substrate (Pierce), using either film or a CCD imaging system. Films were scanned using a Bio-Rad imager. Band intensities were quantified using Quantity One or ImageJ software. Membranes were stripped using Restore Stripping buffer (Pierce) and re-probed using a mouse anti-β-actin antibody (Sigma) to confirm equal total protein loading. Densitometry data are presented as average ± SD.
**Isolation and Western blotting of total rat kidney cortex membranes**

Rat kidney cortex homogenates were cleared of large debris by centrifugation at 250 x g for 10 minutes at 4°C. To obtain total membranes the supernatant was further centrifuged at 100,000 x g for 1 hour at 4°C. The membranes were resuspended in a buffer containing 300 mM mannitol, 16 mM HEPES, 10 mM Tris, pH 7.5 and one Mini-Complete tablet (Roche, Indianapolis, IN) per 50 mL buffer. Western blotting was performed as above except that infrared fluorescence detection was also used, using Li-COR IRDye-conjugated secondary antibody and an Odyssey instrument (Li-COR Biosciences, Lincoln, NE).

**RNA Extraction and Quantitative Real-time PCR**

Total RNA was isolated from kidney samples using the RNeasy Mini Kit from Qiagen (Valencia, CA) and cDNA was synthesized using reverse transcription reagents from Bio-Rad. The mRNA level was quantified using a Bio-Rad iCyCler real-time PCR machine. Cyclophilin was used as internal control, and the amount of RNA was calculated by the comparative C_T method as recommended by the manufacturer. All the data were calculated from triplicate reactions. Primer sequences used are as follows: rat NaPi-IIa forward, 5’ - GCC ACT TCT TCT TCA ACA TC - 3’; rat NaPi-IIa reverse, 5’ - CAC ACG AGG AGG TAG AGG - 3’; rat NaPi-IIc forward, 5’ - TCT TCG CAG TTC AGG TTG - 3’; rat NaPi-IIc reverse, 5’ - GTG AGT AGT AAG TAG ACA ATG G - 3’; rat NHERF-1 forward, 5’ - TCA ACA TTC AAA TCA GCA TCA G - 3’; rat NHERF-1 reverse, 5’ - GAA GAG CAG GGA GTC AGG - 3’; rat PDZK1 forward, 5’ - AAT CAT CAA GGA CAT AGA ACC - 3’; rat PDZK1 reverse, 5’ - CCA GCA CCA ACA GAG TAG - 3’; rat
PiT-2 forward, 5' - GTG GAT GGA ACT CGT CAA G - 3'; rat PiT-2 reverse, 5' - CAG GAT CAG CAC ACC - 3'; mouse NaPi-IIa forward, 5' - AGA CAC AAC AGA GGC TTC - 3'; mouse NaPi-IIa reverse, 5' - CAC AAG GAG GAT AAG ACA AG - 3'; mouse NaPi-IIc forward, 5' - CAT CTT CAA CTG GCT CAC - 3'; mouse NaPi-IIc reverse, 5' - GGT TAT CAC ACT GCT CAC - 3'; mouse Pi T-2 forward, 5' - TGC TCT GCT GTT CGC CTT C - 3'; mouse PiT-2 reverse, 5' - TCT CTA ATC TGC CTG CTA TCT TCC - 3'. RNA data are presented as average relative levels vs. cyclophilin A ± SD.

**Immunofluorescence Microscopy**

Six control and 6 K-deficient rats were anesthetized as described above before retrograde perfusion through the abdominal aorta. The perfusion buffer consisted of 3% paraformaldehyde (diluted from 32% paraformaldehyde ampoules, Electron Microscopy Sciences, Hatfield, PA) in a 6:4 mixture of cacodylate buffer (pH 7.4, adjusted to 300 mOsm with sucrose) and 10% hydroxyethyl starch. The perfusion pressure was 300 mm Hg, and 1 mL perfusion buffer was used per g bodyweight. After 5 minutes the fixative was washed out by perfusion for 5 minutes with the cacodylate buffer. The kidneys were removed, cut into 4 to 6 slabs, embedded in OCT, and frozen in liquid nitrogen. Sections 6 micron thick were cut on a Leica cryostat at -20ºC and stored at -20ºC until ready to use. Sections were washed once with PBS before blocking for 30 minutes in staining solution (PBS containing 0.1% Triton X-100 and 5% milk powder) to which 10% goat serum was added. Sections were then incubated overnight at 4ºC with primary antibodies diluted in the staining solution. After 4 washes with PBS containing 0.1% Triton X-100 sections were incubated with secondary goat antibodies (Invitrogen,
Carlsbad, CA) diluted 1:250 as well as Alexa 633-phalloidin (Invitrogen) in staining solution for 1 hour at room temperature. After 4 washes with PBS containing 0.1% Triton X-100 the sections were mounted in Vectashield (Vector Laboratories, Burlingame, CA). Fluorescence images were acquired on a Zeiss 510 LSM laser scanning confocal microscope (Carl Zeiss Microimaging, Inc., Thornwood, NY).

Electron Microscopy

Tissue preparation. Adult Sprague-Dawley rat were anesthetized with inhalant isoflurane. The kidneys were preserved by in vivo retrograde aortic perfusion with PBS, followed by 4% paraformaldehyde in 0.1M lysine buffer containing 0.01M NaIO₄ and 0.15M sucrose. Samples of renal cortex were dissected and shipped in fixative by overnight courier to the University of Florida College of Medicine Electron Microscopy Core Facility where they were rinsed in PBS, incubated in 0.1M NH₄Cl for 1 hour, then dehydrated in a graded series of ethanols, infiltrated and embedded in Lowicryl K4M resin (Electron Microscopy Sciences, Ft. Washington, PA) and polymerized under UV light for 24 hours at -20°C, followed by approximately 60 hours at room temperature. Ultrathin sections of samples containing well-preserved proximal tubule were mounted on Formvar/carbon coated nickel grids.

Immunogold localization. For immunogold labeling the ultrathin tissue sections were exposed to the primary antibody and then to a colloidal gold-conjugated secondary antibody. For NaPi-IIc localization, the chicken anti-NaPi-IIc primary antibody was diluted 1:100 and the secondary was rabbit anti-chicken IgY conjugated to 5 nm diameter colloidal gold (BB International, Ted Pella, Redding, CA). Unless noted
otherwise, all steps were done by floating the grids on droplets of solution at room temperature. The sections were exposed to 0.1M NH₄Cl for 1 hour, incubated with 1% BSA in PBS, pH 7.4 for 30 min, washed with incubation solution (0.2% acetylated BSA (Aurion BSA-c, Electron Microscopy Sciences), 10 mM NaN₃, in PBS, pH 7.4), and then incubated in a humidified chamber overnight at 4°C with the primary antibody diluted in incubation solution. The sections were then washed with incubation solution and exposed to the secondary antibody diluted in incubation solution for 1.5 h at room temperature. The sections were washed with incubation solution, washed with PBS, postfixed with 1.25% glutaraldehyde in PBS, washed with PBS, and finally washed with glass-distilled water. The sections were air dried over night and counterstained with saturated uranyl acetate. Each group of sections subjected to the immunogold procedure included a control section that was exposed to incubation buffer in place of the primary antibody.

Transmission electron microscopy. Ultrathin sections were examined using a Hitachi 7600 transmission electron microscope (Hitachi High Technologies America, Pleasanton, CA) equipped with a MacroFire slow-scan CCD camera (Optronics, Goleta, CA) and AMT Image Capture software (Advanced Microscopy Techniques, Danvers, MA).

Immunohistochemistry

Transverse slices of 4% paraformaldehyde-lysine-peridoate-sucrose preserved kidney, 2-3 mm thick, were rinsed in PBS, dehydrated in a graded series of ethanols, and infiltrated and embedded in polyester wax, made from polyethylene glycol 400
distearate (Polysciences, Warrington, PA) and 10% acetyl alcohol. Sections 3 µm thick were mounted on gelatin-coated slides and heated at 37°C overnight. The sections were dewaxed in a graded series of ethanols, rinsed in PBS, and treated with 3% H₂O₂ followed by 5% normal goat serum in PBS to prevent non-specific reactivity. The sections were then incubated with the anti-NaPi-IIa primary antibody diluted 1:1000 overnight at 4°C. The sections were then washed, exposed to anti-rabbit polymer-linked peroxidase-conjugated secondary antibody (MACH2, Biocare Medical, Concord, CA), washed, and reacted with diaminobenzidine (Vector Laboratories, Burlingame, CA) for 5 minutes. Finally sections were washed, dehydrated in graded ethanols followed by xylene, and mounted on glass slides with Eukitt Mounting Medium (Hawthorne, NY). Sections were photographed using a Nikon LaboPhot-2 microscope equipped with a Nikon DS-5M digital color camera and NIS Elements software (Nikon USA, Melville, NY). Only sections from the same immunohistochemistry experiment, subjected to identical experimental conditions, were used for comparisons of immunolabel between control and K-deficient rats. Digital images for comparisons were collected in a single session with identical manual settings on both the camera and photomicroscope. No corrections or adjustments in contrast, brightness, or color balance were made to the collected images.

**Statistical Analysis**

Data are expressed as means ± SD. Data were analyzed for statistical significance by unpaired Student’s t-test or one way analysis of variance.
Results

*PiT-2 expression in the proximal tubule BBM is regulated by dietary Pi in both rats and mice.*

The presence of PiT-2 in the rat proximal tubule BBM and its response to changes in dietary Pi intake, establishing PiT-2 as a third player in rat renal Pi re-absorption, was recently reported (28) and is further illustrated by immunofluorescence data shown in Figure 1A. In particular, we wish to illustrate the up-regulation of PiT-2 at the BBM in all segments of the proximal tubule under dietary Pi deprivation. Under normal (0.6%) dietary Pi PiT-2 BBM localization overlaps strongly with NaPi-IIc BBM localization at the S1 segments only, while in the S2 and S3 segments PiT-2 is not concentrated at the BBM (see e.g. Figure 3 in (23) or Figure 6C). When dietary Pi is chronically low (0.1%), PiT-2 is expressed at the BBM not only in S1 but also in S2 and S3 (Figure 1A top right panel, red, S3 segments indicated by asterisks), similarly to NaPi-IIa (Figure 1A, top left panel, red). In contrast, high dietary Pi (1.2%) dramatically decreases the expression levels of NaPi-IIa and PiT-2 in all segments of the proximal tubule (Figure 1A, bottom panels).

Figure 1B establishes PiT-2 as a kidney BBM transporter regulated by dietary Pi intake in mice. BBM were isolated from mice chronically fed either a low (0.1%) Pi diet, a normal (0.6%) Pi diet or a high (1.2%) Pi diet and probed by Western blots for NaPi-IIa, NaPi-IIc and PiT-2. For the three Na⁺-coupled Pi transporters parallel decreases in mouse renal BBM abundance are observed when the dietary Pi content is increased.
Dietary K-deficiency in rats up-regulates renal BBM NaPi-IIa but down-regulates renal BBM NaPi-Ilc and PiT-2.

A summary of the rat parameters measured in this dietary K-deficiency study is presented in Table 1. In addition to decreased body weights but increased kidney weights, rats fed a K-deficient diet for 14 days display hypokalemia and hypophosphatemia and have increased urine P_i output compared to rats fed a control diet.

Total renal cortical BBM was isolated from rats fed either a control diet or a K-deficient diet for 14 days. Cortical BBM Na^+-coupled P_i transport activity was decreased by almost 40% in BBM isolated from K-deficient rats compared to control rats (from 800 ± 200 pmol ^{32}P/10s/mg BBM protein to 480 ± 130 pmol ^{32}P/10s/mg BBM protein, p = 0.002). Separation of the BBM proteins by SDS/PAGE followed by Western blotting indicated a significant increase in NaPi-IIa BBM abundance in dietary K-deficiency (Figure 2A), in agreement with our earlier studies (32). In contrast, NaPi-Ilc BBM abundance in dietary K-deficiency was dramatically decreased (Figure 2B), while BBM PiT-2 abundance was also significantly reduced (Figure 2C). When total renal cortical membranes were assayed, the observed changes in NaPi-IIa, NaPi-Ilc and PiT-2 paralleled the ones seen in the BBM (supplemental Figure S1).

In addition to the significant decreases in NaPi-Ilc and PiT-2 BBM abundance with dietary K-deficiency, a small decrease was observed in BBM abundance of the NaPi-IIa and NaPi-Ilc interacting proteins NHERF-1 (EBP50) and PDZK1 (NHERF3) in dietary K-deficiency (Figure 3). Interestingly, we always observed two specific bands for
NHERF1, and only the lower molecular weight band is decreased in dietary K-deficiency.

We also separated the rat superficial cortex (SC) from the juxtamedullary cortex (JMC) and isolated BBM from both. Western blotting revealed an increase in NaPi-IIa in both SC and JMC BBM with dietary K-deficiency (Figure 4A). For both NaPi-IIc and PiT-2 JMC BBM abundance was much smaller compared to SC BBM abundance, in agreement with their observed localization predominantly in the S1 segments of the proximal tubule with a 0.6% P_i diet. However, for both NaPi-IIc and PiT-2 a significant decrease in BBM abundance was observed with dietary K-deficiency, in both the SC and the JMC BBM (Figures 4B and 4C).

**mRNA levels of the three BBM P_i transporters are decreased in K-deficient rats.** Measurements of mRNA levels by quantitative real-time PCR indicated a decrease in Npt2a transcripts in dietary K-deficiency in both the SC and JMC (Figure 5A), while the Npt2c and PiT-2 transcript decreased in SC only (Figures 5B and 5C). Thus, whereas for NaPi-IIc and PiT-2 BBM protein levels parallel mRNA levels, for NaPi-IIa post-translational mechanisms are at play that increase its BBM protein abundance despite decreases in transcript amount. Transcript levels of NHERF1 and PDZK1 were not altered in dietary K-deficiency (Figure 5D).

**Immunofluorescence confirms the decreased abundance of NaPi-IIc and PiT-2 in the proximal tubule BBM of K-deficient rats.**
The increased abundance of NaPi-IIa and decreased abundance of NaPi-IIc and PiT-2 in the K-deficient BBM was also observed by immunofluorescence of rat kidney sections. We simultaneously used a rabbit anti-NaPi-IIa antibody, a chicken anti-NaPi-IIc antibody and Alexa Fluor-labeled phalloidin to localize NaPi-IIa, NaPi-IIc and F-actin in the same sections. In a parallel section we then used the rabbit anti-PiT-2 antibody, the chicken anti-NaPi-IIc antibody and phalloidin to obtain in addition the localization and abundance of PiT-2. Figure 6 shows representative images obtained in this manner for parallel sections from a kidney from a control rat (Figure 6A) and from a rat on a K-deficient diet (Figure 6B). Whereas the increase in NaPi-IIa BBM staining intensity in K-deficiency is subtle, there is a very clear decrease in both the intensity and the number of proximal tubules that show BBM staining for NaPi-IIc or PiT-2. This is also clearly visible in the pseudo-colored overlay images shown in Figure 6C. These images further indicate that under normal dietary P_i BBM PiT-2 is localized at the same proximal tubule sites as NaPi-IIc, i.e. S1 fragments only (yellow in NaPi-IIc/PiT-2 overlay image, tubule segments indicated by an asterisk) (23), while NaPi-IIa is more widely distributed and present in S1, S2 and S3 proximal tubule BBM. However, an intracellular pool of PiT-2 is visible in all proximal tubule epithelial cells, and when animals are chronically fed a low P_i diet (0.1% P_i) rather than a control P_i diet (0.6% P_i), upregulation of PiT-2 at the BBM of the later proximal tubule segments is visible, resulting in an expression pattern that is similar to NaPi-IIa and encompasses BBM expression in later segments of the proximal tubule (Figure 1A).
**Immunohistochemistry confirms the increased abundance of NaPi-IIa in the K-deficient BBM.**

The abundance of NaPi-IIa in the BBM of rats fed a K-deficient or control diet was further evaluated by immunohistochemistry. Figure 7 compares similar fields in the juxtamedullary cortex in a control (panel a) and K-deficient (panel b) rat. A clear increase in NaPi-IIa staining is seen in the K-deficient BBM. In addition, an undulating luminal surface is observed in many proximal tubules of the rats fed a K-deficient diet (indicated by asterisks), caused by variations in cell height in neighboring cells.

**Electron microscopy analysis of NaPi-IIlc indicates redistribution to intracellular vesicles in dietary K-deficiency.**

To determine whether the decrease in NaPi-IIlc BBM abundance seen by immunofluorescence microscopy (Figure 6) and in Western blots (Figures 2 and 4) is accompanied by internalization of the transporter, we used immunogold labeling and electron microscopy. In control rats, the majority of immunogold label for NaPi-IIlc was associated with the microvilli in the proximal tubule BBM (Figure 8a, arrows). Only a few gold particles were present over cytoplasmic vesicles, primarily in the pits at the base of the microvilli (Figure 8c, arrows) and in small vesicles in the subapical region (Figure 8c, black arrowheads). Gold particles were only rarely found in endosomes or lysosomes (Figure 8e). In K-deficient rats, the BBM immunolabel for NaPi-IIlc was markedly reduced (Figure 8b, arrows) and labeling of cytoplasmic vesicles was prevalent (Figure 8d, black arrowheads). Furthermore, in the K-deficient animals, NaPi-IIlc immunolabel was frequently found in cytoplasmic vesicles deep within the proximal
tubule cell body (Figure 8f, black arrowheads), unlike the controls where cytoplasmic label was almost exclusively subapical.

Gold labeling was also found in lysosomes (black arrows) and in small vesicles associated with lysosomes (black arrowheads) more frequently in K-deficient rats (Figure 9b) than in the control animals (Figure 9a).

The specificity of the immunogold labeling is illustrated in supplemental Figure S2, which shows the complete absence of label in collecting duct intercalated cells.

**Dietary K-deficiency in mice also leads to increased NaPi-IIa and decreased NaPi-IIc and PiT-2 in the renal BBM.**

We next determined whether mice show the same response to a K-deficient diet as rats. The measured mouse parameters are summarized in Table 2 and indicate hypokalemia and hypophosphatemia. Na\(^+\)-coupled P\(_i\) transport in BBM isolated from mice fed the K-deficient diet for 14 days was reduced by almost 30% compared to the transport measured in BBM from mice fed a control diet (from 211 ± 46 pmole \(^{32}\text{P}/10\text{s/mg BBM protein}\) to 155 ± 36 pmole \(^{32}\text{P}/10\text{s/mg BBM protein}\); \(p = 0.04\)).

Furthermore, there was a small but insignificant increase in the amount of NaPi-IIa in the K-deficient mouse BBM compared to the control BBM (Figures 10A and 10B). However, the BBM levels of NaPi-IIc and PiT-2 were both significantly decreased in the K-deficient mice compared to the control mice (Figure 10A and 10B). We further found that the mRNA levels of the type II transporters were significantly decreased in mice put on a K-deficient diet for 14 days, but no significant change was observed in the PiT-2 mRNA level (Figure 10C). Overall these studies indicate that the responses of rats and
mice to a K-deficient diet are very similar with respect to the regulation of the renal BBM $P_i$ transporters.
Discussion

Studies to date have indicated that renal Pi re-absorption in the proximal tubule is mediated by at least two BBM Na⁺-coupled Pi transporters, NaPi-IIa and NaPi-IIc. The relative importance of these two Pi transporters in both physiological and disease states is a matter of intense research and debate (30). Earlier studies focused solely on NaPi-IIa, and from studies in NaPi-IIa knock-out mice it was deduced that NaPi-IIa is responsible for 70% of the total Pi re-absorption, NaPi-IIc or other Pi transporters accounting for the remaining 30% (3). However, these percentages might not reflect the importance of NaPi-IIc in rat or human Pi reabsorption. Indeed, in humans mutations in NaPi-IIc and not NaPi-IIa have been identified as the cause of the Pi-wasting in hereditary hypophosphatemic rickets with hypercalciuria (4, 10, 12, 15). Our study of the phosphaturia associated with dietary K-deficiency in rats and mice further indicates that NaPi-IIc can play an important role in the maintenance of Pi homeostasis since, contrary to NaPi-IIa, its BBM abundance decreases with dietary K restriction (Figures 2, 4, 6, 8 and 10) and thus parallels the observed decrease in renal Na⁺-coupled Pi transport. In addition our study further characterized a third apical BBM Pi transporter, PiT-2, which is also regulated differently from NaPi-IIa in diet-induced hypokalemia (Figures 2, 4, 6 and 10).

Our earlier studies indicated that dietary K-deficiency in rats increases the fractional excretion of Pi despite increases in the proximal tubule BBM abundance of NaPi-IIa (32). Whereas we previously sought to reconcile these two observations by evoking the altered lipid environment of NaPi-IIa in the K-deficient BBM and its effect on
the transporter’s activity (26, 10), our present study suggests that an additional regulatory mechanism, leading to decreased NaPi-IIc abundance in K-deficient BBM, could also contribute to the phosphaturia in K-deficiency. Indeed, we observed a dramatic decrease in BBM NaPi-IIc with diet-induced hypokalemia, both in rats (Figures 2, 4, 6 and 8) and in mice (Figure 10). Furthermore we document the first electron microscopy localization of NaPi-IIc at the proximal tubule brush border and its relocation to deep cytoplasmic vesicles including endosomes and lysosomes with dietary K-deficiency (Figures 8 and 9).

It appears likely that the decrease in BBM NaPi-IIc contributes to the decreased Na\(^+\)-coupled P\(_i\) transport activity measured in BBM vesicles from animals on a K-deficient diet. However, to determine the relative contribution of NaPi-IIa and NaPi-IIc to the measured P\(_i\) transport activity would require knowledge of their relative expression levels in the rodent BBM. The relative importance of the two transporters was deduced from studies in knock-out mice; however, these do not allow accurate determination of protein expression levels since compensatory mechanisms to improve survival might come into effect. However, future studies of dietary K-deficiency in NaPi-IIa or NaPi-IIc knock-out mice could more precisely assess the effect of dietary K-deficiency on each transporter’s expression and activity.

We also found a small decrease in the abundance of one form of the PDZ domain-containing protein NHERF1, a NaPi-IIa and NaPi-IIc interacting protein (Figure 2), which might or might not be related to the decreased abundance of NaPi-IIc. This is of interest since mutations in NHERF1 were recently identified as a cause of phosphaturia and nephrolithiasis or osteopenia in humans (13, 16).
In addition to decreases in BBM NaPi-IIc, we also observed significant decreases in the BBM abundance of a type III Na\(^+\)/Pi\(^-\) co-transporter, PiT-2, in dietary K-deficiency both in rats and mice (Figures 2, 4, 6 and 10). Type III Na\(^+\)/Pi\(^-\) co-transporters were originally identified as retroviral receptors (Glvr-1 and Ram-1) but have since been characterized as ubiquitously expressed Pi transporters (PiT-1 and PiT-2 respectively) that were thought to fulfill housekeeping roles in Pi homeostasis (8, 29). Expression of both PiT-1 (26) and PiT-2 (14) in all regions of the kidney has been described. We have focused on PiT-2 as a recent report describes the specific expression of PiT-2 at the apical BBM of renal proximal tubule epithelial cells and its regulation by dietary Pi in the rat (28). Here, we further document the localization of PiT-2 in the proximal tubule BBM of the rat and the down-regulation of its BBM abundance by chronic high dietary Pi (Figure 1A) and by dietary K-deficiency (Figures 2, 4, 6). We also demonstrate that PiT-2 is localized in mouse BBM where its abundance is markedly regulated by chronic variations in dietary Pi content (Figure 1B) or K content (Figure 10). We note that a low Pi diet seems to induce apical BBM expression of PiT-2 in the later segments of the proximal tubule, whereas under normal dietary Pi the localization of PiT-2 is restricted to the early convoluted and S1 segments (compare Figures 6C, control diet and 1A, low Pi diet).

Our studies also point to the importance of post-translational regulation in the physiology of the Na\(^+\)-coupled Pi transporters. Indeed, whereas for NaPi-IIc and PiT-2 both SC mRNA and BBM protein levels are decreased in K-deficiency, for NaPi-IIa there is increased BBM protein abundance but decreased mRNA expression as determined by quantitative real-time PCR (Figure 5). Thus, for NaPi-IIa, post-
translational mechanisms are at play that increase its retention in the BBM and that could include influences of the altered lipid environment as discussed above.

While dietary K-deficiency causes differential regulation of the Na\(^+\)/P\(_i\) transporters by different transcriptional, translational and post-translational mechanisms, the relevant signal that mediates these changes is not clear. While these animals develop systemic metabolic alkalosis they have evidence for intracellular acidosis. Indeed, dietary K-deficiency has been shown to be associated with increases in Na\(^+\)/H\(^+\) exchange and Na\(^+\)/citrate co-transport activity and decrease in Na\(^+\)/sulfate co-transport activity (17, 18), changes which, together with the decrease in Na\(^+\)/P\(_i\) co-transport activity, have also been reported in models of metabolic acidosis (1, 21). While K-deficiency also results in increased stimulation of the renin-angiotensin-system, the results in the literature suggest that angiotensin II increases rather than decreases Na\(^+\)/P\(_i\) co-transport activity (31).

In conclusion, our studies of the phosphaturia that accompanies dietary K-deficiency have shown that three distinct P\(_i\) transporter proteins can contribute in different ways to observed imbalances of P\(_i\) homeostasis. While NaPi-IIa was long believed to be the main proximal tubule BBM P\(_i\) re-absorber, our studies and others now indicate that NaPi-IIc and PiT-2 might also play important roles in renal P\(_i\) re-absorption.
Table 1. Body weight, kidney weight, urine and serum parameters of rats after 14 days on a K-deficient diet. Starting body weights were 129.1 ± 4.7 g for the rats put on the control diet and 131.1 ± 2.7 g for the rats put on the K-deficient diet (p = 0.34). Data are average ± SD.

<table>
<thead>
<tr>
<th></th>
<th>Control diet</th>
<th>K-deficient diet</th>
<th>p value</th>
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<tbody>
<tr>
<td>Body weight (g)</td>
<td>258.4 ± 9.8</td>
<td>209.7 ± 9.6</td>
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<tr>
<td>Total kidney weight (g)</td>
<td>2.0 ± 0.2</td>
<td>2.9 ± 0.2</td>
<td>&lt;0.001</td>
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<tr>
<td>Serum potassium (mmol/L)</td>
<td>3.9 ± 0.4</td>
<td>2.0 ± 0.2</td>
<td>&lt;0.001</td>
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<td>Serum phosphorus (mg/dL)</td>
<td>7.9 ± 0.8</td>
<td>6.1 ± 0.9</td>
<td>0.003</td>
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<td>Urine volume (mL)</td>
<td>9.6 ± 2.0</td>
<td>59 ± 14</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Urine potassium (mmol/day)</td>
<td>3.1 ± 1.0</td>
<td>0.1 ± 0.1</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Urine phosphate (mg/day)</td>
<td>0.96 ± 0.27</td>
<td>3.07 ± 0.98</td>
<td>&lt;0.001</td>
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</table>
Table 2. Body weight, kidney weight and serum parameters of mice after 14 days on a K-deficient diet. Starting body weights were 22.5 ± 0.9 g for the mice put on the control diet and 22.5 ± 1.9 g for the mice put on the K-deficient diet ($p =0.99$). Data are average ± SD.

<table>
<thead>
<tr>
<th></th>
<th>Control diet</th>
<th>K-deficient diet</th>
<th>$p$ value</th>
</tr>
</thead>
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<td>Body weight (g)</td>
<td>24.5 ± 0.9</td>
<td>22.0 ± 2.1</td>
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<tr>
<td>Total kidney weight (g)</td>
<td>0.29 ± 0.03</td>
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<td>Serum potassium (mmol/L)</td>
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<tr>
<td>Serum phosphorus (mg/dL)</td>
<td>8.7 ± 2.4</td>
<td>8.3 ± 1.2</td>
<td>0.05</td>
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</table>
Figure 1. PiT-2 expression in the proximal tubule BBM is regulated by dietary Pi in both rats and mice. A: Immunofluorescence of kidney sections from rats chronically fed a low (0.1%) or high (1.2%) P_i diet. Parallel sections are shown, stained for NaPi-IIc (pseudo-colored green) and NaPi-IIa (red), or NaPi-IIc (green) and PiT-2 (red). For each combination image acquisition parameters were identical for the rat fed the low or high P_i diet. When rats are fed a low P_i diet, PiT-2 expression in the renal BBM is upregulated and detected in all segments of the proximal tubule (compare with Figure 6C), including in the S3 segments indicated by asterisks. In contrast, high dietary P_i decreases the expression levels of NaPi-IIa and PiT-2 to background levels using the image acquisition parameters used for the animal on a low P_i diet. The scale bar represents 100 μm. B: Western blotting of renal BBM isolated from mice chronically fed a low (0.1%) P_i diet, a normal (0.6%) P_i diet or a high (1.2%) P_i diet and probed for NaPi-IIa, NaPi-IIc, PiT-2 and β-actin. The BBM abundance of all three transporters is down-regulated as the dietary P_i content is increased. Densitometry values were normalized to the low P_i diet values and are plotted ± SD (NaPi-IIa: black filled bars; NaPi-IIc: hatched bars; PiT-2: open bars).

Figure 2: Renal BBM Na^+-coupled phosphate transporters in dietary K-deficiency in rats: NaPi-IIa abundance increases while NaPi-IIc and PiT-2 abundance decreases in K-deficient vs. control rat BBM. A: NaPi-IIa; B: NaPi-IIc; and C: PiT-2 abundance in 6 control and 6 K-deficient rat BBM samples was determined by Western
blotting. The bar graphs show average densitometry values ± SD. The experiment was repeated three times, and a representative Western blot and analysis is shown.

Figure 3. NHERF1 and PDZK1 (NHERF3) protein abundance in renal BBM in dietary K-deficiency in the rat. Western blotting of total kidney BBM isolated from control and K-deficient rats indicates a significant decrease in one of the two bands observed for NHERF1. A small but statistically not significant decrease in PDZK1 BBM abundance is also measured.

Figure 4: Both superficial cortex (SC) and juxtamedullary cortex (JMC) BBM show increased NaPi-IIa and decreased NaPi-IIc and PiT-2 abundance in K-deficient rats compared to control rats. SC and JMC BBM samples from 4 rats on a K-deficient diet and from 4 rats on a control diet were separated by SDS-PAGE and probed for: A: NaPi-IIa and β-actin, B: NaPi-IIc and β-actin, C: PiT-2 and β-actin. The bar graphs show average densitometry values ± SD. The experiment was repeated three times, and a representative Western blot and analysis is shown.

Figure 5: K-deficiency leads to decreases in Npt2a, Npt2c and PiT-2 mRNA levels in the rat kidney. A: Npt2a; B: Npt2c; C: PiT-2 mRNA levels in SC and JMC kidney homogenates were quantified by QPCR as detailed in Methods. Npt2a transcript amounts are decreased significantly in both SC and JMC while Npt2c and PiT-2 transcript levels are decreased in SC only. NS indicates p values > 0.05. D: Rat kidney mRNA levels of NHERF1 and NHERF3 are not altered with dietary K-deficiency.
Figure 6: Immunofluorescence imaging of NaPi-IIa, NaPi-IIc and PiT-2 in kidney sections from K-deficient and control rats. Rat kidneys were perfuse-fixed as detailed in Methods before sectioning. A first section was stained simultaneously for NaPi-IIc (chicken antibody, detected using Alexa Fluor 488-conjugated goat anti-chicken antibodies), NaPi-IIa (rabbit antibody, detected using Alexa Fluor 568 goat anti-rabbit antibodies) and F-actin (detected using Alexa Fluor 633-conjugated phalloidin) while a parallel section was stained simultaneously for NaPi-IIc, PiT-2 and F-actin using the same secondary detection reagents. Panels A and B show representative images from a control (A) and K-deficient (B) rat in grayscale. The NaPi-IIa and NaPi-IIc images are from the first section, while the PiT-2 and F-actin images are from the parallel section. Significant decreases in BBM NaPi-IIc and PiT-2 staining can be observed in dietary K-deficiency. C: Overlay images of NaPi-IIa and NaPi-IIc or of NaPi-IIc and PiT-2 as indicated. In all images NaPi-IIc is pseudo-colored green, while NaPi-IIa or PiT-2 are pseudo-colored red. The images of the control animal illustrate the overlap of NaPi-IIc and PiT-2 staining at the BBM of S1 proximal tubules (yellow in the NaPi-IIc/PiT-2 overlay image, segments indicated by *), as well as the differential distribution of NaPi-IIa and NaPi-IIc (NaPi-IIc predominantly in S1, indicated by *). The scale bar represents 100 μm.

Figure 7: Increase in NaPi-IIa BBM abundance in dietary K-deficiency detected by immunohistochemistry. Light micrographs illustrating NaPi-IIa immunoreactivity in kidney sections of control (panel a) and K-depleted (panel b) rat kidney detected using
immunoperoxidase cytochemistry. NaPiIIa immunoreactivity was present in the brush border of proximal tubules in both conditions, but was more intense in K-depleted animals than in controls. Also, in K-depleted animals, the undulations of the luminal surface of many proximal tubules (*) was exaggerated compared to controls, due to increased variation in cell height.

Figure 8: Representative high magnification transmission electron micrographs of proximal tubule cells in the S1 segment of control (panels a, c, e) and K-depleted (panels b, d, f) rats labeled for NaPi-IIc using immunogold cytochemistry. Three sections of the cell are shown: the brush border (panel a and b), the base of the microvilli and the subapical region (panel c and d) and cytoplasmic vesicles deeper inside the cell (panel e and f). In control rats, NaPi-IIc immunogold labeling was strong in the brush border (panel a, arrows), and was present in large vesicles and apical pits at the base of the brush border (panel c, arrows), but few gold particles (black arrowheads) were associated with the small cytoplasmic vesicles, either in the subapical region (panel c) or deeper in the cell body (panel e). The majority of small cytoplasmic vesicles in control rats had no NaPi-IIc immunolabel (open arrowheads, panels c and e). By comparison, relatively few gold particles labeling NaPi-IIc were present in the brush border of K-depleted rats (panel b, arrows). However, in K-depleted rats, although label was still observed in the large vesicles and apical pits at the base of the brush border (panel d, arrow), the NaPi-IIc immunogold labeling was markedly increased in small cytoplasmic vesicles (black arrowheads) in both the subapical region (panel d) and deeper in the cell (panel f).
Figure 9: Transmission electron micrographs illustrating NaPi-IIc immunolabel in lysosomes of S1 segment proximal tubule cells from control (panels a-c) and K-depleted (panels d-f) rats. In panels a (control) and d (K-depleted), areas containing lysosomes (L) are denoted by white rectangles and illustrated at high magnification in subsequent panels, with correlating letters (control, panels b and c; K-depleted, panels e and f). In control rats, only occasional gold particles were found in lysosomes (panel b, arrows) or in small vesicles in the vicinity of lysosomes (panel c, arrowhead), whereas the majority of lysosomes were not labeled (panel c). In K-depleted rats, NaPi-IIc immunolabel was more prevalent in lysosomes (panels e and f, arrows) compared to control rats.

Figure 10: Dietary K-deficiency in mice: BBM protein levels and mRNA of the Na\(^+\)/Pi transporters NaPi-IIa, NaPi-IIc and PiT-2. A: NaPi-IIa, NaPi-IIc and PiT-2 protein in the renal BBM isolated from control and K-deficient mice as determined by Western blotting. The corresponding β-actin levels are also shown. B: Densitometric analysis of the Pi transporter data presented in panel A; from left to right: NaPi-IIa, NaPi-IIc and PiT-2. p-values for control vs. K-deficiency are indicated and indicate significant decreases in BBM NaPi-IIc and PiT-2 in dietary K-deficiency. C: From left to right: Npt2a, Npt2c and PiT-2 mRNA levels in total kidney homogenates from control and K-deficient mice. Significant decreases in transcript levels are observed for Npt2a and Npt2c.
Acknowledgements

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Supplementary Material

Supplementary Figure S1.

A: NaPi-IIc and PiT-2 protein levels are decreased in K-deficient rat kidney total cortical membranes. Rat kidney cortex homogenates were spun at 100,000 x g for 1 hour to recover all cellular membrane proteins. They were separated on SDS-PAGE and assayed by Western blotting for NaPi-IIa, NaPi-IIc, PiT-2 and β-actin. Samples from rats fed a K-deficient diet for 14 days (lanes 5-8) show increased NaPi-IIa but decreased NaPi-IIc and PiT-2 compared to samples from rats fed a control diet (lanes 1-4). The changes in NaPi-IIa, NaPi-IIc and PiT-2 in these total membrane preparations thus mimic the changes observed in the brush border membrane only (Figure 2).

B: Isolated BBM do not contain early endosome membranes. BBM (lanes 1-8) or total membranes (lanes 9-16) containing equal amounts of total protein were separated by SDS-PAGE and probed for early endosomal antigen 1 (EEA1) and Rab5. No EEA1 was detected in the BBM, neither in control (lanes 1-4) nor in K-deficient (lanes 5-8) BBM. Some Rab5 was detected in the control BBM; however, this is not surprising since Rab5 has been shown to be associated with the apical plasma membrane in addition to early endocytic structures (Bucci C, Wandinger-Ness A, Lütcke A, Chiariello M, Bruno Bruni C and Zerial M. Rab5a is a common component of the apical and basolateral endocytic machinery in polarized epithelial cells. Proc Natl Acad Sci U S A 91: 5061-5065, 1994). It is not clear why less Rab5 seems to be associated with the K-deficient BBM compared to the control BBM.
Supplementary Figure S2. Apical region of an intercalated cell in a cortical collecting duct from a sample labeled for NaPi-IIc, demonstrating specificity of the immunolabel for the proximal tubule. No gold particles are present, which is representative of the findings in renal epithelial cells other than proximal tubule cells.