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ION CHANNELS, TRANSPORTERS

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Impaired PTH-induced endocytotic down-regulation of the renal type IIa Na^+/P_i-cotransporter in RAP-deficient mice with reduced megalin expression

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Abstract Inorganic phosphate (P_i) reabsorption in the renal proximal tubule occurs mostly via the Na^+/P_i cotransporter type IIa (NaP_i-IIa) located in the brush-border membrane (BBM) and is regulated, among other factors, by dietary P_i intake and parathyroid hormone (PTH). The PTH-induced inhibition of P_i reabsorption is mediated by endocytosis of Na/P_i-IIa from the BBM and subsequent lysosomal degradation. Megalin is involved in receptor-mediated endocytosis of proteins from the urine in the renal proximal tubule. The recently identified receptor-associated protein (RAP) is a novel type of chaperone responsible for the intracellular transport of endocytotic receptors such as megalin. Gene disruption of RAP leads to a decrease of megalin in the BBM and to a disturbed proximal tubular endocytotic machinery. Here we investigated whether the distribution of NaP_i-IIa and/or its regulation by dietary P_i intake and PTH is affected in the proximal tubules of RAP-deficient mice as a model for megalin loss. In RAP-deficient mice megalin expression was strongly reduced and restricted to a subapical localization. NaP_i-IIa protein distribution and abundance in the kidney was not altered. The localization and abundance of the NaP_i-IIa interacting proteins MAP17, PDZK-1, D-AKAP2, and NHE-RF1 were also normal. Other transport proteins expressed in the BBM such as the Na^+/H^+ exchanger NHE-3 and the Na^+/sulphate cotransporter NaSi were normally expressed. In whole animals and in isolated fresh kidney slices the PTH-induced internalization of NaP_i-IIa was strongly delayed in RAP-deficient mice. PTH receptor expression in the proximal tubule was not affected by the RAP knock-out. cAMP, cGMP or PKC activators induced internalization which was delayed in RAP-deficient mice. In contrast, both wildtype and RAP-deficient mice were able to adapt to high-, normal, and low-P_i diets appropriately as indicated by urinary P_i excretion and NaP_i-IIa protein abundance.

Keywords Dietary phosphate intake · Endocytosis · Megalin · Na^+/P_i cotransporter · Parathyroid hormone · Receptor-associated protein · Proximal tubule

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Introduction

Phosphate reabsorption in the renal proximal tubule occurs mostly via the secondary active sodium phosphate cotransporter type IIa (NaP_i-IIa) [16]. NaP_i-IIa transport activity is regulated by a variety of hormonal and non-hormonal factors [16]. One of the major regulating factors is parathyroid hormone (PTH) that induces phosphaturia by inducing brush-border membrane (BBM) Na/P_i-cotransporter internalization and subsequent lysosomal degradation [15, 16, 20]. Recent studies have shown that this process requires endocytosis of Na/P_i-cotransporter proteins from the BBM. Internalization of NaP_i-IIa most likely occurs at the invaginated membrane regions at the base of the brush border membrane [23]. Internalized NaP_i-IIa proteins are subsequently directed to the lysosomes for degradation [20, 23].

Megalin, a 600-kDa membrane protein belonging to the LDL receptor family, is strongly involved in the endocytotic pathway of renal proximal tubules [5, 7, 8, 18, 25, 29]. Megalin expression is found in the BBM, at the base of the microvilli and throughout much of the subapical endocytotic apparatus [7, 8, 9] where NaP_i-IIa

protein is also localized [23]. In the proximal tubule megalin serves as a receptor in the brush border membrane for endocytosis of a variety of hormones and endogenous or exogenous substances [8, 9]. Reduction or loss of megalin expression and function is associated in mice models and patients (Fanconi type syndromes) with loss of vitamin D_3, albumin, and other smaller proteins into the urine resulting in the disturbance of whole body calcium homeostasis and bone development [7, 8, 17, 18, 19, 29). Moreover, megalin has been shown to interact with parathyroid hormone and is able to inactivate PTH, thus, controlling PTH activation of PTH receptors [12]. In addition to its role in endocytosis of proteins from the urine, it has been suggested that megalin may directly interact with proteins in the brush border membrane such as the Na^+/H^+-exchanger NHE-3 and may be important for the regulation and expression of this protein by mediating its endocytosis and membrane recycling [4].

The recently identified receptor-associated protein (RAP) is a novel type of chaperone involved in the biosynthesis and intracellular transport of endocytotic receptors of the LDL family, among others megalin [5, 27, 29]. RAP binds to megalin competing with its intracellular ligands and thereby prevents premature targeting to the lysosome and degradation [28]. Gene disruption experiments of RAP demonstrated a reduction and redistribution of megalin in the BBM and a disturbed proximal tubular endocytotic machinery [5]. Hence, RAP serves an important function in the normal renal expression and subcellular distribution of megalin. RAP-deficient mice are relatively healthy compared to megalin-deficient mice and can thus serve as a model for megalin loss in adult animals. This prompted us to use RAP-deficient mice to investigate whether the distribution of NaP_i-IIa and/or its regulation by PTH and dietary phosphate intake is affected in proximal tubules by loss of RAP/megalin.

Materials and methods

Animal studies

Experiments were performed with age matched, male, wildtype mice (RAP+/+) and RAP knockout mice (RAP–/–)(Jackson Laboratory, Maine, USA) weighing between 20–25 g (RAP+/+) and 15–20 g (RAP–/–). The RAP knockout mice were deficient for the low-density RAP 1 and bred in the C57BL/6 J background [27]. Mice were kept on a standard laboratory diet and had free access to food and water. For some experiments the mice received diets (Kliba, NAFAG, Switzerland) with a high (1.2%) or low (0.1%) P_i content as described previously [13].

Spontaneous urine samples were collected daily at the same time and rapidly frozen until analysis. All samples were analyzed for P_i and creatinine using commercial kits (Sigma) according to the protocol provided by the manufacturer.

Injection of PTH and FITC-dextran

Mice were anaesthetized with a combination of ketamine (Narketan 10, Chassot, Belp, Switzerland) and xylazine (Rompun, Bayer, Leverkusen, Germany), injected intraperitoneally (i.p.). 1–34 PTH (25 μg/animal, dissolved in 0.9% saline) and/or 10-kDa FITC-dextran (2.5 mg/animal, dissolved in 0.9% saline) were injected into the tail vein as a single bolus (100 μl). Animals from the control group were injected with 100 μl of 0.9% saline alone. Twenty minutes after injection urine was collected from the urinary bladder and the animals were fixed by intravascular perfusion via the abdominal aorta at a pressure of 1.38 hp as described previously [10]. The fixative consisted of 3% paraformaldehyde and 0.05% picric acid. It was dissolved in a 3:2 mixture of 0.1 M cacodylate buffer (pH 7.4, adjusted to 300 mosmol with sucrose) and 10% hydroxyethyl starch in saline (HAES-steril, Fresenius, Stans, Switzerland). After 5 min of fixation the kidneys were rinsed for 5 min with the 0.1 M cacodylate buffer. Thin tissue slices (1–2 mm thick) were mounted on small cork disks, frozen in liquid propane and stored at −80°C until use.

Detection of FITC-dextran

The relative amount of FITC-dextran in the collected urine was measured with a spectrofluorometer at an excitation wavelength of 488 nm and an emission wavelength of 520 nm. The resulting fluorescence was expressed in arbitrary units.

Kidney slices

For kidney slice experiments mice were kept on a low-phosphate diet for 5 days prior to the experiments to increase NaP_i-IIa expression in kidney [13]. The mice were anaesthetized (i.p.) and perfused through the left ventricle with 50 ml warm (30°C) sucrose/phosphate buffer (140 mM sucrose, 140 mM NaH_2PO_4/NaH_2PO_4, pH 7.4) to remove all blood from the kidneys. Kidneys were rapidly harvested, adhering connective tissue and extrarenal vessels were removed, and thin coronal slices (about 1 mm in thickness) were prepared. From each kidney six to seven slices could be prepared. Slices were transferred into 4 ml of pre-warmed (37°C) Hank's buffer [6] (NaCl 110 mM, KCl 5 mM, $MgSO_4$ 1.2 mM, $CaCl_2$ 1.8 mM, Na-acetate 4 mM, Na-citrate 1 mM, glucose 6 mM, L-alanine 6 mM, NaH_2PO_4 1 mM, Na_2HPO_4 3 mM, $NaHCO_3$ 25 mM, pH 7.4, gassed with 5% CO_2 and 95% O_2) and allowed to adapt for 10 min at 37°C in a water bath prior to the start of the incubation. Slices were then left untreated (control) or incubated with PTH, 8-Br-cAMP, 8-Br-cGMP, or 1,2-dioctanoyl-sn-glycerol (DOG). During the whole course of the experiments all solutions were gassed with 5% CO_2/ 95% O_2 and the pH was kept constant at 7.4±0.1. We have previously shown that the kidney slices/proximal tubule cells retain major morphological characteristics during the incubation time and are responsive to several hormonal treatments demonstrating their viability [2].

All chemicals were obtained from Sigma Chemical (St. Louis, Mo., USA). All experiments were performed at least with two kidneys from two different animals. Untreated slices were used in all experiments as an internal control. For immunohistochemistry, kidney slices were transferred to a fixation solution at the end of the incubation. The same fixative was used as for the in vivo experiments. Slices were fixed for 4 h on ice. After fixation, slices were rinsed few times with PBS, mounted onto thin cork plates and immediately frozen in liquid propane, cooled with liquid N_2.

Immunohistochemistry

For immunofluorescence staining, sections (3–5 μm thick) were cut in a cryostat and placed on chrome alum gelatine-coated glass slides. For NaP_i-IIa and MAP17 immunofluorescence sections were pretreated for 10 min in PBS containing 3% defatted milk powder and 0.02% Triton X-100 ("blocking solution") to block nonspecific binding of antibody. Immunostaining was carried out as described previously [10]. Briefly, sections were incubated with an anti-rat NaP_i-IIa rabbit antiserum diluted 1:500, rabbit anti MAP17 antibody diluted 1:1,000 or anti-megalin Ab (kind gift of Dr. Ziak

and Dr. Roth, Institute of Pathology, University of Zurich) diluted 1:10,000, respectively. For PTH receptor, NHE-3, and NHERF-1 staining sections were pretreated with 0.5% SDS in PBS for 5 min. After repeated rinsing with PBS sections were incubated for 10 min with blocking solution. Sections were then incubated with an affinity-purified polyclonal antibody against PTH receptor (Covance Research Products, USA) at a dilution of 1:50, with polyclonal anti-rat NHE3 antibody (kindly provided by Dr. Orson W. Moe, University of Texas Southwestern Medical Center, Dallas, Tex., USA) at a dilution of 1:1,000, or with affinity-purified anti-NHE-RF1 antiserum (kindly provided by E. Weinman, Baltimore, Md., USA) diluted 1:200. In the case of D-AKAP-2 and PDZK1 sections were microwaved in buffer containing 0.01 M citrate in distilled water at low power for 10 min. After rinsing with PBS and covering for 10 min with "blocking" solution, sections were incubated with polyclonal antibodies against rabbit D-AKAP2 (rabbit) (Gisler, Pribanic, Bacic, Sabourin, Tsuji, Manser, Biber, and Murer, unpublished results) diluted 1:1,000 or with an anti-PDZK1 antibody [11] diluted 1:500. All primary antibodies were diluted in "blocking solution" and incubated over night at 4°C. Sections were then rinsed three times with PBS and covered for 40 min at room temperature in the dark with FITC-conjugated swine anti-rabbit IgG (Dakopatts, Glostrup, Denmark) diluted 1:50 in PBS/ milk powder. Double stainings of NaP_i-IIa and β-actin filaments were achieved by adding rhodamine-phalloidin (Molecular Probes, Eugene, Ore., USA), respectively, at a dilution 1:50 in the solution containing secondary antibodies. Finally, the sections were rinsed three times with PBS, covered with a glass-slip using DAKO-Glicergel (Dakopatts) containing 2.5% 1,4-diazabicyclo [2.2.2.] octane (Sigma, St. Louis, Mo., USA) as a fading retardant, and studied with an epifluorescence microscope. Unspecific binding to the tissue of the secondary antibodies was assessed by omitting the primary antibody (data not shown). All control incubations were clearly negative.

Western blots

For Western blotting, mice were sacrificed, the kidneys rapidly removed, and connective tissue removed and homogenized for 2 min in an ice-cold buffer (140 mM mannitol, containing PMSF, K-EDTA, leupeptin and statpeptin as protease inhibitors). The tissue was then centrifuged for 15 min at 4°C and at 4,500 rpm. The supernatant was again centrifuged for 30 min at 4°C at 41,000 rpm and the pellet resuspended in the membrane preparation buffer [3]. After measurement of the total protein concentration (Biorad Protein kit), 50 μg of total membrane protein were solubilized in Laemmli sample buffer, and SDS-PAGE was performed on 8–15% polyacrylamide gels. For immunoblotting, proteins were transferred electrophoretically from gels to PVDF membranes (Immobilon-P, Millipore, Bedford, Mass., USA). After blocking with 5% milk powder in TRIS-buffered saline/0.1% Tween-20 for 60 min, the blots were incubated with the primary antibodies (rabbit anti-NaP_i-IIa 1:6,000, mouse monoclonal anti-actin (42 kDa, Sigma) 1:5,000, rabbit anti-D-AKAP2 1:5,000, rabbit anti-NHE-3 1:5,000, rabbit anti NaSi 1:5,000, rabbit anti-MAP17 1:3,000, rabbit anti NHERF-1 1:3,000, rabbit anti PDZK1 1:5,000) either for 2 h at room temperature or overnight at 4°C. After washing and subsequent blocking, blots were incubated with the secondary antibodies (donkey anti-rabbit 1:10,000 or sheep anti-mouse 1:10,000, respectively) IgG-conjugated with horseradish peroxidase (Amersham Life Sciences) for 1 h at room temperature. Antibody binding was detected with the peroxidase/ luminal enhancer kit (Pierce, Rockford, USA) before exposure to X-ray film (Kodak). All films were analysed using SCION Imaging software to calculate the protein of interest : actin ratio. All results were tested for significance using the unpaired Student's *t*-test and only results with $P<0.05$ were considered as statistically significant.

Results

Immunohistochemistry using kidneys from RAP+/+ and RAP–/– mice showed that megalin labeling in the microvilli of the brush border was strongly reduced with a strong signal in the subapical compartment similar to what has been reported earlier (Fig. 1, panels A, B, a and b) [5]. This confirms the importance of RAP for the expression and localization of megalin. In contrast, the localization of NaP_i-IIa was comparable in both groups of mice (Fig. 1, panels C, D, c and d). The intensity of the NaP_i-IIa immunostaining was strongest in proximal convoluted tubules of juxtamedullary nephrons. A more detailed view of proximal tubules of the midcortical region (Fig. 1, panels c and d) revealed that the strongest immunostaining for NaP_i-IIa was visible in the BBM and weaker at some intracellular sites. The overall NaP_i-IIa protein abundance was comparable in wildtype and RAP-deficient mice as demonstrated and discussed later (Fig. 9). Moreover, no difference in the relative abundance or localization of the BBM-expressed NaSi and Na^+/H^+ exchanger NHE-3 were found (Fig. 2). Recently, a number of proteins have been identified that interact with NaP_i-IIa some of which influence its abundance, localization and activity ([11, 22]; (Gisler, Pribanic, Bacic, Sabourin, Tsuji, Manser, Biber, and Murer, unpublished results). However, as shown in Fig. 3, the localization of NHE-RF1, PDZK1, MAP 17, and D-AKAP was not altered in kidneys from RAP-deficient mice compared to wildtype (Fig. 3), suggesting that disruption of RAP did not interfere with their expression and function. Also the relative abundance of these proteins as determined by Western blotting was not affected (ratio NHE-RF-1/actin RAP+/+: 1.24±0.05, RAP–/–: 1.37±0.13; ratio PDZK-1/ actin RAP+/+: 0.63±0.18, RAP–/–: 0.98±0.28; ratio MAP17/actin RAP+/+: 0.64±0.06, RAP–/–: 0.80±0.14; ratio D-AKAP/ actin RAP+/+: 0.81±0.01, RAP–/–: 0.86±0.02, for all conditions at least $n=6$ animals, repeated in triplicates).

To test for the PTH induced endocytosis of NaP_i-IIa in vivo, RAP–/– and RAP+/+ mice were injected with PTH into the tail vein and fixed by perfusion after 20 min as described previously [23]. In wildtype animals after PTH injection NaP_i-IIa protein-specific immunofluorescence was scarcely detectable in midcortical and superficial regions whereas in proximal tubules of juxtamedullary nephrons some apical expression of NaP_i-IIa was still observed (Fig. 4B). Cross-sections of midcortical proximal tubules (Fig. 4F) illustrate that NaP_i-IIa immunofluorescence in the BBM was decreased, whereas the intensity of the intracellular staining was increased in the subapical region. These data confirmed those previously published by our group [14, 23]. In contrast, in RAP-deficient mice the PTH injection had no effect on the intensity and distribution of NaP_i-IIa staining (Fig. 4D, H), suggesting defective PTH-induced endocytosis of NaP_i-IIa. To confirm that the PTH injection into the tail vein had been successful, coinjections with FITC-dextran (10 kDa) were performed and urinary FITC excretion was

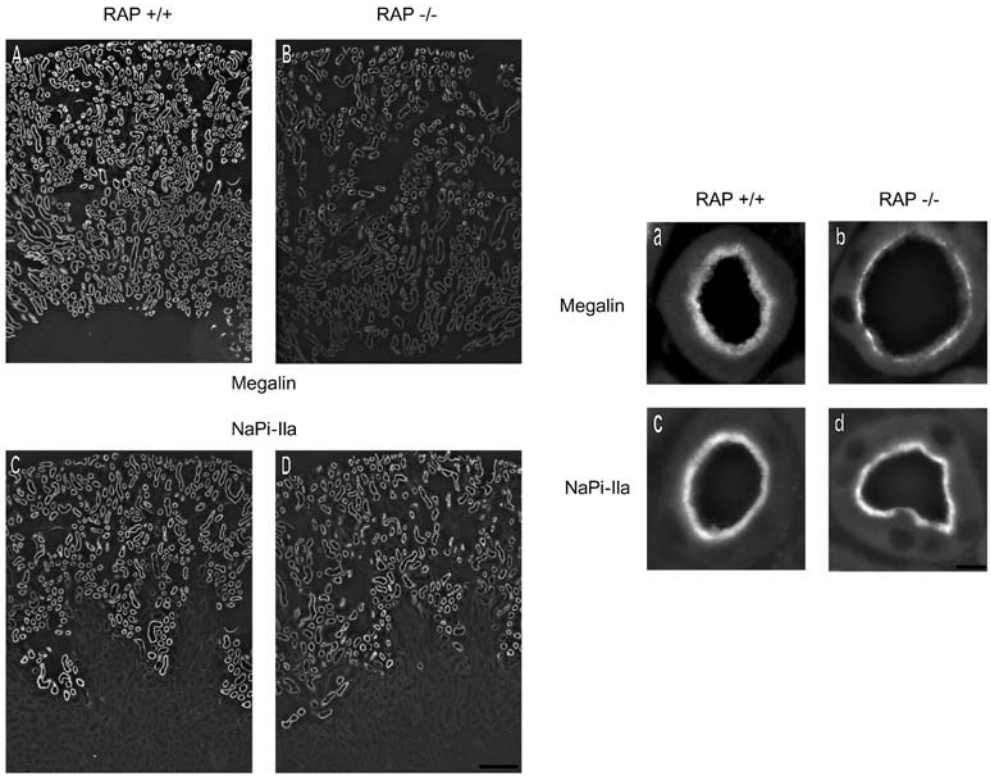

Fig. 1 Localization and expression of megalin and Na^+/P_i cotransporter type IIa (NaP_i-IIa) in kidneys from receptor-associated protein (RAP) wildtype (+/+) and RAP-deficient (–/–) mice. Cryosections (4 μm) from the kidney cortex of wildtype (*panels A, C, a, c*) and RAP-deficient (*panels B, D, b, d*) mice were labelled with antibodies against megalin or NaP_i-IIa. Megalin labelling is strongly reduced in kidneys from RAP–/– mice (*panel B*) and shows subapical staining (*panel b*) in contrast to wildtype animals where megalin is abundant (*panel A*) and seen throughout the entire brush border membrane (*panel a*). No difference was seen for NaP_i-IIa staining between wildtype (*panels C, c*) and RAP-deficient mice (*panels D, d*) under low-phosphate diet. *Scale bars*: panels A–D ~200 μm; panels a–d ~10 μm

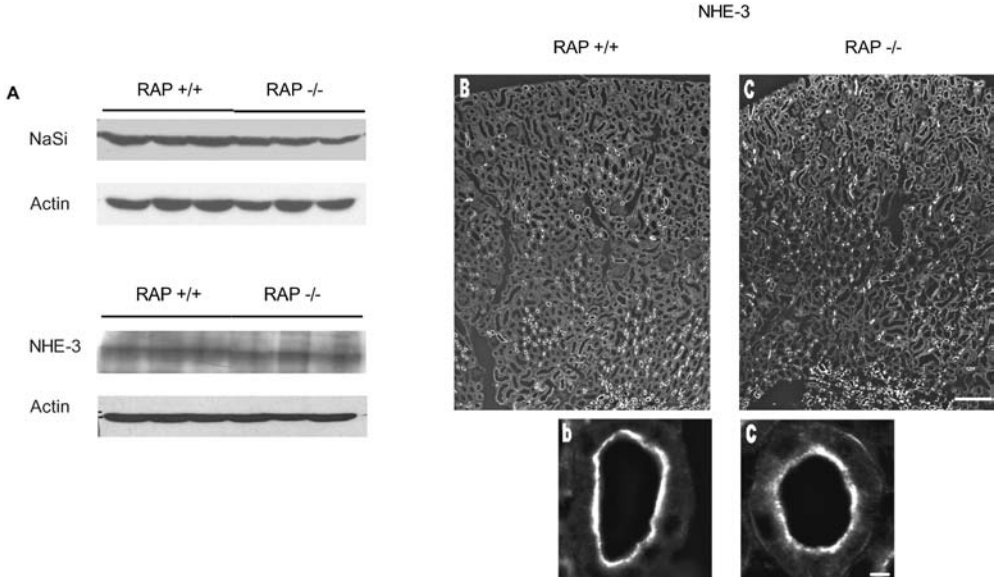

Fig. 2A–C Expression of Na^+/sulphate cotransporter (NaSi) and the Na^+/H^+ exchanger NHE-3 in kidneys from RAP+/+ and RAP–/– mice. **A** Western blotting was performed to assess the relative abundance of NaSi and NHE-3 in crude membrane fractions from kidneys of wildtype (RAP+/+) and RAP-deficient (RAP–/–) mice. All membranes were stripped and reprobed for actin to normalize for loading. The ratio of NaSi or NHE-3 and actin was calculated after densitometry to test for changes in the relative abundance. No significant change was found for NaSi or NHE-3 (data not shown). **B** Immunohistochemistry for NHE-3 demonstrated similar patterns of labelling in kidneys from wildtype and RAP-deficient mice at lower (**A**, **B**) or higher (*panels a and b*) magnification with intense staining of the brush border membrane (BBM). Scale bars: **B**, **C**~200 μm; panels b, c ~10 μm

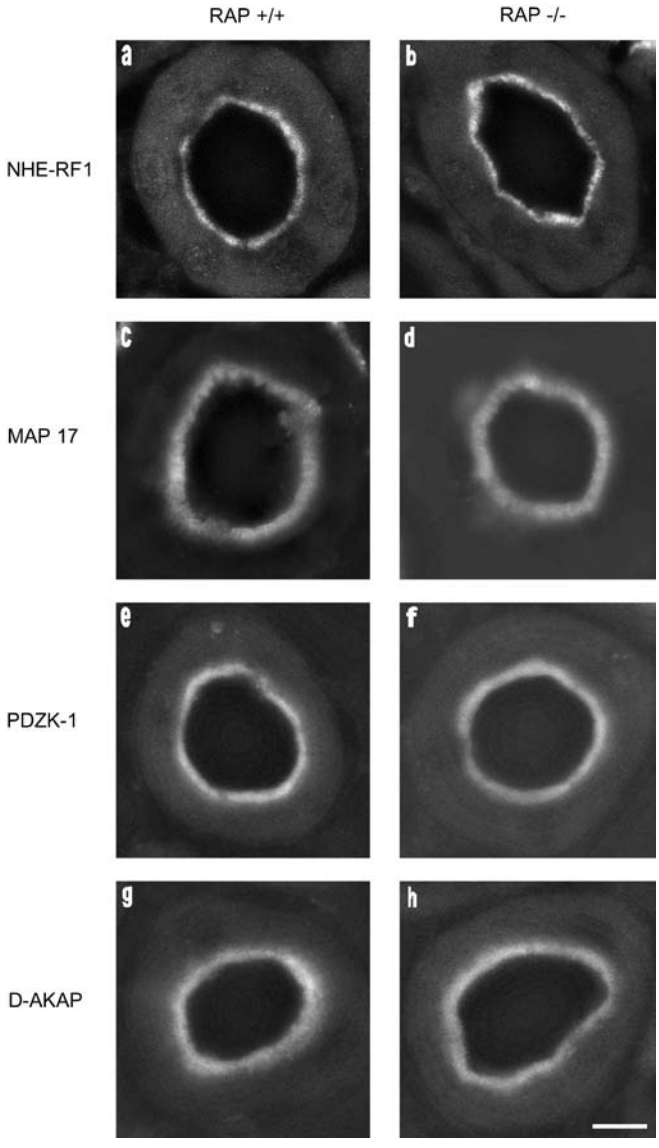

Fig. 3 Normal localization of proteins interacting with NaP_i-IIa in the proximal tubule. In vitro and in vivo data recently demonstrated interaction of NHE-RF1, MAP17, PDZK-1 and D-AKAP with NaP_i-IIa. Immunohistochemical detection of NaP_i-IIa-interacting proteins in superficial kidney cortex cryosections from wildtype (*panels a, c, e, f*) and RAP-deficient mice (*panels b, d, f, h*) demonstrates indistinguishable subcellular localization of these proteins mainly in the brush border membrane. *Scale bar* ~10 μm

measured. Comparable amounts of FITC were found in the urine in both RAP+/+ and RAP–/– mice with similar volumes of urine excreted (Fig. 4I). Furthermore, a similar intensity of staining and pattern of expression of PTH receptors on the apical and basolateral sides of proximal tubules were observed in RAP+/+ and RAP–/– mice (Fig. 5). These experiments, thus, ruled out that PTH did not reach its target or that an altered expression of PTH receptors in the proximal tubule could be responsible for the lack of PTH-induced NaP_i-IIa endocytosis.

In the next set of experiments we used freshly isolated kidney slices from RAP+/+ and RAP–/– mice to investigate the possibility that endocytosis may be delayed in kidneys from RAP–/– mice or that the PTH receptor may not be functional. As shown in Fig. 6, in kidney slices from wildtype mice (RAP+/+) incubated with 100 nM PTH, NaP_i-IIa immunostaining was strongly decreased at all time points (45 min, 90 min, 120 min)(Fig. 6D–F) compared to the control slices (Fig. 6A–C), whereas in RAP-deficient mice the PTH-induced internalization was strongly delayed (Fig. 6d–f). PTH has been shown to activate at least two signalling cascades in proximal tubule cells, the phospholipase C-PKC and the adenylate cyclase-PKA pathways, both leading to internalization of NaP_i-IIa [1, 24]. In addition, the atrial natriuretic peptide induces NaP_i-IIa internalization via cGMP and PKG [1]. In order to test if a lack of activation of these pathways by the PTH receptor may be responsible for the delayed endocytosis of NaP_i-IIa we applied 8-Br-cAMP (100 μM), 8-Br-cGMP (1 mM) or the protein kinase C activator DOG (10 μM) for 45 min directly on the kidney slices [2, 24]. Again, internalization of NaP_i-IIa was strongly reduced in proximal tubules in kidneys from RAP-deficient mice (Fig. 7). These results, thus, demonstrated that endocytosis of NaP_i-IIa is strongly impaired in RAP-deficient mice. Furthermore, the fact that internalization was even delayed after application of cAMP, cGMP or PKC activation shows that the defect is independent of the signalling cascade activated.

Renal phosphate reabsorption, localization and abundance of the NaP_i-IIa protein are strongly regulated by dietary phosphate intake. A diet rich in phosphate induces internalization and degradation of NaP_i-IIa, whereas a low-phosphate diet increases NaP_i-IIa protein abundance [13]. Therefore, we tested the adaptation of RAP+/+ and RAP–/– mice to high and low-phosphate diets. Basal urinary phosphate excretion (phosphate/mg creatinine) and reduction of phosphate excretion in response to dietary phosphate excretion were similar in both mouse groups (Fig. 8). Also the increase in phosphate excretion after switching to a high-phosphate diet was not altered. The lower mean value in the RAP–/– mice was due to two out of ten animals not responding to the high-phosphate diet and is thus not significantly different. Unfortunately, we were not able to determine serum phosphate values to calculate the fractional renal excretion rates of phosphate. Immunohistochemistry showed indistinguishable NaP_i-IIa abundance and localization under both low- and high-phosphate diet (data not shown). Also Western blotting demonstrated the ability of RAP–/– mice to adapt the abundance of NaP_i-IIa protein to the dietary phosphate intake similar to wildtype mice. NaP_i-IIa protein abundance was similar under high-, normal and low-phosphate diet in RAP-deficient and wildtype mice as determined by Western blotting and calculated as NaP_i-IIa/actin ratio (Fig. 9). Furthermore, the ability of RAP–/– mice to downregulate NaP_i-IIa expression in response to an acute switch from low- to high-phosphate diet (4 h after feeding a high-phosphate diet) was not affected as demonstrated by immunohistochemistry (Fig. 10).

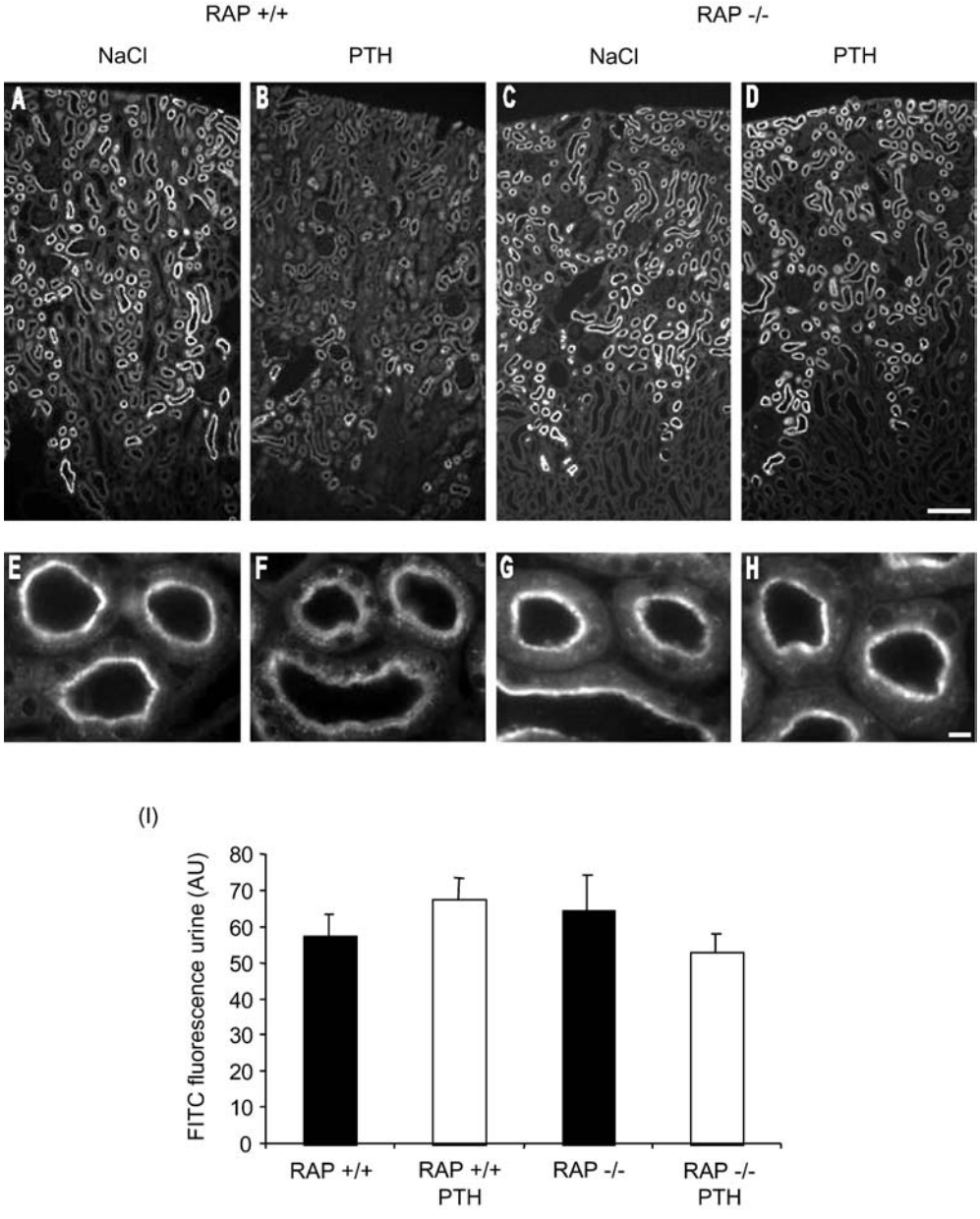

Fig. 4A–I Lack of parathyroid hormone (PTH)-induced NaP_i-IIa internalization in intact animals after PTH injection in RAP-deficient mice. Saline (0.9% NaCl) or PTH were injected into the tail vein of wildtype and RAP-deficient mice. Mice were fixed by perfusion 20 min later and kidneys stained for NaP_i-IIa. In NaCl-injected wildtype mice (**A**, **B**, **E**, **F**) NaP_i-IIa staining was strongest in proximal convoluted tubules of juxtamedullary nephrons (**A**, **E**). In wildtype mice injected with PTH (**B**, **F**) weaker NaP_i-IIa staining demonstrates that PTH induced internalization and degradation of BBM NaP_i-IIa. In contrast, in RAP-deficient mice NaP_i-IIa labeling was found with the same intensity and distribution pattern in NaCl (**C**, **G**) or PTH (**D**, **H**) injected animals. Scale bars: **A**–**D** ~200 μm; **E**–**H** ~10μm. **I** Urinary excretion of FITC-dextran (10 kDa) which was coinjected with PTH or NaCl into the tail vein. Urine was collected from the urinary bladder 20 min after injection and FITC-fluorescence measured. The total urine volume was similar in all groups

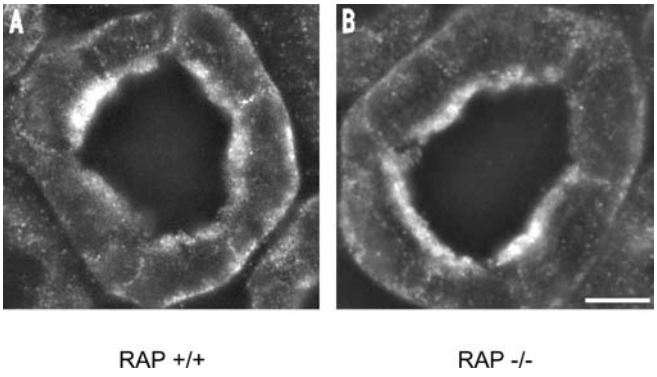

Fig. 5A, B Normal expression and localization of PTH receptors in the proximal tubule of RAP+/+ and RAP–/– mice. Immunostaining for the PTH receptor showed indistinguishable labelling on the apical and basolateral side of proximal tubules in both wildtype (**A**) and RAP-deficient (**B**) mice. *Scale bar* ~10 μm

Discussion

NaP_i-IIa represents the major phosphate-reabsorbing transport system in the kidney [16]. Because of the physiological importance of plasma phosphate levels the activity of NaP_i-IIa is tightly regulated by a variety of factors. In general, factors decreasing renal phosphate reabsorption, such as PTH, atrial natriuretic peptide, or high dietary phosphate intake, lead to a downregulation of NaP_i-IIa protein abundance in the BBM by inducing endocytosis and lysosomal degradation [16, 20, 23]. To date, little has been known about the underlying mechanisms involved in the endocytotic retrieval of NaP_i-IIa transporter proteins.

In the present study we used RAP-deficient mice which have been shown to express strongly reduced

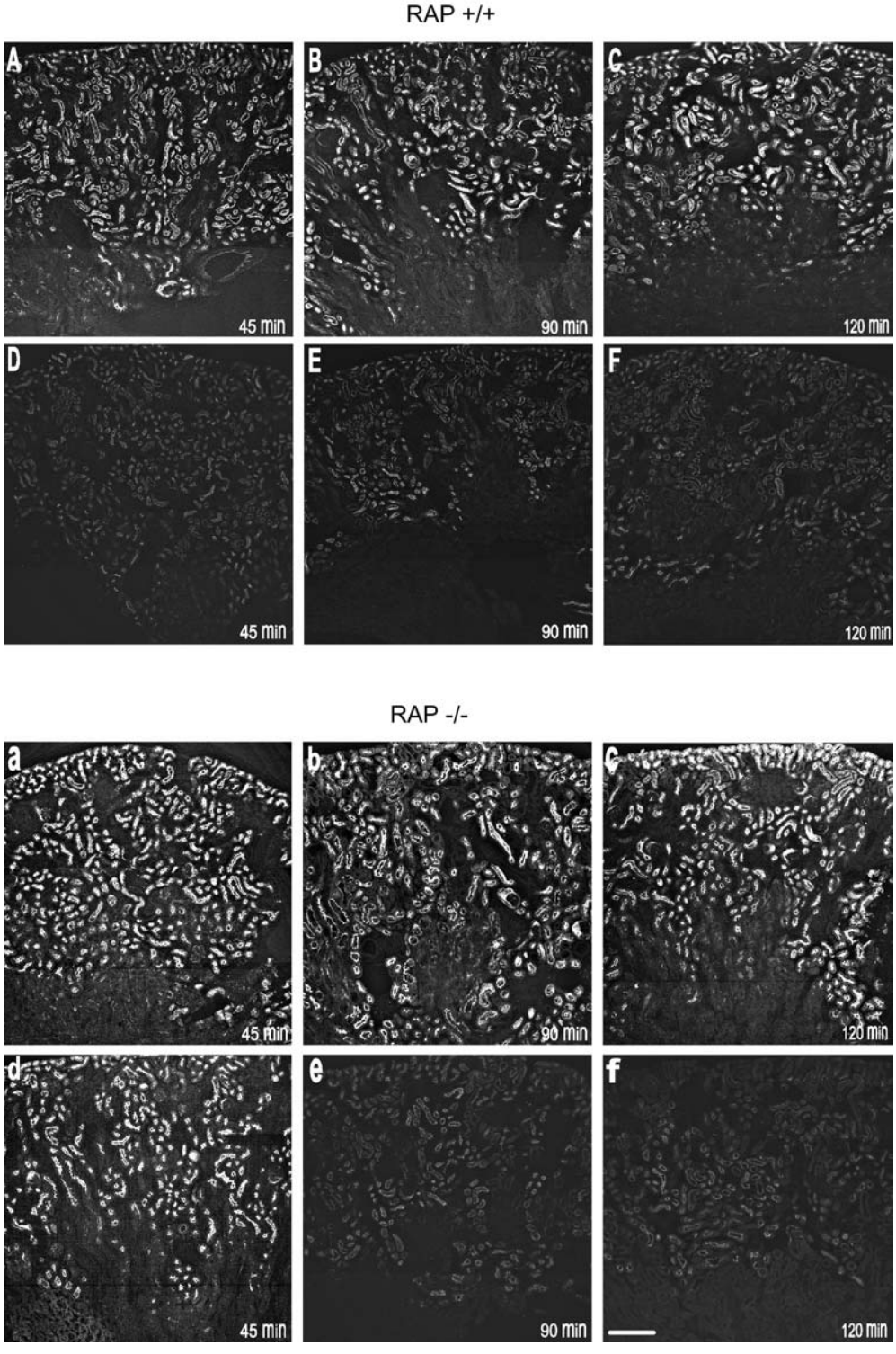

Fig. 6 Delayed PTH-induced internalization of NaP_i-IIa in isolated kidney slices in vitro. Overview pictures over kidney slices prepared from wildtype (*RAP+/+*; *upper panels A–F*) and RAP-deficient (*RAP−/−*; *lower panels a–f*) mice, incubated with 100 nM PTH for several time periods, fixed, and stained for NaP_i-IIa protein. For all time points, 45 min (*panels A, a*), 90 min (*panels B, b*) and 120 min (*panels C, c*), control and PTH-exposed slices were obtained from the same kidneys. The intensity and localization of NaP_i-IIa immunostaining did not change between all three time points in control slices of both wildtype and RAP-deficient mice. Incubation of kidney slices with PTH induced a rapid internalization of NaP_i-IIa in the wildtype and was almost complete after 45 min (*panel D*), whereas in the RAP-deficient mice internalization was delayed and occurred only between 45 and 90 min after PTH exposure (*panels d, f*). *Scale bars*: panels A–F, panels a–f ~200 μm

amounts of megalin in the proximal tubule [5] to examine mechanisms of NaP_i-IIa regulation via endocytosis [5, 17, 18, 28]. Megalin plays an important role in the receptor-mediated endocytosis of substances from the urine, among them vitamin-binding protein, retinol-binding protein, several hormones and hormone-like factors, microglobins and calcium [7, 8, 18]. In addition, it has been suggested that megalin may be important for the regulation or stability of the Na^+/H^+ exchanger NHE-3 in the BBM of the proximal tubule [4]. Our study is the first demonstration of a role for megalin in the regulation of a BBM protein, the NaP_i-IIa. Two lines of evidence support the notion that the endocytotic internalization of NaP_i-IIa is disturbed in RAP-deficient mice but not PTH sig-

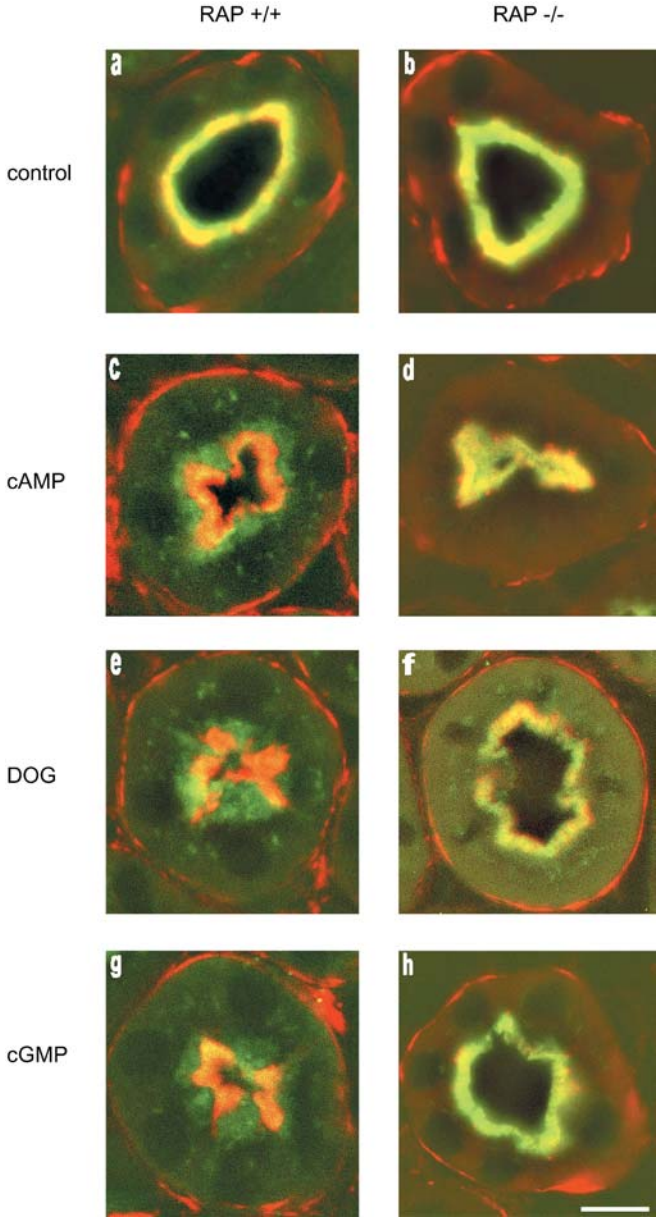

Fig. 7 Stimulation of NaP_i-IIa internalization with cAMP, cGMP and PKC activation in isolated kidney slices. Kidney slices from wildtype (RAP+/+) and RAP-deficient (RAP–/–) mice were prepared and incubated for 45 min either with control solution (*panels a, b*), 8-Br-cAMP (100 μM) (*panels c, d*), the PKC activator 1,2-dioctanoyl-sn-glycerol (DOG) (10 μM) (*panels e, f*), or 8-Br-cGMP (1 mM) (*panels g, h*). The sections were stained with an antibody against NaP_i-IIa (*green*) and with rhodamine-phalloidin against β-actin filaments (*red*). Under control conditions a high degree of overlap (yellow) between NaP_i-IIa (*green*) and actin (*red*) is seen demonstrating localization of NaP_i-IIa in the brush border membrane in both wildtype and RAP-deficient mice (*panels a, b*). After the treatment with cAMP (*panel c*), DOG (*panel e*) or cGMP (*panel g*), respectively, in wildtype mice NaP_i-IIa cotransporter-related fluorescence in the brush border decreases, whereas the subapical cytoplasm shows a weak but distinct signal. In contrast, in kidney slices from RAP-deficient mice NaP_i-IIa still resides in the BBM indicating defective internalization. The pictures were all taken from the S1 segments of superficial nephrons. *Scale bar* ~10 μm

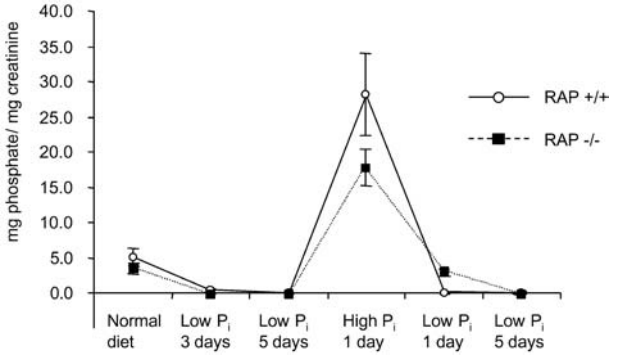

Fig. 8 Urinary phosphate excretion in response to different diets rich or low in phosphate. Urinary phosphate excretion was measured in ten wildtype and ten RAP-deficient mice by collecting spot urine samples. All samples were analysed for phosphate and normalized for creatinine excretion in the same sample. Mice received normal diet, a diet low in phosphate (0.1%) or rich in phosphate (1.2%). For switching to the high-phosphate diet, mice did not receive food the night before to increase food intake. For all time points no significant differences were found between wildtype and RAP-deficient mice

nalling. First, activation of internalization with pharmacological agents (8-Br-cAMP, 8-Br-cGMP, DOG) led to internalization which was also delayed suggesting that not a failure of activation of these pathways was responsible for the delay. Interestingly, this also supports the idea that all these pathways may converge in the same mechanism leading to endocytotic retrieval of NaP_i-IIa. Secondly, it has recently been shown that megalin may regulate activation of PTH receptors by antagonizing PTH [12]. Thus, a stronger or faster response to PTH would be expected rather than a delay. In addition, we found that the localization and relative abundance of PTH receptors was similar in both wildtype and RAP-deficient mice.

The reduction in megalin in RAP-deficient mice seems not to influence the steady-state expression level of NaP_i-IIa as well as of the Na^+/sulphate cotransporter and the Na+/H+ exchanger isoform 3 (NHE-3), despite its shown interaction with megalin [4]. However, the functional significance of the NHE-3 and megalin interaction may need further investigation.

Presently, several questions remain to be answered. It needs to be clarified if the reduction in megalin alone is responsible for the observed impairment of PTH-induced endocytosis or if other factors are also affected by RAP deficiency. The fact that NaP_i-IIa internalization was delayed but not abolished may suggest that the residual megalin expression was sufficient for endocytosis or that other proteins may partially compensate. It can also not be ruled out that loss of RAP which acts as a chaperone for several members of the LDL-receptor family alters the expression or function of other proteins important for NaP_i-IIa function/retrieval. Further experiments using megalin-deficient cells or animals may help to answer this question. Another important question is to determine the potential molecular role of megalin in the endocytosis of NaP_i-IIa, i.e. the nature of interaction and if other

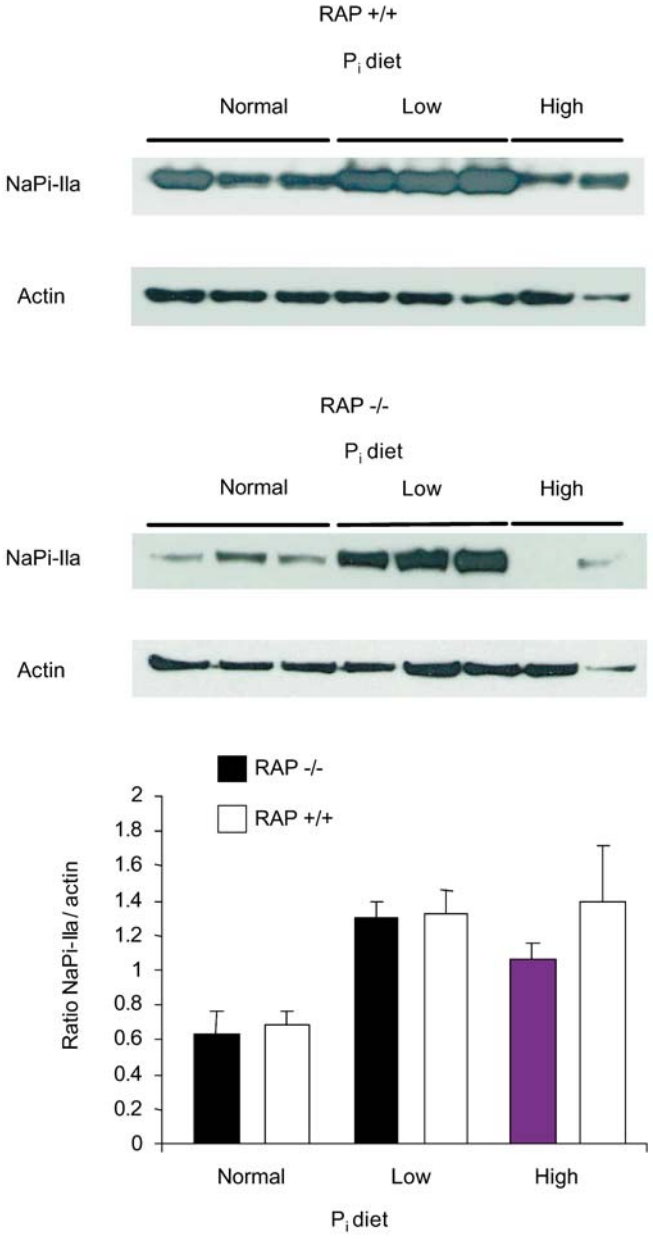

Fig. 9 Chronic adaption of NaP_i-IIa protein expression in whole kidneys to low-, normal, and high-phosphate diets. Wildtype and RAP-deficient mice were adapted for 5 days to diets containing a low, normal or high concentration of phosphate and relative NaP_i-IIa protein abundance was assessed by Western blotting in a crude membrane fraction. All Western blotting membranes were stripped and reprobed against actin to normalize for protein loading. Both wildtype and RAP-deficient mice adapted similarly NaP_i-IIa protein expression to the dietary phosphate intake

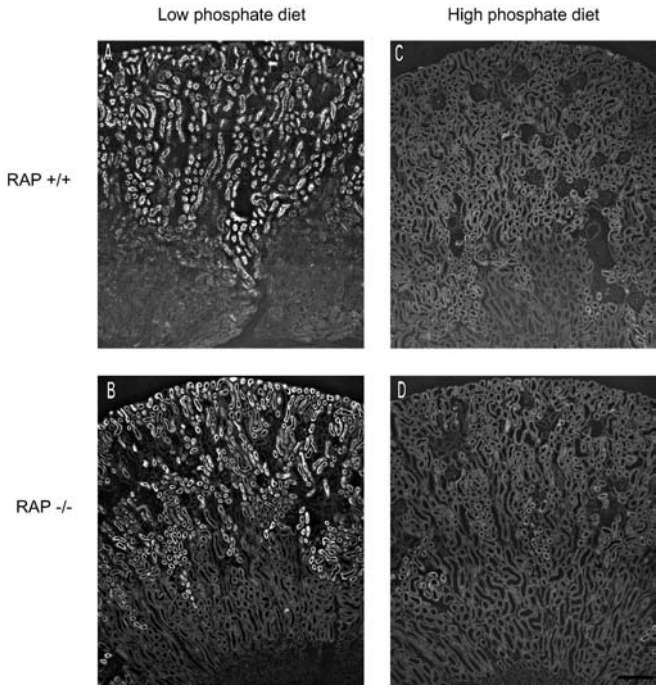

Fig. 10 Acute adaption to a high-phosphate diet induced internalization of NaP_i-IIa in intact animals. Overview pictures from whole kidneys from wildtype and RAP-deficient mice which had been kept for 5 days on a low-phosphate diet (0.1%) (*panels A, B, a, b*). Some animals were acutely switched to a high-phosphate diet (1.2%) and kidneys were collected 4 h afterwards (*panels C, D, c, d*). The decrease in NaP_i-IIa-specific immunoreactivity after acute adaptation to a high-phosphate diet is similarly observed in both animal groups. *Scale bars* ~200 μm

proteins are involved. Based on yeast-two-hybrid experiments in our laboratory where we used several intracellular parts of NaP_i-IIa as baits we did not find any evidence for direct megalin/NaP_i-IIa interactions suggesting that intermediate proteins may be involved (Gisler, Pribanic, Bacic, Sabourin, Tsuji, Manser, Biber, and Murer, unpublished results). Moreover, the endocytotic pathway necessary for NaP_i-IIa routing to the lysosome will need further characterization to better understand the regulation of proximal tubule transport proteins. Recently, two mouse models for Dent's disease lacking the ClC-5 channel, have been shown to suffer from reduced endocytosis along the proximal tubule [21, 26]. Interestingly, phosphaturia, reduced megalin and NaP_i-IIa expression was found in ClC-5-deficient mice [21]. The latter, however, may be due to altered renal handling of PTH and vitamin D. If ClC-5 is also directly involved in the regulation of NaP_i-IIa retrieval is not clear to date.

In summary, we have shown PTH-induced endocytosis is severely impaired in RAP-deficient mice which represent a useful model for megalin deficiency. Endocytosis of NaP_i-IIa is delayed but not abolished, which may be due to residual megalin expression or other compensatory mechanisms. However, our results underline the significance of an intact endocytotic apparatus for the normal regulation and internalization of an important proximal tubule brush border membrane protein such as NaP_i-IIa.

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