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Interactions of the growth-related, type IIc renal sodium/phosphate cotransporter with PDZ proteins

R Villa-Bellosta[1,4], **M Barac-Nieto**[1,2,4], **SY Breusegem**[3,4], **NP Barry**[3], **M Levi**[3], and **V Sorribas**[1]

[1] Laboratory of Molecular Toxicology, Department of Toxicology, University of Zaragoza, Zaragoza, Spain

[2] Department of Physiology, Kuwait University, Kuwait City, Kuwait

[3] Departments of Medicine, Physiology and Biophysics, VA Medical Center and University of Colorado Health Sciences Center, Denver, Colorado, USA

Abstract

Despite similar molecular structures, the growth-related sodium/phosphate cotransporter NaPiIIc is regulated differently than the main NaPiIIa phosphate transporter. Using two-hybrid systems and immunoprecipitation, we identified several proteins that interact with NaPiIIc that might account for this differential regulation. NaPiIIc interacted with the PDZ domain-containing sodium–hydrogen exchange-regulating factor (NHERF) 1 and NHERF3 through novel binding motifs in its C terminus. NaPiIIc from brush-border membranes coprecipitated with both NHERF1 and NHERF3, with more NHERF3 co-precipitated in rats fed a low-phosphorus diet. NaPiIIc colocalizes with both NHERF1 and NHERF3 in brush-border membranes of rats fed either a low- or high-phosphorus diet. When mouse NaPiIIc was transfected into opossum kidney cells, it was localized mainly in apical microvilli and the *trans*-Golgi. Both confocal and total internal reflection microscopy show that NaPiIIc colocalizes with NHERF1 and NHERF3 in the apical microvilli, and this was not altered by truncation of the last three amino acids of NaPiIIc. Interactions of NaPiIIc with NHERF1 and NHERF3 were modulated by the membrane-associated 17 kDa protein (MAP17) similarly to NaPiIIa, but only the MAP17–NaPiIIc–NHERF3 complexes were internalized to the *trans*-Golgi. Our study shows that NaPiIIc interacts with a limited number of PDZ domain proteins, and the mechanisms and consequences of such interactions differ from those of NaPiIIa.

Keywords

phosphate transporter; NaPiIIc; PDZ; protein interaction; OK cells; rat

Type II Na-coupled phosphate transporters are the molecules responsible for tubular reabsorption of inorganic phosphate (Pi) and are the target of hormonal and non-hormonal mechanisms that control phosphate homeostasis.[1,2] NaPiIIa is responsible for about 70% of the Pi reabsorbed in the adult kidney,[3] while NaPiIIc handles the remaining 30%.[4] These percentages depend, however, on the age of the animals, because NaPiIIc seems to be more

Correspondence: V Sorribas, Laboratory of Molecular Toxicology, University of Zaragoza, Miguel Servet 177, 50013 Zaragoza, Spain. E-mail: sorribas@unizar.es.
[4]These authors contributed equally to this work.

DISCLOSURE

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active in the young.[5,6] In spite of sharing a very similar molecular structure, NaPiIIa and NaPiIIc exhibit an increasing list of physiological differences. For example, while NaPiIIa is electrogenic,[7] NaPiIIc is electroneutral.[6] Furthermore, both transporters participate in the renal adaptation to changes in dietary Pi with different characteristics: in response to a high Pi intake, NaPiIIa abundance changes quickly (less than 1 h) throughout the proximal tubule, internalization takes place in a microtubule-independent way, and molecules are targeted to the lysosome via endosomes.[8,9] In contrast, NaPiIIc responds slowly (4 h), abundance changes are observed in the S1 segment only, molecules are internalized through a micro-tubule-dependent pathway, and rather than being degraded, they are accumulated in a subapical compartment.[10] Similar differences have been reported recently for the response of NaPiIIc to parathyroid hormone.[11]

Some of these differences may be explained by the type and number of proteins that the transporters interact with. For example, it is well known that NaPiIIa is integrated in a series of macromolecular complexes whose architecture is based on PDZ-type (PSD-95, discs-large, and ZO-1) protein interactions.[12,13] NaPiIIa participates in this complex by means of a class I PDZ-binding site located at the carboxyl (C) terminus of NaPiIIa, comprising its last three amino acids (TRL). This classical binding site is missing in NaPiIIc, in which QQL is the C terminus of the human, mouse, and rat transporters. Consequently, we have performed a study to identify proteins that interact with NaPiIIc using baits corresponding to different domains of the transporter. We have observed that NaPiIIc, as does NaPiIIa, interacts with PDZ domain-containing proteins, sodium–hydrogen exchange-regulating factor (NHERF) 1 and NHERF3, but not with others such as NHERF2, NHERF4, PIST, (PDZ domain protein interacting specifically with TC10) and SHANK (SH3 domain and ankyrin repeat-containing proteins). The nature, consequences, and functions of the PDZ interactions seem to be very different for the two transporters.

RESULTS

Identification of proteins that interact with NaPiIIc

A two-hybrid strategy in *E. coli* was followed with two baits to identify interacting proteins in a mouse kidney cortex cDNA library. We screened 100 000 clones in several cotransformations using the third extracellular loop, 114-amino-acid long, of NaPiIIc as bait (LB; Figure 1a). The clones that were obtained are listed in Table 1 and, of note, 11 corresponded to the lysosomal acid glucosidase (NM_021517). When a 65-amino-acid-long bait derived from the C terminus (CB) was used, a total of 89 000 clones were screened, from which 25 corresponded again to acid α-glucosidase, 5 to NHERF3[PDZK1],[14] and 4 to napsin (a lysosomal aspartyl protease). The NHERF3 interaction was not expected because NaPiIIc does not contain a classical PDZ binding site at the C terminus.

Some of the interactions obtained with the C-terminal bait were also tested using a mammalian two-hybrid system (Clontech, Mountain View, CA, USA). Opossum kidney (OK) cells were cotransfected with CB bait in pM plasmid, plus a PDZ protein as a prey in pVP16, and secreted alkaline phosphatase as the reporter enzyme of the system. A full-length CB or a truncated C terminus (in which the last three amino acids had been eliminated) was used as bait. We tested interactions with several relevant PDZ proteins directly (Figure 1b) after the identification of NHERF3 in the library screen. The intensities of the interaction signals derived from the activity of the reporter enzyme were impressed in X-ray films and are shown in Figure 1b and c: In addition to confirming the interaction with NHERF3, NHERF1[15] also showed interaction with the carboxy bait. However, other proteins such as NHERF2[16] NHERF4,[17] PIST,[18] and SHANK2E[19] were negative in this assay. Very interestingly, the truncated bait failed to interact with NHERF3 but did interact with NHERF1 (Figure 1b). An analysis of the individual PDZ

domains showed that NaPiIIc C terminus interacts with the first domain of NHERF1, and with the second PDZ domain of NHERF3 (Figure 1c).

Confirmation of the interactions by coimmunoprecipitation

The interactions of NaPiIIc with NHERF1 and NHERF3 were further tested by coimmunoprecipitation in two different model systems. First, OK cells were cotransfected with FLAG-tagged NaPiIIc and with myc-tagged NHERF1 or NHERF3. Forty-eight hours after transfection, NaPiIIc was precipitated with an anti-FLAG antibody and the corresponding NHERF protein detected with an anti-myc antibody in a western blot (Figure 2a). NHERF3 but not NHERF1 successfully coimmunoprecipitated, therefore confirming the interaction observed between NaPiIIc and NHERF3 using both the bacterial and the mammalian two-hybrid systems.

To assess the relevance of these results *in vivo* and to exclude possible artificial interactions that can occur in OK cells expressing various levels of exogenous proteins, we immunoprecipitated NaPiIIc from rat kidney cortex using a specific polyclonal antibody (Figure 2b), and probed for coimmunoprecipitation of NHERF1 and NHERF3. The NaPiIIc antibody recognized a band of 82 kDa of greater intensity in juxtamedullary (JM) than in superficial cortical brush-border membrane (BBM) vesicles from rat kidney cortex (Figure 2c). The antibody also revealed adaptation of NaPiIIc abundance of both superficial and JM BBM to chronic changes in dietary concentration of Pi, as previously reported.[10] NHERF1 and NHERF3 were also expressed in BBM of these animals, and did not show expression changes in response to altered Pi concentration in the diet (Figure 2d, lysates). More importantly, both PDZ-containing proteins were successfully coimmunoprecipitated by the NaPiIIc antibody from superficial and JM BBM (only JM BBM precipitations are shown in Figure 2d). NaPiIIc was more successfully precipitated in BBM of animals eating low-Pi-containing fodder (data not shown); however, the abundance of coprecipitated NHERF1 was similar in BBM of animals eating low- and high-Pi-containing fodder, and the coprecipitated NHERF3 was more abundant in BBM of animals chronically fed a low- compared to a high-Pi-containing diet.

Subcellular distribution of NaPiIIc

The distribution of mouse NaPiIIc expressed in OK cells was studied by fluorescent confocal microscopy (Figure 3). Full-length cyan fluorescent protein (CFP)-NaPiIIc fusion protein was mainly expressed in the apical membrane, showing a patched pattern of distribution typical of several membrane proteins of proximal tubule origin, including NaPiIIa, and representing the apical microvilli (Figure 3a). As in the case of membrane-associated 17 kDa protein (MAP17),[20] most cells also showed a perinuclear distribution of NaPiIIc compatible with its location at the *trans*-Golgi network (TGN). This localization was confirmed by coexpression of NaPiIIc with a yellow fluorescent protein (YFP)-Golgi marker (Figure 3b). An identical distribution was obtained when expressing a FLAG-tagged NaPiIIc and using immunodetection with an anti-FLAG antibody (data not shown).

Next, the role of the C-terminus of NaPiIIc on its cellular distribution was analyzed by expression of a truncated YFP-NaPiIIc fusion protein, in which the last three amino acids had been eliminated (Figure 3c). While this mutation modifies the cell distribution of other transporters, such as NaPiIIa,[21,22] truncated NaPiIIc behaved exactly as the full-length transporter, that is, it was also expressed in apical membrane patches and in the TGN. Not only its distribution but its function as Pi transporter was also unaltered when truncated and complete NaPiIIc were expressed in *Xenopus laevis* oocytes (Figure 3d).

Colocalization of NaPiIIc with NHERF1 and NHERF3 in OK cells

As requisite for validation of *in vitro* interactions, colocalization of NaPiIIc, NHERF1, and NHERF3 was verified, both in OK cells coexpressing mouse NaPiIIc- and NHERF-encoding plasmids (Figure 4) and in rat kidney sections (Figure 5). Confocal imaging shows that NaPiIIc colocalizes with both NHERF1 (Figure 4a) and NHERF3 (Figure 4b) at the apical cell membrane in a patched distribution pattern similar to that of NaPiIIa. In addition, NaPiIIc and NHERF3, but not NHERF1, also colocalize in the TGN.

The colocalization of NaPiIIc and NHERF1/3 in apical microvilli was confirmed by total internal reflection fluorescence (TIRF) microscopy. Using this technique, only fluorescence within 200 nm of the coverslip is excited.[23] Thus, when filter-grown OK cells are turned onto coverslips, images of the apical microvilli and intervillar regions with high signal-to-noise ratio are obtained due to elimination of intracellular background (Figure 4c and d). Both NaPiIIa and NaPiIIc are mainly localized to the microvilli. NHERF1 shows both microvillar as well as intermicrovillar components. The visibility of this intermicrovillar pool, presumably due to NHERF1 within the subapical space, appears to be a function of its expression level. For NHERF3, the same expression pattern is seen; however, the intermicrovillar pool appears more prominent than that of NHERF1. The overlay images show a large degree of colocalization of both Na/Pi cotransporter proteins with NHERF1 and NHERF3 in the apical microvilli.

Colocalization of NaPiIIc with NHERF1 and NHERF3 in kidney cortex

Kidneys from rats chronically adapted to a low-Pi or a high-Pi diet were fixed-perfused and processed for immunofluorescence. A NaPiIIc-specific polyclonal antibody stained the luminal BBMs of proximal tubules, as reported[6,10] (Figure 5), with decreased expression in rats fed a high-Pi diet compared to rats fed a low-Pi diet (Figure 5a). However, this adaptation of NaPiIIc did not modify the pattern of colocalization of NaPiIIc with NHERF1 and NHERF3 as shown in Figure 5b and c: both in kidney sections derived from rats on a 0.1 or 1.2% Pi diet, colocalization of NaPiIIc with NHERF1 and NHERF3 in the proximal tubule BBM was observed (Figure 5b and c, yellow overlay in third column of panels). All three proteins also colocalized with the β-actin cytoskeleton, as revealed by the white color in the three-color merge images (Figure 5b and c, fourth column of panels).

Role of MAP17 in NaPiIIc–NHERF interaction

MAP17, a small membrane protein found in the proximal tubule, ‘activates’ NHERF3 to internalize the ternary complex MAP17 + NHERF3 + NaPiIIa to the TGN.[20] While the role of such a process is still not clear, we tested whether MAP17 would also induce internalization of NaPiIIc through interaction with PDZ proteins. Figure 6a shows that coexpression of MAP17 and NaPiIIc has no effect on the distribution of NaPiIIc, as reported previously for NaPiIIa. However, when cotransfected with NHERF3, the complex MAP17−NHERF3−NaPiIIc is internalized to the TGN (Figure 6c), as in the case of NaPiIIa. This effect is specific for NHERF3, because NHERF1 was not able to induce the translocation (Figure 6b).

DISCUSSION

In this work, we have demonstrated differences in protein/protein interactions between NaPiIIa and NaPiIIc. We have shown that the number of PDZ proteins that interact with NaPiIIc appears to be less than with NaPiIIa. While NaPiIIa interacts with NHERF1–4, CAL^{PIST}, and SHANK2E,[12] NaPiIIc only interacts with NHERF1 and NHERF3. In addition, the last three amino acids of NaPiIIc are not necessary for the interaction with NHERF1, because the reporter enzyme in the mammalian two-hybrid system is still activated with the truncated NaPiIIcΔQQL (Figure 1b). By contrast, a construct missing the last three (TRL) amino acids of NaPiIIa is not able to interact with either NHERF1, NHERF3, or NHERF4.[22] Although

complete NaPiIIc only activated the reporter when cotransfected with the first PDZ domain of NHERF1, this does not preclude weaker interaction with the other domain of NHERF1 (Figure 1c). When expressed in OK cells, NaPiIIc does not coimmunoprecipitate NHERF1 but does coprecipitate NHERF3 (Figure 2a). In the renal BBM, however, NaPiIIc coprecipitates both NHERF1 and NHERF3. This could be explained by differences in the interactions of endogenously expressed proteins compared with those of expressed foreign proteins (mouse proteins in OK cells). In addition, only BBM NHERF3 is more abundantly coprecipitated when NaPiIIc expression increases in response to chronic low dietary Pi (Figure 2d).

In conclusion, these results suggest that the interaction NaPiIIc–NHERF1 differs in nature from the interaction NaPiIIc–NHERF3. To our knowledge, we show for the first time expression of NaPiIIc in an *in vitro* renal cell model system. Confocal fluorescence microscopy reveals that NaPiIIc distributes apically in membrane patches in OK cells, as NaPiIIa does (Figures 3 and 4). In addition, TIRF microscopy demonstrates colocalization of NaPiIIa and NaPiIIc with NHERF1/3 in the apical microvilli but not in the intervilli regions of these cells. The last three amino acids of NaPiIIc (QQL) constitute a C-terminal motif required for its interaction with NHERF3 that cannot be classified within any of the three major groups of PDZ-binding motifs.[24] By contrast, the C terminus of NaPiIIa (TRL) belongs to class I PDZ motifs and has a role in the cellular distribution of the transporter. The truncated mutant, NaPiIIaΔTRL, shows altered expression at the apical membrane, including a loss of the patched distribution.[22] By contrast, NaPiIIcΔQQL distributes exactly as the complete NaPiIIc does in OK cells, and shows the same transport activity as native NaPiIIc when expressed in *Xenopus* oocytes (Figure 3c and d). Since OK cells express an endogenous NHERF1 (V Sorribas, unpublished data) and NaPiIIcΔQQL interacts with NHERF1 (Figure 1b), this interaction could be involved in the maintenance of a dominant apical expression pattern of the truncated NaPiIIc in OK cells, particularly since the $P_{578}R_{579}$ motif in NaPiIIa, mandatory for its apical expression,[22] is absent in NaPiIIc.

Compared to adult NHERF1(−/−) mice, young mice have defective targeting of NaPiIIa to apical membranes in the renal proximal tubule, and manifest hypophosphatemia and phosphaturia.[25] In contrast, NHERF3(−/−) mice only exhibit attenuated expression of NaPiIIa and hyperphosphaturia when fed high-Pi diets.[26] These observations suggest that renal phosphate reabsorption, particularly in the young mice, is more sensitive to deletion of NHERF1 than of NHERF3, consistent with our *in vitro* finding that truncation of NaPiIIc, which obliterates its interaction with NHERF3 but not that with NHERF1, has little effect on its cell distribution or transport properties. In terms of its regulation, however, we observed that NaPiIIc–NHERF3 complexes are more abundant in BBM of animals adapted to low compared to high dietary Pi, while the abundance of NaPiIIc–NHERF1 complexes is not modified (Figure 2d). While this could be due in part to the different nature of the these interactions with distinctive consequences for their coimmunoprecipitation, it could also indicate that the NaPiIIc–NHERF1 interaction is important to maintain a basal level of NaPiIIc apical expression, while the NaPiIIc–N HERF3 interaction is relevant for the adaptation to low dietary Pi. Clearly, important investigative efforts are necessary to understand the roles of the interactions of NHERF1 and NHERF3 with NaPiIIc and NaPiIIa in Pi renal reabsorption.

Finally, the effects of the small membrane protein MAP17 on NaPiIIc and NaPiIIa[20] were similar: when NaPiIIc and MAP17 are coexpressed with or without NHERF1, no apparent effects are observed on the distribution of any of these proteins. However, when coexpressed with NHERF3, the three proteins (MAP17–NaPiIIc–NHERF3) are expressed in the TGN (Figure 6). This could be due to either internalization from the apical membrane, or to failure to export the complex to the BBM. However, the effect of MAP17–NHERF3 on NaPiIIa, also in OK cells, was due to direct internalization of the expressed proteins,[20] and therefore, it is

likely that a similar mechanism is acting on NaPiIIc. Nevertheless, the functional significance of these interactions and translocations are still unclear.

In summary, we have shown that NaPiIIc interacts with PDZ domain containing proteins NHERF1 and NHERF3, but the nature of the physical interactions and their consequences seem to differ from those of NaPiIIa with NHERF1–4. Additional work is necessary to clarify, as in the case of NaPiIIa, the physiological consequences of these interactions.

MATERIALS AND METHODS

Identification of NaPiIIc-interacting proteins

A mouse kidney cDNA library of the Bacteriomatch II Two-Hybrid System (Stratagene, La Jolla, CA, USA) was screened as explained[20] using two baits generated from mouse NaPiIIc (accession number NM_026018). Figure 1a represents the secondary structure of the transporter based on the latest findings for NaPiIIa,[7] and the baits are indicated with arrows. The extracellular bait consisted of the 114 amino acids of the third external loop and was prepared into pBT by directional cloning of PCR amplicons using the following primers: sense 5′-GACGAATTCCGCAGCATTAGAGAGA-3′, antisense 5′-GACCTCGAGTGTGAGTTTTGAGCCG-3′ (underlined are the restriction sites *Eco*RI and *Xho*I added for directional cloning). The second bait included the last 65 amino acids of NaPiIIc and corresponded to the internal C terminus of mouse NaPiIIc. This bait was cloned into pBT with the following primers containing also *Eco*RI and *Xho*I sites: sense 5′-GACGAATTCACAACACCGAC CATCT-3′, antisense 5′-GACCTCGAGTCACAACTGCTGAGAA-3′.

Mammalian two-hybrid interactions

Two-hybrid interactions in OK cells were performed using the Matchmaker Mammalian Assay kit from Clontech as reported.[20] NaPiIIc baits corresponding to the C terminus were cloned into pM by PCR amplification with primers harboring *Eco*RI and *Hin*dIII sites, for the full-length carboxyl bait: sense 5′-GACGAATTCCAAC ACCGACCATCTT-3′; antisense 5′-GACAAGCTTTCACAACTGCT GAGAA-3′. For a truncated bait in which the last three amino acids had been eliminated (IIcΔQQL), a modified antisense primer was used to advance the stop codon (bold) three positions in combination with the same sense primer as for the complete bait: antisense 5′-GACAAGCTTTTCACAACTGCTAAGAAGC-3′. The cDNA preys were inserted as full-length open reading frames into pVP16. The individual PDZ domains of NHERF1 and NHERF3 were also used as explained.[20]

Antibody preparation and western blotting

A chicken polyclonal antibody was prepared against a peptide (AHCYENPQVIASQQL) corresponding to amino acids 561–575 of the cytosolic C terminus of rat NaPiIIc (Davids Biotechnologie, Regensburg, Germany). The 15-aa peptide was conjugated to keyhole limpet hemocyanin, mixed in Freund's complete adjuvant, and injected into hen. Three booster injections were given every 2 weeks to the animals, and then 10 eggs were collected. A monospecific antibody was affinity purified from egg yolk IgG fraction using the antigenic peptide. Specificity of signals was determined by blocking the antibody with the antigenic peptide using a standard procedure. The antibody was used at 1:8,000 dilution for western blotting, and 1:500 dilution for immunofluorescence microscopy.

Coimmunoprecipitations

For coimmunoprecipitation in OK cells, the open reading frame of mouse NaPiIIc cDNA was cloned in frame into pFLAG-CMV-6c (Sigma, St Louis, MO, USA). Open reading frames of

NHERF1 and NHERF3 were cloned into pCMV-Myc (Clontech). Both pFLAG-NaPiIIc and either pCMV-Myc NHERF1 or NHERF3 plasmids were cotransfected in OK cells. Coimmunoprecipitation was performed as described[27,28] with mouse anti-FLAG monoclonal antibody (Sigma) and detected with a rabbit anti-myc polyclonal antibody (Invitrogen, Carlsbad, CA, USA).

Coimmunoprecipitation of NaPiIIc and NHERF proteins from rat kidney was carried out on BBM vesicles from superficial and juxtamedullary cortex[20] and a ProFound Mammalian Co-Immuno-precipitation kit (Pierce, Rockford, IL, USA) following the manufacturer's instructions. Briefly, 20 μg of affinity-purified anti-NaPiIIc antibody was crosslinked to 100 μl coupling gel overnight, quenched, washed in coupling buffer and combined with 30 μg of BBM proteins previously dissolved in T-PER buffer (Pierce). After overnight incubation, the samples were washed, eluted, neutralized, quantified, and analyzed by SDS-PAGE using antibodies against NaPiIIc, NHERF1 (Chemicon, Temecula, CA, USA), and NHERF3 (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Site-directed mutagenesis

Truncation of full-length mouse NaPiIIc was performed by site-directed mutagenesis with a Quickchange II Site-directed Mutagenesis kit (Stratagene). Primers were designed with the online tool of the manufacturer: sense 5′-AGAACCCACAAGTCATAG CTTCTTAGCAGTTGTGAG-3′; antisense 5′-CTCACAACTGCTAAG AAGCTATGACTTGTGGGTTCT-3′.

Cell culture and transfections

OK cell culture was performed as reported.[20] Transfections and cotransfections were achieved with Lipofectamine 2000 (Invitrogen) and cells at 90% confluency, following the manufacturer's instructions.

Cloning of full-length NaPiIIc and expression in *X. laevis* oocytes

A cDNA template was prepared from kidney cortex RNA using a Superscript reverse transcriptase (Invitrogen). A 1,983 bp fragment of NaPiIIc (Slc34a3, accession number NM_080854) including the open reading frame was amplified with Platinum *Taq* DNA Polymerase High Fidelity (Invitrogen), cloned into pPCR-Script (Stratagene) downhill of the T7 RNA polymerase promoter, and sequenced.[29] For *in vitro* transcription, the plasmids were linearized with *Not*I, transcribed using a mMESSAGE mMACHINE kit (Ambion, Austin, TX, USA), and purified with NucAway spin columns (Ambion).

Handling of *X. laevis*, their oocytes, and transport assays were performed as described.[29]

Phosphate adaptation experiments

Wistar male rats (Harlan, Barcelona, Spain) were adapted for 4 days (chronically) to a diet containing either 0.1 or 1.2% Pi as described,[20] with the exception that the animals were fed *ad libitum*.

Fluorescence confocal microscopy

For fluorescence microscopic imaging, mouse NaPiIIc, Na-PiIIcΔQQL, NHERF1, and NHERF3 were cloned in frame, by standard PCR cloning, into the fluorescent protein-encoding plasmids pECFP-C1 and pEYFP-C1 (both from Clontech) or pCu-C1 (a gift from D Piston, Vanderbilt University, Nashville, TN, USA). Cu encodes a brighter variant of CFP. OK cells expressing the fluorescent fusion proteins were grown on poly-$_L$-lysine-coated cover glasses (Nunc, Roskilde, Denmark), and treated for immunocytochemistry as described.[14] For Golgi

apparatus identification, a pEYFP-Golgi plasmid encoding the N-terminal 81 amino acids of human β-1,4-galactosyltransferase (BD Clontech, Mountain View, CA, USA) was used. Preparations were analyzed using a laser scanning confocal microscope (LSM510; Carl Zeiss Inc., Thorn-wood, NY, USA).

Immunohistochemistry in rat kidney sections from adapted animals was performed as described.[8,20] Primary antibodies were our custom-made NaPiIIc chicken antibody (Davids Biotechnologie; see above), rabbit polyclonal anti-NHERF1 (a gift from Dr E Weinman, University of Maryland, MD, USA), and rabbit polyclonal anti-NHERF3 (a gift from Dr D Silver, Albert Einstein College of Medicine, NY, USA) or mouse monoclonal anti-NHERF3 (BD Biosciences, San Jose, CA, USA). They were detected with Alexa Fluor-conjugated secondary antibodies (Molecular Probes, Eugene, OR, USA). In addition, Alexa 633-labeled phalloidin (Molecular Probes) was used to stain the actin cytoskeleton.

Total internal reflection fluorescence microscopy

Details of the imaging of fluorescently tagged apical proteins by TIRF microscopy will be described elsewhere (J Blaine, SY Breusegem, M Asadi-Zeydabadi, M Levi, and NP Barry, manuscript in preparation, see also Barry *et al*.[30]). Briefly, OK cells were grown on polyester filters (12 mm diameter; Corning, Corning, NY, USA) to 90–95% confluency and transfected with plasmids encoding fluorescently tagged proteins using Lipofectamine 2000 as above. The membranes were cut out and turned 180° into a coverslip-bottomed dish (MatTek Corporation, Ashland, MA, USA) with OptiMEM-I (Invitrogen). Cells were imaged by TIRF using a TIRF microscope (Carl Zeiss Inc.) equipped with an Ar^+ laser, at 37 °C, through a 100 × 1.45 numerical aperture (NA) oil immersion objective. Cerulean fluorescence was excited using the 458 nm laser line and imaged through a 470–500 nm band-pass filter onto a Zeiss Axiocam MRm camera. YFP fluorescence was excited using the 514 nm laser line and imaged though a 550–600 nm band-pass filter.

Statistics and data analysis

Analysis of uptake data in oocytes was carried out as previously described.[27,29] Means were compared using unpaired *t*-test.

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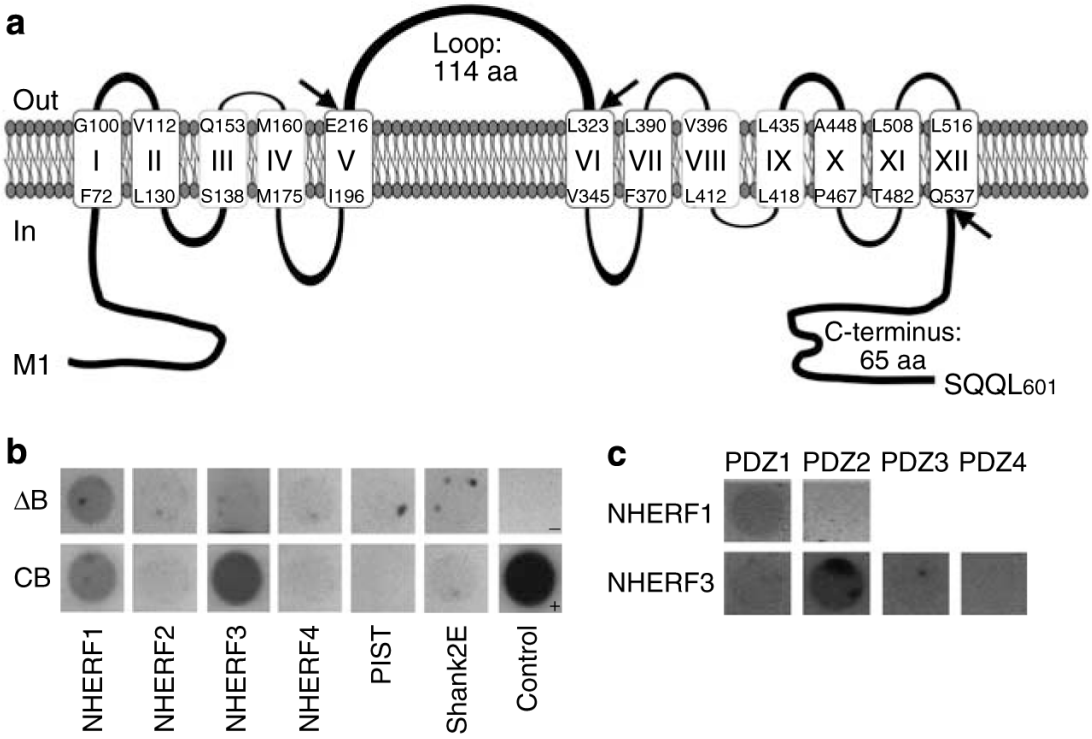

Figure 1. Two-hybrid system analysis of NaPiIIc protein interactions

(**a**) Representation of the secondary structure of mouse NaPiIIc and the baits used. The extracellular loop of 114 amino acids and the C-terminal free cytosolic end of 65 amino acids used as baits are indicated by arrows. This topological model is based on the latest model of NaPiIIa.[7] (**b**) Results of the mammalian two-hybrid system, showing interactions of NaPiIIc CB bait with NHERF1 and 3. In the case of NHERF1, the interaction is independent of the last three amino acids of NaPiIIc (ΔB). (**c**) Interaction of NaPiIIc CB bait with the individual PDZ domains of NHERF1 and NHERF3.

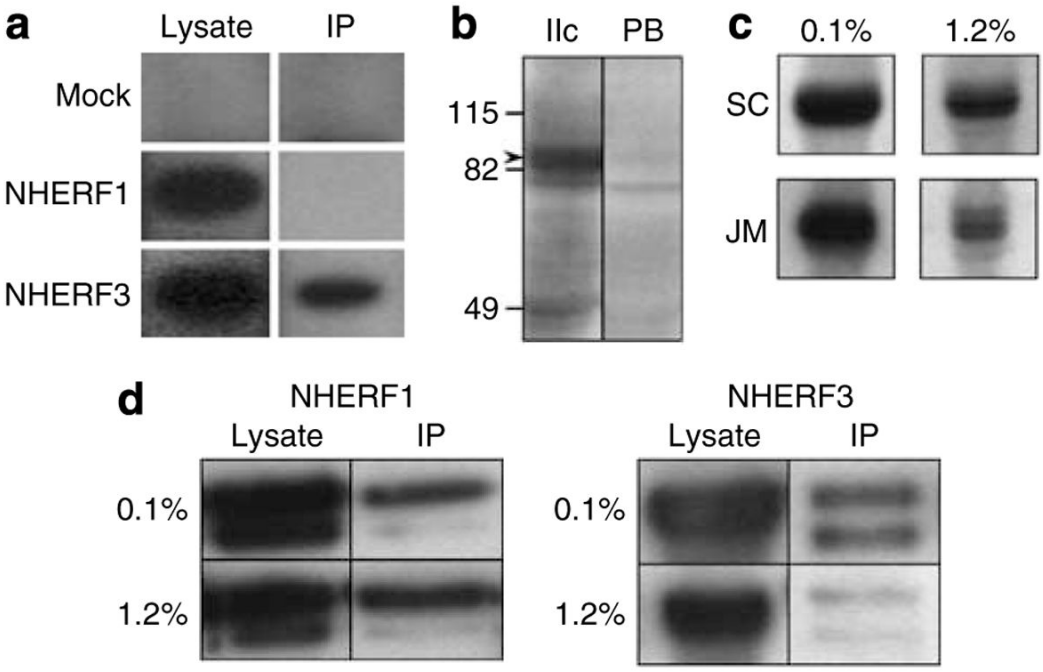

Figure 2. Coimmunoprecipitation of NaPiIIc and NHERF proteins

(**a**) Coimmunoprecipitation of transfected myc-tagged NHERF proteins in OK cells with FLAG-tagged transfected NaPiIIc showing that only NHERF3 is successfully precipitated, while both PDZ proteins were expressed in the initial cell lysate. (**b**) Characterization of the specificity of a polyclonal antibody raised against rat NaPiIIc. IIc, control western blot; PB, peptide blocking of the antibody; arrowhead, specific signal of NaPiIIc. (**c**) Adaptation of NaPiIIc expression to chronic changes in dietary Pi in both superficial (SC) and JM BBM. 0.1 and 1.2%, concentration of Pi in animal fodder. (**d**) Coimmunoprecipitation of NHERF1 and NHERF3 from JM membranes purified from animals chronically adapted to 0.1 and 1.2% Pi.

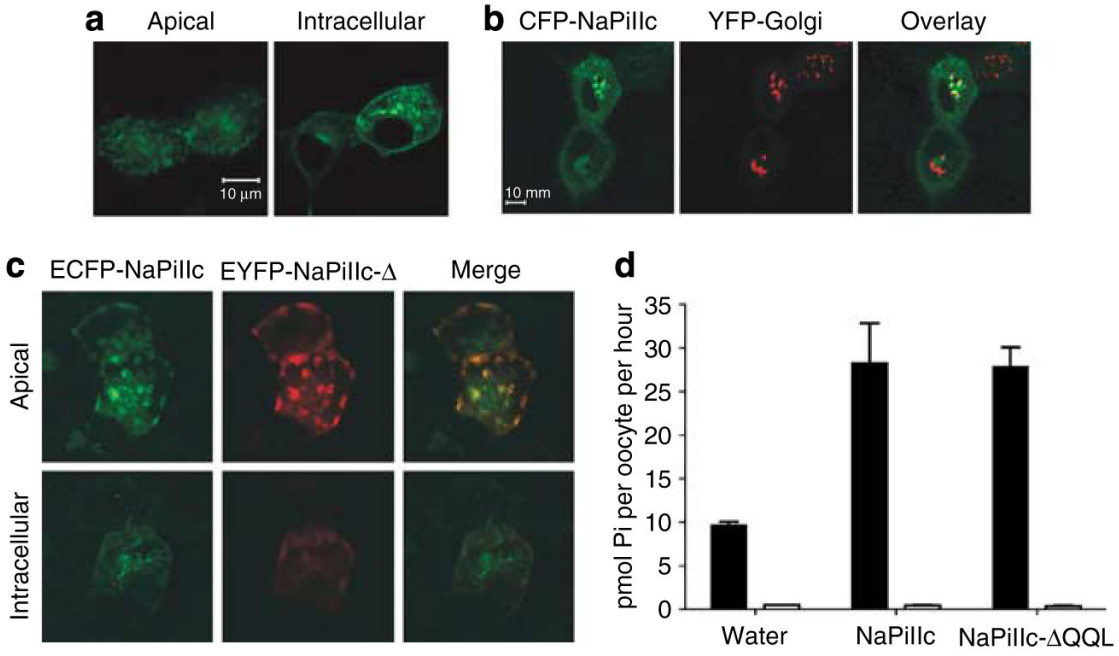

Figure 3. Cellular distribution of NaPiIIc

(**a**) CFP-NaPiIIc expressed in OK cells has an apical distribution in patches as well as a perinuclear staining that resembles *trans*-Golgi localization. (**b**) Colocalization of intracellular CFP-NaPiIIc fluorescence with the Golgi marker YFP-Golgi. (**c**) Cell distribution of truncated EYFP-NaPiIIc-Δ showing complete apical colocalization with the full-length ECFP-NaPiIIc transporter. (**d**) Expression of full-length and truncated NaPiIIc in *Xenopus* oocytes results in identical Pi transport activity. Black and white bars are for Pi uptake in the presence and absence of Na^+, respectively.

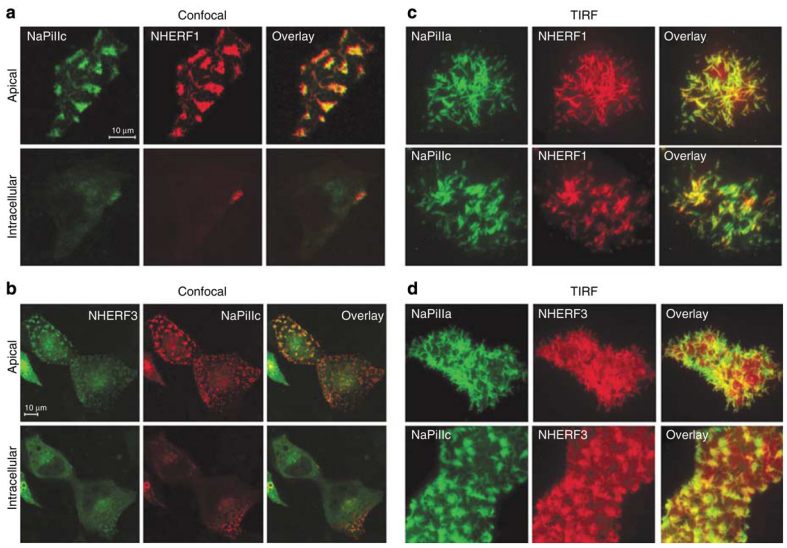

Figure 4. Colocalization of NaPiIIc with PDZ proteins in OK cells by confocal and TIRF microscopy

(**a** and **b**) Colocalization of NaPiIIc with (**a**) NHERF1 and (**b**) NHERF3 by confocal microscopy. In both cases, colocalization takes place in the apical membrane. There is also some minor colocalization of NHERF3 and NaPiIIc in the perinuclear TGN. (**c** and **d**) Apical localization was further analyzed by TIRF microscopy as described in Results. (**c**) OK cells doubly transfected with Cu-NaPiIIa and YFP-NHERF1, or CFP-NaPiIIc and YFP-NHERF1. (**d**) Colocalization of Cu-NaPiIIa and CFP-NaPiIIc with YFP-NHERF3. Colocalization in the apical microvilli is shown in yellow in the overlay images.

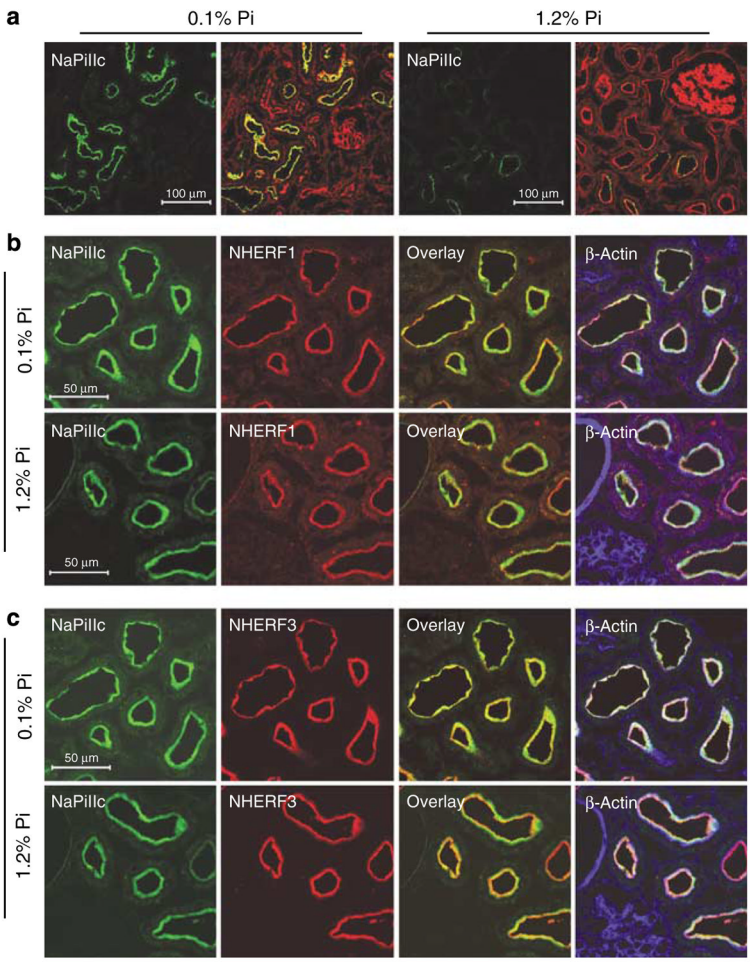

Figure 5. Colocalization of NaPiIIc and NHERF proteins in kidney cortex from rats adapted to low- and high-Pi diets

(**a**) NaPiIIc expression (pseudocolored green) in proximal tubule BBM is decreased in rats fed a 1.2% Pi diet (right) compared to rats fed a 0.1% Pi diet (left). Identical image acquisition and processing settings were used for both images. The corresponding actin staining is shown as well (pseudocolored red) to show the kidney architecture. In both cases, only a subset of proximal tubule sections is stained, and no staining is observed in other compartments (glomeruli, vasculature). (**b** and **c**) NaPiIIc (pseudocolored green) and (**b**) NHERF1 (red) and (**c**) NHERF3 (red) colocalize in rat proximal tubule BBM, both in rats fed 0.1 and 1.2% Pi diets. The high-Pi images were taken with different acquisition settings to clearly show the colocalization of the remaining NaPiIIc with the PDZ proteins as yellow in the two-color overlay images (third column) or white in the three-color overlay images (F-actin, blue). Parallel sections were used for panels b and c.

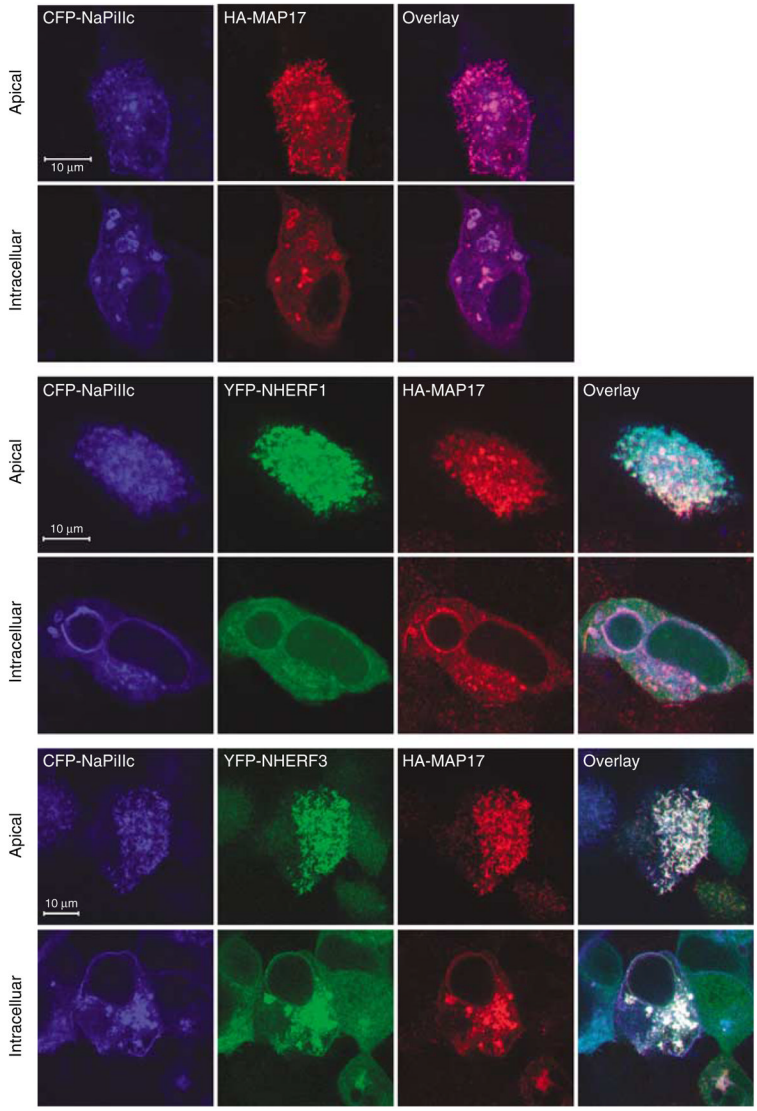

Figure 6. Effect of MAP17 on NaPiIIc–NHERF1 and NaPiIIc–NHERF3 interactions in OK cells

(Top) OK cells cotransfected with CFP-NaPiIIc (blue) and HA-tagged MAP17 (red). Pink in the overlay image in apical patches and in TGN indicates expression in the same compartment. (Middle) OK cells cotransfected with CFP-NaPiIIc (blue), YFP-NHERF1 (green), and HA-MAP17 (red). NHERF1 is not expressed in TGN and no white overlay can be observed; that is, MAP17 has no effect on NaPiIIc when cotransfected with NHERF1. (Bottom) Cotransfection of OK cells with CFP-NaPiIIc (blue), YFP-NHERF3 (green), and HA-MAP17 (red) increases expression of the three proteins in the TGN, as shown by the white TGN in the overlay image.

Table 1

NaPiIIc-interacting proteins identified with Bacteriomatch II two-hybrid system

Clone name	Accession number	Type bait	Early colonies	Late colonies
NHERF3^{PDZK1}	NM_021517	CB	5	—
Acid α-glucosidase	NM_008064	CB	14	11
PP2A regulatory subunit	NM_138748	CB	2	—
Napsin A aspartic peptidase	NM_008437	CB	—	4
Fibulin-containing Efemp2	NM_021474	CB	—	1
Acid α-glucosidase	NM_008064	LB	1	10
Phosphoglycerate kinase 1	NM_008828	LB	1	1
3-OH-butyrate dehydrogenase	NM_175177	LB	—	1
3-OH-AcylCoA dehydrogenase	NM_008212	LB	—	1
Hypothetical neonate kidney	AK085526	LB	—	1
Mouse embryo clone	AL645856	LB	—	1

CB, C-terminal bait; LB, loop bait. Colonies containing prey cDNAs were identified after cotransformation of a mouse kidney cDNA library with baits derived from mouse NaPiIIc C terminus (CB) or from the intracellular loop (LB), using the Bacteriomatch II two-hybrid system. The type of initial bait is indicated in the third column. Number of colonies are divided into two groups, whether they grew fast (24 h) or late (after 3 days).