Interaction of the Type IIa Na/P\textsubscript{i} Cotransporter with PDZ Proteins*

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The type IIa Na\textsuperscript{+}-dependent inorganic phosphate (Na/P\textsubscript{i}) cotransporter is localized in the apical membrane of proximal tubular cells and is regulated by an endocytic pathway. Because molecular processes such as apical sorting, internalization, or subsequent degradation might be assisted by associated proteins, a yeast two-hybrid screen against the C-terminal, cytosolic tail of type IIa cotransporter was designed. Most of the potential proteins found belonged to proteins with multiple PDZ modules and were either identical/related to PDZK1 or identical to NHERF-1. Yeast trap truncation assays confined the peptide-protein association to the C-terminal amino acid residues TRL of type IIa cotransporter and to single PDZ domains of each identified protein, respectively. The specificity of these interactions were confirmed in yeast by testing other apical localized transmembrane proteins. Moreover, the type IIa protein was recovered in vitro by glutathione S-transferase-fused PDZ proteins from isolated renal brush border membranes or from type IIa-expressing oocytes. Further, these PDZ proteins are immunohistochemically detected either in the microvilli or in the subapical compartment of proximal tubular cells. Our results suggest that the type IIa Na/P\textsubscript{i} cotransporter interacts with various PDZ proteins that might be responsible for the apical sorting, parathyroid hormone controlled endocytosis or the lysosomal sorting of internalized type IIa cotransporter.

In kidney, reabsorption of filtered inorganic phosphate (P\textsubscript{i}) takes place along the proximal tubules and is controlled by a variety of hormones (e.g. parathyroid hormone, PTH)\textsuperscript{1} and other factors (e.g. dietary intake of P\textsubscript{i})\textsuperscript{2} (1, 2). Three structurally unrelated sodium-dependent phosphate (Na/P\textsubscript{i}) cotransporter families have been identified (1, 3). By immunohistochemistry, it was apparent that members of the type I and the type IIa Na/P\textsubscript{i} cotransporters are located in the apical membrane of proximal tubular cells (4, 5). Targeted inactivation of the type IIa Na/P\textsubscript{i} cotransporter gene (npt2) provided strong evidence that ~70% of Na-dependent P\textsubscript{i} transport across the brush border membrane is mediated by the type IIa Na/P\textsubscript{i} cotransporter (6). Furthermore, the type IIa cotransporter represents the major target for the many factors described to regulate proximal tubular P\textsubscript{i} reabsorption (2). Additionally, reduced proximal P\textsubscript{i}-reabsorption, as observed in X-linked hypophosphatemia, is due to a decreased abundance of the type IIa Na/P\textsubscript{i} cotransporter (7).

According to the current mechanistic view, inhibition of proximal tubular P\textsubscript{i}-reabsorption, such as by PTH or by a diet of high P\textsubscript{i} content (acutely given), is achieved by a removal of type IIa cotransporters (2) from the apical membrane. Results obtained from in vivo (rats) and in vitro (OK cells) studies indicated that internalized type IIa Na/P\textsubscript{i} cotransporters are subjected to degradation in the lysosomes (8, 9). Besides Na/P\textsubscript{i} cotransport, the activity of the brush border Na/H exchanger, NHE-3, is regulated by PTH as well; however, internalization of NHE-3 seems to occur after a delay and not immediately after binding of PTH to its receptor (10). This kinetic difference of PTH action on the type IIa protein and on the NHE-3 exchanger, respectively, points to a regulatory mechanism specific for the type IIa Na/P\textsubscript{i} cotransporter. Not much is known about the molecular reactions that underlie the internalization of the type IIa cotransporter. Although protein kinases are activated upon the binding of PTH to its receptor (2), the target(s) for activated kinases relevant for the internalization of the type IIa protein has(have) not yet been identified.

The microvillar localization of the type IIa protein and its physiologically controlled abundance in the apical membrane suggest specific interactions of the type IIa protein with other microvillar/subapical proteins. Such interactions may be necessary for the correct apical positioning or may be involved in the signaling pathway that leads to internalization. To identify such candidate proteins, a yeast two-hybrid screen was performed. As a bait, we used the C-terminal 75 amino acid residues (563–637) of the type IIa Na/P\textsubscript{i} cotransporter for the following reasons: (a) based on the current model of the secondary structure, the C terminus is located at the cytoplasmic surface (11); (b) by truncation studies, evidence was obtained that the C terminus is important for apical expression of the type IIa protein.\textsuperscript{2}

We found that the C terminus of the type IIa Na/P\textsubscript{i} cotransporter is a strong interactor to various PDZ proteins. Some of these PDZ proteins were localized in the brush border or in the subapical compartment of proximal tubular cells and thus may be of importance for the polar distribution and/or regulation of the type IIa Na/P\textsubscript{i} cotransporter.

\textsuperscript{2}N. Hernando, J. Biber, and H. Murer, unpublished results.

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\[2\]The abbreviations used are: PTH, parathyroid hormone; Na/P\textsubscript{i}, Na\textsuperscript{+}-dependent inorganic phosphate; OK cell, opossum kidney cell; DNA-BD, DNA binding domain; ADH, anti-diuretic hormone; SD-Trp, synthetic minimal tryptophan dropout medium; GST, glutathione S-transferase; BBMV, brush border membrane vesicle; CT, C terminus; MAST, microtubulin-associated serine/threonine kinase; HSP, heat shock protein; aa, amino acid(s); PCR, polymerase chain reaction.
EXPERIMENTAL PROCEDURES

Bait DNA Constructs—EcoRI and SalI restriction sites were introduced by PCR into cDNA fragments encoding the N- or C-terminal tails of the following proteins: mouse type II Na/Pi cotransporter (aa 1–109 and 563–637, accession no. AAC52361), mouse type I Na/Pi cotransporter (aa 1–23 and 443–465, accession no. CA454459), rat Na/H exchanger NHE-3 (aa 1–53 and 447–531, accession no. AA41702), mouse glycophorin A (accession no. BAA21478), rabbit sodium/glucose cotransporter SGLT-1 (aa 1–31 and 545–662, accession no. CA29727), rat sodium/sulfate cotransporter NaSl-1 (aa 1–17 and 569–595, accession no. AAA41677), mouse peptide transporter Pept-2 (aa 1–51 and 705–746, accession no. AAF42470), and rat calcitonin gene-related peptide receptor b2 (aa 1–34 and 451–487, accession no. AJ249198), rat sulfate/oxalate/bicarbonate anion exchanger Sat-1 (aa 1–73 and 628–703, accession no. AAA17545), rabbit sodium/glucose cotransporter SGLT-1 (aa 1–31 and 545–662, accession no. CA29727), rat sodium/sulfate cotransporter NaSl-1 (aa 1–17 and 569–595, accession no. AAA41677), mouse peptide transporter Pept-2 (aa 1–51 and 705–746, accession no. AAF42470), and rat calcitonin gene-related peptide receptor b2 (aa 1–34 and 451–487, accession no. AJ249198).

To generate LexA DNA binding domain (DNA-BD) fusions, the fragments were inserted in-frame into the vector pBTM116 carrying the TRP1 selection marker (12). All cDNA fragments encoding for N termini were subcloned into the vector pFBL23 (13), where the inserts had reverted orientation relative to LexA. Site-directed mutagenesis (QuickChange, Stratagene) was applied to generate truncations (–3, –14, –16, –36 and –89) of the C terminus from the type II Na/Pi cotransporter by introducing stop codons at the respective positions. As a control bait, the subunit of the reverse transcriptase from human immunodeficiency virus (RTPr51) in pBTM116 was used (generously provided by Prof. U. Hübser; see Ref. 14).

All bait constructs were verified by sequencing and used with upper primers annealing to either the LexA DNA-BD (pBTM116: lexA_upper 5'-CTGGATAATTCCCGTGGCG-3'); pFBL23: lexA_lower 5'-GCCATGCCCGTAGAGGTGT-3' or to the ADH promoter (pBTM116: ADH_lower 5’-GCATGCCGGTAGAGGTGT-3’; pFBL23: ADH-Pro_LV_upper 5’-TCCTGATTGTTGGCTCTTCC-3') from the plasmids.

Prey DNA Constructs—Deletion constructs in pACT2 used for PDZ interaction trap assays in yeast were produced by PCR. cDNA fragments encoding single or multiple PDZ domains (listed below) were obtained using site-directed mutagenesis (Stratagene). Stop codons downstream of PDZ domain 1, 2, and 3, or by standard amplification, using primers harboring Ncol or XhoI restriction sites: mouse NaPi-Cap1 (accession no. AF220100, aa 1–110 for PDZ1, aa 108–240 for PDZ2, aa 217–359 for PDZ3, aa 348–472 for PDZ4, aa 1–229 for PDZ1–2, aa 1–350 for PDZ3–4, mouse NaPi-Cap2 (accession no. AF334612, aa 1–145 for PDZ1, aa 125–270 for PDZ2, aa 243–377 for PDZ3, aa 370–498 for PDZ4, aa 1–354 for PDZ1–2 and aa 1–378 for PDZ3–4), mouse NHERF-1 (accession no. U74079, aa 1–129 for PDZ1–2 oocytes, washed three times with binding buffer and used for diodeoxy sequencing (Microsynth, Switzerland). Identical sequences were grouped via ClustalW at Pôle Bio-Informatique (Lyonnais) or via Fileup from Genetics Computer Group (Oxford) and overlaps connected (Contig assembly program, Baylor College of Medicine). Searches for protein relationships were performed at the National Center for Biotechnology Information at the National Institutes of Health (Bethesda, MD) using BLAST (23). The modular architecture of proteins was determined by SMART (24).

Liquid β-Galactosidase Assays—Interaction of single proteins and constructs thereof were determined by a liquid assay for β-galactosidase from permeabilized cells in the presence of o-nitrophenyl-β-D-galactopyranoside (ONPG, Sigma) (25, 26). The activity of β-galactosidase (A β-gal) was normalized to 1.0 × 107 cells assayed (A β-gal = 1.0 × 107 cells/ml).

GST Fusion Constructs and Protein Expression—The following GST fusion constructs were made in vector pGEX-6P-2 (Amersham Pharmacia Biotech) using EcoRI/XhoI or BamHI/XhoI restriction sites: mouse NaPi-Cap1 (aa 1–519), mouse NaPi-Cap2 (aa 1–498), mouse NHERF-1 (aa 1–355), mouse C2PA (aa 195–375 of C2PA plus 17 N-terminal and 36 C-terminal nonannealing aa), mouse zetin (262 aa), mouse FHL-2 (aa 6–270) and mouse 54Tm (aa 1–281). Mouse NHERF-2 was digested with EcoRI/XhoI and the fragment corresponding to aa 1–289 directly cloned in pGEX-6P-1. Since the full-length mouse MAST205 (1734 aa) was not expressed in E. coli, a known interacting fragment enclosed by Smal/XhoI restriction sites (aa 905–1830) was subcloned in pGEX-6P-1. Finally, the C-terminal Smal/ XhoI fragment of mouse NaPi-Cap1 (aa 394–724) was also in pGEX-6P-1. All constructs were checked by sequencing using primers Gex5' (5'-CCAGCAAGTA-TATAGGATGA-3') and Gex3'rev (5’-GCTTACGACAAAGCTTGTC-3').

Plasmids were transformed in XLI-Blue cells (Stratagene). 50 ml overnight cultures were diluted in 1:500 in 500 ml of LB/Amp and grown to 0.8 A600 at 37 °C. Protein expression was induced by adding 0.2 ml of isopropyl-1-thio-β-D-galactoside (Axon Lab), and the incubation was continued for 5 h at 37 °C. Cells were pelleted at 5,000 × g for 10 min at 4 °C, resuspended in 4 ml of lysozyme buffer (50 mml Tris-HCl, pH 8, 120 mcn NaCl, 0.5% Igepal (Sigma CA-630), 5 mnn diethiolethion, 1 mnn EDTA, 1 mnn phenylmethylsulfonl fluoride, 1 μg/ml leupeptin, 2 mg/ml lysozyme), and immersed on ice for 15 min. The cells were subjected three times to pulsed sonication for 30 s on ice. Aliquots were cleared at 12,000 rpm in a tabletop microcentrifuge for 15 min (4 °C) and frozen at –80 °C.

Lysates were thawed at 37 °C and spun for 3 min as above to remove insoluble debris. Equal amounts of GST fusion proteins (~2 μg) were incubated with 25 μl of precipitated glutathione-agarose beads (Sigma; 50% slurry) in a total volume of 500 μl of binding buffer (50 mml Tris-HCl, pH 8, 120 mml NaCl, 0.5% Igepal CA-630, 5 mnn diethiolethion) by rocking at 4 °C for 30 min. After absorption, beads were collected by brief centrifugation at 12,000 rpm for 10 s (4 °C) and gently washed three times with 500 μl of binding buffer containing 0.075% SDS.

Pull-down Experiments—Pull-down experiments were performed either with isolated proximal tubular brush border membranes (BBM) from mice or with Xenopus laevis oocytes injected with type IIa cRNA. Primers (27) were designed to amplify binding by PCR for 5 min at 4 °C and centrifuged at 16,000 × g for 3 min. Extracts of oocytes injected with Ia cRNA for details, see Ref. 28) were obtained as described (29). GST fusion protein-bound beads were incubated for 4 h at 4 °C with solubilizes corresponding to 0.05 mg of BBMV protein or 1–2 oocytes, washed three times with binding buffer and used for gel-electrophoresis. Western blotting was performed with a polyclonal anti-Ia cotransporter antibody directed against the N terminus (5), and...
immunoreactive bands were detected by ECL using a secondary HRP-coupled IgG (Amersham Pharmacia Biotech).

**Northern Blotting**—Tissue distribution of mRNA expression of identified gene products was studied by Northern blotting using poly(A)+ RNAs of adult mice either purchased from CLONTECH or isolated by standard procedures. Full-length inserts were randomly labeled with [32P]dCTP (oligolabeling kit; Amersham Pharmacia Biotech) and used as probes. After hybridization, all blots were washed sequentially with 3× SSC, 1× SSC, and 0.5× SSC (containing 0.1% SDS) at temperatures up to 55 °C.

**Immunofluorescence and Immunogold Electron microscopy**—Immunohistochemical distribution of NaPi-Cap1 and NaPi-Cap2 in mouse kidney was essentially performed as described (31). Polyclonal antisera were raised against synthetic peptides derived from the N terminus of mouse NaPi-Cap1 and mouse NaPi-Cap2, respectively. β-Actin was visualized by phallolidin-Texas Red. Anti MAST205 antibody was kindly provided by P. Walden (31), and an anti-HSP86 antibody was purchased from Affinity Purified Reagents.

**RESULTS**

**Identification of Type IIa Na/Pi Cotransporter-associated Proteins**—An adult mouse kidney cDNA library was screened in yeast against the C-terminal tail (aa 563–637) of the murine type IIa NaPi cotransporter. Initially, based on selection for growth on synthetic media and on LacZ expression, 138 positive colonies were obtained from 4.4 × 10⁶ Leu/Trp/His protoplasts. To confirm the initial positives, plasmids from 104 positive colonies were obtained from 4.4 × 10⁶ Leu/Trp/His protoplasts. To confirm the initial positives, plasmids from 104 positive colonies were obtained from 4.4 × 10⁶ Leu/Trp/His protoplasts.

**GST Precipitations for in Vitro Corroboration of Interactions**—Some of the interactions found by the yeast two-hybrid assay were verified by pull-down experiments using GST fusion constructs and either solubilized mouse kidney BBMV (Fig. 1A) or lysates of X. laevis oocytes expressing the mouse type IIa NaPi cotransporter (Fig. 1B). From solubilized BBMV, the mature type IIa protein (mass ∼ 85 kDa; see Ref. 5) was precipitated by the following GST fusion constructs (Fig. 1A): GST-NaPi-Cap1, GST fused to the PDZ domain 3 of NaPi-Cap1 (see below), GST-NaPi-Cap2, GST-NHERF-1, GST-NHERF-2, and GST fused to the protein similar to NHERF-2. The type IIa cotransporter was neither pulled down by GST alone nor by GST fused to C2PA, zetin 1, MAST205, and to FHL-2, as shown previously (35, 41, 42), and additionally NaPi-Cap1, but not NaPi-Cap2.

**TABLE I**

Compilation of all proteins identified by the two-hybrid screen as putative associaters to the C terminus of the type IIa Na/Pi cotransporter

| No. of positives | Similarities/identities* | Clone name |
|------------------|--------------------------|------------|
| 25               | 75% to rat diphor-1 or human PDZK1 | Mouse NaPi-Cap1b |
| 35               | 28% to rat diphor-1 | Mouse NaPi-Cap2b |
| 7                | 100% to mouse NHERF-1 | Mouse NHERF-1 |
| 2                | 90% to human NHERF-2 | Mouse NHERF-2 |
| 1                | 75% to human NHERF-2 | ? |
| 1                | 78% to mouse C2PA’ | Mouse zeitin 1 |
| 1                | 86% to rat zeitin 1 | Mouse HSP84 |
| 7                | 100% to mouse HSP84 | Mouse HSP86 |
| 7                | 100% to mouse HSP84 | Mouse HSP86 |
| 5                | 100% to mouse MAST205 | Mouse MAST205 |
| 1                | 100% to mouse FHL-2“ | Mouse FHL-2 |
| 1                | 53% to human 54TmP | Mouse 54TmP |

* Overall similarities/identities of above amino acid sequences were obtained using the Blast or ClustalW algorithm.

b Abbreviation for Na/Pi cotransporter C-terminal-associated protein.

This identified protein is merely identical to the PDZ domain of mouse C2PA (aa 195–375) and probably represents an alternative spliced variant of mouse C2PA.

d Abbreviation for “four and a half lim domains 2.”

serine/threonine kinase (31). Others were found only once, such as the mouse protein FHL-2 having four and half LIM domains (39) or as the mouse homologues of rat zeitin 1 (accession no. AF245225) and of the human putative transmembrane protein 54TmP (accession no. AF004876). Differences in the representation of these clones could be explained by either the low abundance of corresponding mRNAs or by the presence of diverse truncations of inserts existing in the library.

**Specific Binding of Putative Type IIA-associated Proteins in Yeast**—To evaluate the specificity of the identified clones for binding the C terminus of the type IIA cotransporter, various baits derived from a number of proximal tubular, apically localized membrane proteins were constructed and tested in yeast for possible interactions with some of the proteins obtained by the screen (Table II). Most of the baits used did not activate the reporter genes in the presence of the different proteins exhibiting binding capacity for the type IIA cotransporter. Exceptional was the C terminus of the type I NaPi cotransporter (40), which was found to associate with all the PDZ proteins listed as well as weakly with MAST205. Furthermore, the C-terminal tail of the Na/H exchanger, NHE-3, bound NHERF-1, as shown previously (35, 41, 42), and additionally NaPi-Cap1, but not NaPi-Cap2.
membrane proteins (43, 44) and thereby may prevent the pull-down of NaPi-Cap1.

**Table II**

| Bait                  | NaPi-Cap1 | NaPi-Cap2 | NHERF-1 | C2PA | HSP84/86 | MAST205 |
|-----------------------|-----------|-----------|----------|------|----------|----------|
| CT* mouse/NaPi-IIa    | ++        | ++        | ++       | ++   | ++       | ++       |
| NT* mouse/NaPi-IIa    |           |           | ++       | ++   | ++       |          |
| CT* mouse/NaPi-I      | ++        |           | ++       | ++   | (+)      | +        |
| NT* mouse/NaPi-I      |           |           |          | (+)  |          |          |
| CT* rat/NHE-3         | +         |           | +        | ++   | (+)      |          |
| NT* rat NHE-3         |           |           |          | ++   | (+)      |          |

* CT signifies the putative cytosolic C terminus of the protein.

* NT signifies the putative cytosolic N terminus of the protein.

**Fig. 1.** Type IIa cotransporter is recovered in vitro by various proteins identified by the two-hybrid approach. Purified GST alone or GST fusion proteins (see “Experimental Procedures”) immobilized on glutathione-agarose were incubated in the presence of lysates from murine kidneys BBMVs (A) or from X. laevis oocytes expressing the mouse IIa Na/Pi cotransporter (B). After intense washing, the final samples were denatured in the absence of a reducing agent, and transferred material was immunblotted using a polyclonal antibody raised against the type IIa Na/Pi cotransporter. The band of ~85 kDa represents the mature Na/Pi cotransporter (see also Ref. 5). As controls for the stability of the type IIa protein, aliquots of the lysates were analyzed as well.

**Fig. 2.** Expression pattern of mouse NaPi-Cap1 and NaPi-Cap2 mRNA. Poly(A)+ RNA from multiple mice tissues was probed with randomly labeled inserts of NaPi-Cap1 (A) and NaPi-Cap2 (B). The multiple tissue blot was additionally probed for β-actin (C).
was strictly associated with the microvilli, NaPi-Cap2 was predominantly located in the subapical compartment, but was not detected in the microvilli. However, faint immunostaining for NaPi-Cap2 was also found throughout the cytoplasm (Fig. 3A). By immunogold electron microscopy, evidence was obtained that NaPi-Cap2 was associated with vesicular structures within the subapical compartment. Eventually, NaPi-Cap1 was also present in the intermicrovillar clefts (open arrowheads).

Yeast Trap Truncation Assays to Specify the Interaction of Type IIa Cotransporter with Mouse NaPi-Cap1, NaPi-Cap2, and NHERF-1—Both NaPi-Cap1 and NaPi-Cap2 encompass four PDZ domains. To analyze which of the four PDZ domains of NaPi-Cap1 or NaPi-Cap2, respectively, was responsible for the interaction with the C terminus of the type IIa Na/Pi cotransporter, each of the four PDZ domains was tested separately by trap two-hybrid assays. As depicted in Fig. 4A, solely PDZ domain 3 of NaPi-Cap1 and NaPi-Cap2 increased significantly the activity of β-galactosidase, in accordance with GST-NaPi-Cap1/PDZ3 by which the type IIa cotransporter was pulled down (see Fig. 1). The PDZ domain 3 of both NaPi-Caps was also found to be mostly important for the interaction with the C terminus of the type I Na/Pi cotransporter (data not shown). Furthermore, our results indicated that the type IIa cotransporter via its C terminus interacts predominantly with the first PDZ domain of NHERF-1 (Fig. 4B).

The last three C-terminal amino acid residues of the type IIa cotransporter sequence are TRL and thus may represent a PDZ-binding cassette (41). To refine if this putative PDZ binding determinant was responsible for the interaction with identified PDZ proteins, the last three amino acid residues (TRL) of the type IIa cotransporter were deleted and the respective bait was assayed in yeast in the presence of either NaPi-Cap1, NaPi-Cap2 or additionally NHERF-1 and C2PA. As illustrated in Fig. 5A, the removal of the three C-terminal amino acid residues, TRL, completely abolished the interaction of the C-terminal tail of the IIa Na/Pi cotransporter with NaPi-Cap1, NaPi-Cap2, NHERF-1, and C2PA. Similarly, the last three
Regulation of Renal Transporters by PDZ Interactions

Most of the proteins identified (71 out of 93) represented PDZ proteins (see Table I). Two similar proteins with four modular PDZ repeats were identified: NaPi-Cap1, being identical to the previously described proteins diphor-1 and PDZK1 (3, 32, 33), and NaPi-Cap2, being 28% identical to NaPi-Cap1. Other PDZ proteins found belonged either to the NHERF family (NHERF-1, NHERF-2, and a protein similar to NHERF-2) (35–37) or were related to the C2PA protein, which harbors a PDZ domain and a C2 domain in addition. Further, two large groups of proteins comprised the heat shock proteins HSP84 and HSP86 (38, 45) and the microtubulin-associated serine/threonine kinase MAST205 (31). Interaction of above proteins with the C terminus of the type IIa cotransporter was specific (with one exception) since both the N terminus of the type IIa cotransporter and the C or N termini of various other proximal tubular, apically localized membrane proteins failed to stimulate LacZ expression in yeast. Interestingly, all identified PDZ proteins bound also to the C terminus of the type I Na/Pi cotransporter, which has been described to be localized in the apical membrane as well (4). However, if such an interaction with the type I cotransporter reflects any physiological significance, it remains to be shown if the C terminus of the type I Na/Pi cotransporter is faced toward the cytoplasm and not to the extracellular space.

Additional evidence for an interaction of the type IIa cotransporter with identified proteins was obtained from pull-down experiments, demonstrating that at least all PDZ proteins (NaPi-Cap1/2, C2PA, and NHERF isoforms) were (in vitro) binding partners for the mature type IIa Na/Pi cotransporter as present in isolated proximal tubular brush border membranes or/and in oocytes of X. laevis injected with type IIa cRNA. A first mandatory step to establish the physiological relevance of positive interactions in yeast was to determine the location of the putative candidate proteins in renal tissue, i.e., such proteins were required to be expressed in microvilli and/or subapical compartment of proximal tubular cells. The proteins NaPi-Cap1 and NaPi-Cap2 showed strict and NHERF-1 mostly proximal tubular location. NaPi-Cap1 (see also Ref. 44) and NHERF-1 (47) were found exclusively in microvilli, whereas NaPi-Cap2 was distributed predominantly in the subapical compartment of proximal cells. Interestingly, as revealed by immunogold electron microscopy, NaPi-Cap2 appeared to be associated with vesicular structures in the subapical compartment. At present, the nature of such vesicles is not known and vesicular proteins eventually linked to NaPi-Cap2 remain to be determined.

Although the type IIa cotransporter was precipitated by both NHERF-2 and its likely isoform, a conclusion about this kind of association would be premature since the renal localization of NHERF-1 isoforms have not yet been verified by morphological techniques. With respect to C2PA, preliminary immunohistochemical studies indicated that this protein is barely localized in the proximal tubules (data not shown). Furthermore, immunofluorescence data disclosed that the heat shock proteins HSP84/86 and the microtubulin-associated serine/threonine kinase MAST205 (31) were localized along the whole nephron and were neither specifically localized in the brush border nor in the subapical region (data not shown). Therefore, these proteins are probably not implicated in either apical sorting or endocytosis of the type IIa cotransporter. It can, however, not be excluded if HSP84/86 or MAST205 plays a role in protein synthesis or lysosomal routing of the type IIa cotransporter; the latter has been shown previously to be dependent on an intact microtubular network (48). Further studies on other proteins which have emerged from the two hybrid approach were impossible, due to the lack of suitable antibodies. Based
on the criteria discussed above (positive yeast assay, pull-down and immunolocalization), NaPi-Cap1/2 and NHERF-1 were supposed to associate with the type IIa cotransporter. But it remains to be shown if all these interactions also occur under in vivo conditions.

PDZ domains are stretches of 80–90 amino acids and have been assigned to an increasing number of proteins having physical organizer functions in the formation of protein complexes of various sizes. Such protein complexes may allow a proper arrangement of components involved in signal transduction pathways and/or may allow a correct recruitment of membrane-inserted proteins, as has been shown e.g. for synaptic vesicle transporter and channels (41, 42, 49). In addition, evidence for PDZ interactions in the localization of proteins to the apical membrane has been accumulated (50, 51). The proteins NaPi-Cap1, NaPi-Cap2, and NHERF-1, on which our studies concentrated, contain two or four PDZ domains. However, their interactions with the C terminus of the type IIa cotransporter were confined to only one of these domains, i.e. PDZ3 of NaPi-Cap1/2 and PDZ1 of NHERF-1. As shown by others, NaPi-Cap1 also interacts with a small transmembrane, cancer-associated protein, MAP17 (43), and with the multidrug resistance-associated protein, MRPP2 (44). The latter two proteins are supposed to interact with PDZ domains 1 and/or 4 of NaPi-Cap1. Presumably, other PDZ domains of NaPi-Cap1 and NaPi-Cap2 may be occupied by cytoskeletal proteins or by members of the ER/SM system of actin-binding proteins (52). As for NHERF-1, it was initially identified as a protein which binds to the Na/H exchanger NHE-3 (35, 36), but interaction of NHERF-1 with other proteins, such as the β2-adrenergic receptor (53), the cystic fibrosis transmembrane conductance regulator (50, 54), and the B1 subunit of the H-ATPase (47), has been reported as well.

Often, PDZ folds recognize a C-terminal amino acid motif of the D/S/TXV type; however, many variations thereof appeared. The last three amino acid residues TRL, resemble a PDZ binding motif. Indeed, deletion of the amino acid residues TRL is sufficient to completely abrogate the interaction with the PDZ proteins NaPi-Cap1, Napi-Cap2, and NHERF-1.

In summary, a yeast two-hybrid screen with the C terminus of the type IIa Na/Pi cotransporter as a bait revealed several proteins that are likely to be associated with the IIa cotransporter. Physiological relevance of the identified proteins is met by their proximal tubular as well as apical localization. The proteins NaPi-Cap1, NaPi-Cap2, and NHERF-1 are of particular interest and may mediate apical positioning and/or endocytosis of the type IIa Na/Pi cotransporter. These multidomain PDZ proteins are presumably cosequestered by type IIa co-transporters at the membrane and are therefore assumed to build up a complex network consisting of the type IIa Na/Pi cotransporter, other membrane proteins, cytoskeletal proteins, or components of signaling pathways (33, 43). It seems of interest that NHERF-1 recruits ezrin, which in turn functions as a protein kinase A anchoring site for conferring cAMP-mediated regulation of NHE-3 (41, 46, 55). Thus, NHERF-1 seems to serve as an organizer for signaling complexes necessary for the regulation of a variety of proteins, e.g. permitting phosphorylation of NHE-3 (35). It must be determined if NHERF-1 exerts a similar function in the regulation of the type II cotransporter.

The observation that the C terminus of the type IIa cotransporter recognizes PDZ motifs of several proteins (NaPi-Cap1/2 and NHERF-1) suggests that these proteins may differently affect apical sorting and/or endocytosis. Thus, in the case of the type IIa Na/Pi cotransporter, such interactions may not be of static nature but rather may be dynamically regulated by the activation status of specific signaling pathways.

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