Monitoring Protein-Protein Interactions between the Mammalian Integral Membrane Transporters and PDZ-interacting Partners Using a Modified Split-ubiquitin Membrane Yeast Two-hybrid System*

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PDZ-binding motifs are found in the C-terminal tails of numerous integral membrane proteins where they mediate specific protein-protein interactions by binding to PDZ-containing proteins. Conventional yeast two-hybrid screens have been used to probe protein-protein interactions of these soluble C termini. However, to date no in vivo technology has been available to study interactions between the full-length integral membrane proteins and their cognate PDZ-interacting partners. We previously developed a split-ubiquitin membrane yeast two-hybrid (MYTH) system to test interactions between such integral membrane proteins by using a transcriptional output based on cleavage of a transcription factor from the C terminus of membrane-inserted baits. Here we modified MYTH to permit detection of C-terminal PDZ domain interactions by redirecting the transcription factor moieties from the C to the N terminus of a given integral membrane protein thus liberating their native C termini. We successfully applied this “MYTH 2.0” system to five different mammalian full-length renal transporters and identified novel PDZ domain-containing partners of the phosphate (NaPi-IIa) and sulfate (NaS1) transporters that would otherwise not be detectable. Furthermore this assay was applied to locate the PDZ-binding domain on the NaS1 protein. We showed that the PDZ-binding domain for PDZK1 on NaS1 is upstream of its C terminus, whereas the two interacting proteins, NHERF-1 and NHERF-2, bind at a location closer to the N terminus of NaS1. Moreover NHERF-1 and NHERF-2 increased functional sulfate uptake in Xenopus oocytes when co-expressed with NaS1. Finally we used MYTH 2.0 to demonstrate that the NaPi-IIa transporter homodimerizes via protein-protein interactions within the lipid bilayer. In summary, our study establishes the MYTH 2.0 system as a novel tool for interactive proteomics studies of membrane protein complexes. Molecular & Cellular Proteomics 7: 1362–1377, 2008.

The mammalian renal proximal tubule sustains reabsorption of fluid and solutes of the highest variety and magnitude. Vectorial transport in this epithelium is mediated by the polarized distribution of polytopic transport proteins. Strikingly many membrane proteins located at the apical but not at the basolateral side harbor a C-terminal PDZ-binding motif. The acronym PDZ denotes a conserved modular domain of 90 amino acids that recognizes an octapeptide PDZ-binding motif primarily residing at the C termini of target proteins (1–3). PDZ domains are classified into three groups according to their binding specificity. Class I modules represent PDZ proteins that bind the canonical consensus sequence X(S/T)XΦ where X is unspecified and Φ is a hydrophobic residue (4).

There is an ever growing membership of class I PDZ-binding membrane proteins in the apical brush border membrane of proximal tubular cells (5, 6). The Na+/H+ exchanger (NHE)1-3 (C-terminal STHM) contributes to trans- and para-

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Received, February 21, 2008, and in revised form, April 10, 2008
Published, MCP Papers in Press, April 11, 2008, DOI 10.1074/mcp.M800079-MCP200

* The abbreviations used are: NHE, Na+/H+ exchanger; Ade, adenine; 3-AT, 3-amino-1,2,4-triazole; CAL, CFTR-associated ligand; CT, C terminus; Cub, C-terminal part of ubiquitin; lacZ, gene for β-galactosidase; MAP17, membrane-associated protein of 17 kDa; MYTH, membrane yeast two-hybrid; MYTH conventional format, reporters fused to the CT of the bait; MYTH 2.0 format, reporters fused to the N terminus of the bait; MCS, multiple cloning site; minimal SD, minimal synthetic medium consisting of a dropout for the nutritional
cellular absorption of Na\(^+\), Cl\(^-\), HCO\(_3^-\), and water (7). The co-transporters NaPi-IIa (C-terminal ATRL) and NaS1 (C-terminal QTMP) support Na\(^+\)-coupled uptake of Pi (8) and sulfate (9), respectively. The membrane-associated protein MAP17 (C-terminal STPM) of 17 kDa, which likely has multiple unknown functions other than transport, is classified into this group of apical class I PDZ-binding proteins as well (10, 11).

Conventional yeast two-hybrid (YTH) assays and classical biochemical techniques such as GST pulldowns identified proximal tubule brush border class I PDZ proteins as binding partners to the apical transmembrane proteins NHE-3, NaPi-IIa, and MAP17 (5). The Na\(^+\)/H\(^+\) exchanger-regulating factor NHERF-1 and NHERF-2 were characterized as cofactors that confer cAMP-induced inhibition of NHE-3 (12, 13). Despite encompassing two PDZ modules in tandem (PDZ1 and PDZ2), both NHERF isoforms seem to preferentially utilize PDZ2 to interact with NHE-3 (14). Apart from the canonical C-terminal site, an internal PDZ-binding site in NHE-3 has been proposed (15). Via their PDZ1 domains, NHERF-1 and NHERF-2 can also interact with the C-terminal TRL motif of NaPi-IIa (16). Two other related PDZ proteins called PDZK1 and PDZK2 contain four PDZ modules of which the third PDZ domain (PDZ3) interacts with the NaPi-IIa C-terminal canonical site as the sole site of PDZ recognition (6, 16). In addition, PDZ1 and PDZ4 of PDZK1 but not NHERF-1 or NHERF-2 bind to MAP17 (6, 17, 18). Notably a putative connection between PDZ proteins and the sulfate transporter NaS1 has not been determined so far.

Studies performed in cell models have enhanced the understanding of the physiological role of these PDZ proteins. Unlike the strict microvillar localization of NHERF-1/2 and PDZK1/2 in proximal kidney cells (5), CAL was demonstrated to traffic between the Golgi and the plasma membrane (19, 20). In these cells, apical expression of NaPi-IIa partially depends on NHERF-1 (5), and cAMP-mediated regulation of NHE-3 requires NHERF isoforms (12, 13, 21). PDZK1 was proposed to constitute an apical scaffold for the targeting/retention of diverse renal ion transporters (6, 22). As the PDZK1-interacting protein MAP17 is most abundant at the apical membrane of opossum kidney cells, it is also plausible that MAP17 might operate as an apical adaptor of PDZK1 (17). CAL in turn was reported to facilitate the retrograde trafficking of the mature Cl\(^-\) channel CFTR resulting in CFTR degradation (19).

Acquiring valuable information about interactions of integral membrane proteins has posed a major challenge because the hydrophobic nature of the membrane-spanning proteins prevents them from being used in the conventional YTH assay where both bait and prey need to be obligatory soluble polypeptides (23). To overcome this hurdle, we previously developed the conventional split-ubiquitin (Ub) membrane YTH (MYTH) technology based on a split-ubiquitin protein complementation readout (24–27). The small protein Ub, once conjugated to the C termini of its targets, destines proteins for proteasomal degradation in eukaryotic cells. The Ub moiety is then salvaged for recycling byUb-specific proteases (UBPs) resident in the nucleus and cytosol of all eukaryotic cells (28). Split-Ub systems rely on the fact that severed C-terminal (Cub) and N-terminal (Nub) halves of Ub spontaneously resemble to form a functional Ub molecule (29). The mutation of a single residue near the N terminus of Ub from isoleucine (Nubl) to glycine (NubG) renders the two separated Cub and NubG halves incompetent for self-reconstitution. The only way for Cub to associate with NubG and to reconstruct the intact functional Ub is for them to be brought together by an auxiliary mechanism that can be provided by interaction of proteins that are additionally annexed to Cub and NubG (25, 30). The reconstituted Ub, but not its halves alone (Cub or NubG), are recognized by UBPs that cause the release of a transcription factor (TF) strategically placed C-terminal to Cub. The released TF enters the nucleus and activates the reporter cascade in a manner similar to conventional YTH. Therefore, the conventional MYTH system consists of a membrane-inserted bait whose C terminus is fused to a Cub-TF hybrid. The inbound interacting prey (either a membrane or cytosolic protein) is fused to NubG, which will furnish the other half of Ub to permit UBP-mediated release of TF.

In this study, we applied a modified MYTH system to the full-length proximal tubule membrane proteins NaPi-IIa, MAP17, NHE-1, NHE-3, and NaS1 as baits integrated into a plasma membrane environment. We designed a MYTH 2.0 system that redirects the TF and Cub moieties from the C to the N terminus of a transmembrane bait protein (see Fig. 1A) thereby exposing the free C termini of the above polytopic membrane proteins to permit interactions with PDZK1, NHERF-1, NHERF-2, and CAL (see Fig. 2A). Using this approach, we made four important observations. First, we validated the MYTH 2.0 system as a tool to investigate interactions between five distinct mammalian full-length integral membrane proteins and their PDZ domain-containing partners. Second, we discovered novel interacting proteins of the phosphate (NaPi-IIa) and sulfate (NaS1) renal transporters using this MYTH 2.0 approach. Third, we showed that NHERF-1 and NHERF-2, the two novel NaS1 interactors found in this study, lead to increased functional sulfate uptake in Xenopus oocytes when co-injected with NaS1. Fourth, we demonstrated homo-oligomerization of the full-length NaPi-IIa protein within the bilayer by interactions of its transmembrane domains. In summary, our collective results demonstrate that the MYTH 2.0 system provides a useful tool to
promote further analyses of integral membrane proteins from any model organism.

**EXPERIMENTAL PROCEDURES**

**Cloning of the MYTH 2.0 Bait Vectors pTLB-1 and pCLB-1**—The MYTH bait vector pTMBV4 (Dualsystems Biotech AG, Schlieren, Switzerland) allows the fusion of a bait to the N terminus of a Cub-(LexA+VP16) (Cub-TF) reporter module (conventional MYTH system) whose LexA is mutated at R156G to abate the affinity toward the exogenous reporter gene promoters (Dualsystems Biotech AG). As a shuttle vector, pTMBV4 contains a kanamycin resistance gene, a LEU2 gene for auxotrophic selection in yeast, a highly potent TEF1 promoter, and a CEN/ARS origin of replication (Ori) for low copy propagation in yeast (Dualsystems Biotech AG). Related to this vector is pTMBV1\textsuperscript{2} that contains an expanded multiple cloning site (MCS) with among others Aatt and Eco47III sites between the Sfl sites of pTMBV4.

The vector pTMBV1 was modified to pTMBV2 by inserting the portion Aatt-BglII-Stop codon (TAA) upstream of the stop codon from VP16. For this purpose, pTMBV1 was amplified by means of bidirectional PCR using Pfu Turbo DNA polymerase (Stratagene, La Jolla, CA), and its parental DNA was digested with DpnI (Stratagene). The extended and nicked vector fragment was religated with T4 DNA ligase (Roche Applied Science) after treatment with T4 polynucleotide kinase (Promega, Madison, WI). To generate pTLB-1 (pTEF-LexMut-Cub-Bait-CEN/ARS), the following ampiclons were designed by PCR using pTMBV1 as a template: the TF fragment as Xbal-Kozak (GCCACC)-(LexA+VP16)-T-NotI and the Cub fragment as Xbal-Spacer (GAACATA)-NotI-Cub-TT-Sall. The nucleotide T in the TF fragment brings the reporter in-frame to Cub, whereas nucleotides TT in the Cub fragment bypass a stop codon within the preceding region. A Linker-MCS fragment was obtained directly from pTMBV1 by digestion with Sall and AattII. Linearized pTMBV2 (Xbal and AattII) was complemented with fragments derived from Cube (Xbal and Sall) and Linker-MCS (Sall and AattII). Finally, the resulting construct was assembled into pTLB-1 by insertion of the TF fragment between Xbal and NotI sites. Thus, the new MYTH 2.0 bait vector pTLB-1 comprises a very strong TEF1 promoter, a LEU2 gene, a Kozak initiation signal sequence, an inverted cassette of pTMBV1 with the respective C-terminal positions, and flanked with restriction sites by the Expand High Fidelity PCR kit (Roche Applied Science). After digestion, the inserts were ligated in-frame into pTLB-1. The 5'-P- of the linearized plasmid was removed by calf intestinal phosphatase (New England Biolabs, Beverly, MA) in the case of single-cut assembly as follows (all accession numbers reported are from GenBank\textsuperscript{TM}): mouse NHE-3,\textsuperscript{3} mouse NaPi-IIa (accession number BC020529) as well as mouse NaS1 (accession number AF193965) in Stul only, truncations of mouse NaPi-IIa (ΔTRL and ΔCT void of 67 residues) and NHE-3 (ΔTHM and ΔCT void of 370 residues) between Stul and SacII, rat NHE-1 (accession number M85299) as an Ncol-EcoRV embedded fragment between Ncol and Eco47III, and mouse MAP17 (accession number NM_026018) between Ncol and SacII. Human NaS1 (accession number NM_022444) was cloned into pCLB-1 between Ncol and SacII. The bait yeast GPR1 was integrated, and the baits yeast Alg5p and human ErbB3 have been described recently (24, 25).

The ORFs of preys were derived from full-length soluble or membrane proteins and integrated in-frame into pNubG-HA-X (Dualsystems Biotech AG). This vector creates a fusion to the HA tag located at the C terminus of the NubG domain and is maintained via a strong constitutive ADH1 promoter, a TRP1 selection marker, and a 2μ Ori for high copy propagation. The same cloning procedure of the baits was applied to the following preys: mouse NHE-3 between Ncol and Sall; mouse NaPi-IIa as a BglII embedded fragment in BamHI only (compatible ligation of cohesive ends); mouse PDZK1 (accession number AF220100) containing a blunted Ncol site at amino acid position 473 (site-directed mutagenesis; Stratagene); and mouse NHERF-1 (accession number U74079), mouse NHERF-2 (accession number NM_023055), and mouse CAL (accession number BC051171) between Ncol and BamHI. For NubG or Nubl constructs of yeast Os1p and Fur4p, refer to other references (24, 27).

**MYTH Analysis—Host Saccharomyces cerevisiae strain THY AP4 with the genotype MATa, ura3, leu2, lexA::lacZ-trp1, lexA::His3, lexA::Ade2 (31) was grown in YES medium (1% yeast extract, 2% Bacto peptone, 2% glucose, 0.01% adenine hemisulfate, 2% select agar) on plates devoid of Leu (TF-Cub-Baits) or Trp (HA-Preys). The SD medium was composed of 0.67% yeast nitrogen base without amino acids (Difco), dropout supplement powder (Clontech), 2% glucose, 0.01% adenine hemisulfate at pH 5.6, 2% select agar for plates. The bait GPR1 was maintained on YPAD medium-G418 via resistance to kanamycin.**

Co-transformation of preys was always performed sequentially following the above procedure except that overnight cultures were derived from two colonies grown in 50 ml of adequate SD medium and that the two-hybrid selection was achieved on minimal SD plates (–Trp/–Leu/–His/–Ade). To minimize inherent leaky His3 expression, co-transformants of baits with pNubG-HA-X were streaked out on minimal SD plates containing increasing amounts of 3-aminopyridine (3-AR), had been expressed. Thereby 35 TF-Cub-NaS1-dependent clones were identified of which three

\begin{footnotesize}
\textsuperscript{2} I. Staglar, unpublished results.

\textsuperscript{3} D. Fuster, unpublished clone.
\end{footnotesize}
encoded PDZK1, NHERF-1, and NHERF-2, respectively. The nature of 28 TF-Cub-NaS1-dependent clones will be described elsewhere. Lastly the remaining four clones interacted with both TF-Cub-NaS1 and TF-Cub-β2AR and were therefore considered as artifacts.

**Protein Extraction and Immunoblotting**—Proper expression of baits and preys was confirmed by immunoblotting as described previously (33). Cells were propagated to an A650 of 1.0 in the appropriate SD medium to maintain the plasmid and collected at 700 × g for 5 min at 4 °C. A total lysate was produced from 10 ml of culture by vortexing the cells for 5 min in extraction buffer (1× SDS sample buffer, 100 mM DTT, 1 mM EDTA, complete protease inhibitor (Roche Applied Science) containing 200 μl of acid-washed glass beads (425–600 μm; Sigma). To obtain a membrane extract, cells from 50 ml of culture were subjected to repetitive vortex pulses (8 × 30 s) in lysis buffer (50 mM Tris-HCl at pH 7.5, 1 mM EDTA, Complete protease inhibitor) containing 300 μl of glass beads on ice. A debris-cleared fraction (700 × g for 20 min at 4 °C) was centrifuged at 150,000 × g to yield the cytosolic fraction (supernatant). The pellet was resuspended in 150 μl of lysis buffer supplemented with 1% Triton X-100 by passages through a 25-gauge needle. A subsequent ultra-centrifugation as above resulted in the soluble membrane fraction. From both soluble fractions, 150-μl aliquots were taken up in an equal amount of 2× SDS sample buffer (+DTT).

Each sample was denatured at 45 °C for 20 min, and 15 μl was used for SDS-PAGE followed by transfer to nitrocellulose (Schleicher & Schuell). The membrane was blocked (TBS at pH 7.4, 0.25% Triton X-100, 5% milk powder) for 2 h and incubated overnight either with rabbit polyclonal antibodies raised against the C terminus of rat NHE-3 (1:3000; serum 1314), the N terminus of NaPi-IIa (1:6000), and VP16 (1:350; Clontech) or with a mouse monoclonal antibody raised against the N-terminus of NaPi-IIa (1:3000; serum 1314), the N terminus of NaPi-IIa (1:6000), and goat anti-mouse NHE-1 (1:100,000; Amersham Biosciences) were applied for enhanced chemiluminescence development (Pierce).

**Immunofluorescence of Yeast**—Cells were grown overnight to an A650 in 30 ml of SD–Leu and fixed for 30 min in 5% formaldehyde at room temperature. Approximately 106 cells (10 ml of culture) were collected (700 × g for 5 min) and incubated for 2 h at room temperature in PF buffer (100 mM KPO4 at pH 6.5, 0.5 mM MgCl2, 5% formaldehyde). After equilibration to SP buffer (40 mM KPO4 at pH 6.5, 0.5 mM MgCl2, 1.2 M sorbitol), cells were resuspended in 1 ml of 1.35 mg/ml Zymolyase-20T (Seikagaku Corp., Tokyo, Japan) dissolved in SP buffer plus 0.2% (v/v) β-mercaptoethanol and converted to spheroplasts with shaking at 100 rpm for 30 min at 30 °C. Cells were centrifuged at 100 × g for 5 min at 4 °C, washed three times in SP buffer, spotted on precoated poly-L-lysine slides (O. Kindler GmbH and Co., Freiburg, Germany) and subjected to an acetone/methanol treatment as described by Gotta et al. (40). Washed spots were blocked with PBS-T (PBS at pH 7.4, 2% BSA, 0.1% Triton X-100) and incubated overnight with the following polyclonal antibodies: rabbit anti-rat NHE-3 (1:1000, C-terminal; serum 1358), rabbit anti-rat NaPi-IIa (1:1000, N-terminal), and goat anti-mouse NHE-1 (1:200, C-terminal; Santa Cruz Biotechnology). After rinsing in PBS-T, primary antibodies were detected using goat anti-rabbit FITC (1:50; Dako Cytomation) or donkey anti-goat Alexa Fluor 488 (1:200; Molecular Probes). Spots were intensively washed in PBS-T, stained for 3 min with 1 μg/ml 4′,6-diamidino-2-phenylindole, and mounted using Dako Gylcergel. Finally a Leica TCSSP laser scanning microscope (Wetzlar, Germany) equipped with a 63× oil-immersed objective was applied for confocal microscopy.

**Overlay of the Class I PDZ Domain Proteomic Array**—The C-termini 79 (CT) or 76 (ΔTRL) amino acids of mouse NaPi-IIa were expressed between EcoRI and Xhol sites of pGEX-6P-2 (GE Healthcare) and batch-purified on glutathione-agarose beads (1 μl of bead resin/ml of cleared lysate) following the procedure as delineated previously (16). Upon elution in EB buffer (50 mM Tris-HCl at pH 8.0, 120 mM NaCl, 1 mM DTT, 0.1% (gepal CA-630, 20 mM reduced glutathione) for 30 min at 4 °C, supernatants were dialyzed against SB buffer (SB buffer devoid of glutathione) on Microcon YM-10 Centricron concentrators (Millipore; cutoff, 10 kDa). Proteins concentrated to 10 mg/ml in SB buffer plus 10% glycerol were stored at ~80 °C until use. The overlay of the derived array from a 100 nm concentration of each purified GST fusion protein has been described elsewhere (41).

**Transport Measurements in Xenopus Oocytes**—Mature Xenopus laevis females were purchased from the African Xenopus Facility C.C. (Noordhoek, South Africa) and kept under standard conditions. Stage V and VI oocytes from X. laevis were maintained at 18 °C in modified Barth’s solution MBS (88 mM NaCl, 1 mM KCl, 0.82 mM MgSO4, 0.4 mM CaCl2, 0.33 mM Ca(NO3)2, 2.4 mM NaHCO3, 20 mg/liter gentamycin sulfate, 10 mM HEPES/Tris at pH 7.4). Human NaS1 was cloned into plasmid pSPORT, whereas human NHERF-1 (accession number NM_004252) and NHERF-2 (accession number NM_004785) were generated in pGEX-6P-2. For cRNA synthesis, each plasmid was linearized by NotI digestion. The cDNA was in vitro transcribed using the mMessage mMachine (Ambion) T7 RNA polymerase kit, and
the resulting capped cRNA was dissolved in Milli-Q H2O. Oocytes were injected with either 50 nl of water (control), 5 ng of human NaS1 cRNA, 2.5 ng of human NHERF-1 cRNA, 2.5 ng of human NHERF-2 cRNA, or combinations using a Nanoject automatic injector (Drummond Scientific Co., Broomall, PA). Transport measurements were performed by a [35S]sulfate uptake on day 3 postinjection. Briefly 10 oocytes (individual data point) were washed at room temperature for 2 min in solution A (100 mM NaCl, 2 mM KCl, 1 mM CaCl2, 1 mM MgCl2, 10 mM Hepes/Tris at pH 7.5) and placed for 30 min at room temperature into 100 μl of solution A containing 0.1 mM Na2SO4 and 10 mM cysteine ml of 35SO42− (PerkinElmer Life Sciences). The oocytes were washed four times with ice-cold solution A, lysed with 1% SDS, and dissolved in scintillant (Emulsifier Safe, Canberra Packard) before counting by liquid scintillation spectrometry.

**Immunoprecipitation from Xenopus Oocyte Membranes**—Rat NaPi-IIa (±ΔTRL) and rat NaS1 were cloned in-frame between XhoI and Cid sites of pSD-HA or pSD-myc, both descendent vectors of pSD-Easy with additional N-terminal tags. The resulting constructs were linearized with BglII and transcribed on SP6 (Ambion). X. laevis oocytes were (co-)injected with 10 ng of each cRNA and incubated for 3 days at 16 °C. Total membranes were isolated from 30 oocytes and solubilized in 0.5% (w/v) sodium deoxycholate for 1 h at room temperature. The solubilized material was used for immunoprecipitations at 4 °C for 16 h in sucrose buffer containing 0.1% (w/v) Triton X-100 as described by de Jong et al. (42). Modifications from this protocol included the removal of yolk proteins twice by centrifugation at 200 × g (instead of 3000 × g) and the immobilization of 1.25 μl from a mouse anti-HA antibody (clone HA-7; Sigma) on 40 μl of Protein G plus/Protein A-agarose suspension (Calbiochem) in IPP500 (42). After four washes in IPP100 (42), proteins were eluted in 50 μl of 2× SDS sample buffer (+DTT) for 30 min at 37 °C and subjected in equal amounts to immunoblotting as delineated below. Primary antibodies were as follows: mouse anti-HA (clone HA-7, 1:10,000; Sigma), rabbit anti-MYC (2 μg/ml; Upstate, Waltham, MA), or rabbit anti-NaPi-IIa (1:6000).

**RESULTS**

**Construction of MYTH 2.0 Bait Vectors**—The current bait vectors for the conventional MYTH system fuse the Cub-TF reporter to the C terminus of a membrane-associated bait. The appropriate centromeric bait construct pCYC-Bait-Cub-TF permits constitutive low level expression in yeast. 

**Choice, Expression, and Localization of Baits**—The mammalian membrane proteins NaPi-IIa (16), MAP17 (6, 17, 43), and NHE-3 (22, 44), which are all apically localized, require at least their free C termini to bind the previously identified PDZ proteins (6, 14, 16, 17, 45). Along with apical NaS1 (9, 46, 47) and basolateral NHE-1 (7), these particular integral membrane proteins were generated as TF-Cub-Baits in the setting of the MYTH 2.0 system (Fig. 2A) with the goal to verify PDZ protein-membrane protein interactions based herein on full-length membrane proteins. Because only the MYTH 2.0 aligns with the prerequisite of C-terminal unmasked baits, the conventional MYTH system was found to be not applicable in this sense (data not shown).

To illustrate that the five renal epithelial membrane protein baits are (i) properly expressed, (ii) inserted into the yeast membrane, and (iii) not self-activated by triggering reporter genes in the absence of any interacting protein, we conducted genetic, biochemical, and immunofluorescence assays. The five TF-Cub-membrane bait proteins were analyzed in the presence of two non-interacting yeast integral membrane proteins, namely Ost1p as a member of the oligosaccharyltransferase complex in ER and Fur4p as a uracil permease localized to the plasma membrane. They were fused to either NubI (Ost1-NubI and Fur4-NubI) or NubG (Ost1-NubG and Fur4-NubG). Both Nub control fusion constructs are designed to activate the yeast reporter system irrespective of a protein-protein interaction because of the high affinity of Nubl to associate with Cub into active ubiquitin (25). Activation of the reporter system was measured as growth on media lacking histidine and adenine in the presence of the optimal concentration of 3-AT (Fig. 2B). All five mammalian TF-Cub-Bait chimeras exhibited growth on the selective medium in the case of co-expression with either NubI fusion, indicating that they are properly inserted into the membrane. On the other hand, co-expression of the five mammalian TF-Cub-Bait fusions with the non-interacting Ost1-NubG and Fur4-NubG protein fusions did not lead to initiation of the reporter system, indicating that the chosen TF-Cub-Bait chimeras are not self-activating (Fig. 2B).

In addition, membrane targeting of TF-Cub-Baits was analyzed from yeast lysates after sequential high speed centrifugations. Upon segregation of the cytosolic fraction, the residual pellet was solubilized in 1% of Triton X-100 and subsequently ultracentrifuged to obtain the membrane fraction (Fig. 2C). The yeast ER membrane protein Alg5p was included to validate this fractionation procedure. Although the soluble TF-Cub moiety alone from vector pTLB-1 was found in the cytosolic fraction, all five mammalian full-length TF-Cub-Bait proteins were present only in the membrane fraction (Fig. 2C). Furthermore immunoblotting of total extracts from yeast against VP16 from the TF module verified the correct expressions of all TF-Cub-Baits (supplemental Fig. 1A). Two similarly sized immunoreactive bands of NHE-3 and NaPi-IIa were detected by anti-VP16, but
only the upper band of NHE-3 and both bands of NaPi-IIa were recognized by the appropriate transporter-specific antibodies.

To address whether a chosen mammalian protein can fulfill transport activity as a TF-Cub fusion in yeast, NaPi-IIa bait protein was tested for phosphate uptake in yeast strain PM971, which is devoid of high affinity Pi co-transport systems (38). PM971 was transformed with the bait TF-Cub-NaPi-IIa and deprived of Pi before uptake measurements. In this strain, the type IIa NaPi cotransporter was functionally expressed with concomitant Na\(^+\)-dependent Pi transport (Fig. 2D). As shown in the bar graph, the amount of Pi uptake rose relative to time of incubation indicating that the NaPi-IIa transporter is functionally active in the yeast cell membrane.

Lastly we conducted immunofluorescence analysis to localize NHE-3, NHE-1, and NaPi-IIa in yeast. In spheroplasts from yeast cells treated with zymolyase, transporter-specific
Fig. 2. Characterization of various mammalian TF-Cub-Baits in yeast. A, membrane topology of TF-Cub-Baits (NaPi-IIa, MAP17, NHE-1, NHE-3, and NaS1) with N-terminally tagged TF-Cub fusion. B, proper insertion of TF-Cub-Baits into the membrane as exemplified by genetic NubG/NubI experiments. Yeast cells expressing TF-Cub-Baits of NaPi-IIa, MAP17, NHE-1, NHE-3 (pTLB-1 context), and NaS1 (pCLB-1 context) were transformed with control constructs of either yeast Ost1p (ER) or Fur4p (plasma membrane) fused to NubG and NubI. Yeast growth was challenged on minimal SD medium depleted of Trp and Leu (SD/H11002WL) or Trp, Leu, Ade, and His (SD/H11002WLAH) by spotting three independent transformants on the different media. A lack of growth in the presence of the two yeast integral membrane proteins fused to NubG confirms that TF-Cub-tagged mammalian renal baits are not self-activating. In contrast, growth on the same plates in the presence of either NubI construct indicates that the chimeric bait proteins are expressed, and the proteins are properly inserted into the membrane. C, targeting of TF-Cub-Baits to the membrane of yeast. Full-length proteins NaPi-IIa, NHE-1, NHE-3, and NaS1 were expressed in yeast as TF-Cub-Baits from pTLB-1. Lysates thereof were subjected to two consecutive ultracentrifugations to isolate the cytosolic fraction, C, from the detergent-soluble membrane fraction, M. An equal volume from each supernatant was used for the detection of VP16 from the TF-Cub-Bait proteins in immunoblots (IB). The localization of the endogenous ER membrane protein Alg5 to the detergent-soluble membrane fraction validates the centrifugation procedure (n = 2). D, functional phosphate transport activity of TF-Cub-NaPi-IIa in yeast. Yeast strain PM971 with a deficiency of two high affinity Pi transporters was transformed with TF-Cub-NaPi-IIa. In this strain, the bait was properly expressed as a post-translationally modified full-length and single form (left panel, arrowhead). Timed 32P uptake was measured at 30 °C on P-starved PM971 cells. Relative net Na+/H+ dependent transport of Pi was calculated by subtracting the Na+/H+ independent component (~40%) from the total Pi uptake (right panel). Values are means of triplicate. The experiment was repeated twice with similar results (n = 3). E, localization of TF-Cub-Baits in yeast. Fixed and acetone-treated spheroplasts of yeast were processed for confocal microscopy using transporter-specific primary and FITC-conjugated secondary antibodies (green). Nonspecific immunofluorescence is shown in the lower panels from vector-transformed cells (n = 2). Confocal planes were based on the strongest 4',6-diamidino-2-phenylindole stain (blue) of the nuclei, the centers of which are designated by arrows. Secondary antibodies alone yielded no signals (not shown). PM, plasma membrane.
immunofluorescence of TF-Cub-Bait from NaPi-IIa was strictly confined to the plasma membrane. The analog bait of NHE-1 was expressed exclusively in the plasma membrane, whereas that of NHE-3 was distributed in both intracellular and cellular membrane pools (Fig. 2E). In summary, the results of these genetic, biochemical, and cell biological experiments strongly suggest that the N-terminal TF-Cub fusions of NaPi-IIa, MAP17, NHE-3, NaS1, and NHE-1 are properly inserted into the yeast membrane and are not self-activating and that at least the TF-Cub-NaPi-IIa bait is functionally active in yeast.

**Choice of PDZ Targets as Preys—** The interactions of the C termini derived from NHE-3, NaPi-IIa, and MAP17 with the PDZ proteins PDZK1, PDZK2, NHERF-1, NHERF-2, and CAL have been demonstrated using genetic as well as biochemical techniques (6, 16, 17). Recently a proteomic PDZ array was constructed that comprised almost 100 class I PDZ domains from proteins of different tissues (Table I) (41). The interaction between NaPi-IIa and PDZ domains is exemplified in Fig. 3. An overlay with a GST-fused C-terminal tail of NaPi-IIa identified PDZ3 of PDZK1 (bin G4), PDZ3 of PDZK2 (bin G8), and PDZ1 of NHERF-1 (bin B3) with high affinity as well as PDZ2 of NHERF-1 (bin B4), PDZ1 of NHERF-2 (bin B5), CAL (bin B10), and Densin-180 (bin E1) with moderate affinity (Fig. 3A). These interactions depend on TRL from the extreme C-terminal end of NaPi-IIa because removal of this motif resulted in no detectable binding (Fig. 3B). Thus, this array identified only NaPi-IIa-interacting PDZ proteins that had already been published thereby supporting the strikingly high sensitivity and selectivity of NaPi-IIa toward these PDZ proteins.

**PDZ-mediated Protein-Protein Interactions Detected by the MYTH 2.0 System—** Based on the immunoblot against the HA tag from total extracts, fusions of the PDZ proteins PDZK1, NHERF-1, NHERF-2, and CAL to the C terminus of NubG-HA were detected as full-length proteins in yeast (supplemental Fig. 1B). A combination of these NubG-PDZ proteins with various TF-Cub-Baits was then applied to the new MYTH 2.0 system to test whether the entire membrane-inserted mammalian renal baits can still associate with their respective PDZ proteins. Reporter activity was measured by auxotrophic growth selection on media lacking histidine and adenine but

| Bin | PDZ domain | Bin | PDZ domain | Bin | PDZ domain |
|-----|------------|-----|------------|-----|------------|
| A1  | MAGI-1 PDZ1 | C9  | E6TP1 PDZ  | F5  | MUPP1 PDZ7 |
| A2  | MAGI-1 PDZ2 | C10 | ERBIN PDZ  | F6  | MUPP1 PDZ8 |
| A3  | MAGI-1 PDZ3 | C11 | ZO-1 PDZ1  | F7  | MUPP1 PDZ10|
| A4  | MAGI-1 PDZ4+5 | C12 | ZO-1 PDZ2  | F8  | MUPP1 PDZ12|
| A5  | MAGI-2 PDZ1 | D1  | ZO-1 PDZ3  | F9  | MUPP1 PDZ13|
| A6  | MAGI-2 PDZ2 | D2  | ZO-2 PDZ1  | F10 | PTPN13 PDZ1|
| A7  | MAGI-2 PDZ3 | D3  | ZO-2 PDZ2  | F11 | PTPN13 PDZ2|
| A8  | MAGI-2 PDZ4 | D4  | ZO-2 PDZ3  | F12 | PTPN13 PDZ3|
| A9  | MAGI-2 PDZ5 | D5  | ZO-3 PDZ1  | G1  | PTPN13 PDZ4+5|
| A10 | MAGI-3 PDZ1 | D6  | ZO-3 PDZ2  | G2  | PDZK1 PDZ1|
| A11 | MAGI-3 PDZ2 | D7  | ZO-3 PDZ3  | G3  | PDZK1 PDZ2|
| A12 | MAGI-3 PDZ3 | D8  | C2PA PDZ   | G4  | PDZK1 PDZ3|
| B1  | MAGI-3 PDZ4 | D9  | GIPC PDZ   | G5  | PDZK1 PDZ4|
| B2  | MAGI-3 PDZ5 | D10 | MALS-1 PDZ | G6  | PDZK2 PDZ1|
| B3  | NHERF-1 PDZ1 | D11 | MALS-2 PDZ | G7  | PDZK2 PDZ2|
| B4  | NHERF-1 PDZ2 | D12 | MALS-3 PDZ | G8  | PDZK2 PDZ3|
| B5  | NHERF-2 PDZ1 | E1  | Densin-180 PDZ | G9  | PDZK2 PDZ4|
| B6  | NHERF-2 PDZ2 | E2  | Rhophilin PDZ1 | G10 | LNX1 PDZ1|
| B7  | PSD-95 PDZ1+2 | E3  | Rhophilin PDZ2 | G11 | LNX1 PDZ2|
| B8  | PSD-95 PDZ3 | E4  | Harmonin PDZ1 | G12 | LNX1 PDZ3|
| B9  | PDZ-GEF-1 PDZ | E5  | Harmonin PDZ2 | H1  | LNX1 PDZ4|
| B10 | CAL PDZ | E6  | Neurabin PDZ | H2  | LNX2 PDZ1|
| B11 | nNOS PDZ | E7  | Spinophilin PDZ | H3  | LNX2 PDZ2|
| B12 | INADL PDZ5 | E8  | α1 syntrophin PDZ | H4  | LNX2 PDZ3|
| C1  | INADL PDZ6 | E9  | β1 syntrophin PDZ | H5  | LNX2 PDZ4|
| C2  | INADL PDZ7 | E10 | β2 syntrophin PDZ | H6  | LARG PDZ|
| C3  | SAP97 PDZ1+2 | E11 | γ1 syntrophin PDZ | H7  | MAST-205 PDZ|
| C4  | SAP97 PDZ3 | E12 | γ2 syntrophin PDZ | H8  | Tamalin PDZ|
| C5  | SAP102 PDZ1+2 | F1  | PAPIN PDZ1 | H9  | Shank PDZ|
| C6  | SAP102 PDZ3 | F2  | MUPP1 PDZ1 | H10 | Rho-GEF PDZ|
| C7  | Chapsyn110 PDZ1+2 | F3  | MUPP1 PDZ4 | H11 | RA-GEF PDZ|
| C8  | Chapsyn110 PDZ3 | F4  | MUPP1 PDZ6 | H12 | None |

Decryption of bin codes from the class I PDZ domain proteomic array

| Bin | PDZ domain |
|-----|------------|
| A1  | MAGI-1 PDZ1 |
| A2  | MAGI-1 PDZ2 |
| A3  | MAGI-1 PDZ3 |
| A4  | MAGI-1 PDZ4+5 |
| A5  | MAGI-2 PDZ1 |
| A6  | MAGI-2 PDZ2 |
| A7  | MAGI-2 PDZ3 |
| A8  | MAGI-2 PDZ4 |
| A9  | MAGI-2 PDZ5 |
| A10 | MAGI-3 PDZ1 |
| A11 | MAGI-3 PDZ2 |
| A12 | MAGI-3 PDZ3 |
| B1  | MAGI-3 PDZ4 |
| B2  | MAGI-3 PDZ5 |
| B3  | NHERF-1 PDZ1 |
| B4  | NHERF-1 PDZ2 |
| B5  | NHERF-2 PDZ1 |
| B6  | NHERF-2 PDZ2 |
| B7  | PSD-95 PDZ1+2 |
| B8  | PSD-95 PDZ3 |
| B9  | PDZ-GEF-1 PDZ |
| B10 | CAL PDZ |
| B11 | nNOS PDZ |
| B12 | INADL PDZ5 |
| C1  | INADL PDZ6 |
| C2  | INADL PDZ7 |
| C3  | SAP97 PDZ1+2 |
| C4  | SAP97 PDZ3 |
| C5  | SAP102 PDZ1+2 |
| C6  | SAP102 PDZ3 |
| C7  | Chapsyn110 PDZ1+2 |
| C8  | Chapsyn110 PDZ3 |

GEF, guanine nucleotide-exchange factor; nNOS, neuronal nitric-oxide synthase; RA, Ras/Rap1-associating; INADL, INAD-like; GIPC, GAIP-interacting protein C terminus; PAPIN, plakophilin-related armadillo-repeat protein-interacting PDZ protein.
Truncating the entire C terminus (ΔCT) of NHE-3 as a MYTH 2.0 bait disrupted all PDZ interactions (data not shown). Furthermore full-length NHE-3 and its C-terminal PDZ-binding motif deletion ΔTHM did not interact with CAL, but both NHE-3 forms were detected to homodimerize with full-length NHE-3 (Fig. 4A, all panels). A detailed investigation of the NHE-3 homodimerization will be published separately. Pertaining to controls, Ost1-NubG and Ost1-NubI represented negative and positive interactions of NHE-3, respectively.

The interaction pattern of TF-Cub-NaPi-IIa was distinct from that of NHE-3 in terms of MYTH assemblies: it recognized all NubG-PDZ proteins although by different relative reporter activities (Fig. 4B, left and right panels). TF-Cub-NaPi-IIa with a C-terminal TRL deletion (NaPi-IIa ΔTRL) either completely lost its interactions with PDZ proteins or decreased colony survival of PDZ cotransformants to background (Fig. 4B, middle and right panels). In addition, deletion of the entire C terminus (ΔCT) from NaPi-IIa in MYTH showed no binding to all tested PDZ proteins (data not shown). This indicates that putative non-canonical accessory sites within all intracellular domains of NaPi-IIa per se are not implicated in interactions with the applied PDZ proteins. Lastly NaPi-IIa homodimerized with both NaPi-IIa full-length and ΔTRL-truncated proteins in yeast as elucidated in details below (see Fig. 6).

In bait dependence tests, all PDZ proteins displayed high selectivity toward their target membrane bait proteins. The TF-Cub-Bait of NHE-1 that does not possess any of the specified PDZ-binding motifs (C-terminal PKGQ) was negative for all PDZ proteins (Fig. 4C). Analogous results were obtained with unrelated Bait-Cub-TF chimeras of yeast Gpr1p (the yeast G-protein-coupled receptor) and Ost1p (data not shown) as well as with the human receptor tyrosine kinase ErbB3 (supplemental Fig. 2, left panel). In agreement with recent findings (6, 17), TF-Cub-MAP17 might interact more strongly with PDZK1 than with NHERF-1 or NHERF-2 when merely based on semiquantitative ONPG values (Fig. 4D).

Analysis of the Human NaS1 Transporter Using the MYTH 2.0 System—Previous findings of hyposulfatemia and hyper-sulfaturia in NaS1-null mice (48) evinced the apical NaS1 membrane transporter to play a major role in mediating proximal tubule sulfate reabsorption in kidney. Despite its importance, the investigation of NaS1 interactions has not been tackled by any of the available interactive proteomics technologies so far. To counteract this fact, we conducted a MYTH 2.0 screen using a NubG-tagged human kidney cDNA library to find NaS1 protein interactors. To that end, cDNA fragments originating from human kidney of ~0.6–8 kb in length were inserted C-terminally to the NubG sequence generating the prey library in the NubG-X orientation where X stands for any cDNA insert. This library was introduced by transformation into the yeast strain expressing the human

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O. Moe, unpublished data.
A Modified Split-Ub Membrane Yeast Two-hybrid (MYTH 2.0)

TF-Cub-NaS1 bait. A MYTH 2.0 screen of $\sim 8 \times 10^8$ yeast transformants revealed 31 novel NaS1-interacting proteins; among them were NHERF-1, NHERF-2, and PDZK1 (Table II). Although this study focused attention on the aforementioned PDZ proteins, the characterization of the remaining 28 newly identified NaS1 interactors will be described elsewhere. When tested in the MYTH 2.0 system, reporter activity was strong from TF-Cub-NaS1 NHERF-1 and weaker with PDZK1 or NHERF-2 (Fig. 5A). These interactions were specific for the N-terminally TF-Cub-tagged NaS1 protein because none of them were detected using an NaS1 bait (NaS1-Cub-TF) in the conventional MYTH format (supplemental Fig. 2, right panel).

To identify which regions of NaS1 are required for binding to PDZK1, NHERF-1, and NHERF-2, full-length (aa 1–595) TF-Cub-NaS1 bait and several truncations thereof (aa 1–239, 1–345, 1–426, 1–532, and 1–573) were tested for their ability...
to interact with the newly identified PDZ proteins in MYTH dot assays. The correct expression and localization of all TF-Cub-NaS1 deletion mutants were assessed by NubG and NubI assays. The correct expression and localization of all TF-Cub-NaS1 baits (Fig. 5B, C) or by immunoblotting analysis using an anti-VP16 antibody (data not shown). According to our mapping experiments, full-length NaS1 (aa 1–595) possessed epitopes on the transporters although the undersized band at 70 kDa for NaPi-IIa may imply a deficient immunoblotting. In this experiment, sulfate uptake was significantly measured by NHERF-1/2 isoforms. We performed a transport assay in Xenopus oocytes and measured [35S]sulfate uptake. After co-expressing NaS1 with either NHERF-1 or NHERF-2, sulfate uptake was significantly augmented (Fig. 5C), suggesting a functional regulation of NaS1 by NHERF-1/2 isoforms.

**Demonstration of NaPi-IIa Oligomerization via the MYTH 2.0**—Besides testing protein-protein interactions between mammalian transporters and their PDZ partners, the MYTH 2.0 system is also suited to investigate the oligo/dimeric state of membrane proteins. As depicted in supplemental Fig. 1B, NHE-3 and NaPi-IIa proteins in a NubG-Prey setting were labeled on immunoblots against the HA tag or against epitopes on the transporters although the undersized band at 70 kDa for NaPi-IIa may imply a deficient N-glycosylation post-translational modification. As shown in Fig. 6A, NubG-NaPi-IIa as a prey strongly formed homeric dimers with the TF-Cub-Bait of NaPi-IIa or with its C-terminal truncations ΔTRL and ΔCT in dot assays (Fig. 6A). Dimerization was specific because none of the TF-Cub-NaPi-IIa baits induced

### Table II

| Accession no. | Description of identified human proteins | Process |
|--------------|------------------------------------------|---------|
| NM_000146    | Ferritin, light polypeptide (FTL)         | Phosphorylation |
| NM_018245    | Oxoglutarate dehydrogenase-like (OGDHL)  | Metabolism |
| NM_006611    | CD59 molecule, complement-regulatory protein (CD59) | Signal transduction |
| NM_000925    | Serpin peptidase inhibitor, clade A, member 1 (SERPINA1) | Unknown |
| NM_020312    | Coenzyme Q9 homolog (S. cerevisiae) (COQ9) | Metabolism |
| NM_003002    | Succinate dehydrogenase complex, subunit D (SDHD) | Transporter/channel |
| NM_002614    | PDZ domain-containing 1 (PDZK1) | Signal transduction |
| NM_018477    | Actin-related protein 10 homolog (S. cerevisiae) (ACTR10) | Cytoskeleton network |
| NM_013446    | Makorin, ring finger protein, 1 (MKRN1)  | Metabolism |
| NM_006821    | Acyl-CoA thiosterase 2 (ACOT2) | Metabolism |
| NM_003345    | Heat shock 70-kDa protein 1A (HSPA1A)    | Protein transport |
| NM_013336    | Sec61 α₁ subunit (S. cerevisiae) (SEC61A1) | Protein transport |
| NM_004252    | Na⁺/H⁺ exchanger, member 3, regulator 1 (SLC9A3R1) | Signal transduction |
| NM_001664    | Ras homolog gene family, member A (RHOA) | Cell organization/biosynthesis |
| NM_006034    | Tumor protein p53-inducible protein 11 (TP53I11) | Protein degradation |
| NM_003006    | Selectin P ligand (SELPLG) | Unknown |
| NM_006827    | Transmembrane emp24-like trafficking protein 10 (TMED10) | Protein transport |
| NM_000050    | Argininosuccinate synthetase | Metabolism |
| NM_002773    | Protease, serine, 8 (prostasin) (PRSS8)  | Protein biosynthesis |
| NM_032811    | Transforming growth factor β regulator 1 (TBRG1) | |
| NM_015161    | ADP-ribosylation factor-like 6-interacting protein 1 (ARL6IP1) | Protein biosynthesis |
| NM_014220    | Transmembrane 4 L six family member 1 (TM4SF1) | |
| NM_001642    | Amyloid β (A4) precursor-like protein 2 (APLP2) | Signal transduction |
| NM_006702    | Patatin-like phospholipase domain-containing 6 (PNPPLA6) | Metabolism |
| NM_032341    | Coenzyme Q5 homolog, methyltransferase (COQ5) | Metabolism |
| NM_001037494 | Dynel, light chain, LC8-type 1 (DYNL1L1) | Cell organization/biosynthesis |
| NM_002087    | Granulin (GRN) | Signal transduction |
| NM_004552    | NADH dehydrogenase (ubiquinone) Fe-S protein (NDUFS) | |
| NM_001885    | Crystallin, αB (CRYAB) | Signal transduction |
| NM_003006    | Tetraspanin 3 (TSPAN3) | Unknown |
| NM_004785    | Actin-related protein 10 homolog (S. cerevisiae) (ACTR10) | Cytoskeleton network |
| NM_005724    | Tumor protein p53-inducible protein 11 (TP53I11) | Protein degradation |
| NM_000050    | Argininosuccinate synthetase | Metabolism |
| NM_002773    | Protease, serine, 8 (prostasin) (PRSS8)  | Protein biosynthesis |
| NM_032811    | Transforming growth factor β regulator 1 (TBRG1) | |
colony survival in the presence of another renal transporter, NHE-3 (Fig. 6A). These data collectively suggest that the homodimeric property of NaPi-IIa does not involve either the N- or C-terminal ends.

Dimerization of NaPi-IIa was further confirmed in X. laevis oocytes utilizing cRNAs of N-terminal HA- and MYC-tagged NaPi-IIa. The glycosylated and tagged forms of the NaPi-IIa protein were found in the membrane fraction from singly injected oocytes (Fig. 6B, lower panel). An anti-HA immunoprecipitate from membranes of co-injected oocytes contained either MYC-NaPi-IIa or MYC-NaPi-IIa ΔTRL (Fig. 6B). A PDZ scaffold is therefore not required for type IIa NaPi dimerization in oocytes. Homodimerization of NaPi-IIa was specific as neither co-injected MYC-NaS1 nor separately injected HA-NaPi-IIa was retained (Fig. 6B).

DISCUSSION

The fact that most of the widely used genetic tools for studying protein-protein interactions solely accommodate...
soluble domains from proteins poses a significant obstacle to our understanding of membrane-anchored or -inserted proteins. Thus far, conventional YTH only accepts the participation of hydrophilic tails from integral membrane proteins. Such soluble tails of transmembrane proteins may acquire partially altered conformations when unhitched from the integrity of their holoproteins begetting either false positive or negative results. Theoretically the previously described conventional MYTH in the split-Ub format can surmount this hurdle. Since its introduction, the MYTH technology indeed has led to the dissection of quaternary structures from protein complexes such as sucrose transporters (49); a yeast protein translocation machinery (25, 50); a receptor tyrosine kinase, ErbB3 (24); physical interactions between plant K+ channels (31); and a yeast ATP-binding cassette transporter Ycf1p (26).

Scaffolding PDZ proteins usually recognize a few amino acids at the very ends of soluble C-terminal tails from transmembrane proteins (1). Recent studies also showed that PDZ domain-containing proteins recognize and bind non-C-terminal motifs (2, 51, 52). However, the impact of a full-length membrane-inserted protein on a PDZ-mediated association and the possibility of binding sites beyond the C-terminal domain have thus far not been addressed with any interactive proteomics technology. To this end, we modified a conventional MYTH to a new format where the C-terminal tail of a polytopic membrane bait protein is unhindered to interact for instance with PDZ prey proteins (Fig. 1A). This work illustrates five salient features. First, all transmembrane bait proteins examined were targeted to the appropriate location and were processed properly in yeast. Second, we verified interactions of PDZ proteins from proximal tubular cells, some of which were initially identified in conventional YTH traps, but we applied herein full-length membrane proteins instead of only their cytosolic termini as baits. The newly developed MYTH 2.0 system reproduced the PDZ-binding profile with high fidelity. Third, we discovered new interacting candidates such as CAL for NaPi-IIa or PDZK1, NHERF-1, and NHERF-2 for NaS1 by either screening a human kidney NubG-tagged cDNA library or by applying direct trap assays. In this regard, it is worth pointing out the unprecedented and successful subjection of NaS1 to proteomics, which is the merit of the modified MYTH 2.0 system. Fourth, we used the MYTH 2.0 assay to map and confine the regions on NaS1 required for binding to the above PDZ proteins. Finally the modified system disclosed homodimerization of NaPi-IIa that was accomplished by domains other than its cytoplasmic tails. Thus, the MYTH 2.0 represents a useful tool to divulge physiological binding partners/regulators of membrane proteins and establishes a novel horizon for interactive proteomics studies of membrane protein complexes.

When the proteins NHE-3, NHE-1, NaPi-IIa, MAP17, and NaS1 were inserted in the MYTH 2.0 assembly with their C termini uncoupled from reporters, full length and processed TF-Cub-Baits were obtained in yeast (Fig. 2). Several findings...
imply that the fusions of TF-Cub to the transporter proteins were probably embedded in a natural membranous setting of yeast: (i) lack of transporters in a non-solubilized cell fraction; (ii) distribution of NHE-3, NaPi-IIa, and NHE-1 as TF-Cub-Baits in a pattern similar to their native distribution in mammalian cells; and (iii) Na\(^+\)-dependent P\(_i\) entry after transformation with TF-Cub-NaPi-IIa (Fig. 2, C–E). We were not able to show transformation-dependent Na\(^+\)/H\(^+\) exchange activity in this strain because of a multitude of endogenous alkali-metal-cation transporters that are present in yeast as noted previously (53).

The PDZ proteins PDZK1, NHERF-1, NHERF-2, and CAL were first isolated by conventional YTH using soluble C termini derived from NHE-3, NaPi-IIa, and MAP17 as baits (6, 16, 17). These interactions have been mapped to cover C-terminal class I PDZ motifs of the transporters (6, 16, 17). No further interacting PDZ proteins have been unveiled for NHE-3, NaPi-IIa, and MAP17. This is especially true for NaPi-IIa whose C terminus was overlaid on an array of up to 100 different PDZ proteins resulting in the detection of only PDZK1/2, NHERF-1/2, and CAL (Fig. 3).

In the MYTH 2.0 system, relative quantification of β-galactosidase activity was indicative for strong interaction of NHERF-1 and NHERF-2 with NHE-3 (Fig. 4A). Remarkably truncating NHE-3 of the C-terminal PDZ motif (ΔTHM) or of the entire C terminus either marginally or totally abrogated split-Ub formation along with NHERF-1. Hence internal binding sites within the C-terminal domain of NHE-3 must exist for NHERF-1 but not for NHERF-2 whose target binding site on NHE-3 is completely C-terminal (Fig. 4A). Such deviation from the prototypic C-terminal PDZ recognition was initially attributed to both cofactors by in vitro assays (15).

NaPi-IIa exhibited a broader binding spectrum than NHE-3 because all tested PDZ proteins, PDZK1, NHERF-1/2, and CAL, were able to at least moderately induce the reporter genes in the MYTH 2.0 assay. The finding that binary complexes of NaPi-IIa with NHERF-1 and -2 might have binding affinities similar to that of NHE-3, when judged by Miller units from MYTH complementations, may have consequences to infer Na\(^+\), H\(^+\), and P\(_i\)-coupled homeostasis. The modality of internal binding sequences probably does not pertain to NaPi-IIa because the last three C-terminal amino acids TRL were mandatory for all PDZ interactions in the MYTH 2.0 (Fig. 4B) and in the proteomic PDZ array (Fig. 3). Interestingly CAL did not associate with any other bait protein used in this study except NaPi-IIa. Therefore, CAL might be involved in specific regulation of NaPi-IIa in a manner similar to that described for the retrograde trafficking of CFTR (19). Furthermore NaPi-IIa was found to form homodimers. This dimerization was confirmed by immunoprecipitation from an oocyte expression system and was achieved by membrane-inserted domains without any engagement of cytosolic termini or a submembranous PDZ scaffold (Fig. 6A).

MAP17 activated the MYTH system much more strongly in the presence of PDZK1 compared with NHERF-1 and NHERF-2 (Fig. 4D). It may be that MAP17 represents a specific auxiliary adaptor for the scaffolding protein PDZK1 from proximal tubular cells that concomitantly regulates the localization or turnover of PDZK1-coupled proteins such as NaPi-IIa (10, 11, 54). Moreover MYTH provided a valuable implement to identify the ability of intact NaS1 to interact with PDZK1, NHERF-1, and NHERF-2 (Fig. 5), a feature that was not achieved by any other interactive proteomics-based methods until now. The putative physiological relevance of NaS1-NHERF-1 and NaS1-NHERF-2 complexes was further validated by a functional assay showing that NHERF-1 and to a lesser extent NHERF-2 stimulate sulfate uptake in Xenopus oocytes when co-injected with NaS1. It is conceivable that NHERF-1 and/or NHERF-2 increase the protein expression, stability, or substrate turnover rate of NaS1 on the plasma membrane; however, the precise mechanism(s) still needs to be clarified. Of particular significance are our findings from the MYTH 2.0 mapping experiments using serial truncations of NaS1 as baits. The existence of internal PDZ-binding determinants on this sulfate transporter beyond the C-terminal motif, i.e. upstream of the C terminus, was revealed for PDZK1 and NHERF-1/2 isoforms comprising residues 532–555 or 426–532 of the transporters, respectively. Only rare cases of such internal PDZ-binding ligands on integral membrane proteins have been reported previously (3, 55).

Our data also demonstrate compelling evidence that NHE-3 and NaPi-IIa must contain N and C termini both facing the cytosol, whereas at least the N termini of MAP17 and NaS1 must be cytosolic. Otherwise it cannot be explained why co-expression of the corresponding TF-Cub-Baits with Ost1-NubI or Fur4-NubI in yeast resulted in a positive MYTH read-out and why co-expression with Ost1-NubG or Fur4-NubG was the opposite. In other words, the MYTH 2.0 can also be applied to partially characterize the topology of a particular integral membrane protein.

In conclusion, we have developed an altered membrane YTH system that will allow us to study intact polytopic membrane proteins, which are embedded in or anchored to the lipid bilayer as either bait or prey, for the detection of protein interactions involving this difficult class of hydrophobic proteins. This system can reproduce interactions that have been identified previously and can add additional information about new binding partners that were not detectable by any other protein-protein interaction method. Thus, we anticipate that our approach will greatly enhance the ability to disentangle complexes of membrane proteins and their interacting partners.

Acknowledgments—We acknowledge the kind contributions from the following individuals: Bengt Persson for the yeast strain PM971; Alexandru Bobulescu for technical assistance and helpful discussions; Daniel Auerbach, Safia Thaminy, Dawn Edmonds, and Corey Nislow for scores of control constructs and for helpful comments; Reinhart Reithmeier for the human kidney NubG-X cDNA library; and Paul Stehberger for fluorescence microscopy.
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