GABARAP deficiency modulates expression of NaPi-IIa in renal brush-border membranes

Sonja C. Reining,1 Serge M. Gisler,1,2 Daniel Fuster,2 Orson W. Moe,2,3 Gregory A. O’Sullivan,4 Heinrich Betz,4 Jürg Biber,1 Heini Murer,1 and Nati Hernandez1

1Institute of Physiology and Zurich Center for Integrative Human Physiology (ZIHP), University of Zurich, Zurich, Switzerland; 2Department of Internal Medicine and 3Charles and Jane Pak Center of Mineral Metabolism, University of Texas Southwestern Medical Center, Dallas, Texas; and 4Department of Neurochemistry, Max Planck Institute for Brain Research, Frankfurt am Main, Germany

Submitted 14 August 2008; accepted in final form 13 February 2009

Reining SC, Gisler SM, Fuster D, Moe OW, O’Sullivan GA, Betz H, Biber J, Murer H, Hernandez N. GABARAP deficiency modulates expression of NaPi-IIa in renal brush-border membranes. Am J Physiol Renal Physiol 296: F1118–F1128, 2009. First published February 18, 2009; doi:10.1152/ajprenal.90492.2008.—Renal reabsorption of inorganic phosphate (Pi) is mainly mediated by the Na+-dependent Pi-cotransporter NaPi-IIa that is expressed in the brush-border membrane (BBM) of renal proximal tubules. Regulation and apical expression of NaPi-IIa are known to depend on a network of interacting proteins. Most of the interacting partners identified so far associate with the COOH-terminal PDZ-binding motif (TRL) of NaPi-IIa. In this study GABARAP, receptor-associated protein (GABARAP) was identified as a novel interacting partner of NaPi-IIa applying a membrane yeast-two-hybrid system (MYTH 2.0) to screen a mouse kidney library with the TRL-truncated cotransporter as bait. GABARAP mRNA and protein are present in renal tubules, and the interaction of NaPi-IIa and GABARAP was confirmed by using glutathione S-transferase pulldowns from BBM and coimmunoprecipitations from transfected HEK293 cells. Amino acids 36–68 of GABARAP were identified as the determinant for the described interaction. In the vivo effects of this interaction were studied in a murine model. GABARAP+/− mice have reduced urinary excretion of Pi, higher Na+-dependent 32P uptake in BBM vesicles, and increased expression of NaPi-IIa in renal BBM compared with GABARAP+/+ mice. The expression of Na+/H+ exchanger regulatory factor (NHERF)1, an important scaffold for the apical expression of NaPi-IIa, is also increased in GABARAP+/− mice. The absence of GABARAP does not interfere with the regulation of the cotransporter by either parathyroid hormone or acute changes of dietary Pi content.

Membrane transport systems for inorganic phosphate (Pi) are the key players in maintaining whole organism Pi homeostasis. In the kidney, Pi excretion is determined by reabsorption of filtered Pi, that is mediated by the Na+-dependent Pi cotransporters NaPi-IIa and NaPi-IIc. Both transporters are expressed in the brush-border membrane (BBM) of proximal tubules (1, 11, 40). NaPi-IIa and NaPi-IIc use the lumen-to-cell electrochemical gradient of Na+ to accomplish uphill transport of Pi from the urine back into the cells. However, because of their different Na+-Pi stoichiometries, the transport mediated by NaPi-IIa is electrogenic, whereas NaPi-IIc is electroneutral (15, 40). In the adult kidney the amount of reabsorbed Pi is mainly determined by the apical expression of NaPi-IIa, which is regulated by different factors, among them dietary Pi intake and parathyroid hormone (PTH) (for review, see Ref. 16). Upregulation involves de novo protein synthesis and membrane insertion, whereas downregulation occurs by membrane retrieval and lysosomal degradation of NaPi-IIa, with little or no recycling back to the membrane (for review, see Ref. 16). Apical expression of NaPi-IIa depends on the presence of its last three amino acids (TRL) as illustrated by the observation that a ΔTRL cotransporter partially accumulates intracellularly (22). These last three amino acids constitute a PSD95/DlgA/ZO-1 (PDZ)-binding motif, which mediates interactions with various PDZ domain-containing proteins including the four members of the Na+/H+ exchanger regulatory factor (NHERF) family (18). NHERF1 harbors two PDZ domains and a merlin-ezrin-radixin-moesin (MERM)-binding domain. The first PDZ domain of NHERF1 binds to NaPi-IIa, whereas the MERM-binding domain interacts with proteins of the MERM family and thus may link NaPi-IIa to the actin cytoskeleton (38). Disruption of the interaction between NaPi-IIa and NHERF1 leads to a reduced apical expression of NaPi-IIa in cells (20). Moreover, basal expression of NaPi-IIa as well as its upregulation in response to low-Pi diet is impaired in NHERF1−/− mice (41, 51).

Here we used a novel screening technique that enables the detection of interaction between transmembrane proteins. This screen led to the identification of the GABAα receptor-associated protein (GABARAP) as a new interacting partner of NaPi-IIa. In addition to GABAα receptors (48), GABARAP interacts with a variety of other receptors (9, 19), as well as with various cytoplasmic proteins important for intracellular trafficking such as tubulin, gephyrin, the N-ethylmaleimide-sensitive factor (NSF), and the clathrin heavy chain (24, 26, 34, 49). This pattern of interactions suggested a role of GABARAP in protein trafficking by modulating the sorting, the surface expression, or the turnover rate of membrane proteins. Supporting this hypothesis is the finding that overexpression of GABARAP in cultured cells increases the cell surface expression not only of GABAα receptors (27) but also of the angiotensin II type 1 receptor (9). However, the synaptic localization of GABAα receptors is not affected in GABARAP−/− mice (36). In addition to a role in intracellular trafficking, GABARAP may function as an ubiquitin-like protein. The COOH terminus of GABARAP shares strong similarity to ubiquitin (10), and it undergoes a posttranslational modification that resembles ubiquitylation. Thus GABARAP is cleaved at G116, activated by E1- and E2-like enzymes, and finally conjugated to other...
molecules, most likely phospholipids (42, 44). Insertion of lipopeptides into the membrane could influence the membrane expression of proteins.

In the present study, we show that GABARAP is a novel interacting partner of NaPi-IIa. We postulated that GABARAP may regulate the expression of NaPi-IIa in the proximal BBM, similar to its effect on other membrane proteins. To test this hypothesis we compared the expression of the cotransporter in GABARAP+/− and GABARAP−/− mice. Knockout of GABARAP leads to increased expression of NaPi-IIa but not NaPi-IIc in renal BBM. The increased NaPi-IIa abundance in GABARAP−/− mice correlates with a higher Na+-dependent P Conv transport activity in BBM vesicles (BBMV) and reduced urinary excretion of P Conv. The expression of NHERF1 is also increased in GABARAP−/− mice. Finally, we show that the absence of GABARAP does not prevent endocytosis of NaPi-IIa in response to PTH administration or the acute dietary regulation of the cotransporter.

**EXPERIMENTAL PROCEDURES**

**Split-ubiquitin yeast two-hybrid screen.** A mouse kidney cDNA library with inserts linked to the NH2-terminal half of ubiquitin (NubG) in the vector pADSL-Nx was generated with the SMART cDNA construction kit (Clontech). The library was screened, using as bait NaPi-IIa lacking the COOH-terminal PDZ-binding motif (TRL). As recently published (17), the NH2 terminus of NaPi-IIa was fused to a chimera consisting of the COOH-terminal half of ubiquitin (Cub) and a transcription factor (TF) derived from the LexA DNA-binding domain and the VP16 activation domain. Interaction of bait with prey, split ubiquitin was complemented to induce the proteolytic release of TF and to ultimately allow activation of HIS3 and lacZ reporter genes. In this membrane yeast two-hybrid (YTH) system, determination of bait-prey interaction was done by colorimetric measurement of β-galactosidase activity after growth selection in the absence of histidine as reported previously (17).

**Animal studies.** GABARAP+/− mice were derived from a library (Omnibank) of embryonic stem cells in which various genes were inactivated by gene-trap methodology (55). One of the clones was used to generate a heterozygous GABARAP mouse by blastocyst injection (Lexicon Pharmaceuticals). GABARAP−/− mice were generated from transgenic 129SvEvBrd embryonic stem cells, injected into C57BL/6J albino blastocysts and backcrossed for seven generations with the C57BL/6J line. Mice were genotyped by PCR amplification of genomic DNA extracted from tail tissue as indicated previously (36). All experiments were performed with 12- to 14-wk-old male mice.

Mice were injected intraperitoneally with 0.5 μg/g body wt of [1-34]P ConvT (Sigma) or with 0.9% NaCl as control. Kidneys were harvested 45 min after injection and rapidly frozen until further use.

For metabolic studies, mice were housed separately in metabolic cages with free access to food and water. Urine was collected under mineral oil. Blood was withdrawn from the vena cava of anesthetized mice. Urinary and plasma ion composition was analyzed by ion chromatography (Metrohm 820 Professional IC). Creatinine and phosphate concentrations were determined according to the Jaffe (Wako chemicals) and Fiske Subarav methods (Sigma Diagnostics), respectively. Urinary concentration of cAMP was determined with a [3H]cAMP assay system (Amersham).

For dietary adaptation, mice placed in individual cages were trained for 4 days to eat either a high (1.2%)-, or a low (0.1%)-P Conv-content diet (Kliba, NAFAG). Food was provided for only 2 h daily, while animals had free access to water. On the fifth day, each initial group was further divided into two subgroups that were fed for 4 h either with the high- or the low-P Conv diet.

All animal experiments were performed according to Swiss Animal Welfare laws, with approval of the local veterinary authority (Kantonales Veterinäramt Zürich).

**Plasmids.** To obtain glutathione S-transferase (GST) fusion constructs, PCR products encoding either the full length or amino acids 1-68 and 36-117 of GABARAP were subcloned into the BamHI/Sall site of pGEX-TK-Ras (56). To generate myc-fused GABARAP, the nucleotide sequence encoding the myc epitope (EQKLISEEDL) immediately preceded by a Kozak consensus sequence was first introduced into the pcDNA3.1 plasmid (Invitrogen) by bidirectional PCR. Then, full-length GABARAP was subcloned into the myc-pcDNA3.1 plasmid with XhoI/KpnI restriction sites. Mouse NaPi-IIa was subcloned into the mammalian expression vector pHA-MEX (Dualsystems Biotech) with XhoI/EcoRI restriction sites.

**Immunoblots and GST pulldowns.** Kidney homogenate or renal BBM were prepared as described previously (5). For immunoblot analysis, kidney homogenate (40 μg) or BBM (15 μg) were separated on 9% SDS-PAGE gels, transferred to polyvinylidene difluoride (PVDF) membranes, and then incubated with antibodies against NaPi-IIa (15), PDZK1 (18), NHERF-1 (Abcam), GABARAP (19) and β-actin (sigma). After incubation with horseradish peroxidase-linked secondary antibodies (GE Healthcare), immuno reactive signals were detected by chemiluminescence (ECL, Amersham Pharmacia Biotech) with the DIANA III chemiluminescence detection system (Raytest). The densitometry of all images was analyzed with appropriate software (Advanced Image Data Analyser AIDA, Raytest) to calculate, for each given experiment, the ratio of the protein of interest to β-actin.

GST pulldowns were performed with isolated BBM or HEK293 cell lysates as reported previously (18). Solubilized BBM/cell lysates were incubated overnight at 4°C with glutathione beads coupled either to GST alone or to GST fusion proteins. Samples were centrifuged at 13,000 g for 1 min. The pelleted beads were washed five times with Tris-buffered saline (TBS) containing 0.1% (vol/vol) Nonidet P-40 and 0.1% (vol/vol) Tween 20. Proteins were eluted by incubation in loading buffer at 95°C for 5 min. Samples were subsequently analyzed by immunoblot as described above.

**Cell culture and immunoprecipitation.** HEK293 cells were plated in 10-cm dishes and cultured in DMEM supplemented with 10% FCS, 5 mM l-glutamine, 100 U/ml penicillin, and 100 U/ml streptomycin in 5% CO2 at 37°C. Subconfluent cultures were transfected with 6 μg of plasmids encoding myc-GABARAP and/or NaPi-IIa, using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. Two days after transfection, cells were lysed in 1 ml of immunoprecipitation (IP) buffer containing 50 mM Tris pH 7.4, 72 mM NaCl, 0.75% Triton X-100, 0.75% Na-deoxycholate, 1 mM PMSF, 5 μg/ml leupeptin, and 5 μg/ml pepstatin A. After preclearing with 40 μl of protein A/G agarose beads (Calbiochem), anti-myc antibody (Invitrogen) was added in a dilution of 1:250. After incubation overnight at 4°C on a rotary shaker, 30 μl of protein A/G beads was added to the lysates and further incubated at 4°C on a rotary shaker for 60 min. Beads were pelleted by short centrifugation steps and washed four times with IP buffer containing detergents and once with IP buffer without detergents. Proteins were eluted by incubation in loading buffer at 95°C for 5 min. Samples were subsequently analyzed by immunoblot.

**Uptake in isolated BBMV.** The Na+-dependent transport rate of 32P Conv into renal BBMV was determined in the presence of 0.1 mM potassium phosphate as described previously (4). Similar protocols were used to measure uptake of radioactively labeled l-glutamine and d-glucose.

**RNA isolation and real-time PCR.** Total RNA was extracted from kidneys homogenized in RLT buffer with the RNaseasy Mini Kit (Qiagen). Similarly, RNA from dissected nephron segments was extracted with the RNaseasy Micro Kit (Qiagen) according to manufacturer’s instructions. Either 300 ng of RNA from total kidney or 80 ng of RNA from nephron segments was used as template for reverse translation.
transcription with the TaqMan Reverse Transcription Kit (Applied Biosystems). Real-time PCR was performed on the ABI PRISM 7700 Sequence Detection System, with commercial primers and probes for NHERF1 and GABARAP (Taqman Gene Expression Assays) as well as primers and probes for NaPi-IIa and NaPi-IIc (31) and β-epithelial Na+ channel (β-ENaC) (14). Amplification was carried out with TaqMan Universal PCR Master Mix (PCR machine and reagents from Applied Biosystems). The expression of the gene of interest was calculated in relation to hypoxanthine-guanine phosphoribosyltransferase (HPRT). Relative expression ratios were calculated as \( R = 2^{\Delta \Delta Ct\text{[HPRT]} - \Delta \Delta Ct\text{[test gene]}} \), where \( C_t \) represents the cycle number at the threshold 0.2.

**Immunostaining.** Mouse kidneys were perfusion fixed through the left ventricle with a fixative solution, and subsequent immunohistochemistry was performed as described previously (12). Cryosections (6 μm) were taken and pretreated for 5 min with either 0.1% SDS (for NaPi-IIa and NaPi-Iic) or 0.5% SDS (for NHERF1 and CD98/4F2). After blocking with 2% BSA, 0.02% Na-azide in PBS, sections were incubated overnight at 4°C with antibodies against NaPi-IIa (1:600) (11), NaPi-Iic (1:400) (35), NHERF1 (1:300) (52), and CD98/4F2 (1:200) (Santa Cruz) in blocking solution. Binding sites of the primary antibodies were detected with Alexa 488-conjugated anti-rabbit antibodies (Invitrogen). F-actin was visualized with Texas red-coupled phalloidin (Invitrogen). Sections were studied by epifluorescence with a Polyvar microscope (Reichert Jung), and digital images were acquired with a charge-coupled device camera.

**RESULTS AND DISCUSSION**

Using a classical YTH screen, we previously identified (18) several proteins that interact with the COOH-terminal intracellular tail of NaPi-IIa. Most of these interactions were PDZ-based and depended on the last three residues of NaPi-IIa (TRL). Classical YTH analysis requires soluble baits to screen for protein-protein interactions. In comparison, the advantage of the split-ubiquitin membrane YTH system (MYTH) is that full-length integral membrane proteins can be used as baits (17, 43). We used the MYTH 2.0 system to screen a mouse kidney library targeting the identification of interactions independent on the COOH-terminal PDZ-binding domain of NaPi-IIa. To this end, we used the TRL-truncated (instead of full length) cotransporter as bait. Three interacting clones were identified as GABARAP. Two of these clones contained the full-length open reading frame (ORF), whereas the third cDNA was a partial fragment that lacked the first 34 nucleotides of the ORF. The GABARAP protein consists of 117 amino acids and shares

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**Fig. 1.** Renal expression of GABARAP and its interaction with NaPi-IIa. A and B: quantification of GABARAP (A) and NaPi-IIa/β-ENaC (B) mRNAs in dissected nephron segments. The abundance of GABARAP, NaPi-IIa and β-ENaC mRNA was measured by real-time PCR. Values were normalized to hypoxanthine-guanine phosphoribosyltransferase (HPRT). Relative expression ratios were calculated as \( R = 2^{\Delta \Delta Ct\text{[HPRT]} - \Delta \Delta Ct\text{[test gene]}} \), where \( C_t \) represents the cycle number at the threshold 0.2.

**C:** immunoblot analysis to detect the presence of GABARAP in brush-border membranes (BBM). BBM isolated from GABARAP−/− mice were used to show specificity of the antibody. **D:** glutathione S-transferase (GST) pulldown analysis to show interaction between GABARAP and NaPi-IIa. Full length and overlapping halves of GABARAP were used to pull down NaPi-IIa from BBM. **Top:** immunoblot probed with an anti-NaPi-IIa antibody. **Bottom:** Ponceau staining of the polyvinylidene difluoride (PVDF) membrane (as grayscale figure). To demonstate that similar amounts of GST and its fusion proteins were used in the different pulldowns. **E:** communoprecipitation of NaPi-IIa with myc-GABARAP from cotransfected HEK293 cells. Immunoblots of the immunoprecipitated material (IP; top) and HEK293 cell lysates (bottom) were probed with anti-NaPi-IIa (top blot) or anti-myc (bottom blot) antibodies. **F:** GST pulldowns on lysates from HEK293 cells transfected with full-length or truncated NaPi-IIa. **Top:** immunoblot of the pulled down material probed with the NaPi-IIa antibody. **Middle:** Ponceau staining of the PVDF membrane (as grayscale figure). **Bottom:** immunoblot on HEK293 cell lysates probed with the NaPi-IIa antibody.
similarity with light chain-3 of microtubule-associated proteins 1A and 1B (48).

GABARAP mRNA and protein are expressed in kidney. GABARAP mRNA was previously shown to be expressed in all tested tissues, including kidney (53). To further address the distribution of GABARAP mRNA within the kidney, real-time PCR was performed on dissected murine nephron segments. GABARAP mRNA was found to be uniformly expressed (Fig. 1A). To rule out cross-contaminations with mRNA from different nephron segments, we analyzed the profile of known marker genes differentially expressed along the nephron. As a marker for proximal tubules, we chose the putative interacting partner NaPi-IIa. As shown previously (31), NaPi-IIa mRNA was highly abundant in proximal convoluted tubules (S1 + S2) and expressed to a lesser extent in proximal straight segments (S3), whereas expression in more distal segments was hardly detectable (Fig. 1B). To demonstrate the enrichment of distal nephron segments, we determined the expression of β-ENaC mRNA, which has been shown to be present in the distal convoluted tubule, the cortical collecting duct (CCD), and the outer medullary collecting ducts (47). As expected, β-ENaC mRNA was highly abundant in our preparation of CCD and absent in proximal segments (Fig. 1B). Therefore it is unlikely that the uniform GABARAP mRNA distribution along the nephron is due to segment cross-contamination. Importantly, GABARAP mRNA expression was detected in proximal convoluted and proximal straight tubules, those segments where NaPi-IIa mRNA is also present.

Endogenous GABARAP protein expression has been reported in rat brain, liver, kidney, spleen, skeletal muscle, and heart (45) as well as in various human cell lines (19). Immunoblot using an antibody raised against human GABARAP (19) confirmed its expression in murine renal BBM preparations (Fig. 1C). In addition to the band at the expected molecular mass of 17 kDa (48), we observed a second product of faster mobility that most likely represents a lipidated form of GABARAP (45). None of these bands was detected in BBM preparations of GABARAP−/− mice, confirming the specificity of the signal.

Regarding the subcellular localization of GABARAP, a punctate distribution within the cytoplasm of GABARAP has been reported in breast epithelial cells, HeLa cells, and neurons (19, 25, 26, 48). Unfortunately, our attempts to identify the localization of GABARAP in the kidney failed, since similar signals were detected in cryosections from GABARAP+/+ and GABARAP−/− kidneys (data not shown). These signals could represent either nonspecific binding of the antibody to renal tissue or cross-reaction with the GABARAP-like proteins GABARAPL1 and GABARAPL2. Both paralogs are expressed in kidney, albeit at very low levels (39, 50). Despite this technical caveat, the presence of GABARAP in BBM preparations points to a subcellular proximity of GABARAP and NaPi-IIa, making the interaction between both proteins plausible.

NaPi-IIa interacts with an internal sequence of GABARAP. The interaction of GABARAP and NaPi-IIa was verified by pulldowns of NaPi-IIa from BBM using GST-GABARAP and by coimmunoprecipitation from transfected HEK293 cells. In the pulldown assay, a band at the expected molecular mass for NaPi-IIa was detected with GST-GABARAP but not with GST alone (Fig. 1D). The association of GABARAP with the γ-subunit of GABA_R receptors depends on a region between amino acids 36 and 68 of GABARAP (48). To find the molecular determinant of GABARAP that mediates the interaction with NaPi-IIa, we expressed two overlapping halves of GABARAP (aa 1-68 and aa 37-117) fused to GST as described previously (48). As shown in Fig. 1D, both fusion proteins were capable of pulling down NaPi-IIa from BBM, suggesting that, similar to the GABA_A receptor γ-subunit, the interaction of GABARAP with NaPi-IIa involves the region between amino acids 36 and 68 of GABARAP. GABARAP 36-117 has been shown to interact also with the transferrin receptor (19). Therefore, these internal residues might represent a common motif for protein-protein interactions.

The specific interaction between NaPi-IIa and GABARAP was further confirmed by coimmunoprecipitation of myc-tagged GABARAP and NaPi-IIa from transfected HEK293 cells (Fig. 1E). Cells were either transfected with NaPi-IIa alone or cotransfected with NaPi-IIa and myc-GABARAP. An antibody against the myc epitope was then used to immunoprecipitate myc-GABARAP. A band with the expected molecular mass for NaPi-IIa was seen only in the immunoprecipitation from cotransfected cells in the presence of myc-antibody (Fig. 1E, top) and not in negative controls lacking either myc-GABARAP or the anti-myc antibody. Together, the GST pulldown and the coimmunoprecipitation data suggest that NaPi-IIa and GABARAP may interact with each other in vivo.

In an attempt to identify the molecular determinant for the interaction within NaPi-IIa, we fused several discrete intracellular domains of NaPi-IIa to GST in order to pull down GABARAP from transfected HEK293 cells. However, under conditions similar to those used in the pulldown experiments described above, we were unable to detect interaction between both partners (data not shown). The failure of these discrete domains of NaPi-IIa to associate with GABARAP could be due

| Table 1. Metabolic cage studies and urinary/blood analysis of GABARAP−/− and GABARAP+/+ mice |
|----------------------------------|-------------------|
| **Metabolic cages**             | **GABARAP−/−**    | **GABARAP+/+** |
| Body weight, g                  | 28.48±0.56        | 30.53±0.34†   |
| Food intake, mg/g body wt       | 0.137±0.005       | 0.148±0.004   |
| Water intake, ml/g body wt      | 0.203±0.010       | 0.170±0.009*  |
| Stool, g/g body wt              | 0.052±0.004       | 0.052±0.003   |
| Urine, ml/g body wt             | 0.059±0.006       | 0.041±0.002†  |
| **Urine chemistry**             |                   |               |
| pH                              | 6.10±0.04         | 6.15±0.08     |
| Osmolality, mosmol/kgH2O        | 2,494±164         | 3,634±124‡    |
| Creatinine, mg/dl               | 59.28±4.28        | 94.96±5.93‡   |
| cAMP (pmol)/creatinine (mg/dl)  | 25.14±0.91        | 23.68±0.68    |
| Na⁺ (mM)/creatinine (mg/dl)     | 1.95±0.11         | 1.80±0.10     |
| K⁺ (mM)/creatinine (mg/dl)      | 7.15±0.23         | 7.02±0.38     |
| Ca²⁺ (mM)/creatinine (mg/dl)    | 0.037±0.009       | 0.032±0.003   |
| Mg²⁺ (mM)/creatinine (mg/dl)    | 0.62±0.02         | 0.56±0.03     |
| P (mM)/creatinine (mg/dl)       | 1.79±0.07         | 1.24±0.09‡    |
| Cl⁻ (mM)/creatinine (mg/dl)     | 5.13±0.38         | 4.32±0.24     |
| **Blood chemistry, mM**         |                   |               |
| Na⁺ (mM)                        | 143.3±0.33        | 146.8±0.86‡   |
| K⁺ (mM)                         | 3.84±0.09         | 4.39±0.18*    |
| Ca²⁺ (mM)                       | 1.28±0.01         | 1.27±0.01     |
| P (mM)                          | 2.54±0.06         | 2.66±0.14     |
| Cl⁻ (mM)                        | 110.5±0.45        | 112.1±0.64*   |

Values are means ± SE for either n = 15 mice/group (for metabolic cage data and urine analysis) or n = 10 mice/group (for blood analysis). GABARAP, GABA_A receptor-associated protein; Pₐ, inorganic phosphate. *P ≤ 0.05, †P ≤ 0.01, ‡P ≤ 0.0001, unpaired Student’s t-test.
either to the need for more than one single intracellular loop for the association or to the requirement of more physiological conditions. A recent report proposes a consensus sequence for GABARAP-binding sites built around a highly conserved tryptophan residue (46). The COOH-terminal intracellular tail of NaPi-IIa contains a sequence that would fit to this motif. Therefore, pulldowns were performed with lysates from HEK293 cells transfected with either the full length or a truncated cotransporter (Δ571) that lacks the tryptophan-containing peptide. Both the full-length and truncated NaPi-IIa were pulled down by GST-GABARAP (Fig. 1F). Although these data do not rule out its potential implication in vivo, they suggest that the mentioned COOH-terminal peptide of NaPi-IIa is not required for the in vitro interaction with GABARAP.

Metabolic studies with GABARAP+/+ and GABARAP−/− mice. Generation of GABARAP−/− mice has been described elsewhere (36). These animals show a normal phenotype with HPRT (Table 1). Similar levels of cAMP were detected in the urine of GABARAP+/+ and GABARAP−/− mice. This suggests that the basal cAMP status of tubular cells, a major regulator of Pi transport, is not affected by the absence of GABARAP.

Despite the reduced urinary Pi excretion in GABARAP−/− mice, the concentration of Pi in serum did not differ between the groups (Table 1). Whether this is related to a higher consumption of Pi in GABARAP−/− mice, due for instance to their higher body weight, or to some compensatory mechanism(s) such as reduced intestinal absorption or enhanced bone deposition remains unknown. The Ca2+ concentration in blood was also similar in both groups of animals, whereas the circulating levels of Cl−, Na+, K+, Mg2+, and Ca2+ were all increased in GABARAP−/− compared with GABARAP+/+ mice.

A  GABARAP+/+   GABARAP−/−

NaPi-IIa   β-actin

B  GABARAP+/+   GABARAP−/−

NaPi-Ic

β-actin

C  2ΔCt[HPRT]−ΔCt[NaPi-IIa]

GABARAP+/+   GABARAP−/−

Fig. 2. Expression of NaPi-IIa and NaPi-Ic protein and mRNA in GABARAP+/+ and GABARAP−/− mice. A and B: immunoblot on BBM isolated from GABARAP+/+ and GABARAP−/− mice to analyze the expression of NaPi-IIa (A) and NaPi-Ic (B). Expression of both transporters was normalized to the abundance of β-actin for the quantification. C: real-time PCR to determine mRNA levels of NaPi-IIa (left) and NaPi-Ic (right) on mRNA extracted from kidney. NaPi-IIa and NaPi-Ic mRNA expression was normalized to HPRT (n = 6). Data are means ± SE. **P < 0.01, unpaired Student’s t-test.
**NaPi-IIa expression is increased in GABARAP−/− mice.** Urinary excretion of P_i is mostly controlled by the rate of reabsorption in the proximal tubule (16). On the basis of the severe renal wasting phenotype of NaPi-IIa−/− mice (1) as well as on RNA hybrid depletion experiments (40) it has been proposed that NaPi-IIa is the major P_i transporter in the adult murine kidney, whereas NaPi-IIc was considered to play only a minor role. However, mutations of NaPi-IIc identified in families with several hypophosphatemic syndromes and in particular the detectable phenotype in the heterozygotes (2, 21, 30) points to either dramatic differences between humans and mice or an incomplete understanding of renal handling of P_i. Therefore, we next analyzed whether the reduced urinary excretion of P_i detected in GABARAP−/− mice was due to changes in the expression of NaPi-IIa and/or NaPi-IIc. We found that NaPi-IIa abundance was increased approximately twofold in renal BBM of GABARAP−/− compared with GABARAP+/+ mice (Fig. 2A). However, the expression of NaPi-IIc was similar in both groups (Fig. 2B).

Immunohistochemistry was performed to determine whether the loss of GABARAP alters the segmental and/or subcellular distribution of NaPi-IIa and NaPi-IIc. We have shown (11) that

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**Fig. 3. Immunohistochemical analysis of NaPi-IIa, NaPi-IIc, and Na+/H+ exchanger regulatory factor (NHERF)1 in GABARAP+/+ and GABARAP−/− mice.** Immunostaining of NaPi-IIa (A), NaPi-IIc (B), and NHERF1 (C) on kidney cryosections obtained from GABARAP+/+ (left) and GABARAP−/− (right) mice is shown. Renal cortical overviews are displayed as grayscale figures. The images of single proximal tubules show NaPi-IIa (A) and NaPi-IIc (B), both green, costained with β-actin (red) as a marker for BBM. C: NHERF1 (green) was costained with anti-CD98/4F2 (red) as marker for proximal tubules.
NaPi-IIa expression is highest in proximal convoluted tubules (S1, S2) and gradually decreases toward the end of the proximal straight tubule (S3) in superficial and juxtamedullary nephrons. In adults, NaPi-IIc expression is found preferentially in S1 segments of proximal convoluted tubules of juxtamedullary nephrons (37, 40). Compared with GABARAP+/+, the segmental distribution and subcellular localization of NaPi-IIa and NaPi-IIc were not altered in kidneys from GABARAP−/− (Fig. 3, A and B). The twofold increase of NaPi-IIa abundance in BBM of GABARAP−/− mice revealed by immunoblots (Fig. 2A) could not be detected by the immunofluorescence analysis (Fig. 3A).

Changes on NaPi-IIa abundance in response to acute stimuli are due to regulated insertion/retroval from the BBM rather than to changes on mRNA levels (16, 28), whereas chronic adaptation may (28) or may not involve transcriptional regulation (31). Therefore, we quantified the amount of NaPi-IIa mRNA in kidneys from GABARAP+/+ and GABARAP−/− mice by real-time PCR. As shown in Fig. 2C, both groups of animals had similar levels of NaPi-IIa mRNA, suggesting that upregulation of the cotransporter in GABARAP−/− mice is independent of changes in transcription. As reported previously (31), the expression of NaPi-IIc mRNA was very low compared with NaPi-IIa and its levels were similar in GABARAP+/+ and GABARAP−/− kidneys (Fig. 2C). Therefore, under normal conditions GABARAP seems to play a suppressive role in the expression/stabilization of NaPi-IIa in renal proximal tubules, and its absence cannot be compensated by GABARAP-like paralogs. This negative effect is in contrast to the proposed role of GABARAP in the trafficking and recycling of renal proximal tubule NaPi-IIa, perhaps by binding to NHERF1 (31). However, NaPi-IIa mRNA abundance was not altered in kidneys from transfected HEK293 cells with GST-GABARAP did not give evidence for interaction between the proteins (data not shown). This observation, together with the data from the MYTH analysis, where the ΔTRL-NaPi-IIa was found to interact with GABARAP but not with NHERF1, strongly suggests that the association of NaPi-IIa with GABARAP does not require the presence of NHERF1. Surprisingly, no change in the expression of NHERF1 signal intensity or subcellular localization was detected by immunofluorescence or cryosections obtained from GABARAP+/+ and GABARAP−/− mice (Fig. 3C). Although NHERF1 is not an integral membrane protein, the capacity of its COOH terminus to associate with actin-binding proteins from the ezrin-radixin-moesin family may provide a link to the actin cytoskeleton and therefore promote stabilization of membrane proteins (38).

Unlike in NHERF1−/− animals, the expression of NaPi-IIa is not altered in mice lacking PDZK1/NHERF3, a second PDZ partner of the cotransporter (7, 18). As shown in Fig. 5A, the expression of PDZK1/NHERF3 was similar in GABARAP−/− and GABARAP+/+ mice.

**PTh-induced endocytosis of NaPi-IIa is not impaired in GABARAP−/− mice.** PTH is one of the best-understood humoral factors that control the apical expression of NaPi-IIa. PTH is a potent phosphaturic hormone and works by inducing endocytosis of NaPi-IIa, at least partially, via clathrin-mediated

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**Fig. 4.** Na+−dependent uptake into renal BBM vesicles (BBMV). Na+−dependent uptakes of P1 (n = 10), L-glutamine (n = 4), and D-glucose (n = 5) into BBMV isolated from kidneys of GABARAP−/− and GABARAP+/+ mice are shown. Uptakes were analyzed after 1-min incubation. Data are means ± SE. *P ≤ 0.05, unpaired Student’s t-test.
pits (16). GABARAP has been shown to associate with the heavy chain of clathrin (34). To test whether the downregulation of NaPi-IIa in response to PTH is affected by the loss of GABARAP, we injected animals with PTH and quantified the amount of NaPi-IIa remaining in renal BBM. Administration of PTH led to ~50% reduction of NaPi-IIa in BBM of GABARAP+/+ mice (Fig. 6A). PTH also induced a reduction of NaPi-IIa in GABARAP−/− mice. Moreover, despite the higher basal levels in GABARAP−/− animals, the amount of NaPi-IIa remaining upon PTH administration was similar in both groups of mice (Fig. 6A). In agreement with the immunoblot data, similar Na+ dependent uptakes of 32Pi were measured in BBMV isolated from GABARAP+/+ and GABARAP−/− mice upon PTH treatment, despite the higher basal uptake measured in mutant mice (Fig. 6B). Therefore, the absence of GABARAP does not prevent the acute endocytosis of NaPi-IIa in response to PTH.

Acute dietary regulation of NaPi-IIa is not impaired in GABARAP−/− mice. The content of Pi in the diet is a well-known metabolic factor that affects the expression of NaPi-IIa in the apical microvilli. The expression of NaPi-IIa in the BBM increases in response to a low dietary intake of Pi, whereas in conditions of high dietary Pi, the level of NaPi-IIa decreases (3). Both responses are fast and can be detected a few hours (acute adaptation) after Pi ingestion (29). Acute downregulation of NaPi-IIa relies on its endocytic retrieval and lysosomal degradation, similar to the PTH effect (23). Acute upregulation does not require de novo synthesis; instead, it depends on an intact microtubular cytoskeleton, suggesting the sorting to the BBM of intracellularly stored NaPi-IIa. As expected, the acute switch from high- to low-Pi diet results in the upregulation of NaPi-IIa in the BBM of GABARAP+/+ mice (Fig. 8C). In addition, this switch results in a decrease in the urinary.

![Image](http://ajprenal.physiology.org/)

**Fig. 5. Renal expression of NHERF1 and PDZK1 in GABARAP+/+ and GABARAP−/− mice. A: immunoblot analysis of NHERF1 and PDZK1 in homogenates of kidneys extracted from GABARAP+/+ and GABARAP−/− mice. For quantification, expression was normalized to β-actin. B: real-time PCR to determine NHERF1 mRNA levels normalized to HPRT (n = 6). Data are means ± SE. **P ≤ 0.01, unpaired Student’s t-test.**

**Fig. 6. Parathyroid hormone (PTH)-induced endocytosis of NaPi-IIa in GABARAP+/+ and GABARAP−/− mice. A: representative immunoblot of NaPi-IIa on BBM isolated from GABARAP+/+ and GABARAP−/− mice. As expected, the acute switch from high- to low-Pi, diet results in the upregulation of NaPi-IIa in the BBM of GABARAP+/+ mice (Fig. 8C). In addition, this switch results in a decrease in the urinary.
excretion of Pi (Fig. 8A) and the Pi concentration in serum (Fig. 8B). The acute switch from high- to low-Pi diet results in changes in GABARAP mice similar to those described above for GABARAP mice (Fig. 8). These findings indicate that the acute dietary regulation of NaPi-IIa does not require the presence of GABARAP.

Taken together, we have identified GABARAP as a new interacting partner of NaPi-IIa and shown that NaPi-IIa interacts with an internal domain of GABARAP. The apical expression of NaPi-IIa is increased in GABARAP mice under normal dietary conditions. This increased NaPi-IIa abundance results in a higher Na+-dependent Pi uptake into renal BBMV and a reduced urinary excretion of Pi. The upregulation of NaPi-IIa in the BBM is independent of changes on mRNA levels. Instead, it is paralleled by an increased expression of NHERF1 (but not PDZK1/NHERF3). The regulation of the cotransporter by either PTH or acute changes of dietary Pi is not affected by the absence of GABARAP, suggesting that the regulated endo/exocytosis processes are not altered. However, because of the higher expression of NHERF1 in GABARAP mice and its potential to regulate PTH receptor signaling (6, 32), it remains to be investigated whether the intracellular mechanism of the hormonal action is similar in wild-type and GABARAP-deficient mice. Our finding that the abundances of both NaPi-IIa and NHERF1 are increased in GABARAP mice suggests that, under normal conditions, both proteins are negatively regulated by GABARAP. This is in contrast to the proposed role of GABARAP in promoting membrane expression of receptors (9, 27). However, it is worth mentioning that, in addition to its role in intracellular trafficking, GABARAP also functions as an ubiquitin-like protein. Evidence comes not only from the crystal structure of GABARAP (10) but also from the function

**Fig. 7.** Acute adaptation of GABARAP and GABARAP mice to a high dietary Pi content. Mice were adapted to a low-Pi diet (↓) for 4 days as indicated in EXPERIMENTAL PROCEDURES. On the 5th day they were divided into 2 subgroups that either received a low-Pi diet (n = 3) or were switched to a high-Pi diet (↑) (n = 5). A: urinary Pi excretion as ratio to urinary creatinine. B: serum Pi concentration. C: representative immunoblot of NaPi-IIa on BBM. Expression was normalized to β-actin for the quantification. Data are means ± SE. *P ≤ 0.05. **P ≤ 0.01. ***P ≤ 0.001.

**Fig. 8.** Acute adaptation of GABARAP and GABARAP mice to a low dietary Pi content. Mice were adapted to a high-Pi diet (↑) for 4 days as indicated in EXPERIMENTAL PROCEDURES. On the 5th day they were divided into 2 subgroups that either received a high-Pi diet (n = 3) or were switched to a low-Pi diet (↓) (n = 5). A: urinary Pi excretion as ratio to urinary creatinine. B: serum Pi concentration. C: representative immunoblot of NaPi-IIa on BBM. Expression was normalized to β-actin for the quantification. Data are means ± SE. **P ≤ 0.01. ***P ≤ 0.001.
of its yeast ortholog, Atg8. Atg8 is involved in autophagy, an essential process for lysosomal degradation of organelles and proteins in response to starvation (for review see Ref. 45). In this process Atg8 is modified in an ubiquitin-like manner. The E1/E2-like enzymes and the modification of GABARAP in mammalian cells have been identified (44). So far, the only known targets of GABARAP after its modification are phospholipids (42), and the lipid composition of the plasma membrane has been reported to regulate NaPi-IIa (54).

In summary, GABARAP interacts with NaPi-IIa and controls the expression of the cotransporter in the proximal BBM. The absence of GABARAP influences the abundance of NaPi-IIa, and therefore renal reabsorption of P, without affecting its acute regulation. Further work will be required to characterize the molecular mechanism responsible for these findings.

ACKNOWLEDGMENTS

We thank C. A. Enns, Oregon Health and Science University, and E. Weinmann, University of Maryland, for providing us with the antibodies against GABARAP and NHERF1. A. Velić, N. Kampik, and C. A. Wagner, University of Zurich, kindly shared dissected nephron segments and primers/probes for real-time PCR. F. Verrey, University of Zurich, provided primers/probe for the β-ENaC real-time PCR.

Present address of G. A. O’Sullivan: Max Planck Institute for Molecular Cell Biology and Genetics, Pfotenhauerstraße 108, 01307 Dresden, Germany.

GRANTS

This work was supported by Swiss National Science Foundation Grant 4434003 (to H. Murer), Sixth European Frame Work EureGene Project Grant 005083 (to H. Murer), National Institute of Diabetes and Digestive and Kidney Diseases Grants DK-48482 and DK-20543 (to O. W. Moe), the Simmons Family Foundation (to O. W. Moe), and the Fritz-Thyssen-Stiftung.

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