Interaction of a farnesylated protein with renal type IIa Na/P_i co-transporter in response to parathyroid hormone and dietary phosphate

Mikiko ITO*, Sachi IIDAWA*, Michiyo IZUKA*, Sakiko HAITO*, Hiroko SEGAWA*, Masashi KUWAHATA*, Ichiro OHKIDO†, Hiroshi OHNO‡ and Ken-ichi MIYAMOTO*[1]

*Department of Nutrition, Tokushima University, School of Medicine, Kuramoto-cho 3-18-15, Tokushima City 770-8503, Japan, †Division of Nephrology and Hypertension, Jikei University School of Medicine, Tokyo, Japan, and ‡Division of Molecular Membrane Biology, Cancer Research Institute, Kanazawa University Graduate School of Medical Science, Kanazawa, Ishikawa 920-0934, Japan

Treatment with PTH (parathyroid hormone) or a high-P_i diet causes internalization of the type IIa sodium-dependent phosphate (Na/P_i IIa) co-transporter from the apical membrane and its degradation in the lysosome. A dibasic amino acid motif (KR) in the third intracellular loop of the co-transporter is essential for protein's PTH-induced retrieval. To elucidate the mechanism of internalization of Na/P_i IIa, we identified the interacting protein for the endocytic motif by yeast two-hybrid screening. We found a strong interaction of the Na/P_i IIa co-transporter with a small protein known as the PEX19 (human peroxisomal farnesylated protein; PxF, Pex19p). PEX19 can bind to the KR motif, but not to a mutant with this motif replaced with NI residues. PEX19 is highly expressed in mouse and rat kidney. Western blot analysis indicates that PEX19 is located in the cytosolic and brush-border membrane fractions (microvilli and the subapical component). Overexpression of PEX19 stimulated the endocytosis of the Na/P_i IIa co-transporter in opossum kidney cells in the absence of PTH. In conclusion, the present study indicates that PEX19 may be actively involved in controlling the internalization and trafficking of the Na/P_i IIa co-transporter.

Key words: endocytosis, internalization, peroxisomal protein, peroxisomal farnesylated protein (PEX19).

INTRODUCTION

The reabsorption of P_i in the renal proximal tubule is essential for the maintenance of plasma P_i homoeostasis [1]. The Na/P_i IIa (type IIa sodium-dependent phosphate) co-transporter is located in the apical membranes of renal proximal tubule cells and is one of the major co-transporters functioning in renal P_i reabsorption. Two major regulators of the Na/P_i IIa co-transporter are dietary P_i and PTH (parathyroid hormone). Restriction of dietary P_i is associated with an adaptive increase in the overall capacity of the proximal tubule to reabsorb P_i [2–4]. PTH also regulates renal P_i reabsorption and induces phosphaturia [5–7].

A similar mechanism has been proposed to explain the capacity of PTH and dietary P_i to inhibit P_i reabsorption by the proximal tubule [5–7]. Reduced P_i reabsorption is achieved by a removal of the Na/P_i IIa co-transporter from the apical membrane. Acute internalization of the transporter in response to a high dietary P_i intake or the administration of PTH occurs via clathrin-coated structures and is followed by lysosomal degradation of the co-transporter [5,8–11]; apparently no recycling of internalized co-transporters occurs. NaPi-4 is the opossum orthologue of Na/P_i IIa co-transporter. NaPi-4 is expressed in the OK (opossum kidney) cell line. Expression of Na/P_i IIa co-transporter and NaPi-4 is under the control of PTH [9,12–15]. Studies with intact renal proximal tubule tissue and OK cells have shown that protein kinases A and C mediate the intracellular PTH effect [10,11]. PTH does not directly bring about an altered phosphorylation of the co-transporter; however, phosphorylation events are assumed to occur, e.g. at the level of interacting proteins [14,16].

The Na/P_i IIa co-transporter contains several motifs (tyrosine, dileucine and diacidic motifs) that are important for endocytosis and/or lysosomal targeting [15]; however, these motifs are not required for PTH-induced endocytosis and degradation. The renal type IIa and the small-intestinal type IIb co-transporter proteins share approx. 75 % sequence similarity [17] and are thought to have a similar structure. However, PTH-induced retrieval has been observed for the IIa protein but not for the IIb co-transporter [15,18]. Karim-Jimenez et al. [18] used chimaerae to show that the N- and C-termini are not required for PTH-induced retrieval, whereas a 'domain' located between residues 216 and 658 seemed to be necessary for this response. This region contains two putative intracellular loops, and sequences in the last intracellular loop are highly conserved between the type IIa and type IIb proteins. The two charged amino acids in the type IIa co-transporter ($K^{503}R^{504}$) are replaced by uncharged residues in type IIb ($N^{520}I^{521}$). Based on the results of chimaera experiments with type IIb, this KR motif appears to be important for PTH-induced endocytosis [18].

Identifying proteins that interact with the KR motif should help determine the mechanism of PTH-induced endocytosis. To accomplish this, we screened for interaction partners with the yeast two-hybrid system. We tested a defined portion of the human Na/P_i IIa co-transporter containing the KR motif against a cDNA library derived from human kidney tissue. In this report, we discuss the results of this search for proteins interacting with this motif and the effects of such interactions on PTH-induced up-regulation.

MATERIALS AND METHODS

Animals and diets

Male Wistar rats (5 weeks after birth) were purchased from SLC (Shizuoka, Japan). They were housed in plastic cages and

Abbreviations used: PEX19, peroxisomal farnesylated protein; Na/P_i IIa co-transporter, type IIa sodium-dependent phosphate co-transporter; PTH, parathyroid hormone; OK cell, opossum kidney cell; EGFP, enhanced green fluorescence protein; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; BBMV, brush-border membrane vesicle; NHERF-1, Na^+/H^+ exchanger regulatory factor 1; NHE3, Na^+/H^+ exchanger; PMP22, peroxisomal membrane protein 22; AP-2, adaptor protein 2.

[1] To whom correspondence should be addressed (e-mail miyamoto@nutr.med.tokushima-u.ac.jp).

fed a standard rat chow diet (Oriental Yeast Co., Tokyo, Japan) *ad libitum* for the first week. The rats then received a diet containing 1.2 % calcium and 0.6 % P_i for 5 days. On the sixth day, three groups of six rats each were established: a control P_i group chronically fed a 0.6 % P_i diet, a low-P_i group fed a 0.02 % P_i diet and a high-P_i group fed a 1.2 % P_i diet containing 1.2 % calcium [19]. The groups were fed their respective diets for 7 days [19]. A diet for acute adaptation to P_i consisted of feeding rats a low-P_i diet for 10 days followed by a high-P_i diet for 6 h. After a given diet regimen was completed, all of the rats were anaesthetized with intraperitoneal pentobarbital and their kidneys removed rapidly [4].

Plasmid constructs

For NaPi-pEGFP constructs, wild-type human Na/P_i IIa co-transporter (NaP_i-KR) was fused to the N-terminus of the EGFP (enhanced green fluorescence protein) by inserting the full-length cDNA into the pEGFP-C1 vector (Stratagene, La Jolla, CA, U.S.A.). We used PCR to replace residues $K^{506}R^{507}$ (corresponding to residues $K^{503}R^{504}$ in the rat sequence) with residues NI. For NaPi-pFLAG constructs, full-length fragments of the NaP_i-KR and NaP_i-NI co-transporters were fused to the N-terminus of the FLAG peptide by inserting the full-length cDNA into the pFLAG-CMV2 vector (Sigma Chemical Company). For PEX19-pEGFP and PEX19-pFLAG constructs, human PEX19 (peroxisomal farnesylated protein; PxF, Pex19p) obtained by yeast two-hybrid screening was fused to the C-terminus of the EGFP by inserting the cDNA with introduced *Hin*dIII/*Sal*I sites into the pEGFP-C1 (Clontech, Palo Alto, CA, U.S.A.) and pCMV-Tag 2C (Stratagene) vectors. All constructs were confirmed by sequencing, and two independent clones from each construct were used for experiments.

Cell culture and PTH treatment

OK cells were obtained from the American Type Culture Collection and maintained in an appropriate medium [20]. For transfection, OK cells were plated in a 35 mm glass-bottomed dish (Mattek Corp., Ashland, MA, U.S.A.) at a density of 2×10^5 cells. Cultures at subconfluence were transfected with 4 μl of Lipofectamine 2000 (Invitrogen Corp., Carlsbad, CA, U.S.A.) and 1 μg of plasmids according to the manufacturer's instructions. All experiments were carried out with confluent cells 24 h after transfection. Treatment with PTH was carried out as described previously [15]. The cultures of OK cells were maintained at 37 °C after transfection. The medium was changed 2 h prior to the experiment. Living green fluorescing (EGFP) cells expressing either the wild-type (NaP_i-KR) or the mutated (NaP_i-NI) co-transporter protein were selected with a confocal microscope before treatment. PTH peptide (10^{-8} M) was then added to the cell medium as described previously [15]. After 10 min, 30 min and 1 h, the same selected cells were again identified, and the patterns of expression of the different co-transporters were compared with those before treatment. Confocal microscope images were taken with a Leica TCS-SL (Wetzlar, Germany) laser-scanning microscope equipped with a 63 × oil-immersion objective.

Yeast two-hybrid screen

*Eco*RI and *Sal*I restriction sites were introduced by PCR into cDNA fragments encoding the third intracellular loop of human Na/P_i IIa co-transporter protein (residues 483–524). The PCR fragments produced were digested with the restriction enzymes and cloned into the pEG202 or pEG202-NLS vector (OriGene Technology, Rockville, MD, U.S.A.). The pEG202-NLS vector is similar to pEG202 but has the simian virus 40 nuclear localization sequence between LexA and the polylinker. The NaP_i-KR construct was then screened against a human kidney cDNA library in the pJG4-5 vector (Clontech) as described in the manufacturer's manual. To identify the interaction of NaP_i-KR and NaP_i-NI proteins, we twice amplified the NaP_i-NI gene containing *Eco*RI and *Sal*I restriction sites. As a positive control for the binding assay, the rat NHERF-1 (Na^+/H^+ exchanger regulatory factor 1; residues 1–355) was used as bait, and the rat NHE3 (Na^+/H^+ exchanger; residues 598–831) was used as target [21]. To test the effect of the farnesylation signal in PEX19, we removed the C-terminal residues (CLIM) and then inserted the construct into the pJG4-5 vector (PEX19Δfar). These plasmids were co-transformed into the yeast reporter strain EGY48 [p8op-lacZ] and plated on to a synthetic dropout plate [− *his*, − *ura*, − *trp*] to select for transformants. Changing the carbon source of the medium from glucose to galactose could induce the expression of all of these genes. To test the positive interactions between two hybrid proteins, these plasmids were co-transformed into yeast and were plated on to four selective plates; a galactose/raffinose medium without leucine, a glucose medium without leucine, a galactose/raffinose/X-gal medium and a glucose/X-gal medium. Interaction was detected by the colour development caused by LacZ reporter activity for yeast plated on galactose/raffinose/X-gal medium. In addition, to quantify the interactions, yeast colonies were harvested, lysed and activity determined by a liquid assay for β-galactosidase (Yeast Protocols Handbook, Clontech). β-Galactosidase activity was monitored at 420 nm, and 1 unit of β-galactosidase activity was defined as the amount capable of hydrolysing 1 μmol of *o*-nitrophenyl-β-D-galactopyranoside (Roche Diagnostics, Mannheim, Germany) to *o*-nitrophenol and D-galactose per min per cell.

Immunoprecipitation

Cell lysates were prepared from OK cells expressing FLAG–NaP_i-KR or FLAG–NaP_i-NI in the presence or absence of PEX19-pEGFP. Cells were lysed, and proteins were immunoprecipitated with an anti-FLAG M2 affinity gel (Sigma Chemical Company) according to the manufacturer's instructions. PEX19 and Na/P_i IIa co-transporter were resolved by SDS/PAGE on 15 or 8 % gels. The prepared proteins were transferred electrophoretically to a PVDF membrane (Amersham Biosciences). These proteins were detected with a polyclonal anti-PEX19 antibody directed against the C-terminus [22] or anti-Na/P_i IIa antibody directed against the C-terminus [19] and immunoreactive bands were detected by ECL Plus (Amersham Biosciences) using a horseradish peroxidase-coupled IgG.

Confocal microscopy analysis

OK cells were plated on glass coverslips at a density of 4×10^4/well, transfected with 4 μl of Lipofectamine 2000 and 1 μg of plasmids, and probed by immunostaining [9,15]. Cells were incubated with anti-Na/P_i IIa serum (dilution, 1:500) and then incubated with Alexa Fluor 568-conjugated rabbit IgG and Alexa Fluor 488-phalloidin or Alexa Fluor 568-phalloidin (Molecular Probes, Eugene, OR, U.S.A.; dilution, 1:200) to detect actin. The coverslips were mounted with Vectashield H-1000 (Vector Laboratories, Burlingame, CA, U.S.A.). Confocal images were taken with a Leica TCS-SL (Wetzlar, Germany) laser-scanning microscope equipped with a 63× oil-immersion objective.

Western blotting for detection of endogenous protein in OK cells

OK cells were cultured in high-P_i media for 24 h and then replaced with low-P_i media for 6 h prior to the transfection procedure [23]. Cells were transfected with either the empty vector or the PEX19-pFLAG plasmid. The preparation of the crude membrane fraction was carried out as described previously [9]. Western blotting was performed with a polyclonal anti-Na/Pi-4 antibody or anti-Na^+/K^+ ATPase α-1 antibody (Upstate Biotechnology, Lake Placid, NY, U.S.A.).

Reverse transcriptase PCR of PEX19 in OK cells and mouse kidney

Invitrogen's First-Strand Synthesis kit was used to generate full-length cDNAs from 1 μg of total RNA from cultured OK cells or mouse kidney. PEX19 and G3PDH (glyceraldehyde-3-phosphate dehydrogenase) control fragments were then selectively amplified with the following primers: PEX19-p1, 5′-TGCAGAGCATCATGCAGAACC-3′; PEX19-p2, 5′-AGAGCATCCAGGTCAAAGTTG-3′; G3PDH-For, 5′-CTGCACCACCAACTGCTTAGC-3′; and G3PDH-Rev, 5′-GCCTGCTTCACCACCTTCTTG-3′. PCR for PEX19 was carried out with 30 cycles of 94 °C for 30 s, 53 °C for 30 s and 72 °C for 30 s. For G3PDH, PCR was conducted for 25 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s. PCR fragments were subcloned into the pCR2.1 vector (Invitrogen), and the expected amplification products were confirmed by sequencing.

Northern blotting

The tissue distributions of mRNA expression of identified gene products were probed by Northern blotting with poly(A)$^+$ RNAs of adult mouse or rat using standard procedures [19]. Fragments containing the full coding sequence of human PEX19 were used as probes. Autoradiography was performed with the BAS1500 (Fuji Film, Tokyo, Japan), and the mRNA level was calculated.

Preparation of BBMVs (brush-border membrane vesicles) and Western blot analysis

Six individual rats were studied in each of the experimental groups for BBMV isolation [4,19]. On the day of the experiment, at the end of the feeding period, the rats were anaesthetized and the kidneys removed rapidly. BBMVs were prepared from the kidney by the Ca^{2+}-precipitation method as described previously [24] and 20 μg of protein subjected to SDS/PAGE (8 % gels). Western blotting was performed with a polyclonal anti-Na/P_i IIa antibody.

Mammalian two-hybrid analysis

Mammalian two-hybrid analyses were performed using the CheckMate system (Promega). Constructs encoding VP16 transactivating (pACT) and GAL4 DNA-binding (pBIND) proteins were created by cloning PCR-generated fragments into *Bam*HI-/*Kpn*I-digested vectors. The fragments comprised the following amino acids: PEX19, 1–300; μ2 subunit of AP-2 (adaptor protein 2), 1–435; NaPi-Cap1, 1–519; NaPi-Cap2, 1–509. The pBIND-ID and pACT-MyoD plasmids were provided in the CheckMate kit as a positive control for the binding activity. The pG5luc reporter plasmid contains five GAL4-binding sites upstream of the coding sequence for firefly luciferase. The pBIND vector also encodes *Renilla* luciferase downstream of a constitutively active human growth factor promoter; therefore it was possible to use a Dual Luciferase kit (Promega) for both luminometric measurement of the interaction and determination of transfection efficiency. The analyses were carried out on COS-7 cells (American Type Culture Collection; 1.5×10^5 per 12-well dish), which were transfected with the appropriate vectors and the pG5luc reporter (0.4 μg of each plasmid per dish) using Lipofectamine 2000. The experiments were performed in triplicate, and the results were recorded as the protein concentration and the relative luciferase activity (i.e. the ratio of firefly luciferase activity to *Renilla* luciferase activity).

RESULTS

Effect of PTH is dependent on two amino acid residues in the Na/P_i IIa co-transporters

The Na/P_i IIa co-transporter has a sequence of two basic residues ($K^{503}R^{504}$) in the third intracellular loop (Figure 1A). The two charged amino acids in the type IIa co-transporter ($K^{503}R^{504}$) are replaced by uncharged residues in type IIb ($N^{520}I^{521}$). Figure 1(B) shows that wild-type (NaP_i-KR) and mutated (NaP_i-NI) co-transporters were fused to EGFP and were transiently expressed in OK cells. We confirmed that the EGFP-fused Na/P_i IIa co-transporters are fluorescent proteins and that they are expressed predominantly in the apical membrane of OK cells. Their proper apical locations can be visualized as patches of EGFP fluorescence that are localized in the OK cell microvilli. As reported by Pfister et al. [9] and Karim-Jimenez et al. [18], we found that the NaP_i-KR co-transporter is internalized and degraded after 1 h of incubation with PTH (Figure 1B). In contrast, the NaP_i-NI co-transporter is not sensitive to PTH treatment after 1 h. This suggests that the KR motif plays an essential role in the PTH-induced down-regulation of the Na/P_i IIa co-transporter.

Identification of a protein in yeast associated with the NaP_i-KR motif

To identify proteins that interact with Na/P_i IIa co-transporter KR motif, we screened a cDNA library from human kidney tissue by using the LexA yeast two-hybrid system with the last intracellular loop and the KR motif as bait (Figure 2A). Using the KR motif in pEG202-NLS as bait, we isolated 221 clones from 2×10^7 Leu/Trp/His prototrophs, all of which contained overlapping cDNAs segments derived from the same mRNA, as determined by restriction endonuclease mapping. Half of the 221 clones were zinc-finger proteins (promyelocytic leukaemia zinc finger protein; GenBank accession no. AF130255), but in yeast these clones were autoactivated (results not shown). Most of these clones (81) were identified as PEX19 (accession no. AB018541), which is required for peroxisomal biogenesis [25,26]. Although several clones were identified as containing DNA encoding mitochondrial proteins (accession no. NC001807) or unknown small proteins (accession nos. AC027288 and AL035420), these clones did not bind the KR motif specifically in the LexA-binding assay (results not shown).

To confirm the interaction with PEX19, the rescued plasmid from yeast was retransformed in the yeast reporter strain EGY48 harbouring the following bait–prey constructs: (i) wild-type NaP_i-KR or mutant NaP_i-NI co-transporter and PEX19; (ii) PEX19 and a LexA–Bicoid fusion protein from *Drosophila* or pEG202-NLS empty vector as controls; (iii) the NaP_i-KR or NaP_i-NI and empty vector with (pJG4-5 vector) or without (pJG4-6 vector) the B42 transcriptional activation domain; and (iv) the Na^+/H^+ exchanger NHE3 and NHERF-1 as a positive control [21]. In both the colony colour assay (Figure 2B) and the liquid β-galactosidase

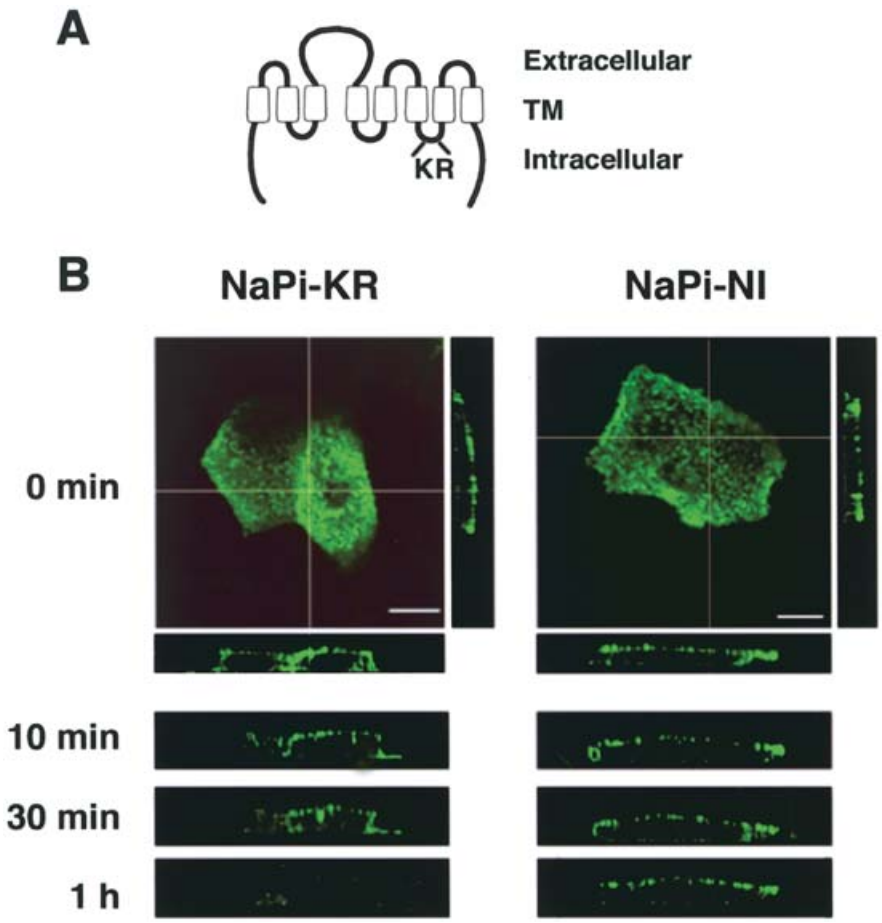

Figure 1 Schematic model of Na/P_i IIa co-transporter and PTH regulation of mutated Na/P_i IIa co-transporters

(**A**) The cytoplasmic tails and extracellular loops are indicated as a black line. The transmembrane domains (TM) are shown as boxes. The Na/P_i IIa co-transporter has eight transmembrane domains and cytoplasmic N- and C-termini. Two amino acid residues (KR) are an important motif for PTH-induced internalization, and its location in the third intracellular loop is indicated. (**B**) OK cells were transfected with wild-type (NaP_i-KR) or mutant (NaP_i-NI) co-transporters fused with EGFP protein and processed in the PTH experiment. Cells were treated with 10^{-8} M PTH (residues 1–34) for 10 min–1 h. The patterns of green fluorescent cells before (0 h) and after (10 min, 30 min and 1 h) the treatment are indicated. The images at 0 min are the confocal cross section (main panels), *yz* section (right-hand panels) and *xz* section (bottom panels) along the white lines shown; only the *xz* sections are shown for 10 min–1 h. In *xz* cross-sections, apical membranes are at the top and basal membranes are at the bottom. Scale bars, 10 μm.

assay (Figure 2C), the interaction of NaP_i-KR with PEX19 was strong and specific, but the interaction of NaP_i-NI with PEX19 was weak.

Interaction of Na/P_i IIa co-transporter and PEX19 in transfected cells

To explore further the interaction of Na/P_i IIa co-transporter and PEX19 in mammalian cells, we transfected OK cells with expression plasmids encoding tagged PEX19 and NaP_i-KR or NaP_i-NI co-transporters and found that the proteins co-immunoprecipitated. In Western blotting, PEX19-protein-fused EGFP was expressed with two bands (61 and 65 kDa) by anti-PEX19 antibody (Figure 3A). Similar observations were detected with anti-EGFP antibody (results not shown). This may be due to post-transcriptional modification of PEX 19 in OK cells. FLAG protein fused to Na/P_i proteins (FLAG–NaP_i-KR or FLAG–NaP_i-NI) were also detected with broad bands at 110 kDa (Figure 3B). PEX19 co-precipitated with FLAG–NaP_i-KR or FLAG–NaP_i-NI (immunoprecipitated with anti-FLAG antibody); however, the amounts of precipitate with FLAG–NaP_i-NI were very low (Figure 3C). PEX19 was not precipitated by either the mock control or by the FLAG protein alone. These results indicated that PEX19 interacts with Na/P_i IIa co-transporter *in vivo* as well as *in vitro*.

Expression of Na/P_i IIa co-transporters and PEX19 in OK cells

Staining OK cells with FLAG–Na/P_i IIa (FLAG antibody) revealed the protein to be expressed in apical patches (Na/P_i IIa co-transporter patches represent clusters of actin where the co-transporter accumulates) [9,18]. Figure 4 shows that the overlap between Na/P_i IIa co-transporter (NaP_i-KR or NaP_i-NI) and actin locations was total, which is indicated by the yellow signal of the merged composite.

In OK cells transfected with the EGFP–PEX19 fusion protein, EGFP fluorescence was detected in the cytoplasm (Figures 4A and 4D). NaP_i-KR and NaP_i-NI co-transporters were predominantly located in the apical membrane (Figures 4B, 4C, 4E and 4F). Co-expression of PEX19 stimulated the migration of NaP_i-KR

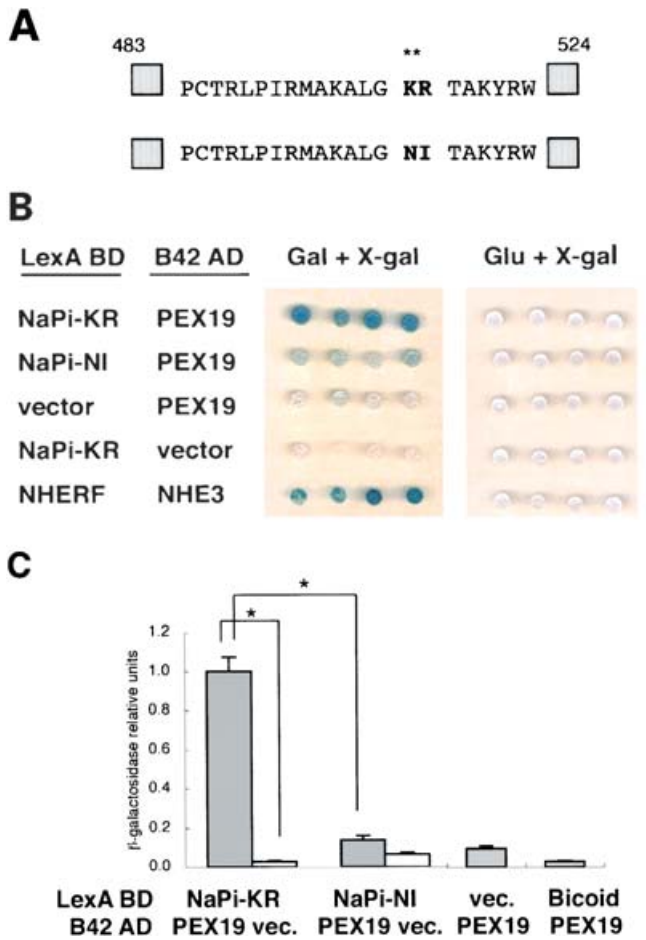

Figure 2 Interaction of the PEX19 protein with the KR motif of the Na/P_i IIa co-transporter in the yeast two-hybrid screen

(**A**) Amino acid sequences of the bait for the third intracellular loop of the Na/P_i IIa co-transporters are shown; the numbers indicate amino acid positions. Asterisks indicate the changed amino acid residues. Wild-type (NaP_i-KR) and mutant (NaP_i-NI) proteins were used as baits in the yeast two-hybrid system. (**B**) Colony colour assay of various two-hybrid constructs. Pairs of constructs used for the assay are shown in the left-hand panel. DNA fragments encoding the peptides shown in (**A**) were fused to the LexA DNA-binding domain (LexA BD) in the vector pEG202 and PEX19 cDNA was fused to the B42 transcriptional activation domain (B42 AD) in the vector pJG4-5. As a positive control, NHERF-1 and NHE3 were fused to the same genes. The negative control was PEX19 fused to the *Drosophila* Bicoid protein. Four independent transformants were selected and spotted on to both galactose (Gal + X-gal) and glucose (Glu + X-gal) solid media. (**C**) Interactions were analysed by liquid β-galactosidase assays with permeabilized liquid cultures of total yeast lysate. The β-galactosidase activity produced by the association of NaP_i-KR with PEX19 was set to 1. $^*P = 0.001$. For all groups, $n = 4$.

co-transporter to the intracellular domain (Figures 4H and 4K), but the co-expression of EGFP vector and NaP_i-KR co-transporter did not affect the migration of the NaP_i-KR protein (Figures 4G and 4J). In contrast, co-expression of PEX19 and the NaP_i-NI co-transporter had no effect on the migration of the NaP_i-NI protein (Figures 4I and 4L). These results indicate that PEX19 stimulates the retrieval of the NaP_i-KR co-transporter protein but not that of the NaP_i-NI mutant.

Down-regulation of endogenous Na/P_i IIa co-transporter in OK cells

To elucidate whether PEX19 can stimulate endogenous Na/P_i IIa co-transporter (NaPi-4) expression, we examined expression with

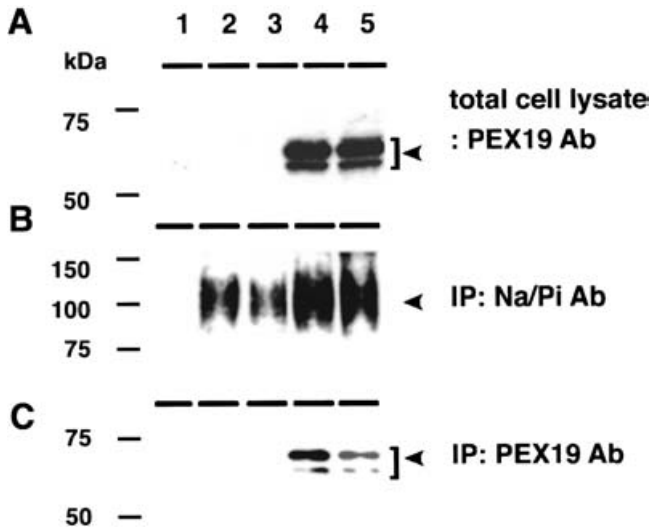

Figure 3 Interaction of Na/P_i IIa co-transporters and PEX19 in OK cells

A full-length Na/P_i IIa co-transporter (KR) and the mutant construct (NI) were fused with FLAG protein via insertion into the pFLAG-CMV2 vector. PEX19 was fused to the EGFP in the pEGFP-C1 vector. OK cells were co-transfected with the following plasmids; lane 1, mock transfection without any plasmids; lane 2, EGFP empty vector and NaP_i-KR; lane 3, EGFP empty vector and NaP_i-NI; lane 4, PEX19 plasmid and NaP_i-KR; lane 5, PEX19 and NaP_i-NI. The protein interaction in lysates was detected by Western blotting (**A**) or immunoprecipitated with FLAG antibody after 48 h (**B** and **C**). (**A**) In total cell lysates, the PEX19 protein was detected by anti-PEX19 antibody. Immunoprecipitated proteins were then detected by Western blotting with the anti-C-terminal Na/P_i antibody (**B**) and the interaction with PEX19 was detected by anti-PEX19 antibody (**C**). Molecular-mass markers are indicated in kDa.

Western blotting (Figure 5). When PEX19 was overexpressed in OK cells, NaPi-4 protein was markedly decreased in the apical membrane of OK cells. In the control study, we investigated whether PEX19 affected the expression of Na^+/K^+ ATPase in the basolateral membrane [27]. As shown in Figure 5, the levels of the Na^+/K^+ ATPase protein were not changed in the OK cells. These findings indicate that PEX19 may be one of the proteins associated with the endocytosis of the Na/P_i IIa co-transporter.

Endogenous expression of PEX19 in OK cells and proximal tubules

To identify the endogenous PEX19 expression in OK cells and mouse kidney, we carried out reverse transcriptase PCR analyses (Figure 6A). PEX19 mRNA transcripts were expressed in OK cells and mouse kidney at similar levels. Opossum PEX19 cDNA was identified, and the partial clone exhibited sequence similarity with human PEX19 at both the mRNA (82.3%) and protein (89.9%) levels (results not shown).

The expression of PEX19 mRNA was also detected by Northern blotting with mouse poly(A)$^+$ RNA [25] (results not shown). The major transcript of PEX19 mRNA was detected in the kidney as strands 2.4 and 4.0 kb in length. We detected weak signals in brain, heart, small intestine, placenta and skeletal muscle. These different-sized bands may be due to alternative-splicing forms (e.g. a larger 3′ end caused by an alternative polyadenylation) [25]. Expression of the PEX19 protein was analysed by immunoblotting with a polyclonal antibody raised against a synthetic C-terminal peptide of rat PEX19. Western blots conducted with isolated cytoplasmic and brush-border membrane fractions of rat kidneys indicated that PEX19 antibody reacted with a band of about 33 kDa in both fractions (Figure 6B).

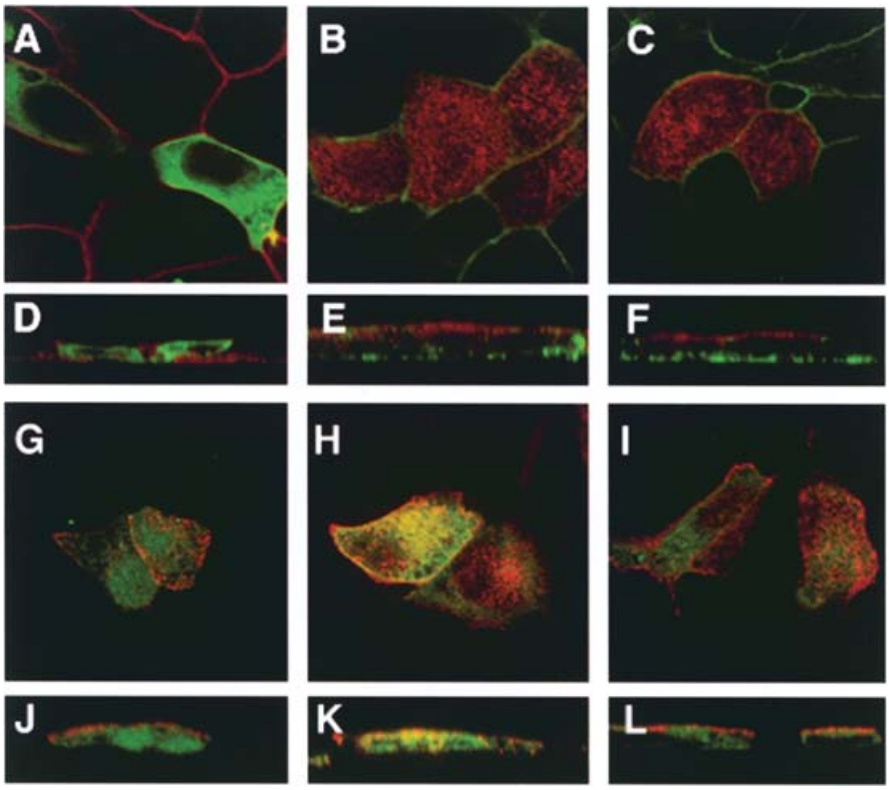

Figure 4 Internalization of Na/P_i IIa co-transporter by PEX19

Cells were transfected and then processed for *xy* cross sections by confocal microscopy (**A**–**C** and **G**–**I**). The confocal *xz* cross sections are shown (**D**–**F** and **J**–**L**) and apical membranes are at the top and basal membranes are at the bottom. OK cells were transfected with various plasmids. Cells in (**B**), (**G**) and (**H**) are expressing full-length NaP_i-KR, whereas cells in (**C**) and (**I**) are expressing NaP_i-NI. In addition, cells in (**G**) are expressing pEGFP empty vector. The cells in (**A**), (**H**) and (**I**) are expressing PEX19-EGFP. (**A**, **D**) PEX19-EGFP is green, actin is red. (**B**, **E**) Actin is green, NaP_i-KR is red. (**C**, **F**) Actin is green, NaP_i-NI is red. (**G**, **J**) EGFP is green, NaP_i-KR is red. (**H**, **K**) PEX19-EGFP is green, NaP_i-KR is red. (**I**, **L**) PEX19-EGFP is green, NaP_i-NI is red.

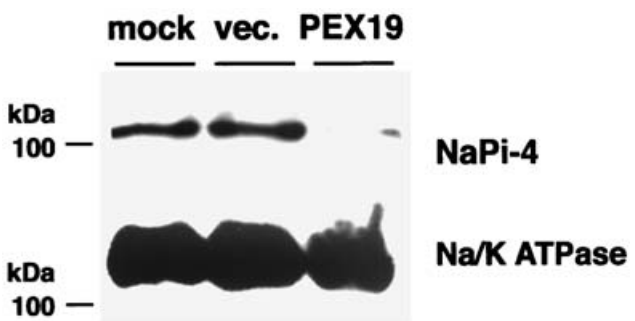

Figure 5 Down-regulation of endogenous Na/P_i IIa co-transporter (NaPi-4) by overexpressed PEX19 in OK cells

OK cells were transfected with empty vector or PEX19-pFLAG plasmid. The crude membrane fraction was prepared and analysed by Western blotting with NaPi-4 or a Na^+/K^+ ATPase antibody. Expression of PEX19 down-regulated that of NaPi-4 in the membrane fraction; PEX19 did not affect the endogenous Na^+/K^+ ATPase expressed in the basolateral membrane of OK cells.

Effect of dietary P_i on PEX19

We next investigated the effect of dietary P_i on PEX19 expression in rat renal proximal tubules. The expression of PEX19 mRNA in rats fed a high-P_i diet was 1.4-fold higher than in rats fed a normal P_i diet (Figure 7A). PEX19 protein levels also clearly increased in rats fed a high-P_i diet (Figure 7B).

Interaction of PEX19 with microvillar PDZ proteins

To date, three PDZ domain proteins have been described in the brush borders (the microvilli and the subapical compartment) of proximal tubule cells: NHERF-1 [28] (also known as EBP50), NaPi-Cap1 [28] (also known as PDZK1 [29], Cap70 [30] and Diphor-1 [31]) and NaPi-Cap2 [28]. These proteins contain PDZ domains and interact with the C-terminal tail (TRL sequence) of Na/P_i IIa co-transporter. Two of these proteins, NaPi-Cap1 and NHERF-1, co-localize with the Na/P_i IIa co-transporter in the proximal brush border. These findings suggest that an interaction with the PDZ domain is necessary for the apical localization of Na/P_i IIa co-transporter [27].

Using a yeast two-hybrid system, we investigated the interaction of PEX19 with these PDZ proteins. As shown in Figure 8(A), a weak but significant interaction of PEX19 and NHERF-1 was identified. This finding may indicate that these proteins form a complex in the apical or subapical fraction. Interactions of PEX19 with NaPi-Cap1 or NaPi-Cap2 were not confirmed because of a self-autoactivation in LexA systems (results not shown). To elucidate whether these proteins interact with PEX19, we examined their ability to bind in a mammalian two-hybrid analysis (Figure 8B). The results show a clear interaction between PEX19 and NaPi-Cap1 but not NaPi-Cap2. Furthermore, we also used this method to examine the interaction of PEX19 with the $\mu 2$ subunit of AP-2 complexes, which is known to be responsible for the clathrin-mediated internalization. This suggests that PEX19 interacts directly with the endocytic complex.

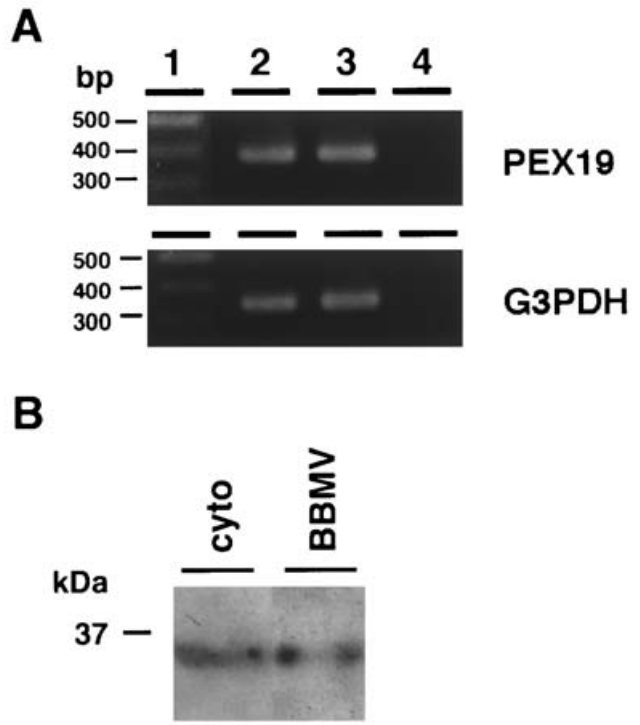

Figure 6 Endogenous expression of PEX19 in OK cells and proximal tubules

(**A**) Reverse transcriptase PCR of PEX19 in OK cells (lane 2) and mouse kidney (lane 3) is shown; G3PDH is the internal control. A 100 bp DNA size marker (lane 1) and no template (lane 4) are also shown. (**B**) PEX19 protein expression was detected by Western blotting. PEX19 was detected as a 33 kDa protein in cytoplasmic (cyto) and BBMV fractions of rat kidney.

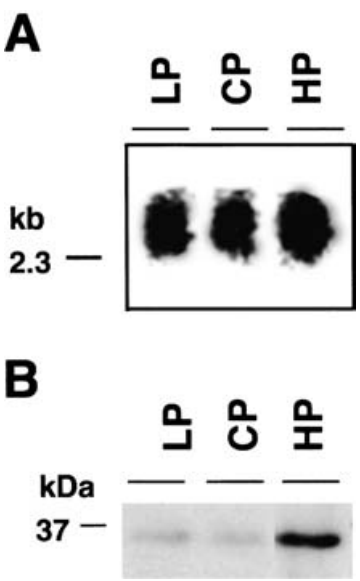

Figure 7 Effect of dietary P_i on PEX19 expression

(**A**) Poly(A)$^+$ RNA from the rat renal cortex with different P_i concentrations in the diet. Rats were fed a low-P_i (0.02 %) diet (LP), a high-P_i (1.2 %) diet (HP) or a normal (0.6 % P_i) diet (CP). Fragments containing the full coding sequence of human PEX19 were used as probes for Northern analysis. The data are representative of three independent experiments. (**B**) Effect of dietary P_i on PEX19 protein expression was examined by Western blotting in the rat BBMV fraction. Each fraction was separated by SDS/PAGE and detected with polyclonal antibody raised against a synthetic C-terminal peptide of PEX19.

Influence of a farnesylation signal in PEX19 on binding with the Na/P_i IIa co-transporter

Farnesylation of the C-terminal CAAX motif (where A means aliphatic) of PEX19 (CLIM) is important for its biological function in yeast and humans [26,32]. We tested in a yeast two-hybrid system the influence of farnesylation on PEX19's interaction with the endocytic motif of the Na/P_i IIa co-transporter (Figure 8C). The mutant PEX19 (a CAAX box-deletion mutant) exhibited less binding activity towards Na/P_i IIa co-transporter than did wild-type PEX19; thus it appears that farnesylation of PEX19 is necessary for its interaction with the endocytic motif of the Na/P_i IIa co-transporter.

DISCUSSION

We have demonstrated that PEX19, a small protein first cloned and described as the human peroxisomal farnesylated protein (PEX19; PxF, Pex19p) [25,33], strongly interacts with the endocytic motif (KR) in the Na/P_i IIa co-transporter. In addition, PEX19 farnesylation was necessary for the binding of the endocytic motif of the Na/P_i IIa co-transporter. PEX19 encodes a farnesylated protein of 299 amino acid residues, and is crucial for peroxisomal biogenesis [32]. Yeast two-hybrid systems have shown that PEX19 interacts with human PMP22 (peroxisomal membrane protein 22) [34] and ATP-binding cassette half-co-transporters, such as the adrenoleukodystrophy protein [35]. PMP22 includes two distinct targeting signals, one in the N-terminus and the other in the C-terminal part of the protein. These signals consist of two transmembrane domains that are adjacent protein loops with almost identical basic clusters, such as KIKKR [34]. The localization of PEX19 is predominantly in the cytosol and slightly in the peroxisome [34]. This suggests that PEX19 may be a docking protein or a peroxisomal receptor. Its function seems to be specific for PMP22 because the mitochondrial protein ornithine-δ-aminotransferase does interact with PEX19. PEX19 recognizes and possibly binds to the charged basic clusters of PMP22 [34]. Therefore, PEX19 may recognize the basic motif KR of the Na/P_i IIa co-transporter and act as a chaperone for membrane insertion or retrieval from the membrane.

In OK cells, PEX19 is expressed predominantly in the cytosomal fraction and stimulates the internalization of NaP_i-KR but not of NaP_i-NI. Although variable expression levels of Na/P_i IIa co-transporter were observed in the transient expression study in each OK cell, co-expression of the NaP_i-NI co-transporter and PEX19 did not affect the endocytosis of the NaP_i-NI protein in OK cells. In contrast, PEX19 affected the internalization of the NaP_i-KR co-transporter in OK cells. In the presence or absence of PTH, PEX19 stimulated the endocytosis of the NaP_i-KR protein in OK cells. These observations indicate that the elevation of PEX19 protein levels may stimulate the endocytic pathway of the Na/P_i IIa co-transporter. It is possible that PTH stimulates the elevation of endogenous PEX19 levels in OK cells.

We next investigated whether PTH stimulates the expression of endogenous PEX19 in OK cells. However, these analyses were not possible because the rat PEX19 antibody failed to react with the endogenous PEX19 protein in OK cells. As an alternative, we investigated whether a high-P_i diet stimulates the expression of PEX19 protein in mouse and rat kidney. Northern blot analysis in various tissues with human PEX19 cDNA indicated that mouse PEX19 mRNA was highly expressed in the kidney. Cell fractionation revealed that PEX19 was localized in the cytosol and BBMV (microvilli and subapical component). Immunohistochemical analysis revealed that PEX19 localizes in the subapical compartment and the cytosomal fraction of the proximal tubule

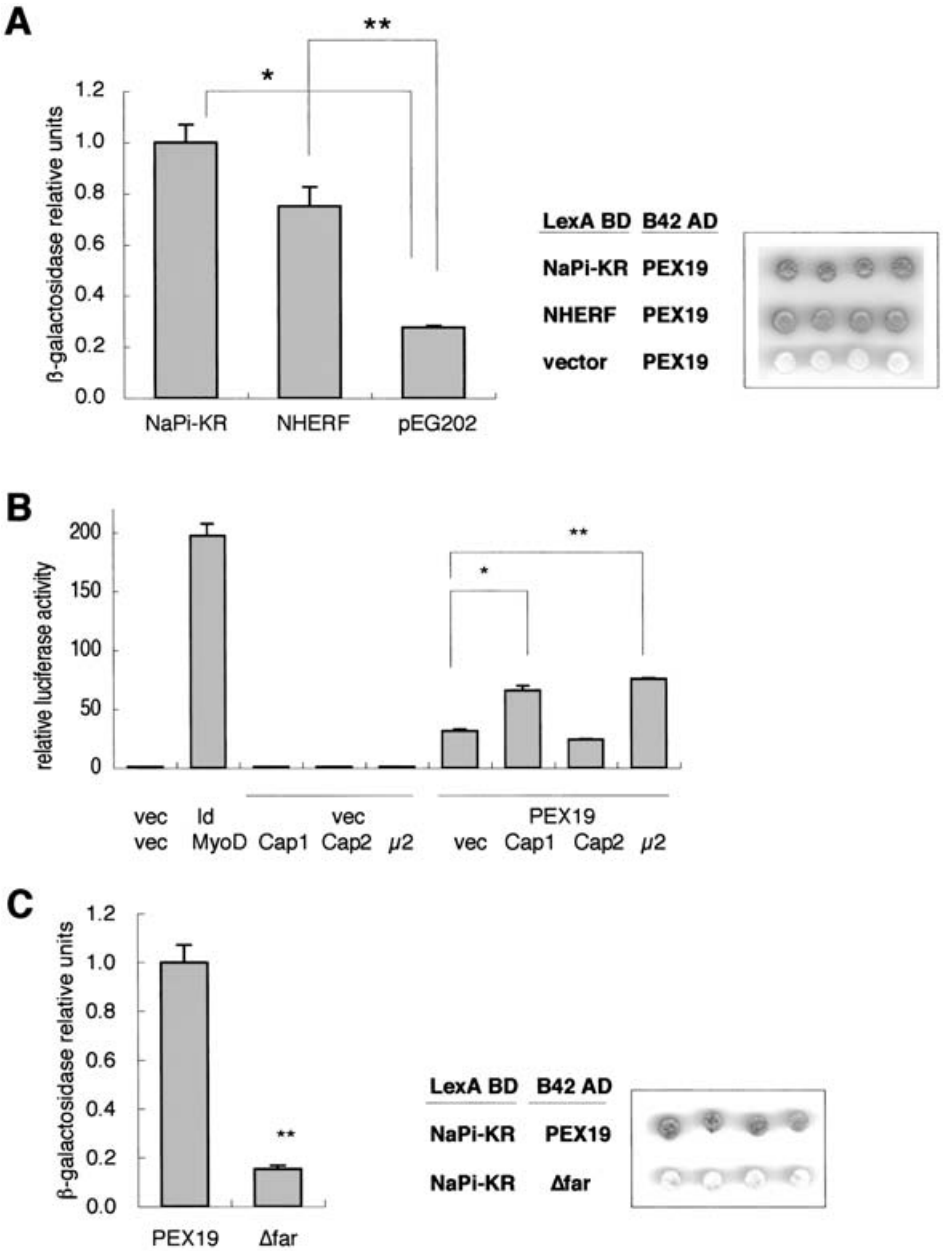

Figure 8 Interaction of PEX19 with various proteins in the yeast two-hybrid assay

(**A**) The interactions of PEX19 with full length of NHERF-1 or empty vector (pEG202-NLS) were analysed by the liquid β-galactosidase assay (left-hand panel). Four independent transformants were selected and spotted on to galactose solid medium (Gal + X-gal; right-hand panel). *$P = 0.002$, **$P = 0.01$ for comparison with control condition. LexA BD, LexA DNA-binding domain; B42 AD, B42 transcriptional activation domain. (**B**) Interaction of PEX19 with NaPi-Cap1, NaPi-Cap2 and μ2 subunit of AP-2 was analysed by the mammalian two-hybrid system in COS-7 cells. The degree of interaction is given as the relative luciferase activity, which was calculated as the ratio of firefly to *Renilla* luciferase activity per mg of protein. The activity produced by the association of pBIND empty vector with pACT empty vector (vec/vec) was set to 1. *$P = 0.04$ **$P = 0.001$ for comparison of luciferase activities with control condition. The Id protein is a member of the helix-loop-helix family of nuclear proteins and acts as a negative regulator of myogenic differentiation. (**C**) Effect of farnesylation signal of PEX19 on its interaction with Na/P_i IIa co-transporter was tested. PEX19Δfar is deficient in the CAAX box at the C-terminus of PEX19. The β-galactosidase assay (left-hand panel) and colony colour assay (right-hand panel) were indicated. The wild-type PEX19 was compared with PEX19Δfar or empty vector. **$P = 0.001$; the β-galactosidase activity produced by the association of NaP_i-KR with PEX19 was set to 1. The histogram represents $n = 4$ separate clones.

cells in the normal rat (results not shown). A high-P_i diet also leads to a transient accumulation of Na/P_i IIa co-transporter in the so-called subapical vacuolar apparatus. In rats acutely changed to a high-P_i diet, the Na/P_i IIa co-transporter was found in the intracellular portion, but the PEX19 protein level rapidly increased, as it did in the PTH study. These findings indicate that the levels of PEX19 are controlled by two major regulators, PTH and P_i levels, and that PEX19 may participate in the internalization of the Na/P_i IIa co-transporter in OK cells.

It is possible that PEX19 interacts with multiple regulatory proteins that stimulate endocytosis of the Na/P_i IIa co-transporter. Based on a yeast two-hybrid screen, three PDZ proteins that interact with the C-terminus of the Na/P_i IIa co-transporter have been identified: NaPi-Cap1, NaPi-Cap2 [28] and NHERF-1. The interaction of PEX19 with NaPi-Cap1/2 was not detected in a yeast two-hybrid assay because these proteins undergo self-autoactivation in yeast. Recently, Shenolikar et al. [36] demonstrated that NHERF-1 plays a key role in the targeting and/or trafficking of the

Na/P_i IIa co-transporter in the mouse kidney, and defects in the apical localization of the Na/P_i IIa co-transporter contributed to P_i wasting seen in NHERF-1-deficient mice. Our yeast two-hybrid assay indicated that PEX19 could interact with NHERF-1. The roles of NHERF-1 and PEX19 on the internalization of the Na/P_i IIa co-transporter remain unknown. Thus we suggest that PTH-induced regulation of the type IIa co-transporter may involve multiple regulatory proteins interacting with the co-transporter. Further studies must be performed to identify proteins that interact with the type IIa protein at the KR dibasic sequence in the intracellular loop.

Acute internalization of the transporter in response to a high dietary P_i intake or administration of PTH occurs via clathrin-coated structures and is followed by lysosomal degradation of the co-transporter [5,8–11]. In the current studies we found that the AP-2 adaptor complex associates directly with the PEX19 via its $\mu 2$ subunit. Because the AP-2 adaptor complex and clathrin triskelions form the major structural components of plasma membrane-coated pits and vesicles [37,38], our results suggest that the interaction with PEX19 mediates Na/P_i IIa co-transporter internalization via the clathrin-coated-pit-dependent pathway. The AP-2 adaptor complex is a heterotetramer consisting of four subunits: α and $\beta 2$ (110–115 kDa), $\mu 2$ (50 kDa) and $\sigma 2$ (17 kDa). However, we did not detect interactions between PEX19 and the α, $\beta 2$ or $\sigma 2$ subunits (results not shown). Further study is needed to clarify the roles of PEX19 in the internalization of Na/P_i IIa co-transporter. In conclusion, the present study indicates that PEX19 is one of the proteins interacting with the endocytic motif ($K^{503}R^{504}$) of the Na/P_i IIa co-transporter and that the co-transporter is regulated as an acute response to high dietary intake of P_i or the administration of PTH.

We thank Y. Fujiki for providing us with the antibody for human PEX19 protein. We thank Dr T. Horio (University of Tokushima, Tokushima, Japan) for his advice concerning yeast two-hybrid experiments and for critically reading the manuscript. This work was supported by grants in aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (14770552).

REFERENCES

1 Murer, H., Hernando, N., Forster, I. and Biber, J. (2000) Proximal tubular phosphate reabsorption: molecular mechanisms. Physiol. Rev. **80**, 1373–1409
2 Levi, M., Lotscher, M., Sorribas, V., Custer, M., Arar, M., Kaissling, B., Murer, H. and Biber, J. (1994) Cellular mechanisms of acute and chronic adaptation of rat renal P(i) transporter to alterations in dietary P(i). Am. J. Physiol. **267**, F900–F908
3 Werner, A., Kempson, S. A., Biber, J. and Murer, H. (1994) Increase of Na/Pi-cotransport encoding mRNA in response to low Pi diet in rat kidney cortex. J. Biol. Chem. **269**, 6637–6639
4 Katai, K., Segawa, H., Haga, H., Morita, K., Arai, H., Tatsumi, S., Taketani, Y., Miyamoto, K., Hisano, S., Fukui, Y. and Takeda, E. (1997) Acute regulation by dietary phosphate of the sodium-dependent phosphate transporter (NaP(i)-2) in rat kidney. J. Biochem. (Tokyo) **121**, 50–55
5 Lotscher, M., Scarpetta, Y., Levi, M., Halaihel, N., Wang, H., Zajicek, H. K., Biber, J., Murer, H. and Kaissling, B. (1999) Rapid downregulation of rat renal Na/P(i) cotransporter in response to parathyroid hormone involves microtubule rearrangement. J. Clin. Invest. **104**, 483–494
6 Murer, H., Lotscher, M., Kaissling, B., Levi, M., Kempson, S. A. and Biber, J. (1996) Renal brush border membrane Na/Pi-cotransport: molecular aspects in PTH-dependent and dietary regulation. Kidney Int. **49**, 1769–1773
7 Green, J., Debby, H., Lederer, E., Levi, M., Zajicek, H. K. and Bick, T. (2001) Evidence for a PTH-independent humoral mechanism in post-transplant hypophosphatemia and phosphaturia. Kidney Int. **60**, 1182–1196
8 Lotscher, M., Kaissling, B., Biber, J., Murer, H. and Levi, M. (1997) Role of microtubules in the rapid regulation of renal phosphate transport in response to acute alterations in dietary phosphate content. J. Clin. Invest. **99**, 1302–1312
9 Pfister, M. F., Lederer, E., Forgo, J., Ziegler, U., Lotscher, M., Quabius, E. S., Biber, J. and Murer, H. (1997) Parathyroid hormone-dependent degradation of type II Na^+/Pi cotransporters. J. Biol. Chem. **272**, 20125–20130
10 Traebert, M., Roth, J., Biber, J., Murer, H. and Kaissling, B. (2000) Internalization of proximal tubular type II Na-P(i) cotransporter by PTH: immunogold electron microscopy. Am. J. Physiol. Renal Physiol. **278**, F148–F154
11 Traebert, M., Volkl, H., Biber, J., Murer, H. and Kaissling, B. (2000) Luminal and contraluminal action of 1–34 and 3–34 PTH peptides on renal type IIa Na-P(i) cotransporter. Am. J. Physiol. Renal Physiol. **278**, F792–F798
12 Pfister, M. F., Ruf, I., Stange, G., Ziegler, U., Lederer, E., Biber, J. and Murer, H. (1998) Parathyroid hormone leads to the lysosomal degradation of the renal type II Na/Pi cotransporter. Proc. Natl. Acad. Sci. U.S.A. **95**, 1909–1914
13 Lederer, E. D., Sohi, S. S., Mathiesen, J. M. and Klein, J. B. (1998) Regulation of expression of type II sodium-phosphate cotransporters by protein kinases A and C. Am. J. Physiol. **275**, F270–F277
14 Jankowski, M., Biber, J. and Murer, H. (1999) PTH-induced internalization of a type IIa Na/Pi cotransporter in OK- cells. Pflugers Arch. **438**, 689–693
15 Hernando, N., Forgo, J., Biber, J. and Murer, H. (2000) PTH-induced downregulation of the type IIa Na/P(i)-cotransporter is independent of known endocytic motifs. J. Am. Soc. Nephrol. **11**, 1961–1968
16 Jankowski, M., Hilfiker, H., Biber, J. and Murer, H. (2001) The opossum kidney cell type IIa Na/P(i) cotransporter is a phosphoprotein. Kidney Blood Press Res. **24**, 1–4
17 Hilfiker, H., Hattenhauer, O., Traebert, M., Forster, I., Murer, H. and Biber, J. (1998) Characterization of a murine type II sodium-phosphate cotransporter expressed in mammalian small intestine. Proc. Natl. Acad. Sci. U.S.A. **95**, 14564–14569
18 Karim-Jimenez, Z., Hernando, N., Biber, J. and Murer, H. (2000) A dibasic motif involved in parathyroid hormone-induced down-regulation of the type IIa NaPi cotransporter. Proc. Nat. Acad. Sci. U.S.A. **97**, 12896–12901
19 Segawa, H., Kaneko, I., Takaashi, A., Kuwahata, M., Ito, M., Ohkido, I., Tatsumi, S. and Miyamoto, K. (2002) Growth-related renal type II Na/Pi cptransporter. J. Biol. Chem. **277**, 19665–19672
20 Reshkin, S. J., Wuarin, F., Biber, J. and Murer, H. (1990) Parathyroid hormone-induced alterations of protein content and phosphorylation in enriched apical membranes of opossum kidney cells. J. Biol. Chem. **265**, 15261–15266
21 Weinman, E. J., Evangelista, C. M., Steplock, D., Liu, M. Z., Shenolikar, S. and Bernardo, A. (2001) Essential role for NHERF in cAMP-mediated inhibition of the Na+-HCO3-co-transporter in BSC-1 cells. J. Biol. Chem. **276**, 42339–42346
22 Fujiki, Y. (2000) Peroxisome biogenesis and peroxisome biogenesis disorders. FEBS Lett. **476**, 42–46
23 Pfister, M. F., Hilfiker, H., Forgo, J., Lederer, E., Biber, J. and Murer, H. (1998) Cellular mechanisms involved in the acute adaptation of OK cell Na/Pi-cotransport to high- or low-Pi medium. Pflugers Arch. **435**, 713–719
24 Minami, H., Kim, J. R., Tada, K., Takahashi, F., Miyamoto, K., Nakabou, Y., Sakai, K. and Hagihira, H. (1993) Inhibition of glucose absorption by phlorizin affects intestinal functions in rats. Gastroenterology **105**, 692–697
25 Braun, A., Kammerer, S., Weissenhorn, W., Weiss, E. H. and Cleve, H. (1994) Sequence of a putative human housekeeping gene (HK33) localized on chromosome 1. Gene **146**, 291–295
26 Matsuzono, Y., Kinoshita, N., Tamura, S., Shimozawa, N., Hamasaki, M., Ghaedi, K., Wanders, R. J., Suzuki, Y., Kondo, N. and Fujiki, Y. (1999) Human PEX19: cDNA cloning by functional complementation, mutation analysis in a patient with Zellweger syndrome, and potential role in peroxisomal membrane assembly. Proc. Natl. Acad. Sci. U.S.A. **96**, 2116–2121
27 Hernando, N., Deliot, N., Gisler, S. M., Lederer, E., Weinman, E. J., Biber, J. and Murer, H. (2002) PDZ-domain interactions and apical expression of type IIa Na/P(i) cotransporters. Proc. Natl. Acad. Sci. U.S.A. **99**, 11957–11962
28 Gisler, S. M., Stagljar, I., Traebert, M., Bacic, D., Biber, J. and Murer, H. (2001) Interaction of the type IIa Na/Pi-cotransporter with PDZ proteins. J. Biol. Chem. **276**, 9206–9213
29 Kocher, O., Comella, N., Tognazzi, K. and Brown, L. F. (1998) Identification and partial characterization of PDZK1: a novel protein containing PDZ interaction domains. Lab. Invest. **78**, 117–125
30 Wang, S., Yue, H., Derin, R. B., Guggino, W. B. and Li, M. (2000) Accessory protein facilitated CFTR-CFTR interaction, a molecular mechanism to potentiate the chloride channel activity. Cell **103**, 169–179
31 Custer, M., Spindler, B., Verrey, F., Murer, H. and Biber, J. (1997) Identification of a new gene product (diphor-1) regulated by dietary phosphate. Am. J. Physiol. Renal Physiol. **273**, F801–F806
32 Gotte, K., Girzalsky, W., Linkert, M., Baumgart, E., Kammerer, S., Kunau, W. H. and Erdmann, R. (1998) Pex19p, a farnesylated protein essential for peroxisome biogenesis. Mol. Cell Biol. **18**, 616–628
33 Kammerer, S., Arnold, N., Gutensohn, W., Mewes, H. W., Kunau, W. H., Hofler, G., Roscher, A. A. and Braun, A. (1997) Genomic organization and molecular characterization of a gene encoding HsPXF, a human peroxisomal farnesylated protein. Genomics **45**, 200–210

34 Brosius, U., Dehmel, T. and Gartner, J. (2002) Two different targeting signals direct human peroxisomal membrane protein 22 to peroxisomes. J. Biol. Chem. **277**, 774–784
35 Gloeckner, C. J., Mayerhofer, P. U., Landgraf, P., Muntau, A. C., Holzinger, A., Gerber, J. K., Kammerer, S., Adamski, J. and Roscher, A. A. (2000) Human adrenoleukodystrophy protein and related peroxisomal ABC transporters interact with the peroxisomal assembly protein PEX19p. Biochem. Biophys. Res. Commun. **271**, 144–150
36 Shenolikar, S., Voltz, J. W., Minkoff, C. M., Wade, J. B. and Weinman, E. J. (2002) Targeted disruption of the mouse NHERF-1 gene promotes internalization of proximal tubule sodium-phosphate cotransporter type IIa and renal phosphate wasting. Proc. Natl. Acad. Sci. U.S.A. **99**, 11470–11475
37 Takei, K. and Haucke, V. (2001) Clathrin-mediated endocytosis: membrane factors pull the trigger. Trends Cell Biol. **11**, 385–391
38 Kirchhausen, T. (2002) Clathrin adaptors really adapt. Cell **109**, 413–416

Received 12 August 2003/13 October 2003; accepted 15 October 2003
Published as BJ Immediate Publication 15 October 2003, DOI 10.1042/BJ20031223