Regulation of intestinal Na^+-dependent phosphate co-transporters by a low-phosphate diet and 1,25-dihydroxyvitamin D_3

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In a study of the rat intestinal P_i transport system, an activator protein for rat Na/P_i co-transport system (PiUS) was isolated and characterized. We also investigated the effects of restriction of vitamin D and P_i (two of the most important physiological and pathophysiological regulators of P_i absorption in the small intestine) on intestinal P_i transport activity and the expression of Na/P_i co-transporters that are expressed in rat small intestine. Rat PiUS encodes a 424-residue protein with a calculated molecular mass of 51463 Da. The microinjection of rat PiUS into *Xenopus* oocytes markedly stimulated Na^+-dependent P_i co-transport activity. In rats fed with a low-P_i diet, Na^+-dependent P_i co-transport activity was increased approx. 2-fold compared with that of rats fed a normal P_i diet. Kinetic studies demonstrated that this increased activity was due to an elevation of V_{max} but not K_m. The PiUS mRNA levels showed an approximate doubling in the rats fed with the low-P_i diet compared with those fed with the normal P_i diet. In addition, after the administration of 1,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$] to vitamin D-deficient animals, the P_i uptake was significantly increased in the Na^+-dependent component in the brush border membrane vesicle (BBMV) at 24 and 48 h. In addition, we found a further high-affinity Na/P_i co-transport system in the BBMV isolated from the vitamin D-replete animals. The levels of type III Na/P_i co-transporter PiT-2 mRNA were increased 24 and 48 h after 1,25-$(OH)_2D_3$ administration to vitamin D-deficent animals, whereas PiUS and the type IIb Na/P_i co-transporter mRNA levels were unchanged. In conclusion, we first cloned a rat activator protein, PiUS, and then studied its role along with that of other type III Na/P_i co-transporters. PiUS and PiT-2 might be important components in the regulation of the intestinal P_i transport system by P_i restriction and 1,25-$(OH)_2D_3$.

Key words: inorganic phosphate, PiUS.

INTRODUCTION

The intestinal absorption of P_i has been characterized in several mammalian and avian species [1–5]. Studies conducted with isolated intestinal brush border membrane vesicles (BBMVs) have demonstrated that the transepithelial uptake of P_i occurs primarily in the proximal small intestine and consists of two components: passive diffusion across the intestinal brush border and Na^+-dependent, carrier-mediated, uptake. The molecular mechanisms of the regulation of P_i transport have been studied by the cloning of several cDNA species corresponding to mammalian renal Na/P_i co-transporters. However, the mechanisms of the adaptation of intestinal P_i transport are not well understood because of a lack of knowledge about the structure of intestinal Na/P_i co-transporters [6,7].

Intestinal P_i transport activity is well known to be controllable by 1,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$] and a low-P_i diet [2,4,8–17]. 1,25-$(OH)_2D_3$ regulates the intestinal absorption of P_i [16,18–21]. The intestinal P_i transport process occurs both by an Na^+-independent, non-saturable process and via an active, Na^+-dependent component of P_i absorption, mainly in the duodenum and jejunum [8]. However, one effect of vitamin D_3 on P_i absorption seems to be the stimulation of Na^+-dependent P_i co-transporters [8–11].

In addition, a low-P_i diet increases brush border membrane Na^+-dependent P_i transport [12–17]. Rat intestinal P_i absorption is decreased when dietary P_i is increased, and is enhanced in animals fed with a low-P_i diet [12]. Adaptive responses to changes in dietary P_i intake have been reported in intestinal preparations from different species [13,16,17]. Like its renal counterpart, the intestinal adaptive response to changes in dietary P_i is specific to the Na/P_i co-transporter, with no change in the transport of amino acids and glucose [12].

Three types of Na/P_i co-transporter have been isolated from several species [18–20]. The type I and type II Na/P_i co-transporters are expressed mainly in renal epithelial cells [21]. Type III transporters are widely expressed in mouse, rat and human tissues [19]. Type III transporters were isolated as receptors for gibbon ape leukaemia virus (GLVR1 or PiT-1) in mice and humans and amphotropic murine retrovirus (Ram-1 or PiT-2) in rats [19], and were shown to have normal cellular functions as Na^+-dependent P_i co-transporters in several tissues [19,22–24]. The amino acid sequences of PiT-1 and PiT-2 transporters are approx. 60% identical [19], and exhibit no significant overall sequence similarity to the type I or type II transporters. More recently, Hilfiker et al. [25] cloned an isoform of the type II Na/P_i co-transporter (type IIb) cDNA from mouse small intestine; its mRNA was found in a variety of tissues. The

Abbreviations used: BBMV, brush border membrane vesicle; BNPI, brain-specific Na/P_i co-transporters; 1,25-$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; PiUS, an activator protein for Na^+-dependent P_i transport system.

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The nucleotide sequence data reported will appear in DDBJ, EMBL and GenBank Nucleotide Sequence Databases under the accession number AB015723.

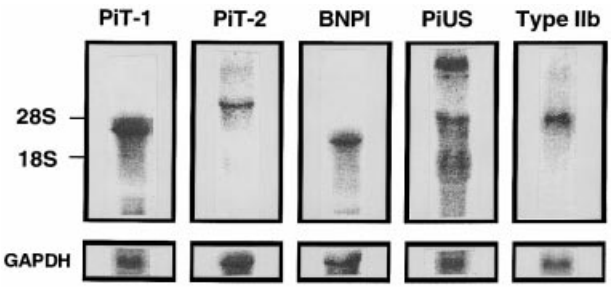

Figure 1 Expression of Na/P_i co-transporter and its activator in the rat small intestine

A Northern blot analysis was performed with the following cDNA probes: rat RNaPi -1 [35], rat NaPi-2 [18], rat PiT-1, rat PiT-2 [22], rat BNPI [20] and rabbit PiUS [26]. Total RNA (20 μg) or poly(A)$^+$ RNA (10 μg) was denatured and loaded on 1.2% (w/v) agarose gels containing formaldehyde. The hybridization was performed as described in the Materials and methods section. The experiments were performed at least three times; the results shown are representative of all the experiments. Abbreviation: GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

physiological role of Na/P_i co-transport mediated by type IIb in the small intestine remains unknown.

A putative activator protein for Na^+-dependent P_i transport (PiUS) has been found in the small intestine [26]. PiUS was cloned from rabbit small intestine by expression cloning. PiUS markedly stimulated Na/P_i co-transport activity in *Xenopus* oocytes [26]. The putative amino acid sequence of PiUS cDNA revealed a highly hydrophilic protein and no membrane-spanning domain, suggesting that PiUS might be an activator for Na^+-dependent P_i transporter [26]. Furthermore, in rat and human brain, a family of brain-specific Na/P_i co-transporters (BNPI) was cloned and was also found to be expressed in the small intestine [20]. However, their functional roles and regulation are unknown.

To elucidate the regulation of intestinal Na/P_i co-transporters, we investigated the effect of dietary low P_i and 1,25-$(OH)_2D_3$ on rat intestinal Na/P_i co-transport and the mRNA levels of the Na/P_i co-transporters.

MATERIALS AND METHODS

Animals and diets

Male Wistar rats weighing 200 g, obtained from SLC (Shizuoka, Japan), were housed in plastic cages and received a supplement containing 0.5% calcium, 0.6% phosphorus and 4.4 i.u. vitamin D_3/g [15]. The animals were pair-fed with either a normal-P_i diet (0.6%) (normal) or a low-P_i diet (0.02%) (low P_i) for 7 days between 09:00h and 24:00h.

Vitamin D-deficent animals

Male Wistar rats (3 weeks of age; body weight 40 g) were fed *ad libitum* with a vitamin D-free diet containing 0.6% calcium (diet 11) [27] for 6 weeks and then with a vitamin D- and calcium-free diet (diet 11-Ca) for an additional week. Rats with a low plasma concentration of calcium and vitamin D at the end of this feeding period were subjected to the experiments. For repletion, the vitamin D-deficient animals were treated intravenously with 1,25-$(OH)_2D_3$ (6.25 μg/kg of body weight) in ethanol/propylene glycol (1:4, v/v). The vitamin D-replete rats were fasted for 12 h in metabolism cages with water *ad libitum* before killing and the removal of tissues. The mean serum Ca^{2+} level for all vitamin D-deficient animals used in this study was 5.3 ± 0.4 mg/dl. The plasma concentrations of 1,25-$(OH)_2D_3$ were below 5 ng/ml, confirming vitamin D deficiency.

Preparation of BBMVs and transport measurements

BBMVs were prepared from rat small intestine (jejunum) by the Ca^{2+} precipitation method as described previously [28]. The purity of the membranes was assessed by measuring the levels of leucine aminopeptidase, Na^+,K^+-ATPase and cytochrome *c* oxidase [27]. The uptake of radiolabelled P_i was measured by the rapid-filtration technique [29]. After 10 μl of the vesicle suspension had been added to 90 μl of the incubation solution (consisting of 100 mM NaCl, 100 mM mannitol, 20 mM Hepes/Tris and 0.1 mM KH_2PO_4), the preparation was incubated at 20 °C. The measurements of Na^+-dependent and Na^+-independent P_i uptake were performed as described previously [15]. Transport was terminated by rapid dilution with 3 ml of an ice-cold solution consisting of 100 mM mannitol, 20 mM Hepes/Tris, 0.1 mM KH_2PO_4, 20 mM $MgSO_4$ and 100 mM choline chloride. The reaction mixture was then immediately transferred to a pre-moistened filter (0.45 μm) maintained under a vacuum.

Transcription and measurement of P_i transport *in vitro* in microinjected *Xenopus* oocytes

The rat PiUS clone was linearized by digestion with *Eco*RI and transcribed into cRNA with T7 RNA polymerase (Promega, Madison, WI, U.S.A.) [30]. The measurements of Na^+-dependent and Na^+-independent P_i uptake in *Xenopus* oocytes were performed as described previously [30].

Northern blot analysis

Total RNA from jejunal mucosa was isolated by extraction with acid guanidinium thiocyanate/phenol/chloroform by the method of Chomczynski and Saachi [31]. Resolved RNA was transferred to a Hybond-N membrane (Amersham, Little Chalfont, Bucks., U.K.) and covalently cross-linked by exposure to UV. Hybridization was performed in a solution containing 50% (v/v) formamide, 5 × SSPE [SSPE being 0.15 M NaCl/10 mM sodium phosphate (pH 7.4)/1 mM EDTA], 5 × Denhardt's solution [0.1% BSA/0.1% (w/v) Ficoll 400/0.1% (w/v) polyvinylpyrrolidone] and 1% (w/v) SDS. The membranes were exposed in a bio-imaging analysis system (BAS1500; Fuji Photo Film Co., Tokyo, Japan). Total RNA from various rat tissues was denatured, fractionated by electrophoresis on a 1.2% (w/v) agarose gel containing formaldehyde, transferred to a nylon membrane and subjected to hybridization with randomly primed ^{32}P-labelled rat PiT-1, rat PiT-2 [22], mouse type IIb [25], rat BNPI [20], rat PiUS [26], rat Na^+-dependent glucose transporter SGLT1 [27] or rat peptide transporter PepT1 [32]. These cDNA clones were prepared as described previously [22,32].

Hybridization to the labelled probes was performed overnight in a solution containing 50% (w/v) deionized formamide, 10 × Denhardt's solution, 40 mM Tris/HCl, pH 7.5, 10 mg/ml salmon sperm DNA and 1% (w/v) SDS at 42 °C. The membranes were washed twice for 10 min each time with 0.1 × SSC/0.1% SDS at 60 °C (SSC is 0.15 M NaCl/0.015 M sodium citrate). The filters were exposed for 2, 12 and 24 h to a bio-imaging plate and quantified by the BAS1500 system mentioned above.

Cloning of rat PiUS

A cDNA library in vector λgt10 (4×10^4 independent recombinants) was constructed from 2.0 μg of the polyadenylated

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GACTCCAGAACCTCTCGCAGCAGCATCAGGCTGCTGTGGAGGAGCTCAGAGAGAAGCATC  240
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    M  R  E  E  Q  A  K  E  I  E  I  L  K  Q  K  L  F  S  K   19
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 E  E  S  G  E  *                                             424
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Figure 2 Nucleotide and amino acid sequences of rat PiUS cDNA isolated from rat intestine

Amino acids are indicated by single-letter abbreviations.

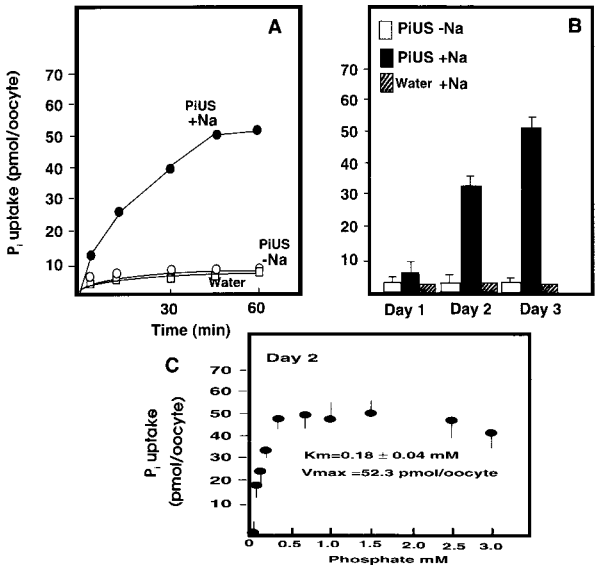

Figure 3 Functional analysis of rat PiUS in *Xenopus* oocytes

(**A**) Oocytes were injected with 50 nl of water (□) or 50 nl of water containing 5.0 ng of rat PiUS cRNA (○, without Na^+; ●, with Na^+). Uptake measurements were performed over a period of 60 min at 18 °C, after which individual oocytes were washed and assayed for associated radioactivity. Results are means ± S.E.M. for eight to ten oocytes. (**B**) Na^+-dependent and Na^+-independent P_i uptake in *Xenopus* oocytes. Uptake measurements were performed for 60 min at 18 °C, after which individual oocytes were washed and assayed for associated radioactivity. Results are means ± S.E.M. for eight to ten oocytes. (**C**) P_i-concentration dependence. Oocytes were injected with 5.0 ng of PiUS cRNA; 2 days after injection, transport (60 min incubation) was measured in the presence of NaCl. The curve was fitted to a Michaelis–Menten equation with the use of non-linear regression, yielding a K_m of 0.18 ± 0.04 mM and a V_{max} of 52.3 pmol per oocyte. Results are means ± S.E.M. for seven oocytes.

RNA [30]. Plaques were screened by hybridization under low-stringency conditions with a ^{32}P-labelled rabbit PiUS cDNA probe [26]. For the preparation of PiUS cDNA probe, PCR was performed as described previously with the following primer pairs: rabbit PiUS, sense, 5′-ATGAGCCCAGCCTTCAGGG-CCATGG-3′ (nt 174–198 relative to the translation start site); anti-sense, 5′-GCGCGTGCCCATCTTGAGGTCCAGG-3′ (nt 824–848 relative to the translation start site) [26]. The amplified fragments were subcloned into pBluescript II KS$^+$ and sequenced with T3 and T7 primers as described previously [30]. Five positive clones were isolated; the corresponding inserts were subcloned into the *Not*I site of pBluescript II SK$^+$ (Stratagene, La Jolla, CA, U.S.A.) and characterized by restriction mapping with *Pst*I, *Eco*RI or *Hin*dIII. Both strands of the cDNA inserts were sequenced by the dideoxy chain-termination method with a T7 sequencing kit (Pharmacia, Uppsala, Sweden).

Serum measurements

Plasma [Ca^{2+}] and [P_i] were measured as described previously [33]. Plasma 1,25-$(OH)_2D_3$ levels were measured by a radio-receptor assay (Incstar, Minneapolis, MN, U.S.A.). Plasma intact parathyroid hormone levels were measured with a rat immuno-radiometric assay (Nichols, Sam Clement, CA, U.S.A.).

Statistical analysis

Values are expressed as means ± S.E.M. The differences between the means of two groups and three or more groups were estimated

by Student's t test and one-way analysis of variance respectively. $P < 0.05$ was considered significant. Non-linear regression analysis was performed with the kinetic software package ENZFITTER [34].

RESULTS

Expression of intestinal P_i co-transporters

To investigate the expression of Na/P_i co-transporter genes in the rat small intestine, we subjected rat intestinal total RNA to a Northern blot analysis with cDNA probes for the following Na/P_i co-transporters and activator: rat RNaPi-1 [35], rat NaPi-2 [18], rat PiT-1, rat PiT-2 [22], rat BNPI [20] and rabbit PiUS [26]. We found that PiT-1, PiT-2 and BNPI were expressed in the rat small intestine (Figure 1). Rabbit PiUS cDNA hybridized to several transcripts. In addition, the 4.0 kb transcript of the type IIb was expressed in rat small intestine.

Cloning of rat PiUS from rat intestinal cDNA library

To isolate a rat PiUS cDNA clone, we screened a rat small-intestine cDNA library with a rabbit PiUS cDNA probe (nt +1 to +420, relative to the transcription start site) [26]. The largest of five positive clones contained an insert of 2583 bp, similar to the size of rat intestinal mRNA recognized by the rabbit PiUS in the Northern analysis. The insert contained a complete open reading frame for a protein that we have termed rat PiUS (Figure 2). The amino acid sequence of rat PiUS shows 93.0 % sequence similarity to rabbit PiUS. The open reading frame continues to the first stop codon (TGA) at nt 1281 and encodes a 424-residue protein with a calculated molecular mass of 51 kDa. The hydropathy analysis of the predicted amino acid sequence revealed a hydrophilic protein and no putative transmembrane domain (results not shown). Potential phosphorylation sites for protein kinase C were detected at amino acid residues 70, 113, 122, 125, 141, 178, 208, 321 and 386. The rat PiUS amino acid sequence does not show significant similarity to other known mammalian sequences. In addition, potential sites for cAMP-dependent protein kinase were detected at residues 59, 163, 276 and 307.

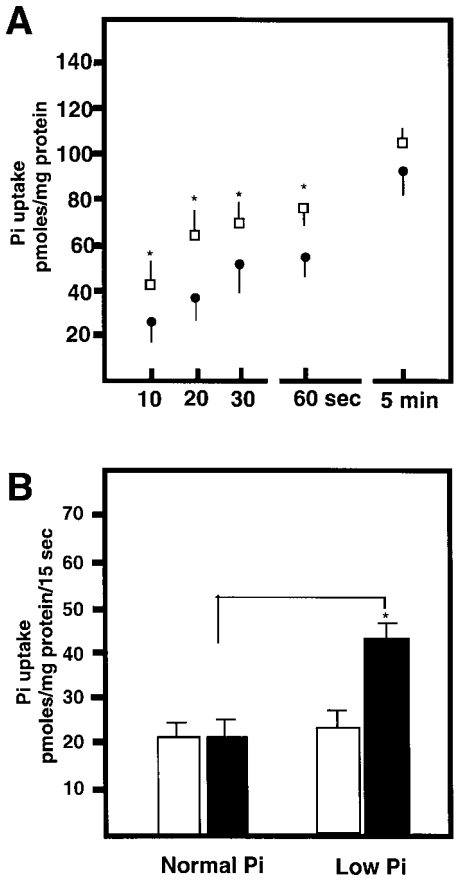

Figure 4 Effects of low-P_i diet on intestinal Na^+-dependent P_i co-transporter activity: time course (A) and Na^+-dependence (B)

Rats fed with a low-P_i diet for 7 days were used for the preparation of BBMVs and mRNA. Na/P_i transport activities were measured at 0.1 mM P_i for 5 min (**A**). In the absence of Na^+, choline chloride was used for P_i uptake. Symbols in (**A**): ●, normal P_i; □, low P_i. Symbols in (**B**): filled columns, Na^+-dependent; open columns, Na^+-independent. Results are means ± S.E.M. ($n = 5$); $^*P < 0.05$.

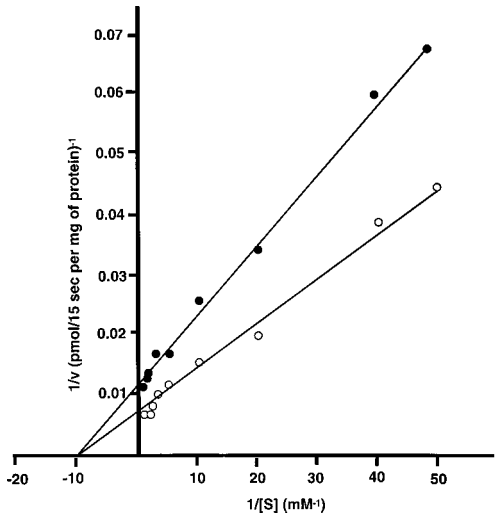

Figure 5 Influence of a low-P_i diet on the kinetic parameters of phosphate transport

Experiments were performed as described in the legend to Figure 4. For each of six vesicle preparations, K_m and V_{max} in the rats fed with a low-P_i diet for 7 days were determined from Lineweaver–Burk diagrams by regression analysis. P_i concentrations in the medium were varied between 0.02 and 10 mM. The uptake values were corrected for uptake in the presence of 0.1 M choline chloride. Symbols: ●, normal P_i; ○, low P_i. Results are means ± S.E.M. ($n = 5$).

The microinjection of PiUS into *Xenopus* oocytes stimulated Na^+-dependent P_i co-transport activity (Figure 3A). The Na^+-dependent P_i uptake at 30 min was stimulated approx. 10-fold compared with that of water-injected controls. In the absence of NaCl, no elevation of P_i uptake was shown in the oocytes injected with rat PiUS cRNA (Figure 3B). To characterize the PiUS-cRNA-induced increase in P_i uptake, we analysed it as a function of different P_i concentrations. The apparent K_m for expressed uptake was 0.18 ± 0.04 mM. These values are similar to those found with rabbit PiUS injection [26].

Effects of low-P_i diet on intestinal Na/P_i co-transport activity

First we examined whether dietary low P_i affects intestinal P_i transport activity (Figure 4A). The rats fed with a low-P_i (0.02 %) diet for 7 days showed markedly lower plasma concentrations of

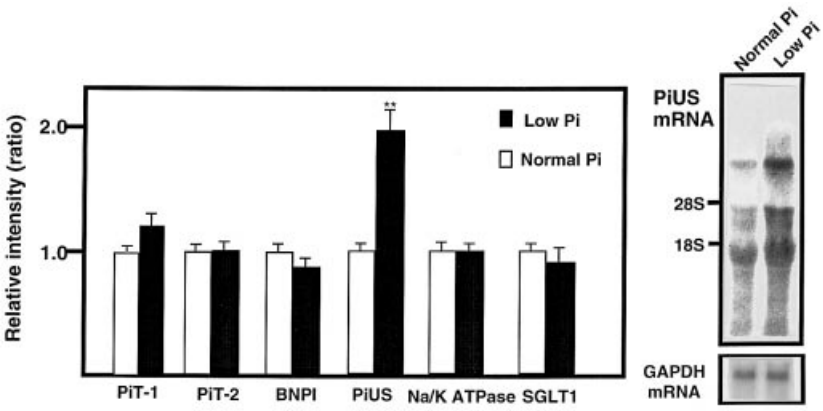

Figure 6 Effects of a low-P_i diet on intestinal Na/P_i co-transporter mRNA levels

Rat intestinal total RNA was prepared and analysed by Northern blotting. Densitometric scanning was done at 2, 12 and 24 h exposure for each mRNA. The results of densitometric scanning are shown as means ± S.E.M. of the ratio with respect to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). $^*P < 0.01$.

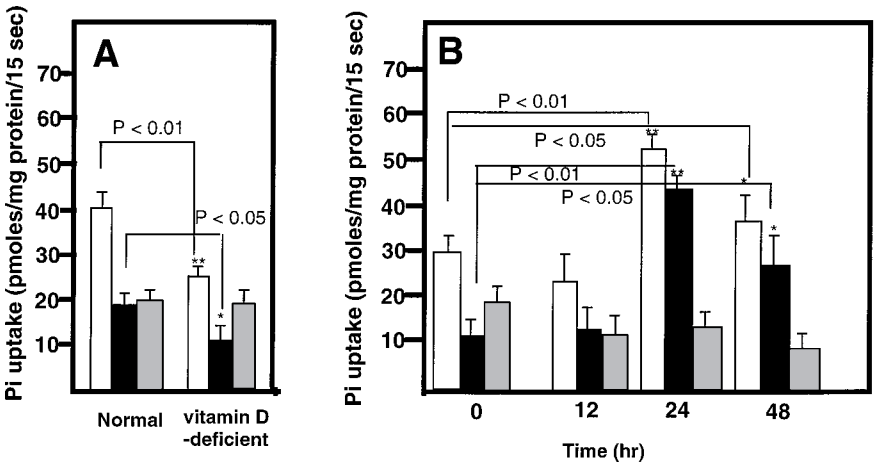

Figure 7 Effects of 1,25-$(OH)_2D_3$ on Na^+-dependent and Na^+-independent P_i co-transport activities in vitamin D-deficient rat small intestine

(**A**) Intestinal Na/P_i transport activity in BBMVs from vitamin D-deficient and normal rats. (**B**) Effects of 1,25-$(OH)_2D_3$ on intestinal P_i transport activity in vitamin D-deficient animals. Vitamin D-deficient rats were injected intravenously with 1,25-$(OH)_2D_3$ (6.25 μg/kg) and killed at various times thereafter. P_i transport activity was determined at 0, 12, 24 and 48 h after an injection of 1,25-$(OH)_2D_3$ to vitamin D-deficient rats. The jejunum was removed and BBMVs were isolated from the animals. The P_i uptake was determined as described in the text. Results are means ± S.E.M. ($n = 5$); $^*P < 0.05$, $^{**}P < 0.01$. White columns, total; black columns, Na^+-dependent; grey columns; Na^+-independent.

P_i than the animals fed with a normal-P_i (0.6 %) diet for the same period (3.36 ± 0.20 mg/dl compared with 7.07 ± 1.6 mg/dl). The serum 1,25-$(OH)_2D_3$ levels in the rats fed with the low-P_i and normal-P_i diet were 520 ± 43 and 75 ± 21 pg/ml, respectively.

As described in the Materials and methods section, we measured Na/P_i co-transport activity in the BBMV isolated from rats fed with a low-P_i diet for 7 days. Na^+-dependent P_i uptake was determined in the isolated BBMV from the jejunum of normal rats. The P_i uptake was linear for up to 30 s and slowly increased to 5 min (Figure 4A). The initial Na^+-dependent P_i co-transport activity (at 15 s) in the rats fed with the low-P_i diet was approximately doubled compared with that in the rats fed with the normal P_i diet. The Na^+-dependent P_i transport component was estimated to be approx. 50 % of the total P_i uptake at 15 s (Figure 4B). The Na^+-dependent component was significantly increased in the BBMVs isolated from rats fed with the low P_i diet, whereas the Na^+-independent component was not significantly changed (Figure 4B).

Kinetic analysis showed that the K_m values for P_i were 0.1 ± 0.04 and 0.1 ± 0.02 mM for the normal and the low-P_i diet respectively. The elevation of net P_i uptake in the rats fed with the low-P_i diet was due to an increase in the V_{max} (normal-P_i, 82 ± 19 pmol/15 s per mg of protein; low-P_i, 164 ± 22 pmol/15 s per mg of protein) (Figure 5).

To investigate further the expression of Na/P_i co-transporters in the rats fed with the low-P_i diet, a Northern blot analysis was performed. As shown in Figure 6, the PiUS mRNA levels were approximately doubled in the rats fed the low-P_i diet compared with those fed the normal-P_i diet. However, PiT-1, PiT-2, BNPI, Na/K-ATPase and SGLT1 mRNA levels were not changed. In addition, the type IIb mRNA levels were not affected by the feeding of a low-P_i diet (results not shown).

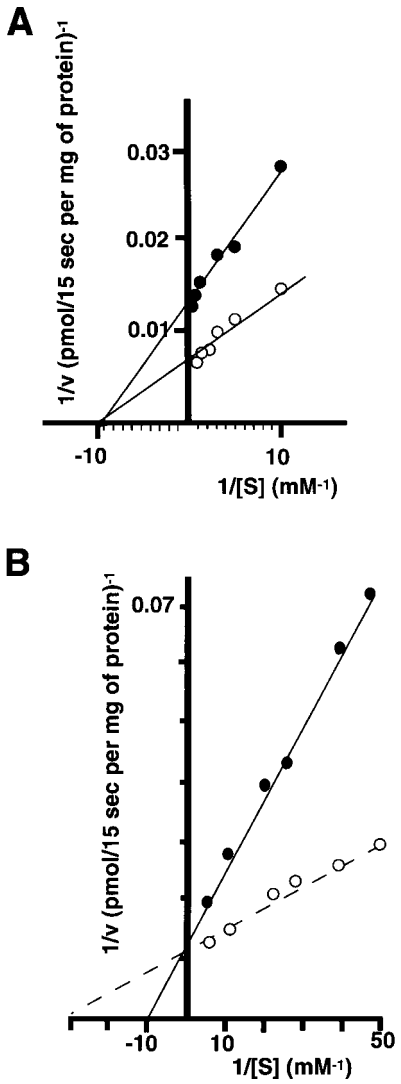

Figure 8 Influence of 1,25-$(OH)_2D_3$ on the kinetic parameters of P_i transport

Experiments were performed as described in the legend to Figure 5. For each of six vesicle preparations, K_m and V_{max} values 24 h after the administration of 1,25-$(OH)_2D_3$ were determined from Lineweaver–Burk diagrams by regression analysis. P_i concentrations in the medium were varied between 0.02 and 10 mM. Data are representative of five separate experiments. Symbols: ●, without 1,25-$(OH)_2D_3$; ○, with 1,25-$(OH)_2D_3$ after 24 h.

P_i uptake in BBMVs isolated from the small intestine of vitamin D-deficient animals

In the vitamin D-deficient animals, the total P_i transport activity was significantly decreased in the BBMVs isolated from the jejunum (Figure 7A); the Na^+-dependent component showed a greater decrease in net P_i transport activity than in the normal animals (Figure 7A). After the administration of 1,25-$(OH)_2D_3$ to vitamin D-deficient animals, the P_i uptake was significantly increased in the Na^+-dependent component in the BBMVs and was unchanged in the Na^+-independent component (Figure 7B). We performed a kinetic analysis of P_i transport in the vitamin D-deficient rats 24 h after the administration of vitamin D. The kinetic analysis revealed that the elevation of net P_i uptake was due to an increase in V_{max} (vitamin D-deficient, 45 ± 11 pmol/15 s per mg of protein; vitamin D-replete, 143 ± 24 pmol/15 s per mg

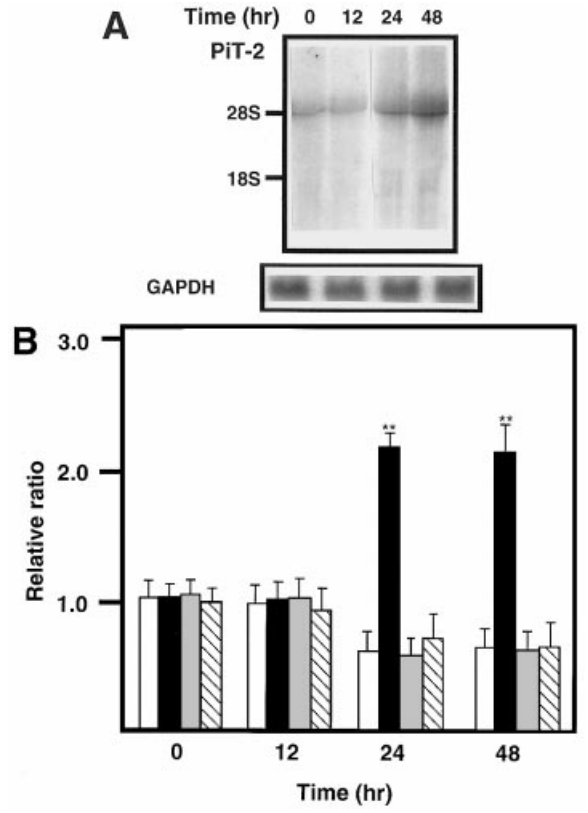

Figure 9 Expression of Na/P_i co-transport mRNA in vitamin D-deficient rats

After the administration of 1,25-$(OH)_2D_3$, we detremined the level of each transcript in the vitamin D-replete animals by Northern blotting (**A**). Densitometric scanning was done at 2, 12 and 24 h exposure for each total mRNA [5 μg of poly(A)$^+$ RNA] (**B**). The results of densitometric scanning are shown as means $\pm$ S.E.M. ($n = 5$) of the ratio with respect to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). $^{**}P < 0.01$. White columns, PiT-1; black columns, PiT-2; grey columns, BNPI; hatched columns, PiUS.

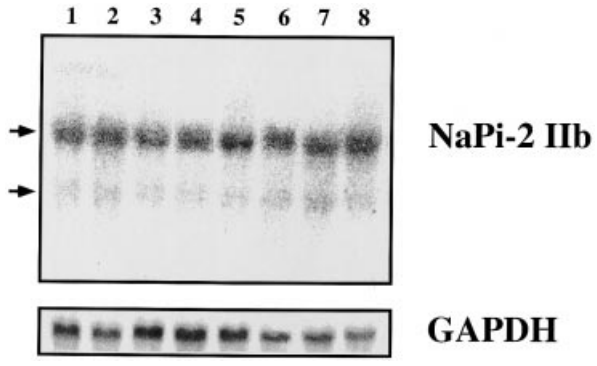

Figure 10 Expression of type IIb mRNA in vitamin D-deficient rats

After the administration of 1,25-$(OH)_2D_3$, the level of the type IIb transcript in the vitamin D-replete animals was determined. Total RNA (10 μg) was denatured and loaded on 1.2% (w/v) agarose gels containing formaldehyde. The hybridization was performed as described in the Materials and methods section. The experiment were performed at least three times; the results shown are representative of all the experiments. Lane 1, normal rat; lane 2, vitamin D-deficient rats; lane 3, vitamin D-deficient rats 12 h after the administration of 1,25-$(OH)_2D_3$; lane 4, 24 h after the administration of 1,25-$(OH)_2D_3$; lane 5, 48 h after the administration of 1,25-$(OH)_2D_3$; lane 6, 12 h after the administration of ethanol; lane 7, 24 h after the administration of ethanol; lane 8, 48 h after the administration of ethanol. The arrows indicate the position of 28 S and 18 S rRNA. Abbreviation: GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

of protein) in the Na^+-dependent component but not in K_m (vitamin D-deficient, 0.11 mM; vitamin D-replete, 0.12 mM) (Figure 8). In the BBMV isolated from the vitamin D-replete rats, we found the appearance of an additional high-affinity Na/P_i co-transporter system (K_m 0.033 mM) (Figure 8). The plasma Ca^{2+} levels of the vitamin D-deficient rats increased to the normal range 24–48 h after the vitamin D injection. The 1,25-$(OH)_2D_3$ injection decreased the plasma intact parathyroid hormone levels significantly, from the initial hyperparathyroidism to the normal range 24–48 h after the injection (vitamin D-deficient, 231 ± 45 pg/ml; vitamin D-replete 24 h, 38 ± 9 pg/ml).

To identify the expression of intestinal P_i co-transporters, all types of Na/P_i co-transporter (types I–III) cDNA were used for the Northern blot analysis (Figure 9). The levels of PiT-2 mRNA were increased (2.3-fold) 24 h after the administration of $1,25(OH)_2D_3$ to vitamin D-deficient animals (Figure 9). However, no fluctuation was shown in the amounts of PiT-1, BNPI, PiUS or the type IIb mRNA (Figures 9 and 10).

DISCUSSION

The intestinal Na/P_i co-transporter has properties similar to those of the renal Na/P_i co-transporter [5,12,36,37]. In contrast with the kidney, where the Na^+-independent component of P_i transport is negligible, this component comprises a much higher portion of the intestinal P_i transport. The Na^+-independent diffusional component of intestinal P_i transport represents approx. 40–50 % of the total uptake [37]. This component is significantly higher in the ileum than in the jejunum (results not shown). In addition, the capacity of the intestinal Na/P_i co-transport, as measured in rat jejunal BBMVs, was found to be significantly lower than that measured in renal BBMVs. These observations are consistent with those of other investigators [7,36–39]. In the kidney, at least three types of Na/P_i co-transporter have been isolated and the properties of the transporters have been well characterized. In contrast, no major functional Na/P_i co-transporter in the small intestine has been identified.

In *Xenopus* oocytes, PiUS markedly stimulated Na^+-dependent P_i transport activity. PiUS seems to be a non-hydrophobic membrane protein that activates a endogenous Na/P_i co-transporter in *Xenopus* oocytes. To characterize the expression of the Na^+-dependent P_i transporter, we used mRNA fractions collected from sucrose gradients with increased activity in comparison with total poly(A)$^+$-rich RNA preparations. We performed a kinetic analysis to examine differences in the expressed activity from this intrinsic activity. In water-injected and mRNA-injected oocytes, K_m values for P_i interaction (PiUS-injected, 0.18 mM; water-injected, 0.2 mM [26]) were observed that were close to those reported for Na^+/P_i co-transport in isolated rat jejunum BBMVs (0.1–0.2 mM). In this context, we suspect that rat PiUS could stimulate a major functional Na/P_i co-transporter in the small intestine, in addition to endogenous Na/P_i co-transporter in *Xenopus* oocytes.

Intestinal P_i absorption is decreased when dietary P_i is increased, and is enhanced in animals fed with a low-P_i diet [12]. Adaptive responses to changes in dietary P_i intake have been reported in intestinal preparations from different species [16,17]. Similarly to its renal counterpart, the intestinal adaptive response to changes in dietary P_i is specific to the Na/P_i co-transporter, with no change in the transport of D-glucose and L-glycine as measured in the same BBMV preparations [12]. The increased P_i transport after a low-P_i diet is associated with an increased V_{max} [8]. Changes in the dietary P_i content result in appropriate changes in the renal tubular P_i reabsorption to restore P_i homoeostasis [18,21]. P_i transport at the proximal tubule BBMV increases or decreases in response to a low-P_i diet or a high-P_i diet respectively. In the present study, similar adaptive changes were seen in the rat small intestine. The adaptive increase in the intestinal Na^+-dependent P_i transport in response to dietary P_i restriction occurred as early as 24 h and persisted for the duration of the diet. Thus the intestinal adaptation is generally slower than that of the kidney [8]. No early, rapid phase of adaptation has been described for intestinal BBMV P_i transport.

The intestinal absorption of P_i is enhanced by vitamin D metabolites and specifically by 1,25-$(OH)_2D_3$, which also increases the absorption of Ca^{2+} [2,40]. The activities of P_i transport in the rat small intestine have been studied in vitamin D-deficient rats. The P_i uptake in BBMVs from vitamin D-deficient rat jejunum showed an overshoot phenomenon in the presence of NaCl. This activity was markedly increased in the vitamin D-deficient rat after the administration of 1,25-$(OH)_2D_3$ [41]. Similar results were obtained in *Xenopus* oocytes micro-injected with duodenum poly(A)$^+$ RNA isolated from the rabbit intestine [42].

One effect of vitamin D_3 on P_i absorption seems to be the stimulation of the synthesis of additional co-transporter units [41]. The Na^+-dependent uptake of P_i by rabbit mucosa was shown to be stimulated by 1,25-$(OH)_2D_3$, whereas the Na^+-independent entry of P_i was unaffected [10]. The uniqueness of vitamin D-mediated P_i absorption is also supported by the observation that arsenate, an analogue of the P_i ion, inhibited P_i absorption but only in vitamin D-replete chicks, suggesting that the vitamin D-dependent process was indeed different from that occurring in the absence of vitamin D [43]. The dependence of $1,25(OH)_2D_3$-mediated intestinal P_i transport on protein synthesis was shown by the inhibitory effect of cycloheximide [44]; in a sense, this confirmed the finding of Ferraro et al. [45] that the maintenance of the P_i absorption system of the intact rat was dependent on continuous protein synthesis. Cycloheximide and actinomycin D also block the $1,25(OH)_2D_3$-stimulated uptake of P_i by isolated chick renal cells [45,46].

In the BBMVs isolated from the vitamin D-replete rats, we found that an additional high-affinity Na/P_i co-transport system was present in the vitamin D-replete rat small intestine. Although the expression of PiT-2 mRNA was extremely low in the vitamin D-deficient rat intestine, it was markedly increased in the vitamin D-deficient rats after the intravenous administration of 1,25-$(OH)_2D_3$. This result suggests that PiT-2 might be one of the candidate high-affinity Na/P_i co-transporters in the vitamin D-responsive system.

In addition, Hilfiker et al. [25] reported that the injection of type IIb cRNA into oocytes resulted in the expression of Na-dependent P_i transport with characteristics similar to those observed for Na/P_i-co-transport mediated by the renal type II Na/P_i co-transporter. However, the most striking difference of type IIb-mediated Na/P_i co-transport is its pH dependence. Na/P_i co-transport in mouse small intestine is highest at a more acidic pH and exhibits a K_m for P_i of approx. 50 μM [25]. The functional characteristics observed for type IIb-mediated Na/P_i co-transport are in agreement with these results and support the notion that the type IIb co-transporter might represent a candidate for a small-intestine Na/P_i co-transporter. However, the type IIb mRNA levels were not affected by restriction of vitamin D and P_i (two of the most important physiological and pathophysiological regulators) [25]. Further study is needed to clarify the physiological role of Na/P_i co-transport mediated by the type IIb in rat small intestine.

Studies of vitamin D-replete rats have shown a temporal

relationship between the rise in plasma 1,25-$(OH)_2D_3$ and the stimulation of Na^+-dependent P_i transport [8]. The adaptation of intestinal P_i transport was shown in BBMVs from rats fed with a vitamin D-deficient diet in the present study. In addition, the levels of serum 1,25$(OH)_2D_3$ were significantly increased in the rats fed with the low-P_i diet compared with those of the rats fed with the normal diet. However, we did not detect an elevation of the PiT2 mRNA level in the rats fed with the low-P_i diet. Therefore 1,25-$(OH)_2D_3$ and P_i restriction might give different signals in the up-regulation of intestinal Na/P_i co-transport.

In conclusion, intestinal P_i transport activity and its related transcript levels were examined in rats. Vitamin D stimulated the PiT2 mRNA levels; a low-P_i diet stimulated PiUS mRNA levels. These observations suggest that multiple components might be present in the rat intestinal P_i co-transport system.

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