Regulation of intestinal Na\textsuperscript{+}-dependent phosphate co-transporters by a low-phosphate diet and 1,25-dihydroxyvitamin D\textsubscript{3}

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In a study of the rat intestinal P\textsubscript{i} transport system, an activator protein for rat Na/P\textsubscript{i} co-transport system (PiUS) was isolated and characterized. We also investigated the effects of restriction of vitamin D and P\textsubscript{i} (two of the most important physiological and pathophysiological regulators of P\textsubscript{i} absorption in the small intestine) on intestinal P\textsubscript{i} transport activity and the expression of Na/P\textsubscript{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\textsuperscript{+}-dependent P\textsubscript{i} co-transport activity. In rats fed with a low-P\textsubscript{i} diet, Na\textsuperscript{+}-dependent P\textsubscript{i} co-transport activity was increased approximately 2-fold compared with that of rats fed a normal P\textsubscript{i} diet. Kinetic studies demonstrated that this increased activity was due to an elevation of V\textsubscript{max} but not K\textsubscript{m}. The PiUS mRNA levels showed an approximate doubling in the rats fed with the low-P\textsubscript{i} diet compared with those fed with the normal P\textsubscript{i} diet. In addition, after the administration of 1,25-dihydroxyvitamin D\textsubscript{3} [1,25-(OH)\textsubscript{2}D\textsubscript{3}] to vitamin D-deficient animals, the P\textsubscript{i} uptake was significantly increased in the Na\textsuperscript{+}-dependent component in the brush border membrane vesicle (BBMV) at 24 and 48 h. In addition, we found a further high-affinity Na/P\textsubscript{i} co-transport system in the BBMV isolated from the vitamin D-replete animals. The levels of type III Na/P\textsubscript{i} co-transporter PiT-2 mRNA were increased 24 and 48 h after 1,25-(OH)\textsubscript{2}D\textsubscript{3} administration to vitamin D-deficient animals, whereas PiUS and the type Ib Na/P\textsubscript{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\textsubscript{i} co-transporters. PiUS and PiT-2 might be important components in the regulation of the intestinal P\textsubscript{i} transport system by P\textsubscript{i} restriction and 1,25-(OH)\textsubscript{2}D\textsubscript{3}.

Key words: inorganic phosphate, PiUS.

INTRODUCTION

The intestinal absorption of P\textsubscript{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\textsubscript{i} occurs primarily in the proximal small intestine and consists of two components: passive diffusion across the intestinal brush border and Na\textsuperscript{+}-dependent, carrier-mediated, uptake. The molecular mechanisms of the regulation of P\textsubscript{i} transport have been studied by the cloning of several cDNA species corresponding to mammalian renal Na/P\textsubscript{i} co-transporters. However, the mechanisms of the adaptation of intestinal P\textsubscript{i} transport are not well understood because of a lack of knowledge about the structure of intestinal Na/P\textsubscript{i} co-transporters [6,7].

Intestinal P\textsubscript{i} transport activity is well known to be controllable by 1,25-dihydroxyvitamin D\textsubscript{3} [1,25-(OH)\textsubscript{2}D\textsubscript{3}] and a low-P\textsubscript{i} diet [2,4,8–17]. 1,25-(OH)\textsubscript{2}D\textsubscript{3} regulates the intestinal absorption of P\textsubscript{i} [16,18–21]. The intestinal P\textsubscript{i} transport process occurs both by an Na\textsuperscript{+}-independent, non-saturable process and via an active, Na\textsuperscript{+}-dependent component of P\textsubscript{i} absorption, mainly in the duodenum and jejunum [8]. However, one effect of vitamin D\textsubscript{3} on P\textsubscript{i} absorption seems to be the stimulation of Na\textsuperscript{+}-dependent P\textsubscript{i} co-transporters [8–11].

In addition, a low-P\textsubscript{i} diet increases brush border membrane Na\textsuperscript{+}-dependent P\textsubscript{i} transport [12–17]. Rat intestinal P\textsubscript{i} absorption is decreased when dietary P\textsubscript{i} is increased, and is enhanced in animals fed with a low-P\textsubscript{i} diet [12]. Adaptive responses to changes in dietary P\textsubscript{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\textsubscript{i} is specific to the Na/P\textsubscript{i} co-transporter, with no change in the transport of amino acids and glucose [12].

Three types of Na/P\textsubscript{i} co-transporter have been isolated from several species [18–20]. The type I and type II Na/P\textsubscript{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\textsuperscript{+}-dependent P\textsubscript{i} co-transporters in several tissues [19,22–24]. The amino acid sequences of PiT-1 and PiT-2 are 60% identical [19], and exhibit no significant overall sequence similarity to the type I or type II transporters. More recently, Hilfinger et al. [25] cloned an isoform of the type II Na/P\textsubscript{i} co-transporter (type Ib) cDNA from mouse small intestine; its mRNA was found in a variety of tissues. The
physiological role of Na/Pi-co-transport mediated by type IIb in the small intestine remains unknown.

A putative activator protein for Na\(^+\)-dependent Pi transport (PiUS) has been found in the small intestine [26]. PiUS was cloned from rabbit small intestine by expression cloning. PiUS has been found in the small intestine [26]. PiUS was brain, a family of brain-specific Na\(^+\) co-transporters. A putative activator protein for Na\(^+\)-dependent Pi transport (PiUS) 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/Pi-co-transporters, we investigated the effect of dietary low P\(_i\) and 1,25-(OH)\(_{2}\)D\(_3\) on rat intestinal Na/Pi-co-transport and the mRNA levels of the Na/Pi-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\(_2\)/g [15]. The animals were pair-fed with either a normal-P, diet (0.6% Na\(^+\)) or low-P, diet (0.02% Na\(^+\)) for 7 days between 09:00h and 24:00h.

Vitamin D-deficient 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 1) [27] for 6 weeks and then with a vitamin D- and calcium-free diet (diet 11a) 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)\(_{2}\)D\(_3\) (6.25 mg/kg body weight) in ethanol/proplylene 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 plasma concentrations of 1,25-(OH)\(_{2}\)D\(_3\) 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 Pi was measured by the rapid-filtration technique [29]. After 10\(\mu\)l of the vesicle suspension had been added to 90\(\mu\)l of the incubation solution (consisting of 100 mM NaCl, 100 mM mannitol, 20 mM Hepes/Tris and 0.1 mM KH\(_2\)PO\(_4\)), the preparation was incubated at 20°C. The measurements of Na\(^+\)-dependent and Na\(^+\)-independent Pi 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\(_2\)PO\(_4\), 20 mM MgSO\(_4\) and 100 mM choline chloride. The reaction mixture was then immediately transferred to a pre-moistened filter (0.45 \(\mu\)m) maintained under a vacuum.

Transcription and measurement of P, transport in vitro in microinjected Xenopus oocytes

The rat PiUS clone was linearized by digestion with EcoRI and transcribed into cRNA with T7 RNA polymerase (Promega, Madison, WI, U.S.A.) [30]. The measurements of Na\(^+\)-dependent and Na\(^+\)-independent Pi 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 (Amersharm, Little Chalfont, Bucks., U.K.) and covalently cross-linked by exposure to UV. Hybridization was performed in a solution containing 50\% (w/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 random 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 Agt10 (4 x 10\(^5\) independent recombinants) was constructed from 2.0 \(\mu\)g of the polyadenylated
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 Pi 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) Pi 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′-ATGAGCCCAGCCTTCAGGGCCCATGG-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 PstI, EcoRI or HindIII. 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] were measured as described previously [33]. Plasma 1,25-(OH)$_2$D$_3$ levels were measured by a radio-receptor assay (Incstar, Minneapolis, MN, U.S.A.). Plasma intact parathyroid hormone levels were measured with a rat immunoradiometric 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 using the Student’s t-test and one-way ANOVA respectively, with a significance level of $p < 0.05$.
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 Pi co-transporters

To investigate the expression of Na\(^+\)/Pi 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\(^+\)/Pi 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.

The microinjection of PiUS into Xenopus oocytes stimulated 
Na\(^+\)-dependent Pi uptake activity (Figure 3A). The Na\(^+\)-
dependent Pi uptake at 30 min was stimulated approx. 10-fold 
compared with that of water-injected controls. In the absence of 
NaCl, no elevation of Pi uptake was shown in the oocytes 
injected with rat PiUS cRNA (Figure 3B). To characterize the 
PiUS-cRNA-induced increase in Pi uptake, we analysed it as a 
function of different Pi 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-Pi diet on intestinal Na/Pi co-transport activity

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

Figure 6 Effects of a low-Pi diet on intestinal Na/Pi 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)2D3 on Na+-dependent and Na+-independent Pi co-transport activities in vitamin D-deficient rat small intestine

(A) Intestinal Na/Pi transport activity in BBMVs from vitamin D-deficient and normal rats. (B) Effects of 1,25-(OH)2D3 on intestinal Pi transport activity in vitamin D-deficient animals. Vitamin D-deficient rats were injected intravenously with 1,25-(OH)2D3 (6.25 μg/kg) and killed at various times thereafter. Pi transport activity was determined at 0, 12, 24 and 48 h after an injection of 1,25-(OH)2D3 to vitamin D-deficient rats. The jejunum was removed and BBMVs were isolated from the animals. The Pi 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, than the animals fed with a normal-Pi (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)2D3 levels in the rats fed with the low-Pi and normal-Pi, diet were 520 ± 43 and 75 ± 21 pg/ml, respectively.

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

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

To investigate further the expression of Na/Pi co-transporters in the rats fed with the low-Pi 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-Pi diet compared with those fed the normal-Pi 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-Pi diet (results not shown).
Figure 8 Influence of 1,25-(OH)\textsubscript{2}D\textsubscript{3} on the kinetic parameters of Pi transport

Experiments were performed as described in the legend to Figure 5. For each of six vesicle preparations, \( K_m \) and \( V_{\text{max}} \) values 24 h after the administration of 1,25-(OH)\textsubscript{2}D\textsubscript{3} were determined from Lineweaver–Burk diagrams by regression analysis. Pi concentrations in the medium were varied between 0.02 and 10 mM. Data are representative of five separate experiments. Symbols: ○, without 1,25-(OH)\textsubscript{2}D\textsubscript{3}; □, with 1,25-(OH)\textsubscript{2}D\textsubscript{3} after 24 h.

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

In the vitamin D-deficient animals, the total Pi transport activity was significantly decreased in the BBMVs isolated from the jejunum (Figure 7A); the Na\textsuperscript{+}-dependent component showed a greater decrease in net Pi transport activity than in the normal animals (Figure 7A). After the administration of 1,25-(OH)\textsubscript{2}D\textsubscript{3} to vitamin D-deficient animals, the Pi uptake was significantly increased in the Na\textsuperscript{+}-dependent component in the BBMVs and was unchanged in the Na\textsuperscript{+}-independent component (Figure 7B). We performed a kinetic analysis of Pi transport in the vitamin D-deficient rats 24 h after the administration of vitamin D. The kinetic analysis revealed that the elevation of net Pi uptake was due to an increase in \( V_{\text{max}} \) (vitamin D-deficient, 45 ± 11 pmol/15 s per mg of protein; vitamin D-replete, 143 ± 24 pmol/15 s per mg protein).

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

After the administration of 1,25-(OH)\textsubscript{2}D\textsubscript{3}, we determined 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)\textsuperscript{+} RNA] (B). The results of densitometric scanning are shown as means ± 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)\textsubscript{2}D\textsubscript{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)\textsubscript{2}D\textsubscript{3}; lane 4, 24 h after the administration of 1,25-(OH)\textsubscript{2}D\textsubscript{3}; lane 5, 48 h after the administration of 1,25-(OH)\textsubscript{2}D\textsubscript{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)\(_2\)D\(_3\) 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 Pi 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)\(_2\)D\(_3\) 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 Pi transport is negligible, this component comprises a much higher portion of the intestinal Pi transport. The Na\(^+\)-independent diffusional component of intestinal Pi 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 Pi transport activity. PiUS seems to be a non-hydrophobic membrane protein that activates an endogenous Na/P\(_i\) co-transporter in *Xenopus* oocytes. To characterize the expression of the Na\(^+\)-dependent Pi 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 Pi interaction (PiUS-injected, 0.18 mM; water-injected, 0.2 mM) (26) were observed that were close to those reported for Na\(^+\)/Pi co-transport in isolated rat jejunal 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 Pi absorption is decreased when dietary Pi is increased, and is enhanced in animals fed with a low-P diet [12]. Adaptive responses to changes in dietary Pi 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 Pi is specific to the Na/P\(_i\) co-transporter, with no change in the transport of D-glucose and D-glycine as measured in the same BBMV preparations [12]. The increased Pi transport after a low-P diet is associated with an increased \(V_{\text{max}}\) [8]. Changes in the dietary Pi content result in appropriate changes in the renal tubular Pi reabsorption to restore Pi homeostasis [18,21]. Pi transport at the proximal tubule BBMV increases or decreases in response to a low-P diet or a high-P diet respectively. In the present study, similar adaptive changes were seen in the rat small intestine. The adaptive increase in the intestinal Na\(^+\)-dependent Pi transport in response to dietary Pi 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 Pi transport.

The intestinal absorption of Pi is enhanced by vitamin D metabolites and specifically by 1,25(OH)\(_2\)D\(_3\), which also increases the absorption of Ca\(^2+\) [2,40]. The activities of Pi transport in the rat small intestine have been studied in vitamin D-deficient rats. The Pi 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)\(_2\)D\(_3\) [41]. Similar results were obtained in *Xenopus* oocytes microinjected with duodenum poly(A)\(^+\) RNA isolated from the rabbit intestine [42].

One effect of vitamin D\(_3\) on Pi absorption seems to be the stimulation of the synthesis of additional co-transporter units [41]. The Na\(^+\)-dependent uptake of Pi by rabbit mucosa was shown to be stimulated by 1,25(OH)\(_2\)D\(_3\), whereas the Na\(^+\)-independent entry of Pi was unaffected [10]. The uniqueness of vitamin D-mediated Pi absorption is also supported by the observation that arsenate, an analogue of the Pi ion, inhibited Pi 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)\(_2\)D\(_3\)-mediated intestinal Pi 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 Pi absorption system of the intact rat was dependent on continuous protein synthesis. Cycloheximide and actinomycin D also block the 1,25(OH)\(_2\)D\(_3\)-stimulated uptake of Pi 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-repletate 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)\(_2\)D\(_3\). 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 Pi 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 Pi of approx. 50 \(\mu\)M [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 Pi (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)_{2}D_{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)_{2}D_{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)_{2}D_{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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