Regulation of small intestinal Na-P_i type IIb cotransporter by dietary phosphate intake

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Hattenhauer, Olaf, Martin Traebert, Heini Murer, and Jürg Biber. Regulation of small intestinal Na-P_i type IIb cotransporter by dietary phosphate intake. *Am. J. Physiol.* 277 (*Gastrointest. Liver Physiol.* 40): G756–G762, 1999.—Dietary restriction of phosphate is a well-known stimulator (acting indirectly via vitamin D_3) of small intestinal apical Na-P_i cotransport. In the present study, we document by Western blots and immunohistochemistry that, in mice, a low-P_i diet given for several days leads (in parallel to a stimulation of Na-P_i cotransport) to an increase of the abundance of the type IIb Na-P_i cotransporter in the brush-border membrane of mouse enterocytes. Similar results were also obtained by an injection of cholecalciferol. The abundance of the type IIb transcript was investigated by Northern blots. These results indicated that the amount of the type IIb transcript was not changed by either low-P_i diet or cholecalciferol. It is concluded that stimulation of intestinal Na-P_i cotransport by low-P_i diet and vitamin D_3 can be explained by an increased amount of type IIb Na-P_i cotransporters in the brush-border membrane and that augmentation of type IIb Na-P_i cotransporters is not related to an increased rate of transcription of the type IIb gene.

Na-P_i cotransport; intestine; adaptation; vitamin D_3

ABSORPTION OF P_i in small intestine occurs largely via a transcellular pathway that is initiated by transport of P_i across the brush-border membrane. There is a large body of evidence that transport of P_i through the apical membrane is secondary active and in nonruminants dependent on Na (Na-P_i cotransport). Until recently, small intestinal Na-P_i cotransport has been characterized on the basis of functional criteria with the use of whole tissue preparations and isolated brush-border membrane vesicles (1, 3, 6, 8, 28, 29). Three families (types I, II, and III) of vertebrate Na-P_i cotransporters have been identified in the past few years (22, 32). In mouse and human members of the type II family, type IIb Na-P_i cotransporters have recently been identified (10, 14), and it was shown that in mice the type IIb Na-P_i cotransporter is expressed in the enterocytes and located in the brush-border membranes (14). Apical location, kinetic characteristics, and pH dependency (14) suggested that the type IIb Na-P_i cotransporter is involved in small intestinal P_i reabsorption.

Dietary deprivation of phosphate and 1,25-dihydroxyvitamin D_3 (vitamin D_3) represents important physiological regulators of small intestinal P_i absorption (for review see Refs. 6 and 8). Both factors result in an increase of brush-border Na-P_i cotransport that is characterized by an increase of the maximal velocity (V_{max}) value (4, 12, 21, 24, 26). Until now, the molecular mechanisms of the regulation of small intestinal absorption of P_i by a low-P_i diet and/or vitamin D_3 have not been established. In particular, it has not been shown whether by low-P_i diet and/or by vitamin D_3 the amount of Na-P_i cotransporters in the brush-border membrane is increased, as would be suggested by the observed increase of the V_{max} value.

In the present study, we demonstrate that the increase of brush-border Na-P_i cotransport induced by a low-P_i diet as well as by vitamin D_3 can be explained by an increase in the amount of type IIb Na-P_i cotransporters in the brush-border membrane. Furthermore, because neither by low-P_i diet nor by vitamin D_3 was the abundance of the type IIb transcript found to be changed, it is concluded that the observed upregulation of the amount of type IIb Na-P_i cotransporters may be explained by a nongenomic action of low-P_i diet or vitamin D_3.

MATERIALS AND METHODS

Animals. All experiments were performed with adult (8- to 10-wk-old) male mice (National Medical Research Institute). For chronic adaptation, mice were fed for 5 days with diets (Kliba, Switzerland) containing either 1.1% (high) or 0.09% (low) P_i. For acute adaptation to low-P_i diet, mice were first fed for 1 wk with high-P_i diet (4 h daily). On the day of the experiment, low-P_i diet (and, as a control, high-P_i diet) was given for 4 h.

To study the effect of vitamin D_3, mice kept on normal laboratory chow (0.85% P_i) were injected intraperitoneally 24 h before the experiment with cholecalciferol or with vehicle (ethanol-propylene glycol-150 mM NaCl at 1:4:5) alone. Cholecalciferol was purchased from WDT (Germany) and diluted so that 24 IU of cholecalciferol were injected per 0.1 ml.

Each experiment was performed with four animals per group, and each experiment was repeated at least two times.

Isolation of brush-border membranes, transport studies, and Western blotting. Small intestines were removed and rinsed with ice-cold NaCl (150 mM). Scraped mucosa of the upper small intestine (first 12 cm, two animals per preparation) was homogenized in a solution of (in mM) 300 mannitol, 5 EGTA, and 10 Tris·HCl, pH 7.2, at 4°C and afterward prepared by the Mg-precipitation technique as described (13). Final membranes were stored in the above buffer at a concentration of 5–10 mg protein/ml.

Uptake of phosphate was measured at pH 7.2 at 25°C in the presence of inwardly directed gradients of 100 mM NaCl or 100 mM KCl and with the use of 0.5 mM [^{32}P]K_2HPO_4 as described (28, 30). Uptake of phosphate was determined after 15 s, representing initial near-linear conditions, and after 60 min, to determine the equilibrium values.

For gel electrophoresis (SDS-PAGE, 9% gels), brush-border membranes (50 μg protein) were denatured in the absence of a reducing agent by heating at 95°C for 2 min. Separated proteins were transferred onto nitrocellulose and analyzed as

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described (7) with the use of custom-made (Eurogentec, Belgium) polyclonal antibodies raised against synthetic peptides derived from the COOH and NH_2 terminals of the type IIb protein. In pilot experiments, the specificity of the immunoreaction was controlled by inclusion of the antigenic peptides at 50 μg/ml (not shown). Immunodetection was performed by ECL (Pierce) with the use of a horseradish perodixdase-conjugated goat anti-rabbit IgG (Amersham). In renal brush-border membranes, the type IIa Na-P_i cotransporter was detected as described (7).

Immunohistochemistry. Small intestinal rings (~1 cm, taken 5 cm apart from the pylorus) were fixed by immersion in 3% paraformaldehyde-0.05% picric acid in 0.1 M cacodylate buffer, pH 7.4. Afterward, the specimens were frozen in liquid propane and stored at −70°C. For immunohistochemistry, cryosections of 5-μm thickness were prepared and stained as described (7), with the use of either of the anti-type IIb polyclonal antiserum or with phalloidin-rhodamine to stain for β-actin. The specificities of the antisera were tested by peptide protection and by the use of the corresponding preimmune sera. All controls were clearly negative (data not shown and Ref. 14).

Isolation of mRNA and Northern blotting. Total RNA of upper small intestines (one animal per isolation) was isolated by using TRIzol (GIBCO BRL). Poly(A)$^+$ mRNA was isolated with the polyATract system (Promega) according to the manufacturer's protocol, and 2–4 μg were separated by formaldehyde agarose electrophoresis. Detection of the type IIb transcript was performed with a full-length probe randomly labeled with [^{32}P]dCTP (oligolabeling kit, Pharmacia). Northern blots were washed sequentially with 2× standard saline citrate (SSC), 1× SSC and 0.5× SSC (all containing 0.1% SDS) while raising temperatures up to 55°C. As controls for loading, we used a probe for the ribosomal protein L28 or a probe for β-actin (not shown). Intensities of the signals were analyzed with ImageQuant software (Molecular Dynamics).

RESULTS

In the first set of experiments, Na-P_i cotransport was determined in small intestinal brush-border membrane vesicles (BBMV) isolated from mice fed for 5 days with diets containing either 1.1% P_i (high P_i) or 0.09% P_i (low P_i) and from mice fed a normal laboratory chow and injected either with cholecalciferol or vehicle alone. In agreement with earlier reports (4, 12, 21, 24, 26), low-P_i diet and injection of cholecalciferol resulted in an increased rate of Na-P_i cotransport in isolated BBMV compared with BBMV isolated from animals fed a high-P_i diet or from animals injected with vehicle alone (Fig. 1). In all experiments, P_i uptake in the presence of Na into BBMV isolated from mice fed a high-P_i diet or from control-injected animals was not significantly different from P_i uptake in the presence of K. Compared with the corresponding controls, Na-independent P_i uptakes and the equilibrium values (not shown) were not changed by either low P_i or by injection of cholecalciferol.

BBMV were further analyzed by immunoblotting with polyclonal antibodies raised against synthetic peptides derived from the COOH or NH_2 terminals of the recently described type IIb Na-P_i cotransporter (14). On Western blots, the type IIb protein was detected as a band of ~108 kDa by both antisera. Specificity of this reaction was confirmed by inclusion of the corresponding peptides, which blocked the reaction with the 108-kDa band (data not shown; see Ref. 14). Both antisera used exhibited a nonspecific reaction at ~100 kDa; the intensity of this nonspecific reaction varied from preparation to preparation. As illustrated in Fig. 1, the type IIb cotransporter (band at 108 kDa) was detected in BBMV of animals fed a low-P_i diet and BBMV isolated from animals injected with cholecalciferol. However, in BBMV from animals fed a high-P_i diet, the type IIb protein was not detected and could only barely be detected in BBMV of animals injected with vehicle. Equal loading of the gels was confirmed by the intensity of the β-actin band, which was visualized by staining with ponceau rouge S.

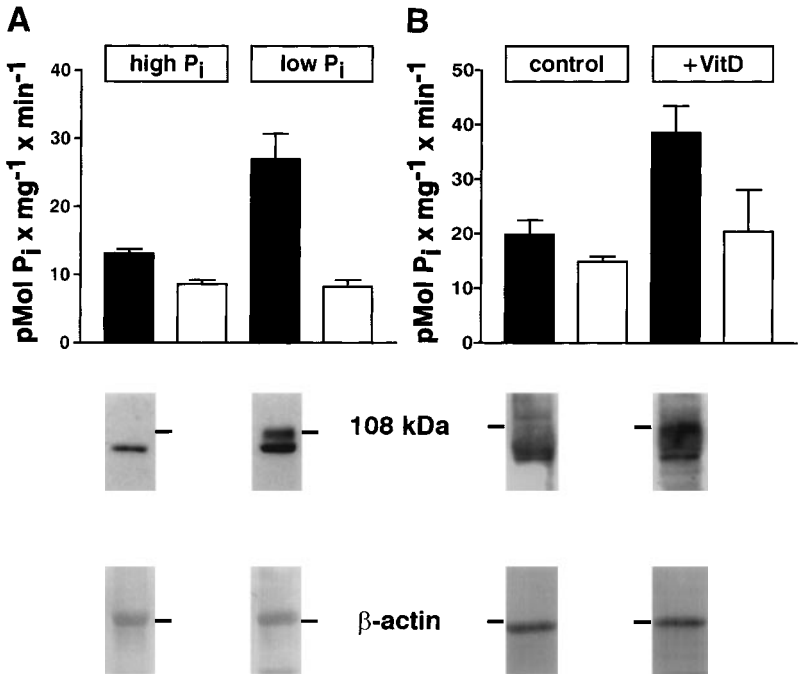

Fig. 1. Na-P_i cotransport and type IIb protein detection (Western blots) performed with brush-border membrane vesicles (BBMV) isolated from small intestinal mucosa of mice chronically fed a high- or low-P_i diet (*A*) and from animals injected with cholecalciferol [plus vitamin D (+VitD)] or vehicle (control) (*B*). Uptake of phosphate was performed for 15 s either in the presence of NaCl (solid bars) or KCl (open bars). On Western blots, type IIb protein was detected as a band of 108 kDa by both polyclonal antisera used. Band below type IIb protein represents a nonspecific reaction as revealed by inclusion of antigenic peptide (not shown). As loading controls, β-actin band was stained with ponceau rouge S. Results shown were obtained from 1 of 3 experiments. Bars represent means ± SD of 4 uptake determinations. Uptakes in presences of Na (high P_i vs. low P_i and control vs. +VitD) were significantly different ($P < 0.01$).

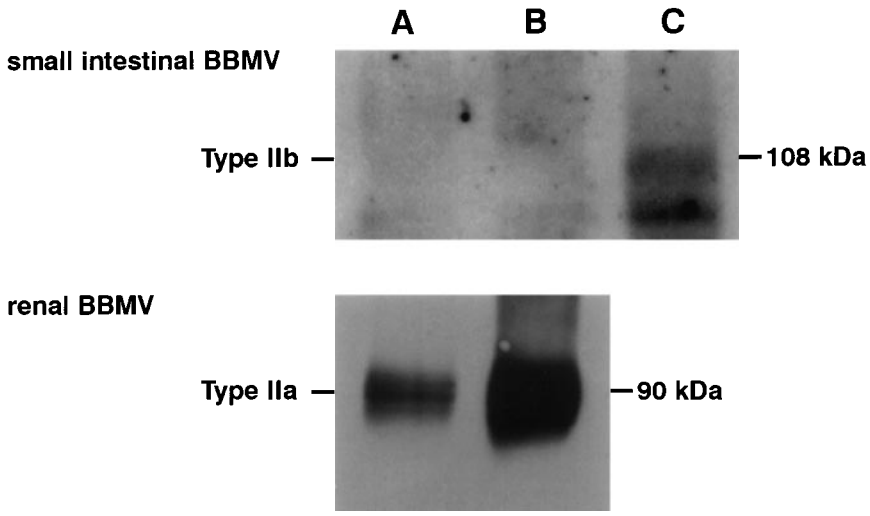

Fig. 2. Western blot analysis of small intestinal and renal BBMV isolated from mice acutely adapted to a low-P_i diet. Animals were kept for 1 wk on a high-P_i diet and then divided into 2 groups. On day of experiment, 1 group (*lane A*) received a high-P_i diet and 1 group (*lane B*) received a low-P_i diet, both for 4 h. In addition, BBMV were isolated from mice kept for 5 days on high-P_i diet (*lane C*). To control for physiological response of acute low-P_i diet, renal proximal BBMV were isolated and probed for type IIa Na-P_i cotransporter.

Because in renal proximal tubules Na-P_i cotransport is upregulated by a low-P_i diet already after a few hours (acute adaptation; Refs. 18–20), it was of interest to ask whether in small intestine a similar rapid adaptive response on the abundance of the type IIb cotransporter may occur. For acute adaptation, mice were fed with a high-P_i diet for 1 wk and on the day of experiment for 4 h with a low-P_i diet or, as a control, with a high-P_i diet. As illustrated in Fig. 2, in BBMV isolated from mice fed acutely with a low-P_i diet the type IIb protein (band at 108 kDa) could not be detected, indicating that this time period of low-P_i diet was not sufficient to upregulate the type IIb cotransporter. As a control for the physiological response of the acute low-P_i diet, proximal tubular BBMV were isolated from the same animals and analyzed for the abundance of the renal type IIa Na-P_i cotransporter. As illustrated, in renal BBMV the adaptive response on the type IIa cotransporter was significant and of similar extent as reported (18, 20).

To further confirm that dietary P_i intake and cholecalciferol altered the abundance of the type IIb Na-P_i cotransporter in the brush borders of enterocytes, small intestinal tissues were analyzed by immunohistochemistry. As previously shown, in mouse enterocytes the type IIb Na-P_i cotransporter was detected in the apical membrane only (14). Here, we further show that the type IIb cotransporter is expressed uniformly along the entire villi but is absent in the crypts, which were visualized by staining for β-actin (Fig. 3). Figure 4 illustrates the immunostaining observed with small intestinal tissues of animals chronically fed a high-P_i diet or a low-P_i diet (Fig. 4, *A* and *B*) and tissues of animals injected with vehicle or cholecalciferol (Fig. 4, *C* and *D*). Consistent with the Western blots shown in Fig. 1, the type IIb protein was most abundant in brush-border membranes of animals fed a low-P_i diet and in tissues obtained from animals injected with cholecalciferol. After the chronic high-P_i diet, no type IIb-related immunostaining was detected, and in animals kept on a normal laboratory chow (0.85% P_i) and injected with vehicle, the intensity of the immunostaining was weak but always higher compared with the high-P_i diet condition, indicating that the high-P_i diet conditions (1.1% P_i) resulted in a decrease of the type IIb cotransporter abundance. The increase of the abundance of the type IIb protein by both low-P_i diet and cholecalciferol was observed to occur homogeneously along the entire villi and was not observed in the crypts.

To investigate whether the increase of the abundance of the type IIb Na-P_i cotransporter is due to transcriptional mechanisms, the relative abundance of the type IIb transcript in small intestinal mucosas of the differently treated mice was analyzed by Northern blots (Figs. 5 and 6). By immunofluorescence, all tissues from which poly(A)$^+$ RNAs were isolated showed the same changes (not shown) of the type IIb abundance as

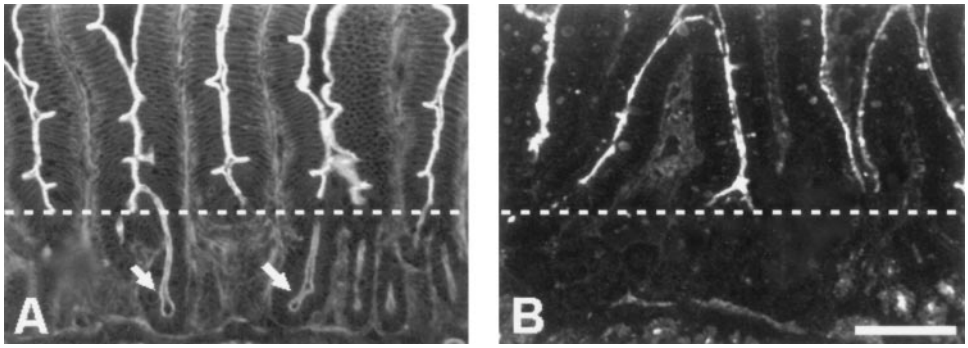

Fig. 3. *A*: appearance of β-actin stained with phalloidin-rhodamine. Punctuated line marks border between mature enterocytes and clefts (marked by arrows). *B*: immunodetection of type IIb Na-P_i cotransporter in small intestinal mucosa of mice fed a low-P_i diet. A specific immunosignal was detected in the brush borders only. Bar = 100 μm.

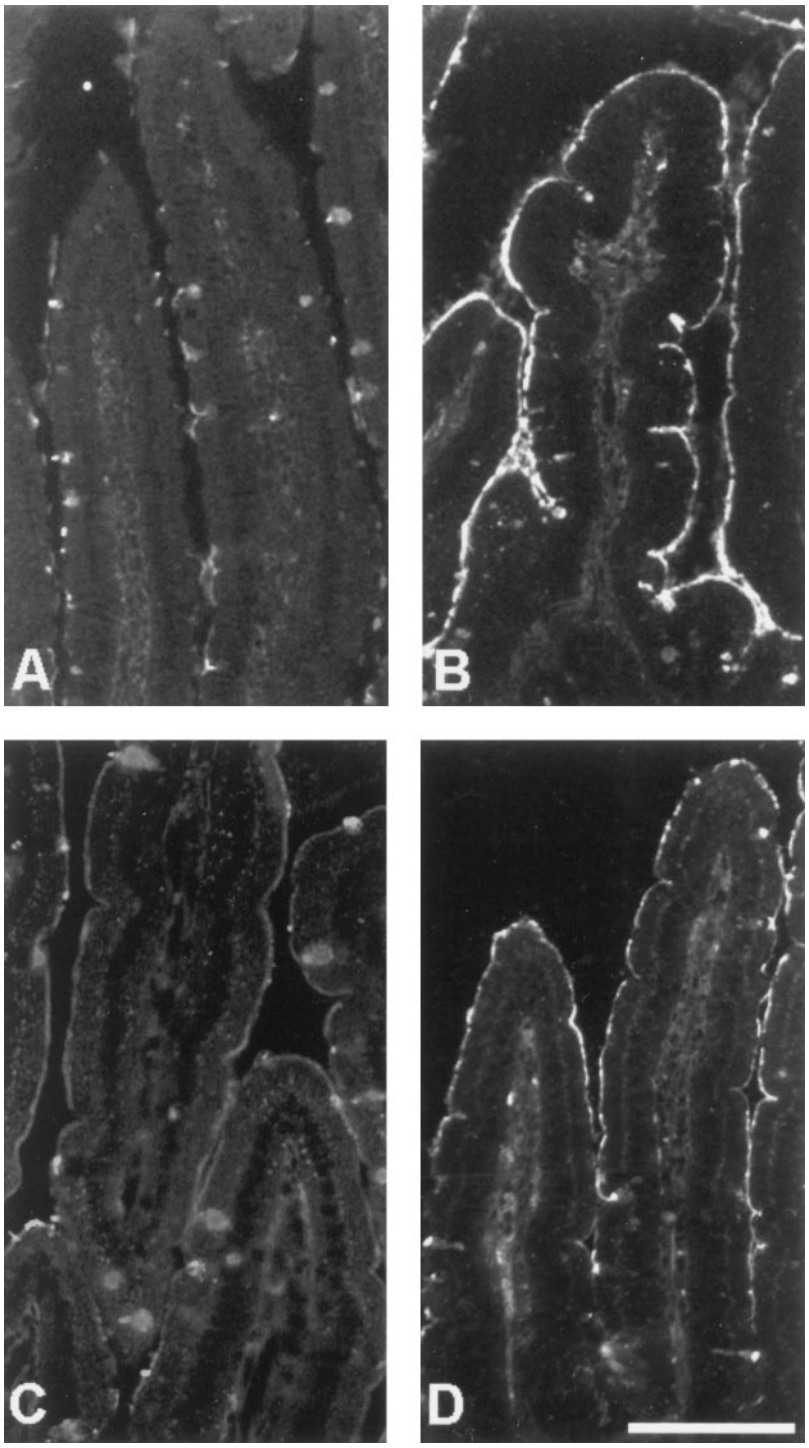

Fig. 4. Immunohistochemical detection of the type IIb Na-P_i cotransporter in small intestines of mice chronically fed a high-P_i diet (*A*) or a low-P_i diet (*B*) and animals fed a normal chow and injected with vehicle (*C*) or injected with cholecalciferol (*D*). Apical staining was clearly detected after chronic low-P_i diet and injection of cholecalciferol but was absent after chronic high-P_i diet. A faint staining was observed in tissues obtained from animals fed a normal diet. Punctuated, randomly distributed signals represent nonspecific staining. Bar = 150 μm.

shown in Fig. 4 (*A* vs. *B*; *C* vs. *D*). In all poly(A)$^+$ mRNA samples isolated from mice fed a low-P_i diet, no significant changes of the type IIb transcript compared with poly(A)$^+$ mRNAs isolated from mucosas of mice fed a high-P_i diet were observed (Fig. 5). Similarly, after injection of cholecalciferol, no significant changes of the type IIb transcript compared with control injections were observed (Fig. 6). All Northern blots were quantified relative to the transcript of the ribosomal protein L28. Identical results were obtained when the transcript of β-actin was used as a reference (data not shown).

DISCUSSION

On a functional level, upregulation of small intestinal absorption of phosphate due to a diet of low-P_i content has been described to occur in many species (4, 6, 8). In all of these studies, it has been established that the adaptive response to a low P_i diet leads to an

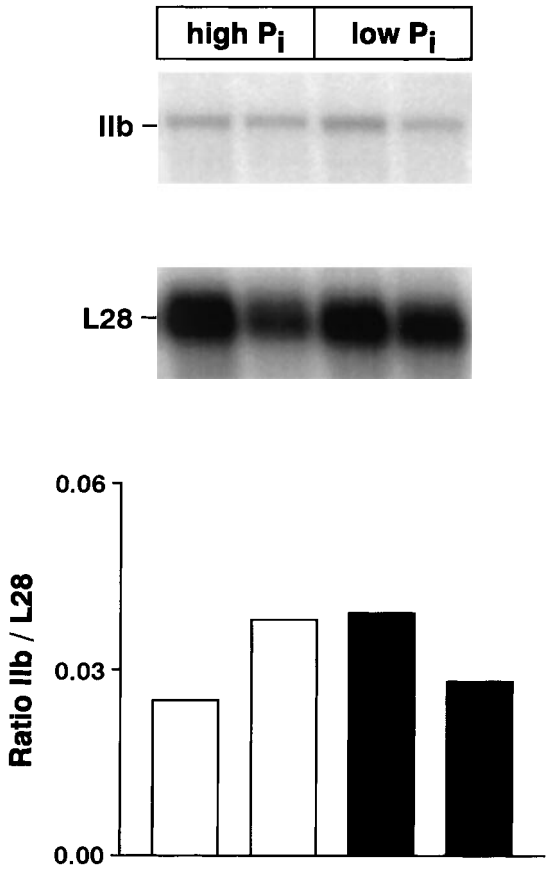

Fig. 5. Detection of type IIb transcript (~4 kb) on Northern blots in poly(A)$^+$ mRNA samples isolated from mice chronically fed with high- or low-P_i diet. Relative abundance of type IIb transcript compared with L28-related signal is given at *bottom*. Open bars, high P_i; solid bars, low P_i. Results of 1 of 3 independent experiments are shown.

increased V_{max} value of the apically located Na-P_i cotransport. To date, this adaptive phenomenon has not been analyzed with respect to possible alterations of the amounts of defined Na-P_i cotransporters. Only recently, Na-P_i cotransporters expressed in mammalian small intestine have been identified and characterized (10, 14). It was shown that this Na-P_i cotransporter (named type IIb) transports P_i in a Na-dependent and electrogenic manner and further that the type IIb protein is localized in the apical membrane of mouse enterocytes (14). In this study, we further show that the type IIb Na-P_i cotransporter was detected in the brush borders of mature enterocytes and could not be detected in the clefts.

Our results indicate that the increase of small intestinal Na-P_i cotransport provoked by a chronic (5-day) low-P_i diet correlated with an increased abundance of the type IIb protein. These results suggest that upregulation of intestinal P_i absorption by a low-P_i diet can be explained by an increase in the abundance of the type IIb Na-P_i cotransporter in the apical membrane. Because after the chronic high-P_i diet the type IIb protein could not be detected by immunological methods, and because in BBMV isolated from animals kept on a high-P_i diet Na-dependent cotransport of P_i was minimal or absent, it is concluded that the type IIb Na-P_i cotransporter may represent a major pathway by which P_i is transported across the small intestinal brush-border membrane under upregulated conditions.

In renal proximal tubules, a chronic low-P_i diet results in an upregulation of the type IIa Na-P_i cotransporter (18, 20). This effect of a low-P_i diet was observed also after 2–4 h (acute adaptation, Refs. 18–20). To see whether in small intestine a similar rapid adaptation as in kidney may occur, the type IIb protein was also analyzed after a short period (4 h) of feeding with a low-P_i diet. Although this maneuver resulted in an increased amount of the renal type IIa cotransporter as reported in other studies (18, 20), we could not detect such an acute effect on the type IIb cotransporter. This indicates that the cellular mechanisms involved in the rapid renal response to low-P_i diet may not be existent in the small intestine and furthermore that a low-P_i diet most likely indirectly (see below) provokes an increase of P_i absorption.

A low-P_i diet leads to a rapid decrease of the plasma concentration of P_i and activation of the renal 1,25-hydroxylase, resulting in an increased level of vitamin D_3 (19, 25, 31). Therefore, adaptation of small intestinal Na-P_i cotransport by low-P_i diet has been explained to occur via vitamin D_3 (6). Indeed, various reports showed that a treatment with vitamin D_3 resulted in an increase of small intestinal P_i absorption via an in-

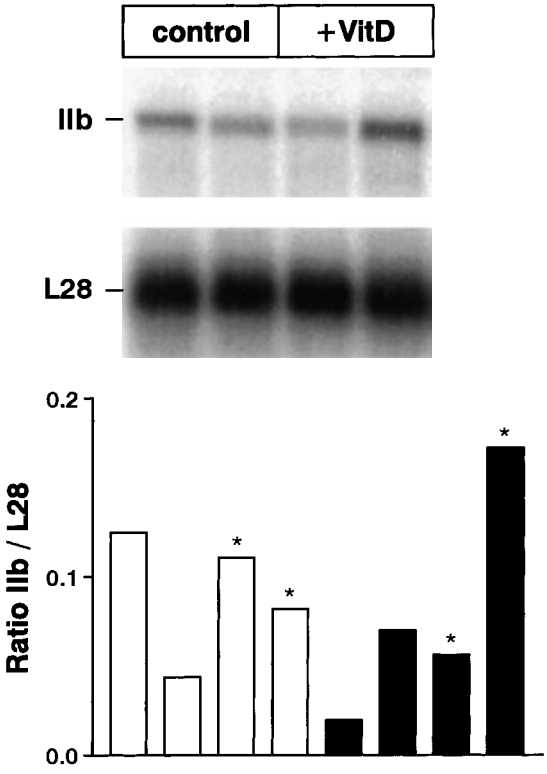

Fig. 6. Northern blots performed with poly(A)$^+$ mRNA isolated from upper small intestines of mice injected with vehicle (control) or with cholecalciferol (+VitD) 24 h before preparation. Two independent experiments were performed, each with 2 animals per group. Quantification of type IIb transcript relative to transcript of L28 protein is presented at *bottom*. Open bars, controls; solid bars, cholecalciferol. Bars marked with asterisk correspond to Northern blot shown.

crease of the V_{max} value of apical Na-P_i cotransport (12, 16, 21, 24). As shown in this study, treatment with cholecalciferol resulted in a stimulation of BBMV Na-P_i cotransport that is similar to the case of the adaptive response correlated with an increased amount of the type IIb protein. These data confirm earlier reports on the effect of vitamin D_3 and in addition ascribe the effect of vitamin D_3 to an increased abundance of the type IIb Na-P_i cotransporter in the brush borders.

For the stimulatory effects of vitamin D_3 on small intestinal absorption, such as for the absorption of P_i or Ca, genomic and nongenomic mechanisms have to be considered (2, 5, 15). With regard to a possible genomic action of vitamin D_3 (and low-P_i diet, respectively), besides changes in the type IIb protein abundance, changes in the amount of the type IIb transcript would have been expected as well. However, Northern blot analysis indicated that neither by chronic low-P_i diet nor by a treatment with cholecalciferol was the amount of the type IIb transcript altered. Therefore, we conclude that an altered rate of transcription of the type IIb gene may not be the primary cause that stimulates small intestinal P_i absorption via an increase in the amount of the type IIb cotransporter. However, an effect of vitamin D_3 on transcription of other genes cannot be excluded entirely because *1*) in some studies the vitamin D_3 response on Na-P_i cotransporter was reported to be dependent on protein synthesis (21) and *2*) a study on expression of small intestinal poly(A)$^+$ mRNA in oocytes of *Xenopus laevis* described a possible augmentation by vitamin D_3 of gene products that may activate Na-P_i cotransport (33).

Besides a genomic action, various nongenomic mechanisms of the action of vitamin D_3 have been described, such as a change of membrane fluidity and activation of protein kinase pathways (2, 9, 11, 17, 27). With respect to a possible nongenomic action of vitamin D_3, it is of interest that in the duodenal loops of chickens P_i uptake was stimulated by vitamin D_3 within 1 h (23). Whereas a change of membrane fluidity per se seems unlikely to directly account for the observed increase of the amount of type IIb cotransporter, a change of membrane fluidity may indirectly be involved, for example, via altered influx of Ca (9, 27). On the other hand, activation of protein kinase C activity by vitamin D_3 may also represent a candidate mechanism to explain our observations (17). Yet to establish whether the abundance of the type IIb protein within the brush-border membrane is influenced by vitamin D_3 via a genomic or nongenomic mechanism, a cellular model would be needed that allows investigation of the vitamin D_3 action on the type IIb Na-P_i cotransporter in the presence of inhibitors of transcription as well as of protein synthesis.

In conclusion, our data demonstrate that the type IIb Na-P_i cotransporter that is expressed in brush borders of enterocytes is upregulated by a low-P_i diet and similarly by vitamin D_3. Because the extent of upregulation of Na-P_i cotransport correlated with the amount of the type IIb cotransporter, we conclude that adaptation of intestinal Na-P_i cotransport by a low-P_i diet and vitamin D_3 is mostly via the type IIb cotransporter. As upregulation of the type IIb cotransporter by a low-P_i diet and vitamin D_3 did not involve alterations in the amount of the type IIb transcript, the effects of a low-P_i diet and vitamin D_3 seem to be unrelated to a transcriptional control of the transporter gene, but may be via a nongenomic mechanism. Genomic mechanisms, however, cannot be excluded entirely and might involve factors controlling the expression of the type IIb Na-P_i cotransporter at a posttranscriptional level.

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