Intestinal Na-P\textsubscript{i} cotransporter adaptation to dietary P\textsubscript{i} content in vitamin D receptor null mice

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INTESTINAL ABSORPTION of P\textsubscript{i} has been characterized in several mammalian and avian species (2, 3, 5–7, 10, 12, 16–18, 21–23, 32, 36, 38, 39, 41). Studies conducted with isolated intestinal brush-border membrane (BBM), Na\textsuperscript{+}-dependent P\textsubscript{i} transport mechanism in the intestine (5, 8, 10, 22, 36, 41). Extensive clinical and experimental studies support the existence of an active, 1,25(OH)\textsubscript{2}D\textsubscript{3}-responsive P\textsubscript{i} transport mechanism in the intestine (9, 11–13, 17, 18, 21–24, 28, 32, 38, 39, 51, 54). Vitamin D\textsubscript{3}, a steroid hormone, plays a central role in modulating phosphate homeostasis and P\textsubscript{i} uptake by the small intestine. The active form of vitamin D\textsubscript{3} is 1,25(OH)\textsubscript{2}D\textsubscript{3}, which binds the vitamin D receptor (VDR) and induces changes in gene expression. Hattenhauer et al. (19) demonstrated that stimulation of intestinal Na-P\textsubscript{i} cotransport by a low-P\textsubscript{i} diet or vitamin D\textsubscript{3} is mediated via an increase in type IIb transporter expression in BBMVs. The goal of the present study was to determine whether vitamin D action is an essential factor for adaptation of intestinal Na-P\textsubscript{i} cotransport to dietary P\textsubscript{i} deficiency.

MATERIALS AND METHODS

Animals and diet. VDR null mutant mice were generated by gene targeting as described previously (25, 56). The locus targeted for the disruption of the VDR gene included exon 2, and the mutant locus contained the neomycin-resistant gene. VDR genotypes were determined by analyzing DNA obtained from each mouse at ~10 days after birth. Genomic DNA was extracted from tail clippings and amplified by PCR using primers specific for VDR (+/-) exon 2 or for the neomycin-resistant gene. Novel primers with the sequences of 5'-GATGTGTGTATCTGAGCTTGTC-3' and 5'-CGAGGTCGACGGTATCGATA-3' were prepared for VDR (+/-) exon 2, and 5'-TTGCTCTTCCGCTTTCTGG-3' and 5'-CGATACCGTAAAGCAGGG-3' were prepared for detection of the neomycin-resistant gene (Fig. 1).

Mice [VDR (+/-) and VDR (-/-)] were weaned at 3 wk of age and given free access to water and a control diet containing 0.5% P\textsubscript{i}, and 0.5% Ca for 6 days (30, 31). On day 7, mice were assigned to one of two groups: the control group, which was fed a diet containing 0.5% P\textsubscript{i}, 0.5% Ca, and 20% lactose; and the low-P\textsubscript{i} group, which was fed a diet containing 0.25% P\textsubscript{i}, 0.5% Ca, and 20% lactose (30, 31). After 4 wk of test diet administration, mice were anesthetized with intraperitoneal pentobarbital sodium, and the tissues were rapidly removed.

Northern blot analysis. Poly(A)\textsuperscript{+} RNA (3 μg/lane) isolated from mouse intestine or kidney was separated on a 1% agarose gel in the presence of 2.2 M formaldehyde and blotted onto a Hybond-N\textsubscript{+} membrane (Amerham Pharmacia Biotech) as described previously (37, 44). The specific probes for each phosphate transporter subtype were labeled with [\textsuperscript{32}P]dCTP using the Megaprint DNA Labeling System (Amerham Pharmacia Biotech). Hybridization proceeded for 3 h at 65°C in Rapid-hyb buffer (Amerham Pharmacia Biotech). The final stringent wash of the membrane was performed with 0.1× standard sodium phosphate-EDTA, 0.1% SDS at 65°C, and the blot was autoradiographed using a Fujix bioimaging analyzer (BAS-1500, FujiFilm, Tokyo, Japan).

Preparation of BBMVs and transport assay. BBMVs were prepared from mouse kidney or intestine by the Ca\textsuperscript{2+} precipitation

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method as described previously (37, 44, 45). Levels of leucine aminopeptidase, Na⁺-K⁺-ATPase, and cytochrome c oxidase were measured to assess the purity of the membranes. The uptake of 32P⁻ was measured by the rapid filtration technique. Next, 10 μl of vesicle suspension were added to 90 μl of incubation solution that was composed of (in mM) 100 NaCl, 100 mannitol, 20 HEPES/H9251/Tris, and 0.1 KH2H9251PO₄. The incubation was performed at 10°C. Na⁺−dependent Pi uptake was measured as described previously (44, 45). Transport was terminated by rapid dilution with ice-cold saline. The reaction mixture was immediately transferred to a premoistened filter (0.45 μm) and maintained under vacuum.

Immunoblotting. Protein samples were heated at 95°C for 5 min in sample buffer in the presence of 5% 2-mercaptoethanol and subjected to SDS-PAGE (37, 44). The separated proteins were transferred by electrophoresis to Hybond-P polyvinyliden difluoride transfer membranes (Amersham Pharmacia Biotech). The membranes were treated with diluted anti-type IIb Na-Pi cotransporter antiserum (1:200), or rabbit anti-PepT1 antibody (1,000:1) for 1 h and washed three times in 0.1% Triton X-100-Tween 20. They were incubated with a primary rabbit or guinea pig anti-type IIa (1:4,000), or type IIc (1:1,000) Na-Pi cotransporter antibody. Type IIb Na-Pi cotransporter polyclonal antibody was generated in rabbits against a COOH-terminal peptide corresponding to amino acid residues 682-697 (QVEVLSMKAL-SNFTVFC) of mouse type IIb Na-Pi cotransporter. The COOH-terminal cysteine residue was introduced for conjugation with keyhole limpet hemocyanine. Type IIa or type IIc cotransporter antibodies were generated as described previously (37, 44). Mouse anti-actin monoclonal antibody (Chemicon) was used as an internal control. Horseradish peroxidase-conjugated anti-rabbit IgG was utilized as the secondary antibody (Jackson ImmunoResearch Laboratories), and signals were detected using the ECL Plus system (Amersham Pharmacia Biotech).

Immunohistochemistry. Mice were anesthetized with pentobarbital sodium (50 mg/kg body wt) and perfused via the left ventricle with PBS followed by paraformaldehyde-lysine-periodate (PLP). Tissues were postfixed with 10 and 20% sucrose at 4°C and embedded in OCT compound (Miles, Elkhart, IN). Frozen sections (5 μm) were thaw mounted onto silane-coated slides and air dried. For immunofluorescence, serial sections were incubated with rabbit anti-type IIb Na-Pi cotransporter antiserum (1:200), rabbit anti-type IIa (1:4,000), guinea pig anti-type IIc (1:200), or rabbit anti-PepT1 antibody (1,000:1) overnight at 4°C (37, 44, 47). Thereafter, they were treated with Alexa Fluor 568 anti-rabbit IgG (Molecular Probes) or Alexa Fluor 488 anti-guinea pig IgG (Molecular Probes) as the secondary antibody for 60 min (37).

Serum Ca, Pi, PTH, and 1α,25(OH)2D3. The serum concentrations of Ca and Pi were determined by the Calcium-E test (Wako, Osaka, Japan) or Phospha-C test (Wako). The serum concentration of parathyroid hormone (PTH) was determined by a mouse PTH ELISA Kit (Immunotopics, San Clemente, CA). Serum 1α,25-(OH)2D3 was determined by radio receptor assay (Mitsubishi, Tokyo, Japan). The fractional excretion index for Pi (FEi Pi) was calculated as follows: urine Pi/(urine creatinine × serum Pi).

Statistical analysis. Data are expressed as means ± SE. Differences among multiple groups were analyzed by ANOVA. The significance of differences between two experimental groups was established by Student’s t-test. P < 0.05 was considered significant.

RESULTS

Serum Ca, Pi, PTH, and vitamin D in VDR (−−) mice. Serum Ca, Pi, PTH, and vitamin D in VDR (+/+ and −−) mice that were fed a control diet (0.5% Pi) are shown in Fig. 2. Serum Ca and Pi levels were significantly decreased in VDR (−−) mice compared with VDR (+/+) mice (Fig. 2, A and B). In contrast, plasma PTH and 1α,25(OH)2D3 levels were markedly increased in VDR (−−) mice compared with VDR (+/+) mice (Fig. 2, C and D). These observations are consistent with previous reports (25, 30, 31, 56).
Na-P$_i$ cotransport activity in VDR (−/−) mice. Intestinal BBM Na-P$_i$ cotransport activity was assessed in VDR (+/+ and VDR (−/−) mice fed a control P$_i$ diet (Fig. 3A). In contrast, renal BBM Na-P$_i$ cotransport activity was linear up to 30 s, with a slow increase persisting to 5 min (data not shown). In the VDR (−/−) mice, intestinal Na-P$_i$ cotransport activity was reduced to 60% of that seen in wild-type (+/+) mice (Fig. 3A). In contrast, renal BBM Na-P$_i$ cotransport activity did not differ between VDR (+/+) and VDR (−/−) mice (Fig. 3B).

Expression of type II Na-P$_i$ cotransporter mRNA in VDR (−/−) mice. Type II Na-P$_i$ cotransporter mRNA levels were determined in VDR (−/−) or VDR (+/+ mice fed a control P$_i$ diet (Fig. 4). Intestinal type IIb Na-P$_i$ cotransporter mRNA did not differ in a comparison of VDR (−/−) mice and wild-type VDR (+/+) mice (Fig. 4, A and B). In contrast, calbindin D$_{9k}$ mRNA levels were significantly lower in VDR (−/−) mice compared with VDR (+/+) mice (Fig. 4, A and C). Furthermore, renal type IIa or type IIc mRNA levels did not differ in a comparison of VDR (−/−) and VDR (+/+ mice (Fig. 4, D–F).

Expression of the type II Na-P$_i$ transporter proteins in VDR (−/−) mice. Type IIb Na-P$_i$ cotransporter protein was detected as a 108-kDa band on Western blotting. Intestinal BBMV type IIb protein expression was significantly decreased in VDR (−/−) mice compared with VDR (+/+ mice (Fig. 5, A and B).

Furthermore, type IIa protein expression was slightly but significantly decreased in VDR (−/−) mice compared with VDR (+/+ mice (Fig. 5, C and D). In contrast, there was no difference in type IIc protein expression in a comparison of VDR (−/−) and VDR (+/+ mice (Fig. 5, E and F). The amount of P$_i$ excretion was not different in a comparison of VDR (−/−) mice and VDR (+/+ mice [VDR (+/+)] vs. VDR (−/−); FELP$_i$: 11.8 ± 1.9 vs. 10.1 ± 0.2].

Immunohistochemical analysis of the type II Na-P$_i$ cotransporters in VDR (−/−) mice. To confirm the reduction in type IIb or type IIa Na-P$_i$ cotransporter expression in VDR (−/−) mice, immunohistochemical analysis was performed (Fig. 6). The type IIb Na-P$_i$ cotransporter-immunoreactive signals were clearly present at the apical membrane of enterocytes from VDR (+/+ mice (Fig. 6A), whereas enterocytes from VDR (−/−) mice showed no immunoreactivity (Fig. 6B). In contrast, oligopeptide transporter PepT1-immunoreactive signals showed a similar distribution in a comparison of VDR (−/−) and VDR (+/+ mice (Fig. 6, C and D).

Type IIa Na-P$_i$ cotransporter-immunoreactive signals were slightly reduced at the apical membrane of the superficial nephrons in VDR (−/−) mice compared with VDR (+/+ mice (Fig. 6, E–H). In contrast, type IIc immunoreactivity showed a similar distribution in a comparison of VDR (+/+ and VDR (−/−) mice (data not shown).

Effect of low-P$_i$ diet on phosphate transport activity and type IIb Na-P$_i$ cotransporter expression. VDR (−/−) mice fed a low-P$_i$ diet displayed an intestinal sodium-dependent phos-

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**Fig. 3.** Intestinal and renal Na$^+$-dependent P$_i$ cotransport activity in VDR (−/−) mice. Na$^+$-dependent P$_i$ cotransport activity was assessed by measurement of P$_i$ uptake into intestinal (A) or kidney basolateral basement membrane vesicles (BBMVs; B; n = 6). Values are means ± SE. *P < 0.05.

**Fig. 4.** Assessment of type II Na-P$_i$ cotransporter mRNA levels. Poly(A)$^+$ RNA (3 µg) isolated from intestine (A) or kidney (D) from mice fed a control P$_i$ diet (0.5% P$_i$) was loaded onto each lane. GAPDH was used as an internal control. The relative intensity of the VDR (+/+ transcript was 1.0. type IIb Na-P$_i$ cotransporter (B), calbindin D$_{9k}$ (C), type IIa Na-P$_i$ cotransporter (E), and type IIc Na-P$_i$ cotransporter (F; n = 6–10). NS, not significant. *P < 0.05.

**Fig. 5.** Calbindin D$_{9k}$ and type II Na-P$_i$ cotransporter protein expression in VDR (−/−) mice. Western blot analysis was performed on VDR (−/−) and VDR (+/+ mice (A). Calbindin D$_{9k}$ and type II Na-P$_i$ cotransporter protein levels were reduced by 25% and 52%, respectively, in VDR (−/−) mice compared with VDR (+/+ mice (C). A: 100 µg protein sample; B: 50 µg protein sample. GAPDH was used as an internal control. The relative intensity of the VDR (+/+ transcript was 1.0. *P < 0.05.

**Fig. 6.** Immunohistochemical analysis of type II Na-P$_i$ cotransporters in VDR (−/−) mice. (A) Type IIb Na-P$_i$ cotransporter-immunoreactive signals were clearly present at the apical membrane of enterocytes from VDR (+/+ mice (B) and VDR (−/−) mice (C). In contrast, type IIc immunoreactive signals showed a similar distribution in a comparison of VDR (−/−) and VDR (+/+ mice (D–H).
phosphate cotransport activity and intestinal type IIb Na-Pi cotransporter mRNA level that were significantly increased compared with those fed the control P_i diet (Fig. 7, A and B). Type IIb transporter protein expression was also increased in VDR (-/-) mice fed a low-P_i diet (Fig. 7C). Furthermore, type IIb-immunoreactive signals were observed at the apical membrane of enterocytes from VDR (-/-) mice fed a low-P_i diet (Fig. 7D). Similar observations were detected in VDR (+/+ ) mice fed a low-P_i diet.

Calbindin D_9k mRNA levels were significantly decreased in VDR (+/+ ) and VDR (-/-) mice that were fed a low-P_i diet compared with those animals fed a control P_i diet (Fig. 8). These data suggest that the effect of a low-P_i diet was specific to the Na-P_i cotransporter system and had no effect on transcellular Ca^{2+} transport system.

**Effect of a low-P_i diet on serum Ca, P_i, PTH, and vitamin D in VDR (-/-) mice fed a low-P_i diet.** Serum Ca levels were significantly increased in VDR (-/-) mice fed a low-P_i diet compared with those fed a control P_i diet (7.7 ± 0.3 vs. 8.2 ± 0.2 mg/dl) (Fig. 9A). In contrast, serum levels of P_i were lower in VDR (-/-) mice fed a low-P_i diet compared with VDR (+/-) mice fed a control P_i diet (3.5 ± 0.3 vs. 5.5 ± 0.5 mg/dl) (Fig. 9B). Serum PTH levels were significantly decreased in VDR (-/-) mice fed a low-P_i diet compared with those fed a control P_i diet (Fig. 9C). This may be due to the elevation of serum calcium levels in VDR (-/-) mice fed the low-P_i diet. In VDR (+/+ ) mice fed a low-P_i diet, serum 1α,25(OH)_2D_3 levels were significantly increased compared with those mice fed a control P_i diet. In contrast, there were no differences in serum 1α,25(OH)_2D_3 levels between VDR (-/-) mice fed a low- and control P_i diet (Fig. 9D). Furthermore, FE_P was significantly decreased in VDR (-/-) mice fed a low-P_i diet compared with those mice fed a control P_i diet (control P_i vs. low-P_i, 11.2 ± 1.5 vs. 2.4 ± 0.2).

**DISCUSSION**

Regulation of intestinal P_i absorption by dietary P_i content has been extensively studied using isolated BBM and cell cultures (4, 8, 35, 40). Specifically, low-P_i diets result in increased intestinal Na-P_i cotransport activity, rapid decreases in plasma P_i, activation of renal 1,25-hydroxylase, and an increase in vitamin D_3 levels (29, 40). Several studies suggest that adaptation of small intestinal Na-P_i cotransport to a low-P_i diet is mediated by vitamin D_3 by demonstrating that changes in apical Na^+-dependent P_i transport rates, but not in the apparent K_m value for P_i, respond to different levels of dietary P_i content (5, 17, 24, 32, 36, 41). Similar observations were reported in the renal P_i reabsorption system (35). While the precise mechanisms remain unclear, adaptation in the renal Na-P_i cotransporter system may involve PTH, vitamin D, growth hormone, thyroid hormone, calcitonin, or other agents (33). Taken in concert, the above findings suggest that vitamin D_3 may also play a role in the adaptation of the intestinal Na-P_i system to dietary P_i. However, the present study demonstrated that adaptation to a low-P_i diet occurred even in VDR (-/-) mice, demonstrating that vitamin D action is not necessary for this phenomenon. A low-P_i diet stimulated expression of type IIb transporter protein in enterocytes and type IIa and type IIc transporter proteins in renal cells from VDR (-/-) mice. We previously demonstrated that upregulation of renal type IIa Na-P_i cotransporter by a low-P_i diet (26) was mediated via increased expression of transcription factor E3 (TFE3). This transcription factor promotes expression of the type IIa transporter protein gene via phosphate-response elements in its promoter sequence. Furthermore, Moz et al. (34) described a posttranscriptional modification of the type IIa gene product by a low-P_i diet; renal proteins from rats fed a low-P_i were able to stabilize type IIa mRNA in vitro.
Similar mechanisms may mediate upregulation of intestinal type IIb Na-P_i cotransporter by a low-P_i diet. Hattenhauer et al. (19) and Xu et al. (53) demonstrated that stimulation of intestinal Na-P_i cotransport by 1,25(OH)_2D_3 can be explained by an increased number of type IIb Na-P_i cotransporter proteins without an increased rate of transcription of the type IIb gene. The present study also demonstrated that stimulation of intestinal P_i absorption by 1,25(OH)_2D_3 may be not mediated by increases in type IIb transporter gene expression.

Furthermore, Hattenhauer et al. (19) demonstrated that dietary P_i restriction increased type IIb protein but not type IIb mRNA. However, the present study indicates that dietary P_i restriction increased intestinal type IIb mRNA in VDR (+/+)) and VDR (-/-) mice. Although we do not have a clear explanation, this difference might be due to the content of P_i in the diets or the period of P_i restriction. In any case, vitamin D may not be involved in the upregulation of the type IIb mRNA by dietary P_i restriction.

Another potential mediator of dietary P_i adaptation is fibroblast growth factor 23 (FGF23) (42, 49, 50). We previously reported that injection of FGF23 DNA into rats blunted adaptation of renal Na-P_i cotransport to dietary P_i (44). Other studies have demonstrated that FGF23 administration resulted in decreased intestinal Na-P_i cotransport activity (43) and that administration of FGF23 suppressed expression of renal type II Na-P_i cotransporters (type IIa and type IIc) (44). A recent study reported that a low-P_i diet resulted in decreased plasma FGF23, whereas a high-P_i diet resulted in increased plasma FGF23 (55). Measurements of serum FGF23 are needed to clarify the mediators of dietary P_i adaptation.

VDR (-/-) mice exhibit features similar to those of patients with hereditary vitamin D-resistant rickets, which results from genetic mutations in the VDR gene (25, 30, 31, 56). VDR

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**Fig. 6.** Immunoﬂuorescent detection of type II Na-P_i cotransporters protein. A and B: type IIb Na-P_i cotransporter. C and D: peptide transporter 1 (PepT1). E–H: type IIa Na-P_i cotransporter. A, C, E, and F: VDR (+/+) mice. B, D, G, and H: VDR (-/-) mice. Magnification: ×400 (A–D, F, and H) and ×100 (E and G).
mice display retarded growth, hypocalcaemia, hypophosphatemia, and severely impaired bone mineralization (25, 56). However, Masuyama et al. (30, 31) demonstrated that administration of a low-Pi VDR (low-Pi diet; 0.25% P_i) resulted in normalization of serum calcium and phosphate concentration and improved bone mineralization and turnover in VDR (-/-) mice. In the present study, a low-Pi diet resulted in attenuation of hypocalcaemia but had no effect on phosphate levels in VDR (-/-) mice. An increase in intestinal calcium transport appeared to occur via acceleration of passive absorption of calcium transport rather than by vitamin D-dependent absorption, as calbindin D_9k mRNA levels remained unchanged. Improvements in plasma P_i were likely mediated via upregulation of the type IIb Na-P_i cotransporter in VDR (-/-) mice.

In addition, the present data showed that a low-P_i diet (0.25% P_i) stimulates intestinal BBM phosphate transport and type IIb protein synthesis in VDR (-/-) mice comparable to that in VDR (+/+). To determine the degree of P_i restriction necessary to stimulate intestinal Na-P_i cotransport activity in VDR (+/+), groups of mice were fed a diet containing 0.02, 0.25, or 0.6% (control) P_i (data not shown). Severe (0.02%) P_i restriction induced a 2.0-fold increase in intestinal Na-P_i cotransport activity compared with the moderately P_i-restrictive diet (0.25% P_i). In addition, serum 1,25(OH)_2D_3 levels with the 0.02% P_i diet were significantly increased compared with the 0.25% P_i diet (336 ± 32 vs. 108 ± 45 pg/ml). These data suggest that changes in serum 1,25(OH)_2D_3 concentration per se are sufficient to determine the activity of intestinal Na-P_i cotransport in VDR (+/+) mice (52). In contrast, both severe (0.02%) and moderate (0.2%) P_i restriction induced 4.2- and 4.4-fold increases, respectively, in intestinal Na-P_i cotransport in VDR (-/-) mice compared with the control diet (0.6% P_i) (data not shown).
remains as to the underlying mechanisms for the regulation of Na-Pi cotransport by the degree of the P_{3} restriction in VDR (−/−) mice. One possible explanation is that P_{3} demand may be increased in VDR (−/−) mice compared with wild-type animals, because of impaired bone mineralization and reduced bone turnover (4, 27, 42). However, the role of bone mineralization and turnover in controlling intestinal Na-Pi cotransport by P_{3} restriction is not yet established.

The present study also showed that the PTH levels in VDR (−/−) animals are much higher than in controls, but urinary F{E}_{Pi} is unchanged. Forte et al. (15) demonstrated that the blunted phosphaturic response to PTH observed in vitamin D-deficient animals is associated with the reduced responsiveness of renal cortical adenylate cyclase to the hormone. Furthermore, in a previous study, we demonstrated that renal type IIa transporter expression was decreased in the deep cortex of vitamin D-deficient rats, and administration of 1,25-dihydroxyvitamin D_{3} in these animals resulted in normalization of type IIa transporter expression (46). The present study demonstrated that type IIa transporter mRNA levels were similar in a comparison of VDR (+/+) and VDR (−/−) mice. In addition, immunohistochemical analysis indicated that the reduction of type IIa immunoreactive signals was in the superficial cortex rather than the deep cortex. The discrepancy between these studies may be due to a species-dependent difference in vitamin D metabolism (1, 6, 7).

In conclusion, the present study demonstrated an elevation of intestinal P_{3} transport activity and type IIb protein content in mice fed a low-P_{3} diet. Furthermore, this phenomenon was independent of vitamin D_{3}.

![Fig. 8. Effect of a low-P_{3} diet on calbindin D_{9k} (CaBP-9k) mRNA levels in VDR (−/−) mice. Top: Northern blot analysis of calbindin D_{9k} was performed. Bottom: relative intensity was calculated using GAPDH as the internal control. Values are means ± SE (n = 6). *P < 0.01.](image)

![Fig. 9. Effect of a low-P_{3} diet on the serum levels of Ca (A), phosphate (B), PTH (C), and 1α,25(OH)_{2}D_{3} (D) in VDR (−/−) mice 4 wk after administration of test diet, as described in the MATERIALS AND METHODS. Values are means ± SE (n = 6–8). **P < 0.01, ***P < 0.0001.](image)

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