Relative contributions of Na^+-dependent phosphate co-transporters to phosphate transport in mouse kidney: RNase H-mediated hybrid depletion analysis

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Reabsorption of P_i in the proximal tubule of the kidney is an important determinant of P_i homoeostasis. At least three types (types I–III) of high-affinity Na^+-dependent P_i co-transporters have been identified in mammalian kidneys. The relative roles of these three types of Na^+/P_i co-transporters in P_i transport in mouse kidney cortex have now been investigated by RNase H-mediated hybrid depletion. Whereas isolated brush-border membrane vesicles showed the presence of two kinetically distinct Na^+/P_i co-transport systems (high K_m–low V_{max} and low K_m–high V_{max}), *Xenopus* oocytes, microinjected with polyadenylated [poly(A)$^+$] RNA from mouse kidney cortex, showed only the high-affinity P_i uptake system. Kidney poly(A)$^+$ RNA was incubated *in vitro* with antisense oligonucleotides corresponding to Npt-1 (type I), NaPi -7 (type II) or Glvr-1 (type III) Na^+/P_i co-transporter mRNAs, and then with RNase H. Injection of such treated RNA preparations into *Xenopus* oocytes revealed that an NaPi-7 antisense oligonucleotide that resulted in complete degradation of NaPi-7 mRNA (as revealed by Northern blot analysis), also induced complete inhibition of P_i uptake. Degradation of Npt-1 or Glvr-1 mRNAs induced by corresponding antisense oligonucleotides had no effect on P_i transport, which was subsequently measured in oocytes. These results indicate that the type II Na^+/P_i co-transporter NaPi-7 mediated most Na^+-dependent P_i transport in mouse kidney cortex.

INTRODUCTION

Reabsorption of P_i in the proximal tubule of the kidney is important for P_i homoeostasis in vertebrates [1,2]. Various Na^+-dependent P_i co-transporters have been identified in the kidney cortex of humans, rabbits, rats and mice [3–11], and they have been classified into two different types on the basis of their predicted amino acid sequences: type I, represented by NaPi-1 (rabbit), NPT-1 (human), Npt-1 (mouse) and RNaPi-1 (rat); and type II, represented by NaPi-2 (rat), NaPi-3 (human), NaPi-4 (OK cell), NaPi-6 (rabbit) and NaPi-7 (mouse) [3–11]. In addition, the proteins Ram-1, Glvr-1 and GLVR-1, originally identified as retrovirus receptors, have been shown to constitute a third type (type III) of high-affinity Na^+-dependent P_i co-transporter, expressed in rat, mouse and human kidney [12–14]. We have now evaluated the relative contributions of various Na^+/P_i co-transporters in P_i transport in mouse kidney cortex with the use of *in vitro* RNase H-mediated hybrid depletion. In this approach, polyadenylated [poly(A)$^+$] RNA from mouse kidney cortex was incubated *in vitro* first with synthetic antisense oligonucleotides specific for the transcripts of transporter genes and then with RNase H, which catalyses the degradation of hybrids formed between the oligonucleotides and the corresponding transporters mRNAs [15]. The effect of this specific degradation on P_i uptake was then measured in *Xenopus* oocytes injected with the RNA preparation.

EXPERIMENTAL

Animals

Mice (ICR strain; body mass 30–50 g) were obtained from SLC (Shizuoka, Japan), housed in plastic cages and fed a diet containing 1.2% (w/w) calcium, 0.6% (w/w) phosphorus and 4.4 i.u. of vitamin D_3/g [16]. They were killed by decapitation at an age of 2–3 months.

Preparation of brush-border membrane vesicle (BBMV) and transport measurements

BBMVs were prepared from mouse kidney cortex by the Ca^{2+} precipitation method as described previously [17]. The purity of the membranes was assessed by measurement of leucine aminopeptidase, Na^+- and K^+-dependent ATPase and cytochrome-*c*-oxidase activities [17]. Uptake of [^{32}P]P_i was measured by a rapid filtration technique [17]. Incubation at 37 °C was initiated by the addition of a 10 μl BBMV (50 μg of protein) suspension to 100 μl of a solution containing 100 mM NaCl, 100 mM mannitol, 20 mM Hepes/Tris (pH 7.5), 0.01–10 mM $KH_2{}^{32}PO_4$ (9000 Ci/mmol; DuPont–NEM). Transport was terminated by rapid dilution of the reaction mixture with 1 ml of ice-cold stop solution (100 mM mannitol/20 mM Hepes/Tris/0.1 mM KH_2PO_4/20 mM $MgSO_4$/100 mM choline chloride) and application to a pre-moistured filter (0.45 μm pore size) maintained under vacuum. The filters were washed and associated ^{32}P was measured by liquid-scintillation spectroscopy.

Northern blot analysis

Total RNA was isolated from mouse kidney cortex by acid guanidine thiocyanate/phenol/chloroform extraction [18], after which poly(A)$^+$ RNA was separated with an oligo(dT) column, denatured by heating for 5 min at 70 °C in a solution containing 10 mM Mops (pH 7.0), 5 mM sodium acetate, 1 mM EDTA, 2.2 M formaldehyde and 50% (v/v) formamide, and subjected to electrophoresis in a 1.2% (w/v) agarose gel containing 2.2 M formaldehyde. Resolved RNA was transferred to Hybond-N membranes (Amersham) and covalently cross-linked by exposure to UV light. Hybridization with a ^{32}P-labelled cDNA probe was

Abbreviations used: BBM, brush-border membrane; BBMV, brush-border membrane vesicle; poly(A)$^+$, polyadenylated.
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performed in a buffer containing 50 % formamide, 5 × SSPE [0.15 M NaCl/10 mM sodium phosphate (pH 7.4)/1 mM EDTA], 2 × Denhardt's solution and 1 % (w/v) SDS, after which the membranes were analysed with a Fuji BAS-2000 system. Npt-1 [10], NaPi-7 [9], and Glvr-1 [13] cDNA probes were prepared by PCR with mouse-kidney total cDNA and specific oligonucleotide primers. Glyceraldehyde-3-phosphate dehydrogenase mRNA was used as an internal standard.

Oocyte injection and P_i transport assay

Xenopus laevis females were obtained from Hamamatsu Jikken (Shizuoka, Japan). Small clumps of oocytes were treated twice for 90 min each time with collagenase (2 mg/ml) in a Ca^{2+}-free solution, ORII (82.5 mM NaCl/2 mM KCl/1 mM $MgCl_2$/10 mM Hepes/Tris, pH 7.5) to remove the follicular layer [7,19]. After extensive washing with ORII solution and then with a modified Barth's solution [19], the oocytes were maintained in the latter overnight at 18 °C. Healthy stage-V oocytes were then injected with poly(A)$^+$ RNA from mouse kidneys (dissolved in water at a concn. of 1 mg/ml) or with water using a manual injector (Narishige, Tokyo, Japan). After 4 days, six to eight injected oocytes were incubated in Na^+-containing [^{32}P]P_i uptake solution for 0–90 min at room temperature [19]. They were then washed three times with ice-cold uptake solution lacking ^{32}P but containing 5 mM KH_2PO_4, and each oocyte was transferred to a scintillation vial and its content of ^{32}P determined.

RNase H-mediated hybrid depletion

Oligonucleotides (see Table 1) were synthesized, purified with an Applied Biosystems DNA synthesizer (model 380B) and purified by HPLC. Poly(A)$^+$ RNA (5 μg) from mouse kidney cortex was incubated for 5 min at 70 °C with 40 ng of oligonucleotide in 20 μl of 100 mM KCl [15]. The annealing mixture was allowed to cool to room temperature over a period of 10 min, placed on ice, and diluted with 20 μl of a solution containing 70 units of RNase inhibitor (TaKaRa, Kyoto, Japan), 1 unit of RNase H (Gibco–BRL), 5 μg of tRNA, 6 mM $MgCl_2$, 25 mM Tris/HCl (pH 7.5) and 0.75 mM dithiothreitol. The resulting mixture was incubated for 30 min at 37 °C, placed on ice, and extracted once with phenol and once with chloroform. RNA was then precipitated with 70 % (v/v) propan-2-ol in 20 mM potassium acetate to remove free oligonucleotides. The precipitates were washed twice with 70 % (v/v) ethanol, dissolved in 40 μl of diethyl-pyrocarbonate-treated water and then centrifuged through a column containing Sephacryl-400 (Pharmacia) to remove any residual free oligonucleotides. The resulting flowthrough fraction was recovered, and its RNA was precipitated twice with 70 % ethanol and finally dissolved in 2 μl of diethyl-pyrocarbonate-treated water. A portion (50 nl) of the RNA precipitation was injected into oocytes.

Eadie–Hofstee analysis

Eadie–Hofstee transformation of the data was used to calculate kinetic parameters. The plots were not linear but could be resolved into straight lines: one at low concentrations of P_i, corresponding to the high-affinity system only, and one at high concentrations of P_i, corresponding to the sum of the high-affinity and low-affinity processes. This was accomplished with an iterative process that minimized the sum of the squares of the standard deviations of the experimental points from the line. The apparent K_m and V_{max} values of the two processes were obtained directly from the slopes and intercepts of the two linear portions. The standard errors of the slopes and intercepts were calculated with the help of published equations [20]. The slopes (apparent K_m) and intercepts (V_{max}) of regression lines were compared independently by Student's t test.

Statistical analysis

Values were 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. A P value of < 0.05 was considered statistically significant.

RESULTS

P_i transport in mouse renal BBMVs

The time course of P_i uptake in mouse-kidney BBMVs was linear for at least 20 s in both NaCl and KCl (Figure 1A). The Na^+-dependent component of P_i transport, used to derive kinetic parameters, is also linear for 20 s. The initial rate of P_i transport was measured over a wide range of P_i concentrations (0.01–10 mM), and an Eadie–Hofstee plot was generated from the data derived from 10 different BBMV preparations (Figure 1B). The plot was clearly non-linear and could be resolved into two linear components: one characterized by high capacity (V_{max}, 2245 ± 143 pmol/10 s per mg of protein) and relatively low affinity (apparent K_m 1.05 ± 0.14 mM), and the other of lower capacity (V_{max} 524 ± 50 pmol/10 s per mg of protein) and higher affinity (apparent K_m 0.12 ± 0.02 mM).

Microinjection of *Xenopus* oocytes

Microinjection of mouse-kidney poly(A)$^+$ RNA into *Xenopus* oocytes resulted in a significant increase in P_i uptake (Figure 2). The extent of P_i uptake in RNA-injected oocytes after incubation for 90 min was approx. 5 times that in water-injected oocytes.

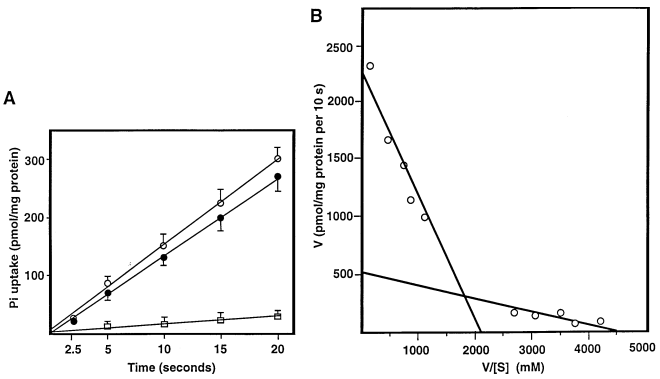

Figure 1 Time course (A) and Eadie–Hofstee plot (B) of P_i transport in mouse-kidney BBMVs

(**A**) Uptake media contained 100 mM KCl, 100 mM mannitol and 20 mM Hepes/Tris, pH 7.5 (□) and 100 mM NaCl, 100 mM mannitol and 20 mM Hepes/Tris, pH 7.5 (○). The sodium-dependent component of P_i transport, obtained by subtracting uptake in KCl medium from uptake in NaCl medium, is also shown (●). Data are means ± S.E. (n = 8–10). (**B**) Eadie–Hofstee plot of the Na^+-dependent component of P_i transport. Renal BBM P_i transport was measured at 10 s at P_i concentrations between 0.01 mM and 10 mM. Uptake measured in the presence of KCl was subtracted from that in the presence of NaCl to obtain the Na^+-dependent component of P_i flux. Computer transformation of the data was used to determine the regression lines [20] and to calculate the kinetic parameters, as described in the Experimental section. Data shown were derived from 10 different BBMV preparations from normal mouse kidney cortex, with uptake measurements performed in quadruplicate. K_m (high affinity) = slope of the right limb; V_{max} (high affinity) = extrapolated intercept of the right limb, $V/S = 0$; K_m (low affinity) = slope of the left limb; V_{max} = intercept of left limb.

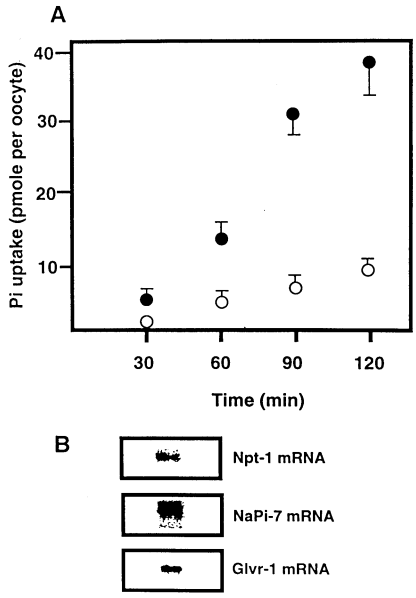

Figure 2 Time course of P_i transport in *Xenopus* oocytes microinjected with (A), and Northern blot analysis of (B), mouse-kidney poly(A)$^+$ RNA

(**A**) Kidney poly(A)$^+$ RNA (50 ng) dissolved in water (●), or water alone (○), was microinjected as described in the Experimental section. Data are means ± S.E.M. ($n = 5$). (**B**) Northern blot analysis of mouse-kidney poly(A)$^+$ RNA (2 μg) with ^{32}P-labelled cDNA probes specific for Npt-1, NaPi-7 or Glvr-1 mRNAs as indicated.

Kinetic analysis revealed K_m values of 0.35 mM and a V_{max} of 38 nmol/10 min per oocyte, similar to those of the high-affinity P_i uptake system in BBMVs; the low-affinity system of BBMVs was not apparent. Northern blot analysis revealed that Npt-1 (type I), NaPi-7 (type II) and Glvr-1 (type III) mRNAs are abundant in mouse kidney.

Effects of RNase H-mediated hybrid depletion on P_i transport

To investigate the effect of RNase H-mediated hybrid depletion for the three Na^+/P_i co-transporter mRNAs detected by Northern blot analysis, we selected three specific sequences in the coding region of each transcript (Table 1). Treatment of mouse-kidney poly(A)$^+$ RNA *in vitro* with NaPi-7 antisense oligonucleotide B and RNase H resulted in approx. 50% inhibition of P_i uptake subsequently measured in microinjected *Xenopus* oocytes, compared with that apparent in oocytes injected with untreated RNA or RNA exposed to the corresponding sense oligonucleotide (Table 2). Furthermore, NaPi-7 antisense oligonucleotide C completely inhibited P_i-uptake-induced mouse-renal poly(A)$^+$ RNA in *Xenopus* oocytes. NaPi-7 antisense oligonucleotide A also appeared to inhibit P_i uptake, but the

Table 2 Effect of RNase H-mediated hybrid depletion *in vitro* on Na^+/P_i co-transporter-mediated P_i uptake in *Xenopus* oocytes

RNase H-mediated hybrid depletion was performed as described in the Experimental section with mouse-kidney poly(A)$^+$ RNA and the sense and antisense versions of the oligonucleotides (Oligo) shown in Table 1. *Xenopus* oocytes were then injected with either distilled water, untreated poly(A)$^+$ RNA, or poly(A)$^+$ RNA subjected to hybrid depletion, and P_i transport was assayed. Data are means ± S.E. ($n = 4$). $^*P < 0.01$, $^{**}P < 0.001$ versus corresponding sense oligonucleotide.

	P_i uptake (pmol/120 min per oocyte)
Npt-1	
Water	8.4 ± 1.7
Poly(A)$^+$ RNA	30.2 ± 2.9
Oligo A (Npt-1) sense	33.9 ± 3.0
Antisense	28.8 ± 7.9
Oligo B (Npt-1) sense	30.4 ± 2.3
Antisense	28.4 ± 5.1
Oligo C (Npt-1) sense	32.7 ± 3.6
Antisense	30.1 ± 4.8
Npt-1	
Water	9.5 ± 1.8
Poly(A)$^+$ RNA	37.8 ± 2.4
Oligo A (Npt-1) sense	36.5 ± 3.4
Antisense	25.9 ± 16.7
Oligo B (NaPi-7) sense	34.8 ± 5.3
Antisense	15.3 ± 9.6*
Oligo C (NaPi-7) sense	37.3 ± 4.9
Antisense	6.9 ± 3.2**
Glvr-1	
Water	14.5 ± 2.7
Poly(A)$^+$ RNA	30.2 ± 2.2
Oligo A (Glvr-1) sense	35.7 ± 3.0
Antisense	30.1 ± 7.3
Oligo B (Glvr-1) sense	25.5 ± 5.8
Antisense	31.1 ± 2.8
Oligo C (Glvr-1) sense	27.5 ± 4.5
Antisense	25.6 ± 13.8

Table 1 Sequence of oligonucleotides used for RNase H-mediated hybrid depletion

Nucleotide numbers (positions) are relative to translation start site.

Oligonucleotide (sense)	Sequence	Position
Npt-1		
A	5′-GAGACCAAGCCCAGCAA-3′	+3 to +22
B	5′-GAACCAGTGCCTCCCCAAGA-3′	+43 to +62
C	5′-GTCCTTGCTCATCCCACCTG-3′	+393 to +413
NaPi-7		
A	5′-GCCCCTGTGGGGAGGTCCTGGAGGAGCGCC-3′	+174 to +210
B	5′-GCTGGTGCAGAGCTCCAGCACCT-3′	+473 to +495
C	5′-GTTCTGTGTTCACCTCGGCCATCAC-3′	+1255 to +1279
Glvr-1		
A	5′-GCTCATCCTGGGCTTCATCATTGC-3′	+83 to +106
B	5′-GTGTAGTGACCCTGAAGCAAGCCTG-3′	+175 to +199
C	5′-CTGTGGGCTCCGCCTTGCTGGGGG-3′	+223 to +246

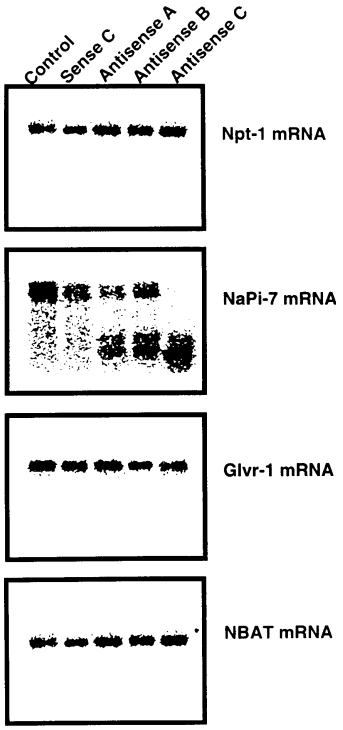

Figure 3 Effect of RNase H-mediated hybrid depletion *in vitro* on Na^+/P_i transporter mRNA abundance

Northern blot analysis of poly(A)$^+$ RNA subjected to hybrid depletion with NaPi-7 sense oligonucleotide C or NaPi-7 antisense oligonucleotides A, B or C. Blots were probed with ^{32}P-labelled cDNAs corresponding to Npt-1, NaPi-7, Glvr-1 or NBAT mRNAs, as indicated.

effect was not significant. None of the antisense oligonucleotides targeted to Npt-1 or Glvr-1 inhibited P_i uptake.

We determined by Northern blot analysis the amount of transporter mRNAs remaining after RNase H-mediated hybrid depletion. Treatment with NaPi-7 antisense oligonucleotide C resulted in complete degradation of NaPi-7 mRNA (Figure 3). In contrast, NaPi-7 sense oligonucleotide C did not induce the degradation of the type-II transporter mRNA transcripts. Treatment with NaPi-7 antisense oligonucleotides A and B also resulted in degradation of NaPi-7 mRNA but to a lesser extent than antisense oligonucleotide C. None of the NaPi-7 antisense oligonucleotides affected the amount of mRNAs encoding Npt-1, Glvr-1 or the basic amino acid transporter (NBAT).

Similarly, Npt-1 and Glvr-1 antisense oligonucleotides induced degradation of the corresponding transporter mRNAs but not that of those encoding the other two transporters (results not shown).

DISCUSSION

The kinetics of P_i transport in isolated BBMVs revealed the presence of at least two components: one of high affinity and one of low affinity. However, previous studies have shown that expression of type I, II or III Na^+/P_i co-transporters in *Xenopus* oocytes confers the property of high-affinity P_i uptake [3,5,7,21,22]. Our data now indicate that the type II co-transporter NaPi-7 mediates most high-affinity P_i transporters in mouse kidney, a conclusion that is consistent with various previous observations. Thus electrophysiological analysis of the rabbit type-I Na^+/P_i co-transporter in *Xenopus* oocytes showed that this protein also functions as an anion channel that is permeable not only to Cl^{*-} but also to organic anions [23]. Moreover, type I transporter mRNA and protein do not respond to the amount of P_i in the diet or to parathyroid hormone, the two most important physiological and pathophysiological regulators of renal proximal-tubular P_i reabsorption [1,2]. In X-chromosome-linked hypophosphataemic mice, which manifest abnormal P_i reabsorption in the proximal tubule, the amount of the type II transporter NaPi-7 protein is markedly decreased, whereas that of the type-I transporter, Npt-1, is unaffected [9,10,24].

Immunohistochemical analysis has also shown that the type-I transporter NaPi-1 is expressed in the straight part of proximal tubules (S_3 segments), but not in convoluted proximal tubule [25,26]. The regulation of P_i reabsorption by parathyroid hormone and serum P_i concentration occurs mainly in convoluted proximal tubule (S_1 segments). In contrast, the type-II transporter NaPi-2 is expressed in convoluted proximal tubule [25].

The type III transporters, originally isolated as virus receptors, show a wide tissue distribution, are regulated by changes in medium P_i concentration *in vitro* [21], and show a high affinity for P_i [21,22]. In the present study, we observed that Glvr-1 mRNA is abundant in mouse kidney. However, P_i depletion or parathyroidectomy did not affect the amount of Glvr-1 mRNA in mouse kidney (results not shown). Together, these observations suggest that type II, rather than type I or type III, Na^+/P_i co-transporters mediate most Na^+/P_i co-transporters in the mouse kidney and serve as the physiological and pathophysiological regulation of proximal-tubular reabsorption of P_i.

Two kinetically distinct P_i transport processes have been identified in brush-border membrane (BBM) of rat and pig kidney [27–29]: a low-affinity and high-capacity system, present only in the early proximal tubule in a position to reabsorb the bulk of filtered P_i, and a high-affinity and low-capacity system located throughout the proximal tubule to reclaim residual, filtered P_i [27–29]. We also provide evidence for the presence of both P_i transport systems in BBM of mouse kidney. However, a low-affinity and high-capacity system was not detected in *Xenopus* oocytes expressing mouse renal mRNA. *Xenopus* oocytes provide a useful system for translating heterologous mRNA microinjected into them, and have already been used to isolate cDNAs that encode transporter proteins. However, they have higher endogenous transport activity for P_i [low-affinity system(s)] rather than amino acids and glucose. In addition, when several P_i transport systems were co-expressed in *Xenopus* oocytes, transport properties of the individual transporter proteins might be difficult to distinguish. Further studies are needed to understand Na^+-dependent P_i transport systems in mouse kidney.

In the present study, RNase H-mediated hybrid depletion *in vitro* was effective in inducing degradation of specific transporter mRNAs, as revealed by Northern blot analysis. We selected antisense oligonucleotides located in 5′ and internal coding regions of the transporter mRNAs. Whereas hybrid depletion with NaPi-7 antisense oligonucleotide C resulted in complete inhibition of P_i uptake in oocytes and complete degradation of NaPi-7 mRNA, the effect of NaPi-7 antisense oligonucleotides A and B was less marked. Thus the effectiveness of hybrid depletion was dependent on the sequence targeted by each oligonucleotide.

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