Cloning and functional expression of a Na⁺-dependent phosphate co-transporter from human kidney: cDNA cloning and functional expression

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A cDNA clone encoding a protein 69 % identical in amino acid sequence with that of the Na/P_i co-transporter NaP_i-1 was isolated from a human kidney cDNA library. The DNA sequence was identical with that of NPT-1 cDNA published by Chong, Kristjansson, Zoghbi and Hughe (1993) (*Genomics*, **18**, 355–359). In the present study, we have characterized the function of the encoded protein and the tissue distribution of its mRNA. Injection of RNA transcribed from NPT-1 into *Xenopus* oocytes resulted in expression of Na/P_i co-transport activity showing a high affinity for P_i transport (K_m 0.29 mM). Kinetic character-

ization ([P_i], [Na⁺]) demonstrated that the expressed transport activity has properties similar to those displayed by oocytes injected with human kidney poly(A)⁺ RNA. Northern blotting demonstrated that NPT-1 mRNA is expressed in renal cortex, liver and brain but not in other tissues. Hybrid depletion with antisense oligonucleotides to NaP_i-3 and NPT-1 completely inhibited poly(A)⁺ RNA-induced Na⁺-dependent P_i uptake in oocytes. These findings indicate that two high-affinity Na/P_i co-transporters (NaP_i-3 and NPT-1) are present in human kidney cortex.

INTRODUCTION

Proximal-tubular reabsorption of P_i is regulated by modulation of apical Na/P_i co-transporter systems [1–3]. Reabsorption by these systems is a key determinant of P_i homeostasis and is regulated by a variety of hormones as well as by dietary phosphate intake [1–3].

Recently, Murer and co-workers have isolated cDNAs encoding Na/P_i co-transporters from rabbit kidney cortex (NaP_i-1), rat kidney cortex (NaP_i-2) and human kidney cortex (NaP_i-3) [4,5]. NaP_i-2 and NaP_i-3 show high homology to each other (81 % identity) but share only 19 % identity with NaP_i-1. mRNAs encoding NaP_i-2/3 are abundant in renal cortex and NaP_i-2/3-related proteins represent the major Na/P_i co-transport system in the proximal-tubule brush-border membrane in various species [5,6]. Nonetheless, there is evidence for multiple phosphate-transport systems along the length of the proximal tubule [7–10]. A high-capacity system is located early in the proximal tubule where the bulk of the filtrate is absorbed, and a high-affinity system is located more distally, in a suitable position to reclaim the final tubular fluid solute [1–3]. In addition a low-affinity high-capacity system is located upstream in the most proximal tubule [1–3].

Recently, Chong et al. [11] have isolated a cDNA clone (NPT-1) encoding a Na⁺-dependent P_i co-transporter from a human kidney cDNA library; however, the function of the encoded protein and its tissue distribution have not been determined [11].

In the present study we report on the isolation of a cDNA clone from a human kidney cortex cDNA library using homology-based PCR. The predicted amino acid sequence of this clone is identical with that of NPT-1 [11]. Expression of NPT-1 complementary RNA (cRNA) in *Xenopus laevis* oocytes results in high-affinity Na⁺-dependent P_i transport. Hybrid depletion with antisense oligonucleotides directed against NaP_i-3 and NPT-1 completely inhibited the mRNA-induced Na⁺-dependent phosphate uptake in oocytes. These findings indicate

that two high-affinity Na/P_i co-transporters (NaP_i-3 and NPT-1) are present in human kidney cortex.

MATERIALS AND METHODS

Cloning of cDNA encoding a human kidney Na/P_i co-transporter

RNA was prepared using caesium chloride/guanidinium isothiocyanate purification followed by oligo(dT)-cellulose affinity chromatography as previously described [12,13]. A cDNA library in vector λgt 10 (5 × 10⁵ independent recombinants) was constructed from human kidney cortex poly(A)⁺ RNA by oligo(dT)-primed cDNA synthesis (Gibco, Gaithersburg, MD, U.S.A.). Plaques were screened with ³²P-labelled NP-1 [150 bp PCR product produced by degenerate primers from the Na⁺-binding motif (SOB) region of NaP_i-1] by high-stringency hybridization [12]. Three 1.8 kb clones (NPT-1) were isolated and subcloned into the *NotI* site of pBluescript II SK⁺ (Stratagene, LaJolla, CA, U.S.A.). NPT-1 was digested with *Bam*HI, *AccI* or *Eco*RV and subcloned into pUC18 and pGEM5Z (Promega, Madison, WI, U.S.A.). Both strands of the cDNA inserts were sequenced by the dideoxy-chain-termination method using a T7 sequencing kit (Pharmacia, Milwaukee, WI, U.S.A.). Synthetic oligonucleotides were used as primers to complete the sequence. To ensure the validity of our analysis, sequence reaction and migrations were performed at least three times and on both strands of cDNA. Resolution was improved in some regions by replacing dGTP by deaza-dITP in the nucleotide mix.

In vitro transcription

The NPT-1 cDNA clone was linearized by digestion with *Eco*RI and transcribed into NPT-1 cRNA using T3 RNA polymerase (Promega) [14].

Abbreviations used: SOB, sodium binding; poly(A)⁺, polyadenylated; cRNA, complementary RNA.

The nucleotide sequence from which the amino acid sequence reported in this paper was deduced has been deposited in the EMBL/GenBank/DBJ Nucleotide Sequence Databases under the accession number D28632.

Oocyte injections and transport assay

Xenopus laevis females were obtained from Hamamatsu Jikkenn (Sizuoka, Japan). Small clumps of oocytes were treated twice for 90 min with 2 mg/ml collagenase in a Ca^{2+} -free solution (ORII solution: 82.5 mM NaCl, 2 mM KCl, 1 mM MgCl_2 , 10 mM Hepes/Tris, pH 7.5) in order to remove the follicular layer [5]. After extensive washing, first with ORII solution and then with modified Barth's solution [88 mM NaCl, 1 mM KCl, 0.82 mM MgSO_4 , 0.4 mM CaCl_2 , 0.33 mM $\text{Ca}(\text{NO}_3)_2$, 2.4 mM NaHCO_3 , 10 mM Hepes/Tris, pH 7.5], the oocytes were maintained in modified Barth's solution overnight at 18 °C. Healthy stage V oocytes were then injected with poly(A)⁺ RNA, cRNA (dissolved in water at concentrations from 1 mg/ml) or water using a manual injector (Narishige, Tokyo, Japan). Six to eight oocytes were washed in Na^{+} -free uptake solution (100 mM choline chloride, 2 mM KCl, 1 mM CaCl_2 , 1 mM MgCl_2 , 10 mM Hepes/Tris, pH 7.5, 0.5 mM KH_2PO_4 (30 $\mu\text{Ci}/\text{ml}$)] for 0–60 min at room temperature, and the other half were used for measurement of Na^{+} -independent uptake, with incubation as above but substituting 100 mM choline chloride for the NaCl. After incubation, the oocytes were washed three times with cold Na^{+} -free uptake solution containing 5 mM KH_2PO_4 . Each oocyte was then transferred to a scintillation vial, dissolved in 0.2 ml of 10% SDS and counted for radioactivity in 5 ml of liquid-scintillation fluid.

Northern-blot analysis

Poly(A)⁺ RNA (5 μg) from various human tissues (Clontech, Palo Alto, CA, U.S.A.) and human kidney (provided by Dr. Kanayama, Tokushima University) was denatured and subjected to electrophoresis on a 1.2% agarose gel containing formaldehyde, transferred to a nylon membrane (Gene Screen, DuPont–New England Nuclear) and hybridized to ^{32}P -labelled randomly primed full-length NPT-1 cDNA [12,13].

Hybrid depletion

Poly(A)⁺ RNA (1 mg/ml) from human kidney cortex was denatured at 65 °C for 5 min in a solution containing 50 mM NaCl and 40 μM of each of the following primers: NaP_i -3 sense A (5'-CCCGCTCACACTGGGTTCACATC-3') or antisense A (5'-GATGTTGCAACCCAGTGTGAGCGGG-3'), complementary to the human NaP_i -3 mRNA sequence from nucleotides 1404–1428 [5], NaP_i -3 and NPT-1 sense B [5'-CCAGC(C/A)CCAGG(A/G)AGAAG(G/C)TG-3'] or antisense B [5'-CA(G/C)CTTCT(C/T)CCTGG(G/T)GCTGG-3'] oligonucleotides complementary to the human NaP_i -3 and NPT-1 mRNA sequence from nucleotides position 1463–1482 (NaP_i -3) and 1497–1516 (NPT-1) [5,11] with high homology. It was then further incubated at 42 °C for 30 min before injection. Oocytes were injected with a volume of 50 nl and incubated at 18 °C for 3 days in modified Barth's solution. Uptake of P_i was then determined as described above.

RESULTS AND DISCUSSION

Screening of a human kidney cDNA library with a probe derived from NaP_i -1 yielded three overlapping cDNA clones. Two full-length cDNA clones, 3 and 4, were isolated and sequenced, revealing an encoded reading frame of 467 amino acids; the encoded protein was nearly identical with that of NPT-1 cDNA published by Chong et al. [11]. The following amino acid

Figure 1 Northern-blot analysis of RNA from various human tissues

Poly(A)⁺ RNA (5 μg) from various human tissues was resolved on a 1.2% agarose gel, transferred to a nylon membrane, and hybridized with ^{32}P -labelled NPT-1 cDNA (a) or β -actin (b). Lane 1, heart; lane 2, small intestine; lane 3, skeletal muscle; lane 4, kidney; lane 5, liver; lane 6, brain.

Table 1 P_i uptake in oocytes injected with NPT-1 and renal poly(A)⁺ RNA

Poly(A)⁺ RNA (50 ng) or cRNA (5 ng) was injected into oocytes. After 3 days of incubation, P_i transport was measured for 60 min. Values are means $\pm$ S.D. ($n = 5$).

Injection	P_i uptake (pmol/60 min per oocyte)	
	+ Na^{+}	– Na^{+}
Water	11.6 $\pm$ 2.6	5.6 $\pm$ 1.5
Poly(A) ⁺ RNA	32.9 $\pm$ 8.4	15.8 $\pm$ 4.6
NPT-1 cRNA	556 $\pm$ 142	18.9 $\pm$ 8.1

substitutions were noted in our clones: R37L, A38M, V79I, A90G, R206G and G209C.

The protein encoded by NPT-1 shows amino acid identity of 69%, 30% and 39% when compared with rabbit NaP_i -1 [4], rat NaP_i -2 [5] and human NaP_i -3 [5] respectively. No sequence homology was found with other P_i -transport systems from mitochondria [15] or *Escherichia coli* [16]. In NaP_i -1, alignment of five amino acid residues (G³⁴²/A³⁸¹-X-X-X-X-L³⁸⁶-X-X-X-P³⁹⁰-R³⁹¹) is consistent with a motif (SOB) proposed for Na^{+} -dependent transport systems [4,5]. In clone NPT-1, the G, L and R residues of this motif are present.

The expression of NPT-1 mRNA was analysed by Northern blotting of RNAs from various human tissues (Figure 1). A prominent RNA 2.0 kb in length was detected in kidney, liver and brain. Recently, Ghishan et al. [17] reported the presence of Na^{+} -dependent P_i transport in the hepatic basolateral membrane. In addition, injection of liver mRNA into *Xenopus* oocytes resulted in Na^{+} -dependent P_i transport. These results suggest that NPT-1 may also function as a prominent P_i transporter in the liver. NPT-1-specific mRNAs were not detected in heart, small intestine or skeletal muscle. These observations indicate that NPT-1 does not represent a ubiquitous housekeeping Na^{+} -dependent transport system for P_i [18].

NPT-1 and poly(A)⁺ RNA from human kidney cortex specifically stimulated Na^{+} -dependent P_i uptake in *Xenopus* oocytes (Table 1). Under conditions described in the Materials and methods section, Na^{+} -dependent P_i transport was also linear

Figure 2 Dependence of NPT-1 cRNA-induced P_i uptake on P_i concentration

Oocytes were injected with either 5 ng of NPT-1 cRNA per oocyte or 50 ng of poly(A)⁺ RNA from human kidney cortex per oocyte. At 3 days after injection, P_i uptake was measured within initial linear-uptake conditions (at 5 min) as a function of P_i concentration (a). The apparent K_m was obtained using the Michaelis-Menten equation [K_m for NPT-1-induced uptake, 0.29 ± 0.055 mM; K_m for poly(A)⁺ RNA-induced uptake, 0.22 ± 0.023 mM] (b). The data represent means $\pm$ S.D. obtained from six to eight oocytes per group of a representative experiment. ●, NPT-1 cRNA-induced uptake; ○, poly(A)⁺ RNA-induced uptake; □, water-induced uptake.

over the 60 min incubation period. A water-injected control showed little increase over this period.

We have compared the kinetic properties of P_i transport in NPT-1 cRNA- and poly(A)⁺ RNA-injected oocytes. Oocytes were injected with 5 ng of NPT-1 cRNA or 50 ng of poly(A)⁺ RNA, incubated for 3 days and assayed for P_i uptake (Figure 2). Substrate concentrations were varied between 0.01 and 50 mM P_i to determine K_m and V_{max} values for P_i transport in cRNA- and poly(A)⁺ RNA-injected oocytes. The values for the latter show significant variability as a result of low transport activity because of the short incubation time (5 min) and differences between individual oocytes. However, the K_m for P_i transport was not significantly different between cRNA- and poly(A)⁺ RNA-injected oocytes (0.29 and 0.22 mM respectively); V_{max} values were 1.2 and 6.8 pmol of P_i/min per oocyte, respectively.

In a second set of experiments, Na⁺ in the uptake solution was gradually replaced by choline, and P_i transport was determined in these oocytes (Figure 3a). First, we determined the K_d for Na⁺ in water-injected oocytes. Water-injected oocytes showed significantly different K_m and V_{max} values from those for cRNA-injected oocytes. Based on a kinetic analysis of Na⁺ interaction, P_i transport expressed by the cloned cRNA could be distinguished from intrinsic P_i transport of the oocytes, which exhibits an approximate K_d for Na⁺ of 10–20 mM (results not shown). The approximate K_d for Na⁺ after injection of NPT-1 cRNA was found to be around 45–50 mM, which is in good agreement with

Figure 3 Dependence on Na⁺ concentration and pH of NPT-1 cRNA-induced uptake

Oocyte injections were performed as described in Figure 2. At 3 days after injection, P_i uptake was measured within initial linear-uptake conditions (at 5 min) as a function of Na⁺ concentration (a). In the study of Na⁺-dependence, choline was used for iso-osmotic ionic replacement. The apparent K_d and Hill coefficient for Na⁺ interaction were obtained using the Hill equation [for NPT-1-induced uptake, $K_d = 44 \pm 6$ mM and $n = 2.4$ and for poly(A)⁺ RNA-induced uptake, $K_d = 58 \pm 10$ mM and $n = 2.2$]. In pH-dependence experiments (b), uptake was assayed in NaCl medium adjusted to different pH values as indicated [5]. The data represent the mean $\pm$ S.D. obtained from six to eight oocytes per group of a representative experiment. ●, NPT-1 cRNA-induced uptake; ○, poly(A)⁺ RNA-induced uptake; □, water-induced uptake.

the value for injection of total poly(A)⁺ RNA of human kidney cortex (Figure 3a). This suggests that NPT-1 is a brush-border-membrane Na⁺-dependent P_i co-transporter similar to NaP_i-3, with an approximate K_d for Na⁺ of 57 mM and K_m for P_i of 0.17 mM [5]. Magagnin et al. [5] reported that renal brush-border membrane Na⁺-dependent P_i transport has a particular pH-dependence; oocytes injected with NPT-1 cRNA clearly showed pH-dependent P_i transport activity (Figure 3b). The specificity of these effects was measured using Na⁺-dependent uptake of [¹⁴C]leucine as a control. The observed pH effect is specific for P_i uptake and is not observed for [¹⁴C]leucine uptake (results not shown); the pH effect on NaP_i-3 is greater than for NPT-1 [5].

Human kidney cortex poly(A)⁺ RNA was subjected to hybrid depletion with NaP_i-3 and NPT-1 antisense oligonucleotides before injection into oocytes and assay of P_i uptake. Hybrid depletion of NaP_i-3 mRNA resulted in 72% inhibition of P_i transport induced by poly(A)⁺ RNA (Table 2). In addition, injection of NaP_i-3 and NPT-1 antisense oligonucleotides resulted in almost complete inhibition of P_i transport. Sense oligomers had little effect on P_i transport. As a control, we measured uptake of L-[¹⁴C]leucine. Induction of L-leucine uptake into oocytes by kidney poly(A)⁺ RNA was unaffected by hybrid depletion of NaP_i-3 and NPT-1 mRNA.

Recently, Biber et al. [19,20] reported the immunological localization of NaP_i-1 in rabbit kidney. NaP_i-1 antibody-mediated fluorescence was restricted to the brush border of proximal tubular cells (S1, S2 and S3); no significant differences

Table 2 Effect of hybrid depletion of NaP_i-3 and NPT-1 mRNA on uptake of P_i and leucine reduced by human kidney poly(A)⁺ RNA in *Xenopus* oocytes

Oocytes were injected with water or 20 ng of human kidney. Before injection, poly(A)⁺ RNA was hybridized with each of the following primers (each at 40 μ M): a NaP_i-3 sense A (5'-CCCGCTCACACTGGGTCCAACATC-3') or antisense A (5'-GATGTTGCAACCCAGTGTGAGCGGG-3'), NaP_i-3 and NPT-1 sense B (5'-CCAGC(C/A)CCAGG(A/G)AGAAG(G/C)TG-3') or antisense B (5'-CA(G/C)CTTCT(C/T)CCTGG(G/T)GCTGG-3') oligonucleotide. This region was highly conserved between the two P_i transporters. At 3 days after injection, [³²P]P_i uptake was determined as described in the Materials and methods section. To assess L-[¹⁴C]leucine uptake, oocytes were incubated for 60 min at room temperature in assay medium containing 50 μ M L-[¹⁴C]leucine. Values represent means $\pm$ S.E.M. (P_i and leucine uptake, $n = 4$) per incubation of six to eight oocytes. * $P < 0.05$, ** $P < 0.005$ compared with control (water injection). ND, not determined.

Injection	Uptake (pmol/60 min per oocyte)	
	P _i	Leucine
Water	12.4 $\pm$ 4.8	134 $\pm$ 44
Poly(A) ⁺ RNA	44.9 $\pm$ 10.2**	988 $\pm$ 189**
Water	11.6 $\pm$ 5.1	ND
Poly(A) ⁺ RNA + antisense A	20.4 $\pm$ 6.6*	ND
Water	15.8 $\pm$ 2.9	ND
Poly(A) ⁺ RNA + sense A	46.2 $\pm$ 11.6**	ND
Water	14.2 $\pm$ 3.6	109 $\pm$ 31
Poly(A) ⁺ RNA + antisense B	10.9 $\pm$ 5.9	788 $\pm$ 194**
Water	14.9 $\pm$ 7.4	206 $\pm$ 84
Poly(A) ⁺ RNA + sense B	59.2 $\pm$ 19.6**	642 $\pm$ 150**

could be detected between the S1, S2 and S3 segments. Furthermore, the functional expression experiments in oocytes showed that NaP_i-1 might be a high-affinity transporter in rabbit kidney cortex [5]. Using an NaP_i-1-specific cDNA probe, they could not obtain hybridization signals with mRNA samples isolated from kidney cortex of rat, pig or mouse [5]. These results suggest that proteins encoded by genes with high homology to NaP_i-1 do not play a dominant role in P_i reabsorption in these other species [5]. In contrast, NaP_i-2/3 is expressed in human, rat, mouse and rabbit kidney cortex. Functional expression of NaP_i-2/3 in *Xenopus* oocytes has implicated two closely related proteins in renal brush-border Na/P_i co-transport in these species. In contrast with these results, we found NaP_i-1-related mRNA in rat and mouse kidney cortex by homology-based PCR (results not shown). More recently, Delisle et al. [21] reported that antibodies for NaP_i-1 specifically reacted with the proteins of brush-border membranes from rat, rabbit and bovine kidney cortex. These results suggest that at least two Na/P_i co-transporters might be present in kidney cortex in these species. Recent reports have suggested that such a multiplicity/heterogeneity in Na/P_i co-transporter does not necessarily reflect species differences, but might be explained by internephron differences (superficial versus deep) and intranephron locations (early versus late proximal tubules) [19–22]. Studies on functional expression in *Xenopus* oocytes have shown that NPT-1, like NaP_i-3, may be a component of the high-affinity system. In addition, hybrid depletion of human kidney poly(A)⁺ RNA with a NaP_i-3 and NPT-1 antisense oligonucleotide completely inhibits the poly(A)⁺ RNA-induced P_i uptake in oocytes. These findings strongly suggest the presence of two high-affinity Na/P_i co-transporters in human kidney cortex.

Recently, Werner et al. [23] reported that chronic P_i deprivation leads to increased brush-border-membrane P_i transport via an increase in the level of NaP_i-2 mRNA in rat kidney. Delisle et al. [21] have shown that an increase in renal P_i transport caused by dietary phosphate intake may occur without any change in the

amount of NaP_i-1 immunoreactive protein in the brush-border membrane from rat kidney cortex. We also suggest that NaP_i-2/3, but not NaP_i-1, may be regulated by a variety of hormones and by dietary phosphate (K. Miyamoto, unpublished work). Further studies will be needed to clarify the role of NaP_i-1-related protein in renal P_i transport.

People with X-linked hypophosphataemia show selectively impaired Na⁺-dependent P_i co-transport, and the mutant phenotype is confined to the brush-border membrane [24–26]. About half of the P_i transport function is lost; the $V_{\max}$, but not the K_m value is altered [24–26]. The mutation therefore affects high-affinity low-capacity P_i transport. Na⁺ affinity and stoichiometry for the transporter, response of transport to membrane potential, and pH-dependence are all similar in mutant and normal membrane [24–26]. Chong et al. [11] reported that NPT-1 maps to human chromosome 6p21.3-p23. NaP_i-3 maps the location to human chromosome 5 [27]. These results indicate that high-affinity Na/P_i co-transporters in human kidney are not mutated in X-linked hypophosphataemia. Scriver and Tenenhouse [25] have suggested that an inhibitory *trans*-acting product of the X-linked locus, affecting P_i transport and vitamin D metabolism, could explain the renal phenotype. Further studies will be needed to identify the mutant gene product in this disease.

Finally, the role of high-affinity Na/P_i co-transporters in hereditary hypophosphataemic rickets with hypercalciuria and hypophosphataemic bone disease, which are autosomally inherited disorders caused by defective reabsorption of P_i in the proximal tubule of the kidney, is under study.

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