Growth-related Renal Type II Na/P\textsubscript{i} Cotransporter*

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Inorganic phosphate (P\textsubscript{i}) is of critical importance to body functions, particularly during periods of growth. The kidneys contribute to the maintenance of the positive P\textsubscript{i} balance required for growth by reabsorbing a high fraction of the filtered P\textsubscript{i} (1). The capacity for Na\textsuperscript{+}-dependent phosphate cotransport across the luminal brush border membrane of renal proximal tubular cells is higher in juveniles than in adults (2, 3).

Several mammalian renal Na\textsuperscript{+}-dependent P\textsubscript{i} cotransporters have recently been isolated and characterized (4). The cDNAs of these transporters can be divided into three types (types I–III) in the kidney cortex (4). Type II Na/P\textsubscript{i} cotransporters belong to a unique class of Na\textsuperscript{+}/Pi cotransporter (type IIc). Microinjection of type IIc cRNA demonstrated sodium-dependent Pi cotransport activity. Affinity for P\textsubscript{i} was 0.07 mM in 100 mM Na\textsuperscript{+}. The transport activity was dependent on extracellular pH. In electrophysiological studies, type IIc Na/Pi cotransport was electroneutral, whereas type IIa was highly electrogenic. In Northern blotting analysis, the type IIc transcript was only expressed in the kidney and was highly expressed in weaning animals. In immunohistochemical analysis, the type IIc protein was shown to be localized at the apical membrane of the proximal tubular cells in superficial and midcortical nephrons of weaning rat kidney. Hybrid depletion experiments suggested that type IIc could function as a Na/P\textsubscript{i} cotransporter in weaning animals, but its role is reduced in adults. The finding of the present study suggest that the type IIc is a growth-related renal Na/P\textsubscript{i} cotransporter, which has a high affinity for P\textsubscript{i} and is electroneutral.

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In the present study, we isolated a cDNA from the human and rat kidney that encodes a growth-related Na\textsuperscript{+}-dependent inorganic phosphate (P\textsubscript{i}) cotransporter (type IIc). Microinjection of type IIc cRNA into Xenopus oocytes demonstrated sodium-dependent P\textsubscript{i} cotransport activity. Affinity for P\textsubscript{i} was 0.07 mM in 100 mM Na\textsuperscript{+}. The transport activity was dependent on extracellular pH. In electrophysiological studies, type IIc Na/P\textsubscript{i} cotransport was electroneutral, whereas type IIa was highly electrogenic. In Northern blotting analysis, the type IIc transcript was only expressed in the kidney and was highly expressed in weaning animals. In immunohistochemical analysis, the type IIc protein was shown to be localized at the apical membrane of the proximal tubular cells in superficial and midcortical nephrons of weaning rat kidney. Hybrid depletion experiments suggested that type IIc could function as a Na/P\textsubscript{i} cotransporter in weaning animals, but its role is reduced in adults. The finding of the present study suggest that the type IIc is a growth-related renal Na/P\textsubscript{i} cotransporter, which has a high affinity for P\textsubscript{i} and is electroneutral.

Experimental procedures

Animals and Diets—Male Wistar rats (3 weeks after birth) were purchased from Shizuoka Laboratory Animal Center (Shizuoka, Japan). They were housed in plastic cages and fed standard rat chow diet (Oriental, Osaka, Japan) ad libitum for the first week. After that period, they received a diet containing 1.2% calcium and 0.6% phosphorus for 5 days. On the 6th day, the following three groups of six rats each were established: the control Pi group, rats that were chronically treated with Pi for 5 days; the low Pi group, rats that were chronically fed a diet containing 0.6% P\textsubscript{i}; the low P\textsubscript{i} group, rats that received a diet containing a low percentage (0.02%) of P\textsubscript{i}; and the high P\textsubscript{i} group, in which the rats received a high percentage (1.2%) P\textsubscript{i} diet. After 7 days of the given diet, all of the rats were anesthetized with intraperitoneal pentobarbital, and their kidneys were removed rapidly.

cDNA Cloning—cDNAs for human expressed sequence tags (EST) (GenBankTM/EBI/DBJ accession no. AI792826), which we found in the course of EST database searches to show nucleotide sequence similarity to human type IIa Na/P\textsubscript{i} cotransporter, were obtained using IMAGE (integrated and molecular analysis of genomes and their expression). The ~0.8-kb Satt1 fragment was excised from human cDNA (IMAGE cDNA clone 1535299) and labeled with 32P using the Megaprime DNA labeling system, dCTP (Amersham Biosciences) for use as a probe to screen a human kidney 5'-Stretch Plus cDNA library (CLONTECH). Screening of the cDNA library and isolation of positive plaques were performed as described previously (10, 11).

The type IIc Na/P\textsubscript{i} cotransporter fragment (corresponding to nucleotides 89–600 of the nucleotide sequence) was used to isolate a rat cDNA for type IIc Na/P\textsubscript{i} cotransporter. The oligo(dT)-primed cDNA library was prepared from rat kidney poly(A)\textsuperscript{+} RNA using the SuperScript Choice system (Invitrogen) (12). The synthesized cDNA was ligated to AZIPLX EcoRI arms (Invitrogen). Screening of the cDNA library and isolation of the positive plaques were performed as described previously (12).

Xenopus Oocyte Expression—cRNAs obtained by in vitro transcrip-
Fig. 1. Cloning of Na/Pi cotransporter (type IIc). a, sequence alignment of type II Na/Pi cotransporters. The deduced amino acid sequence of type IIc Na/Pi cotransporter (human) is shown aligned with those of types IIa, IIb, and IIc cotransporters. Residues identical in at least two sequences are shaded. Lines under the sequences show predicted transmembrane regions of type IIc Na/Pi cotransporter, numbered 1–8. In type IIc Na/Pi cotransporter, putative N-linked glycosylation sites are marked by the # sign. Putative protein kinase C-dependent phosphorylation sites are located at residues 24, 152, 481, and 581 (labeled with *). The residue numbers are indicated beside the aligned sequences.

b, Northern blotting analysis in human tissues. High stringency Northern hybridization analysis using a human type IIc probe was performed against poly(A)^+ RNA from human tissues.

c, Northern blotting analysis in rat tissues. High stringency Northern hybridization analysis using a rat type IIc probe was performed against poly(A)^+ RNA from rat tissues.

d, developmental changes in rat renal type IIc mRNA levels. Lane 1, 5 days old; lane 2, 15 days; lane 3, 22 days; lane 4, 60 days.
tion using T7 RNA polymerase for the human type IIc cDNA (hNPIIc) and rat type IIa (NaPi-2) in plasmid pBluescript SK (-Stratagene) were linearized with XbaI as described previously (12). Xenopus oocyte expression studies and uptake measurements were performed as described previously (11, 12). The uptake rates of [32P]phosphorus were measured 2-3 days after injection of cRNA. For expression experiments, 25 ng of cRNA was injected into each oocyte. Xenopus oocyte expression was performed as described previously (11, 12).

P Uptake Measurements—Groups of six to eight oocytes were incubated in 500 μl of standard uptake solution (100 mM NaCl, 2 mM KCl, 1 mM CaCl2, 1 mM MgCl2, 10 mM HEPES, and 5 mM Tris, pH 7.4) or Na+-free uptake solution in which Cl- was replaced by choline chloride containing 0.1 M NaCl and rat type IIa Na/Pi cotransporter (control solution) or cRNA of human type IIc Na/Pi cotransporter (open bar) (10) were assayed after 2 days for uptake of Pi (100 μM) in 96 mM NaCl medium (n = 8 experiments). Values are means ± S.E. 3, 10, 30, 100, 300, and 1000 μM Pi, in standard uptake solution and plotted against the Pi concentration. The Pi uptake was saturable and fit the Michaelis-Menten curve. Values are means ± S.E. (n = 6 experiments). d, Cl concentration dependence of type IIc Na/Pi cotransporter-mediated Pi uptake was measured at 3, 10, 30, 100, 300, and 1000 μM Pi, in standard uptake solution and plotted against the Pi concentration. The Pi uptake was saturable and fit the Michaelis-Menten curve. Values are means ± S.E. (n = 6 experiments). e, pH dependence of type IIc Na/Pi cotransporter-mediated Pi uptake. The type IIc cotransporter-mediated uptake of Pi (100 μM) was measured in the standard uptake solution at various pH values. The uptake value was greatest at pH 7.5. Values are means ± S.E. (n = 5 experiments).

Antisense Hybrid Depletion—For hybrid depletion experiments, rat kidney poly(A)⁺ RNA (5 μg/μl) was denatured at 65 °C for 5 min in solution A (50 mM NaCl and a 20 μM concentration of a 16-mer oligonucleotide complementary to rat type II phosphate transporters) and further incubated at 42 °C for 30 min (13). The positions of sense oligonucleotide type IIa (5’-GTCCAGGGGAGAGGCC-3’, nucleotides +1004–1019), antisense type IIa (5’-GGCCCTCTACCCCTGGAC-3’, nucleotides +1004–1019), sense type IIc (5’-ATGCCCCCTGCTGACT-3’, nucleotides +134–149), and antisense type IIc (5’-AGTCCACGGCCCAA-3’, nucleotides +134–149) are complementary to the rat type IIa and type IIc mRNA sequence (7). The sample of poly(A)⁺ RNA was injected into the oocytes, and uptake measurements were performed as described previously (10, 11).

Immunoblotting Analysis—Brush-border membrane vesicles (BBMV) were prepared from rat kidney by the Ca²⁺ precipitation method as described previously (14). The levels of leucine aminopeptidase, Na⁺K⁺-ATPase, and cytochrome c oxidase were measured to assess the purity of the membranes. Protein samples were heated at 95 °C for 5 min in sample buffer in either the presence or absence of 5% 2-mercaptoethanol and subjected to SDS-polyacrylamide gel electrophoresis. The separated proteins were transferred electrophoretically on Hybond-P polyvinylidene difluoride transfer membranes (Amer sham Biosciences). The membranes were treated with diluted affinity-purified anti-type IIa (1:1000) (14) or type IIc (1:1000) NaPi2 cotransporter antibody and then with horseradish peroxidase-conjugated anti-rabbit IgG as the secondary antibody (Jackson ImmunoResearch Laboratories, Inc.). The signals were detected using the ECL Plus system (Amer sham Biosciences) (15).

The abbreviation used is: BBMVs, brush border membrane vesicles.
Immunohistochemistry—Immunohistochemical analysis of the rat kidney was performed as described previously with minor modification (15). For immunostaining, serial sections (5 μm) were incubated with affinity-purified anti-type IIa (1:4000) or type IIc (1:1000) Na/Pi co-transporter antibodies overnight at 4°C. Thereafter, they were treated with Envision rabbit peroxidase (Dako) for 30 min. To detect immunoreactivity, the sections were treated with diaminobenzidine (0.8 mM).

Anti-peptide Antibody—An oligopeptide (CYENPQVIASQQL) corresponding to amino acid residues 590–601 of rat type IIc Na/Pi cotransporter was synthesized. The C-terminal cysteine residues were introduced for conjugation with keyhole limpet hemocyanin. Rabbit anti-peptide antibodies were produced as described previously (14).

Statistical Analysis—Data are expressed as the mean ± S.E. Differences between experimental groups were determined by analysis of variance, and p values of <0.05 were accepted as indicating a significant difference.

RESULTS

Cloning of Type IIc Na/Pi Cotransporter—The human type IIc cDNA was 2020 bp long with an open reading frame of 1797 bp encoding 599 amino acids. Hydropathy analysis of the predicted amino acid sequence revealed the presence of eight putative transmembrane domains. The extracellular segments of human type IIc cotransporter contained four putative N-linked glycosylation sites. Potential intracellular phosphorylation sites for protein kinase C was detected at residues 24, 152, 481, and 581 (Fig. 1a).

Amino acids in the membrane-spanning regions were especially well conserved among the three isoforms. Amino acid comparisons revealed that the newly identified protein was 36–38% homologous to Na/Pi cotransporters identified in human type IIa and type IIb amino acid sequences, respectively (16, 17). Overall homology to types I and III Na/Pi cotransporters was ~10% (10, 18). The highest degrees of homology were detected in regions that have been suggested to be the membrane-spanning domains. The most striking difference in the newly identified protein compared with the type II Na/Pi cotransporters was found in the C-terminal region containing clusters of cysteine residues. A similar clustering of cysteine residues was also present in the type IIb Na/Pi cotransporters of human, mouse, and flounder kidney.

Tissue Distribution of Type IIc Na/Pi Cotransporter—The expression of type IIc mRNA was analyzed by Northern blotting using human multiple tissue Northern blot and poly(A)+ RNA from rat tissues (Fig. 1, b and c). Using the type IIc cDNA as a probe, a strong 2.4-kb signal was observed only in the kidney. No signals were detected in the brain, heart, skeletal muscle, thymus, spleen, lung, or peripheral blood leukocytes. In addition, the expression of the type IIc mRNA was significantly higher in weaning animals (22 days old) compared with those in adults (60 days old) (Fig. 1d). The levels of type IIc mRNA were lowest in suckling animals.

Functional Analysis of Type IIc Na/Pi Cotransporter—The
functional properties of human type IIc Na/Pi cotransporter were examined in *Xenopus* oocytes. As shown in Fig. 2, the microinjection of *Xenopus* oocytes with human type IIc Na/Pi cotransporter resulted in a marked increase relative to the level apparent in water-injected oocytes (Fig. 2a). [32P]Phosphate uptake mediated by human type IIc was dependent on Na\(^+\) but not Cl\(^-\) (Fig. 2b), and it increased in a concentration-dependent manner in the presence of Na\(^+\) (Fig. 2b). The uptake was saturable, and the Michaelis-Menten constant (K\(_M\)) for P\(_i\) was 70 \(\mu\)M (Fig. 2c). Type IIc-mediated Na/Pi uptake was stimulated by a more alkaline pH, a hallmark of proximal tubular Na/Pi cotransport (Fig. 2d). The apparent K\(_M\) and Hill coefficient for Na interaction was K\(_M\) = 48 ± 9 \(\mu\)M and n = 1.73, respectively (Fig. 2e).

**Electrophysiology of Type IIc Na/Pi Cotransporter**—Fig. 3 shows typical time courses of currents at a membrane potential of −60 mV during the addition of Pi. Superfusion of oocytes expressing the type IIa Na/Pi cotransporter with P\(_i\) exhibited currents that depended on the presence of external Na\(^+\). Such currents were not observed when the same protocol was applied to water or noninjected oocytes (data not shown). Washout of P\(_i\) was also accompanied by a similar biphasic return to the base-line values. Reversal potential shifted from −22 mV to +16 mV during stimulation with 1 mM P\(_i\), in type IIa Na/Pi cotransporter-expressing oocytes. These observations suggest that the currents stimulated by 1 mM P\(_i\) were Na\(^+\) currents. These findings confirmed that the previous observation that the Na/Pi cotransport by the type IIa cotransporter was electrogenic (19). In contrast, a superinfusion of oocytes expressing the type IIc Na/Pi cotransporter with P\(_i\) (0.1–3 mM) did not exhibit the currents. These observations suggested that, unlike type IIa, Na/Pi cotransport by the type IIc Na/Pi cotransporter is electroneutral.

**Western Blotting Analysis**—The molecular weight of type IIc Na/Pi cotransporter protein was determined by Western blotting analysis (Fig. 4a). In BBMVs isolated from the rat kidney (22 days old), the specific antibody reacted with a band of 80–85 kDa under reducing conditions (Fig. 4a). As measured by the presence of antigen peptides in the absorption experiments, the 80–85 kDa band disappeared (Fig. 4a). In addition, FLAG-fused type IIc Na/Pi cotransporter in COS-7 cells was observed as 85- and 160-kDa bands using FLAG-specific monoclonal antibody (Fig. 4b). The type IIc antibodies reacted with the 80–85 kDa protein band (data not shown). In addition, we examined whether the type IIc antibodies react with type IIa Na/Pi cotransporter protein. The type IIc antibodies did not react with any bands in the COS 7 cells expressing the type IIa or type IIb Na/Pi cotransporters (Fig. 4b).

Next, we investigated developmental changes in rat renal type IIc protein levels (Fig. 4c). Western blotting demonstrated that the amount of type IIc protein in the BBMVs was highest in weaning rats, lower in adult rats, and lowest in suckling rats. In Fig. 4c, BBMVs isolated from the kidney of a rat (40 days old) fed a diet low in P\(_i\) for 7 days were prepared and used for Western blotting. The amounts of type IIc transporter protein (80–85 kDa band) were significantly increased (by about 5-fold for the 80–85 kDa band) compared with those in rats fed the control diet. In contrast, the high P\(_i\) diet markedly suppressed the level of type IIc transporter protein.

**Immunohistochemistry**—Immunolocalization of type IIc Na/Pi cotransporter protein was performed with the kidneys of weaning rats (22 days old). In Fig. 5, a and h, expression of type IIc cotransporter immunoreactive protein was detected exclusively in the superficial and juxtamedullary nephron. The control antibodies did not stain it (data not shown). The highest expression was observed in convoluted proximal tubules. At higher magnification, it was evident that type IIc antibody-mediated immunoreactivity was localized in the brush border of proximal tubular cells and was completely absent in the basolateral membrane domain (Fig. 5g). Brush-border staining was slightly weaker in superficial nephrons than in juxtamedullary nephrons. In contrast, in weaning rats, type IIa-related immunoreactivity was detected only in juxtamedullary nephrons (Fig. 5e and d) but not in the superficial and midcortical regions. Type IIa-related immunostaining was observed in a subapical vesicular structure, which likely belongs to the vacuolar endocytic apparatus, in weaning rat kidney (Fig. 5h). In the adult kidney (Fig. 5e and f), type IIa-related immunoreactivity was detected only in juxtamedullary nephrons and not in the superficial and midcortical regions. Type IIa-related immunostaining was observed in midcortical and juxtamedullary nephrons in adult rats.

**Hybrid Depletion**—Evidence for the type IIc Na/Pi cotransporter was obtained by antisense experiment (Fig. 6). As described under “Experimental Procedures,” when pA\(^+\) mRNA isolated from the kidney of adult rats was treated with type IIa...
transporter antisense oligonucleotides of type IIa-specific mRNA, Na\(^+\)-dependent Pi uptake was completely suppressed in injected oocytes (Fig. 6a). In contrast, when poly(A)\(^+\) RNA isolated from the kidney of weaning rats was treated with type IIa antisense oligonucleotides, Pi uptake was still detected in injected oocytes (Fig. 6b). In contrast, type IIc antisense oligonucleotides significantly suppress Pi uptake in oocytes expressing poly(A)\(^+\) RNA from weaning rat kidney (Fig. 6d). However, similar treatment did not affect Pi uptake in oocytes expressing poly(A)\(^+\) RNA from adult rat kidney (Fig. 6c).

**DISCUSSION**

Tubular Pi reabsorption decreases during aging as has been indicated by metabolic balance studies, clearance studies, and studies with isolated vesicles (1–3). This decrease is due to a reduction in the \(V_{\text{max}}\) without a change in the apparent \(K_s\) for \(P_i\) of the brush border membrane Na/P\(_i\) cotransport. Kinetic properties and pH dependence of type IIc-mediated Na/P\(_i\) cotransport favor this protein as a candidate for a Na/P\(_i\) cotransporter involved in a high Pi transport activity in weaning animals (4).

In addition, our characterization of the kinetics of the type IIc transporter gave findings consistent with those reported in the BBMV studies (20, 21). As reported for the renal type IIa Na/P\(_i\) cotransporter, superfusion of oocytes expressing the type IIa Na/P\(_i\) cotransporter with \(P_i\) exhibited an inwardly directed current that was dependent on the presence of Na\(^+\) and the steady-state holding potential (19). However, type IIc mediated Na/P\(_i\) cotransport was electroneutral. The apparent \(K_s\) and Hill coefficient for Na\(^+\) interaction were obtained using the Hill equation (for human type IIc-mediated uptake, \(K_s = 48 + 9\) mm, and \(n = 1.73\)). Busch et al. (22) characterized the electronegativity by expressing the type IIa Na/P\(_i\) cotransporter (NaPi-2) cloned from rat kidney in Xenopus oocytes. They showed that in the mandatory presence of extracellular Na\(^+\), Pi induced an inward current (I\(_p\)) for membrane potentials (V) in the range of \(-80 < V < +10\) mV. Consistent with the findings from BBMVs, the magnitude of I\(_p\) depended on the substrate concentrations, extracellular pH, and membrane potential. However, in contrast to the 2:1 stoichiometry to Na/P\(_i\) at pH 7.4 proposed from BBMV studies (23, 24), findings of a Hill slope close to 3 for the Na\(^+\) dose response at saturating Pi suggested a 3:1 stoichiometry for type IIa Na/P\(_i\) cotransport at \(-50\) mV. In contrast, the present findings suggest that type IIc has the 2:1 stoichiometry to Na/P\(_i\) at pH 7.4 as proposed from BBMV studies (23, 24).

The physiological significance of an electroneutral Na/P\(_i\) co-
transporter during growth is unknown. An electroneutral transporter would transport less $P_i$ across the apical membrane of the proximal tubule, as the driving force for $Na^+$ would be less. Two factors oppose the entry of $P_i$ from the tubular lumen into the cell, the inside negative cell potential and the high intracellular $P_i$ concentration. The intracellular $P_i$ concentration measured in isolated perfused kidneys using nuclear magnetic resonance (NMR) was significantly lower in growing animals than in adults. This provides a greater driving force for an electroneutral $Na/P_i$ cotransporter in growing animals. However, further studies are needed to clarify the role of the electroneutral $Na/P_i$ cotransporter in $P_i$ transport.

The type IIc transporter protein is detected in the apical membrane of renal proximal tubular cell in adult rats. Western blot analysis also shows that type IIc $Na/P_i$ cotransporter is present in the BBMVs from adult rat kidneys. However, in the hybrid depletion experiment, type IIc antisense oligonucleotide did not affect the $P_i$ uptake in oocytes induced by microinjection of renal poly(A)$^+$ RNA from adult rat kidneys. However, in the BBMVs from adult rat kidneys. The present findings suggest that a high $P_i$ diet sup-

expression of type IIc $Na/P_i$ cotransporter in the kidney of weaning rats may support high $P_i$ transport activity in weaning animals during down-regulation of the type IIa $Na/P_i$ cotransporter.

It is possible that the induction of type IIc protein in superficial nephrons in the weaning rats not only may be related to the developmental stage but also may be affected by the different $P_i$ contents of the available food. The sucking rats were fed exclusively with rat milk (0.2% $P_i$), and from day 20, the rats were fed the standard laboratory diet with a $P_i$ content of 0.6% (29). Supplementing the $P_i$ content may induce the type IIc protein in the weaning kidney. We therefore investigated the effect of dietary $P_i$ on the amount of type IIc protein. The findings of the present study suggest that a high $P_i$ diet sup-

Age dependence was also observed at the level of type IIa $Na/P_i$ cotransporter protein expression (1, 26–28). In the kid-

expression of type IIc $Na/P_i$ cotransporter in the kidney of weaning rats may support high $P_i$ transport activity in weaning animals during down-regulation of the type IIa $Na/P_i$ cotransporter.

Recently, Hoag et al. (9) examined the effect Npt2 gene knock-out on age-dependent BBMVs $Na/P_i$ cotransport and expression of $Na/P_i$ cotransporter genes Npt1, Glr-I, and Ram-1 (9). At all ages, $Na/P_i$ cotransport in Npt2--/-- mice is 15% of that in Npt2+/+ littermates. They concluded that Npt2--/-- mice cannot be compensated for by the age-depend-

Finally, the findings presented herein illustrate the mechanism by which the weaning kidney achieves the high rates of $P_i$ reabsorption required for the maintenance of a positive external balance. In this study, the type IIc was a growth-related
renal Na/Pi cotransporter, which is highly expressed in the weaning kidney.

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