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Expression cloning of human and rat renal cortex Na/P_i cotransport

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ABSTRACT We have isolated two cDNA clones, NaPi-2 and NaPi-3, by screening rat kidney cortex and human kidney cortex cDNA libraries, respectively, for expression of sodium-dependent phosphate transport in *Xenopus laevis* oocytes. Substrate specificity and a detailed kinetic analysis (Na, P_i, H^+ concentrations) suggested that expressed uptake activities relate to proximal tubular brush border membrane Na/P_i cotransport. NaPi-2 cDNA contains 2464 bp encoding a protein of 637 aa; NaPi-3 cDNA contains 2573 bp encoding a protein of 639 aa. NaPi-2- and NaPi-3-deduced protein sequences show high homology to each other but are different from the protein sequence deduced from the previously cloned NaPi-1 cDNA (from rabbit proximal tubules). Hydropathy profile predictions suggest at least eight membrane-spanning regions in NaPi-2/3-related proteins. *In vitro* translation results in proteins of the expected size and suggests glycosylation. Northern blot analysis shows corresponding mRNA species (≈2.7 kb) in kidney cortex of various species but no hybridization with RNAs isolated from a variety of other tissues (including intestinal segments); a hybridization signal (≈4.8 kb) was observed only in the lung (human). We conclude that we have structurally identified two closely related proteins most likely involved in human and rat renal brush border Na/P_i cotransport.

Brush border membrane Na/P_i cotransport is crucially involved in renal proximal tubular P_i reabsorption and is also a target for different cellular mechanisms involved in physiological regulation of tubular P_i reabsorption (for review, see refs. 1 and 2); some genetic defects in renal P_i handling might also be related to alterations in this transport step (3). On the basis of studies with isolated brush border membrane vesicles, it can be assumed that there is a "multiplicity" (heterogeneity) in Na/P_i cotransport, similar to that suggested for other brush border membrane Na/solute cotransport systems (e.g., Na/D-glucose; refs. 4–7; for review, see refs. 1 and 2).

We have recently cloned a cDNA (NaPi-1) from a rabbit kidney cortex cDNA library that, after *in vitro* transcription [complementary RNA (cRNA)] and oocyte injection (*Xenopus laevis*), induced Na-dependent P_i transport (8, 9). We documented that NaPi-1-related mRNA is expressed only in the rabbit kidney proximal tubules (10) and that a NaPi-1-specific antibody recognizes only a brush border protein (11, 12). Thus, NaPi-1 seems to be a likely candidate for one of the brush border membrane Na/P_i cotransporters, at least in the rabbit. Using a NaPi-1-specific cDNA probe, we could not obtain "clear" hybridization signals (to allow a "homology-based" cloning) with mRNA samples isolated from kidney cortex of other species (e.g., rat, pig, and mouse), suggesting that proteins with high homology to NaPi-1 do not play a dominant role in P_i reabsorption in these other species (A.W., J.B., and H.M., unpublished observations). Therefore, we reinitiated an expression cloning strategy, starting from rat and human kidney cortex cDNA libraries. We have isolated rat (NaPi-2) and human (NaPi-3) cDNAs,† based on their potency to express Na-dependent P_i transport after injection of cRNA into *X. laevis* oocytes. The corresponding mRNA species (≈2.7 kb) are predominantly present in kidney cortex of various species, and the corresponding proteins (NaPi-2/3; ≈70 kDa) are highly homologous to each other. Because the characteristics of expressed activities closely resembled brush border membrane transport properties (e.g., characteristic pH dependence), we conclude that NaPi-2/3-related proteins represent major brush border membrane Na/P_i cotransport systems present in the proximal tubules of various species.

METHODS

***X. laevis* Oocytes and Transport Assay.** All techniques and methods concerning the handling of oocytes as well as the assay for transport have been described (8, 9, 13). Oocytes were injected with 50 nl of H_2O containing no or 0.005–1 μg of cRNA per ml. Occasionally we also injected total and/or size-fractionated rat or human kidney cortex poly(A)$^+$ RNA (maximally 20 ng per oocyte). After 1–5 days, uptake of the substrates $H^{32}PO_4^{2-}$, $^{35}SO_4^{2-}$, and L-[2,3-^{3}H]arginine (New England Nuclear Radiochemicals) was measured either in the presence or absence of sodium as described (8, 9, 13–15).

Isolation of RNA and mRNA. RNA was extracted from different tissues according to the protocol described elsewhere (13, 14), and when specified poly(A)$^+$ RNA was isolated and size fractionated by sucrose density gradient centrifugation as described (8, 13, 16). Human kidney cortex mRNA was isolated from histologically normal cortical tissue obtained from a tumor-nephrectomized patient. For Northern blot analysis using mRNAs from different human tissues, we used human multiple tissue Northern blots (Clontech; no. 7760-1) containing poly(A)$^+$ RNA from eight tissues (2 μg per lane).

Construction and Screening of cDNA Libraries. Directional cDNA libraries were constructed by using size-selected rat and human poly(A)$^+$ RNA that had been shown to maximally induce expression of Na-dependent P_i-transport activity in oocytes (size fraction containing mostly 2- to 3-kb fragments; data not shown). cDNA libraries were constructed by using a commercial kit (SuperScript plasmid system, pSPORT 1 vector; GIBCO/BRL) and precisely following the supplier's instructions. Libraries contained about 2×10^5 colonies, of which 40,000 were screened by sib selection procedures; initial pools for screening contained about 1000 colonies. Plasmid DNA was isolated by standard procedures (alkaline lysis and using Qiagen columns; Kontron, Zürich). Plasmids

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Abbreviation: cRNA, complementary RNA.
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†The sequences discussed in this paper have been deposited in the GenBank data base (accession nos. L13257 and L13258).

were linearized with *Not* I and used for *in vitro* synthesis of cRNA, including capping using T7 RNA polymerase (Promega) (17). Synthesized cRNA was dissolved in H_2O for further use.

DNA Sequencing. Sequencing was carried out by the chain-termination method using a T7 polymerase sequencing kit (Pharmacia). NaPi-2/3 cDNAs were digested with either *Alu* I, *Pal* I, *Sau*3A, or *Rsa* I and subcloned into Bluescript SK^+ (Stratagene). The cDNA inserts were sequenced in both directions. Synthetic oligonucleotides were used as primers to complete the sequence.

Northern Analysis. Total RNA (≈20 μg) or $poly(A)^+$ RNA (2–5 μg) was denaturated, electrophoresed in 1% agarose/formaldehyde gels, and transferred to GeneScreen*Plus* (DuPont/NEN). cDNA probes of NaPi-2/3 (full length) were labeled by random priming (Pharmacia) using [α-^{32}P]dCTP (Amersham). Blots were prehybridized and hybridized in a buffer containing 5× standard saline phosphate/EDTA, 1% SDS, 5× Denhardt's solution, herring sperm DNA at 0.2 mg/ml, and 50% (vol/vol) formamide overnight at 42°C. After hybridization, blots were washed four times in 2× standard saline citrate (SSC)/0.1% SDS at room temperature and then twice for 10 min each in 0.4× SSC/0.1% SDS at 50°C.

***In Vitro* Translation.** *In vitro* translation of NaPi-2 and NaPi-3 cRNAs was performed with a rabbit reticulocyte lysate system in the absence and presence of canine pancre-

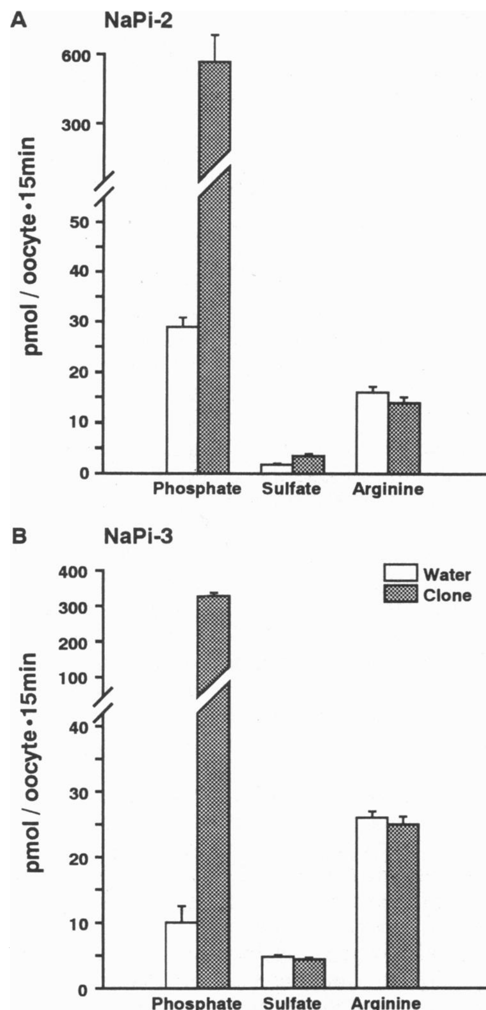

FIG. 1. Expression of transport activities by NaPi-2 (rat) and NaPi-3 (human) cRNAs. Oocytes injected either with water (open bars) or cRNA (1 ng per oocyte) related to NaPi-2 (*A*) or NaPi-3 (*B*) (stippled bars) were assayed after 2 days for uptake of phosphate (0.5 mM), sulfate (0.5 mM), and arginine (0.05 mM) in 100 mM NaCl medium. The bars represent the mean ± SE obtained from six to eight oocytes per group of a representative experiment. The cRNA-dependent increases in P_i uptake are entirely related to an increase in Na-dependent uptake (data not shown; see Fig. 3).

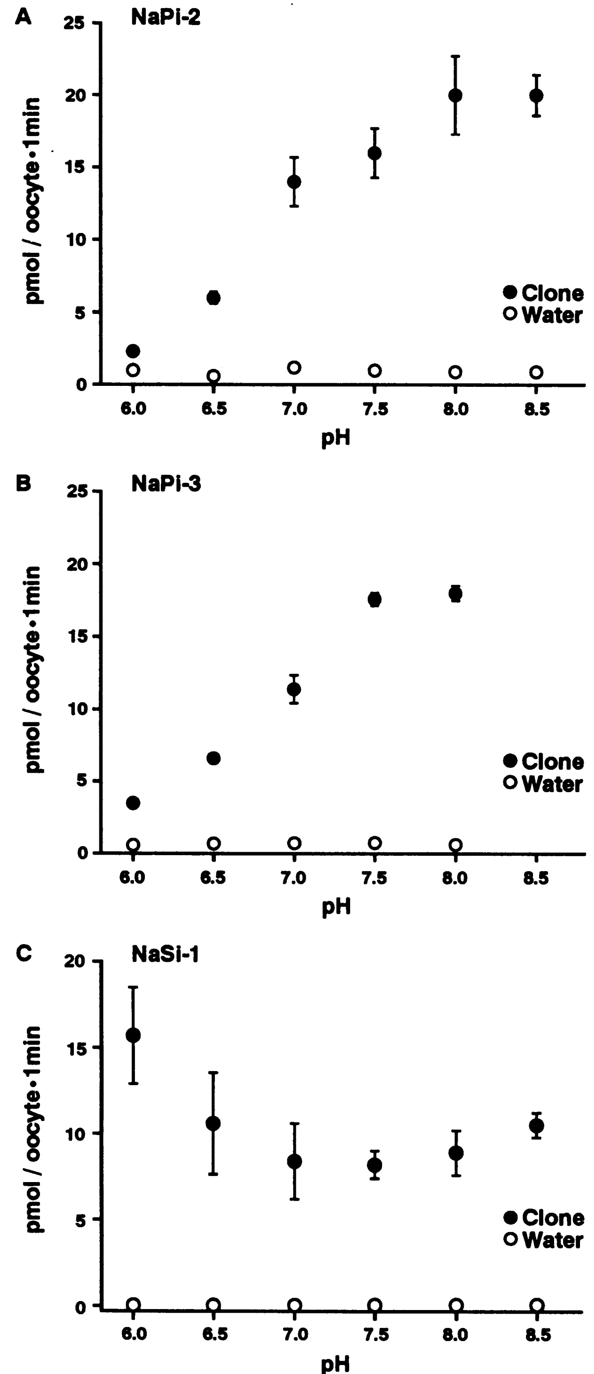

FIG. 2. pH dependence of NaPi-2 (rat) and NaPi-3 (human) cRNA-induced Na-dependent P_i uptake and comparison with NaSi-1 (rat) cRNA-induced Na-dependent sulfate uptake. Oocytes injected with either water (○) or 1 ng of cRNA per oocyte (●) were assayed 4 days later for uptake of 0.5 mM phosphate (*A* and *B*) and 0.5 mM sulfate (*C*). Uptake was assayed in NaCl medium adjusted to different pH values as indicated. The data represent the mean ± SE obtained from six to eight oocytes per group of a representative experiment. NaSi-1 cRNA was transcribed from the NaSi-1 cDNA recently cloned from the same library as NaPi-2; NaSi-1 is most likely related to brush border membrane Na/sulfate cotransport (25). Expressed uptake entirely relates to Na-dependent uptake (data not shown; see also Fig. 3; ref. 25).

atic microsomes (Promega); we followed the supplier's protocols with minor modifications as described (18).

RESULTS AND DISCUSSION

First, we injected poly(A)$^+$ RNA isolated from either human or rat kidney cortex into oocytes and observed, similar to our previous studies on rabbit kidney cortex mRNA (8, 9), expression of Na-dependent P_i uptake in oocytes. The activity was 3- to 4-fold greater than the intrinsic activity when total mRNA was used and 7- to 10-fold greater when size-fractionated mRNA (fractions containing mostly 2- to 3-kb mRNA species; injection of 15–20 ng, 3–4 days after injection; data not shown) was used.

We screened rat and human cDNA plasmid libraries by sib selection procedures on the basis of expression of Na-dependent P_i transport (in *X. laevis* oocytes). As shown in Fig. 1, we obtained cDNA clones that specifically stimulate Na-dependent P_i uptake (NaPi-2 from rat, Fig. 1*A*; NaPi-3 from man, Fig. 1*B*). Injection of 1 ng of cRNA led to >20-fold stimulation of P_i uptake (compared to intrinsic uptake of H_2O-injected oocytes) within 2 days. Transport of sulfate or L-arginine (Fig. 1) or L-leucine and 3-*O*-methyl-D-glucose (data not shown) was not stimulated. The observed increase in P_i transport after injection of NaPi-2 or NaPi-3 cRNA was entirely due to an increase in Na-dependent uptake (data not shown; see also Fig. 3). Initial characterization of cRNA-induced Na-dependent P_i uptake documented that the magnitude of expressed uptake is linearly related to the amount of cRNA injected (up to 10 ng, assayed 2 days after injection; data not shown) and to the time of expression (up to 3 days after injection 1 ng of cRNA injected; data not shown). Furthermore, under standard conditions used throughout the present study (1–2 ng of cRNA injected, up to 4 days of expression), Na-dependent P_i transport was also linear in the time of uptake (up to 60 min; data not shown).

Renal brush border membrane Na-dependent P_i transport has a particular pH dependence (increased flux rates with increasing pH; refs. 19 and 20), which is different from nonpolarized plasma membrane Na/P_i cotransport (e.g., refs. 21 and 22) and small intestinal brush border membrane Na/P_i cotransport (e.g., refs. 23 and 24). Therefore, a study of the pH dependence of expressed uptake represents a most useful criterion to relate expressed Na-dependent P_i uptake to renal brush border membrane Na/P_i cotransport. Fig. 2 *A* and *B* documents a strong increase of expressed NaPi-2/3-related Na-dependent P_i uptake with increasing pH of the uptake medium. Because these experiments do have the risk of unspecific effects (e.g., related to altered driving forces), (*i*) we made these experiments at a very brief incubation period (5 min; initial linear uptake), and (*ii*) we used as a control expressed Na-dependent sulfate uptake (related to a recently cloned cDNA; ref. 25). As observed, this particular pH effect is specific for P_i uptake and not observed for sulfate uptake; in the pH range between 6.5 and 8, transport of sulfate is practically unaffected while phosphate transport is more than doubled by increasing the pH. Thus, the observed pH dependence of NaPi-2 and NaPi-3 cRNA-induced Na-dependent P_i

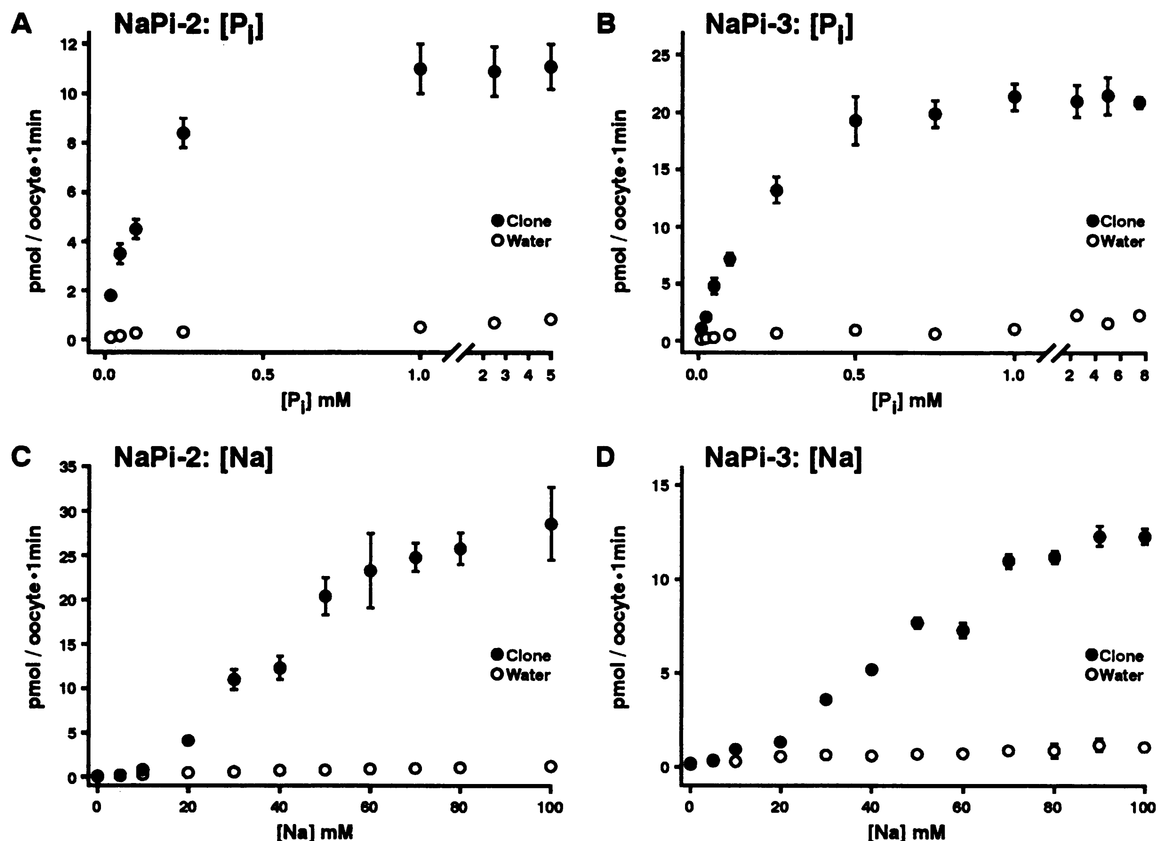

FIG. 3. Na and P_i concentration dependence of NaPi-2 and NaPi-3 cRNA-induced uptake. Oocytes were injected with either 1 ng of NaPi-2 cRNA (*A* and *C*) per oocyte or with 1 ng of NaPi-3 cRNA (*B* and *D*) per oocyte. Three days after injection, P_i uptake was measured within initial linear uptake conditions as a function of P_i concentration (*A* and *B*) or as a function of Na concentration (*C* and *D*). In the study of Na dependence, choline was used for isoosmotic ionic replacement. For P_i interaction, the apparent K_m was obtained by curve fitting according to the Michaelis–Menten equation (K_m for NaPi-2-induced uptake, 0.130 ± 0.015 mM; K_m for NaPi-3-induced uptake, 0.170 ± 0.023 mM). The apparent affinity and the Hill coefficient for Na interaction were obtained by curve fitting on the basis of the Hill equation (for NaPi-2 induced uptake, K_d = 42 ± 7 mM and n = 2.5; for NaPi-3 induced uptake, K_d = 57 ± 13 mM and n = 2.1). The values given are means ± SE obtained from a group of six to eight oocytes for each condition and are from a representative experiment.

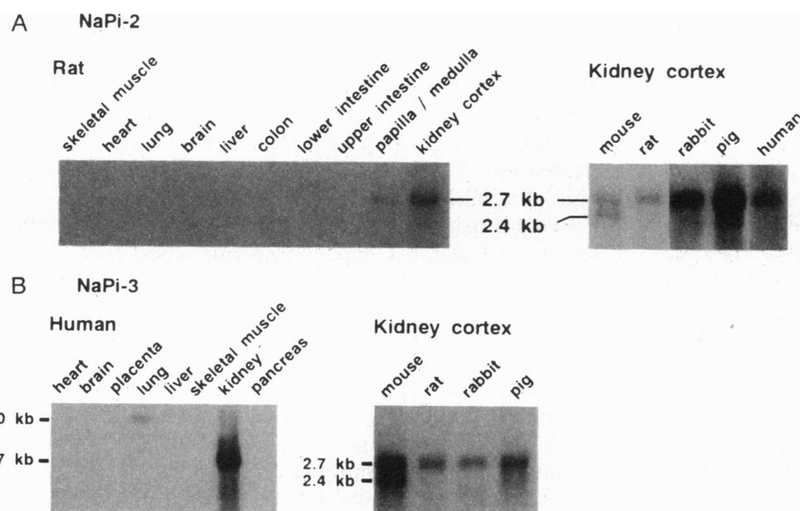

FIG. 4. Northern blot hybridizations using NaPi-2 cDNA and NaPi-3 cDNA probes. (*A Left*) In the rat, only mRNA species of 2.7 kb from kidney cortex and kidney medulla/papilla hybridize with the NaPi-2 cDNA probe; the signal obtained with kidney cortex is much more intense. (*A Right*) Mouse kidney cortex transcripts of two sizes (2.4 and 2.7 kb) hybridized with the NaPi-2 cDNA probe, whereas rabbit, pig, and human kidney cortex mRNA showed only one signal (2.7 kb), even after a longer exposure. (*B Left*) A NaPi-3 cDNA probe hybridized to mRNA species present in human kidney (2.7 kb) and human lung (5 kb). (*B Right*) The hybridization of a NaPi-3 cDNA probe with kidney cortex mRNAs from different species is similar to that shown for NaPi-2.

uptake suggests a close structural relationship of the cloned cDNAs (proteins) to the brush border transport function.

To further characterize NaPi-2 and NaPi-3 cRNA-induced uptake, we studied Na dependence as well as P_i dependence (Fig. 3); P_i dependence showed "simple" saturation kinetics, whereas Na dependence was sigmoidal. The calculated kinetic constants (K_m and K_d values and Hill coefficients; see legend to Fig. 3) were very similar to those obtained in studies with isolated brush border membrane vesicles (for review, see refs. 1, 2, 26, and 27).

Fig. 4*A* shows Northern blots using a NaPi-2 (rat) cDNA probe; a hybridization signal of ≈2.7 kb was observed in only renal tissue, with a high signal in cortex as compared to papilla/medulla. In Northern blots using RNA samples from kidney cortex of different species, a 2.7-kb signal was present in mouse, rabbit, pig, and human; in mouse a second band at 2.4 kb was seen. Using a NaPi-3 (human) cDNA probe, a similar (human) tissue distribution and similar species homologies were observed (compare *A* and *B* in Fig. 4); a signal at ≈5 kb was observed in human lung, suggesting the existence of a renal brush border type Na/P_i cotransport in alveolar epithelia (similar to the presence of Na/D-glucose cotransport; ref. 28). The 2.7-kb signal observed with either NaPi-2 or NaPi-3 cDNA probes with rabbit mRNA (Fig. 4) is different from the 1.9-kb signal observed with a NaPi-1 cDNA probe (data not shown; ref. 9).

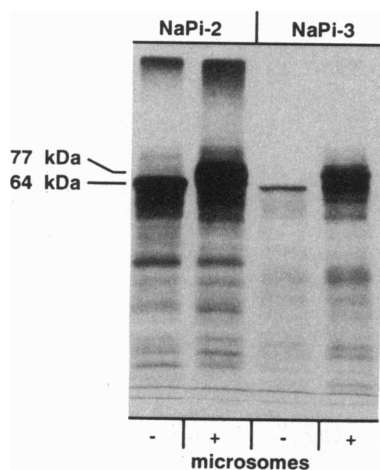

FIG. 5. *In vitro* translation of NaPi-2 and NaPi-3 cRNA. The reaction was performed in the absence (−) or presence (+) of canine pancreatic microsomes. Core-glycosylated forms (≈77 kDa for NaPi-2 and NaPi-3) and the nonglycosylated forms (≈64 kDa for NaPi-2 and NaPi-3) are indicated.

Fig. 5 shows the *in vitro* translation products of NaPi-2 and NaPi-3 cRNAs. In the absence of glycosylation (without microsomes), the major NaPi-2- and NaPi-3-dependent translation products are slightly >60 kDa, and in the presence of microsomes, the two proteins seem to be core-glycosylated.

DNA sequencing of NaPi-2 and NaPi-3 cDNA inserts in pSPORT 1 plasmids was performed. The sequences were deposited in the GenBank data base (accession numbers: NaPi-2, L13257; NaPi-3, L13258). NaPi-2 cDNA is 2464 bp long, and NaPi-3 cDNA is 2573 bp in length. The open reading frame of NaPi-2 cDNA (nucleotides 54–1964) encodes a protein of 637 aa (calculated M_r of 68,703; Fig. 6), and the open reading frame of NaPi-3 cDNA (nucleotides 82–1998) encodes a protein of 639 aa (calculated M_r of 68,933; Fig. 6).

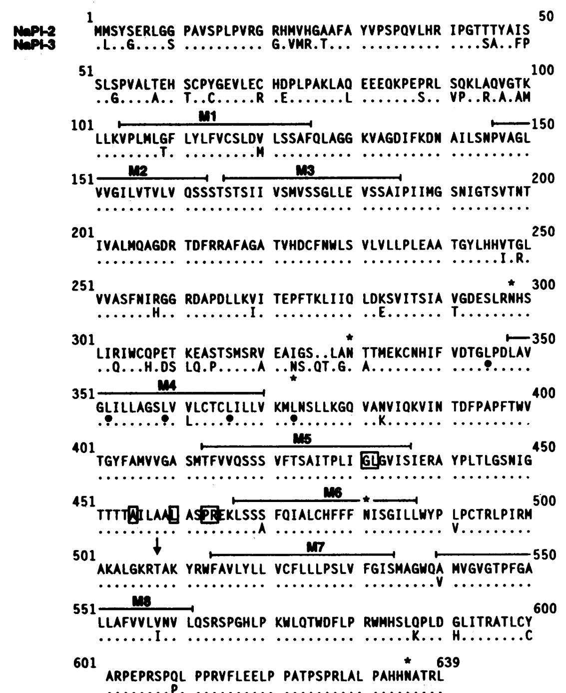

FIG. 6. Deduced amino acid sequences of the Na/P_i cotransporters of rat (NaPi-2) and human (NaPi-3) kidney cortex. The proposed membrane-spanning domains are numbered M1–M8. Stars are placed at potential N-glycosylation sites. The 6 aa of a likely SOB motif (29) are boxed. The membrane-spanning domain M5 contains five leucine residues representing a leucine zipper motif (●). A potential phosphorylation site for protein kinase C exposed to the cytoplasmic surface and common to NaPi-2 and NaPi-3 (Thr-508) is marked by an arrow.

Comparison of NaPi-2 and NaPi-3 cDNAs revealed 81% identity within the open reading frames and 40% identity in the 3′ untranslated regions.

The deduced amino acid sequences of the renal Na/Pi cotransporters NaPi-2 (rat) and NaPi-3 (human) are shown in Fig. 6. The two sequences are highly homologous: 95% similarity and 81% identity. The major differences between the amino acid sequences of NaPi-2 and NaPi-3 are found in the N-terminal region and between amino acids 300 and 330. A secondary structure analysis of the NaPi-2/3-related proteins, which was based on a combination of hydropathy analysis (30, 31) and the inside-positive rule (32, 33), predicts at least eight membrane-spanning domains, with both termini exposed at the cytoplasmic surface. Such a secondary structure prediction would locate two potential N-glycosylation sites within the 190- to 340-aa stretch at the extracellular surface.

Sequence comparison with proteins in the data bases (European Molecular Biology Laboratory and Swiss-Prot) did not show significant identity to other nonmammalian or mammalian membrane transport systems, such as the Na/D-glucose cotransporter and Na/Cl γ-aminobutyric acid transporter families (34–36). Direct comparison with other membrane transport systems (BESTFIT) revealed some identity to the Na/glutamate transporter of *Escherichia coli* (23%; ref. 29) and the mitochondrial H/P_i symporter of rat (24%; ref. 37) and *Saccharomyces cerevisiae* (22%; ref. 38). Only 20% overall identity of the NaPi-2/3 proteins to the recently cloned Na/P_i cotransport system of rabbit kidney cortex (NaPi-1; ref. 9) was found; highest identity (40%) was found between aa 418–473 of NaPi-2/3 and 331–386 of NaPi-1. The regions of highest identity between the NaPi-2/3 and NaPi-1 proteins both contain an SOB-like motif (boxed in Fig. 6), represented by 6 aa conserved among several bacterial and mammalian Na-coupled transporters (29, 34), which suggests a possible functional significance of these regions in the Na-coupled transport of P_i.

On the basis of the above data, we conclude that we have cloned human and rat cDNAs closely related to human and rat kidney proximal tubular brush border Na/P_i cotransporters; tissue distribution as well as characteristics of expressed activity supports this conclusion. NaPi-2 (rat) and NaPi-3 (human) cDNAs and their related proteins are highly homologous, and the small differences between them seem to be due to species variations. However, although some structural similarities between NaPi-1 (ref. 9; rabbit) and NaPi-2/3 do exist, it must be concluded that these are different molecules and therefore may reflect (at least in the rabbit) further evidence for a multiplicity in renal brush border membrane Na/P_i cotransporters (see above). In this respect, future studies on the physiological regulation of renal P_i reabsorption (e.g., by parathyroid hormone or due to P_i deprivation) as well as studies on genetic abnormalities in renal phosphate handling will also have to take into account such a multiplicity of Na/P_i cotransporters at the genetic level.

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