Identification of Three Isoforms for the Na⁺-dependent Phosphate Cotransporter (NaPi-2) in Rat Kidney*

(Received for publication, May 28, 1998, and in revised form, July 16, 1998)

Sawako Tatsumi, Ken-ichi Miyamoto‡, Tomoko Kouda, Keiko Motonaga, Kanako Katai, Ichiro Ohkido, Kyoko Morita, Hiroko Segawa, Yoshiko Tani, Hironori Yamamoto, Yutaka Taketani, and Eiji Takeda

From the Department of Clinical Nutrition, School of Medicine, Tokushima University, Tokushima 770, Japan

We have isolated three unique NaPi-2-related protein cDNAs (NaPi-2a, NaPi-2b, and NaPi-2γ) from a rat kidney library. NaPi-2a cDNA encodes 337 amino acids which have high homology to the N-terminal half of NaPi-2 containing 3 transmembrane domains. NaPi-2b encodes 327 amino acids which are identical to the N-terminal region of NaPi-2 containing 4 transmembrane domains, whereas the 146 amino acids in the C-terminal region are completely different. In contrast, NaPi-2γ encodes 268 amino acids which are identical to the C-terminal half of NaPi-2. An analysis of phage and cosmid clones indicated that the three related proteins were produced by alternative splicing in the NaPi-2 gene. In a rabbit reticulocyte lysate system, NaPi-2 α, β, and γ were found to be 36, 36, and 29 kDa amino acid polypeptides, respectively. NaPi-2a and NaPi-2γ were glycosylated and revealed to be 45- and 35-kDa proteins, respectively. In isolated brush-border membrane vesicles, an N-terminal antibody was reacted with 45- and 40-kDa, and a C-terminal antibody was reacted with 37-kDa protein. The sizes of these proteins corresponded to those in glycosylated forms.

A functional analysis demonstrated that NaPi-2γ and -2α markedly inhibited NaPi-2 activity in Xenopus oocytes. The results suggest that these short isoforms may function as a dominant negative inhibitor of the full-length transporter.

Renal phosphate (Pᵢ) reabsorption is an essential aspect of the maintenance of plasma Pᵢ homeostasis (1). Several mammalian renal Na⁺-dependent Pᵢ cotransporters have recently been isolated and well characterized (2–7). The cDNA of these transporters can be divided into at least three types (types I–III) in the kidney cortex. It has been demonstrated that the type II transporter is a major functional Na⁺-dependent phosphate cotransporter in the proximal tubules (8–12). The rat (NaPi-2), human (NaPi-3), and murine (NaPi-7) type II Na/Pᵢ cotransporter showed common characteristics in electrophysiological studies (13, 14). The apparent Kᵢ for Pᵢ and Na⁺ in the type II Na/Pᵢ transporter are in good agreement with previous Pᵢ uptake studies in vesicles of renal brush-border membranes (15, 16).

In contrast, there are several conflicting results regarding the molecular structure and regulation of type II Na/Pᵢ cotransporter (8, 17–19). Concerning the molecular structure of NaPi-2, its related proteins have been identified and partly characterized from the kidney proximal tubules of rat and mouse (19). These proteins were analyzed with the Western blot technique using polyclonal antibodies raised against the C- and N-terminal proteins of the rat NaPi-2 as deduced from the nucleotide sequence of its cloned cDNA (3). A novel protein of 40 kDa (p40) was detected and appears to be derived from a protein of 75 kDa (p70), which is closer to the predicted molecular mass of 68.7 kDa deduced from NaPi-2 cDNA (3). The p40 and p70 proteins possessed similar physicochemical properties and p40 was regulated in the same fashion as p70 in rats given a low-Pᵢ diet, suggesting that p40 may play an important role in the regulation of the renal Na/Pᵢ cotransport system (19). Similar results using polyclonal antibodies directed against the deduced N- and C-terminal amino acid sequences of NaPi-2 have been obtained by other workers (20).

To address the molecular structure of renal Na⁺-dependent Pᵢ cotransporter, we have cloned NaPi-2α, β, and γ which have partially conserved NaPi-2 cDNA. The present results suggest that NaPi-2α, β, and γ are splicing variants of the NaPi-2 gene and could modulate the function of NaPi-2 in renal proximal tubules.

EXPERIMENTAL PROCEDURES

cDNA Library Construction and Screening—A cDNA library in vector λgt10 (5 × 10⁶ independent recombinants) was constructed from rat kidney poly(A)⁺ RNA by oligo(dT)-primed cDNA synthesis (Life Technologies, Inc., Gaithersburg, MD). Plaques were screened by hybridization under low-stringency conditions as described previously (11). The S²P-labeled rat NaPi-2 cDNA probes were used as the following set of primers: 1) SA-AA; 2) SB-AB; 3) SC-AC, primer SA, 5’-CTCCCT-GGGCTGGCCATGGCTGC-3’ (nucleotide positions +1359 to +1382); primer SB, 5’-ATTAGTGCTCTACAGGAGGAGATTGG-3’ (nucleotide positions +1 to +25); primer SC, 5’-CAGTCGCAAGGTAATACACATACGAGGAGGAGGAACTT-3’ (nucleotide positions +1888 to +1911) (3). Positive clones of four types (NaPi-2, NaPi-2α, NaPi-2β, and NaPi-2γ) were isolated and subcloned into the EcoRI site of pBluescript II SK(+) and characterized by restriction mapping with proper restriction enzymes. Both strands of the cDNA inserts were sequenced by using vector-derived primers, and synthetic oligonucleotides derived from the cDNA sequence (21). NaPi-2 cDNA clones (pNaPi-2: A2 and pNaPi-2: B2) were isolated containing 2440 and 2527 base pairs of insert, respectively. Sequencing data indicated that the two clones have the same open reading frame, encoding a 637-amino acid protein, but have dif-
different polyadenylation signals (data not shown). The sequence of the pNaPi-2-A clone was completely identical to that of the previous report (3). pNaPi-2-B is approximately 87 base longer in 3'-untranslated sequence upstream of its poly(A) signal. pNaPi-2-B was used for functional analysis.

RNA Isolation and Northern Blot Analysis—Kidneys were obtained from male Wistar rats (body weight, 170 to 200 g). The animals were anesthetized with Nembutal (50 mg/kg of body weight, intraperitoneally) and killed by aortic puncture. Total RNA was isolated from rat kidney cortex by acid guanidine thiocyanate/phenol/chloroform extraction (12). Total RNA samples were separated by electrophoresis on 1.2% agarose gels containing 2.2M formaldehyde and transferred to Hybond-N membranes (Amersham, Buckinghamshire, United Kingdom) and covalently cross-linked by exposure to UV light. We synthesized oligonucleotide primers specific for the NaPi-2, NaPi-2a, NaPi-2b, and NaPi-2c cDNA sequences. These sequences of the upstream and downstream primers were: NaPi-2a, 5'-GTTCAGAGCCAGGTAAGACGATAC-3' (nucleotide positions 1941 to 1964 relative to the translation start site of NaPi-2a cDNA) and 5'-CAGCTCTTTGAAAGCCACTGGGCC-3' (nucleotide positions 11139 to 11162 relative to the translation start site of NaPi-2a cDNA); NaPi-2b, 5'-GCAACCTCCTCTTCTGGCTTTGG-3' (nucleotide positions 1647 to 1669 relative to the translation start site of NaPi-2b cDNA) and 5'-GCTTCGCAAGCGTGGCGCCACAGGCCAAACATGTTATGG-3' (nucleotide positions 1912 to 1935 relative to the translation start site of NaPi-2b cDNA); NaPi-2g, 5'-GAGCCATATGTCCTGAGGTATTTCG-3' (nucleotide positions 2600 to 2578 relative to the translation start site of NaPi-2g cDNA) and 5'-GCAACCTCCTCTTCTGGCTTTGG-3' (nucleotide positions 1647 to 1669 relative to the translation start site of NaPi-2b cDNA).
to the translation start site of NaPi-2 and 5'-CACAGCCTGGGGGCG-GAGCTAAG-3' (nucleotide positions -469 to -447 relative to the start site of NaPi-2).

Hybridization with the 32P-labeled NaPi-2, NaPi-2a, NaPi-2b, and NaPi-2-specific cDNA probes was performed in a buffer containing 50% (v/v) formamide, 5% (v/v) sodium dodecyl sulfate (SDS), (0.15 M sodium phosphate, pH 7.4), 1 mM EDTA, 2 × Denhardt’s solution and 1% (w/v) SDS, after which the membranes were analyzed with a Fuji (Tokyo) BAS-2000 image analysis system.

Cloning Cosmid and Phage Clones—To isolate genomic DNA encoding NaPi-2, we synthesized oligonucleotide primers specific for the NaPi-2 mRNA sequence described by Magagnin et al. (5). The sequences of the upstream and downstream primers were 5'-CGTGGTTGCTGTTGTTAAACGTCCTGCAG-3' (nucleotide positions +1665 to +1680 relative to the translation start site of NaPi-2 cDNA) and 5'-CTAGAGCGCCGGTTGGCATTGTG-3' (nucleotide positions +1891 to +1914 relative to the translation start site), respectively. Rat genomic DNA was subjected to polymerase chain reaction (PCR) amplification with the two primers and Taq DNA polymerase (Takara, Kyoto, Japan) for 30 cycles of denaturation at 95 °C for 1 min, annealing at 55 °C for 2 min, and extension at 72 °C for 2 min. The PCR product was subcloned into pBluescript II SK(+) (Stratagene, La Jolla, CA) with the TA cloning system (Promega, Madison, WI). The plasmid was digested with PstI and Xhol, and the released DNA fragment was labeled with [α-32P]dCTP (110 TBq/mmol) (ICN) by the Megaprime DNA labeling system (Amersham) (11).

We screened a genomic DNA library (CLONTECH, Palo Alto, CA) constructed in EMBL3 from fragments of rat kidney DNA generated by Sau3AI digestion. Plaques (1 × 109) were transferred to a nitrocellulose membrane (Hybond-C extra; Amersham), and hybridization and washing were performed as described previously (21). Positive clones were purified and DNA was extracted with the use of large-scale liquid cultures.

A cosmids library (pWE15, CLONTECH) was used for the screening of the NaPi-2 gene. Colonies (1.5 × 108) were transformed on nylon-based membranes (Colony/Plaque Screen, NEN Research Products, Boston, MA). Positive clones were isolated as described previously (22).

In Vitro Translation of cRNA—Rat NaPi-2, NaPi-2a, NaPi-2b, and NaPi-2c cDNA clones were subjected to in vitro translation in the presence or absence of pancreatic microsomes using a rabbit reticulocyte lysate translation system. In the absence of microsomes, the reaction was set up as follows: 1 μg of cRNA, 17.5 μl of rabbit reticulocyte lysate, 0.5 μl of amino acid mixture minus methionine (1 μM), 2.0 μl of [35S]methionine (1200 Ci/mmol), 1 μl of RNasin ribonuclease inhibitor (40 units/μl), and nuclease-free water up to 25 μl. Both reactions were incubated at 37 °C for 90 min and then placed on ice. The samples were heated at 100 °C for 3 min and subjected to a 10% SDS-polyacrylamide gel electrophoresis (PAGE). For autoradiography, the gels were dried and exposed to x-ray film overnight at room temperature (23, 24).

Western Blot Analysis—Brush-border membrane vesicles (BBMV) were prepared from the rat kidney by the Ca2+-free preparation method previously described (25). The antibodies were raised against a peptide that represented an amino acid sequence (Leu-Ala-Leu-Pro-Ala-His-His-Asn-Ala-Thr-Arg-Leu, amino acids 626–637) in the C-terminal region of NaPi-2 or an amino acid sequence (Met-Met-Ser-Tyr-Ser-Glu-Arg-Leu-Gly-Gly-Pro-Ala-Val-Ser, amino acids 1–15) in the N-terminal region of NaPi-2 (3). An N-terminal cysteine residue was introduced for conjugation with keyhole limpet hemocyanin (Sigma) using m-maleimidobenzoyl-N-hydroxysuccinimide ester. For the Western blot analysis, membrane proteins were separated by SDS-PAGE and electrotransferred onto nitrocellulose sheets (16). The samples were mixed with sample buffer containing a final concentration of 20% (v/v) glycerol, 1% (w/v) 2-mercaptoethanol, 0.025 M Tris-HCl (pH 6.8), with or without 0.5% dithiothreitol.

Oocyte Injections and Transport Assay—Xenopus laevis females were obtained from Hamamatsu Jikkenn (Shizuoka, Japan). Small clumps of oocytes were treated twice for 90 min with 2 mg/ml collagenase in a Ca2+ -free solution (ORII solution: 82.5 mM NaCl, 2 mM KCl, 10 mM MgCl2, 10 mM Hepes/Tris, pH 7.5) in order to remove the follicular membranes (11).

After extensive washing, first with ORII solution and then with modified Barth’s solution overnight at 18 °C, healthy oocytes were injected with cRNA (dissolved in water at concentrations from 1 mg/ml) or water using a manual injector (Narishige, Tokyo, Japan). Twenty oocytes were washed in Na+-free solution using the above protocol. Both reagents were used for the measurement of Na+-independent uptake, with incubation as above but substituting 100 mM choline for NaCl.

RESULTS

Isolation of NaPi-2a, β, and γ cDNAs from Rat Kidney cDNA Library—We screened a rat kidney cDNA library by using N- and C-terminal-specific cDNA fragments as described under “Experimental Procedures.” Eight cDNA clones were obtained and sequenced. Three clones, termed NaPi-2a, NaPi-2b, and NaPi-2c encoded the N-terminal region of NaPi-2, showing the amino acid sequence in Fig. 1A. NaPi-2a cDNA was 2389 bp in length, including 1011 bp of the open reading frame which encodes 337 amino acids. NaPi-2a was found to have high homology to the N-terminal region of NaPi-2, but 25 amino acids in the C-terminal region of NaPi-2a had a quite different. The putative structure showed that this clone has three trans-membrane domains and one glycosylation site (Figs. 2 and 3).

The NaPi-2β cDNA was 1166 bp. The open reading frame was 981 bp and encoded 327 amino acids (Fig. 1B). The 174-amino acid in the N-terminal region of NaPi-2β were identical to those of NaPi-2, while the 153-amino acid region in the C-terminal region of NaPi-2β were quite different from those of NaPi-2. However, the putative structure of NaPi-2β showed four transmembrane domains and a leucine zipper motif in the third transmembrane domain (Figs. 2 and 3).

NaPi-2γ cDNA was 1961 bp. The open reading frame was 804 bp encoding 268 amino acids (Fig. 1C). The hydrophyt plot analysis shows that NaPi-2γ has four transmembrane protein and one glycosylation site, and one protein kinase C phosphorylation site in the intracellular domain. NaPi-2γ was completely identical to the NaPi-2 C-terminal region of 268 amino acids (Figs. 2 and 3).

Identification of the NaPi-2 Gene—We screened approximately 1.0 × 108 plaques of a human genomic DNA library and detected three positive clones. These clones were purified and are referred to as λNP-1, λNP-2, and λNP-3. DNA from the three clones was further analyzed by restriction enzyme mapping, and the insert lengths were estimated to be about 15 and 14 kb, respectively. λNP-1 was identical to λNP-3. A partial sequence analysis and Southern blot hybridization revealed that both λNP-1 and λNP-2 encompass the entire coding region of the NaPi-2 cDNA. Clone λNP-1 also contained an extensive 5′-flanking region, whereas λNP-2 contained only exon 9 to exon 13 of the NaPi-2 gene (Fig. 4).

Similarly, we have isolated a cosmold clone (CNP-1) which has about 40 kb of the NaPi-2 gene. This clone, we also mapped the phage genome and each exon (data not shown).

Mapping of the Sequences of NaPi-2a, b, and γ cDNAs—The sequences of the intron-exon junctions between exons 1 and 13 are compatible with the consensus sequences (AG-GT) for splicing junctions. Sequence data were obtained by analyzing the DNA insert of clone ANP-1 and the PCR products of this clone obtained with primers based on the cDNA sequence. Intron sizes were estimated by comparison either with a DNA size
marker on agarose gel electrophoresis after digestion with restriction enzymes or with PCR products (data not shown). The detailed analysis of the gene mapping showed that NaPi-2a uses 1400 bp of intron 8 as the exon and that this region also encodes the 3'-noncoding region. NaPi-2b uses exon 1 to exon 5, and skips exon 6 to exon 13. The mapping of NaPi-2b in the cosmid clone indicated that the new exon of the 3'-region of NaPi-2b is used; 10 kb downstream of exon 13 of the NaPi-2 gene. The gene structure of NaPi-2g is more unique. The 5'-untranslated sequence is mapped at intron 8. The putative capping site of NaPi-2g cDNA is present 278 bp upstream of the exon/intron junction of exon 9. NaPi-2g uses intron 9 of the NaPi-2 gene as the 5'-untranslational region. The 3'-nontranslational region of NaPi-2g used exon 13 of the NaPi-2 gene.

**Northern Blot Analysis of NaPi-2a, b, and g**—To identify the transcripts of each isoform, the specific sequence was chosen in each clone. We determined the size of each transcript for the NaPi-2g isoforms (Fig. 5). The NaPi-2g cDNA full-length probe hybridized to four transcripts (9.5, 4.6, 2.6, and 1.2 kb). The specific probe for NaPi-2b hybridized at 1.2 kb. In contrast, NaPi-2g was hybridized at 9.5 and 2.6 kb.

**In Vitro Synthesis of NaPi-2a, b, and g**—As shown in Fig. 6.
we performed \textit{in vitro} translation for NaPi-2\textsubscript{a}, \textit{b}, and \textit{g}. A, putative membrane spanning regions (M1-M8) are depicted as cylinders. Putative N-glycosylation sites and protein kinase C sites are marked. The leucine zipper motif was marked as L-L-L. In the locations of the intra- and extracellular loops of M4-M8, NaPi-2\textit{y} is reversed to NaPi-2\textit{a}. NaPi-2\textit{a} has three transmembrane domains and protein phosphorylation site. NaPi-2\textit{b} has three transmembrane domains and a leucine-zipper motif in the C-terminal region. NaPi-2\textit{y} has four transmembrane domains and one glycosylation site. \textit{B}, the locations of the leucine zipper motif of NaPi-2 and NaPi-2\textit{b}. NaPi-2\textit{b} is a novel leucine zipper motif in the fourth transmembrane domain.

In rabbit reticulocyte lysate, NaPi-2\textit{a} was 36 kDa, NaPi-2\textit{b} was 36 kDa, and NaPi-2\textit{g} was 29 kDa in SDS-PAGE. In the presence of microsome membranes, NaPi-2\textit{a} and NaPi-2\textit{g} were glycosylated and migrated to the following molecular masses: NaPi-2\textit{a} migrated to 45 kDa and NaPi-2\textit{g} migrated to 35 kDa, but NaPi-2\textit{b} was not glycosylated.

\textbf{Detection of NaPi-2\textit{a}, \textit{b}, and \textit{g} in Isolated Brush-border Membrane Vesicles from Rat Kidney Cortex—}

To detect the presence of the NaPi-2\textit{a}, \textit{b}, and \textit{g} isoforms in BBMV isolated from rat renal proximal tubular cells, a Western blot analysis was carried out using the N- and C-terminal specific antibodies as described under "Experimental Procedures" (Fig. 7). The C-terminal antibodies reacted with 180, 70–90, and 37 kDa proteins in the presence of a reducing reagent (\textit{dithiothreitol}). In the absence of the reducing reagent, we did not detect the 45- and 40-kDa proteins, but a prominent 180-kDa protein appeared. In the case of the type I Na/Pi cotransporter RNaPi-1, we could not detect the dissociation of the protein band (66 kDa) regardless of the presence or absence of reducing regent.

\textbf{Functional Analysis of NaPi-2\textit{a}, \textit{b}, and \textit{g} in Xenopus Oocytes—}

To elucidate the functional roles of NaPi-2\textit{a}, \textit{b}, and \textit{g}, we analyzed Na\textsuperscript{1}\textit{P} transport activity in Xenopus oocytes (Fig. 8). \textit{In vitro} transcribed RNA of these isoforms was injected into \textit{X. laevis} oocytes either separately or combined in equimolar proportions. When 5 ng of NaPi-2\textit{c}RNA was microinjected into \textit{Xenopus} oocytes, the Na\textsuperscript{1}\textit{P} dependent Pi transport activity was stimulated to an approximate 40-fold increase compared with that in the water-injected controls. Na\textsuperscript{1}\textit{P} cotransport activity was not observed in \textit{Xenopus} oocytes expressing NaPi-2\textit{a}, \textit{b}, or...
Identification of Three NaPi-2 Related Proteins

In the present study, we isolated three types of NaPi-2-related cDNA clones. The mapping of each cDNA clone showed that the novel sequence is present in the isolated λ clone including the NaPi-2 gene. A Northern blot analysis showed that each transcript of the cDNA was consistent with the four transcripts hybridized by NaPi-2 cDNA, suggesting that these three NaPi-2-related clones are splicing variants of the NaPi-2 gene.

In addition, in the in vitro translation analysis, NaPi-2α, β, and γ were revealed at 36, 36, and 29 kDa, respectively. These protein sizes were increased when the reaction was added to the mixture of microsomal membrane; the protein sizes of glycosylated NaPi-2α, β, and γ were 45, 36, and 35 kDa, respectively. Indeed, the sizes of the in vitro translation products and glycosylated proteins were very similar to those in the BBMV proteins (45, 40, and 35 kDa) reacted with NaPi-2 N- or C-terminal antibodies. Previous studies with polyclonal antibodies have demonstrated that NaPi-2 is present as proteins of 80–90 kDa in the rat renal BBM (20). Under reducing conditions, additional proteins of 45–49 (p45) and 40 kDa (p40) were detected with N-terminal and C-terminal antibodies, respectively (19, 27). Beliveau and co-workers (19, 27) demonstrated that p40 and p45 have been shown to be glycosylated and up-regulated by a low Pi diet. The amounts of NaPi-2α, β mRNA were significantly increased in rats fed a low Pi diet compared with those in rats fed a normal Pi diet (data not shown). These results suggest that NaPi-2α and NaPi-2γ may correspond to...
Identification of Three NaPi-2 Related Proteins

**Fig. 8. Functional assay of NaPi-2α, β, and γ in Xenopus oocytes.** Plasmid encoding NaPi-2, NaPi-2α, NaPi-2β, and NaPi-2γ cDNA were linearized using NotI and used for the *in vitro* synthesis of cRNA. Five nanograms of each cRNA were microinjected into healthy oocytes. After 3 days, 20 oocytes were washed in Na

|    | NaPi-2 | NaPi-2α | NaPi-2β | NaPi-2γ |
|----|--------|---------|---------|---------|
| Pi uptake (pmol/oocyte/30 min) | 500 | | | |
| + | | | | |
| − | | | | |

**A**

p45 and p40, respectively. In the present study, the N-terminal antibodies were reacted with 40-kDa protein, in addition to 45-kDa protein described previously (27). The 40-kDa protein was not detected in the BBMV isolated from rats fed a high P<sub>i</sub> diet (data not shown). The size of NaPi-2β in the BBMV (40 kDa) is larger than the predicted 36 kDa. It is possible that the protein detected in the BBMV is not a product of NaPi-2β. To clarify the presence of NaPi-2β, we performed Western blotting experiments with antibodies directed against the region of these isoforms that are not conserved. We evaluated the generation of the specific antibody recognition of 3 different epitopes (C-terminal region) of NaPi-2β. The antibodies obtained from rabbit immunized with the peptides did not produce any positive results (data not shown). Further study is needed to clarify the presence of NaPi-2β protein in the BBMV, in addition to the characterization of the 40-kDa protein.

In a previous study, we isolated the human NaPi-3 gene and characterized its structure (21). The structure of the rat NaPi-2 gene was highly similar to those of the human NaPi-3 and mouse NaPi-7 genes (21, 26). The 13 exons were mapped in the two λ phages and one cosmid clone. The gene structural feature showed that the large intron is present between exon 8 and exon 9 among three species: human, mouse, and rat (21, 26). Kohl et al. (28) recently reported that the flounder type II transporter gene was divided by two independent genes. The hydrophobic analysis of NaPi-2 predicted eight transmembrane regions (3). Obviously, the membrane spanning regions 1 to 3 and 4 to 8 are separated by a large hydrophilic loop into two distinct domains (27, 28). This division is reflected on the genomic level regarding the exon/intron organization. Kohl et al. (28) suggest the presence of the duplicated gene products in renal proximal tubular cells. Indeed, NaPi-2α is splicing products from exon 1 to exon 8, and NaPi-2γ is from 9 to 13 of the NaPi-2 gene.

Xiao et al. (27) suggested that the lower weight proteins (40–45 kDa) resulted from a specific post-translational proteolytic cleavage of the NaPi-2 polypeptide and that the cleavage site could thus be located between Asp-298 and Asn-328, which have been shown to constitute the only two N-glycosylated residues in NaPi-2 (29). Kohl et al. (30) tested the functional consequences of an interrupted protein backbone in the type II Na/P<sub>i</sub> cotransporter as proposed by Xiao et al. (27). The fragments were denoted 1–3 plus 4–8, and 1–5 plus 6–8 referring to the putative membrane-spanning segments in the proposed topological model of type II Na/P<sub>i</sub> cotransporter. The *in vitro* translation experiments prove the integrity of the different cRNAs resulting in correctly translated protein fragments. However, none of the truncated transporters of the 1–5 plus 6–8 combination was efficiently processed in *Xenopus* oocytes. The coexpression experiments revealed that the complementing fragments 1–3 plus 4–8 could stabilize each other resulting in proper membrane delivery (30). This implies a direct interaction of the two cognate constructs and correct folding of the individual fragments. This assumption is supported by the functional integrity of the combined fragments 1–3 and 4–8 (30).

*In vitro* transcribed RNA of these isoforms was injected into *X. laevis* oocytes either separately or combined in equimolar proportions and assayed for P<sub>i</sub> transport. However, we failed to detect the enhancement of P<sub>i</sub> uptake in *Xenopus* oocytes co-expressing NaPi-2α/β and NaPi-2γ (1–3 plus 5–8/1–4 plus 5–8) referring to the putative membrane-spanning segments). In contrast, the co-injection of NaPi-2 and NaPi-2γ into *Xenopus* oocytes completely inhibited the Na<sup>+</sup>-dependent P<sub>i</sub> uptake. NaPi-2α also partially inhibited the P<sub>i</sub> uptake. However, NaPi-2β did not affect NaPi-2 function. These results suggest that NaPi-2 αγ are dominant negative inhibitors of NaPi-2 rather than a functional complex from two independent isoforms. Indeed, in hypophosphatemic mice (Hyp), P<sub>i</sub> deprivation
caused an 8-fold increase in immunoreactive type II transporter protein at the BBMV, but NaPi cotransport activity was a 2-fold increase in the BBMV, suggesting that the majority of this BBM protein is inactive (20). The finding of the dominant negative isoforms of NaPi type II transporters may have important physiopathological implications in X-linked hypophosphatemia.

In our analysis of NaPi-2α, β, and γ, NaPi-2α was found to have three transmembrane domains and involve Asn-298, but not Asn-328. On the basis of the NaPi-2 putative membrane structure, the extracellular domains of NaPi-2γ have three transmembrane domains and involve Asn-328. On the basis of the NaPi-2 putative membrane, suggesting that the structure of NaPi-2 may be in reverse orientation to that of NaPi-2. Using immunohistochemical approaches we are now determining whether epitopes are located in the intra- or extracellular compartments.

In addition, X-linked hypophosphatemia and hereditary hypophosphatemic rickets with hypercalcuria (HHRH) are Mendelian disorders of Pi homeostasis characterized by rachitic bone disease, hypophosphatemia, and impaired renal Pi reabsorption (31). The mutant gene in patients with X-linked hypophosphatemia has recently been identified by positional cloning and was designated PEX to signify a P-preventing gene with homology to endoproteindase maps that to the X chromosome (32). In contrast, the molecular basis for the renal defect in P-reabsorption in HHRH has not yet been addressed. However, a recent study suggested that HHRH arises from a primary defect in the NaPi transporter (33). We have analyzed the human NaPi-3 gene, but could not find any mutation in Japanese HHRH patients. The presence of regulatory proteins such as NaPi-2 might be helpful to resolve the molecular basis of HHRH.

Finally, we investigated the molecular structure and regulation of the type II NaPi cotransporter. NaPi-2α, β, and γ have partially conserved NaPi-2 cDNA and are splicing variants of the NaPi-2 gene. These NaPi-2-related proteins may modulate the function of NaPi-2 in the renal proximal tubules.

Acknowledgments—We are grateful to Drs. H. Murer and J. Biber for providing rat NaPi-2 cDNA clone.

REFERENCES

1. Murer, H., and Biber, J. (1992) in The Kidney: Pathophysiology (Selkirk, D. W., and Giebisch, G., eds) 2nd Ed., pp. 2481–2509, Raven Press, New York.

2. Werner, A., Moore, M. L., Mantei, N., Biber, J., Semenza, G., and Murer, H. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 9608–9612.

3. Magagnin, S., Werner, A., Markovich, D., Sorribas, V., Stange, G., Biber, J., and Murer, H. (1995) Proc. Natl. Acad. Sci. U. S. A. 90, 5979–5983.

4. Sorribas, V., Markovich, D., Hayes, G., Stange, G., Fogo, J., Biber, J., and Murer, H. (1994) J. Biol. Chem. 269, 6615–6621.

5. Verri, T., Markovich, D., Perego, C., Norbis, F., Stange, G., Sorribas, V., Biber, J., and Murer, H. (1995) Am. J. Physiol. 268, F626–F633.

6. Collins, J. F., and Ghishan, F. K. (1994) FASEB J. 8, 862–868.

7. Michael, P. K., and Kabat, D. (1995) Kidney Int. 49, 959–963.

8. Biber, J., Custer, M., Magagnin, S., Hayes, G., Werner, A., Lotscher, M., Kaisling, E., and Murer, H. (1996) Kidney Int. 49, 981–985.

9. Murer, H., and Biber, J. (1996) Annu. Rev. Physiol. 58, 607–618.

10. Murer, H., and Biber, J. (1997) Pflugers Arch. 433, 379–389.

11. Miyamoto, K., Tatsumi, S., Sonoda, T., Yamamoto, H., Minami, H., Taketani, Y., and Takeda, E. (1995) Biochem. J. 305, 81–85.

12. Miyamoto, K., Segawa, H., Morita, K., Nii, T., Tatsumi, S., Takeda, Y., and Takeda, E. (1997) Biochem. J. 327, 735–739.

13. Busch, A., Waldegger, S., Herzer, T., Biber, J., Markovich, D., Hayes, G., Murer, H., and Lang, F. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 8205–8208.

14. Hartmann, C. M., Wagner, C. A., Busch, A. E., Markovich, D., Biber, J., Lang, F., and Murer, H. (1995) Pflugers Arch. 430, 830–836.

15. Logman Adham, M., Motock, G. T., Wilson, P., and Levi, M. (1995) Am. J. Physiol. 269, F93–F102.

16. Kataki, K., Segawa, H., Haga, H., Morita, K., Arai, H., Tatsumi, S., Takanai, Y., Miyamoto, K., Hisano, S., Fukui, Y., and Takeda, E. (1997) J. Biochem. (Tokyo) 121, 50–55.

17. Werner, A., Kempson, S., Biber, J., and Murer, H. (1994) J. Biol. Chem. 269, 6637–6639.

18. Levi, M., Lotscher, M., Sorribas, V., Custer, M., Arrar, M., Kaisling, B., Murer, H., and Biber, J. (1994) Am. J. Physiol. 267, F900–F908.

19. Boyer, C. J., Xiao, Y., Dugre, A., Vincent, E., Delisle, M. C., and Beliveau, R. (1996) Biochim. Biophys. Acta 1291, 117–123.

20. Collins, J. F., Bulus, N., and Ghishan, F. K. (1995) Am. J. Physiol. 268, G917–G924.

21. Takeda, Y., Miyamoto, K., Tanaka, K., Kataki, K., Chikamori, M., Tatsumi, S., Segawa, H., Yamamoto, H., Morita, K., and Takeda, E. (1997) Biochem. J. 324, 927–934.

22. Miyamoto, K., Kesterson, R. A., Tamamoto, H., Taketani, Y., Nishiwaki, E., Tatsumi, S., Inoue, Y., Morita, K., Takeda, R., and Pike, J. W. (1997) Mol. Endocrinol. 11, 1165–1179.

23. Pelham, R. B., and Jackson, R. J. (1987) Eur. J. Biochem. 67, 247–256.

24. Walter, P., and Blobel, G. (1983) Methods Enzymol. 96, 84–111.

25. Minami, H., Kim, K. R., Tada, K., Takahashi, F., Miyamoto, K., Nakabou, Y., Sakai, K., and Hagihira, H. (1993) Gastroenterol. 105, 692–697.

26. Hartmann, C. M., Hewson, A. S., Kos, C. H., Hilfiker, H., Soumounou, Y., Murer, H., and Tenenhouse, H. S. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 7409–7414.

27. Xiao, Y., Boyer, C. J., Vincent, E., Dugre, A., Vachon, V., Potier, M., and Beliveau, R. (1997) Biochem. J. 323, 401–408.

28. Kohl, B., Hulseweh, B., Strundk, U., and Werner, A. (1996) FASEB J. 10, A90.

29. Hayes, G., Busch, A., Lotscher, M., Waldegger, S., Lang, F., Verrey, F., Biber, J., and Murer, H. (1994) J. Biol. Chem. 269, 24143–24149.

30. Kohl, B., Wagner, C. A., Hulseweh, B., Busch, A. E., and Werner, A. (1998) J. Physiol. 506, 341–350.

31. Tenenhouse, H. (1997) J. Bone Miner. Res. 12, 159–164.

32. The Hyp Consortium (1995) Nat. Genet. 11, 130–136.

33. Beck, L., Karaplis, A. C., Amiezuka, N., Hewson, A. S., Ozawa, H., and Tenenhouse, H. S. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 5372–5377.