Evolution of the Na-Pi cotransport systems

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Werner, Andreas, and Rolf K. H. Kinne. Evolution of the Na-Pi cotransport systems. Am J Physiol Regulatory Integrative Comp Physiol 280: R301–R312, 2001.—Membrane transport systems for Pi transport are key elements in maintaining homeostasis of Pi in organisms as diverse as bacteria and human. Two Na-Pi cotransporter families with well-described functional properties in vertebrates, namely NaPi-II and NaPi-III, show conserved structural features with prokaryotic origin. A clear vertical relationship can be established among the mammalian protein family NaPi-III, a homologous system in C. elegans, the yeast system Pho89, and the bacterial Pi transporter Pit. An alternative lineage connects the mammalian NaPi-II-related transporters with homologous proteins from Caenorhabditis elegans and Vibrio cholerae. The present review focuses on the molecular evolution of the NaPi-II protein family. Preliminary results indicate that the NaPi-II homologue cloned from V. cholerae is indeed a functional Pi transporter when expressed in Xenopus oocytes. The closely related NaPi-II isoforms NaPi-IIa and NaPi-IIb are responsible for regulated epithelial Na-dependent Pi transport in all vertebrates. Most species express two different NaPi-II proteins with the exception of the flounder and Xenopus laevis, which rely on only a single isoform. Using an RT-PCR-based approach with degenerate primers, we were able to identify NaPi-II-related mRNAs in a variety of vertebrates from different families. We hypothesize that the original NaPi-IIb-related gene was duplicated early in vertebrate development. The appearance of NaPi-IIa correlates with the development of the mammalian nephron.

phosphate; comparative physiology; gene structure

EVERY CELL, whether of prokaryotic or eukaryotic origin, depends on Pi for structural and metabolic needs. The energy inherent in electrical and chemical potentials is convertible into ATP and vice versa (66, 95, 96). Given the negative electrochemical potential across the cell membrane, the anionic Pi cannot be accumulated within the cytosol by simple diffusion. Because availability of Pi is rate limiting to growth, a transport system capable of accumulating Pi against the prevailing electrochemical gradient is a vital prerequisite for evolutionary success. Theoretically, an increase of the extracellular Pi concentration by one or two orders of magnitude could drive the anion into the cell. However, the very limited solubility of Pi in combination with divalent ions such as Ca\(^{2+}\) effectively limits the upper Pi concentrations available to all organisms and cells. Therefore, the uptake of Pi is obligatorily coupled to a downhill movement of H\(^+\) or Na\(^+\), depending on the ionic currency used.

Functional studies using a variety of model organisms have revealed a number of different strategies to deal with the problem of Pi accumulation. A common theme is that all organisms do not rely on a single transport system. In unicellular organisms at low external Pi concentrations, a membrane transporter with high affinity for the substrate combined with a low transport capacity would be present (PstA/C in Esche-
E. coli, Pho84 in yeast; Refs. 3, 84, 88). In addition, a low-affinity, high-capacity system would cover P$_i$ uptake at normal or increased external P$_i$ concentrations (84, 88, 90). In contrast, for multicellular organisms with a balanced internal milieu, the housekeeping membrane transport system (NaPi-III) is complemented by one or more highly regulated system(s) at epithelial surfaces responsible for the control of P$_i$ homeostasis (NaPi-II; Refs. 76, 109).

The techniques of molecular genetics combined with the recent explosion of sequence information, including the entire genomes of E. coli (12), Saccharomyces cerevisiae (18), Caenorhabditis elegans (102), and Drosophila (1), have rendered the structural background of a number of P$_i$-transporting genes and added to the understanding of the phylogenetic relations between the different systems. However, there is a lack of physiological data to complement the increasing number of candidate genes identified by database searches. Many such queries are assigned as “putative” or “similar to” P$_i$ transporters. Wherever possible, functional and physiological correlates for the accumulation of P$_i$ to the related query sequences are included.

This review focuses on comparative aspects of P$_i$ transport systems, namely on the two protein families, NaPi-III (Pit, Pho89) and NaPi-II, respectively (Fig. 1). Sequence comparisons have established homologies between the vertebrate systems NaPi-III and transport proteins in bacteria and yeast. In vertebrates, these transport proteins are thought to represent the ubiquitously expressed housekeeping P$_i$ transport system (52).

From a purely homocentric point of view, the phylogenetic relationship within the epithelial Na-P$_i$ cotransporter family NaPi-II is of major interest. The members of this protein family are involved in regulated P$_i$ uptake in various epithelia and control P$_i$ uptake and loss from the organism. Thus, in vertebrates, the slowly adapting intestinal P$_i$ absorption as well as the acutely regulated renal P$_i$ reabsorption are both mediated by NaPi-II-related proteins (45, 76). Our own work has characterized NaPi-II-related sequences in various vertebrate species to study the molecular evolution of NaPi-II. Homolog proteins in nonvertebrates, namely C. elegans and V. cholerae, have been identified by sequence comparison. In addition, new evidence for bidirectional transcription of the NaPi-II encoding gene in C. elegans, winter flounder, and zebrafish provides a novel distinct molecular mechanism for regulation of NaPi-II gene expression (48, 79).

**NAPI-III, PHO89, PIT FAMILY**

This family of proteins has homolog forms in various model organisms, namely E. coli, S. cerevisiae, C. elegans, and Homo sapiens. In bacteria, it is called Pit (27); in yeast, is it Pho89 (84); in human, it includes the two NaPi-III-related retroviral receptors Glvr and Ram (Pit-1, Pit-2; Ref. 53); and in C. elegans, six related isoforms are referred to as phosphate permeases (24). The close homology between these proteins implies that the transporters share comparable membrane topologies including 10–12 transmembrane spanning segments (TM). The apparent ambiguous number of TM helices may reflect the result of different topology predictions rather than truly divergent structures (51, 68, 104). The hydrophilic loop in rat Pit-2 between TM 6 and 7 (10 TM) or TM 7 and 8 (12 TM), respectively, has been experimentally assigned to the cytoplasmic space (17).

The structural parallels within the protein family are only partly reflected on a functional level. E. coli, which mostly depends on a bioenergetic H$^+$ cycle (66), couples the uptake of P$_i$ via the Pit system to protons. The Pit transporter, a low-affinity, high-capacity system, contributes 90% to the total phosphate uptake of cells grown in high P$_i$ medium (1 mM P$_i$, Ref. 114). The Michaelis constants ($K_m$s) for the anion binding were reported to be in the low micromolar range (9.2 $\mu$M, Ref. 70; 11.9 $\mu$M, Ref. 103; 38.3 $\mu$M, Ref. 114). Extensive functional characterization of the transporter in whole cells, membrane vesicles, and proteoliposomes revealed that to act as a substrate P$_i$ needs to be complexed with a divalent cation. The requirement of divalent cations for the Pit system to transport P$_i$ is underlined by the fact that pitA mutants show resistance to toxic medium concentrations (2.5 mM) of zinc(II) (5). The metal-HPO$_4^{2-}$ complex is cotransported with one proton with a 1:1 stoichiometry as reflected by the pronounced pH dependence of metal phosphate uptake (92, 103, 114). Where extracellular pH is increased and/or membrane potential is depolarized, P$_i$ influx and exchange occur (92, 103).

In contrast to the bacterial H$^+$-MeHPO$_4^-$ cotransport system Pit, Pho89 of S. cerevisiae shows the characteristics of a high-affinity Na-P$_i$ cotransport system. The $K_m$s for P$_i$ binding were reported as 0.5 and 0.6 $\mu$M. Roomans et al. (91) proposed two Na-binding sites with apparent affinities of 0.04 and 29 mM, whereas Martinez and Persson (68) proposed a maximal stimulatory
effect on transport rate at a concentration of 25 mM sodium. Interestingly, a stimulatory effect of divalent cations on P transport rate has been reported for Pho89. However, this finding was attributed to a decrease in surface potential, and MeHPO₄⁻ transport was not considered (91). Pho89 shows a pronounced pH dependence with virtually no activity at pH 4.5 with an optimum at 9.5 (68). Therefore, Pho89 maintains Pi supply in alkaline environments where the proton-driven system Pho84 is inactive (84).

The six membrane proteins homologous to NaPi-III-Pit-Pho89 in C. elegans have not been characterized on a functional base.

The mammalian homologues to the Pit-Pho89 systems comprise the two retroviral receptors Glvr and Ram (80, 104). The functional properties of both human isoforms and rat Ram have been addressed using different expression systems, i.e., Xenopus oocytes and fibroblasts (53, 81). The proteins show comparable functional characteristics. A two-electrode voltage clamp using Xenopus oocytes showed a strict Na-dependence of P₋ mediated current by Glvr and Ram, with 1–2 Na⁺ ions translocated with one P₋ per cycle. Half-maximal transport rate was at concentrations of 40–50 mM Na⁺. The apparent affinity for P₋ was reported to be 24 and 25 μM for human Glvr and rat Ram, respectively (53). With transfected fibroblasts assayed by flux measurements, a Kᵣ of 53 μM P₋ was reported for human Glvr (81). However, this difference is likely to reflect experimental rather than functional discrepancies. Both isoforms show increased transport rates at low pH, which may reflect a preference for H₂PO₄⁻ over HPO₄²⁻ (52).

Interestingly, both the expression level and thus the transport activity of the NaPi-III protein family are regulated by the level of extracellular P, (17, 53, 68, 114). However, this phenomenon is poorly studied. This is largely due to the presence of alternative P-transporting systems that seemed of higher scientific interest. In E. coli, this is the high-affinity P₋-uptake system composed of the proteins PstA, PstC, PetB, and PstS (88, 108). In S. cerevisiae, the regulated pathway via Pho84, which mediates P₋ uptake at low external P, concentrations, has been thoroughly investigated (16, 61, 84). In vertebrates, research on P₋ transport has concentrated mostly on the highly regulated epithelial Na-P, cotransport system NaPi-II (76–78, 109).

**NaPi-II FAMILY: PROTEIN STRUCTURE**

Two Na-dependent P, cotransport systems can be functionally distinguished and are expressed in intestinal and renal epithelia. These transport proteins prominently influence mammalian P, homeostasis. The cloning of the relevant proteins from human (28, 67, 73) and mouse (20, 45) revealed a close structural similarity between the systems. Both are members of the NaPi-II protein family, with the renal isoforms belonging to the subfamily NaPi-IIa and the intestinal transporters assigned to the subfamily NaPi-IIb, respectively. The attribute “intestinal,” however, is misleading, because this protein is highly expressed in a variety of other tissues, such as lung, prostate, and pancreas (28). This aspect of NaPi-IIb function is discussed extensively below. The division into the subfamilies NaPi-IIa and IIb allowed the reorganization of the previously rather heterogeneous protein family comprising NaPi-II-related isoforms from human and rat (IIa; Ref. 67), mouse (IIa; Ref. 20), opossum (IIa; Ref. 98), rabbit (IIa; Ref. 106), bovine (IIb; Ref. 41), flounder (IIb; Ref. 111), Xenopus (IIb; Ref. 49), and zebrafish (IIb; Ref. 79). All these transporters show virtually identical hydropathy profiles, suggesting a common three-dimensional structure. Initial topology prediction suggested 11 hydrophobic helices, which are potentially crossing the membrane. However, experimental evidence using rat NaPi-IIa and flounder NaPi-IIb contradicts a simple in-out arrangement of the predicted helices. Both NH₂ and COOH termini have been confirmed to face the intracellular space, which excludes an uneven number of transmembrane segments (57, 60). Epitope tags introduced within the first and the fourth hydrophilic “spacer” were found to be accessible by antibodies without permeabilization of the cell membrane (60). The fourth intervening sequence, often referred to as “large, extracellular loop,” is glycosylated in vivo; however, the modification is not required for proper sorting and function of the transporter (40).

There is still a controversy about the exact number of transmembrane helices in NaPi-II: most of the published models comprise eight membrane passages with one or two hydrophobic helices tilted to form hinge regions; these are consistent with the experimental data reported so far (Fig. 2; Refs. 77, 83).

Despite obvious parallels with regard to topology, the two isoforms NaPi-IIa and NaPi-IIb show distinct differences in three hydrophilic regions (Fig. 3). These include the two termini and a segment within the glycosylated extracellular loop (Fig. 2). The NH₂ terminus in either isoform lacks structural hallmarks and may be deleted without affecting expression in Xenopus oocytes (A. Werner, unpublished). The COOH terminus of both isoforms includes a PDZ binding domain

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**Fig. 2. Model of NaPi-II based on secondary structure predictions and experimental findings as suggested by Murer et al. (Ref. 77).** The predicted topology displays 8 transmembrane helices and 2 hydrophobic hinge regions. Both NH₂ and COOH termini have been confirmed experimentally to be cytoplasmic, whereas the first loop and the glycosylated loop (G) are extracellular (shown in black). The number of putative NH₂-glycosylation sites varies between NaPi-II proteins from different species. The conserved PDZ binding domain is shown striped.
at the very end of the protein (A/X-T-X-L/F). In addition, the IIb-related proteins contain a stretch of six to ten cysteine residues that are absent in NaPi-IIa. The divergent structure in the extracellular loop consists of 45 amino acids (NaPi-IIb) and 27 or 28 amino acids (NaPi-IIa), respectively (Fig. 3).

To obtain insights into the stage in vertebrate evolution at which gene duplication and the subsequent development of the two distinct isoforms occurred, we characterized a number of species for NaPi-II expression. The divergent regions between NaPi-IIa and NaPi-IIb are boxed. Two structural features are highlighted: the COOH-terminal PDZ binding motif is shown in bold, and the cysteine cluster is represented in the window near the COOH terminus.

Fig. 3. A: sequence alignment of NaPi-IIa and NaPi-IIb isoforms from human (GenBank accession numbers AAA36354 and AAC98695), mouse (AAC42026 and AAC80007), flounder (AAB16821), and the related sequences from *C. elegans* (AAA81148) and *V. cholerae* (CACA04943). The divergent regions between NaPi-IIa and NaPi-IIb are boxed. Two structural features are highlighted: the COOH-terminal PDZ binding motif is shown in bold, and the cysteine cluster is represented in the window near the COOH terminus. B: prediction of transmembrane helices in the NaPi-II homologues from *V. cholerae*, *C. elegans*, and human (NaPi-IIa). A web-accessible program based on a hidden Markov model was used (97, www.cbs.dtu.dk/services/TMHMM-1.0). Note that the helices do not necessarily have to cross the membrane. Thin lines connect structures with high sequence similarities. The number of hydrophobic domains is not constant during evolution; whether this affects the topology of the related proteins remains to be established (top). Schematic picture of the human NaPi-IIa cDNA with the exon/intron organization indicated. The given length of exons and introns are from Hartmann et al. (Ref. 37; bottom).
(106), and rat (67), as well as the NaPi-IIb sequences from bovine (41), mouse (46), flounder (111), and zebrafish (79).1 We used the primers to amplify NaPi-II-related fragments of renal and/or intestinal RNA from chicken, carp, flounder, shark, skate, trout, Xenopus, and Drosophila. The amplified DNA fragments were cloned and sequenced. The results, including all vertebrate NaPi-II-related sequence information available to date, are summarized in Fig. 4. The occurrence of two NaPi-Iib isoforms in most of the fish species (shark, skate, trout, and zebrafish) establishes Iib as the ancient isoform. Because C. elegans expresses a single NaPi-II homologue, we hypothesize duplication of the original NaPi-Iib-related gene early in the development of vertebrates. Such a scenario is in agreement with the so-called 1-2-4 rule in early vertebrate evolution (71). The model assumes, on the basis of symmetry in gene clusters, that the entire genome underwent two rounds of duplications, with most of the duplicated copies eventually developing to pseudogenes or even junk DNA. However, a number of genes have apparently avoided such fate due to, for example, a specialized expression pattern of the isoforms (4). Whether the presence of a single transporter in Xenopus and flounder represents the consequence of a gene loss or is due to a lack of the proposed duplication(s) is purely speculative.

The expression of the NaPi-IIa isoform is restricted to mammals and chicken. Interestingly, the avian kidney contains two types of nephrons. The first one is similar to reptilian nephrons, the other type has a loop of Henle that enables birds to produce hyperosmotic urine (13, 23). A putative expression of NaPi-IIa in the latter nephron type would corroborate the hypothesis that the evolution of NaPi-IIa paralleled the development of the mammalian-type nephron.

We could not detect an NaPi-II homologue in Drosophila, a fact that has now been corroborated by the full sequence of the fly genome (1). A blast search using the highly conserved putative substrate-binding region as a query sequence failed to identify an NaPi-II homologue in the Drosophila genome. However, a comparable strategy identified an NaPi-II homologue in both C. elegans and V. cholerae. It seems that NaPi-II was not required in Drosophila P homeostasis, resulting in a degeneration of the gene. This raises the question whether NaPi-II candidate genes in C. elegans and V. cholerae represent P carriers or precursor structures with undefined function (Fig. 3). We have cloned the relevant bacterial gene, and preliminary results indicate that the protein enhances Pi uptake after expression in oocytes, although at a very low level. Such a rudimentary form of NaPi-II would not only provide a useful tool to study aspects of function and topology but also open up possibilities for overexpressing the protein.

**NAPI-II FAMILY: STRUCTURE-FUNCTION RELATIONSHIP**

The functional properties of a variety NaPi-II-related transporters have been reported (14, 15, 30, 32). Only studies based on cloned transporters will be included at this point, although previous experiments using isolated brush-border vesicles and cell culture models show comparable results (35, 78). This reflects the primary influence of NaPi-II in these preparations (72, 101). The oocytes of the clawed frog Xenopus laevis are the expression system of choice for the characterization of transport proteins. Consequently, the overwhelming majority of functional data on NaPi-II-mediated Na-Pi cotransport is based on oocyte expression. Alternative expression systems have been investigated, but with the intentions to study other aspects rather than functional characterization (e.g., reconstitution of physiological/regulatory features; protein overexpression; Refs. 9, 33, 87). The initial experiments in Xenopus oocytes relied on radiotracer flux measurements, because electroneutrality of the overall transport was assumed. Busch et al. (15), however, showed that rat NaPi-IIa-mediated transport elicited a Pi-inducible inward current into oocytes in the presence of Na+ ions. Electrophysiological methods have subsequently been used to characterize a variety of NaPi-II members, including human NaPi-IIa (15), rat NaPi-IIa (14, 30), mouse NaPi-IIa (38) and NaPi-Iib (45), flounder NaPi-Iib (32), and zebrafish NaPi-Iib (79). To summarize, the systems show comparable apparent substrate affinities, 30–250 μM for Pi, and 35–60 mM for Na, respectively, independent of their origin and subtype. The coupling ratio is 3 Na+/1 Pi (31). The maximal transport rate as well as the affinity for Na+ was found to be dependent on the membrane potential. For a detailed description of the functional characteristics of rat NaPi-IIa and flounder NaPi-Iib, including pre-steady-state analysis of single transport steps and kinetic modeling refer to the publications by Forster et al. (30–32). Interestingly, there are no functional correlates of the structural differences noted between the two isoforms. The characteristic difference in susceptibility to protons of renal and intestinal transport as observed in brush-border membrane vesicles (8, 47) could be reconstituted in Xenopus oocytes expressing murine NaPi-Iia and NaPi-Iib, respectively (14, 45). With the use of chimeras of the latter two isoforms, the domain responsible for pH dependence of the transport could be narrowed to just three amino acids. The sequence 478REK in mouse NaPi-IIa was shown to confer increased activity with rising pH, i.e., to mediate Iia or “renal” characteristics. Orthologous replacement of the amino acids 478REK by 500GNT from murine NaPi-Iib resulted in the loss of proton

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1The sequences of the degenerate primers used in screening for NaPi-II-related sequences in different species are: TMI-1, TCCAAT TGC AGT TCC/TG GTCAAGCATGGT; TMI-2, GGCACCTCTCTAAAG AC GAA AACAAC; TMI-3, CGGTGCGATGCTTGCTTG TTG TCACCT; TMI-1, TAGAG GAC ACG AC A/GC GC AAG AACCAAGGCT; TMI-2, GAA GAA AAGA GAA TA GCT GC GAA ATCTG; TMI-3, GGGTGCTGCGATC CGAA CT GAA GAA GAT CAT. Wobbles are shown in bold. After oligo(dT) primed reverse transcription, a touch-down PCR protocol was used. If no distinct product was identifiable, a nested PCR was performed under similar cycling conditions.
sensitivity, i.e., in “intestinal” pH dependence (25). These experiments, as well as the different voltage dependence of P_i transport between flounder and zebrafish NaPi-IIb (79), corroborate the hypothesis that functional hallmarks of the two NaPi-II isoforms are not related to the divergent structures at the termini and the extracellular loop. Therefore, these structural differences between NaPi-IIa and NaPi-IIb must carry alternative functional characteristics, perhaps on a regulatory level.

NaPi-II FAMILY: GENE STRUCTURE

The genomic structure and chromosome localization of a variety of different NaPi-II genes from human (37, 100), mouse (37), rat (93), winter flounder (48), and C. elegans (24) have now been determined. These data are summarized in Table 1. The size of the NaPi-II genes parallels the apparent complexity of the organism’s genome, according to the general relationship for intron-genome suggested by Vinogradov (107). The differences in NaPi-II between C. elegans and vertebrates are too pronounced to provide insights into the relationship between protein structure and gene development. However, if attention is restricted to the vertebrate NaPi-II genes, possible relationships may indeed be dissected.

Complete gene structures are available for human (Fig. 3B; Refs. 37, 100), mouse (37), and flounder (48).

Table 1. NaPi-II gene structure and chromosomal localization

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Genomic information of NaPi-II-related genes from human, mouse, winter flounder, and C. elegans. The approximate length of the gene, the number of exons/introns, the chromosomal assignment, and the related references are indicated. Information from additional organisms, rat (93), opossum (43) and zebrafish (Werner, unpublished) are not included because only fragments of the gene are sequenced.
The two NaPi-IIa-related genes show only minor differences with fluctuations in the size of the longer introns. The location of the introns with respect to the protein sequence is identical (37, 100). The flounder gene shows similar features. Interestingly, the divergent protein structure between human/mouse NaPi-IIa and flounder NaPi-IIb is associated with a difference in the size of exons 1 and 2 and, consequently, a difference in the localization of introns I and II. This suggests that splice site mutations may account for the divergent NH\textsubscript{2} terminus of the NaPi-IIa and NaPi-IIb isoforms. A similar mechanism has been proposed for the smaller NH\textsubscript{2} terminus, which increases the hydrophilicity of transthyretin isoforms (2). The fact that a deletion of the complete NH\textsubscript{2} terminus of flounder NaPi-II did not interfere with the expression of the truncated protein in Xenopus oocytes questions whether the NaPi-II NH\textsubscript{2} terminus bears any functional significance (Werner, unpublished). The next five exons and the relevant splice sites in the homologous genes of human, mouse, and flounder are conserved. The picture changes at exon 9 (Fig. 3), where unrelated protein sequences are expressed in NaPi-IIa and NaPi-IIb, respectively. The fact that divergence is restricted to exon 9 again suggests that splice-site mutations account for the observed changes in protein structure. Note that exon 9 encodes mostly hydrophilic amino acids, which are integrated in the large extra-cellular loop. The remaining gene structure is well conserved between NaPi-IIa and NaPi-IIb, including the large exon 13, which encodes ~150 amino acids for the terminal. Because 50–60 amino acids at the COOH terminus of the two transporter families are divergent, this may have resulted from a frame shift mutation. Interestingly two prominent motifs are found within this region: a string of cysteine residues in the NaPi-IIb protein family and a PDZ domain binding motive at the very COOH terminus of both isoforms. The changes are likely to contribute to differences in cellular expression of the two NaPi-II isoforms (Figs. 2 and 3).

The promoter region of the NaPi-II gene is of considerable interest because of the likely control of gene transcription to maintain P\textsubscript{i} homeostasis. It is plausible that an increase in NaPi-II mRNA expression accounts for the long-term stimulation of renal P\textsubscript{i} reabsorption via brush-border NaPi-IIa function (54, 63, 110); NaPi-IIb transcription in contrast seems to be unaffected (39). Apparently contradictory results from adaptation experiments with mice and opossum kidney (OK) cells show no alteration in NaPi-II mRNA in response to P\textsubscript{i} restriction (85, 101). However, experimental differences (Northern blot vs. ribonuclease protection) or cell-specific ambiguities related to the phenotype of OK cells may account for these differences. Interestingly, an increase in NaPi-IIa mRNA in OK cells was observed in response to high bicarbonate-carbon dioxide tension (50). Immunocytochemical demonstration of cell-specific NaPi-IIa protein expression indicates that a recruitment of new cells expressing NaPi-IIa mRNA accounts for the observed overall increase of mRNA rather than an upregulation of the transcription rate in cells already expressing NaPi-IIa (62, 63). Northern blot experiments using mRNA from superficial and medullary tissue, respectively, corroborate the expression of NaPi-IIa in medullary nephrons in response to long-term stimulation of P\textsubscript{i} reabsorption (99).

Initial attempts to identify 5′-sequences involved in the regulation of the NaPi-IIa gene (NPT2/Npt2 according to Hartmann et al., Ref. 37) were inconclusive, suggesting that a constitutively active promoter drives the expression of NaPi-II (37, 43). In more recent studies, successful combinations of promoter length and inducing stimuli [parathyroid hormone (PTH), P\textsubscript{i}, vitamin D\textsubscript{3}] and cell lines (OK cells, COS-7 cells, fibroblasts) have been reported. Hilfiker et al. (44) identified a C/EBP-like region in the promoter of the human NaPi-IIa gene that drives cell-specific expression of the luciferase reporter gene. On the basis of similar experiments, Shachaf et al. (93) established an effect of tandemly repeated AP2 sites on promoter activity of the rat NaPi-IIa gene. Furthermore, the identification of two specific elements responsive to either 1,25-dihydroxyvitamin D\textsubscript{3} or dietary P\textsubscript{i}, respectively, has added significant complexity to the transcriptional regulation of human NaPi-IIa gene (55, 99). Using a COS-7 cell line expressing the human vitamin D receptor, a fragment of 15 nucleotides in the NaPi-IIa promoter (−1977 to −1963) was shown to bind to the vitamin D receptor resulting in the stimulation of the expression of a reporter gene (99). In the same lab, a phosphate response element (PRE) (at −1010 to −985) was identified. In addition, it was shown that the expression of the murine transcription factor TFE3, which binds to PRE, was increased in response to P\textsubscript{i} restriction (55).

An unusual but highly interesting feature of some NaPi-II genes is the recent evidence of the transcription of the complement of the NaPi-II DNA strand giving rise to a fully processed antisense mRNA. This phenomenon has been clearly documented in the NaPi-II homologues of both C. elegans and flounder and has recently been reported in zebrafish (24, 48, 79). The C. elegans transcript encodes a transposase without a characterized function. The flounder antisense mRNA exhibits a short open reading frame, and the translation product is a 68-amino acid peptide (48). The presence of an antisense transcript, which shows partial complementarity to the transporter encoding mRNA, raises the question of biological relevance. Is the translation product relevant for Na-P\textsubscript{i} cotransporter function, or is the antisense mRNA itself regulatory? The recent cloning of the homologous antisense product from zebrafish, however, revealed that the open reading frame as detected in the flounder was not conserved. This suggests a biological function not at a protein level but via RNA-RNA interaction. The newly identified antisense transcript related to one of the two zebrafish NaPi-IIb genes shows a reciprocal expression pattern compared with the Na-P\textsubscript{i} cotransporter mRNA (79). Our interpretation of these data is that the formation of sense/antisense duplexes effectively eliminates NaPi-II protein expression. The biology and
mechanism of RNA interference (Refs. 29, 115) is of increasing interest, and the occurrence of the NaPi-II antisense transcript raises the prospect of a related mechanism to regulate NaPi-II gene expression.

A COMPARATIVE APPROACH TO EXPLAIN RENAL EXPRESSION OF NAPI-IIa AND NAPI-IIb

The principle of whole body homeostasis of $P_i$ requires a balanced intestinal absorption and renal excretion of $P_i$ (7, 22), the latter closely being related to water availability (13, 23). The need either to retain water (in terrestrial and marine environments) or to excrete water (in a freshwater environment) is reflected by specific adaptations in renal structure and function (13, 23). Whether the molecular physiology of NaPi-II parallels such broad adaptations will be discussed by comparing just three of our model organisms, namely winter flounder (seawater), zebrafish (freshwater), and rat (terrestrial). Bijouvet and Reitsma (11) suggested both fish show net tubular $P_i$ secretion by simply comparing the daily intake of $P_i$, plasma $P_i$ level, and the glomerular filtration rate (GFR). Using this analysis, the rat, and mammals in general, shows tubular $P_i$ reabsorption (11). The prediction was confirmed by clearance studies using whole animals (13, 23) and primary cell culture models (36, 89). The molecular identification of the relevant NaPi-II homologues and subsequent immunocytochemical studies have now resulted in a detailed picture of how NaPi-II participates in reabsorption/secretion of $P_i$ in the different systems. The flounder (which is an euryhaline teleost) shows a small number of fully functional glomeruli, which are poorly vasculated. Consequently, the volume of the filtered primary urine is low (26). Flounder renal tubules consist of a short proximal tubular segment, denoted PI, which shows morphological features characteristic for a reabsorbing epithelium. The much longer proximal tubular portion PII is presumed to be involved in ion secretion (10). The adjacent collecting tube and collecting duct lead to the bladder, whose epithelium has morphological and functional similarities with the mammalian distal tubule. This is exemplified by the expression of the thiazide-sensitive Na-Cl cotransporter in flounder bladder epithelial cells; Ref. 34). A series of immunocytochemical studies using an anti-NaPi-IIb antiserum as well as RT-PCR experiments with isolated tubular segments showed that the transporter was confined to tubular segment PII and the collecting tube. Interestingly, the Na-Pi cotransporter was found in the basolateral membrane of the secreting tubule PII and in the apical membrane in the collecting tubule (26, 56). The membrane localization of the protein is consistent with a mechanism of secondary active secretion in PII followed by reabsorptive fine tuning of the urine in the adjacent collecting segment.

It must be emphasized that such an hypothesis is based on immunocytochemical evidence. Flounder renal tubular primary cultures in a reabsorptive/secretive state express NaPi-II-related mRNA, although immunohistochemical detection of the transporter failed (H. Hentschel, L. Renfro, and A. Werner, unpublished observation). Efficient secretion of $P_i$ may interfere with another physiological task of the proximal tubular segment PII, namely the excretion of $Mg^{2+}$ (10). Efficient secretion of both ions in the same segment would result in the formation of a precipitate. However, the nephrons of flounder and other fish do not represent a uniform population (26, 82). Such heterogeneity in length may reflect functional differences, i.e., that not all the nephrons secrete divalent cations and $P_i$ with the same efficiency.

The renal tubule of zebrafish shows a similar tubular morphology compared with flounder, although zebrafish nephrons contain functional glomeruli. The recently reported NaPi-IIb-related transporter from zebrafish could be detected only in the apical membrane of the collecting tubule (Nalbant and Werner, unpublished observation). Both the reabsorptive segment PI and the secreting segment PII were negative. This suggests that zebrafish excrete $P_i$ by glomerular filtration followed by NaPi-IIb-mediated reabsorption in the collecting tubule. Because renal $P_i$ handling in rats has been extensively studied and reviewed (7, 62, 76–78), only a brief summary is given here. Rat NaPi-IIa is expressed apically in the convoluted proximal tubule (21, 63). Regulation of tubular $P_i$ reabsorption by PTH or $P_i$ availability is achieved by either incorporation or retrieval of functional NaPi-IIa transporter from the brush-border membrane (19, 64, 65, 85, 86). With the use of immunocytochemical methods, NaPi-IIa has not been detected in other tubular segments, i.e., the distal tubule, which still accounts for ~15% of the absorption of the filtered $P_i$ load. Interestingly, NaPi-II mRNA was detected in rat collecting duct by RT-PCR; however, the cognate protein could not be detected (21).

Gene knockout experiments corroborated the central role of NaPi-IIa in renal $P_i$ handling. Npt2$^{-/-}$ mice (NaPi-IIa knockouts) were viable but showed severe phosphaturia and skeletal abnormalities (6). Interestingly, the remaining renal $P_i$ reabsorption in these animals was no longer sensitive to PTH (117).

The expression pattern of NaPi-II in the kidney of the three model organisms reflects a fundamental difference between fish and mammals with respect to renal $P_i$ handling: the site of $P_i$ reabsorption shifted from the collecting tubule to the proximal convoluted tubule in mammals. The proximal $P_i$ reabsorption in mammals may be required due to the background of the high GFR and the reabsorptive capacity of the proximal tubule. Because terrestrial animals conserve water by efficient tubular reabsorption, drastic reduction in urinary volume without a parallel reduction of $P_i$ by reabsorption would result in elevated concentrations of the anion in later tubular segments, and, concomitantly, this would increase the risk of precipitate formation. The divergent expression pattern obviously parallels the structural evolution of the NaPi-IIa and NaPi-IIb isoforms as suggested in Fig. 4.
NAPI-IIa AND NAPI-IIb, WHAT DOES THE ONE DO AND THE OTHER CANNOT?

At the present time, NaPi-IIa has been detected in the kidney and the brain (46). As already discussed, NaPi-IIa is responsible for the controlled renal reabsorption of P_i. What is the function of NaPi-IIa in the brain? It was argued that NaPi-IIa would cover the increased demands of P_i in metabolically active cells. Recent evidence, however, suggests that even in the brain NaPi-IIa function is related to P_i homeostasis. Mulroney et al. (75) established a link between the P_i concentration in the cerebrospinal fluid (CSF) and renal P_i clearance, i.e., experimentally elevated P_i concentration in the CSF resulted in decreased renal P_i reabsorption despite low or normal levels of P_i in plasma. Immunocytochemistry has revealed NaPi-IIa expression in cells lining the third ventricle and amygdala (46, 75). It is hypothesized that NaPi-IIa in the brain is involved in P_i sensing and consequently that renal expression of NaPi-IIa can be regulated centrally (75).

In contrast to the isoform NaPi-IIa, NaPi-IIb is expressed in a variety of tissues, including lung, small intestine, kidney, prostate, and salivary gland in mammals (28). In zebrafish, a relatively broad expression pattern of NaPi-IIb was also found (79). In addition, NaPi-IIb is thought to be instrumental in mediating P_i secretion in the flounder nephron (26, 56). A reversal of the P_i flux is achieved by the basolateral sorting of the transporter. Interestingly, in a number of secreting human tissues, such as human mammary gland or prostate, NaPi-IIb-related mRNA was detected (28, 112). Firm evidence has demonstrated Na-dependent P_i transport in basolateral membrane vesicles from sheep parotid gland (105). In human milk, sperm fluid, or in the saliva of ruminants, P_i is an essential component for buffering and nutritional and/or metabolic needs (94). It is likely that NaPi-IIb may mediate secondary active P_i accumulation at the basolateral membrane and consequently reverse P_i flux in mammalian secretory tissue.

An alternative approach to address the issue of protein sorting includes the exogenous expression of NaPi-IIa and NaPi-IIb in established cell lines. Such strategy does not necessarily unravel physiologically relevant sorting mechanisms but may eventually lead to the identification of sorting motifs within the transport proteins or the cognate interacting factors. Hernando et al. (42) showed that rat NaPi-IIa is sorted correctly to the brush-border membrane in OK cells but not in an established pig kidney cell line (LLC-PK1), Madin-Darby canine kidney cells, and CaCo-2 cells. Mouse NaPi-IIb and flounder NaPi-IIb used in the same study were sorted apically in all the cell lines used. Molecular components responsible for this difference in sorting have yet to be identified.

Vertebrates other than mammals tend to vary the GFR to maintain water homeostasis and to secrete accumulating metabolic by-products (13, 23). Therefore, tubular transport processes are not the primary regulatory sites and are thus not required to be very efficiently controlled. In contrast, mammals tend to keep the GFR constant and adapt the rate of reabsorption. Because the filtered load in a mammalian nephron is high, such regulation has to be both rapid and efficient to minimize the loss to urine. The evolution of NaPi-IIa may reflect such a tendency: NaPi-IIa, the phylogenetically younger isoform, harbors the molecular information to participate in rapid regulatory steps such as endo- and exocytosis. The protein is expressed in only a limited number of cells and is committed to the control of whole body P_i homeostasis. NaPi-IIb, the phylogenetically older isoform, shows a relatively broad expression pattern and exhibits plasticity with regard to intracellular sorting with the task of accumulating intracellular P_i in a variety of epithelial tissues.

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