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REVIEW

Na^+-DEPENDENT PHOSPHATE COTRANSPORTERS: THE NaPi PROTEIN FAMILIES

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Summary

In vertebrates, the level of inorganic phosphate (P_i) is tightly balanced both inside the cell and in the whole organism. A number of different Na^+-dependent P_i cotransport systems involved in P_i homeostasis have been identified and characterized at the molecular level in the past 7 years. The transporters constitute three different protein families denoted NaPi-I, NaPi-II and NaPi-III. NaPi-I from the rabbit was the first member of this family to be cloned. However, it still resists efforts to unravel its physiological role and a clear-cut functional identity: is it a Cl^- channel, a Na^+/P_i cotransporter, a regulator, or does it perform a combination of these functions? These questions provide a slight taste of the problems associated with orphan genes derived from sequencing projects. The members of the NaPi-II protein family are crucially involved in tightly controlled renal P_i excretion and, as recently discovered, intestinal P_i absorption. The expression and the cellular distribution of NaPi-II in the proximal tubular epithelium are affected by hormonal and metabolic factors known to influence extracellular fluid P_i homeostasis. Recently, the expression of NaPi-II has been demonstrated in osteoclasts and brain; however, the physiological roles of NaPi-II in these tissues remain to be established. The members of the third protein family, NaPi-III, have been identified on the basis of their function as viral receptors. The widespread expression of this family suggests that NaPi-III is involved in supplying the basic cellular metabolic needs for P_i.

Key words: Na^+/P_i cotransporter, homeostasis, inorganic phosphate, transporter, kidney.

Introduction

Inorganic phosphate (P_i) is involved in a large number of biochemical processes and contributes to the structure of DNA, RNA, proteins and phospholipids. Because of its limited solubility in the presence of divalent cations, the concentration of free P_i is balanced in the millimolar range in both intra- and extracellular fluids. Bacteria, yeast, plants and vertebrates have developed their own strategies to control P_i homeostasis, with different membrane transporters being involved. Since P_i translocation does not primarily contribute to acute cellular responses to stress (changes in pH or osmolarity, hormonal stimuli, alterations in membrane potential), there is a limit, at least theoretically, to the number of differently regulated P_i transport systems.

Several organs play a crucial role in vertebrate P_i homeostasis. The daily need for P_i is covered by intestinal absorption from the diet in the proximal intestine. Bones represent the major storage compartment of P_i, and P_i is needed in the intracellular space for metabolic and structural purposes. The extracellular concentration of P_i is controlled *via* tightly regulated renal excretion. Hormones and metabolic factors influencing P_i homeostasis operate primarily at this step (Berndt and Knox, 1992). For this reason, the molecular components involved in renal P_i transport, especially the Na^+/P_i cotransport system, have attracted scientific interest. In a series of elegant studies, the physiological, regulatory and functional aspects of this membrane transport system have been unravelled (Murer *et al.* 1991). However, the isolation of the first cDNA encoding a Na^+/P_i cotransporter was achieved as late as 1991 by expression cloning using the *Xenopus laevis* oocyte system (Werner *et al.* 1991). It soon became clear that the protein, then termed NaPi-1, did not match the physiological and functional characteristics reported for renal Na^+/P_i cotransport (Biber *et al.* 1993). The search was reinitiated, and 2 years later the first two members of a protein family denoted NaPi-II were reported (Magagnin *et al.* 1993). These proteins expressed in oocytes mimicked the hallmarks of mammalian renal P_i reabsorption, i.e. pH-dependency and regulation by parathyroid hormone and by P_i availability (Murer and Biber, 1996: Biber *et al.* 1996). The protein family NaPi-III includes viral receptors which were shown to mediate Na^+-dependent P_i transport after expression in *Xenopus laevis* oocytes (Kavanaugh *et al.* 1994). This review summarizes the overwhelming impact of the molecular

approach on our understanding of P_i homeostasis and tries to integrate the details of P_i handling at the cellular and whole-body levels.

NaPi-I

The first success in the search for the regulated renal Na^+/P_i cotransport system was a cDNA clone derived from rabbit kidney cortex (Werner *et al.* 1991). It encoded a membrane protein of 465 amino acid residues which stimulated Na^+-dependent P_i uptake after expression in *Xenopus laevis* oocytes. Experimental inconsistencies (the cDNA did not match the mRNA size-fraction that stimulated Na^+/P_i uptake in *Xenopus* oocytes) indicated the presence of an additional transport protein. Furthermore, the transport activity expressed in oocytes could not be correlated with the functional characteristics of renal P_i reabsorption, e.g. pH-dependency and regulation of transport activity in response to P_i availability (Biber *et al.* 1993).

Members of the NaPi-I protein family have now been cloned from human (Chong *et al.* 1993; Miyamoto *et al.* 1995; Ni *et al.* 1996), rat (Li and Xie, 1996; Ni *et al.* 1994), rabbit (Werner *et al.* 1991) and mouse (Chong *et al.* 1995) tissue and are predominantly expressed in the kidney, liver and brain. Similar proteins have been identified in *Caenorhabditis elegans* in a large sequencing project (Wilson *et al.* 1994*b*), but the sequences have not been further characterized. The different proteins range in size from 465 to 576 amino acid residues. They are proposed to span the membrane 6–10 times. However, the topology of NaPi-I has never been addressed experimentally.

The functional properties of NaPi-I isolated from rabbit kidney and from human kidney have been characterized after expression in *Xenopus* oocytes, with somewhat ambiguous results. Werner *et al.* (1991) and later Bröer *et al.* (1998) reported rabbit NaPi-I-induced Na^+/P_i uptake in *Xenopus* oocytes, as determined by radiotracer flux measurements. However, the transport activity could not be linearly correlated with the amount of RNA injected or with the incubation time. Electrophysiological measurements revealed that P_i transport was paralleled by a Cl^- conductance sensitive to Cl^- channel blockers but also to organic anions such as uric acid, benzylpenicillin, Phenol Red and probenecid (Busch *et al.* 1996). In these studies, a P_i-induced current was observed only at P_i concentrations greater than 1 mmol l^{-1} in the superfusate. Because of the predominant Cl^- conductance, it was concluded that rabbit NaPi-I does not itself represent a Na^+/P_i cotransporter, but that its overexpression stimulates an intrinsic transport activity. The increased Na^+/P_i transport activity after the expression of rabbit NaPi-I in MDCK (Madin-Darby-canine-kidney) and LLC-PK_1 (porcine kidney) cells could also involve an intrinsic system (Quabius *et al.* 1995). The hypothesis that NaPi-I is a modulator was challenged by Miyamoto *et al.* (1995), who cloned human kidney NaPi-I and expressed the protein in *Xenopus* oocytes. In contrast to the rabbit clone, human NaPi-I cRNA stimulated Na^+/P_i cotransport activity dramatically. The increase in Na^+/P_i cotransport activity was consistent with the data obtained after expressing the rat brain isoform in oocytes (Li and Xie, 1996).

It may be an extremely difficult task to establish a clear functional picture of NaPi-I-related proteins. A bifunctional protein is conceivable in view of recent reports of 'multifunctional' systems such as SGLT1 (Na^+/glucose transporter 1), which mediates glucose uptake and water permeability (Loo *et al.* 1996), and EAAT 5 (excitatory amino acid transporter 5)/ASCT 1 (ASC-type amino acid transporter), which exhibit both glutamate/amino acid transport activity and a Cl^- conductance (Zerangue and Kavanaugh, 1996*a*,*b*). It is also possible that there is a protein network with different clustered permeabilities (i.e. a P_i transporter and a Cl^- conductance) that interfere with each other at a functional level. Such an assumption is promoted by the C-terminal motif (–T/HTRL) found in renal and liver isoforms of NaPi-I. Related short C-terminal peptides are known to bind to so-called PDZ-domains mediating the protein–protein interactions which are involved in the clustering of membrane proteins (Staub and Rotin, 1997).

The physiological relevance of the NaPi-I proteins is not clear. Several reports have ruled out a prominent role for NaPi-I proteins in regulating body P_i homeostasis. Li *et al.* (1996) have suggested a link between rat liver NaPi-I and glucose metabolism. They showed that insulin stimulated NaPi-I expression and Na^+/P_i uptake in rat hepatocytes. The effect was reversed by glucagon. An increase in the level of NaPi-I

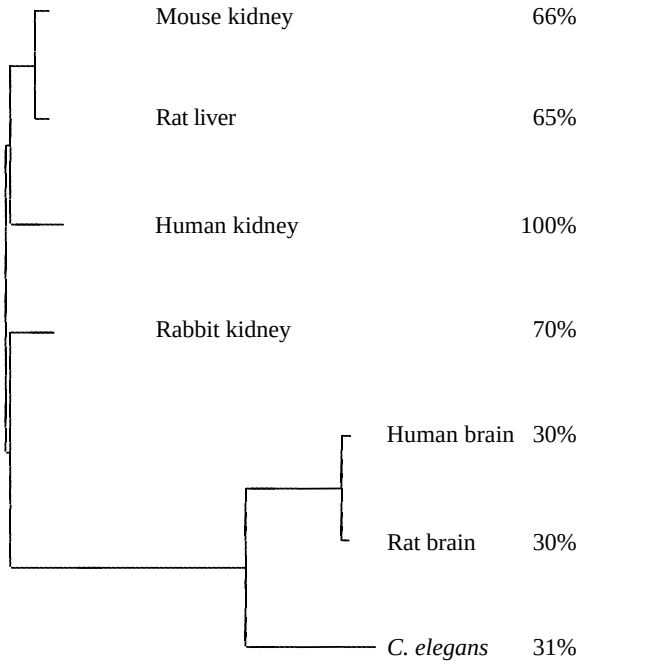

Fig. 1. Evolutionary tree of the different members of the NaPi-I protein family. The values on the right indicate the degree of identity relative to the human kidney clone. The database accession numbers are as follows: mouse kidney, X77241; rat liver, U28504; human kidney, X71355 and D28532; rabbit kidney, M76466; human brain, I73259; rat brain, U07609; *Caenorhabditis elegans*, Z22177.

mRNA in response to insulin could also be measured in the kidney. Other P_i-transporting systems (NaPi-II, NaPi-III) were not directly affected (Li *et al.* 1996). Unfortunately, brain was not included in the study. These intriguing data suggest the hypothesis that NaPi-I serves to supply the great demand of P_i in the liver, kidney and brain as a result of their high level of glucose metabolism.

NaPi-II

Soon after its identification, convincing functional and physiological evidence demonstrated the dominant role of NaPi-II-related proteins in renal P_i reabsorption. Nucleic acid probes and/or peptide-derived antisera were used to quantify the level of NaPi-II-related mRNA and protein in response to physiological changes (Levi *et al.* 1994; Kempson *et al.* 1995). Immunohistochemical approaches demonstrated that the Na^+/P_i cotransporter is confined to the brush border of proximal tubular cells (Custer *et al.* 1994). In a series of elegant experiments, Lötscher *et al.* (1996, 1997) established a link between the action of parathyroid hormone and P_i availability and brush-border membrane integration/retrieval of NaPi-II protein. The issue of regulation has been addressed in recent review articles and will not be discussed here in detail (Levi *et al.* 1996; Murer *et al.* 1998).

Aspects of the structure and function of NaPi-II

The functional properties of NaPi-II-related proteins expressed in *Xenopus* oocytes were found to match those of experiments using brush-border membrane vesicles and cell culture models (Amstutz *et al.* 1985; Hoffmann *et al.* 1976; Malmström *et al.* 1987). The binding of P_i shows Michaelis–Menten characteristics with a K_m in the range 20–100 $\mu mol\,l^{-1}$ depending on species (at 100 $mmol\,l^{-1}$ Na^+). Na^+ interacts in a cooperative way with the transporter with a stoichiometry of $3Na^+$ to $1P_i$. The K_m was found to be approximately 50 $mmol\,l^{-1}$ at resting potential and neutral pH. Protons decreased the affinity of the transporter for Na^+, resulting in the characteristic pH-dependency of Na^+/P_i reabsorption (Busch *et al.* 1994). The kinetic properties of NaPi-II have been investigated in detail by electrophysiological means, including pre-steady-state analysis with flounder and rat NaPi-II (Busch *et al.* 1994; Forster *et al.* 1997, 1998). The closely related functional characteristics within the protein family are paralleled by highly conserved hydrophobic domains. Consequently, the members of the NaPi-II family identified to date probably share a common three-dimensional structure.

The model for NaPi-II proposes eight membrane-spanning domains (Fig. 2). The topology of membrane proteins is notoriously difficult to investigate and, according to our own findings, the NaPi-II proteins are particularly intractable to analysis, because modifications that are tolerated in other proteins (insertion of epitopes or C-terminal truncations) interfere with protein trafficking and Na^+/P_i transport activity. Three regions of NaPi-II have been experimentally assigned to either cytoplasmic or extracellular domains. The large loop between hydrophobic regions three and four was found to be glycosylated and therefore extracellular (Hayes *et al.* 1994). The N and C termini were shown to face the cytoplasm by immunohistochemical means using oocytes expressing epitope-tagged NaPi-II from flounder (Kohl *et al.* 1998). They were assayed either without permeabilizing the membrane or in thin sections. Only the transporters carrying an epitope in the glycosylated loop gave rise to increased fluorescence in the whole-cell assay. In oocyte sections, all the tagged functional transporters were detected (Fig. 3; Kohl *et al.* 1998). These findings are of particular importance since the two termini and the extracellular loop represent the poorly conserved parts of the protein family. Any differences observed in the cellular handling of NaPi-II isoforms are probably due to these parts of the protein.

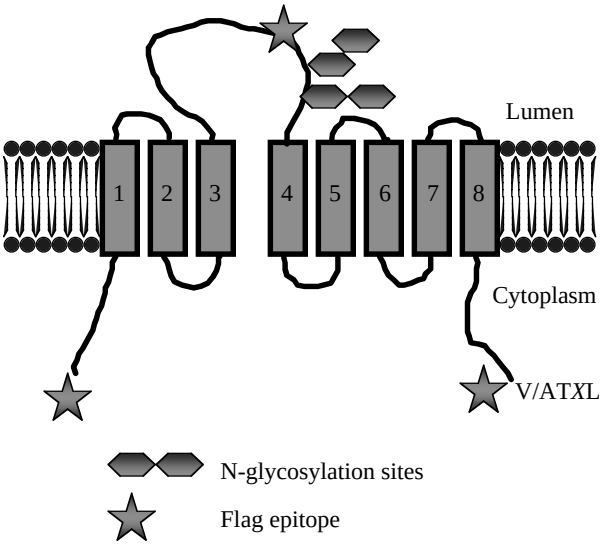

Fig. 2. Topological model of a NaPi-II protein showing eight membrane-spanning domains with both the N and C terminus inside the membrane. Flag epitopes for immunodetection of the protein are indicated (Kohl *et al.* 1998). The number of N-glycosylation sites varies among species; however, they are consistently localized within the large extracellular loop. V/AT*X*L represents the potential PDZ-interacting motif at the C terminus, which may influence protein sorting.

An integrative approach

Under non-pathological conditions, the mammalian kidney exclusively reabsorbs P_i, whereas the kidney in fish and birds reabsorbs or secretes P_i according to the physiological requirements (Renfro and Gupta, 1990). To investigate the role of NaPi-II under these circumstances, a homologous cDNA clone was isolated from flounder kidney (Werner *et al.* 1994). The localization of NaPi-II within the renal structures of the flounder was of particular interest. The euryhaline winter flounder (*Pleuronectes americanus*) exhibits low rates of glomerular filtration in order to minimize water loss. A short reabsorbing segment, PI, follows the glomerulus (this part

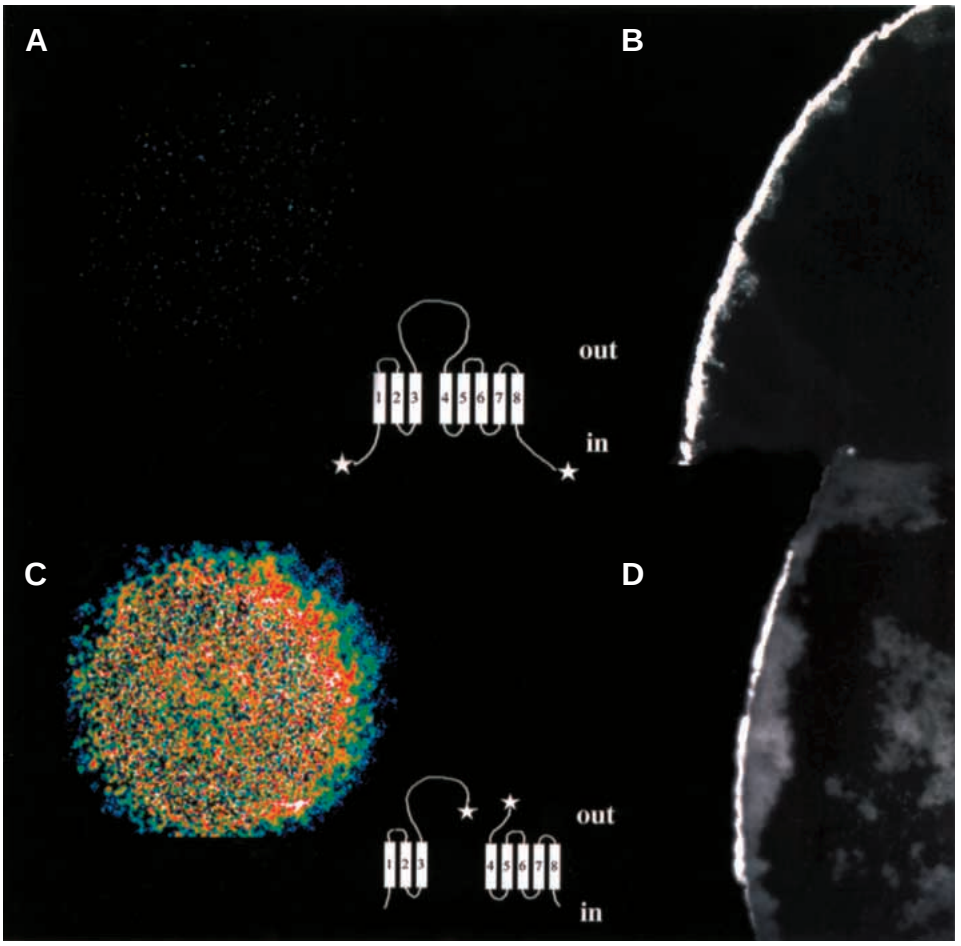

Fig. 3. Topological analysis of the flounder NaPi-II protein after expression in *Xenopus laevis* oocytes. The left-hand panels (A,C) show experiments using intact oocytes including pictograms of the proposed model with the relevant Flag epitopes. The transporters carrying an epitope in the glycosylated loop (C) give rise to increased levels of fluorescence in the whole-cell assay. The right-hand panels (B,D) represent cryosections of oocytes from the same batch. All the tagged functional transporters were detected. The oocytes in A and B express a NaPi-II construct carrying Flag epitopes (stars) at both termini. The expressed proteins in C and D carry an epitope in the glycosylated loop.

shows functional and morphological similarities to the mammalian proximal tubule). A long adjacent segment, PII, presumably mediates secretion of solutes into the urine (Beyenbach and Liu, 1996; Elger *et al.* 1998). The collecting tubule connects to the bladder (homologous to the mammalian distal tubule). Interestingly, NaPi-II was absent in segment PI but expressed at a high level in PII and the collecting tubule. In the secreting segment PII, the transporter was confined to the basolateral membrane, but in the collecting tubule it was detected apically (Fig. 4; Kohl *et al.* 1996). NaPi-II is hypothesized to mediate P_i secretion in segment PII, whereas the same transporter is thought to fine-tune the excretion of P_i by modulating reabsorption in the collecting tubule (Elger *et al.* 1998).

The cellular and tubular distribution of NaPi-II in the flounder is not reflected in the mammalian kidney. Consistent with a unidirectional P_i flux from the lumen to the blood, NaPi-II is present exclusively in the apical cell compartment. However, the situation is reversed when the Na^+/P_i cotransporter from both rat and flounder is expressed in MDCK cells (Quabius *et al.* 1996). Whereas the rat cotransporter showed no preference in sorting, the flounder homologue was confined specifically to the apical membrane (B. Kohl, B. Huelseweh and A. Werner, in preparation). The production of rat/flounder transporter chimeras followed by heterologous expression in MDCK cells should clarify this issue. There is a structural motif (V/AT*X*L) (see Fig. 2) located at the very end of the C terminus which may influence protein sorting. However, these four amino acids are conserved in all NaPi-II proteins and are therefore unlikely to contribute to the specific localization of rat and flounder NaPi-II. Recent results from a two-hybrid screen established the C-terminal interaction of mouse NaPi-II with a protein containing the PDZ domain (L. Dehmelt and A. Werner, in preparation). Such an interaction might be important for the clustering of NaPi-II with other transport proteins and/or for anchoring it to the cytoskeleton. Most interestingly, a comparable PDZ-interacting motif is also found in the renal forms of NaPi-I protein.

The kidney and beyond

To date, the primary structures of the renal NaPi-II proteins from human, rat (Magagnin *et al.* 1993), rabbit (Verri *et al.* 1995), mouse (Collins and Ghishan, 1994), bovine (Helps *et al.* 1995), opossum (Sorribas *et al.* 1994), carp, flounder (Werner *et al.* 1994) and zebrafish (P. Nalbant, C. Boehmer, L. Dehmelt, F. Werner and A. Werner, manuscript submitted) tissues have been determined (Fig. 5). In the flounder, it has been demonstrated that the same Na^+/P_i cotransporter is expressed in the kidney and intestine (Kohl *et al.* 1996). This is in contrast to mammalian systems where no intestinal NaPi-

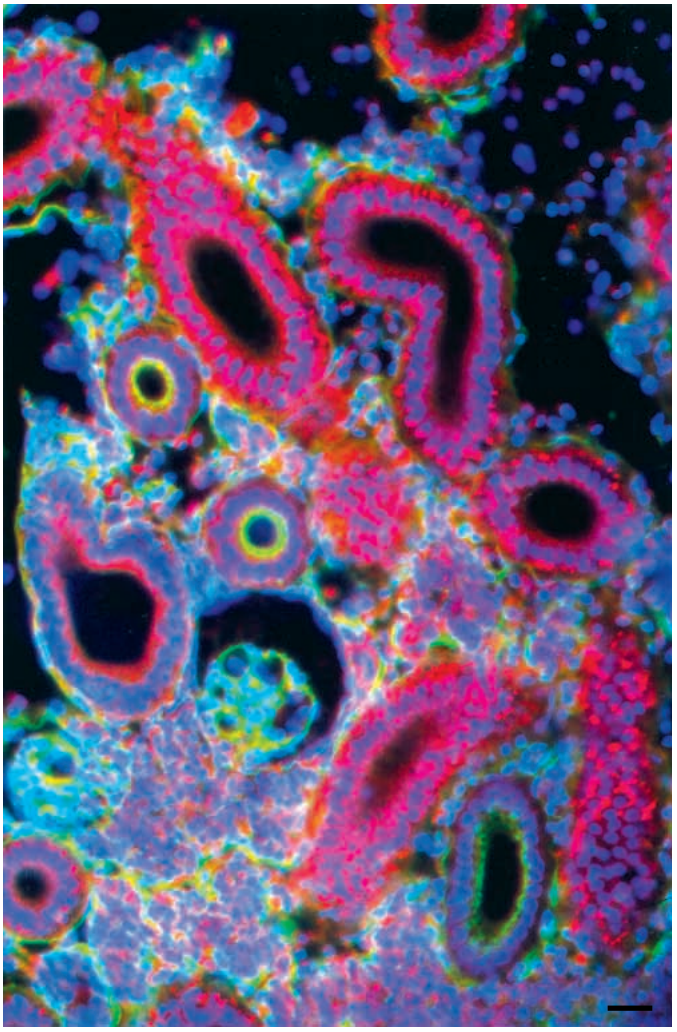

Fig. 4. Thin section of a flounder kidney showing a glomerulus, reabsorbing proximal tubule (PI), secreting tubule (PII) and collecting tubule. The blue coloration (Dapi-staining) indicates nuclear structures. The green fluorescence arises from lens culinaris lectin and is localized to the brush border of the reabsorbing segment PI. The flounder NaPi-II-derived red fluorescence stains the basolateral side of the secreting segment PII and, less intensely, the apical side of the brush border of the collecting tubule. Scale bar, 20 μm.

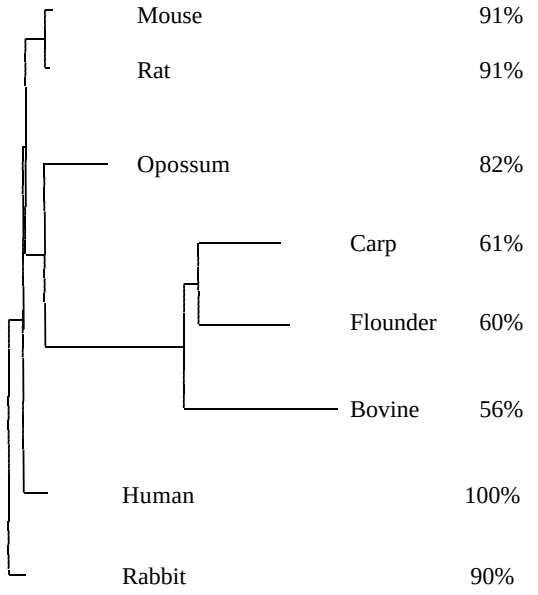

Fig. 5. Evolutionary tree of the different members of the NaPi-II protein family. The values on the right indicate the degree of identity relative to the human kidney clone. The database accession numbers are as follows: mouse kidney, L33878 and U22465; rat kidney, L13257; opossum, L26308; carp (A. Werner, unpublished results); flounder kidney and intestine, U13963; bovine kidney, X81699; human kidney, L13258; rabbit kidney, U20793.

II homologue has yet been reported. Interestingly, such an intestinal isoform was recently cloned from zebrafish, predicting a NaPi-II homologue in mammalian intestine as well (P. Nalbant and A. Werner, in preparation). A comparison of the different renal isoforms revealed that two subfamilies can be distinguished. The first group comprises all mammalian species, with the exception of the bovine transporter which, together with the fish transporters, constitutes the second group. The reason for this peculiar marriage is not clear at present. In ruminants, P_i is secreted very efficiently in the salivary glands and later reabsorbed in the intestine. It supports the microorganisms in the rumen and serves as a buffering system against volatile fatty acids (Shirazi-Beechey, 1996). Whether the ability to secrete P_i, i.e. the potential to direct the Na^+/P_i cotransporter to the basolateral membrane, correlates with the expression of this special NaPi-II isoform needs to be tested.

Recent reports by Gupta *et al.* (1997) and Hisano *et al.* (1997) have added a new component to the current view of NaPi-II-mediated P_i transport. Gupta *et al.* (1997) established a link between NaPi-II-mediated P_i transport and bone resorption by osteoclasts from chick and rabbit. In active osteoclasts, solute flux is directed from the site of bone resorption, the acidic compartment between the bone matrix and the ruffled border, to the blood. However, NaPi-II activity is inhibited at high proton concentrations (Amstutz *et al.* 1985; Hoffmann *et al.* 1976). Consequently, NaPi-II is unlikely to be involved in generating a transcellular P_i flux as observed in renal epithelia. This assumption was corroborated by Gupta *et al.* (1997), who confined the transporter to the basolateral compartment in osteoclasts. Transcytosis is involved in the vectorial transport of bone degradation products across the osteoclasts (Salo *et al.* 1997; Nesbitt and Horton, 1997). If P_i is translocated *via* the same pathway, then a basolateral P_i supply for the high metabolic needs may be necessary. A similar line of argument (increased P_i supply for metabolically active cells) was followed to explain the expression of NaPi-II in brain (Hisano *et al.* 1997).

NaPi-III

The most recent family of Na^+/P_i cotransporters to be described was originally identified as a retroviral receptor for

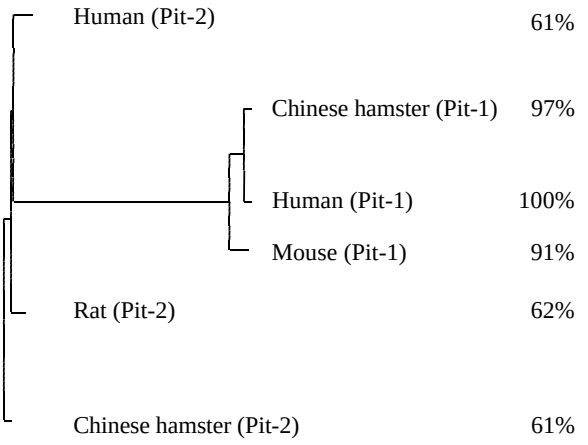

Fig. 6. Evolutionary tree of the different members of the NaPi-III protein family. The values on the right indicate the degree of identity relative to the human clone denoted Pit-1. The database accession numbers are as follows: Human Pit-1, L20859; human Pit-2, L20852; chinese hamster Pit-1, U13946; chinese hamster Pit-2, U13945; mouse Pit-1, M73696; rat Pit-2, L19931.

gibbon ape leukaemia virus (Glvr-1; O'Hara *et al.* 1990) and for rat amphotropic virus (Ram-1; van Zeijl *et al.* 1994; Miller *et al.* 1994). A slight similarity with a putative phosphate permease from *Neurospora crassa* suggested that the membrane proteins might exhibit P_i transport activity (Kavanaugh *et al.* 1994). Both receptors, human Glvr-1 and rat Ram-1, were found to induce P_i transport in a Na^+-dependent manner after expression in *Xenopus* oocytes. The affinity for P_i (at pH 7.5) obeyed the Michaelis–Menten equation with a K_m of approximately 25 μmol l^{-1}. The dependence on Na^+ concentration was sigmoidal, with a K_m between 40 and 50 mmol l^{-1} and a Hill coefficient of 1.5–2. In contrast to the NaPi-II-related proteins, these novel P_i transporters showed decreased activity at alkaline pH. This was explained by a preference for monovalent phosphate as a substrate. Referring to the newly discovered functional characteristics of the viral receptors, Glvr-1 and Ram-1 were denoted Pit-1 and Pit-2, respectively (Kavanaugh and Kabat, 1996).

The members of the NaPi-III protein family are almost ubiquitously expressed in rat tissues. Only spleen was entirely negative in northern blots with both specific probes. Interestingly, the level of Pit-related mRNA increased three- to fivefold in response to a 24 h incubation in P_i-free medium (long-term adaptation). This effect was not dependent on transcription, but could be related to enhanced mRNA stability (Chien *et al.* 1998). cDNA sequences related to Pit-1 and Pit-2 have been cloned from human (O'Hara *et al.* 1990; van Zeijl *et al.* 1994), rat (Miller *et al.* 1994), Chinese hamster (Wilson *et al.* 1994*a*) and mouse (B. O'Hara, unpublished results; Fig. 6) tissue. Both NaPi-III-related subfamilies show a similar topology with two hydrophobic domains, each spanning the membrane 5–6 times. The major structural differences are found in a hydrophilic loop between the two hydrophobic domains.

The broad expression range and the ability to adapt to changes in extracellular P_i concentration suggest a housekeeping role for NaPi-III at the cellular level. Whether it is responsible for other functions is still an open question, but recent reports have demonstrated that the expression of NaPi-III is compatible with the presence of other NaPi-related P_i transporters (Boyer *et al.* 1998; Tenenhouse *et al.* 1998). Since housekeeping proteins are located basolaterally, determining the cellular distribution of NaPi-III would be of great interest. The fact that the two viruses, GALV and RAM, mediate wide-range blood-borne infections suggests a basolateral occurrence of NaPi-III in epithelia. Upon infection, a virus interferes with the surface expression of its receptor. In the case of GALV and RAM, this leads to a downregulation of Na^+/P_i uptake, leading to severe P_i deprivation. This effect of viral infection is not only of (patho)physiological importance but represents a tool with which to study cellular P_i homeostasis. Furthermore, the dual function of NaPi-III as a Na^+/P_i cotransporter and a viral receptor opens up new strategies for investigating the extracellular structures of the transporter and its topology in general.

Conclusion

Three different families of Na^+/P_i cotransporter have been identified. The members of the NaPi-I family have been linked with insulin-stimulated glucose metabolism in liver, kidney and brain. NaPi-II proteins in the kidney and intestine are responsible for intracellular P_i accumulation in order to establish a transepithelial flux of P_i. The third protein family, NaPi-III, exhibits the characteristics of a housekeeping system. However, attempts to establish a correlation between the structure and a physiological function, such as an 'accumulating system' (NaPi-II) or 'housekeeping system' (NaPi-III), seem to oversimplify the situation. Job-sharing was obviously invented long ago and holds for P_i transporters. This flexibility complicates a detailed description of P_i homeostasis and the possibility of interfering in pathophysiological situations.

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