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The Renal Type II Na^+/Phosphate Cotransporter

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A sodium-dependent phosphate transporter (type II Na/Pi-cotransporter) was isolated which is expressed in apical membranes of proximal tubules and exhibits transport characteristics similar as described for renal reabsorption of phosphate. Type II associated Na/Pi-cotransport is electrogenic and results obtained by electrophysiological measurements support a transport model having a stoichiometry of 3 $Na^+/HPO_4^=$. Changes of transport such as by parathyroid hormone and altered dietary intake of phosphate correlate with changes of the number of type II cotransporters in the apical membrane. These data suggest that the type II Na/Pi-cotransporter represents the main target for physiological and pathophysiological regulation.

KEY WORDS: Proximal tubule; transport; phosphate; regulation.

INTRODUCTION

Renal reabsorption of inorganic phosphate (Pi) is a key process in the control of the extracellular concentration of Pi (Pi-homeostasis). Under normal physiological conditions most (up to 80%) of the freely filtered ionic phosphate is reabsorbed along the proximal tubular segments S1, S2, and S3; up to 10% is handled along the more distal nephron segments and around 10% of the filtered load is excreted in the urine. In the proximal tubule, reabsorption of Pi is initiated via an apically localized secondary active transport process (Na/Pi-cotransport) and transepithelial transport is completed by an exit step at the basolateral side of the proximal tubular cell. There is considerable evidence to suggest that the rate-limiting step in renal Pi-reabsorption is the apical sodium-coupled transport mechanism and, moreover, that the rate of this transport system is adjusted to the body needs by a variety of hormonal and nonhormonal factors (Berndt and Knox, 1992).

In recent years several proximal tubular apical Na/Pi-cotransporters have been cloned which have been classified as type I and type II based on their homologies (Murer and Biber, 1996). It is the aim of this article to summarize current knowledge on one of the cloned Na/Pi-cotransporters, the type II proximal tubular apical Na/Pi-cotransporter. We will focus on available structural information and, based on recent electrophysiological measurements, present a model of how this transporter may function. Furthermore, we will briefly discuss the role of the type II Na/Pi-cotransporter in the physiological regulation of proximal Pi-reabsorption. The role of two other phosphate transporters in proximal tubular Pi-reabsorption is discussed elsewhere; for the type I Na/Pi-cotransporter see Busch *et al.* (1996) and Werner *et al.* (1991), and for the gibbon ape leukemia virus human membrane receptor, GLVR-1, see Kavanaugh and Kabat (1996).

THE TYPE II Na/Pi-COTRANSPORTER

Derived from cDNA libraries of rat and human kidney cortex, type II Na/Pi-cotransporters have been identified using an expression-cloning strategy (Magagnin *et al.*, 1993). Subsequently isoforms of this transporter have been cloned from cDNA libraries of mouse and rabbit kidney cortex (Collins and Ghishan, 1994; Hartmann *et al.*, 1995; Verri *et al.*, 1995), from opossum kidney (OK) cells (Sorribas *et al.*, 1994),

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from flounder kidney (Werner *et al.*, 1994), and from bovine renal NBL-1 cells (Helps *et al.*, 1995). The open reading frames of these various mammalian and nonmammalian type II Na/Pi-cotransporter cDNA's code for proteins of around 640 amino acids in length which share more than 80% overall homology. Highest homologies are found within predicted transmembrane regions (see Fig. 1), and lowest homologies are found in the N- and C-terminal tails and within a large extracellular loop containing several N-glycosylation sites. Since a comparison with current databank entries does not show significant homologies of type II Na/Pi-cotransporters with other sodium-dependent cotransporters, cloned type II Na/Pi-cotransporters are likely to represent a unique family of sodium-dependent cotransport proteins.

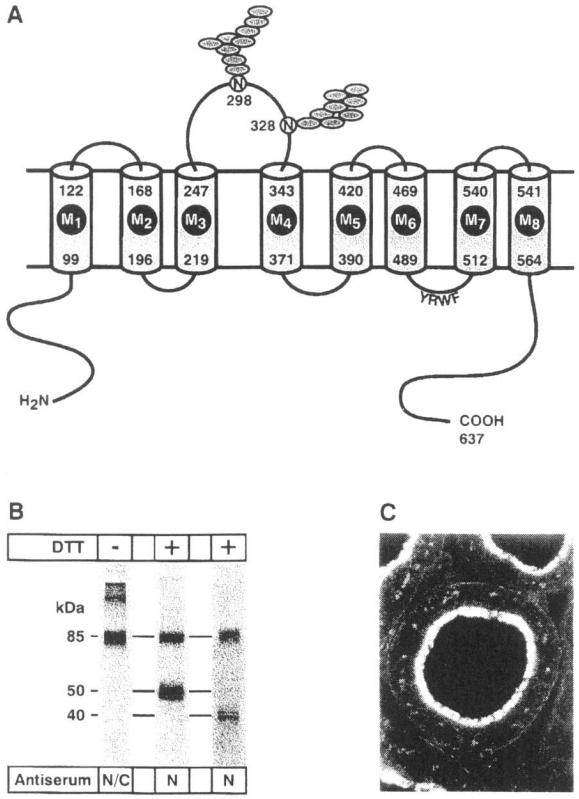

Fig. 1. (A) Putative secondary structure of the type II Na/Pi-cotransporter (rat isoform). Indicated are the two asparagine residues demonstrated to be glycosylated and a tyrosine-containing motif possibly involved in internalization and lysosomal degradation mediated by PTH. (B) Western blot analysis of the type II Na/Pi-cotransporter in rat brush border membranes under nonreducing and reducing (± DTT) conditions. Immunoreactions were performed with polyclonal antisera raised against synthetic peptides derived from the N- and C-terminus respectively. (C) Apical localization of the type II Na/Pi-cotransporter in the proximal tubule (S1-segement) of rat kidney. Apart from the brush border membrane itself immunoreaction also occurred in various intracellular vesicular structures.

A putative model of the structure of type II Na/Pi-cotransporters is shown in Fig. 1 and predicts eight transmembrane segments flanked by stretches of approximately 100 amino acids each at the N-terminus and C-terminus. As illustrated the molecule likely is composed of two hydrophobic cores (M1–M3 and M4–M8) connected by a large extracellular loop containing a number of potential N-glycosylation sites (NXT/S). For the rat isoform N-glycosylation of the two asparagine residues N 298 and N 328 has been demonstrated directly by site-directed mutagenesis (Hayes *et al.*, 1994). Indirect data obtained by immunofluorescence (unpublished) suggest that the N-terminus is located cytoplasmatically. Apart from these structural features, no additional details are presently available about the topology of this cotransporter.

On Western blots and under nonreducing conditions, the rat type II Na/Pi-cotransporter appears as a protein with an apparent molecular mass of 80–90 kDa which is in agreement with the predicted molecular mass of 68 kDa being N-glycosylated. However, when analyzed under reducing conditions it became apparent that the type II cotransporter as contained in isolated renal proximal brush border membranes exists in part also as a cleaved form held together by (a) disulfide bridge(s) (Biber *et al.*, 1996; Boyer *et al.*, 1996). The exact site of proteolytic cleavage is currently not known. The cleavage site is suggested to be between the cysteine residues C306, C334, C361, or C363 as deduced from the apparent molecular mass of the fragments (Fig. 1). Currently it is not known if this observation represents an artefact introduced by the preparation of the membranes or if a proteolytic cleavage of the type II Na/Pi-cotransporter may also occur *in vivo*. The following two observations are of interest in this respect:(i) high sensitivity of Na/Pi-cotransport to proteases such as trypsin and papain has been reported in transport experiments with isolated renal brush border membranes (Rohn *et al.*, 1983) and (ii) by injection of cRNA's coding for either the fragment M1–M3 or M4–M8 of the flounder isoform into oocytes of *Xenopus laevis*, no Na/Pi-cotransport was observed, whereas reconstitution of Na/Pi-cotransport was achieved by coinjection of both cRNA's together (A. Werner, unpublished), suggesting that the function of the type II Na/Pi-cotransporter is not impaired by a proteolytic cleavage between the transmembrane segments M3 and M4.

Based on irradiation experiments performed with isolated renal brush border membrane vesicles it has been suggested that the functional unit of type II Na/Pi-cotransporter associated Na/Pi-cotransport is most probably a homotetramer. However, transport experiments performed under different conditions (e.g., in the presence or absence of sodium) indicated that phosphate transport involves both monomers and tetramers (Jette *et al.*, 1996).

ELECTROPHYSIOLOGICAL CHARACTERIZATION OF TYPE II Na/Pi COTRANSPORTERS

The finding that a net inward flux of Na^+ accompanies Pi transport by type II Na/Pi-cotransporters suggests the involvement of an electrogenic mechanism, in common with other Na-dependent cotransporters (reviewed in Wright *et al.*, 1996). Electrogenicity implies that a net transfer of charge occurs with each transport cycle and it follows that transmembrane voltage is also a determinant of the transport kinetics. In previous kinetic studies of Na/P_i -cotransporter function using brush border membrane vesicles, a Na/P_i stoichiometry of 2:1 was favored, implying an electroneutral transport cycle at neutral pH when P_i is predominantly divalent (Hoffmann *et al.*, 1976). Nevertheless, evidence for electrogenicity was indicated by a small alteration in P_i transport rates when the transmembrane potential was shifted by means of anion replacement (Hoffmann *et. al.*, 1976). Furthermore, using a potential-sensitive dye, Burkhardt *et al.* (1981) showed that a greater change in membrane potential during P_i transport occurred at lower pH values. This was interpreted as being due to transport of monovalent Pi resulting in a net influx of positive charge; however, transport at neutral pH was still assumed to be electroneutral. In the case of Na/P_i -cotransporters, the use of brush-border membrane vesicles to quantitate electrogenic behavior is limited by the lack of direct control of both vesicle transmembrane voltage and current and the possible heterogeneity of Na/P_i -cotransporter types present and therefore might lead to misinterpretation of experimental findings.

Recently, the expression of cloned type II Na/P_i cotransporters in *Xenopus laevis* oocytes has enabled one to establish the electrogenic properties of several members of this family. These studies (Busch *et al.*, 1994, 1995; Forster *et al.*, 1997; Hartmann *et al.*, 1995) have confirmed that: (i) a net transfer of charge accompanies P_i transport over a wide pH range (6.3–8.0); (ii) isoforms share common kinetic features; (iii) the electrophysiological assay of steady-state kinetic behavior supports many of the findings previously established by uptake studies with brush border membranes.

As shown in Fig. 2a, when the transmembrane potential of an oocyte expressing the rat Na/P_i isoform is monitored, a membrane depolarization accompanies the external superfusion with P_i, which is consistent with the net influx of positive charges. Under voltage clamp conditions (Fig. 2b), in which the transmembrane potential is clamped to different holding potentials (V_h), application of P_i induces an inward current (I_p), the magnitude of which is a function of V_h. This behavior confirms that type II Na/P_i -cotransport is electrogenic and is observed for all type II Na/P_i-cotransporter isoforms cloned so far. Furthermore, it is neither observed in the absence of external sodium nor in noninjected or water-injected oocytes.

To investigate the determinants of electrogenicity and the mechanisms by which the membrane potential can influence the transport flux, measurements are made in the steady-state (i.e., at constant V_h) and in the presteady-state immediately following a rapid step change in V_h. Both approaches provide complementary information which can be used to develop a kinetic model of the type II Na/P_i -cotransport mechanism.

Steady-State Properties

Electrophysiological studies of the rat, human, and murine isoforms (Busch *et al.*, 1994, 1995; Hartmann *et al.*, 1995) in the steady state have shown that at $V_h = -50$ mV, pH 7.4 and 100 mM NaCl, the apparent K_m for P_i (K_m^{Pi}) lies in the range 0.07–0.3 mM, and the Hill coefficient is close to unity, implying noncooperative binding of P_i with the transporter. These findings agree with results from brush border membrane studies (e.g., Hoffmann *et al*, 1976; Burkhardt *et al*, 1981). Furthermore K_m^{Pi} is found to decrease with reduced Na, in support of an ordered model for substrate binding in which binding of sodiumions precedes P_i binding. In contrast, at saturating P_i (1 mM) and pH 7.4, the apparent K_m for Na (K_m^{Na}) lies typically in the range 40–60 mM at -50 mV and the dose response relation with respect to Na indicates a Hill coefficient of approximately 3. This result supports a transport model having a Na/P_i stoichiometry of at least 3:1 per transport cycle. At neutral pH it therefore

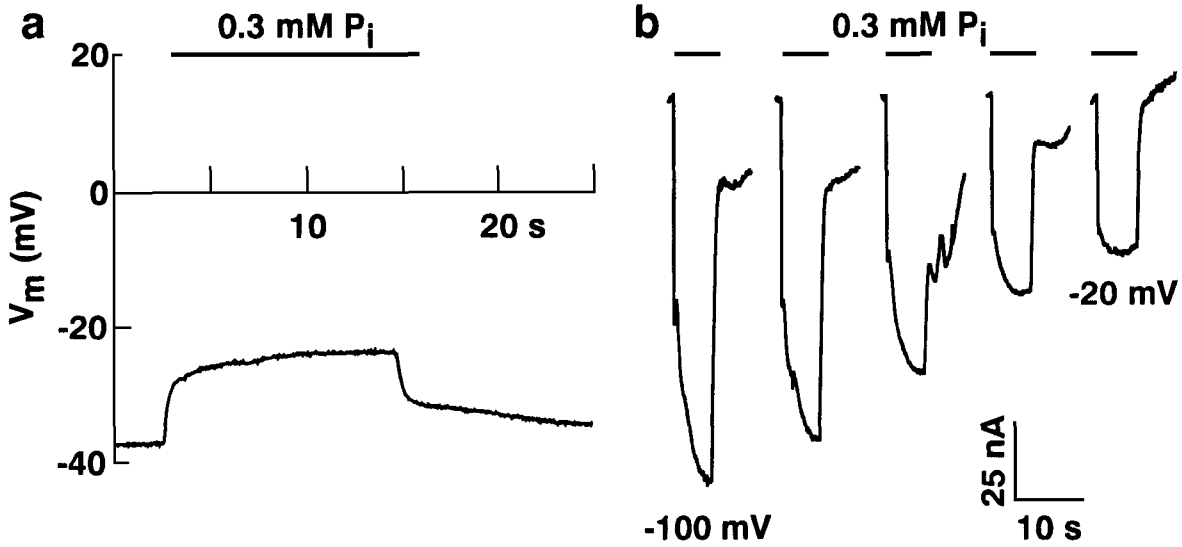

Fig. 2. Electrogenic behavior of type II Na/P_i-cotransporter. (a) Recording of transmembrane potential (V_m) from an oocyte expressing the rat renal type II Na/P_i-cotransporter when 0.3 mM P_i is externally applied in the presence of 96 mM NaCl. (b) Recording of membrane current under voltage clamp conditions from the same oocyte with 0.3 mM P_i applied as indicated and for five different holding potentials. A downward deflection indicates inward current flow.

suggests an excess charge transfer of at least one per cycle which manifests itself as the measured steady-state current. Also of importance is the finding that K_m^{Na}, but not K_m^{Pi}, is strongly V_h-dependent, whereas the respective Hill coefficients are both voltage-independent, indicating that the stoichiometry of substrate binding is unaffected by membrane potential.

The dependence of steady-state P_i-induced currents on pH has also been quantified electrophysiologically. The principal findings that acidification of the external medium results in a concomitant decrease in I_p and an increase in K_m^{Na} (Forster *et al.*, 1997; Hartmann *et al.*, 1995) agree with brush border membrane studies (Amstutz *et al.*, 1995). The observation that the reduction in I_p with pH does not follow a P_i-titration curve indicates that: (i) type II Na/P_i -cotransporters transport both mono- and divalent P_i and (ii) H^+-interaction with the cotransporter results from a reduced affinity for sodium (Busch *et al.*, 1994).

Presteady-State Properties

As shown in Fig. 3, when a depolarizing voltage step is applied to an oocyte expressing the rat type II Na/P_i cotransporter in the presence of 100 mM NaCl and absence of P_i, the measured current comprises a rapid transient corresponding to the charging of the oocyte membrane together with a slower relaxation to a steady state. This latter component is: (i) strongly suppressed in the presence of saturating P_i (1 mM); (ii) reduced when both substrates are absent, and (iii) absent in control oocytes. The P_i-dependent current found from the difference between records with and without P_i (see Fig. 3) comprises transient relaxations at each voltage transition, superimposed on the respective steady currents. Similar, presteady-state relaxations have been reported for a number of Na-dependent cotransporters (e.g., Na/glucose: Loo *et al.*, 1993; Na/GABA: Mager *et al.*, 1993; Na/glutamate: Wadiche *et al.*, 1995).

Presteady-state relaxations recorded from mammalian type II Na/P_i-cotransporters are small in magnitude and quantification over an extended range of voltage and substrate concentration necessitates extensive signal averaging. Recently, the relaxations have been studied in detail by expressing the Na/P_i-cotransporter cloned from the winter flounder (Kohl *et al.*, 1996; Werner *et al.*, 1994) in *Xenopus* oocytes (Forster *et al.*, 1997). The P_i-induced currents are up to an order of magnitude larger than those obtained from the mammalian isoforms, with concomitantly larger presteady-state relaxations. Since oocytes expressing the flounder isoform display steady-state kinetic characteristics consistent with type II Na/P_i-cotransport, this isoform therefore offers a convenient model for

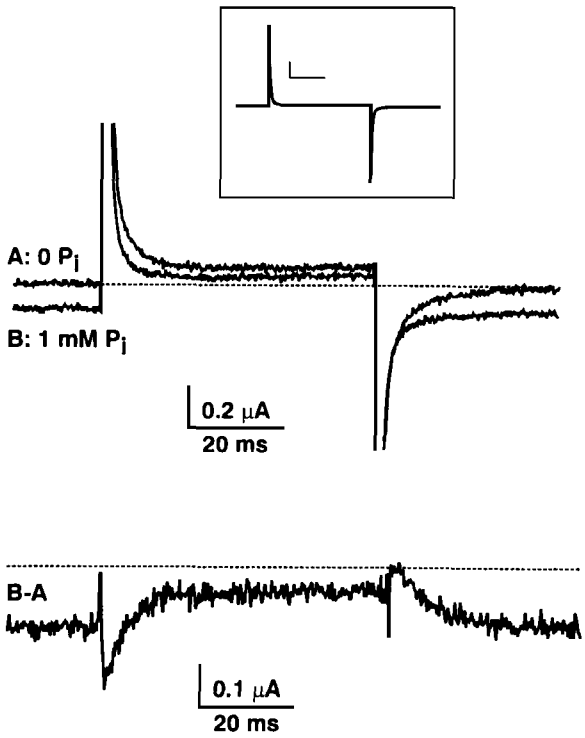

Fig. 3. Presteady-state relaxations recorded from an oocyte expressing the rat renal type II Na/P_i cotransporter. Inset: superimposed records of oocyte capacitive charging transient in the presence and absence of 1.0 mM P_i for a voltage pulse from a holding potential of −100 mV to 0 mV. Center: the same records expanded 10-fold to show the altered relaxation kinetics in the presence of 1 mM P_i. Dotted line indicates baseline steady-state current at −100 mV in the absence of P_i. Bottom: the difference between the records with and without 1 mM P_i showing the relaxations at voltage transitions superimposed on the steady-state P_i-induced currents. Dotted line indicates zero Pi-induced current.

electrophysiological studies in both the steady and presteady state. By quantitating the presteady-state relaxations in terms of a single exponential time constant (τ) and the apparent charge (Q) associated with this kinetic component, it is found that: (i) the charge transfer is balanced for equal depolarizing and hyperpolarizing voltage steps, (ii) Q reverses at the holding potential, (iii) the Q–V characteristic shows saturation at extreme de- and hyperpolarizing potentials; (iv) the τ–V relation is dependent only on the target potential, and (v) there is a linear correlation obtained between the apparent total charge transfer and maximum I_p. Taken together, these findings establish that the relaxations are directly associated with functional flounder type II Na/Pi-cotransporters and have kinetic properties attributable to nonlinear charge translocations occurring within the transmembrane electric field.

To establish the origin of the presteady-state relaxations, their properties were determined under different superfusion conditions (Forster *et al.*, 1997). Although increasing P_i was found to suppress the relaxations in a dose-dependent manner, the relaxation voltage dependence given by the Q–V and τ–V data was unaffected. In contrast, reducing the sodium concentration led to a significant negative shift in the Q–V midpoint voltage (from 0 mV at 100 mM Na to −60 mV at 25 mM Na) and faster relaxation time constants over the voltage range −140 to + 100 mV. Furthermore, like P_i, changes in pH did not affect the relaxation voltage dependence, suggesting that protons do not interact with Na-binding within the transmembrane field.

The voltage dependence of the presteady-state currents could be described by a model based on Eyring–Boltzmann transition rate theory involving two voltage-dependent steps which give rise to presteady-state charge movements: (i) the reorientation of the charged, empty carrier between the cytosolic and external face of the membrane (apparent valence 0.5) and (ii) the binding/release of external Na at a site within the transmembrane field (apparent valence 0.2). These transitions constitute part of a complete kinetic model involving at least six states having mirror symmetry, analogous to schemes proposed for other Na-dependent cotransporters (e.g., Loo *et al.*, 1993; Wadiche *et al.*, 1995) (see Fig. 4). In the case of type II Na/P_i-cotransporters, the remaining kinetic steps of the complete transport cycle (external P_i binding/release and transitions between states with the loaded carrier oriented toward the cytosol) have yet to be defined. Based on the predicted behavior of models for the Na/glucose-transporter, it is likely that the overall scheme for type II Na/P_i-cotransporter will be similar, with differences in the individual rate constants and apparent valences for voltage-dependent transitions.

THE TYPE II Na/Pi-COTRANSPORTER REPRESENTS A TARGET FOR REGULATION OF RENAL PROXIMAL Pi-REABSORPTION

So far expression of type II Na/Pi-cotransporter mRNA and protein has been detected in proximal tubules only (Collins and Ghishan, 1994; Custer *et al.*, 1994; Magagnin *et al.*, 1993). Immunofluorescence studies clearly demonstrated an apical localization of this protein in the proximal tubular epithelial cell

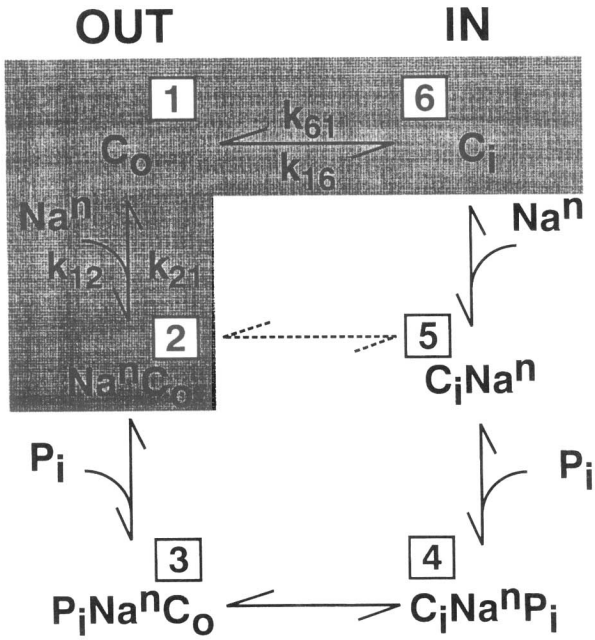

Fig. 4. Kinetic model of type II Na/P_i-cotransporter. Under normal physiological conditions, the transporter cycles anticlockwise around the loop between at least six states with the binding of n sodium ions followed by P_i and corresponding release to the cytosol in the reverse order (mirror symmetry). Three states represent conformations of the unloaded (1), partially loaded (2), and fully loaded (3) carrier facing the external medium. The other three states correspond to the carrier facing the cytosol. The shaded transitions (6⇔1, 1⇔2) represent voltage-dependent transitions so far identified by analysis of presteady-state records. A Na^+ leak (2⇔5) has not yet been confirmed. Current modeling for the flounder isoform suggests that only one sodium ion binds to a site within the transmembrane field (n = 1), and the remaining sodium ions bind at a separate site external to the field. A finding of Na dose response Hill coefficient <3 for the flounder isoform (Forster *et al.*, 1997) also suggest the possibility of multiple binding sites for sodium. (An updated version of the kinetic model (Fig. 4) has been presented by Forster *et al., J. Membrane Biol.* **160**, 9–25 (1997)).

(Fig. 1). Under normal physiological conditions expression of the Na/Pi-cotransporter has been observed in brush border membranes throughout the whole proximal tubule, whereas highest expression was generally observed in S1 segments. These morphological observations are in support of a role of this cotransporter in proximal Pi-reabsorption. Functional evidence for such a role has also been obtained from kinetic characteristics determined by isotope flux and electrophysiological measurements (see above and references in Busch *et al.*, 1994, 1995; Hartmann *et al.*, 1995; Murer *et al.*, 1991). These results indicated that the type II Na/Pi-cotransporter exhibits apparent K_m values for Pi (around 0.07 to 0.3 mM) and Na (40–60 mM) which are similar in magnitude to those reported from microperfusion studies and transport experiments performed with isolated brush border membranes (reviewed in Murer *et al.*, 1991). Additional evidence that the type II Na/Pi-cotransporter is involved in proximal Pi-reabsorption was obtained by using antisense oligonucleotides injected intravenously (Oberbauer *et al.*, 1996). By this *in vivo* approach it has been demonstrated that by antisense oligonucelotides, proximal tubular Pi-reabsorption is partly suppressed due to a reduced expression of the type II cotransporter within the apical membrane. Similarly, in OK-cells, apical Na/Pi-cotransport could be partly "knocked out" after an incubation of the cells with antisense oligonucleotides derived from the type II Na/Pi-cotransporter nucleotide sequence (Sorribas *et al.*, 1994). Furthermore reduced proximal tubular Pi-reabsorption, as occurring in X-linked hypophosphatemia (e.g., *Hyp* mice model), could be correlated to a reduced expression of the type II Na/Pi-cotransporter in the proximal apical membranes (Tenenhouse and Beck, 1996). Taken together, these data support the notion that approximately 50% of proximal tubular Pi-reabsorption occurs via the type II Na/Pi-cotransporter.

Parathyroid hormone (PTH) and dietary content of phosphate are the major regulators of proximal Pi-reabsorption (Berndt and Knox, 1992). Using antibodies against cloned renal Na/Pi-cotransporters it has been demonstrated that by the above-mentioned regulators, the number of type II Na/Pi-cotransporter molecules within the brush border is altered and correlates with changes in Pi-transport.

Low Pi-diet given for several days (chronic adaptation) typically leads to increased proximal Na/Pi-cotransport. The high rate of Na/Pi-cotransport achieved by this nutritional condition is reversible within a few hours after feeding the animal with a diet containing a normal (or slightly higher) phosphate content. This rapid change of proximal Na/Pi-cotransport has been correlated with a decrease of the number of type II Na/Pi-cotransporters within the brush border membrane (reviewed in Levi *et al.*, 1996). A similar observation has been reported after intravenous injection of phosphate to animals chronically adapted to a low Pi-diet (Chenh *et al.*, 1984). Interestingly, increased Na/Pi-cotransport has also been observed after a few hours following gavage of a low Pi-diet which also has been correlated with an increase of Na/Pi-cotransporter molecules within the brush border. Since the latter phenomenon has been described as being independent of protein syn-

thesis (Levine *et al.*, 1986), it is suggested that under acute conditions low Pi-diet leads to a recruitment of existing Na/Pi-cotransporters from an intracellular pool (Levi *et al.*, 1996). In fact, immunohistological observations suggest the existence of immunoreactive intracellular membranous structures possibly serving such a function. The nature of these structures and their role in the suggested rapid recruitment of type II Na/Pi-cotransporters remains to be determined. Furthermore the nature of the cellular signaling mechanism(s) involved in the rapid regulation of the type II Na/Pi-cotransporter by the dietary Pi content is not yet known.

Parathyroid hormone is the major hormonal regulator of proximal Pi-reabsorption. As demonstrated with microperfusion experiments, with transport studies using isolated proximal brush border membranes and cultured cells (OK-cells), PTH leads to an inhibition of Na/Pi-cotransport through the proximal tubular brush border membrane (reviewed in Murer *et al.*, 1991). Having the appropiate antibodies at hand, it became clear that PTH leads to a reduction of the number of type II Na/Pi-cotransporters in the brush border membrane (Kempson *et al.*, 1994). After a short period of time following injection of PTH, a transient accumulation of type II Na/Pi-cotransporters was evident in a subapical compartment, suggesting that PTH mediates an internalization with subsequent degradation of Na/Pi-cotransporters. Direct evidence for a PTH-mediated lysosomal degradation of type II Na/Pi-cotransporters has been obtained recently with OK cells. In these cells PTH leads to a complete disappearance of Na/Pi-cotransporters (the intrinsic and transfected rat isoform) which could be prevented completely by inhibitors of lysosomal degradation (Pfister *et al.*, 1997). The molecular mechanisms of PTH action leading to the degradation of the type II Na/Pi-cotransporter are not yet understood. Since PTH, via its receptor, leads to an activation of protein kinase C and A activities (reviewed in Murer *et al.*, 1991), it has been suggested that the type II Na/Pi-cotransporter may be phosphorylated by one of these kinases.

Despite the presence of a number of consensus phosphorylation sites (Hayes *et al.*, 1995), phosphorylation of the type II Na/Pi-cotransporter has not yet been related directly to the observed action of PTH. Various amino acid motifs have been reported to direct internalized membrane proteins to the lysosomal pathway (Marks *et al.*, 1997). Among others the tyrosine-containing motif YXXΦ (where X stands for any amino acid and Φ represent a bulky hydrophobic amino acid) has gained some interest. Interestingly, such a motif is contained at position 509-512 (YRWF) and it will be of interest to elucidate a possible role of this domain in the regulation of type II Na/Pi-cotransport by PTH.

SUMMARY AND OUTLOOK

By an expression cloning strategy a family of Na/Pi-cotransporters (type II family) has been identified and demonstrated to be involved in proximal tubular Pi-reabsorption. Direct evidence was obtained that this Na/Pi-cotransporter is a key target for the physiological regulation of proximal Pi-reabsorption. Future experimental acitivities will be needed to establish the topology of this cotransporter and to elucidate the molecular mechanisms involved in the regulations by PTH and phosphorous content of the diet.

Despite the lack of structural homology between type II Na/Pi-cotransporters and members of the Na/glucose and Na/neurotransmitter families, a common mechanism of action seems to be shared between them. The challenge for the future will be to relate the transition states predicted by kinetic models to specific structural entities and conformational changes of the Na/Pi-cotransporter molecule itself.

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