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Phosphate deprivation induces overexpression of two proteins related to the rat renal phosphate cotransporter NaPi-2

Christian J.C. Boyer, Yansen Xiao, André Dugré, Éric Vincent, Marie-Claude Delisle, Richard Béliveau *

Laboratoire d'Oncologie Moléculaire, Département de Chimie-Biochimie, Université du Québec à Montréal, P.O. Box 8888, Centre-Ville Station, Montreal, Que., H3C 3P8, Canada

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Abstract

Polyclonal antibodies were raised in rabbits against the C-terminal portion of the rat renal brush-border membrane sodium/phosphate cotransporter NaPi-2. Antibody specificity and molecular sizes of proteins related to NaPi-2 were assayed by Western blot analysis. Proteins of 40 and 70–75 kDa (p40 and p70) were immunodetected in rat and mouse brush-border membranes and proteins of 72 and 82 kDa were detected in rabbit. The absence or presence of β-EtSH in the samples before electrophoresis greatly influenced the immunodetection profile of the rat proteins. Since the 40 kDa protein (p40) can only be detected under reducing conditions, it probably originates from reduction of disulfide bonds in p70. Tryptic cleavage of p40 and p70 revealed identical protein fragments showing the close structural identity of those proteins. Both proteins were more abundant in the outer cortex portion of the rat kidney than in the juxtamedullary portion. Furthermore, rats fed a low-phosphate diet for 24 h showed a 20- and 14-fold increase in the amount of p40 and p70, respectively, compared to control rats, showing that the adaptation to P_i deprivation by increasing renal phosphate reabsorption is not only the result of overproduction of p70, as previously shown, but is also due to the novel p40 which most probably derives from p70.

Keywords: NaPi-2; Phosphate cotransporter; Immunodetection; Polyclonal antibody; (Kidney)

1. Introduction

Renal reabsorption of inorganic phosphate (P_i) from the glomerular filtrate takes place essentially across the brush-border membrane (BBM) of the proximal tubule. This process, under hormonal and cellular control [1–3], involves a specific sodium gradient-dependent transport system [4,5]. Phosphate most likely leaves the cell via an anion-exchange mechanism located in the basolateral membrane [6,7].

The recent expression cloning in *Xenopus laevis* oocytes of NaPi-1 [8] and NaPi-6 [9] cDNA from rabbit, NaPi-2 from rat [10], NaPi-3 from human [10], NaPi-4 from OK cells [11,12] and NaPi-5 cotransport system from flounder [13] constitutes a major step in our understanding of the molecular structure, function and regulation of the sodium/phosphate cotransporter. Since all systems except NaPi-1 seem to belong to the same family of cotransporters, it has been suggested that NaPi-1 be designated type I and the other systems be called type II (NaPi-2, 3, 4, 5 and 6) [3,9]. Also, according to its predicted molecular mass, NaPi-1 is a smaller protein (~ 52 kDa) than type II systems (~ 70 kDa) and shows very little homology with them [10,14]. In vitro translation experiments have demonstrated that both types of NaPi cotransporters are *N*-glycosylated [8,10]. In addition, a recent study has identified two sites of *N*-glycosylation (Asn-298 and Asn-328) on an extracellular domain of NaPi-2 using site-directed mutagenesis [15]. According to this report, glycosylation of NaPi-2 does not appear to be essential for sodium/phosphate cotransport. Another point of comparison between both types of cotransport systems is that type II systems have been shown to play a role in the adaptive response to low-P_i diet [9,16] but NaPi-1 does not appear to be in-

Abbreviations: β-EtSH, β-mercaptoethanol; BBM, brush-border membrane; HPLC, high performance liquid chromatography; IgG, immunoglobulin G; KLH, keyhole limpet hemocyanin; PAGE, polyacrylamide gel electrophoresis; P_i, inorganic phosphate; PVDF, polyvinylidene difluoride.

* Corresponding author. Fax: +1 (514) 9874054.

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volved [9,14,17]. Immunodetection studies have shown that NaPi-1-related proteins are present predominantly in the juxtamedullary portion of rat kidney cortex compared to the superficial cortex [17,18].

Recent studies have demonstrated that the level of NaPi-2-related mRNA and protein is reduced by 50% in the X-linked *Hyp* mouse, an animal model for hypophosphatemic vitamin D-resistant rickets in humans and animals [19–21]. This reduction is correlated with lower phosphate transport in BBM vesicles isolated from *Hyp* mice [22].

In the present study, we identified and partly characterized NaPi-2-related proteins from the kidney's proximal tubule of rat, mouse and rabbit. These proteins were analyzed with the Western blot technique using polyclonal antibodies raised against the C-terminal portion of the rat NaPi-2 protein as deduced from the nucleotide sequence of its cloned cDNA [10]. A novel protein of 40 kDa (p40) was detected and appears to derive from a protein of 70–75 kDa (p70), which is closer to the predicted molecular mass of 68.7 kDa deduced from the cDNA [10]. Both proteins possessed similar physico-chemical properties and p40 was regulated in the same fashion as p70 during low-phosphate diet, suggesting that p40 may play an important role in the regulation of renal sodium/phosphate cotransport.

2. Materials and methods

2.1. Membrane preparations and cell fractionation

Sixteen-week-old male New Zealand rabbits (2 kg) and 2-month-old male Sprague–Dawley rats (300–350 g) were purchased from Charles River (St-Constant, Que.); beef kidneys were obtained from a local abattoir; mice were purchased from Jackson Laboratory (Bar Harbor, ME); and partially purified plasma membranes from OK cells were kindly provided by Dr. Andrew Baines (Toronto, Canada). In order to examine the effect of phosphate deprivation, rats were fed either a low-phosphate (No. 86128) or a standard (No. 86129) diet from Teklad (Madison, WI) containing 0.03% (w/w) or 1% (w/w) phosphate for 3 days. Brush-border membrane vesicles were prepared from outer and juxtamedullary cortex according to the method of Booth and Kenny [23]. The final pellet containing purified brush-border membranes was resuspended in 300 mM mannitol/20 mM Hepes/Tris, pH 7.5, and the following protease inhibitors: chymostatin A (10 μg/ml), bacitracin (10 μg/ml), pepstatin (10 μg/ml) and aprotinin (1.85 μg/ml). The outer cortex was defined as the zone extending from the surface of the kidney to the middle of the cortex, and juxtamedullary cortex as the other half of the cortex. The purity of the membrane preparations was evaluated by measuring alkaline phosphatase activity [24] which was enriched 10- to 15-fold over the cortex homogenate. Cytosol was prepared by differential centrifugation at 4°C. After disruption of the tissue in a Potter tissue homogenizer (Braun, Melsungen, Germany), intact cells were removed by centrifugation at $3000 \times g$ for 10 min. The supernatant was then centrifuged at $100\,000 \times g$ to yield a pellet enriched in microsomes and a supernatant containing the cytosolic fraction.

2.2. Peptide synthesis

The fifteen-amino acid peptide CPRLALPAHHNATRL was synthesized with an Applied Biosystems automated peptide synthesizer model 431A (Mississauga, Ont.) using Fmoc (9-fluoronyl-methyloxycarbonyl) chemistry following the recommendations of the manufacturer. This peptide corresponds to the 14-amino acid C-terminal portion of the rat sodium/phosphate symporter as deduced from the nucleotide sequence of the cloned NaPi-2 gene [10] to which an N-terminal cysteine residue was added to allow coupling of the peptide to a carrier protein. The peptide was purified on a semi-preparative Vydac C18 high performance liquid chromatography (HPLC) column (Hesperia, CA) and its identity was confirmed by fast atom bombardment mass spectroscopy.

2.3. Immunization

The HPLC-purified peptide was coupled to keyhole limpet hemocyanin (KLH) (Pierce, Rockford, IL) through the free sulfhydryl group of its cysteine residue with MBS (Pierce) as described by Mumby and Gilman [25]. On day 0, rabbits were injected subcutaneously at 2 sites on the back and intramuscularly at 2 sites on the rear hinds with 400 μg of the KLH-coupled peptide mixed homogeneously with Freund's complete adjuvant (Pierce) in a 1:1 volume ratio. A booster injection of antigen was administered on day 14 using Freund's incomplete adjuvant. The final injection (day 26) consisted of 400 μg of KLH-coupled peptide adsorbed to aluminum hydroxide (Imject™ Alum, Pierce) administered intraperitoneally at two sites in the lower abdomen. The rabbits were bled 33 days after the final injection. The blood was allowed to coagulate at room temperature for 1 h and stored overnight at 4°C to allow the clot to retract. The clot was discarded and the serum was collected after blood cells were removed by centrifugation at $\sim 1000 \times g$ for 10 min.

2.4. Antibody purification

The immunoglobulin G (IgG) of the serum (adjusted to pH 8.6 with Tris/HCl) was purified on a 5-ml HiTrap protein A fast protein liquid chromatography column (Pharmacia, Dorval, Que.) equilibrated with 50 mM Tris/HCl, pH 8.6. The column was washed extensively with the same buffer and the IgG was eluted with 100 mM citrate, pH 3. The fractions (1 ml) were collected in tubes

containing 100 μl of 1 M Tris/HCl, pH 9, to neutralize the citrate. The antibodies were further purified by affinity chromatography on a peptide-Sepharose column (2 ml) prepared as described by Mumby and Gilman [25] and equilibrated with 100 mM NaCl, 20 mM Tris/HCl, pH 7.5 (buffer A). After extensive washing with the same buffer, the antibodies were eluted from the column with 0.2 M glycine, pH 2.2. The fractions were collected as mentioned above for the protein A column. Neutralized fractions that contained protein, as determined by absorbance at 280 nm, were pooled and dialyzed overnight against buffer A.

2.5. Western blot analysis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed with a Mini-Protean II (Bio-Rad, Mississauga, Ont.) electrophoresis apparatus and the Laemmli buffer system [26]. The separating gel contained 7.5% (w/v) acrylamide/bis-acrylamide (29.1:0.9). Unless indicated otherwise, prior to electrophoresis, the samples were mixed with sample buffer containing a final concentration of 1.0% (w/v) SDS and 2.5% (v/v) β-EtSH, without boiling. Electrophoresis was carried out at a constant voltage of 120 V for about 1.5 h. Unless indicated otherwise, each well contained 12 μg of protein. Molecular mass determinations were based on a calibration curve prepared with protein standards (Bio-Rad).

Following electrophoresis, the gels were equilibrated in transfer buffer (10% (v/v) methanol, 25 mM Tris, 192 mM glycine, pH 8.3) for 15 min. The proteins were transferred onto polyvinylidene difluoride (PVDF) microporous (0.45-μm pore diameter) membranes (Millipore, Mississauga, Ont.) by electroelution in a Mini Trans-Blot electrophoresis transfer cell (Bio-Rad) at a constant voltage of 100 V for 3 h at 4°C. The non-specific binding sites on PVDF membranes were blocked by overnight incubation in 150 mM NaCl, 0.05% (w/v) Tween 20, 0.1% (w/v) bovine serum albumin and 50 mM Tris/HCl, pH 7.0 (buffer B) containing 5% (w/v) powdered milk, at 4°C. The membranes were washed three times by gentle agitation in buffer B for 10 min and incubated for 1 h with a 1/6000 dilution of the anti-peptide antibody in buffer B. The membranes were then washed three more times in buffer B and incubated for 1 h with a 1/1000 dilution of anti-rabbit IgG horseradish peroxidase-linked whole antibody from donkey (Amersham, Oakville, Ont.) in buffer B. The membranes were finally washed three times for 10 min in buffer B and labelled antigens were revealed with enhanced chemiluminescence (ECL) Western blotting solutions (Amersham) following the recommendations of the manufacturer. The membranes were exposed for appropriate times to Fuji or Kodak films which were preflashed with a Sensitize flashgun unit (Amersham). The films were developed with a Mini-Med/90 X-ray film processor (AFP Imaging, Elmsford, NY). The X-ray films were analyzed with an Ultroscan XL laser densitometer (Pharmacia).

2.6. Tryptic cleavage

Brush-border membrane proteins (250 μg) were applied on a two-well 1-mm gel containing 7.5% (w/v) acrylamide/bis-acrylamide (29.1:0.9) and resolved using SDS-PAGE. Prior to electrophoresis, the samples were mixed with sample buffer at a final concentration of 1.0% (w/v) SDS and 2.5% (v/v) β-EtSH, without boiling. Electrophoresis was carried out at a constant voltage of 100 V for about 1.5 h. Following their separation, proteins of 40 and 70 kDa were cut from the gel and homogenized using a Potter tissue homogenizer (Braun, Melsungen, Germany). Both samples were treated as described by Cleveland et al. [27]. Protein samples were deposited on a second SDS-PAGE gel (1.5 mm) containing 15% (w/v) acrylamide/bis-acrylamide (29.1:0.9) and before starting electrophoresis, 1 μg trypsin (12.7 U) (Sigma) was added into each sample well. Electrophoresis, Western blot and immunodetection procedures were carried out as previously described.

3. Results

Polyclonal antibodies directed against the 14-amino acid C-terminal portion of the cloned rat renal brush-border membrane sodium/phosphate cotransporter NaPi-2 [10] were raised in rabbits, affinity-purified and used to determine the presence of related proteins in rat, mouse, rabbit and beef renal cortex brush-border membranes and in

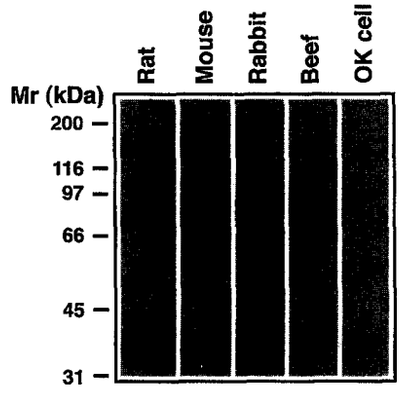

Fig. 1. Immunodetection of renal brush-border membrane proteins from rat, mouse, rabbit, beef, as well as in partially purified plasma membranes from OK cells using polyclonal antibodies directed against the rat renal sodium/phosphate cotransporter NaPi-2. Brush-border membrane proteins were resolved by SDS-PAGE using 7.5% polyacrylamide gels. Immunoblots were performed as described under Section 2 and antibody binding was detected using donkey horseradish peroxidase-linked anti-rabbit IgG antibodies. Labelled antigens were revealed by autoradiography with ECL Western blotting solutions. Comparable data were obtained in three independent experiments performed in duplicate.

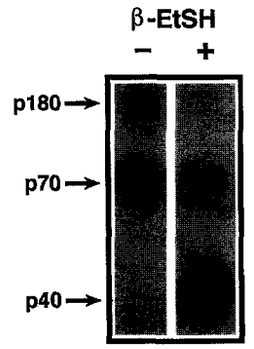

Fig. 2. Effect of β-mercaptoethanol on the immunodetection of rat renal brush-border membrane proteins. Samples were prepared either in the absence (−) or presence (+) of β-EtSH. Proteins were analyzed as described in the legend of Fig. 1. Comparable data were obtained in five independent experiments.

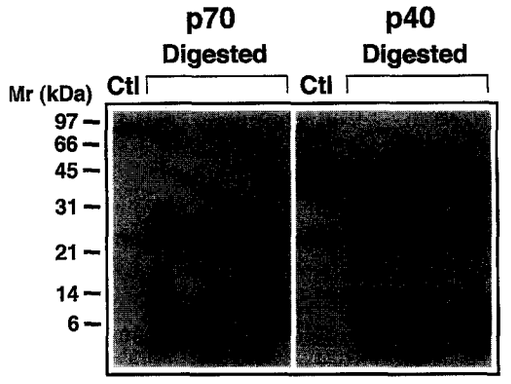

Fig. 3. Comparison of peptide fragments obtained by tryptic cleavage of BBM rat 40- and 70-kDa proteins. Proteins of 40 and 70 kDa were separated and cut from a first gel (7.5% polyacrylamide); both gel slices were homogenized and loaded on a second gel (15% polyacrylamide) in the presence (Digested) or absence (Ctl) of 1 μg trypsin (12.7 U) as described under Section 2. Proteins were analyzed as described in the legend of Fig. 1. Both digestions were performed in triplicate. Comparable data were obtained in two independent experiments performed in triplicate.

plasma membranes from OK cells (Fig. 1). Western blots of rat and mouse BBM revealed proteins of 40, 70–75 (designated p40 and p70), 116 and 176 kDa. When 10^{-6} M of the antigenic peptide was added during incubation with the antibodies, those rat proteins were not detected (not shown). The 176-kDa protein was more strongly detected in mouse than rat BBM. However, detection of both 116- and 176-kDa proteins was not systematically observed in all BBM preparations and therefore no further analysis of those proteins was pursued. Proteins of 72 and 82 kDa were detected in BBM prepared from rabbit kidney cortex. No protein could be detected in BBM from beef and in plasma membranes purified from OK cells.

The influence of β-mercaptoethanol (β-EtSH) on the immunodetection profile of rat BBM proteins was investigated (Fig. 2). Immunoblots of BBM proteins were analyzed after electrophoresis of samples which had been prepared either in the absence or presence of β-EtSH. Exclusion of the reducing agent from the samples before electrophoresis resulted in Western blots where p40 was absent while detection of p70 was enhanced. Under these non-reducing conditions, an additionnal protein of about 180 kDa (p180) was also detected. Addition of β-EtSH to the samples before electrophoresis resulted in Western blots where only p40 and p70 were immunodetected. Boiling of the samples prior to electrophoresis had no effect on the immunodetection of any of these proteins (not shown). To further study p40, the addition of β-EtSH to protein samples before electrophoresis was used in following Western blot experiments.

To investigate the relationship between the rat p40 and p70 proteins, we performed limited trypsin proteolysis on each of these proteins, following their separation by SDS-PAGE. After enzymatic cleavage, eight fragments were obtained from p70, identical to those found in the p40 fragment pattern, demonstrating that these proteins are very similar in their amino acid sequence (Fig. 3).

The presence of p40 and p70 proteins was investigated in outer cortical and juxtamedullary fractions from rat kidney cortex (Fig. 4). Both proteins were enriched in the BBM portion compared to the homogenate where they are present in a much smaller proportion of total protein. The detection of these proteins in the brush-border membrane was 7- to 8-fold stronger in the outer cortex region than in the juxtamedullary region. These proteins were absent from the cytosolic fraction.

The expression of NaPi-2 was studied under a condition previously shown to alter phosphate reabsorption. Renal adaptation to chronic dietary phosphate deprivation is well known to involve an adaptive increase of sodium/phosphate cotransport in brush-border membranes from the proximal tubule [28,29]. Under these low dietary phosphate conditions, the initial rate of Na^+-dependent phosphate transport into vesicles is increased 3- to 4-fold [17].

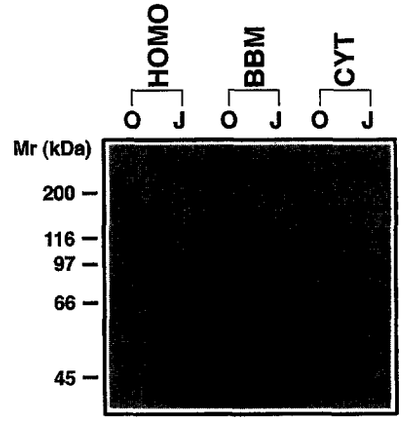

Fig. 4. Western blot analysis of fractions from rat kidney cortex. Proteins from the cell homogenate (HOMO), brush-border membrane (BBM) and cytosolic (CYT) fractions from outer (O) and juxtamedullary (J) cortex were analyzed as described in the legend of Fig. 1. Comparable data were obtained in three independent experiments performed in duplicate.

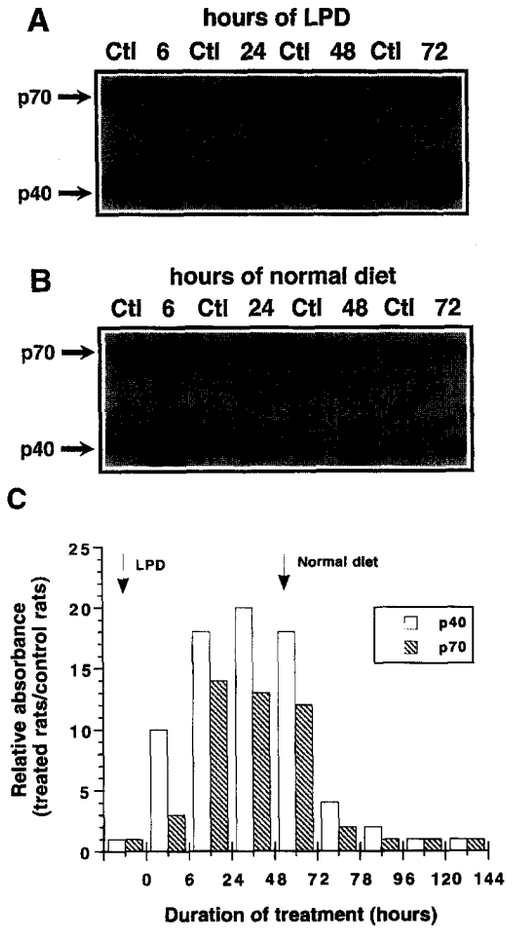

Fig. 5. Effect of a low-phosphate diet on the immunodetection of p40 and p70 in rat brush-border membranes. BBM were isolated from the outer cortex portion of rats either fed a low-phosphate diet (LPD) for 6, 24, 48 and 72 h (A), or submitted to a LPD for 72 h and then given a normal diet (B). In B, the hours indicated represent the time after the LPD was stopped. There was a control (Ctl) for each group of rats. Proteins were analyzed as described in the legend of Fig. 1 and the resulting relative absorbance (treated rats/control rats) was plotted as a function of the time of treatment (C). Comparable data were obtained in three independent experiments.

The low dietary phosphate intake led to a maximum 20- and 14-fold increase in the intensity of the immunolabelled p40 and p70 proteins, respectively (Fig. 5A and C) after 24 to 48 h. Upon return to a normal diet, p40 and p70 levels decreased to control levels within 24 to 48 h (Fig. 5B and C).

4. Discussion

To study the type II sodium/phosphate symporter, polyclonal antibodies were raised against a synthetic peptide corresponding to the 14-amino acid C-terminal portion of the recently cloned rat kidney cortex sodium/phosphate cotransporter designated NaPi-2 [10]. This C-terminal segment was chosen because hydropathy analysis of the amino acid sequence of NaPi-2 [3,10,30] according to Kyte and Doolittle [31] suggests that it is fairly hydrophilic and predicted to be exposed. The affinity-purified antibodies were used in Western blot experiments to identify immunoreactive membrane proteins. Proteins of 40 and 70–75 kDa (p40 and p70) were specifically detected by the antibodies in membranes obtained from rat and mouse kidney cortex. Addition of protease inhibitors during BBM purification or incubation of purified BBM with or without protease inhibitors were without any effect on the detection of p70 and p40, indicating that p40 is not a degradation artifact occurring during membrane isolation. The apparent molecular mass of 70–75 kDa (p70) is in good agreement with that calculated from the amino acid sequence of non-glycosylated NaPi-2 which is 68.7 kDa [10], but not the p40 which most probably derives from p70. Similar reports using polyclonal antibodies directed against the deduced N- and C-terminal amino acid sequences of NaPi-2 have demonstrated the detection of proteins of apparent molecular masses of ~ 80–90 kDa in rat BBM and ~ 80–110 kDa in cRNA-injected oocytes [15,32]. Our antibodies cross-reacted with rabbit BBM proteins of 72 and 82 kDa because the C-terminal sequence of the rat NaPi-2 cotransporter [10] is identical to the one from NaPi-6 type II cotransporter from rabbit [9]. The apparent molecular masses of these two proteins is in good agreement with that calculated from the amino acid sequence of non-glycosylated NaPi-6 which is 69.4 kDa [9]. The reactivity of our antibodies with rat, mouse and rabbit BBM proteins indicates that this carrier possesses similar epitopes in these species. The absence of detected proteins from partially purified OK cell plasma membranes served as a negative control since the type II sodium/phosphate cotransporter cloned from this species (NaPi-4) does not possess the same antigenic C-terminal sequence as NaPi-2 from rat [12]. No protein related to NaPi-2 could be detected in beef BBM using these antibodies, but the C-terminal portion of the recently cloned type II sodium-dependent phosphate cotransporter from the bovine renal epithelial cell line NBL-1 shares only 3 identical amino acids with the rat renal NaPi-2 cotransporter [33].

The detection of p180 when β-EtSH is omitted from samples before electrophoresis suggests that the phosphate transporter may be formed of subunits linked together with disulfide bonds to constitute a protein complex, as previously suggested for NaPi-1 [17]. A few studies using the radiation inactivation technique have reported high molecular mass proteins for the renal sodium/phosphate symporter [34–36]. Thiol oxidation was also suggested to be involved in the regulation of P_i uptake by BBM [37]. Furthermore p40, which can only be detected under reducing conditions, probably originates from reduction of disulfide bonds in p70. However, the effects of reduction on NaPi-2-related proteins are still unclear and deserve further study.

The eight identical fragments obtained for both p40 and p70 after tryptic cleavage demonstrate the very close structural relation between these proteins. These fingerprinting experiments show that both proteins possess a common portion of their amino acid chains that contains the same tryptic cleavage sites. This may suggest that the regulation of this cotransport could involve a step where the 70 kDa protein is specifically cleaved to give a 40 kDa protein.

Also, the outer cortex BBM co-localization of both proteins (p40 and p70) agrees with recent findings obtained using immunohistochemistry [38]. Thus, as mentioned above, this contributes to the possibility that p40 could derive from p70.

Adaptation to P_i restriction is dependent on de novo protein synthesis but it is still unclear whether the proteins involved are sodium/phosphate cotransporters or other proteins playing a role in the regulation of this transport. In this study, a 20- and 14-fold increase in the amount of NaPi-2-related proteins detected (p40 and p70, respectively) was observed for BBM of rats submitted to a low-phosphate diet for 24 to 48 h. The sodium/phosphate cotransport was 3- to 4-fold higher than controls (results not shown). The increase in NaPi-2 related proteins thus appears to be associated with an adaptive increase in sodium/phosphate cotransport in response to a low-P_i diet. This is the first report showing the apparent involvement of a 40-kDa protein (p40) in this type of regulation. However, the reason why the increase in p40 and p70 expression under low-phosphate conditions is much higher than the increase in transport remains unknown, but suggests a possible pool of inactive transporters regulated to remain metabolically quiescent. A low ratio BBM vesicles/leaflets could also be responsible for these discrepancies: the immunodetection of NaPi-2 does not distinguish between BBM vesicles and leaflets, but sodium/phosphate cotransport assays can only be accomplished through BBM vesicles. Previous reports have demonstrated by Northern and Western blot analysis an increase of NaPi-2-related mRNA in the kidney cortex of rats fed a low-phosphate diet compared to control rats [16,38]. Furthermore, the injection of mRNA from rats adapted to a low-P_i diet into oocytes resulted in a higher expression of sodium/phosphate cotransport [30]. In contrast, previous studies have shown that a low-phosphate diet does not affect the level of expression of the type I (NaPi-1) renal sodium/phosphate cotransporter [9,14,17].

In conclusion, the proteins detected in rat, rabbit and mouse possess an epitope sufficiently similar to the C-terminal region of NaPi-2 to be recognized by this antibody. The rat and mouse BBM introduce, in addition to p70, an unexpected p40 possessing similar physico-chemical properties. Both proteins present an adaptive response to a low-phosphate diet. This study reports evidence suggesting that along with p70, p40 may play an important role in the renal sodium/phosphate cotransport and its regulation.

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