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Immunodetection and characterization of proteins implicated in renal sodium/phosphate cotransport

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Abstract

Polyclonal antibodies raised against the 14-amino acid C-terminal portion of the rabbit renal brush-border membrane Na^+/P_i cotransporter, as deduced from the nucleotide sequence of the cloned NaPi-1 gene, were used for Western blot analysis of renal brush-border membrane proteins from rat, rabbit and beef. Proteins of 65 kDa from the rat, 64 kDa from the rabbit, and 38, 66, 77, 92, 110, 176 and 222 kDa from the beef were specifically labelled. The affinity of the antibodies was much greater, however, for the proteins of the rat and rabbit than for those of the beef. The rat 65-kDa antigen was readily detected in brush-border membranes isolated from kidney cortex, but was absent from the basolateral membrane and the cytosolic and microsomal fractions of this tissue, in agreement with the subcellular localization of the Na^+/P_i cotransporter. This antigen was however several-fold more abundant in the juxtamedullary portion of the cortex than in the outer portion. Despite a strong stimulation in phosphate transport, a low-phosphate diet had little influence on the amount of antigen detected. An additional peptide-displaceable band corresponding to a protein of 250 kDa appeared when β-mercaptoethanol was omitted during electrophoresis, in agreement with the possibility that disulfide bonds may be involved in the regulation of renal phosphate transport activity.

Key words: Immunodetection; Anti-peptide antibody; Sodium ion/phosphate cotransporter; Kidney; (Rat); (Rabbit); (Bovine)

1. Introduction

Reabsorption of phosphate occurs mainly across the apical brush-border membrane of the proximal tubule and is carried out by a specific sodium gradient-dependent transport system [1–4]. The kinetic properties of this transport activity and its physiological regulation by a variety of hormones and by dietary phosphate intake have been the object of intensive research which has been summarized in excellent recent reviews [5–7].

Despite a wealth of information concerning its functional properties, the structural identity of the carrier molecule remains to be ascertained [8]. A number of proteins have been proposed to be involved in renal sodium/phosphate cotransport. These include proteins of 31, 53, 104 and 176 kDa from an opossum kidney established cell line (OK cells) which were affinity-labelled with *N*-acetyl[^{3}H]imidazole [9] and proteins of 70 and 97 kDa from rat kidney cortex which were photoaffinity-labelled with a ^{32}P-labelled azidobenzoic acid derivative of NAD [10]. A 55-kDa glycoprotein from a bovine renal epithelial cell line (NBL-1) was labelled with [^{3}H]mannose during adaptation to a low-phosphate medium [11]. Phosphate-binding proteolipids of 3 [12] and 70 kDa [13] were both purified from chloroform extracts of rabbit brush-border membrane vesicles. The latter protein has recently been shown to carry out sodium-dependent phosphate transport upon reconstitution in proteoliposomes [14]. Finally, a cDNA clone designated NaPi-1 and coding for a 52-kDa sodium-dependent phosphate carrier was obtained from rabbit kidney cortex mRNA, expressed in *Xenopus laevis* oocytes and sequenced [15,16].

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Abbreviations: ECL, enhanced chemiluminescence; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid; IgG, immunoglobulin G; KLH, keyhole limpet hemocyanin; PVDF, polyvinylidene difluoride.

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Using a different approach, the size of the functional unit of the carrier molecule was estimated with the radiation-inactivation technique. Based on the rate of decay of their transport activities in function of the radiation dose, sizes of 234, 242 and 172 kDa were obtained for the phosphate carriers of the rat [17], mouse [18] and beef [19], respectively. These results have led to the suggestion that the renal sodium/phosphate symporter functions in situ as an oligomeric protein complex, probably a homotetramer.

In the present study, polyclonal antibodies raised against the C-terminal portion of the protein encoded by the NaPi-1 gene were used for Western blot analysis of brush-border membrane proteins from three animal species. Single proteins were detected specifically in rat and rabbit membranes, but the bovine carrier appears to be more distantly related. The subcellular distribution of the labelled antigen corresponded to that expected for the phosphate carrier. However, the labelled antigen was mainly detected in the juxtamedullary portion of the cortex and was not quantitatively influenced by the phosphate content of the diet, indicating that the stimulation of phosphate transport induced by the diet could involve a phosphate carrier other than NaPi-1 or a mechanism in which the number of phosphate carriers present in the brush-border membrane does not increase.

2. Materials and methods

2.1. Membrane preparations and cell fractionation

16-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.). Bovine kidneys were obtained from a local abattoir. 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 whole cortex, outer cortex and juxtamedullary cortex with an $MgCl_2$ precipitation method [20] or with slight modifications for the preparation of large batches of bovine membranes as described earlier [21]. 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. Basolateral membranes were purified with a self-orienting Percoll-gradient method [22]. Cytosol and microsome fractions were prepared by differential centrifugation at 4°C. After disruption of the tissue in a Potter (Braun, Melsungen, Germany) tissue homogenizer, intact cells were removed by centrifugation at $3000 \times g$ for 10 min. Following removal of the mitochondria by centrifugation at $8500 \times g$ for 10 min, plasma membranes were precipitated by centrifugation at $28000 \times g$ for 20 min. The supernatant was finally centrifuged at $100000 \times g$ to yield a pellet enriched in microsomes and a supernatant containing the cytosolic fraction.

2.2. Peptide synthesis

The fifteen-amino acid peptide CEIQDWAKEKQHTRL was synthesized using Fmoc chemistry and an Applied Biosystems (Mississauga, Ont.) automated peptide synthesizer following the recommendations of the manufacturer. This peptide corresponds to the 14-amino acid C-terminal portion of the rabbit sodium/phosphate symporter as deduced from the nucleotide sequence of the cloned NaPi-1 gene [16] to which an N-terminal Cys residue was added to allow coupling of the peptide to a carrier protein. The peptide was purified on a semi-preparative Vydac (Hesperia, CA) C18 high performance liquid chromatography column and its identity was verified by fast atom bombardment mass spectrometry.

2.3. Immunization

The purified peptide was coupled to keyhole limpet hemocyanin (KLH) (Pierce, Rockford, IL) through the free sulfhydryl group of its cysteine residue with *m*-maleimidobenzoyl-*N*-hydroxysuccinimide ester (Pierce) as described by Mumby and Gilman [23]. Rabbits were injected subcutaneously at four sites on the back with 200 μg of the KLH-coupled peptide mixed homogeneously with Titermax adjuvant (CytRx, Norcross, GA) (1 : 1 volume ratio). A booster injection of antigen was administered after 14 days. The rabbits were bled twelve days after the booster injection. The blood was allowed to coagulate at room temperature for 1 h and stored overnight at 4°C. The clot was removed and the serum was collected after sedimenting blood cells by centrifugation at $10000 \times g$ for 10 min. Reactivity of the antiserum against the peptide and KLH was assayed by enzyme-linked immunosorbent assay [23].

2.4. IgG purification

The immunoglobulin G (IgG) of the serum (adjusted to pH 8.6 with Tris/HCl) was purified on a Pharmacia (Dorval, Que.) HiTrap protein A fast protein liquid chromatography column (5 ml) 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. Antibodies were further

purified by affinity chromatography on a peptide-Sepharose column (2 ml) prepared as described by Mumby and Gilman [23] and equilibrated with 100 mM NaCl, 20 mM Tris/HCl (pH 7.5). After extensive washing with the same buffer, the antibodies were eluted with 0.2 M glycine (pH 2.2). The fractions were collected as mentioned above for the protein A column.

2.5. Immunodetection

SDS-polyacrylamide gel electrophoresis was performed with a Mini-Protean II (Bio-Rad, Mississauga, Ont.) electrophoresis apparatus and the Laemmli [24] buffer system. The separating gel contained 10% (w/v) acrylamide and 0.3% (w/v) *N,N'*-methylene-bisacrylamide. Unless indicated otherwise, prior to electrophoresis, the samples were boiled 3 min in sample buffer containing 1.0% (w/v) SDS and 2.5% (v/v) β-mercaptoethanol. Electrophoresis was carried out at a constant voltage of 150 V for 1 h. Each well contained 12 μg of protein. Molecular mass determinations were based on a calibration curve prepared with the following proteins (Bio-Rad): myosin (200 kDa), β-galatosidase (116 kDa), phosphorylase *b* (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa) and soybean trypsin inhibitor (21 kDa).

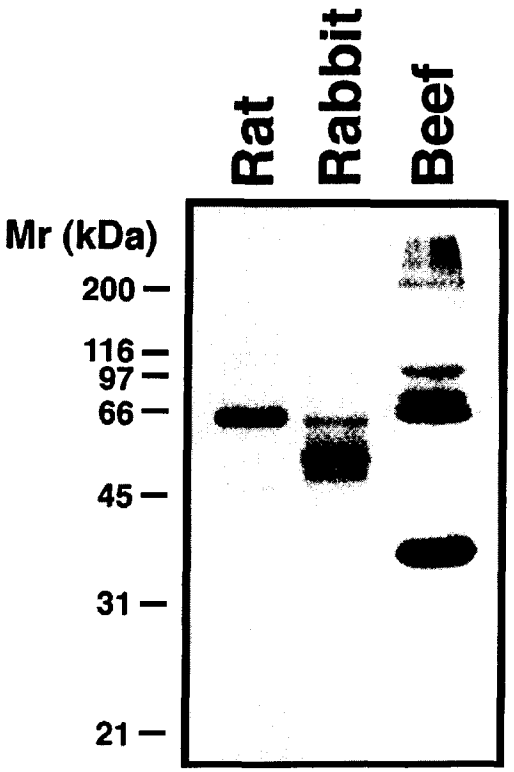

Fig. 1. Western blot analysis of renal brush-border membrane proteins from rat, rabbit and beef using polyclonal antibodies directed against the rabbit renal Na^+/P_i cotransporter. Brush-border membrane proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred to PVDF membranes. The Western blots were incubated with affinity-purified antibodies directed against the 14-amino acid C-terminal portion of the rabbit Na^+/P_i cotransporter, followed by donkey anti-rabbit IgG horseradish peroxidase-linked antibodies. Labelled antigens were revealed by autoradiography with ECL Western blotting solutions.

Following electrophoresis, the gels were equilibrated in transfer solution (10% (v/v) methanol, 25 mM Tris, 192 mM glycine (pH 8.3)). The proteins were transferred to 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 during 3 h at 4°C. The remaining binding sites on PVDF membranes were saturated 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 A) containing 5% (w/v) powdered milk, at 4°C. The membranes were washed three times by gentle agitation in buffer A for 10 min and incubated for 1 h with appropriate dilutions of the antipeptide antibody (as specified in the figure legends) in buffer A. The membranes were washed three more times in buffer A 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 A. The membranes were finally washed 5 times for 5 min in buffer A. Labelled antigens were revealed with enhanced chemiluminescence (ECL) Western blotting solutions (Amersham) following the recommendations of the manufacturer. The membranes were exposed for different times to preflashed (1/16 opening) Fuji films which were developed with standard methods. The autoradiograms were analyzed with an Ultroscan XL (Pharmacia) densitometer.

2.6. Phosphate transport

Uptake of radiolabelled phosphate into brush-border membrane vesicles containing 300 mM mannitol and 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (Hepes)/Tris (pH 7.5), was measured at 25°C with a rapid filtration method [25] by adding 5 μl of membrane suspension (about 30 mg protein/ml) to 25 μl of a solution containing 200 μM $[^{32}P]KH_2PO_4$-K_2HPO_4, 5 mM Hepes/Tris (pH 7.5), and 150 mM $NaNO_3$ or KNO_3. After a 5 s incubation, the reaction was stopped with 1 ml of ice-cold stop solution containing 150 mM KCl and 5 mM Hepes/Tris (pH 7.5). The suspension was filtered immediately under vacuum through a nitrocellulose (Millipore) filter (0.45-μm pore diameter). The filter was rinsed with an additional 7 ml of stop solution and the radioactivity was measured by liquid scintillation counting in water.

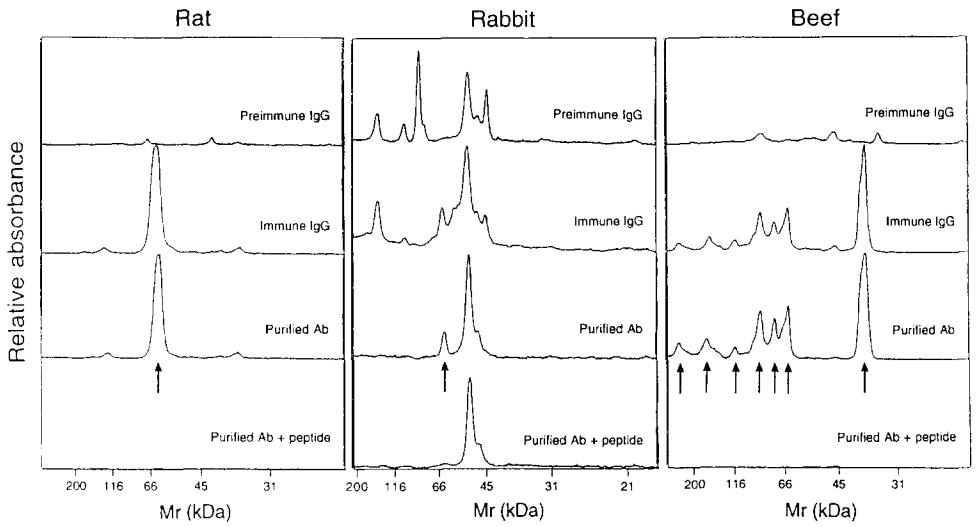

Fig. 2. Specificity of the antibody labelling reaction. The profiles obtained by densitometric scanning of the autoradiograms after incubation of the Western blots with pre-immune serum IgG, the crude IgG fraction prepared by chromatography on protein A-Sepharose, antibodies purified with a peptide-Sepharose affinity chromatography column, and purified antibodies with 10^{-4} M peptide are compared. Proteins which were specifically labelled are indicated by arrows.

3. Results

Polyclonal antibodies directed against the 14-amino acid C-terminal portion of the cloned rabbit renal brush-border membrane sodium/phosphate symporter [16] were raised in rabbits, affinity-purified and used for the immunodetection of related proteins in the brush-border membranes of three animal species (Fig. 1). Autoradiograms of rat membranes revealed a single intensely labelled band corresponding to a protein of 65 kDa. In rabbit membranes, a major band corresponding to a protein of 55 kDa and fainter bands corresponding to proteins of 50 and 64 kDa were detected. The profile obtained with bovine membranes was more complex with proteins of 38, 66, 77, 92, 110, 176 and 222 kDa being readily labelled.

The specificity of the labelling reaction was examined by comparing the profiles obtained after incubation of the Western blots with pre-immune serum IgG, the crude immune IgG fraction, affinity-purified antibodies alone, and purified antibodies in the presence of 10^{-4} M peptide (Fig. 2). Most of the minor bands detected in the rat membranes could be attributed to non-specific labelling since they were present in the profiles obtained with pre-immune serum IgG. The major band corresponding to a protein of 65 kDa was only detected with the antipeptide antibodies and was displaced by the peptide. In the case of the rabbit, non-specific labelling was rather extensive as evidenced by the presence of several bands with pre-immune serum IgG and by the inability of the peptide to prevent the labelling of most of the bands detected by

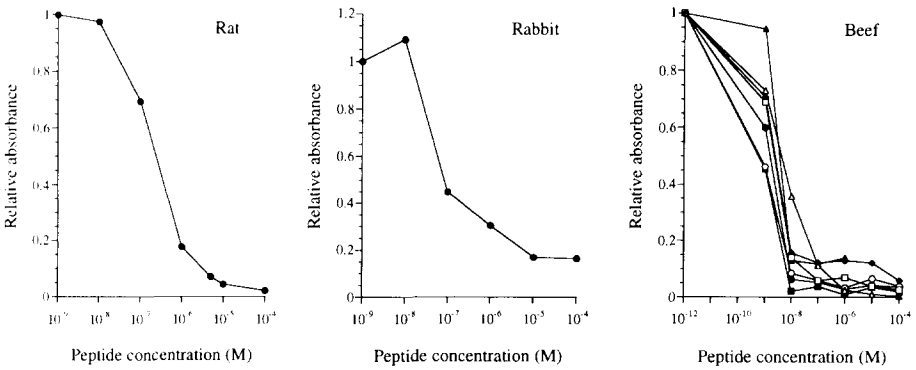

Fig. 3. Antibody displacement studies. Brush-border membrane proteins were labelled as described in the legend of Fig. 1 with affinity-purified antibodies diluted 1/250 for the rat and 1/1000 for the rabbit and beef in the presence of different concentrations of peptide. The intensity of each of the specifically labelled protein bands, corresponding to proteins of 65 kDa from the rat, 64 kDa from the rabbit, and 38 (△), 66 (□), 77 (○), 92 (◆), 110 (▲), 176 (■) and 222 (●) kDa from the beef, was measured by densitometric scanning of the autoradiograms.

the purified antibody. The only protein which was specifically labelled was that of 64 kDa. For bovine membranes, little labelling was observed with pre-immune serum IgG. Identical proteins of 38, 66, 77, 92, 110, 176 and 222 kDa were labelled with crude immune IgG and purified antibodies. All these bands disappeared when the incubation with the antibodies was carried out in the presence of the peptide.

The affinity of the purified antibodies was much greater for the proteins of the rat and rabbit than for those of the beef (Fig. 3). The labelling of the 65-kDa protein from rat was reduced 50% by the addition of about $5 \cdot 10^{-7}$ M peptide for a 1/250 dilution of the antibodies. In the case of the 64-kDa protein from rabbit, an equivalent displacement of the label was obtained with about 10^{-7} M peptide for a 1/1000 dilution of the antibodies. In contrast, the amount of peptide required to halve the intensity of the labelled protein bands from bovine membranes ranged from 10^{-9} to 10^{-8} M for a 1/1000 dilution of the antibodies.

The specificity of the antibodies was further examined by testing whether the 65-kDa protein from rat was detected in different subcellular fractions from kidney cortex (Fig. 4). In agreement with the cellular distribution of the sodium/phosphate symporter, this antigen was readily labelled in the brush-border membrane, but was not detectable in the cell homogenate, basolateral membrane, or cytosolic and microsomal fractions, where it is either absent or present in a much smaller proportion of total protein. Surprisingly however, the 65-kDa antigen was always 2–6 times more intensely labelled in the juxtamedullary portion of the cortex than in the outer portion (Fig. 4).

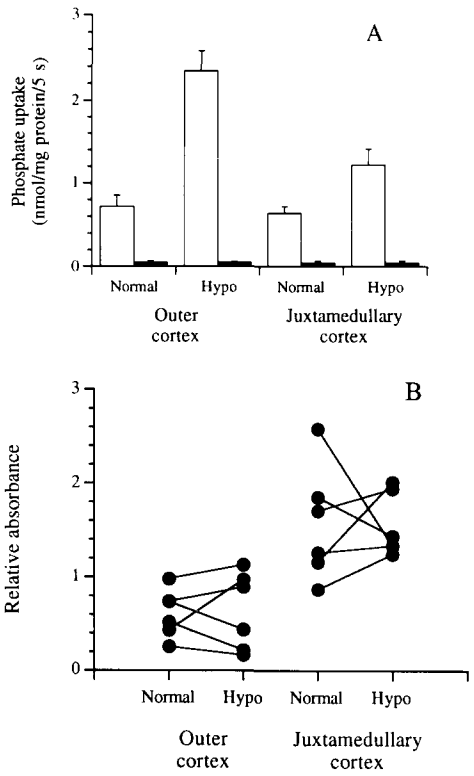

Fig. 5. Comparison between phosphate transport activity and amount of 65-kDa antigen detected in brush-border membranes isolated from the outer and juxtamedullary portions of rats fed normal and low-phosphate (Hypo) diets. (A) Initial rates of phosphate uptake were measured as described in the Materials and methods section. The incubation medium contained 200 μM [^{32}P]KH_2PO_4-K_2HPO_4, 5 mM Hepes/Tris (pH 7.5), and 150 mM $NaNO_3$ (□) or KNO_3 (■). (B) Proteins were analyzed as described in the legend of Fig. 1. The intensity of the 65-kDa labelled antigen was measured by densitometric scanning of the autoradiograms.

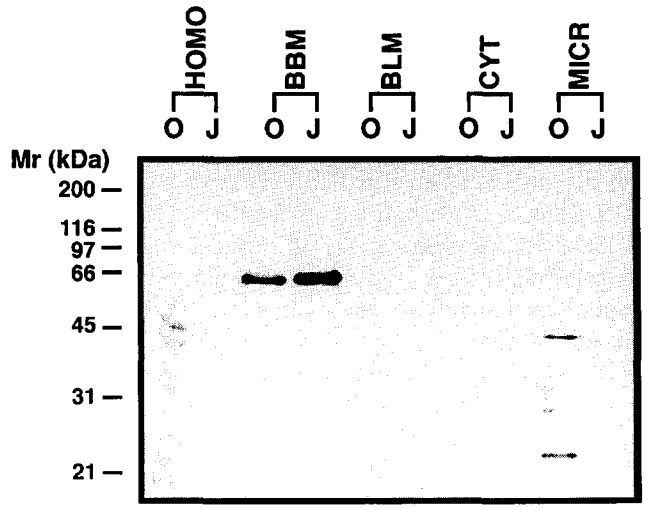

Fig. 4. Western blot analysis of different subcellular fractions from rat kidney cortex. Proteins from the cell homogenate (HOMO), brush-border membrane (BBM), basolateral membrane (BLM), and cytosolic (CYT) and microsomal (MICR) fractions of outer (O) and juxtamedullary (J) cortex were analyzed as described in the legend of Fig. 1.

Dietary phosphate deprivation is well known to elicit an adaptive increase in brush-border membrane phosphate transport [26,27]. We therefore investigated whether the level of expression of the 65-kDa antigen was correlated with the phosphate transport activity of brush-border membrane vesicles isolated from rats fed normal- and low-phosphate diets (Fig. 5). Phosphate deprivation led to 3.4- and 2.0-fold increases in the initial rate of sodium-dependent phosphate transport into vesicles prepared from outer and juxtamedullary cortex, respectively (Fig. 5A). In contrast, dietary phosphate intake had no consistent effect on the intensity of the immunolabelled 65-kDa protein band from either portion of the cortex (Fig. 5B).

The functional unit of the rat renal sodium/phosphate symporter was suggested to consist of a protein complex of about 234 kDa, probably a homotetramer [17]. The possibility was thus investigated that its subunits could be linked by disulfide bonds (Fig. 6).

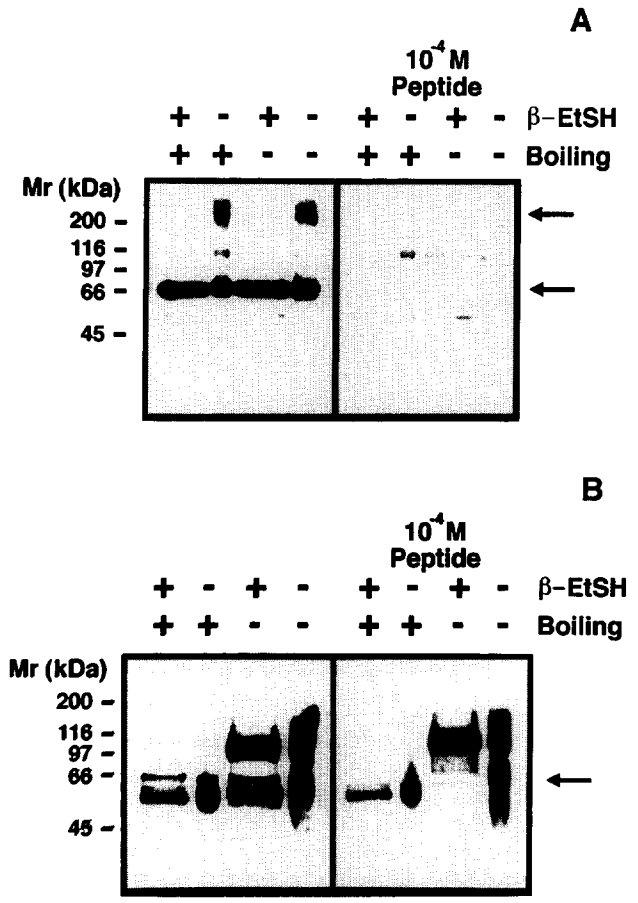

Fig. 6. Effect of β-mercaptoethanol and boiling on the immunodetection of rat (A) and rabbit (B) renal brush-border membrane proteins. (Left) Samples were either boiled or not in the presence or absence of β-mercaptoethanol (β-EtSH) as indicated and analyzed as described in the legend of Fig. 1. (Right) Duplicate Western blots were analyzed with an identical protocol except that 10^{-4} M peptide was added to the antibodies during the labelling reaction. Protein bands that were displaced by the peptide are indicated by arrows.

Western blots of brush-border membrane proteins were analyzed after electrophoresis of samples which had been boiled or not in the presence and absence of β-mercaptoethanol. The 65-kDa protein from the rat was the major labelled species under all four experimental conditions, but in the absence of the reducing agent, an additional labelled band appeared corresponding to a protein of about 250 kDa. This protein, like that of 65 kDa, was not visible when the peptide was added to the antibodies during the labelling reaction (Fig. 6A). Densitometric scanning of the autoradiograms indicated that the 250-kDa protein band accounts for about 30% of the total peptide-displaceable label. In contrast with these clear-cut results, a larger peptide-displaceable protein band could not be demonstrated in rabbit brush-border membranes when β-mercaptoethanol was omitted from the electrophoresis sample buffer (Fig. 6B).

4. Discussion

The antibodies described in the present report were directed against a synthetic peptide corresponding to the 14-amino acid C-terminal segment of the recently cloned putative rabbit kidney cortex sodium/phosphate cotransporter designated NaPi-1 [16]. This segment was chosen because it is hydrophilic and predicted to be exposed on the cytoplasmic side of the membrane [16]. Furthermore, C-terminal peptides are known to often yield antibodies which can recognize the native protein [28]. Western blot experiments were performed with these antibodies in an effort to identify brush-border membrane proteins which could be related to the renal phosphate carrier in different animal species. Single proteins of 65 and 64 kDa were specifically recognized by the antibodies in membranes isolated from rat and rabbit kidney cortex, respectively. Their apparent molecular masses are in good agreement with that calculated from the amino acid sequence of NaPi-1 (52 kDa) since this protein is most probably glycosylated [16]. In contrast, several proteins were immunodetected in bovine membranes, but the peptide concentration required to reduce the labelling of these proteins was 10–100 times lower than for those of the other two species. These results indicate that the epitopes detected in the brush-border membranes of the rat and rabbit are more similar to each other than to those which were labelled in the bovine membrane.

The 65-kDa protein from the rat was found only in brush-border membranes as expected for the sodium/phosphate symporter. Surprisingly the amount of antigen detected was 2–6 times higher in the juxtamedullary portion of the cortex than in the outer portion although transport rates were similar. If NaPi-1 were the only phosphate carrier of the kidney, this would indicate that it is the level of regulation and not the density of the carrier molecules in the membrane that is responsible for the transport activity in brush-border membranes from juxtamedullary and outer cortex. The possibility remains however that at least one other sodium/phosphate symporter could exist in kidney cortex in addition to NaPi-1 which was found predominantly in the juxtamedullary cortex. Several groups have reported the existence of two sodium/phosphate cotransporters in kidney cortex: one with high affinity and low capacity and the other with low affinity and high capacity for phosphate [29–33]. However, the low-affinity carrier cannot correspond to NaPi-1 since it was only detected in the outer cortex [29]. In addition, another phosphate carrier, designated NaPi-2, has recently been cloned from rat kidney cor-

tex and sequenced [34]. Although this carrier shares little sequence homology with NaPi-1, it should have similar kinetic properties since the K_m for phosphate was either similar [29] in both cortical fractions or only slightly lower in the juxtamedullary fraction [35].

In addition to the discrepancy between the distribution of the detected antigen and phosphate transport activity in the different fractions of the cortex, the amounts of antigen detected did not correlate with the differences in phosphate transport measured in the brush-border membranes isolated from rats fed low-phosphate and control diets. It cannot be entirely excluded that covalent modifications of some amino acids near the C-terminal end of NaPi-1, containing the epitope against which our antibodies were developed, could prevent their detection and preclude an accurate estimate of the number of transporters in the membrane. The mechanism by which the epithelial cells of the kidney adapt to a low-phosphate diet is still unclear [8]. On the one hand, an increase in the number of phosphate carriers in the brush-border membrane is suggested by the fact that the increase in phosphate transport is associated with an increased Vmax rather than with a change in K_m for phosphate [2,3] and requires de novo protein synthesis [36]. On the other hand, an alternative mechanism by which the enhanced phosphate transport activity would result from an increase in the activity of the transporters already present in the membrane is suggested by the fact that the sodium-dependent binding of phosphonoformic acid, a specific inhibitor of sodium/phosphate cotransport [37], is unaffected by the phosphate content of the diet [38,39]. Among possible mechanisms for adaptation to dietary phosphate, modulation of the activity of the phosphate cotransporter by the cholesterol content and fluidity of the membrane has been extensively documented [38,40,41]. The exact contribution of this mechanism to the regulation of phosphate transport is still uncertain however since increases in phosphate transport were found to precede detectable changes in membrane fluidity and cholesterol content [42]. The adaptive increase in phosphate transport could also involve an increase in the proportion of active transporters that would occur without changing the actual number of transporter molecules present in the membrane by, for example, a redistribution of functional monomers as was suggested earlier [39]. Recent results suggest that adaptation to a low phosphate diet could be due to a modulation of NaPi-2 expression [43].

A high-molecular mass protein (250 kDa) was detected when β-mercaptoethanol was omitted during electrophoresis. The phosphate carrier therefore appears to form a disulfide-linked oligomeric complex with other protein molecules. Thiol oxidation was previously suggested to be involved in the regulation of phosphate uptake by renal brush-border membranes since it was increased by the addition of a thiol reducing agent, dithiothreitol, and decreased by the addition of a thiol oxidant, diamide [44]. The significance of this correlation is still unclear, however, especially since such oligomeric complexes could not be demonstrated in membranes isolated from the rabbit although these may have been masked by the much higher level of non-specific labelling found with rabbit membranes.

In conclusion, the results of the present study indicate that regulation of renal phosphate transport by dietary phosphate intake may occur without changes in the number of transporter molecules (NaPi-1) in the brush-border membrane.

5. Acknowledgments

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