Identification and Epitope Analysis of the Renal Na⁺/Pᵢ Cotransport Protein Using Monoclonal Antibodies*

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Hanna Debiec† and Pierre M. Ronco
From the Institut National de la Santé et de la Recherche Médicale Unité 64, Hôpital Tenon, 75970 Paris Cedex 20, France

Seven monoclonal antibodies (mAbs) were raised against a rabbit renal brush-border glycoprotein (molecular mass, 63–66 kDa), presumably involved in Na⁺/Pᵢ cotransport, which we had previously purified and reconstituted in active form in proteoliposomes (Debiec, H., Lorenc, R., and Ronco, P. M. (1992) Biochem. J. 286, 97–102). Antibody specificity for the 63–66-kDa protein was analyzed by enzyme-linked immunosorbent assay and confirmed by Western blotting and immunofluorescence spectroscopy of solubilized brush-border membranes (BBM), which both yielded a single 63–66-kDa band. Enzyme-linked immunosorbent assay and immunoblotting of renal cortical cell fractions localized the immunoreactive protein to the brush-border membrane. This location was confirmed by indirect immunofluorescence of kidney cortex sections. Binding of two of the seven mAbs (63A20 and 206A126) to native BBM only occurred when the related epitope was exposed in the presence or absence of Na⁺, respectively; the other mAbs did not react with native BBM probably because of intramembranous orientation of the epitopes. mAb 63A20 inhibited dose-dependently Na⁺/Pᵢ cotransport when preincubation of BBM was carried out in the presence of Na⁺ but did not affect Na⁺/d-glucose cotransport. Proteoliposomes formed from BBM proteins depleted of the 63–66-kDa protein by affinity chromatography with mAb 63A20 showed an 85% reduction in Na⁺/Pᵢ cotransport, whereas Na⁺/d-glucose cotransport was not modified. These results thus establish that the 63–66-kDa BBM protein is the essential component of the Na⁺/Pᵢ cotransport system. The present study also provides the first immunologic tools available for immunohistochemical localization of the Na⁺/Pᵢ cotransporter. Finally, the identification of a functional epitope by mAb 63A20 opens up new ways to explore the molecular aspects of Pᵢ uptake.

Phosphate (Pᵢ) is transported into eukaryotic cells by a Na⁺-coupled cotransport process probably mediated by an intrinsic membrane protein and driven by an inwardly directed sodium gradient. This type of Na⁺/Pᵢ cotransport has been first described in renal (1) and intestinal (2) epithelia and most extensively studied in these epithelia (3–6) where it is the rate-limiting step in transepithelial Pᵢ transport, controlled by many hormonal and dietary factors. Detailed studies using renal membrane vesicles suggest the presence of multiple systems for Pᵢ cotransport along the nephron, differing in their affinities and capacities (7, 8). Recently, a similar Na⁺/Pᵢ cotransport process has been detected in other cell types including cardiac and skeletal muscle (9, 10), bone (11), liver (12), and placenta (13) cells. In these cells, Pᵢ transport is also hormonally regulated and shares some properties with the kidney Na⁺/Pᵢ cotransporter, but substantial differences were also observed (14). The main question that then arises concerns the unity or multiplicity (isoforms) of the Na⁺/Pᵢ membrane cotransporter.

Membrane transport proteins have been difficult to purify, mainly because of their hydrophobicity and low abundance. Moreover, in the case of the Na⁺/Pᵢ cotransporter, there is no available specific covalent label that could be used as a marker during purification. Considerable and various efforts have therefore been accomplished to identify the cotransporter(s) at the molecular level as attested by photoaffinity labeling (15–17), radiation inactivation (18), chemical modification (19, 20), and purification-reconstitution (21, 22) experiments, but these techniques have provided conflicting results. Recently, using a different approach based on expression cloning, a presumed component of the rabbit kidney Na⁺/Pᵢ cotransporter was identified as a 61-kDa glycoprotein with a protein moiety of about 52 kDa (23). An alternative strategy for identification of the Pᵢ membrane transport systems is the production of monoclonal antibodies. This technology has been used successfully to identify many membrane cotransporters including Na⁺/glucose (24, 25) and Na⁺/amino acid (26, 27) carriers. Furthermore, monoclonal antibodies are valuable tools to identify crucial functional segments (epitopes) in the molecule; to define cellular localization, tissue specificity, and ontogeny of the cotransporter; and to study protein processing during biosynthesis.

We have previously isolated from rabbit renal brush-border membranes (BBM) a reconstitutively active form, a 63–66-kDa glycoprotein (the molecular mass of the protein component is approximately 50 kDa) presumably involved in Na⁺/Pᵢ cotransport (28, 29). To establish unequivocally the identity of the cotransporter, this purified membrane protein was used as an antigen to immunize mice for the production of monoclonal antibodies. This report describes the screening process and the utilization of selected monoclonal antibodies to confirm that the 63–66-kDa BBM protein is the essential component of the Na⁺/Pᵢ cotransport system in the kidney. It

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† Recipient of a fellowship from the Association pour la Recherche sur le Cancer. To whom correspondence should be addressed: Unité INSERM U.64, Hôpital Tenon, 4, rue de la Chine, 75970 Paris Cedex 20, France.

† The abbreviations used are: BBM, brush-border membrane(s); BBMV, brush-border membrane vesicle(s); BLM, basolateral membrane(s); mAb, monoclonal antibody; ELISA, enzyme-linked immunosorbent solid phase assay; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; DTT, dithiothreitol.

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Preparation of IgMs purified by gel filtration on Sephacryl S-300 were routinely 25-fold and 6-fold enriched in marker enzymes. All other materials were of analytical grade and commercially available.

**EXPERIMENTAL PROCEDURES**

**Materials**—All reagents and standard proteins for electrophoresis were from Bio-Rad; Nonidet P-40, phenylglyoxal, and lipids (phosphatidylcholine type XI-E, phosphatidylethanolamine type III-A (egg yolk), phosphatidyl-L-serine (bovine brain), and cholesterol) were purchased from Sigma. CNBr-activated Sepharose 4B was from Pharmacia and streptavidin supplied by Amersham International. All other reagents were analytical grade and commercially available.

**Production of Monoclonal Antibodies**—A set of monoclonal antibodies was raised against the 63-66-kDa protein extracted from rabbit renal BBM with chloroform/methanol and purified by hydroxyapatite chromatography as described previously (29). BALB/c mice were injected subcutaneously three times at 2-week intervals with approximately 100 μg of the purified protein incorporated into complete Freund's adjuvant. The best responses were boosted intraperitoneally with the same antigen preparation but without Freund's adjuvant. Spleen cells were collected 4 days after the last immunization and fused with the NS-1 mouse myeloma cells in the presence of 30% polyethylene glycol (31). Hybridoma culture supernatants were screened for the presence of antibodies by an ELISA using the isolated protein immobilized on polyvinyl microtiter plates. Sheep recognizing antibodies reactive with the immunogen were selected, subcloned by limiting dilution, and expanded in mouse ascitic fluid for large scale antibody production.

The antibodies characterized in the present study were shown to be of the IgM type by Ouchterlony analysis using class- and subclass-specific anti-mouse Ig antibodies. IgMs were purified from ascites by gel filtration on a Sephacryl S-300 column. Purity of the IgMs was established by SDS-PAGE using the Laemmli's system (37) as described below.

**ELISA**—Polyvinyl microtiter wells were coated by overnight incubation with 50 μl of the purified protein (10 μg/ml) or solubilized membrane fractions (400 μg/ml) in sodium carbonate-bicarbonate buffer containing protease inhibitors, pH 9.5. All incubations were carried out at 37 °C. After aspiration of the coating solution, the wells were washed with PBS containing 0.05% (v/v) Tween 20 (PBS-T) for 2 h with 200 μl of PBS-T. The wells were then incubated for 2 h with hybridoma supernatants or with the purified monoclonal antibody to be tested at a concentration given in figure legends. The antibody bound specifically to the antigen was quantitated using the biotin-streptavidin system (biotinylated species-specific anti-mouse Ig followed by horseradish peroxidase-labeled streptavidin). Each incubation step was preceded by extensive washing in PBS-T. The color development was performed for 10 min at room temperature with 0.4 mg/ml o-phenylenediamine dihydrochloride and 0.015% H2O2 in 0.1 M citrate buffer. The reaction was stopped by the addition of 1 N H2SO4, and absorbance values were measured in a two-wavelength microplate reader at 492 nm, with a reference wavelength of 630 nm.

**Preparation of Cell and Mitochondria Membranes**—BBM were prepared from male rabbit (6 months old) renal cortex by the magnesium precipitation method and differential centrifugation (32). The density of the BBM fraction was established by ultracentrifugation of alkaline phosphatase in the BBM fraction compared with the crude homogenate. The basolateral membranes (BLM) and mitochondria were prepared from rabbit kidney cortex, using a self-orienting Percoll gradient (34). (Na+ - K+)-ATPase (35) and succinic dehydrogenase (36) were used as basolateral and mitochondrial markers, respectively. The BLM and mitochondria fractions were routinely 25-fold and 8-fold enriched in marker enzymes compared with the initial homogenate, respectively.

**Immunofluorimetry Chromatography**—For immunofluorimetry column preparation, IgMs purified by gel filtration on Sephacryl S-300 were adjusted to the appropriate concentration (0.5 mg/ml) and applied on CNBr-activated Sepharose 4B according to the manufacturers' instructions. BBM were treated with 0.1 M EDTA to remove extrinsic membrane proteins (24) and were then solubilized with 1.0% Nonidet P-40 in buffer KM (0.1 M KCl, 0.2 M mannitol, 10 mM HEPES/Tris, pH 7.4, 0.5 mM DTT and 0.1 mM MnCl2) containing 2 mM KF, 2 mM phenylmethylsulfonyl fluoride, and 1 mM benzamidine. The mixture was incubated for 1 h at 4 °C and then centrifuged at 100,000 × g for 1 h. The supernatant was applied to the monoclonal antibody column and incubated overnight at 4 °C. After incubation, the effluent was collected, and the column was washed with the following solutions in chronological order: (i) 50 mM Tris/HCl, pH 8.0, containing 1.0% Nonidet P-40 and 0.5% NaCl; (ii) 50 mM Tris/HCl, pH 8.0, containing 0.5 mM NaCl; (iii) 50 mM Tris/HCl, pH 7.0. Elution was carried out with 100 mM glycine buffer, pH 2.5, containing 0.2% Nonidet P-40. Aliquots of eluates were immediately adjusted to pH 7.0 with 2 mM Tris and concentrated 5-fold with a Bio-Gel concentrator. Concentrated fractions were dialyzed against 10 mM Tris/HCl, pH 7.0, and then subjected to SDS-PAGE.

**SDS-PAGE and Western Blot Analysis**—SDS-PAGE was performed according to Laemmli (37) using an 8% polyacrylamide separation gel with 0.1% SDS and 6 M urea. Sample buffer contained 2% SDS, 6 M urea, and 5 mM DTT. All other components were as in the Laemmli sample buffer (37). Samples were run for 60 min at 200 volts. After fixation, the gels were stained (38). For Western blot analysis, electrophoretically resolved proteins were electrotransferred (50 min, 100 volts) onto nitrocellulose membrane in 25 mM Tris/HCl, pH 8.3, 192 mM glycine, and 20% methanol. The blots were incubated for 2 h with PBS containing 10% (w/v) nonfat dry milk and 0.1% (v/v) Tween 20 (blocking buffer) and then overnight with the selected monoclonal antibodies, with or without detergent before development with 4-chloro-1-naphthol and H2O2.

**Immunomorphological Studies**— Binding of monoclonal antibodies to renal tissue was studied on frozen transversal kidney cortex sections by indirect immunofluorescence. Samples of kidney cortex were taken from normal New Zealand White rabbits after pentobarbital anesthesia, immediately frozen in liquid nitrogen and stored at -70 °C until use. Cryostat frozen sections (4 μm) were briefly fixed in acetone and saturated with 3% gelatin in PBS for 2 h. Then the sections were incubated overnight at room temperature with the various monoclonal antibodies diluted in PBS containing 1.5% gelatin. After intensive washing in PBS, bound antibody was revealed with biotinylated species-specific anti-mouse Ig followed by fluorescein-labeled streptavidin. Preparations were observed with a fluorescence microscope (Leitz Wetzlar, Ortholux II) equipped with epifluorescence optics.

**Brush-border Membrane Radioimmunoeassay**—Binding of antibodies to BBM was measured in the presence or absence of sodium. BBM suspended in 10 mM triethanolamine HCl, 0.15 M KCl, 2 mM EDTA, 10% (v/v) glycerol, pH 7.4 (T-K buffer), were immobilized on nitrocellulose filters (diameter = 18 mm, 60 μg of protein/well). The filters were incubated in the wells of plastic plates previously saturated with T-K buffer containing 10% nonfat dry milk. After a 2-h incubation at room temperature, the BBM solution was removed, and the filters were divided into two groups referred to as group K+ and group Na+ according to the presence of K+ or Na+ in the incubation solutions. The subsequent experimental steps including the washing and blocking steps and the binding of the first antibody were performed for group K+ in the T-K buffer and for group Na+ in the T-Na buffer (10 mM triethanolamine HCl, 0.15 M NaCl, 2 mM EDTA, 10% (v/v) glycerol, pH 7.4). To block nonspecific binding sites on the nitrocellulose, the filters were incubated for 2 h with T-K or T-Na buffer containing 0.5% Tween 20. Unbound detergent was removed by three further 5-min washings at room temperature in the respective buffer without detergent. Remaining nonspecific binding sites on the nitrocellulose were blocked by overnight incubation at 4 °C with T-K or T-Na buffer containing 10% nonfat dry milk. Thereafter, the filters were washed, warmed up to 37 °C, and incubated for 2 h with the various monoclonal antibodies dissolved in the appropriate buffer, T-K and T-Na, respectively. The next washing procedure was done in T-K or T-Na buffer supplemented with 10% nonfat dry milk and 0.3% Tween 20 (37°C). To quantify bound antibody, all filters were incubated for 1 h (37°C) in the supplemented T-K buffer containing biotinylated species-specific anti-mouse Ig. After three further 5-min washings at room temperature in the respective buffer without detergent, remaining nonspecific binding sites on the nitrocellulose were blocked by overnight incubation at 4 °C with T-K or T-Na buffer containing 10% nonfat dry milk and 0.3% Tween 20 (37°C). To quantify bound antibody, all filters were incubated for 1 h (37°C) in the supplemented T-K buffer, the filters were incubated for 1 h (room temperature) in the same buffer containing 125I-streptavidin (0.2 μCi/ml). After...
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Production and Characterization of Specific Monoclonal Antibodies—Fourteen out of the 354 hybridomas resulting from the fusion with the myeloma partner NS-1 of splenocytes from the mouse immunized with the 63–66-kDa BBM protein produced antibodies against the plastic-immobilized immunogen as detected by ELISA. Seven of these could be stabilized by limiting dilution cloning and expanding in ascites. The produced antibodies, all of the IgM class, were purified from ascites fluid on a Sephacryl S-300 column. Their specificity for the purified 63–66-kDa protein was first tested by ELISA. When incubated with 0.5 µg of the protein or 0.5 µg of control myoglobin in a range of concentration from 0.1 to 2 µg/ml (5–100 ng/well) (Fig. 1), all seven mAbs bound dose dependently to the 63–66-kDa protein and failed to react with myoglobin. Binding was totally inhibited by preincubation of the mAbs with an excess of 63–66-kDa protein (Fig. 1). The irrelevant antibodies TEPC183 and 50A5 did not show any reactivity with the 63–66-kDa protein (Fig. 1). mAb specificity was further evaluated by Western blotting (Fig. 2). Five (85A127, 185A77, 206A126, 225A188, and 304A191) of the seven mAbs reacted with the purified protein electroblotted onto nitrocellulose after SDS-PAGE (Fig. 2, lanes 1–7). Specificity of the reaction was established by absence of reactivity of TEPC183 and 50A5 (Fig. 2, lanes 9 and 10) with the transblotted protein and complete inhibition of antibody binding after preincubation with an excess of purified 63–66-kDa protein (shown in Fig. 2, lane 8, for mAb 85A127). Two mAbs, 63A20 and 157A21, failed to react with the protein by Western blotting although they showed strong reactivity by ELISA. To discriminate between reactivity of these two mAbs with a contaminating antigen and SDS denaturation of the two related epitopes during electrophoresis, these antibodies (as well as the five others) were assessed for their ability to purify a specific protein with the expected molecular mass from renal BBM solubilized under mild detergent conditions (1% Nonidet P-40). As shown in Fig. 3 (lanes 1–7), all seven mAbs (including 63A20, lane 1, and 157A21, lane 3) coupled to CNBr-activated Sepharose 4B immunoabsorbed from solubilized renal BBM a single protein with electrophoretic mobility

RESULTS

Fig. 1. Specificity of monoclonal antibodies raised against the 63–66-kDa protein tested by ELISA. The purified 63–66-kDa protein (panel A) or myoglobin (panel B) (0.5 µg/well) was adsorbed to the bottom of the wells of a microtiter plate. ELISA was performed with the indicated concentrations of mAbs 63A20 (●) and 85A127 (●), preincubated (+) or not (−) with an excess of pure 63–66-kDa protein. TEPC183 (●) and 50A5 (●) are two control antibodies; the former is an IgM without kidney reactivity, and the latter is specific for a 70-kDa BBM protein not involved in Na\textsuperscript{+}/P\textsuperscript{-} cotransport (45). A representative experiment is shown. The same profile of reactivity was observed with the five other mAbs (data not shown).
bodies with the purified 63–66-kDa protein. The latter was submitted to SDS-PAGE and electrotransferred onto a nitrocellulose membrane that was cut into strips and immunostained with the purified mAbs (10 μg/ml). Lanes 1–8, mAbs: lane 1, 63A20; lane 2, 85A127; lane 3, 157A21; lane 4, 185A77; lane 5, 206A126; lane 6, 225A188; lane 7, 304A191; lane 8, 85A127 preincubated with an excess of pure 63–66-kDa protein. Lanes 9 and 10, control mAbs: lane 9, TEPC183; lane 10, 50A5. Lane 11, marker proteins. Molecular masses of marker proteins are indicated on the left.

Similar to that of the 63–66-kDa antigen used for immunization (Fig. 3, lane 9). Nonimmune mouse IgM TEPC183 gave nonspecific absorption under the same experimental conditions (Fig. 3, lane 9). Taken together, the results described above indicate that the seven mAbs produced were specific for the 63–66-kDa protein and that this antigen was specifically identified in the solubilized BBM preparation.

Cellular Localization of the Antigen Recognized by the mAbs—To analyze the subcellular distribution of the 63–66-kDa protein, we disrupted renal cortical cells, separated cell fractions, and analyzed their reactivity with the mAbs by ELISA (Fig. 4) and immunoblotting (Fig. 5). Fig. 4 compares in the same experiment the reactivity of the two mAbs previously tested in Fig. 1 (63A20 and 85A127) with the same amount (20 μg) of BBM, BLM, and mitochondria proteins adsorbed to the bottom of plastic wells. Both antibodies bound dose-dependently to the BBM preparation; specificity of the reaction for the 63–66-kDa protein was demonstrated by dramatic reduction of the absorbance after preincubation with the protein. In marked contrast, minimal reactivity was obtained with BLM and mitochondria and was not affected by preincubating the mAbs with the 63–66-kDa protein, thus indicating nonspecific binding to BLM and mitochondria fractions. Further analysis of mAb reactivity was carried out by Western blotting (Fig. 5). The five mAbs that remained reactive with the 63–66-kDa protein after SDS-PAGE and electroblotting (Fig. 2), identified a single band (~66 kDa) in the BBM preparation (Fig. 5, lanes 1–5). Specificity of the reaction was attested by preincubating the mAbs with the 63–66-kDa protein (Fig. 5, lane 7) and by replacing them by the control IgM TEPC183 (Fig. 5, lane 6) which both yielded negative results. The five mAbs all failed to react either with BLM and mitochondria (not shown). Molecular masses of marker proteins are indicated on the left.
were incubated with renal cortical tissue sections. By immunofluorescence (Fig. 6), the mAbs bound predominantly to the apical domain of renal proximal-tubule cells. No significant reaction was observed on the basolateral membrane or in nuclei and cytoplasm. Immunostaining was specific since it was abolished when the primary antibodies were omitted, replaced by the irrelevant IgM TEPC183, or pretreated with an excess of antigen (data not shown).

**Membrane Topology of the 63–66-kDa Protein Epitopes—**

The cotransporter is an intrinsic membrane protein with only small domain(s) protruding at the external membrane surface. Based on kinetic studies, it has been suggested that when Na* binds to the cotransporter, the latter undergoes significant conformational changes of the cotransporter. For this purpose, a “native” radioimmunoassay was set up in which nonsolubilized BBM was immobilized on nitrocellulose filters, and then antibody binding was performed in the presence or absence of Na* in the medium. The results are depicted in Table I. Non-specific effects on mAbs binding of Na* activation of the cotransporter were eliminated by using two control antibodies. We first verified that non-specific binding of the mouse IgM TEPC183 which does not recognize BBM was not influenced by ionic composition of the buffer. Second, we checked that mAb 50A5, an antibody reactive with a 70-kDa BBM protein (45) not involved in Na*/P* cotransport, bound to the same extent to immobilized BBM in the presence of K* or Na*. Binding of five of the seven anti-63–66-kDa protein mAbs (85A127, 157A21, 185A77, 225A188, and 304A191) to native BBM was not significantly higher than non-specific antibody binding to the filters whatever the experimental conditions (presence of K* or Na*), suggesting intramembranous localization of the epitopes that were not exposed to the surface even after Na*-induced conformational changes of the cotransporter. In contrast, binding of mAbs 206A126 and 63A20 to immobilized BBM was influenced by the presence of Na* in the medium; mAb 63A20 reacted with native BBM only in the presence of Na*; conversely, mAb 206A126 bound to the same preparation only in the absence of Na* (isosmotically replaced by K*). These results suggest that these two antibodies were directed against epitopes that change their localization in the membrane when Na* binds to the cotransporter.

**Effects of Monoclonal Antibodies on P* Uptake into BBMV: Identification of a Functional Epitope Defined by mAb 63A20—**

mAbs specific for the 63–66-kDa protein epitopes exposed on the external membrane surface in the presence (63A20) or absence (206A126) of Na* in the medium were further characterized with regard to their effects on Na*/P* cotransport into BBMV. The membrane vesicles were preincubated with each of the two mAbs in the presence or absence of Na*, washed and then tested for Na*/P* as well as for Na*/D-glucose cotransport activities. Non-specific effects on cotransporters were excluded by control experiments (Table II) with (i) mouse IgM TEPC183; (ii) mAb 50A5; and (iii) the anti-63–66-kDa mAbs that recognized epitopes localized inside the BBM (85A127, 157A21, and 185A77); none of these antibodies affected Na*/P* cotransport after preincubation either in the presence or in the absence of Na*. In contrast, preincubation of BBMV with mAb 63A20 (1 mg/ml) in the presence of Na* reduced by 30% Na*/P* cotransport (Table II). This effect

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**Table I**

**Binding of monoclonal antibodies to brush-border membrane proteins**

| Antibody | Presence of K* | Presence of Na* |
|----------|----------------|----------------|
|          | Binding to filters | **cpm × 10^3** | Binding to filters | **cpm × 10^3** |
| 63A20    | 6.4 ± 1.5 | 6.3 ± 1.6 | 5.9 ± 1.1 | 9.8 ± 0.8* |
| 85A127   | 4.6 ± 1.1 | 5.5 ± 1.3 | 3.6 ± 0.8 | 4.6 ± 1.4 |
| 157A21   | 5.1 ± 0.3 | 5.9 ± 0.4 | 5.3 ± 1.0 | 6.9 ± 0.7 |
| 185A77   | 1.7 ± 0.6 | 2.9 ± 0.9 | 0.9 ± 0.2 | 1.1 ± 0.3 |
| 206A126  | 8.1 ± 1.9 | 15.6 ± 2.5* | 8.9 ± 1.2 | 9.5 ± 1.2 |
| 225A188  | 10.1 ± 2.3 | 11.0 ± 1.8 | 10.8 ± 1.7 | 11.5 ± 1.6 |
| 304A191  | 12.5 ± 0.9 | 12.7 ± 1.1 | 14.2 ± 2.9 | 13.9 ± 1.2 |
| 50A5     | 11.6 ± 1.5 | 38.4 ± 2.6* | 9.8 ± 2.1 | 37.5 ± 3.8 |
| TEPC183  | 14.4 ± 1.8 | 15.9 ± 1.7 | 16.5 ± 3.2 | 17.5 ± 2.4 |

*Significantly different (p < 0.01, paired t test) from control (filter without BBM).

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**Table II**

**Effect of monoclonal antibodies on Na*/Pi cotransport into brush-border membrane vesicles**

Brush-border membranes were resuspended in Na*/mannitol buffer or K*/mannitol buffer containing different monoclonal antibodies (1 mg/ml). After incubation with antibodies, the vesicles were washed with mannitol buffer three times by ultracentrifugation. [32P]Pi uptake was determined in the presence of NaCl or a KCl gradient (out > in). The Na*/P* cotransport figures presented in the table are corrected for uptake in the presence of KCl (see “Experimental Procedures”). Values are means ± S.D. from three experiments.

| Antibody | Preincubation with mAb in Na*/mannitol buffer |
|----------|---------------------------------------------|
| Control  | 620 ± 62 | 630 ± 65 |
| 63A20    | 430 ± 35* | 642 ± 55 |
| 206A126  | 625 ± 58 | 705 ± 60 |
| 85A127   | 632 ± 59 | 648 ± 60 |
| 157A21   | 630 ± 55 | 650 ± 58 |
| 185A77   | 635 ± 58 | 630 ± 60 |
| 50A5     | 615 ± 60 | 625 ± 62 |
| TEPC183  | 620 ± 55 | 645 ± 65 |

*Significantly different (p < 0.01, paired t test) from control.
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was dose-dependent, reaching a plateau of 33\% inhibition at 1 mg/ml (Fig. 7), and specific for the Na\(^{+}/P\) cotransporter as in the same range of concentrations of mAb 63A20 (0.12-2 mg/ml), Na\(^{+}/D\)-glucose cotransport was not affected (Fig. 7). Substitution of K\(^{+}\) for Na\(^{+}\) in the preincubation medium suppressed the inhibitory effect of mAb 63A20 on Na\(^{+}/P\) cotransport (Table II). These data suggest that the epitope recognized by 63A20 revealed upon Na\(^{+}\) activation of the protein plays an important role in Na\(^{+}/P\) cotransporter activity. In contrast, the second antibody, mAb 206A126, directed against an epitope that also changed its membrane exposure in the presence of Na\(^{+}\), did not alter Na\(^{+}/P\) cotransport after preincubation either in the presence or in the absence of Na\(^{+}\) (Table II).

**Immunodepletion of Na\(^{+}/P\) Cotransport Activity by mAb 63A20**—To demonstrate that the 63-66-kDa protein is the essential component of the Na\(^{+}/P\) cotransport system, the solubilized BBM preparation was chromatographed on the Sepharose 4B-mAb 63A20 column, and the effluent and the starting solubilized BBM were reconstituted into liposomes. Pi and D-glucose uptakes were measured in the presence of a Na\(^{+}\) or K\(^{+}\) gradient (out > in). As depicted in Fig. 8A showing the time course of Pi, and D-glucose uptakes in proteoliposomes formed from solubilized membrane proteins, uptakes were substantially enhanced in the presence of a Na\(^{+}\) gradient compared with a K\(^{+}\) gradient, suggesting appropriate reconstitution of both Na\(^{+}/P\) and Na\(^{+}/D\)-glucose cotransport activities. When proteoliposomes were prepared from the effluent of the column devoid of the 63-66-kDa protein (as checked by immunoblot), they exhibited dramatically reduced Pi uptake in the presence of a Na\(^{+}\) gradient, whereas P\(_i\) uptake in the presence of a K\(^{+}\) gradient was not affected (Fig. 8B). Hence the Na\(^{+}/P\) cotransport activity, calculated as the difference between P\(_i\) uptake in the presence of Na\(^{+}\) and P\(_i\) uptake in the presence of K\(^{+}\), was reduced by 85-87\% at each time point in comparison with the nonimmunodepleted BBM preparation (Fig. 8A). As a control, BBM incubated with Sepharose 4B coupled to nonimmune mouse IgM and reconstituted into liposomes did not show any decrease in transport capacity (data not shown). The specificity of the 63-66-kDa protein for the Na\(^{+}/P\) cotransport system was further determined by measuring the reconstitutable activity of Na\(^{+}/D\)-glucose cotransport. Results presented in Fig. 8B showed no decrease in Na\(^{+}/P\)-glucose cotransport in the proteoliposome preparation immunodepleted of the 63-66-kDa protein, suggesting that the observed loss of Na\(^{+}/P\) cotransport activity was directly related to the elimination of the 63-66-kDa protein.

**FIG. 8. Initial rates of phosphate and D-glucose uptakes into proteoliposomes before and after immunoaffinity depletion of the 63-66-kDa protein.** Proteoliposomes were prepared with solubilized BBM (panel A) and BBM depleted of the 63-66-kDa protein by immunoaffinity on Sepharose 4B beads coupled to mAb 63A20 (panel B). Pi and D-glucose uptakes were measured in the presence of a NaCl (●) or a KCl gradient (○). Values are means ± S.D. from three proteoliposome preparations. The upper part of the figure shows immunoblots with mAb 85A20 of the preparations used for reconstitution. mAb 63A20 could not be used for immunostaining because it recognizes an epitope destroyed during SDS electrophoresis.

**DISCUSSION**

In a previous work (29) we characterized the renal BBM Na\(^{+}/P\) cotransport protein by purification and liposome reconstitution procedures. The results indicated that an acidic glycoprotein with an apparent molecular mass of 63-66 kDa estimated under denaturing conditions, was involved in this cotransport process. In the present study, to identify unequivocally the Na\(^{+}/P\) cotransporter, we decided to follow a combined immunologic and functional approach that consisted in producing mAbs against the purified 63-66-kDa protein and testing the activity of immunodepleted BBM fractions in proteoliposome reconstitution experiments. This approach, used to our knowledge for the first time in the identification of a membrane P\(_i\) transporter, was rewarding since (i) it confirmed that the 63-66-kDa protein plays a crucial role in the Na\(^{+}/P\) cotransport system; (ii) it resulted in the description of a functional epitope on the molecule, and (iii) it provided the first immunologic tools for immunohistochemical localization of the Na\(^{+}/P\) cotransporter.

Confirmation that the 63-66-kDa protein is a crucial piece of the Na\(^{+}/P\) cotransporter relies on four lines of evidence. First, it seems to be exclusively localized in the brush-border membrane as shown by ELISA, immunoblotting of renal
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cortical cell subfractions, and indirect immunofluorescence of rabbit kidney cortex sections. Our data confirm and extend previous physiological and biochemical results derived from micropuncture experiments in intact and excised nephrons and from uptake experiments using BBMV (3, 5) demonstrating that the site of Na⁺/Pᵢ cotransport is the apical membrane of proximal-tubule cells. Recently however, a Na⁺/Pᵢ cotransporter has also been detected in the basolateral domain of epithelial cells (46, 47), differing in affinity and hormonal sensitivity from its apical counterpart (48). The role of this basolateral cotransporter might be to meet metabolic needs of the cell under some physiological circumstances such as Pᵢ depletion. The fact that our mAbs did not recognize any immunoreactive material in the basolateral membrane preparation suggests that the two cotransporters are not cross-reactive and are probably borne by two distinct molecular entities. Alternatively, concentration of the Na⁺/Pᵢ cotransporter might be too small to be detected in the basolateral membrane of proximal-tubule cells under normal conditions. However when Pᵢ reabsorption across the brush-border membrane is insufficient to yield adequate intracellular Pᵢ, concentration, significant amounts of Na⁺/Pᵢ cotransporters might be sorted to the basolateral cell domain. We are currently testing this hypothesis.

The second evidence in favor of the Na⁺/Pᵢ cotransport activity of the 63-66-kDa protein is provided by the Na⁺ dependence of mAbs 63A20 and 206A126 binding to their respective epitopes in “native” BBM absorbed to nitrocellulose filters. Indeed, we expected that reactivity of some mAbs with the presumed Na⁺/Pᵢ cotransport would be influenced by the presence (or the absence) of Na⁺ in the incubation medium because it was known from Pᵢ uptake kinetics studies that the Na⁺/Pᵢ cotransporter could undergo Na⁺-dependent conformational changes (43, 44, 49). Na⁺ appears to act as an allosteric modulator of Pᵢ transport, and it has been proposed that Na⁺ first interacts with the cotransporter system and then modifies its conformation to allow Pᵢ binding (49), thus favoring accumulation of active conformers of the cotransporter unit. These modifications are associated with exposure, or hiding, of previously buried, or exposed, parts of the cotransporter (43, 44, 49). The immunologic approach that we used supports this allosteric model. (i) The epitope defined by mAb 206A126 is only accessible when the cotransporter exists in the inactive state (K⁺ buffer) but becomes most likely hidden after transition to the Na⁺-activated state of the carrier. (ii) Reciprocally, the epitope recognized by mAb 63A20 seems to be exposed to the BBM surface only in the active conformation of the cotransporter and is no longer available for antibody binding in the resting state; that mAb 63A20 can bind to its epitope in the presence of 1% Nonidet P-40 (in immunofluorescence and immunoaffinity chromatography experiments) confirms that the antibody does not recognize a “Na⁺ conformation” of the epitope but an epitope normally buried in the membrane which can be exposed either by Na⁺ binding to the cotransportor or by detergent.

Third, since Na⁺-dependent conformational changes have also been reported for the Na⁺/D-glucose transporter (50) and seem to be the rule for most, if not all, Na⁺-coupled organic solute cotransporters (51), it was important to bring about more specific proofs of the identity of the Na⁺/Pᵢ cotransporter. This was achieved by transport experiments in which Pᵢ or D-glucose uptake was assessed after preincubation of BBMV with the various anti-63-66-kDa mAbs. Only mAb 63A20 induced significant inhibition of Na⁺/Pᵢ cotransport. Specificity of this effect was assessed as follows. (i) Inhibition was not caused by steric hindrance as it was not reproduced by mAb 50A5, although the latter strongly binds to BBM. (ii) Inhibition was observed when preincubation was carried out in the presence of Na⁺ (then, the epitope is exposed) and disappeared when Na⁺ was replaced by K⁺ (then, the epitope is not available to mAb binding). (iii) Inhibition was dose-dependent. (iv) In the same range of concentrations, mAb 63A20 did not reduce Na⁺/D-glucose cotransport activity. mAb 63A20 thus defines a crucial epitope involved in the functioning of the Na⁺/Pᵢ cotransporter.

Fourth, definitive proof that the 63-66-kDa protein is required for Na⁺/Pᵢ cotransport activity has been obtained by reconstituting the cotransport system in proteoliposomes. We utilized solubilized BBM proteins previously depleted of the 63-66-kDa protein by immunoaffinity chromatography on a Sepharose 4B-mAb 63A20 column. mAb 63A20 was selected for this study because it recognizes a functional epitope. In 63-66 kDa-depleted proteoliposome preparations, 85-87% of the Na⁺/Pᵢ cotransport activity was lost. In contrast, depletion of the 63-66-kDa protein had no effect on Na⁺/D-glucose cotransport, thereby establishing the specificity of the immunoaffinity procedure. Although, reassociation of solubilized BBM over the mAb 63A20 immunoaffinity column seemed to remove totally the 63-66-kDa protein as determined by immunoblotting, a residual (13-15%) Na⁺/Pᵢ cotransport activity was measured. The inability to immunodeplete all of the cotransport activity may be because of the persistence of small amounts of the 63-66-kDa protein undetected by Western blotting. Alternatively, this remaining activity may be supported by Na⁺/Pᵢ cotransport isoforms not recognized by mAb 63A20; this hypothesis is in keeping with the detection in kidney cortex of two Na⁺/Pᵢ cotransport systems differing in affinity and capacity (7, 8), a finding reminiscent of the expression of multiple isoforms of Na⁺/D-glucose cotransporters (52) or Na⁺/K⁺ exchangers (53) in the same tissue.

In conclusion, we have demonstrated that the 63-66-kDa protein plays a key role in Na⁺/Pᵢ cotransport. The identity of this molecule with the Na⁺/Pᵢ cotransporter is further supported by recent studies performed on proteoliposomes reconstituted with the immunopurified protein only, showing sodium requirement, kinetic parameters, substrate specificity, and sensitivity to inhibitors similar to those expected for the Na⁺/Pᵢ cotransport in intact renal BBM. In addition, the molecular masses of the Na⁺/Pᵢ cotransporters identified by our immunologic approach and by expression cloning (23) are very similar. The molecular mass of the peptide deduced from the cDNA sequence is approximately 52 kDa, and the cDNA sequence is approximately 48 kDa. The role of this protein plays a key role in Na⁺/Pᵢ cotransport.

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