HYBRIDOMA
Volume 30, Number 1, 2011

DOI: 10.1089/hyb.2010.0064

Generation of Monoclonal Antibodies Against Tumor-Associated Antigen MX35/sodium-Dependent Phosphate Transporter NaPi2b

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Tumor-associated antigen MX35, which is overexpressed in 70–90% of epithelial ovarian cancers, has been recently identified as phosphate transporter NaPi2b. This finding has raised significant interest in understanding NaPi2b function under physiological conditions and its deregulation in human pathologies, such as cancer. As a member of the sodium-dependent phosphate transporter family, NaPi2b is primarily involved in the maintenance of phosphate homeostasis in the human body. The role of NaPi2b in oncogenic transformation and malignant growth is not well understood. To date, several monoclonal antibodies specific to NaPi2b have been reported. However, available monoclonal antibodies are not very efficient in recognizing endogenous NaPi2b under reducing conditions. In addition, these antibodies could not recognize the mutant form of transporter (NaPi2b-T330V). In this study we describe the production of monoclonal antibodies raised against the N-terminal region of NaPi2b. One of them, designated N-NaPi2b(15/1), possesses very useful immunological characteristics. We found that N-NaPi2b(15/1) specifically recognizes NaPi2b protein in immunohistochemical analysis and immunoprecipitation assay. Importantly, N-NaPi2b(15/1) antibody detects very efficiently endogenous and expressed wild-type and mutant forms of NaPi2b under both reducing and non-reducing conditions in Western blot analysis. These features make N-NaPi2b(15/1) antibody a very useful tool for studying the pattern of NaPi2b expression in health and pathologies.

Introduction

Inorganic phosphate (P_i) plays essential structural and metabolic roles in living cells. P_i homeostasis in mammals is strictly controlled through the balance of intestinal absorption and renal reabsorption of P_i, which are mediated by proteins of solute carrier family SLC34 (also known as type II Na/P_i co-transporters).[1] NaPi2b (*SLC34A2*) belongs to the SLC34 family and is expressed in small intestine at the apical side of the enterocyte's brush-border membranes, where it is responsible for transcellular P_i absorption.[2,3] The studies on conditional knockout mice demonstrated that NaPi2b contributes to >90% of total active phosphate absorption, thus indicating the crucial role of NaPi2b in maintaining phosphate homeostasis.[4] Attempts to generate NaPi2b homozygous-deficient mice resulted in the death of embryos *in utero* soon after implantation, indicating that NaPi2b is the major P_i transporter during mouse embryonic development.[5]

Northern blot and RT-PCR analysis revealed that the expression of *SLC34A2* gene is very high in human lung, while low to moderate levels are detected in other tissue, such as trachea, kidney, small intestine, ovary, placenta, uterus, testis, prostate, pancreas, mammary, and thyroid gland.[1,2,6] Notably, increased expression of NaPi2b at the mRNA level was reported in ovarian and papillary thyroid tumors.[7,8]

The expression of NaPi2b at protein level was confirmed in different normal tissue and organs with the use of polyclonal antibodies directed against the C- or N-termini of rat/mouse NaPi2b transporter. These studies showed the expression of NaPi2b in various tissues, including lung, kidney, testis, liver, and mammary gland, where it takes part in the maintenance of local P_i concentration.[9–12] The use of polyclonal antibodies against the extracellular loop of human NaPi2b (188–361 aa) revealed NaPi2b expression in human salivary gland.[13] Phosphate transporter NaPi2b was found to be highly expressed in human ovarian tumors, when its expression was analyzed with the use of monoclonal antibody MX35.[14] This antibody was obtained by immunizing mice with ovarian carcinoma cells and screening for tumor-associated antigens. Further analysis revealed that approximately 90% of human ovarian cancers exhibit strong immunoreactivity with the MX35 antibody.[15] It is important to emphasize that the

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number of monoclonal antibodies generated to different domains of human NaPi2b protein is very limited.

The crystal structure of NaPi2b has not been solved so far; the proposed model suggests that NaPi2b possesses eight transmembrane domains with both N- and C-terminal ends facing the cytoplasm and regulatory loops at both sides of the membrane.[6,16,17] Notably, multiple glycosylation sites are predicted within the largest extracellular loop. We have previously generated and characterized a monoclonal antibody (MAb) L2(20/3) against human NaPi2b with the epitope located within the extracellular loop of the transporter (188–361 aa).[18] L2(20/3) recognizes endogenous NaPi2b in various immunoassays, including Western blotting, immunoprecipitation, and immunohistochemistry.[18] With the use of this antibody we demonstrated heterogenous expression of NaPi2b transporter in different types of epithelial ovarian tumors and established a possible association of NaPi2b expression with the differentiation status of ovarian epithelium during cancerogenesis.[19] Interestingly, antibody L2(20/3) does not recognize NaPi2b under reducing conditions on Western blots, implicating the importance of disulfide bridges for the structure of the epitope. In addition, this antibody is not able to recognize a mutant form of transporter (NaPi2b-T330V), which carries amino acid substitution within the epitope of L2(20/3). These features restrict the use of antibody L2(20/3) for the investigation of NaPi2b expression in normal and pathological states, including ovarian cancer. To overcome these limitations, we decided to generate monoclonal antibodies against the N-terminal region of NaPi2b.

Here, a bacterially expressed N-terminal fragment of human NaPi2b (1–101 aa) was used to generate monoclonal antibodies, which recognized specifically recombinant expressed and endogenous NaPi2b. Furthermore, the new antibodies detected both wild-type and NaPi2b-T330V under reducing and non-reducing conditions in Western blotting.

Materials and Methods

Cloning of recombinant plasmids

All cDNA sequences were derived from the original clone DKFZp6860655Q2 (received from RZPD gene bank). Plasmid DNA was purified using DNA purification kit (Promega, Madison, WI). For the generation of the pGEX-4T1-N-NaPi2b recombinant plasmid, NaPi2b cDNA was amplified with primers containing *BamH1* and *EcoR1* restriction sites. The amplicon spanning the nucleotides 1–303 of the coding sequence was cloned into the pGEX-4T1 vector (Novagen, Darmstadt, Germany) in frame with the C-terminal GST-tag.

Production and purification of GST-N-NaPi2b fusion protein

pGEX-4T1-N-NaPi2b recombinant plasmid was transformed into BL21(DE3)lysE *Escherichia coli* cells. The expression of recombinant GST-fused NaPi2b (1–101 aa) protein, designated GST-N-NaPi2b, was induced by 1 mM iso-propyl-b-D(2)-thiogalactopyranoside (IPTG) for 3 h at 30°C. Purification of GST-N-NaPi2b recombinant protein from the soluble fraction of bacterial lysate was carried out using Glutathione Sepharose 4B (Amersham, Uppsala, Sweden) according to the manufacturer's recommendations.

Production of hybridoma cells

Generation of monoclonal antibodies was performed as described above using GST-N-NaPi2b as an antigen.[18]

Cell lysate preparation and immunoblotting

Generation of HEK293 cells stably overexpressing wild-type and mutant forms of NaPi2b has been previously described.[20] HEK293, OVCAR-3, and SKRC18 cells were lysed in buffer containing 10 mM Tris-HCI (pH 7.5), 150 mM NaCl, 10 mM $MgCl_2$, 0.5% NP-40, and a mixture of Halt Protease Inhibitor Cocktail (Pierce, Rockford, IL). Protein concentration was measured by Coomassie assay (Pierce).

Western blot analysis

Bacterially expressed GST-N-NaPi2b or mammalian cell lysates were separated by 10% or 8% SDS/PAGE and electrotransferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Temecula, CA). The membranes with GST-N-NaPi2b were blocked with 5% non-fat milk in 1x PBS, containing 0.1% Tween-20 (PBST) for 1 h at room temperature (RT), and divided into strips followed by a single wash with PBST. Each strip was incubated with 1x PBS, post-immune serum (1:1000), culture media of positive clones, or cell culture media alone for 2 h at RT. Membranes with mammalian cell lysates were blocked with 3% BSA in 1x PBST for 1 h at RT. Anti-NaPi2b MAbs were incubated with membranes at 4°C overnight. After washing with PBST, HRP-conjugated goat anti-mouse lgG 1:5000 (Promega) was added to the membrane for 1 h at RT. Western blots were developed using the ECL system (Amersham) and then exposed to Agfa X-ray film.

Production and purification of MAbs from ascitic fluid

BALB/c mice were injected with 0.5 mL of pristane and 7–10 days later inoculated with 5×10^6 of hybridoma cells. The ascitic fluid was collected after 7–10 days, centrifuged at 14000 rpm for 20 min, filtered through 0.4 μm filter

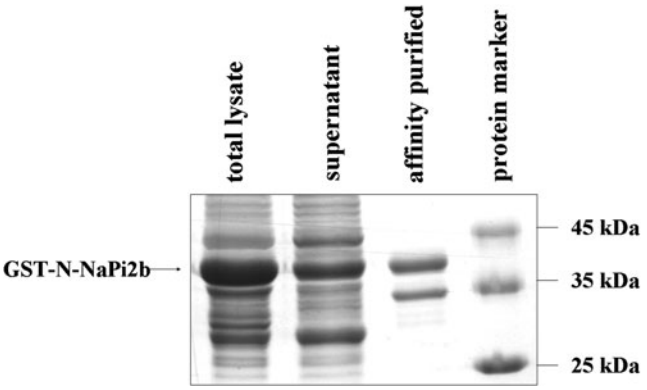

FIG. 1. SDS-PAGE analysis of bacterially expressed and affinity purified GST-N-NaPi2b. Expression of GST-N-NaPi2b protein in BL21(DE3)lysE cells was induced by IPTG for 4 h at 37°C. Purification of GST-N-NaPi2b protein from soluble fractions was carried out by affinity purification on Glutathione Sepharose. Position of recombinant GST-N-NaPi2b is indicated by arrow.

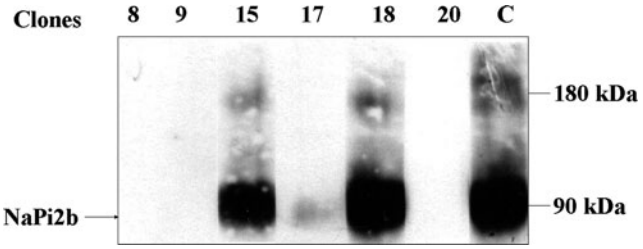

FIG. 2. Western blot analysis of HEK293 cells expressing wild-type NaPi2b with culture media of selected hybridoma clones. Anti-NaPi2b polyclonal serum was used as a positive control (c). Designated numbering of hybridoma clones is shown at top of immunoblot.

(Millipore), and used for affinity purification by Protein A Sepharose CL-4B (Amersham) chromatography. The IgG fractions were pooled and dialyzed in a phosphate-buffered saline (PBS, pH 7.4). The aliquots of purified antibodies were stored at −20°C in 50% glycerol.

Immunohistochemical analysis

Anti-NaPi2b MAbs were used for immunohistochemical analysis of ovarian cancer samples according to established protocols.[18] Briefly, representative sections of ovarian tumors were prepared from paraffin blocks. Endogenous peroxidase was quenched with H_2O_2 (3%) in 0.01% PBS. After blocking non-specific staining with avidin-biotin blocking solution (Vector Laboratories, Burlingame, CA), tissue sections were incubated overnight at 4°C with anti-NaPi2b MAb (10 μg/mL). Then sections were incubated with biotinylated secondary antibodies for 2 h at RT (1:400, goat anti-mouse biotinylated IgG, Sigma, St. Louis, MO), followed by incubation with avidin-biotin-peroxidase complex (Vector) for 30 min at RT, and developed with diaminobenzidine solution. Hematoxylin was used for counterstaining. Standard microscopy was performed using a Zeiss Universal microscope (Jena, Germany), and images were captured using digital Axiocam software.

Immunoprecipitation

25 μL of 50% suspension of Protein A Sepharose CL-4B (Amersham) was incubated with 1 mL of hybridoma media from selected positive clones for 1 h at 4°C at rotation. Supernatants (500 μg of total protein) from the lysates of HEK293 cells transfected with pcDNA3.1/NaPi2b or pcDNA3.1 alone were added to beads. After incubation on the wheel at 4°C for 3 h, beads were washed and boiled in Laemmli sample buffer. Immune complexes were separated by 8% SDS-PAGE for further immunoblotting with anti-NaPi2b antibodies, as described above.

Results and Discussion

The expression profile of MX35 tumor-associated antigen/NaPi2b in normal and cancer tissue revealed its potential as a prospective target for cancer immunodiagnostics and immunotherapy. The monoclonal antibody MX35 against NaPi2b transporter was used in clinical trials as a carrier of alpha-particles for radioimmunotherapy of ovarian cancer.[21] The role of NaPi2b in ovarian cancer and other pathologies is not well known and a wide panel of specific antibodies will help to investigate this important aspect of P_i transport.

In this study we focused on the generation and characterization of monoclonal antibodies directed against the N-terminal (1–101 aa) region of NaPi2b that would be able to recognize expressed wild-type and mutant forms of NaPi2b, as well as endogenous NaPi2b in different immunoassays.

Initially the first 303 bp of the NaPi2b coding sequence were amplified by PCR and cloned into the pGEX-4T1 expression vector. The resulting plasmid was transformed into BL21(DE3)lysE cells, and the expression of recombinant GST-N-NaPi2b was induced by IPTG. SDS-PAGE analysis of bacterial cell lysates indicated that GST-N-NaPi2b protein was highly expressed and presented mainly in a soluble form. Therefore, affinity purification on Glutathione Sepharose was used to purify recombinant proteins. According to the data presented in Figure 1, the purity of affinity-purified GST-N-NaPi2b was approximately 95%. This preparation of GST-N-NaPi2b was used as an antigen for the immunization of BALB/c mice, hybridoma cell screening, and further analysis of antibodies. Hybridoma cells were generated by a standard procedure as described previously.[18] Primary ELISA screening of hybridoma clones resulted in the selection of 37 hybridoma clones with reactivity against GST-N-NaPi2b. Further analysis showed that 11 clones produced

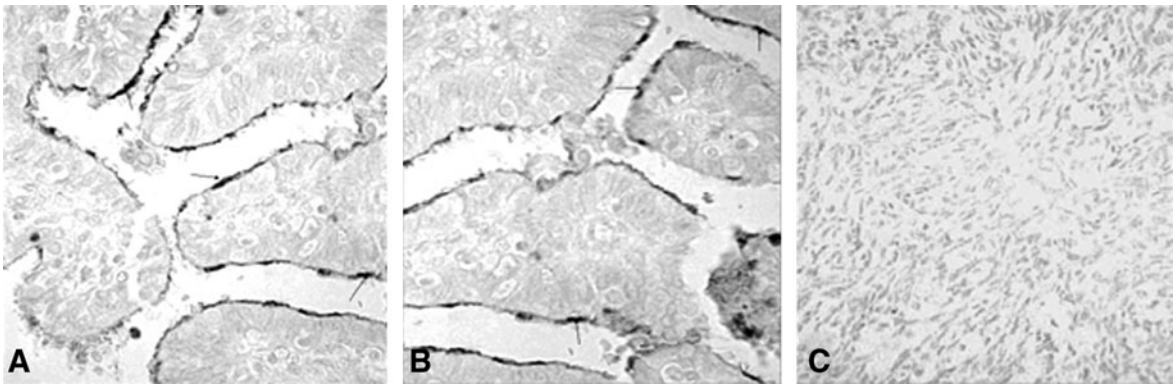

FIG. 3. Immunohistochemical analysis of human ovarian carcinoma with N-NaPi2b(15/1) (**A**) and L2(20/3) (**B**) MAbs. Arrows indicate the position of immunoreactive staining at the apical surface of cancer cells. (**C**) Staining of normal ovary with NaPi2b(15/1) MAb. Magnification, x40.

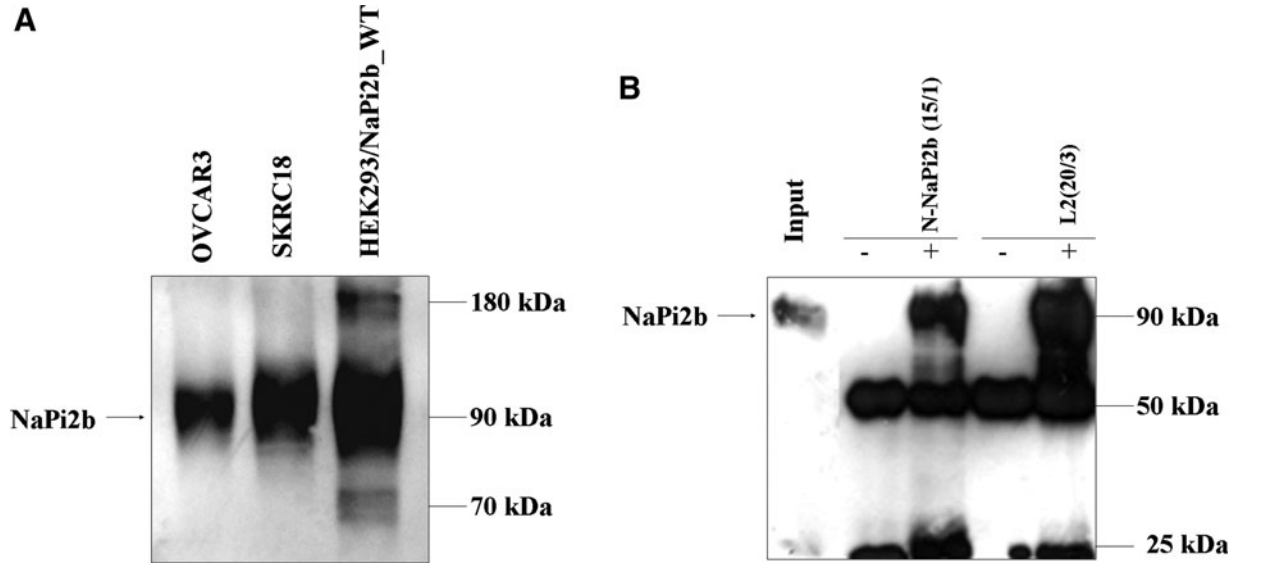

FIG. 4. (**A**) Immunoblot analysis of NaPi2b expression in OVCAR3 and SKRC18, which express endogenous NaPi2b. HEK293 cells stably expressing NaPi2b were used as a positive control. Affinity purified N-NaPi2b(15/1) antibody was used in this study. (**B**) Immunoprecipitation of expressed NaPi2b with N-NaPi2b(15/1) antibody. Supernatants of HEK293-NaPi2b cells were pre-incubated with Protein A Sepharose containing IgGs N-NaPi2b(15/1) and L2(20/3) against the extracellular loop of NaPi2b (188–361 aa).

antibodies to GST (data not shown). The remaining 26 clones were further examined by Western blot analysis of recombinant GST-N-NaPi2b and GST alone. These experiments allowed us to identify six hybridoma clones producing antibodies specific only to the recombinant N-terminal part of NaPi2b. Culture media from the selected clones were analyzed by Western blotting using lysates of HEK293 cells stably expressing wild-type NaPi2b (Fig. 2). This analysis indicated that among selected clones only two produced antibodies that specifically recognized full-length NaPi2b. Both clones, termed N-NaPi2b(15) and N-NaPi2b(18), were subcloned to monoclonality and used for ascitic fluid production.

Purified from ascitic fluid by Protein A Sepharose chromatography, N-NaPi2b(15/1) and N-NaPi2b(18/1) antibodies were initially tested in immunohistochemical analysis of ovarian cancer samples. The results indicate that antibody N-NaPi2b(15/1) detected NaPi2b antigen on the apical side of ovarian cancer cells (Fig. 3A). In contrast, antibody N-NaPi2b(18/1) revealed no immunoreactivity (data not shown) and was therefore excluded from further analysis. As a positive control, the well-characterized antibody L2(20/3) was included in the analysis (Fig. 3B). Immunohistochemical staining of ovarian tumors with the antibodies N-NaPi2b(15/1) and L2(20/3), as well as MX35,[14] gave comparable results. Additional evidence of specificity N-NaPi2b(15/1) antibodies came from negative staining of normal ovary where NaPi2b is not expressed.

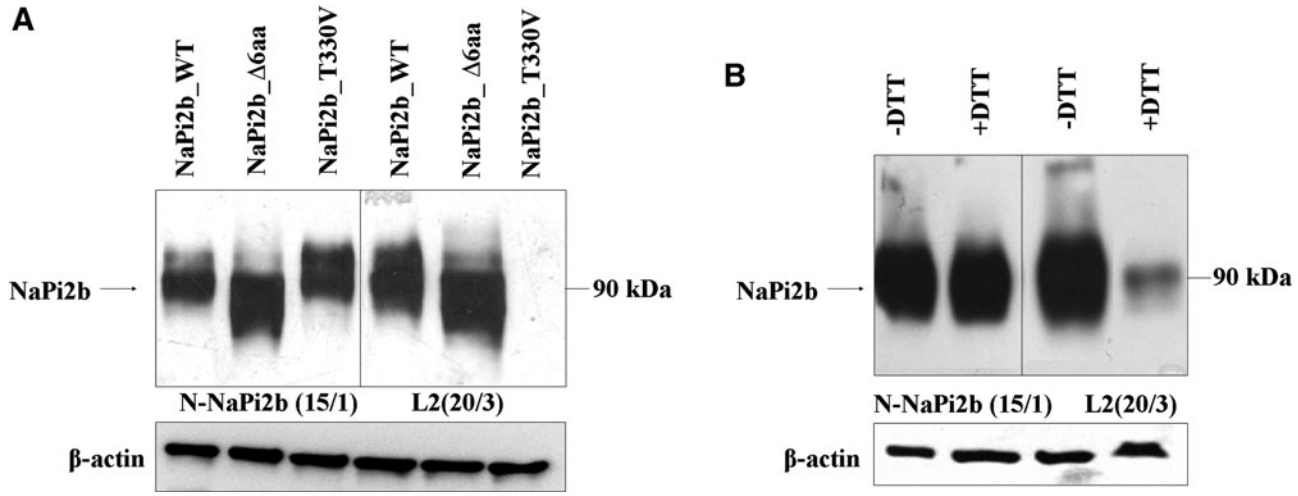

FIG. 5. (**A**) Western blot analysis of HEK293 cells expressing wild-type and mutant forms of NaPi2b (NaPi2b-T330V, NaPi2b-Δ6) with N-NaPi2b(15/1) and L2(20/3) MAbs. (**B**) Western blot analysis of HEK293 cells expressing wild-type NaPi2b under reducing (+DTT) and non-reducing (-DTT) conditions with N-NaPi2b(15/1) and L2(20/3) MAbs.

Next, we tested N-NaPi2b(15/1) MAb in Western blot analysis with lysates of OVCAR3 and SKRC18 cells, which are known to express high levels of endogenous NaPi2b. In this study, we used HEK293 cell line stably expressing wild-type NaPi2b as a positive control. As shown in Figure 4A, N-NaPi2b(15/1) MAb recognized specifically NaPi2b in transfected HEK293 cells, as well as endogenous NaPi2b in OVCAR3 and SKRC18 cells. Furthermore, the results of the immunoprecipitation assay clearly indicate that N-NaPi2b(15/1) antibody had the potential to immunoprecipitate wild-type NaPi2b from HEK293-NaPi2b cell lysates with the same efficiency as L2(20/3) antibody (Fig. 4B).

Western blot analysis of mutant forms of NaPi2b with N-NaPi2b(15/1) MAb demonstrates that two mutant proteins—NaPi2b-T330V (amino acid substitution in extracellular loop of NaPi2b within the epitope of L2(20/3) MAb) and NaPi2b-Δ6 (6 aa deletion in the C-terminus of NaPi2b (591–596 aa) were specifically recognized by antibody N-NaPi2b(15/1) (Fig. 5A). These mutants were identified previously in ovarian cancer cell lines[20] and possibly in ovarian cancer *in vivo* as well.[22] Importantly, in contrast to NaPi2b (15/1) MAb, L2(20/3) MAb did not recognize the T330V mutant form of NaPi2b (Fig. 5A).

In previous studies, we have observed a significant decrease in the immunoreactive signal corresponding to the full-length NaPi2b protein in Western blotting with L2(20/3) or MX35 antibodies when cell lysate were prepared under reducing conditions. For further analysis of the specificity of NaPi2b(15/1) MAb, we performed Western blot analysis of cell lysates positive for NaPi2b under reducing and non-reducing conditions. According to the presented data (Fig. 5B), the epitope recognized by antibody N-NaPi2b(15/1) is not sensitive to reducing conditions, in contrast to L2(20/3) and MX35 antibodies.[18]

In summary, we have generated a monoclonal antibody N-NaPi2b(15/1) specific to the N-terminal region of sodium-dependent phosphate transporter NaPi2b. The new antibody recognizes human endogenous and expressed NaPi2b in various immunoassays, including Western blotting, immunoprecipitation, and immunohistochemistry. In contrast to previously generated monoclonal antibodies, N-NaPi2b (15/1) works well under reducing and non-reducing conditions in Western blotting and effectively recognizes currently known NaPi2b mutants. These features make N-antibody NaPi2b(15/1) a useful tool for the investigation of NaPi2b functions under normal and pathological conditions.

Acknowledgments

This work was supported in part by grants from the National Academy of Sciences of Ukraine, and a short-term fellowship (to V.G.) from Boehringer Ingelheim Fonds.

Author Disclosure Statement

The authors have no financial conflicts to declare.

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Received: June 11, 2010
Accepted: September 2, 2010