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Development of Monoclonal Antibodies Specific for the Human Sodium-dependent Phosphate Co-transporter NaPi2b

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Abstract

Homeostasis of inorganic phosphate in human body is maintained by regulated absorption, metabolism, and excretion. Sodium-dependent phosphate transporters (NaPi) mediate the transport of inorganic phosphate (P_i) in cells in response to dietary phosphate consumption, hormones, and growth factors. NaPi2b is a member of the sodium-dependent phosphate transporter family, with a distinct pattern of expression and regulation. Signaling pathways activated by mitogens, glucocorticoids, and metabolic factors have been implicated in regulating P_i transport via NaPi2b. Inactivation of NaPi2b function by mutations has been linked to human pathologies, such as pulmonary alveolar microlithiasis. In this study, we describe the generation and characterization of monoclonal antibodies against human NaPi2b. The monoclonal antibodies were shown to recognize specifically transiently overexpressed and endogenous NaPi2b in commonly used immunoassays, including Western blotting, immunoprecipitation, and immunohistochemistry. These properties make them particularly valuable reagents for elucidating NaPi2b function in health and disease.

Introduction

Cellular concentration of inorganic phosphate (P_i) should be maintained constant for proper regulation of growth, metabolism, and signaling pathways, as well as for bone formation. Inorganic phosphate homeostasis is maintained by various mechanisms, mainly through the regulation of its absorption and metabolism. Cellular intake of P_i is mediated by sodium-dependent phosphate transporters (solute carrier series, SLC), which could be grouped into three subtypes based on sequence identity: NaPi-I (*SLC17*), NaPi-II *(SLC34)*, and NaPi-III *(SLC20)*. Type II Na/Pi cotransporters are expressed in various tissues of different organisms including mammals and play a major role in the homeostasis of inorganic phosphate.[1,2] NaPi2b (NaPi-IIb, SLC34A2, NaPi3b, NPT-2) belongs to the type II family of sodium-dependent phosphate transporters, which also includes NaPi2a (NaPi-IIa) and NaPi2c (NaPi-IIc). Human NaPi2b was identified by two independent laboratories by employing bioinformatic homology screens[3,4] one year later than mouse NaPi2b,[5] and most of the data available so far are derived from studies with mice. Mouse NaPi2b is a transmembrane protein that is predicted by Hilfiker et al. to be anchored to the cellular membranes through at least eight highly hydrophobic α-helical regions.[5] In the predicted topology of NaPi2b, both N- and C-terminal tails face the cytoplasm, exposing various length loops to the extracellular and intracellular compartments. The largest extracellular loop contains several potential sites of glycosylation and a region rich in cysteine residues, which might be involved in disulfide bond formation. Expression studies were performed for human, mouse, and rat NaPi2b on the mRNA and protein levels.[3–11] It was shown that human NaPi2b is expressed in lung, small intestine, prostate, liver, kidney, etc. by Northern blot analysis.[3,4]

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At the protein level sodium dependent phosphate transporter 2b expression was analyzed with polyclonal antibodies against mouse NaPi2b and detected in different tissues: small intestine,[5] lung,[6] epididimus,[7] liver,[8] mammary gland,[9] and osteoblasts.[10] Analysis of NaPi2b expression in human tissues using polyclonal anti-human NaPi2b antibodies was restricted to the parotid gland.[11] The approximate molecular weight of NaPi2b according to SDS gel electrophoresis was estimated to be in the in 76–110 kDa range.[5–11] Furthermore, it was demonstrated that NaPi2b could also form homodimers.[11,12]

The level of NaPi2b was found to be controlled by several hormones and metabolic factors according to the body's P_i needs. It has been reported that the increased level of NaPi2b is induced by low phosphate diet, vitamin D, and estrogens.[13,14] Downregulation of NaPi2b levels in response to FGF 23, EGF, and glucocorticoids was demonstrated in cellular models.[15–17] In addition, P_i and carbonate are known to buffer protons in the blood during metabolic acidosis. To serve in proton buffering, P_i is released from bone together with carbonate or replenished by NaPi2b intestinal absorption.[18] Immunohistochemical studies showed that NaPi2b is localized mainly in brush borders of enterocytes or in apical membranes of mammary epithelial cells.[5,19] NaPi2b transports divalent P_i together with three Na^+ ions by electrogenic mechanism. Signaling pathways that regulate the function of NaPi2b are not well understood. Recently, the ability of the serum and glucocorticoid inducible kinase (SGK1) to stimulate phosphate transport via NaPi2b was demonstrated.[20] Furthermore, the activity of NaPi2b was shown to be inhibited by rapamycin, indicating the involvement of mTor signaling pathway in the regulation of phosphate homeostasis.[21]

Deregulation of NaPi2b function has been linked to human pathologies. It has been recently demonstrated that mutations in NaPi2b cause pulmonary alveolar microlithiasis.[22] Furthermore, the expression of NaPi2b was observed in ovarian, breast, and papillary thyroid tumors.[23–25] A further link of NaPi2b to cancer has been recently established. The consortium led by the Ludwig Institute for Cancer Research identified NaPi2b as the MX35 cancer antigen, which has been associated with ovarian cancer for many years.[26]

In this study, we describe the generation and characterization of monoclonal antibodies against human NaPi2b. A fragment corresponding to extracellular loop of human NaPi2b[26] was expressed in bacteria as GST fusion protein and used for immunization and screening procedures. Several rounds of screening allowed us to select two monoclonal antibodies, termed L2(20/3) and L3(28/1), which recognize specifically endogenous human NaPi2b in various immunological assays, including Western blotting, immunoprecipitation, and immunohistochemistry. These well characterized antibodies may be useful reagents for studying the physiological and pathological function of NaPi2b in humans.

Materials and Methods

Cell lines

All cell lines were obtained from the cell bank of the Ludwig Institute for Cancer Research at the New York Branch at Memorial Sloan-Kettering Cancer Center.

Preparation and purification of GST-NaPi2b fusion protein

The extracellular domain (loop) of human NaPi2b (L, loop, 188–361aa) and various fragments of this domain (1L, 188–300aa; 2L 291–361aa; 3L, 291–340aa; 4L, 311–340aa; 5L, 311–361aa) were PCR amplified using specific oligonucleotide primers with BamH1 and EcoR1 restriction sites and cloned into pGEX4T1 vector (Novagen, Darmstadt, Germany) in frame with the C-terminal GST-tag sequence. The resulting constructs, designated as pGEX-4T1-NaPi2b-L, pGEX-4T1-NaPi2b-1L, GEX-4T1-NaPi2b-2L, pGEX-4T1-NaPi2b-3L, pGEX-4T1-NaPi2b-4L, and pGEX-4T1-NaPi2b-5L were transformed into BL21(DE3) *Escherichia coli* strain cells. The expression of recombinant proteins were induced with 1 mM iso-propyl-b-D(2)-thiogalactopyranoside (IPTG) for 1–3 h at 30°C. Purification of GST-NaPi2b recombinant proteins from the soluble fraction of bacterial lysate was carried out using GST agarose (Qiagen, Crawley, United Kingdom) according to the manufacturer's recommendations. Purification of GST-NaPi2b-L from the insoluble fraction was performed using electroelution from the gel.

Generation of hybridoma cells

Six- to 8-week-old female BALB/c mice were immunized by intraperitoneal (i.p.) injection with 15 μg of recombinant GST-NaPi2b-L (188–361aa) fusion protein in complete Freund's adjuvant four times in 2-week interval. Then, immunized mice (with serum titer no less than 10^{-5}–10^{-6}) were boosted with 20 μg of antigen in PBS by i.p. injection. Three days later splenocytes from immunized mice were fused in the presence of PEG (MW2000, Merck, Darmstadt, Germany) with SP2/0 myeloma cells cultured in RPMI 1640 medium containing 20% fetal calf serum (FCS). Primary screening of hybridoma supernatants was performed using the ELISA technique, and isolated positive clones were subcloned by limiting dilution method using GST-NaPi2b-L as antigen.[27]

Western blot analysis of recombinant proteins

For hybridoma screening bacterially expressed GST-NaPi2b recombinant proteins: GST-NaPi2b-L 188–361aa, GST-NaPi2b-1L 188–300aa, or GST-NaPi2b-2L 291–361aa were separated by a 10% SDS/PAGE and electrotransferred to Immobilon-P membrane (Millipore, Billerica, MA). The membrane was blocked by 5% nonfat milk in PBS, containing 0.1% Tween-20 (PBST) for 1 h at room temperature and divided into strips followed by a single wash with PBST. Strips were incubated with PBS, postimmune serum (1:1000), hybridoma media from positive clones, or cell culture media alone for 2 h at room temperature. Peroxidase-conjugated secondary antibody (Promega, Madison, WI) was added to the strips after three washes and incubated for 1 h at room temperature. Strips were washed three times, and the immunoreactivity was detected by ECL system (Amersham, Uppsala, Sweden).

For MAb epitope mapping, recombinant peptides (GST-NaPi2b-L, GST-NaPi2b-1L, GST-NaPi2b-2L, GST-NaPi2b-3L, GST-NaPi2b-4L, GST-NaPi2b-5L) have been analyzed by Western blotting as described above and incubated with L2(2/1), L2(20/3), and L3(28/1) MAbs. Anti-GST MAbs were used as a control for recombinant protein expression.

Purification of MAbs

BALB/c mice were injected with 0.5 mL of pristane and 7–10 days later inoculated with 5×10^6 of hybridoma cells.[18] The ascitic fluid was collected after 7–10 days, and after centrifugation for at 14000 rpm for 20 min and filtration by 0.4 μm filter (Millipore) used for affinity purification by Protein A-Sepharose CL-4B (Amersham) chromatography. The IgG fractions were pulled together and dialyzed in a phosphate-buffered saline (PBS, pH 7.4). The aliquots of purified antibodies were stored at $-20°C$.

Cell lysate preparation and immunoblotting

OVCAR3, SK-RC-18, and SK-RC-01 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 estimated by BSA assay (Pierce), and equal amounts of proteins (10 μg) were run on 8% SDS-PAGE gels. Proteins were transferred toa polyvinylidene difluoride (PVDF) membrane (Millipore). The membrane was blocked with 5% BSA in 1× PBST for 1 h at room temperature (RT). Anti-NaPi2b MAbs were incubated with the 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.

Immunohistochemical analysis

Anti-NaPi2b MAbs were used for immunohistochemical analysis of ovarian cancer samples according to a standard protocol. 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 mkg/mL). Sections were incubated with biotinylated secondary antibodies for 2 h at room temperature (1:400, goat anti-mouse biotinylated IgG, Sigma, Steinheim, Germany), followed by incubation with avidin-biotin-peroxidase complex (Vector Laboratories) for 30 min at room temperature and developed with diaminobenzidine solution. Hematoxylin was used for counterstaining. Standard microscopy was performed using a Zeiss Universal microscope (Zeiss, Jena, Germany), and images were captured using digital Axiocam software.

Transient transfection of HEK293 cells

Transfection of HEK293 cells was performed with FuGene transfection reagent (Roche, Basel, Switzerland) according to the manufacturer's instruction. Briefly, for each transfection, 5 mkg of plasmid DNA (pcDNA3.1/Glu-NaPi2b or empty vector) and 10 mkL of FuGene was used. After 48 h of incubation, cells were lysed and analyzed.

Immunoprecipitation

Immunoprecipitation assay was performed with lysates of HEK293 cells transfected with pcDNA3.1/NaPi2b or pcDNA3.1 alone and hybridoma media from selected positive clones as described.[28]

Mixed hemadsorption assays

The mixed hemadsorption assay (MHA), which detects surface-bound IgG by adherence of rabbit anti-mouse IgG-coated human red blood cells (blood group O) to target cells, was performed as described.[29]

Isotype antibody detection

Isotypes of antibodies were defined with a mouse monoclonal isotyping test kit (AbD Serotec, Oxford, United Kingdom) using cultures supernatants of KL2(2/1), KL2(20/3), and KL3(28/1) clones.

Results and Discussion

Membrane transport of P_i by NaPi cotransporters is recognized as a key regulatory event in maintaining phosphate

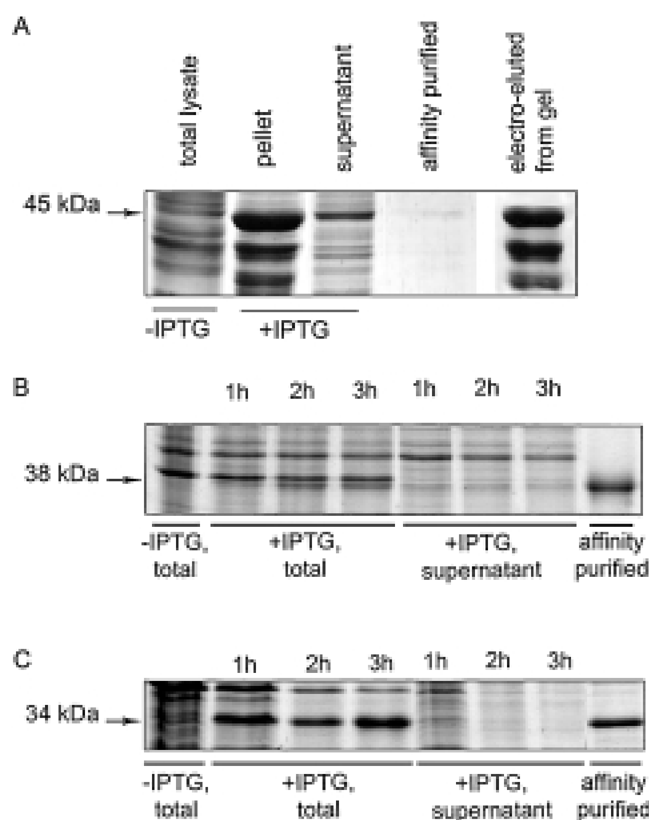

FIG. 1. SDS-PAGE analysis of bacterially expressed and affinity purified GST/NaPi2b fusion proteins. **(A)** Expression analysis and purification of GST/NaPi2b-L from insoluble fraction of bacterial cell lysate. Expression of GST/NaPi2b-L in BL21(DE3) cells was induced by IPTG for 3 h at 27°C. The pellet of bacterial cells was lysed and centrifuged to remove the insoluble fraction. Purification of GST/NaPi2b-L from the insoluble fraction was carried out by electroelution from the SDS-PAGE gel. (**B and C**) Expression profiles and the quality of affinity purified GST/NaPi2b-1L (188–300aa) (**B**) and GST/NaPi2b-2L (291–361aa) (**C**) analyzed by SDS-PAGE electrophoresis. The expression of GST/NaPi2b-1L and GST/NaPi2b-2L was induced by IPTG for 1, 2, and 3 h. Harvested cells were lysed and centrifuged at 13,000 rpm to remove insoluble fraction. The extract of soluble proteins was used for purification by affinity chromatography on glutathione sepharose.

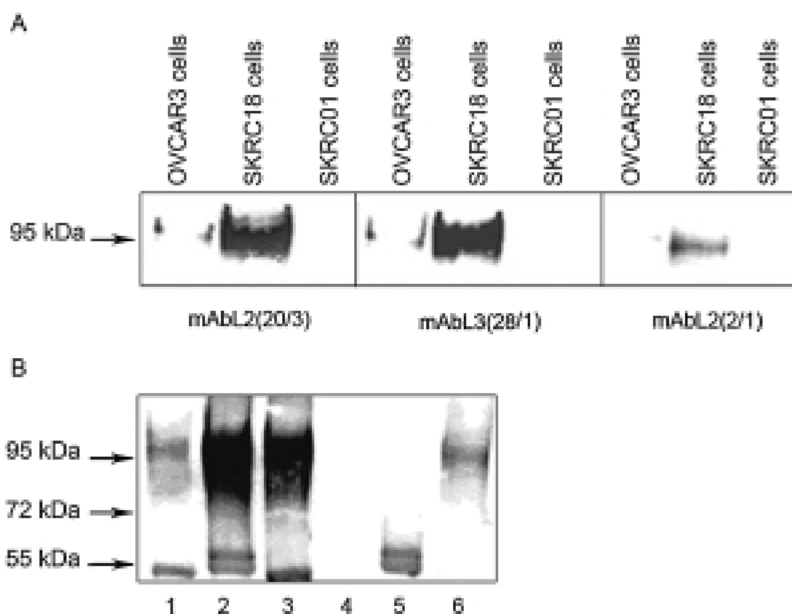

FIG. 2. Western blot analysis (**A**) and immunoprecipitation (**B**) of transiently overexpressed and endogenous NaPi2b. (**A**) Specific recognition of endogenous NaPi2b by generated antibodies in Western blot analysis. Total cell lysates (10 μg) from OVCAR3, SKRC18, and SKRC01 cell lines were separated by SDS-PAGE under non-reducing (without DTT) conditions and probed with generated monoclonal antibodies. (**B**) Immunoprecipitation of transiently overexpressed NaPi2b. HEK293 cells were transiently transfected with pcDNA3.1+/NaPi2b. The supernatants of transfected cells were incubated with protein A sepharose containing IgGs from L2(2/1), L2(20/3), L3(28/1) hybridoma clones (lines 1–3) or with protein A sepharose alone (line 4). The immune complexes were resolved by SDS-PAGE and immunoblotted with L2(20/3) MAb. Line 5, the supernatant of cells transfected with pcDNA3.1+ alone were incubated with protein A sepharose containing IgGs from L2(20/3) hybridoma clones. Line 6, supernatant of stably transfected HEK293 cells with pcDNA3.1/NaPi2b.

homeostasis in organisms as diverse as bacteria and human. Phosphate transporter NaPi2b is responsible for mediating epithelial P_i transport in several human tissues. Deregulation of NaPi2b function has been linked to pulmonary alveolar microlithiasis.[22] Recently NaPi2b has been found to be expressed in different tumor types, including ovarian cancer, breast, and thyroid.[23–25] The mechanism and the importance of NaPi2b expression in human tumors, especially ovarian cancer, are not known so far. These findings identified NaPi2b as an important target molecule and a suitable candidate for the development of novel diagnostic and therapeutic interventions in human pathologies.

The main focus of this study was to generate monoclonal antibodies against human NaPi2b, which could be used in various immunological assays, including Western blotting, immunoprecipitation, and immunohistochemistry to allow a more detailed study of the physiological and pathophysiological role of NaPi2b on the protein level. For this purpose we felt an antibody that specifically reacts with the extracellular region of NaPI2b would be most suited. In order to obtain such antibodies we prepared recombinant fusion proteins of the largest extracellular loop (188–361aa) of NaPi2b. A fragment of NaPi2b, corresponding to amino residues 188–361 was amplified by PCR and cloned into pGEX4T1 vector in frame with the N-terminally located GST sequence. The fusion protein from the resulting plasmid pGEX4T1/NaPi2b-L was expressed in *E. coli* BL21 (DE3) cells. Expression analysis indicated that GST/NaPi2b-L was expressed at high levels after induction with IPTG, with the majority of the protein found in inclusion bodies (Fig. 1A). GST/NaPi2b-L from the insoluble fraction was purified by electroelution from gel and yielded sufficient protein (purity >80%) for immunization and ELISA screening (Fig. 1A). In addition, the N- and C-terminal regions of the largest extracellular loop were expressed in bacteria as GST fusion and designated as GST/NaPi2b-1L (188–300aa) and GST/NaPi2b-2L (291–361aa). As shown in Figure 1B and C, both fusion proteins expressed well, but were only partially soluble. Affinity purification on glutathione sepharose allowed us to obtain significant quantities of recombinant GST/NaPi2b-1L and GST/NaPi2b-2L of approximately 95% purity (Fig. 1B and C).

Hybridomas were generated by a standard procedure as described in Materials and Methods. Primary ELISA screening of hybridoma media from generated hybrids with GST/NaPi2b-L fusion protein resulted in 110 positive clones. In a second round of ELISA screening, 83 clones were selected with reactivities against GST/NaPi2b-L of which 27

TABLE 1. MIXED HEMADSORPTION ASSAY OF NEW MONOCLONAL ANTIBODIES AGAINST NaPi2b

Antigen Cell line	*Titer (μg MAb/mL)*		*Secondary Ab only*
	L2(20/3)	*L3(28/1)*	
OVCAR-3	0.04	0.16	Negative
SK-RC-18	0.04	0.04	Negative
SK-RC-01	Negative	Negative	Negative

clones recognized GST alone. The specificity of the remaining 56 positive hybridoma clones was examined by Western blot using GST/NaPi2b-L. This analysis identified 24 clones, which recognized specifically, but with different intensity, a 45 kDa band corresponding to GST/NaPi2b-L (data not shown). Positive clones that showed the strongest reactivity towards GST/NaPi2b-L were further tested by immunoblotting against GST/NaPi2b-1L and GST/NaPi2b-2L. Interestingly, none of the selected clones recognized the GST/NaPi2b-1L fusion protein (data not shown). These data clearly indicate that the C-terminal region of the NaPi2b extracellular loop appears to be more immunogenic than the N-terminal region. This finding correlates with a bioinformatic analysis of the predicted NaPi2b immunogenicity (www.imtech.res.in/raghava/bcepred). The best responders (seven hybridoma clones) were subcloned to monoclonality and their specificity was confirmed again by ELISA and Western blotting.

To determine whether selected MAbs can recognize endogenous NaPi2b, we tested their specificity in Western blotting, immunoprecipitation, mixed hemadsorption assay, and immunohistochemistry using mammalian cell lines and tissues. Initially, the lysates of NaPi2b positive (OVCAR3 and SK-RC-18) and negative (SK-RC-01) cell lines were probed in Western blotting with hybridoma supernatant from selected clones. All three cell lines have been typed previously by RT-PCR for the expression of SLC34A2 mRNA.[26] This analysis allowed us to identify three clones that recognized specifically endogenous NaPi2b by immunoblotting. As shown in Figure 2A, monoclonal antibodies termed L2(20/3), L3(28/1), and to a less extent L2(2/1) detect a diffuse band of approximately 95 kDa in OVCAR3 and SK-RC-18 cells, but not in SK-RC-01 cells under non-reduced conditions (Fig. 2A). When total cell extracts were resolved under reducing conditions and probed with NaPi2b MAbs, the 95 kDa band recognized in endogenously expressed NaPi2b in OVCAR3 and SK-RC-18 cell lysates was less diffuse and was observed only with MAbs L2(20/3) and L3(28/1), but not with MAb L2(2/1) (data not shown). These results indicate that all three monoclonal antibodies recognize conformational epitope(s) in the C-terminal region of the largest extracellular loop.

Then, we tested the ability of selected hybrid clones to immunoprecipitate transiently overexpressed NaPi2b. In this experiment, HEK293 cells were transiently transfected with pcDNA3.1/Glu-NaPi2b or pcDNA3.1 alone. We found that monoclonal antibodies L2(20/3) and L3(28/1) worked particularly well in immunoprecipitating transiently overexpressed NaPi2b (Fig. 2B). Furthermore, we found that both monoclonal antibodies L2(20/3) and L3(28/1) could specifically immunoprecipitate endogenous NaPi2b from SK-RC-18 cells (data not shown). We have tested monoclonal antibodies L2(20/3) and L3(28/1) in a mixed hemadsorption assay where they showed strong reactivity with the cell lines OVCAR-3 and SK-RC-18, which expressed NaPi2b on their cell surface but did not react with SK-RC-1, which does not express NaPi2b (Table 1).

The newly generated antibodies were also examined for their capability to recognize NaPi2b by immunohistochemistry. Taking into account that NaPi2b was strongly expressed in a large number of human ovarian cancers, we probed samples of normal ovary and ovarian carcinoma. The results presented in Figure 3A and B indicate that clones L2(20/3) and L3(28/1) recognized the corresponding antigen in paraffin-embedded sections of ovarian carcinoma with positive staining localized mostly to the luminal edge of tumor sample. No or very little immunoreactive signal was detected in normal ovary under the same experimental conditions. These results support previously published data indicating that NaPi2b is expressed in ovarian cancer.[23]

To map the epitopes recognized by the monoclonal antibodies, we utilized a panel of GST/NaPi2b fusion constructs, representing various regions of the largest extracellular loop. The schematic presentation of generated fusion proteins used in this study is shown in Figure 4A. GST/NaPi2b fusion proteins and GST alone were resolved by SDS-PAGE under non-reduced conditions, transferred to the PVDF membrane, and probed with L2(2/1), L2(20/3), and L3(28/1) MAbs. This analysis indicated that the binding sites for all three antibodies were located in the region between amino acid residues 311 and 340. Figure 4B illustrates the epitope mapping for monoclonal antibody L2(20/3). These results are not surprising, since a bioinformatic analysis identified this region as the potentially most antigenic site in the NaPi2b sequence. Moreover, this region contains two cysteine residues (C322 and C328), which might be involved in disulfide bond formation. The presence of three potential

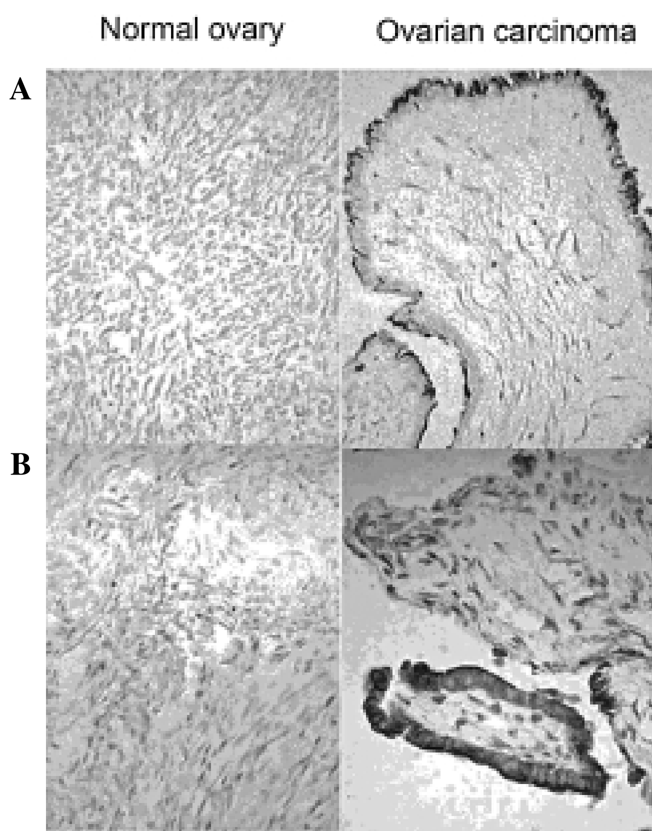

FIG. 3. Immunohistochemical analysis of ovarian tissues with NaPi2b/(20/3) and NaPi2b/(28/1) MAb. Immunohistochemical staining of paraffin-embedded sections of normal ovary and ovarian carcinoma with L2(20/3) (**A**) and L3(28/1) (**B**) antibodies. Immunohistochemistry was carried out as described in the Materials and Methods section.

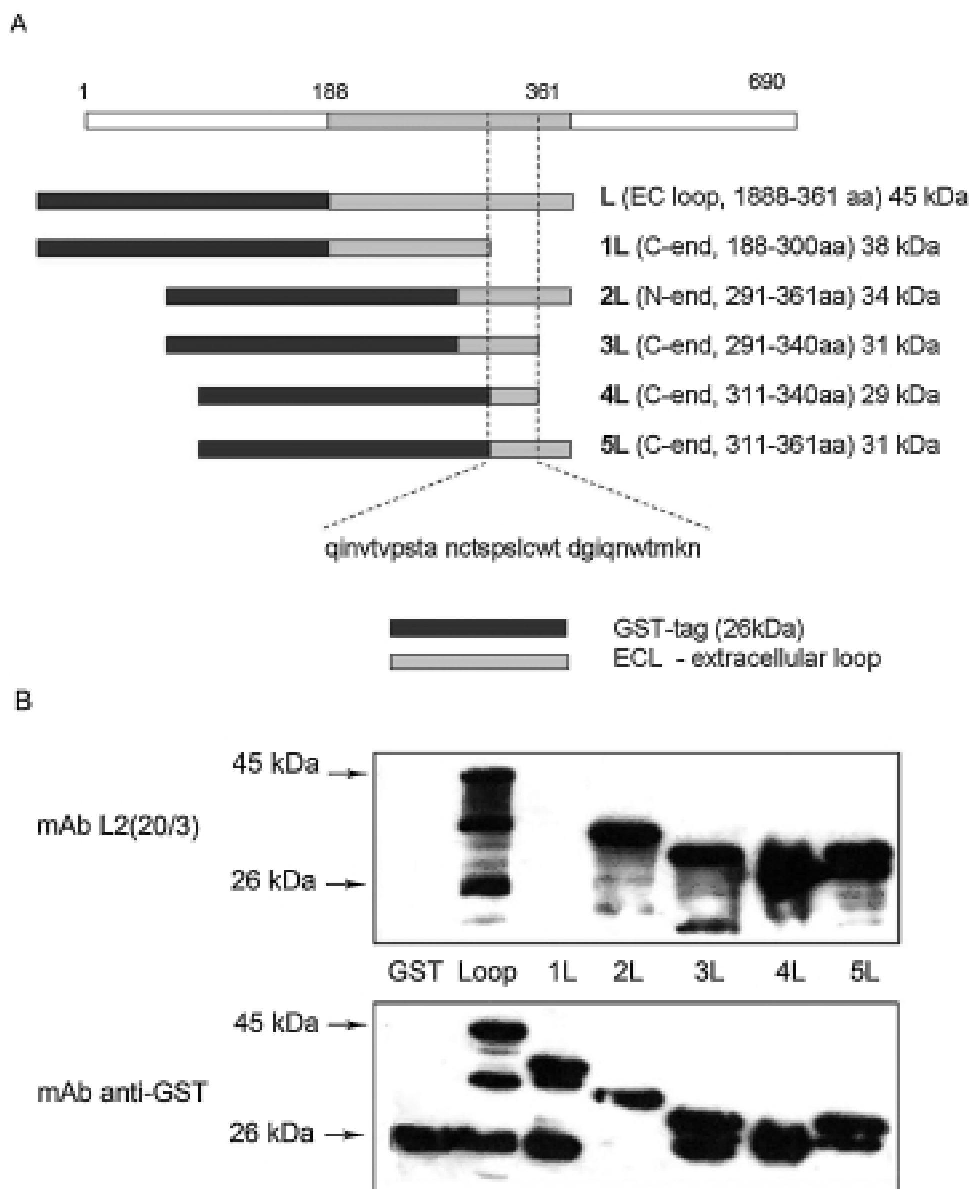

FIG. 4. Epitope mapping for anti-NaPi2b monoclonal antibodies. (**A**) Schematic of GST/NaPi2b fusion proteins used in this study. The region of NaPi2b that possesses the epitopes for generated antibodies is marked and shown by amino acid sequence. (**B**) Western blot analysis of GST alone and GST/NaPi2b fusion proteins with L2(20/3) and anti-GST monoclonal antibodies. Equal amounts of GST alone or various GST/NaPi2b fusion proteins (2 μg each) were resolved by SDS-PAGE, electro-blotted to PVDF membrane, and probed with L2(20/3) and anti-GST monoclonal antibodies.

sites of glycosylation (N313, N321, and N340) within the 311–340aa region has been also predicted by the bioinformatic analysis. Therefore, the pattern of glycosylation, as well as the formation of disulfide bridges, might determine the efficiency and the specificity of the epitopes recognized in this highly antigenic region under physiological and pathological conditions.

The three antibodies were also typed for their Ig isotype. Clones L2(2/1) and L3(28/1) produced IgG1, while clone L2(20/3) produced IgG2b antibodies.

In summary, we have generated a panel of monoclonal antibodies against bacterially expressed extracellular domain of the human sodium-dependent phosphate transporter NaPi2b. Two hybridomas, termed L2(20/3) and L3(28/1), produced antibodies that specifically and efficiently recognized endogenous human NaPi2b in various immunoassays, including Western blotting, immunoprecipitation, and immunohistochemistry. These properties make these two antibodies particularly useful for studying the physiological and pathophysiological function of human NaPi2b.

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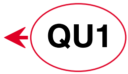

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QU1
Uneven columns OK?