Expression of the Na/P$_i$-cotransporter Type IIb in Sf9 Cells: Functional Characterization and Purification

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Abstract. In mammals the type IIb Na/P$_i$-cotransporter is expressed in various tissues such as intestine, brain, lung and testis. The type IIb cotransporter shows 51% homology with the renal type IIa Na/P$_i$-cotransporter, for which a detailed model of the secondary structure has emerged based on recent structure/function studies. To make the type IIb Na/P$_i$-cotransporter available for future structural studies, we have expressed this cotransporter in Sf9 cells. Sf9 cells were infected with recombinant baculovirus containing 6His NaPi-IIb. Infected cells expressed a polypeptide of ∼90 kDa, corresponding to a partially glycosylated form of the type IIb cotransporter. Transport studies demonstrated that the type IIb protein expressed in Sf9 cells mediates transport of phosphate in a Na-dependent manner with similar kinetic characteristics (apparent K_ms for sodium and phosphate and pH dependence) as previously described. Solubilization experiments demonstrated that, in contrast to the type IIa cotransporter, the type IIb can be solubilized by nonionic detergents and that solubilized type IIb Na/P$_i$-cotransporter can be purified by Ni-NTA chromatography.

Key words: Na-dependent phosphate cotransport — NaPi-IIb — Sf9 cells — Baculoviruses — Solubilization — Purification

Introduction

Type II Na/P$_i$-cotransporters, members of the solute carrier family 34 (www.gene.ucl.ac.uk/nomenclature), are expressed in polarized cells such as renal proximal tubular cells (NaPi-IIa), enterocytes or alveolar type II cells type (NaPi-IIb) (Custer et al., 1994; Hilfiker et al., 1998; Traebert et al., 1999). In renal proximal tubules and in small intestine, type II Na/P$_i$ cotransporters play important roles in the maintenance of the overall phosphate homeostasis. In particular, the renal type IIa cotransporter was shown to represent a major player in the control of extracellular concentration of phosphate (for review, *see* Murer et al., 2000). Similarly, the type IIb cotransporter is regulated according to the body needs of inorganic phosphate. Its abundance is upregulated by 1,25-dihydroxyvitamin D$_3$ and low-phosphate diet (Hattenhauer et al., 1999; Katai et al., 1999). On the other hand, administration of epidermal growth factor and glucocorticoids decreases expression of NaPi-IIb in the intestine (Xu et al., 2001; Arima et al., 2002).

As demonstrated for the type IIa isoform, type II Na/P$_i$-cotransporters likely span the membrane eight times, are N-glycosylated at a large extracellular loop and exhibit two similar regions, which are supposed to be part of the translocation pathway for one phosphate ion together with three sodium ions (Hayes et al., 1994; Kohler et al., 2002; Forster et al., 2002). Other structural features were obtained by epitope-tagging experiments and the use of antibodies, demonstrating that both the NH$_2$- and the COOH-termini are located at the cytoplasmic side (Lambert et al., 1999). Apart from these topological aspects, other structural features of type II Na/P$_i$-cotransporters are not known.

To purify a type IIb Na/P$_i$-cotransporter and possibly make it available for high-resolution structural analysis, we have expressed the Na/P$_i$-cotransporter IIb of mouse in Sf9 cells. We demonstrated that NaPi-IIb can be functionally expressed in these cells and, most importantly, that NaPi-IIb can be solubilized by non-ionic detergents and purified based on Ni-NTA affinity chromatography.

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Materials and Methods

PLASMID CONSTRUCTION

Mouse NaPi-IIb cDNA contained in pSPORT1 (Hilfiker et al., 1998; accession number: AAC52361) was amplified using the primers 5′-TCTGGAATTCATGGCTCCTTGGCCCGAGTTGG (sense) and 5′-GATTACGCCAAGCTCTAAT- ACGACTCAC-TATAGG (antisense). The PCR product of 4 kb was subcloned in *Eco*RI and *Sal*I sites of the vector pFastBac-Hta (Gibco BRL). Site-directed Mutagenesis (Quick Change, Stratagene) was used to mutate the starting methionine of NaPi-IIb to alanine (primer: CCATGGATCCGGAATTCGCGGCTCCTTGGCCCGAGTTG). The final sequence was verified by sequencing (Microsynth, Switzerland).

CELL CULTURE AND PRODUCTION OF RECOMBINANT BACULOVIRUSES

Sf9 cells were maintained in 75 cm^2 culture flasks (at 27°C) containing Grace's Insect Medium (Gibco BRL), 10% foetal bovine serum (Gibco BRL) and 10 µg/ml gentamicin sulfate (Sigma).

Recombinant bacmid DNA was obtained after transfection of the pFastBac-Hta/NaPi-IIb constructs into DH10Bac cells (Gibco BRL). Recombinant baculoviruses were recovered from Sf9 cells (9×10^5 cells per 35-mm well) 3 days after transfection and stored at −80°C. All procedures were performed according to the manufacturer's protocol. For routine infections, cells grown to log phase (approximately 9×10^5 cells/35-mm well) were infected with recombinant baculoviruses containing NaPi-IIb (10^{-3} dilution of the original stock) in 0.5 ml Grace's Insect Medium. 1.5 ml of virus-free medium was added after 1 h of incubation. Cells were harvested 3 days after infection for the experiments described below.

IMMUNOBLOTTING

Total cells lysates were obtained by solubilizing cells (approximately 50 mg total protein) in 1 ml of TBS (120 mM NaCl, 50 mM Tris-HCl, pH 7.3) containing 0.5% Igepal (Sigma). After sonication (400 W; 10×3 s), cell debris was removed by centrifugation ($12,000 \times g$; 2 min). Supernatants were analyzed by western blotting, using 20 µg of total protein. Denaturation was performed at 96°C for 2 min in 2% SDS, 1 mM EDTA, 10% glycerol, 85 mM Tris/HCl (pH 6.8). After blocking with 5% non-fat milk powder and 1% Triton X-100 in TBS, immunodetection of electrotransferred protein was performed according to standard procedures. Untagged NaPi-IIb was detected with a rabbit polyclonal antibody raised against a synthetic peptide derived from the NH$_2$-terminus of NaPi-IIb at a dilution of 1:4,500 (Hattenhauer et al., 1999). The histidine-tagged NaPi-IIb was detected with a mouse monoclonal anti-(His)$_6$ tag antibody (Dianova, Italy; dilution 1:400). Binding of primary antibodies was visualized with anti-rabbit or anti-mouse IgGs conjugated to horse radish peroxidase (Amersham Pharmacia Biotech) and enhanced chemiluminescence (Pierce).

IN VITRO TRANSLATION

In vitro translation in the absence or presence of canine microsomes (TNT translation kit, Promega) was performed with NaPi-IIb cRNA synthesized according to standard protocols (Ambion MEGAscript SP6 kit). Products of translation were separated on 9% SDS-PAGE and analyzed by autoradiography.

PHOSPHATE-UPTAKE STUDIES WITH INTACT CELLS

Sodium-dependent phosphate transport was determined 3 days after infection with NaPi-IIb. Cells (grown in 6-wells plates) were washed once with phosphate-free uptake medium (in mM: 137 NaCl, 5.4 KCl, 2.8 CaCl$_2$, 1.2 MgSO$_4$, 10 HEPES, pH 7; room temperature) and afterwards incubated with 1 ml of the same buffer but containing 100 µM K$_2$HPO$_4$ and [^{32}Pi]K$_2$HPO$_4$ (1 µCi/ml). After 6 min, the uptake medium was removed and the cells were washed 4 times with 2 ml of ice-cold stop solution (137 mM choline chloride, 14 mM Tris-HCl, pH 7). Cells were lysed with 1% Triton X-100 and aliquots were taken for scintillation counting and determination of the protein concentration (*DC* Protein Assay, BioRad). To determine sodium-independent transport, NaCl was replaced by choline-chloride. To determine the apparent K_m-value for Na$^+$ ions, NaCl was replaced with an equimolar amount of N-methyl-glucamine. Determination of the apparent K_m value for phosphate was done by varying the extracellular phosphate concentration between 25 µM and 200 µM. External pH values were adjusted between 6.5 and 8 to determine pH dependence of Na/P$_i$ cotransport. All uptakes were determined in four independent experiments.

PHOSPHATE-UPTAKE STUDIES WITH ISOLATED MEMBRANES

Sf9 cells or Sf9 cells expressing 6His NaPi-IIb were harvested by centrifugation ($1,000 \times g$ for 10 minutes at 4°C) and resuspended in 50 ml of ice cold buffer (50 mM D-mannitol, 2 mM EGTA, 5 mM Tris-HCl, pH 7). Cell lysis was achieved by nitrogen cavitation after equilibration for 30 min at 600 p.s.i. The homogenate was centrifuged first at $500 \times g$ for 10 min to remove cellular debris. Membranes were collected by centrifugation at $70,000 \times g$ for 30 min and then resuspended in 300 mM D-mannitol, 20 mM HEPES-Tris (pH 7.2).

Transport of phosphate was measured as described (Stoll, Kinne & Murer, 1979; Hatttenhauer et al., 1999) at 25°C in the presence of inward gradients of 100 mM NaCl or 100 mM KCl and 0.2 mM K-phosphate.

SOLUBILIZATION AND Ni-NTA CHROMATOGRAPHY

Sf9 cells grown to log phase (approximately 3×10^6 cells/75 cm^2 flask) were infected with recombinant baculoviruses containing 6His NaPi-IIb (50 µl from the original stock) in 3 ml Grace's Insect Medium. 10 ml of virus-free medium was added after 1h of incubation. After 3 days, infected cells from 4 flasks were harvested by centrifugation at $1,000 \times g$ for 10 min at 4°C. The pellets were resuspended in 40 ml ice-cold buffer (50 mM mannitol, 2 mM EGTA, 5 mM Tris-HCl, pH 7). Membrane preparations were performed as described above and resuspended in 300 mM D-mannitol, 20 mM HEPES-Tris (pH 7.2). The membranes were then solubilized in the same buffer supplemented with 1% n-decyl-β-D-maltopyranoside or 1% octyl-β-D-glucopyranoside. After sonication (400 W; 10×3 s) insoluble material was removed by centrifugation at $100,000 \times g$ for 45 min (4°C). Soluble histidine-tagged proteins contained in the supernatant were purified by Ni-NTA affinity chromatography on 0.4 ml of 50% "slurry" beads (Qiagen), according to the manufacturer's instructions (Gibco BRL). After the first flow-through, columns were washed with 10 volumes of phosphate-based buffer (50 mM sodium phosphate, pH6.0, 10 mM imidazole, 300 mM NaCl, 10% glycerol) and eluted with the same buffer but containing 100 mM imidazole. Fractions (0.5 ml) were collected and analyzed by western blotting.
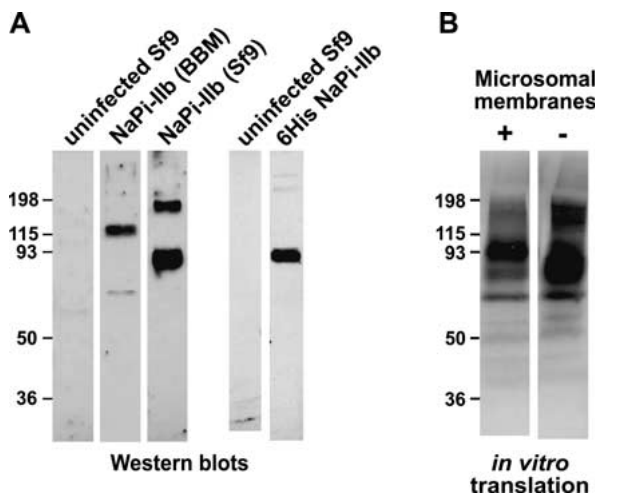
Fig. 1. Expression of NaPi-IIb in Sf9 cells. (*A*) Uninfected Sf9 cells, isolated mouse small intestinal brush-border membranes (BBM) and Sf9 cells infected with NaPi-IIb were analyzed by western blotting using a polyclonal antibody directed against the NH$_2$-terminus of NaPi-IIb. 6His NaPi-IIb was detected using a monoclonal antibody directed against the 6His-tag. (*B*) In vitro translation of NaPi-IIb cRNA in the absence and presence of microsomal membranes.

Alternatively, membranes were solubilized in buffer containing 500 mM NaCl, 10% glycerol, 10 mM Tris-HCl (pH 8), supplemented with 1% octyl-β-D glucopyranoside and immobilized on Ni-NTA beads by a 4-h "end over end" incubation. Bound proteins were washed with phosphate-based buffer and eluted in one step by centrifugation (batch elution) at 3000 × *g* for 2 min (4° C) with 0.5 ml of 250 mM imidazole in the same buffer. The eluent was analyzed both by Coomassie Blue staining and western blotting.

Results

PRODUCTION OF RECOMBINANT BACULOVIRUSES

Initially, Sf9 cells were infected with recombinant baculovirus DNA containing histidine-tagged NaPi-IIb cDNA including also the start codon ATG of the original open reading frame of NaPi-IIb (Hilfiker et al., 1998). However, after expression of this construct in Sf9 cells, the expected histidine-tagged NaPi-IIb protein could not be detected. Yet, as evidenced by western blots, NaPi-IIb was fully expressed (*see* below). This indicated that instead of the ATG upstream of the 6His tag, the start codon of the NaPi-IIb sequence initiated translation. Therefore, to obtain the 6His-tagged NaPi-IIb protein, the start codon contained in the original sequence of NaPi-IIb was mutated (Met to Ala).

EXPRESSION OF NaPi-IIb IN Sf9 CELLS

Three days after infection of Sf9 cells with recombinant baculoviruses, expression of wild type (untagged, *see* above) or histidine-tagged NaPi-IIb was
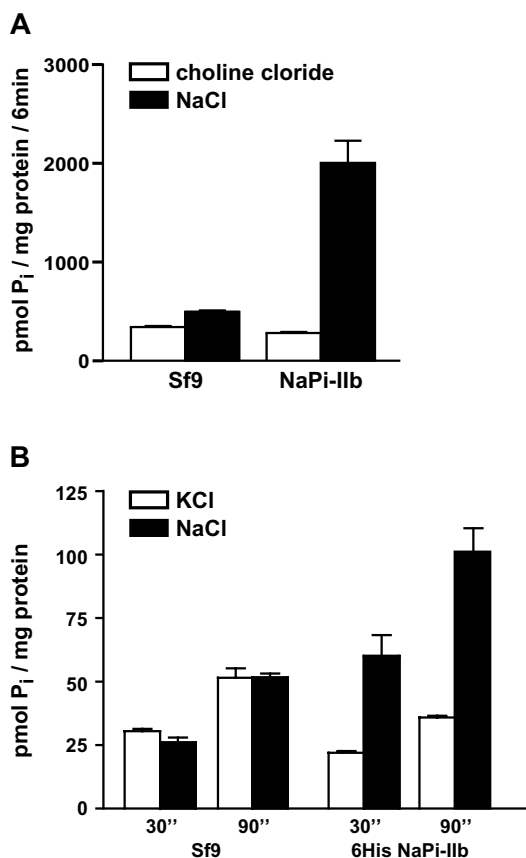

Fig. 2. NaPi-IIb expressed in Sf9 cells mediates phosphate uptake in a sodium-dependent manner. (*A*) Uptake measurements were performed in uninfected Sf9 cells and Sf9 cells infected with NaPi-IIb three days after infection at room temperature in the presence of 137 mM NaCl or 137 mM choline chloride at pH 7. The data represent the mean ± SD of 12 dishes. (*B*) Phosphate uptake measurements were performed with membranes isolated from Sf9 cells or Sf9 cells infected with 6His NaPi-IIb. Uptakes were performed in the presence of 100 mM NaCl or 100 mM KCl. The data represents the mean ± SD of 3 measurements.

determined by western blotting. As shown in Fig. 1*A*, both the untagged and the histidine-tagged NaPi-IIb protein were detected as a band of ~90 kDa. Occasionally and independently of the denaturation procedure (*data not shown*), using either whole cells or isolated membranes, NaPi-IIb also appeared as a band of approximately 200 kDa, most likely representing a dimer.

Based on a comparison of the apparent size of NaPi-IIb expressed in Sf9 cells (~90 kDa) with NaPi-IIb contained in small intestinal brush-border membranes of adult mice (108 kDa; Fig. 1*A*), it was assumed that in Sf9 cells N-glycosylation of NaPi-IIb was incomplete. To confirm this assumption, NaPi-IIb cRNA was translated in vitro in the absence and presence of microsomes. As illustrated in Fig. 1*B*, unglycosylated NaPi-IIb (absence of microsomes) was detected as a band of approximately 75 kDa,
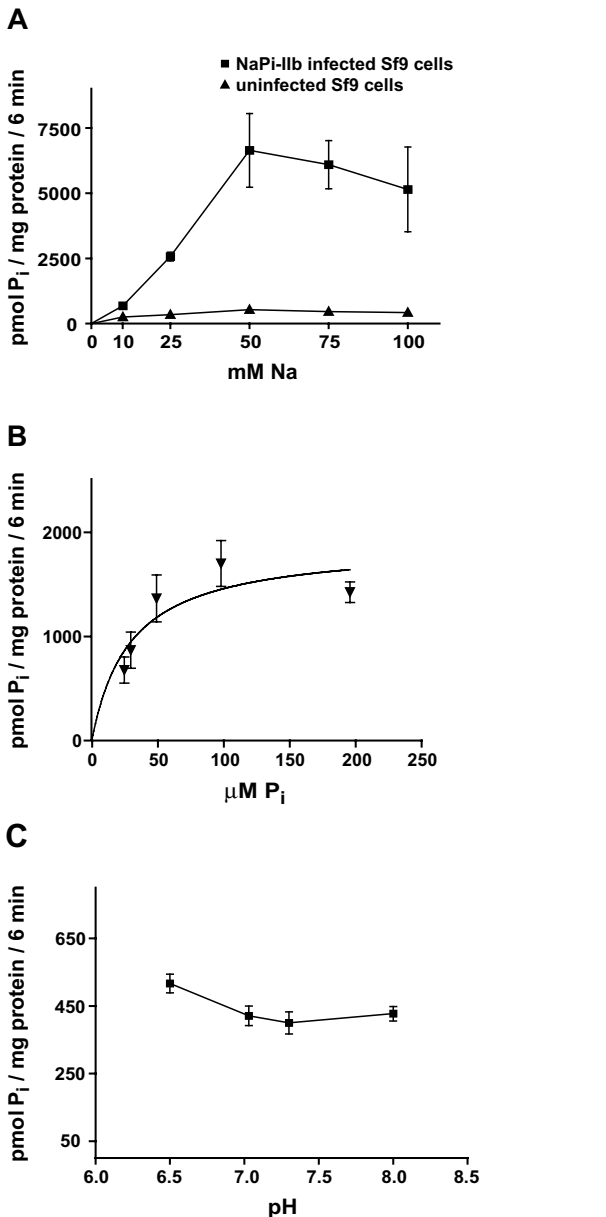
Fig. 3. Characterization of Na/P$_i$-cotransport mediated by NaPi-IIb expressed in Sf9 cells. (*A*) Uptake measurements performed as function of external NaCl concentration showed strong sodium-dependence with an apparent $K_m \approx 30$ mM. (*B*) Phosphate uptake was measured as a function of phosphate concentration. The data represent the difference obtained from uptake measurements performed in the presence or absence of sodium and the curve was fitted according to Michaelis-Menten equation (apparent $K_m \approx 30$ μM). (*C*) Phosphate uptake at different pH values showed that Na/P$_i$-transport mediated by NaPi-IIb is not pH-dependent. The data represent the difference obtained from uptake measurements performed in the presence or absence of sodium. The data represent the mean ± SD of 12 dishes. Each experiment was repeated at least 3 times.
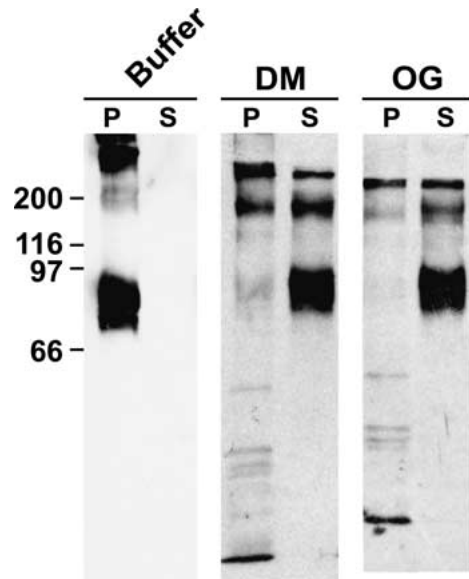
Fig. 4. Solubilization of NaPi-IIb from Sf9 cells. Sf9 cells infected with NaPi-IIb were collected by centrifugation ($1000 \times g$, 10 min, 4°C), resuspended in 10 mM Hepes-NaOH, pH 7.6 without (buffer) or with 1% (w/v) detergents *n*-decyl-β-D-maltopyranoside (DM) or octyl-β-D-glucopyranoside (OG). After incubation on ice for 1h and centrifugation at $100,000 \times g$ for 45 min at 4°C, equal aliquots of pellets (*P*) and supernatants (*S*) were analyzed by western blotting. The same observations were made when isolated membranes were used instead of intact Sf9 cells.

Transport Studies of NaPi-IIb Expressed in Sf9 Cells

Phosphate (P$_i$) uptake measurements with intact cells and membranes isolated from uninfected and Sf9 cells infected with NaPi-IIb were performed in the absence and presence of sodium (Fig. 2). In agreement with earlier observations (Fucentese et al., 1995), neither uninfected Sf9 cells nor membranes isolated thereof did exhibit significant net sodium-dependent phosphate transport. On the other hand, sodium-dependent phosphate uptake was observed in Sf9 cells infected with NaPi-IIb and in membranes isolated from cells infected with 6His NaPi-IIb. Induced sodium-dependent phosphate transport was entirely blocked by 3 mM phosphonoformic acid (*not shown*), which has been described as a general inhibitor of Na/P$_i$-cotransporters (Loghman-Adham, Szczepanska-Konkel & Dousa, 1992).

Na/P$_i$-cotransport of infected Sf9 cells was further characterized in terms of its dependences on the concentrations of sodium and phosphate ions and the external pH value (Fig. 3). The results indicated that the characteristics of Na/P$_i$-cotransport mediated by NaPi-IIb or 6His NaPi-IIb expressed in Sf9 cells were comparable to the ones observed after expression of NaPi-IIb in oocytes of *Xenopus laevis* oocytes (Hilfiker

whereas in the presence of microsomes, a partially glycosylated form at ~90 kDa was observed, which corresponded with the apparent molecular weight of NaPi-IIb after expression in Sf9 cells.
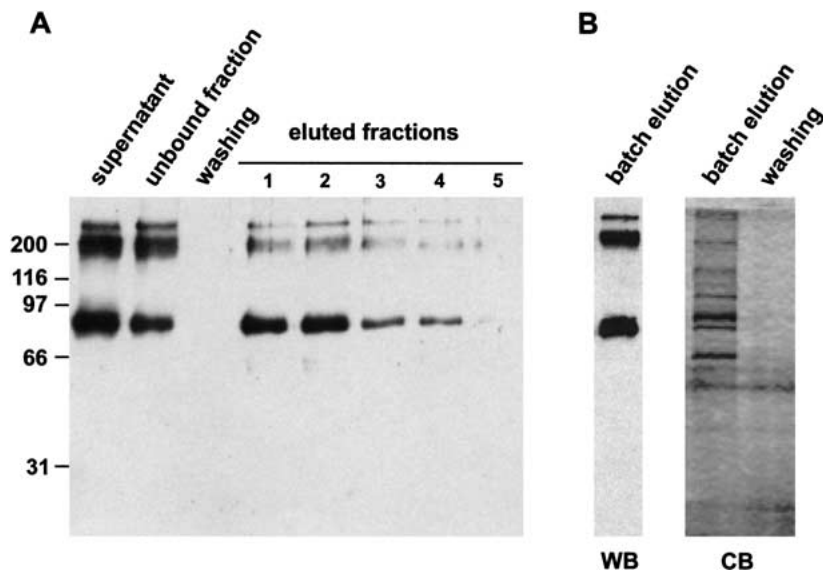
Fig. 5. Purification of 6His NaPi-IIb by Ni-NTA affinity chromatography. (*A*) Supernatants obtained after solubilization of membranes isolated from Sf9 cells infected with 6His NaPi-IIb were loaded on a Ni-NTA column. Samples were taken from all steps and analyzed by western blotting. (*B*) The sample obtained after batch elution of NI-NTA beads with 250 mM imidazole was analyzed by western blotting (*WB*) and Coomassie Blue staining (*CB*).

et al., 1998). In infected cells, the apparent K_m values for the interactions with sodium and phosphate ions were determined as 30 mM and 30 μM respectively. And, in agreement with earlier studies (Hilfiker et al., 1998), no significant dependence of Na/P$_i$-cotransport upon the external pH value was observed.

PARTIAL PURIFICATION OF 6His NaPi-IIb

To solubilize wild-type or 6His-tagged NaPi-IIb, infected Sf9 cells or isolated membranes were treated with nonionic detergents. After high-speed centrifugation, the effectiveness of solubilization was analyzed in the supernatants. Among several detergents tested (*data not shown*), n-decyl-β-D-maltopyranoside and octyl-β-D-glucopyranoside were found to be the most effective ones. As illustrated in Fig. 4, the majority of the NaPi-IIb protein contained in infected cells or purified total membranes was recovered from high-speed supernatants. Also, after solubilization, for reasons not yet known, the amount of the dimeric form observed at ~200 kDa varied from experiment to experiment.

Based on the above described results, total membranes isolated from cells infected with 6His NaPi-IIb were solubilized with 1% n-decyl-β-D-maltopyranoside or 1% octyl-β-D-glucopyranoside and the resulting supernatant was subjected to Ni-NTA chromatography (Fig. 5A). As illustrated, part of the applied 6His NaPi-IIb was recovered from the flow-through. After extensive washing (no detectable western blot signal) 6His NaPi-IIb could be eluted by imidazole. Figure 5B illustrates western blot analysis and protein staining of the eluted material, which was obtained by a batch elution. These parallel stainings indicated that the western-blot signals for NaPi-IIb corresponded to a prominent band of ~90 kDa and a faint band around

200 kDa stained by Coomassie Blue. Besides, several additional bands were observed, likely originating from Sf9 cell proteins, which unspecifically bind to Ni-NTA beads such as the band observed at ~66 kDa.

Discussion

As is the case for the majority of membrane proteins, the type IIb Na/P$_i$-cotransporter is present in native tissues at very low concentrations. In this study, we have used the Sf9-baculovirus system to express the NaPi-IIb protein with the goal to obtain purified NaPi-IIb in larger quantities. Expression of NaPi-IIb in Sf9 cells was monitored by western blots and by Na/P$_i$-cotransport determinations. In addition, initial attempts demonstrated that it is feasible to purify the NaPi-IIb cotransporter by Ni-NTA chromatography after solubilization with nonionic detergent.

On western blots, NaPi-IIb expressed in Sf9 cells appeared as a band of ~90 kDa. In contrast, in small intestinal brush-border membranes of adult mice, NaPi-IIb was observed at 108 kDa (Hilfiker et al., 1998).

This observation suggested that in Sf9 cells NaPi-IIb is glycosylated only partially or not at all. Evidence that the ~90 kDa band indeed represented a partially glycosylated NaPi-IIb was obtained by an in vitro translation of NaPi-IIb cRNA in the presence of microsomes. In the absence of microsomes, ungycosylated NaPi-IIb appeared as a band of ~75 kDa, which was in agreement with the observation obtained after the treatment of mouse small intestinal brush-border membranes with F-glucosidase (Arima et al., 2002). Interestingly, in brush-border membranes of small intestine of mice during the transition of suckling to weaning, NaPi-IIb was reported as being par-

tially glycosylated and appearing at ~90 kDa (Arima et al., 2002). If such incomplete N-glycosylation of the six potential N-glycosylation sites of the NaPi-IIb cotransporter as observed in Sf9 cells or during the suckling/weaning transition in mice, affects Na/P$_i$-cotransport or eventually the apical sorting, cannot be answered definitively. Since the basic characteristics of NaPi-IIb-mediated Na/P$_i$-cotransport in Sf9 were comparable to the ones reported after expression of NaPi-IIb in *Xenopus laevis* oocytes (Hilfiker et al., 1998), we conclude that the degree of N-glycosylation of NaPi-IIb does not affect the phenomenological parameters such as the apparent K_m-values for sodium and phosphate ions nor the pH dependence. Similar observations have been made with the type IIa Na/P$_i$-cotransporter after expression in Sf9 cells (Fucentese et al., 1995) or after having removed the N-glycosylation sites by site directed mutageneisis and expression in *Xenopus laevis* oocytes (Hayes et al., 1994).

With *n*-decyl-β-D-maltopyranoside or octyl-β-D-glucopyranoside up to 90% of the total amount of NaPi-IIb contained in whole cells or isolated membranes could be solubilized. Interestingly, we were not able to solubilize the type IIa Na/P$_i$-cotransporter after expression in Sf9 cells although NaPi-IIa and NaPi-IIb are 51% homologous and show almost identical hydropathy plots within the transmembrane regions (Fucentese et al., 1997; Forster et al., 2002). Several reasons may explain the different solubilization behavior of NaPi-IIa and NaPi-IIb: a) Since expression in Sf9 cells resulted in a partially glycosylated NaPi-IIb protein but unglycosylated NaPi-IIa (Fucentese et al., 1995), it cannot be excluded that N-glycosylation may influence the solubilization behavior; b) The biggest sequence differences between NaPi-IIa and NaPi-IIb are found within the hydrophilic NH$_2$- and COOH-termini (Hilfiker et al., 1998). It is, however, not known if these termini are of importance for the formation of mixed micelles.

In conclusion, we have expressed and characterized NaPi-IIb in Sf9 cells. In contrast to the cotransporter NaPi-IIa (Fucentese et al., 1997), we have demonstrated that NaPi-IIb can be solubilized and partially purified by Ni-NTA affinity chromatography. Although after Ni-NTA chromatography, NaPi-IIb was still partially contaminated by other proteins, our data represent first steps aiming to obtain purified NaPi-IIb protein in amounts necessary for future structural studies.

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