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Functional Expression and Purification of Histidine-Tagged Rat Renal Na/Phosphate (NaPi-2) and Na/Sulfate (NaSi-1) Cotransporters

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Abstract. Two mammalian sodium-dependent anion-cotransporters (NaPi-2 for phosphate and NaSi-1 for sulfate) have been expressed in Sf9 insect cells using the baculovirus expression system. A histidine tag was introduced at the C-termini in order to facilitate purification by metal-affinity chromatography. Sf9 cells infected with the histidine-tagged Ni/P_i-cotransporter exhibited more than 60-fold higher sodium-dependent transport of phosphate compared to noninfected cells. Expressed Na/P_i-cotransport exhibited a K_m of P_i of 0.21 mM and an apparent K_m of sodium of 92 mM. Infected cells expressed a 65 kDa polypeptide as detected by Western blotting and immunoprecipitation. Sf9 cells infected with the histidine-tagged NaSi-1 or untagged NaSi-1 protein expressed sodium-dependent sulfate cotransport up to 60-fold higher compared to noninfected cells. Transport of sulfate was highly dependent on sodium exhibiting a K_m of SO_4^{2-} of about 0.3–0.4 mM and a K_m of sodium of 55 mM. By Western blotting and immunoprecipitation expressed NaS_i-1 proteins were detected at 55–60 kDa. These studies demonstrate that histidine tagged proximal tubular Na-dependent cotransporters for phosphate and sulfate can be expressed functionally in Sf9 cells and that the kinetic characteristics were not altered by the introduction of a histidine tag at the C-termini. Furthermore, it is demonstrated that after solubilization under denaturing conditions histidine-tagged cotransporter proteins can be purified by metal-chelate affinity chromatography.

Key words: Renal Na/P_i-cotransport — Renal Na/sulfate-cotransport — Sf9 cells — Purification — Ni^{2+}-chelate chromatography

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Introduction

Reabsorption of phosphate (P_i) and sulfate (SO_4^{2-}) in proximal tubules utilizes Na/phosphate and Na/sulfate cotransporters localized in the brush border membrane [16, 17, 18]. Such cotransporters have been identified recently by expression cloning using oocytes of *Xenopus laevis;* e.g., from rat kidney cortex the type II Na/P_i-cotransporter NaPi-2 [14] and the Na/sulfate-cotransporter NaS_i-1 [15]. Injection of NaPi-2 or NaSi-1 cRNA into *Xenopus laevis* oocytes leads to expression of Na-dependent transport of phosphate or sulfate respectively, with characteristics similar to those observed in proximal tubular brush border membrane vesicles [for NaPi-2 *see* refs. 6, 14, 16; for NaSi-1 *see* refs. 13, 15, 18]. In further studies we demonstrated that NaPi-2 and NaSi-1 mRNAs are predominantly expressed in the proximal tubules and that the corresponding proteins are localized in the apical membrane of proximal tubular cells [2, 12]. These data documented that the cloned cotransporters, NaPi-2 and NaSi-1 represent apically located Na-dependent phosphate and sulfate transport systems of proximal tubules. Furthermore, evidence was obtained that the type II Na/P_i-cotransporter represents a major target for the physiological and pathophysiological regulation of proximal tubular P_i-reabsorption [7, 11, 17, 20].

The two cotransporters NaPi-2 and NaSi-1 have been expressed in Sf9 insect cells using recombinant baculoviruses. To facilitate efficient purification by metal affinity chromatography a histidine tag has been introduced at the C-termini. In this paper we describe that introduction of a histidine tag at the C-termini of the cotransporters NaPi-2 and NaSi-1 does not alter the transport characteristics for phosphate and sulfate respectively and that histidine-tagged NaPi-2 and NaSi-1

cotransporters after expression in Sf9 cells can be purified by metal affinity chromatography.

Materials and Methods

Plasmid Construction

From the plasmid pSport 1 containing NaPi-2 cDNA [14] a fragment containing the last 240 bp at the 3′-end of the NaPi-2 coding region, a nucleotide sequence coding for six histidines and a stopcodon was obtained by PCR. This fragment was constructed such that it contained a *Hpa*I restriction site at the 5′-end and a *Bam*HI restriction site at the 3′-end. Together with the remaining coding region of the NaPi-2 cDNA (1720 bp) which was isolated by digestion with *Sal*I and *Hpa*I the PCR fragment was ligated into the vector pSport 1 (Gibco, BRL).

A similar procedure was applied to construct histidine-tagged NaSi-1. From the plasmid pSport 1 containing NaSi-1 cDNA [15] a fragment was obtained by PCR containing the last 160 bp at the 3′-end of the NaSi-1 coding region, a nucleotide sequence coding for 6 histidine residues, a stop codon and in addition a *Nde*I site at the 5′-end and a *Bam*HI site at the 3′-end. The rest of the coding region of the NaSi-1 cDNA (1640 bp) was isolated by digestion using *Sal*I and *Nde*I and ligated together with the PCR fragment into pSport 1. From the pSport 1 vectors histidine-tagged cDNA sequences were cut out (*Eco*RI and *Bam*HI for NaPi-2; *Pst*I and *Bam*HI for NaSi-1) and ligated into the baculovirus expression vector pVL 1392 (Pharmingen, San Diego, CA). To obtain a construct of untagged NaSi-1, NaSi-1 cDNA as contained in pSport 1 [15] was isolated by digestion with *Sa*/I and *Not*I and ligated into the baculovirus expression vector pAcSG-His NT C (Pharmingen).

Clones containing either NaPi-2 or NaSi-1 inserts in the proper orientation relative to the polyhedrin promotor [19] were identified by dideoxy-sequencing. Plasmids used for transfections were purified with Wizard Minipreps (Promega).

Production of Recombinant Baculoviruses

Sf9 cells (derived from *Spodoptera frugiperda*) were cultured in Grace's Medium (Gibco, BRL) supplemented with 10% fetal bovine serum (Flow Laboratories) and 50 μg/ml gentamycin at 27°C.

To generate recombinant baculoviruses, Sf9 cells (2×10^6 cells per 60 mm culture dish) were transfected according to the manufacturers instructions with 3 μg of plasmid containing 6His/NaPi-2, 6His/NaSi-1 or untagged NaSi-1 together with 0.5 μg of a modified Baculovirus (*Autographica californica*) viral DNA (BaculoGold, Pharmingen, San Diego, CA) which contained a lethal deletion that is replaced by the DNA of the transplacement plasmid. To clone recombinant baculoviruses Sf9 cells were infected with serial dilutions of viruses. After 3 days cell lysates were screened for recombinant viruses by DNA hybridization as described [19]. Recombinant viruses were amplified 2 times.

Uptake Measurements

Approximately 5×10^5 Sf9 cells/35 mm dish (grown to log-phase) were infected with recombinant viruses at a MOI of 0.1 to 1. After 1 hr of incubation the medium (0.5 ml) was replaced with 2 ml of fresh virus-free medium. After 5 days, infected and noninfected cells were analyzed for sodium-dependent transport of phosphate or sulfate as described [1]. Cells were washed once with substrate-free uptake medium and incubated at room temperature with 1 ml of uptake medium containing 0.1 mM P_i and 1 μCi/ml [^{32}P]K_2HPO_4 (NEN 053) or 0.1 mM K_2SO_4 and 1 μCi/ml [^{35}S]Na_2SO_4 (NEN 041H). After 5 min the uptake medium was removed and cells were washed four times with ice-cold stop solution and solubilized with 0.5 ml of 1% (w/v) Triton X-100. Aliquots were used to determine the radioactivity by liquid scintillation counting and to determine total protein content using the BioRad Dye binding assay and γ-globulin as standard. To determine sodium-independent transport of phosphate or sulfate, sodium was replaced by an equimolar amount of N-methyl-glucamine. The apparent K_m of P_i was determined at extracellular P_i-concentration between 0.05 mM and 1 mM and the apparent K_m of SO_4^{2-} was determined at SO_4^{2-}-concentration between 0.25 mM and 4 mM. To determine the K_m for Na^+, extracellular Na^+-concentration was varied between 0 mM and 137 mM. All transport studies were performed at pH 7.4 and 25°C.

SDS-Polyacrylamide Gel Electrophoresis and Western Blotting

Polyacrylamide gels were prepared essentially as described [10] using 9% running gels. Proteins were loaded in 2×-loading buffer (0.19 M Tris-HCl, 2 mM EDTA, 4% SDS, 20% glycerol and bromphenol blue; pH 6.8). Immunoprecipitated proteins were additionally boiled in the presence of 100 mM DTT. Molecular weight standards (BioRad) were run in parallel.

For immunoblotting the proteins were transferred to nitocellulose (Schleicher & Schuell, BA 83) according to [21]. The membranes were blocked with 150 mM NaCl, 20 mM Tris-HCl (TBS; pH 7.3) containing (5% w/v) low fat milk and 1% Triton X-100 (w/v) (Blotto) for 2 hr and then incubated overnight with a rabbit anti(NaPi-2) antiserum [2] or a rabbit anti(NaSi-1) antiserum [12] or with a monoclonal mouse anti(his-tag) antibody (Dianova). To visualize primary antibody binding, donkey anti-rabbit IgG (for the anti(NaPi-2) or anti(NaSi-1) antiserum) or sheep anti-mouse IgG (for the anti(his-tag) antibody) horseradish peroxidase conjugates (Amersham, GB) were added for an additional 2 hr in Blotto. Finally, blots were washed three times with TBS and the bands were visualized by an ECL detection system (Amersham) using Kodak X-OMAT AR films. Quantification was performed with the ImageQuant software package (Molecular Dynamics).

Purification of Tagged Proteins

7×10^7 Sf9 cells grown in 150-mm culture dishes were infected either with wild-type baculovirus or with recombinant baculovirus containing 6His/NaPi-2 or 6His/NaSi-1 sequences. After 4 days infected Sf9 cells were harvested by centrifugation at $150 \times g_{av}$ for 8 min and resuspended in 30-ml ice-cold buffer containing in mM: 20 Hepes, 150 NaCl, 5 EDTA, 2 DTT (pH 8.0) and in addition the protease inhibitors p-methylsulfonylfluoride (1) and TPCK (0.1). Cells were lysed by nitrogen cavitation after an equilibration for 30 min at 600 p.s.i. Nuclei and cell debris were removed by centrifugation at $500 \times g_{av}$ for 10 min. Membranes were then harvested by centrifugation at $70{,}000 \times g_{av}$ for 30 min, resuspended in 200 mM sucrose, 20 mM HEPES (pH 8.0; buffer A) plus the protease inhibitors to a concentration of 5 mg/ml and either used immediately or stored at −70°C.

The following steps were performed at 4°C: Membrane preparations (2 mg in 400 μl) were centrifuged for 20 min at $100{,}000 \times g_{av}$, resuspended in 7.5 ml of 0.1 M Na_2CO_3, 0.1 mM DTT, 0.1 mM EDTA (pH 11.5) and incubated for 30 min on ice. After centrifugation for 1 hr at $100{,}000 \times g_{av}$ the supernatant was removed and stripped membranes were solubilized with 40 μl solubilization buffer (1% SDS, 10% glycerol, and 10 mM Tris-HCl, pH 7.5) for 5 min at 65°C and cooled

down to roomtemperature. The SDS was diluted to 0.08% by adding 460 μl buffer A containing protease inhibitors and 1% (w/v) Triton X-100. After sonication in a bath sonicator for 20 sec, the insoluble material was removed by centrifugation for 1 hr at 100,000 × g_{av}. The supernatant was mixed 1:1 with 20 mM HEPES, 1 M NaCl, 2 mM imidazole (pH 8.0) and incubated over night at 4°C with Ni^{2+} charged His Bind resin (Novagen) by end-over-end rotation at 4°C. The resin was subsequently packed onto a column and washed with 15 bed volumes (15 ml) of 20 mM Hepes, 0.5 M NaCl, 0.5% Triton X-100, 40 mM imidazole (pH 8.0; buffer B). Bound proteins were eluted with 4 bed volumes of buffer B containing in addition 110 mM imidazole (150 mM final). Each fraction was analyzed by Western blotting or (after concentration using Millipore ultrafree-MC centrifugal filters) for total protein content using PRO-1 (Promega) according to the manufacturer's instructions. Fluorescence was recorded and quantified using a fluorimager (Molecular Dynamics).

Metabolic Labeling and Immunoprecipitation

Sf9 cells were seeded at a density of 5×10^5 cells/well in 24 well plates and infected with either wild-type or recombinant baculovirus containing 6His/NaPi-2 or 6His/NaSi-1 sequences. At day 4 after infection, the cells were starved for 30 min with 500 μl of methionine- and glutamine-free minimum essential medium (MEM, Gibco BRL, pH adjusted to 6.2) in a 5% CO_2 atmosphere at 27°C. The medium was carefully removed and 200 μl MEM supplemented with 20 μCi [^{35}S]-methionine (NEN 009T) were added for 4 hr. Afterwards, the cells were rinsed twice with PBS (pH 6.2) and harvested in 50 μl of 2% SDS, 50 mM Tris-HCl (pH 7.4) containing the protease inhibitors p-methylsulfonylfluoride (PMSF, 1 mM) and TPCK (0.1 mM) and heated for 5 min at 65°C. Samples were diluted with 11 volumes of TENT buffer (1% Triton X-100, 5 mM EDTA, 150 mM NaCl, 50 mM Tris-HCl, pH 7.4, PMSF and TPCK). Anti(NaPi-2) and anti(NaSi-1) antisera were added at dilutions of 1:100 and 1:60 respectively. After an overnight incubation at 4°C, 2.5 μl of protein A-sepharose (1:1 slurry of beads preswollen in TENT buffer) were added for every μl of primary antibody. Incubation was continued for 2 hr and the antibody-protein A sepharose complexes were collected by centrifugation. Finally, the beads were washed twice in TENT buffer and twice in SET buffer (0.1% SDS, 2 mM EDTA, 10 mM Tris-HCl, pH 7.4). Immunoprecipitated proteins were eluted by boiling for 3 min in 2×-loading buffer containing 100 mM DTT and analyzed by Western blotting with the anti(his-tag) antibody and by autoradiography.

Abbreviations

cDNA, complementary DNA; CHAPS, 3-[(3-cholamidopropyl)-dimethylammonium]-1-propane-sulfonate; cRNA, complementary RNA; DTT, 1,4-dithio-DL-threitol; EDTA, ethylenediamine-tetraacetic acid; kb, kilobase; MOI, multiplicity of infection; OK-cells, opossum kidney cells; P_i, inorganic phosphate; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; TBS, tris-buffered saline; TPCK, tosyl-L-phenylalanine chloromethyl ketone; Tris, tris(hydroxymethyl)-aminomethane.

Results

Expression of 6His/NaPi-2, 6His/NaSi-1 and Untagged NaSi-1 Cotransporters in Sf9 Cells

The cDNA's coding for the rat renal type II Na/P_i-cotransporter, NaPi-2 [14], and the Na/sulfate-cotransporter, NaSi-1 [15], were cloned into the transfer vectors pVL 1392 or pAcSG-His NT C to express these cotransporters in Sf9 insect cells. A histidine tag was introduced at the C-terminis to facilitate purification by metal-affinity chromatography [5]. The plasmid constructs were cotransfected with a modified baculovirus DNA containing a lethal deletion which is restored after homologous recombination with the transplacement plasmid. Recombinant viruses were identified by Southern blotting (*data not shown*) and amplified two times. Noninfected cells or cells infected with wild-type viruses were used as controls and showed no reaction. Routinely, Sf9 cells were infected with viruses at a MOI of 0.1–1 and harvested 4–5 days after infection.

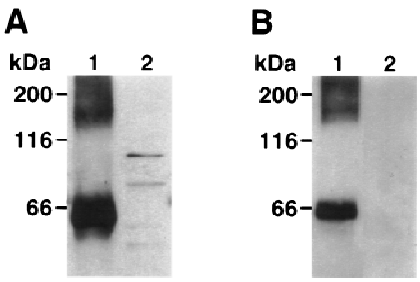

Fig. 1. Western blot analysis of membrane preparations derived from Sf9 cells expressing 6His/NaPi-2. Membranes of infected cells were prepared 4 days after infection, separated by SDS-PAGE and analyzed by Western blotting. (*A*) The Western blot was performed using anti(NaPi-2) antiserum raised against a synthetic N-terminal peptide [2]. Lane 1 contained membrane preparation of cells infected with 6His/NaPi-2 and lane 2 contained membranes prepared from cells infected with wild-type virus. (*B*) Western blot with anti(his-tag) antibody. Lane 1 contained membrane preparation of cells infected with 6His/NaPi-2. As a control lane 2 contained membranes prepared from cells infected with wild-type virus.

Expression of the cotransporters in Sf9 cells was first analyzed by immunoblotting. In cells infected with 6His/NaPi-2 recombinant baculoviruses a protein of approximately 65 kDa was detected with the anti(NaPi-2) antiserum (Fig. 1*A*) which was in agreement with the molecular mass of the untagged NaPi-2 cotransporter after expression in Sf9 cells [4]. No specific immunoreaction was observed using membranes isolated from cells infected with wild-type virus (Fig. 1*A*, lane 2). In parallel, immunoblots were performed with the anti(his-tag) antibody (Fig. 1*B*). As illustrated, the immunoreactive protein pattern of membranes isolated from Sf9 cells infected with 6His/NaPi-2 was identical to the pattern as observed with the anti(NaPi-2) antiserum. No reaction of the anti(his-tag)antibody was detected with membranes isolated from cells infected with wild-type virus (Fig. 1*B*, lane 2).

Expression of the tagged and untagged NaSi-1 protein was monitored using a polyclonal anti(NaSi-1) antiserum [12]. In crude membranes isolated from Sf9 cells infected with either the histidine-tagged or the un-

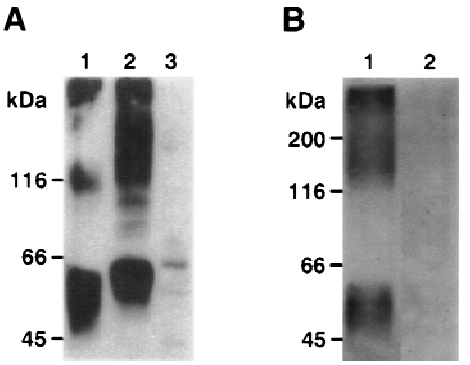

Fig. 2. Western blot analysis of membrane preparations from Sf9 cells expressing 6His/NaSi-1 or untagged NaSi-1 cotransporter. Membranes of infected cells were prepared 4 days after infection, separated by SDS-PAGE and analyzed by Western blotting. (*A*) The Western blot was performed using the anti(NaSi-1) antiserum [12]. Membranes were prepared from cells infected with 6His/NaSi-1 (Lane 1) or untagged NaSi-1 (Lane 2). Lane 3 contained a membrane preparation obtained from cells infected with wild-type virus. (*B*) Western blot performed with the anti(6his) antibody. Membrane preparations were obtained from Sf9 cells infected with 6His/NaSi-1 (Lane 1) or with the untagged NaSi-1 protein (Lane 2).

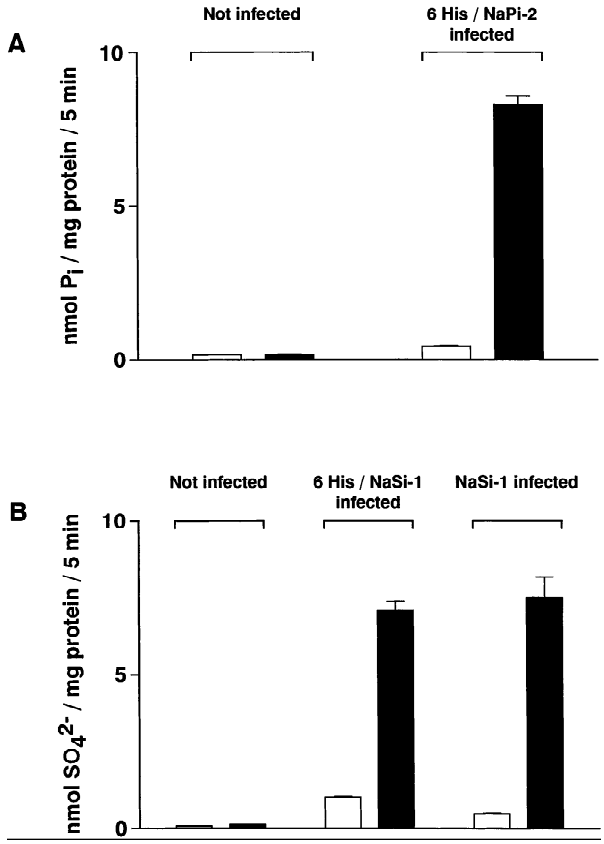

Fig. 3. Na^+-dependent transport of phosphate or sulfate in infected and in noninfected Sf9 cells. Transport was determined 5 days after infection at room temperature for 5 min either in the presence of sodium (filled bars) or in the absence of sodium (empty bars). Data are given as the mean ± SD of four individual dishes obtained from one single infection. (*A*) Uptake of inorganic phosphate by noninfected Sf9 cells and Sf9 cells infected with 6His/NaPi-2. (*B*) Sulfate-uptake by noninfected cells and cells infected with untagged NaSi-1 or 6His/NaSi-1.

tagged NaSi-1 protein, a band of 55–60 kDa was detected (Fig. 2*A*) which was in good agreement with the size of the product obtained after in vitro translation of NaSi-1 cRNA [15] and with the size of the NaSi-1 protein as contained in rat proximal tubular brush border membranes [12]. No corresponding band was observed in lysates of Sf9 cells infected with wild-type virus (Fig. 2*A*). The presence of the histidine tag was confirmed using an anti(his-tag) antibody by which the NaSi-1 histidine-tagged protein but not the untagged NaSi-1 protein was detected (Fig. 2*B*).

In contrast to the appearance of the NaPi-2 and NaSi-1 proteins on Western blots performed with isolated renal brush border membranes [2, 12], besides the immunoreactive bands representing the monomeric proteins higher order aggregates of the expressed cotransporters were observed with the specific antisera as well as with the anti(his-tag)antibody (Figs. 1 and 2). Various attempts to resolve these aggregates such as by various heat treatments or by using reducing agents failed (*data not shown*).

To determine if the cotransporters, 6His/NaPi-2, 6His/NaSi-1 and untagged NaSi-1, when expressed in Sf9 cells are functionally active and exhibit Na-dependent transport of either phosphate or sulfate, uptake experiments were performed. As illustrated in Fig. 3*A* Sf9 cells expressing the 6His/NaPi-2 protein exhibited a significant Na-dependent P_i-transport which was not observed in noninfected cells. The rate of 6His/NaPi-2 associated phosphate-transport was linear over the first 10 min of incubation (*data not shown*) similar to what has been demonstrated with Sf9 cells expressing the untagged NaPi-2 cotransporter [4]. The kinetic parameters of 6His/NaPi-2 associated Na/P_i-cotransport (listed in the Table) were found to be in good agreement with the transport data obtained with Sf9 cells infected with the untagged NaPi-2 cotransporter [4], using *Xenopus laevis* oocytes injected with NaPi-2 cRNA [14] or using brush border membranes isolated from rat kidney cortex [6, 16].

Sf9 cells expressing the histidine-tagged or the untagged Na/sulfate-cotransporter exhibited approximately 50-fold higher Na-dependent sulfate-transport compared to noninfected cells (Fig. 3*B*). In both cases, linear uptake of sulfate was observed over the first 10 min of incubation (*data not shown*). To further characterize Na-dependent transport of sulfate of infected Sf9 cells the K_m-values for sulfate and sodium were determined (listed in the Table). The results obtained were in good agreement with the kinetic parameters obtained by transport studies performed with *Xenopus laevis* oocytes injected

Table 1. Characterization of Na/P_i- and Na/sulfate cotransports associated to 6His/NaPi-2, 6His/NaSi-1 and NaSi-2 proteins expressed in Sf9 cells

Parameter	6His/NaPi-2	NaPi-2*	6His/NaSi-1	NaSi-1
K_m (P_i)	0.21 ± 0.02 mM	0.114 mM		
K_m (SO_4^{2-})			0.29 ± 0.03 mM	0.45 ± 0.07 mM
K_m (Na^+)	92 ± 15.5 mM	63 ± 8 mM	60.8 ± 11.8 mM	52.5 ± 8.2 mM
Hill-coefficient	2.5 ± 0.44	3 ± 0.9	1.66 ± 0.28	2.3 ± 0.65

Transport experiments were performed 5 days after infection at room. The apparent K_m values were calculated by curve fitting according to the Michaelis-Menten or the Hill equation respectively. The data shown represent the means ± SD of four individual dishes obtained from one single infection. Each experiment has been performed twice. *Values are taken from ref. 4.

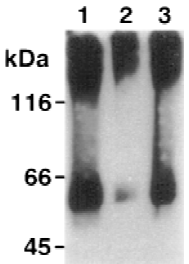

Fig. 4. Solubilization of 6His/NaPi-2. After solubilization, equal volumes of the pellet (Lane 2) and of the supernatant (Lane 3) were analyzed using the anti(NaPi-2) antiserum by immunoblotting. Densitometric analysis showed, that the supernatant contained about twofold more of the monomeric and aggregated form of the NaPi-2 protein compared to the pellet. Immunoreactivity of isolated membranes before the solubilization step is shown in lane 1.

with NaSi-1 cRNA [15] or with rat renal proximal tubular brush border membrane vesicles [13, 18].

As evident from the results shown in Fig. 3, Sf9 cells expressing the cotransporters NaPi-2 or NaSi-1 exhibited slightly increased sodium-independent transports of phosphate or sulfate when compared to noninfected cells. This slight increase of Na-independent solute transport may be explained by a small amount of residual sodium due to an incomplete washout or by a small Na-independent transport component of these cotransporters. The latter possibility however seems rather unlikely because negligible transport of these solutes was detected in the absence of sodium in studies using oocytes injected with corresponding cRNAs [14, 15].

Solubilization and Purification of 6His/NaPi-2 and 6His/NaSi-1 Cotransporters

Crude membrane preparations obtained from infected Sf9 cells were used as starting material to purify the 6His/NaPi-2 and 6His/NaSi-1 cotransporters. The membrane preparations were first extracted at high pH using Na_2CO_3. By this treatment the total protein content was reduced by more than 50%, whereas 60–80% of the tagged cotransporters were retained in the membrane fractions (*data not shown*). Initially, solubilization of the cotransporters was performed with nondenaturing detergents such as CHAPS, Triton X-100, octylglucoside or dodecylmaltoside. When used under different conditions (concentration, ionic strength) the yield of solubilization obtained with the nonionic detergents was never more than 10% (*data not shown*). Therefore, the ionic detergent SDS was used by which more than 60% of the histidine tagged cotransporters (NaPi-2, Fig. 4) and NaSi-1 (*data not shown*) were solubilized. Interestingly, the ratios of the monomeric and the aggregated forms as described in Figs. 1 and 2 of expressed 6His/NaPi-2 and 6His/NaSi-1 proteins were similar after a solubilization with 1% SDS (Fig. 4).

Solubilized proteins were incubated with Ni^{2+}-charged His Bind overnight to achieve optimal binding. A variety of washing and elution conditions were tested to optimize the removal of as much as possible of contaminating cellular proteins without diminishing the yield of the tagged cotransporters (*data not shown*). The purification protocol finally applied consisted in a wash step using 40 mM imidazole followed by elution of the bound proteins with 150 mM imidazole. The Western blots shown in Fig. 5*A* and *C* indicate that both histidine-tagged proteins were bound to the Ni^{2+}-charged His Bind resin since only small amounts were detected in the flowthrough fractions (Fig. 5*A* and *C;* lanes 1). During the wash step using 40 mM imidazole only small amounts of bound proteins were initially eluted. The last wash fractions were virtually void of cotransporter proteins and did not contain significant amounts of protein. Subsequent elution with 150 mM imidazole resulted in a complete recovery of bound proteins since by stripping of the columns with 0.1 M EDTA no further elution of the histidine-tagged proteins was detected. After elution with 150 mM imidazole, total protein staining revealed the presence of a band corresponding to the position of the 6His/NaPi-2 protein as detected on Western blots. In addition a band of 30 kDa was observed in these frac-

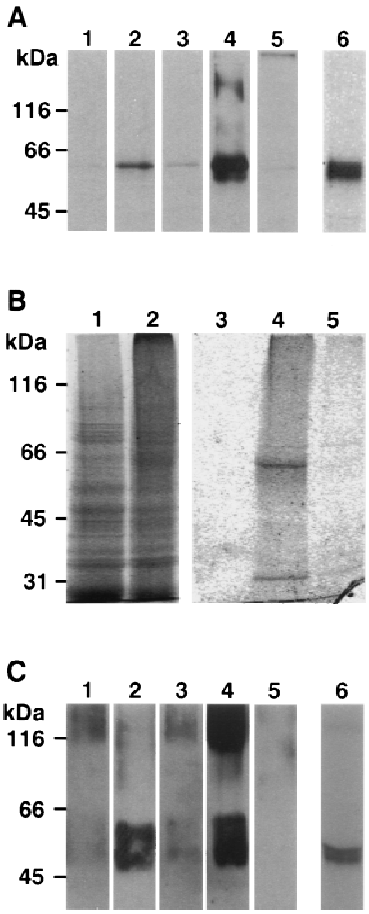

Fig. 5. Purification and immunoprecipitation of 6His/NaPi-2 and 6His/NaSi-1 cotransporters. Aliquots of different fractions obtained by Ni-chelate chromatography were analyzed by immunoblotting; the purification profile for NaPi-2 is shown in panel *A* and the one for NaSi-1 in panel *C*. For total protein staining one third of each fraction (NaPi-2 purification) was concentrated and analyzed for total protein content (*B*). Immunoprecipitations (Lanes 6 in *A* and *C*) were performed as described in Materials and Methods. Lanes 1: flowthroughs; lanes 2 and 3: first and last wash fractions respectively; lanes 4; fractions obtained by elution with 150 mM imidazole; lanes 5: fractions obtained after stripping the column with 0.1 M EDTA. Lanes 6 represent autoradiographs of immunoprecipitated 6His/NaPi-2 (*A*) and 6His/NaSi-1 proteins.

tions. Since this band was also observed after a control chromatography using solubilized membranes isolated from noninfected cells it is concluded that the 30 kDa band is not related to the expressed cotransporter but represents a Sf9 cell protein interacting specifically or nonspecifically with the matrix used. It is of note that in the fractions containing purified cotransporters still both the monomeric and aggregated forms of the 6His/NaPi-2 and 6His/NaSi-1 proteins were observed. Using bovine serum albumin as a standard it was estimated that the total amount of purified 6His/NaPi-2 cotransporter as shown in Fig. 5*B* (lane 4) ranged between 100 and 200 nanogramms.

To test if the 6His-tagged proteins did not undergo substantial degradation during the purification procedure expressed histidine-tagged cotransporters were metabolically labeled and immunoprecipitated from total cell lysates. As illustrated in Fig. 5*A* and *C* (lanes 6) immunoprecipitated histidine-tagged cotransporters exhibited the same molecular mass as after the whole purification procedure indicating that during the purification steps no significant degradation occurred.

Discussion

In a previous study we have shown that the baculovirus system can be used to express the rat renal type II Na/Pi-cotransporter NaPi-2 [4]. In the present study we have extended this observation by showing that the Na/sulfate cotransporter (NaSi-1) can be expressed functionally in Sf9 cells as well. Furthermore, we have tagged these two cotransporters (NaPi-2 and NaSi-1) at the C-termini with a histidine tag to establish procedures for a purification of these proteins by metal chelate chromatography.

Expression of the cotransporters was monitored by immunoblots using specific antibodies and additionally by using an anti(his-tag)-antibody. Similar to the untagged Na/Pi-cotransporter NaPi-2 [4], the 6His/NaPi-2 protein was expressed as a 65 kDa protein. The discrepancy between the molecular mass of the expressed NaPi-2 protein and the one observed in isolated renal brush border membranes (approx. 85 kDa, ref. 2) is explained by the lack of or by an incomplete glycosylation [3, 4]. The Na/sulfate cotransporter, NaSi-1 (both histidine-tagged and untagged), was detected with the anti(NaSi-1) antiserum as a band of approximately 55–60 kDa. This size is in good agreement with the size of the native NaSi-1 protein expressed in proximal tubular apical membranes [12] and with the molecular mass of the NaSi-1 cotransporter observed after in vitro translation [15]. Since thus far no evidence for a significant glycosylation of the NaSi-1 protein has been obtained, no conclusions about the state of glycosylation of the NaSi-1 cotransporter in Sf9 cells can be made at present. The presence of the histidine tags was confirmed by immunoblots using an anti(his-tag)antibody by which the same protein patterns of expressed cotransporters were observed as with the specific antisera. Interestingly, and in contrast to the native cotransporters as expressed in proximal tubular apical membranes [2, 12], the two cotransporter proteins expressed in Sf9 cells appeared also as aggregates that could not be resolved and that were retained after solubilization with SDS and metal affinity chromatography. It is assumed that these aggregates may be due to the high amounts of expressed cotransporters which may not have been folded properly and therefore may not have been inserted into the plasma-

membrane. Incomplete insertion of expressed proteins into the plasmamembrane of Sf9 cells has also been reported for the shaker K-channel [9] or the gastric K/H-ATP'ase [8] and for the Na/Pi-cotransporter NaPi-2 [4].

Functional integrity of expressed histidine-tagged cotransporters was analyzed by transport experiments. Based on kinetic parameters (apparent K_ms for the solutes phosphate, sulfate and sodium and Hill coefficients) it is concluded that the histidine tags introduced at the C-termini of the NaPi-2 and the NaSi-1 proteins did not alter the basic functional characteristics of tagged cotransporters when compared to the untagged (Table and Ref. 4). In addition, a comparison of the rates of phosphate and sulfate transport of Sf9 cells expressing the untagged cotransporters with Sf9 cells expressing the tagged cotransporters suggests that the histidine-tagged cotransporters were inserted equally well into the plasmamembrane as the untagged proteins.

Various nonionic detergents were tested to solubilize the expressed histidine-tagged cotransporters. However, none of the tested nonionic detergent yielded in more than approximately 10% solubilization. On the other hand, over 60% of expressed cotransporters could be solubilized by the ionic detergent SDS. At present, it is not known if SDS may denature the cotransporters irreversibly or not and it remains to be shown if the replacement of SDS by the nonionic detergent Triton X-100 during the described purification step will result in native and functional cotransporters. Solubilized histidine-tagged cotransporters were found to be almost completely bound to Ni^{2+}-resin. After an extensive wash step, a fraction containing substantial amounts of immunoreactive cotransporters in the absence of significant amounts of other proteins was obtained by an elution using a high imidazole concentration. Since immunoprecipitated cotransporters exhibited the same molecular mass as the purified ones it seems likely that during the purification steps the cotransport proteins were not substantially degraded. It was estimated that from 10 mg of isolated plasmamembranes of infected Sf9 cells it was possible to purify up to one microgram of cotransporter protein. Therefore, with the purification protocol described, reasonably high amounts of purified cotransporters which would be needed for further studies can be obtained.

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