Identification and localization of sodium-phosphate cotransporters in hepatocytes and cholangiocytes of rat liver

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Submitted 23 June 2004; accepted in final form 18 November 2004

Frei, Pascal, Bo Gao, Bruno Hagenbuch, Alfonso Mate, Jürg Biber, Heini Murer, Peter J. Meier, and Bruno Stieger: Identification and localization of sodium/phosphate cotransporters in hepatocytes and cholangiocytes of rat liver. Am J Physiol Gastrointest Liver Physiol 288: G771–G778, 2005. First published November 24, 2004; doi:10.1152/ajpgi.00272.2004.—Hepatocytes and cholangiocytes release ATP into bile, where it is rapidly degraded into adenosine and Pi. In rat, biliary Pi concentration (0.01 mM) is ~100-fold and 200-fold lower than in hepatocytes and plasma, respectively, indicating active reabsorption of biliary Pi. We aimed to functionally characterize canalicular Pi reabsorption in rat liver and to identify the involved Pi transport system(s). Pi transport was determined in isolated rat canalicular liver plasma membrane (LPM) vesicles using a rapid filtration technique. Identification of putative Pi transporters was performed with RT-PCR from liver mRNA. Phosphate transporter protein expression was confirmed by Western blotting in basolateral and canalicular LPM and by immunofluorescence in intact liver. Transport studies in canalicular LPM vesicles demonstrated sodium-dependent Pi uptake. Initial Pi uptake rates were saturable with increasing Pi concentrations, exhibiting an apparent K_m value of ~11 μM. Pi transport was stimulated by an acidic extravesicular pH and by an intravesicular negative membrane potential. These data are compatible with transport characteristics of sodium-phosphate cotransporters NaPi-IIb, PiT-1, and PiT-2, of which the mRNAs were detected in rat liver. On the protein level, NaPi-IIb was detected at the canalicular membrane of hepatocytes and at the brush-border membrane of cholangiocytes. In contrast, PiT-1 and PiT-2 were detected at the basolateral membrane of hepatocytes. We conclude that NaPi-IIb is most probably involved in the reabsorption of Pi from primary hepatic bile and thus might play an important role in the regulation of biliary Pi concentration.

NaPi; PiT; bile formation; transport

MATERIALS AND METHODS

Chemicals. Radiolabeled orthophosphoric acid (KP) was obtained from New England Nuclear (Boston, MA). All other chemicals were of the highest degree of purification.

Animals. Male Sprague-Dawley rats weighing 200–250 g were obtained from RCC (Füllinsdorf, Switzerland). They were kept under standard conditions and in accordance with the regulations of the veterinary authority of Zurich.

Isolation of canalicular and basolateral rat LPM vesicles. Canalicular LPM (cLPM) and basolateral LPM (bLPM) vesicles were isolated from rat liver as described (28). The vesicles were resuspended for all experiments in 300 mM mannitol and 20 mM HEPES-Tris (pH 7.4) and were stored frozen in liquid nitrogen (protein concentration > 5 mg/ml). Protein was determined by a modification of the method of Lowry (4) using bovine serum albumin as a standard.

Vesicle transport studies. Frozen membrane suspensions were quickly thawed by immersion of the tubes in a 37°C water bath. The thawed membranes were diluted to the desired protein concentration, revesiculated with a tight Dounce homogenizer (type B; 30 up and down strokes), and placed on ice. Uptake of KP into cLPM vesicles was measured by a rapid filtration technique. Uptake studies were routinely done at 25°C by adding 80 μl incubation medium to 20 μl vesicle suspension (3–4 μg protein/μl). Unless stated otherwise, the uptake medium consisted of (in mM) 0.125 KP, 50 mannitol, 20 HEPES-Tris (pH 7.4), and 125 NaCl or KCl. After the indicated time intervals, uptakes were terminated by the addition of 3 ml ice-cold stop solution consisting of (in mM) 100 mannitol, 100 NaCl, and 10 Tris·HCl (pH 7.4). Membrane vesicle-associated Pi was separated from free Pi by immediate rapid filtration through a 0.45-μm nitrocellulose filter (Sartorius, Göttingen, Germany), which had been presoaked in 5 mM KH_2PO_4. After two washes with 3 ml cold stop solution, the filters were dissolved in 5 ml liquid scintillation cocktail (Filter-Count; Canberra-Packard Instruments, Zurich, Switzerland), and the radioactivity was counted in a tri-Carb 2200 CA liquid scintillation counter (Canberra-Packard). Nonspecific binding to filters and membranes was determined in each experiment by adding cold (0–4°C) incubation and stop solutions to 20 μl cold membrane suspension. This membrane/filter blank was subtracted from all determinations.

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RT-PCR. Total rat liver RNA was isolated from male Sprague-Dawley rats according to the method of Chomczynski and Sacchi (13). mRNA was extracted from total RNA using the polyATract mRNA Isolation System from Promega (Madison, WI). A 1.25-μg mRNA sample was retrotranscribed with 15 units AMV reverse transcriptase (Promega) using random primer. PCR was performed with Dynazyme (Finzymes, Espoo, Finland) using the following primers: NaPi-IIb sense/antisense, 5'-GGGATTGGGAATTCATCC/TTCACACAAAGGTGTCTC-3', expected product size 462 bp; PI-1 sense/antisense, 5'-CATCTCGGTGGATGTGC/TGTTGGTC-3', 526 bp; and PI-2 sense/antisense, 5'-GCTTAC-CATTGGCTTTCG/ACAGAGGGAATGGCGGA-3'; 198 bp.

Western blot analysis. The polyclonal rabbit anti-mouse NaPi-IIb antibody has been characterized in detail elsewhere (20). The antigenic peptide close to the NH2 terminus of mouse NaPi-IIb shows 91% sequence identity with the corresponding rat protein sequence. The two members of the NaPi-III family, PI-1 and PI-2, were studied with two specific antibodies. The polyclonal antibody against mouse PI-1 was obtained from Alpha Diagnostics (San Antonio, TX). The rabbit polyclonal serum against the intracellular loop of human PI-2 (anti-PI-2) was a generous gift of S. Kuhnmann, D. Kabat, and J. M. Heard (Oregon Health and Science University, Portland, OR) and was described in Ref. 35. The antigenic peptide for production of the anti-PI-2 antibody consisted of the intracellular loop of human PI-2, which shows 88% identity with the corresponding rat PI-2 sequence. For gel electrophoresis, cLPM and bLPM samples were mixed with loading buffer on ice. Dithiothreitol was added as a reducing agent for PI-1 and PI-2 but not for NaPi-IIb immunoblotting. Gels were run overnight and transferred onto nitrocellulose transfer membranes (Protran; Schleicher & Schuell, Dassel-Reilliehausen, Germany). The latter were blocked with 5% nonfat milk-TBS-0.1% Tween 20 (TBS-T) and were incubated with diluted primary antibodies to NaPi-IIb (1:2,500), PI-1 (1:50), and PI-2 (1:1,000) in 5% nonfat milk-TBS-T. Before and after the addition of primary antibodies to NaPi-IIb (1:2,500), PI-1 (1:500), and PI-2 (1:500) in 5% nonfat milk-TBS-T, overnight at 4°C with the polyclonal rabbit antibodies against NaPi-IIb as well as PI-1 and PI-2 were incubated with diluted secondary antibody (horseradish peroxidase-conjugated goat anti-rabbit IgG; Amersham Biosciences, Oelfingen, Switzerland), membranes were washed three times for 10 min in TBS-T. After the incubation of the secondary antibody, immunodetection was performed by ECL (Amersham). Immunofluorescence staining of NaPi in rat liver. For NaPi-IIb IF, livers were removed, cut into small pieces, and frozen on dry ice. Cryosections (6 μm thick) were cut and fixed for 20 min in 2% (wt/vol) paraformaldehyde. For PI-1 and PI-2 IF, livers were fixed by perfusion for 12 min with cold HBSS containing 3% paraformaldehyde and 0.1% glutaraldehyde and then fixed by immersion in PBS containing 3% paraformaldehyde and 0.1% glutaraldehyde at 4°C up to 2 h. Liver samples were cryoprotected in 30% sucrose overnight, and cryosections were cut. For all transporters, slides were incubated overnight at 4°C with the polyclonal rabbit antibodies against NaPi-IIb (1:400), PI-1 (1:50), and PI-2 (1:250) in PBS containing 2% normal goat serum and 0.2% Triton X-100. The following monoclonal mouse antibodies were used for double-labeling experiments: an antibody against human multidrug resistance protein-2 (Mrp2) (1:100; Alexis Biochemicals, Lausen, Switzerland) to label the canalicular membrane of hepatocytes; an antibody against rat aminopeptidase N (1:500) (32) to label the brush-border membrane of cholangiocytes (27); and the monoclonal antibody 1–18 (36) (1:2,000) to label the basolateral membrane of hepatocytes. Sections were washed in PBS and incubated for 30 min at room temperature with the secondary antibodies Cy3-labeled goat anti-rabbit (1:300) and Cy2-labeled goat anti-mouse (1:100) (Jackson ImmunoResearch, West Grove, PA). Thereafter, sections were washed in PBS, coverslipped (Immumount; Shandon, Pittsburgh, PA), and analyzed by confocal laser microscopy (Leica SL confocal microscope). The specificities of the immunoreaction against NaPi-IIb and PI-1 were verified by incubating sections with the primary antibodies preabsorbed with 20 μg/ml of the corresponding antigen used for immunization.

RESULTS

Functional properties of Pi transport in cLPM vesicles. To delineate the functional properties of the hypothesized canalicular Pi transport system, we investigated the sodium, pH, and potential dependency of Pi uptake into rat liver cLPM vesicles.

An inwardly directed Na+ gradient stimulated 0.1 mM Pi uptake into cLPM vesicles ~40-fold compared with a similarly directed K+ gradient and induced a 9.0 ± 4.6-fold (mean ± SD, 3 independent experiments) transient intravesicular accumulation of Pi (overshoot) compared with the equilibrium values at 60 min (0.67 ± 0.49 nmol/mg protein, mean ± SD, 3 independent experiments) (Fig. 1). This sodium-dependent Pi uptake activity was saturable with increasing substrate concentrations and yielded an apparent Km value of ~11 μM (Fig. 2). Furthermore, sodium-dependent Pi uptake (10 s) into cLPM vesicles was ~80% higher at an extravesicular acidic pH of 6.5 than at an extravesicular alkaline pH of 8.0 (Fig. 3). Finally, sodium-dependent Pi uptake (10 s) into cLPM vesicles was ~50% higher at a transient negative intravesicular K+ diffusion potential than at an inside-positive K+ diffusion potential (Fig. 4). These data are consistent with the presence of a canalicular sodium-coupled Pi cotransport system that exhibits a high affinity for Pi, is stimulated by an extravesicular acidic pH, and has a Na-to-Pi stoichiometry of >1. These functional transport properties are typical for the three inorganic phosphate transporters NaPi-IIb (published Km ~ 0.05 mM), PI-1 (published Km ~ 0.025 mM), and PI-2 (published Km ~ 0.025 mM) (30). To find out which of these three transporters might be involved in canalicular Pi reabsorption, we next analyzed their hepatic expression by RT-PCR, Western blot analysis, and IF.

Identification of putative NaPis in rat liver by RT-PCR. On the mRNA level, NaPi-IIb as well as PI-1 and PI-2 were found to be expressed in rat liver (data not shown).

Western blot analysis of NaPi expression in rat liver. Western blot analysis showed selective expression of NaPi-IIb in cLPM but not in bLPM vesicles (Fig. 5). The polyclonal
NaPi-IIb antibody raised against a peptide close to the NH₂ terminus of mouse NaPi-IIb recognized two protein bands, one at 80 kDa and one at 90 kDa (Fig. 5). Both protein bands completely disappeared with addition of antigenic peptide to the primary antibody (data not shown). The same two protein bands of 80 and 90 kDa were obtained when a polyclonal antibody against a peptide close to the COOH terminus of mouse NaPi-IIb was used (data not shown). In contrast to NaPi-IIb, Western blot analysis showed that both PiT-1 and PiT-2 are predominantly if not selectively expressed in blLPM vesicles; only weak bands were obtained in cLPM vesicles (Fig. 5). Thereby, immunoreactive PiT-1 and PiT-2 exhibited apparent molecular weights of ~85 and ~70 kDa, respectively, which is similar to previous reports (6, 12, 35). To find out whether 1) NaPi-IIb is also apically expressed in cholangiocytes and 2) PiT-1 and PiT-2 are also expressed at the canalicular membrane of rat hepatocytes, we next studied NaPi protein expression by IF in intact rat liver.

**IF analysis of NaPi expression in rat liver.** On sections incubated with the NaPi-IIb antibody, immunoreactivity was observed at the canalicular membrane of rat hepatocytes. The canalicular NaPi-IIb staining colocalized with Mrp2 staining (Fig. 6, A–C) and was absent on sections incubated with the antibody preabsorbed with the peptide used for immunization.

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**Fig. 2.** Saturation kinetics of sodium-dependent Pᵢ uptake in canalicular cLPM vesicles. Separate experiments demonstrated linearity of initial sodium-dependent Pᵢ uptake values up to 6 s at 10 μM Pᵢ (data not shown) and at 1 mM Pᵢ (inset). Uptakes of increasing Pᵢ concentrations were determined at 4 s as described in MATERIALS AND METHODS. Kinetic parameters were fitted to the uptake data using Michaelis-Menten equation with nonlinear regression analysis (Systat; Systat, Evanston, IL). The indicated Kᵢ and Vᵢ values represent the means ± SD of 4 separate experiments. The figure shows a representative uptake experiment of increasing Pᵢ concentrations in the presence (●) and absence (○) of an inwardly directed sodium gradient (100 mM). The fitted saturation curve shows the sodium-dependent portion of Pᵢ uptake (total NaCl-minus KCl-gradient-driven Pᵢ uptakes).

**Fig. 3.** Effect of pH on sodium-dependent Pᵢ transport in cLPM vesicles. Sodium-dependent Pᵢ uptake was determined under standard conditions as described in MATERIALS AND METHODS, with the exception that the pH of the incubation media was varied between 6.5 and 8.0. The pH was adjusted with HEPES-Tris. Data represent means ± SD of triplicate determinations in 1 out of 2 membrane preparations.

**Fig. 4.** Effects of valinomycin-induced K⁺ diffusion potentials on sodium-dependent Pᵢ uptake in cLPM vesicles. cLPM vesicles were diluted 1:1 in loading buffer containing (in mM) 150 mannitol, 20 HEPES-Tris (pH 7.4), and either 50 choline gluconate or K gluconate. The K⁺ ionophore valinomycin was added to the vesicle suspension (1 μg/100 μl diluted vesicles) 5 min before Pᵢ uptakes were started. Valinomycin-treated vesicles (20 μl) were added to 80 μl incubation medium, which consisted of (in mM) 125 Na gluconate, 0.125 KP, 20 HEPES-Tris (pH 6.5 or pH 8.0), and either 50 K gluconate or choline gluconate. Bars represent Pᵢ uptake during 10 s in the presence of transient positive (50 mM K gluconateout/50 mM choline gluconatein; black bars) or negative (50 mM choline gluconateout/50 mM K gluconatein; striped bars) K⁺ diffusion potentials. Data represent means ± SD of 6 determinations in 2 separate membrane preparations. The stimulation of uptake with an in-to-out K⁺ gradient over an out-to-in K⁺ gradient is significant (P < 0.01, Student’s t-test) at pH 6.5 and pH 8.0.

**Fig. 5.** Western blot analysis of phosphate transporter expression in cLPM and basolateral liver plasma membrane (blLPM) vesicles. Samples were processed as described in MATERIALS AND METHODS. Electrobotted proteins were incubated with polyclonal rabbit antibodies against NaPi-IIb, PiT-1, and PiT-2. Protein amounts loaded were as follows: NaPi-IIb, 100 μg/lane; PiT-1, 200 μg/lane; PiT-2, 80 μg/lane. Molecular mass markers are in kilodaltons.
A weak intracellular NaPi-IIb staining in hepatocytes was also present after antibody absorption (Fig. 6D). Additionally, NaPi-IIb expression could be shown at the brush-border membrane of cholangiocytes where NaPi-IIb immunoreactivity colocalized with aminopeptidase N in double-staining experiments. NaPi-IIb could be detected in cholangiocytes of the interlobular bile ducts (Fig. 6, E–G) as well as of the extrahepatic bile ducts (Fig. 6, H–K). In contrast, on sections incubated with PiT-1 and PiT-2 antibody, immunoreactivity was observed at the basolateral membrane of rat hepatocytes (Fig. 7). The basolateral PiT-1 and PiT-2 stainings colocalized with staining for the basolateral marker 1–18. Basolateral hepatocyte membrane staining was not observed after absorption of the PiT-1 antibody with the corresponding antigenic peptide, indicating specific basolateral surface labeling of PiT-1 (Fig. 7G). No colocalization of PiT-1 and PiT-2 immunoreactivities with canalicular aminopeptidase N were observed, further confirming the basolateral surface localization of PiT-1 and PiT-2 in rat hepatocytes (Fig. 8, A and C). Whereas PiT-1 and PiT-2 immunoreactivities also did not overlap with apical aminopeptidase N reactivity, no clear basolateral localization of PiT-1 and PiT-2 could be obtained in cholangiocytes (Fig. 8, B and D). Results from IF analysis are summarized in Fig. 9.

DISCUSSION

In this study, we provide evidence that NaPi-IIb is involved in the reabsorption of Pi from rat bile into hepatocytes and cholangiocytes. We demonstrate the presence of a high-affinity Pi uptake system ($K_m \sim 0.01$ mM) in isolated rat cLPM
vesicles. This canalicular P\textsubscript{i} uptake system exhibits very similar transport characteristics to the sodium-phosphate cotransporters NaPi-IIb, PiT-1, and PiT-2 (30). However, only NaPi-IIb protein could be localized to the canalicular membrane of hepatocytes and to the apical membrane of cholangiocytes, whereas both PiT-1 and PiT-2 were found to be expressed at the basolateral plasma membrane of hepatocytes. Other NaPis that have so far been characterized cannot account for the observed canalicular Pi uptake, because NaPi-I is a pH-independent, low-affinity Pi transporter (\(K_m \sim 1.0 \text{ mM}\)) that is expressed at the basolateral plasma membrane of mouse hepatocytes (46), and the high-affinity P\textsubscript{i} transporter NaPi-IIa is only expressed in kidney and brain (43). Thus only NaPi-IIb fulfills the required transport properties and localization for Pi reabsorption from rat bile.

The identification of NaPi-IIb as the canalicular and cholangiocyte Pi\textsubscript{i} uptake system adds to the few biliary uptake processes that have so far been characterized on the molecular level. The latter include for instance SPNT1 in hepatocytes (11) or the ileal sodium-dependent bile acid transporter Asbt, which is apically expressed in cholangiocytes (25). The canalicular Pi\textsubscript{i} reabsorption by NaPi-IIb, which might help to prevent the loss of P\textsubscript{i} into bile and to maintain the P\textsubscript{i} concentration in hepatocytes and cholangiocytes, represents a new physiological function of NaPi-IIb in mammals. So far, murine NaPi-IIb has been shown to be involved in intestinal (19), alveolar (39), and epididymal (45) Pi reabsorption. Most interestingly, Graham et al. (18) have recently described the expression of two NaPi-IIb isoforms in zebrafish bile ducts. However, in contrast to our study in rat liver, NaPi-IIb was not reported to be expressed in hepatocytes of the zebrafish.

Surprisingly, Western blot analysis of NaPi-IIb in cLPM vesicles showed protein bands with a molecular weight of \(\sim 80\) and \(90 \text{ kDa}\). This is unexpected because studies using mouse intestinal membranes (20) showed an apparent molecular weight for NaPi-IIb of \(>100 \text{ kDa}\). The reason for this discrepancy is currently unknown. Proteolytic cleavage of NaPi-IIb in rat liver can be excluded as cause for the apparent difference in mobility because the same result was achieved with both COOH- and NH\textsubscript{2}-terminal NaPi-IIb antibodies (data not shown). Since the size of mouse and rat NaPi-IIb differs only in two amino acids and since mouse NaPi-IIb has only one putative N-glycosylation site more than rat NaPi-IIb (3), this does not explain the observed difference in electrophoretic mobility either. However, there is evidence that this difference represents a species rather than an organ variability: Arima et al. (3) have described a NaPi-IIb molecular weight of 78 kDa by Western blot analysis in rat duodenum brush-border membranes, which is in agreement with the observed molecular weight of NaPi-IIb in rat cLPM vesicles.

The concentration of P\textsubscript{i} in rat bile is low, reaching only 14 \(\mu\text{M}\) under normal circumstances. This concentration is nearly

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**Fig. 7.** Basolateral localization of PiT-1 and PiT-2 in rat hepatocytes. Stainings were done on cryosections of perfusion-fixed rat liver (3% paraformaldehyde/0.1% glutaraldehyde). A: red staining for PiT-1. D: red staining for PiT-2. B and E: green staining for the basolateral antigen 1–18 (36). C: superimposition of A and B showing colocalization of PiT-1 and 1–18 at the basolateral membrane of rat hepatocytes (yellow). F: superimposition of D and E showing colocalization of PiT-2 and 1–18 at the basolateral membrane of rat hepatocytes (yellow). G: after antibody absorption with the antigenic peptide, there is only a diffuse intracellular background staining in hepatocytes and no PiT-1 staining at the basolateral membrane. Bars, 10 \(\mu\text{m}\).
canalicular Pi reabsorption. IF analysis showed that both PiT-1 and PiT-2 are selectively expressed at the basolateral membrane of rat hepatocytes. This indicates that the weak PiT-1 and PiT-2 protein bands observed by Western blot analysis in rat hepatocytes showed \( K_m \) values between \( \sim 250 \) and \( 630 \) \( \mu M \) (2, 17, 47). These \( K_m \) values contrast somewhat with values determined for rat PiT-1 and PiT-2 (25–200 \( \mu M \), depending on the experimental setup) (23, 38). We assume that the apparent difference in \( K_m \) values between these determinations is caused by different experimental procedures. Hence, these data suggest that PiT-1 and PiT-2 play an important role in basolateral Pi uptake in rat liver. Although it has been suggested that NaPi-I serves to supply the great demand of Pi in the liver required for the high level of intracellular glucose metabolism (26), the physiological role of NaPi-I as sodium-phosphate cotransporter remains unclear (42).

Expression of PiT-1 and PiT-2 has previously been found by Northern blot analysis in a wide variety of tissues (23). On the basis of this finding, it has generally been thought for many years that PiT-1 and PiT-2 are expressed at the basolateral membranes of polarized epithelial cells and that they are involved in cellular Pi homeostasis (6, 14, 30). So far, however, the only experimental support for this hypothesis was the finding that PiT-2 is basolaterally (and apically) expressed in polarized human airway epithelial cells (41). We now show that PiT-1 and PiT-2 are specifically expressed at the basolateral membrane of the polarized hepatocytes in intact liver by IF analysis. Our data also indicate that cholangiocytes do not express members of the NaPi-III family, although expression levels below our detection limit cannot be excluded at the moment. A detailed knowledge about the (sub)cellular distribution pattern of PiT-1 and PiT-2 in other tissues might be relevant for future gene transfer protocols, since both PiT-1 and PiT-2, besides their function as Pi transporters, serve as virus receptors. PiT-1 and PiT-2 are the receptors for the gibbon ape leukemia virus and the amphotropic murine leukemia virus, respectively (23). In the past, several studies have

![Fig. 8. Double labeling of hepatocytes and cholangiocytes with PiT-1/PiT-2 and aminopeptidase N. Stainings were done on cryosections of perfusion-fixed rat liver (5% paraformaldehyde/0.1% glutaraldehyde). Only superimpositions are shown. A and B: red staining for PiT-1, green staining for aminopeptidase N. C and D: red staining for PiT-2, green staining for aminopeptidase N. There is no colocalization of PiT-1 and PiT-2 with aminopeptidase N either at the canalicular membrane of hepatocytes (arrow) or at the apical brush border of cholangiocytes (A). *, portal vein. Bars, 10 \( \mu M \).

200-fold lower than the Pi concentration in rat plasma (29). This Pi gradient supports a recent hypothesis that the physiological role of NaPi-IIb in bile ducts is to scavenge biliary Pi to prevent formation of precipitates (18). Interestingly, it has been found in humans that calcium phosphates are present in the nuclei of cholesterol stones and also in substantial amounts in black pigment gallstones (8). It has been postulated that the precipitation of calcium phosphate can form the nidus of a gallstone as well as accelerate gallstone growth (24). Consequently, NaPi-IIb-mediated Pi reabsorption might help to prevent gallstone initiation and growth by calcium phosphate. However, it is important to realize that our data have been obtained in rat liver. Although the NaPi-IIb transcript has been detected in human liver (44), nothing is known about the cell types expressing NaPi-IIb or its subcellular localization in human liver and gallbladder. It is also not known whether canalicular Pi is reabsorbed in human liver. Unlike in rat, no or only a small Pi gradient between plasma and bile seems to be present in humans. Whereas the Pi concentration in human plasma is 0.71–1.36 mM (33), a Pi concentration of 0.45–0.9 mM has been determined in common bile duct bile obtained from gallstone patients (1, 37). It therefore remains to be elucidated whether there are substantial species differences between mammals concerning canalicular Pi reabsorption.

Our results do not support a role of PiT-1 and PiT-2 in canalicular Pi reabsorption. IF analysis showed that both PiT-1 and PiT-2 are selectively expressed at the basolateral membrane of rat hepatocytes. This indicates that the weak PiT-1 and PiT-2 protein bands observed by Western blot analysis in cLPM vesicles resulted from cross-contamination of cLPM vesicles with bLPM vesicles. However, our data strongly support a role of PiT-1 and PiT-2 in basolateral Pi uptake of hepatocytes. Pi uptake into LPM vesicles was previously reported to be stimulated by an inwardly directed sodium gradient, a negative membrane potential, and an acidic extravesicular pH (2, 17, 47). Pi uptake into LPM vesicles or by primary rat hepatocytes showed \( K_m \) values between 200 and 1000 \( \mu M \) (2, 17, 47). These \( K_m \) values contrast somewhat with values determined for rat PiT-1 and PiT-2 (25–200 \( \mu M \), depending on the experimental setup) (23, 38). We assume that the apparent difference in \( K_m \) values between these determinations is caused by different experimental procedures. Hence, these data suggest that PiT-1 and PiT-2 play an important role in basolateral Pi uptake in rat liver. Although it has been suggested that NaPi-I serves to supply the great demand of Pi in the liver required for the high level of intracellular glucose metabolism (26), the physiological role of NaPi-I as sodium-phosphate cotransporter remains unclear (42).

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![Fig. 9. Scheme of hepatocellular and cholangiocytic phosphate transporter expression. The cellular and subcellular localization of NaPi-IIb, PiT-1, and PiT-2 have been investigated in this study. Basolateral expression of NaPi-I in hepatocytes has been previously reported by Yabuuchi et al. (46).]
been published in which amphotropic or gibbon ape pseudo-type recombinant retroviruses were used for gene therapeutic purposes (5, 7, 15, 22, 31, 40, 41). Based on our data showing that both PiT-1 and PiT-2 are only basolaterally expressed in rat hepatocytes, an intravenous (systemic or portal venous) injection of these retroviral vectors instead of a retrograde injection into the biliary system (7) seems to be favorable.

In conclusion, our results indicate that rat liver most likely regulates Pi concentration in bile through Pi reabsorption by NaPi-IIb. NaPi-IIb, which is expressed at the canalicular membrane of hepatocytes and at the brush-border membrane of cholangiocytes, can prevent loss of Pi in bile and helps to maintain the Pi concentration in hepatocytes and cholangiocytes. This indicates that NaPi-IIb may play an important role in the overall Pi homeostasis in rat liver, together with PiT-1 and PiT-2, which are expressed at the basolateral hepatocyte plasma membrane.

ACKNOWLEDGMENTS

We thank Brigitte O’Neill for expert technical assistance. Human PiT-2 antiserum was a generous gift of S. Kahmann, D. Kabat (Oregon Health and Science University), and J. M. Heard (Institute Pasteur, Paris, France). Partial this work was presented at the 54th Annual Meeting of the American Association for the Study of Liver Diseases (Boston, MA, October 24–28, 2003).

GRANTS

This work was supported by Swiss National Science Foundation Grant 31–6414.00 and the Bonizzoni-Theler-Stiftung (to P. Frei).

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