Bile Acid Transport in Sister of P-Glycoprotein (ABCB11) Knockout Mice†

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ABSTRACT: In vertebrates, bile flow is essential for movement of water and solutes across liver canalicular membranes. In recent years, the molecular motor of canalicular bile acid secretion has been identified as a member of the ATP binding cassette transporter (ABC) superfamily, known as sister of P-glycoprotein (Spgp) or bile salt export pump (Bsep, ABCB11). In humans, mutations in the BSEP gene are associated with a very low level of bile acid secretion and severe cholestasis. However, as reported previously, because the s*pgp*$^{-/-}$ knockout mice do not express severe cholestasis and have substantial bile acid secretion, we investigated the "alternative transport system" that allows these mice to be physiologically relatively normal. We examined the expression levels of several ABC transporters in *spgp*$^{-/-}$ mice and found that the level of multidrug resistance Mdr1 (P-glycoprotein) was strikingly increased while those of Mdr2, Mrp2, and Mrp3 were increased to only a moderate extent. We hypothesize that an elevated level of Mdr1 in the s*pgp*$^{-/-}$ knockout mice functions as an alternative pathway to transport bile acids and protects hepatocytes from bile acid-induced cholestasis. In support of this hypothesis, we showed that plasma membrane vesicles isolated from a drug resistant cell line expressing high levels of P-glycoprotein were capable of transporting bile acids, albeit with a 5-fold lower affinity compared to Spgp. This finding is the first direct evidence that P-glycoprotein (Mdr1) is capable of transporting bile acids.

Bile acids are synthesized from cholesterol in the liver, transported across the canalicular membrane, and secreted into the small intestine where they facilitate the absorption of dietary lipids and fat-soluble vitamins. The bile canaliculus contains a number of membrane proteins that utilize ATP to transport bile acids and organic compounds into the bile canaliculus. They are members of the ATP-dependent binding cassette (ABC) transporter superfamily such as multidrug resistance MDR1 P-glycoprotein (ABCB1) for organic cations, MDR3 (ABCB4) for phosphatidylcholine, multidrug resistance-associated protein MRP2 (ABCC2) for non-bile acid organic anions, and sister of P-glycoprotein Spgp[1] (ABCB11) for bile acids.

It was reported previously that Spgp-transfected insect cells mediate bile acid transport with an efficacy similar to that of its ATP-dependent transport across canalicular membranes (*1*, *2*). Also, patients with an inherited Spgp defect, type 2 progressive familial intrahepatic cholestasis (PFIC2) exhibit a very low level of biliary bile acid secretion (*3*, *4*). As a consequence of this defect, bile acids accumulate in the liver and cause severe liver toxicity that ultimately leads to cirrhosis, hepatic failure, and death. We have shown that in Spgp "knockout" (*spgp*$^{-/-}$) mice, biliary transport of the bile acid taurocholate was greatly inhibited due to the inactivation of Spgp, but overall, the *spgp*$^{-/-}$ mice are only mildly cholestatic (*5*). The lack of a severe phenotype is likely due to the ability of these mice to detoxify hydrophobic bile acids by hydroxylation and to utilize another mechanism (currently undefined) independent of Spgp to transport bile acids across the canalicular membranes. In a follow-up study, we fed the *spgp*$^{-/-}$ mice a diet of 0.5% cholic acid (CA) in an effort to induce a more severe cholestatic phenotype (*6*). A surprising observation was that the bile acid output and bile flow in the CA-fed mutant mice were elevated to a level similar to those of the wild type. This confirms the presence of a Spgp-independent mechanism for translocating cholic acid at a high capacity, yet the efficacy of this system in reducing the level of bile acids in the hepatocytes is insufficient to protect the mutant mice from cholestasis. A number of ATP-dependent transporters in the canalicular membrane are potential candidates for this Spgp-independent mechanism. These include Mdr1, Mdr2, Mrp2, and ecto-ATPase. In this paper, we characterize the properties of bile acid transport and gene expression in the canaliculus of *spgp*$^{-/-}$ mice and test the hypothesis that Mdr1 can function as an alternative transporter for cholic acid.

EXPERIMENTAL PROCEDURES

Animals. Mice with a targeted deletion of the *spgp* gene were produced as described previously (*5*). Animals were maintained in a 12 h light−dark cycle, at 25 °C, with free access to food and water. Mice were fed ad libitum standard mouse diet (Pico Lab Rodent Diet 20, PMI LabDiet, Richmond, IN) containing 41.6% carbohydrate, 21.0% protein, 9.9% fat, 4.4% fiber, 5.7% ash, and 10% moisture (control diet). The experimental diet consisted of the control diet supplemented with 0.5% (w/w) cholic acid (CA). CA

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[1] Abbreviations: Spgp, sister of P-glycoprotein; Bsep, bile salt export pump; Mdr, multidrug resistance; Pgp, P-glycoprotein; PFIC, progressive familial intrahepatic cholestasis; CA, cholic acid; TCA, taurocholic acid.

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is a common bile acid found in humans and mice. Such a CA-supplemented diet has been used to mimic different human liver diseases (*7*−*9*). Experiments were performed under approved protocols of the Committee on Animal Care, University of British Columbia (Vancouver, BC), according to the guidelines of the Canadian Council on Animal Care.

Bile Duct Cannulation and Collection of Bile. Bile samples were collected as described previously (*5*). Briefly, mice were anesthetized by intraperitoneal injection of Ketamine (112.5 mg/kg) and Xylazine (11.3 mg/kg) after a 2−4 h fast. The abdomen was opened, and gallbladder was cannulated using a PE-10 catheter after distal common bile duct ligation. After bile flow equilibration for 20 min, bile was collected into prepared tubes at 5 min intervals for 10 min. A bolus of taurocholate (100 mol/kg of body weight) was then infused into the tail vein over an interval of 20 s. Bile was then further collected through the cannula at 2 min intervals for 10 min, followed by 10 min intervals for 20 min.

High-Performance Liquid Chromatography (HPLC). Different components in bile samples were separated and quantified by HPLC using the technique described previously (*6*).

Purification of Liver Membrane Vesicles. Plasma membranes enriched in bile canaliculi from *spgp*$^{-/-}$ and wild-type mice were prepared by a modification of the Percoll and discontinuous sucrose gradient methods as described by Bohme et al. (*10*). Briefly, 10 g of pooled mice livers was homogenized in 100 mL of buffer A [250 mM sucrose, 5 mM HEPES-HCl (pH 7.4), 0.5 mM $CaCl_2$, 2 tablets of EDTA-free protease inhibitors, and 0.1 mM PMSF], and the pink gelatinous nuclear and plasma membrane pellet was collected after centrifugation at 1500*g* for 10 min. The pellet was then homogenized (glass/glass) in 50 mL of buffer B [250 mM sucrose, 5 mM HEPES-HCl (pH 7.4), and 1 mM EGTA] with 20 up-and-down strokes. The homogenate was mixed with 12 mL (14%) of Percoll and centrifuged in a SW28 rotor at 37000*g* for 40 min. Crude membrane sheets (∼25−30 mL) were recovered from the middle of the tube, diluted to 40 mL with buffer B, and homogenized by 10 up-and-down strokes. The homogenate was layered on 15 mL of 38% (w/w) sucrose (density = 1.164 g/mL). The gradients were then centrifuged in a SW28 rotor at 95000*g* for 30 min at 4 °C. Plasma membrane sheets were recovered from the 38% (w/w) sucrose interface. The membranes were snapped frozen in liquid N_2 and stored at −80 °C. Separation of canalicular and sinusoidal membranes was performed by sucrose density gradient centrifugation (*11*).

Biochemical Analysis of Canalicular Membrane Vesicles. The following enzyme activities were analyzed in various membrane fractions. Alkaline phosphatase was measured using a commercial kit (Sigma) using *p*-nitrophenyl 5′-monophosphate as the substrate; Mg^{2+}-ATPase and the oubain-sensitive Na^+K^+-ATPase activities were measured according to the method of Scharschmidt et al. (*12*).

Protein Analysis. Using membrane-rich microsomal fractions, protein expression of transporters in liver tissue from control and treated animals in all models of cholestasis was assessed as previously described (*9*). In brief, samples of fresh liver tissue were taken from multiple liver lobes, membrane-rich microsomal fractions were prepared, and protein concentrations were determined according to the manufacturer's instructions (Pierce, Woburn, MA). Western analysis was performed using standard techniques. Nitrocellulose membranes (Optitran, Schleicher & Schuell, Keene, NH) were probed with the following antibodies: a polyclonal rabbit anti-Bsep (IW antibody) raised against the peptide 1170-MDNIKYG- - - - -RGE-1224 (*13*), a polyclonal rabbit anti-Mrp2 (EAG15 antibody) raised against the C-terminal 12-amino acid sequence (kindly provided by D. Keppler), a monoclonal mouse anti-rat Mdr recognizing all the isoforms (C219; Signet Signal Transduction Laboratories, Dedham, MA), a polyclonal rabbit antibody raised against synthetic peptides derived from the "linker" region recognizing specifically Mdr1a isoforms (kindly provided by P. Gros), a polyclonal rabbit anti-Ntcp (kindly provided by F. Suchy), and a polyclonal rabbit Mrp3 antibody (kindly provided by G. Kruh). Immune complexes were detected using horseradish goat anti-rabbit or sheep anti-mouse immunoglobulin (Ig) G G(ab′)2 fragments using the ECL Western blotting kit (Amersham, Arlington Heights, IL) according to the manufacturer's instructions. Immunoreactive bands were quantified by laser densitometry (Personal Densitometer SI; Molecular Dynamics, Sunnydale, CA).

Radiolabeled Taurocholate Uptake by Isolated Canalicular Membrane Vesicles. Transport experiments were performed using a rapid filtration assay. Purified frozen canalicular membrane vesicle (CMV) samples were thawed quickly at 37 °C and incubated on ice for 30 min until use. Reaction buffer (110 μL) [10 mM Hepes-Tris (pH 7.4), 250 mM sucrose, 5 mM ATP disodium acid or AMP monosodium acid, 10 mM $MgCl_2$, 10 mM creatine phosphate Tris acid, and 100 μg/mL creatine kinase] containing 3 μM [^{3}H]-taurocholic acid (Perkin-Elmer, Boston, MA) was incubated at 37 °C for 5 min and then rapidly mixed with 50 μL of a 0.8 μg/μL membrane vesicle suspension (40 μg of protein). At the indicated times, 20 μL aliquots were removed and added to 1 mL of ice-cold washing buffer A [10 mM Hepes-Tris (pH 7.4), 250 mM sucrose, and 1 mM cold taurocholic acid] and filtered through 0.45 μm pore size prewetted nitrocellulose (HAWP045 Millipore) filters. To reduce the level of nonspecific binding to the filters, the filters were first prewashed with water and then with buffer A. The filters were then washed with 5 mL of buffer A and rinsed twice with 5 mL of buffer B [10 mM Hepes-Tris-HCl (pH 7.4) and 250 mM sucrose]. Radioactivity was measured by liquid scintillation counting in 5 mL of liquid scintillation fluid. Inhibition of taurocholate uptake was studied by preincubation with various inhibitors for 5 min at 37 °C prior to the start of the [^{3}H]taurocholate uptake assay. [^{3}H]Glycocholate, [^{3}H]tauroursodeoxycholate (kindly provided by A. Hoffmann), and [^{3}H]leukotriene C4 (NEN) were also used for uptake experiments as described above.

Mdr1-Containing Plasma Membrane Vesicles. Plasma membrane vesicles were prepared from Chinese hamster ovary CH^{R}B30 cells as previously described (*14*). These cells express a high level of class I P-glycoprotein, equivalent to mouse mdr1 or human MDR1 protein representing ∼15% of total plasma membrane proteins and negligible amounts of class II and III P-glycoprotein (*14*). The plasma membrane fraction was collected from the 16%−31% interface of the sucrose gradient. Protein concentrations were determined using the BCA assay as described earlier. The protein concentration was determined individually for each experiment (usually 0.4−0.6 mg/mL depending on the number of

Table 1: Comparison of Biliary Clearance of Bile Acids in Wild-Type and *spgp*$^{-/-}$ Mice on the CA Diet[a]

	wild type	*spgp*$^{-/-}$	significance (*p* value)
bile flow rate [μL min^{-1} (g of liver)$^{-1}$]	2.02 ± 0.29	1.82 ± 0.24	0.35
total bile acid secretion [nmol min^{-1} (g of liver)$^{-1}$]	86.36 ± 14.10 (100%)	77.05 ± 15.98 (100%)	0.38
taurocholic acid	73.97 ± 15.07 (75%)	42.61 ± 19.64 (52%)	0.02
tauromuricholic acids	20.41 ± 2.27 (21%)	31.76 ± 3.19 (39%)	0.01
tauroursodeoxycholic acid	1.04 ± 0.28 (1%)	1.71 ± 1.02 (2%)	0.20
tetrahydroxylated bile acids	0.73 ± 0.70 (0.01%)	6.14 ± 1.22 (7%)	0.001

[a] The bile acid secretion rate (nanomoles per minute per gram of liver) was calculated from the determined concentration (millimolar) times the bile flow rate measured as the volume of bile collected per unit time per gram of liver. The percentage of total bile acid secretion is reported in parentheses. Four wild-type male mice and five knockout male mice were used in this analysis. Statistical significance was determined using a two-tailed *t*-test and a *p* value of 0.05.

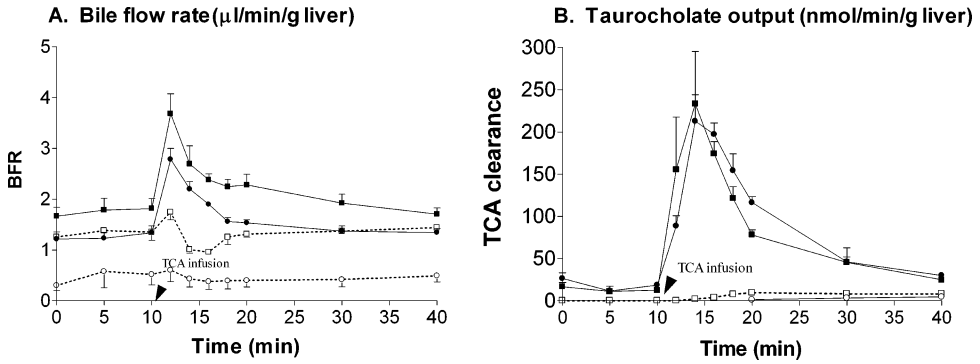

FIGURE 1: Changes in (A) bile flow rate (BFR) and (B) taurocholate clearance as a function of liver weight in wild-type and *spgp*$^{-/-}$ mice. A bolus of taurochlate (100 μmol/kg of body weight) was infused into the tail vein over a 20 s interval 10 min after the initiation of bile flow measurement: male wild type (■), female wild type (●), male knockout (□), and female knockout (○). Data are represented as means ± the standard error of the mean (n = 4−5).

cells used for preparation). The vesicles are ~50% inside-out based on enzyme activity.

RESULTS

Effect of Cholic Acid Feeding on Bile Acid Output. We observed previously that bile acid output for wild-type mice on normal diet was 20 nmol min^{-1} (g of liver)$^{-1}$, and it was 4 nmol min^{-1} (g of liver)$^{-1}$ for *spgp*$^{-/-}$ mice (*5*). The bile flow rate was 0.6 μL min^{-1} (g of liver)$^{-1}$ in *spgp*$^{-/-}$ mice, and it was not significantly different from that in wild-type mice (*5*). The output rate of total bile acids in mice fed with CA for 2 days was not significantly different between the mutant and wild type [77 and 86 nmol min^{-1} (g of liver)$^{-1}$, respectively] (*6*). However, there was a significant difference in the distribution of bile acid species secreted (Table 1). The bile of *spgp*$^{-/-}$ mice contained a greater proportion of hydrophilic bile acids. Cholic acid output in wild-type and mutant mice was 75 and 52% of the total, respectively, while muricholic acids represented 21 and 39% and tetrahydroxylated bile acids 0.01 and 7%, respectively (Table 1).

Clearance of Infused Taurocholate in spgp$^{-/-}$ *Mice.* To investigate in greater detail the ability of *spgp*$^{-/-}$ mice to secrete bile acids, a high concentration of taurocholic acid was injected into the tail vein of the mice and biliary taurocholate output was determined for each mouse. Because bile acids are a major driving force for bile flow, we determined if the injected taurocholate was able to stimulate bile flow in *spgp*$^{-/-}$ mice. We observed that in both male and female wild-type mice, taurocholate greatly stimulated the bile flow rate, peaking within 5 min of injection and returning to near normal 10 min later (Figure 1A). A similar response was not observed in *spgp*$^{-/-}$ mice (Figure 1A, bottom time trace). There was no significant "peak" of bile flow activity. When biliary taurocholate output was examined in wild-type mice (Figure 1B), we observed a dramatic increase. For example, 3 min after injection, taurocholate output was increased ~20-fold, from the basal level of 12.3 ± 5.2 nmol min^{-1} (g of liver)$^{-1}$ to the stimulated level of 233.3 ± 124.0 nmol min^{-1} (g of liver)$^{-1}$. A similar response was not observed in *spgp*$^{-/-}$ mice. The taurocholate output rate was at least 25-fold lower in knockout mice than in controls, and no rapid clearance was observed. Taken together, these data confirm that the ability to clear rapidly infused taurocholate in mice is mediated by Spgp and that an alternative system with similar rapid clearance properties is not present in the *spgp*$^{-/-}$ mice.

Cholic Acid Transport in Isolated Canalicular Membrane Vesicles (CMVs). We next assessed the ability of isolated canalicular membrane vesicles (CMVs) from wild-type and *spgp*$^{-/-}$ mouse livers to mediate bile acid transport. CMVs were prepared as described in Experimental Procedures. We obtained an average yield of 0.08−0.10 mg of protein/g of liver, which was similar to results published for rat liver (*10*, *11*, *15*). The protein yield was similar between normal and mutant mice (Table 2). Enzyme markers indicated enrichment of membrane vesicles derived predominantly from the canalicular domain of the hepatocyte plasma membrane (Table 2). Alkaline phosphatase activity, a marker of the canalicular membrane, was enriched ~60-fold compared with that of the homogenate (Table 2). Na^+K^+-ATPase, a marker for basolateral membrane, was enriched ~6−8-fold (Table 2). On the basis of the orientation of ectoenzyme nucleotide pyrophosphatase and its activity, the isolated membrane vesicles were 35−43% inside-out (*10*).

To confirm the functionality of CMVs isolated from *spgp*$^{-/-}$ mice, the ATP-dependent leukotriene C_4 transport,

Table 2: Protein Yield and Enzyme Enrichment of Canalicular Membrane Vesicles in Wild-Type and *spgp*$^{-/-}$ Mice[a]

	protein yield (mg/g of liver)		alkaline phosphatase (RE)		NaK-ATPase (RE)		membrane sidedness (% inside-out)	
	+/+	−/−	+/+	−/−	+/+	−/−	+/+	−/−
male	0.08 ± 0.03 (26)	0.08 ± 0.03 (13)	68.7 ± 36.7 (26)	74.2 ± 31.7 (13)	6.0 ± 2.5 (6)	5.9 ± 3.1 (6)	43 ± 12 (10)	41 ± 11 (9)
female	0.10 ± 0.04 (9)	0.08 ± 0.03 (5)	61.3 ± 13.8 (9)	47.4 ± 11.3 (5)	6.3 ± 0.8 (4)	7.4 ± 1.6 (4)	35 ± 6 (6)	39 ± 5 (4)

[a] The homogenate and the plasma membrane samples were obtained as described in Experimental Procedures. Enzyme specific activities are reported as micromoles per hour per milligram of protein at 37 °C. The total recovery of enzymes was in the range of 86−110%. Data are given as mean ± the standard deviation with the number of experiments in parentheses. Relative enrichment (RE) is defined as the ratio of specific activity in the subfractions to specific activity in the homogenate. +/+ represents wild-type mice, and −/− represents *spgp*$^{-/-}$ mice.

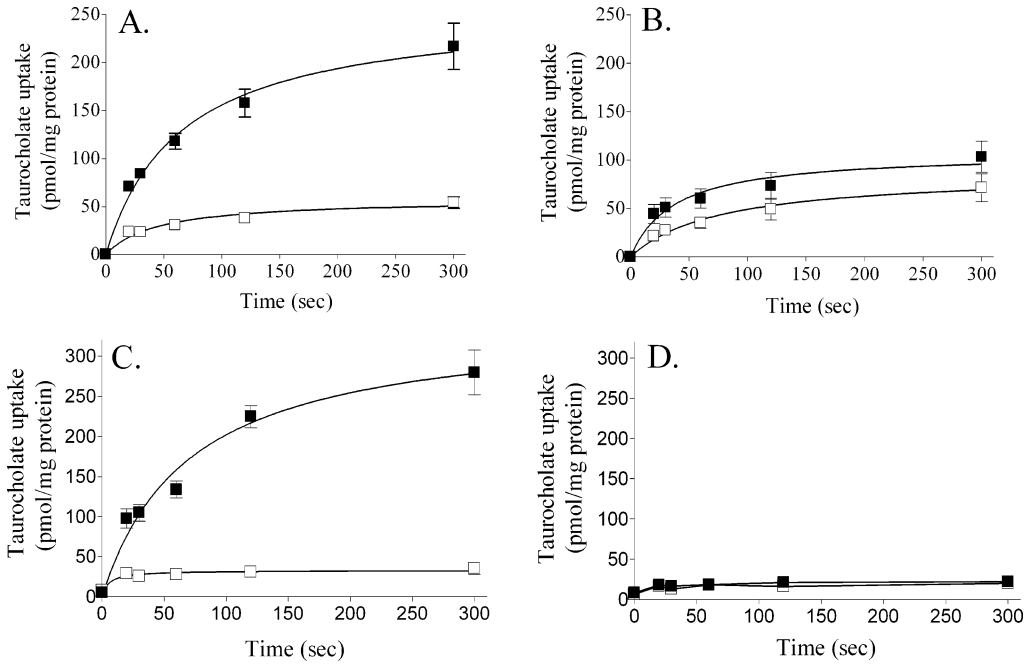

FIGURE 2: Time-dependent taurocholate uptake into CMV isolated from wild-type and *spgp*$^{-/-}$ mice. Transport studies were undertaken using canalicular-enriched membrane vesicles isolated from (A) wild-type mice, (B) *spgp*$^{-/-}$ mice fed with normal diet, (C) wild-type mice, and (D) *spgp*$^{-/-}$ mice fed with CA diet. Taurocholate uptake was assessed as described in Experimental Procedures. Values are rates measured in the presence of 5 mM MgATP (■) or 5 mM MgAMP (□).

which has been demonstrated to be mediated by Mrp2 in membrane vesicles from transfected cells, was assessed. The initial rate of transport of leukotriene C_4 was 5.0 ± 1.3 pmol min^{-1} (mg of protein)$^{-1}$ in the CMVs from *spgp*$^{-/-}$ mice, whereas the initial rate of transport was 2.4 ± 0.9 pmol min^{-1} (mg of protein)$^{-1}$ in wild-type mice. These results reflect quantitatively the ∼2-fold increased level of expression of Mrp2 in the CMVs of *spgp*$^{-/-}$ mice (see below and Figure 5) and confirm that the CMVs of the mutant mice were functional and their purity was approximately the same as the purity of those from wild-type animals.

The enriched CMVs of wild-type mice exhibit ATP-dependent taurocholate uptake at 37 °C as shown in Figure 2A. At 4 °C, there was no significant difference between taurocholate uptake in the presence of MgATP or MgAMP (data not shown), confirming that the measured rate at 37 °C was due to an energy-dependent process. The rate of ATP-dependent uptake of taurocholate was very low (transport activity with MgATP compared to that with MgAMP) in CMVs from *spgp*$^{-/-}$ mice (Figure 2B). ATP-dependent taurocholate uptake was also observed in membrane vesicles isolated from wild-type mice fed a CA-supplemented diet (Figure 2C). Surprisingly, significant ATP-dependent uptake of taurocholate was not observed in isolated CMVs from the knockout mice fed a CA-supplemented diet (Figure 2D) given that the bile acid output in vivo is similar in wild-type and *spgp*$^{-/-}$ mice (Table 1). Kinetic analyses of transport showed that the K_m and V_{max} values of ATP-stimulated taurocholate transport were 14 μM and 800 pmol min^{-1} (mg of protein)$^{-1}$, respectively, in wild-type CMVs (Figure 3A). We were not able to measure reliably the K_m and V_{max} of ATP-dependent taurocholate transport in CMVs from *spgp*$^{-/-}$ mice because of the relatively low uptake rate and high background, presumably due to nonspecific binding of radiotracer taurocholate (Figures 2B and 3B). Thus, under the conditions of our assay, we were not able to demonstrate a Spgp-independent transport activity using isolated CMVs from the mutant mice.

The ATP-dependent taurocholate uptake in wild-type CMVs could be inhibited by the Spgp-specific polyclonal antibody (IW), cyclosporin A (CsA), and bile acids such as tauro-β-muricholic acid, α-muricholic acid, tauroursodeoxycholic acid, and glycoursodeoxycholic acid (Figure 4). We found in this study that CsA is a potent inhibitor of Spgp-mediated ATP-dependent taurocholate uptake. For example, 1 μM CsA inhibited ∼50% of taurocholate transport activity (Figure 4B). We did not observe inhibition by estradiol-17β-glucuronide at 10 or 100 μM, as has been observed in purified rat CMVs previously (*16*). Also, paclitaxel did not inhibit taurocholate transport at 10 and 100 μM. These

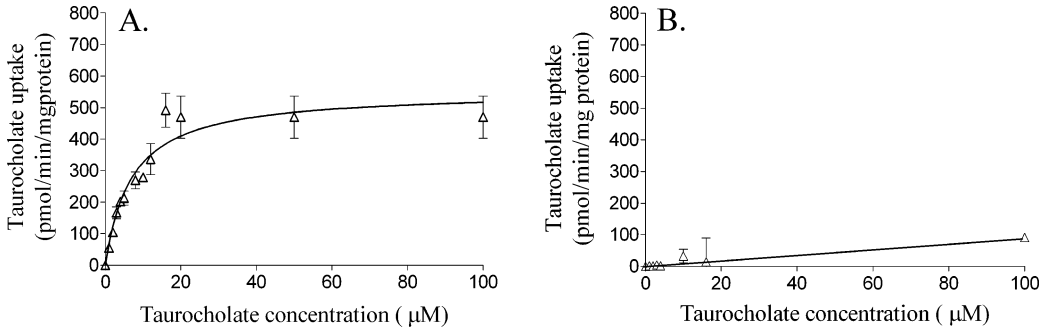

FIGURE 3: Concentration dependence of [^{3}H]taurocholate uptake in CMV from wild-type and *spgp*$^{-/-}$ mice. The rates of MgATP-dependent uptake of different concentrations of taurocholate (3 μM [^{3}H]taurocholate tracer) into membrane vesicles were measured for 30 s at 37 °C in uptake media containing 5 mM MgATP or 5 mM MgAMP as described in Experimental Procedures. Values are rates measured in the presence of MgATP minus rates measured in the presence of MgAMP for triplicate determinations; bars represent the standard error. The lines of best fit and kinetic parameters were computed by nonlinear least-squares analysis. Kinetic parameters for taurocholate uptake into CMV are as follows: $K_m \sim 14$ μM and $V_{max} \sim 800$ pmol mg^{-1} min^{-1}.

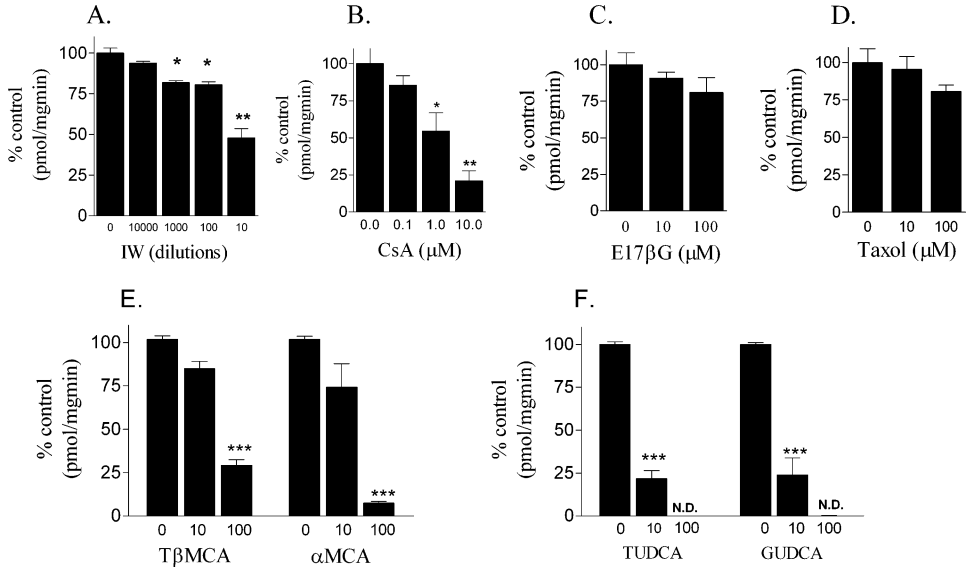

FIGURE 4: Effects of bile acids and xenobiotics on the modulation of Spgp-mediated taurocholate uptake transport in CMV: (A) Spgp polyclonal antibody, IW, (B) cyclosporin A, (C) ethinylestradiol-17β-glucuronide (E17βG), (D) paclitaxel, (E) tauro-β-muricholic acid (TβMCA) and α-muricholic acid (αMCA), and (F) tauroursodeoxycholic acid (TUDCA) and glycoursodeoxycholic acid (GUDCA). The data are means ± the standard deviation of three experiments. N.D. means not detectable. *$P < 0.05$, **$0.01 < P < 0.05$, and ***$P < 0.01$ indicates a significant difference from the corresponding control.

discrepancies might reflect differences in the level of protein expression in mice compared to rat liver or differences in the selectivity for substrates between rat and mice Spgp protein.

Expression of ABC Transporters in spgp$^{-/-}$ *Mice.* Cholestasis results in transcriptionally induced alterations in the expression of bile acid transporter in cholestatic animal models and in cell culture systems. We have reported previously that among 36 genes that have been examined, the expression of nine genes was significantly upregulated and seven downregulated in CA-fed *spgp*$^{-/-}$ mice compared with the wild type of the same sex (*6*). For instance, the mRNA of canalicular mdr1a and mdr1b P-glycoproteins responsible for biliary clearance of various cations was upregulated, as were the basolateral efflux pumps (i.e., mrp3 and mrp4). The mRNA level of a number of ABC transporters involved in bile formation, such as mdr2 and mrp2, was not changed significantly. To determine whether the changes in mRNA expression are associated with changes in protein level, we measured the expression of a number of liver transporters under normal and cholestasis-induced conditions. Western blotting analysis indicated that the expression of Mdr1a (labeled with a specific antibody for this isoform) was significantly upregulated in *spgp*$^{-/-}$ mice on a normal diet, and it was greatly upregulated under CA feeding conditions. A number of other liver ABC transporters such as Mdr2, Mrp2, and Mrp3 were also upregulated, but to a much lesser extent. As expected, the basolateral bile acid-transporting polypeptide Ntcp was downregulated in *spgp*$^{-/-}$ mice (Figure 5). The level of expression of Ntcp was further reduced in both mutant and wild-type mice upon CA feeding. The levels of the other proteins (Mrp2 and Mdr2) did not significantly increase after CA feeding in mutant mice (Figure 5).

ATP-Dependent Uptake of Taurocholate into Mdr1 Plasma Membrane Vesicles. The apparent bile flow and bile acid output, and the dramatic overexpression of Mdr1 in CA-fed *spgp*$^{-/-}$ mice, suggest that Mdr1 is possibly an alternative bile acid transporter in the canaliculus. However, we did not detect significant ATP-dependent taurocholate uptake in isolated CMVs from *spgp*$^{-/-}$ mice. This may be due to a low affinity for bile acid transport in CMVs from *spgp*$^{-/-}$ mice. Therefore, we took advantage of a highly efficient in vitro system that we developed in the past to confirm and

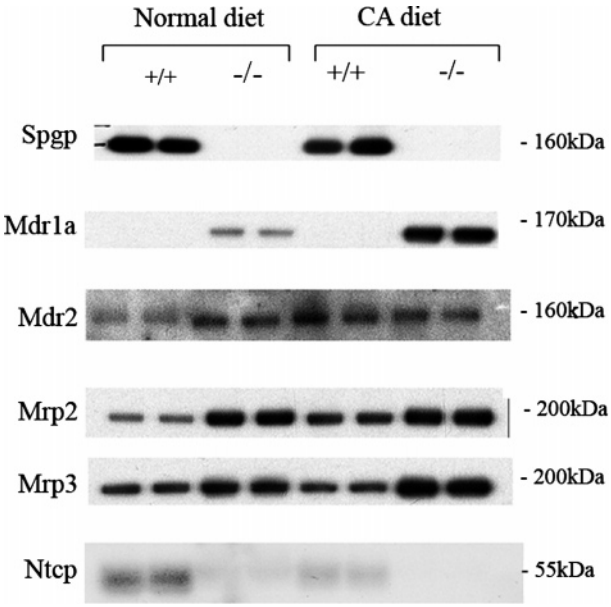

FIGURE 5: Effect of disruption of *spgp* on the expression of other canalicular membrane proteins. Representative Western blots after probing for Spgp (1:5000), mdr1a (1:100), Mrp2 (1:5000), Mdr2 (1:100), and Ntcp (1:1000) are shown.

characterize Mdr1-mediated bile acid transport (*17*−*20*). As shown in Figure 6A, we were able to detect a time-dependent ATP-stimulated taurocholate uptake in plasma membrane vesicles (PMV) from CH^{R}B30 cells, which contain a very high level of Chinese hamster P-glycoprotein 1 (Pgp1, an Mdr1 orthologue). It is noteworthy that the background level in this assay system is relatively high, and this may be due to the hydrophobic nature of the substrate that was used. Saturation of ATP-dependent taurocholate uptake was established at a concentration gradient of 3−4-fold (Figure 6B). We determined that this Pgp1-mediated taurocholate transport has K_m and V_{max} values of 69 μM and 585 pmol min^{-1} (mg of protein)$^{-1}$, respectively (Figure 6B). The affinity of Pgp1 in the PMVs for taurocholate is ∼5-fold lower than that observed with Spgp in CMVs. Thus, Mdr1 or Pgp1 has the properties of a low-affinity bile acid transporter. The involvement of Pgp1 in taurocholate transport was further confirmed by 50% inhibition of ATP-dependent taurocholate uptake by cyclosporin A (CsA), a potent Mdr inhibitor, at a concentration of 3 μM (Figure 6C). We also observed a stimulation of taurocholate uptake at low concentrations of CsA (0.1−0.5 μM), demonstrating a similar effect on bile acid and drug transport as reported by Lu et al. (*21*).

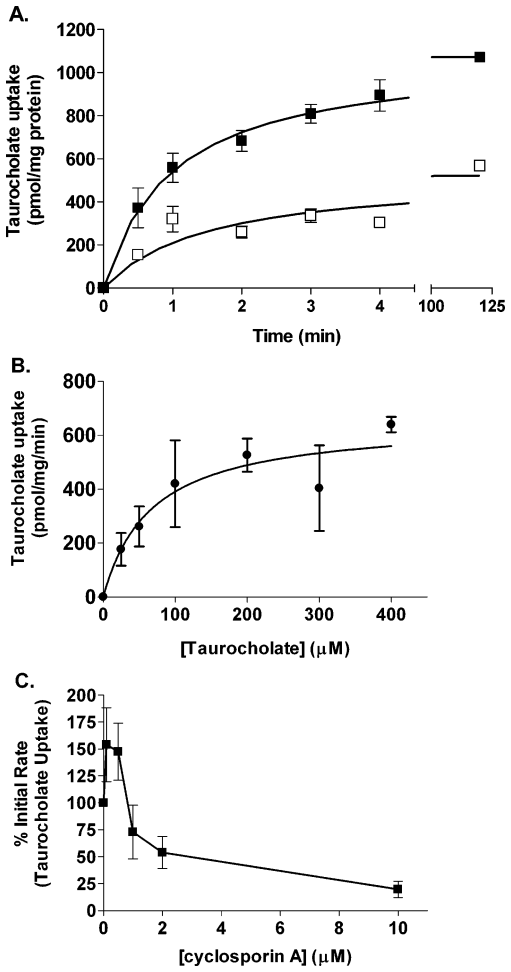

FIGURE 6: ATP-dependent taurocholate uptake into Mdr-containing plasma membrane vesicles. (A) Time-dependent taurocholate uptake in the presence of 5 mM ATP (■) or 5 mM AMP (□) from Mdr1-containing CH^{R}B30 cells. (B) ATP-dependent taurocholate uptake (●) is the difference between taurocholate uptake in the presence of ATP and AMP. (C) Inhibition of cyclosporin A on ATP-dependent taurocholate uptake at the 1 min time point in Mdr1-containing plasma membrane vesicles.

DISCUSSION

We show in this study that transport of bile acids in the CMVs of wild-type mice is ATP-dependent with a relatively high affinity (K_m = 14 μM) and capacity (V_{max} ∼ 800 pmol mg^{-1} min^{-1}) for taurocholate. The kinetic parameters are consistent with values reported for transport in other systems, including membrane vesicles isolated from insect cells transfected with rat, mouse, and human Spgp (*1*, *22*−*25*). The wild-type CMV activity can be attributed to Spgp since very little ATP-dependent taurocholate transport activity was detected in the CMVs of *spgp*$^{-/-}$ mice. We thus confirm that by the knockout of Spgp in mice Spgp is the physiological bile acid transporter.

A surprising observation was that despite a lack of the physiological bile acid transporter, the *spgp*$^{-/-}$ mice were capable of secreting bile acid into the bile (*5*), and on a CA-supplemented diet, this level of secretion was elevated to a level similar to that of wild-type mice (*6*). Evidently, a high-capacity alternative bile acid transport system is present in *spgp*$^{-/-}$ mice. Since this alternative system is insufficient to protect the mutant mice from severe cholestasis under the CA feeding conditions, we reasoned that this alternative system must have a low affinity (despite a high capacity) for bile acid transport. In this context, we have proposed a "rain barrel" model to explain how a high-capacity bile acid transport system can still lead to cholestasis (*26*). The model takes into account the fact that multiple transporters are potentially capable of secreting bile acids under cholestatic conditions. The contribution of each transporter to the total amount of bile acid transported is dependent on transporter concentration, and transport kinetics. Under physiological conditions, bile acids are removed efficiently from the portal circulation by hepatocytes and actively pumped across the canalicular membrane by Spgp or the high-affinity, high-capacity system (the downspout of a rain barrel). A low-affinity alternative system comes into play when the high-

affinity system is inactivated and the level of bile acids rises in the hepatocytes, reaching the K_m of the alternative system (equivalent to the overflow pipe of a rain barrel). This higher level of accumulated bile acids in the hepatocytes results in cholestasis even though the alternative system may have the capacity to transport a wild-type level of bile acids as seen in our mutant mice.

While the identity of these potential alternative systems in mutant mice is unknown, expression data revealed that a number of ABC transporters, including Mdr1, Mrp2, Mrp3, and Mdr2, were significantly upregulated in the CMVs of *spgp*$^{-/-}$ mice. The most striking was the elevation of the level of Mdr1 at both mRNA and protein levels (*6*). This protein was further upregulated after CA feeding (Figure 5). These observations are consistent with findings in three experimental models of cholestasis showing coordinated upregulation of Mdr1 (Pgp) along with Spgp/Bsep and other proteins (*27*). While the molecular basis of this dramatic upregulation of Mdr1 is not defined at present, it is likely a response to cholestatic stress. It is noteworthy that the expression of Spgp is transcriptionally and posttranslationally regulated by the concentration of its own substrates and other intracellular lipid products (*28*, *29*). Thus, it would not be surprising if Mdr1 were similarly regulated.

In this study, we provide several lines of evidence which show that the taurocholate transport is due to Mdr1 (Pgp). First, Mdr1 is related to Spgp in sequence and so is an obvious candidate for being the alternative transporter. Second, Pgp is highly overexpressed in the canalicular membranes of the Spgp knockout mice. Third, we were able to consistently detect a time-dependent, ATP-stimulated taurocholate uptake in another system expressing a high level of Pgp, the plasma membrane vesicles from CHO B30 cells. This system is very well characterized for Pgp-mediated drug transport (*14*, *17*−*20*). Although we cannot completely disregard the possibility of the involvement of other transporters in taurocholate transport in this system, we have shown that Spgp is not detected in B30 membranes and the level of transport observed in the B30 vesicles is over and above that of the control vesicles from drug-sensitive wild-type cells. Fourth, the level of Mdr1 expressed in B30 membranes is close to 15% of total membrane proteins, consistent with a relatively high V_{max} of transport. There is no membrane protein whose level is significantly increased that could account for this low-affinity, high-capacity transport. Fifth, taurocholate transport is inhibited by cyclosporine A at a concentration relatively specific for Mdr1 (Figure 6C). The specificity and involvement of Mdr1 are further demonstrated by inhibition of drug transport in the drug-resistant CHO cell line by taurocholate and a number of physiologically relevant bile acids (data not shown). Sixth, it should be noted that even with this high level of Mdr1 expression, ATP-dependent transport of taurocholate was only a little more than 2-fold above background. This may explain why we were unable to assess reliably Mdr1-mediated taurocholate transport in the CMVs of *spgp*$^{-/-}$ mice (Figure 2) because of the high background of nonspecific binding of the hydrophobic substrate to the membrane vesicles.

Taken together, the results described above are completely consistent with the hypothesis that Mdr1 (Pgp) is capable of transporting bile acids and that it is likely the low-affinity and high-capacity alternative bile acid transport system that allows the Spgp knockout mice to be physiologically "normal". Our results thus illustrate the well-known broad substrate specificity of Mdr1 and for the first time show that Mdr1 can tolerate bile acids as biologically relevant substrates. This may have physiological implications. For instance, Mdr1 is a major drug pump in the blood−brain barrier to protect the central nervous system (CNS) from neural toxicity (*30*). The bile acid transport activity of Mdr1 could be a defense mechanism against bile acid accumulation in CNS. In addition, we believe that our findings have potential medical implications for a fatal childhood disease, PFIC2. It has been possible to manipulate Mdr1/Pgp expression and affinity in different systems (*18*, *31*−*34*). One can speculate that such an approach could prove to be useful for PFIC2, a genetic disease that would otherwise require liver transplantation.

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