Localization of the PE methylation pathway and SR-BI to the canalicular membrane: evidence for apical PC biosynthesis that may promote biliary excretion of phospholipid and cholesterol

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Abstract To better understand the regulation of biliary phospholipid and cholesterol excretion, canalicular membranes were isolated from the livers of C57BL/6J mice and abundant proteins separated by SDS-PAGE and identified by matrix-assisted laser desorption/ionization mass spectrometry. A prominent protein revealed by this analysis was betaine homocysteine methyltransferase (BHMT). This enzyme catalyzes the first step in a three-enzyme pathway that promotes the methylation of phosphatidylethanolamine (PE) to phosphatidylcholine (PC). Immunoblotting confirmed the presence of BHMT on the canalicular membrane, failed to reveal the presence of the second enzyme in this pathway, methionine adenosyltransferase, and localized the third enzyme of the pathway, PE *N*-methyltransferase (PEMT). Furthermore, immunfluorescence microscopy unambiguously confirmed the localization of PEMT to the canalicular membrane. These findings indicate that a local mechanism exists in or around hepatocyte canalicular membranes to promote phosphatidylethnolamine methylation and PC biosynthesis. Finally, immunoblotting revealed the presence and immunofluorescence microscopy unambiguously localized the scavenger receptor class B type I (SR-BI) to the canalicular membrane. Therefore, SR-BI, which is known to play a role in cholesterol uptake at the hepatocyte basolateral membrane, may also be involved in biliary cholesterol excretion. Based on these findings, a model is proposed in which local canalicular membrane PC biosynthesis in concert with the phospholipid transporter mdr2 and SR-BI, promotes the excretion of phospholipid and cholesterol into the bile.—Sehayek, E., R. Wang, J. G. Ono, V. S. Zinchuk, E. M. Duncan, S. Shefer, D. E. Vance, M. Ananthanarayanan, B. T. Chait, and J. L. Breslow. **Localization of the PE methylation pathway and SR-BI to the canalicular membrane: evidence for apical PC biosynthesis that may promote biliary excretion of phospholipid and cholesterol. *J. Lipid Res.* 2003.** 44: **1605–1613.**

Supplementary key words phosphatidylethanolamine • phosphatidylcholine • methionine adenosyltransferase • scavenger receptor class B type I

The excretion of the biliary lipids, bile acids, phospholipids, and cholesterol, is a complex process and is regulated at multiple levels. Bile acids are synthesized in hepatocytes, excreted into the bile, and enter the enterohepatic circulation, where they are taken up from the portal system by the hepatocyte basolateral membrane transporter Ntcp. The uptake of bile acids is followed by their reexcretion, along with newly synthesized bile acids, back into the bile. This recycling is considered a major driving force behind biliary excretion of phospholipids and cholesterol (1). However, the importance of the bile acid recycling in determining biliary phospholipid and cholesterol excretion has been challenged by studies in mdr2 knockouts

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Abbreviations: BHMT, betaine homocysteine methyltransferase; MAT, methionine adenosyltransferase; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PEMT, phosphatidylethanolamine *N*-methyl transferase; SR-BI, scavenger receptor class B type I.

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where normal bile acid excretion is associated with a complete defect in biliary phospholipid and cholesterol excretion (2). This animal model provides unequivocal proof that mdr2, a flippase that localizes specifically to the canalicular membrane and transfers phosphatidylcholine (PC) from the inner to the outer leaflet, has a critical role in biliary phospholipid excretion. Moreover, the failure of mdr2 knockouts to excrete cholesterol suggests that biliary phospholipid excretion plays a critical role in driving the excretion of cholesterol into the bile. The complex relationships between biliary cholesterol and phospholipid excretion has been further exemplified by studies in mice with liver and intestinal overexpression of hemitransporters implicated in the enterohepatic metabolism of plant sterols and cholesterol, ABCG5 and ABCG8 (3). In this model, transgenic males display a 4- to 5-fold increase in biliary cholesterol concentrations that was associated with a modest but significant increase in phospholipid concentration, suggesting that under certain conditions, increased excretion of cholesterol may drive phospholipid excretion. Finally, Cyp27a1 knockout mice, an animal model with a severely depleted bile acid pool, maintain their biliary cholesterol levels (4). When taken together, these studies exemplify the complexity of biliary lipid excretion and call for studies that clarify the details of these processes at the molecular level.

To increase our understanding of biliary lipid excretion, canalicular membranes from the livers of C57BL/6J mice were isolated and abundant proteins separated by SDS-PAGE and identified by matrix-assisted laser desorption/ionization mass spectrometry (MALDI-TOF MS). One of the proteins identified was the enzyme betaine homocysteine methyltransferase (BHMT). This enzyme catalyzes the first in a three-step metabolic pathway that promotes the methylation of phosphatidylethanolamine (PE) to PC. Immunoblotting confirmed the presence of BHMT and also revealed the enzyme that catalyzes the third step in this pathway, PE *N*-methyltransferase (PEMT). Furthermore, immunofluorescent microscopy clearly showed that PEMT is localized to the canalicular membrane. These findings suggest that canalicular localization of BHMT and PEMT contributes to local PC synthesis and secretion into the bile. In addition, immunoblotting of canalicular membranes and immunfluorescent microscopy studies clearly revealed the presence of scavenger receptor class B type I (SR-BI), which may play a role in directly transferring cholesterol from the canalicular membrane into the biliary space. These findings suggest a model in which local canalicular membrane PC biosynthesis, in concert with mdr2 and SR-BI, promotes phospholipid and cholesterol excretion into the bile.

MATERIALS AND METHODS

Animals

Wild-type C57BL/6J males were purchased from The Jackson Laboratories (Bar Harbor, ME) and studied at 10–13 weeks of age. Animals were housed in a temperature- and humidity-controlled room with a 12 h light-dark cycle (6 AM–6 PM light) and fed Picolab Rodent Chow 20 (5053) pellets for 3 weeks. The Rockefeller University Institutional Animal Care and Research Advisory Committee approved all experiments.

Membrane preparation

Mouse liver canalicular and basolateral membranes were isolated by a minor modification of the nitrogen cavitation/calcium precipitation method described by Kipp and Arias for the rat (5). Twenty mouse livers were rapidly perfused with an ice-cold SHCa buffer (0.25 M sucrose, 10 mM HEPES/Tris, pH 7.4, 0.2 mM $CaCl_2$) to which 2 μg/ml aprotinin, 2 μg/ml pepstatin A, 2 μg/ml leupeptin, 5 μg/ml benzamidine, and 20 μg/ml phenylmethylsulfonyl fluoride were added. The livers were minced with a razor blade and groups of 10 livers homogenized in 50 ml of SHCa buffer with four strokes of a loose-fitting Dounce homogenizer. The liver suspensions were combined and filtered through a double layer of cheesecloth. The filtrate was homogenized with an additional 15 strokes, diluted with SH buffer (Ca-free SHCa buffer) to 270 ml, supplemented with EDTA (pH 7.4) to a final concentration of 1 mM, and centrifuged for 10 min at 1,880 *g* (Beckman Allegra 6R, 2868 rpm). After centrifugation, the tubes were allowed to come to a stop with the brake off. The pellet and fluffy layer just above the pellet were collected and used to prepare canalicular membranes, while the supernatant was used to prepare basolateral membranes.

Canalicular membrane preparation

Pellet and fluffy layer were resuspended in 50 ml of SHCa buffer, homogenized with six strokes of a loose-fitting Dounce homogenizer, and centrifuged for 10 min at 3,000 *g* (Beckman Allegra 6R, 3,624 rpm). The resulting pellet was suspended in 50 ml of SHCa buffer, homogenized with six strokes of a loose-fitting Dounce homogenizer, placed in a beaker within an ice-cold high-pressure chamber (Parr Instrument Model 4635), and equilibrated for 15 min with nitrogen at 900 pounds per square inch by stirring. After 15 min the chamber's pressure was brought within 3 min to atmospheric pressure. The suspension was then homogenized with six strokes in a tight-fitting Dounce homogenizer, diluted with SHCa buffer to 120 ml, supplemented with $CaCl_2$ to a final concentration of 10 mM, incubated for 10 min on ice, and centrifuged for 20 min at 7,600 *g* (Beckman L6 centrifuge, SW28 rotor, 6,500 rpm at 4°C, brake on). After centrifugation, the supernatant was filtered through fine nylon mesh and the filtrate centrifuged for 30 min at 47,000 *g* (Beckman SW28, 16,200 rpm at 4°C). The pellet was then homogenized in 30 ml of SHCa buffer with six strokes in a tight-fitting Dounce homogenizer and centrifuged for 10 min at 3,000 *g* (Beckman Allegra 6R, 3,624 rpm). The supernatant was collected and centrifuged for 30 min at 47,000 *g* (Beckman SW28, 16,200 rpm at 4°C) and the pellet suspended in SHCa buffer using a 1 ml syringe attached to a 28-gauge needle at a concentration of 1–2 mg/ml protein and stored at −80°C until used. In a typical preparation, the livers from 20 mice yielded 150–250 μg of canalicular membrane protein.

Basolateral membrane preparation

The first supernatant was separated and centrifuged for 10 min at 5,500 *g* (Sorval GSA rotor, 5,800 rpm) and supernatant and fluffy layer collected and centrifuged for 30 min at 22,000 *g* (Sorval GSA rotor, 11,600 rpm). The pellet was resuspended in 12 ml of calcium-free SH buffer containing 1 mM EDTA (pH 7.4), homogenized with six strokes in a loose-fitting Dounce homogenizer, and layered on a discontinuous gradient consisting of 1 ml 60% sucrose, 23 ml 23% sucrose, 4% Ficoll 400, and 7 ml 20% sucrose. After 90 min centrifugation at 130,000 *g* (Beckman

swinging bucket rotor SW 28, 27,000 rpm), the interphase between the 20% sucrose and 23% sucrose 4% Ficoll layers was aspirated, diluted 6-fold with SHCa buffer, and centrifuged for 30 min at 47,000 *g* (Beckman SW-28 rotor, 20,000 rpm). The pellet was resuspended in SHCa buffer and centrifugation repeated as above. The resulting pellet was resuspended in SHCa buffer with a syringe and 28-gauge needle at a concentration of ~10 mg/ml protein and stored at −80°C until used. A typical preparation yielded 2.5–5 mg of basolateral membrane protein.

Preparation of liver cytosol

Liver tissue (0.5 g) was homogenized on ice in 7 ml of ice-cold homogenization buffer (100 mM K_2HPO_4, 1 mM EDTA, 5 mM DTT, 50 mM KCl, 5% glycerol, pH 7.4) by seven strokes of a tight-fitting Dounce homogenizer. The homogenate was spun for 10 min at 2,000 *g* at 4°C (Beckman Allegra 6R, 2,960 rpm), and the resultant supernatant spun for 20 min at 10,000 *g* (Beckman SW55 rotor, 10,300 rpm at 4°C). The supernatant of the second spin was subjected to ultracentrifugation at 100,000 *g* for 90 min (Beckman SW55 rotor, 32,500 rpm at 4°C), and cytosol (supernatant) was collected and stored at −80°C.

Alkaline phosphatase activity

Alkaline phosphatase activity was measured in whole-liver homogenate and canalicular or basolateral membrane fractions. Aliquots containing 10–20 μg of protein were assayed colorimetrically using a commercially available kit (Sigma kit Cat # 245-10). Activity was expressed as units/mg protein/min.

Biliary protein extraction

The gallbladder bile of C57BL/6J males was aspirated as previously described (6) and biliary proteins were isolated following bile delipidation. Briefly, 50 μl of bile were mixed in an Eppendorf tube with 1 ml of ice-cold ethyl-ether/ethanol (1:3; v/v), vortexed, incubated for 1 h at −20°C, spun for 30 min at room temperature, the supernatant discarded, and the pellet resuspended in 1 ml of ice-cold ethyl-ether/ethanol (2:3; v/v). Resuspended pellet was incubated for 1 h at −20°C, spun for 30 min at 4°C, the supernatant discarded, and the pellet resuspended in 1 ml of ice-cold ethyl-ether, vortexed, incubated for 1 h at −20°C, spun for 30 min at 4°C, and the last ethyl-ether extraction repeated one more time as above. The final pellet was allowed to evaporate at room temperature, dissolved in ddH_2O, and protein concentration measured using the bicinchoninic protein assay (Pierce Biotechnology, Rockford, IL).

SDS-PAGE, Coomassie staining, zinc staining, and Western blotting

Ten to fifty micrograms of biliary proteins or canalicular or basolateral membrane fractions were resuspended in an equal volume of DTT containing Novex Tris-Glycine Sample Buffer, boiled for 5 min, and loaded on a Novex polyacrylamide Tris-Glycine gel and subjected to electrophoresis at 100 V for 2–3 h. For Coomassie staining, gels were soaked in a Coomassie blue solution for 1 h and destained with 10% PBS-methanol. For zinc staining, a Bio-Rad zinc staining and destaining kit was used according to the manufacturer's Instruction Manual (Bio Rad Laboratories, Hercules, CA). For Western blots, proteins were subjected to overnight transfer at 110 mA onto a nitrocellulose membrane, prehybridized in casein blocker, and hybridized with either anti-mdr2 (monoclonal C219, Signet Laboratories, Dedham, MA), anti-Ntcp, anti-SR-BI (polyclonal NB 400-104, Novus Biologicals, Inc., Littleton, CO), anti-apolipoprotein A-I (apoA-I) (polyclonal K23500R, BIODESIGN International, Saco, ME), anti-BHMT (a generous gift of Dr. Timothy A. Garrow, University of Illinois, Urbana, IL), anti-methionine adenosyltransferase (MAT) (a generous gift of Dr. Jose M. Mato, Universidad de Navarra, Pamplona, Spain), or anti-PEMT2 antibodies, washed in PBS Tween buffer, incubated with the appropriate secondary antibody, and detected by chemiluminescence using NEN Luminol and Oxidizing reagents.

Mass spectrometry

The protein bands separated by SDS-PAGE were visualized by zinc staining (Bio Rad Laboratories). The bands of interest were excised, subjected to in-gel digestion with trypsin, and the resulting peptide mixtures extracted as described (7). Peptide mixtures were analyzed with MALDI-TOF MS using a delayed ion extraction and ion mirror reflector mass spectrometer (Voyager-DE STR; Perseptive Biosystems). The measured masses of the tryptic peptides (tryptic peptide map) were used to search for protein candidates in the nonredundant protein sequence database with the program ProFound (ProteoMetrics, New York, NY) (8). To confirm the protein identification results obtained from tryptic peptide mapping, peptide mixtures were analyzed by tandem MS fragmentation analyses using HPLC-ion trap mass spectrometry (LCQ, Finnigan MAT, San Jose, CA) equipped with a capillary HPLC (Magic 2002, Michrom BioResources, Auburn, CA) (9) and the protein search algorithm PepFrag (ProteoMetrics) (8).

Immunofluorescent microscopy and confocal imaging

Livers were cut into pieces less than 1 mm thick, embedded in OCT compound (Miles, Elkhart, IN), and frozen in liquid nitrogen. Cryostat sections (8–10 μm in thickness) were picked up on poly-L-lysine-coated glass slides, air-dried, and fixed in acetone for 15 min at −20°C. Ten percent goat serum in Tris-buffered saline was applied to block nonspecific binding. Samples were then incubated with individual or mixtures of anti-Mrp2 (Alexis Biochemicals, San Diego, CA), anti-PEMT2, and/or immunopurified anti-SR-BI antibodies (Novus Biologicals) diluted 1:100, 1:200, and 1:400, respectively, for 30–40 min (PEMT studies) and 1 h (SR-BI studies) at room temperature. After rinsing with Tris-buffered saline, the samples were incubated with the mixture of corresponding secondary antibodies (conjugated with Alexa 594 and Alexa 488, respectively) (Molecular Probes, Eugene, OR), and diluted 1:400 for 1 h at room temperature. In controls, specimens were either incubated with nonimmune IgG or the primary antibodies were omitted from the labeling process. After a final washing step, the sections were mounted with a ProLong antifade reagent (Molecular Probes, Leiden, The Netherlands), covered with glass, and examined using a confocal laser scanning microscope LSM 410 (Carl Zeiss, Jena, Germany). Obtained images were saved to MO disk and processed on a Macintosh Dual G4 Power PC (Apple Computer, Cupertino, CA). Adobe Photoshop 7 and Adobe Illustrator 10 software (Adobe Systems, San Jose, CA) was used to adjust brightness and contrast of the images and to compose them in plates. Plates were printed directly using a Fuji Pictrography 3000 printer (Fuji Photo Film, Tokyo, Japan).

RESULTS

A number of tests were performed to assess the quality and purity of the isolated liver canalicular membrane fraction. Kipp and Arias reported that liver canalicular membranes are enriched in alkaline phosphatase (5). Therefore, this enzymatic activity was assessed in whole-liver homogenate and canalicular or basolateral membrane fractions and found to be 0.06 ± 0.05, 2.66 ± 1.36, and 0.11 ± 0.03 U/mg

protein/min, respectively (mean ± SD of 10 different preparations). Thus the nitrogen cavitation/calcium precipitation method yielded canalicular membranes that were 44- and 24-fold enriched in alkaline phosphatase compared with whole-liver homogenates and basolateral membrane fractions, respectively. To assess cross-contamination between membrane fractions, immunoblotting was carried out for the canalicular membrane marker, mdr2, and the basolateral membrane marker, Ntcp, the sodium-dependent bile acid transporter. As shown in **Fig. 1**, mdr2 localized exclusively to the canalicular membrane, and Ntcp is mainly in basolateral membrane (276-fold enrichment by scan densitometry). These results clearly indicate that the mouse liver canalicular membrane fraction isolated using the protocol described in this paper is highly concentrated with no evidence of major cross-contamination with basolateral membrane fractions.

Proteins residing in the canalicular membrane fraction were next identified. Two different preparations of canalicular and basolateral membrane fractions were subjected to SDS-PAGE and the results shown in **Fig. 2**. As shown, many differences exist in the protein-banding pattern between the two membrane fractions. Three discrete canalicular protein bands labeled in this figure as 1 (108 kDa), 2 (54 kDa), and 3 (50 kDa) were analyzed with MALDI-TOF MS. As shown in **Table 1**, these bands corresponded to radixin, BHMT, and γ-actin, respectively. The identities of the BHMT and γ-actin bands were confirmed by trypsinization and tandem-MS fragmentation analysis. Radixin is an actin barbed-end capping protein present at the undercoat of the cell-to-cell adherence junction and has been previously shown to occur at high concentrations in canalicular membrane preparations (10). γ-Actin is a cytoskeletal protein. BHMT is an enzyme that catalyzes the transfer of a methyl group from betaine to homocysteine. Immunoblotting was used to further characterize the subcellular localization of BHMT and the results shown in **Fig. 3**. BHMT is present in canalicular membranes, to a lesser extent in basolateral membranes, and, as expected, to a greater extent in cyotsol (11).

BHMT catalyzes the first step in a pathway that promotes the methylation of PE to PC. Immunoblotting was also used to localize the other two enzymes of this pathway. As shown in Fig. 3, the second enzyme, MAT, is cytosolic and not present on either the canalicular or basolateral membranes, whereas the third enzyme, PEMT, is present in canalicular membranes, to a lesser extent in basolateral membranes, and not present in cytosol (12).

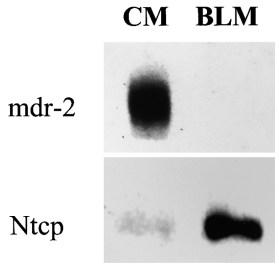

Fig. 1. Membrane localization of the apical phospholipid transporter mdr2 and basolateral bile acid transporter Ntcp to isolated canalicular membrane (CM) and basolateral (BLM) membrane preparation. CM and BLM were isolated using the nitrogen cavitation/calcium precipitation method and subjected to Western blotting with specific anti-mdr2 and anti-Ntcp antibodies as detailed in Materials and Methods.

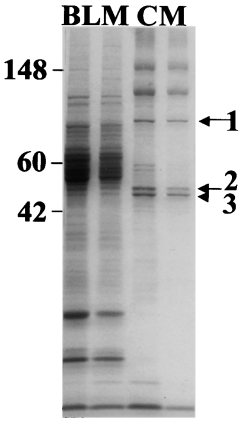

Fig. 2. SDS-PAGE protein profiles of BLM and CM preparations. Samples (10 μg protein/lane) of two different BLM preparations and two different canalicular membrane preparations were loaded on a 10–20% tris-glycine gel and Coomassie stained as described in Materials and Methods. CM bands 1 (Radixin), 2 [betaine homocysteine methyltransferase, (BHMT)], and 3 (γ-actin) were characterized using mass spectrometry analysis as described in Materials and Methods and detailed in Table 1.

For further characterization of PEMT localization, we applied immunofluorescent microscopy and compared the hepatocytic localization of PEMT to that of MRP2 (multidrug resistance-associated protein 2), an ABC transporter that is confined to the canalicular membrane. As shown in **Fig. 4A**, anti-MRP2 antibodies specifically stained the canalicular membranes (arrows). It is of note that the staining with anti-PEMT antibodies revealed a pattern that resembled the staining with anti-MRP2, with the most intense staining localized to the canalicular membranes (Fig. 4B, arrows). Moreover, as shown in Fig. 4C, the colocalization of PEMT and MRP2 is most evident when the staining of these proteins is overlapped. These results unequivocally indicate that PEMT localizes to the canalicular membrane. It is important to note, however, that the staining with anti-PEMT antibodies is not confined to the canalicular membrane and staining was clearly detected in areas that may correspond to a specific endoplasmic reticulum (ER) membrane referred to as mitochondria-associated membrane (MAM) (13). Finally, to exclude the possibility that the immunofluorescent microscopy studies detected PEMT in the biliary canalicular space, we extracted the bile proteins and tested for the presence of PEMT. Whereas immunoblotting studies clearly revealed the presence of the biliary resident protein apoA-I, anti-PEMT antibodies failed to find PEMT in the bile (data not shown).

Several studies provide evidence that SR-BI is involved in biliary cholesterol excretion. Induced mutant mice overexpressing SR-BI in liver have increased biliary cholesterol concentrations (14, 15), and SR-BI-mediated HDL

TABLE 1. Mass spectrometry analysis of canalicular membrane proteins (see also Fig. 2)

Band	Protein Identification by MS (GenBank Acc. #)	Molecular Weight (kDa[a])	Protein Identification Confirmed by MS/MS	
			Peptide Molecular Weight	Sequence
1	Radixin (gi|131820|)	68.4 (108[b])	—	—
2	BHMT (gi|2645804|)	45.0 (54[b])	1,097.6	AIAEELAPER
			1,136.6	KEYWQNLR
			1,860.9	QGFIDLPEFPFGLEPR
			1,934.0	LNAGEVVIGDGGFVFALEK
3	γ-Actin (gi|809561|)	41.8 (50[b])	1,131.5	GYSFTTTAER
			1,160.6	EITALAPSTMK
			1,170.6	HQGVMVGMGQK
			1,197.5	DSYVGDEAQSK
			1,789.9	SYELPDGQVITIGNER
			1,959.9	YPIEHGIITNWDDMEK

BHMT, betaine homocysteine methyl transferase; MS/MS, tandem mass spectrometry.
[a] Molecular weight based on SWISS-PROT database.
[b] Molecular weight based on SDS-PAGE (Fig. 2).

particle uptake by liver results in selective sorting of HDL cholesterol from protein and polarized cholesterol excretion (16). As shown in **Fig. 5**, immunoblotting indicates that SR-BI is present at roughly equal concentrations in both the canalicular as well as the basolateral membrane. Moreover, to validate our immunoblot findings, we applied immunofluorescent microscopy to mouse liver sections and tested for the cellular localization of MRP2 and SR-BI (**Fig. 6**). As shown in Fig. 6B, anti-SR-BI antibodies stained membranes that are both common to (arrows) and different from (arrow heads) membranes that are stained by anti-MRP2 antibodies (Fig. 6A). Moreover, as shown in Fig. 6C, a colocalization of SR-BI and MRP2 is most evident when the staining of these proteins is overlapped. These results unequivocally show that SR-BI localizes to the canalicular membrane, which suggests that this receptor may participate in the transfer of canalicular-membrane cholesterol to the adjacent biliary compartment.

DISCUSSION

In the present study, the canalicular membrane fraction from mouse liver was isolated for the first time and MALDI-TOF MS was used to identify the presence of the BHMT enzyme. This enzyme catalyzes the first step in a three-enzyme pathway that promotes the methylation of PE to PC, a major biliary excretory product. Immunoblotting of canalicular membranes confirmed the presence of BHMT along with PEMT, an enzyme that catalyzes the third step in the conversion of PE to PC, and immunofluorescent microscopy confirmed the localization of PEMT to the canalicular membrane. Immunoblotting also revealed the presence of SR-BI in canalicular membranes, and these findings were confirmed by immunofluorescent microscopy. These findings indicate that the PE methylation pathway operates at or in close proximity to the canalicular membrane and possibly contributes to phospholipid and cholesterol excretion into bile. A schematic of this pathway is provided in **Fig. 7**.

The flux of phospholipid through the liver each day is large and, in the mouse, is estimated to approximate the entire liver phospholipid pool (17). This phospholipid pool is derived largely from de novo synthesis by two pathways. In one pathway present in all nucleated cells, CDP-choline and diacylglycerol react to form PC catalyzed by CDP-choline:diacylglycerol phosphocholinetransferase. In the PE methylation pathway, which is quantitatively significant only in liver, betaine transfers a methyl group to homocysteine via BHMT to form methionine. Methionine then transfers a methyl group to adenosine via MAT to form *S*-adenosylmethionine. Finally, three methyl groups are transferred from *S*-adenosylmethionine to PE to form PC. Under normal metabolic conditions, it is thought that the CDP-choline pathway accounts for 60–80% of liver PC biosynthesis (18). The CDP-choline and the PE methylation pathways can both provide PC for lipoprotein and membrane biosynthesis and bile secretion (19). In the current study, we show that BHMT and PEMT also localize to basolateral and canalicular membranes, suggesting they may serve to convert PE, known to be present in relatively high concentrations in the inner leaflet of plasma membranes, to PC for specific needs (20, 21). We propose that the PC formed at the inner leaflet of the canalicular mem-

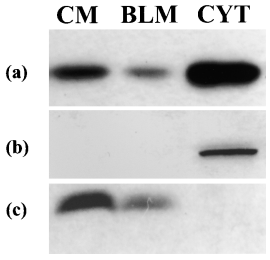

Fig. 3. Cellular localization of (A) BHMT, (B) methionine adenosyltransferase (MAT), and (C) phosphatidylethanolamine *N*-methyltransferase (PEMT). Samples (10 μg protein/lane in BHMT and MAT and 50 μg protein/lane in PEMT blots) of CM, BLM, and cytosol (CYT) used for Western blot using specific antibodies as described in Materials and Methods.

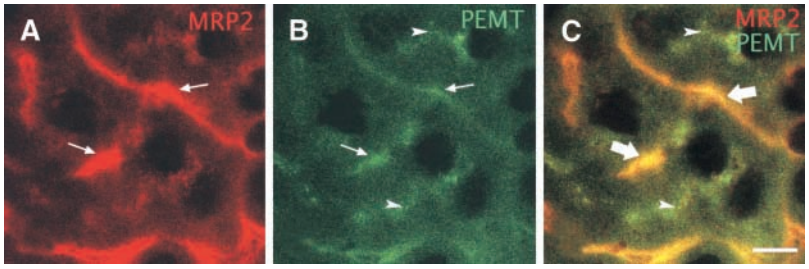

Fig. 4. Colocalization of PEMT and MRP2 to liver canalicular membranes. Liver sections where prepared and processed for immunofluorescent microscopy as described in Materials and Methods. Confocal laser scanning micrographs show fluorescence for MRP2 (A, red signals) and PEMT (B, green signals). The protein specifically visualized is indicated by colored text. C: Overlapped staining for both MRP2 and PEMT (yellow staining). Thin and thick arrows point to hepatocytes canaliculi, whereas arrowheads point to intracellular localization of PEMT, presumably to mitochondrial-associated membranes. Bar, 10 μm.

brane plays an important role in biliary phospholipid excretion. The convenient juxtaposition of a PC synthesis pathway to mdr2, which in turn flips PC to the outer leaflet, could efficiently contribute to biliary phospholipid excretion.

Phospholipid excretion into the bile can be regulated by the activity of mdr2 as well as by the availability of newly synthesized PC. The mdr2 knockout mouse completely fails to excrete phospholipid into the bile, and EM studies show these mice fail to bud, and pinch off vesicles normally involved in phospholipid excretion (22). Evidence for regulation by newly synthesized phospholipid is present but less direct. For example, it has been shown that biliary phospholipid and cholesterol excretion are tightly coupled. In the mdr2 knockout mouse, failure to excrete biliary phospholipid is accompanied by a failure to excrete biliary cholesterol (2). Conversely, cholesterol excretion can also affect phospholipid excretion. In a recent study, we showed that feeding a 0.5% cholesterol diet to C57BL/6J mice increased biliary cholesterol excretion, and this was accompanied by an increase in biliary phospholipid excretion (6). Furthermore, more recent studies in ABCG5/ABCG8 transgenics have shown that the stimulation of cholesterol excretion increases the excretion of biliary phospholipids (3). It is possible that the canalicular membrane synthesis of PC from PE is involved in this increase in phospholipid excretion.

The majority of BHMT is located in the cytosol (11), and we cannot absolutely rule out that in our immunoblotting studies some of the canalicular and basolateral membrane BHMT represents contamination by other cellular compartments. As for PEMT, previous immunohistochemistry studies using electron microscopy have placed this enzyme on a special ER membrane fraction associated with mitochondrial preparations (13) now referred to as MAM. Moreover, recent studies by Shields et al. could show that PEMT is an integral membrane protein with four transmembrane domains (23). Our immunofluorescent microscopy studies unambiguously show that, in addition to its localization to MAM, PEMT localizes to the canalicular membrane (Fig. 4). These findings are supported by previous studies in hamsters' canalicular membrane preparations (24). It is of note that studies in PEMT knockout mice revealed no change in biliary phospholipid levels (25). These findings apparently challenge the model in Fig. 7. However, it is also important to note that in PEMT knockout animals, the exclusion of the PE methylation pathway was associated with a 60% increase in the activity of the CDP-choline pathway. Because the PE methylation pathway accounts for 20–40% of hepatocytic phospholipid synthesis, a 60% increase in biosynthesis may fully compensate for the elimination of PEMT-dependent canalicular membrane PC production, thereby maintaining a normal biliary phospholipid excretion. Therefore, it should be noted that although our study unambiguously localized the PE pathway to the canalicular membrane, further experimentation is needed to prove the authenticity of the model in Fig. 7. In addition, the specific docking mechanisms for BHMT to the basolateral and canalicular membranes have not been studied. A search for potential *N*-myristoylation or palmitoylation sites, which may anchor BHMT to cellular membranes, did not disclose the unambiguous consensus sequences necessary for these modifications. Therefore, further experimentation is warranted to address the docking mechanisms.

The localization of SR-BI to the canalicular membrane

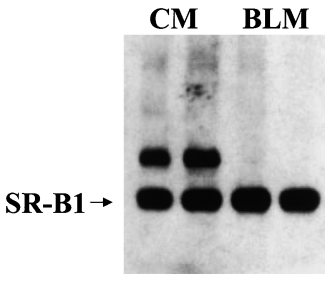

Fig. 5. Scavenger receptor class B type I (SR-BI) localization to CM and BLM. Samples (10 μg protein/lane) of two different CM preparations and two different BLM preparations were subjected to SDS-PAGE and Western blots using anti-SR-BI antibodies as described in Materials and Methods. Arrow points to the SR-BI band. In CM preparations the band just above SR-BI represents a cross-reacting protein and is currently under investigation.

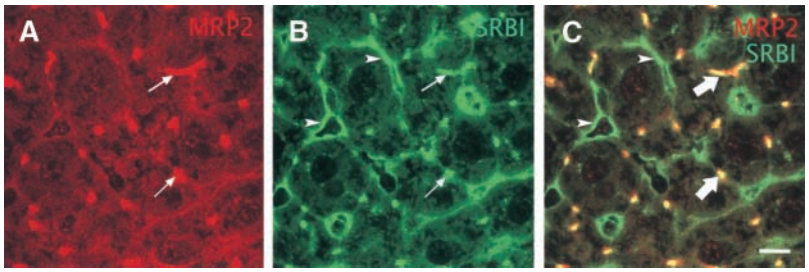

Fig. 6. Colocalization of SR-BI and MRP2 to liver canalicular membranes. Liver sections where prepared and processed for immunofluorescent microscopy as described in Materials and Methods. Confocal laser scanning micrographs show fluorescence for MRP2 (A, red signals) and SR-BI (B, green signals). The protein specifically visualized is indicated by colored text. C: Shows overlapped staining for both MRP2 and SR-BI (yellow staining). Thin and thick arrows point to hepatocytes canaliculi, whereas arrowheads point to localization of SR-BI to basolateral membranes. Bar, 10 μm.

has intriguing physiological implications. Studies in SR-BI transgenics and knockout mice show stimulated and suppressed biliary cholesterol excretion, respectively (14, 15, 26–28). Utilizing confocal microscopy, Silver et al. have recently shown in primary mouse hepatocytes that basolateral SR-BI is internalized, localizes to the endocytic recycling and subapical compartments, and appears to localize to the canalicular membrane (16). Furthermore, Ikemoto et al. could show that SR-BI is localized to canalicular membrane preparations in the rat (29). In contrast, two recent studies that applied immunocytochemistry and single-staining immunofluorescence microscopy claimed that SR-BI is not associated with the canalicular membrane (30, 31). To settle the controversy in the literature, we subjected liver sections to double-staining immunofluorescence microscopy. Our results unambiguously show that SR-BI is localized to the canalicular membrane (Fig. 6). Because SR-BI has been implicated in selective uptake of HDL cholesterol by the hepatocyte basolateral membrane, its presence in the canalicular membrane implies a role in biliary cholesterol excretion. Evidence that SR-BI plays a role in the unidirectional flux of cholesterol has been presented in cell culture studies (32, 33). In the model presented in Fig. 7, the SR-BI hairpin structure projects into the canalicular space and interacts with biliary vesicles or micelles to promote the transfer of cholesterol from the external leaflet of the canalicular membrane into the bile. Recent studies in ABCG5/ABCG8

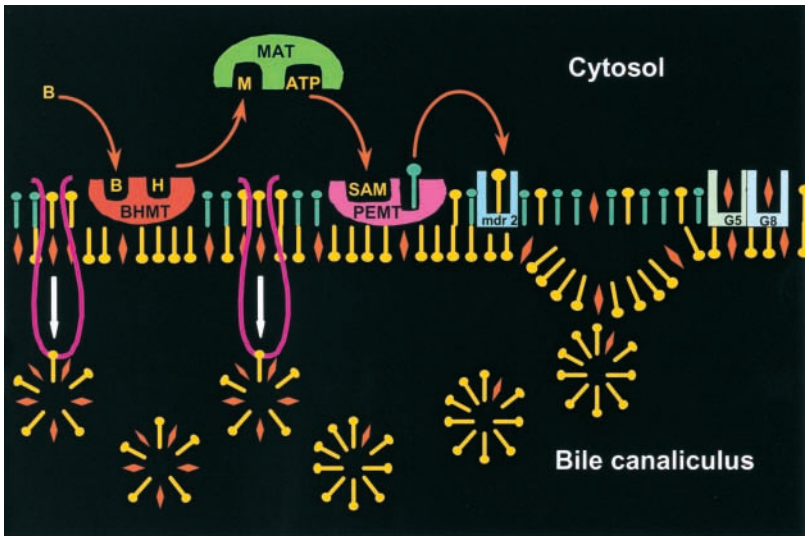

Fig. 7. Proposed model of how canalicular membrane phosphatidylcholine synthesis, mdr2, and SR-BI coordinately promote biliary cholesterol excretion. According to this model, BHMT is associated with the cytoplasmic leaflet of the canalicular membrane, where it catalyzes the transfer of a methyl group from betaine (B) to homocysteine (H). Methionine (M), the product of this reaction, becomes a substrate for cytoplasmic MAT that utilizes adenosine triphosphate (ATP) to generate *S*-adenosylmethionine (SAM). SAM becomes a methyl group donor and a substrate for canalicular membrane-associated PEMT. The methylation of PE to phosphatidylcholine (PC) culminates by mdr2 translocation of PC from the cytoplasmic to the canalicular-leaflet, budding, and secretion of PC and cholesterol-containing vesicles into the canalicular space. Once in the bile canaliculus, vesicles interact with hairpin-structured SR-BI (probably through their phospholipid moiety), which further promotes (white arrows) the transfer of canalicular membrane cholesterol to biliary vesicles. G5 = ABCG5, G8 = ABCG8.

knockouts could show that these genes play a major role in the excretion of cholesterol into the bile (34). It should be noted however that *i*) the disruption of the ABCG5/ABCG8 pathway largely reduced but did not abolish the excretion of cholesterol into the bile, and *ii*) ABCG5/ABCG8 may play a role in flipping cholesterol from the inner to the outer leaflet of the canalicular membrane, but do not preclude a role for SR-BI in facilitating the transfer of cholesterol from the outer leaflet to vesicles and micelles in the canalicular space.

In summary, in the present study, we have shown that BHMT and PEMT, enzymes involved in the methylation of PE to PC, localized to canalicular membranes. We speculate that this localization enables the synthesis of PC at close proximity to its biliary excretion site. Moreover, we provide clear evidence that SR-BI is localized to the canalicular membrane and believe that local synthesis of PC, together with the presence of SR-BI, facilitates the excretion of cholesterol into the bile. The elucidation of the regulation of this process at the molecular level may shed new light on factors that modulate biliary phospholipid and cholesterol excretion.

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