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The role of *mdr2* P-glycoprotein in hepatobiliary lipid transport

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In the series on the hepatocyte canalicular membrane, this review is the first addressing one of the important canalicular proteins that belong to the superfamily of the ATP-binding cassette (ABC) transporters. These proteins mediate the primary-active ATP-dependent unidirectional transport of substrates across membranes. ABC transporters in the canalicular membrane provide a driving force for bile flow and play a key role in the hepatobiliary elimination of endogenous and xenobiotic substances. The genes encoding ABC transporters include the multidrug resistance (MDR) genes, and one of them is the murine *mdr2* gene. The *mdr2* gene product has been identified as a major protein in the hepatocyte canalicular membrane. The function of the *mdr2* gene product has been elucidated following its homozygous disruption, which leads to a lack of phosphatidylcholine secretion from the hepatocyte across the canalicular membrane into bile, and to liver disease. The review describes the fascinating progress made within a few years in our understanding of the physiological role of this canalicular ABC transporter and its likely relevance for human liver disease.

—Dietrich Keppler, Coordinating Editor

ABSTRACT The small apical (canalicular) domains of hepatocytes form a luminal meshwork of tubules between adjacent hepatocytes and are the sites of primary bile formation. Organic compounds are transported across this membrane domain against high concentration gradients. It has been recognized in recent years that the hepatocyte is harnessed with a set of canalicular ATP-dependent transport proteins, specialized in this uphill transport. Bile salts, organic anions, cations, and neutral amphipaths are all pumped into the bile via such primary active transporters. Functionally, these transporters resemble ABC transporters overexpressed in cells with the multidrug resistance phenotype. Indeed, those transporters that have been characterized at the molecular level turn out to be

new, or already recognized, members of this family. Phospholipid secretion across the canalicular membrane of the mouse is also mediated by a member of this family, *mdr2* P-glycoprotein. This was demonstrated by the absence of phospholipid secretion into bile of mice with a disrupted *mdr2* gene and by subsequent demonstration of phospholipid translocation in cells that overexpress this protein. The recognition of *mdr2* P-glycoprotein as a phospholipid flippase sheds new light on the function of P-glycoproteins and is an important step in understanding the mechanism of biliary lipid secretion. — Oude Elferink, R. P. J., Tytgat, G. N. J., and Groen, A. K. The role of *mdr2* P-glycoprotein in hepatobiliary lipid transport. *FASEB J.* 11, 19–28 (1997)

Key Words: bile · canalculus · phospholipid · cholesterol · ABC transporters · bile salts · flippase · multidrug resistance

ON A MASS BASIS, bile salts are the most important components of bile; their function is to emulsify dietary lipids in the intestine. The second most important components are the biliary lipids phosphatidylcholine and cholesterol. The human liver secretes about 1 g cholesterol and 4 g phosphatidylcholine into bile per day. A substantial part of these lipids is reabsorbed in the intestine, thereby constituting an enterohepatic cycle. The functions of biliary lipid secretion are multiple. First, biliary secretion of cholesterol represents the only way the body can dispose of this molecule, apart from its metabolism to bile salts. Second, the secretion of phosphatidylcholine assists the solubilization of cholesterol and thereby prevents crystallization of this hydrophobic molecule in the biliary tract, and thus formation of cholesterol gallstones. A third important function of phosphatidylcholine is to inactivate the detergent action of bile salts in order to prevent damage to epithelial cells lining the bile duct and the gallbladder.

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Figure 1. Diverse types of bile salts. *A)* The basic structural formula of the bile salt molecule is depicted in a flat formula (top) and one that shows the 3-dimensional configuration (bottom). The latter clearly shows that hydroxyl groups in the α -configuration have a different position than in the β -configuration. *B)* The space-filling models of deoxycholate, and ursodeoxycholate demonstrate the importance of the stereospecific localization of the hydroxyl groups; in deoxycholate, the hydroxyl groups (blue) are concentrated on one side of the steroid nucleus (orange), which makes it more hydrophobic.

The mechanism of biliary lipid secretion is poorly understood. This lack of insight is largely caused by the unique architecture of the liver, with the canalicular lumen as a virtually inaccessible space. In addition, biliary secretion is a unique, specialized function of the liver; no proper *in vitro* models exist so far in which the canalicular lipid secretion process can be mimicked. Probably for this reason, an important breakthrough in the understanding of lipid secretion had to come from a transgenic mouse model. In an attempt to define the physiological function of the mouse *mdr* gene products, knockout mice were produced for each of these genes. In knockouts for the *mdr2* gene, biliary lipid secretion turned out to be completely

abrogated, and this pointed to a function of *mdr2* P-glycoprotein (Pgp)² in lipid transport (1). This function would never have been found in an *in vitro* model like a transfected cell line; this demonstrates the value of transgenic animal models in the elucidation of processes for which the integrity of a complex organ is essential.

BILIARY LIPID SECRETION: THE ROLE OF BILE SALTS

Biliary lipid secretion is driven by bile salts. The tight coupling between lipid and bile salt secretion has been demonstrated in numerous animal species (for a recent review, see ref 2), although the ratio of lipid to bile salt, as well as the type of secreted bile salt, varies considerably. **Figure 1** and **Table 1** display some of the most important bile salt species. The common denominator is the steroid nucleus; the different types of bile salt have one, two, or three hydroxyl groups at various sites in the molecule. The number of hydroxyl groups and the stereospecific position in the molecule determine the hydrophobicity of the molecule and its detergent properties. The carboxyl group is always conjugated with either glycine or taurine. Human bile contains predominantly glycine conjugates of cholate, chenodeoxycholate,

TABLE 1. The diversity of bile salts

Bile salt (taurine conjugate)	R ₁	R ₂	R ₃	R ₄	H _i
Lithocholate	α OH	H	H	H	1.00
Deoxycholate	α OH	H	α OH	H	0.59
Chenodeoxycholate	α OH	α OH	H	H	0.46
Cholate	α OH	α OH	α OH	H	0.00
Ursodeoxycholate	α OH	β OH	H	H	-0.47
β -muricholate	α OH	β OH	H	β OH	-0.78

The table gives the location of the hydroxyl groups on the steroid nucleus of various physiological bile salt molecules. The location of the hydroxyl groups is indicated by R₁ to R₄ and corresponds with those in Fig. 1. The number and the stereospecific localization of the hydroxyl groups determine the hydrophobicity of the molecule; this is indicated by the hydrophobicity index (H_i), which was based on its behavior in reverse-phase HPLC as performed by Heuman et al. (55). These indices were related to the behavior of taurocholate as (H_i/i = 0).

² TC, taurocholate; Pgp's, P-glycoproteins; MDR, multidrug resistance; PC, phosphatidylcholine; TDC, taurodeoxycholate; PE, phosphatidylethanolamine; TP, transfer protein; NBD, 7-chloro-4-nitrobenz-2-oxa-1,3-diazole.

and deoxycholate, whereas rat and mouse bile contains only the taurine conjugates. Rat bile contains mainly taurocholate (TC), whereas tauromuricholate is the most important constituent of mouse bile.

In many studies, a clear-cut relation is observed between the rate of bile salt secretion and that of biliary lipids. There is a hyperbolic increase in phospholipid secretion with increasing bile salt output, and the slope of this relation is steeper during secretion of hydrophobic bile salts compared to more hydrophilic bile salts (2) (see also below). These observations suggest that bile salts in some way solubilize phospholipid (and cholesterol) from the canalicular membrane.

A second key observation made in various species is the striking difference between the composition of phospholipids in bile and in the canalicular membrane. Biliary phospholipids consist almost exclusively of phosphatidylcholine (PC; >95%) whereas the canalicular membrane also contains sphingomyelin ($\pm 20\%$), phosphatidylethanolamine (PE) ($\pm 20\%$), and phosphatidylserine ($\pm 20\%$). Furthermore, PC in bile is more hydrophilic than the membrane PC. It contains predominantly palmitate (16:0) at the sn₁ position and oleate (18:1) or linoleate (18:2) at the sn₂ position, whereas the sn₁ position of canalicular membrane PC contains more stearate (18:0) (for review, see ref 2). Despite its different composition, the canalicular membrane is thought to be the source of biliary phospholipids. Retrograde injection of bile salt into the biliary tree elicits a burst of "bile-type" lipids, indicating that the mere contact of detergent with the canalicular membrane is sufficient to drive lipid secretion (3).

On the basis of these observations, the most commonly accepted model for biliary phospholipid secretion involved passive extraction of canalicular membrane PC by micellar bile salts in the canalicular lumen (for review, see ref 2). PC was thought to be transported to the canalicular membrane via vesicles particularly rich in "bile-type" PC, which are synthesized in the endoplasmic reticulum. After insertion in the membrane, these PC molecules were thought to reside in microdomains. Interaction of bile salt monomers or micelles with these microdomains would lead to budding of the microdomain bilayer and subsequent release of PC-rich vesicles. The latter was necessary to explain the observed presence of phospholipid vesicles in bile (4, 5). It has, however, remained extremely difficult to prove that these vesicles represent the earliest event in canalicular release of lipid. The postulate of the existence of *bilayer* microdomains was a crucial problem in this theory; although it is well accepted that lipid microdomains exist in single leaflets, it is unlikely they can exist in a bilayer, because the lateral mobilities of lipids in the two leaflets of a bilayer are independent (see ref 6).

THE PHYSIOLOGICAL FUNCTION OF MDR2 P-GLYCOPROTEIN

The model of biliary lipid secretion described above needed adjustment as a result of the recent observation that *mdr2* P-glycoprotein plays a central role in biliary lipid secretion (1). This transporter protein is a member of the small family of P-

glycoproteins (Pgp's), which in turn belong to the superfamily of ATP-binding cassette (ABC) transporters. Members of this superfamily mediate the primary active translocation of all types of compounds across membranes. In mice, three genes were found to encode for Pgp's *mdr1a*, *mdr1b*, and *mdr2*. In humans, two genes were characterized: *MDR1* and *MDR3*. High homology was observed between the mouse *mdr2* and the human *MDR3* genes. Transfection of cells with the human *MDR1* or the mouse *mdr1a* or *mdr1b* gene conferred multidrug resistance (MDR) against various amphipathic drugs, which was mediated by the extrusion of these compounds from cells (for review, see ref 7). These Pgp's are predominantly expressed in epithelial cells of the intestine and kidney, in the adrenal gland, and in the endothelial cells that constitute the blood-brain barrier. The mouse *mdr2* and the human *MDR3* gene clearly deviate from the former Pgp's in that they are not involved in transport of amphipathic drugs. Furthermore, these Pgp's have a different tissue distribution; they are predominantly expressed in the canalicular membrane of the hepatocyte and to a much lower extent in muscle and spleen (8, 9). Their function remained obscure, however, for many years.

The elucidation of *mdr2* Pgp function was resolved when knockout mice for the *mdr* genes were produced (1). Bile from *mdr2* knockout mice was almost devoid of both phospholipid and cholesterol whereas bile salt secretion was normal. Fortunately, it could be readily inferred which of these changes represented the primary defect: bile of mice heterozygous for *mdr2* gene disruption had a 40% decreased phospholipid but normal cholesterol content. Infusion of taurodeoxycholate (TDC), which is more hydrophobic than the endogenous pool ($\pm 70\%$ muricholate and 30% cholate), also did not induce significant phospholipid secretion in *mdr2* (−/−) mice. In the heterozygotes, phospholipid secretion increased with TDC infusion but always remained reduced compared to the control mice infused with TDC at identical rates (Fig. 2). This strongly suggested that *mdr2* Pgp catalyzes a rate-controlling step in the biliary secretion of phospholipids, which is separate from the role of bile salt secretion.

MDR2 P-GLYCOPROTEIN IS A FLIPPASE

Based on its homology with the other P-glycoproteins it was hypothesized that *mdr2* Pgp is a transporter which functions as a flippase that translocates phosphatidylcholine from the inner to the outer leaflet of the canalicular membrane (1). This hypothesis was supported by subsequent experiments of Ruetz and Gros (10) who transfected the *mdr2* gene in a yeast secretion mutant. Isolation of secretory vesicles, in which the expressed *mdr2* Pgp had accumulated, provided an experimental model to assay the translocation of 7-nitrobenz-2oxa-1,3-diazole (NBD)-labeled phosphatidylcholine from the outer to the inner leaflet of the vesicular membrane. NBD-PC is a fluorescent derivative of PC, in which the fluorophore (NBD) is incorporated in one of the fatty acyl chains. ATP-dependent translocation of NBD-PC was observed that was specific for *mdr2* Pgp. No ATP-dependent NBD-PC translocation could

Figure 2. Relation between phospholipid and bile salt secretion in *mdr2* (+/+), (+/-), and (-/-) mice. *A*) This relation during secretion of endogenous bile salts (70% taurochenodeoxycholate and 30% taurocholate); there is a hyperbolic relation between phospholipid and bile salt excretion. *B*) Biliary phospholipid secretion during infusion of taurodeoxycholate, which is a more hydrophobic bile salt. It is clear that the amount of phospholipid per bile salt molecule is increased during secretion of the more hydrophobic taurodeoxycholate compared to the situation with endogenous bile salts. Under both conditions phospholipid excretion is virtually absent in *mdr2* (-/-) mice and reduced in *mdr2* (+/-) mice.

be observed in vesicles from a yeast strain that expressed *mdr1a* (*mdr3*) P-glycoprotein. The fraction of PC molecules that was translocated was very low; this could suggest that *mdr2* Pgp has a low affinity for NBD-PC. Alternatively, in the absence of subsequent extraction of phospholipid from the *trans* side of the bilayer, translocation may be largely halted by the phospholipid surplus on the *trans* side of the vesicles, which is created by *mdr2* Pgp. It was subsequently shown that TC is able to induce an increase in *mdr2*-mediated translocation, and this might be caused by extraction of NBD-PC from the inner leaflet of the vesicles by TC molecules. Indeed, under the same conditions, the nonmicelle-forming bile salt taurodehydrocholate had no effect (11). The intravesicular concentrations of TC that elicited this effect were very small (about 50 μ M) and far below the critical micellar concentration of TC (about 5 mM), suggesting that the observed phenomenon is not extraction by TC micelles, but rather aggregation of NBD-PC with TC molecules in the intravesicular lumen. These low intravesicular concentrations would be quite unable to extract normal PC from any type of membrane. Thus, if the reported induction of translocation indeed represents extraction by bile salts, it must be explained by the relatively hydrophilic character of the NBD-PC molecule as compared to normal PC. These experiments, therefore, do not yet provide information about the physiological mechanism of extraction of PC molecules from the canalicular membrane. Surprisingly, *mdr2*-mediated translocation could be inhibited by low concentrations of verapamil, an inhibitor of *mdr1a/1b* Pgp; this would imply that verapamil is recognized by both types of P-glycoproteins. In contrast, other *mdr1a/1b* substrates (colchicine and vinblastine) were without effect (10). The same assay was subsequently applied by Nies et al. (12) using rat canalicular membranes labeled with NBD-PC by incubation with fluorescent donor liposomes. In this system, a similar ATP-dependent translocation of NBD-PC was observed; as with yeast secretory vesicles, translocation was relatively slow (steady state reached in ± 10 min) and the translocation represented a minor fraction of the incorporated NBD-PC. In this system, too, translocation could be modestly stimulated (1.5-fold) by addition of taurocholate but not by taurodehydrocholate. The extent of ATP-dependent NBD-PC

translocation from external to internal leaflet was much lower in right side-out vesicles compared to inside-out vesicles, which is consistent with the asymmetric translocator function of *mdr2* P-glycoprotein.

Evidence supporting the flippase function of *mdr2* Pgp was also provided by Smith et al. (13), who used fibroblasts from transgenic mice that express MDR3, the human homolog of *mdr2* Pgp. After metabolic labeling of intracellular phosphatidylcholine with radioactive choline, translocation from the inner to the outer leaflet was assayed and compared with normal mouse fibroblasts, which do not express *mdr2*. Translocation of radioactive phosphatidylcholine to the outer leaflet was measured by the potential to exchange with phosphatidylcholine transfer protein (PC-TP) and liposomes in the medium. In MDR3 expressing fibroblasts, a threefold more rapid translocation of PC was observed than in control fibroblasts.

These studies suggest that *mdr2* Pgp is indeed an ATP-dependent phosphatidylcholine translocator. Berr et al. (14) had previously described a PC-flippase activity in rat liver canalicular membranes. Similar to the classical studies of Bishop and Bell (15) which described a flippase activity in the endoplasmic reticulum, they used radioactive dibutyryl-PC (diC_4PC) as ligand for transport. Considerable translocation activity was observed in canalicular membranes which was even higher than in microsomal preparations. Kullack-Ublick et al. (16) recently extended this work by the isolation of a 1.8–2.6 kb mRNA fraction from rat liver, which gives rise to diC_4PC uptake when injected into *Xenopus laevis* oocytes. The K_m for diC_4PC (10 mM) was similar to that described in rat liver canalicular membranes. The observed PC-translocating activity in canalicular membranes was ATP-independent, and it is therefore unlikely that it was mediated by *mdr2* Pgp. Indeed, the isolated mRNA size class responsible for this activity was too small to code for *mdr2* P-glycoprotein. In addition, identical experiments were performed with canalicular membranes from control and *mdr2* knockout mice, and in both preparations similar translocation rates with diC_4PC as substrate were observed (C. Frijters, unpublished observations). The uptake of diC_4PC by mRNA-injected oocytes (16) suggests that this gene product is capable of trans-

location in the opposite direction as *mdr2* Pgp-mediated PC translocation (i.e., from the external to the internal leaflet of the plasma membrane), but it is also possible that this protein has a bidirectional translocation capacity. What this translocation activity represents is, as yet, unclear. It was suggested that the ATP-independent translocation activity ensures membrane integrity by passive compensatory lipid flux during active *mdr2*-mediated PC extrusion into the outer leaflet. Whatever the role of this second canalicular PC-translocator is, its putative function in overall PC secretion is strictly dependent on that of *mdr2* Pgp activity, because under all conditions tested, phospholipid secretion into bile is negligible in the *mdr2* knockout mice.

The flippase function of *mdr2* Pgp coincides with the postulated mechanism for *mdr1a/1b* Pgp as a flippase-like transporter or "membrane vacuum cleaner" (17). This hypothesis was based on indirect evidence from studies such as that of Homolya et al. (18), which showed that membrane-permeant esters of fluorescent indicators are extruded from the cell by MDR1 Pgp before they can be hydrolysed to their impermeable counterparts. It was postulated that MDR1 Pgp can bind substrates within the inner leaflet of the bilayer as well as from the cytosolic water phase. Whether translocation leads to release into the outer leaflet of the bilayer or into the water phase is not yet clear.

RESISTANCE OF THE CANALICULAR MEMBRANE AGAINST DETERGENT BILE SALTS

The incorporation of a flippase activity in the model of biliary lipid secretion suggests an important role for the asymmetry of the canalicular membrane bilayer. In the absence of PC translocation activity (in the *mdr2* (−/−) mouse), there is no lipid secretion (1, 19). As a consequence, the outer leaflet of the canalicular membrane must be highly resistant to the high concentrations of micellar bile salts present in the canalicular lumen. Similarly, the apical membrane of bile duct epithelial cells must have a similar intrinsic resistance toward bile salts. Even when as much as 50–60% of the bile salts consist of the very hydrophobic bile salt TDC, secretion of phospholipid remains negligible (see Fig. 2 and ref 20). Any other cell membrane incubated with the concentrations of TDC found in these bile samples would be solubilized quite rapidly. The mechanism of this resistance of the canalicular membrane is unknown. The outer leaflet content of cholesterol and sphingomyelin may be crucial in this context. Addition of cholesterol to erythrocyte membranes confers considerable resistance of these membranes toward bile salts (ref 21 and references therein). In addition, it was demonstrated that sheep erythrocytes are more resistant toward bile salts than pig or human erythrocytes. The main difference in membrane lipid composition between these species is the higher sphingomyelin content of sheep erythrocytes (for review, see ref 2). Glycosphingolipids, which are specifically present in the outer leaflet of apical membranes of epithelial cells, can also contribute to resistance against bile salts. Studies of the biosynthetic route of glycosyl-phosphatidylinositol (GPI)-anchored

proteins have revealed that these proteins reside in glycolipid-rich membrane domains ("rafts") and that these domains are relatively resistant toward detergents (22). In these studies, incubation with Triton X100 at 0°C is generally used as the model system, but a similar resistance has been observed with the bile salt deoxycholate (23, 24). In a recent study, Schroeder et al. (25) incubated liposomes of various lipid compositions with Triton X100 and demonstrated that the resistance of lipid bilayers against this detergent roughly correlates with the melting temperature (T_m) of the constituent lipids. The T_m is the temperature at which a phospholipid goes from the gel state into the liquid crystalline state. Thus, liposomes made of dioleoyl-PC (monounsaturated $C_{18:1}$ fatty acid, $T_m = -20^\circ\text{C}$) were completely solubilized by Triton X100 at 0°C whereas dipalmitoyl-PC liposomes (saturated $C_{16:0}$ fatty acid, $T_m = 41^\circ\text{C}$) were resistant. Liposomes that contained a large proportion of sphingolipids (sphingomyelin and cerebroside), which have long, saturated acyl chains and therefore higher melting temperatures (30° to 60°C resp.) were largely resistant to detergent treatment. This study also demonstrated that detergent treatment of liposomes that consisted of PC, SM, cerebroside, and cholesterol led to the selective extraction of PC from the liposomes, providing a possible physicochemical explanation for the highly preferential appearance of PC in bile.

Unfortunately, little information is available concerning the lipid composition of canalicular membranes because the conventional methods of isolation do not produce sufficiently pure membranes (26). At best, information can be drawn from studies regarding the lipid composition of apical membranes from other epithelial cells. Both sphingomyelin and glycosphingolipids display a very low rate of flip-flop across the membrane bilayer (27). Therefore their localization is confined to the leaflet at which their terminal synthetic steps occur: the luminal (exoplasmic) leaflet of the Golgi apparatus (28). Thus, transport of these lipids to the plasma membrane leads to asymmetric localization in the outer leaflet (29). Analysis of kidney and intestinal brush border membranes has yielded levels of sphingomyelin and especially glycolipids (up to 33 mol %), which, considering their asymmetric distribution between inner and outer leaflet, may be high enough to cover the complete outer apical membrane leaflet (for reviews, see refs 30, 31). The content of cholesterol is particularly high in plasma membranes; the ratio of cholesterol to phospholipid may be as high as 0.8 (32).

In conclusion, data from apical membrane compositions of other epithelial cells allow the suggestion that the outer leaflet of the hepatocyte apical membrane may have a high content of glycolipids, sphingomyelin, and cholesterol, and that this may confer the extreme resistance of this leaflet towards detergent bile salts. Translocation of phosphatidylcholine into this leaflet by *mdr2* Pgp may lead to selective extraction by bile salts.

INTRACELLULAR PC SUPPLY IN CONJUNCTION WITH A FLIPPASE FUNCTION

De novo synthesis of PC in the hepatocyte occurs via the CDP-choline pathway, which uses phosphocholine and di-

acylglycerol as precursors. A second, minor pathway involves methylation of PE, but this will contribute only very little because PE contains predominantly longer fatty acyl chains than "bile-type" PC (33). The majority of biliary PC is not derived from de novo synthesis but from preexisting microsomal and extrahepatic pools (34). HDL may constitute a major source of extrahepatic PC (35). Within the hepatocyte, PC molecules can be extensively remodeled with regard to their fatty acyl chains (34). Whatever the contribution of different sources of PC may be, the mobilization of PC must be tightly regulated, because in the *mdr2* ($-/-$) mouse no hepatic accumulation of PC is observed (19).

A model in which lipid secretion involves only the outer leaflet of the membrane clearly imposes restraints on the mechanism of lipid supply to the canalicular membrane. The conventional model of biliary lipid secretion inferred supply in the form of PC-rich bilayer vesicles that fuse with the membrane and thereby deliver PC to both leaflets of the membrane (2). This contrasts with the idea that PC has to be translocated from the inner to the outer leaflet: in such a vesicular supply model, 50% of the delivered PC is already in the outer leaflet and available for extraction; in the *mdr2* ($-/-$) mouse, one would then expect a reduction of PC secretion but not a complete absence. Alternatively the lipid composition of the PC-supplying vesicles may be asymmetric, with detergent-resistant lipids primarily in the inner exoplasmic leaflet of the vesicle. Fusion of such vesicles with the canalicular membrane would not lead to the presence of PC in the outer leaflet. Transcytosis, as monitored by biliary IgA and horseradish peroxidase secretion, is normally functional in the *mdr2* ($-/-$) mouse. In addition, the exocytosis of lysosomal enzymes mediated by endogenous bile salt secretion is intact (36). A model of a resistant canalicular outer membrane leaflet requires that the exoplasmic leaflet of transcytotic and exocytotic vesicles is also of a specific "bile salt-resistant" composition. Data from model epithelial cell lines suggest that this is, indeed, the case (for review, see ref 30).

Cohen et al. (37) provided data suggesting that the cytosolic PC-TP may function in the supply of PC to the inner canalicular leaflet. This protein is abundantly present in liver cytosol. Using the natural, fluorescent PC (1-palmitoyl, 2-parinaroyl PC) in an in vitro model, they demonstrated transfer of this phospholipid from small unilamellar vesicles to model canalicular membranes. The donor vesicles had a lipid composition mimicking the endoplasmic reticulum. The acceptor vesicles were devoid of PC in order to obtain net transfer from the ER-like vesicles to the acceptor membranes. The transfer of PC could be strongly accelerated by submicellar concentrations of bile salts (37). Although definite proof for this mechanism is still lacking, it suggests that PC may be (partly) supplied directly by an asymmetric process involving PC-TP. Whatever the contribution of PC-TP in PC supply to the canalicular membrane may be, it is not likely that it determines the striking selectivity of bile-type PC. PC-TP has a higher affinity for PC species in which the second acyl chain is long ($>C_{18}$) whereas biliary PC typically contains shorter acyl chains ($\leq C_{18}$) (38).

TWO MODELS OF BILIARY LIPID SECRETION INVOLVING MDR2 P-GLYCOPROTEIN

On the basis of the arguments described above, two models may be proposed for the mechanism of biliary PC secretion. In both models the function of *mdr2* Pgp as a flippase plays a central role. **Figure 3A** depicts a model in which Pgp translocates PC from the inner to the outer leaflet, where it becomes available for extraction by bile salts. Bile salts are continuously pumped into the canaliculus, but in the absence of *mdr2* Pgp no extraction occurs, because the outer leaflet is covered with "resistant" phospholipids and cholesterol (Fig. 3A, left panel) and therefore no phospholipid is secreted. When *mdr2* Pgp is present and active, PC will be translocated to the outer leaflet but when bile salts are absent this translocation will soon halt; without net extraction, translocation by *mdr2* Pgp creates a surplus of phospholipid in the outer leaflet, which is thermodynamically highly unfavorable (Fig. 3A, middle panel). The translocated PC can diffuse in the lateral plane of the outer leaflet. When both bile salts and *mdr2* Pgp are present (Fig. 3A, right panel), bile-type PC with relatively short fatty acyl chains will be extracted and *mdr2* Pgp-mediated translocation will proceed as long as there is a supply of PC on the cytoplasmic side and PC is extracted on the canalicular side. PC supply may occur either in the form of asymmetric vesicles with PC on the cytoplasmic leaflet or by delivery via PC-TP, or both. In this model, the selective secretion of bile-type PC may depend entirely on the physicochemical behavior of bile salt (micelles) toward the complex canalicular outer leaflet. Although this concept has not been tested in detail, some studies suggest that such selective extraction can indeed occur. Incubation of erythrocyte membranes with bile salts was shown to induce a more or less specific extraction of biliary type PC (2). Similarly, Yousef et al. (39) incubated isolated bile canalicular membrane vesicles with different bile salts and observed that PC and PE were selectively extracted from the membranes whereas PS and SM were selectively retained. Extraction of the membranes with low concentrations of bile salts yielded phospholipids with the fatty acid composition of bile-type PC. The extraction of PE from the membranes contradicts with its near absence in bile. This may, however, be due to scrambling of this phospholipid in the membrane preparation; in vivo PE is present almost exclusively in the inner leaflet of the membrane.

A problem of this model is that it does not directly explain the observed presence of lipid vesicles in bile. Vesicles containing PC and cholesterol besides mixed micelles of PC, cholesterol, and bile salts have been unequivocally demonstrated in human bile samples collected downstream in the biliary tree (40–42). Hence, its composition does not necessarily reflect that of primary bile in the canaliculus. It could be that modification of bile during its passage through the biliary tree leads to the formation of vesicles. Indeed, water is added in the bile ductules and the resulting decrease in total lipid concentration could induce a transition from the micellar to the vesicular phase (41, 42). Two morphological studies have, however, suggested that lipid vesicles also exist at the canalicular level. In an electronmicroscopic study

Figure 3. Two models (A, B) for the mechanism of mdr2 Pgp-mediated phospholipid secretion. For clarification, see text.

Ulloa et al. (4) observed lipid vesicles within the canalicular lumen of human liver specimens. In a recent report, Crawford et al. (43) used an ultrarapid freezing technique to study the morphology of the canalicular lipid secretion process. They observed characteristic electron-lucent vesicular structures that were clearly distinct from microvilli and attached to the canalicular membrane by an electron-dense base. The presence of these vesicular structures depended on ongoing bile salt secretion at the moment of freezing of the tissue. Furthermore, these vesicular structures were not observed during infusion of the nonmicelle-forming bile salt taurodehydrocholate, which is incapable of driving lipid secretion. The fact that these "stalked" vesicles are absent in liver specimens from *mdr2* ($-/-$) mice (44) suggests that they may represent intermediate structures of lipid secretion.

These considerations lead to a second, more complex model (Fig. 3B). In this model, PC remains in microdomains after translocation into the outer leaflet (Fig. 3B, middle panel). The active pumping by *mdr2* Pgp causes a phospholipid excess in the outer leaflet that destabilizes these microdomains; however, this is not sufficient to induce PC secretion in the absence of bile salts (Fig. 3B, middle panel). When bile salt molecules are present, these will further destabilize the microdomain, possibly by the formation of inverted micelles in the membrane (Fig. 3B, right panel). Through the ongoing translocation of PC, the domains grow into vesicular structures that can pinch off to yield biliary lipid vesicles. According to this model, all lipids that are translocated are also excreted into the lumen as vesicles; therefore, the selective excretion of bile-type PC must be determined by the substrate specificity of *mdr2* Pgp in this model.

A complicating problem of the second model is that the formation and maintenance of the postulated microdomains have not been demonstrated. As described above, in analogy with apical membranes from other epithelia, the content of glycolipids in the outer leaflet may be very high. Glycolipids have a strong tendency to cluster by hydrogen bonding (45). If the glycolipid content of the canalicular membrane is high enough, PC might be repulsed from the glycolipid-containing areas into microdomains.

Another question that arises from the second model is whether pinching of these vesicles from the outer leaflet of the membrane can occur without complete lysis or at least substantial leakage from the cells. The vesicular secretion model (Fig. 3B) remains speculative as long as evidence from in vitro model systems is lacking.

mdr2 P-GLYCOPROTEIN FUNCTION AND CHOLESTEROL SECRETION

Many studies have demonstrated that PC and cholesterol secretion are tightly coupled; therefore, they were always thought to be released into bile as intact units (2). In a model involving PC-translocation into microdomains in the outer leaflet of the canalicular membrane, it might be envisioned that cholesterol diffuses laterally into these domains. The fact that in rodents the biliary cholesterol to PC ratio is about fivefold lower than

that of the canalicular membrane should then be explained by assuming that relatively little cholesterol has time to diffuse into these domains before they are released from the membrane. The initial data obtained from the *mdr2* ($-/-$) knockout mice seemed to support this contention: the absence of phospholipid secretion is accompanied by a very marked decrease in cholesterol secretion. In heterozygotes for the gene disruption, however, a 40% decrease in PC secretion was accompanied by a normal cholesterol secretion (1). It was further demonstrated that at different rates of endogenous bile salt secretion ($+/-$), mice displayed the same near-linear dependence of cholesterol on bile salt output as did control mice (19). Thus, only a complete or nearly complete absence of phospholipid secretion impairs cholesterol output. More important, cholesterol secretion could be induced in the *mdr2* ($-/-$) by increasing the hydrophobicity of the bile salt pool, i.e., infusion of taurocholate (19) or taurodeoxycholate (20). These observations suggest that, at least in the absence of *mdr2* Pgp function, phospholipid and cholesterol can be uncoupled to a considerable extent depending on the hydrophobicity of the secreted bile salt. It is likely that in the ($-/-$) animals taurocholate-induced cholesterol secretion occurs by simple extraction of cholesterol by taurocholate micelles, and that this is less efficient than in normal animals because these micelles do not contain PC, which markedly decreases their capacity to solubilize cholesterol (46). Direct extraction of cholesterol from the membrane by bile salts is more compatible with the extraction model (Fig. 3A) than with the vesiculation model (Fig. 3B).

As discussed above, our revised model of biliary lipid secretion requires an asymmetric supply of PC to the membrane. Is this also the case for cholesterol? It is often stated that cholesterol has a much higher rate of spontaneous flip-flop across membrane bilayers than phospholipids. Indeed, rates on the order of seconds have been reported in experimental systems with liposomes and cholesterol oxidase (47). However, direct measurement with fluorescent cholesterol analogs have yielded much lower rates, and this issue remains unsettled. As a consequence, it is not clear whether, in analogy with PC, a canalicular translocator for cholesterol should be postulated as well. Lipids present in the inner leaflet can laterally diffuse through the tight junctions (48). As a consequence, all cholesterol present in the entire plasma membrane is available for extraction from the canalicular outer leaflet. It is known that a large part of the total free cholesterol of the cell is present in the plasma membrane; estimations range from 40 to 90% of the pool (49). The remainder is predominantly present in endosomes, *trans* Golgi apparatus, and lysosomes. The lipids of these organelles are in a dynamic contact with the plasma membrane via vesicular traffic. It may be, therefore, that cholesterol is indirectly supplied to the canaliculus via vesicular traffic. Replenishment of the free cholesterol pool in the cell may be via *de novo* synthesis in the ER, but quantitatively more important is the lysosomal hydrolysis of esterified cholesterol from endocytosed LDL. The sterol carrier protein 2 has been implicated in the transport of cholesterol through the cytosol (see ref 50), but this function is still under debate (51).

LIVER PATHOLOGY IN A MOUSE MODEL DEFICIENT IN BILIARY LIPID SECRETION

An important observation from the *mdr2* knockout model is that the mice suffer from liver disease that starts at a few weeks of age and progresses throughout life (1, 52). Histological studies showed some hepatocyte necrosis, and electronmicroscopic studies revealed dilated canaliculi. The most striking feature, however, is represented by inflammation of the portal tract and a very prominent proliferation of the bile ducts and ductules. Portal inflammation and ductular proliferation are progressive through the first 3 months of age. At the age of 4 to 6 months, the *mdr2* ($-/-$) mice start to develop nodular outgrowths in the liver parenchyma, which histologically resemble the picture of chemically induced hepatocarcinogenesis. These nodules develop into hepatocellular carcinomas, and in older ($-/-$) mice (>1 year) metastases were observed in the lungs. The cause of tumor formation is unclear. It could be related to the increased replication of hepatocytes as a consequence of cell damage. It is striking, however, that this does not hold for the cholangiocytes, which proliferate even stronger. Alternatively, the absence of *mdr2* Pgp could, either directly or indirectly, lead to accumulation of carcinogenic compounds in the hepatocytes. The latter mechanism could explain the observation that tumors seem to be derived from hepatocytes rather than from cholangiocytes.

The absence of *mdr2* Pgp function clearly has deleterious effects for cells lining the biliary tree. It is our hypothesis that this is caused by the cytotoxic bile that flows along these cells. Several studies have demonstrated that addition of phospholipid and cholesterol diminishes the toxicity of the otherwise cytolytic bile salt micelles (21, 53, 54). Evidently, the type of secreted bile salts is an important factor in the cytotoxicity of the lipid-free bile (21, 53, 55). In this respect it should be emphasized that the bile salt composition of mouse bile is of a highly hydrophilic character: 60–70% of the secreted bile salts consist of muricholate, which is very hydrophilic (56, 57). Hence, the pathology observed in the ($-/-$) mice may be relatively mild because of the relatively hydrophilic bile salt composition in this animal. Experiments in which *mdr2* knockout mice were fed bile salts of different hydrophobicity seemed to confirm this hypothesis: increasing the hydrophobicity of the bile salt pool by cholate feeding leads to a more severe liver pathology, whereas further decreasing its hydrophobicity by ursodeoxycholate feeding improves the histology (58). The recognition of a deficiency in hepatic lipid secretion as the cause of cholangiopathy urges one to reconsider a number of liver diseases in human neonates, as well as acquired cholangiopathies. In the past, little attention was given to hepatobiliary lipid secretion in such conditions. In view of the fact that the bile salt pool in humans is much more hydrophobic than in mice, a deficiency of lipid secretion in humans could have much more dramatic consequences than in the mouse. A mutation in the human *MDR3* gene that leads to a complete absence of function may therefore not be compatible with life. However, acquired or inborn, partial deficiencies of *MDR3* activity might cause or predispose

to hitherto poorly defined forms of liver pathology. Very recently, a patient with progressive familial intrahepatic cholestasis (PFIC) was reported with high serum γ -glutamyltransferase activity. Histological examination of a liver specimen from this patient revealed severe fibrosis and ductular proliferation. Northern blotting of the liver mRNA suggested the complete absence of *MDR3* mRNA as opposed to other control samples. Unfortunately, no analysis of biliary phospholipids and bile salts was available from this patient (59). It is clear that further research will have to determine to what extent defects in biliary phospholipid secretion contribute to several forms of liver disease. [F]

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