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Abstract

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ABC-Transporters are localized in Caveolin-1 positive and Reggie-1 and -2 negative Microdomains of the Canalicular Membrane in Rat Hepatocytes

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List of abbreviations:

Bsep, bile salt export pump; multidrug resistance associated protein, Mrp2; canalicular plasma membrane, cLPM; ATP binding cassette, ABC; Progressive Familial Intrahepatic Cholestasis, PFIC; basolateral plasma membrane, bLPM; alkaline phosphatase, AP; polyclonal antiserum, pABs; monoclonal antibody, mAb; aminopeptidase N, APN; dipeptidylpeptidase IV, DPPIV; detergent resistant microdomains, DRMs;

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Abstract

The canalicular plasma membrane is constantly exposed to bile acids acting as detergents. Bile acids are essential to mediate release of biliary lipids from the canalicular membrane. Membrane microdomains (previously called lipid rafts) are biochemically defined by their resistance to detergent solubilization at cold temperature. We aimed to investigate the canalicular plasma membrane for the presence of microdomains, which could protect this membrane against the detergent action of bile acids. Highly purified rat liver canalicular plasma membrane vesicles were extracted with 1% TritonX-100 or 1% LubrolWX at 4°C and subjected to flotation through sucrose step gradients. Both detergents yielded detergent resistant membranes containing the microdomain markers alkaline phosphatase and sphingomyelin. However, cholesterol was resistant to LubrolWX solubilization, while it was only marginally resistant to solubilization by TritonX-100. The microdomain marker caveolin-1 was localized to the canalicular plasma membrane domain and was resistant to LubrolWX, but to a large extent solubilized by TritonX-100. The two additional microdomain markers reggie-1 and reggie-2 were localized to the basolateral and canalicular plasma membrane and were partially resistant to LubrolWX but resistant to TritonX-100. The canalicular transporters bile salt export pump, multidrug resistance protein 2, multidrug resistance-associated protein 2 and Abcg5 were largely resistant to LubrolWX but were solubilized by TritonX100.

Conclusion: These results indicate the presence of two different types of microdomains in the canalicular plasma membrane: "Lubrol-microdomains" and "Triton-microdomains". "Lubrol-microdomains" contain the machinery for canalicular bile formation and may be the starting place for canalicular lipid secretion.
Bile formation involves vectorial secretion of bile acids and other cholephilic compounds across hepatocytes from the sinusoidal blood plasma into bile canaliculi (1, 2). The main bile constituents are bile salts, organic anions, phospholipids and cholesterol which aggregate in bile into mixed micelles (3). Hence, bile fluid is exquisitely suited for the excretion of water insoluble substances. The most important transporters involved in canalicular secretion of bile salts, organic anions and phosphatidylcholine are the bile salt export pump Bsep (Abcb11) (BSEP in humans) (4, 5), the multidrug resistance associated protein 2 Mrp2 (Abcc2) (MRP2 in humans) (6), and the phosphatidylcholine translocator Mdr2 (Abcb4) (MDR3 in humans), (7), respectively. Export of cholesterol from the canalicular plasma membrane (cLPM) is mediated by the heterodimeric protein Abcg5/Abcg8 (ABCG5/ABCG8 in humans) (8). All these transporters are members of the ATP-binding cassette (ABC) protein superfamily and utilize ATP-hydrolysis for transport function.

Canalicular phospholipid secretion has been extensively studied both in inherited human liver disease and in animal models. The results indicate that, once secreted into the bile canaliculi, bile salts extract phosphatidylcholine from the outer leaflet of the cLPM and solubilize it within mixed micelles (3). This principle has been worked out in various animal models, which demonstrated that in the absence of bile salt secretion canalicular phospholipid secretion ceases. However, in mice with disrupted Mdr2 function, phospholipid secretion cannot be stimulated, even at high rates of bile salt output (9). This highlights the importance of functional Mdr2 for canalicular phospholipid secretion. Furthermore, inherited forms of human liver diseases with defective expression and/or function of BSEP (e.g. Progressive Familial Intrahepatic Cholestasis type 2, PFIC 2) or of MDR3 (PFIC 3) provide strong evidence that the same mechanism of biliary phospholipid secretion is also valid in human liver (10).
Furthermore, the finding that animals without biliary phospholipid secretion display no biliary cholesterol secretion (9) demonstrates that the presence of mixed bile salt/phospholipid micelles is required in bile in order to maintain normal cholesterol secretion. In addition, the heterodimeric ABC transporter Abcg5/Abcg8 is involved in canalicular cholesterol secretion, since 1) mice with disrupted Abcg5 or Abcg8 display a marked reduction in biliary cholesterol secretion (11), and 2) patients with mutations in the ABCG5/ABCG8 gene display hypercholesterolemia and β-sitosterolemia (8). Hence, at least the three ABC-transporters Bsep, Mdr2 and Abcg5/Abcg8 are needed for the maintenance of normal biliary cholesterol secretion (3). The exact role of an ATP-independent phosphatidylcholine translocator that is also expressed at the cLPM, is not understood at present (12, 13).

In vitro, bile salts preferentially extract phosphatidylcholine from the cLPM, albeit phosphatidylcholine represents only 35% of total canalicular phospholipids (14, 15) and the cLPM has an exceptionally high sphingomyelin content (15). This unique property of the cLPM could be due to the presence of lipid microdomains such as for example detergent sensitive membrane regions enriched in phospholipids and detergent resistant sphingomyelin/cholesterol clusters, which are also called lipid rafts (16, 17). In such microdomains, sphingomyelin and cholesterol are arranged in a tightly packed, liquid-order state (16) forming highly dynamic structures, which exist in short length and time scales (18). Recently, evidence has been provided for the presence of coexisting raft and non-raft microdomains in both the basolateral plasma membrane domain (bILPM) and the cLPM domain of rat hepatocytes (19, 20). In these studies the TritonX-100 resistant lipid microdomains were enriched in alkaline phosphatase (AP), caveolin-1, cholesterol, sphingomyelin, the ganglioside GM1 and the aquaporins 8 (canalicular) and 9 (basolateral) (20). Other studies have indicated
that the hepatocyte plasma membrane might contain at least two different pools of cholesterol and caveolin-1 enriched microdomains, the major one (~ 90%) being soluble and the minor one (~ 10%) being insoluble in TritonX-100 (1%) (21). Furthermore, the coexistence of different cholesterol-enriched lipid microdomains has been proposed in the apical membrane of MDCK cells based on its relative solubility in the non-ionic detergents TritonX-100 ("Triton microdomains") and LubrolWX ("Lubrol microdomains") (22). And finally, in neurons and astrocytes, caveolin-1 negative lipid microdomains have been identified that are associated with the marker proteins reggie-1/flotillin-2 and reggie-2/flotillin-1 (23-26). Reggie proteins are widely expressed, form oligomers at the cytoplasmic face of the plasma membrane (27) and scaffolds of plasma membrane microdomains, which are clearly distinct from caveolae (28). Reggie microdomains represent platforms for multiprotein complex formation and signal transduction in a cell-type and situation-specific manner (29, 30). They communicate, for instance, with Rho-GTPases and regulate actin cytoskeleton dynamics (31). Hence, heterogeneous lipid microdomains might coexist at the two polar plasma membrane domains of epithelial cells and differentially influence vectorial transport processes such as hepatocellular bile formation.

In this study, we investigated the hypothesis that distinct lipid microdomains with different protein and lipid compositions and different sensitivities toward non-ionic detergents (LubrolWX, TritonX-100) might coexist at the cLPM of rat hepatocytes. Furthermore, we wondered whether the canalicular ABC transporters are partially or even completely compartmentalized into distinct lipid microdomains.
Experimental Procedures

Antibodies and the methods describing the extraction and analysis of lipids, SDS-polyacrylamide gel electrophoresis and immunoblotting, immunofluorescence and immunoelectron microscopy are given in a file containing supplementary material.

Animals: Male Sprague-Dawley rats (180-200g) were obtained from Harlan (Horst, The Netherlands). They received humane care in accordance with local and federal guidelines and regulations were kept under standard conditions.

Chemicals: LubrolWX and TritonX-100 were purchased from Serva Feinbiochemica (Heidelberg, Germany) and MP Biomedicals (Irvine, CA), respectively. Lipid standards were from Sigma-Aldrich (St. Louis, MO). All other chemicals were of the highest grade and readily available from commercial sources.

Isolation of detergent resistant microdomains (DRMs) from highly purified cLPM: cLPM were isolated as described (32) and stored in liquid nitrogen until use. DRMs were isolated by performing all steps at 4°C with minor modifications as described (33). Briefly: cLPM (1mg protein) were thawed on ice and diluted to 1ml in TNE (150mM NaCl, 1mM EDTA, 20mM Tris-HCl pH7.4) containing antipain and leupeptin 2µg/ml each and 1mM phenylmethylsulfonylfluoride. The suspension was mixed with 1ml of either 2%(w/vol) LubrolWX or 2%(w/vol) TritonX-100 in TNE (yielding a detergent to protein ratio (w/w) of 20) and homogenized by passing it ten times through a 25-gauge needle. After incubation for 30min on ice, the suspension was brought to 40%(w/vol) sucrose by adding 2ml 80%(w/vol) sucrose in TNE. This mixture was overlaid with 5ml 35%(w/vol) and 4ml 5%(w/vol) sucrose both in TNE.
The DRMs were floated by centrifugation for 18h at 40,000rpm (270,000gav), 4°C in a TST41.14 swinging bucket rotor from Kontron Instruments (Schlieren, Switzerland). Twelve 1ml fractions were harvested from the top and the pellet was resuspended in 1ml TNE by vigorous vortexing and 10 passages through a 25-gauge needle. AP activity in the presence of 0.2mM ZnSO₄ was measured immediately as described (34). For all other analyses, the fractions were stored in aliquots at −80°C until used.
Results:

In order to test whether the cLPM of rat liver contains DRMs, we exposed cLPM vesicles from rat liver to the two non-ionic detergents LubrolWX and TritonX-100. As indicated in Fig.1, after cold extraction of cLPM with 1%(w/vol) LubrolWX 24% of total cLPM protein (Fig.1A) and 88% of total canalicular AP activity (Fig.1B) floated to the top of the 35% sucrose layer during ultracentrifugation (fractions 3-5, Fig.1), i.e. they were not solubilized by LubrolWX and, thus, associated with LubrolWX-resistant membrane microdomains (so called "Lubrol-microdomains"). For 1%(w/vol) TritonX-100, the corresponding numbers were 10% (total protein) and 85% (AP activity), respectively (Fig.1). Hence, the vast majority of the canalicular AP activity was recovered in the DRMs after extraction with either detergent. Using 2%(w/vol) instead of 1%(w/vol) TritonX-100 showed no difference in the distribution of AP activity after flotation, while extraction with 0.5%(w/vol) TritonX-100 resulted in a lower recovery of detergent resistant AP activity (data not shown). Consequently, subsequent experiments were performed with 1%(w/vol) TritonX-100. Increasing LubrolWX concentration to 2%(w/vol) did not alter the amount of protein floating to the DRMs, nor the distribution pattern of canalicular proteins between solubilized and DRM fractions on the gradient (data not shown). Therefore, a LubrolWX concentration of 1%(w/vol) was chosen for this study. These data indicate that the cLPM of rat liver contains indeed DRMs that harbor a significant proportion of membrane-associated proteins and are highly enriched in GPI-anchored AP, which represents a typical marker enzyme for DRMs (35).

To further characterize the canalicular "Lubrol microdomains" and "Triton microdomains", we analyzed the low-density membrane material recovered from fractions 3-5 with respect to additional DRM marker proteins such as caveolin-1 and
reggie-1 and reggie-2. In addition, the cytoskeletal protein actin was probed for to
investigate a possible association of cytoskeletal elements with the two canalicular
DRMs. As shown in Fig.2, caveolin-1 was mostly resistant to LubrolWX (fractions 3-
5), but almost completely solubilized by TritonX-100 (fractions 8-12). In contrast,
reggie-1 and reggie-2 were completely resistant to TritonX-100 in this system (Fig.2,
fractions 3-5). And finally, actin was found to be associated with detergent soluble
and detergent resistant cLPM subfractions indicating no preferential interaction with
canalicular DRMs. These data support the presence of distinct DRMs at the cLPM of
rat liver. They demonstrate that solubilization of cLPM with LubrolWX or TritonX-
100 results in cLPM microdomains of different marker protein composition. "Lubrol-
microdomains" contain quantitatively caveolin-1, and "Triton-microdomains" are
associated with reggie-1 and reggie-2.

The strong association of caveolin-1 with detergent-insoluble (LubrolWX) and
detergent-soluble (TritonX-100) cLPM subfractions (Fig.2) was surprising, since the
liver has been reported to express only low levels of caveolin-1 (36) and the
subcellular distribution of caveolin-1 in rat liver has remained controversial (37-39).
Therefore, we investigated the exact hepatocellular surface distribution of caveolin-1
in more detail and compared it with established basolateral and canalicular marker
proteins as well as with the surface distribution of reggie-1 and reggie-2. As
illustrated in Fig.3A, in Western blot analysis of isolated bLPM and cLPM caveolin-
1 exhibited an exclusive canalicular localization as especially evidenced by its
colocalization with the canalicular marker enzyme APN. In contrast, the expression of
reggie-1 and reggie-2 was not domain-specific, but evenly distributed between bLPM
and cLPM of rat hepatocytes. The antigen 1/18 has been previously shown to
represent a valid basolateral marker protein in rat hepatocytes (40).
The canalicular localization of caveolin-1 was further confirmed by immunofluorescence studies using Mrp2 as a canalicular marker (Fig.3B). Immunopositive caveolin-1 was clearly associated with the cLPM (Fig.3C) and colocalized with canalicular Mrp2 (Fig.3D). However, green caveolin-1 immunoreactivity was also seen along the sinusoidal lining of hepatocytes (Figs.3C,D). Since isolated bLPM were virtually devoid of immunopositive caveolin-1 (Fig.3A), the apparent sinusoidal caveolin-1 positivity was most probably due to caveolin-1 with endothelial cells, where it is highly expressed (41), although low level expression of caveolin-1 at the bLPM of hepatocytes cannot be definitely excluded (38). In any case, the data demonstrate that caveolin-1 is a highly expressed and intrinsic protein of the cLPM domain of rat hepatocytes and, thus, can be used as a marker protein of canalicular DRMs in rat liver. The non-polar expression of reggie-1 in hepatocytes (Fig.3A) was confirmed by immunofluorescence localization (Fig.3E).

We next used immunoelectron microscopy to more precisely localize Mrp2 and caveolin-1 in the cLPM. Since aldehyde fixation of liver tissue abolished reactivity of the anti-caveolin-1 antibody, methanol fixation had to be applied. This resulted in a satisfactory structural preservation of bile canaliculi in ultrathin frozen sections (Fig.4A) and at the same time permitted simultaneous immunogold localization of caveolin-1 and Mrp2 (Fig.4A,B). As previously reported (42), immunogold labeling for Mrp2 was intense at the microvilli of the bile canaliculi. As expected from the confocal immunofluorescence result for caveolin-1, immunogold labeling for caveolin-1 in ultrathin frozen sections of methanol-fixed liver was sparse but also detectable at microvilli and sub-plasma membrane regions of the bile capillaries. Inspecting the cLPM at higher magnification revealed again expression of both proteins and they were observed in proximity and hence corroborating the finding.
from the immunofluorescence experiment. However, the distance between the gold particles precludes a true colocalization. This might, at least in part, be the result of the methanol fixation method, which had to be used to detect caveolin-1.

In addition to caveolin-1, reggie-1 and reggie-2, sphingomyelin represents an additional DRM marker (16). In fact, and as illustrated in Fig.5, sphingomyelin segregated quantitatively into both "Lubrol-microdomains" and "Triton-microdomains" (fractions 3-5). This was not the case for cholesterol and various phospholipids. Hence, while the caveolin-1 positive "Lubrol-microdomains" (Fig.2) contained most canalicular phospholipids (except part of phosphatidylcholine) and all the cholesterol (Fig.5), the reggie-1 and reggie-2 positive "Triton-microdomains" (Fig.2) were devoid of any phospholipids and contained only a portion of canalicular cholesterol (Fig.5). While these data are consistent with LubrolWX being a less selective detergent for DRMs than TritonX-100 (43), they support further the presence of distinct canalicular DRMs. More specifically, the partial solubilization of cholesterol, but not sphingomyelin, by TritonX-100 indicates that part of the cLPM cholesterol is soluble independent of sphingomyelin and, thus, might represent a mobilizable cLPM cholesterol pool that can be extracted from the canalicular membrane together with phospholipids (especially phosphatidylcholine, Fig.5) by the detergent action of intracanalicular bile salts.

Finally, we wondered about the segregation of some typical canalicular marker enzymes and canalicular ABC transporters within and outside "Lubrol-microdomains" and "Triton-microdomain". As illustrated in Fig.6, the canalicular marker enzymes APN, ectoATPase and DPPIV were almost equally sensitive to solubilization by LubrolWX and TritonX-100. While APN and DPPIV minimally partitioned into both types of DRMs, ectoATPase could not be detected in DRMs at
all (Fig.6). In contrast, the ABC transporters Abcg5, Bsep, Mrp2, Mdr2 and Mdr1 were significantly associated with "Lubrol-microdomains" (Fig.7), while "Triton-microdomains" contained only some minor portions of Mdr1 and even less so of Mrp2 (Fig.7). These data, while further supporting the existence of different types of canalicular DRMs, strongly indicate that canalicular ABC transporters function within phospholipids and cholesterol containing cLPM membrane microdomains (the caveolin-1 positive "Lubrol-microdomains") in rat hepatocytes. In contrast, reggie-1 and reggie-2 positive "Triton-microdomains" might represent transporter free and sphingomyelin enriched, more rigid membrane microdomains that are important for the maintenance of the overall structural integrity and functional compartmentalization of the cLPM.

Discussion

The present study provides evidence for the presence of two different types of DRMs in the cLPM of rat hepatocytes. Based on the detergents used for solubilization of isolated cLPMs we named the two distinct canalicular DRMs "Lubrol-microdomains" and "Triton-microdomains". "Lubrol-microdomains" are associated quantitatively with caveolin-1 (Fig.2), contain the majority of canalicular cholesterol and phospholipids (Fig.5), portions of the marker enzymes APN and DDPIV (Fig.6) and large portions of all ABC transporters tested (i.e. Abcg5, Bsep, Mrp2, Mdr2 and Mdr1) (Fig.7). "Triton-microdomains" are associated quantitatively with AP (Fig.1), reggie-1 and reggie-2 (Fig.2), sphingomyelin (Fig.5) and contain also minor fractions of canalicular cholesterol (Fig.5), APN and DDPIV (Fig.6) as well as Mdr1 and Mrp2 (Fig.7). Hence, our study supports the concept of distinct canalicular cholesterol-
enriched lipid microdomains that can be distinguished by the marker proteins caveolin-1 and reggie-1 and reggie-2. Furthermore, a large part of canalicular ABC transporters reside within the phospholipid and cholesterol enriched membrane regions ("Lubrol-microdomains") suggesting that they require a complex membrane lipid environment for proper functioning.

By using the same two non-ionic detergents (e.g. LubrolWX and TritonX-100), two distinct cholesterol-based lipid microdomains have previously been proposed in the apical plasma membrane of MDCK cells (22). Our findings support this previous study and extend the concept of the coexistence of multiple, distinct types of raft-like assemblies of lipids and proteins to the cLPM (apical) of hepatocytes. Although we have not tested further the exact localization of the distinct DRMs along the cLPM, due to a lack of suitable antibodies for immunoelectron microscopy, it is tempting to speculate that, similar to MDCK cells, the caveolin-1 specific and lipid and transporter enriched "Lubrol-microdomains" may correspond to the microvillar portions, and the reggie-1 and reggie-2 and sphingomyelin specific "Triton-microdomains" to the more rigid planar portions of the cLPM. This interpretation would be compatible with several other previous reports: 1) Bsep, which is present in "Lubrol-microdomains", but absent from "Triton-microdomains" (Fig.7), has been shown to be preferentially localized in microvilli and to be virtually absent from planar portions of the cLPM (4), which is paralleled by scarce Mrp2 labeling observed in this study, 2) reggie-1 and reggie-2 containing membrane microdomains ("Triton-microdomains" in this study, Fig.2) are clearly different from caveolin-1 containing membrane microdomains. They might represent stable membrane scaffolds or platforms with possible own regulatory functions that are
distinct from caveolae (25), and 3) other ABC transporters (e.g. MDR1, MRP1) have been found to be associated with "Lubrol-microdomains" (44, 45).

The association of a significant portion of ABC transporters (Fig.7) and all different lipid species (Fig.4) with "Lubrol-microdomains" indicates that ATP-dependent canalicular bile salt, organic anion, phospholipid and cholesterol secretion requires a complex lipid environment for proper functioning. In this regard the high cholesterol content of "Lubrol-microdomains" appears especially interesting, since it has recently been shown in a heterologous expression system that the ATPase activity of the canalicular ABC-transporter ABCG2 is stimulated by cholesterol loading (46). As the methodology used to isolate DRMs has an inherent considerable quantitative variability (47), we could not quantitatively assess the enrichment of cholesterol in DRMs compared to cLPM. Nevertheless, by inserting or retrieving canalicular export systems into cholesterol enriched microdomains (i.e. "Lubrol-microdomains"), hepatocytes could regulate the activity of the ABC transporters and thus canalicular bile formation. Such a putative regulatory mechanism could also involve actin (Fig.2), as it was shown for insulin in rat liver plasma membrane microdomains (21, 48), as well as the cytoskeletal web and intermediate filaments underneath the cLPM (49, 50). Furthermore, stimulation of the choleretic activity of hepatocytes leads to an upregulation of aquaporin-8 in caveolin-1 enriched canalicular microdomains (20). Also, partitioning of the serotonin transporter and the sodium-phosphate cotransporter into membrane microdomains has been demonstrated as a regulatory mechanism for these transporters (51, 52). Finally, and most importantly, the observation that LubrolWX was able to partially and preferentially solubilize phosphatidylcholine (Fig.5) mirrors the finding that taurocholate preferentially releases phosphatidylcholine from cLPM in vitro (15). Hence, caveolin-1 and ABC transporter
positive "Lubrol-microdomains" might represent the canalicular microdomains from where biliary phospholipids are preferentially solubilized by intracanalicular bile salts. Although this latter conclusion remains to be experimentally verified, our data strongly suggest that "Lubrol-microdomains" are representative for cLPM microdomains that localize the entire functional machinery for maintenance of ongoing canalicular bile formation including bile salt (Bsep), organic anion (Mrp2), phospholipid (Mdr2) and cholesterol (Abcg5/8) secretion and therefore could also be called "bile salt microdomains".

In contrast to "Lubrol-microdomains", "Triton-microdomains" appear to represent more stable non-caveolin-1 (caveolae) associated canalicular DRMs, which might not be directly involved in bile secretory processes. "Triton microdomains" contain notably fewer different lipid species than "Lubrol microdomains". To what extent different biophysical properties of the two different DRMs and to what extent the presence of inside-out and right-sight out oriented cLPM during detergent extraction contribute to this difference remains open at this moment. In addition, TritonX-100 is more selective in DRM isolation, such that it disrupts more lipid-protein interactions than LubrolWX (43). While the association of "Triton microdomains" with AP, sphingomyelin and some cholesterol (Figs.1,5) is in agreement with previous findings (53), the observation of a specific association of reggie-1 and reggie-2 with "Triton-microdomains" is novel. Reggie-2/flotillin-1 upon its identification as a DRM component was found to be extracted together with caveolin-1 after cold extraction of 3T3-L1 adipocytes with TrionX-100 (54). However, electron microscopy studies have since shown that reggie proteins demarcate microdomains ("reggie-microdomains") distinct of caveolae in all cell types analyzed so far (23, 24, 55). Preliminary experiments showed only partial
colocalization of reggie-1 and Mrp2 (data not shown), indicating a distinct expression of reggie-1 and caveolin-1 in the cLPM. In contrast, caveolin-1 and Mrp2 showed complete colocalization in the cLPM (Fig. 3D). Opposite to 3T3-L1 cells, hepatocytes are highly polarized cells. Hence, the dissociation of Lubrol-microdomains and Triton-microdomains may be cell-type specific and/or related to the degree of cell polarity. This view is supported by the identification of distinct microdomains in the apical membrane of MDCK cells (22), which is paralleled by an expression of caveolin-1 and reggie-1 and reggie-2 in different membrane subdomains (56). While the expression of reggie-2 in mouse liver has been reported (57), in the same study expression of reggie-1 could not be demonstrated. The present study reports to our knowledge for the first time the expression of reggie-1 in liver and the localization of reggie-1 and reggie-2 to both plasma membrane domains of hepatocytes. The lack of detection of reggie-1 in the previous study may be due to the use of tissue lysates compared to highly purified and enriched plasma membrane fractions used in this study for Western blotting.

In conclusion, by using LubrolWX and TritonX-100, we have presented strong evidence for the presence of to different microdomains in the cLPM of rat liver: "Lubrol-microdomains" and Triton-microdomains". The "Lubrol-microdomains" may play an essential role in canalicular bile formation, since they contain the entire machinery for the generation of canalicular bile salt dependent and independent bile flow.

**References**


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**Figure legends:**

**Figure 1:** Distribution of total protein (A) and AP (B) in detergent resistant microdomains (DRMs) isolated from cLPM. cLPM were extracted with 1%(w/vol) LubrolWX or 1%(w/vol) TritonX-100 and floated on discontinuous sucrose gradients as described in Materials and Methods. Twelve 1ml fractions were collected from the top and analyzed for protein content and AP activity. Low density fractions 3-5 contained detergent resistant microdomains (i.e. "Lubrol-microdomains" (left panels) and "Triton-microdomains" (right panels)), high-density fractions 9-12 contain solubilized proteins. **Fig. 1A:** The protein concentration in each fraction was determined as described in materials and methods. The total recovery in relation to the input was 95% for LubrolWX and 93% for TritonX-100. **Fig. 1B:** AP activity was measured in the various fractions as described in Materials and Methods. AP was quantitatively recovered in both "Lubrol-microdomains" and "Triton-microdomains". The total recovery of AP relative to the total inputs was 95% for LubrolWX and 96% for TritonX-100. One representative result out of at least two independent experiments is shown.
Figure 2: Distribution of the DRM markers caveolin-1, reggie-1 and reggie-2 between canalicular "Lubrol-microdomains" and "Triton-microdomains".

cLPM were extracted with 1%(w/vol) LubrolWX (left panel) or 1%(w/vol) TritonX-100 (right panel) and floated on discontinuous sucrose gradients as described in Materials and Methods. Fractions and the resuspended pellets (P) were subjected to Western blot analysis for marker proteins and actin. Untreated cLPM served as controls (C). Apparent molecular weights are given on the right. Caveolin-1 (Cav-1) was found to be associated predominantly with "Lubrol-microdomains", while reggie-1 and reggie-2 were found to be distinct marker proteins for "Triton-microdomains". The cytoskeletal protein actin is only minimally associated with both types of DRMs. One representative result out of at least two independent experiments is shown.

Figure 3: Immunological localization of caveolin-1 at the cLPM domain in isolated rat liver plasma membrane vesicles and in intact rat liver. A: bLPM and cLPM rat liver plasma membrane vesicles were isolated as described in Materials and Methods and probed with specific antibodies (Western blotting) against the indicated proteins. APN and 1/18 are established marker proteins for the cLPM and bLPM domains of rat liver, respectively. B to E: Rat liver was fixed and processed for immunofluorescence using antibodies against caveolin-1, reggie-1 and the established canalicular marker Mrp2 (B) as described in Materials and Methods. C/D: Caveolin-1 labeled the cLPM domain (C) and colocalized with Mrp2 (D). Additional caveolin-1 labeling was also seen along the sinusoidal lining of hepatocytes (C,D) most probably reflecting caveolin-1 expression in sinusoidal lining endothelial cells. Nuclei (blue) were stained with DAPI. E: Reggie-1 labeling was seen in the bLPM and cLPM of hepatocytes.
Figure 4: **Immunoelectron microscopic localization of Mrp2 and caveolin-1 in the canalicus**: Low power micrograph showing a bile canaliculus with immunogold labeling for MRP2 and caveolin-1 (A). At higher magnification (B), immunogold labeling for MRP2 (small gold particles, arrowheads) and for caveolin-1 (large gold particles, arrows) of the microvilli can be seen. Caveolin-1 immunogold labeling is also observed beneath the bile capillary plasma membrane. Original magnifications: x38'500 (A), x107'000 (B).

Figure 5: **Lipid composition of canicular DRMs**: cLPM were treated with 1%(w/vol) LubrolWX or 1%(w/vol) TritonX-100 and floated on discontinuous sucrose gradients as described in Materials and Methods. Lipids from the recovered 12 fractions, the resuspended pellets (P) and untreated cLPM (C) were extracted and analyzed as described in Materials and Methods. Individual lipid species were identified by comigration with the purified lipids sphingomyelin (SM), phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidylethanolamine (PE) and cholesterol (Ch). While "Lubrol-microdomains" (fractions 3-5) contained almost quantitatively all canicular lipid species, "Triton-domains" (fractions 3-5) were selectively enriched in sphingomyelin, contained a minor portion of cholesterol and were completely devoid of phospholipids. A representative result of two independent experiments is shown.
Figure 6: Distribution of canalicular marker enzymes within and outside "Lubrol-microdomains" and "Triton-microdomains". cLPM were extracted with 1%(w/vol) LubrolWX or 1%(w/vol) TritonX-100 and floated on discontinuous sucrose gradients as described in Materials and Methods. The recovered 12 fractions and the resuspended pellets (P) were subjected to immunoblot analyses using antibodies against APN, ectoATPase and DPPIV as indicated. Untreated cLPMs were used as positive control (C). Apparent molecular weights are given on the right. The marker enzymes studied were only minimally (APN, DPPIV) or not at all (ectoATPase) associated with canalicular DRMs. One representative result out of at least two independent experiments is shown.

Figure 7: Distribution of canalicular ABC transporters within and outside "Lubrol-microdomains" and "Triton-microdomains". cLPMs were extracted with 1%(w/vol) LubrolWX or 1%(w/vol) TritonX-100 and floated on discontinuous sucrose gradients as described in Materials and Methods. The recovered 12 fractions and the resuspended pellets (P) were subjected to Western blot analysis using antibodies against the ABC transporters indicated on the left side. Untreated cLPMs were used as positive control (C). Apparent molecular weights are given on the right. Canalicular ABC transporters were found to reside in part in "Lubrol-microdomains" and to be virtually absent from "Triton-microdomains". Only Mdr1 and Mrp2 remained associated to some degree with "Triton-microdomains". One representative result out of at least two independent experiments is shown.
Figure 1A

Protein Recovery:

- F3-5 (DRMs): 24%
- F9-12 (soluble): 56%
- Pellet: 2%

Protein Recovery:

- F3-5 (DRMs): 10%
- F9-12 (soluble): 72%
- Pellet: 3%

275x190mm (300 x 300 DPI)
Figure 1B

Recovery of AP

F3-5 (DRMs): 88%
F9-12 (soluble): 3%
Pellet: 0.1%

Recovery of AP

F3-5 (DRMs): 85%
F9-12 (soluble): 6%
Pellent: 1%

Lubrol WX

Triton X-100

286x190mm (300 x 300 DPI)
Figure 2

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Lubrol WX

Triton X-100

Mr (kDa)

48

42

21

46

255x190mm (300 x 300 DPI)
Figure 3

A

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B

Mrp2

C

Caveolin-1

D

merge

275x190mm (300 x 300 DPI)
Figure 3, continued

E

Reggie-1

275x190mm (300 x 300 DPI)
Figure 5

281x190mm (300 x 300 DPI)
Figure 6

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275x190mm (300 x 300 DPI)
Figure 7

Lubrol WX       Triton X-100

Abcg5          Abcg5  ~Mr (kDa)
Bsep           Bsep   70
Mrp2           Mrp2   150
Mdr2           Mdr2   200
Mdr1           Mdr1   150

275x190mm (300 x 300 DPI)
Supplementary Material

Antibodies: A polyclonal antiserum (pABs) was raised in rabbits against an oligopeptide corresponding to the N-terminal 15 amino acids of rat Abcg5 coupled at the C-terminus via an additional C-terminal cysteine to keyhole limpet hemocyanine (Neosystems, Strasbourg, France) as described in (1). The production of pABs against Bsep and Mrp2 has been described previously (2, 3). Hybridomas producing monoclonal antibodies (mABs) against aminopeptidase N (APN) (BB4/33) and dipeptidylpeptidase IV (DPPIV) (CLB 4/44) were provided by Dr. A. Quaroni (Cornell State University, Ithaca, NY) (4). The mABs recognizing ectoATPase and the bLPM marker 1/18 have been described previously (4). mABs against Mdr1 (C219) and Mdr2 (P3II-26) were from Alexis Biochemicals (Lausen, Switzerland). Anti-Caveolin-1 pABs and mABs, anti-reggie1/flotillin-2 mABs and anti-reggie2/flotillin-1 mABs were purchased from BD Transduction Laboratories (Heidelberg, Germany) and anti pan-actin pABs (pan Ab-5) were from Lab Vision (Basel, Switzerland). Goat anti mouse-Alexa488 was obtained from Invitrogen Molecular Probes (Carlsbad, CA) and donkey anti rabbit-Cy3 was from Jackson ImmunoResearch Laboratories (West Grove, PA).

Extraction and analysis of lipids: Lipids were extracted from cLPM and DRMs according to Bligh and Dyer as described (5). Briefly, 205µg cLPM or 200µl DRM-fractions were brought to 800µl with water and extracted with 3ml methanol:trichloromethane (2:1(vol/vol)) for 20min. at room temperature. The extracts were centrifuged for 5min. at 1400g, the lipid containing supernatants collected and the remaining pellets reextracted with 1ml trichloromethane. After another centrifugation, the pooled supernatants were mixed with 1ml 0.1M KCl and centrifuged again to obtain phase separation. The upper phase was completely removed and the lipids from the lower phase were dried under a stream of nitrogen, redissolved in 500µl trichloromethane, and stored at –20°C until used.
Lipids were separated by high performance thin layer chromatography using a modified method of Schmitz and Assmann as detailed in (5) for phospholipids. For the separation of cholesterol, the plates were developed twice in \( n \)-hexane: \( n \)-heptane:diethyl ether: acetic acid (63:18.5:18.5:1(vol/vol)) and subsequently processed as for separation of phospholipids. Quantification of individual lipids was performed with a Camag Scanner II (CAMAG, Muttenz, Switzerland).

Protein concentration was measured using the bicinchoninic acid assay (Interchim, Montluçon, France) with bovine serum albumin as a standard.

**SDS-polyacrylamide gel electrophoresis and immunoblotting:** For protein analysis, aliquots of membrane fractions were processed by electrophoresis and transferred to nitrocellulose membranes as described (4). For detection of proteins, nitrocellulose membranes were blocked for 1h with 5%(w/vol) non-fat dry milk dissolved in TBS-T (10mM Tris-HCl pH7.6, 150mM NaCl, 0.1 %vol/vol) Tween20), incubated with primary antibodies in TBS-T for either 2h at room temperature or over night at 4°C followed by three 10min. washes in TBS-T. The blots were then incubated for 1h at RT with the appropriate secondary antibodies diluted in TBS-T/5%(w/vol) dry milk, washed three times in TBS-T and developed with the UptiLight chemiluminescence reagent (Interchim).

**Immunofluorescence:** Rat livers were quickly removed after decapitation, processed into small cubic pieces, frozen in dry ice and stored at -80°C until use. 6\( \mu \)M cryosections were fixed for 10min. in methanol at 4°C. After 3 rinses for 5min. in PBS, sections were air-dried for 20min. at RT and thereafter incubated with the primary antibodies at appropriate dilutions in PBS containing 0.2%(vol/vol) TritonX-100 and 2%(vol/vol) donkey serum at 4°C over night. Following 3 rinses with PBS for 5min., the sections were incubated for 60min. at RT with secondary antibodies in the buffer used for primary antibodies. Finally, the sections were
rinsed again 3 times for 5 min. with PBS and cover-slipped with Shandon immumount (Thermo Electron Corp., Pittsburgh, PA). Sections were analyzed with fluorescence microscopy using a Zeiss Axiovert (Zeiss, Göttingen, Germany). Alternatively (Fig. 3E), cryosections were fixed for 5 min in methanol at 4°C. After rinsing with PBS, sections were transferred to the blocking solution (1% (w/vol) BSA in PBS) for 30 min and rinsed again. The sections were incubated with the primary antibody overnight at 4°C and after rinsing with PBS exposed to the secondary antibody. Analysis of antibody staining was performed with a Zeiss LSM510 system.

**Immunoelectron microscopy.** Thin slices (about 1 mm) of rat liver were fixed by immersion in ice-cold methanol for 30 min. Afterwards, they were placed in 0.6 M sucrose in PBS (10 mM phosphate buffer - 0.1M NaCl, pH 7.2) at ambient temperature and cut into 1 mm³ pieces. After 20 min, the tissue pieces were transferred in 1.2 M sucrose in PBS for 40 min and finally in 2.3 M sucrose in PBS for 4 hours. Tissue pieces mounted on aluminium pins were frozen and stored in liquid nitrogen. Ultrathin frozen sections (about 70 nm thin) were prepared with a cryo diamond knife (Diatome, Biel, Switzerland) using a Leica EM UC6 ultramicrotome equipped with a Leica EM LC6 cryochamber and picked up on nickel grids according to Tokuyasu (6, 7) and stored overnight on gelatin at 4°C. Prior to immunolabeling, gelatin was liquefied at 37°C, nickels grids removed and washed by floating them on droplets of PBS (pH 7.4). For double immunogold labeling, grids with the attached sections were floated on droplets of a mixture of appropriately diluted anti-Mrp2 and anti-caveolin-1 antibodies for 2 hours at ambient temperature. After rinses with PBS, grids were incubated on droplets of a mixture of gold-labeled affinity-purified goat anti-rabbit IgG (6 nm gold particles) and gold-labeled affinity purified goat anti-mouse IgG (12 nm gold particles) for 1 hour at ambient temperature. Finally, grids with the attached thin sections were rinsed in PBS, fixed with 2% (w/vol) glutaraldehyde in PBS for 10-20 min, rinsed with PBS and distilled
water, and embedded and stained with methylcellulose and uranyl acetate according to Tokuyasu (6, 7).

References


