YALE JOURNAL OF BIOLOGY AND MEDICINE 70 (1997), pp. 435-445.

Regulation of Organic Anion Transport in the Liver

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(Received May 12, 1997; accepted September 16, 1997)

In several liver diseases the biliary transport is disturbed, resulting in, for example, jaundice and cholestasis. Many of these symptoms can be attributed to altered regulation of hepatic transporters. Organic anion transport, mediated by the canalicular multispecific organic anion transporter (cmoat), has been extensively studied. The regulation of intracellular vesicular sorting of cmoat by protein kinase C and protein kinase A, and the regulation of cmoat-mediated transport in endotoxemic liver disease, have been examined. The discovery that the multidrug resistance protein (MRP), responsible for multidrug resistance in cancers, transports similar substrates as cmoat led to the cloning of a *MRP* homologue from rat liver, named *mrp*2. Mrp2 turned out to be identical to cmoat. At present there is evidence that at least two mrp's are present in hepatocytes, the original mrp (mrp1) on the lateral membrane, and mrp2 (cmoat) on the canalicular membrane. The expression of mrp1 and mrp2 in hepatocytes appears to be cell-cycle-dependent and regulated in a reciprocal fashion. These findings show that biliary transport of organic anions and possibly other canalicular transport is influenced by the entry of hepatocytes into the cell cycle. The cloning of the gene for cmoat opens up new possibilities to study the regulation of hepatic organic anion transport.

HEPATIC TRANSPORT OF ORGANIC ANIONS

Mutant rat strains, named TR^- [1], GY [2] and EHBR [3, 4], with a conjugated hyperbilirubinemia, led to the discovery of the canalicular multispecific organic anion transporter (cmoat)[b]. This ATP-dependent transporter [5] mediates the biliary excretion of non-bile acid organic anions. Substrates for cmoat include bilirubin diglucuronide [6], oxidized glutathione (GSSG) [5, 7], leukotreine C_4 (LTC_4) [8], and a range of other glutathione *S*-conjugates [9, 10]. Also sulfated and glucuronidated bile salts are transported by cmoat [11, 12]. The latter studies suggest that cmoat transports organic anions with at least two separate negative charges. Recently it was demonstrated that the multidrug resistance protein (MRP), responsible for non-P-glycoprotein-dependent multidrug resistance, transports similar substrates as cmoat in an ATP-dependent fashion [13-16]. Substrates

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[b] *Abbreviations:* mrp1, mrp2, multidrug resistance proteins 1 and 2; cmoat, canalicular multispecific organic anion transporter; cBAT, canalicular bile acid transporter; Ntcp, Na^+ -dependent bile acid transporter; CDNB, 1-chloro-2,4-dinitrobenzene; GS-DNP, glutathione-dinitrophenyl; CMFDA, chloromethylfluorescein diacetate; GS-MF, glutathione-methylfluorescein; GS-B, glutathione-bimane; GSH, reduced glutathione; GSSG, oxidized glutathione; PKC, protein kinase C; PKA, protein kinase A; DBcAMP, dibutyryl cAMP; LTC_4, leukotriene C_4; TNFα, tumor necrosis factor α; IL-1, interleukin-1; IL-6, interleukin-6; PMA, phorbol myristyl tetraacetate; LPS, lipopolysaccharide; BSP, bromosulfophthalein.

include leukotriene C_4 (LTC_4), glutathione-dinitrophenyl (GS-DNP) and oxidized glutathione (GSSG). The *MRP* gene has been cloned from a doxorubicin-resistant small cell lung cancer cell line that exhibits non-P glycoprotein-mediated multidrug resistance [17]. MRP is an 1531-amino acid, 190 kD, N-glycosylated integral membrane protein, encoded by a 6.5 kilobase mRNA (for reviews see Refs. [18-21]). The similarity in substrate specificity suggested that MRP may be identical to CMOAT. However, expression of MRP in normal liver is very low [22, 23] and MRP is localized to the lateral membrane in hepatocytes [24]. These similarities between MRP and CMOAT led to the cloning of a homologue of *mrp*, called *cmrp* or *mrp*2, from rat liver [25, 26]. The mrp2 protein is located on the canalicular membrane and is absent in the TR^- and the EHBR rat liver. Rat mrp2 consists of 1541 amino acids with a molecular mass of 190-200 kD [25, 26]. The mutation in the TR^- rat is due to a one-base pair deletion resulting in a frame shift and the introduction of a stop codon. The untranslated mutated *mrp2* mRNA appears rapidly degraded [25]. The human *MRP*2 has also been cloned [27] and has been shown to be defective in livers from Dubin-Johnson patients [28]. These data suggest that mrp2 is identical to cmoat. Therefore, at least two mrp's are present in the rat hepatocyte: one lateral form (mrp1) and one apical form (mrp2, cmrp or cmoat). To avoid confusion the "mrp1" and "mrp2" nomenclature will be used in the rest of this paper.

Several studies suggest that, besides mrp1 and mrp2, other, mrp-like, organic anion transporters with different substrate specificities probably exist in the hepatocyte. For example, impairment of biliary organic anion transport in the TR^- rat differs for different substrates: transport of bilirubin-diglucuronide is more affected than the transport of bilirubin-ditaurate, which is almost normal in the TR^- rat [29]. Studies with isolated membrane vesicles also indicate that organic anion transporters other than mrp2/cmoat probably are present on the canalicular membrane [30, 31]. These transporters may provide additional or alternative biliary secretion routes.

ASSAYS TO MEASURE AND DETECT ORGANIC ANION TRANSPORT ACTIVITY

Before *cmoat* was cloned, most data were derived from changes in transport activity, since no antibody was available. Using radioactive labeled glutathione-dinitrophenyl (GS-DNP) as a cmoat substrate, organic anion efflux can be measured in freshly isolated [7] and cultured hepatocytes [32]. GS-DNP is formed intracellularly from [^{14}C]1-chloro-2,4-dinitrobenzene (CDNB). Excretion from isolated hepatocytes is mainly mediated via mrp2, since GS-DNP excretion from isolated mutant TR^- rat hepatocytes is considerably lower. A more convenient assay, avoiding the use of radioactivity, determines the efflux of the fluorescent glutathione *S*-conjugate, glutathione-methylfluorescein (GS-MF) [22]. Cells are incubated with the non-fluorescent chloromethylfluorescein diacetate (CMFDA) at 10°C. The CMFDA is taken up by the cells via diffusion, and the acetate groups are removed intracellularly by esterases and the chloromethyl group is conjugated with GSH by glutathione *S*-transferases. When the 10°C medium is changed to a medium at 37°C, the initial efflux of the fluorescent product GS-MF can be determined. Quantitation of the fluorescence can be done in a microtiter plate using a fluorescence ELISA plate reader.

Under certain conditions, mrp activity may be visualized intracellularly using fluorescent mrp substrates such as carboxyfluorescein [33], glutathione-methylfluorescein (GS-MF) [22] the calcium indicator fluo-3 [34], calcein [35] and glutathione-bimane (GS-B) [36]. GS-B, formed from the non-fluorescent monochlorobimane (MCB), was shown to be transported into intracellular structures in 24 hr cultured rat hepatocytes [36]. Also, in HepG2 hepatoma cells and hepatocytes immortalized with SV-40 large T

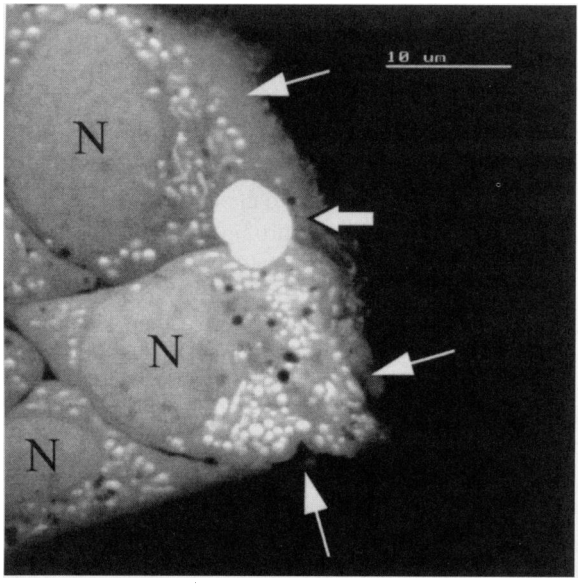

Figure 1. Accumulation of glutathione-methylfluorescein (GS-MF) in intracellular vesicular compartments in HepG2 cells. HepG2 cells, grown on coverslips, were loaded with 2.5 μM chloromethylfluorescein diacetate (CMFDA) for 15 min at 37°C. Cover slips were transferred to fresh medium and stored on ice. Intracellular accumulation of the MRP-substrate GS-MF was visualized by confocal microscopy. GS-MF accumulates in small intracellular vesicles (small arrows) and large (apical) vacuoles (large arrow), indicating MRP transport activity is present. N: nucleus.

antigen, fluorescent organic anion-accumulating vesicles are present, as visualized using GS-MF [22] (see also Figure 1). These vesicles may either originate from endocytosed plasma membrane or from the Golgi compartment. Both MRP1 and MRP2 can mediate this vesicular transport.

CYCLIC AMP STIMULATED SORTING OF ENDOCYTOSED MRP2-CONTAINING VESICLES

The above-mentioned GS-B accumulating vesicles found in normal cultured rat hepatocytes are not observed in mutant TR^- cells [36]. This indicates that mrp2 is also active in intracellular vesicles. In freshly isolated hepatocytes, these organic anion-accumulating vesicles are not detected. However, they gradually appeared upon prolonged culture, concomitantly with a decrease in GS-DNP efflux from the cells. After 24 hr of culture, 70 percent of mrp2 activity has disappeared from the plasma membrane [32]. These results indicate that after disruption of cell polarity by collagenase isolation of the hepatocytes, remnants of apical membrane containing mrp2 activity are endocytosed and accumulate intracellularly. Disruption of cell polarity also takes place during biliary obstruction. Hepatocytes isolated from bile duct ligated rats show a similar reduction of GS-DNP transport of 67 percent within 48 hr after obstruction [37]. Therefore, the disappearance of transport activity in this *in vivo* model may result at least partly from apical endocytosis of mrp2.

The fate of these endocytosed mrp2-containing apical membranes is different in isolated hepatocyte couplets, which have retained their polarity [38]. This was studied with GS-MF in order to visualize intracellular compartments containing mrp2 activity. Using confocal microscopy, it could be shown that in 3 hr cultured hepatocyte couplets, GS-MF accumulates in the canalicular lumen, in an intracellular (vesicular) network and in pericanalicular vesicles, identified as lysosomes. Incubation of the cells with dibutyrylcAMP (DBcAMP) for the last 2 hr of culture strongly stimulates GS-MF secretion into the canalicular lumen and significantly increased canalicular membrane circumference.

Under these conditions, the GS-MF containing intracellular structures have almost disappeared. A similar effect of DBcAMP has been observed on the apical targeting of the canalicular Cl^-/HCO_3^- exchanger [39] and on canalicular bile acid transport activity [40]. These data suggest that cAMP stimulates the fusion of vesicles containing canalicular transporters with the remaining canalicular membrane of the couplet. This phenomenon is inhibited by nocodazole [38, 40] and by bafilomycin A1 [41]. Nocodazole is an inhibitor of microtubule polymerization, and bafilomycin inhibits vesicular acidification. These results indicate a dependency of cAMP-stimulated sorting of mrp2 on microtubules and acidic compartments. A similar effect of cAMP on apical exocytosis from a subapical compartment has been observed in MDCK cells [42-44]. In contrast to the hepatocyte couplet model, DBcAMP did not effect efflux from non-polarized hepatocytes [45]. Thus, an intact apical domain may be essential for this effect. Stimulation of apical exocytosis by DBcAMP is not confined to the couplet system. Hayakawa et al. [46] showed that DBcAMP stimulates exocytosis of horseradish peroxidase into bile of the isolated perfused rat liver. Gatmaitan et al. [47], measured a 2-fold increase in both GS-DNP and taurocholate transport, in canalicular membrane vesicles isolated from rats injected with DBcAMP. The effect of DBcAMP was inhibited by colchicine. These data also suggest that *in vivo* cAMP stimulates a microtubule-dependent movement of mrp2 and cBAT to the canalicular membrane. Therefore, stimulation of PKA may activate a regulatory system that determines the secretory capacity of the canalicular membrane.

REGULATION OF MRP1 AND MRP2 BY PROTEIN KINASE C

Mrp2-mediated transport can be manipulated by activators and inhibitors of protein kinase C (PKC) [45]. Short-time incubations (3 min) of freshly isolated hepatocytes with the phorbol ester PMA and the hormone vasopressin, both activators of PKC, stimulated GS-DNP efflux from the cells by more than 50 percent. An inhibitor of PKC, staurosporine, inhibited efflux by 53 percent. In contrast, incubation of the cells with glucagon, forskolin and dibutyryl-cAMP (DBcAMP), all stimulators of PKA, did not influence organic anion transport. These results suggest that PKC is somehow involved in the regulation of mrp2-mediated organic anion transport. However, the nature of this regulation is not clear. Two possible mechanisms may be considered. First, a direct phosphorylation of the carrier by PKC may result in an increased transport activity as was suggested for P-glycoprotein [48]. However, phosphorylation studies of mrp2 have not yet been performed. Secondly, activation of PKC may regulate the number of carriers on the plasma membrane by mobilization of an intracellular pool of transporters to fuse with the plasma membrane as was shown for the insulin-responsive glucose transporter and the cystic fibrosis transmembrane conductance regulator (CFTR) (for reviews see Refs. [49, 50]). Evidence for the latter mechanism comes from observations made by Bruck et al. [51] who showed that both vasopressin and phorbol dibutyrate were able to stimulate apical exocytosis of the fluid phase marker horseradish peroxidase into bile of the isolated perfused liver. This process was inhibitable by the PKC inhibitor H7, suggesting that PKC is involved in apical exocytosis.

PKC also appears to regulate MRP1 transport activity. Ma et al. [52] found that drug accumulation in the MRP1 overexpressing leukemia cell line HL60/ADR is increased by treating the cells with H-7, staurosporine or chelerythrine, all more or less specific PKC inhibitors. MRP1 is phosphorylated, mainly on serine residues, in these cells. Treatment of cells with the above-mentioned PKC inhibitors reduces or even abolishes MRP1 phosphorylation, suggesting a correlation between PKC-mediated phosphorylation and the observed inhibition of transport activity. In another study with HL60/ADR and

GLC4/ADR cells, a reduction of MRP1 activity has been reported upon treatment with the specific PKC inhibitor bisindolylmaleimide [53]. No such studies have been performed with hepatic MRP1. These results suggest that both MRP1 and MRP2 may be regulated by a PKC-dependent mechanism. The importance of this regulatory mechanism to biliary organic anion secretion and bile formation has to be determined.

REGULATION OF MRP2 DURING ENDOTOXEMIA

Impairment of hepatic bilirubin transport, leading to hyperbilirubinemia, is frequently observed during sepsis [54-57]. Several factors may be involved in the inhibition of hepatobiliary organic anion transport. Acute effects of endotoxin were studied in the isolated perfused rat liver. Endotoxin present in the perfusate of a recirculating perfused rat liver causes an acute reduction (36 percent) of the biliary BSP excretion, accompanied by a decrease in both bile salt-independent bile flow and perfusate flow [58, 59]. Other models have been developed to study more long-term effects of LPS on biliary transport. Studies in perfused endotoxemic livers, isolated from rats 18 hr after injection with a single, relatively low dose of LPS, show diminished bilirubin (– 49 percent) and taurocholate (– 28 percent) transport [60]. Bile salt-independent bile flow is reduced by 42 percent in this study. This has been confirmed in recent transport studies with basolateral and canalicular membrane vesicles, isolated from endotoxemic rat livers [61, 62]. Also, the canalicular excretion of another mrp2 substrate, LTC_4, is reduced by 50 percent in the perfused endotoxemic rat liver [63]. Others report an 86 percent inhibition of bile acid transport, using a higher dose of endotoxin in *in situ* perfused livers [64]. These data indicate that with a low doses of endotoxin, bile acid transport is less affected than organic anion transport.

The reduction of bilirubin transport in endotoxemic livers can be partially attributed to impaired uptake [60]. However, the inhibition of organic anion transport is largely due to a reduction in canalicular cmoat/mrp2 transport activity as was demonstrated in hepatocytes isolated from endotoxemic livers [65]. The down-regulation of mrp2 and cBAT activity during endotoxemia is a gradual process with a maximal inhibition 12 hr after endotoxin injection [62, 65]. This is followed by a slow recovery in 4 to 5 days. Evidence from kinetic studies suggests that the reduction in mrp2 and cBAT-mediated transport is due to a reduced number of transporters [62]. Trauner et al. recently reported a 96 percent decrease in mrp2 mRNA and a 75 percent decrease in mrp2 protein levels, 16 hr after LPS injection [66]. Also bile acid uptake via the Na^+-dependent bile acid transporter (Ntcp) is substantially decreased due to decreased transcription and translation [61, 67].

How down-regulation of hepatic transport during endotoxemia is brought about is still an open question. Altered regulation of mrp2 by PKC appears not to be responsible, since stimulation of canalicular organic anion transport could not restore normal transport activity in endotoxemic hepatocytes [65]. Interestingly, Wettstein et al. found a 20 percent stimulation of biliary LTC_4 transport in endotoxemic livers by hypotonic media- or glutamine-induced cell swelling [63]. No significant effect was observed in normal liver. This suggest that in endotoxemic hepatocytes an intracellular population of mrp2-containing vesicles exist, able to fuse with the canalicular membrane, which is not present in normal hepatocytes. The nature of this intracellular population is unclear but probably represents mrp2 on their way to lysosomes to be degraded. Thus, inhibition of canalicular transport appears to result from inhibition of mRNA synthesis, which causes a reduced synthesis of transporters. This, in turn, leads to a reduced number of transporters on the canalicular membrane, because endocytosed mrp2 is not replenished with newly synthesized transporters.

Increasing evidence suggests that endotoxin-induced cytokines such as tumor necrosis factor α (TNFα) and interleukin-1 (IL-1) play an important role in mediating the down-regulation of hepatic transport: 1) Pretreatment of rats with dexamethasone, an inhibitor of cytokine production, largely prevents down-regulation of organic anion transport [65]; 2) Antibodies against TNFα prevent the down-regulation of bile acid transport during endotoxemia [64]; 3) TNFα and IL-1, but not interleukin-6 (IL-6), induce down-regulation of bile acid uptake and excretion [61, 67]. Cytokines may directly or indirectly, influence the expression of hepatic transporters. The signal transduction pathways involved in the down regulation of transcription of these transporters are currently unknown. If cytokines are the main mediators of down-regulation of hepatobiliary transport in sepsis, this will have implications for other situations where cytokines are produced, e.g., it may explain the impaired biliary transport in viral hepatitis.

CELL CYCLE-DEPENDENT REGULATION OF MRP1 AND MRP2

The presence of organic anion transporters on both the lateral (mrp1) and the canalicular (mrp2) membrane of the hepatocyte [24] seems incompatible with vectorial transport into bile. However, compared to mrp2, mrp1 expression is very low in normal hepatocytes. With our antibodies, mrp1 protein could be detected only in purified plasma membrane fractions from isolated hepatocytes but not in crude membranes and on frozen sections [22]. In contrast, mrp1 staining was detectable in the basolateral membrane of bile duct epithelial cells (Figure 2). No mrp2 staining was observed in these cells (unpublished observation), which may indicate that in bile duct epithelial cells mrp1 is the main glutathione *S*-conjugate transporter. Expression of mrp1 in hepatocytes is linked to proliferation. Transfection of hepatocytes with the SV40 large T antigen induces MRP1 expression. Also, in HepG2 hepatoma cells, MRP1 is highly expressed [22]. In these cells, MRP1 can be found in lateral membranes, but not at the basal membrane. Also MRP2 is expressed in these cells and is localized on the membranes of apical vacuoles formed

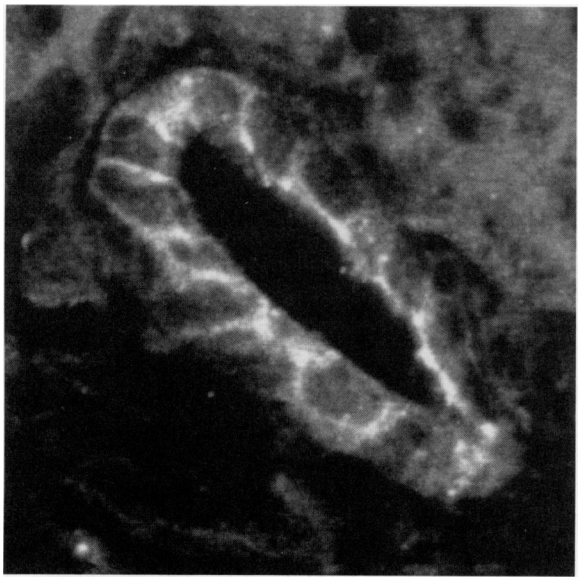

Figure 2. Localization of mrp1 in the basolateral membrane of bile duct epithelial cells. Frozen sections of rat liver were incubated with the pAb mrpk5 specific for mrp1 and with a secondary FITC-labeled anti-rabbit antibody. Staining was visualized by confocal microscopy. Bile duct epithelial cells show relatively strong staining of mainly basolateral membranes. Occasionally membranes facing the lumen are labeled. This may be due to poor histology.

between the cells (Figure 3). Therefore, MRP1-mediated transport may be important in proliferating hepatocytes but not in quiescent cells. Preliminary data in cultured hepatocytes confirmed this hypothesis [23]. TR^- hepatocytes cultured for 4 days show a gradual increase in organic anion (GS-MF) transport activity, which coincides with increased mrp1 protein expression. Similar experiments with normal hepatocytes show a decreased expression of mrp2, which has almost disappeared at day 4. Correlation with cell cycle markers c-myc and cyclin D1 indicate that an increase of mrp1 expression concomitantly with a decrease in mrp2 expression occurs in the G1 phase of the cell cycle. Thus, the expression of mrp1 and mrp2 appears to be regulated in a reciprocal fashion. Surprisingly, the localization of mrp1 depends on cell-cell contact. In hepatocytes cultured at low density, mrp1 is mainly present on intracellular vesicular structures. In high density cultures, mrp1 is located in the lateral membrane, where two adjacent cells make contact (unpublished observation). The function of mrp1 in proliferating hepatocytes is not clear but may involve transport of GSSG and lipid peroxidation products in order to maintain a proper redox status [18, 19], which is determined by the GSSG/GSH ratio [68, 69]. These findings show that biliary transport of organic anions and possibly other canalicular transport is negatively influenced by the entry of hepatocytes into the cell cycle. This may be due to a transient loss of cell polarity. Based upon these results, one can hypothesize that entry of hepatocytes into the cell cycle as a result of regeneration of a diseased liver will lead to a diminished capacity to generate bile. This may turn out to be an important mechanism for cholestasis in liver disease.

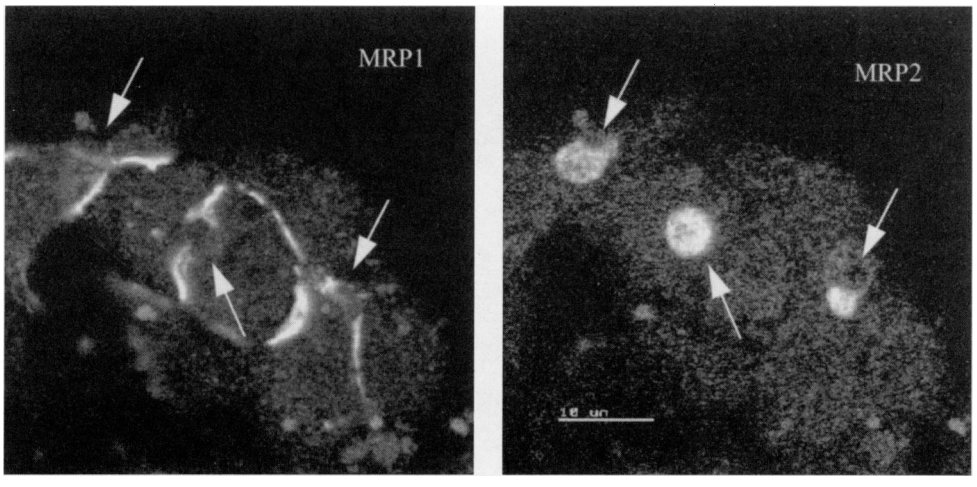

Figure 3. Localization of MRP1 and MRP2 in HepG2 hepatoma cells. HepG2 cells were stained for MRP1 (mrpk5 pAb) and MRP2 (mAb, provided by R.P.J. Oude Elferink). MRP1 staining can be observed on membranes where two cells make contact. The mrp2 antibody stains apical vacuoles in HepG2 (see arrows), suggesting that MRP2 is present in these vacuoles and probably is responsible for the observed accumulation of GS-MF (see Figure 1).

CONCLUSIONS

The cloning of *cmoat/mrp2* has provided new possibilities to study the mechanism of the impaired canalicular excretion of organic anions observed in many liver diseases. For example, the inhibition of organic anion transport during endotoxemia appears to be caused by an almost complete blockade of the *mrp2* mRNA synthesis. The regulatory mechanism behind this blockade remains unclear but may involve cytokines. This brings us to the question of how mrp2 expression is regulated on both the mRNA and protein level, and which factors are involved in the sorting of mrp2 to the canalicular membrane. Evidence obtained with cultured hepatocytes suggest that PKA stimulates apical sorting of mrp2. PKC may stimulate transport activity by either phosphorylation of the carrier and/or stimulate sorting to the canalicular membrane. Besides mrp2, a transporter with a similar substrate specificity, called mrp1, is present in liver and located in the basolateral membrane in hepatocytes and in bile duct epithelial cells. The expression of mrp1 and mrp2 in hepatocytes appears to be cell-cycle-dependent and regulated in a reciprocal fashion: In resting, differentiated hepatocytes mrp2 is highly expressed while mrp1 expression is low, in proliferating hepatocytes this expression pattern is reversed.

These data illustrate that hepatic organic anion transport is a highly regulated process. The underlying mechanisms are not yet clear. Future studies on the signal transduction pathways involved in the regulation and disregulation of these and other transporters in the liver may provide us with new insights in the mechanism of cholestatic liver disease and may lead to new therapies.

ACKNOWLEDGEMENTS: Supported by the Biomed and Health Research Program of the European Community (BMHI-CT93-1436) and the Netherlands Foundation for Scientific Research (NWO) (902-23-208).

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