P-glycoproteins and hepatobiliary secretion
Hooiveld, Guido Jacobus Etienne Johannes

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FUNCTION AND REGULATION OF ATP-BINDING CASSETTE TRANSPORT PROTEINS INVOLVED IN HEPATOBILIARY TRANSPORT

Guido JEJ Hooiveld*‡
Jessica E van Montfoort*§
Dirk KF Meijer*
Michael Müller‡

Groningen University Institute for Drug Exploration
*Department of Pharmacokinetics and Drug Delivery
‡Division of Gastroenterology and Hepatology
University of Groningen, Groningen, The Netherlands

§Division of Clinical Pharmacology and Toxicology
University Hospital Zürich, Zürich, Switzerland

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ABSTRACT

Hepatobiliary transport of endogenous and exogenous compounds is mediated by the coordinated action of multiple transport systems present at the sinusoidal (basolateral) and canalicular (apical) membrane domains of hepatocytes. During the last few years many of these transporters have been cloned and functionally characterized. In addition, the molecular bases of several forms of cholestatic liver disease have been defined. Combined, this has greatly expanded our understanding of normal physiology of bile formation, the pathophysiology of intrahepatic cholestasis, as well as of drug elimination and disposition processes. In this review recent advances, with respect to function and regulation of ATP-binding cassette (ABC) transport proteins expressed in liver, are summarized and discussed.
INTRODUCTION

Formation of bile is an important function of the liver because bile is essential for the digestion and absorption of lipids from the intestinal lumen, and it provides an excretory route for endogenous and xenobiotic compounds. Bile is formed by a process of osmotic filtration in response to osmotic gradients created within the lumen of the bile canaliculus between adjacent hepatocytes. This osmotic gradient is established by ongoing active secretion of solutes into the canalicular lumen. The main organic constituents of bile are bile salts, phospholipids, cholesterol, a variety of proteins, small peptides, amino acids, and bilirubin. Essential for the proper formation of bile are transport proteins located in the basolateral (sinusoidal) and apical (canalicular) membrane domains of hepatocytes. The hepatocellular localization of these transport proteins is depicted in Figure 1, and their corresponding functions are summarized in Table 1.

In this review we will focus on function and regulation of ATP-binding cassette (ABC) transport proteins expressed in liver. The first ABC transport proteins identified in liver (MDR1 and MDR3) are localized at the apical membrane domain of hepatocytes. Under normal physiological conditions, they mediate the last and rate-limiting step in hepatobiliary transport, i.e. the secretion of its substrate(s) into the canalicular lumen. More recently, it became clear that some of the additionally identified ABC transporter proteins are expressed at the basolateral membrane domain. This demonstrates that ABC transport proteins are also involved in the basolateral efflux of compounds.

Transport proteins localized at the basolateral membrane domain mediate the first step in hepatobiliary transport, the uptake of compounds into hepatocytes. We therefore begin this review with a brief outline of uptake carriers present in hepatocytes. However, for more in-depth information regarding hepatic uptake systems, the reader interested is kindly referred to recent papers covering this subject.

BASOLATERAL TRANSPORT SYSTEMS

Uptake of potentially toxic compounds into hepatocytes is mediated by a variety of transport proteins, belonging to the solute carrier superfamily (SLC). These transporters are not directly ATP-dependent for their function. In Figure 2, the molecular structures of various organic model compounds are presented. The Na+/taurocholate cotransporting polypeptide (NTCP, Ntcp in rodents) represents the major bile salt uptake system of hepatocytes [reviewed in Hagenbuch and Meier and Hagenbuch].

A variety of small (type I) organic cations, including drugs, choline, or monoamine neurotransmitters, are translocated by the organic cation transporter (OCT)-1. Until now, OCT1/Oct1 is the only electrogenic cation transporter identified that is strongly expressed at the basolateral membrane domain of hepatocytes, even though it belongs to a solute carrier family that comprises of more than 18 different gene products [reviewed in Koepsell and Koepsell et al.]. Other members of this gene family include OCT2/Oct2 and Oct3, the novel organic cation transporters (OCTN)-1 and OCTN-2, and the organic anion transporters (OAT)-1 to 3.

Members of the organic anion transporting polypeptide (OATP) gene family include human OATP1 [alternatively named OATP or OATP-A], human OATP2 [OATP-C or LST-1], rat Lst-1, and rat Oatp1-3. These polyspecific carrier systems mediate the uptake of a variety of organic anions, including bile salts and glutathione conjugates, but also accommodate neutral steroids and even bulky (type II) organic cations [reviewed in Meier et al., see also Van Montfoort et al.]. Additional members of the OATP gene
family include the prostaglandin transporter PGT/Pgt\textsuperscript{36,37}, and the organic anion transporters Oat-k1 and Oat-k2, originally cloned from kidney\textsuperscript{38,39}.

**CANALICULAR ATP-DEPENDENT TRANSPORT SYSTEMS**

Most canalicular transport systems involved in bile formation are ATP-binding cassette (ABC) transport proteins\textsuperscript{6}, one of the largest superfamilies of proteins in prokaryotes and eukaryotes\textsuperscript{40-43}. ABC transporters are membrane proteins that mediate transport of substrates across membranes and are driven by ATP hydrolysis\textsuperscript{12}. A typical ABC transporter consists of 12 or more membrane spanning domains and two intracellular
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nucleotide-binding loops, as outlined in Figure 3. The nucleotide-binding domains are highly conserved and contain the Walker A and B motifs that are involved in the binding and subsequent hydrolysis of ATP. The membrane spanning domains are thought to determine the substrate specificity of the transporter.

With respect to bile formation, two subclasses of the ABC superfamily are important: the P-glycoprotein (Pgp) subfamily [also named the ABC subfamily B based on the new system of nomenclature for ABC transporter genes (see: http://www.med.rug.nl/mdl/humanabc.htm)], and the multidrug-resistance protein (MRP) subfamily (ABC-C). Another subfamily, ABC-A, may also be of importance since at least three members (ABC1, ABC2, and ABC3) are expressed in liver at high level. However, the function and subcellular localization of these apparent transporters in liver is presently unknown.

Recently, it was reported that mutations in the human ABC1 gene are the molecular defect in Tangier disease, a rare recessive disorder characterized by the accumulation of cholesteryl ester in various tissues.

THE P-GLYCOPOPROTEIN (PGP) SUBFAMILY

P-glycoproteins, encoded by the human MDR1 or rodent Mdr1a/1b genes, are ATP-dependent drug transporters, and were initially isolated from multidrug resistant cells [reviewed in Gottesman and Pastan]. However, tissue distribution studies revealed that drug transporting Pgps are not only expressed in drug resistant cells, but also at the apical domain of cells in normal tissue with excretory functions, such as liver (canalicular membrane of hepatocytes), small intestine (brush border membrane of enterocytes), kidney (brush border membrane of proximal tubule cells), and at the blood-brain barrier (capillary endothelial cells). The drug transporting Pgps were therefore the first ABC transporters recognized in canalicular membranes of normal hepatocytes. Subsequently, it was demonstrated that the closely related human MDR3 and rodent Mdr2 proteins, that do not confer multidrug resistance, are predominantly expressed in liver. Another member of the Pgp subfamily was identified in pig liver and was (hence) named ‘sister of Pgp’ (Spgp).

MDR1 and Mdr1a/1b are present at low levels in normal liver. Considering its distribution pattern in excretory organs, combined with the capacity to export drugs, it has been suggested that MDR1 and Mdr1a/1b are involved in the body elimination of amphiphilic substrates and also may largely influence distribution patterns of drugs in the body. To investigate the physiological and pharmacological role of Mdr1a and Mdr1b, researchers at the Netherlands Cancer Institute developed Mdr1 gene knockout mice. Mice with a disruption of the Mdr1 genes grow and develop normally, are fertile, and have no obvious abnormality under laboratory conditions. These results indicate that Mdr1-type Pgps are not essential for basic physiological functions. However, these mice are very sensitive to xenobiotics, including chemotherapeutic agents, neurotoxins and cardiac glycosides, due to profoundly reduced clearance of these agents in liver, intestine, and brain. Interestingly, recent studies from our laboratory, using Mdr1 gene knockout mice, show the importance of Mdr1a/1b in the body distribution and elimination not only of typical MDR1 substrates, but also of relatively small, aliphatic and aromatic, permanently charged cationic drugs such as tri-n-butylmethylammonium, azidoprocainamide methiodide, and N-(4,4-azo-n-pentyl)-21-deoxyajmalinium. Taken together, it is clear that a major function of Mdr1a/1b is the protection of hepatocytes against toxic effects of many substances, e.g. those present in the diet. Mdr1a/1b confers protection by mediating the active secretion of toxic agents into bile. Besides just being drug transporters, additional functions have been proposed for Mdr1-type...
### Table 1: Functional Characteristics of Transport Proteins Expressed in Membranes of Human or Rat Hepatocytes

<table>
<thead>
<tr>
<th>Superfamily</th>
<th>Gene Symbol</th>
<th>Gene Product</th>
<th>Species</th>
<th>Alternative Name</th>
<th>Localization</th>
<th>Typical Substrates</th>
<th>Inhibitors</th>
<th>Driving Force</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solute Carrier Family 10</td>
<td>SLC10A1</td>
<td>NTCP</td>
<td>Human</td>
<td>NTCP</td>
<td>BM</td>
<td>Conjugated bile salts, such as TC</td>
<td>Secondary active, Na⁺ gradient</td>
<td>Exclusively expressed in liver</td>
<td></td>
</tr>
<tr>
<td>Solute Carrier Family 22</td>
<td>SLC22A1</td>
<td>OCT1</td>
<td>Human</td>
<td>OCT1</td>
<td>BM</td>
<td>Small (type 1) organic cations, including TEA, MPP, choline</td>
<td>Chemical gradient, membrane potential dependent</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>SLC22A7</td>
<td>OAT2</td>
<td>Human</td>
<td>Oat2</td>
<td>BM</td>
<td>Organic anions, including salicylate, MTX, PAH</td>
<td>BSP</td>
<td>Presently unknown, Na⁺ independent</td>
<td></td>
</tr>
<tr>
<td>Solute Carrier Family 21</td>
<td>SLC21A3</td>
<td>OATP1</td>
<td>Human</td>
<td>Oatp1</td>
<td>BM</td>
<td>Structurally unrelated and differently charged compounds, including unconjugated and conjugated bile salts (cholate, TC), uncharged compounds (ouabain), cardiac glycosides (digoxin), and permanently charged bulky (type 2) organic cations (APDA, mQd, mQn)</td>
<td>Presently unknown, Na⁺, Cl⁻ independent</td>
<td>May function bidirectionally</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SLC21A4</td>
<td>Oat-k1</td>
<td>Rat</td>
<td></td>
<td>BM</td>
<td>MTX, folate</td>
<td>BSP, TC</td>
<td>Presently unknown, Na⁺, Cl⁻ independent</td>
<td>Low expression in liver, may function bidirectionally</td>
</tr>
<tr>
<td>ABC Subfamily B</td>
<td>ABCB1</td>
<td>MDR1</td>
<td>Human</td>
<td>Pgp1/Pgp2</td>
<td>CM</td>
<td>Structurally unrelated, mainly amphipathic cationic compounds, including daunorubicin and vinristine, but also small (type 1) cations</td>
<td>Cyclosporin A, SDZ-PSC833, verapamil, vanadate, quinidine, quinine</td>
<td>Primary active, ATP hydrolysis</td>
<td>Expressed in many excretory organs</td>
</tr>
<tr>
<td></td>
<td>ABCB1a/1b</td>
<td>Mdr1a/1b</td>
<td>Rat</td>
<td></td>
<td>CM</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>ABCB4</td>
<td>MDR3</td>
<td>Human</td>
<td>MDR2</td>
<td>CM</td>
<td>Phosphatidylkholine</td>
<td>Cyclosporin A, SDZ-PSC833, verapamil, vanadate</td>
<td>Primary active, ATP hydrolysis</td>
<td>(Almost) exclusively expressed in liver. MDR3 protein is absent in patients with PFIC type 3</td>
</tr>
<tr>
<td></td>
<td>ABCB11</td>
<td>BSEP</td>
<td>Human</td>
<td>SPGP</td>
<td>CM</td>
<td>Monovalent bile salts, such as TC, tauroursodeoxycholate</td>
<td>SDZ-PSC833</td>
<td>Primary active, ATP hydrolysis</td>
<td>Exclusively expressed in liver. BSEP protein is absent in patients with PFIC type 2</td>
</tr>
</tbody>
</table>
### Table 1:

<table>
<thead>
<tr>
<th>super family</th>
<th>gene symbol</th>
<th>gene product</th>
<th>species</th>
<th>alternative name</th>
<th>localization</th>
<th>typical substrates</th>
<th>inhibitors</th>
<th>driving force</th>
<th>remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABC sub-family C</td>
<td>ABCC1</td>
<td>MRP1</td>
<td>human</td>
<td>MRP</td>
<td>BM</td>
<td>anionic conjugates with GSH, glucuronate, or sulfate, such as E17βG, LTC4, GSSG, but also cationic drugs in the presence of GSH</td>
<td>MK571, cyclosporin A</td>
<td>primary active, ATP hydrolysis</td>
<td>low expression in normal liver</td>
</tr>
<tr>
<td></td>
<td>ABCC2</td>
<td>MRP2</td>
<td>human</td>
<td>cMOAT, cMRP</td>
<td>CM</td>
<td>anionic conjugates with GSH, glucuronate, or sulfate, including bilirubin glucuronides, LTC4, E17βG, taurolithocholate 3-sulfate, but also anionic drugs such as pravastatin, MTX</td>
<td>MK571, cyclosporin A</td>
<td>primary active, ATP hydrolysis</td>
<td>MRP2 protein is absent in patients with Dubin-Johnson syndrome</td>
</tr>
<tr>
<td></td>
<td>ABCC3</td>
<td>MRP3</td>
<td>human</td>
<td>MOAT-D, MOAT-2</td>
<td>BM</td>
<td>conjugates with glucuronate or sulfate, including E17βG, E3040-glucuronide, taurolithocholate 3-sulfate</td>
<td>primary active, ATP hydrolysis</td>
<td>low affinity for GSH-conjugates</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MRP6</td>
<td>BM</td>
<td>BM</td>
<td>BM</td>
<td>BQ123</td>
<td>primary active, ATP hydrolysis</td>
<td>besides BQ123, currently no other substrate identified</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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a. Table is restricted to transport proteins expressed in liver.
b. See text for details and references.
c. Substrates mentioned are indicative but not complete.
Pgps. These range from transport of endogenous agents, such as steroid hormones, to secretion of cytokines, from a role in cytotoxicity of natural killer cells to intracellular transport of cholesterol [reviewed in Borst and Schinkel\textsuperscript{60,61} and Borst et al.\textsuperscript{62}]. However, the precise role of drug transporting Pgps in the above-mentioned processes remains to be elucidated.

In contrast to the drug transporting Pgps, the role of human MDR3 (and its rodent orthologue Mdr2) seems crucial for basic liver physiology. Although human MDR1 and MDR3 are 77% identical at the amino acid level, transport of drugs by MDR3/Mdr2 was not found in several experiments with transfected cells \textit{in vitro}\textsuperscript{63}. The \textit{MDR3/Mdr2} genes are almost exclusively expressed in the liver, and by immunohistochemistry it was demonstrated that MDR3/Mdr2 is located at the canalicular membrane domain of hepatocytes\textsuperscript{64,65}. In hepatobiliary lipid transport MDR3/Mdr2 functions as an ATP-dependent phosphatidylcholine (PC) translocase. This function became apparent when mice were generated with an disruption of the \textit{Mdr2} gene\textsuperscript{66}. These mice displayed a complete absence of PC and a dramatic decrease in cholesterol in bile. Mice heterozygous for \textit{Mdr2} gene had a 40% decreased PC but normal cholesterol output, demonstrating that absence of PC secretion is the primary defect by \textit{Mdr2} gene disruption. Further evidence supporting a role for Mdr2 as the PC translocase was provided by Ruetz and Gros\textsuperscript{67}, who demonstrated that Mdr2 could transport a fluorescent PC analogue through the membranes of yeast secretory vesicles. Recently it was shown that hepatocyte-specific expression of the human \textit{MDR3} could fully restore the secretion of PC into the bile in \textit{Mdr2} knockout mice\textsuperscript{68}. Additional evidence for a critical role of MDR3 in biliary PC secretion has been provided by De Vree et al.\textsuperscript{69}. These authors reported on mutations in the \textit{MDR3} gene and absence of MDR3 protein in patients with progressive familial intrahepatic cholestasis (PFIC) associated with elevations of both serum bile salts and \(\gamma\)-glutamyltranspeptidase levels. These data, taken together with a previous observation that patients deficient in biliary phospholipid secretion also lacked MDR3 expression\textsuperscript{70}, led to the classification of PFIC type 3 for cholestasis secondary to mutations in the \textit{MDR3} gene\textsuperscript{69}.

Following the partial cloning of Spgp in pig liver\textsuperscript{54}, the full-length cDNAs encoding rat\textsuperscript{71,72} and mouse\textsuperscript{73,74} Spgp were isolated. After functionally expressing rat Spgp cDNA in Sf9 insect cells, Gerloff \textit{et al.}\textsuperscript{71} were the first to demonstrate that Spgp functions as an ATP-dependent bile salt transporter. Rat Spgp-mediated transport rates of the various bile salts tested have the same order of magnitude as ATP-dependent transport in canalicular rat liver plasma membrane vesicles. The encoded protein was localized by immunofluorescence microscopy and immunogold labeling studies to canalicular microvilli and to a canalicular sub-population of membrane vesicles. Gerloff \textit{et al.}\textsuperscript{71} therefore speculated that Spgp represents the major bile salt export pump (Bsep) of mammalian liver. This proposition is supported by the recent findings that the \textit{BSEP/SPGP} gene is mutated in patients with PFIC type 2 (PFIC 2)\textsuperscript{75,76}, a syndrome characterized by extremely low biliary bile salt concentrations, elevated serum bile salt concentrations and normal serum levels of \(\gamma\)-glutamyltranspeptidase. Analysis of the \textit{BSEP/SPGP} coding region in affected patients revealed mutations resulting in the absence of functional \textit{BSEP/SPGP}\textsuperscript{75}. In clonogenic assays it was found that rat Bsep/Spgp transfected cells had a low level of resistance to taxol only, but not to other drugs (e.g. vinblastine or digoxin) that form part of the multidrug resistance phenotype\textsuperscript{72}. Similar results were reported by Lecureur \textit{et al.}\textsuperscript{73}, who evaluated transport function of mouse Bsep/Spgp using transfected cells. They reported that only vinblastine efflux was slightly enhanced in transfected cells whereas no effect was observed for vincristine or digoxin. Consequently, these studies
indicate that it is unlikely that Spgp/Bsep plays an important role in detoxification of xenobiotics.

**THE MULTIDRUG RESISTANCE-ASSOCIATED PROTEIN (MRP) SUBFAMILY**

In the MRP subfamily, the expression of (at least) four members has been demonstrated in liver until now [reviewed in Borst et al.77]. MRP1 (Mrp1 in rodents) was initially cloned from a multidrug resistant small lung cancer cell line78, and was the first transporter of the MRP subfamily identified in liver. *Mrp1* gene knockout mice that have been generated are viable and fertile, but their response to an inflammatory stimulus is impaired. This is attributed to a decreased secretion of leukotriene C4 (LTC4) from leukocytes79. Moreover, *Mrp1* knockout mice are hypersensitive to the anticancer drug etoposide79,80. In hepatocytes and transfected polarized cells MRP1/Mrp1 is located at the basolateral membrane domain81,82, but its expression level in liver is low under normal conditions7. In contrast, Mrp2, a homologue of Mrp1, is highly expressed in the canalicular membrane domain of hepatocytes, and functions as a canalicular multispecific organic anion transporter83-85. Even though both transport proteins are expressed in different membrane domains, the substrate specificity of Mrp1 and Mrp2 is similar, if not identical. However, major kinetic differences have been established [reviewed in König et al.86]. Physiologically important substrates for Mrp1 and Mrp2 include glutathione S-conjugates such as leukotriene C4, bilirubin- and estrogen-glucuronides, taurolithocholate 3-sulfate, and glutathione disulfide (GSSG)86. In addition, transport of several cationic drugs in the presence of GSH has been observed87-89. Mrp2 was shown to be absent in the so-called Groningen Yellow/Transport Deficient (GY/TR') and Eisai hyperbilirubinemic (EHBR) rats83,90. These are two different rat strains with a naturally occurring hereditary defect in non-bile salt organic anions91,92. Absence of Mrp2 in these rats is due to mutations that introduce a stop codon in the coding sequence of *Mrp2* gene83,90. Similarly, MRP2 protein is not expressed in patients with Dubin-Johnson syndrome93,94, an inheritable disorder that is associated with deficient biliary secretion of amphiphilic anionic conjugates, including bilirubin glucuronides. These patients have missense mutations or nucleotide deletions in the *MRP2* gene, resulting in the absence of MRP2 protein94,95. The elucidation of all exon-intron boundaries of the human *MRP2* gene96,97 will further facilitate the analysis of additional mutations. Recently, using cell viability assays it was demonstrated that MRP2 confers resistance to various cytotoxic drugs98-100. However, it remains to be clarified whether this also occurs in vivo.

By screening databases of human expressed sequence tags, Kool et al.101 identified 4 additional members of the MRP transporter family (MRP3 to MRP6) and examined the expression of these MRP homologues in human tissue. *MRP3* mRNA is expressed at relatively low levels in normal human liver and intestine, *MRP4* mRNA is present at low level in only a few tissues, but not in liver, *MRP5* mRNA is ubiquitously expressed at intermediate levels, whereas *MRP6* mRNA is easily detectable in liver and kidney only101-103. The full length human and rat *MRP3* cDNAs have been cloned by several laboratories104-108. Immunohistochemical analyses revealed that MRP3/Mrp3 protein is most likely located in the basolateral membrane of hepatocytes and cholangiocytes106,107, although one paper reported on an apical localization105. Recently, the transport properties of rat Mrp3 have been characterized using membrane vesicles from transfected cells109. It was demonstrated that several kinds of organic anions, in particular glucuronide conjugates, are transported via Mrp3. However, the substrate specificity of Mrp3 differs from that of
Mrp1 and Mrp2 since glutathione S-conjugates are poor substrates for Mrp3. Moreover, it was reported that rat Mrp3 is able to transport bivalent as well as monovalent bile acids that are also substrates for Mrp2 or Bsep, respectively, such as tauroliothocholic acid 3-sulfate and taurocholic acid. The full-length cDNAs encoding human MRP4 and human MRP5 have been cloned. The expression of MRP4 and MRP5 proteins in liver is low or even absent and neither the physiological function nor cellular localization of these proteins are yet known. The cDNAs of both human and rat MRP6 have been
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isolated\textsuperscript{102-104,114}. Using a polyclonal antiserum, Madon et al.\textsuperscript{114} located rat Mrp6 protein mainly to the lateral membrane domain of hepatocytes. Mrp6 was demonstrated to transport the anionic cyclopentapeptide BQ123, an endothelin receptor antagonist, but transport of leukotriene C\textsubscript{4} or taurocholic acid, ‘classic’ substrates for respectively Mrp1/2 or Spgp/Bsep was not observed\textsuperscript{114}. The physiological function of MRP6/Mrp6 remains to be clarified.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure2}
\caption{(CONTINUED). Molecular structures of various frequently used cationic, anionic, and neutral organic model compounds. C. Cardiac glycosides, such as digoxin and ouabain, are uncharged compounds that interact with the hepatic uptake of type 2 cations and are likely substrates for MDR1 P-glycoprotein. D: Cholic acid is a major primary bile acid and its uptake into hepatocytes is mediated by members of the OATP superfamily. Taurocholic acid, a monovalent conjugated primary bile acid, is an excellent substrate for Ntcp and Bsep, as well as for Mrp3. The secretion by hepatocytes of taurolithocholic acid 3-sulfate, a bivalent conjugated and sulfated secondary bile acid, may be mediated by both Mrp2 and Mrp3. E: Glutathione (GSH), glutathione disulfide (GSSG), leukotriene C\textsubscript{4}, 17b-estradiol 17-(beta-D-glucuronide), and bilirubin glucuronides are substrates for Mrp1 and Mrp2, whereas only the latter two substances are efficiently transported by Mrp3.}
\end{figure}
In recent years important advances have been made in the understanding of the regulation processes involved in bile formation. Of particular importance has been the analysis of transporter gene expression in animal models of intrahepatic and obstructive cholestasis. In rats, cholestasis can be experimentally induced by e.g. endotoxin (LPS)- or estrogen (EE)-treatment, or common bile duct ligation (BDL). In addition, liver regeneration can be mimicked by two-thirds partial hepatectomy (PH). However, it should be emphasized that although many of these models may represent aspects of physiological regulation processes, they may also (at least partially) reflect pathological or drug-induced perturbations that are basically different from physiological regulatory processes.

The level of Mdr1a mRNA is not affected by endotoxin treatment and increases only slightly after bile duct ligation or partial hepatectomy. In contrast, Mdr1b gene expression is markedly enhanced during LPS and BDL induced cholestasis, and even more in the remnant liver after partial hepatectomy. Combined with earlier studies, these results demonstrate that the expression of the two rodent Mdr1 genes is differentially regulated. In vitro studies revealed that expression of the human MDR1 gene is induced by a variety of toxic agents, UV irradiation, and heat shock. This implies that MDR1 promoter activation may be part of a general stress response in many cells. Similar observations have been made with reference to rodent Mdr1a/1b gene expression.

Expression of the PC translocase Mdr2 gene is not affected after LPS treatment, and is only slightly enhanced after bile duct ligation and partial hepatectomy. Continuous exposure of rats to simvastatin or pravastatin, inhibitors of 3-hydroxy-3-methylglutaryl-coenzyme A reductase, results in increased biliary phospholipid output and Mdr2 gene expression and protein levels. Similar observations have been made in mice that were fed the peroxisome proliferators ciprofibrate and clofibrate. When mice were fed a
diet supplemented with the hydrophobic bile salt cholate, it was observed that \textit{Mdr2} mRNA levels were induced, which was functionally reflected in a concomitant increase in the maximal phospholipid secretion capacity\textsuperscript{135,136}. Feeding the (relatively) hydrophilic bile salt ursodeoxycholate did not influence the \textit{Mdr2} mRNA levels nor maximal PL output capacity\textsuperscript{135}. These latter findings imply that the type of bile salt in plasma may influence the expression level of \textit{Mdr2} protein and therefore the rate of PL secretion. Initially, functional studies with canalicular membrane vesicles prepared from endotoxemic animals revealed that the ATP-dependent transport of substrates for Bsep and Mrp2 was greatly decreased\textsuperscript{137-139}. In subsequent studies it has been demonstrated that in rats treatment with LPS or EE, and BDL results in a marked decrease of \textit{Mrp2} and, to a lesser extent, of \textit{Bsep} mRNA levels\textsuperscript{115,140}. Besides a decreased \textit{Mrp2} gene expression, a redistribution of Mrp2 protein from the canalicular membrane to an intracellular subapical compartment is also observed after LPS treatment\textsuperscript{141}. Interestingly, the decreases of \textit{Bsep} mRNA and protein in the before mentioned cholestatic models is less prominent than those observed for the basolateral bile salt carriers Ntcp and Oatp1, or the canalicular transporter \textit{Mrp2}\textsuperscript{115,140,142-146}. Thus, Bsep protein expression is relatively preserved during cholestatic liver injury and therefore it may continue to excrete bile salts, although at impaired rates\textsuperscript{146,147}. Remarkably, after partial hepatectomy the mRNA levels of \textit{Mrp2} and \textit{Bsep} are only slightly decreased, while the proteins levels of Mrp2 and Bsep were unaffected or were even somewhat upregulated\textsuperscript{116,148}. This is likely the molecular explanation for the fact that the remnant liver after partial hepatectomy is not cholestatic, whereas cholestasis induced by LPS, EE, or BDL treatment is due to, apart from redistribution of intracellular Mrp2 protein, downregulation of \textit{Mrp2} and \textit{Bsep} mRNA levels. A dose- and time-dependent induction of \textit{Mrp2} gene expression was observed in isolated rat hepatocytes cultured in the presence of xenobiotics, including vincristine, tamoxifen, rifampicin, or cycloheximide\textsuperscript{149,150}.

As mentioned earlier, the level of \textit{MRP1/Mrp1} mRNA and protein levels in normal, resting hepatocytes is very low\textsuperscript{7}, but mRNA levels of \textit{Mrp1} are considerably increased after endotoxin administration and partial hepatectomy\textsuperscript{115,116}. Furthermore, \textit{Mrp1} mRNA and protein levels were found to be increased in human hepatoblastoma HepG2 cells and SV40 large T antigen-immortalized human hepatocytes\textsuperscript{81}. These results suggested that \textit{MRP1/Mrp1} gene expression and function may be associated with cell proliferation. Indeed, in a recent study it was reported that in isolated rat hepatocytes that have entered the cell cycle \textit{Mrp1} gene expression is induced while expression of \textit{Mrp2} is decreased\textsuperscript{151}. This switch in expression occurred in the mid-G\textsubscript{1} phase of the cell cycle, and appeared associated with a decrease in cell polarity. Yamane \textit{et al.}\textsuperscript{152} showed that \textit{Mrp1} gene expression is induced when rat hepatoma H4IIE cells are exposed to reactive oxygen-generating compounds. This was coupled to an increased expression of \(\gamma\)-glutamylcysteine synthetase (\(\gamma\)-GCS), a rate-limiting enzyme in the biosynthesis of GSH. As mentioned before, GSH is an important factor in Mrp1 function as well as in the defense against metabolites generated by oxidative stress [reviewed in Wang and Ballatori\textsuperscript{153} and Lu\textsuperscript{154}]. Based on these results, it is proposed that expression of \textit{Mrp1} as well as \(\gamma\)GCS gene is, at least partially, regulated by the intracellular reduction-oxidation (redox) status\textsuperscript{152}. Moreover, a parallel expression pattern of \textit{MRP1} and \(\gamma\)GCS mRNA has been reported for many drug-resistant cell lines, colon tumors from patients, and normal mouse tissues\textsuperscript{155-159}.

\textit{Mrp3} is another candidate that could provide basolateral export of organic anions from hepatocytes. Interestingly, \textit{Mrp3} mRNA and protein levels are induced in Mrp2 deficient EHBR rats and in BDL- and \(\alpha\)-naphthilisothiocyanate-induced cholestasis\textsuperscript{104,160}. All these animal models are characterized by increased concentrations of biliary constituents in
systemic blood, including unconjugated bilirubin and bilirubin conjugates. These latter compounds were shown to induce the hepatic expression of Mrp3 mRNA levels\textsuperscript{160}. Increased amounts of MRP3 are also detected in livers of Dubin-Johnson patients\textsuperscript{107}. Considering the cellular localization of Mrp3, its upregulation during cholestasis, and its substrate specificity, it is hypothesized that Mrp3 may play a significant role in the basolateral export of bile acids under conditions in which Mrp2 is absent or downregulated\textsuperscript{104,107,160}. In addition, Mrp3 may be involved in the cholehepatic circulation of bile salts\textsuperscript{110}.

**MOLECULAR MECHANISMS INVOLVED IN REGULATION OF HEPATIC ABC PROTEINS**

Cytokines, such as interleukin-1\(\beta\) (IL-1\(\beta\)), tumor necrosis factor-\(\alpha\) (TNF-\(\alpha\)), and IL-6, play an important role in the regulation of hepatic transport proteins\textsuperscript{161,162}. It is known that a major part of the LPS-induced changes in hepatocytes is due to the combined action of the before mentioned cytokines, released from Kupffer cells\textsuperscript{162,163}. For example, administration of anti-TNF-\(\alpha\) antibodies to, or pretreatment of rats with dexamethasone, an inhibitor of LPS-mediated cytokine release, neutralizes the effects of LPS on Mrp2 expression and restored biliary bile salt secretion and bile flow, whereas downregulation of Ntcp after LPS-administration could be mimicked by injection of rats with TNF-\(\alpha\) or IL-1\(\beta\)\textsuperscript{138,141,142,164}.

After the isolation of the human \textit{MDR1} promoter, it became clear that it contains an inverted CCAAT box, identified as core sequence of the so-called Y-box, and a number of putative recognition sites for transcription factors, including those for activator protein (AP)-1, stimulating protein (SP)-1, nuclear factor (NF)-Y, and Y-box binding protein (YB)-1\textsuperscript{165-168}. NF-Y and SP1 interact with the Y-box and the GC-rich region, respectively. These two transcription factors cooperate in controlling the basal \textit{MDR1} promoter activity\textsuperscript{169}. Recently, an important role for both NF-Y and SP1 in the transcriptional activation of the \textit{MDR1} gene after genotoxic stress was demonstrated\textsuperscript{168}. In contrast, YB-1 that has been identified by others as important for \textit{MDR1} gene upregulation after UV-exposure\textsuperscript{170}, was found not to be sufficient to mediate \textit{MDR1} gene activation after genotoxic stress\textsuperscript{168}. As in many other promoter regions, the SP1 binding site in the \textit{MDR1} 5’-flanking region significantly overlaps with binding sites for transcription factors of the early growth response (EGR) family\textsuperscript{171}. Binding of SP1 or EGR1 to these GC-rich DNA sequences is often mutually exclusive, and the result can be different depending on the cellular ‘context’\textsuperscript{172}. The rat \textit{Mdr1b} promoter is positively regulated by SP1, whereas overexpression of EGR1 decreased \textit{Mdr1b} promoter activity\textsuperscript{173}. Therefore, it was concluded that competitive interactions between SP1 and EGR1 determine the constitutive expression of \textit{Mdr1b} mRNA in rat hepatoma cells\textsuperscript{173}. P53 is another factor shown to be involved in the basal regulation of \textit{MDR1} and rat \textit{Mdr1a/1b} gene expression\textsuperscript{174,175}. Interestingly, opposing effects were found when the effect of p53 status on \textit{MDR1} and \textit{Mdr1a/1b} gene expression was studied: whereas wild-type p53 repressed \textit{MDR1}/\textit{Mdr1a} gene expression, overexpression of mutant p53 resulted in markedly elevated levels of \textit{MDR1} promoter activity as well as rat \textit{Mdr1a} mRNA and protein levels\textsuperscript{174}. In contrast, Zhou and Kuo\textsuperscript{175} identified a p53-binding site in the rat \textit{Mdr1b} promoter, and reported that wild-type p53 upregulated \textit{Mdr1b} promoter activity and endogenous gene expression of rat \textit{Mdr1b}. These opposing results may provide an explanation for the observed different regulation of rodent \textit{Mdr1a/1b} gene expression. The induction of rat \textit{Mdr1b} mRNA levels by endogenous signaling molecules such as TNF-\(\alpha\) or insulin has recently been
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demonstrated\textsuperscript{176,177}. Induction of \textit{Mdr1b} gene expression after exposure to insulin is mediated by binding of the transcription factor nuclear factor (NF-κB) to a functional NF-κB-like binding site in the rat \textit{Mdr1b} promoter\textsuperscript{176}. This transcription factor may also be involved in regulation of \textit{Mdr1b} gene expression in response to reactive oxygen species\textsuperscript{130}.

Until now, SP1 is the only transcription factor that is identified to functionally interact with the promoter of the phosphatidylcholine translocase MDR3/\textit{Mdr2}, and seems necessary for basal expression\textsuperscript{178-180}. Since in rodents \textit{Mdr2} mRNA and protein levels are enhanced after treatment with statins, it is speculated that sterol regulatory element-binding proteins (SREBPs) are involved in the regulation of \textit{Mdr2} expression\textsuperscript{131}.

Analysis of the promoter region of the \textit{MRP1} gene has identified consensus binding sites for numerous transcription factors including the activator proteins AP1 and AP2, stimulating protein-1 (SP1), and glucocorticoid response elements\textsuperscript{181}. It was shown that the SP1 binding sites present in the promoter are essential for optimal \textit{MRP1} transcriptional activity\textsuperscript{182}, and that the tumor suppressor gene p53 suppressed \textit{MRP1} promoter activity: SP1 alone stimulated \textit{MRP1} promoter activity up to approximately 200-fold; the latter was attenuated by coexpression of p53\textsuperscript{183}.

The promoter regions of the human and rat \textit{MRP2} gene have been isolated\textsuperscript{184,185}. Sequence analysis of the human \textit{MRP2} promoter showed a number of consensus binding sites for both ubiquitous and liver-enriched transcription factors, including AP1, SP1, and hepatocyte nuclear factor (HNF)-1 and -3β. Based on studies with various deletion constructs it appears that important regulatory elements are localized within a fragment ranging from 431 to 251 base pairs upstream of the transcription initiation site\textsuperscript{184}. In this region a putative binding site for the liver-abundant transcription factor CCAAT-enhancer binding protein β (C/EBPβ) is present, and mutations in this site result in a 50% decrease of promoter activity\textsuperscript{184}. Analysis of the rat \textit{Mrp2} promoter region revealed that two sequences, one containing an inverted CCAAT element and the other a GC-box, are required for basal expression of rat Mrp2 in H4IE rat hepatoma cells\textsuperscript{185}.

The human \textit{MRP3} promoter sequence has recently been isolated and several putative binding sites for transcription factors, including AP1, AP2, and SP1, have been identified\textsuperscript{186}. Future experiments, however, have to clarify which transcription factors functionally interact with the \textit{MRP3} promoter region.

CONCLUSIONS AND PERSPECTIVES

Hepatobiliary transport of endogenous and exogenous compounds is mediated by the coordinated action of multiple transport systems present at the basolateral and canalicular membrane domains of hepatocytes. Many of these transporters have been cloned and functionally characterized. However, the relative contribution of each individual protein in transmembrane transport of substrates \textit{in vivo} remains to be established. Such information will be instrumental for the design of pro-drugs for organ- and cell-selective drug delivery\textsuperscript{187} and may provide additional insights in the potential role of transport proteins, such as MDR1\textsuperscript{188}, in clinically significant drug-drug interactions. The generation of and studies with transporter gene knockout mice address this topic and will contribute to a further elucidation of elimination patterns of substrates. However, due to compensatory upregulation of other transporter genes the information obtained may not always be unequivocal. For these transporters the use of conditional knockout mice appears to be more appropriate.

During the last few years the molecular bases of several forms of cholestatic liver disease have been defined. Combined with the analysis of transporter gene expression in animal
models of intrahepatic and obstructive cholestasis, this has resulted in better understanding of normal physiology of bile formation as well as the pathophysiology of cholestasis.

The isolation and characterization of hepatic transcription factors that govern aspects of major metabolic and developmental pathways is of particular interest for liver physiology, since the liver fulfills a pivotal role in controlling whole body metabolism. These factors include sterol regulatory element binding proteins (SREBPs), controlling transcription of various genes in the fatty acid and cholesterol metabolic pathways [reviewed in Brown and Goldstein189,190], and certain nuclear hormone receptors. The latter comprise a large superfamily of ligand-modulated transcription factors that mediate responses to both endogenous and exogenous cofactors, such as oxysterols, bile acids, all-trans retinoic acid and 9-cis retinoic acid, or unsaturated fatty acids and fibrates. These molecules are ligands for the liver X receptor (LXR), farnesoid receptor (FXR), the retinoic acid receptors RAR and RXR, and peroxisome proliferator-activated receptor (PPAR), respectively [reviewed in Willy and Mangelsdorf191 and Giguere192]. The elucidation of their role in transcriptional control of transporter gene expression will shed further light on the mechanisms of adaptive responses as well as short and long term regulation of transport proteins in health and disease.

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