Regulation of Expression of the Multidrug Resistance-Associated Protein 2 (MRP2) and Its Role in Drug Disposition

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ABSTRACT
The multidrug resistance protein 2 (MRP2; ABCC2) is an ATP-binding cassette transporter accepting a diverse range of substrates, including glutathione, glucuronide, and sulfate conjugates of many endo- and xenobiotics. MRP2 generally performs excretory or protective roles, and it is expressed on the apical domain of hepatocytes, enterocytes of the proximal small intestine, and proximal renal tubular cells, as well as in the brain and the placenta. MRP2 is regulated at several levels, including membrane retrieval and reinsertion, translation, and transcription. In addition to transport of conjugates, MRP2 transports cancer chemotherapeutics, uricosurics, antibiotics, leukotrienes, glutathione, toxins, and heavy metals. Several mutagenesis studies have described critical residues for substrate binding and various naturally occurring mutations that eliminate MRP2 expression or function. MRP2 is important clinically as it modulates the pharmacokinetics of many drugs, and its expression and activity are also altered by certain drugs and disease states.
Fig. 1. A, phylogenetic tree showing the human MRP family, redrawn from Borst et al. (1999). SUR, sulfonylurea receptor; CFTR, cystic fibrosis transmembrane regulator. B, proposed membrane topology of Mrp2, adapted and redrawn from Ito et al., (2001). C, amino acid sequence alignment for human MRP2 (Q99887), MRP1 (P33527), and MRP6 (Q51438), and rat Mrp2 (Q63120), and Mrp3 (Q88563), with respective NCBI protein accession numbers. Sequences were aligned using multiple sequence alignments on BCM Search Launcher (http://dot.imgen.bcm.tmc.edu:9331/multi-align/multi-align.html) and formatted using BoxShade 3.21 (http://www.ch.embnet.org/software/BOX_form.html). Approximate locations of putative TMs were obtained from the NCBI protein entries indicated above and may differ from those assumed in the references. Dots indicate mutations in MRP2 or Mrp2 discussed in the text.
due to distinct mutations in the mrp2 gene, both of which create premature termination codons. Cloning of mrp2 has made possible an understanding of its structure function relationships, localization and regulation of expression, and characterization of the defect in patients with the Dubin-Johnson Syndrome (see below).

**Structure and Function of MRP2**

The membrane topology predicted for MRP2 is like that of MRP1 and contains 17 transmembrane (TM) helices, which form three membrane-spanning domains (MSD1, -2, and -3) connected by poorly conserved linker regions (L0 and L1) and highly conserved nucleotide-binding domains (NBD1 and NBD2) (Fig. 1B) (Borst et al., 1999; Konig et al., 1999a). Both the predicted odd number of transmembrane domains and immunofluorescence studies indicate the extracellular localization of the amino terminus (Konig et al., 1999a).

Studies in patients with Dubin-Johnson syndrome have revealed valuable information about MRP2 genomic organization and the structure and function of MRP2 protein. The classic Dubin-Johnson syndrome (DJS) consists of elevated total and direct bilirubin, increased urinary coproporphyrin I fraction (>80%), and deposition of a dark pigment in the liver (Tob et al., 1999). Patients with DJS may also have a decreased biliary clearance of bromosulfophthalein and some degree of jaundice (Tob et al., 1999). DJS is linked to mutations in the MRP2 gene; these are summarized in Table 1 (Paulusma et al., 1997; Wada et al., 1998; Tob et al., 1999; Ito et al., 2001e; Mor-Cohen et al., 2001). Homozygous mutations lead to classic DJS, whereas heterozygous mutants have moderately elevated urinary coproporphyrin 1 fraction (~40%) with normal total and direct bilirubin (Tob et al., 1999). Many of these mutations are localized to NBD1 or NBD2. Unlike other mutations, R1150H mutants of the MRP2 protein mature and are properly localized, but transport activity is impaired (Mor-Cohen et al., 2001). Future studies are needed to identify any polymorphisms and their effect on MRP2 function.

Characterization of the substrate recognition/transport sites of MRP2 has been based on the importance of amino acid residues located in the MSD of other ATP-binding cassette transporters, MDR1, and cystic fibrosis transmembrane regulator, and the closely-related family members MRP1 and MRP3. MRP3 was cloned as a homolog of MRP1 and 2 and is highly expressed on the basolateral membrane of rat and human liver under cholestatic and hyperbilirubinemic conditions (Hirohashi et al., 1998; Kiuchi et al., 1998; Konig et al., 1999b). Hydropathy plot analysis has shown that the structures of MRP1 to 3 are very similar. Initial studies showed that the amino terminal MSD1 of MRP1 is nonessential for substrate transport (Bakos et al., 1998), although the linker region connecting MSD1 and MSD2 is essential for MRP1 transport of leukotriene C4. Likewise, no mutations in MSD1 of MRP2 have been identified in patients with DJS, consistent with this region lacking a critical function for MRP2 in humans (Table 1). Efforts have therefore focused on transmembrane segments TM6 to TM17 of MSD2 and MSD3 (Fig. 1, B and C).

To characterize binding sites for typical anionic MRP2 substrates, studies altering charged (especially cationic) amino acids were performed. Ryu et al. (2000) used site-directed mutagenesis to examine the participation of basic residues in TM6 to TM17 of MRP2 in the transport of a fluorescent substrate, glutathione-methylfluorescein. Thirteen basic residues (His, Arg, Lys) in these regions were substituted with alanine; four mutants (K324A in TM6, K483A in TM9, R1210A in TM16 and R1257A in TM17) were all delivered appropriately to the cell surface when expressed in COS-7 cells yet showed decreased efflux of the substrate (Ryu et al., 2000). Similar studies substituting 10 charged amino acids in rat Mrp2 have exploited the differences in substrate specificity between Mrp2, which transports glucuronide, sulfate, and glutathione conjugates with great efficiency versus Mrp3, which efficiently transports glucuronide and sulfate conjugates but not glutathione conjugates (Ito et al., 2001c). Site-directed mutagenesis of Lys to Met (K325M) and Arg to Leu (R586L) of rat Mrp2 markedly reduced transport of DNP-SG and leukotriene C4, without affecting transport capacity of model glucuronide and sulfate conjugates yet increased the affinity for transport of E217G (Ito et al., 2001c). Unlike other MRPs, Mrp3 also transports taurocholate, a bile acid. Site-directed mutagenesis studies substi-

**TABLE 1**

Mutations in human MRP2

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Location</th>
<th>Translation</th>
<th>Domain</th>
<th>Phenotype</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>−24(C-T)</td>
<td>Promoter</td>
<td>Promoter</td>
<td>5′-UTR</td>
<td>Not reported</td>
<td>(Ito et al., 2001e)</td>
</tr>
<tr>
<td>1249G-A/wt</td>
<td>Exon 10</td>
<td>V4171</td>
<td>MSD2</td>
<td>Not reported</td>
<td>(Ito et al., 2001e)</td>
</tr>
<tr>
<td>1815−2(T-A)/1815+2(T-A)</td>
<td>Exon 13</td>
<td>147-bp deletion</td>
<td>NBD1</td>
<td>DJS</td>
<td>(Ito et al., 1999); (Wada et al., 1998)</td>
</tr>
<tr>
<td>2002del67/2002del67</td>
<td>Exon 16</td>
<td>Premature termination codon</td>
<td>NBD1</td>
<td>DJS</td>
<td>(Ito et al., 1999); (Wada et al., 1998)</td>
</tr>
<tr>
<td>2302(T-C)/2302(C-T)</td>
<td>Exon 18</td>
<td>R768W/R768W</td>
<td>NBD1</td>
<td>DJS</td>
<td>(Wada et al., 1998); (Ito et al., 1999); (Mor-Cohen et al., 2001)</td>
</tr>
<tr>
<td>2303(T-C)/2439+2(T-C)</td>
<td>Exon 18</td>
<td>R768W/168-bp deletion</td>
<td>NBD1</td>
<td>DJS</td>
<td>(Ito et al., 1999)</td>
</tr>
<tr>
<td>2303(T-C)/wt</td>
<td>Exon 18</td>
<td>R768W/wt</td>
<td>NBD1</td>
<td>Increased UCP1</td>
<td>(Ito et al., 1999)</td>
</tr>
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<td>2366(C-T)/wt</td>
<td>Exon 18</td>
<td>S789F/wt</td>
<td>NBD1</td>
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<td>(Ito et al., 1999)</td>
</tr>
<tr>
<td>2439+2(T-C)/2439+2(T-C)</td>
<td>Exon 18</td>
<td>168-bp deletion/168-bp deletion</td>
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<td>DJS</td>
<td>(Wada et al., 1998); (Ito et al., 1999)</td>
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<td>2439+2(T-C)/414N-A-G</td>
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<td>NBD1/2</td>
<td>DJS</td>
<td>(Ito et al., 1999)</td>
</tr>
<tr>
<td>2439+2(T-C)/wt</td>
<td>Exon 18</td>
<td>168-bp deletion/wt</td>
<td>NBD1</td>
<td>Increased UCP1</td>
<td>(Ito et al., 1999)</td>
</tr>
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<td>3196(C-T)/G196(C-T)</td>
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<td>DJS</td>
<td>(Ito et al., 1999)</td>
</tr>
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<td>3449G-A/3449G-A</td>
<td>Exon 25</td>
<td>R1150H/R1150H</td>
<td>MSD3</td>
<td>DJS</td>
<td>(Mor-Cohen et al., 2001)</td>
</tr>
<tr>
<td>3517A-T/3517(A-T)</td>
<td>Exon 25</td>
<td>I1173F/I1173F</td>
<td>MSD3</td>
<td>DJS</td>
<td>(Mor-Cohen et al., 2001)</td>
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<tr>
<td>3972C-T/wt</td>
<td>Exon 28</td>
<td>I1324I/wt</td>
<td>NBD2</td>
<td>None</td>
<td>(Ito et al., 2001e)</td>
</tr>
<tr>
<td>4175delT</td>
<td>Exon 30</td>
<td>Loss of R1392 and M1393</td>
<td>NBD2</td>
<td>DJS</td>
<td>(Ito et al., 2001e)</td>
</tr>
<tr>
<td>4348G-A/wt</td>
<td>Exon 31</td>
<td>A1450T/wt</td>
<td>NBD2</td>
<td>Not reported</td>
<td>(Ito et al., 2001e)</td>
</tr>
</tbody>
</table>

UTR, untranslated region; wt, wild type; bp, base pair; UCP1, urinary coproporphyrin fraction 1.
tutting the cationic amino acid Arg<sup>586</sup> and Arg<sup>1096</sup> in rat Mrp2 with neutral amino acids (R586L, R586I, R1096L, and R1096M) or a cationic amino acid (R1096K) led to acquisition of taurocholate transport and retention of glucuronide and glutathione conjugate transport by Mrp2 (Ito et al., 2001d). These authors suggest that the presence of Arg at amino acids 586 and 1096 prevents taurocholate transport by Mrp2 (Ito et al., 2001d).

In other recent studies, a highly conserved tryptophan residue, Trp<sup>1254</sup>, in the last transmembrane segment (TM17) of MSD3 of Mrp1 has been shown to be essential for transport of E<sub>2</sub>17G (Ito et al., 2001b). Mutation of the analogous Trp<sup>1254</sup> of MRp2 showed this amino acid to be essential for MRp2 transport of methotrexate; nonconservative substitutions (Ala, Cys) eliminated E<sub>2</sub>17G transport, whereas conservative substitutions (Tyr, Phe) were without effect (Ito et al., 2001d). Only the most conservative substitution (W1254Y) retained leukotriene C<sub>4</sub> transport activity. These data indicate not only that Trp<sup>1254</sup> is essential for MRp2-mediated transport of methotrexate but also demonstrate the presence of more than one substrate binding site in MRp2. The ability of substrates to inhibit and stimulate transport of other substrates, as discussed below, is consistent with this demonstration of distinct transport/binding sites and is a complexity of MRp2 function that will require significantly more attention.

**Localization of MRp2**

Characterization of the distribution of MRp2 mRNA in tissues other than liver showed that MRp2 is also expressed in the apical membranes of the proximal tubule of the kidney and in the apical membrane of the duodenum and jejunum. Mrp2 is expressed in the brush-border membrane domain of segments S<sub>1</sub>, S<sub>2</sub>, and S<sub>3</sub> of proximal tubule epithelia in rat kidney (Schaub et al., 1997) and in proximal tubules of normal human kidney and clear-cell carcinomas, originating from proximal tubule epithelium (Schaub et al., 1999). When expressed in human embryonic kidney cells (HEK293, Mrp2 mediates the ATP-dependent transport of p-aminohippurate with a K<sub>m</sub> value of 880 µM (Leier et al., 2000); however, little else is known regarding the role of MRp2 in renal secretion of organic anions. In the rat small intestine, Mrp2 expression is concentrated at the tip of the villus, with the highest concentrations seen in the proximal jejunum, with little Mrp2 protein detected in the distal ileum (Mottino et al., 2000). A similar distribution of the phase II conjugating enzymes, UDP-glucuronosyltransferase and glutathione S-transferase, suggests that metabolism and subsequent efflux of the organic anion conjugates act coordinately to decrease the intestinal absorption of food contaminants and drugs that enter the enterocytes via the digestive tract. Functional studies in vivo and in Ussing chambers demonstrate a serosal-to-mucosal flux of DNP-SG and that this flux is significantly decreased in EHBR (Gotoh et al., 2000). In postpartum lactating rats, the intestinal mass is increased, particularly in late lactation, presumably due to the 2- to 3-fold increase in food consumption at this time. Mrp2 protein expression in jejunum (expressed per milligram of brush-border membrane protein) is also increased 2- to 3-fold in late lactation, and this is reflected in increased secretion of DNP-SG in an inverted intestinal sac model (Mottino et al., 2001). Increased expression of Mrp2 may be an adaptive mechanism that serves to protect the organism from absorption of dietary toxins at a time when food consumption is dramatically increased. Mrp2 has been shown to mediate the transport of the abundant food-derived carcinogen 2-aminono-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) (Dietrich et al., 2001a). These authors further found that the absorption of PhIP is 2-fold higher in the TR<sup>-</sup> rat, which is deficient in Mrp2, thus clearly demonstrating that Mrp2-mediated extrusion reduces the oral bioavailability of this carcinogen (Dietrich et al., 2001a). Mrp2 is also expressed in the gall bladder epithelium (Rost et al., 2001).

In addition to the liver, kidney, and small intestine, MRp2 is expressed in brain endothelial cells, possibly forming a functional component of the blood-brain barrier (Kusuhara and Sugiyma, 2002). MRp2 has also been detected in several other tissues, including the placenta, where it serves to protect the fetus from toxins and to excrete endogenous conjugates from the fetus (St-Pierre et al., 2000). Additionally, Mrp2 mRNA transcripts have been detected in low levels in other tissues in the rat, including the lung and the stomach (Cherrington et al., 2002), but the impact of its transcription in these tissues is unknown.

**Regulation of Expression**

MRp2 expression is responsive to a number of drug treatments and diseases affecting the liver, particularly cholestatic liver disease. Recent studies indicate that regulation of MRp2 function occurs at least at three distinct levels. These include its endocytic retrieval from the canalicular membrane, its translational regulation, and its transcriptional regulation.

**Endocytic Retrieval.** MRp2 is synthesized in the endoplasmic reticulum, processed in the Golgi and then translocated to the apical plasma membrane, where it must be inserted into the membrane to mediate transport of substrates across the canalicular membrane into bile. Transporter function is regulated by the dynamic endocytic retrieval and exocytic insertion of transporters between the canalicular membrane and an intracellular pool of vesicles (Hausssinger et al., 2000; Kipp and Arias, 2000). Canalicular membrane proteins (e.g., Mrp2 and the bile salt export pump (Bsep)) are present in a pericanalicular pool of vesicles that disappear upon treatment of rats with dibutyryl cAMP due to their rapid sorting to the canalicular domain (Bayer and Soroka, 1995; Gatmaitan et al., 1997; Roelofsen et al., 1998). Conversely, Mrp2 and Bsep transport activity is increased in canalicular membranes isolated from livers treated with dibutyryl cAMP; prior administration of colchicine, which disrupts microtubules, blocks these actions of dibutyryl cAMP. Mrp2 and Bsep also colocalize with the transcytotic marker polymeric IgA receptor on microtubule-associated transcytotic vesicles, indicating that these proteins must traffic together (Soroka et al., 1999). Treatment of rats with lipopolysaccharide, which induces cholestasis, induces endocytic retrieval of Mrp2 (Dombrowski et al., 2000; Hausssinger et al., 2000); this retrieval is reversible if the liver is perfused with hypo-osmotic buffer within 3 h of lipopolysaccharide (Kubitz et al., 1999b). Perfusion of the liver with hyperosmotic buffer also causes rapid retrieval of Mrp2 and Bsep into a subcanalicular compartment and decreases bile flow; subsequent perfusion with hypo-osmotic buffer induces reinsertion of Mrp2 into the canalicular membrane and restores bile flow (Kubitz et al., 1997) and biliary excretion of DNP-SG, an Mrp2 substrate (Ito et al., 2001c). Phallolidin, the potential

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*Reference*:

mushroom hepatotoxic and cholestatic agent derived from *Amanita phalloides*, causes a rapid (within 30 min) retrieval of Mrp2 and other canalicular proteins into intracellular sites; retrieval of Mrp2 coincides with decreased bile flow and decreased biliary excretion of leukotriene C4 (Rost et al., 1999). Finally, E217G causes a rapid inhibition of bile flow and retrieval of Mrp2 into intracellular sites; bile flow recovers spontaneously and is followed by the exocytic insertion of Mrp2 into the canalicular membrane (Mottino et al., 2002). Pretreatment of rats with dibutyryl cAMP partially attenuates both the inhibition of bile flow and endocytic retrieval of Mrp2 and significantly accelerates the recovery of flow and exocytic insertion of Mrp2 (Mottino et al., 2002). These data indicate that the function of Mrp2 at the canalicular membrane can be regulated over a very short (i.e., minutes) time frame by its rapid endocytic retrieval from and exocytic insertion into the canalicular membrane. It follows that a breakdown in this process could lead to a decreased amount or absence of Mrp2 (and other transporters) in the canalicular membrane, and hence, cholestasis (Kipp and Arias, 2000).

**Translational Regulation.** Treatment of rats with ethynylestradiol markedly decreases Mrp2 protein expression but has no effect on Mrp2 mRNA expression, affecting neither total Mrp2 mRNA nor the relative abundance of the three Mrp2 mRNA transcripts that contain differing 3'-untranslated regions (Trauner et al., 1997). Similarly, expression of hepatic Mrp2 mRNA is unchanged, whereas expression of Mrp2 protein is decreased by 50% in pregnant versus control rats (Cao et al., 2001), indicative of posttranscriptional control of Mrp2. Conversely, treatment of rats with pregnenolone-16α-carbonitrile (PCN) increases Mrp2 protein expression markedly but has no effect on Mrp2 mRNA expression (Vore et al., 2001; Johnson et al., 2002). To assess the role of translational regulation of Mrp2, we examined the polysomal distribution of Mrp2 mRNA in livers from control and pregnant rats and rats treated with PCN or vehicle (corn oil). Polysomal distribution analysis entails separation of polyribosomes from single ribosomes and their subunits by differential sedimentation through a sucrose gradient. If the protein encoded by a specific DNA sequence is being actively translated regions of both rat and human MRP2 significantly decreased luciferase expression (Li and Vore, 2002). Although these data strongly suggest that the 5'-untranslated region contains sites that decrease the rate of translation of MRP2, further studies are needed to identify the mechanism(s) of translational regulation.

**Transcriptional Control.** Transcriptional regulation of MRp2 expression has been characterized both with respect to decreased expression in disease and increased expression by treatment with various classic enzyme inducers. Activation of hepatic inflammation by conditions such as sepsis, alcoholic, autoimmune and viral hepatitis, and parenteral nutrition-associated liver disease is associated with cholestasis and hyperbilirubinemia (Hill et al., 1997). The liver is a principal target of inflammatory mediators, such as tumor necrosis factor-α and interleukin-6 and -1β, and regulates changes in hepatic protein synthesis during the acute phase response. Denson et al. (2000) have identified an RXRα/RARα response element in the Mrp2 promoter and have shown that transcriptional suppression of Mrp2 by acute phase proteins occurs via interleukin-1β-induced reduction in nuclear RXR/RAR heterodimers.

Characterization of increased transcriptional expression of Mrp2 has proven to be more complex. The promoter regions of the human MRp2 and the rat Mrp2 gene contain a number of putative consensus binding sites for AP1, SP1, HNF1, and HNF3β (Kauffmann and Schrenk, 1998; Tanaka et al., 1999). The promoter region of Mrp2 mRNA contains important elements for control of expression in HepG2 cells, particularly the CCAAT-enhancer binding protein β. In primary cultures of rat hepatocytes, dexamethasone, 2-acetylaminofluorene, cisplatin, cycloheximide, phenobarbital, clotrimazole, and PCN all increase Mrp2 mRNA and protein within 24 h (Kauffmann et al., 1997; Courtois et al., 1999; Kubitz et al., 1999a,b). Courtois et al. (1999) further showed that mifepristone did not inhibit Mrp2-induction by dexamethasone in cultured rat hepatocytes, eliminating a glucocorticoid receptor-mediated induction, and that treatment of rats in vivo with dexamethasone also induced Mrp2 mRNA. The inducibility of Mrp2 gene expression in primate liver was investigated in rhesus monkeys treated with tamoxifen or rifampin (Kaufmann et al., 1998). Both tamoxifen and rifampin strongly induced Mrp2 mRNA in two male and two female rhesus; tamoxifen induced Mrp2 protein both in male and female rhesus, whereas rifampin showed some inducing effect in a female but was inactive in a male monkey.

Recent studies demonstrated that ligands for FXR, PXR, and CAR (chenodeoxycholic acid, PCN, dexamethasone, and phenobarbital) all induced Mrp2 mRNA in primary cultures of rat hepatocytes and characterized a putative proximal promoter (−1034 to −376) of the rat Mrp2 promoter that bound the corresponding FXR/RXR, PXR/RXR, and CAR/RXR heterodimers (Kast et al., 2002). Cotransfection of HepG2 cells with the Mrp2 proximal promoter (−1034 to −15) with plasmids encoding PXR, CAR, or FXR and RXR followed by treatment with receptor-specific ligands led to ligand-specific activation of PXR and FXR, whereas CAR was constitutively active, as expected. When HepG2 cells were cotransfected with pTk2xER-8, containing two copies of the wild-type ER-8, the reporter was activated over 100-fold in a PXR- and PCN-dependent manner. However, treatment of rats in vivo with phenobarbital leads to inconsistent effects on Mrp2 expression. Thus, Hagenbuch et al. (2001) were unable to show a significant effect of phenobarbital treatment on Mrp2 expres-
sion, consistent with the work of others (Fernandez-Checa et al., 1993; Kiuchi et al., 1998; Ogawa et al., 2000) showing a lack of effect on ATP-dependent transport of Mrp2 substrates (e.g., leukotriene C4) or Mrp2 expression. In contrast, Johnson et al. (2002) recently reported that phenobarbital treatment of rats had no effect on Mrp2 mRNA expression but increased Mrp2 protein expression. As indicated above, treatment of rats with PCN has no significant effect on Mrp2 mRNA expression but increases Mrp2 protein expression severalfold (Johnson et al., 2002). We have also observed that PCN treatment of rats in vivo has no significant effect on Mrp2 mRNA but markedly (2- to 3-fold) increases Mrp2 protein expression (Vore et al., 2001). Clearly, further studies are needed to understand the factors regulating Mrp2 expression in vitro versus in vivo. The fact that Mrp2 expression and activity is regulated at three levels, resulting in altered expression within minutes (endocytic retrieval, exocytic insertion), several hours (translational regulation), or days (transcriptional regulation) suggests that control of Mrp2 activity is critical for cell function. The likely role of Mrp2 in efflux of oxidized and reduced glutathione and regulation of intracellular redox status may be such a critical cellular function.

Transport and ATPase Activity of Mrp2

MRP2 is known to transport a wide variety of compounds, including various endobiotics and xenobiotics. The substrate specificities of Mrp1 and Mrp2 are similar, with both extruding glutathione, glucuronide, and sulfate conjugates (Keppler et al., 1997). Mrp1 has a higher affinity for leukotriene C4, but bilirubin mono- and bis-glucuronides are better substrates for Mrp2 (Keppler et al., 1997). The transport efficiency (Vmax/Km) of substrates for rat and human MRPs have been ranked as follows: leukotriene C4 > leukotriene D4 > 2,4-DNP-SG > monoglucuronosyl bilirubin > E217G > taurolithocholate sulfate > oxidized glutathione (Keppler et al., 1997).

The substrates and inhibitors of Mrp2 have been thoroughly reviewed elsewhere (König et al., 1999a; Suzuki and Sugiyama, 1999). Much of this work defining the substrates of Mrp2 has been done by comparing functional activity in the Mrp2-deficient GY/TR- or EHBR rats to control rats. Briefly, leukotrienes C4, D4, and E4, and numerous glutathione conjugates, including oxidized glutathione, DNP-SG, bromosulphophthalein glutathione, conjugates of heavy metals including arsenic, and cadmium are substrates as shown in either in vitro or in vivo studies (Suzuki and Sugiyama, 1999). Glucuronide conjugates of bilirubin, estradiol, triiodothyronine, grepafloxacin, and SN-38 are also MRP2 substrates (Suzuki and Sugiyama, 1999), as are numerous conjugates of other compounds, such as acetalaminophen glucuronide (Xiong et al., 2000). Additionally, Mrp2 transports glucuronide and sulfate conjugates of several bile salts, a range of unconjugated organic anions, including bromosulphophthalein, reduced folates, methotrexate, irinotecan, and its metabolite SN-38, ampicillin, ceptraxone, pravastatin, temocaprilat, and BQ-123, as well as Flu-3 and p-aminophippurate (König et al., 1999a; Suzuki and Sugiyama, 1999; Kusuhara and Sugiyama, 2002). Recently, a chemoprotective role for Mrp2 against carcinogens has been shown for the tobacco-specific carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol and for the food carcinogen PhIP (Dietrich et al., 2001a; Leslie et al., 2001). Also, the fungal toxin ochratoxin A is an MRP2 substrate (Leier et al., 2000), and transport of the tea flavonoid epicatechin was inhibited by MK-571, an antagonist of Mrp2, suggesting it also may be an MRP2 substrate (Vaidyanathan and Walle, 2001). These and other data show that Mrp2 can transport conjugates, unconjugated compounds, or certain agents in association with glutathione.

In addition to the Mrp2 deficient GYTR- or EHBR rats, transport activities of Mrp2 and mechanisms have been examined in several different expression systems, including transfected mammalian cells and baculovirus infected insect cells. When human Mrp1 and Mrp2 are expressed in Sf9 insect cells using the baculovirus expression vector system, Mrp2 has a lower affinity for leukotriene C4 and N-ethylmaleimide-glutathione (NEM-SG) than does Mrp1; also, methotrexate is a substrate for both transporters, but its transport by Mrp2 is more efficient (Bakos et al., 2000). The effects of several organic anions on NEM-SG uptake by Mrp1/2 were also examined (Bakos et al., 2000). Notably, sulfinpyrazone (∼300 μM) stimulated Mrp2-mediated transport but inhibited Mrp1 transport; benzylpenicillin and indomethacin also stimulated Mrp2 under certain conditions; methotrexate and glutathione were low potency inhibitors of Mrp1/2 (Bakos et al., 2000).

Aside from transporting covalent glutathione, glucuronide, and sulfate conjugates, Mrp2 can transport certain compounds in the presence of glutathione. Thus, when expressed in Sf9 cells, rabbit Mrp2 did not transport vinblastine in the absence of reduced glutathione, whereas in the presence of 5 mM reduced glutathione, there was significant vinblastine uptake (Van Aubel et al., 1999). Furthermore, leukotriene C4 uptake was stimulated by reduced glutathione and inhibited by vinblastine in the presence, but not absence, of reduced glutathione. Additionally, uptake of reduced glutathione alone was not ATP-dependent, unlike the uptake of conjugates or cotransport of vinblastine and reduced glutathione (Van Aubel et al., 1999).

In a separate study, Evers et al. (2000) further examined vinblastine and sulfinpyrazone efflux associated with glutathione in confluent MDCKII cells expressing Mrp1 or Mrp2. Apical efflux of reduced glutathione was inhibited by benzylamine and probenecid but stimulated by low concentrations of sulfinpyrazone and indomethacin (Evers et al., 2000). The transport ratio of sulfinpyrazone (0.2 to 3.2 mM) to reduced glutathione ranged from 3.1 to 91, suggesting that at low concentrations sulfinpyrazone transport is coupled to reduced glutathione but at high concentrations is transported without reduced glutathione (Evers et al., 2000). Furthermore, reduced glutathione apical export increased with increasing vinblastine concentrations, and the transport ratio of vinblastine to reduced glutathione ranged from 2 to 3 (Evers et al., 2000). Unlike Mrp2, Mrp1 transports daunorubicin (Bakos et al., 1998), but this transport is not stimulated by reduced glutathione (Evers et al., 2000). Mrp2-mediated reduced glutathione-coupled transport has recently been shown for other toxic compounds as well, including arsenite and α-naphthylisothiocyanate (Kala et al., 2000; Dietrich et al., 2001b). These two compounds form reversible complexes with reduced glutathione, which dissociate in bile so that the parent compound recycles back to hepatocytes (Dietrich et al., 2001b). This recycling can lead to depletion of intracellular reduced glutathione and also leads to very high
Table 2
Factors altering MRP2/Mrp2 mRNA or protein expression and function in vitro or in vivo, as discussed in the text

<table>
<thead>
<tr>
<th>Regulation of expression</th>
<th>Inhibition or stimulation of activity</th>
<th>ATP depletion</th>
<th>Estrogen glucuronides</th>
<th>Cholestasis</th>
<th>MRP2 mutation: R1150H</th>
<th>Cancer chemotherapeutics: irinotecan, methotrexate, and vinblastine</th>
<th>Uricosurics: sulfipyrazone, probenecid, and benzbromarone</th>
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Concentrations of α-naphthylisothiocyanate in the biliary tree, its primary site of toxicity.

Excretion into bile involves several steps, including uptake into the hepatocyte, metabolism and transport across the canalicular membrane. Therefore, compounds excreted from the blood into bile may require protein-mediated transport across both the sinusoidal and canalicular membranes. To investigate the functional interplay between uptake and excretion processes, the basolateral transporter human organic anion transporting polypeptide OATP8 (SLC21A8) and the apical transporter MRSP2 were coexpressed in MDCKII cells (Cui et al., 2001). When the cells were grown to confluent monolayers, transcellular transport of bromosulphophthalein, leukotriene C₄, E₂₁₇G, dehydroepiandrosterone sulfate, Fluo-3, and rifampin was higher in double-transfected (OATP8/MRP2) cells than in single-transfected (MRP2 only) cells (Cui et al., 2001). Similarly, Sasaki et al. (2002) coexpressed OATP8 (SLC21A6) and MRP2 in MDCKII cells and demonstrated vectorial transport of E₂₁₇G, leukotriene C₄, and taurocholic acid sulfate but not dehydroepiandrosterone sulfate or estrone-3-sulfate. These models may be useful to study transcellular transport for compounds excreted into bile.

MRP2 has been implicated in drug and estrogen induced cholestasis. To investigate the mechanism, Mrp2 was coexpressed with the major canalicular bile salt transporter Bsep. When both rat transporters were coexpressed in Sf9 cells, the IC₅₀ values for the bile acids taurocholate sulfate and taurochenodeoxycholate sulfate for inhibition of Bsep-mediated taurocholate transport resembled those in Sprague-Dawley rats, whereas when Bsep alone was expressed, IC₅₀ values resembled those in Mrp2-deficient EHBR rats (Akita et al., 2001). They also found that taurochenodeoxycholate sulfate and taurocholate sulfate trans-inhibit Bsep, requiring transport by Mrp2 into the intravesicular space (Akita et al., 2001). Similarly, E₂₁₇G inhibited Bsep in Bsep/ Mrp2 coexpressing Sf9 membrane vesicles but not in Bsep-alone expressing Sf9 membrane vesicles (Stieger et al., 2000). These findings indicate that Mrp2 functions coordinately with other transporters in cells and that its activity can influence other transport processes.

ATP dependence of MRP2-mediated substrate transport has clearly been demonstrated for most substrates. Although the ATPase activity of MRP2 is only about 20% of that of MDR1, Bakos et al. (2000) observed 0.5- to 4.5-fold substrate-stimulated MRP2 ATPase activity for NEM-SG, methotrexate, reduced glutathione, indomethacin, probenecid, and sulfipyrazone using MRP2-baculovirus infected Sf9 insect cells. Indomethacin, probenecid, and sulfipyrazone inhibited MRP1 ATPase activity but stimulated MRP2 ATPase activity (Bakos et al., 2000). However, reduced glutathione had no effect on MRP2 ATPase activity stimulated by indomethacin, probenecid, and sulfipyrazone (Bakos et al., 2000). Furthermore, unlike the other low affinity MRP2 substrates (NEM-SG, methotrexate, and reduced glutathione), p-aminohippurate had no effect on MRP2 ATPase activity (Bakos et al., 2000), although p-aminohippurate is an MRP2 substrate (Leier et al., 2000). This suggests that MRP2 transport activity is not necessarily coupled to measurable ATPase stimulation by the same substrate. Similarly, although E₂₁₇G is a substrate for MDR1 expressed in Sf9 insect cells, there is no measurable E₂₁₇G-stimulated ATPase activity (Huang et al., 1998). Whether this phenomenon is an artifact of the ATPase background of the baculovirus-Sf9 expression system is unknown. Recently, MRP2 was isolated, purified, and reconstituted into liposomes; in this system, MRP2 substrates oxidized glutathione, reduced glutathione, and 5-decylglutathione stimulated ATPase activity (Hagmann et al., 1999). This preparation may give further insights into the actual MRP2 ATPase activity.

Clinical Impact and Conclusions

A number of factors can alter MRP2 by changing its level of expression or by directly inhibiting (or stimulating) its activity, as discussed in the text above. In clinical studies, expression of MRP2 mRNA and protein was decreased in patients with obstructive cholestasis who were poorly drained by percutaneous transhepatic biliary drainage (Shoda et al., 2001). In another clinical study, rifampin treatment of normal human subjects increased MRP2 mRNA and protein in the duodenum (Fromm et al., 2000). Additionally, induction of chronic renal failure in rats increased Mrp2 mRNA and protein levels in both the kidney and the liver (Laouari et al., 2001). This may represent a compensatory mechanism during renal failure, although the human response has not yet been documented. These and other changes discussed in the text above are summarized in Table 2.

In summary, alterations in MRP2 expression and/or function could have a variety of clinically important effects. First, decreased MRP2 function can impair normal hepatic function including the capacity to excrete endogenous compounds, such as conjugates of bilirubin, steroids, and leukotrienes. For example, Dubin-Johnson syndrome patients lacking functional MRP2 have hyperbilirubinemia and dark pigment deposition in the liver. Next, altered MRP2 function can change the clearance of many clinically important drugs, including cancer chemotherapeutics (irinotecan, methotrexate, and vinblastine), antibiotics (ampicillin, ceftriaxone, and rifampin), antiherpetic drugs, and antigensens-converting enzyme inhibitors, as well as many toxins and their conjugates, such as α-naphthylisothiocyanate, heavy metals, 4-(methyl nitrosamino)-1-(3-pyridyl)-1-butanol, and some dietary compounds, such as ochratoxin A, epicatechin, and PhIP. Since MRP2 seems to act as a protective barrier in the
brain, intestine, and placenta, MRP2 alterations may also affect the absorption and distribution of these compounds, thus affecting therapeutics or toxicity. MRP2 also has a role in drug- and estrogen-induced cholestasis, although the exact mechanism is unclear. Finally, since both reduced and oxidized glutathione are MRP2 substrates, stimulation or inhibition of MRP2 expression or activity may play an important role in cell redox status or response to oxidative stress. Future studies are needed to clarify these complex interactions and the molecular details of MRP2 regulation, structure, and function. Also, further studies are needed to reveal any polymorphisms and their effect on MRP2 function.

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References


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