MRP2-4, from drug resistance to physiology

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Chapter 5

Generation and characterization of mice lacking the multidrug resistance protein 3 (Abcc3)
Generation and Characterization of Mice Lacking the Multidrug Resistance Protein 3 (Abcc3)

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Summary

To study the physiological role of Mrp3 and its possible role in protecting tissues against drugs we generated mice deficient in Mrp3 by embryonic stem cell technology. The Mrp3\(^{\text{+/+}}\) mice are viable, healthy and show no overt phenotype. Characterization of the tissue distribution of Mrp3 in the mouse revealed that it is expressed throughout the gastrointestinal tract, liver, stomach, spleen, pancreas, lung, bladder and the adrenal gland. In mammalian cells MRP3 confers resistance to etoposide, but the Mrp3\(^{\text{+/+}}\) mice do not display any altered sensitivity to this drug. We tested further the hypothesis that Mrp3 participates in the transport of bile acids in vivo. Trans-ileal transport of taurocholate was unaltered in the Mrp3\(^{\text{+/+}}\) mice. In a model of obstructive cholestasis, bile duct ligation (BDL), we find that Mrp3\(^{\text{+/+}}\) mice do not have increased histological liver damage compared with wildtype mice and display only a marginal increase in hepatic Mrp3, concomitant to an approximate 50% decrease in Mrp2 levels. BDL treatment (3 days) results in substantially elevated serum bile acid levels, but the levels in Mrp3\(^{\text{+/+}}\) mice were not different than those observed in control mice. Serum levels of bilirubin glucuronide, however, were significantly lower in Mrp3\(^{\text{+/+}}\) mice after 3 days of BDL suggesting that bilirubin glucuronide is an endogenous substrate of Mrp3 and is transported across the sinusoidal membrane into the circulation by Mrp3 in BDL mice.

Introduction

Members of the ATP binding cassette (ABC) family of transporters are membrane transporters that couple the energy released from ATP hydrolysis to transport of a substrate across the membrane\(^1\). Among these, the ABCC subfamily contains 12 members thus forming the largest subfamily of human ABC transporters\(^2\). The ABCC subfamily contains Multidrug Resistance
Proteins 1-9 (MRP1-9) along with SUR1, SUR2 and CFTR. MRP1s transport organic anions with broad substrate specificity placing them in a position to participate in diverse physiological processes. Their importance is further emphasized by the fact that mutations in two members of this family, MRP2 and MRP6, are the cause of two inherited human diseases, the Dubin-Johnson syndrome and Pseudoxanthoma elasticum, respectively.

The physiological roles of MRP3 (ABCC3) are unknown. Human and rat MRP3 were cloned by several independent groups in rapid succession. Compared with MRP1, MRP3 has a more restricted tissue distribution. In humans MRP3 is limited to the gut, liver, pancreas, kidney, and the adrenal gland (see chapter 1 for a detailed discussion). In these tissues, MRP3 localizes to the basolateral membranes of epithelial cells.

Initial studies on the physiological role of MRP3 focused on the liver. The basal hepatic expression of Mrp3 in rats is very low but is highly induced in the naturally occurring Mrp2-deficient rat strains TR and EHBR and in livers of Dubin-Johnson patients. Moreover, treatments that induce cholestasis (e.g. common bile-duct ligation; BDL) also result in a substantial induction of hepatic Mrp3 in rats, and, as shown recently, to some induction in mice as well.

A possible explanation for these findings comes from transport assays with rat MRP3. In these assays rat Mrp3 transports glycocholic acid (GCA), taurocholic acid (TCA), and taurolithocholic-3-sulfate (TLCS), the latter two with high affinity. This has led to the suggestion that Mrp3 may be important in the absorption of bile acids in the terminal ileum and in protecting the liver under conditions of impaired bile flow, cholestasis, by transporting toxic organic anions (e.g. bile acids) across the sinusoidal membrane into the circulation for subsequent excretion in the urine.

Studies on the substrate specificity of human MRP3, however, revealed a different picture. Transport of bile acids by MRP3 was studied in vesicular transport assays and in cells that overproduce MRP3 that were additionally transfected with an ileal apical sodium-dependent bile acid transporter (ASBT) cDNA construct to allow entry of bile acids into the cell. These studies demonstrated that, unlike rat Mrp3, human MRP3 transports GCA and TCA with low affinity. To study the role of Mrp3 in bile acid physiology we generated mice with a homozygous disruption of Mrp3 by embryonic stem cell technology. The generation and initial characterization of the Mrp3<sup>−/−</sup> mice is reported here.
Methods

Generation of Mrp3\textsuperscript{(-/-)} mice

A sequenced mouse Mrp3 cDNA fragment (unpublished data) was used to screen an EMBL3 genomic 129/Ola DNA phage library. A single phage clone (total length 20 kb) was picked up and characterized by southern blotting and exon-intron positions were mapped. A targeting vector was constructed by joining a 3.8 kb EcoRV-BgIII 5' Mrp3 fragment, a fragment containing a hygromycin resistance gene driven by the mouse phosphoglycerate kinase promoter, and a 2.8 kb Sacl-PstI 3' Mrp3 fragment (figure 1). Correct targeting deleted 4.9 kb of Mrp3 sequence containing exons 2-8 encoding amino acids 16-331. Two independent transfections of the targeting construct into 129/Ola derived E14 embryonic stem cells resulted in 0.7% homologous recombinants. Targeted clones with the predicted replacement event were identified by using probes 5' or 3' to the homology region, and a hygromycin probe (data not shown). Two ES clones (clone numbers 6510 and 218) with normal karyotype were injected into mouse blastocysts and both resulted in chimeric mice that transmitted the Mrp3\textsuperscript{-/-} allele through the germ line to F\textsubscript{1}, offspring. The heterozygous, homozygous and wildtype mouse stocks were maintained as a cross of FVB and 129/Ola (50%/50%) as the 129/Ola mice breed poorly. All animals were housed in constant temperature rooms with a 12-h light/12-h dark cycle, and were fed a pelleted chow diet and given acidified water ad libitum. Mouse handling and experimental procedures were conducted in accordance with institutional guidelines for animal care and use.

Generation and characterization of antibodies against mMrp3

Fragments of the L\textsubscript{0} and linker regions of mMrp3 corresponding to amino acids 230-296 and 818-952, respectively, were cloned by RT-PCR from total liver RNA using primers based on rat Mrp3. The sequence of both cloned fragments showed the highest homology to rat and human Mrp3/MPR3 of all MRPs. The fragments were cloned into the pMalC expression plasmid and fusion proteins were isolated as previously reported\textsuperscript{21}. Polyclonal serum from rabbits injected with the L\textsubscript{0} (A54) and linker- (A66) fusion proteins was collected after 4 boosts. Both polyclonal sera recognize mouse and rat Mrp3, but not human MRP3 (not shown).
Immunoblotting and Immunohistochemistry

Total tissue lysates were homogenized in a hypotonic lysis buffer containing 10 mM KCl, 1.5 mM MgCl\textsubscript{2}, 10 mM Tris-HCl, pH 7.4, supplemented by a mixture of protease inhibitors used at the dilution recommended by the manufacturer (Roche Molecular Biochemicals). Samples containing varying amounts of protein were size-fractionated in a 7.5% SDS-polyacrylamide gel. Subsequently, gels were blotted overnight in a tank blotting system. Mrp3 was detected with the rabbit polyclonal serum A66 (1:250) or A54 (1:100) followed by a goat anti-rabbit horseradish peroxidase conjugate (1:10000) and visualized with chemiluminescence (ECL, Amersham Pharmacia Biotech). Mrp2 was detected with the rabbit polyclonal sera K13, as previously described \textsuperscript{22}. To control for gel loading the rat α-tubulin monoclonal antibody YL 1/2 (1:1000) was used \textsuperscript{23}.

For immunolocalization of Mrp3 in tissue sections, cryosections (4 μm) were air dried overnight and fixed for 7 minutes in acetone at room temperature, incubated with the primary antibody (1:200; 1 h room temperature), and detected using HRP-labeled swine-anti-rabbit antiserum (Dako, Copenhagen, Denmark; 1:200). For double stainings, slides were incubated simultaneously with anti-Mrp3 polyclonal antiserum (A66) and the rat anti-Bcrp monoclonal antibody BXP-53. First, the Mrp3 staining was completed using HRP-labeled swine-anti-rabbit serum (in the presence of 1% normal rat serum) and tyramine-rhodamine (1:1000; Pierce, Rockford, USA) and 0.01% H\textsubscript{2}O\textsubscript{2}. Then the double staining was finalized using biotin-labeled rabbit-anti-rat (in the presence of 1% normal rabbit serum) followed by Alexa488-labeled streptavidin (1:1000; Molecular probes, Leiden, the Netherlands). DNA was counterstained with DAPI (Molecular probes, Leiden, the Netherlands). In control stainings the primary antibodies were replaced by appropriate normal sera. The slides were examined under a fluorescence microscope (Leica DMRB, Rijswijk, the Netherlands).

Trans ileal TCA transport experiments

Terminal ileum sections were mounted in an Ussing chamber apparatus, as previously described \textsuperscript{24}. Briefly, the tissue sections were bathed in a Krebs-bicarbonate solution (in mM: 120 NaCl, 4.8 KCl, 1.2 KH\textsubscript{2}PO\textsubscript{4}, 1.2 MgSO\textsubscript{4}, 24 NaHCO\textsubscript{3}, 1.3 CaCl\textsubscript{2}, 2 L-Glutamine, 10 Hepes/NaOH, pH 7.4) in both compartments. The experiment was initiated by adding TCA (1 mM) to the mucosal compartment and following its appearance in the serosal compartment. Samples from both compartments were taken over time and the mean TCA transport rate was calculated. Transport of
TCA in the opposite direction was minimal (<10% of the mucosal to serosal transport) under these experimental conditions (not shown).

**BDL experiments**

Animals were anaesthetized with a mixture of hypnorm/dormicum, the common bile duct was ligated and the gall bladder removed. After 3 or 7 days the mice were lightly anaesthetized with metofan and sacrificed by heart puncture collection of blood. Tissues were collected and frozen in liquid nitrogen for further Immunoblot and immunolocalization studies or fixed in ethanol/acetic acid/formaldehyde in 0.9% NaCl (40:5:10:45) for immunohistology. Serum levels of liver enzymes, bilirubin, and bile acids were determined as mentioned below.

**Etoposide sensitivity**

Etoposide-phosphate (100 mg effective etoposide, ETOPOPHOS®; Bristol Myers-Squibb Pharmaceuticals, Princeton, NJ) was dissolved in 0.9% NaCl to obtain a stock solution of 20 mg/ml. Concentrations were adjusted to inject intravenously 5-7 µl/g body weight (80-120 mg/kg) in a tail vein of male mice (12-16 weeks old). Mice were weighed and observed daily.

**Determination of serum levels of bilirubin, bile acids and clinical chemistry**

Serum levels of liver enzymes and bilirubin were determined at the clinical chemistry department of the Netherlands Cancer Institute following standard procedures and serum levels and species of bile acids were determined by mass spectrometry as previously described

**Statistical evaluation**

Results are presented as means ± SD. Differences between groups were evaluated with a Student’s t test and a P value <0.05 was considered significant.
Chapter 5

Results

Generation of Mrp3<sup>(−/−)</sup> mice and etoposide sensitivity

Using a mouse Mrp3 cDNA fragment we isolated a single phage clone from a 129/Ola mouse genomic DNA library. The genomic DNA fragment contained exons 2-8 of mMrp3. A gene-targeting construct was made in which exons 2-8 were deleted and replaced by a marker gene to select for hygromycin resistance (figure 1a). The deleted region (amino acids 16-331) form the TMD<sub>0</sub>, L<sub>0</sub>, and the first putative transmembrane helix of the TMD<sub>1</sub> segment of Mrp3. Additionally, deletion of exons 2-8 results in a frame shift that will lead to an early stop codon immediately after exon 1. We obtained two independent properly targeted embryonic stem cell clones and injected these into mouse blastocysts. This resulted in chimeric mice that transmitted the Mrp3<sup>−/−</sup> allele through the germ line to F1 offspring (figure 1b). Offspring from Mrp3<sup>(+/−)</sup> intercrosses were born at the expected Mendelian ratio indicating that there is no selection against the mutant allele during development. The homozygous null mice were healthy, fertile and displayed no overt phenotype (see also table 1). Extensive histological analysis of 3- and 12-month old male and female mice revealed no substantial differences between Mrp3<sup>(+/−)</sup> and Mrp3<sup>(−/−)</sup> mice. Absence of intact Mrp3 was also verified by immunoblotting with two independent rabbit polyclonal sera (figure 1c and not shown).

Cells that overproduce MRP3 are resistant to the anticancer drug etoposide. We tested whether the Mrp3<sup>(−/−)</sup> mice display increased sensitivity to this drug by injecting mice with the water soluble etoposide prodrug, etoposide-phosphate (ETOPOPHOS<sup>®</sup>). At concentrations up to 120 mg/kg the Mrp3<sup>(−/−)</sup> male and female mice (12-16 wk old) did not show increased sensitivity to drug compared with control mice. In contrast, this concentration was previously shown to be toxic to Mrp1<sup>(−/−)</sup> mice<sup>26</sup>. At this dose there was already sub-lethal toxicity (e.g. weight loss) in wildtype mice. Higher concentrations were not tested as they result in undue toxicity in the control mice as well.

Tissue distribution of Mrp3

We analyzed the tissue distribution of Mrp3 with immunoblotting, using Mrp3<sup>(−/−)</sup> mice as negative controls. In samples prepared from total tissue lysates Mrp3 was detected throughout the gastrointestinal tract, liver, spleen, lung, pancreas, bladder, gall bladder and at low levels in the adrenal gland (figure 2a and b). The highest levels of Mrp3 were found in the colon and liver. Mrp3
was not detected in the kidney, brain and testis (figure 2). In the tissues where Mrp3 was present, its absence in the Mrp3 (−/−) mice was confirmed (not shown).

**Figure 1.** Targeting of the Mrp3 gene by homologous recombination. (a) Genomic structure of exons 2-8 of the Mrp3 gene and the targeting vector. The targeting vector contains the Hygromycin cassette, flanked by 5' and 3' homologous arms. The location of the 5' and 3' probes flanking the homologous sequences, used to detect correct targeting is indicated. The mutant allele carries an additional Ncol site within the Hygro cassette and has lost a BglII site due to cloning of the targeting construct. Restriction sites: N, Ncol; B, BglII; Hyg, hygromycin resistance gene driven by the mouse phosphoglycerat kinase promoter. (b) Ncol southern blot analysis using the 3' probe. (c) Liver and colon homogenates (30 μg) from two wildtype (wt) and two knockout (ko) mice were size-fractionated in a 7.5% SDS-polyacrylamide gel and Mrp3 was detected using the A66 rabbit polyclonal serum. A monoclonal against α-tubulin was used as a control for gel loading.
Table 1. Clinical chemistry analysis of serum from Mrp3<sup>+/+</sup> and Mrp3<sup>−/−</sup> mice. Serum was collected from 14-week old mice (n=5 per group) and analyzed for clinical chemistry parameters. Each value represents the mean±SD.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Mrp3&lt;sup&gt;+/+&lt;/sup&gt;</th>
<th>Mrp3&lt;sup&gt;−/−&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>General:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weight (g)</td>
<td>30 ± 2</td>
<td>32 ± 2</td>
</tr>
<tr>
<td>Hemoglobin (mM)</td>
<td>9 ± 0.5</td>
<td>8 ± 1</td>
</tr>
<tr>
<td>Hematocrit</td>
<td>0.5 ± 0.0</td>
<td>0.5 ± 0.1</td>
</tr>
<tr>
<td>Total bilirubin (μM)</td>
<td>2 ± 0.5</td>
<td>2 ± 0.4</td>
</tr>
<tr>
<td>Direct bilirubin (μM)</td>
<td>&lt;1 ±</td>
<td>&lt;1 ±</td>
</tr>
<tr>
<td>Creatinine (μM)</td>
<td>14 ± 8</td>
<td>16 ± 5</td>
</tr>
<tr>
<td>Urea (mM)</td>
<td>12 ± 2</td>
<td>11 ± 1</td>
</tr>
<tr>
<td>Sodium (mM)</td>
<td>152 ± 2</td>
<td>151 ± 1</td>
</tr>
<tr>
<td>Potassium (mM)</td>
<td>5 ± 0.5</td>
<td>5 ± 0.5</td>
</tr>
<tr>
<td>Total protein (g/L)</td>
<td>46 ± 2</td>
<td>46 ± 2</td>
</tr>
<tr>
<td>Cholesterol (mM)</td>
<td>4 ± 0.5</td>
<td>4 ± 0.5</td>
</tr>
<tr>
<td><strong>Serum enzymes:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alkaline phosphatase (U/L)</td>
<td>65 ± 10</td>
<td>71 ± 13</td>
</tr>
<tr>
<td>Gamma glutamyltransferase</td>
<td>&lt;5 ±</td>
<td>&lt;5 ±</td>
</tr>
<tr>
<td>Lactate dehydrogenase (U/L)</td>
<td>366 ± 84</td>
<td>387 ± 222</td>
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<td><strong>Blood cell profile:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Erythrocytes (10&lt;sup&gt;12&lt;/sup&gt;/L)</td>
<td>9 ± 0.5</td>
<td>9 ± 1</td>
</tr>
<tr>
<td>Leucocytes (10&lt;sup&gt;9&lt;/sup&gt;/L)</td>
<td>3 ± 1</td>
<td>3 ± 1</td>
</tr>
<tr>
<td>Thrombocytes (10&lt;sup&gt;9&lt;/sup&gt;/L)</td>
<td>928 ± 88</td>
<td>980 ± 196</td>
</tr>
<tr>
<td><strong>Leucocyte differentiation:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eosinophil granulocytes (%)</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>Basophil granulocytes (%)</td>
<td>0.0 ± 0.0</td>
<td>0.5 ± 1</td>
</tr>
<tr>
<td>Neutrophil granulocytes (%)</td>
<td>15 ± 4</td>
<td>17 ± 67</td>
</tr>
<tr>
<td>Lymphocytes (%)</td>
<td>84 ± 3</td>
<td>82 ± 6</td>
</tr>
<tr>
<td>Monocytes (%)</td>
<td>1 ± 0</td>
<td>1 ± 1</td>
</tr>
</tbody>
</table>

We also looked at the localization of Mrp3 in several of these tissues (figure 3a,b). In pancreas, Mrp3 is localized to pancreatic ductal cells, as was seen for human MRP3<sup>11</sup>. Human MRP3 is not detected in the lung and spleen<sup>11</sup>, in contrast to mMrp3. In the lung Mrp3 is localized to the basolateral membrane of bronchial epithelial cells (figure 3c,d) and in the spleen Mrp3 is found at high levels in erythropoietic cells throughout the red pulp (figure 3e,f).
Characterization of the Mrp3(−/−) mouse

The transport of bile acids by both rat and human Mrp3/MRP3 led to the suggestion that Mrp3 may be important for bile acid reclamation in the terminal ileum and in the liver under conditions of impaired bile flow. In the small intestine and colon murine Mrp3 is localized to the basolateral membrane of enterocytes (figure 4a-d), as recently also demonstrated for rat Mrp3. This localization is compatible with a possible role for Mrp3 in trans-ileal transport of bile acids across the enterocytes. Hepatic expression of rat Mrp3 is low, but is highly induced in several models of cholestatic liver disease. In contrast to this, basal hepatic expression of mouse Mrp3 is high (figures 1 and 2). Mrp3 is localized to the basolateral membrane of hepatocytes and is therefore in a position to remove (toxic) organic anions from the liver, as previously proposed (figure 5).

Figure 2. Tissue distribution of Mrp3 in mice. (a) Total tissue lysates were prepared and size-fractionated in a 7.5% SDS-polyacrylamide gel. (30 μg per lane). Mrp3 was detected with the A66 rabbit polyclonal sera. (b) Detection of Mrp3 in gall bladder (g.b). Mrp3 was detected as above in gall bladder and is absent in the Mrp3(−/−) mouse. Equal gel loading was verified with ponceau coloring of blotted membranes.
Figure 3. Immunolocalization of Mrp3 in tissue sections. Mrp3 was analyzed in pancreas (a,b), lung (c,d), and spleen (e,f) of Mrp3<sup>−/−</sup> (a,c,e) and Mrp3<sup>+/+</sup> mice (b,d,f) with the rabbit polyclonal serum A66, as described in the methods section.
**Figure 4.** Immunolocalization of Mrp3 in tissue sections of the gastrointestinal tract. Mrp3 was analyzed in the small intestine (a,b), and colon (c,d) of Mrp3<sup>(−/−)</sup> (a,c) and Mrp3<sup>(+/−)</sup> mice (b,d) with the rabbit polyclonal serum A66, as described in the methods section.

**Figure 5.** Immunolocalization of Mrp3 to the basolateral membrane of mouse liver. (a) Mrp3 was analyzed in cryosections of liver from Mrp3<sup>(−/−)</sup> using the A66 rabbit polyclonal serum (red). Apical Bcrp1 was counterstained with the rat monoclonal antibody BXP-53 (green) and DNA (blue) with DAPI. (b) higher magnification.

Characterization of the Mrp3<sup>(−/−)</sup> mouse
Trans-ileal transport of TCA

Bile acids are taken up from the gut lumen, predominantly at the terminal ileum via Asbt\textsuperscript{28,29}. Within the enterocytes, bile acids are bound by the ileal bile acid binding protein (Ibap)\textsuperscript{30} and are transported across the lateral membrane by an unidentified transporter. As Mrp3 is localized to the basolateral membrane of enterocytes (figure 4) and is able to transport bile acids we tested the possibility that Mrp3 represents this transporter using an Ussing chamber setup with terminal ileum sections. The trans-ileal transport of TCA (1 mM) was measured over time and the mean rate calculated (figure 6). As figure 6 shows, no difference in the rate of trans-ileal TCA transport from the luminal to serosal compartment was seen between Mrp3\textsuperscript{+/−} and Mrp3\textsuperscript{−/−} mice.

![Figure 6](image)

**Figure 6.** Trans-ileal transport of TCA. Terminal ileum sections from Mrp3\textsuperscript{+/−} and Mrp3\textsuperscript{−/−} mice (n=6) were mounted in an Ussing chamber setup. Transport was initiated by adding TCA (1 mM) to the mucosal compartment and drawing samples from the serosal compartment over a time period of 90 minutes. Each bar represents the mean ± SD rate of TCA transport across the terminal ileum sections. TCA was determined as described in methods. TCA was determined as described in methods section.

**BDL experiments with Mrp3\textsuperscript{−/−} mice**

The marked induction of rat Mrp3 in models of cholestasis and the apparently low affinity of human MRP3 for TCA and GCA point to a possible role of Mrp3 under conditions where normal bile flow is impaired. To test this hypothesis we used a BDL model of obstructive cholestasis to study the
contribution of Mrp3 to removal of (toxic) organic anions from the liver. Histological inspection of 3-day BDL livers revealed a similar extent of necrotic foci in the liver and of bile-duct epithelium hyperplasia in Mrp3−/− and control mice (not shown and table 2). Three days of BDL resulted in a 50% reduction in hepatic Mrp2 levels and in a marginal increase in Mrp3 hepatic level that was evident in tissue staining but not in Western analysis (figure 7). No induction of Mrp3 was seen in Western blot analysis after 7 days of BDL either (not shown). We do not detect Mrp1 in normal and BDL livers (not shown).

A major consequence of BDL is a substantial increase in serum levels of bile acid. In both groups of mice this was observed (figure 8A), but no difference in the serum levels of bile acids were seen between the Mrp3−/− and control mice. Additionally, no differences in the bile acid species in the serum of 3-day BDL mutant and wildtype mice were observed (not shown).

An additional consequence of BDL is elevated conjugated bilirubin in serum as a result of its impaired biliary secretion. 3 days of BDL resulted in a sharp increase in serum levels of this conjugate in both mutant and control mice. However, in the mutant mice, the levels of conjugated bilirubin were only 50% of those seen in the control group (figure 8B).

Table 2. Summary of enzyme activities in serum of BDL mice 3 days after ligation. All values are the mean of activity (units/liter) ± SD (n=8-10).

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Mrp3−/−</th>
<th>Mrp3+/+</th>
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<tbody>
<tr>
<td>Alkaline phosphatase</td>
<td>492 ± 199</td>
<td>514 ± 205</td>
</tr>
<tr>
<td>Aspartate aminotransferase</td>
<td>642 ± 156</td>
<td>1239 ± 964</td>
</tr>
<tr>
<td>Alanine aminotransferase</td>
<td>666 ± 323</td>
<td>843 ± 543</td>
</tr>
<tr>
<td>Lactate dehydrogenase</td>
<td>2122 ± 2182</td>
<td>2513 ± 2479</td>
</tr>
</tbody>
</table>

Discussion

We have generated mice lacking Mrp3. These mice are viable, fertile and healthy indicating that Mrp3 is dispensable in the protected environment of our animal facility. The absence of Mrp3 in the Mrp3−/− mice was verified by genomic analysis and with rabbit polyclonal antisera generated against two distinct regions of Mrp3 (figure 1).

In transfection experiments with mammalian cells MRP3 confers resistance to a narrow spectrum of anticancer drugs including etoposide, teniposide and methotrexate. When we challenged mice with an increasing dose of etoposide-phosphate (an etoposide prodrug) we found no difference in sensitivity of the Mrp3−/− mice compared with control at concentrations up to 120
Figure 7. Detection of Mrp3 in BDL livers. Mrp3 was analyzed in liver sections from Mrp3\(^{-/-}\) (a) and Mrp3\(^{+/+}\) after 3-day BDL (c), or from untreated Mrp3\(^{+/+}\) (b) using the A66 rabbit polyclonal serum. (d) Total liver lysates from untreated Mrp3\(^{+/+}\), 3-day BDL Mrp3\(^{+/+}\), and 3-day BDL Mrp3\(^{-/-}\) (n=3 mice per group) were prepared and 40 μg protein loaded per lane. Mrp3 was detected with the A66 rabbit polyclonal serum and Mrp2 with the K13 rabbit polyclonal serum, as described in the methods section. L.c, Gel loading was controlled by ponceau staining of blotted membranes (not shown) and with a non-specific cross-reacting band.

mg etoposide per kg. Higher concentrations were not tested as they result in undue toxicity in the control mice. Several other MRPs as well as MDR1 P-gp and BCRP transport etoposide and the lack of hypersensitivity in the Mrp3\(^{-/-}\) mice probably reflects an overlap with these transporters in tissues where Mrp3 is expressed. In line with this idea, the toxicity of etoposide in the Mrp1\(^{+/+}\) mice was predominantly due to extensive damage of the oropharyngeal epithelium as well as to depression of bone marrow where Mrp1 is highly expressed. We detect Mrp3 in the tongue of wildtype mice (not shown), but Mrp1 expression in this tissue is likely to compensate for its loss in the Mrp3\(^{-/-}\) mice. Whether Mrp3 is expressed in bone marrow remains to be tested.

The tissue distribution of mouse Mrp3 is similar to that of human MRP3, with several exceptions (figure 2): we do not detect Mrp3 in mouse kidney, where human MRP3 is found. Whereas no MRP3 is detected in the lung and spleen of humans, relatively high levels of Mrp3 are
Characterization of the Mrp3'''' mouse

Figure 8. (A) Serum bile acid levels after 3 days of BDL. Serum from BDL mice was collected after 3 days and was subjected to analysis of bile acid composition and levels, as described in the methods section. Each bar and error represent the mean ± SD of total serum bile acid level (n=10). The serum bile acids level of untreated Mrp3'''' and Mrp3'''' were 9.6±9.0 µM and 1.9±1.2, respectively (n=5, p=0.05). (B) Serum bilirubin glucuronide levels after 3 days of BDL. Serum from BDL mice was collected as above and subjected to analysis of total and direct bilirubin, as described in the methods section. Each bar and error represent the mean ± SD of the serum bilirubin glucuronide (n=10, p<0.01). The serum bilirubin levels of untreated Mrp3'''' and Mrp3'''' are not different (see table 1).

present in these tissues in the mouse (figure 3). In the lung, Mrp3 may play a role in protection of the bronchiolar epithelium against damage induced by environmental compounds present in the air. The role of Mrp3 in spleen cells that are undergoing erythropoiesis is presently unclear.

Several lines of evidence suggest that MRP3 may be involved in bile acid physiology: (1) MRP3 is detected in tissues involved in the enterohepatic circulation of bile acids (figures 1-4) 11,27; (2) human 18-20 and rat 17 MRP3/Mrp3 transport bile acids, albeit with markedly different affinities; (3) rat Mrp3 is strongly induced (±30-fold) in several models of cholestasis and in the naturally occurring Mrp2-deficient rat strains, EHB R and TR', suggesting that Mrp3 may be involved in the removal of (toxic) organic anions (e.g. bile acids) under cholestatic conditions 12-15; and (4) the hepatic level of rat Mrp3 partially correlates with TCA transport across the sinusoidal membrane in perfused livers 33,34. We critically tested the contribution of mouse Mrp3 to the in vivo transport of bile acids in the terminal ileum and in the cholestatic liver.

The molecular identity of the transporter that transports bile acids across the basolateral membrane of enterocytes in the terminal ileum, is not yet known. Proposed candidates include an anion exchanger 35 and a truncated form of Asbt (t-Asbt) that localizes to the basolateral membrane of enterocytes 36. The basolateral localization of Mrp3 (figure 4) could allow it to participate in this process. However, using an Ussing chamber setup we find no difference in the
trans-ileal transport of TCA (1 mM) across terminal ileum sections between the Mrp3<sup>-/-</sup> mice and wildtype mice. The simple interpretation of these results is that Mrp3 does not contribute to the basolateral efflux of bile acids from enterocytes. We cannot rule out, however, complex gene expression alterations in the knockout mice that may mask the contribution of Mrp3 to this process.

Under cholestatic conditions complex gene-expression alterations occur that help to minimize the damage to hepatocytes<sup>37,38</sup>. Our results with mouse Mrp3 demonstrate, in contrast to what has been observed in the rat, that the induction of Mrp3 is only marginal under this condition (figure 7). Moreover, whereas in healthy rat liver Mrp3 is undetectable, in mouse livers its basal level is high. Similarly, expression of rat Mrp2 is practically abolished in BDL rats<sup>39</sup> whereas we see only an approximately 2-fold reduction of its expression in mice. The 3-day BDL treatment results in a substantial increase in serum levels of bile acids and hepatic marker enzymes, that is similar in the Mrp3<sup>-/-</sup> and control mice (figure 8A and table 2). The conclusion from these results is that in cholestatic mouse liver Mrp3 is not a major contributor to sinusoidal bile acid efflux and protection of the liver after 3 days of BDL.

These results are challenged by the recent report by Bohan et al.<sup>16</sup> who studied the effect of a two-week BDL treatment on mice. This treatment results in a 6-fold increase of the basal hepatic Mrp3 levels concomitant to a strong increase in serum bile acid levels. Induction of hepatic Mrp3 by this treatment proceeds through a TNFα-dependent induction of the nuclear receptor liver receptor homolog-1 (LRH1). Accordingly, in mice lacking the TNF receptor, Tnfr<sup>-/-</sup> mice, induction of Mrp3 by BDL is abrogated and serum levels of bile acids are elevated to a lower extent than in control mice. The authors interpret this as evidence for a major role of Mrp3 in the basolateral clearance of bile salts after BDL in mice. A simple reason for this discrepancy can be the duration of BDL. Bohan et al. studied the effect of 14 days of BDL whereas we did not extend our studies beyond 7 days. However, we do not think that this is a likely explanation. More likely, the differences can be attributed to experimental methodology. LRH1 regulates many aspects of hepatocyte functions including metabolism and transport processes. Repression of LRH1-mediated transcription in BDL treated Tnfr<sup>-/-</sup> mice is likely to induce numerous hepatic gene alterations, as this is a key hepatic nuclear receptor. Additionally, we do not find strong induction of Mrp3 after BDL treatment whereas this is reported in that study. This discrepancy may be easier to reconcile. The studies were done in mice from different genetic backgrounds and this can impact the endogenous hepatic Mrp3 levels. Possibly, our mice may display a constitutively induced Mrp3 phenotype, for reasons unclear to us, preventing it from being further induced. Analysis of mice with other genetic backgrounds is required to address this possibility.

In a recent paper MRP4 was shown to transport bile acids. Similar to what has been postulated for MRP3, the authors raise the possibility that MRP4 may play a role in mitigating
Characterization of the Mrp3\(^{-/-}\) mouse

cholestasis\(^{40,41}\). Our initial attempts to detect Mrp4 in normal and cholestatic livers with our current antibodies were unsuccessful and additional studies are required to address this issue.

An additional consequence of BDL-treatment is elevation of serum levels of conjugated bilirubin (figure 8B). We find that the Mrp3\(^{-/-}\) mice have a 50% lower level of this conjugate compared with the control. Determination of direct transport of bilirubin glucuronide in transport assays is technically difficult. This compound is highly unstable and therefore any transport that is observed is difficult to interpret. Nevertheless, high affinity transport of bilirubin mono- and diglucuronide by MRP2 (and MRP1)\(^42\) has been demonstrated and we assume that basolateral Mrp3 compensates for the reduction in canalicular Mrp2 activity in the BDL-treated mice and transports this conjugate. Loss of Mrp3 results only in 50% reduction of serum levels of bilirubin glucuronide, indicating that other basolateral transporters may also transport this conjugate.

In conclusion, our results with the Mrp3\(^{-/-}\) mice indicate that Mrp3 does not play a major role in bile acid transport in the terminal ileum and in the cholestatic liver. In cholestatic liver, Mrp3 transports bilirubin glucuronide from hepatocytes into the circulation, but accounts only for about 50% of this process. Other transporters than Mrp3 must therefore contribute to this process as well.

Acknowledgments

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References

Chapter 5

Characterization of the Mrp3<sup>+/−</sup> mouse


Addendum to chapter 5

Addendum to chapter 5

Introduction
Since finishing the writing of chapter 5, we have observed that in liver samples from 7 days BDL Mrp3\(^{(-/-)}\) mice we detect a specific band with the A66 rabbit polyclonal serum by western blot analysis that is absent in samples from wildtype liver (not shown). This band has a higher mobility in SDS-PAGE gel and is approximately 30-40 Kd smaller than wildtype Mrp3. The intensity of this specific band is substantially lower than that of wildtype Mrp3, but we have not yet quantified this precisely. To test the possibility that a truncated form of Mrp3 is made in livers of the Mrp3\(^{(-/-)}\) mice we tested by RT-PCR if we can detect truncated Mrp3 transcripts.

Methods
Isolation of total RNA from liver tissue and synthesis of first strand cDNA using random hexa-nucleotides was done according to standard procedures. PCR reactions with the different oligonucleotides (table 1) were carried out with the following program: 30" 94 °C, 30" 58 °C, 45" 72 °C, X35 cycles. A tenth of the amplification reaction was size fractionated on a 1.2% agarose DNA gel and viewed after staining with ethidium bromide.

Table 1. List of oligonucleotides used to amplify Mrp3 cDNA fragments

<table>
<thead>
<tr>
<th>Name</th>
<th>Position(^a)</th>
<th>Sequence</th>
</tr>
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<tbody>
<tr>
<td>1. ex1-fwd</td>
<td>27</td>
<td>5' gctgggctccaagttctg</td>
</tr>
<tr>
<td>2. ex2-fwd</td>
<td>168</td>
<td>5' gagacaccatcagctcgg</td>
</tr>
<tr>
<td>3. ex8-rev</td>
<td>969</td>
<td>5' gatgtagttcgatcagg</td>
</tr>
<tr>
<td>4. ex8-fwd</td>
<td>952</td>
<td>5' cgtcagatccacactctcg</td>
</tr>
<tr>
<td>5. 1497-rev</td>
<td>1497</td>
<td>5' cacttgatgcgccaggg</td>
</tr>
<tr>
<td>6. 1990-fwd</td>
<td>1990</td>
<td>5' tgtgggaagtctctctgg</td>
</tr>
<tr>
<td>7. 2487-rev</td>
<td>2487</td>
<td>5' agacacctgtccaccagc</td>
</tr>
<tr>
<td>8. 3510-fwd</td>
<td>3510</td>
<td>5' tactaagttggacaacaacc</td>
</tr>
<tr>
<td>9. 4242-rev</td>
<td>4242</td>
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</tr>
<tr>
<td>10. actin-fwd</td>
<td>-</td>
<td>5' gactcctatgtgggtgagcag</td>
</tr>
<tr>
<td>11. Actin-rev</td>
<td>-</td>
<td>5' ccactcctgtgctgaagtct</td>
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\(^a\) position (bp) in Mrp3 cDNA of 5' of the oligonucleotide
Results

We used 5 different oligonucleotide pairs to test the presence of truncated Mrp3 transcripts in the liver (table 1, figure 1a). We were able to amplify fragments of the correct size with 4 of the Mrp3 specific primer sets using cDNA from liver samples from wildtype mice (figure 1b). No specific fragment corresponding to the deleted region in the Mrp3 knockout mouse could be amplified, but unexpectedly, fragments downstream of the deleted region were amplified correctly. Additionally, no amplified fragments were obtained with the ex1-fwd oligonucleotide (table 1) using either wildtype or knockout derived liver cDNA, but this probably reflects a technical problem. A 520 bp actin fragment was used as positive control in these reactions and was correctly amplified from all cDNA sources.

![Figure 1](image_url)

**Figure 1.** RT-PCR analysis of Mrp3 cDNA from mouse liver. (a) Schematic representation of Mrp3 cDNA depicting the location of the oligonucleotides used to analyze Mrp3 transcripts. The oligonucleotides numbering corresponds to that used in table 1. The hatched box represents the deleted region in the Mrp3<sup>−/−</sup> mice (b) Amplified DNA fragments from the indicated reactions were size fractionated on a 1.2% agarose and visualized with ethidium bromide. SM, size marker.

Discussion

Using RT-PCR analysis we confirm that exons 2-8 have been deleted in the Mrp3 knockout mouse, but unexpectedly detect cDNA fragments downstream of the deleted region. We are now...
characterizing the Mrp3 transcript in the knockout mouse and determining its sequence and transcriptional start site. The presence of a truncated transcript may explain why we detect a truncated Mrp3 in liver samples from knockout mice.

We are analyzing additional tissues to see if we can detect this truncated Mrp3. Notably, in our immunolocalization studies of Mrp3 in mouse tissues (Chapter 5), we did not detect Mrp3 staining in tissues from the knockout mouse, including liver, using the same A66 rabbit polyclonal. This suggests that the level of this truncated protein is low in these tissues.

The implications of this finding to our studies of the Mrp3 knockout mouse are still unclear. Nevertheless, the cDNA that can be formed in the knockout mouse will encode an Mrp3 that lacks TMD0, L0 and the first putative transmembrane helix of TMD1. In analogy with previous studies with human MRP1 1-3, this protein is likely to be transport incapable and not be routed properly to the plasma membrane, but this remains to be tested.

References