

Analysis of the In Vivo Functions of Mrp3

Martin G. Belinsky, Paul A. Dawson, Irina Shchaveleva, Lisa J. Bain, Renxue Wang, Victor Ling, Zhe-Sheng Chen,¹ Alex Grinberg, Heiner Westphal, Andres Klein-Szanto, Anthony Lerro, and Gary D. Kruh

Medical Science (M.G.B., I.S., Z.-S.C., A.K.-S., G.D.K.) and Basic Science Divisions (A.L.), Fox Chase Cancer Center, Philadelphia, Pennsylvania; Department of Internal Medicine, Wake Forest University School of Medicine, Winston-Salem, North Carolina (P.A.D.); Department of Biological Sciences, University of Texas at El Paso, El Paso, Texas (L.J.B.); British Columbia Cancer Agency, Vancouver, British Columbia, Canada (R.W., V.L.); and National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, Maryland (A.G., H.W.)

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ABSTRACT

Multidrug resistance protein 3 (MRP3) is an ATP-binding cassette transporter that is able to confer resistance to anticancer agents such as etoposide and to transport lipophilic anions such as bile acids and glucuronides. These capabilities, along with the induction of the MRP3 protein on hepatocyte sinusoidal membranes in cholestasis and the expression of MRP3 in enterocytes, have led to the hypotheses that MRP3 may function in the body to protect normal tissues from etoposide, to protect cholestatic hepatocytes from endobiotics, and to facilitate bile-acid reclamation from the gut. To elucidate the role of Mrp3 in these processes, the *Mrp3* gene (*Abcc3*) was disrupted by homologous recombination. Homozygous null animals were healthy and physically indistinguishable from wild-type mice. *Mrp3*^{-/-} mice did not exhibit enhanced lethality to etoposide phosphate, although an analysis of transfected human embry-

onic kidney 293 cells indicated that the potency of murine Mrp3 toward etoposide (~2.0- to 2.5-fold) is comparable with that of human MRP3. After induction of cholestasis by bile duct ligation, *Mrp3*^{-/-} mice had 1.5-fold higher levels of liver bile acids and 3.1-fold lower levels of serum bilirubin glucuronide compared with ligated wild-type mice, whereas significant differences were not observed between the respective sham-operated mice. Bile acid excretion, pool size, and fractional turnover rates were similar in *Mrp3*^{-/-} and wild-type mice. We conclude that Mrp3 functions as an alternative route for the export of bile acids and glucuronides from cholestatic hepatocytes, that the pump does not play a major role in the enterohepatic circulation of bile acids and that the lack of chemosensitivity is probably attributable to functional redundancy with other pumps.

MRP3 belongs to a group of nine related ATP-binding cassette transporters that constitute the MRP family. Members of this family function as efflux pumps for lipophilic anions and hydrophobic compounds (Kruh and Belinsky, 2003). The in vivo functions of the first two members of this family have been investigated in gene-disrupted mice in the case of Mrp1 and hereditarily deficient rats (EHBR and TR⁻ rats) and humans (Dubin-Johnson syndrome) in the case of Mrp2. These studies

indicate that Mrp1 functions as an in vivo resistance factor for anticancer agents and in inflammatory responses mediated by leukotriene C4 (Lorico et al., 1997; Wijnholds et al., 1997; Johnson et al., 2001; Schultz et al., 2001) and that Mrp2 is involved in the hepatobiliary elimination of endogenous compounds, such as bilirubin glucuronide, and in the hepatobiliary and renal elimination of xenobiotics (Gerk and Vore, 2002). In contrast to MRP1 and MRP2, the in vivo functions of MRP3 have not been established. However, studies in our and other laboratories on the pump's substrate selectivity, resistance capabilities, and tissue-expression pattern have allowed speculation as to its functions in the body. In cellular models, ectopic expression of human MRP3 is able to confer resistance to etoposide and methotrexate, results which are consistent with the hypothesis that the pump is an in vivo resistance factor that protects normal tissues from chemotherapeutic agents

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¹ Current address: College of Pharmacy and Allied Health Professions, St. John's University, Jamaica, New York.

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ABBREVIATIONS: MRP, multidrug resistance protein; ASBT, apical sodium-dependent bile acid transporter; BSEP, bile salt export pump; HEK, human embryonic kidney; kb, kilobase; IBABP, ileal bile acid binding protein; PCR, polymerase chain reaction; bp, base pair(s); OST, organic solute carrier.

(Kool et al., 1999; Zeng et al., 1999, 2001). The substrate selectivity of human and rat Mrp3 is similar to that of MRP1 and MRP2 with respect to the ability to transport glucuronate and glutathione conjugates, but in contrast to the latter two pumps, monoanionic bile acids are also good substrates of MRP3 (Hirohashi et al., 1999, 2000; Zeng et al., 2000). MRP3 assumes basolateral subcellular localization in polarized cells and is expressed in a variety of tissues including kidney, gut, and pancreas (Belinsky et al., 1998; Rost et al., 2002; Scheffer et al., 2002). It is noteworthy that its expression is dramatically increased at the hepatocyte sinusoidal membrane in cholestatic conditions (Donner and Keppler, 2001; Soroka et al., 2001). The ability of MRP3 to transport bile acids, glucuronides, and other compounds that are normally transported across the canalicular membrane by MRP2 and the bile salt export pump in combination with the induction of MRP3 in cholestasis have led to the hypothesis that MRP3 protects hepatocytes by functioning as an alternate route of elimination when the canalicular route of detoxification is impaired. This notion is also supported by studies showing that Mrp3 is induced by bile acids and that Mrp3 expression is linked to signal transduction and transcriptional pathways that govern bile acid homeostatic mechanisms (Inokuchi et al., 2001; Bohan et al., 2003; Zollner et al., 2003). Although mice deficient in some of these transcriptional pathways are more susceptible to liver damage when made cholestatic, the extent to which attenuation of Mrp3 induction may contribute to this process, as opposed to impairments in the pleiotropic mechanisms of bile acid homeostasis that are regulated by these transcriptional pathways, is unknown (Bohan et al., 2003; Zhang et al., 2004). MRP3 has also been implicated in the enterohepatic circulation of bile acids. Bile acids are transported from the intestinal lumen across the enterocyte brush-border membrane by the well characterized ileal apical bile acid transporter (ASBT). However, the mechanisms responsible for bile acid transport across the enterocyte basolateral membrane have not been fully identified. By virtue of its basolateral membrane expression in enterocytes and its bile acid transport properties, it has been proposed that MRP3 functions as the intestinal basolateral bile acid transporter that moves bile acids from the enterocyte into the portal circulation.

Here, *Mrp3*^{-/-} mice were generated and analyzed to investigate the in vivo functions of Mrp3. *Mrp3*^{-/-} mice did not exhibit increased sensitivity to etoposide phosphate, although transfection experiments showed that expression of the murine protein in HEK293 cells is capable of conferring resistance to this agent. Although *Mrp3* null mice are healthy and have normal bile flow, these animals accumulate increased levels of hepatic bile acids and have reduced serum levels of bilirubin glucuronide after bile duct ligation. Measurements of overall bile acid metabolism, including fecal bile acid excretion, bile acid pool size, and bile acid fractional turnover rate were similar in *Mrp3*^{-/-} and wild-type mice. From these findings, we concluded that Mrp3 functions as an alternative route for the export of bile acids and glucuronides in cholestatic hepatocytes, that the pump does not play a major role in the enterohepatic circulation of bile acids, and that the lack of chemosensitivity in *Mrp3*^{-/-} mice may be attributable to functional redundancy with other efflux pumps.

Materials and Methods

Targeted Disruption of the *Mrp3* Gene and Generation of *Mrp3*^{-/-} Mice. A 1-kb fragment containing the 5' end of the *Mrp3* coding sequence was used to screen a mouse strain 129-derived λ phage genomic library, and a ~9-kb *Mrp3* clone was isolated. The *Mrp3* genomic clone was sequenced using an ABI 377 DNA sequencer (Applied Biosystems, Foster City, CA) and encompassed exons 2 to 11 of the *Mrp3* gene, corresponding to nucleotides 100 to 1482 of the coding sequence. The left and right arms of a targeting vector were generated by polymerase chain reaction and were inserted, respectively, to the 5' and 3' of the pgk-neo cassette in the PNT plasmid. The vector was designed to delete exons 6 to 8, encoding nucleotides 613 to 996 of the coding sequence, and to introduce a frame-shift into the transcribed RNA sequence. The nucleotide sequence of the cloned arms was confirmed, and the resulting ~12-kb vector was digested with NotI. The linearized DNA was electroporated into strain 129-derived R1 embryonic stem cells. Individual colonies isolated after positive/negative selection with G418 and gancyclovir were screened by Southern blot analysis using 5' and 3' probes and genomic DNA digested with XhoI/XmnI. In addition, the absence of randomly integrated vector sequences was confirmed by Southern blot analysis using a neo probe. Two correctly targeted ES cell lines were injected into C57BL/6J blastocysts, and the blastocysts were implanted in pseudopregnant females. Male chimeric progeny were crossed with female C57BL/6J (in-house-bred) mice. Germ-line transmission of the targeted allele was confirmed by Southern blot analysis, and subsequent genotyping was accomplished by PCR analysis of tail DNA. The latter reaction was carried out in a single tube using three primers: 5'-gttctgtgccttcctgtcc-3', 5'-gggagggg-ggcaagtcaggcc-3', and 5'-aattgacctgcaggggccctcg-3'. The former two primers generate a 790-bp wild-type product, and the latter two generate a 330-bp product from the targeted allele. The *Mrp3* null allele was subsequently backcrossed for eight generations into the C57BL/6J background. As indicated below, *Mrp3* null mice in the mixed C57BL/6J \times 129 background and the C57BL/6J background were used for these studies.

Isolation of Mrp3 cDNA, Expression Vector Construction, and Transfection. A PCR product corresponding to nucleotides 600 to 1440 of the human MRP3 coding sequence (AF104943) was used to screen a bacteriophage library prepared from mouse testis, and a clone encompassing the entire coding region was isolated. The cDNA was sequenced, and the resulting sequences were assembled using the Sequencer program (Gene Codes Corporation, Ann Arbor, MI). The *Mrp3* cDNA sequence has been deposited in GenBank (accession no. AY841885). A 4.8-kb fragment encompassing the ~4.6-kb coding sequence was cloned into the PEAK10 vector (Edge Biosystems, Gaithersburg, MD) to create PEAK10-Mrp3. Human embryonic kidney cells (HEK293/EBNA) were electroporated with 10 μ g of either PEAK10-Mrp3 or the parental plasmid, and individual puromycin-resistant colonies were isolated and expanded for analysis of Mrp3 protein expression. Cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum.

Generation of Mrp3 Polyclonal Antibody and Immunoblot Analysis. A cDNA fragment encoding amino acids 858 to 945 of the linker region of Mrp3 was inserted downstream of the glutathione *S*-transferase coding sequence in the vector pGEX2T, and the induced fusion protein was purified using glutathione Sepharose beads (Amersham Biosciences Inc., Piscataway, NJ). Rabbits were immunized with the recombinant protein, and the immune sera were used for immunoblot analysis. Total cellular lysates prepared from cultured cells, and membrane fractions prepared from liver were subjected to SDS-polyacrylamide gel electrophoresis and were electrotransferred to nitrocellulose filters. Mrp3 antibody was used at a 1:500 dilution, and horseradish peroxidase-conjugated secondary antibody (PerkinElmer Life and Analytical Sciences, Boston, MA) was used at 1:2500. Mrp2 monoclonal antibody M₂III-5 (kindly provided by George Scheffer, Free University, Amsterdam, The Netherlands)

and a previously described polyclonal antibody against the bile salt export pump (Wang et al., 2001) were used at 1:1000 dilutions. Affinity-purified Mrp4 antisera were raised against amino acids NVDPRTDELIIQQKIREK conjugated to keyhole limpet hemocyanin (Invitrogen, Carlsbad, CA) and were used at a dilution of 1:500. Antibody to β -actin (Sigma-Aldrich, St. Louis, MO) was used at a dilution of 1:1000. Immunoblots were developed using the enhanced chemiluminescence method (Amersham).

Blood Chemistries, Hematology, and Histopathology. Animals (mixed 129 \times C57BL/6J background from the F3 and F4 generation) were maintained in the Fox Chase laboratory animal facility and were housed in a temperature- and humidity-controlled environment under 12-h light/dark cycles. Mice were fed a standard rodent diet (Lab Diet 5013; PMI Nutrition, Brentwood, MO) and had free access to water. The Fox Chase Institutional Animal Care and Use Committee approved the protocol. Peripheral blood was obtained by orbital bleeding of anesthetized mice. Blood chemistry and hematology parameters were determined at Antech Diagnostics (Farmingdale, NY). For histological analysis, tissues were fixed in 10% phosphate-buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin/eosin.

In Vivo Etoposide Toxicity. Groups of male mice (mixed 129 \times C57BL/6J background) aged 8 to 12 weeks were treated with single intraperitoneal injections of etoposide phosphate (Bristol-Myers Squibb Co., Princeton, NJ). Mice were observed daily for a period of 4 weeks. For analysis of white blood cell counts, groups consisting of five male mice were treated with a single intraperitoneal injection (150 mg/kg body weight), and blood samples (20 μ l) were taken daily by orbital bleeding for a period of 6 days. White blood cell counts were analyzed using a Coulter Z1 Series Particle Counter (Beckman Coulter, Miami, FL).

In Vitro Cytotoxicity Assays. Drug sensitivity was analyzed using the CellTiter 96 Cell Proliferation Assay (Promega, Madison WI). Cells seeded overnight in triplicate at \sim 3000 cells/well were treated with drugs at various concentrations, and the proliferation assays were performed after 72 h of drug exposure. The nonparametric two-tailed Wilcoxon test was used to make inferences about the significance of the data.

Bile Duct Ligation and Analysis of Liver and Serum Bile Acids. Mice (mixed 129 \times C57BL/6J background) were anesthetized using vaporized isoflurane applied through a nose cone, and surgery was performed aseptically, using steam-sterilized instruments within a disinfected surgical area. The abdomen was shaved and disinfected with an iodine-based surgical scrub and was covered with sterile drapes. A 2.0-cm transverse incision was made through the skin of the ventral abdomen at a point starting just below the sternum. A similar incision was made through the body wall. The liver was displaced upward to expose the bile duct, which was tied off using 6-0 silk, and the body wall was closed with interrupted sutures of 6-0 Vicryl. The skin was closed with wound clips, and mice were placed on a warming tray until they recovered from the anesthesia. After 3 days, peripheral blood was sampled from fasting (4 h) animals, and livers were removed from animals that were sacrificed. Livers were washed in saline, flash-frozen in liquid nitrogen, and stored at -80°C . Bile acids from preweighed, pulverized liver samples were extracted overnight in *tert*-butanol/water (1:1), and liver and serum bile acids were determined using a colorimetric assay (Sigma Diagnostics, St. Louis MO). Serum bilirubin was determined at Antech Diagnostics.

Kruskall-Wallis tests, a nonparametric analog of the analysis of variance, were used to compare the distributions of measurements across the four mouse type-treatment combinations. These tests were conducted at a 5% significance level. Next, two-sample Wilcoxon tests were used to make comparisons between all possible pairs of mouse type-treatment combinations. To account for multiple comparisons, Bonferroni adjustment was used to control the experiment-wise type I error. In particular, each individual null hypothesis was rejected if the significance level was less than $\alpha = (0.05/6) =$

0.0083. These analyses were performed separately for serum-conjugated bilirubin, serum bile acid, and liver bile acid measurements.

Analysis of Bile Flow. Bile duct cannulation of mice (mixed 129 \times C57BL/6J background) and collection of bile were performed as described previously (Wang et al., 2001). The gallbladder was cannulated after ligation of the common bile duct, and bile was collected at 5-min intervals. At 10 min, taurocholate (100 μM /kg body weight) was injected as a bolus into the jugular vein. Bile was then collected at 2-min intervals for 10 min and then at 10-min intervals for 20 min.

Analysis of Fecal Bile Acid Excretion, Bile Acid Pool Size, and Composition. Wild-type and *Mrp3*^{-/-} male mice (3–4 months; C57BL/6J background) were individually housed in wire-bottom cages, and stools were collected for 3 days. The stools were extracted as described previously (Turley et al., 1996) and were used to determine the total bile acid content by an enzymatic method (Mashige et al., 1981). Pool size was determined as the bile acid content of the small intestine, liver, and gallbladder. These tissues were removed and extracted in ethanol as described previously (Schwarz et al., 1998). The extract was filtered, and bile acid composition was determined using high-performance liquid chromatography as described previously (Torchia et al., 2001). Individual bile acid species were measured using an evaporative light-scatter detector (Alltech ELSD 800; Alltech-Applied Science Labs, State College, PA). Bile acids were identified and quantified by comparison to known amounts of authentic standards purchased from Steraloids (Newport, RI).

Analysis of Asbt, Ost α , Ost β , and Ibabp Expression in Intestine. RNA was prepared from intestines taken from wild-type and *Mrp3*^{-/-} male mice aged 3 to 4 months (C57BL/6J background). cDNA synthesis was initiated from 1 μg of RNA using random hexamer primers and Omniscript transcription reagents (QIAGEN, Valencia, CA). For each real-time PCR reaction, cDNA synthesized from 25 ng of RNA was mixed with 2 \times SYBR Green PCR Master Mix (Applied Biosystems) containing 500 nM concentrations of specific primers. The PCR reactions were carried out in triplicate, and samples were analyzed on an ABI 7900 sequence detection system. The oligonucleotide primer sequences were the following: Asbt, 5'-tggtt-tctctctgtagact-3' and 5'-tggttctgattccagtttccaa-3'; Ost α , 5'-tacaagaacaccctttgccc-3' and 5'-cgaggaatccagagacacaaa-3'; Ost β , 5'-gtatttctgtgcagaagatgcg-3' and 5'-tttctgtttgcccaggtatgctc-3'; Ibabp, 5'-caggctaccgtgaagatgga-3' and 5'-cccacgacctccgaagtct-3'; and GAPDH, 5'-tgtgtccgtcgtggatctga-3' and 5'-cctgcttcaccaccttctgtat-3'.

Results

Inactivation of the *Mrp3* Gene in Mice. A targeting construct designed to delete a \sim 2.1-kb fragment encoding exons 6 to 8 of the *Mrp3* gene was generated (Fig. 1A). The intended deletion removes three exons encoding amino acids 205 to 332 of the *Mrp3* protein and introduces a frame shift in the reading frame should splicing take place between the exons that immediately border the neo marker. The construct was electroporated into R1 embryonic stem cells, and G418/gancyclovir-resistant colonies were isolated. Southern blot analysis of DNA preparations from two ES clones in which proper targeting took place (Fig. 1B) revealed the predicted 5.0- (left) and 5.7-kb (right) bands for the disrupted allele, in addition to the 10.7-kb band for the wild-type allele (left and right). Chimeric mice were generated, and germ-line transmission of the disrupted allele was achieved in animals derived from each cell line. Homozygous null mice were identified in litters of backcrossed heterozygote animals (Fig. 1C). Inactivation of the gene was confirmed by immunoblot analysis using polyclonal antisera directed toward *Mrp3* protein. Immunoreactive protein of the expected molecular weight was readily detected in crude membranes prepared from

liver tissue of wild-type mice, whereas the Mrp3-specific band was absent in *Mrp3*^{-/-} mice (Fig. 1D). Haploinsufficiency was apparent in heterozygote mice in which the level of Mrp3 protein in liver was ~50% of that in wild-type mice (Fig. 1D, lane 4).

Heterozygote crosses yielded a distribution of genotypes approximating the expected Mendelian ratios (24.8% wild type, 49.9% heterozygote, 25.4% null; *n* = 351). *Mrp3*^{-/-} mice were grossly indistinguishable from their wild-type counterparts, had normal viability, and produced litters that were similar in size to those of wild-type mice. The average weight at weaning of male and female *Mrp3*^{-/-} mice was not significantly different from wild-type mice. Histopathological analysis of tissues taken from *Mrp3*^{-/-} mice at 6 weeks and 6 months of age did not reveal obvious abnormalities (data not shown). Serum chemical and hematological parameters measured in ~10-week-old (Table 1) and ~9-month-old mice (data not shown) did not differ significantly between wild-type and *Mrp3*^{-/-} mice.

Sensitivity of Mrp3-Deficient Mice to Etoposide. The sensitivity of *Mrp3*^{-/-} mice toward anticancer agents was analyzed to ascertain the contribution of the protein to protecting normal tissues. Etoposide was selected as the chemotherapeutic agent in these experiments because human

MRP3 is capable of conferring resistance to this compound (Kool et al., 1999; Zeng et al., 1999). Etoposide phosphate, a water-soluble ester of etoposide that is converted to etoposide in plasma, was administered via a single intraperitoneal injection, and the sensitivities of wild-type and *Mrp3*^{-/-} mice were compared. Preliminary experiments indicated that the LD₅₀ values of wild-type and *Mrp3*^{-/-} mice were similar and fell between 125 and 250 mg/kg (data not shown). When this dosage range was examined in detail, significant differences in the chemosensitivity of wild-type and *Mrp3*^{-/-} mice were not observed (Table 2). The estimated LD₅₀ for both groups of mice was ~193 mg/kg body weight.

Damage to hematopoietic tissue is a result of the toxicity of etoposide. Therefore, white blood cell counts were analyzed in the two groups of treated mice. In accordance with the results described above, the effects of this agent on white blood cell counts of wild-type and *Mrp3*^{-/-} mice were indistinguishable with respect to the time and depth of white blood cell count depression and the time until recovery (Fig. 2).

Analysis of the in Vitro Drug Resistance Activity of Mrp3. Although the drug-resistance activity of human MRP3 has been determined, the activity of the rodent pump has not been reported. To confirm that the results of the chemosensitivity experiments were not attributable to spe-

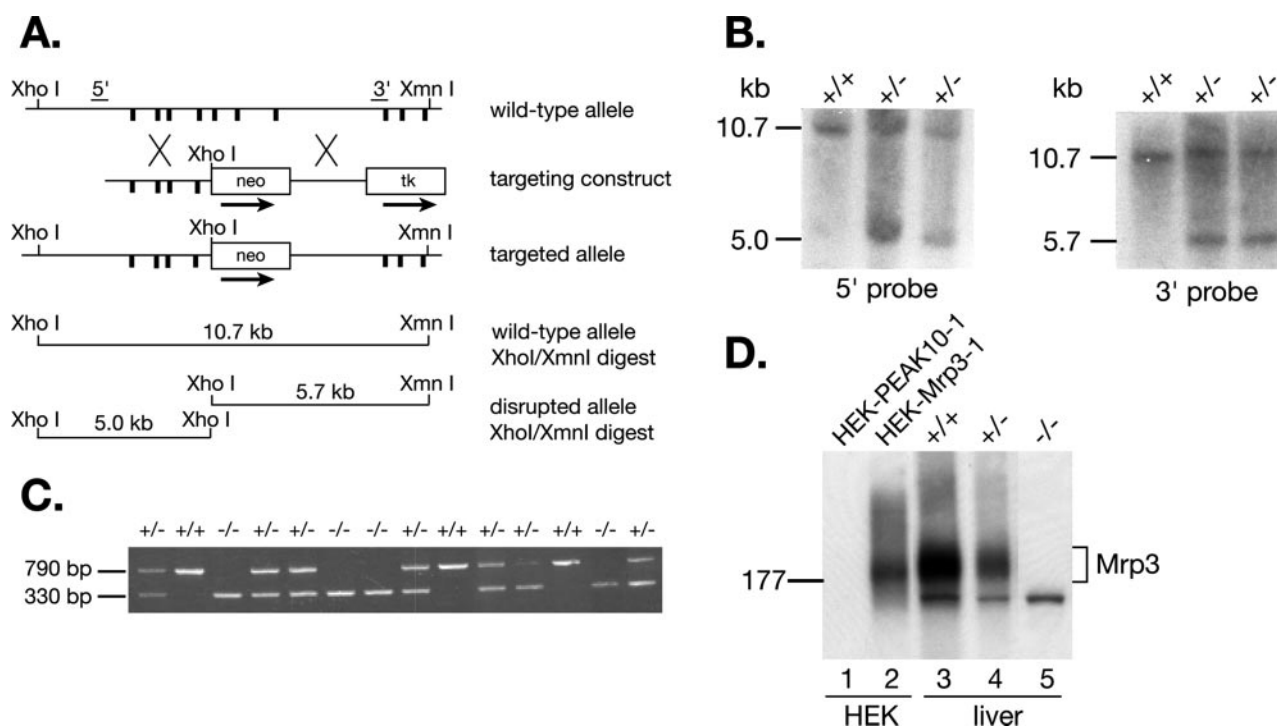


Fig. 1. Targeted disruption of the *Mrp3* gene in mice. **A**, schematic showing a portion of the 5' end of the *Mrp3* gene, the targeting construct, the targeted allele, predicted endonuclease restriction products, and location of 5' and 3' probes used in Southern blot analysis. The neomycin cassette (neo) is designed to replace three exons of the *Mrp3* locus (corresponding to nucleotides 613–995 of the *Mrp3* coding region). Splicing around these three exons is predicted to result in a frame shift in the coding sequence. Proper integration introduces a Xho I site. The predicted products of Xho I/Xmn I digestion in the wild-type and targeted alleles are shown. The construct also contains a thymidine kinase gene cassette used in the positive/negative selection procedure. **B**, Southern blot analysis of ES cells with 5' (left) and 3' (right) probes. The expected wild-type and targeted fragments are shown for two ES clones in which targeting was successful. **C**, three-primer PCR analysis of tail DNA prepared from the progeny of crosses between *Mrp3*^{+/-} mice. A forward primer common to both wild-type and targeted alleles and reverse primers derived from the targeted portion of the wild-type allele and the 5' region of the neomycin cassette were used. The wild-type allele and targeted allele generate 790- and 330-bp products, respectively. **D**, immunoblot analysis of Mrp3 in wild-type, heterozygous, and *Mrp3*-null mice. Proteins were resolved by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose filters. Mrp3 was detected by immunoblotting with anti-Mrp3 polyclonal antibody. Lane 1, HEK293 cells transfected with parental plasmid (HEK-PEAK10-1; 2 μ g of total protein); lane 2, HEK293 cells transfected with Mrp3-expression vector (HEK-Mrp3-1; 2 μ g of total protein). Lanes 3 to 5, crude membranes (20 μ g) prepared from liver tissue of wild-type, *Mrp3*^{+/-}, and *Mrp3*^{-/-} mice, respectively. The Mrp3 band is indicated by the bracket. A nonspecific protein band is also present in the liver samples.

cies-specific differences in resistance properties, the Mrp3 cDNA was isolated [Mrp3 protein was 81% identical with human MRP3 (Belinsky et al., 1998) and 92% identical with rat Mrp3 (NM080581)] and expressed in HEK293 cells. HEK-Mrp3-1 and HEK-Mrp3-2, two Mrp3-transfectants (Fig. 1D, lane 2; data not shown), exhibited 2.5- and 2.0-fold resistance toward etoposide, respectively, compared with parental vector-transfected cells ($p < 0.05$; data not shown). This activity was comparable with that of human MRP3 expressed in the same cellular background, for which we previously reported 3- to 4-fold resistance to this agent (Zeng et al., 1999).

Analysis of Liver Function in Bile Duct-Ligated $Mrp3^{-/-}$ Mice. To determine whether Mrp3 is able to protect cholestatic liver by functioning as a basolateral export system for bile acids and other conjugates, the effects of bile duct ligation in wild-type and $Mrp3^{-/-}$ mice were analyzed. Bile acids and glucuronides, including bilirubin glucuronide, are established substrates of MRP3 (Hirohashi et al., 1999, 2000; Zeng et al., 2000; Lee et al., 2004). Therefore, levels of serum bilirubin glucuronide, a compound that is formed in the liver by glucuronidation, and of liver and serum bile acids were analyzed. The $Mrp3$ null mice exhibited abnormalities in each of these parameters that were consistent with the proposed hepatic basolateral export function.

Cholestasis was achieved after 3 days of bile duct ligation, as indicated by marked elevations in liver bile acids in both bile duct-ligated wild-type and $Mrp3$ null mice by comparison with their respective sham-ligated controls (Fig. 3A). However, under these conditions, liver bile acid levels in the bile duct-ligated $Mrp3^{-/-}$ mice were elevated 1.5-fold in comparison with the bile duct-ligated wild-type mice ($p = 0.0074$). Under non-bile duct ligation (i.e., sham surgery) conditions, liver bile acids were also elevated in $Mrp3^{-/-}$ mice compared with wild-type mice (Fig. 3A), and serum bile acids were depressed in bile duct-ligated $Mrp3^{-/-}$ mice compared with bile duct-ligated control mice (Fig. 3B), but these differences did not reach statistical significance. Serum levels of conjugated bilirubin, which were too low to be measured under non-bile duct ligation conditions, were significantly de-

pressed ($\sim 67\%$; $p = 0.0005$) in bile duct-ligated $Mrp3^{-/-}$ mice compared with bile duct-ligated wild-type animals (Fig. 3C). Comparable levels of necrosis, inflammation, and portal expansion were observed in the livers of the bile duct-ligated $Mrp3^{-/-}$ and corresponding wild-type mice (data not shown).

The effects of bile duct ligation on protein expression levels of Mrp3 (wild-type mice), as well as on the expression of other ATP-binding cassette transporters whose activities could impact liver parameters, were analyzed (Fig. 4). These pumps included Mrp2 and the bile salt export pump (Bsep), two canalicular transporters that efflux glucuronides and bile acids, respectively, and Mrp4, a sinusoidal pump that transports both of these types of compounds (Gerloff et al., 1998; Cui et al., 1999; Chen et al., 2001; Rius et al., 2003). Mrp3 levels were comparable in the sham and bile duct-ligated wild-type mice. Mrp2 levels were slightly depressed in both wild-type and $Mrp3^{-/-}$ bile duct-ligated mice compared with the respective sham-operated control mice, as expected (Paulusma et al., 2000), whereas expression levels of the bile salt export pump and Mrp4 were similar in the four groups of animals. No statistically significant differences were observed when protein levels were quantified and normalized to the actin control.

Analysis of Bile Flow in $Mrp3^{-/-}$ Mice. In view of the abundance of Mrp3 in liver (Fig. 4), measurements were

TABLE 2

Toxicity of etoposide phosphate in wild-type and Mrp3-deficient mice

Groups of male mice (aged 8–12 weeks) were treated with varying amounts of etoposide phosphate administered as a single intraperitoneal injection. Dose values shown are milligrams of etoposide phosphate per kilogram of body weight. The values represent the number of surviving animals per group 4 weeks after treatment.

Dose	Survival	
	$Mrp3^{+/+}$	$Mrp3^{-/-}$
125 mg/kg	6/6	6/6
150 mg/kg	13/13	11/13
175 mg/kg	10/14	11/14
200 mg/kg	7/18	7/18
225 mg/kg	6/17	4/17
250 mg/kg	0/5	0/5

TABLE 1

Chemical and hematological parameters of wild-type and $Mrp3^{-/-}$ mice

Blood and serum were analyzed for the indicated parameters. Groups consisted of three to five 10-week-old mice.

Parameter	Male Mice		Female Mice	
	$Mrp3^{+/+}$	$Mrp3^{-/-}$	$Mrp3^{+/+}$	$Mrp3^{-/-}$
Hemoglobin (g/dl)	15.5 \pm 0.4	17.0 \pm 0.6	17.2 \pm 1.0	14.6 \pm 3.7
Hematocrit (%)	49.6 \pm 5.2	55.2 \pm 3.7	51.6 \pm 3.3	46.0 \pm 8.0
White blood cells ($\times 10^3/\text{mm}^3$)	3.3 \pm 0.6	5.1 \pm 1.5	4.8 \pm 1.2	5.0 \pm 1.0
Red blood cells ($\times 10^6/\text{mm}^3$)	9.1 \pm 0.1	10.1 \pm 0.8	10.5 \pm 0.6	8.9 \pm 1.2
Glucose (mg/dl)	158 \pm 32	181 \pm 41	174 \pm 13	160 \pm 26
Urea nitrogen (mg/dl)	26 \pm 2	27 \pm 2	25 \pm 6	27 \pm 3
Creatinine (mg/dl)	0.2 \pm 0.1	0.3 \pm 0.2	0.5 \pm 0.1	0.3 \pm 0.1
Total protein (g/dl)	5.8 \pm 0.1	5.8 \pm 0.3	5.4 \pm 0.2	5.9 \pm 0.3
Albumin (g/dl)	2.6 \pm 0.1	3.2 \pm 0.3	3.2 \pm 0.1	3.4 \pm 0.2
Bilirubin, total (mg/dl)	0.2 \pm 0.1	0.2 \pm 0.1	0.3 \pm 0.1	0.3 \pm 0.1
Alkaline phosphatase (U/l)	75 \pm 63	132 \pm 20	138 \pm 10	152 \pm 24
Alanine aminotransferase (U/l)	37 \pm 14	62 \pm 49	52 \pm 16	33 \pm 25
Aspartate aminotransferase (U/l)	151 \pm 39	229 \pm 116	100 \pm 45	184 \pm 141
Cholesterol (mg/dl)	99 \pm 12	123 \pm 7	80 \pm 7	118 \pm 7
Calcium (mg/dl)	9.4 \pm 1.0	9.7 \pm 0.6	9.4 \pm 0.3	9.7 \pm 0.4
Phosphorus (mg/dl)	7.7 \pm 2.1	8.9 \pm 2.6	7.3 \pm 0.9	7.8 \pm 1.4
Sodium (mEq/l)	160 \pm 5	156 \pm 5	148 \pm 1	157 \pm 7
Potassium (mEq/l)	7.7 \pm 0.7	7.8 \pm 1.2	6.8 \pm 0.2	7.8 \pm 1.3
Chloride (mEq/l)	98 \pm 16	99 \pm 13	87 \pm 5	93 \pm 10
Globulin (g/dl)	3.2 \pm 0.4	2.6 \pm 0.2	2.3 \pm 0.2	2.5 \pm 0.1

made to determine whether deficiency of Mrp3 affects bile flow. Differences in bile flow were not detected. Basal bile flow rates were 6.0 ± 1.4 and 6.7 ± 1.5 $\mu\text{l}/\text{min}/100$ g body weight ($p > 0.4$), and taurocholate-stimulated bile flow rates increased to 13.0 ± 2.5 and 13.1 ± 2.3 $\mu\text{l}/\text{min}/100$ g of body weight ($p > 0.9$) in wild-type and *Mrp3*^{-/-} mice, respectively.

Analysis of Bile Acid Pool Size and Fecal Excretion in *Mrp3*^{-/-} Mice. It has been proposed that Mrp3 functions as an intestinal basolateral transporter in the enterohepatic circulation of bile acids. To test that hypothesis, bile acid metabolism was examined in the wild-type and *Mrp3*^{-/-} mice. As shown in Fig. 5A, fecal bile acid secretion was not significantly different in the wild-type and *Mrp3*^{-/-} mice (11.40 ± 2.85 versus 12.96 ± 3.96 ; $p > 0.33$). Further analysis revealed that the bile acid pool size and composition were also similar in wild-type and *Mrp3*^{-/-} mice (Fig. 5B). The mass and percentage composition values for all of the major bile acid species, including taurocholate, tauro- β -muricholate, taurodeoxycholate, tauroursodeoxycholate, and taurochenodeoxycholate, were similar for the two genotypes, with taurocholate and tauro- β -muricholate accounting for $\sim 80\%$ of the pool. A crude fractional turnover rate can be calculated, because the daily rate of fecal bile acid excretion and bile acid pool size were measured in the same animals. The bile acid fractional turnover rate (daily fecal excretion per pool size) was also similar in the two genotypes (Fig. 5C; wild-type, 0.27 ± 0.09 versus *Mrp3*^{-/-}, 0.33 ± 0.08 ; $p > 0.15$).

Analysis of Intestinal Bile Acid Transporter Gene Expression in *Mrp3*^{-/-} Mice. To confirm that the lack of impairment in intestinal bile acid absorption by *Mrp3*^{-/-} mice is not attributable to compensatory alterations in the expression of other intestinal basolateral bile acid transporters, mRNA expression of Asbt, which is responsible for the uptake of bile acids across the brush border, the recently described Ost α/β , which is a candidate basolateral bile acid transporter (Dawson et al., 2005), and Ibabp, an established

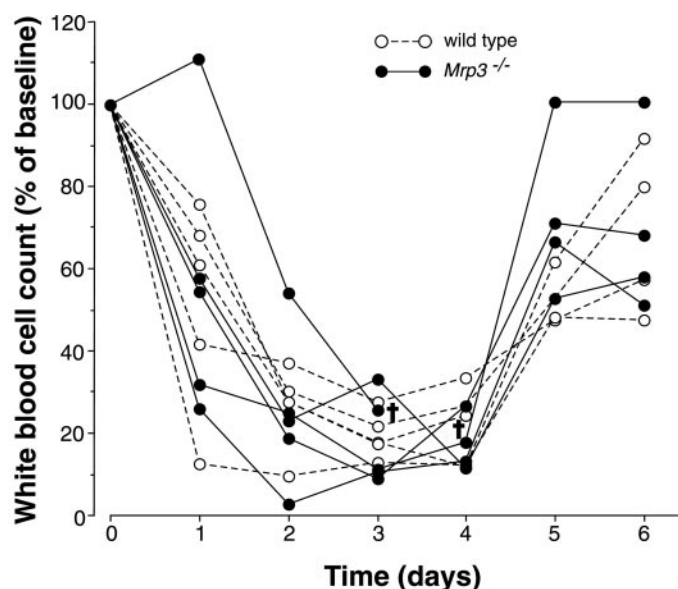


Fig. 2. Analysis of white blood cell counts in *Mrp3*^{-/-} and wild-type mice treated with etoposide. *Mrp3*^{-/-} and wild-type mice ($n = 5$) were treated with a single intraperitoneal injection of 150 mg/kg etoposide phosphate, and white blood cell counts were measured daily over the next 6 days. In the experiment shown, one *Mrp3*^{-/-} mouse died on day 3 and one wild-type mouse died on day 4 after injection (deaths are indicated by crosses). ●, *Mrp3*^{-/-} mice; ○, wild-type mice.

farnesoid X receptor target gene and an indirect indicator of bile acid flux through the ileum (Grober et al., 1999; Chen et al., 2003), were analyzed in wild-type and *Mrp3*^{-/-} mice. Asbt, Ost α , Ost β , and Ibabp mRNA expression did not significantly differ between the wild-type and *Mrp3* null animals (data not shown).

Discussion

In the present study, *Mrp3*^{-/-} mice generated by targeted disruption were analyzed to determine the in vivo functions of the transporter. Although human MRP3 is an established cellular resistance factor for etoposide, our experiments did not reveal enhanced chemosensitivity of *Mrp3*^{-/-} mice. In addition, *Mrp3*^{-/-} mice did not exhibit increased hematopoi-

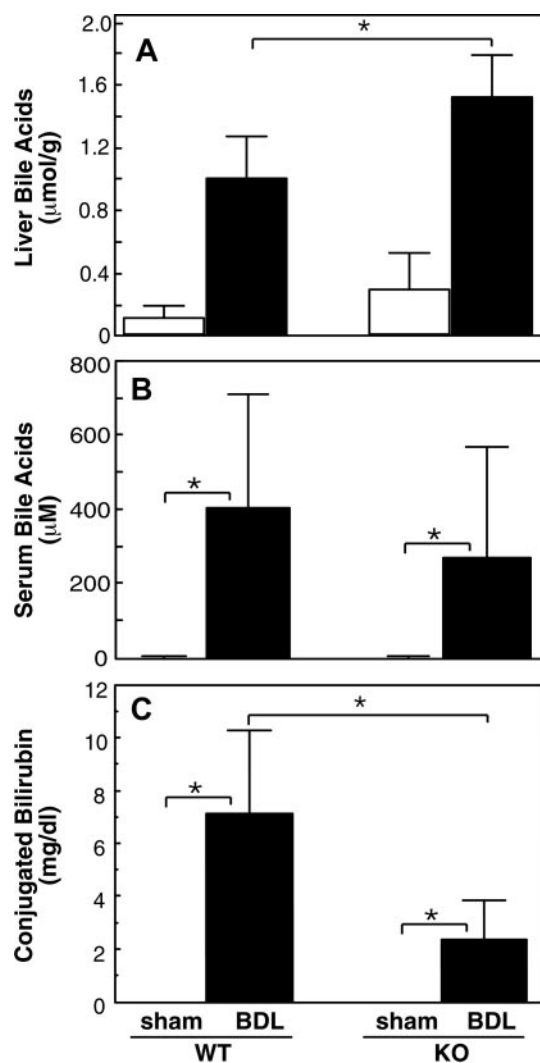


Fig. 3. Analysis of liver and serum bile acids and serum bilirubin glucuronide in bile duct-ligated wild-type and *Mrp3*^{-/-} mice. Liver and serum bile acid and serum bilirubin glucuronide levels were analyzed in wild-type and *Mrp3*^{-/-} male animals after 3 days of bile duct ligation. A, total liver bile acids. *, $p = 0.0074$. B, serum bile acids. *, $p = 0.0009$; C, serum-conjugated bilirubin. *, $p = 0.0005$ for wild-type bile duct-ligated versus knockout bile duct-ligated mice, $p = 0.0007$ for wild-type sham versus wild-type bile duct-ligated mice, $p = 0.0017$ knockout sham versus knockout bile duct-ligated mice. (See *Materials and Methods* for statistical analyses). In A, $n = 8$ to 9 for bile duct-ligated animals and $n = 3$ for sham-ligated animals; in B and C, $n = 12$ to 13 for bile duct-ligated animals and $n = 6$ for sham-ligated animals. BDL, bile duct-ligated; WT, wild-type mice; KO, *Mrp3*^{-/-} mice.

etic damage, a major toxicity of etoposide. These findings contrast with *Mrp1*^{-/-} mice, which exhibit etoposide sensitivity associated with increased hematopoietic damage (Lorico et al., 1997; Wijnholds et al., 1997). The experiments showing that ectopic expression of Mrp3 in HEK293 cells is able to confer 2.0- to 2.5-fold resistance to etoposide, a potency that is only ~2-fold lower than that reported for Mrp1 expressed in HEK293 cells (Stride et al., 1997), indicate that the lack of chemosensitivity is not attributable to the inability of the murine protein to transport etoposide and instead suggest that the presence of redundant functions mask the contribution of Mrp3 to protecting normal tissues. A report showing that MRP1 is expressed at higher levels than MRP3 in human hematopoietic cells suggests that relatively low levels of Mrp3 expression in chemosensitive cells may be an additional factor that bears on the lack of sensitization in *Mrp3*^{-/-} mice (Laupeze et al., 2001). It is worth mentioning that our experiments on the impact of the pump on the in vivo chemosensitivity of normal tissues do not preclude the possibility that MRP3 may function as a resistance factor in tumors, a notion that is supported by studies showing that MRP3 is expressed in several cancers, and a report in which a correlation between MRP3 expression and clinical outcome was found (Nies et al., 2001; Steinbach et al., 2003).

A major finding of our study is that Mrp3 functions to protect cholestatic hepatocytes from endobiotics. Serum levels of hepatic constituents such as bile acids and bilirubin glucuronide increase in cholestatic conditions, indicating that hepatocytes are able to deploy basolateral systems to efflux these compounds into sinusoidal blood. Several observations suggested that MRP3 functions as one of these systems. Conjugated bile acids, such as glycocholate in the case of human MRP3 and glycocholate and taurocholate in the case of the rat protein, are established substrates of MRP3 (Hirohashi et al., 2000; Zeng et al., 2000), and MRP3 is induced in sinusoidal membranes of hepatocytes in humans with Dubin-Johnson syndrome, a disorder caused by hereditary deficiency of MRP2 and whose principal manifestation is jaundice (Konig et al., 1999). Induction of Mrp3 has also been reported in rats with experimentally induced obstructive jaundice, in *Bsep*^{-/-} mice fed a diet supplemented with cholic acid, and in rat strains that are deficient in Mrp2, the latter of which facilitated the isolation of the rat Mrp3 cDNA from liver (Hirohashi et al., 1998; Donner and Keppler, 2001;

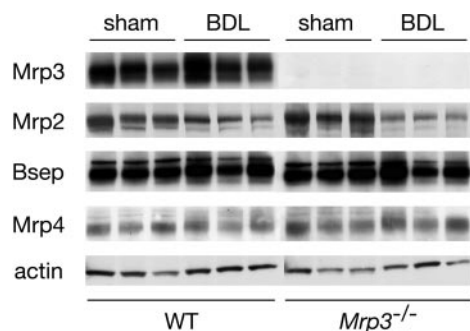


Fig. 4. Expression of Mrp3, Mrp2, Bsep, and Mrp4 in liver of sham or bile duct-ligated wild-type and *Mrp3*^{-/-} mice. Crude membranes (50 µg) prepared from livers of *Mrp3*^{-/-} or wild-type mice 3 days after sham surgery or bile duct ligation were resolved by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose filters. Mrp3, Mrp2, Bsep, and Mrp4 were detected as described under *Materials and Methods*.

Soroka et al., 2001; Wang et al., 2003). In addition, the induction of MRP3 is mediated by transcriptional pathways associated with bile acids. Bile acids and CAR activators induce the expression of Mrp3 in vivo, and induction of MRP3

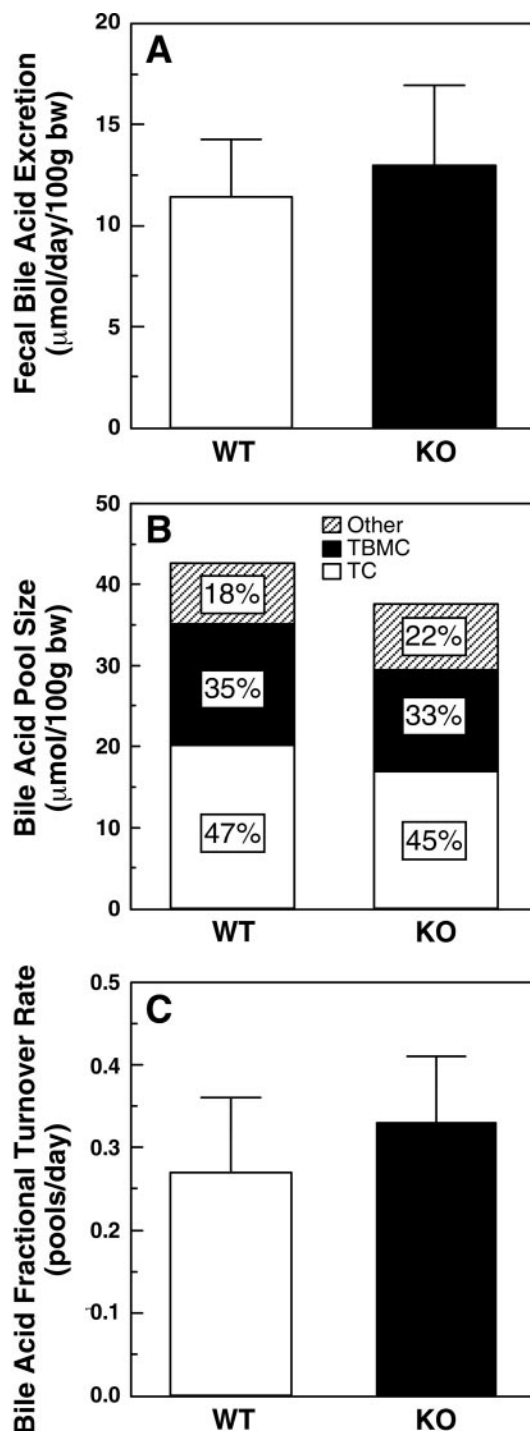


Fig. 5. Analysis of bile acid pool size and fecal excretion in *Mrp3*^{-/-} mice. Mean values ± S.D. are shown. A, fecal bile acid excretion was measured in male mice as described under *Materials and Methods* ($n = 10$). B, the mass of bile acid in the enterohepatic circulation was determined by extraction, purification, and quantification using high-performance liquid chromatography ($n = 8$). C, the bile acid fractional turnover rate was calculated from the data in A and B. Fecal bile acid excretion, total bile acid pool size and bile acid pool composition, and bile acid fractional turnover rate in *Mrp3*^{-/-} mice were not significantly different from wild-type (WT) mice. TBMC, tauro-β-muricholate; TC, taurocholate; KO, *Mrp3*^{-/-} mice.

in Caco2 cells was attributed to FTF-like elements in the MRP3 promoter (Inokuchi et al., 2001; Xiong et al., 2002; Zollner et al., 2003). Furthermore, *Tnfr*^{-/-} mice fail to induce Mrp3, and induction is impaired in *Car*^{-/-} mice (Bohan et al., 2003; Zhang et al., 2004). Although these observations suggested that MRP3 may be an alternate pathway of bile-acid export from hepatocytes, a direct analysis of MRP3 function in vivo has not been reported. Here, we provide direct evidence that Mrp3 protects cholestatic hepatocytes from bile acids by showing that *Mrp3*^{-/-} mice subjected to bile duct ligation have elevated levels of liver bile acids. Moreover, the finding that serum bilirubin levels are elevated in *Mrp3*^{-/-} mice indicates that the protective function of Mrp3 extends to glucuronides as well. These differences in liver parameters were detectable in our experiments after only 3 days of bile duct ligation, a relatively short time frame that was selected so as to minimize the impact of progressive adaptive changes in liver homeostatic mechanisms on the phenotype of *Mrp3*^{-/-} mice. It is noteworthy that this short time frame may explain why differences in liver pathology were not observed between wild-type and *Mrp3*^{-/-} mice. In connection with our assessment of Mrp3 function in liver, it should be mentioned that in the absence of induction, Mrp3 expression in liver is very low or undetectable in rats and humans (Donner and Keppler, 2001; Soroka et al., 2001), whereas in mouse, Mrp3 expression is readily detectable in liver (Fig. 3). Hence, our data suggest that Mrp3-mediated protection of cholestatic mouse liver is afforded by the basal levels of murine Mrp3 expression. In addition, it is important to bear in mind that Mrp3 is likely to represent only one of the basolateral membrane systems that are deployed to protect cholestatic hepatocytes. Mrp4 may be another system, as suggested by reports showing that human MRP4 is able to transport glucuronides and bile acids and that mouse Mrp4 is localized to sinusoidal hepatocyte membranes and induced 7 to 14 days after bile duct ligation (Chen et al., 2001; Rius et al., 2003; Denk et al., 2004).

In contrast to apical transport across the intestinal apical brush-border membrane, there is limited information regarding the mechanisms and transporters responsible for bile acid efflux across the basolateral membrane (Shneider, 2001). Previous in vitro studies using rat ileal basolateral membrane vesicles have demonstrated bile acid anion exchange (Weinberg et al., 1986) as well as ATP-dependent bile acid transport activity (Shoji et al., 2004). However, the relative in vivo contribution of these transport activities to basolateral bile acid export is unknown. Although Mrp3 was considered to be a candidate for the intestinal basolateral bile acid transporter on the basis of its intestinal expression, localization to the basolateral surface, and ability to transport various bile acid species (Rost et al., 1999; Hirohashi et al., 2000; Zeng et al., 2000; Scheffer et al., 2002), we found that disruption of *Mrp3* did not result in significant changes in intestinal bile acid absorption, as indicated by the lack of significant changes in fecal bile acid excretion, bile acid pool size, or the calculated fractional turnover rate for bile acids. These results indicate that Mrp3 is not essential for efficient intestinal absorption of bile acids and that additional transporters must be present to mediate basolateral bile acid efflux in ileum and other Asbt-expressing tissues. One such carrier is the recently described organic solute carrier (OST) α/β (OST α/β) (Seward et al., 2003). In the mouse, Ost α/β

fulfills many of the criteria for a dedicated basolateral bile acid transporter, including tissue expression that closely parallels that of Asbt, basolateral membrane localization, positive regulation by bile acids, and the ability to efflux all the major species of bile acids (Dawson et al., 2005). Although it is possible that Mrp3 functions in conjunction with Ost α/β to mediate intestinal basolateral bile acid transporter, the lack of any compensatory increase in Ost α/β expression suggests that Mrp3 contributes little to the intestinal reclamation of bile acids.

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Address correspondence to: Dr. Gary D. Kruh, Medical Science Division, Fox Chase Cancer Center, 333 Cottman Avenue, Philadelphia, PA 19111.
E-mail: Gary.Kruh@fccc.edu