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CHARACTERIZATION OF TRANSPORT PROTEIN EXPRESSION IN MULTIDRUG RESISTANCE-ASSOCIATED PROTEIN (Mrp) 2-DEFICIENT RATS

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Abbreviations: Multidrug resistance-associated protein, Mrp; EHBR, Eisai hyperbilirubinemic rats; Oatp, organic anion transporting polypeptide; CYP, cytochrome P450; UGT, UDP-glucuronosyl transferase; PMSF, phenylmethanesulfonyl fluoride; P-gp, P-glycoprotein; BCRP, breast cancer resistance protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Ntcp, sodium-dependent taurocholate cotransporting polypeptide; Bsep, bile salt export pump; Ibat, ileal bile acid transporter; SDS, sodium

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dodecyl sulfate; TBS-T, Tris buffered saline with Tween-20; PBS, phosphate buffered saline; BSA, bovine serum albumin; MW, molecular weight.

ABSTRACT

Multidrug resistance-associated protein (Mrp) 2-deficient TR⁻ rats, together with their transport-competent Wistar counterparts (wild-type), have been used to examine the contribution of Mrp2 to drug disposition. However, little is known about potential variation in expression of other transport proteins between TR⁻ and wild-type rats, or whether these differences are tissue specific. Sections of liver, kidney, brain, duodenum, jejunum, ileum, and colon were obtained from male TR⁻ and wild-type Wistar rats. Samples were homogenized in protease inhibitor cocktail and ultracentrifuged at 100,000g for 30 min to obtain membrane fractions. Mrp2, Mrp3, Mrp4, P-glycoprotein (P-gp), sodium-dependent taurocholate cotransporting polypeptide (Ntcp), organic anion transporting polypeptide (Oatp) 1a1 and 1a4, bile salt export pump (Bsep), breast cancer resistance protein (Bcrp), ileal bile acid transporter (Ibat), UDP-glucuronosyl transferase (UGT1a), glyceraldehyde-3-phosphate dehydrogenase, and β -actin protein expression were determined by Western blot. Mrp3 was significantly up-regulated in the liver (~6-fold) and kidney (~3.5-fold) of TR⁻ rats compared to wild-type controls. Likewise, the expression of UGT1a enzymes was increased in the liver and kidney of TR⁻ rats, by ~3.5-fold and ~5.5-fold, respectively. Interestingly, Mrp3 expression was down-regulated in the small intestine of TR⁻ rats, but expression was similar to wild-type in the colon. Mrp4 was expressed to varying extents along the intestine. Expression of some transport proteins and UGT1a enzymes differ significantly between TR⁻ and wild-type rats. Therefore, altered drug disposition in TR⁻ rats must be interpreted cautiously because up- or down-regulation of other transport proteins may play compensatory roles in the presence of Mrp2 deficiency.

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Multidrug resistance-associated protein (Mrp) 2 is the primary transport protein mediating excretion of organic anions, including endogenous and exogenous drug conjugates, across canalicular membranes from the hepatocytes into bile. Mrp2 also is expressed significantly on the brush border membrane of the small intestine and proximal tubule of the kidney, and therefore plays an important role in drug disposition (Borst et al., 1999). Transport-deficient (TR⁻) rats, naturally occurring mutants of the Wistar rat that are Mrp2-deficient (Jansen et al., 1985; Paulusma et al., 1996), have been used to elucidate the role of Mrp2 in drug disposition (Dietrich et al., 2001; Xiong et al., 2002; Chen et al., 2003a; Potschka et al., 2003). A similar mutation also exists in the Sprague-Dawley strain of rats, where the Mrp2-deficient mutant is referred to as Eisai hyperbilirubinemic rats, or EHBR (Hirohashi et al., 1998).

TR⁻ and EHBR rats provide an animal model for human Mrp2 deficiency, Dubin-Johnson syndrome, a condition characterized by mild chronic conjugated hyperbilirubinemia (Dubin and Johnson, 1954; Kartenbeck et al., 1996). Mrp2-deficient rats also exhibit elevated serum bile acid concentrations, alterations in bile composition, and reduced bile flow (Jansen et al., 1985). A compensatory hepatic basolateral organic anion transport mechanism, Mrp3, is induced in TR⁻ rats, EHBRs, and in patients with Dubin-Johnson syndrome (König et al., 1999; Ortiz et al., 1999; Akita et al., 2001). Recently, Mrp3 induction on the basolateral membrane of renal proximal tubular cells also was observed in EHBRs (Kuroda et al., 2004). In addition, Kuroda et al. (2004) demonstrated that hepatic organic anion transporting polypeptide (Oatp) 1a1 and 1a4 protein levels were reduced by ~50% in EHBRs. An increase in bilirubin glucuronidation and cytochrome

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P450 (CYP) 2a1 activity has been noted in EHBRs, while the CYP 2b1/2 and 3a1/2 isoforms appear to be decreased, and CYP 1a2, 2d, and 2e1 remain equivalent (Ohmori et al., 1991; Jäger et al., 1998). Jäger et al (1997) reported that UGT activity was equivalent between TR⁻ and wild-type rat livers. These observations demonstrate that tissues other than the liver, and other transport proteins (such as those responsible for bile acid transport) and enzymes may be altered in Mrp2-deficient animals and humans.

Therefore the objective of the current study was to characterize expression of several key transport proteins of endogenous and exogenous compounds in the liver, kidney, brain, and intestine of TR⁻ and wild-type Wistar rats. The transport proteins examined included those primarily involved in the transport of organic anions [Mrp2, Mrp3, and Mrp4; Oatp1a1 and Oatp1a4; and breast cancer resistance protein (Bcrp)], organic cations [P-glycoprotein (P-gp)], and bile salts [sodium-dependent taurocholate cotransporting polypeptide (Ntcp); bile salt export pump (Bsep); and ileal bile acid transporter (Ibat)]. More detailed information on the structure and function of these transport proteins in various tissues can be found in recent reviews (Kim, 2003; Trauner and Boyer, 2003; Chan et al., 2004; Chandra and Brouwer, 2004; Kushura and Sugiyama, 2004; Lee and Kim, 2004; Leslie et al., 2005). Altered expression of UGT1a in several tissues also was examined as a function of Mrp2 deficiency.

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METHODS

Materials and Antibodies

Phenylmethanesulfonyl fluoride (PMSF), iodoacetamide, Tween-20, and beta-mercaptoethanol were obtained from Sigma Chemical Co. (St. Louis, MO). Complete Protease Inhibitor Cocktail was purchased from Roche Diagnostics (Indianapolis, IN) and molecular weight marker (SeeBlue Plus2) was obtained from Invitrogen (Carlsbad, CA). Primary antibodies were obtained from the following commercial sources: P-glycoprotein (P-gp, Ab-1) and breast cancer resistance protein (BCRP, Ab-1 from clone BXP-21) were obtained from Oncogene (San Diego, CA), Mrp2 (M₂-III-6) from Alexis Biochemicals (San Diego, CA), organic anion transporting polypeptide (Oatp) 1a4 (AB3572P) and β -actin (MAB1501R) from Chemicon International (Temecula, CA), UDP-glucuronosyl transferase (UGT) 1a (WB-UGT1A) from BD Gentest (Woburn, MA), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 6C5) from Abcam (Cambridge, MA). Primary antibodies to Oatp1a1 (K10), sodium-dependent taurocholate cotransporting polypeptide (Ntcp, K4), and bile salt export pump (Bsep, K12) were generously provided by Dr. Peter Meier. Primary antibodies to Mrp3 and ileal bile acid transporter (Ibat) were kind gifts from Dr. Yuichi Sugiyama and Dr. Paul Dawson, respectively. All other chemicals were readily available from commercial sources and were of analytical grade or higher. Water was obtained from a Barnstead NANOpure Infinity water purification system (Dubuque, IA).

Tissue Procurement and Sample Preparation

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All animal procedures were approved by the Institutional Animal Care and Use Committee (University of North Carolina at Chapel Hill). Male wild-type Wistar rats (275-325 g; Charles River Laboratories, Raleigh, NC) and male TR⁻ Wistar rats (275-325 g; in-house breeding colony obtained from Dr. Mary Vore, University of Kentucky, Lexington, KY). Rats were anaesthetized with ketamine and xylazine (60 and 12 mg/kg, respectively) by intraperitoneal injection, and after midline incision, the portal vein was isolated and cannulated. The liver was perfused at 30 ml/min with filtered (0.45 μ m) ice-cold 0.9% NaCl until the liver lobes were devoid of blood. The liver was then removed and immediately immersed in liquid nitrogen. The kidneys were removed and the outer capsule discarded, the rat was decapitated, and the whole brain was isolated. Both the brain and kidneys were placed immediately in liquid nitrogen once they were removed.

Intestinal tissue samples were obtained from separate rats due to the observed instability of transport proteins obtained from the upper regions of the rat intestine. Rats were anaesthetized as described above and the whole small intestine from the pylorus to the ileo-cecal junction was removed. The intestine was sectioned into duodenum (uppermost 10 cm), jejunum (a 10 cm section starting approximately 25 cm from the pylorus), and terminal ileum (a 10 cm section immediately proximal to the ileo-cecal junction). The lumen of each section was flushed with freshly prepared 1 mM PMSF in 0.9% NaCl before placement in a tube of the same solution. The colon was removed, hard fecal matter was removed by gentle rolling, and the lumen was flushed with PMSF solution and placed in a tube of the same solution. The four intestinal segments were everted, and

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mucosal scrapings (~400 mg of tissue per intestinal segment) were obtained using a glass slide and frozen in liquid nitrogen.

Samples (300-400 mg of tissue) of liver, kidney, brain, and scrapings from the duodenum, jejunum, ileum, and colon were thawed in a polypropylene tube under 2 ml of 1X Complete Protease Inhibitor cocktail in TrisHCl (0.1 M, pH 7.4). Samples were homogenized on ice for two 30 sec periods at 30,000 rpm (Tissue-Tearor, Biospec Products Inc, Racine, WI) and then sonicated for 30 sec using a probe Sonic Dismembrator (Model 100, Fisher Scientific, Pittsburgh, PA). Samples were centrifuged at 1500g at 4°C for 10 min, the supernatant was transferred to an ultracentrifuge tube, and centrifuged at 100,000g (XL-80, Beckman-Coulter, Palo Alto, CA). The supernatant was discarded, and the pellet was resuspended in 200 µl of Protease Inhibitor Cocktail solution by homogenization and sonication (described above) and stored at -80°C until Western Blot analysis

Western Blot Procedure

Homogenates were suitably diluted to ~5 mg/ml total protein using a bicinchoninic acid protein assay method as per manufacturer's instructions (Pierce, Rockford, IL). Dilute homogenates were re-assayed and samples prepared for Western Blot as per manufacturer's instructions (Invitrogen, Carlsbad, CA) at a concentration of 50 µg/30 µl, except for samples from the colon, which were prepared at 25 µg/30 µl. Samples prepared specifically for analysis of Ibat were diluted 1:3 with an iodoacetamide solution [iodoacetamide, 1 M; Tris, 500 mM (pH 8.7); glycerol, 10%; sodium dodecyl sulfate

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(SDS), 2.5%] and incubated for 30 min to minimize disulfide bond reformation and maintain Ibat in its monomeric form. Molecular weight marker was loaded in the first lane of every gel. Proteins were separated using 4-12 % Bis-Tris PAGE under denaturing conditions at 150 V for 120 min, and then transferred to polyvinylidene fluoride membranes (Immobilon-P, Millipore, Bedford, MA) at 30 V for 90 min as per manufacturer's instructions (Invitrogen, Carlsbad, CA). Membranes were blocked with 5% skim milk powder (Bio-Rad, Hercules, CA) in Tris buffered saline (pH 7.4) with 0.3% Tween-20 (TBS-T) for 30 min at room temperature. TBS-T contained 0.1% Tween-20 for blots from intestinal segments.

Membranes were incubated with primary antibody solution (1:1,000-2,000 in TBS-T) for 2 h, rinsed 3 times for 10 min each in TBS-T, incubated in an appropriate (anti-rabbit or anti-mouse) horseradish peroxidase conjugated secondary antibody (1:3,000-10,000 in TBS-T) for 2 h, and again rinsed 3 times for 10 min each in TBS-T. Bands were visualized using enhanced chemiluminescence reagents (SuperSignal West Dura, Pierce, Rockford, IL) and a VersaDoc instrument (Bio-Rad, Hercules, CA).

Membranes commonly were stripped of primary and secondary antibodies, allowing reprobing of the same membrane, by incubation in a solution of beta-mercaptoethanol (0.1 M), SDS (70 mM), and TrisHCl (62 mM) at pH 6.8 for 30-40 min at 70°C. After stripping, membranes were rinsed in TBS-T and reblocked in 5% skim milk powder as described above.

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Immunohistochemistry

Intestinal tissues collected for immunohistochemical localization of Mrp4 were obtained as described above. Small segments of duodenum, jejunum, ileum, and colon were everted, fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) for 24 h, rinsed in purified water, and placed in 70% ethanol. Segments were embedded in paraffin and sectioned. Immunohistochemical staining was performed essentially as described previously (Assem et al., 2004). Briefly, deparafinized slides were treated with 3% hydrogen peroxide in water for 5 minutes. Slides were then incubated for 30 min at room temperature with Mrp4 antibody diluted in 1% BSA, washed 3 times with PBS/Tween-20 (0.05 %), and incubated with a polymer labeled anti-rabbit secondary antibody (Dako, Carpinteria, CA). Slides were counterstained with Mayer's Hemotoxylin (Sigma Chemical Co., St Louis, MO).

Data and Statistical Analysis

Integrated optical densities for bands of interest were determined using Quantity One software (Bio-Rad, Hercules, CA), and were normalized to β -actin (or GAPDH when blotting for Ibat) determined from the same membrane. Normalized data from n=3 wild-type and TR⁻ rats were compared using the Student's *t*-test at an $\alpha=0.05$ level of significance. All plots illustrate the mean and standard deviation from 3 wild-type and 3 TR⁻ rats.

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RESULTS

Liver

Western Blots prepared from wild-type and Mrp2-deficient TR⁻ rat liver homogenates (n=3) are shown in Figure 1. Bands of interest were quantified using integrated optical density and normalized to β -actin (Figure 2). Mrp3 protein expression was significantly higher (~6-fold, $P<0.01$) in TR⁻ rats compared to wild-type. This increase was similar to that described previously in EHBRs (Ogawa et al., 2000; Akita et al., 2001). Interestingly, expression of members of the UGT1a family was increased ~3.5-fold in TR⁻ rats compared to wild-type. No other transport proteins studied were significantly different between wild-type and TR⁻ rats; however, Ntcp was reduced by almost 60% in TR⁻ rat livers ($P=0.06$). Although Bsep expression also appeared to be reduced in TR⁻ rats livers (Figure 1), when the data were normalized to β -actin, only a 30% reduction in Bsep expression was noted ($P>0.3$).

Kidney

Representative Western Blots of Mrp3 and UGT1a expression in wild-type and TR⁻ rat kidney homogenates is shown in Figure 1. Expression of both Mrp3 and UGT1a was significantly increased in TR⁻ rat kidney, when compared to wild-type. Densitometric analysis for this blot and others (data not shown) are shown in Figure 2B. Mrp3 and UGT1a expression were ~3.5-fold and ~5.5-fold higher, respectively, in kidneys from TR⁻ compared to wild-type rats ($P<0.001$). The expression of other transport proteins examined did not differ significantly between wild-type and TR⁻ rat kidneys.

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Brain

Densitometric analysis of Western Blots (data not shown) prepared from Wistar and TR⁻ whole rat brain homogenates are plotted in Figure 2C. No significant differences in transport protein expression between Wistar control and TR⁻ rat brains were noted. While Bcrp protein expression in rat brain capillary fractions was reported recently (Hori et al., 2004), a signal only slightly stronger than background staining was detected using the current antibody with whole brain homogenate.

Intestine

A Western Blot of mucosal scrapings from intestinal segments of a representative Wistar and TR⁻ rat is shown in Figure 3A. Mrp2 expression in Wistar control rats decreased along the length of the small intestine, and no detectable expression of Mrp2 was found in the colon. Conversely, Mrp3 expression appeared to increase distally, with high (relative) expression in the colon. Interestingly, Mrp3 expression was decreased in the small intestine of TR⁻ rats when compared to Wistar controls. These results were confirmed from blots of n=3 samples each from Wistar control and TR⁻ rats (data plotted in Figure 3B), where Mrp3 expression was decreased by 55% ($P>0.05$), 81% ($P<0.05$), and 71% ($P<0.05$), in the duodenum, jejunum, and ileum, respectively, in TR⁻ compared to Wistar control rats. In the colon, Mrp3 and Mrp4 expression was relatively high (Figure 3A), with no significant difference between wild-type and TR⁻ rats detected (Figure 3B). Mrp4 expression was low in the jejunum and ileum, with slightly higher expression in the duodenum, where Mrp4 appeared as a double band with approximate molecular weights of 160 and 170 KDa. P-gp also appeared as a double band in the

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ileum, and expression along the gastrointestinal tract was in the order colon>ileum>jejunum>duodenum (Figure 3A), consistent with previous reports (Brady et al., 2002). No significant difference in P-gp expression was observed between wild-type and TR⁻ rats (Figure 3B). Ibat expression in the terminal ileum also was assessed (blot not shown); expression was almost 2-fold higher in TR⁻ rats when compared to Wistar controls (Figure 3B), however this difference was not statistically significant (P=0.057). Expression of UGT1a was similar in all sections of the intestine, and no significant differences in expression were observed between wild-type and TR⁻ rats (Figure 3B).

Immunohistochemistry

Preliminary immunohistochemical localization of Mrp4 was conducted because this is the first study to detect Mrp4 protein in the rat intestine. Results of immunohistochemical staining of Mrp4 in the intestine are shown in Figure 4. Positive staining for Mrp4 in the small intestine appeared to be confined to the cytoplasm of enterocytes at the tips of villi. The staining was somewhat stronger in the basal portion of the cells, and individual strongly positive cells in the crypts appear to be neuroendocrine cells. No differences in staining patterns were apparent between small intestinal samples from wild-type and TR⁻ rats. Positive staining in the colon was confined strictly to the surface epithelium with no staining in the glands. The colon sample from the wild-type rat showed strong staining of surface epithelium while the TR⁻ sample had faint staining which was of similar intensity to the smooth muscle artifactual staining; however, Western Blot analysis from 3 separate rats suggested that the expression of Mrp4 in the colon was similar between wild-type and TR⁻ rats.

DISCUSSION

Mrp2-deficient rats (TR⁻ and EHBR) and their transport-competent counterparts serve as useful models to study the effects of Mrp2 on drug disposition, and provide a rodent model for human Dubin-Johnson Syndrome (Dubin and Johnson, 1954; Jansen et al., 1985). However, several other important biochemical differences exist between the mutant and wild-type rats that must be considered, and the current study was designed to characterize the differences in protein expression of several key transport proteins and UGT1a in multiple tissues from Wistar transport-competent and Mrp2-deficient (TR⁻) rats using semiquantitative Western Blot analysis.

As reported previously (Hirohashi et al., 1998; Ogawa et al., 2000; Akita et al., 2001), Mrp3 is significantly upregulated in the liver of EHBR and TR⁻ rats. The current studies confirm these findings; a significant (~6-fold) increase in Mrp3 protein expression was observed in TR⁻ relative to wild-type rats. Ntcp expression was decreased by 60% in TR⁻ rat livers, suggesting a compensatory mechanism to reduce already elevated hepatocyte bile acid concentrations (Jansen et al., 1985; Trauner and Boyer, 2003). Oatp1a1 and Oatp1a4 were not significantly altered in TR⁻ rats compared to Wistar controls, similar to a previous study in EHBRs (Akita et al.). The similar expression of these Oatps in control and TR⁻ rats may be related to the vital function that these promiscuous transporters play in the hepatic uptake of a variety of endogenous and exogenous compounds (Kim, 2003). These observations, however, are not consistent with a recent report demonstrating a ~50% reduction in Oatp1a1 and Oatp1a4 protein expression in livers from EHBRs compared to Sprague-Dawley controls (Kuroda et al., 2004).

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Possible reasons for this discrepancy between the current and previous studies in EHBR rats are not known (Akita et al., 2001).

Expression of the UGT1a family of drug conjugating enzymes (detected with a cross-reactive human UGT1A family antibody (Kessler et al., 2002)) was significantly upregulated in TR⁻ rats (~3.5-fold). While previous studies have demonstrated that an apparent functional increase in bilirubin glucuronidation is evident in EHBRs (Ohmori et al., 1991), other reports suggested similar UGT activity between wild-type and TR⁻ rat livers (Jäger et al., 1997). No direct evidence for an increase in UGTs at the protein level has been described prior to the findings presented here. Recently it was shown that Mrp3, and members of the UGT family, were both inducible by 1,7-phenanthroline (Wang et al., 2003), however, UGT1a1, the predominant member of the UGT1a family expressed in the liver (Shelby et al., 2003), was not significantly induced.

Upregulation of both Mrp3 and UGT1a in the liver of TR⁻ rats may result from the activation of a common nuclear hormone receptor, such as the farnesoid X receptor, pregnane X receptor, or constitutive androstane receptor (Schuetz et al., 2001; Chen et al., 2003b; Guo et al., 2003; Zollner et al., 2003). This may provide a coordinated mechanism to glucuronidate and transport potential toxicants out of the hepatocyte for renal excretion. The concomitant increase in UGT1a expression observed in the kidney of TR⁻ rats is consistent with this hypothesis. However, further investigation is required before the increase in basolateral Mrp3 expression in the kidney (Figure 1 and 2) can be incorporated into this hypothesis.

No significant differences in transport protein expression were found in whole brain homogenates from Wistar and TR⁻ rats (Figure 2C). Expression of Oatp1a1 and Oatp1a4 in the brain are consistent with previous reports (Angeletti et al., 1997; Gao et al., 1999; Rausch-Derra et al., 2001). Whether Mrp2 is expressed in rat brain still remains somewhat controversial, with mRNA expression and some functional evidence demonstrating the presence of Mrp2 at the level of the blood brain barrier in rats (Miller et al., 2000; Potschka et al., 2003). Although mRNA and Western Blot analysis of bovine brain microvessel endothelial cells did not confirm these findings (Zhang et al., 2000), this may reflect species differences or culturing effects. In the current study, only a weak signal at the molecular weight of Mrp2 was detected in samples from both wild-type and TR⁻ rat brain, suggesting that this band may be the result of non-specific binding to other cellular constituents (data not shown). Thus, expression of Mrp2 protein in the rat brain was not confirmed.

Interestingly, Mrp3 expression in the small intestine of TR⁻ rats was significantly decreased compared to wild-type; however, expression in the colon was similar. In contrast, expression of UGT1a enzymes was similar in the intestine of TR⁻ and wild-type rats, suggesting that mechanisms governing hepatic, renal, and intestinal expression of Mrp3 and UGT1a may not be coordinately regulated. The lack of coordinate regulation of Mrp3 and UGT1a between these organs is similar to that of hepatic and intestinal P-gp and CYP 3A expression (Lown et al., 1994; Paine et al., 1997; Brady et al., 2002). Relatively high expression of both Mrp3 and Mrp4 was evident in the colon, and may

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suggest a physiological role for these transport proteins in this tissue. The down-regulation of basolateral Mrp3 expression in the small intestine of TR⁻ rats appears to be consistent with the TR⁻ phenotype, such that intestinal (re)uptake of organic anions, including conjugated bile salts, would be attenuated. Intestinal expression of Mrp4 mRNA previously was demonstrated (Chen and Klaassen, 2004). However, the current study is the first to detect Mrp4 protein by Western Blot, therefore, preliminary cellular localization studies of Mrp4 in the intestine were conducted. Immunohistochemical staining (Figure 4) demonstrated that Mrp4 was localized primarily to the basal cytoplasmic region of enterocytes at the tips of villi. Although the staining pattern of Mrp4 was not distinctly localized to the basolateral membrane as previously observed in the liver (Assem et al., 2004), Mrp4 may still have a role in the basolateral membrane transport of sulfate-conjugated bile salts and nucleotides (Schuetz et al., 2001; Sampath et al., 2002).

In summary, several important differences in transport protein and UGT1a enzyme expression were evident in Mrp2-deficient TR⁻ rats compared to transport-competent Wistar controls when examined using the semiquantitative Western Blot technique. Also, important tissue-specific differences in protein expression were noted along with regional differences in protein expression in the gastrointestinal tract. Mrp3 was significantly upregulated in the liver and kidney of TR⁻ rats compared to Wistar controls, as described previously, but was down-regulated in the small intestine. Expression of Mrp4 was unchanged in the liver, kidney, brain and intestine of TR⁻ rats. Interestingly, members of the UGT1a family were upregulated in the liver and kidney of TR⁻ rats, but

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were unchanged in the intestine. Taken together, the alterations in Mrp3 and UGT1a expression in TR⁻ rats may reflect a coordinated mechanism to aid in the elimination of compounds through glucuronidation and renal excretion. It is clear that in knockout models, such as the TR⁻ rat, numerous adaptive changes in protein expression occur. These changes can significantly confound interpretation of pharmacokinetic and drug disposition data, and in the case of the TR⁻ rat, differences in drug disposition may not be ascribed solely to Mrp2 deficiency. Clearly, hepatic, renal, and intestinal alterations in expression of other proteins, particularly Mrp3 and UGT1a, compensate for loss of Mrp2 function.

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FOOTNOTES

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FIGURE LEGENDS

Figure 1. Western Blots of liver (A) and kidney (B) homogenates from wild-type (WT) and TR⁻ rats. Blots were prepared as described in Methods from 3 separate wild-type and TR⁻ rats and were loaded with 50 µg of total protein per lane. In Panel B, liver samples from a Wistar and TR⁻ rat are included for reference. Approximate molecular weights (MW) are shown, and semiquantitative assessment of blots is plotted in Figure 2.

Figure 2. Results of integrated optical density analyses of Western Blots prepared from liver (A), kidney (B), and brain (C) homogenates from wild-type (solid bars) and TR⁻ (open bars) rats. All data were normalized to β-actin probed on the same blot as the respective protein. Bars represent the mean and standard deviation from n=3 preparations; absence of a bar represents no detectable expression. Statistically significant differences between wild-type and TR⁻ rats are indicated (*, P<0.01).

Figure 3. A) Western Blot of mucosal scrapings prepared from intestinal segments of representative wild-type (WT) and TR⁻ rats. Blot was prepared as described in Methods and gels were loaded with 50 µg of total protein per lane, with the exception of colon samples, which were loaded with 25 µg per lane. A wild-type liver sample is included in the last lane for reference (50 µg of total protein). Approximate molecular weights (MW) are shown. B) Integrated optical density analyses of Western Blots prepared from mucosal scrapings of duodenum (i), jejunum (ii), ileum (iii), and colon (iv) from wild-type (solid bars) and TR⁻ (open bars) rats. All data were normalized to β-actin probed on the same blot as the respective protein. Bars represent the mean and standard deviation

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from n=3 preparations; absence of a bar represents no detectable expression. Statistically significant differences between Wistar and TR⁻ are indicated (*, P<0.05).

Figure 4. Immunohistochemical staining of Mrp4 (red) in the duodenum (A), jejunum (B), ileum (C), and colon (D) of wild-type (left-hand column) and TR⁻ (right-hand column) rats. Slides were counterstained with hemotoxylin and eosin and images were taken at 200X magnification. Bar represents 50 μ m. Color images are available as Supplemental Data at dmd.aspetjournals.org.

FIG 1

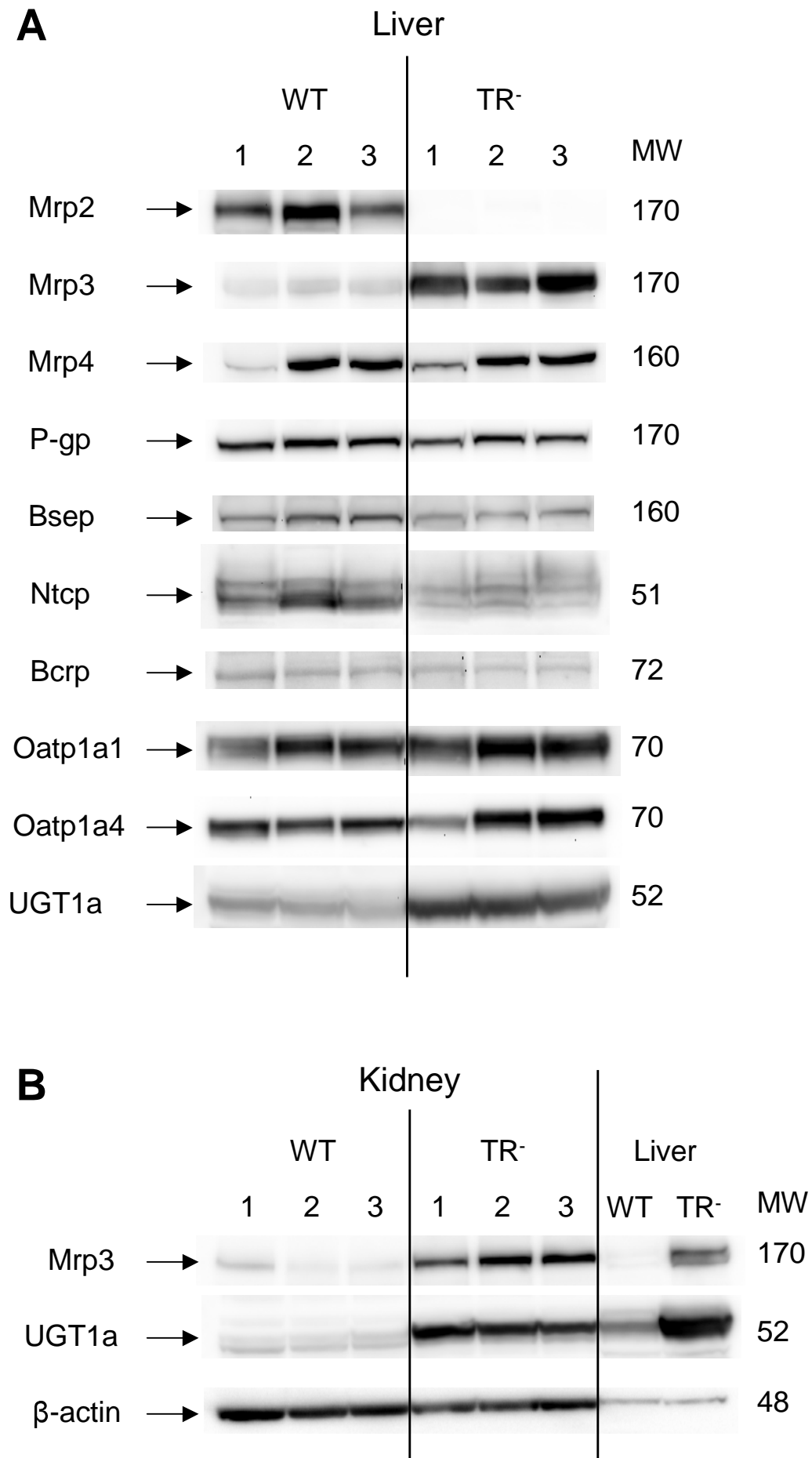


FIG 2

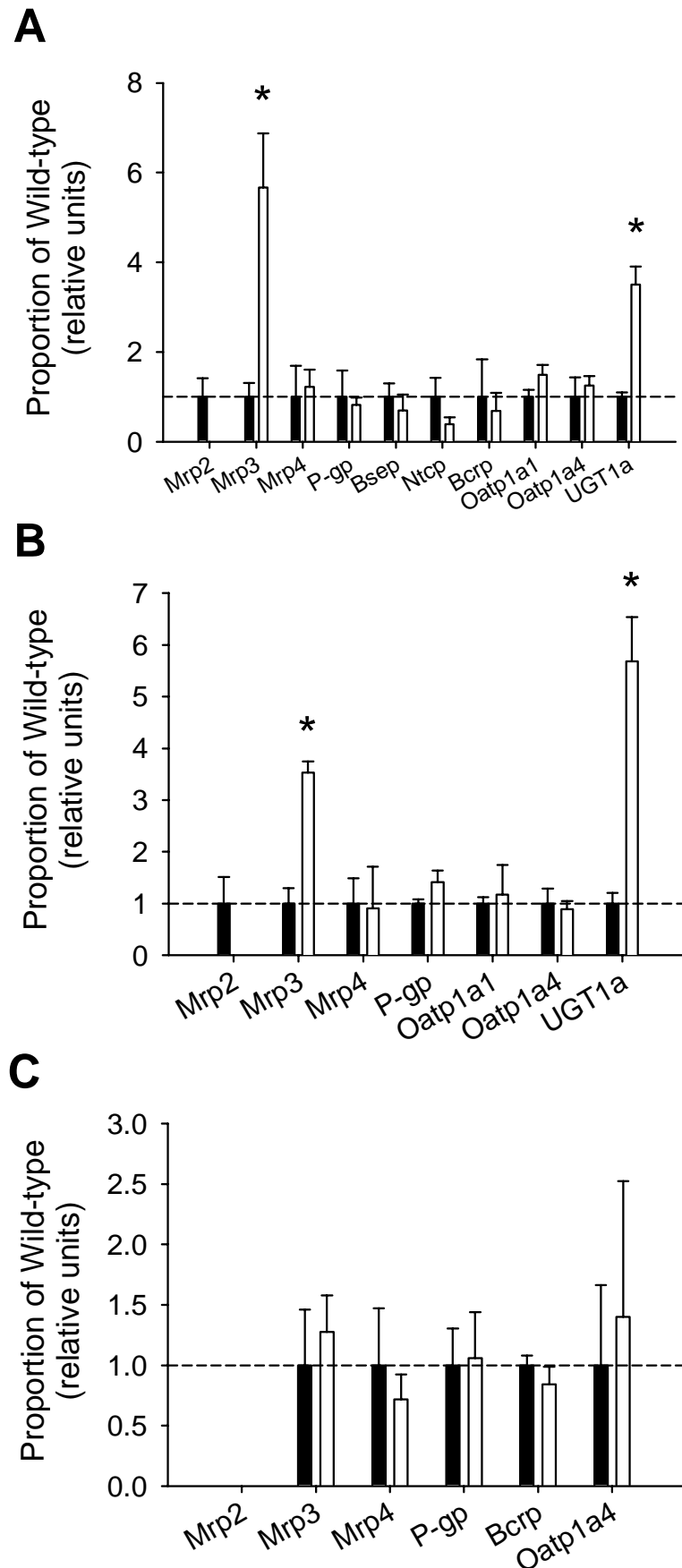


FIG 3

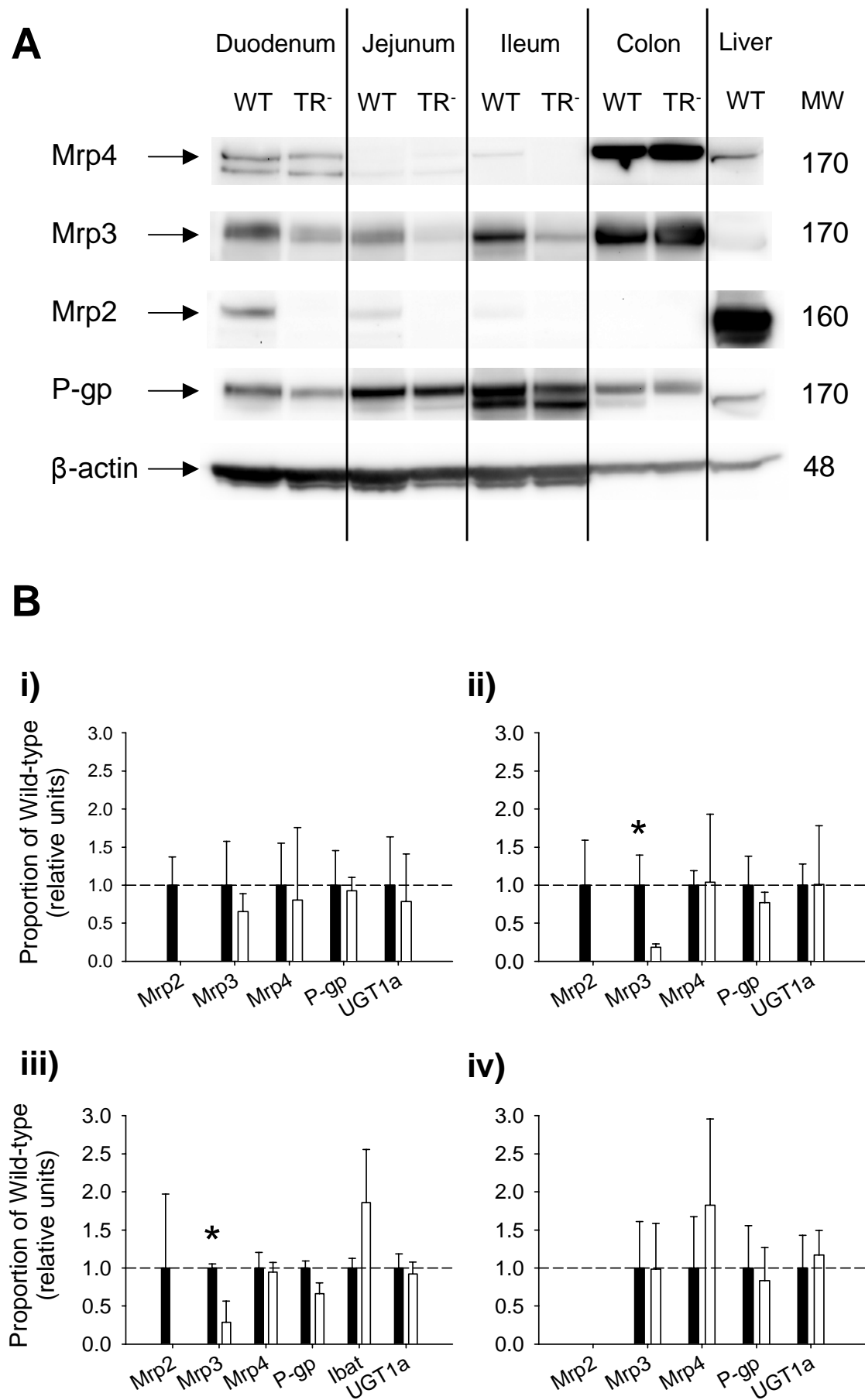


FIG 4

