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Up-regulation of Mrp4 expression in kidney of Mrp2-deficient TR$^-$ rats

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Abstract

Multidrug resistance-associated proteins (Mrps) are a group of ATP-dependent efflux transporters for organic anions. Mrp2 and Mrp4 are co-localized to the apical (brush-border) membrane domain of renal proximal tubules, where they may function together in the urinary excretion of organic anions. Previous reports showed that urinary excretion of some organic anions is not impaired in transport-deficient (TR$^-$) rats, which lack Mrp2, suggesting that up-regulation of other transporter(s) may compensate for the loss of Mrp2 function. The purpose of this study was to determine whether Mrp4 expression in kidney is altered in TR$^-$ rats. Mrp4 mRNA expression was quantified using the high-throughput branched DNA signal amplification assay. Mrp4 protein expression was determined by Western blot and immunohistochemical analysis. Mrp4 mRNA in kidney of TR$^-$ rats was 100% higher than normal Wistar rats. Western blot analysis showed a 200% increase in Mrp4 protein expression in kidney of the mutant rats compared to normal rats. Immunohistochemical analysis of Mrp4 protein demonstrated apical localization of Mrp4 on renal proximal tubules, and that the immunoreactivity was more intense in kidney sections from TR$^-$ rats than those from normal rats. In summary, the results of the present study demonstrate that renal Mrp4 expression is up-regulated in TR$^-$ rats, which may explain why urinary excretion of some organic anions remains normal in the mutant rats.

Keywords: Mrp4; Mrp2; TR$^-$ rat; Kidney; Up-regulation; Gunn rat

1. Introduction

Multidrug resistance-associated proteins (Mrps) are efflux transporters for many structurally diverse amphipathic chemicals and organic anions, including glucuronide conjugates, glutathione *S*-conjugates, as well as a variety of anticancer drugs (i.e., anthracyclines, vinca alkaloids, and methotrexate) [1]. The human MRP family is currently composed of nine members, MRP1–9. Several Mrps participate in the hepatic efflux transport process. Mrp2 is localized to the canalicular membrane domain of hepatocytes, where it transports organic anion compounds into bile. Deficiency of Mrp2 protein in transport-deficient (TR$^-$) rats, as well as in Dubin–Johnson syndrome patients, causes chronic conjugated hyperbilirubinemia [2,3]. Mrp1, Mrp3, and Mrp4 are localized to the basolateral membrane of hepatocytes. However, under normal physiological conditions, these three basolateral transporters are expressed at relatively low levels in liver of rats [1,4]. Hepatic expression of Mrp3 is increased in the Mrp2-deficient TR$^-$ rats [5], as well as in bile-duct ligated rats in which a significant decrease in Mrp2 expression is observed [6,7]. The inducible nature of rat Mrp3 suggests that in liver, Mrp3 is important in the efflux transport of organic anions under conditions where normal biliary excretory functions are altered.

Mrps are also expressed in kidney, another organ essential for the maintenance of body homeostasis. Both rat and human MRP2 have been shown to localize to the apical (brush-border) membrane of proximal tubular cells, where it may function in the efflux transport of organic anions across the luminal membrane [8,9]. Despite its low abundance in liver, rat Mrp4 is highly expressed in kidney [4]. Mrp4

Abbreviations: Mrp, multidrug resistance-associated protein; TR$^-$, transport-deficient rat; UGT, UDP-glucuronosyltransferase; bDNA, branched DNA; PAH, *p*-aminohippurate; CYP, cytochrome P450

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sub-cellular localization is unique among the Mrp family of transporters. In renal proximal tubular cells, Mrp4 is found to be localized to the apical membrane domain [10,11], whereas in hepatocytes, Mrp4 is routed to the basolateral membrane domain [11]. The substrate profile of Mrp4 is also unique in that besides transporting glucuronide- and glutathione *S*-conjugates [10], Mrp4 also transports cyclic nucleotides and nucleotide analogues [12–14], which have not been shown to be transported by Mrp1, Mrp2, and Mrp3.

The Mrp2-deficient TR$^-$ rats are commonly used to examine the role of Mrp2 in the efflux transport of compounds into bile. For example, the findings that organic anions such as cysteinyl leukotrienes [15], acetaminophen–glutathione conjugate [16], reduced and oxidized glutathione [17] are almost absent in bile from TR$^-$ rats strongly indicate that Mrp2 is the predominant transporter in liver that mediates hepatobiliary transport of these compounds. Interestingly, impaired biliary excretion of cysteinyl leukotrienes [15,18] and acetaminophen–glucuronide conjugate [16] in TR$^-$ rats is associated with increased urinary excretion of these compounds. One possible explanation for these finding is that the loss of Mrp2 function may be compensated by (an)other organic anion transporter(s). In light of the fact that Mrp2 and Mrp4 have overlapping substrate profiles, and are co-localized to the apical membrane domain of proximal tubules, we hypothesize that the loss of Mrp2 in TR$^-$ rats is associated with up-regulation of renal expression of Mrp4, which explains why the capacity for the urinary excretion of some organic anions is retained in TR$^-$ rats. To test this hypothesis, we compared Mrp4 expression in kidney and liver from normal Wistar and TR$^-$ rats at both mRNA and protein levels. Mrp4 mRNA levels were quantified using the high-throughput Quantigene® branched DNA (bDNA) signal amplification assay. Mrp4 protein expression was determined by Western blot and immunohistochemical analysis. Hepatic and renal expression of Mrp3 is elevated not only in Mrp2-deficient mutant rats with chronic conjugated hyperbilirubinemia [19], but also in UDP-glucuronosyltransferase (UGT) 1A-deficient Gunn rats with unconjugated hyperbilirubinemia [7,20]. Therefore, we also compared Mrp4 expression between normal Wistar and Gunn rats. In vitro transport studies showed that Mrp4 transports cAMP [12–14]. To determine whether changes in Mrp4 expression in renal proximal tubular cells of Mrp2-deficient TR$^-$ rats alter urinary excretion of endogenous cAMP, cAMP levels in urine and kidney tissue extracts from normal Wistar and TR$^-$ rats were compared.

2. Materials and methods

2.1. Materials

The anti-human MRP4 polyclonal antibody was obtained from Alexis Biochemicals (Lausen, Switzerland). Vectastain Elite ABC kit for rat IgG and Avidin/Biotin blocking kit were purchased from Vector Laboratories (Burlingame, CA, USA). HistoMark Black Substrate system was purchased from Kierkegaard and Perry Laboratories (Gaithersburg, MD, USA). Donkey anti-rabbit horseradish peroxidase-linked antibodies were from Amersham Life Science (Arlington Heights, IL, USA). A rat Mrp4 peptide (20 amino acid residues) homologous to the peptide antigen that was used to generate anti-human MRP4 antiserum (sequences listed in Fig. 2A) was synthesized and high-performance liquid chromatography-purified by the University of Kansas Medical Center Biotech Support Facility (Kansas City, KS, USA). All other chemicals were obtained from the above vendors and Sigma–Aldrich (St. Louis, MO, USA).

2.2. Animals

The breeding pairs of homozygous TR$^-$ rats were obtained from Dr. Mary Vore (University of Kentucky, Lexington, KY, USA). Male Wistar rats (used as controls for the TR$^-$ rats), as well as homozygous UGT1A-deficient Gunn rats and their controls, were purchased from Harlan (Indianapolis, IN, USA). The breeding colony of TR$^-$ rats, along with other animals used in this study, were housed in the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC)-accredited facility at the University of Kansas Medical Center. Liver and kidneys were excised from these rats (body weight between 265 and 330 g, $n = 5$ per strain) and immediately frozen in liquid nitrogen. Samples were stored at −80 °C until use. For immunohistochemistry, kidneys were fixed in 4% paraformaldehyde.

2.3. Twenty-four hour urine collection

Urine samples were collected from male normal Wistar and TR$^-$ rats (body weight between 265 and 330 g, $n = 5$ per strain) housed in metabolic cages ad libitum. Urine samples were collected into sterile tubes for 24 h. Volume of urine samples was determined gravimetrically assuming specific density of urine as 1.0. After centrifugation at 500 × *g* for 10 min, the supernatant of urine samples was stored at −20 °C until analysis.

2.4. Total RNA isolation

Total RNA was isolated from frozen tissue samples using RNA-Bee reagent (Tel-Test, Friendswood, TX, USA) according to the manufacturer's instructions. After spectrophotometric quantification of RNA concentrations, samples were diluted with diethyl pyrocarbonate-treated water to a final concentration of 1 μg/μl. The integrity of the diluted RNA samples was determined by visual examination of the 18S and 28S rRNAs separated on 1.2% denaturing agarose gel.

2.5. Measurement of rat Mrp4 mRNA levels with Quantigene® branched DNA (bDNA) signal amplification assay

Rat Mrp4 cDNA (GenBank Accession Number: AY533525) was cloned by our laboratory [4]. Oligonucleotide probes specific for rat Mrp4 used in Quantigene® bDNA signal amplification assay (Genospectra, Fremont, CA, USA) were described in a previous study [4]. Ten micrograms of total RNA was used to determine the levels of Mrp4 transcript in a 96-well format as described in detail previously [21]. Data are presented as relative light units (RLU) per 10 μg total RNA.

2.6. Membrane preparations for Western blot

Crude membrane samples (a mixture of plasma membrane and intracellular organelle membrane) were prepared from hepatic and renal tissues according to the method described previously [22]. Briefly, 0.2–0.3 g tissue was minced in 10 ml ice-cold buffer A (0.25 M sucrose, 10 mM Tris–HCl [pH 7.4–7.6], containing 25 μg/ml leupeptin, 50 μg/ml aprotinin, 40 μg/ml PMSF, 0.5 μg/ml pepstatin, and 50 μg/ml antipain). The minced tissue was poured into a Dounce homogenizer (Kontes, Vineland, NJ, USA) and homogenized on ice for 20 strokes. The crude homogenate was further homogenized on ice for five strokes with a Teflon homogenizer. Homogenate was filtered through two layers of cheesecloth, and then centrifuged at 100,000 × *g* for 60 min at 4 °C. The resulting pellet was resuspended in buffer B (0.25 M sucrose, 10 mM HEPES [pH 7.5], and 40 μg/ml PMSF). Protein concentration of each sample was determined with the *Dc* protein assay kit from Bio-Rad (Hercules, CA, USA).

2.7. Western blot analysis

Membrane samples mixed with sample loading buffer (75 μg protein/lane) were loaded without heating onto a 7.5% SDS-polyacrylamide gel. Following electrophoresis, proteins in the gel were electrotransferred to nitrocellulose membrane overnight at 20 V at 4 °C. Membranes were blocked for 1 h at room temperature with 5% non-fat milk in Tris–buffered saline containing 0.05% Tween-20 (TBS-T). Blots were then incubated with anti-human MRP4 polyclonal antibody (1:1000 dilution in 2% non-fat milk in TBS-T) for 3 h at room temperature. For the immunocompetition experiment, antiserum (14 μl) was preincubated with rat Mrp4 peptide dissolved in TBS-T (100 mg/ml) in a total volume of 28 μl for 1 h at room temperature, at which point this mixture was diluted with 14 ml of 2% non-fat milk in TBS-T (1:1000 dilution of antiserum), and applied to blot for 3 h at room temperature. After thorough washing (three 15-min washes with excess TBS-T), blots were incubated with donkey anti-rabbit IgG horseradish peroxidase-linked secondary antibody (1:10,000 dilution with 2% non-fat milk in TBS-T) for 1 h. Blots were washed again. Immunoreactive bands were detected with enhanced chemical luminescence (ECL) kit (Amersham). Mrp4 protein was visualized by exposure to Fuji Medical X-Ray film. Immunoreactive intensity of Mrp4 protein in the blots was quantified by densitometric analysis using a personal densitometer from Molecular Dynamics (Sunnyvale, CA, USA).

2.8. Immunohistochemistry

Four percent paraformaldehyde-fixed paraffin-embedded tissues were used for immunohistochemical analysis of Mrp4 expression. Sections on slides were deparaffinized, and rehydrated with xylene and ethanol. Antigen retrieval was performed by autoclaving the slides for 30 min in 1× Target Retrieval Solution (Dako, Copenhagen, Demark). Slides were then incubated with 3% hydrogen peroxide for 5 min followed by incubation with 5% normal rabbit serum for 45 min. The endogenous biotin-containing sites were blocked by incubation with avidin and biotin solutions. Slides were incubated overnight at 4 °C with the monoclonal anti-Mrp4 antibody M_4I-10 [23]. Following incubation with biotinylated secondary antibody for 30 min, slides were incubated with ABC solution, HistoMark Black diaminobenzidine solution, and counter-stained with methyl green.

2.9. cAMP assay

cAMP levels in 24-h urine samples and kidney tissue from normal Wistar and TR^- rats were measured using cAMP Biotrak Enzyme Immunoassay System (Amersham Biosciences, Piscataway, NJ, USA). cAMP in kidney tissue was extracted using a liquid-phase extraction method described in manufacturer's instructions. A non-acetylation procedure was used for the measurement of cAMP in urine and tissue extracts.

2.10. Statistical analysis

Data were expressed as the mean ± standard error and analyzed with Student's *t*-test. Differences were considered significant at $p < 0.05$.

3. Results

3.1. Mrp4 mRNA expression in Mrp2-deficient TR^- rats

As shown in Fig. 1, mRNA of Mrp4 was much more abundant in kidney than in liver. Mrp4 expression in both kidney and liver of Mrp2-deficient TR^- rats was about twice that of Wistar rats.

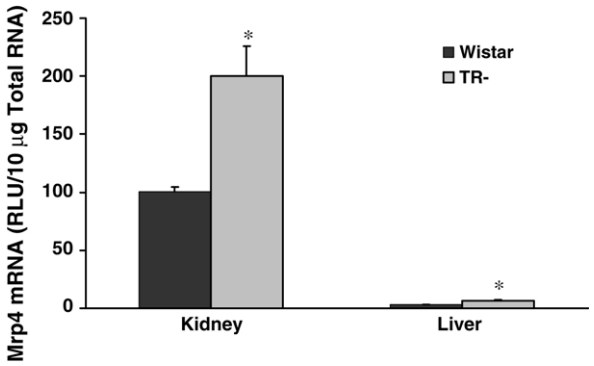

Fig. 1. Mrp4 mRNA expression in kidney and liver of normal Wistar and Mrp2-deficient TR$^-$ rats. Total RNA was isolated from tissues of normal Wistar and TR$^-$ rats. Mrp4 mRNA levels were quantified using QuantiGene® bDNA assay. Results were expressed as means ± S.E. of five rats. Asterisk indicates values significantly different from those of normal Wistar rats ($p < 0.05$).

3.2. Western blot analysis of Mrp4 protein expression in Mrp2-deficient TR$^-$ rats

To determine whether the differences in Mrp4 mRNA expression translate into corresponding changes in Mrp4 protein, membrane fractions were prepared from kidney and liver of both strains of rats and Western blot analysis was performed using polyclonal anti-human MRP4 antibody as primary antibody. The peptide immunogen that was used to generate the anti-human MRP4 antibody is aligned with its homologous rat Mrp4 peptide sequence in Fig. 2A. The two peptides exhibit high sequence homology: 90% identity and 95% similarity. In Western blot analysis, when the blot was exposed to X-ray films for 5–10 min, the anti-human MRP4 antibody detected a 170-kDa protein in membrane preparations from kidney of TR$^-$ and normal rats, but did not detect any proteins of similar sizes in membrane fractions from liver tissue (Fig. 2B). Following prolonged exposure (>1 h), however, a 170-kDa protein band was detected in the crude liver membrane fractions (data not shown). Densitometric analysis indicates that the intensity of the 170-kDa protein band in the kidney of TR$^-$ rats is about three times that of normal Wistar rats (Fig. 2C).

Because there was no previous report on whether the anti-human MRP4 antibody cross-reacts with rat Mrp4, a peptide competition experiment as described in Section 2 was conducted to further characterize the specificity of the immunoreactive signals. As shown in Fig. 2D, preincubation of the primary antibody with the rat Mrp4 peptide described in Fig. 2A completely abolished any immunoreactive signals, including the band with a molecular weight of approximately 170 kDa. Therefore, we conclude that the anti-human MRP4 polyclonal antibody cross-reacts with rat Mrp4 protein under our conditions of Western blot analysis.

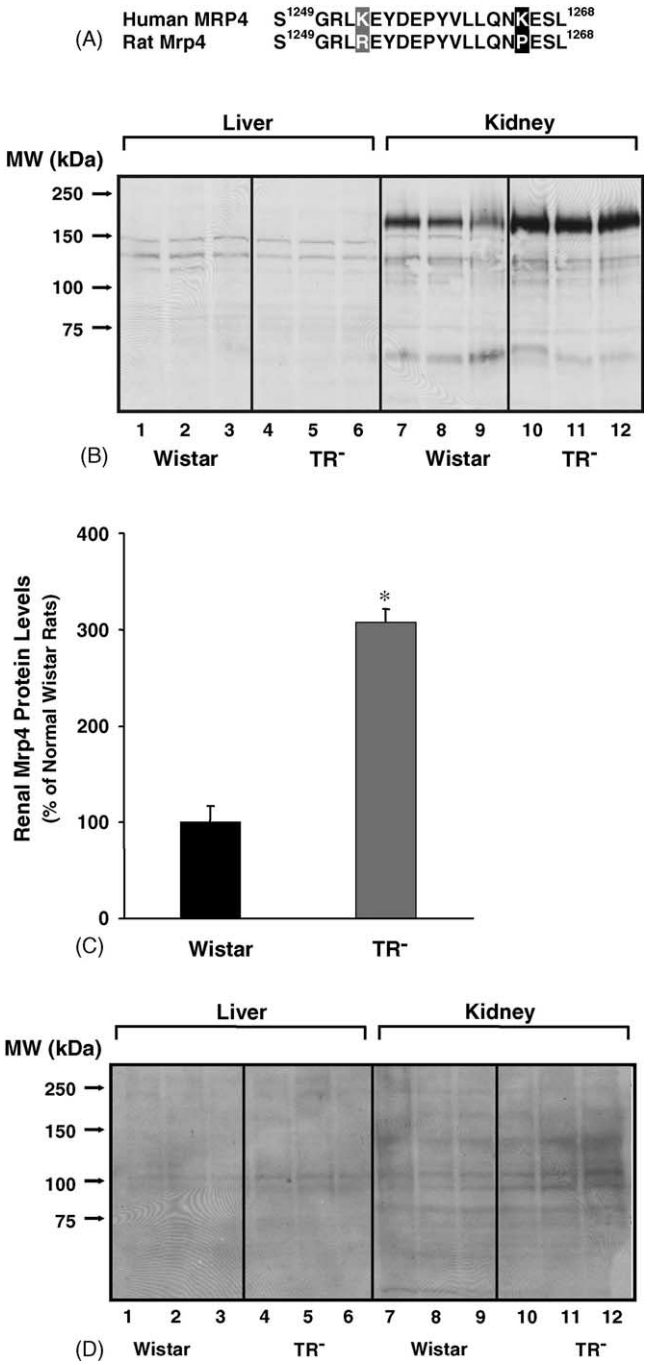

Fig. 2. Western blot analysis of Mrp4 protein expression in kidney and liver of normal Wistar and Mrp2-deficient TR$^-$ rats. An anti-human MRP4 polyclonal antibody was used as primary antibody. (A) Alignment of amino acid sequences of the peptide immunogen used to generate the anti-human MRP4 antibody and its homologous rat Mrp4 peptide. Identical amino acid residues are indicated with black letters on white background. Similar residues are indicated with white letters on gray background, whereas dissimilar residues are indicated with white letters on black background. (B) Western blot of rat Mrp4 protein. Western blot analysis was performed using kidney and liver membrane preparations of normal Wistar and TR$^-$ rats ($n = 5$ rats) as described in Section 2. Representative blot containing three samples per group was shown. (C) Densitometric analysis of Western blot. Asterisk indicates values significantly different from those of normal Wistar rats ($p < 0.05$). (D) Peptide competition experiment. Western blot was performed following preincubation of the anti-human MRP4 antibody and the homologous rat Mrp4 peptide (shown in (A) as described in Section 2).

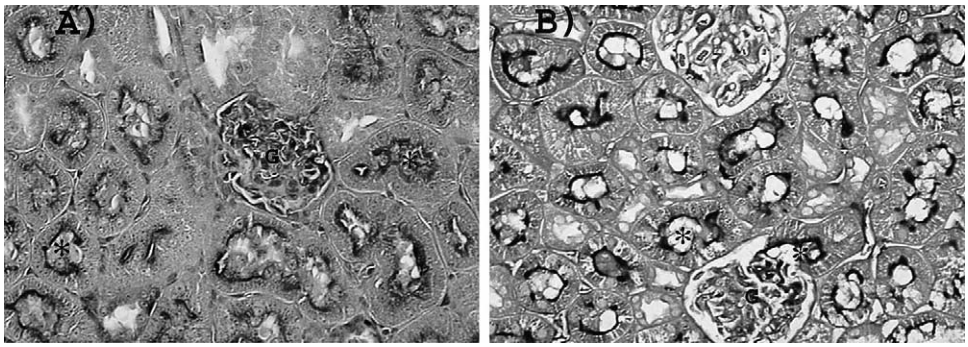

Fig. 3. Immunochemical analysis of Mrp4 protein expression in kidney sections of normal Wistar and Mrp2-deficient TR$^-$ rats. The monoclonal anti-Mrp4 antibody M_4I-10 was used as primary antibody. Immunochemical staining was performed as described in Section 2. (A) Representative photomicrograph of Mrp4 immunohistochemical staining in kidney sections from normal Wistar rats (magnification: 400×). (B) Representative photomicrograph of Mrp4 immunohistochemistry staining in kidney sections from TR$^-$ rats (magnification: 400×). Renal proximal tubules (*). Glomeruli (G).

3.3. Immunohistochemical analysis of Mrp4 expression in kidneys of Mrp2-deficient TR$^-$ rats

Membrane-associated transporter proteins need to assume their normal sub-cellular localization in order to be functional. To determine whether there are differences in the amount of Mrp4 protein routed to the brush-border membrane of renal proximal tubular cells between normal Wistar and TR$^-$ rats, immunohistochemical analysis of Mrp4 expression in kidneys was performed with the monoclonal anti-Mrp4 antibody M_4I-10. This antibody was previously shown to be highly specific, and has been successfully used in immunohistochemical staining of paraffin-embedded tissues from rat, mouse, and human to detect Mrp4 protein expression [23]. As shown in Fig. 3, immunoreactive staining was observed on the apical membrane domain of proximal tubular cells in both normal Wistar and TR$^-$ rats. Immunoreactivity was not seen in other portions of nephrons, including glomeruli, distal convoluted tubules, collecting ducts, etc. In agreement with the mRNA and Western blot analysis data shown earlier, the staining was much more intense in sections from TR$^-$ rats than those from normal Wistar rats. In negative control slides, which were not incubated with primary antibody, no such immunoreactive staining was seen (data not shown).

3.4. Mrp4 expression in UDP-glucuronosyltransferase (UGT) 1A-deficient Gunn rats

In contrast to Mrp2-deficient TR$^-$ rats, Mrp4 mRNA in kidney and liver of UGT1A-deficient Gunn rats was expressed at similar levels as in normal Wistar rats (Fig. 4A). Furthermore, Western blot analysis indicated no differences in Mrp4 protein levels in kidney membrane preparations from the two strains of rats. Similarly, the polyclonal anti-human MRP4 antibody did not detect any immunoreactive bands with similar molecular weight as Mrp4 in membrane preparations from liver after 5–10 min exposure of the blots to X-ray films (Fig. 4B). Densitometric analysis of Western blots confirmed that there were no differences in renal Mrp4 protein expression between normal and Gunn rats (Fig. 4C).

3.5. cAMP in urine and kidney tissue extracts from Mrp2-deficient TR$^-$ rats

As shown in Table 1, urinary concentration of cAMP in TR$^-$ was about twice that in normal Wistar rats. Because the 24-h urine volume in TR$^-$ rats was about half of that in normal Wistar rats, there was no difference in the 24-h urinary excretion rate of cAMP. Furthermore, cAMP concentration in kidney tissue extracts of TR$^-$ rats was found to be similar to that of normal Wistar rats.

4. Discussion

The data from the present study demonstrate that renal Mrp4 expression is significantly increased in Mrp2-deficient TR$^-$ rats compared to normal Wistar rats (Figs. 1 and 2). Our immunohistochemical analysis of Mrp4 expression (Fig. 3) shows that (1) immunostaining is localized to the apical membrane domain of proximal tubules, and (2) staining intensity in kidney of TR$^-$ rats is higher than that in normal Wistar rats. These results strongly suggest that Mrp4 functions as an efflux transporter for organic anions on the apical membrane domain of proximal tubules in kidney. We also compared hepatic and renal expression of Mrp1, Mrp3, Mrp5, and Mrp6 between normal Wistar and TR$^-$ rats. No differences in hepatic and renal expression of Mrp1, Mrp5, and Mrp6 were observed between the two strains of rats (see Supplementary data). In agreement with previous reports [5,19], hepatic and renal expression of Mrp3 is significantly higher in Mrp2-deficient mutant rats than normal rats (liver: 700% increase; kidney: 50% increase) (see Supplementary data).

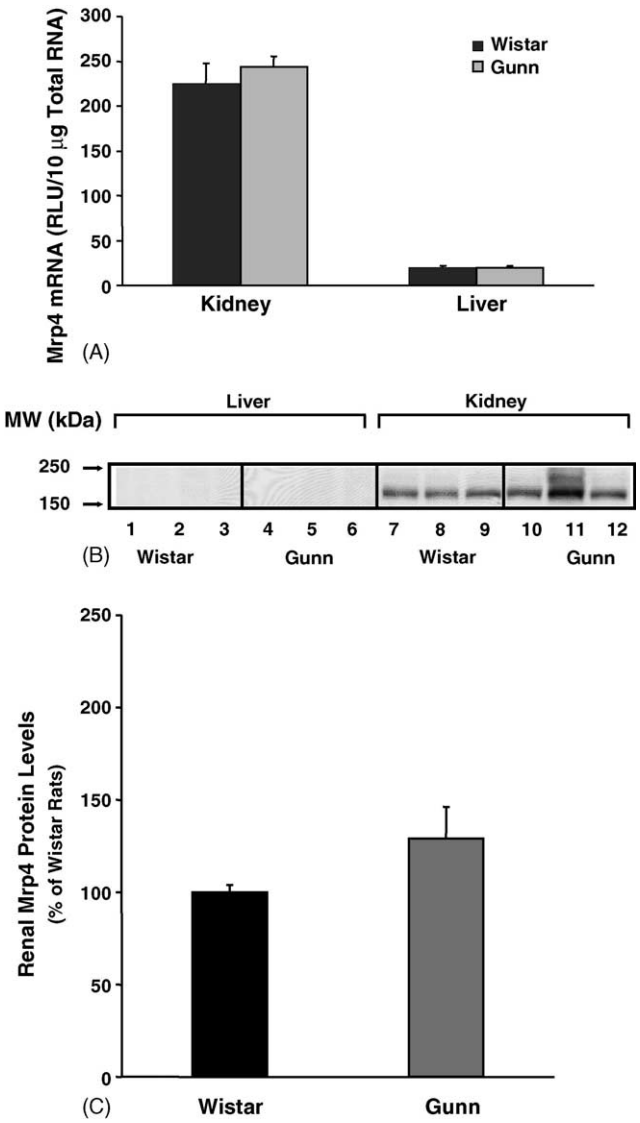

Fig. 4. Mrp4 expression in kidney and liver of normal Wistar and hyperbilirubinemic UGT-1A-deficient Gunn rats. (A) Mrp4 mRNA expression in kidney and liver of Wistar and Gunn rats. Total RNA was isolated from tissues of Wistar and Gunn rats. Mrp4 mRNA was quantified using QuantiGene® bDNA assay. Results were expressed as means ± S.E. of five rats. (B) Western blot of rat Mrp4 protein. Western blot analysis was performed using membrane preparations from kidney and liver of Wistar and Gunn rats ($n = 5$ rats) as described in Section 2. Representative blot containing three samples per group was shown. (C) Densitometric analysis of Western blot.

Many organic anions undergo urinary excretion through carrier-mediated secretion, hydrodynamic filtration, or both. Renal proximal tubules are equipped with multiple transport systems that mediate uptake of organic anions from blood across the basolateral membrane and subsequent secretion across the apical membrane into urine [24]. Organic anion transporter 1, 2, and 3 are basolateral transporters of proximal tubules, responsible for uptake transport of organic anions. Several other transporters on apical membrane domain of proximal tubules, such as rodent organic anion transporting polypeptide (Oatp) 1a1 (also known as Oatp1), OAT4, and sodium phosphate transporter 1 are thought to contribute to reabsorption or secretion of organic anions. Mrp2 and 4 are co-localized to the apical membrane domain of renal proximal tubular cells, but the relative contribution of these two efflux transporters in the urinary excretion of organic anions is not well understood. Mrp2 deficiency in TR$^-$ rats and patients with Dubin–Johnson syndrome results in impaired hepatobiliary excretion of cysteinyl leukotrienes, which are potent mediators for inflammation and smooth muscle contraction. Interestingly, urinary excretion of cysteinyl leukotrienes is elevated in the mutant rats and Dubin–Johnson syndrome patients [15,18]. Recently, Mrp2 was shown to be a low-affinity transporter for *p*-aminohippurate (PAH) [25,26], whereas Mrp4 was reported to transport PAH with higher affinity [27]. PAH is widely used as a model substrate to investigate renal handling of organic anions. Interestingly, urinary excretion of PAH and lucifer yellow, a fluorescent substrate for both Mrp2 and 4 in Mrp2-deficient TR$^-$ rats, do not differ from that of normal Wistar rats, whereas urinary excretion of two other fluorescent Mrp2 substrates, calcein and fluo-3, are significantly reduced in TR$^-$ rats [27,28]. These data indicate that whereas Mrp2 is largely responsible for the urinary excretion of some of its substrates, the capacity to excrete other organic anions is retained in the proximal tubules of TR$^-$ rats. Because Mrp4 is more abundantly expressed in kidney than Mrp2 [10,29], Mrp4 may play a more important role than Mrp2 in the urinary excretion of organic anions like cysteinyl leukotrienes, PAH, and lucifer yellow. As a result, loss of Mrp2 function in the kidney of TR$^-$ rats had little impact on the urinary excretion of some organic anions. Alternatively, up-regulation of other efflux transporter(s) in renal proximal tubules of TR$^-$ rats may compensate for the loss of Mrp2 function in kidney, resulting in the apparent normal urinary excretion of some organic anions in TR$^-$ rats. In the present study, a one-fold induction of Mrp4 mRNA and a two-fold increase of Mrp4 protein expression in kidney of TR$^-$ rats compared to normal Wistar rats were observed. Renal expression of

Table 1
cAMP in urine and kidney tissue extract

	Urine volume (ml/day/kg b.w.)	Urinary [cAMP] (μmol/l)	Urinary cAMP excretion rate (μmol/day/kg b.w.)	Kidney tissue [cAMP] (pml/g)
Normal Wistar	54 ± 7	10 ± 1	0.52 ± 0.01	492 ± 29
TR$^-$	28 ± 3[a]	23 ± 1[a]	0.62 ± 0.04	485 ± 47

[a] Indicates significantly different from normal Wistar rats.

other Mrps, namely Mrp1, Mrp5, and Mrp6 in TR$^-$ rats are not different than normal Wistar rats (see Supplementary data). A 50% increase in renal expression of the basolateral transporter, Mrp3 was observed in the present study (see Supplementary data). However, the impact of renal Mrp3 induction on urinary excretion of organic anions is likely to be limited because of the low constitutive expression of Mrp3 in kidney [29]. Taken together, among the five Mrp family members examined, only Mrp4 expression is up-regulated to a meaningful extent in kidney of TR$^-$ rats, such that this change could impact the urinary excretion of organic anions.

Our finding of Mrp4 up-regulation in kidney of TR$^-$ rats disagrees with two previous reports [10,30], in which Western blot and immunohistochemical analysis showed no differences in renal Mrp4 protein expression between the two strains of rats. In those studies, Mrp4 mRNA expression in kidney was not examined. The discrepancy may be due to the fact that the antibodies used in the previous studies are different from those used in the present study. Our finding is based on both mRNA and protein expression data. Mrp4 up-regulation at the protein-expression level is corroborated by Western blot and immunohistochemical analysis using two different antibodies against Mrp4.

Hepatic and renal expression of Mrp3 is up-regulated in Mrp2-deficient mutant rats [5,19], as well as in UGT 1A-deficient Gunn rats [7,20]. Both strains of rats exhibit the phenotype of hyperbilirubinemia. In addition, treatment of rats with bilirubin, or xenobiotics, such as phenobarbital, trans-stilbene oxide, and oltipraz, induces Mrp3 expression in liver [7,31,32]. Therefore, Mrp3 is a highly inducible member of the Mrp family, despite the lack of understanding of the exact mechanism(s) for Mrp3 induction [33]. In the present study, no differences in renal and hepatic Mrp4 expression were seen between normal Wistar and Gunn rats. Therefore, up-regulation of renal Mrp4 expression in TR$^-$ rats may not be due to hyperbilirubinemia. We found that renal Mrp4 expression is not induced in rats treated with 15 microsomal enzyme inducers [4], which are categorized into six groups based on the mechanisms for cytochrome P450 (CYP) induction, namely, aryl hydrocarbon receptor ligands, constitutive androsterone receptor activators, pregnane X receptor ligands, peroxisome proliferators-activated receptor α ligands, NF-E2-related factor 2 activators, and CYP2E1 inducers. Because renal Mrp4 expression is not readily inducible in rats treated with these prototypical microsomal enzyme inducers, these transcriptional regulation pathways are probably not involved in the up-regulation of renal Mrp4 expression in TR$^-$ rats. Further studies are needed to better understand the mechanisms for the up-regulation of Mrp4 in kidney and Mrp3 in liver of Mrp2-deficient mutant rats.

Denk et al. [30] reported a seven-fold increase in Mrp4 protein in liver of bile-duct-ligated rats, which was accompanied by elevated urinary and plasma concentrations of the Mrp4 substrate, cAMP. In the present studies, up-regulation of Mrp4 expression in kidney resulted in a one-fold increase in urinary cAMP concentration; however, the urinary excretion rate of cAMP remained unchanged between the two strains of rats due to a 50% decrease in 24-h urine output in the mutant rats (Table 1). To the best of our knowledge, this marked reduction in 24-h urine output in TR$^-$ rats was not reported previously. Glomerular lesions have been reported in Mrp2-deficient mutant rats of Sprague–Dawley background [34]. We speculate that similar lesions are also present in the TR$^-$ rats and could contribute to the reduction in urine output. In addition, no differences in cAMP concentration in tissue extracts from the two strains of rats were observed. Because 25–40% of total urinary cAMP is secreted from renal proximal tubular cells under the influence of parathyroid hormone [35], differences in glomerular filtration rate and renal blood flow can affect urinary cAMP excretion. If the decrease in urine output in TR$^-$ rats is due to a decrease in glomerular filtration rate and/or renal blood flow, our result that total urinary excretion of cAMP in the mutant rats remains similar to that in normal rats would indicate that more cAMP is secreted from tubular cells in the mutant rats, which is associated with the up-regulation of renal expression of Mrp4 in these rats.

In conclusion, the results of the present study demonstrate that in parallel to the up-regulation of hepatic Mrp3 expression in Mrp2-deficient TR$^-$ rats [5], renal Mrp4 expression is up-regulated in TR$^-$ rats, which may explain why urinary excretion of some organic anions remains normal in the mutant rats [15,16,27,28].

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2005.06.019.

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