Expression and localization of the multidrug resistance-associated protein 3 in rat small and large intestine

DANIEL ROST,1 SVEN MAHNER,1 YUICHI SUGIYAMA,2 AND WOLFGANG STREMMEL1

1Department of Gastroenterology, University of Heidelberg, 69115 Heidelberg, Germany; and 2Graduate School of Pharmaceutical Sciences, The University of Tokyo, 113–0033 Tokyo, Japan

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Rost, Daniel, Sven Mahner, Yuichi Sugiyama, and Wolfgang Stremmel. Expression and localization of the multidrug resistance-associated protein 3 in rat small and large intestine. Am J Physiol Gastrointest Liver Physiol 282: G720–G726, 2002. First published October 10, 2001; 10.1152/ajpgi.00318.2001.—Multidrug resistance-associated protein 3 (MRP3; symbol ABCC3), has been shown to mediate ATP-dependent transport of organic anions including 17β-glucuronosyl estradiol, glucuronosyl bilirubin, monovalent, and sulfated bile salts. MRP3 mRNA expression was reported in rat intestine suggesting a role of MRP3 in the intestinal transport. We examined the expression and localization of MRP3 in rat small and large intestine by RT-PCR, immunofluorescence, and immunoblot analysis. MRP3 was identified in all intestinal segments by RT-PCR. MRP3 expression was low in duodenum and jejunum but markedly increased in ileum and colon. With the use of a rat MRP3 specific antibody, MRP3 was localized to the basolateral domains of enterocytes. Immunofluorescence analysis and immunoblot analysis confirmed a strong expression of rat MRP3 in ileum and colon. In contrast, MRP2 was predominantly expressed in the proximal segments of rat small intestine. Our findings demonstrate a high expression of rat MRP3 in ileum and colon and provide evidence for an involvement of MRP3 in the ATP-dependent transport of organic anions, including bile salts from the enterocyte into blood.

enterohepatic circulation; intestinal transport; organic anions; bile acids

MEMBERS OF THE MULTIDRUG RESISTANCE-ASSOCIATED PROTEIN (MRP) family have been identified to mediate ATP-dependent transport of organic anions across membranes. MRP substrates include xenobiotic and endogenous lipophilic substances conjugated with glutathione, glucuronate, and sulfate (3, 22). Nine MRP isoforms (ABCC1–6 and ABCC10–12) have been identified in humans and have been shown to exhibit a tissue-specific distribution (19, 26, 27, 39). In humans, MRP1, MRP2, MRP3, and MRP5 are expressed to different extents in small and large intestine, as revealed by RNase protection assay and Northern blot analysis (1, 26, 31). Differential expression of MRP1 and MRP2 proteins in the intestine has been studied in more detail in rodents (30, 31, 33). MRP1 expression in the small intestine was low and was shown to be limited to undifferentiated enterocytes at the base of the crypts (33). The apical MRP isoform MRP2 was predominantly expressed in the proximal part of rat small intestine (11, 31) and has been suggested to contribute to organic anion excretion from the enterocyte into the lumen of the gut (11, 30). Thus in rat, neither MRP1 nor MRP2 can account for the well-recognized basolateral transport of endogenous and xenobiotic substances including hormones, bile salts, and drugs from the small intestine into blood.

Recently, MRP3 has been cloned from rat and human liver (14, 23) and was subsequently localized to the basolateral membrane of hepatocytes (25, 28), cholangiocytes (28, 37), and MRP3-tranfected polarized cells (24, 28). MRP3 substrates include 17β-glucuronosyl estradiol (E217βG) (15, 44), glucuronosyl bilirubin (22), monovalent bile salts (i.e., taurocholate and glycocholate) (16, 44), and sulfated bile salts (i.e., taurochenodeoxycholate-3-sulfate, tauroliothocholate-3-sulfate) (16). In addition to liver and kidney, high MRP3 mRNA expression was found in rat and human intestinal tissues (14, 23, 26), suggesting a possible role of MRP3 in the intestinal secretion of organic anions (13, 14).

To further characterize the role of MRP3 in the intestine, we examined the expression and localization of rat MRP3 in duodenum, jejunum, ileum, and colon using RT-PCR, immunofluorescence microscopy, and immunoblot analysis. Our results demonstrate that MRP3 is localized to the basolateral domain of rat enterocytes and is expressed at high levels in the ileum and colon of rat intestine. MRP3 may, therefore, represent a major export pump for organic anions in the rat distal intestine and may be involved in the enterohepatic circulation, mediating the ATP-dependent secretion of bile salts, steroids, and drugs from the enterocytes into blood.

MATERIALS AND METHODS

Rat tissue samples. Adult male Wistar rats weighing 180–220 g were purchased from Charles River Wiga (Sulzfeld, Germany) and were kept according to the Guide for the Care
MRP2-specific primers, RT-PCR products were obtained only in rat intestine with the strongest band appearing in the rat jejunum. MRP3-specific amplification products were obtained in all segments of small and large intestine. The intensity of the bands gradually increased from proximal to distal parts of the intestine with the strongest bands appearing in the distal colon.

Fig. 1. RT-PCR analysis of MRP2 and MRP3 mRNA expression in rat intestine. Total RNA was extracted from mucosa of duodenum (Duod), jejunum (Jejun), ileum, proximal colon (p Col), and distal colon (d Col) as described in MATERIALS AND METHODS. With the use of MRP2-specific primers, RT-PCR products were obtained only in rat small intestine with the strongest band appearing in the rat jejunum. MRP3-specific amplification products were obtained in all segments of small and large intestine. The intensity of the bands gradually increased from proximal to distal parts of the intestine with the strongest bands appearing in the distal colon.

Antibodies. Rat MRP2-specific EAG15 antibody was raised in rabbit against a synthetic peptide containing the 15 COOH-terminal amino acids of the deduced rat MRP2 sequence (4). The polyclonal MRP3 antibody was obtained by immunizing rabbits with a maltose-binding fusion protein containing the 136 amino acids corresponding to 838–973 of the deduced rat MRP3 amino acid sequence (32, 37). The mouse monoclonal antibody C219 was purchased from Centocor (Malvern, PA) and reacts with rat P-glycoproteins including MDR1a, MDR1b, and MDR2. Cy2-conjugated goat anti-rabbit antibody and Cy3-conjugated goat anti-mouse antibody were purchased from Dianova (Hamburg, Germany).

RNA isolation, RT-PCR, and sequencing. Total RNA was isolated from mucosa samples using the RNAclean solution (Hybaid-AGS, Heidelberg, Germany) according to the manufacturer’s manual. RT-PCR was performed with Ready-To-Go RT-PCR Beads (Amersham Pharmacia Biotech) according to the manufacturer’s manual. The RNA was reverse transcribed with pd(T)12–18-primer using 2 μg total RNA at 42°C for 30 min. For PCR, 35 cycles of denaturation (94°C for 60 s), annealing (52°C for 60 s), and elongation (72°C for 90 s) were performed using the following primer pairs selective for the respective MRP family member: okoplus3 (5’-GGGATAAATCTCAGTGGT-3’) against okorev2 (5’-ATATGTCCTCA-CAGAGTTG-3’) (NIH/GenBank accession no. X96,393) for rat MRP2 and omrp3rat.for1 (5’-TAAGGTTGGATAGCACG-3’) against omrp3rat.rev1 (5’-GGCTAGGCACACGAGCT-3’) (NIH/GenBank accession no. AB010467) for rat MRP3. The β-actin control PCR was performed using a commercially available β-actin primer mixture (Stratagene, Amsterdam, The Netherlands). Amplified fragments were analyzed by agarose gel electrophoresis, purified with GFX PCR DNA and Gel Band Purification Kit (Amersham Pharmacia Biotech) and sequenced by the dideoxynucleotide chain termination method of Sanger using the PE DNA-Sequencing Kit (Perkin-Elmer Applied Biosystems, Warrington, United Kingdom).

Immunoblot analysis. Mucosa samples were homogenized on ice with PBS in the presence of protease inhibitors (Protease inhibitor cocktail; Sigma, Deisenhofen, Germany) using a glass homogenator (Wheaton, Millville, NJ). Samples obtained from different rat intestinal segments (50 μg protein) or rat liver homogenate (30 μg protein) were mixed with sodium dodecyl sulfate sample loading buffer, incubated at 37°C for 60 min, and separated on 7.5% polyacrylamide gels in the presence of β-mercaptoethanol (29). After transfer to polyvinylidene difluoride membranes, blots were blocked for anti-rabbit antibody and Cy3-conjugated goat anti-mouse antibody were purchased from Dianova (Hamburg, Germany).

Fig. 2. Immunolocalization of MRP2 and MRP3 in rat intestine. Immunofluorescence analysis was performed on sections of jejunum (A-C) and ileum (D-F) from Wistar rats. Incubation of cryosections with the MRP2-specific EAG15 antibody revealed apical localization of MRP2 in the rat small intestine with the strongest fluorescent signals observed in the jejunum (B). Apical domains were identified by double labeling with the C219 antibody against P-glycoproteins (P-gps, A). Co-localization of MRP2 and P-glycoproteins results in the yellow color on superimposition of both pictures (C). Goblet cells are marked by arrowheads. Immunofluorescence using a rat MRP3-specific antibody revealed a basolateral membrane localization of MRP3 in enterocytes with the strongest expression in ileum (E and F) and colon (not shown). Double labeling for P-glycoproteins (D and F) demonstrated the absence of MRP3 from the apical membrane domain. Nuclei (blue) were stained using Hoechst 33258.
1 h in 5% low fat dried milk dissolved in Tris-buffered saline containing 0.05% Tween 20 (TTBS). Membranes were incubated for 1 h with the primary and secondary antibody, respectively, diluted in 5% dried milk dissolved in TTBS. After each incubation, blots were washed three times with TTBS. Antibody binding was detected using horseradish peroxidase-conjugated secondary antibodies (Bio-Rad, Muenchen, Germany) and the enhanced chemiluminescence technique (Amersham-Buchler, Braunschweig, Germany). The following dilutions of antibodies were used: EAG15 at 1:5,000; anti-MRP3 antibody at 1:500; horseradish peroxidase-conjugated goat anti-rabbit antibody at 1:2,500. Immunoreactive bands on autoradiography films were scanned (Epson GT 9600; Epson, Tokyo, Japan) and quantified using Raytest image software (Raytest, Straubenhardt, Germany).

**Immunofluorescence microscopy.** For immunofluorescence microscopy, rat intestinal tissue was mounted in Tissue-Tek (Miles, Elkhart, IN) and deep-frozen in liquid nitrogen. Tissue sections (2–4 μm) were prepared with a cryotome (model 2800E FrigoCut; Leica, Nussloch, Germany), air-dried for 2 h, and fixed for 10 min with 3% paraformaldehyde/PBS followed by cold methanol (−20°C) for 10 s. After rehydration with PBS, sections were incubated with the primary antibodies for 60 min and then washed three times for 10 min with PBS and incubated with the combined secondary antibodies for 30 min. After being washed three times with PBS, sections were rinsed with distilled water, air-dried, and mounted with Moviol (Hoechst, Frankfurt, Germany). All antibodies were diluted in PBS containing 5% fetal calf serum and 0.05% Tween at the following dilutions: EAG15 at 1:100; anti-MRP3 antibody at 1:100; C219 at 1:10; Cy2-
conjugated anti-rabbit IgG at 1:200; and Cy3-conjugated anti-mouse IgG at 1:500. Nuclei were stained with Hoechst 33258 (Molecular Probes, Eugene, OR). Micrographs were taken using an Olympus AX70 microscope on Kodak Elite II 400 ASA films (Kodak, Rochester, NY).

RESULTS

RT-PCR analysis of MRP3 and MRP2 mRNA expression. MRP3 and MRP2 mRNA expression in duodenum, jejunum, ileum, and colon was analyzed by RT-PCR using rat-specific primers against MRP3 and MRP2. Amplification of a 661-bp fragment of β-actin was used as an internal control, demonstrating the integrity of the isolated total RNA (Fig. 1). The MRP amplification products showed the expected size of 784 and 868 bp for rat MRP3 and MRP2, respectively. The specificity of the PCR products was confirmed by sequencing. MRP3 expression was low in the proximal part of the small intestine showing a gradual increase towards the ileum. The strongest MRP3 expression was detected in rat colon (Fig. 1). MRP2 expression was detected in all segments of the small intestine, with the strongest bands observed in jejunum (Fig. 1). In contrast to MRP3, no MRP2-specific products were obtained in rat colon.

Localization of MRP3 to the basolateral domain rat enterocytes. Double-label immunofluorescence microscopy was used to study localization and distribution of MRP2 and MRP3 in the different parts of rat intestine. MRP2 expression was restricted to the apical domains in enterocytes of the small intestine (Fig. 2B) as described previously (31). The C219 antibody was used to identify the apical domains of enterocytes (Fig. 2A). Superimposition of both images revealed colocalization of both signals yielding in a yellow color (Fig. 2C). MRP2 expression increased from duodenum to jejunum, where the strongest immunostaining was observed (Fig. 2B). In the terminal ileum, the immunofluorescent staining was less intense. In rat colon, no specific apical signals were obtained. In general, the fluorescence intensity in the small intestine decreased from the tip of the villus to the crypt, where no staining was observed (not shown).

In contrast to MRP2, MRP3 was localized to the lateral and basal membrane of enterocytes (Figs. 2, E and F, and 3). MRP3 was absent from the apical domains as revealed by double-label immunofluorescence microscopy using the apical marker C219 antibody against P-glycoproteins (Figs. 2, D and F, and 3). In duodenum and jejunum, MRP3 expression was weak and limited to enterocytes around the lumen of the gut (Fig. 4). However, in ileum and colon MRP3 expression was markedly increased (Fig. 4). Similar to MRP2 distribution, no MRP3 staining was observed at the bottom of the crypts (arrows in Fig. 4) and fluorescent intensity increased toward the surface of the gut.

Differential expression of MRP2 and MRP3 in rat intestine. Immunoblots on mucosa samples from duodenum, jejunum, ileum, and colon were probed with polyclonal antibodies directed against rat MRP2 and rat MRP3. Incubation with the EAG15 antibody against rat MRP2 resulted in the specific 190-kDa band in duodenum, jejunum, and, much weaker, in ileum of rat small intestine (Fig. 5). Compared with jejunum (100%), where MRP2 expression was the highest, MRP2 expression decreased to 76 ± 24 and 56 ± 16% in duodenum and ileum, respectively. The lowest MRP2 expression was found in colon (6 ± 1%). MRP3 expression in the different parts of rat intestine was analyzed using a rat MRP3 specific polyclonal antibody (32, 37). Immunoblot analysis of mucosa samples revealed a band at 190 kDa in all segments of rat intestine. As reported previously (32, 37), an additional band at ∼120 kDa was observed in all mucosa preparations (not shown). The intensity of this band corresponded to the intensity of the 190-kDa band; however, it is unclear how this band is related to the full-size transporter. In contrast to the MRP2 distribution, the highest MRP3 expression was found in rat ileum.
enzyme expression increases from crypt to villus (8), whereas MRP3 expression in duodenum (26 ± 13%) and jejunum (28 ± 9%) was considerably low.

**DISCUSSION**

Transport of organic anions across the enterocyte basolateral membrane into the blood represents an important step in intestinal absorption and has been studied in vivo using the intestinal loop model (10, 21, 36). Furthermore, the recycling of biliary constituents such as bile salts, steroids, vitamins, and drugs, known as enterohepatic circulation (reviewed in Ref. 5), involves reabsorption and transport across the enterocyte basolateral membrane and depends on distinct transport systems at the apical and basolateral membrane of enterocytes (38). However, the molecular identity of the transport proteins involved is largely unknown.

Transport studies using multidrug resistant cancer cell lines and transfected cell lines have identified members of the MRP-family as ATP-dependent transporters for lipophilic substances conjugated with glutathione, glucuronate, and sulfate (3, 22). Recent findings have demonstrated extensive expression of MRP3 mRNA in tissues of rat and human intestine (13, 23, 25, 26). Functional analysis indicated that gluturonate conjugates are preferred MRP3 substrates and that, in contrast to MRP1 and MRP2, glutathione conjugates are poor substrates for MRP3 (15). Moreover, rat MRP3 exhibits an unique ability to transport monovalent (taurocholate and glycocholate) and sulfated bile salts (taurlithocholate-3-sulfate and taurochenodeoxycholate-3-sulfate) (16, 44). To further characterize the role of MRP3 in intestinal transport, we studied the expression and localization of MRP3 in different segments of rat intestine using RT-PCR analysis, immunofluorescence analysis, and immunoblot analysis.

In the present study, we have localized rat MRP3 to the basolateral membrane domain of enterocytes (Figs. 2, E and F, and 3). The basolateral localization enables this conjugate export pump to contribute to the secretion of organic anions from enterocytes into blood. Moreover, our results were consistent with a high expression of MRP3 in ileum and colon, suggesting an important role for MRP3-mediated uptake in the distal part of rat intestine. MRP3 expression in duodenum and jejunum was low (Fig. 4). In contrast, MRP2 was mainly expressed in the proximal part of rat small intestine with a maximum expression in jejunum. The latter results are in good agreement with previous findings on MRP2 expression in rat intestine (11, 30, 31), indicating the reliability of the mucosa isolation technique used (6). Differential expression of MRP3 with respect to the villus crypt axis resembles MRP2 distribution in the proximal rat intestine, for which increasing protein expression has been demonstrated from the crypt to the villus (31, 40). Similarly, phase II enzyme expression increases from crypt to villus (8, 34), underlining the coordinate expression and cooperative function of conjugation enzymes with conjugate export pumps.

Considering the transport characteristics, localization, and expression pattern of rat MRP3, the uptake of the following compounds from intestine into blood may be, in part, attributed to ATP-dependent transport across the enterocyte basolateral membrane via MRP3. First, bile salts, known to undergo extensive enterohepatic circulation (reviewed in Ref. 17), are mainly reabsorbed in the ileum and have been identified as MRP3 substrates (16, 44). Second, ethinylestradiol and other steroids are efficiently glucuronidated in the intestine (7, 9). Steroid glucuronidation activity increases along the length of the intestine with the highest levels found in terminal ileum and colon (35). Transport of E217G from mucosa to serosa has been described in rat intestine (36) and ATP-dependent transport of E217G was markedly enhanced in MRP3-transfected membrane vesicles (15).

Third, 1-naphthol and other phenolic compounds are glucuronidated by the intestinal mucosa (2, 8, 12). Vectorial transport of 1-naphthol glucuronide from mucosal to serosal side occurs mainly in the ileum and colon of rat intestine (20). In transport studies, 1-naphthol glucuronide has been shown to inhibit MRP3-mediated transport of E217G, indicating that this compound is recognized by MRP3 (15). Fourth, flavonoids may be glucuronidated to different extent in the intestine (7). Transport of flavonoids from mucosa to serosa has been demonstrated in vitro using intestinal Caco-2 cells (41, 42). Intestinal loop experiments demonstrated carrier-mediated transport of riboflavin in rat small and large intestine (43). Furthermore, flavonoids may be potent inducers of MRP-ATPase activity (18), and flavonoid transport can be inhibited by the MRP-inhibitor MK-571 (41, 42). In summary, this study is the first demonstration that MRP3 protein is expressed in the rat intestine. Unlike MRP2, which is predominantly expressed at the apical domain of enterocytes from the proximal small intestine, MRP3 is highly expressed at the basolateral membrane of enterocytes from ileum and colon, where it may be involved in the ATP-dependent transport of organic anions including bile salts, steroids, vitamins, and drugs from enterocytes into blood.

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