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European Journal of Pharmacology 400 (2000) 195–198

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Short communication

Expression and immunolocalization of multidrug resistance protein 2 in rabbit small intestine

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Received 27 March 2000; received in revised form 24 May 2000; accepted 29 May 2000

Abstract

Multidrug resistance protein 2 (MRP2) is an ATP-dependent transporter of anionic drugs and conjugates. It functions as an efflux pump in the apical membranes of liver and kidney cells, but its membrane localization in small intestine has not yet been defined. The present study demonstrates exclusive localization of Mrp2 to the brush-border (apical) membrane of villi, decreasing in intensity from the villus tip to the crypts. In immunoblot analysis of crude membranes of various rabbit tissues, Mrp2 was only found in small intestine, kidney and liver. These results are in-line with the supposed function of Mrp2 in drug excretion.

Keywords: Multidrug resistance protein; MRP2 (multidrug resistance protein 2); Intestine

1. Introduction

Multidrug resistance protein 2 (MRP2) is an ATP-dependent drug transporter belonging to a subgroup of six MRPs within the ATP-binding cassette superfamily (Borst et al., 1999; König et al., 1999). The specificity of rat Mrp2 comprises a variety of organic anions, including unconjugated (*p*-aminohippurate, bromosulphophthalein, folates) and conjugated (leukotriene C_4, estradiol-17β-D-glucuronide, bilirubin-glucuronides) substrates (König et al., 1999; Van Aubel et al., 2000). Comparative studies between wildtype rats and the hyperbilirubinemic rat strains EHBR and GY/TR$^-$, which are defective for functional Mrp2, have identified a number of substrates. Furthermore, substrate specificity has been characterized by using various types of cell lines overexpressing recombinant human MRP2, rat or rabbit Mrp2 (Ito et al., 1998; Van Aubel et al., 1998, 1999; Evers et al., 1998; Cui et al., 1999). MRP2 also confers resistance to various cationic anti-cancer drugs, such as cisplatin, vincristine, etoposide and methotrexate (Koike et al., 1997; Cui et al., 1999; Hooijberg et al., 1999).

Most abundant expression of human and rat *MRP2/mrp2* mRNA is found in liver, whereas lower levels are found in kidney and small intestine (Ito et al., 1997; Kool et al., 1997). In human and rat, MRP2/Mrp2 is located at the renal proximal tubule brush-border membrane and the hepatocyte canalicular membrane (Paulusma et al., 1997; Schaub et al., 1997, 1999). However, the membrane localization in small intestine has not yet been established. In contrast to the rat and human ortholog, rabbit *mrp2* mRNA is highly expressed in small intestine (Van Kuijck et al., 1996). Therefore, we investigated expression and localization of Mrp2 in rabbit small intestine.

2. Materials and methods

2.1. Antibodies

Affinity-purified rabbit polyclonal anti-actin antibodies (20–33) were purchased from Sigma Immunochemicals (St. Louis, MO, USA). Polyclonal antibody GP8 was obtained by immunizing guinea pigs with a glutathione

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0014-2999/00/$ - see front matter
PII: S0014-2999(00)00391-5

S-transferase fusion protein containing the 159 carboxyl-terminal amino acids (1405–1564) of rabbit Mrp2.

2.2. Membrane isolations

All membrane preparations were performed at 4°C. Rabbit tissues were excised and homogenized in TS-buffer (250 mM sucrose, 10 mM Tris-HEPES, pH 7.4). After centrifugation at $200 \times g$, the supernatant was centrifuged at $100{,}000 \times g$ for 30 min and the pellet of crude membranes was dissolved in TS-buffer. Sf9 cells were infected with a recombinant baculovirus encoding rabbit Mrp2 or the β-subunit of the rat H^+/K^+ ATPase (mock) and membranes were isolated as described (Van Aubel et al., 1998). Membranes were treated with PNGase F (*N*-glycosidase F) according to the manufacturer (New England Biolabs, Leiden, The Netherlands).

2.3. Immunoblotting

Protein equivalents (5 μg) of membrane preparations were solubilized in Laemmli sample buffer, heated at 65°C for 10 min, separated on a 6% (for detection of Mrp2) or 10% (for detection of β-actin) polyacrylamide gel and transferred to Hybond-C pure nitrocellulose membrane (Amersham, Buckinghamshire, UK). Transfer of proteins was confirmed by the reversible staining of the membrane with Ponceau Red. Subsequently, the blot was blocked for 60 min with 5% nonfat dry milk powder in Tris-buffered saline supplemented with 0.3% Tween-20 (TBS-T) and washed twice with TBS-T. The membrane was incubated at 4°C for 16 h with polyclonal antibodies GP8 or 20–33 diluted 1:1000 in TBS-T. After two times of washing for 5 min with TBS-T, the blot was blocked for 30 min as described above. The blot was then washed twice with TBS-T and incubated at room temperature for 60 min with affinity-purified horseradish peroxidase conjugated goat anti-guinea pig antibody or affinity-purified horseradish peroxidase conjugated goat anti-rabbit antibody (Sigma Immunochemicals, St. Louis, MO) diluted 1:5000 in TBS-T. Finally, the blot was washed twice for 5 min with TBS-T and TBS, respectively. Proteins were visualized using enhanced chemiluminescence (Pierce, Rockford, IL).

2.4. Immunohistochemistry

Infected Sf9 cells were fixed in 100% methanol for 10 min at −20°C and air-dried. Slices of rabbit small intestine were fixed in 1% (v/v) periodate–lysine–paraformaldehyde fixative for 2 h, washed with 20% (w/v) sucrose

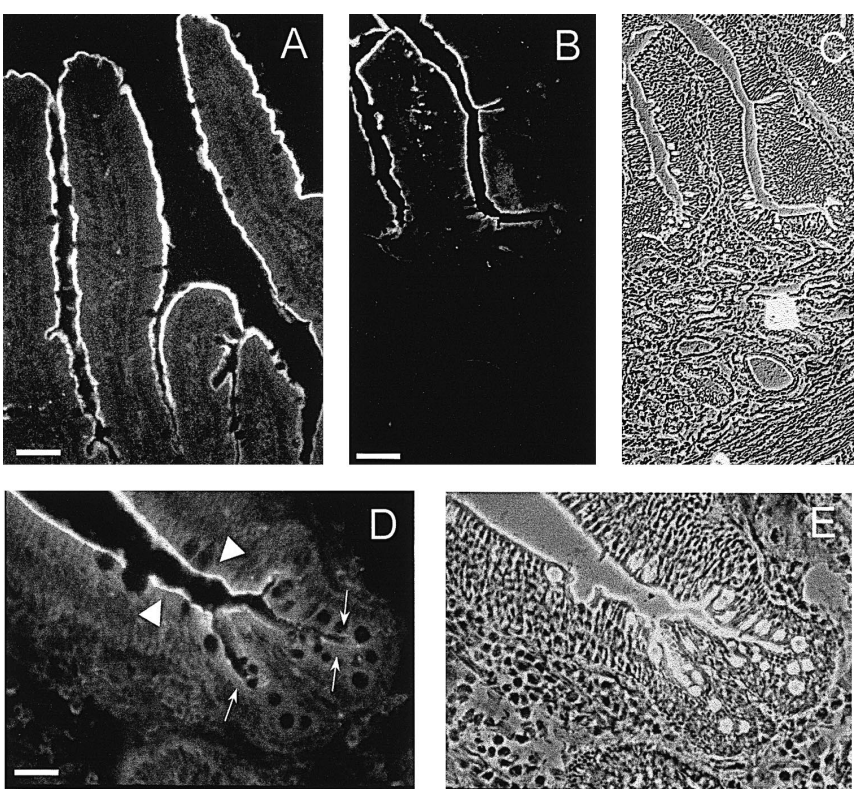

Fig. 1. Immunolocalization of Mrp2 in rabbit small intestine. PLP-fixed sections of rabbit small intestine were incubated with polyclonal antibody GP8. (A and B) Mrp2-specific immunofluorescence was located to the apical membrane of the whole villus. (D) High magnification of a crypt area showing decreasing Mrp2-specific immunofluorescence from the lower part of the villus (*arrow heads*) towards the crypt (*arrows*). (C and E) Phase-contrast microscopy of the section identical to (B) and (D), respectively. White marker bar at the *bottom left* equalizes in length to 216 μm (A and B) and 54 μM in (D).

in phosphate-buffered saline and subsequently frozen in liquid N_2. Coverslips with Sf9 cells and tissue sections (7 μM) were blocked with 10% (v/v) goat serum in TBS for 15 min, washed three times with TBS and incubated with GP8 (diluted 1:50 in TBS) for 16 h at 4°C. After washing in TBS, coverslips and tissue sections were incubated with 1:50 diluted fluorescein isothiocyanate (FITC)-conjugated affinity-purified goat anti-guinea pig antibody (Sigma Immunochemicals, St. Louis, MO, USA), dehydrated by 100% methanol, mounted in mowiol (Hoechst) and analyzed by immunofluorescence microscopy.

3. Results

Expression of human *MRP2* and rat/rabbit *mrp2* mRNA in small intestine has been shown in various reports, but localization of the gene product is still not defined. Indirect immunofluorescence using a polyclonal antibody (GP8) raised against rabbit Mrp2 showed intense immunopositive fluorescence at the brush-border (apical) membrane of villi in periodate–lysine–paraformaldehyde fixed sections of rabbit duodenum (Fig. 1A and B). The intensity of fluorescence decreased along the length of the villus towards the crypts, where no staining was observed (Fig. 1B and D). Other parts of the duodenum, i.e. submucosa and muscle layers were negative (Fig. 1B and C). No staining was found in sections incubated with preimmune serum or with GP8 preincubated with the fusion protein antigen (not shown). In agreement with previous reports for human/rat MRP2/Mrp2, localization was found at the brush-border membrane of renal proximal tubules and the hepatocyte canalicular (apical) membrane (not shown).

To confirm the specificity of the polyclonal antibody GP8, crude membranes from various rabbit tissues were subjected to immunoblot analysis. In crude membranes from only liver, kidney and small intestine, a single protein was detected with a molecular weight of approximately 190 kDa (Fig. 2A), which represents glycosylated Mrp2, as revealed by PNGase F digestion (Fig. 2B). Loading of equal amounts of membranes was confirmed by detection of β-actin. Furthermore, immunocytochemistry and immunoblot analysis showed expression of recombinant Mrp2 in Sf9 cells infected with an Mrp2-encoding baculovirus (Fig. 2C).

4. Discussion

In the present study, we demonstrate the immunolocalization of Mrp2 to the brush-border membrane of rabbit small intestinal villi. The expression level of Mrp2 was highest at the villus tip and decreased from the tip towards the crypts. Other parts of the small intestine were negative.

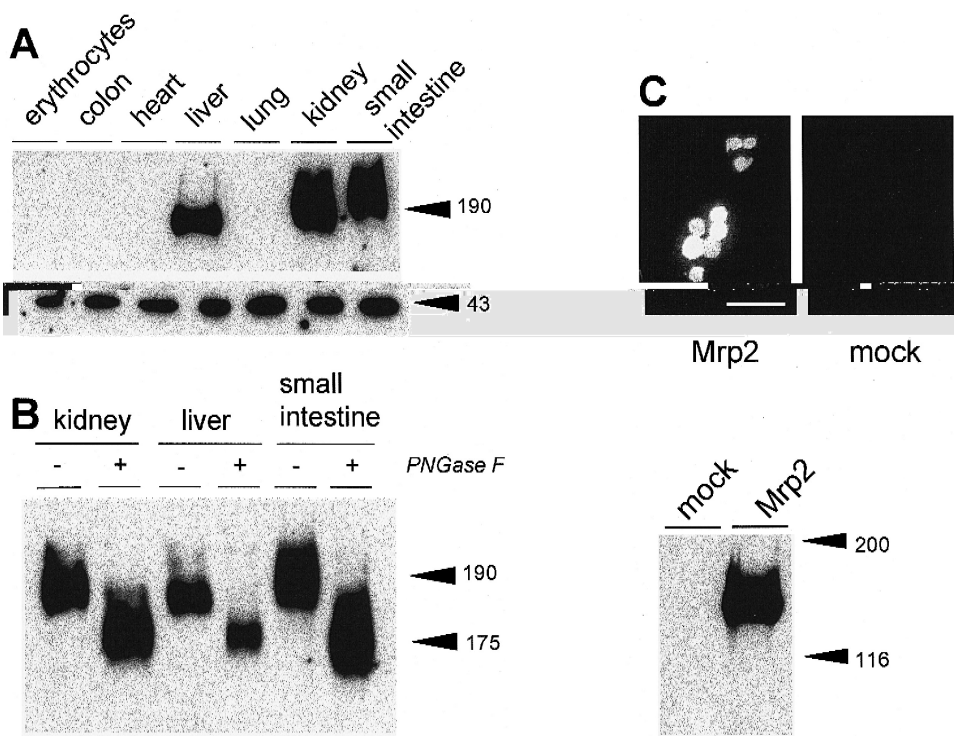

Fig. 2. Expression of Mrp2 in Sf9 cells and rabbit tissues. (A) crude membranes prepared from rabbit erythrocytes, colon, heart, liver, lung, kidney and small intestine were subjected to immunoblot analysis for detection of rabbit Mrp2 (*upper panel*) and β-actin (*lower panel*), respectively. (B) crude membranes of kidney, liver and small intestine were treated with PNGase F. (C) Sf9 cells were cultured and mock-infected or infected with an Mrp2-encoding baculovirus. Crude membranes were isolated and subjected to immunoblot analysis and cells were fixed and analyzed by immunofluorescence microscopy. White marker bar at the *bottom left* (C) equalizes in length to 216 μm. *Arrow heads* indicate molecular weight in kilodaltons.

The specificity of the antibody used for immunohistochemistry was confirmed with immunoblot analysis. Glycosylated Mrp2 was only found in crude membranes of rabbit liver, kidney and small intestine, whereas other tissues, in which various other MRPs are expressed (Kool et al., 1997), were negative. Furthermore, expression of underglycosylated Mrp2 in Sf9 cells was shown both by immunoblotting and immunocytochemistry. The clear immunolocalization for Mrp2 in rabbit small intestine as demonstrated here is in contrast with the apparent difficulty to detect its ortholog in sections of human and rat small intestine (Gotoh et al., 2000). This may result from the relatively low expression of MRP2/Mrp2 in human/rat small intestine compared to liver, in which MRP2/Mrp2 has been clearly demonstrated (Ito et al., 1997; Paulusma et al., 1997; Kool et al., 1997). Still, comparative studies using everted sac isolated from wildtype and GY/TR$^-$ rats have proven the presence of intestinal Mrp2 at the functional level (Gotoh et al., 2000). Furthermore, brush-border membrane vesicles from the Caco-2 cell line, which shows characteristics of normal enterocytes, exhibit ATP-dependent uptake of *S*-(dinitrophenyl)-glutathione and estradiol-17β-D-glucuronide with similar K_m values as has been reported for recombinant human MRP2 (Cui et al., 1999; Hirohashi et al., 2000).

The localization of Mrp2 to the intestinal brush-border membrane is in accordance with a role in drug excretion from blood to lumen, as has been described for various drugs (Saito et al., 1996). Mrp2 might also limit the oral bioavailability of anionic drugs (Saitoh et al., 1997), analogous to the barrier function of intestinal P-glycoprotein for cationic drugs (Schinkel et al., 1997).

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