Ezrin regulates the expression of Mrp2/Abcc2 and Mdr1/Abcb1 along the rat small intestinal tract

Takafumi Nakano, Shuichi Sekine, Kousei Ito, and Toshiharu Horie

Laboratory of Biopharmaceutics, Graduate School of Pharmaceutical Sciences, Chiba University, Chuou-ku, Chiba, Japan
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Nakano T, Sekine S, Ito K, Horie T. Ezrin regulates the expression of Mrp2/Abcc2 and Mdr1/Abcb1 along the rat small intestinal tract. Am J Physiol Gastrointest Liver Physiol 305: G807–G817, 2013. First published October 3, 2013; doi:10.1152/ajpgi.00187.2013.—Multidrug resistance (MDR1/ABCB1) and multidrug resistance-associated protein 2 (MRP2/ABCC2) are well-known efflux transporters that are involved in the prevention of xenobiotic absorption into the blood and the expression of efflux transporters is regulated by head-to-tail folding of the ezrin/radixin/moesin (ERM) family proteins, which are involved in the regulation of membrane localization. However, the specific regulation of Mrp2/Abcc2 and Mdr1/Abcb1 by ERM proteins is not well understood. The current study investigated the expression of Mrp2/Abcc2 and Mdr1/Abcb1 in the rat small intestine to elucidate the regulation of efﬂux transporters by ERM proteins.

Molecular biology and molecular docking of ezrin, MRP2/ABCC2, MDR1/ABCB1, and ezrin

The small intestine is a well-differentiated organ that selectively absorbs substances needed for the survival of the organism, while also functioning as a barrier to prevent the free passage of xenobiotics (i.e., toxins, carcinogens, and drugs). Specific efflux transporters are found within the small intestinal barrier system (11), including members of the ABC protein (ATP-binding cassette) superfamily (e.g., MRP2/ABCC2 and MDR1/ABCB1). These transporters are located on the apical membrane of the small intestinal epithelium, where they prevent the absorption of xenobiotics into the blood (6, 17).

MRP2/ABCC2 and MDR1/ABCB1 are characterized by specific expression patterns in the small intestinal epithelium. In the rat intestine, Mrp2/Abcc2 is expressed along a gradient, with the highest levels of protein in the proximal intestine (duodenum and jejunum) and the lowest levels in the terminal ileum and colon. On the other hand, Mdr1/Abcb1 expression is lowest in the proximal region and increases in the distal direction.

Notably, a divergence between transporter mRNA and protein expression was observed in some of these investigations. For example, Berggren et al. (2) demonstrated a relatively poor correlation between mRNA and protein levels for MRP2/ABCC2 and MDR1/ABCB1 in human specimens. Furthermore, Mottino et al. (26) showed that the Mrp2/Abcc2 mRNA content was similar in proximal and distal segments of the rat small intestine, despite the significant decrease in protein levels along the small intestinal tract. Although the precise mechanisms are unknown, these reports suggest that intestinal MRP2/ABCC2 and MDR1/ABCB1 are postranscriptionally and/or posttranslationally regulated.

Posttranslational regulation of efflux transporters involves dynamic changes in the localization of the efflux transporters from the intracellular vesicular compartment to the apical membrane. In hepatocytes, the cellular localization of MRP2/ABCC2 is reversibly regulated under pathological and physiological conditions (e.g., oxidative stress and lipopolysaccharide-induced cholestasis; Refs. 30, 33). Radixin, a member of the ERM (ezrin/radixin/moesin) family of proteins, appears to be essential for the modulation of drug transporter localization. Radixin exerts its actions by serving as a molecular anchor, connecting many membrane proteins to the actin cytoskeleton (21).

Recently, we and others reported that the apical localization of intestinal MRP2/ABCC2 is regulated by ezrin instead of radixin (27, 40). Additional studies also demonstrated that ERM proteins are critical modulators of MDR1/ABCB1 membrane localization and expression in various experimental models (3, 23, 25). While Yano et al. (41) also suggested that radixin is contributed to the expression and activity of Mdr1/Abcb1 in the mice intestine, others and our past report confirmed ezrin (but not other ERM family) is predominantly expressed in the small intestine (16, 27). Therefore, in this study, we focus on ezrin, which has a central role in the anchoring function in intestinal transporters because of the lack of, or at least very scarce, expression of ERM proteins other than ezrin. The function of ERM proteins is conformationally regulated by head-to-tail folding (binding of the NH2-terminal 4.1-erm-radixin-moesin domain to the COOH-terminal ERM-associated domain). An important conserved phosphorylation site in the COOH-terminal region of ezrin, radixin, and moesin (Thr567 in ezrin, Thr564 in radixin, and Thr558 in moesin) is related to the transformation of ERMs from the dormant closed form to the active open form (13, 34). The active open form efficiently binds to membrane proteins through the NH2-termi-
nal domain and also to the cytoskeleton through the COOH-terminal domain.

The present study focused on the characteristic expression pattern of MRP2/ABCC2 and MDR1/ABCB1 along the small intestinal tract, as well as that of ezrin and phosphorylated ezrin (p-ezrin). To clarify the causality between ezrin expression/COOH-terminal phosphorylation and efflux transporter expression, we also explored the impact of wild-type (WT) ezrin and a nonphosphorylatable mutant, T567A, on MRP2/ABCC2 and MDR1/ABCB1 expression and localization in Caco-2 human intestinal epithelial cells.

MATERIALS AND METHODS

Chemicals and antibodies. Rabbit anti-protein kinase C (PKC)α and mouse anti-β-actin antibodies were obtained from Sigma-Aldrich (St. Louis, MO). Rabbit anti-phospho-PKCα/βII antibody, which recognizes the T638/641 phosphorylated form of PKCα/βII was from Cell Signaling Technology (Beverly, MA). Mouse anti-MDR/ABC1 (1+3 antibody (C219) was obtained from Calbiochem (San Diego, CA). Mouse anti-MRP2/ABCC2 antibody (M2II6) was from Millipore (Bedford, MA). The rabbit anti-Mrp2/Abcc2 polyclonal antibody was raised against a 12-amino acid sequence at the carboxy terminus of rat Mrp2/Abcc2. The ezrin-specific monoclonal antibody (M11) was kindly provided by S. Tsukita (Kyoto University Graduate School of Medicine, Kyoto, Japan). Rabbit anti-ERM and COOH-terminally phosphorylated ezrin (Thr567)/radixin (Thr654)/moesin (Thr558; p-ERM) antibodies were purchased from Chemicon (Temecula, CA). The mouse anti-ezrin monoclonal antibody (4A5) and the horseradish peroxidase-linked secondary antibodies used in the immunoblot analysis were from Santa Cruz Biotechnology (Santa Cruz, CA). The Alexa Fluor 488-labeled anti-rat IgG, Alexa Fluor 546-labeled anti-mouse IgG, and Alexa Fluor 633-labeled anti-mouse IgG used in the immunofluorescence analysis were from Life Technologies (Geithsburg, MD), as were the pcDNA 3.1 Directional TOPO Expression Kit and TRIZol. The pEGFP-N1 plasmid was obtained from Clontech (Palo Alto, CA). EZ-Link Sulfo-NHS-Biotin and immobilized streptavidin beads were from Pierce Chemical (Rockford, IL). ReverTra Ace was obtained from Toyobo (Osaka, Japan). Pfu turbo DNA polymerase and Power SYBR Green were from Stratagene (La Jolla, CA). EZ-Link Sulfo-NHS-Biotin and immobilized streptavidin were from Life Technologies (Gaithersburg, MD) and the pcDNA 3.1 Directional TOPO Expression Kit and TRIZol. The small intestinal mucosa homogenate and the brush border membrane sample were subjected to SDS-PAGE and immunoblot analysis, as described below.

SDS-PAGE and immunoblot analysis. Proteins were lysed in Laemmli sample buffer and subjected to SDS-PAGE, with the exception of the biotinylated samples (described below). In the latter case, the eluents were directly subjected to analysis. Proteins were loaded into a polycrylamide slab gel containing 0.1% SDS, electrophoresed, and transferred onto an Immobilon-P Transfer Membrane filter (Millipore, Bedford, MA). The membrane was blocked for 1 h at room temperature or overnight at 4°C with Tris-buffered saline containing 0.05% Tween 20 (TTBS) and 3% BSA. Blocked membranes were then probed at room temperature for 1 h or 4°C overnight with primary antibodies diluted in TTBS containing 0.1% BSA. The membrane was incubated for 1 h at room temperature with horseradish peroxidase-conjugated secondary antibodies diluted in TTBS containing 0.1% BSA. Bound antibodies were detected by using an LAS-1000 Luminesc Image Analyzer (Fuji Photo Film, Tokyo, Japan) and an enhanced chemiluminescence detection system (GE Healthcare, St. Louis, MO).

Semiquantitative real-time PCR. Total RNA was prepared from the rat small intestine by using TRIZol (Life Technologies) according to the manufacturer’s instructions. Reverse transcription was performed with ReverTra Ace reverse transcriptase (Toyo Bios). Real-time PCR was performed by using Power SYBER Green PCR Master Mix (Eurogentec, Seraing, Belgium) and primer sets for Mrp2/Abcc2, Mdr1a, and ezrin (Mrp2/Abcc2: forward, 5'-TCTGCGATTCGCGC-3' and reverse, 5'-GTCCCCACACAGCCACCAG-3'; Mdr1a: forward, 5'-TCTGCGCGCTTCCAT-3' and reverse, 5'-TACATCGG5'TTCTCTATTG-3'; and ezrin: forward, 5'-CGTCGCTGACTCTGAGCAATG-3' and reverse, 5'-TTAATCTTGTCGCCGCGCTG-3'). Expression of each mRNA was quantified relative to 28S ribosomal RNA (rRNA; forward, 5'-ACTGCGGGCTGGGGGAG-TAAC-3' and reverse, 5'-CCATGCGGGCCACTGCTG-3'). Real-time PCR amplification was determined by utilizing an ABI Prism 7000 machine (Applied Biosystems, Foster City, CA).

dDNA cloning and plasmid construction. Total RNA was isolated from Caco-2 cells with TRIZol (Life Technologies) and mRNA was obtained by using Oligotex-dT30> Super (Takara) according to the manufacturer’s instructions. Full-length ezrin cDNA was obtained via reverse transcriptase-PCR by using PrimeSTAR Max DNA polymerase and isolated Caco-2 mRNA as the template and cloned into pcDNA 3.1D/V5-His-TOPO without the tag peptide at the COOH terminus. The following primers were used for amplification: forward, 5'-CACCATGGCCACACCATGACTCCAGATCC-3' and reverse, 5'-TTACAGGCCTCGGATCTCGTTTGTCGGC-3'.

To create the ezrin constitutively dephosphorylated mutant (T567A), cloned WT ezrin cDNA was subjected to PCR-mediated mutagenesis by using the following primers to mutate codon 567 from Thr to Ala: forward, 5'-CCGCGAACAAGTACAAGGCTCTGGCGGACATCG-3' and reverse, 5'-CCGATGTCGCGCGGAGGCCAGGCACCGT-3'.

To create the ezrin constitutively phosphorylated mutant (T567D), the following primers were used to mutate codon 567 from Thr to Asp: forward, 5'-CGGAGAAGTCGATCTGAGGCTCTGGGACATCG-3' and reverse, 5'-CGGATGTCGCGCGGAGGCCAGGCACCGT-3'.
The cloned WT, T567A, and T567D cDNAs were amplified by using the following primers: 5'-CTAGCTAGCCCCCATCGGAAACCAATTCAATG-3' and 5'-CTGCTAGCTACGAGCCGCTCCGAGACCCGCTG-3'. The amplified cDNAs were digested with the restriction enzymes EcoRI and NheI and cloned into the EcoRI and NheI sites of the expression vector pEGFP-N1. The resulting plasmids were used to obtain transiently transfected WT ezrin- and mutant ezrin-EGFP-expressing cells.

Confocal laser scanning microscopy analysis of Caco-2 cells transiently expressing WT ezrin- and mutant ezrin-EGFP. Caco-2 cells were seeded onto collagen-coated glass coverslips in 24-well plates and cultured for 12 h. They were then transfected with the plasmids constructed as described above (1 µg) by using the lipofection method and FuGENE HD Transfection Reagent (3 µl; Promega, Madison, WI) according to the manufacturer’s instructions. At 72 h after the initiation of transfection, the cells were fixed in phosphate-buffered saline without CaCl$_2$ and MgCl$_2$ [PBS(–)] containing 4% paraformaldehyde for 20 min at room temperature. The cells were washed twice with PBS(–), incubated in PBS(–) containing 0.2% Triton X-100 for 10 min at room temperature to permeabilize the plasma membrane, washed twice with PBS(–), and incubated for 1 h at room temperature with rhodamine phalloidin (Life Technologies). The samples were then analyzed using a confocal laser scanning microscope, LSM510 type (Carl Zeiss, Jena, Germany).

Establishment of Caco-2 cells stably expressing recombinant ezrin. Caco-2 cells were seeded into 10-cm dishes at a density of 1.0 x 10$^6$ cells/dish, cultured for 12 h, and transfected with the constructed plasmids (1 µg) by using the lipofection method and FuGENE HD Transfection Reagent (3 µl). At 24 h after the initiation of transfection, the cells were selected in cell culture medium containing G418 (1.0 mg/ml) to obtain stable transfecteds. The G418-resistant population was then maintained in the presence of G418 (0.5 mg/ml). Considering the heterogeneous nature of Caco-2 cells (31), the G418-resistant cells were not subjected to single cell cloning.

Cell surface biotinylation. Caco-2 cells were seeded into 12-well plates at a confluent density and grown for 14 or 15 days. Forty-eight hours before the biotinylation reaction was performed, the cells were incubated with sodium butyrate (3 mM) for 24 h to enhance the expression of recombinant ezrin, and the compound was then removed and cells were incubated for a further 24 h. Cells were then washed with ice-cold phosphate-buffered saline containing 0.1 mM CaCl$_2$ and 1 mM MgCl$_2$ [PBS(–)] and incubated for 20 min at 4°C with freshly prepared EZ-Link Sulfo-NHS-Biotin dissolved in PBS(–). Cells were washed with PBS(–) containing 100 mM glycine, incubated for 15 min at 4°C, and then washed with PBS(–). Following biotinylation, cells were lysed in lysis buffer [25 mM HEPES/Tris pH 7.4, 10% (vol/vol) glycerol, 1% (vol/vol) Triton X-100, 1 mM PMFS, 5 µg/ml leupeptin, 1 µg/ml pepstatin, and 5 µg/ml aprotinin] for 20 min at 4°C, sonicated, and centrifuged at 20,630 g for 15 min at 4°C. The protein concentration of the resulting supernatant was determined by using the BCA (bicinchoninic acid) Protein Assay Reagent Kit (Pierce). Next, immobilized streptavidin beads were added to the supernatant and were incubated at 4°C overnight with end-over-end rotation. The beads were washed with lysis buffer and incubated in elution buffer [10 mM Tris-HCl pH 6.5, 3% (wt/vol) SDS, 10% (vol/vol) glycerol, 5% (vol/vol) β-mercaptoethanol, 8 M urea, and 0.001% (wt/vol) bromophenol blue] for 5 min at 50°C. The eluent was subjected to immunoblot analysis, as described below.

Statistical analysis. All data are represented as the means ± SE. Data were statistically analyzed by using one-way ANOVA followed by the Bonferroni correction or Dunnett’s post hoc test, as appropriate. Differences between means at the level of P < 0.05 were considered significant.

RESULTS

Expression profiles of Mrp2/Abcc2 and Mdr1/Abcb1 along the proximal to distal axis in the rat small intestine. The rat small intestine (duodenum to ileum) was divided into six equivalent segments (or fractions), numbered from fractions 1 to 6 along the proximal to distal axis. Mrp2/Abcc2 and Mdr1/Abcb1 mRNA levels were quantified in the intact intestine by real-time PCR analysis, and Mrp2/Abcc2 and Mdr1/Abcb1 protein levels were quantified in the intestinal mucosa and the brush border membrane portions of each fraction by immunoblot analysis. Mrp2/Abcc2 mRNA expression was lowest in fraction 4 (corresponding to the proximal ileum), at 24.2 ± 8.1% of the expression level in fraction 1 (designated as 100%; Fig. 1A). On the other hand, Mrp2/Abcc2 protein expression in the small intestinal mucosa was lowest in fraction 5 (38.9 ± 4.9%; Fig. 1B), thus demonstrating a progressive decrease along the small intestinal tract. However, the brush border membrane showed a clearer and larger continuous decrease in Mrp2/Abcc2 protein content along the tract, with the lowest expression in fraction 6 (15.7 ± 5.5%; Fig. 1C).

The mRNA expression of Mdr1a/Abcb1a, a rat orthologue of human Mdr1/Abcb1, increased along the proximal to distal axis, yielding the highest expression level in fraction 6 (485.3 ± 27.7%; Fig. 2A). Mdr1/Abcb1 protein expression was similar to Mdr1a/Abcb1a mRNA expression, with the highest expression in fraction 5 (354.4 ± 58.4%; Fig. 2B). Compared with total protein expression in the small intestinal mucosa, the brush border membrane showed a clearer and larger continuous increase in Mdr1/Abcb1 content along the tract, with the highest expression in fraction 6 (1,113.3 ± 331.8%; Fig. 2C). On the other hand, Mdr1b/Abcb1b, another orthologue of human MDR1/ABCB1, was expressed at extremely low levels in the rat small intestine relative to Mdr1a/Abcb1a (data not shown). It is therefore likely that the Mdr1/Abcb1 protein derived from the Mdr1a/Abcb1a gene is dominant in rat small intestine.

Expression profiles of ezrin and p-ezrin. Given the demonstrated capacity of ezrin to regulate the expression patterns of Mrp2/Abcc2 and Mdr1/Abcb1, we next investigated the expression profiles of ezrin and p-ezrin along the rat small intestinal tract. Ezrin mRNA and protein levels were both low in the proximal region of the tract and increased distally, with the highest expression in fraction 6 (mRNA, 464.8 ± 190.8%; protein, 205.4 ± 20.6%; Fig. 3, A and B). In contrast to total ezrin, COOH-terminally p-ezrin content was highest in the proximal region and decreased along the tract, with the lowest level in fraction 6 (52.5 ± 9.9%; Fig. 3C). Therefore, the expression profile of ezrin along the rat intestinal tract was similar to that of Mdr1/Abcb1, whereas the expression profile of p-ezrin was similar to that of Mrp2/Abcc2.

Expression profile of conventional PKC isoforms. Three conserved phosphorylation sites are involved in PKC maturation, stabilization, and activation (19, 12). PKC activation leads to rapid alterations in the subcellular localization of ezrin and especially in its translocation from the soluble cytosolic fraction to the membranous fraction. We also reported that thymeleatoxin, an activator of conventional PKC (cPKC), induces translocation of PKCα, a cPKC isoform, as well as dephosphorylation of ezrin in the rat small intestine (27). Thus, to gain better insight into the molecular mechanism of the
divergent expression profiles of ezrin and p-ezrin along the proximal to distal tract, we next investigated the localization of PKCα and the intramolecular phosphorylation status of PKCα and PKCβII another cPKC isoform as an index of cPKC activity.

Total PKCα expression was similar throughout the small intestinal mucosa (Fig. 4A). Levels of phosphorylated PKCα and PKCβII were lowest in the proximal region of the small intestinal tract and highest in the distal region (fraction 5, 188.3 ± 23.2%; fraction 6, 168.8 ± 8.0%; Fig. 4B). The content of PKCα in the brush border membrane was similarly increased along the intestinal tract (fraction 5, 152.6 ± 16.7%; fraction 6: 151.7 ± 21.0%; Fig. 4C). These results are suggestive of the translocation of PKCα to the brush border membrane and further indicate that cPKC is activated in the distal region rather than the proximal region of the rat small intestine.

Fig. 1. Expression and localization profiles of Mrp2/Abcc2 along the proximal to distal axis of the rat small intestine. A: relative mRNA expression of Mrp2/Abcc2 normalized to 28S rRNA. B: representative immunoblot (top) and graph (bottom) showing Mrp2/Abcc2 protein expression in the small intestinal mucosa and relative band densities in fractions 1–6, respectively. C: representative immunoblot (top) and graph (bottom) showing Mrp2/Abcc2 protein expression in the brush border membrane and relative band densities in fractions 1–6, respectively. In each experiment, the band density of fraction 1 was designated as 100%. Each value represents means ± SE [n = 4 (A) and n = 5 (B and C)]. *P < 0.05, **P < 0.01 vs. fraction 1.

Fig. 2. Expression and localization profiles of Mdr1/Abcb1 along the proximal to distal axis of the rat small intestine. A: relative mRNA expression of Mdr1/Abcb1 normalized to 28S rRNA. B: representative immunoblot (top) and graph (bottom) showing Mdr1/Abcb1 protein expression in the small intestinal mucosa and relative band densities in fractions 1–6, respectively. C: representative immunoblot (top) and graph (bottom) showing Mdr1/Abcb1 protein expression in the brush border membrane and relative band densities in fractions 1–6, respectively. In each experiment, the band density of fraction 1 was designated as 100%. Each value represents means ± SE [n = 4 (A) and n = 5 (B and C)]. *P < 0.05, **P < 0.01 vs. fraction 1.
Localization of recombinant ezrin-GFP in Caco-2 cells. The following set of in vitro experiments aimed to investigate the causality of the phosphorylation/expression profile of ezrin and the expression profiles of MRP2/ABCC2 and MDR1/ABCB1 along the small intestinal tract. We first investigated the localization of recombinant ezrin-EGFP fusion proteins in transfected Caco-2 cells to confirm the effect of COOH-terminal phosphorylation on ezrin localization. EGFP-expressing Caco-2 cells were used as a control and revealed a diffuse localization of the fluorescent protein in the cytosol and the nuclei, whereas WT-EGFP was primarily localized to the plasma membrane. T567A-EGFP (constitutively dephosphorylated ezrin mutant) was also localized to the plasma membrane without cytoplasmic staining, showing a similar pattern to WT-EGFP (Fig. 5, A and B).

Fig. 3. Expression and phosphorylation profiles of ezrin along the proximal to distal axis of the rat small intestine. A: relative mRNA expression of ezrin normalized to 28S RNA. B: representative immunoblot (top) and graph (bottom) showing ezrin protein expression in small intestinal mucosa and relative band densities in fractions 1–6, respectively. C: representative immunoblot (top) and graph (bottom) showing p-ezrin content in the small intestinal mucosa and relative band densities in fractions 1–6, respectively. In each experiment, the band density of fraction 1 was designated as 100%. Each value represents means ± SE [n = 4 (A) and n = 5 (B and C)]. *P < 0.05, **P < 0.01 vs. fraction 1.

PKCα phospho-PKCα/βII PKCα

Fig. 4. Expression, phosphorylation and localization profile of cPKC along the proximal to distal axis of the rat small intestine. A: representative immunoblot (top) and graph (bottom) showing PKCα protein expression in the small intestinal mucosa and relative band densities in fractions 1–6, respectively. B: representative immunoblot (top) and graph (bottom) showing phosphorylated PKCα and PKCβII isoforms in the small intestinal mucosa and relative band densities in fractions 1–6, respectively. C: representative immunoblot (top) and graph (bottom) showing the localization of PKCα in the brush border membrane and relative band densities in fractions 1–6, respectively. In each experiment, the band density of fraction 1 was designated as 100%. Each value represents means ± SE (n = 3). *P < 0.05, **P < 0.01 vs. fraction 1.
the other hand, T567D-EGFP (constitutively phosphorylated ezrin mutant) showed a markedly distinct localization compared with WT-EGFP and T567A-EGFP, with aggregation in the intracellular region of Caco-2 cells (Fig. 5A). Moreover, many of the T567D-EGFP-expressing cells showed a very different cell morphology compared with WT-EGFP- and T567A-GFP-expressing cells.

**Effect of T567A ezrin mutant on MRP2 and MDR1 expression patterns.** To investigate the effect of ezrin expression and phosphorylation on the cell surface localization of MRP2/ABCC2 and MDR1/ABCB1, the expression patterns of MRP2/ABCC2 and MDR1/ABCB1 were explored in transfected Caco-2 cells that stably expressed recombinant WT ezrin and T567A ezrin constructs. This was done by using cell surface membrane biotinylation techniques. The expression of total ezrin [active (phosphorylated) plus inactive (unphosphorylated) forms] in whole cell lysates of WT- and T567A-stably expressing Caco-2 cells increased to 162.3 ± 6.6 and 166.2 ± 7.5%, respectively, of the value in control LacZ-expressing Caco-2 cells (Fig. 6, A and B). Moreover, the expression of COOH-terminally phosphorylated ezrin in WT-transfected Caco-2 cells increased to 120.9 ± 3.1% of the value in LacZ-transfected Caco-2 cells, whereas its expression did not change in the phosphorylation-deficient T567A-transfected Caco-2 cells (Fig. 6, A and C).

Total expression of MRP2/ABCC2 increased to 140.8 ± 5.0% in the whole cell lysates of WT-transfected Caco-2 cells (Fig. 7A), while biotinylated cell surface/membrane-localized MRP2/ABCC2 content increased to 234.8 ± 30.1% (Fig. 7B). However, cell surface/membrane-localized MRP2/ABCC2 was not significantly enhanced in T567A-expressing cells relative to control cells (Fig. 7B). By contrast, total expression of MDR1/ABCB1 increased to 128.5 ± 2.6 and 132.7 ± 8.7% in WT- and T567A-expressing cells, respectively (Fig. 7C), while cell surface/membrane-localized MDR1/ABCB1 increased to 203.3 ± 12.5 and 258.0 ± 21.2% in WT- and T567A-expressing cells, respectively.
expressing cells, respectively (Fig. 7D). These results suggest that ezrin COOH-terminal phosphorylation has a causative effect on MRP2/ABCC2 membrane localization but that the ezrin protein directs membrane localization of MDR1/ABCB1 independently of its phosphorylation status.

**DISCUSSION**

The expression profiles of MRP2/ABCC2 and MDR1/ABCB1 reveal a characteristic expression pattern along the small intestinal tract. Although the physiological importance of this expression profile is not well understood, Panwala et al. (28) demonstrated that Mdr1a-knockout mice are susceptible to the development of severe, spontaneous intestinal inflammation similar to that of human inflammatory bowel disease when maintained under specific pathogen-free animal facility conditions. Mdr1a-knockout mice present with a commensal bacterial flora that participates in the development of colitis. Because intestinal microflora are more copious in the distal region than in the proximal region of the small intestine, the abundant expression of Mdr1/Abcb1 in the distal region is appropriate for the direct/indirect defense of the organism against intestinal bacteria or bacterial products/toxins. MRP2/ABCC2 and their orthologue have been shown to play important roles in the detoxification of toxic metals including cadmium (24), mercury (42), and arsenite (4). Other reports confirmed intestinal MRP2/ABCC2 has barrier function against free absorption of the nutraceutical compound (18) and food-derived carcinogen (9, 10). In these regards, the characteristic expression patterns of MRP2/ABCC2 and MDR1/ABCB1 are also likely to safeguard the organism against xenobiotics and excess nutrients. In addition, information concerning the expression profiles of efflux transporters and metabolic enzymes along the small intestinal tract is beneficial for the effective development of orally administrated drugs. Indeed, many investigations into the localization of drug transporters and metabolic enzymes have been conducted with this goal in mind (7, 8).

The present study focused on the distinct localizations of MRP2/ABCC2 and MDR1/ABCB1 along the gastrointestinal tract, as well as the COOH-terminal phosphorylation and activation of ezrin, in an attempt to shed further light on the relationship between ezrin and the efflux transporters. Mrp2/Abcc2 mRNA and protein expression levels were higher in the proximal portion than in the distal portion of the organ (Fig. 1, A and B). Conversely, Mdr1/Abcb1 (Mdr1a/Abcb1a) mRNA and protein expression levels increased along the gastrointestinal tract, with the highest expression in the distal ileum (Fig. 2, A and B). The mRNA and total protein expression of ezrin also increased distally along the tract, showing a positive correlation with Mdr1/Abcb1 expression (Fig. 3, A and B). These results are consistent with earlier work concerning ezrin expression in the rat intestine (1). By contrast, COOH-terminally phosphorylated ezrin expression was negatively correlated with Mdr1/Abcb1 expression and positively correlated with Mrp2/Abcc2 expression, with more pronounced expression in the proximal region than in the distal region of the small intestine (Fig. 3C). The factor organizing characteristic expression pattern of MRP2/ABCC2 and MDR1/ABCB1 along gastrointestinal tract is unclear. It may be possible that multiple processes are involved. Interestingly, although both mRNA and total protein expression of Mrp2/Abcc2 and Mdr1/Abcb1 showed gradient pattern to some extent along the rat intestinal tract, the gradient of localized Mrp2/Abcc2 and Mdr1/Abcb1 in the brush border membrane showed clearer and higher magnitude than mRNA and total protein (Figs. 1 and 2). Considering a role of ezrin as molecular anchor, the expression profile of ezrin described in this study is one of the key factor in forming the expression profile of Mrp2/Abcc2 and Mdr1/Abcb1 in rat intestine.

We next focused on the contribution of cPKC to the phosphorylation profile of ezrin. Activation of cPKC induces dephosphorylation of ezrin in the rat small intestine (27). PKC isoforms are classified into Ca\(^{2+}\)-dependent cPKCs (e.g., PKC\(\alpha\), PKC\(\beta\)I, PKC\(\beta\)II, and PKC\(\gamma\)); Ca\(^{2+}\)-independent novel PKCs (e.g., PKC\(\delta\),
PKCα, PKCβ, and PKCγ; and atypical PKCs (e.g., PKCε, PKCθ, and PKCζ). Molecular species of PKC, once stimulated or activated via phosphorylation of three intramolecular threonine or serine residues, translocate to specific intracellular compartments, often the plasma membrane (5, 12, 19). Our previous study revealed that activation of the PKC/H9251 subtype results in its translocation to the brush border membrane in the rat small intestinal tissue (27).

At least five PKC isoforms (PKCα, PKCβIII, PKCδ, PKCε, and PKCζ) are found in the rat intestine (32). Therefore, we investigated the translocation of PKCα to the brush border membrane and the intracellular phosphorylation status of cPKC (α/βII) as an indicator of cPKC activity. PKCα translocation to the membrane and the intramolecular phosphorylation status of PKCα/βII increased along the proximal to distal axis, although the expression profile of PKCα in the small intestinal mucosa was similar in all regions of the organ (Fig. 4). This suggests that cPKC activity is normally elevated in the distal portion of the small intestine. Given that cPKC activation leads to dephosphorylation of ezrin (27), we hypothesize that ezrin is dephosphorylated in the distal region of small intestine due to the high activity of cPKC.

To confirm the link between the phosphorylation status of ezrin and the Mrp2/Abcc2 and Mdr1/Abcb1 expression profiles in the rat small intestine, the effect of COOH-terminal phosphorylation of ezrin on MRP2/ABCC2 and MDR1/ABCB1 localization was investigated by expressing recombinant phosphorylation or dephosphorylation ezrin mutants in Caco-2 cells. Caco-2, a cell line derived from human colon cancer, shares certain characteristics with small intestinal epithelial cells (15). For instance, numerous transporters for glucose, amino acids, phosphoric acid, and bile acids are found in Caco-2 cells. Furthermore, Caco-2 cells also express endogenous MRP2/ABCC2 and MDR1/ABCB1 (35).

To examine the effect of COOH-terminal phosphorylation of ezrin on its own localization, we prepared plasmids expressing EGFP-fusion proteins and transiently introduced the plasmids into Caco-2 cells. WT-EGFP and constitutively dephosphorylation-
lated T567A-EGFP were both primarily localized to the brush border membrane and the plasma membrane region of the cell-cell adhesion site in transfected cells (Fig. 5, A and B). On the other hand, several reports showed predominant localization of endogenous ezrin in the brush border membrane rather than at the plasma membrane of Caco-2 cells (38, 39). This discrepancy between our study and the earlier studies may be due to the effect of the EGFP tag or the immature nature (72 h after transfection) of the Caco-2 cells used in the current work. Regardless, the similar localization of T567A-EGFP and WT-EGFP suggests that the dephosphorylation of ezrin is irrelevant to translocation of ezrin to the apical membrane. Along the same lines, Zhu et al. (44) demonstrated a similar localization of WT ezrin and mutant T567A ezrin in rabbit gastric parietal cells, and our previous work indicated that dephosphorylation of ezrin at the COOH terminus has no effect on ezrin content in the brush border membrane of the rat small intestine (27). Therefore, it is likely that ezrin can exist near the cell membrane compartment independently of its COOH-terminal phosphorylation. In contrast, the localization of the constitutively phosphorylated mutant ezrin (T567D-EGFP) in Caco-2 cells was distinct from that of WT ezrin and T567A ezrin (Fig. 5A). In another report, T567D-expressing cells showed abnormal localization of the constitutively activated protein and a cell shape different to that of WT- and T567A-expressing cells (43). For these reasons, we excluded the T567D mutant from the efflux transporter localization experiments in stably transfected Caco-2 cells.

The p-ezrin content was higher in stably transfected Caco-2 cells expressing WT ezrin than in those expressing T567A mutant ezrin, while the two cell types had similar expression levels of total (phosphorylated plus unphosphorylated) ezrin (Fig. 6). Furthermore, the significant decrease in MRP2/ABCC2 expression at the plasma membrane of T567A-expressing cells compared with WT-expressing cells (Fig. 7B) indicates a causative association between ezrin dephosphorylation/inactivation and MRP2/ABCC2 localization. By contrast, MDR1/ABCB1 expression at the plasma membrane was higher in both WT- and T567A-expressing cells than in control cells (Fig. 7D). These results suggest that ezrin can increase the membrane localization of MDR1/ABCB1 independently of its COOH-terminal phosphorylation. Notably, our earlier work demonstrated that ezrin dephosphorylation fails to reduce the apical localization of MDR1/ABCB1 (or its transporter activity) in the rat small intestine (27). Therefore, a biochemical mechanism other that ezrin phosphorylation may be involved in the membrane localization of MDR1/ABCB1. This is in contrast to MRP2/ABCC2, where the phosphorylated (open) form of ezrin functions as a cross-linker between MRP2/ABCC2 in the plasma membrane and the cytoskeleton. In the latter case, the NH2- and COOH-terminal regions of the open form bind to MRP2/ABCC2 and F-actin, respectively.

The NH2-terminal region of ERM proteins forms a cloverlike molecular structure that is composed of three subdomains with multiple peptide-recognition sites (14, 36). In addition, the COOH-terminal region has multiple actin-binding sites (29, 38) and also interacts with cytoskeletal factors other than F-actin (38). These observations again suggest that ezrin contributes to the stabilization of MDR1/ABCB1 within the membrane by a mechanism that differs from that used for MRP2/ABCC2. Recently, Brambilla et al. (3) reported that binding of MDR1/ABCB1 to ezrin plays a key role in the establishment of multidrug resistance by the efflux transporter. However, the detailed molecular mechanism of how ezrin, independently of its phosphorylation status, increases the membrane localization of MDR1/ABCB1 and modulates its function is still unclear.

Changes in total protein expression of both MRP2/ABCC2 and MDR1/ABCB1 were observed in recombinant ezrin-transfected Caco-2 cells that correlated with changes in membrane-localized protein expression (Fig. 7). Efflux transporters are maintained within intracellular pools (22, 37), and their functions are regulated according to prevailing physiological conditions by translocation between the intracellular pool and the plasma membrane. Thus constitutive expression and translocation of MRP2/ABCC2 and MDR1/ABCB1 within the cell is driven by at least four processes: 1) novel biosynthesis, 2) translocation from the intracellular pool to the plasma membrane, 3) internalization to the intracellular pool from the plasma membrane, and 4) degradation. Because stably transfected cells expressing recombinant ezrin were used as a model in the current study, it is possible that elevated levels of ezrin-bound efflux transporters on the plasma membrane resulted in an imbalance in these four processes of protein translocation, especially degradation, ultimately leading to the changes in total MRP2/ABCC2 and MDR1/ABCB1 protein levels. This possibility should be clarified in further study.

In conclusion, this study provides important information regarding the expression profiles of MRP2/ABCC2 and MDR1/ABCB1 in the small intestine. Even though it is possible that another mechanism, for example, transcriptional modification, is also involved, our results indicate that the phosphorylation and expression levels of ezrin along the gastrointestinal tract can rationally account for the expression pattern of MRP2/ABCC2 and MDR1/ABCB1. For example, high expression of p-ezrin in the proximal portion of the small intestine enabled MRP2/ABCC2 binding and stabilization within the cell membrane, whereas high expression of total ezrin elevated MDR1/ABCB1 expression in the distal portion of the small intestine independently of its COOH-terminal phosphorylation. Thus the phosphorylation and overall expression of ezrin differentially regulate the apical localization and expression of MRP2/ABCC2 and MDR1/ABCB1, respectively, along the small intestinal tract.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: T.N., S.S., and T.H. conception and design of research; T.N. performed experiments; T.N. analyzed data; T.N., S.S., K.I., and T.H. interpreted results of experiments; T.N. prepared figures; T.N., S.S., and K.I. drafted manuscript; T.N., S.S., and K.I. edited and revised manuscript; T.H. approved final version of manuscript.

