Transport of fluorescein methotrexate by multidrug resistance-associated protein 3 in IEC-6 cells

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Li, Tiesong, Kousei Ito, and Toshiharu Horie. Transport of fluorescein methotrexate by multidrug resistance-associated protein 3 in IEC-6 cells. Am J Physiol Gastrointest Liver Physiol 285: G602–G610, 2003; 10.1152/ajpgi.00424.2002.—The transport characteristics of fluorescein methotrexate (F-MTX) were studied by using the rat intestinal crypt cell line IEC-6. Enhanced accumulation of F-MTX at 4°C suggests the existence of an active efflux system. MK-571, an inhibitor of the multidrug resistance-associated protein/ATP binding cassette C (MRP/ABCC) family, also enhanced the accumulation of F-MTX. Transcellular transport of F-MTX from the apical to the basolateral compartment was 2.5 times higher than the opposite direction. This vectorial transport was also reduced by MK-571, indicating the presence of Mrp-type transporter(s) on the basolateral membrane. Mrp3 mRNA was readily detectable, and the protein was localized on the basolateral membrane. Uptake of F-MTX into membrane vesicles from IEC-6 cells and Spodoptera frugiperda-9 cells expressing rat Mrp3 were both ATP dependent and saturable as a function of the F-MTX concentration. Similar $K_m$ values (11.0 ± 1.8 and 4.5 ± 1.1 μM) and inhibition profiles by MK-571, estradiol-17β-d-glucuronide, and taurocholate for the ATP-dependent transport of F-MTX into these vesicles were obtained. These findings suggest that the efflux of F-MTX is mediated by Mrp3 on the basolateral membrane of IEC-6 cells.

MRP3; intestine; crypt

METHOTREXATE (MTX) is widely used as a chemotherapeutic agent for the treatment of many cancers, demonstrating consistent activity against a number of malignant tumors (18). MTX has also been found to play a major therapeutic role in nonneoplastic diseases as an anti-inflammatory and immunosuppressive agent (38). MTX is taken up by the cells via carriers such as reduced folate carrier (RFC) and folate receptor (5, 39) and, once inside the cell, MTX acts as a potent competitive inhibitor of dihydrofolate reductase (DHFR), a major intracellular target of MTX-type drugs. Inhibition of DHFR results in a depletion of the intracellular-reduced folate pools required for the biosynthesis of purines and thymidine. MTX treatment is often accompanied by side effects such as nausea, vomiting, diarrhea, stomatitis, gastrointestinal ulceration, and mucositis (8, 35). The therapeutic use of MTX is limited by its toxic effects on proliferating cells, especially the rapidly dividing cells of intestinal crypts. To combat the cytotoxic action of MTX in the small intestine, it is important to investigate the transport of MTX including influx, efflux, and binding to the intracellular organelles.

The intestinal epithelial cell (IEC)-6 is an immortalized epithelial cell line derived from neonatal rat ileum (32). IEC-6 cells have the characteristics of crypt-type intestinal cells but do not exhibit differentiated morphology or gene expression (32) by the lack of transcription factor Drosophila caudal (Cdx2) expression (42). It has been sometimes used as an in vitro intestinal model for the study of MTX transport, especially involving RFC-mediated uptake (33). RFC has been reported to be expressed in IEC-6 as well as small intestine (37), and optimum transport at low pH for MTX and folic acid, typical substrates for RFC, has also been observed both in vitro and in vivo (39). Although drug accumulation depends on the balance of cellular influx and efflux, little is known about the efflux of MTX in this cell line. Rajgopal et al. (33) found that uptake of MTX in RFC-transfected IEC-6 cells was enhanced when energy metabolism was blocked by azide and was reversed when glucose was present. They supposed that endogenous transporter(s), most likely the multidrug resistance-associated protein/ATP binding cassette C (MRP/ABCC) family, was involved in the efflux of MTX from IEC-6 cells, although there is still little evidence about which MRP member(s) is involved in the efflux. The MRP family has at least nine members in human, MRP1–9 (3, 15, 20, 24), all belonging to the ABC transporter superfamily. Overexpression of these transporters, especially MRP1–3 (ABCC1–3), often results in acquired resistance to cytotoxic drugs, such as the commonly used natural product antineoplastic agents, as well as MTX, by lowering the intracellular concentration of these compounds (4). MTX has actually been reported to be a typical nonconjugated substrate for MRP families including MRP1, MRP2, and MRP3 (21).

Fluorescein methotrexate (F-MTX), a fluorescent derivative of MTX, was synthesized by coupling the carboxyl of the glutamate moiety of MTX through a dia-
minopentane spacer to fluorescein isothiocyanate (9). F-MTX has been used to study the DHFR and membrane-associated MTX transport protein, by using the fluorescence moiety in the molecule (2, 6, 11). Fluorescence labeling of the transporter in situ could be used for detecting and isolating cells by flow cytometry and further monitoring localization and conformation of the transporter within the membrane (7). Recently, F-MTX transport by RFC in human leukemia cell lines and leukemiablasts has also been demonstrated by confocal laser scanning microscopy (19). We (30) have also suggested that the transport of F-MTX in the small intestine is mediated partly by RFC by using the in vitro everted intestine segments of rats. Recently, F-MTX has also been suggested to be a fluorescent substrate for energy-driven efflux transporter, possibly Mrp2, in fish (10). Moreover, rabbit Mrp2-mediated transport of [3H]estradiol-17β-glucuronide (E217βG) was inhibited by F-MTX (49). These reports imply the interaction between F-MTX and Mrp families.

In the present study, to identify the efflux transporter(s) for MTX and its derivatives in IEC-6, we used F-MTX as a model compound. We were able to demonstrate that F-MTX is a fluorescent substrate of rat Mrp3 and 2) F-MTX is preferentially transported in the apical-to-basolateral direction with the aid of Mrp3 on the basolateral membrane of IEC-6 cells.

MATERIALS AND METHODS

**Materials.** Rat intestinal epithelial cells, IEC-6, were obtained from American Type Culture Collection (Manassas, VA) and were used between passage 18 and 25. Cells were grown in 100-mm-diameter tissue culture plates in DMEM (Sigma, St. Louis, MO) containing 4.5 g/l glucose, 1.5 g/l sodium bicarbonate, 5% FBS, 100 U/l bovine insulin (Wako Pure Chemical Industries, Tokyo, Japan) and 20 mg/l gentamicin sulfate (Nacalai Tesque, Kyoto, Japan) and incubated at 37°C in 5% CO2-95% air. Culture media were changed every 2 days. Spodoptera frugiperda (Sf9) cells were maintained as a suspension culture at 27°C with serum-free Ex-Cell 420 (Nihirei, Tokyo, Japan). F-MTX was obtained from Molecular Probes (Eugene, OR). MK-571 was obtained from Cayman Chemical (Ann Arbor, MI). E217βG and taurocholate (TC) were obtained from Sigma. Anti-rat Mrp1, Mrp2, Mrp3, and GAPDH for Northern blot analysis were obtained from American Type Culture Collection (Manassas, VA) and were used between passage 328 and 740 (413 bp) of rat Mrp1 partial cDNA (GenBank accession no. X96394), full-length (4880 bp) rat Mrp2 (16), full-length (5174 bp) rat Mrp3 (12), and bases 523–1003 (481 bp) of rat GAPD, respectively. Northern blot hybridization was performed according to the method described previously (16). Poly(A)+RNA was separated on 0.7% agarose gel containing 3.7% formaldehyde and transferred to a nylon membrane (Biodyne A; Pall Gelman Laboratory, Ann Arbor, MI) before fixation by baking for 2 h at 80°C. Membranes were prehybridized in buffer containing 4 × SSC (1 × SSC consists of 150 mM NaCl and 150 mM sodium citrate, pH 7.0), 5% Denhardt’s solution (Wako Pure Chemical Industries), 0.2% SDS, 0.1 mg/ml sonicated salmon sperm DNA, and 50% formamide for 2 h at 42°C. Hybridization was performed overnight in the same buffer with [32P]-labeled cDNA probes prepared by a random-primed labeling method (Rediprime; Amersham Biosciences, Piscataway, NJ) according to the manufacturer’s instructions. The hybridized membrane was washed in 2 × SSC and 0.1% SDS at room temperature for 20 min, followed by washing in 2 × SSC and 0.1% SDS at 55°C for 20 min and then in 0.1 × SSC and 0.1% SDS at 55°C for 20 min. Filters were exposed to Fuji imaging plates (Fuji Photo Film, Kawasaki, Japan) and analyzed by a BAS 2000 imaging analyzer (Fuji Photo Film).

**Semiquantitative RT-PCR analysis of Mrp expression in IEC-6 cells.** Single-stranded cDNA was synthesized from 1 μg mRNA by using 50 pmol random 9-mer and 50 units RT XL (Takara Bio, Shiga, Japan) in a volume of 20 μl. The tube was incubated in a PC-9600 Microplate gradient thermal cycler (Corbett Research) at 30°C for 10 min, 42°C for 30 min, 50°C for 30 min, 60°C for 30 min, and 99°C for 5 min. The resulting cDNA product (1 μl) was subjected to a step-down PCR reaction with rTaq (Takara Bio) and respective primer sets. The tubes (10 μl each) were incubated at 95°C for 1 min to denature the cDNA and primers. The subsequent step-down cycling program was 95°C for 30 s, 74°C for 15 s, 72°C for 45 s involving 3 cycles, 95°C for 30 s, 70°C for 15 s, 72°C for 45 s involving 3 cycles, 95°C for 30 s, 66°C for 15 s, 72°C for 45 s involving 3 cycles, 95°C for 30 s, 74°C for 15 s, 72°C for 45 s involving 3 cycles, 95°C for 30 s, 58°C for 15 s, 72°C for 45 s involving 3 cycles, 95°C for 30 s, 54°C for 15 s, 72°C for 45 s involving 11–30 (26–45 in total) cycles. Primers specific for rat Mrp1 (sense: 5'-CTGATCTCTGCTGATATCTGATG-3’; antisense: 5'-GACTGTAAGACAGACAGAAGA-3’), rat Mrp2 (sense: 5'-CACGACTCTTCTGAAAAATTTAACGATCC-3’; antisense: 5'-ACATTAGGAATGGCAAGTATAGCCAAACC-3’), and rat Mrp3 (sense: 5’-GACAGCTGCTGACTGAAACCTG-3’, antisense: 5’-GGCCGATCTCTGATTTTTG-3’) were used resulting in amplified products of 413, 463, and 449 bp, respectively. Each PCR product was separated on an 8% polyacrylamide gel and stained with ethidium bromide.
Immunofluorescence microscopy. IEC-6 cells were seeded on a cover glass (15 mm in diameter, uncoated) in a 12-well plate at a density of 5 × 10^4 cells/well and cultured for 2 to 5 days postconfluence. For confocal laser scanning microscopy, cells were washed in PBS and fixed for 10 min in 4% (vol/vol) formaldehyde in PBS at room temperature, followed by permeabilization for 5 min in 1% (vol/vol) Triton X-100 in PBS at room temperature. Cells were incubated with anti-rat Mrp3 rabbit antiserum (1:100) (14) for 1 h at room temperature. Antibody binding was detected with fluorescein-labeled anti-rabbit IgG (1:250) (Vector Laboratories, Burlingame, CA). Nucleic acids were stained by using SYTO61 (1:1,000) (Molecular Probes). Cells were examined by LSM510 confocal laser scanning microscopy (Carl Zeiss, Jena, Germany). The 488-nm wavelength of an argon laser and the 633-nm wavelength of a Helium-Neon laser were used with appropriate combinations for excitation and emission filters.

Production of recombinant baculovirus and viral infection. Recombinant baculovirus was prepared as described previously (17). SF9 cell suspension was poured into a 150-mm glass plate at a density of 5 × 10^6 cells/ml and Sf9 cells or RBCs were size fractionated in an 8.5% pore, Cellulose Acetate (Millipore) by electrophoresis. Blotted membranes were incubated with Tris-buffered saline containing 0.05% Tween 20 and 3% BSA for 1 h at room temperature and probed for 1 h at room temperature with anti-rat Mrp3 rabbit antiserum (1:1,000) or anti-MRP1 monoclonal antibody (MRP1, 1:200; Kamiya Biomed, Seattle, WA) diluted with Tris-buffered saline containing 0.05% Tween 20 and 0.1% BSA. The membranes were then allowed to bind donkey anti-mouse (Santa Cruz Biotechnology, Santa Cruz, CA) or anti-rabbit IgG conjugated with horseradish peroxidase (Amersham Biosciences, Piscataway, NJ) (1:3,000) at room temperature for 1 h and analyzed by using an enhanced chemiluminescence kit (Amersham Biosciences).

Data analysis. Uptake rates were fitted to the Michaelis-Menten equation by using a nonlinear least-squares program, MULTI (44), to calculate the kinetic parameters.

RESULTS

Characteristics of F-MTX uptake by IEC-6 monolayers. The time-dependent uptake of 1 μM F-MTX into IEC-6 monolayers was examined. The uptake of F-MTX at pH 5.5 was significantly higher, by a factor of two, than at pH 7.5 (Fig. 1). The optimal transport at low pH is similar to the MTX transport observed in IEC-6 cells (33). We also examined the uptake of F-MTX into IEC-6 monolayers at 37°C and at 4°C at pH 5.5. Surprisingly, a significantly higher uptake of F-MTX was observed at 4°C than at 37°C (Fig. 1). The enhanced accumulation of F-MTX in the cells at a lower temperature suggests the existence of an active efflux system. Based on the recent reports that MTX is the substrate of MRP transporters (21), we tested leukotriene D4 (LTD4)-receptor antagonist MK-571, a potent inhibitor of the MRP family, to see whether it increased F-MTX accumulation. In the presence of 10 μM MK-571, the accumulation of F-MTX in IEC-6 cells

![Fig. 1. Effect of pH, temperature, and MK-571 on fluorescein methotrexate uptake by intestinal epithelial cell (IEC-6) monolayers.](http://ajpgi.physiology.org/)

IEC-6 monolayers were incubated in 37°C Krebs-Ringer buffer containing 1 μM F-MTX at pH 5.5 (●) or pH 7.5 (○). A. Uptake of F-MTX (1 μM) in 4°C Krebs-Ringer buffer at pH 5.5 (●). B. Uptake of F-MTX (1 μM) in 37°C Krebs-Ringer buffer at pH 5.5 in the presence of 10 μM MK-571. Each data point is the mean ± SD of 3 separate uptake determinations.
was significantly increased by a factor of four compared with that in its absence (Fig. 1). This suggests that the active efflux system for F-MTX in IEC-6 cells may be Mrp-type transporter(s).

Transcellular transport of F-MTX by IEC-6 monolayers. To obtain insight into the functional localization of Mrp-type transporter(s) in IEC-6 cells, the transcellular transport experiment was performed. IEC-6 cells were grown on transwell membrane inserts and incubated with F-MTX at a concentration of 1 μM in the apical or basolateral compartments with or without 10 μM MK-571. As shown, transport of F-MTX in the apical-to-basolateral direction was significantly higher than that in the basolateral-to-apical direction (Fig. 2). The apical-to-basolateral transport of F-MTX was significantly reduced in the presence of 10 μM MK-571, whereas the basolateral-to-apical transport of F-MTX was slightly but significantly enhanced in the presence of 10 μM MK-571. These results indicate that the transporter, likely a member of the Mrp family, localized on the basolateral membrane of IEC-6 monolayers is responsible for F-MTX efflux and is blocked by MK-571. Transport of F-MTX from the apical to the basolateral compartment is still twice as high as transport from basolateral to the apical compartment in the presence of MK-571 (Fig. 2). One of the possible reasons is the ability of inefficient concentration of MK-571 (10 μM) to completely block the Mrp3-mediated transport (see Table 1). However, MK-571 is usually used at ~10 μM because of its cytotoxicity at higher concentration.

Expression of Mrp1, Mrp2, and Mrp3 in IEC-6 cells. Until recently, three members of the Mrp family, including Mrp1, Mrp2, and Mrp3, were reported likely to accept MTX as a substrate (21). Among them, Mrp2 was also reported to accept F-MTX as a substrate (10, 43). To examine the expression of Mrp1, Mrp2, and Mrp3 in IEC-6 cells, Northern blot analysis was performed. As shown in Fig. 3A, an Mrp1 and Mrp3 transcript was detectable in IEC-6 cells. In contrast, no Mrp2-specific bands were obtained in IEC-6 cells. Fur-

![Graph](Image)

**Fig. 2.** Effect of MK-571 on the transcellular transport of F-MTX by IEC-6 monolayers. IEC-6 monolayers were incubated in 37°C Krebs-Ringer buffer containing 1 μM F-MTX at pH 5.5 in the apical or basolateral compartment with (○, △) or without (●, ▲) 10 μM MK-571. Transport of F-MTX in the apical-to-basolateral direction (○, △) or in the basolateral-to-apical direction (●, ▲) was measured as described in MATERIALS AND METHODS. Each data point is the mean ± SD of 3 separate uptake determinations. *P < 0.05, **P < 0.01, significantly different from that in the absence of MK-571.

![Graph](Image)

**Table 1. Effect of MK-571, E217βG, and TC on the ATP-dependent uptake of F-MTX by membrane vesicles from IEC-6 cells and Mrp3-Sf9**

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Concentration, μM</th>
<th>Uptake, % control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IEC-6</td>
<td>Mrp3-Sf9</td>
</tr>
<tr>
<td>MK-571</td>
<td>10.0</td>
<td>52.9 ± 2.9</td>
</tr>
<tr>
<td></td>
<td>100.0</td>
<td>9.5 ± 8.9</td>
</tr>
<tr>
<td>E217βG</td>
<td>7.5</td>
<td>58.3 ± 9.8</td>
</tr>
<tr>
<td></td>
<td>75.0</td>
<td>41.7 ± 6.1</td>
</tr>
<tr>
<td>TC</td>
<td>20.0</td>
<td>53.2 ± 8.5</td>
</tr>
<tr>
<td></td>
<td>200.0</td>
<td>42.0 ± 4.0</td>
</tr>
</tbody>
</table>

Uptake values are means ± SD in % control; n = 3 separate uptake determinations. Membrane vesicles were incubated at 37°C in uptake media containing 5 μM fluorescein methotrexate (F-MTX) with or without MK-571, estradiol-17β-d-glucuronide (E217βG) and taurourcholate (TC). Uptake of F-MTX was measured as described in MATERIALS AND METHODS. Inhibition is expressed as the percentage of the ATP-dependent uptake observed in the absence of inhibitors.

![Blot](Image)

**Fig. 3.** Expression of multidrug resistance-associated protein (Mrp) transporters in IEC-6 cells. A: Northern blot analysis of the expression of Mrp1, Mrp2, and Mrp3. Three micrograms of mRNA were applied to each lane. Autoradiographs of the blots, probed with 32P-labeled cDNA of Mrp1, Mrp2, and Mrp3, are shown. Rehybridization of the same membrane with GAPDH probe indicated the presence of the same amount of mRNA in each lane. Arrowheads indicate respective mRNA product. B: semiquantitative RT-PCR analysis of the expression of Mrp1 and Mrp3. Messenger RNA used in Northern blot hybridization was also used for RT-PCR as described in MATERIALS AND METHODS. PCR products were separated on an 8% polyacrylamide gel and stained with ethidium bromide.
thermore, Mrp3 expression seems to be dominant in IEC-6 cells as assessed by semiquantitative RT-PCR (Fig. 3B). The expression of Mrp3 was readily detectable after 26 cycles, whereas Mrp1 was detected to an equal extent only after 42 cycles. Mrp2 was not detected even after 40 cycles (data not shown), which was consistent with the data obtained by Northern blot analysis.

**Immunolocalization of Mrp3 protein in IEC-6 cells.**

The localization of Mrp3 protein was determined in IEC-6 monolayers with anti-Mrp3 antiserum by using confocal laser scanning microscopy. Mrp3 was predominantly found to be localized on the basolateral membrane domain of IEC-6 monolayers (Fig. 4B). No fluorescence was observed in IEC-6 monolayers when primary antibody was omitted (Fig. 4A). Basolateral localization of Mrp3 was consistent with that found in vitro by using the canine kidney epithelial cell line Madin-Darby canine kidney transfected with human MRP3 (23) as well as under in vivo conditions present in the rat intestinal epithelium (36). Mrp1 was not detected under the same culture condition in IEC-6 cells.

**Expression of Mrp3 protein in IEC-6 cells.**

The expression of Mrp3 protein in IEC-6 cell membrane vesicles was also confirmed by Western blot analysis. A 190-kDa band was detected with anti-Mrp3 antiserum and was consistent with the size reported previously in liver and intestine (36). As positive and negative controls, membrane vesicles from Mrp3-expressing (Mrp3-Sf9) and GFP-expressing (GFP-Sf9) Sf9 cells were loaded (6 μg/lane), respectively. A 175-kDa band was detected in Mrp3-Sf9 but not in GFP-Sf9 (Fig. 5A) as reported previously (1). The difference in the molecular weight between IEC-6 cells and Sf9 cells may be accounted for by the lower degree of sugar modification in insect cells as reported for rabbit Mrp2 expressed in Sf9 cells (43). Densitometric analysis revealed a 55-fold higher expression of Mrp3 protein in Mrp3-Sf9 compared with membrane vesicles from IEC-6 cells. Expression of Mrp1 protein (190 kDa) in IEC-6 cells was only a tracer level compared with that in RBCs (Fig. 5B). The intense bands detected around 70 kDa in IEC-6 cells (Fig. 5B) are unknown proteins cross-reactive to the secondary antibody because they were still detected in the absence of primary antibody (data not shown).

**Transport of F-MTX by Mrp3-Sf9 and membrane vesicles from IEC-6 cells.**

To further characterize the F-MTX efflux directly, membrane vesicles were isolated from IEC-6 monolayers. The time-dependent uptake of F-MTX into membrane vesicles was significantly stimulated fourfold in the presence of ATP compared with that in the presence of AMP (Fig. 6A). In addition, there was clear Mrp3 and ATP depen-
dence in the uptake of F-MTX into Mrp3-Sf9, indicating that F-MTX is a good substrate for rat Mrp3 (Fig. 6B). No endogenous ATP-dependent uptake was observed in control GFP-Sf9 (Fig. 6B). To compare the transport characteristics observed in IEC-6 cells and Mrp3 expressed in Sf9 cells, the concentration dependence and inhibitor sensitivity were examined. As shown in Fig. 7, the ATP-dependent transport was saturable with similar \( K_m \) values of 11.0 ± 1.8 and 4.5 ± 1.1 \( \mu M \) for IEC-6 and Mrp3-Sf9, respectively, whereas the ATP-independent transport was not saturated ≤40 \( \mu M \). The maximum uptake rate \( (V_{max}) \) values obtained were 96.6 ± 9.7 pmol·mg\(^{-1}\)·min\(^{-1}\) and 1,226 ± 155 pmol·mg\(^{-1}\)·min\(^{-1}\) for IEC-6 and Mrp3-Sf9, respectively. The large difference in the \( V_{max} \) values may be due to the protein expression level and other factors as described in DISCUSSION. The LTD\(_4\)-receptor antagonist MK-571, a potent inhibitor of human MRP1 (\( K_i \) ~ 0.6 \( \mu M \)) (25) and rat Mrp2 (\( K_i \) ~ 2.6 \( \mu M \)) (16), E\(_217\)βG (\( K_m \) ~ 67 \( \mu M \)) (13, 14), and TC (\( K_m \) ~ 3.0 \( \mu M \)) (13, 14), both typical substrates of rat Mrp3, all inhibited the ATP-dependent uptake of F-MTX in IEC-6 membrane vesicles and Mrp3-Sf9 with comparable sensitivity (Table 1).

DISCUSSION

The aim of the present study was to identify the efflux transporter(s) for MTX and its derivative in the IEC-6 intestinal crypt model. With the use of F-MTX, a fluorescent derivative of MTX, as a model compound, we confirmed that the F-MTX uptake in IEC-6 cells was optimal at a low pH, and significantly enhanced accumulation of F-MTX in the cells was observed at a lower temperature. These observations are consistent with the data reported by Rajgopal et al. (33). They found that the uptake of MTX into IEC-6 cells at pH 5.5 was twofold higher than that at pH 7.5. Moreover, the uptake of MTX in RFC-transfected IEC-6 cells was enhanced when energy metabolism was blocked by azide and reversed when glucose was present. The
present results and those reported by Rajgopal et al. (33) suggest that F-MTX and MTX may be taken up by the similar uptake transporter with a low pH optimum, likely RFC, and are also pumped out by active efflux transporter(s). However, until now, there has been no information about the molecular identity of the efflux transporter(s) in IEC-6 cells. In this study, stimulation of the F-MTX uptake in IEC-6 by MK-571, an inhibitor of the MRp family, indicated Mrp(s) as a likely candidate. The transcellular transport study further demonstrated specific localization of this putative Mrp-type transporter(s) on the basolateral membrane (Fig. 2). Among the members of the Mrp family, Mrp1, Mrp2, and Mrp3 have been reported to be expressed in intestinal epithelia and able to efflux organic anions including MTX, whereas the localization of these Mrps in the small intestine is quite different. Mrp1 is reported to be localized on the basolateral membrane of intestinal crypt cells, most abundantly in Paneth cells followed by partially differentiated crypts, but not expressed in the differentiated intestinal epithelial cells in the mouse (31). On the other hand, no Mrp2 and Mrp3 expression was observed in the crypt region, whereas it increased along the crypt in the tip direction and reached a maximum at the top of the villus in the rat (29, 36). Moreover, the intracellular distribution is quite different between Mrp2 and Mrp3. Mrp2 is localized on the apical membrane, whereas Mrp3 is found on the basolateral membrane of polarized epithelial cells (29, 36). Although we could not compare the absolute expression levels among these transporters by using different cDNA probes, primers, and antibody, Mrp3 seems to be the dominant Mrp-type transporter expressed in IEC-6 cells (Fig. 3). As far as the expression of the Mrp family is concerned, IEC-6 seems to be a partially differentiated crypt model of the small intestine, although it is reported to reflect immature crypt cells unless an appropriate differentiation-promoting factor, such as Cdx2, is introduced (42). A similar expression profile of the Mrp family was observed in hepatocytes. Mrp1 and Mrp3 are poorly expressed under normal conditions in human and rodent hepatocytes, whereas Mrp2 is abundantly expressed on the apical canalicular membrane. In proliferating hepatocytes after partial hepatectomy, Mrp2 is down-regulated and Mrp1 is inversely induced (34). Similarly, Mrp3 is induced, whereas Mrp2 is down-regulated in obstructive cholestasis (40) or in the liver of Dubin-Johnson syndrome patients (22). Predominant expression of Mrp1 and Mrp3 rather than Mrp2 seems reasonable in undifferentiated IEC-6 cells. Protein expression of Mrp3 was also confirmed by Western blot and confocal laser scanning microscopy. Basolateral-specific staining of Mrp3 is consistent with the in vivo intestinal epithelia and the in vitro vectorial transport property of F-MTX from the apical to the basolateral compartment in IEC-6 monolayers.

The functional characteristics of F-MTX transport into IEC-6 membrane vesicles were also similar to those into Mrp3-Sf9 membrane vesicles, implying the involvement of Mrp3 in the transport of F-MTX in IEC-6 cells. The $K_m$ values (11 vs. 4.5 μM) were similar in both membrane vesicles. Hirohashi et al. (13) have reported that the IC₅₀ value of MTX on the ATP-dependent uptake of $[^{3}H]$E₂₁⁷G by rat Mrp3-expressing vesicles is between 50 and 100 μM, which is nearly equal to the $K_m$ value under tracer experiment conditions. Because MTX is also a transport substrate of Mrp3 (1, 13), the $K_m$ value is also supposed to be between 50 and 100 μM. The affinity of MTX for human MRP3 was as low as 776 μM (45). Thus our data clearly demonstrate that F-MTX is a substrate with much higher affinity for Mrp3 compared with MTX itself (13, 45). Although information about structure-recognition relationships is limited, the addition of the FITC moiety with a monocarboxylate and a degree of hydrophobicity may be favorable as far as Mrp3 recognition is concerned. A similar phenomenon has been observed in rabbit Mrp2 (43). The ATP-dependent uptake of $[^{3}H]$E₂₁⁷G by rabbit Mrp2 was reduced to 94 and 55% in the presence of fluorescein (1 mM) and MTX (1 mM), respectively, whereas it was reduced to 22% in the presence of as little as 100 μM F-MTX (43).

Inhibitor sensitivity of F-MTX was similar but not completely identical for IEC-6 and Mrp3-Sf9. For example, 75 μM E₂₁⁷G and 200 μM TC inhibited the ATP-dependent uptake of F-MTX into Mrp3-Sf9 vesicles by 80% but only inhibited it into IEC-6 vesicles by 60%. These differences may be derived from a contribution by other ATP-dependent transporter(s) in IEC-6 cells whose inhibitor sensitivity is lower than that of Mrp3. As far as human MRP1 is concerned, the $K_m$ value for E₂₁⁷G is reported to be 2.5 μM (26), a rather higher affinity than that of rat Mrp3 (67 μM) (13). Our data showing that 75 μM E₂₁⁷G was not enough to inhibit the ATP-dependent uptake of F-MTX into IEC-6 vesicles (Table 1) supports the hypothesis that Mrp1 is a minor and Mrp3 a major efflux pump for F-MTX in IEC-6 cells given that rat Mrp1 has a similar affinity to human MRP1 for E₂₁⁷G. Although the expression of Mrp3 protein in Mrp3-Sf9 was 55-fold higher than that in IEC-6 vesicles, the $V_{max}$ value of the transport in Mrp3-Sf9 was only 12-fold higher than that in IEC-6 vesicles. Such a difference may be due to the following: 1) the contribution of other transporter(s) may not be negligible, 2) the transport activity is reduced by immature sugar modification (Fig. 5A) or the different membrane environment in insect cells (28), and 3) the difference in the content of inside-out vesicles between Mrp3-Sf9 and IEC-6 vesicles may affect the net transport activity. Because ATP is membrane impermeable and the ATP-hydrolysis domain is located in the cytosolic region of the Mrp molecule, only inside-out vesicles but not right-side-out vesicles are involved in the ATP-dependent transport. Although we do not know which is the case, the above factors may account for such a discrepancy.

In conclusion, F-MTX is also a good substrate for rat Mrp3 and will be of great use for the study of Mrp transporters. The vectorial transport of F-MTX from the apical to the basolateral compartment in IEC-6 is
mainly mediated by Mrp3 expressed on the basolateral membrane.

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