Differential Multidrug Resistance-Associated Protein 1 through 6 Isoform Expression and Function in Human Intestinal Epithelial Caco-2 Cells

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ABSTRACT

Multidrug resistance-associated protein (MRP) isoforms 1 through 6 mRNA are expressed in the human intestine and Caco-2 cells. In Caco-2 cells, the rank order for mRNA expression was MRP2 > MRP6 > MRP4 > MRP3 > MRP1 = MRP5. The functional expression of MRP-like activity was quantified as the efflux of the fluorescent probe calcein from confluent, polarized monolayers of Caco-2 cells. Calcein efflux was sensitive to temperature, energy depletion, and the MRP antagonist MK571 [3-[3-[2-(7-chloroquinolin-2-yl)vinyl]phenyl]-(2-dimethylcarbamoylethylsulfanyl)methylsulfanyl] propionic acid]. Calcein efflux across the apical membrane of Caco-2 cells exceeded that across the basolateral by approximately 2-fold, correlating with the apical localization of MRP2 visualized by immunocytochemical staining. T84 cells do not express MRP2 and show a predominance of basolateral calcein efflux over apical efflux. MRP3 was localized by immunocytochemical staining to the basolateral membrane. MRP1 staining was not localized to either membrane domain and MRP5 staining was not detected. Thus, basolateral calcein efflux may reflect a function of MRP3 or MRP4 and 6 inferred by their basolateral localization in other tissues. Basolateral, but not apical, calcein efflux was sensitive to glutathione depletion with buthionine-sulfoximine, indicating that whereas MRP2-mediated apical efflux is independent of glutathione, basolateral efflux is glutathione-dependent. Benz bromarone, probenecid, pravastatin, and diclofenac were able to inhibit both apical and basolateral calcein efflux. The apical calcein efflux in Caco-2 cells was selectively sensitive to indomethacin and propranolol, but not verapamil or erythromycin, whereas the converse was observed for basal efflux. The differential pharmacological sensitivity of apical (MRP2) and basolateral calcein efflux provides tools for dissecting MRP isoform functional roles.

The intestinal epithelium provides a selective barrier, limiting the access of toxins and other xenobiotics, including orally administered drugs, taken in with the diet, while presenting an optimal surface for nutrient absorption. The intestinal epithelium is also an important site for excretion of specific classes of xenobiotics and endogenous toxins (Hunter and Hirst, 1997; Chan et al., 2004). The multidrug resistance MDR1 (ABCB1) gene product P-glycoprotein subserves an important part of this barrier function. P-glycoprotein provides for ATP-dependent efflux of a wide spectrum of chemically unrelated xenobiotics and drugs, in addition to the classic cytotoxics such as vinblastine and daunomycin. The apical localization of P-glycoprotein in the enterocyte allows it to function both in limiting xenobiotic absorption and in mediating intestinal xenobiotic secretion (Hunter et al., 1993a,b). Whereas P-glycoprotein has wide substrate specificity, it is recognized that other membrane transport proteins provide a complimentary role, allowing for a greater spectrum of xenobiotics to be handled (Chan et al., 2004).

The multidrug resistance-associated proteins (MRPs) belong to the same ATP-binding cassette (ABC) gene superfamily of membrane transporters as P-glycoprotein but together...
with the cystic fibrosis transmembrane conductance regulator and sulfonlurea receptor form a distinct gene family (ABCC). MRPs and P-glycoprotein have a tandem repeat of six membrane spanning domains linked to an ATP-binding cassette, although MRP1 through 3 and 6 through 7 have an N-terminal extension of an additional five membrane spanning domains (Tusnady et al., 1997; Chan et al., 2004). The MRP family has been shown to transport a diverse range of substrates. They predominantly transport anionic substances, but nonanionic organic drugs have also shown to be transported; it is thought this may be accomplished via either cotransport or conjugation with glutathione (Evers et al., 1998, 2000; Gerk and Vore, 2002; Chan et al., 2004). This contrasts with P-glycoprotein, which favors lipophylic compounds of a cationic nature. Nevertheless, it is recognized that P-glycoprotein and MRP isofoms illustrate wide overlap in both their substrate specificity and inhibitors. To this extent, P-glycoprotein and MRPs provide both complimentary and overlapping functionality as drug efflux pumps.

MRP1, 4, and 5 (ABCC1, 4, and 5) mRNAs show relatively ubiquitous expression, whereas the expression of MRP2, 3, and 6 (ABCC2, 3, and 6) is more restricted; expression of the latter is notable in renal, intestinal, and hepatic epithelia (Chan et al., 2004). MRP2 is the only MRP isofom identified to date as localizing to apical membranes (Büchner et al., 1996), where it may be postulated to provide for apical drug efflux, analogous to P-glycoprotein (Chan et al., 2004). In contrast, other MRP isofoms are described to localize to basolateral membranes in a variety of epithelia (Kool et al., 1999a,b; Peng et al., 1999; Chan et al., 2004).

In the present study, we have used the Caco-2 cell model of the human intestinal enterocyte to investigate the molecular expression of MRP1 through 6 and their membrane localization. We have then quantified the functional expression of MRP-like activity with the fluorescent probe calcein (Verantvoort et al., 1995; Essadaigue et al., 1998) and probed for functional differences in MRP-like activities at apical and basal membranes with a range of pharmacological tools. T84 cells, a model of human colonocytes that lack MRP2 expression (Lowes and Simmons, 2001, 2002), are used in comparison. We report that MRP2 is expressed apically in Caco-2 cells, correlating with the predominant apical efflux of calcine. This apical-mediated MRP function is sensitive to indomethacin and propanolol, but not to verapamil or erythromycin, whereas the converse is observed for basal efflux. The differential pharmacological sensitivity of apical (MRP2) and basolateral (MRP3 and/or 4 and 6) provides important tools for investigation of their different functional roles.

### TABLE 1

Sequence and position in the relevant cDNA of each MRP isoform oligonucleotide primers used in PCR analyses

<table>
<thead>
<tr>
<th>MRP Isoform</th>
<th>Primer Sequence (Position)</th>
<th>NCBI GenBank Accession No.</th>
<th>Product Size</th>
</tr>
</thead>
<tbody>
<tr>
<td>MRP1 (ABCC1)</td>
<td>Sense</td>
<td>63AGTGGACCTTCTTCTGAC...113</td>
<td>NM_004996</td>
</tr>
<tr>
<td>MRP2 (ABCC2)</td>
<td>Sense</td>
<td>875ACCTGTTGACCCACCAAGA...454</td>
<td>NM_005845</td>
</tr>
<tr>
<td>MRP3 (ABCC3)</td>
<td>Sense</td>
<td>374AGTTTGCGATTTCTCCGTO...150</td>
<td>NM_003276</td>
</tr>
<tr>
<td>MRP4 (ABCC4)</td>
<td>Sense</td>
<td>371AGCTGTTGACCTCTTAAA...413</td>
<td>NM_005845</td>
</tr>
<tr>
<td>MRP5 (ABCC5)</td>
<td>Sense</td>
<td>519AGCTGTTGACCTCTTAAA...180</td>
<td>NM_005845</td>
</tr>
<tr>
<td>MRP6 (ABCC6)</td>
<td>Sense</td>
<td>537AGCTGTTGACCTCTTAAA...217</td>
<td>NM_005845</td>
</tr>
</tbody>
</table>

### Materials and Methods

#### Materials

Cell culture reagents were obtained from Sigma Chemical (Poole, Dorset, UK). Culture plastic ware was from Corning Costar (Buckinghamshire, UK). Specific oligonucleotide primers (Table 1) were synthesized by the Molecular Biology Unit, University of Newcastle upon Tyne (MRP2–5) or Sigma Chemical (MRP1 and 6). Monoclonal antibodies to MRP1, 2, 3, and 5 were from Alexis Biochemicals (Nottingham, UK). Calcine acetoxyethyl ester was purchased from Molecular Probes (Leiden, The Netherlands). Unless otherwise indicated, Western blot reagents were obtained from Novex. All other reagents unless stated otherwise were supplied from Sigma Chemical.

#### Cell Culture.

Caco-2 cells (passage numbers 25–45) were from American Type Culture Collection (Manassas, VA) and cultured in Dulbecco’s modified Eagle’s medium (DMEM) with 4.5 g/l glucose, supplemented with 20% (v/v) fetal bovine serum, penicillin-streptomycin (100 U/ml and 100 μg/ml, respectively), and 1% (v/v) nonessential amino acids. Cells were maintained at 37°C in a humidified atmosphere of 5% CO₂ in air and subcultured weekly. T84 cells (passage 52–62) from American Type Culture Collection were maintained as for Caco-2 cells in 1:1 Ham’s F-12/DMEM (glucose 4.5 g/l) supplemented with 5% (v/v) newborn calf serum, 1% (v/v) nonessential amino acids, 14 mM HEPES, and 30 mg/ml gentamicin. Cell monolayers were prepared by seeding (2.0 × 10^6 cells/24-mm insert or 1 × 10^6 cells/12-mm insert for Caco-2 and 1.0 × 10^6 cells/24-mm insert for T84 cells) on tissue culture inserts (Transwell polycarbonate, 0.4-μm pore size) and maintained as described above for 14 to 21 days, with media replacement every 2 to 3 days. Transport studies were carried out on 24-mm-diameter monolayers with transepithelial electrical resistance (voltohmmeter; WPI, Sarasota, FL) values >300 and >1000 Ω cm² for Caco-2 and T84 monolayers, respectively, after subtraction of resistance values of inserts alone.

#### Polymerase Chain Reactions (PCRs) for MRP1 through 6.

Flask-grown Caco-2 cells (2 weeks postseeding) were washed and then lysed by addition of lysis buffer [50 μg/ml proteinase K, 5 mM EDTA, 0.25 M NaCl, 0.25 M Tris-HCl, pH 7.5, 2 mM MgCl₂, and 10% (v/v) SDS]. The lysed cell solution was passed four times through a 21-gauge needle and incubated at 45°C for 45 min. NaCl (317 μl of 5 M stock) was added and passage through a needle repeated. Poly A⁺ RNA was then isolated from the cell lysate via overnight incubation with oligo(dT) cellulose (Nacalamesh Biosciences UK, Ltd., Little Chalfont, Buckinghamshire, UK). The solution was centrifuged at 4000g for 8 min, and the oligo(dT) cellulose-poly A⁺ RNA pellet was subjected to a series of washes in high (500 mM NaCl, 10 mM Tris-HCl, pH 7.5) to medium (250 mM NaCl, 10 mM Tris-HCl, pH 7.5) salt buffers. Poly A⁺ RNA was finally obtained after elution in a spin column after addition of 10 mM Tris-HCl and then precipitation by storage overnight at −80°C in sodium acetate and absolute ethanol.

Snap-frozen human small intestine and colon samples were obtained from specimens taken for diagnostic purposes (Newcastle
NHS Hospital Trusts), with ethical approval and written informed consent. Total RNA was isolated using the RNAzol B isolation kit (Biogenesis Ltd., Poole, Dorset, UK) after which poly A- RNA was isolated by the method described above.

Reverse transcription was performed by incubating 0.1 µg of poly A+ RNA, 0.5 mM each of the four dNTPs (MBI Fermentas, Vilnius, Lithuania), 0.001 U/µl random hexanucleotide primers (Amersham Biosciences UK, Ltd.), 16 U of ribonuclease inhibitor (MBI Fermentas), 20 U of Moloney murine leukemia virus reverse transcriptase, and 1X reaction buffer (both MBI Fermentas) in a final volume of 20 µl for 2 h at 42°C. Expression of MRP1 through 6 was analyzed by standard PCR by using a hot-start protocol with Platinum TaqDNA polymerase (Invitrogen, Carlsbad, CA), 2 mM MgCl₂, 0.2 mM dNTP mix, and oligonucleotide primers (Table 1: 0.5 µM) for each MRP isoform. PCR amplification consisted of 30 cycles of denaturation at 94°C for 30 s, extension at 55.4–65.3°C (primer specific) for 30 s, and 72°C for 30 s. PCR products were separated by gel electrophoresis through 1% agarose, stained with ethidium bromide, and imaged under UV light (AlphaImager; Flowgen, Shenstone, UK). PCR products were subcloned (TOPO-TA; Invitrogen) for subsequent sequencing.

The relative abundance of MRP mRNA was then determined by semiquantitative reverse transcription-PCR. Total RNA was isolated from confluent flasks of Caco-2 cells or 12-mm Transwell inserts using the SV total RNA extraction kit (Promega. Madison, WI). Reverse transcription was performed as described above with the following exceptions: 1 µg of total RNA, 40 U of RNasin (Promega), and 100 U of Moloney murine leukemia virus reverse transcriptase with 1X reaction buffer (Promega) were used. Expression of MRP1 through 6 was then compared with 18S ribosomal RNA using PCR. PCR was performed as described above with the following exceptions: MgCl₂ (1.5–2.5 mM) and gene-specific primers (Table 1: 0.4–1.0 µM) were optimized for each MRP isoform and an 18S rRNA primer: competitor ratio of 1:10 (Quantum RNA kit; Ambion, Austin, TX) was used. PCR amplification consisted of 28 cycles of denaturation at 94°C for 30 s, extension at 55.9–64.3°C (primer specific) for 30 s, and 72°C for 30 s.

Expression of MRP1 through 6 was also analyzed using a human multiple tissue cDNA panel (BD Biosciences Clontech, Palo Alto, CA) containing samples pooled from five or more male and female donors (except liver, one female and stomach male donors only). This was used to assess the distribution of MRP1 through 6 through the gastrointestinal tract. PCR conditions were as for semiquantitative analysis, with the inclusion of glyceraldehyde-3-phosphate dehydrogenase to “standardize” for cDNA (BD Biosciences Clontech).

Immunofluorescent Staining for MRP. Caco-2 cells were grown on 12-mm diameter Transwell inserts for the purpose of immunostaining. Inserts were washed three times in phosphate-buffered saline (PBS); similar washes were included between each of the following stages. Cells were fixed in 3% paraformaldehyde for 15 min and then permeabilized with 0.1% Triton X-100 for 10 min. Nonspecific binding sites were blocked by incubation for 30 min with 5% normal sheep serum (MRP2 and 3) or 5% normal mouse serum (MRP1 and 5). Inserts were incubated in primary antibody (MRP1 = MRP1, MRP2 = M₁-III-6, MRP3 = M₁-II-9, and MRP5 = M₁-I-I) diluted to 12.5 µg/ml with PBS, for 60 min. Primary antibody was detected by incubation with fluorescein isothiocyanate-conjugated sheep anti-mouse antibody for 60 min (MRP2 and 3) or with biotin-conjugated mouse anti-rat for 60 min followed by streptavidin-cy5 for 60 min (MRP1 and 5). Cell nuclei were stained with propidium iodide, 5 µg/ml, for 5 min. Inserts were washed and mounted in Vectashield (Vector Laboratories, Burlingame, CA). Staining was viewed using a Leica NT5 confocal laser scanning microscope. As a control for nonspecific binding, the above-mentioned procedure was carried out on matching filters with omission of the primary antibody.

MRP2 Protein (Western Blot). Cell protein was obtained by lysing Caco-2 cells with 2% (v/v) Igepal, 0.2% (w/v) SDS, and 1 mM dithiothreitol made in PBS containing a mini protease inhibitor cocktail tablet. The protein concentration was determined using a Bio-Rad DC protein assay kit. Samples were separated on NUPAGE Tris-acetate 3–8% gels alongside a high range molecular weight marker (Sigma Chemical). Proteins were transferred to Hybond-P membrane (Amersham Biosciences UK, Ltd.) by overnight electroblotting, and nonspecific binding was blocked (5% milk powder with 0.1% Tween 20, 60 min). Primary antibody, diluted in blocking solution (MRP2 = M₁-III-6, 10 µg/ml, or β-actin antibody, A5060, 1:125 dilution; Sigma Chemical) was added and incubated for 120 min. Membranes were washed in Tween/Tris-buffered saline and secondary antibody diluted in blocking solution (anti-mouse or -rabbit peroxidase-conjugated antibodies, 1:30,000 and 1:45,000, respectively; Amersham Biosciences UK, Ltd.) added for 60 min. Membranes were washed in Tween/Tris-buffered saline and developed using an enhanced chemiluminescence plus (ECL+) kit from Amersham Biosciences UK, Ltd.

Transport Studies. MRP functional activity was investigated by quantifying calcein efflux from confluent layers of Caco-2 cells. The principle of the assay is to load the cells with calcein using the membrane-permeant form calcein acetoxyethyl ester (AM), which is cleaved by intracellular esterases to release the fluorescent metabolite calcein. The efflux of free calcein (the fluorescent form) is then determined after washing away excess calcein-AM. Whereas calcein AM is a substrate for MDR1 and MRP, calcein is not a substrate for MDR1, thus allowing specific MRP efflux activity to be determined (Versantvoort et al., 1995; Essodaigui et al., 1998).

Inserts were washed three times in Krebs' buffer (137 mM NaCl, 5.4 mM KCl, 1.0 mM MgSO₄, 0.3 mM KH₂PO₄, 10 mM HEPES, 2.8 mM CaCl₂, 10 mM glucose, pH to 7.4 with Tris) and were loaded with calcein via incubation with the acetoxyethyl ester derivative (30 min at 37°C with 2 µM calcein-AM). Inserts were washed again, placed in new cluster plates at 37°C, and Krebs' buffer was added apically and basally. Efflux was determined by measuring the appearance of fluorescence in the bathing solutions (λₑ 492 nm and λₘ 518 nm; LS-5 spectrofluorometer; PerkinElmer Life and Analytical Sciences, Boston, MA). At the end of the experiment, cells were lysed in distilled water, and calcein fluorescence was determined spectrofluorometrically. Calcein efflux was calculated as a percentage of the available substrate at each time point.

For transport studies in the presence of inhibitors, both apical and basal solutions contained the inhibitor; high concentrations, based on literature information, were used with the concentrations indicated in the appropriate figure legend. None of the inhibitors used interfered with the fluorescence of calcein.

Results are shown as means ± 1 standard error of mean. Where appropriate (and unless otherwise indicated), results were analyzed by Student’s t test using non-normalized data with a significance level of P < 0.05. Calcein efflux inhibition data are shown as percentage of control values at time point 60 min.

Results

Intestinal Expression of MRP mRNA. The expression of MRP1 through 6 was analyzed in the human digestive tract using a multiple tissue cDNA panel consisting of samples pooled from healthy donors. Bands of the predicted sizes were amplified for MRP1, 3, 4, and 5 in all of the tissues (Fig. 1). In addition, MRP1 through 6 all showed significant levels of expression in the jejunum and ileum, with lower expression in the duodenum (Fig. 1). MRP2 showed only low levels of expression in the distal intestine, contrasting to very high levels of expression in liver (Fig. 1). MRP6 also showed high expression in liver with low expression in the distal intestine, and no product in esophagus (Fig. 1). PCR products of the

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predicted sizes were also obtained for MRP1 through 6 in Caco-2 cells (Fig. 2A). Sequencing of the PCR products from Caco-2 cells revealed 100% identity with the sequence in GenBank (Table 1). In addition, sequencing of the PCR products from human small intestine (MRP1–6) and human colon (MRP1, 3, 5, and 6) also revealed 100% identity with the sequence in GenBank (Table 1). The low yield of PCR product for MRP2 and 4 in human colon prevented sequencing to confirm identity of the product.

The expression of MRP1 through 6 in Caco-2 cells was determined relative to that of 18S rRNA. The expression of MRP isoforms was compared for cells grown in flasks with cells grown on tissue culture inset filters (Fig. 2). MRP2 and 6 were most abundant PCR products, followed by MRP3 and 4, with expression of MRP1 and 5 lowest (Fig. 2).

**Immunofluorescent Staining for MRP1, 2, 3, and 5.** The localization of MRP1 through 3 and 5 was investigated by indirect immunofluorescent staining and confocal laser scanning microscopy. Cell nuclei were revealed with propidium iodide. Monolayers stained with anti-MRP5 antibody M5-I-I did not show a detectable staining above background (omission of primary antibody). Positive staining was observed for MRP1 through 3 (Fig. 3). When viewed en face, in the plane of the monolayer, MRP1 staining seemed cytoplasmic and distributed throughout the cells, and strong staining was observed for MRP2 localized above the cell nuclei and MRP3 staining outlined cell junctions. Viewed in the plane perpendicular to the monolayer confirmed MRP1 staining as intracellular and not associated with the cell membrane. MRP2 staining localized to the apical membrane, with some intracellular staining also apparent, whereas MRP3 staining localized at the lateral membrane with very little intracellular staining (Fig. 3). Antibodies to MRP4 or 6 were unavailable.

**Calcein Efflux from Caco-2 Cell Monolayers.** Efflux of calcein from Caco-2 cells was greater to the apical compartment than the basal [approx. 2-fold greater at 60 min; 0.27 ± 0.02 and 0.12 ± 0.01% of available substrate/min (n = 12), respectively; Fig. 4B]. Calcein efflux into both apical and basolateral compartments was sensitive to ATP depletion [apical efflux, 0.41 ± 0.01 and 0.05 ± 0.003; basal efflux, 0.08 ± 0.01 and 0.04 ± 0.01% available calcein/min at 60 min (n = 12), in the absence or presence, respectively, of an ATP-depleting solution (15 mM sodium azide/50 mM 2-deoxyglucose)]. The efflux of calcein into the apical and basolateral compartments was also both significantly (two-way analysis of variance) inhibited by reduction of temperature to 4°C [0.11 ± 0.01 and 0.06 ± 0.004% available calcein/min at 60 min (n = 12), apical and basal efflux, respectively]. In the presence of 10 μM MRP inhibitor MK571 (Gekeler et al., 1995), both apical and basal efflux was significantly reduced [37.1 ± 4.7 and 66.5 ± 7.1% of control efflux (n = 12), respectively; Fig. 4B]. These data are consistent with MRP-like activity localized at both apical and basolateral membranes of Caco-2 cells.
Role of MRP2 in Apical Calcein Efflux. The greater magnitude of the apical calcein efflux, considered together with the apical localization of MRP2 (Fig. 3) and the relative abundance of MRP2 mRNA in Caco-2 cells (Fig. 2), leads to the hypothesis that MRP2 is the isoform mediating apical calcein efflux. This hypothesis was tested by use of human colonic T84 cells. Western blot analyses confirmed that Caco-2 cells express MRP2 protein, whereas MRP2 was not detectable in T84 cells (Fig. 4A), in agreement with the low levels of MRP2 mRNA in the distal intestine (Fig. 1). In T84 cells, in contrast to Caco-2 cells (Fig. 4B), basal calcein efflux was greater than apical efflux (0.07 ± 0.01 and 0.03 ± 0.01% of available substrate/min at 60 min (n = 12), respectively; Fig. 4C). MK571 (10 μM), in contrast to its more potent effect on apical efflux in Caco-2 cells (Fig. 4B), failed to result in a significant reduction in apical efflux in T84 cells (0.02 ± 0.002% available calcein/min (n = 12)), whereas basal efflux was sensitive to the MRP inhibitor 0.02 ± 0.003% available calcein/min (n = 12)]. Therefore, absence of MRP2 in the T84 cells leads to a decrease in the apical calcein efflux, which is no longer sensitive to the MRP inhibitor MK571, whereas basal calcein efflux is still sensitive to MK571.

Differential Pharmacological Sensitivity of Apical and Basal Calcein Efflux. The differential sensitivity of apical and basal calcein efflux to some common pharmacological agents was investigated in Caco-2 cells (Fig. 5). A range of agents inhibited both apical and basal efflux of calcein, including benz bromarone, probenecid, pravastatin, and diclofenac (Fig. 5). This inhibition of apical and basal calcein efflux was associated with a significant increase in cellular retention of calcein (Fig. 5). In contrast, verapamil (100 μM) and erythromycin (1 mM) had no significant effect on apical efflux (95.6 ± 12.5 and 96.0 ± 2.0% of control, respectively) but significantly reduced basal efflux to 31.7 ± 8.6 and 63.8 ± 5.0% of control, respectively. Verapamil and erythromycin inhibition of basal calcein efflux was not associated with an increased cellular retention of calcein (Fig. 5), consistent with the predominant apical efflux not having been affected. In contrast, indomethacin (500 μM) and propranolol (1 mM) significantly reduced apical efflux of calcein without significantly reducing efflux of calcein into the basal solution. Indomethacin and propranolol, similar to the agents that reduced both apical and basal efflux, resulted in significant enhancement of calcein retention by the cells. Quinidine, a P-glycoprotein inhibitor, had no significant effect on the efflux of calcein into either the apical or basal compartments, while also not influencing cellular calcein retention. Thus, pharmacological tools that inhibit the dominant calcein efflux into the apical solution (Fig. 4B), but not those that inhibit only the smaller efflux into the basal solution, enhance cellular retention of calcein.

Buthionine Sulfoximine Treatment. Caco-2 cells were pretreated for 24 h with dl-buthionine-(S,R)-sulfoximine (BSO) (9 mM), a specific irreversible inhibitor of γ-glutamylcysteine synthetase (Griffith and Meister, 1979), to reduce intracellular glutathione for 24 h (Arttamangkul et al., 1999) before loading with calcein. Apical calcein efflux was unaffected by BSO pretreatment [0.25 ± 0.01 and 0.26 ± 0.01% available substrate/min at 60 min for control and BSO-pretreated cells (n = 12), respectively]. In contrast, basal calcein efflux was significantly (two-way analysis of variance) reduced by BSO pretreatment [0.08 ± 0.01% available substrate/min (n = 12)] compared with control (0.10 ± 0.02% available substrate/min (n = 12)).
Discussion

Differential localization of MRP isoforms to the apical and basolateral membrane domains of intestinal enterocytes allows for complimentary roles in detoxification of enterocytes, by excretion into the intestinal lumen or blood, respectively. In addition, MRP2, localized to the apical membrane, may play a role, in parallel with MDR1 and the breast cancer resistance protein (BCRP), in limiting oral drug absorption. In contrast, MRP isoforms localized to the basolateral membrane domain may enhance intestinal absorption by maintaining a favorable gradient for drug absorption. Transport of the prototypic MRP substrate calcein across the apical and the basolateral membranes of intestinal Caco-2 cell layers shows differential sensitivity to a range of pharmacological agents. Thus, the function of different MRP isoforms may be separated on the basis of pharmacological sensitivity.

The human small intestine and Caco-2 cells express MRP isoforms 1 through 6 (ABCC1–6) at medium to low levels (Taipalensuu et al., 2001; Langmann et al., 2003). Additional MRP isoforms 7, 8, and 9 have been described (ABCC10, 11, and 12, respectively). There are no data to date to indicate significant expression in the intestine, whereas the functional characterization is incomplete (Chan et al., 2004). The liver expresses considerably higher levels of MRP3, 6, and in particular MRP2, compared with the intestine. In general terms, duodenal expression of MRP isoforms is lower than that in the jejunum and ileum, with MRP2 and MRP6 being particularly restricted to these latter two segments. This may be a reflection of greater exposure of the distal small intestine to digested food components, including those of a potentially toxic nature, and/or a greater role in the intestinal excretory pathway. In Caco-2 cells, based upon the ratio of MRP:18S rRNA expression, the rank order for expression was MRP2 ≥ MRP6 > MRP4 ≥ MRP3 > MRP1 = MRP5 in the present study, similar to that found in other studies using slightly different techniques and normalization methods, with MRP2 being consistently the most abundant mRNA and lower levels of MRP1 and MRP5 (Hirohashi et al., 2000; Taipalensuu et al., 2001). We found no difference whether Caco-2 cells were grown on plastic or on porous tissue culture inserts. The parallel expression of MRP in the small intestine (jejunum/ileum) and Caco-2 cells emphasizes the utility of the latter as a cellular model for transport studies.

MRP2 is localized to the luminal membrane of the small intestine (Fromm et al., 2000; Mottino et al., 2000) and Caco-2 cells (Fig. 3; Walgren et al., 2000). Additional intracellular MRP2 staining may reflect intracellular vesicular pools or trafficking pathways. Calcein efflux from polarized confluent layers of Caco-2 cells was predominantly into the apical solution, paralleling calcein efflux from and secretion across the rodent intestinal mucosa (Fujita et al., 1997; Stephens et al., 2002). MDR1 (ABCB1) and BCRP (ABCG2) are both highly expressed in the small intestine (Taipalensuu et al., 2001; Langmann et al., 2003) where they are localized to the apical membrane (Thiebault et al., 1987; Maliepaard et al., 2001). MDR1 expression in Caco-2 cells parallels that in the jejunum (Taipalensuu et al., 2001) with clear apical localization in Caco-2 cells (Hunter et al., 1993a). In contrast, expression of BCRP in Caco-2 cells is 100-fold lower than in the jejunum (Taipalensuu et al., 2001). Neither MDR1 nor BCRP count calcein among their diverse substrates (Hollo et al., 1994; Litman et al., 2000); in the present study, calcein efflux was not influenced by inclusion of the MDR1 substrate quinidine, similar to results in the mouse intestine (Stephens et al., 2002). Together, these pieces of evidence argue for apical calcein efflux mediated by MRP2 in Caco-2 cells and by inference in the small intestine. In T84 cells, which lack MRP2 expression (Fig. 4; Lowes and Simmons, 2001, 2002), basolateral calcein efflux dominates, consistent with apical calcein efflux mediated by MRP2 in Caco-2 cells. T84 cells express MDR1, functionally localized to the apical membrane (Hunter et al., 1991), again confirming that calcein efflux is independent of MDR1 expression.

In contrast to MRP2, other MRP isoforms have only been reported to localize to the basolateral membranes of a variety of tissues. We were unable to visualize MRPI at either plasma membrane, consistent with its absence from plasma membrane fractions of Caco-2 cells (Walle et al., 1999). MRPI3 demonstrated pronounced staining of the lateral membranes...
of Caco-2 cells (Fig. 3; Bock-Hennig et al., 2002), consistent with lateral membrane localization of endogenous MRP3 in HepG2 and heterologously expressed MRP3 in polarized cells (Kool et al., 1999b). MRP4-mediated prostaglandin transport is sensitive to indomethacin, whereas MRP1-mediated transport is insensitive (Reid et al., 2003). If MRP4-mediated calcein transport is similarly sensitive to indomethacin, it would exclude MRP4 as the transporter mediating the indomethacin-insensitive basolateral calcein efflux in Caco-2 cells. Direct studies of the affinity of MRP4 for calcein transport and its indomethacin sensitivity are required to confirm this speculation; MRP4 localization in Caco-2 was not possible due to lack of availability of antibodies. MRP5 may also be excluded from a role in the efflux because calcein is not a substrate for MRP5 (McAleer et al., 1999), whereas only low levels of MRP5 mRNA were observed and MRP5 protein was not detected in Caco-2 cells (Figs. 2 and 3). Thus, MRP3 may be a strong candidate for basolateral MRP function in Caco-2 cells, but the involvement of MRP6, localized to the basolateral membrane in kidney and liver (Kool et al., 1999a), should also be considered (MRP6 antibodies were unavailable for the present study).

The present study has identified pharmacological tools that enable at least partial dissection of the functional roles of MRP isoforms. Thus, apical, MRP2-mediated calcein efflux, although sensitive to indomethacin and propranolol, was insensitive to verapamil and erythromycin. In contrast, the basolateral, non-MRP2-mediated, calcein efflux demonstrated a mirror image sensitivity/insensitivity profile. Such differential inhibitory profiles points to hitherto unidentified (Stephens et al., 2002) tools that may be useful in dissecting the roles of specific MRP isoforms in intestinal drug transport and toxicity and that may have wider utility in discerning the functional roles of MRP isoforms in other tissues. It is likely that these tools are imprecise, allowing differential, rather than absolute, discrimination. In addition, the differential inhibitory profiles described here are for human MRP isoforms and caution may be needed before extension to studies in other species.

The role of glutathione conjugation in MRP-mediated transport is complex (Evers et al., 1998, 2000; Gerk and Vore, 2002; Chan et al., 2004). Thus, some substrates require glutathione conjugation, whereas for others cotransport of substrate with glutathione is noted, and for yet others, independence of glutathione is noted. Basal, but not apical, calcein efflux was sensitive to glutathione depletion with BSO. This indicates that although MRP2-mediated apical efflux is independent of glutathione, basolateral, non-MRP2-mediated efflux is glutathione-dependent. MRP1 transport of calcein is reported to be glutathione-independent (Feller et al., 1995), again arguing against a role for MRP1 in basolateral calcein efflux in Caco-2 cells. Thus, an additional experimental paradigm to reveal the roles of different MRP isoforms in intestinal drug transport and detoxifying mechanisms is revealed.

We have identified key pharmacological tools to investigate the role of different MRP isoforms in the detoxification and membrane transport in intestinal enterocytes. Apical MRP function dominates through the localized, relatively high level expression of MRP2. Apical, MRP2-mediated function is selectively sensitive to indomethacin and propranolol. In contrast, MRP-mediated transport at the basolateral membrane is lower in magnitude; selectively sensitive to verapamil, erythromycin, and glutathione-depletion; and probably mediated by MRP3 and/or 6. Identification of the differential
sensitivities of the intestinal apical and basolateral MRP-mediated efflux systems allows for the development of selective agents to modify intestinal drug absorption or drug excretion.

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References


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