0026-895X/99/050929-09$3.00/0

MOLECULAR PHARMACOLOGY, **55:**929–937 (1999).

Drug Resistance and ATP-Dependent Conjugate Transport Mediated by the Apical Multidrug Resistance Protein, MRP2, Permanently Expressed in Human and Canine Cells

YUNHAI CUI, JÖRG KÖNIG, ULRIKE BUCHHOLZ, HERBERT SPRING, INKA LEIER, and DIETRICH KEPPLER

Deutsches Krebsforschungszentrum, Heidelberg, Germany

Received September 25, 1998; accepted February 19, 1999

This paper is available online at http://www.molpharm.org

ABSTRACT

The multidrug resistance protein MRP1 functions as an ATP-dependent conjugate export pump and confers multidrug resistance. We cloned MRP2 (symbol ABCC2), a MRP family member localized to the apical membrane of polarized cells. Stable expression of MRP2 in transfected human embryonic kidney (HEK-293) and Madin-Darby canine kidney (MDCK) cells was enhanced by inhibitors of histone deacetylase. In polarized MDCK cells, both rat and human MRP2 were sorted to the apical plasma membrane. An antibody raised against the amino terminus of rat MRP2 recognized the recombinant protein on the apical surface of nonpermeabilized cells, providing direct evidence for the extracellular localization of the amino terminus of MRP2. ATP-dependent transport by recombinant human and rat MRP2 was measured with membrane vesicles from stably transfected cells. The K_m value of human MRP2 was 1.0 ± 0.1 μM for leukotriene C_4 and 7.2 ± 0.7 μM for 17β-glucuronosyl estradiol; the K_m values of human MRP1 were 0.1 ± 0.02 μM for leukotriene C_4 and 1.5 ± 0.3 μM for 17β-glucoronosyl estradiol. Thus, the conjugate-transporting ATPases MRP2 and MRP1 differ not only by their domain-specific localization but also by their kinetic properties. Drug resistance conferred by recombinant MRP2 was studied in MDCK and HEK-293 cells using cell viability assays. Expression of human and rat MRP2 enhanced the resistance of MDCK cells to etoposide 5.0-fold and 3.8-fold and to vincristine 2.3- and 6.0-fold, respectively. Buthionine sulfoximine reduced resistance to these drugs. Human MRP2 overexpressed in HEK-293 cells enhanced the resistance to etoposide (4-fold), cisplatin (10-fold), doxorubicin (7.8-fold), and epirubicin (5-fold). These results demonstrate that MRP2 confers resistance to cytotoxic drugs.

Membrane proteins mediating the ATP-dependent transport of conjugates of lipophilic compounds with glutathione, glucuronate, or sulfate have been recognized as members of the multidrug resistance protein (MRP) family. This family of conjugate-transporting ATPases includes MRP (Cole et al., 1992), also known as MRP1, and the canalicular isoform of MRP (Büchler et al., 1996), also known as canalicular multispecific organic anion transporter (Paulusma et al., 1996; Taniguchi et al., 1996; Ito et al., 1997) or apical MRP, now widely termed MRP2; as well as related ATP-binding cassette transporters from yeast, plants, and *Caenorhabditis elegans* (reviewed by Keppler and König, 1997). Substrate-dependent ATPase activity of the purified protein has been demonstrated for MRP1 (Chang et al., 1997). ATP-dependent transport of conjugates and other amphiphilic anions into inside-out-oriented membrane vesicles from MRP1-overexpressing drug-selected and MRP1-transfected cells has been established (Jedlitschky et al., 1994, 1996; Leier et al., 1994; Müller et al., 1994; Loe et al., 1996).

The apical isoform of MRP was originally recognized in the rat hepatocyte canalicular membrane by immunofluorescence microscopy and by cloning of a novel 347-bp cDNA fragment that was distinct from rat MRP (Mrp1); this cDNA fragment was not expressed in the liver from mutant rats that lack ATP-dependent transport of conjugates across the canalicular membrane (Mayer et al., 1995). Subsequent cloning of the full-length cDNA revealed rat apical MRP (Mrp2; Paulusma et al., 1996; Büchler et al., 1996; Ito et al., 1997;

This work was supported in part by grants from the Deutsche Forschungsgemeinschaft (Grants SFB 352/B3 and SFB 601/A2); the Forschungsschwerpunkt Transplantation, Heidelberg; and the Tumorzentrum, Heidelberg/Mannheim.

[1] The nucleotide sequences reported in this paper have the GenBank/EBI Data Bank accession numbers X96395 (human MRP2), X96393 (rat Mrp2), and X90643 (347 bp fragment of rat Mrp2).

ABBREVIATIONS: MRP, human multidrug resistance protein (symbol ABCC1); BSO, buthionine sulfoximine; HEK-293, cell line derived from human embryonic kidney; LTC_4, leukotriene C_4; LY335979, 4-(1,1-difluoro-1,1a,6,10b-tetrahydrodibenzo[a,e]cyclopropa[c]cyclohepten-6-yl)-α-[(5-quinolinyloxy)methyl]-1-piperazineethanol; MDCK II, cell line derived from Madin-Darby canine kidney cells; MRP2, human apical multidrug resistance protein (symbol ABCC2); Mrp2, rat apical multidrug resistance protein; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PCR, polymerase chain reaction; TMAP, transmembrane topology analysis program.

Madon et al., 1997). The human ortholog, MRP2, was cloned from liver tissue (Büchler et al., 1996; Paulusma et al., 1997) and from drug-resistant tumor cells (Taniguchi et al., 1996). The amino acid identity between human MRP1 and MRP2 is 49% (Keppler and König, 1997). *MRP1* and *MRP2* have been localized to chromosomes 16p13.1 (Cole et al., 1992) and 10q24 (Taniguchi et al., 1996), respectively. The absence of MRP2 from the human hepatocyte canalicular membrane has been recognized as the cause of the Dubin-Johnson syndrome (Kartenbeck et al., 1996; Keppler and Kartenbeck, 1996), and several mutations in the *MRP2* gene were reported in this hereditary disorder (Paulusma et al., 1997; Wada et al., 1998). The predominant localization of rat and human MRP2 in the hepatocyte canalicular membrane has been consistent with a hepatic canalicular transporter. However, the additional localization of this transport protein to the apical membrane of kidney proximal tubules (Schaub et al., 1997) indicates that the characteristic expression and sorting of MRP2 is to the apical domain of polarized cells.

The permanent expression of human MRP2 in stably transfected cell lines enables studies on the sorting of this integral membrane protein, and membrane vesicles prepared from *MRP2*-transfected cells provide an important tool for establishing the substrate specificity of this ATP-dependent transporter, both with respect to physiological endogenous substrates and with respect to anticancer drugs and their derivatives. Previously, some information on the substrate specificity of rat Mrp2 has been gathered from determinations of ATP-dependent transport into inside-out hepatocyte canalicular membrane vesicles from normal rats in comparison with membrane vesicles from mutant rats selectively lacking Mrp2 (Ishikawa et al., 1990; Oude Elferink et al., 1995; Jedlitschky et al., 1997). Direct information on the substrate specificity of recombinant rat Mrp2 has been obtained recently after transient expression in COS7 cells and *Xenopus laevis* oocytes (Madon et al., 1997) and in transfected NIH3T3 cells (Ito et al., 1998). Evers et al. (1998) most recently showed that membrane vesicles from *MRP2*-transfected cells derived from Madin-Darby canine kidney (MDCK) cells exhibit ATP-dependent transport of the glutathione *S*-conjugates of 2,4-dinitrophenol and ethacrynic acid and that recombinant human MRP2 expressed in these polarized cells is sorted to the apical membrane. Furthermore, recombinant rabbit Mrp2 expressed in insect cells was recognized as an ATP-dependent transporter for leukotriene C_4 (LTC_4) and 17β-glucuronosyl estradiol (van Aubel et al. 1998). However, a kinetic characterization allowing for the direct comparison of K_m values between recombinant human MRP1 and MRP2 has been lacking, and, importantly, it remained an open question whether MRP2 confers multidrug resistance. Our results demonstrate for the first time that recombinant MRP2 confers resistance to etoposide, vincristine, cisplatin, doxorubicin, and epirubicin. As a prerequisite for these studies, we describe that inhibitors of histone deacetylase, such as butyrate and trichostatin A, induce a manifold overexpression of recombinant MRP2.

Experimental Procedures

Materials. [14,15,19,20-^{3}H]LTC_4 (6.1 TBq/mmol) and 17β-D-glucuronosyl [6,7-^{3}H]estradiol (2 TBq/mmol) were obtained from DuPont/New England Nuclear (Boston, MA). Unlabeled LTC_4 was from Cascade Biochem Ltd. (Reading, Berkshire, UK), and unlabeled 17β-D-glucuronosyl estradiol was obtained from Sigma Chemical Co. (St. Louis, MO). Nitrocellulose filters (pore size 0.2 μm) were obtained from Schleicher & Schüll (Dassel, Germany). Cell culture media and supplements were obtained from Sigma-Aldrich Chemie (Deisenhofen, Germany). G418 (Geneticin) was purchased from Calbiochem (Bad Soden, Germany). Sodium butyrate was obtained from Merck-Schuchardt (Hohenbrunn, Germany). Trichostatin A (4,6-dimethyl-7-[*p*-dimethylaminophenyl]-7-oxohepta-2,4-dienohydroxamic acid) and buthionine sulfoximine (BSO) were purchased from Sigma-Aldrich Chemie. LY335979 (4-(1,1-difluoro-1,1a,6,10b-tetrahydrodibenzo[a,e]cyclopropa[c]cyclohepten-6-yl)-α-[(5-quinolinyloxy)-methyl]-1-piperazineethanol; Dantzig et al., 1996) was kindly provided by Drs. J. J. Starling and A. Dantzig from the Eli Lilly Research Laboratories (Indianapolis, IN).

Antibodies. EAG5 is a polyclonal antibody raised in rabbits against the carboxy-terminal sequence of human MRP2 (Büchler et al., 1996; Jedlitschky et al., 1997). The polyclonal antibody EAG15 was raised against the corresponding sequence of rat Mrp2 (Büchler et al., 1996). MDK is a polyclonal antibody raised in rabbits against the amino-terminal sequence of rat Mrp2 (residues 1–25) coupled to keyhole limpet hemocyanin (Büchler et al., 1996) via the carboxyl end of the peptide. QCRL-1, an MRP1-specific monoclonal antibody, was kindly provided by Drs. R. G. Deeley and S. P. C. Cole (Queen's University, Kingston, Ontario, Canada). Monoclonal antibody C219 against MDR *P*-glycoproteins was purchased from Centocor (Malvern, PA).

Cloning of Human *MRP2* and Vector Constructions. Using 5 μg of poly(A)$^+$-enriched RNA (mRNA) and the Zap cDNA synthesis kit (Stratagene, Heidelberg, Germany), a unidirectional human liver cDNA library was constructed according to the manufacturer's instructions. The screening and plaque purification was performed as described (Büchler et al., 1996) with a fragment of human *MRP2* as probe. The hybridization yielded several *MRP2* clones, the longest being 2.3 kb (pMRP2.3′). A reverse transcription-polymerase chain reaction (PCR) strategy was used to obtain the 5′-end of *MRP2* cDNA. Human liver total RNA (5 μg) was reverse-transcribed in 50 μl of transcription buffer [50 mM Tris (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, 1 mM 2′-deoxynucleoside 5′-triphosphate, 40 U of RNasin] in the presence of 50 pmol hcrev3200 (5′-ATT TGA TGC ATG GAC GA-3′; bases 3162–3146) with 50 U of StrataScript reverse transcriptase at 37°C for 1 h. The resulting single-stranded cDNAs were purified by centrifugation through Microcon-100 (Millipore, Eschborn, Germany) and used for the subsequent PCR. PCR was performed in a total volume of 50 μl of PCR buffer (provided by manufacturer) containing 2.5 U TaqPlus DNA polymerase (Stratagene), and a 0.25-mM concentration of each sense and antisense primer. For the amplification of the 5′-fragment of human *MRP2*, the following primer pair was used: the sense primer hcfor5′, 5′-ATA GAA GAG TCT TCG TTC-3′ (bases −37 to −20); and the antisense primer revdeg-I, 5′-TTT GTC CTT TCA CTA GTT C-3′ (bases 2848–2830). The 5′-proximal sequence information needed for the design of the sense primer hcfor5′ was obtained from the expressed sequence tag (EST) library by searching for putative human *MRP2* sequences using the homologous 5′-proximal sequence of rat *mrp2* (Büchler et al., 1996). A clone (no. 124379; GenBank/EBI Data Bank no. R02250) was found to share an 80.7% identity with the rat *mrp2* sequence. With the assumption that this sequence was the 5′ part of human *MRP2*, we designed the primer hcfor5′ in front of the ATG start codon. The PCR was run at a denaturing temperature of 94°C for 1 min, at an annealing temperature of 50°C for 1 min, and at an elongation temperature of 72°C for 3 min, for a total of 35 cycles. The reaction was completed by a 10-min incubation at 72°C. The amplified fragment was cloned into the vector pCR2.1 (Invitrogen, NV Leek, the Netherlands). The resulting clone pMRP2.5′ and the clone pMRP2.3′ were sequenced by the dideoxynucleotide chain termination method of Sanger et al. (1980) using the T7 sequencing kit of Pharmacia Biotech (Freiburg, Germany) and [α-^{35}S]dATP (Du-

Pont/NEN). For the construction of the full-length *MRP2* cDNA, the clone pMRP2.3′ was digested with *Not*I and *Spe*I and ligated with the 5′ 2900-bp fragment obtained by digesting the clone pMRP2.5′ with *Not*I and *Spe*I. This full-length human *MRP2* cDNA is available under the GenBank/EBI Data Bank accession no. X96395.

For transfection studies, the entire human *MRP2* cDNA (bases −37 to 4861) was subcloned between the *Not*I and *Apa*I restriction sites of the mammalian expression vector pcDNA3.1(+) (Invitrogen). The 4.9-kb (bases −49 to 4839) cDNA fragment of rat *mrp2* (Büchler et al., 1996) was inserted into the mammalian expression vector pcDNA3 (Invitrogen) between the restriction sites *Eco*RI and *Xho*I. Successful subcloning was verified by restriction map analysis and DNA sequencing. Both human *MRP2* and rat *mrp2* were, thus, under the control of the cytomegalovirus promoter and enhancer elements.

Cell Culture and Transfection. MDCK II cells were obtained from Dr. K. Simons (European Molecular Biology Laboratory, Heidelberg, Germany). HEK-293 cells were obtained from the American Type Culture Collection (Manassas, VA). MDCK II and HEK-293 cells were cultured in minimum essential medium containing 5 and 10% fetal bovine serum, respectively, supplemented with L-glutamine (2 mM), penicillin (100 U/ml), and streptomycin (100 μg/ml; 0.17 mM). The parental vector pcDNA3.1 or the constructs described above were transfected into the cells by electroporation (280 V; 1050 μF; automatic pulse length). After transfection, cells were selected with 600 μg/ml (0.8 mM) G418 for 2 to 3 weeks. G418-resistant clones were screened for human MRP2 or rat Mrp2 expression by immunoblot analysis and immunofluorescence microscopy. Expression of MRP2 and Mrp2 in positive clones was further enhanced by culturing the cells with sodium butyrate or trichostatin A, both of which are inhibitors of histone deacetylase and known to activate viral promoter and enhancer elements (Chen et al., 1997).

Preparation of Membrane Vesicles and Crude Membrane Fractions. Inside-out membrane vesicles from transfected HEK-293 and MDCK II cells were prepared as described previously (Keppler et al., 1998). Membrane vesicles were frozen and stored in liquid nitrogen. Crude membrane fractions were prepared as follows. Cells were disrupted by sonication in hypotonic buffer (1 mM EDTA, 5 mM sodium/potassium phosphate; pH 7.0). After centrifugation (100,000*g*; 4°C; 45 min) pellets were resuspended in Tris buffer (50 mM; pH 7.4). Crude membrane fractions were frozen and stored at −20°C. All membranes were prepared in the presence of proteinase inhibitors (0.3 μM aprotinin, 1.0 μM leupeptin, 0.1 mM phenylmethylsulfonyl fluoride, and 1.0 μM pepstatin).

Immunoblot Analysis. Membrane fractions were diluted with sample buffer and incubated at 37°C for 30 min before separation on 5% stacking and 7.5% resolving polyacrylamide gels. Immunoblotting was performed with a tank blotting system from Bio-Rad (Munich, Germany) and enhanced chemiluminescence detection (Amersham-Buchler, Braunschweig, Germany). Primary antibodies were diluted in TTBS [10 mM Tris/HCl (pH 8.0), 150 mM NaCl, 0.05% Tween 20] to the following final concentrations: EAG5, 1:5,000; EAG15, 1:10,000. The secondary antibody was a horseradish peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad) used at a 1:2000 dilution.

Northern Blot Analysis. Total RNA was isolated from transfected HEK-293 cells with a RNA-Clean kit (Angewandte Gentechnologie Systeme, Heidelberg, Germany). Total RNA samples (20 μg each) were separated by formaldehyde-agarose gel electrophoresis and transferred onto Duralon UV membranes (Stratagene) as described previously (Büchler et al., 1996). Sample loading and efficiency of transfer were determined by staining of the membranes with ethidium bromide before prehybridization. A 347-bp (Mayer et al., 1995) and a 585-bp (Büchler et al., 1996) cDNA probe were used to detect rat *mrp2* and human *MRP2*, respectively. Autoradiographs were exposed for 2 days.

Vesicle Transport Studies. Transport of 100 nM [^{3}H]LTC$_4$ (0.17 TBq/mmol) or 1.5 μM 17β-glucuronosyl [^{3}H]estradiol (0.20 TBq/mmol) into membrane vesicles was measured by the rapid filtration method (Keppler et al., 1998). Briefly, membrane vesicles (20 μg of protein) were incubated in the presence of 4 mM ATP, 10 mM creatine phosphate, 100 μg/ml creatine kinase, and labeled substrate in an incubation buffer (250 mM sucrose, 10 mM Tris/HCl; pH 7.4) at 37°C. The final volume was 55 μl. Aliquots (15 μl) were taken at the indicated time points, diluted in 1 ml of ice-cold incubation buffer, and immediately filtered through presoaked nitrocellulose membrane (0.2-μm pore size). Filters were rinsed twice with 5 ml of incubation buffer, dissolved in liquid scintillation fluid, and counted for radioactivity. In control experiments, ATP was replaced by an equal concentration of 5′-AMP. ATP-dependent transport was calculated by subtracting values obtained in the presence of 5′-AMP from those in the presence of ATP. For determination of kinetic constants, initial transport rates were measured at five different substrate concentrations (25–1000 nM for LTC$_4$ and 0.5–8 μM for 17β-glucuronosyl estradiol). The concentrations of the labeled substrate were kept constant and varying concentrations of unlabeled substrate were added. K_m values are determined as the substrate concentration at half-maximal velocity of transport under the experimental conditions described above. Similar results were obtained by direct curve-fitting to the Michaelis-Menten equation and by the use of double-reciprocal plots according to Lineweaver and Burk (1934).

Immunofluorescence and Confocal Laser Scanning Microscopy. For immunolocalization of MRP2, transfected MDCK cells were grown on Transwell membrane inserts (pore size 3 μm; Costar, Cambridge, MA) for 7 days. Butyrate, at a final concentration of 2 mM, was added to the culture medium 24 h before use of the cells. Cells were fixed with 4% paraformaldehyde in PBS and permeabilized with 0.5% Triton X-100 in PBS containing 5% fetal bovine serum. Membranes were incubated with polyclonal antibody EAG5 or EAG15 (both diluted 1:25 with PBS) at room temperature for 30 min. After three washes with PBS, membranes were reincubated with Cy2-conjugated goat anti-rabbit IgG (Dianova, Hamburg, Germany; dilution 1:200 in PBS). Nuclei were stained with 0.2 μg/ml propidium iodide added to the solution of the secondary antibody. Confocal laser scanning fluorescence microscopy was performed under conditions described recently (Mayer et al., 1995; Büchler et al., 1996) with an LSM 410 apparatus (Carl Zeiss, Jena, Germany). For localization of the amino terminus of rat Mrp2, nonpermeabilized cells were used for immunofluorescence studies. Briefly, MDCK transfectants grown on glass coverslips were either first fixed with 4% paraformaldehyde/PBS and then incubated with the MDK antibody (1:25) at room temperature for 30 min, or first incubated with MDK antibody (1:25) at 4°C for 1 h and then fixed with 4% paraformaldehyde/PBS. After permeabilization with 1% Triton X-100, coverslips were reincubated with Cy2-conjugated goat anti-rabbit IgG (1:200) at room temperature for 30 min.

Cytotoxicity Assays. Sensitivity to cytotoxic drugs was determined using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) cell viability assay according to the method of Mosmann (1983). Briefly, MDCK or HEK-293 cells were seeded in 96-well plates at a density of 5×10^4 and 1.5×10^4 cells per well, respectively. The histone deacetylase inhibitor trichostatin A (125 nM) was added to enhance the expression of recombinant MRP2 in MDCK cells. Drugs were added 24 h after seeding. After 3 days of incubation under normal culture conditions, MTT was added at a final concentration of 250 μg/ml (0.6 mM). In the case of vincristine, minimum essential medium without riboflavin was used to avoid radical-mediated degradation of vincristine (Granzow et al., 1995). The IC$_{50}$ value was defined as the drug concentration required to reduce cell survival, as determined by the relative absorbance of reduced MTT, to 50%. Relative resistance factors (RRs) were calculated by dividing the IC$_{50}$ value of cells transfected with MRP2 expression vectors by the IC$_{50}$ of cells transfected with the control vector. The effect of MDR1 *P*-glycoprotein modulator LY335979 (Dantzig et al., 1996), at a concentration of 0.25 μM, and the glutathione synthesis inhibitor BSO (20 μM) on the sensitivity of trans-

fectants to the cytostatic agents was studied by including them in the cell viability assays.

Results

Cloning of Human *MRP2*. Human *MRP2* cDNA was obtained after screening a λ-ZAP cDNA library combined with a reverse transcription-PCR amplification of the 5′ part. The full-length cDNA of human *MRP2* contains a single open reading frame of 4635 bp that encodes a predicted protein of 1545 amino acids. The deduced amino acid sequence shares a 78% identity with rat Mrp2 (Büchler et al., 1996; Paulusma et al., 1996; Ito et al., 1997) and an 82% identity with rabbit Mrp2 (van Aubel et al., 1998). The molecular mass of unglycosylated human MRP2 is 174,090 Da. After an alignment of the three currently known orthologs of MRP2, 13 transmembrane segments were predicted by a transmembrane topology analysis program (TMAP) (Persson and Argos, 1994). Four transmembrane segments were predicted between both nucleotide-binding domains and nine transmembrane segments between the amino terminus and the first nucleotide-binding domain. This topology prediction suggests an extracellular localization of the amino terminus. A similar topology with 13 transmembrane segments was predicted by the TMAP program (Persson and Argos, 1994) when human MRP1 and mouse Mrp1 were included in the alignment, together with human MRP2, rabbit Mrp2, and rat Mrp2. However, epitope insertion studies favor six transmembrane segments between both nucleotide-binding domains of MRP1 (Kast and Gros, 1998).

Expression of MRP2 in HEK and MDCK Cells. HEK-293 and MDCK II cells were stably transfected with the vector constructs containing human *MRP2* or rat *mrp2*, as well as the parental vector pcDNA3.1(+). G418-resistant clones were screened for MRP2 and Mrp2 expression. The expression level of MRP2 and Mrp2 in the positive clones was markedly enhanced by culturing the transfected cells with sodium butyrate for 24 h. As shown in Fig. 1A, Mrp2 expression in HEK-Mrp2 cells (HEK-293 cells stably transfected with rat *mrp2*) was increased by sodium butyrate in a concentration-dependent manner. No cytotoxicity of butyrate was observed at the optimal concentration of 5 mM. The maximal effect of butyrate was achieved at a concentration of 10 mM in MDCK transfectants. However, concentrations above 2 mM caused visible cytotoxicity in this cell line. Therefore, HEK-293 transfectants and MDCK transfectants were cultured with 5 mM and 2 mM butyrate, respectively, for 24 h before use in immunofluorescence studies. The expression of both MRP2 and Mrp2 was stable when G418 was omitted from the culture medium.

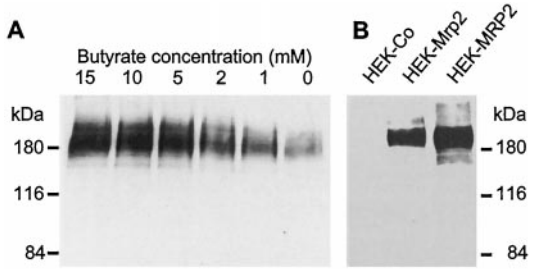

Fig. 1. Immunoblot analysis of human MRP2 and rat Mrp2 in transfected HEK-293 cells. HEK-293 cells were stably transfected with vector pcDNA3.1(+) (HEK-Co), human *MRP2* (HEK-MRP2), or rat *mrp2* (HEK-Mrp2). A, HEK-Mrp2 cells treated with different concentrations of sodium butyrate for 24 h. Crude membrane fractions (10 μg of protein per lane) were analyzed for Mrp2 using the polyclonal antibody EAG15 (Büchler et al., 1996). B, Membrane vesicles prepared from butyrate-treated HEK-transfectants expressing human MRP2 or rat Mrp2 (10 μg of protein per lane). The immunoblot was probed with the EAG5 polyclonal antibody (Jedlitschky et al., 1997) which reacts with both rat and human MRP2.

Immunoblot analysis of the membrane vesicles prepared from the HEK-293 transfectants using the polyclonal antibody EAG5, which recognizes the carboxyl terminus of both human MRP2 and rat Mrp2, showed a 1.5- to 2-fold higher expression level in HEK-MRP2 (HEK-293 cells stably transfected with human MRP2) than in HEK-Mrp2 (Fig. 1B). Both human recombinant MRP2 and rat recombinant Mrp2 showed an apparent molecular mass of about 190 kDa, which is the same mass found in human and rat liver (Büchler et al., 1996; Jedlitschky et al., 1997; Evers et al., 1998). Deglycosylation indicated that the MRP2 overexpressed in HEK-293 cells was glycosylated to the same extent as in human liver (data not shown). The expression of MRP2 was much higher in HEK-293 transfectants than in MDCK transfectants as determined by immunoblot analysis. It is noticeable that endogenous MRP2 was not detectable in HEK-Co cells transfected with the vector alone (Fig. 1B). Moreover, MRP1 was not detected in HEK-293 cells by immunoblot analysis using the monoclonal antibody QCRL-1 directed against MRP1 (not shown).

The expression of *MRP2* and *mrp2* in HEK-293 transfectants was further verified by Northern blotting analysis performed on 20 μg of total RNA. Only one mRNA species with a length of about 5.3 kb was detected in HEK-MRP2 cells using the 585-bp cDNA fragment (Büchler et al., 1996) as probe for *MRP2* (Fig. 2A). In HEK-Co cells, no signal was detected with this cDNA probe. A 5.3-kb mRNA was also found in HEK-Mrp2 cells by using the 347-bp cDNA fragment (Mayer et al., 1995) as a probe for *mrp2* (Fig. 2B).

Localization of MRP2 in Transfectants. Indirect immunofluorescence with HEK-MRP2 cells with the antibody EAG5 indicated that MRP2 was localized to a major portion to intracellular membranes (not shown). However, in polarized MDCK transfectants, the localization of recombinant MRP2 was different. Immunofluorescence and confocal laser scanning microscopy using the antibodies EAG5 or EAG15 revealed a homogenous plasma membrane staining in MDCK transfectants expressing MRP2 or Mrp2 (Fig. 3, A and C). Vertical sections showed intense green fluorescence for MRP2 and Mrp2 on the apical membrane (Fig. 3, B and D). Under the same conditions, no specific staining was observed in MDCK cells transfected only with the parental vector pcDNA3.1(+).

The predicted topology with an extracellular localization of

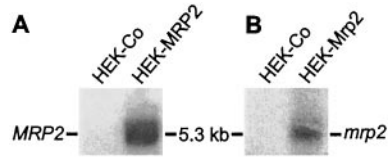

Fig. 2. Northern blot analysis of human *MRP2* and rat *mrp2* expression in transfected HEK-293 cells. A, Total RNA (20 μg per lane) from HEK-293 transfectants was hybridized with a 585-bp fragment of human *MRP2* (Büchler et al., 1996). B, A 347-bp fragment of rat *mrp2* (Mayer et al., 1995) served to detect *mrp2*.

the amino terminus of Mrp2 was studied by means of the antibody MDK raised against the amino terminus of the protein. Cross-reactivity of this antibody was tested by immunoblot analysis. The MDK antibody recognized a glycoprotein of 190 to 200 kDa in crude membranes from MDCK cells transfected with *mrp2* but gave no signal in crude membranes from MDCK cells transfected with the vector alone (data not shown). As shown in Fig. 3E, nonpermeabilized MDCK transfectants expressing Mrp2 were stained with the MDK antibody and a punctuate fluorescence of the apical domain was observed. Nonpermeabilized MDCK cells transfected with the vector alone did not react with the MDK antibody (Fig. 3F). Moreover, nonpermeabilized MDCK cells expressing recombinant rat Mrp2 did not react with the antibody EAG15 directed against the carboxyl terminus of Mrp2 predicted to be localized intracellularly (Fig. 3G).

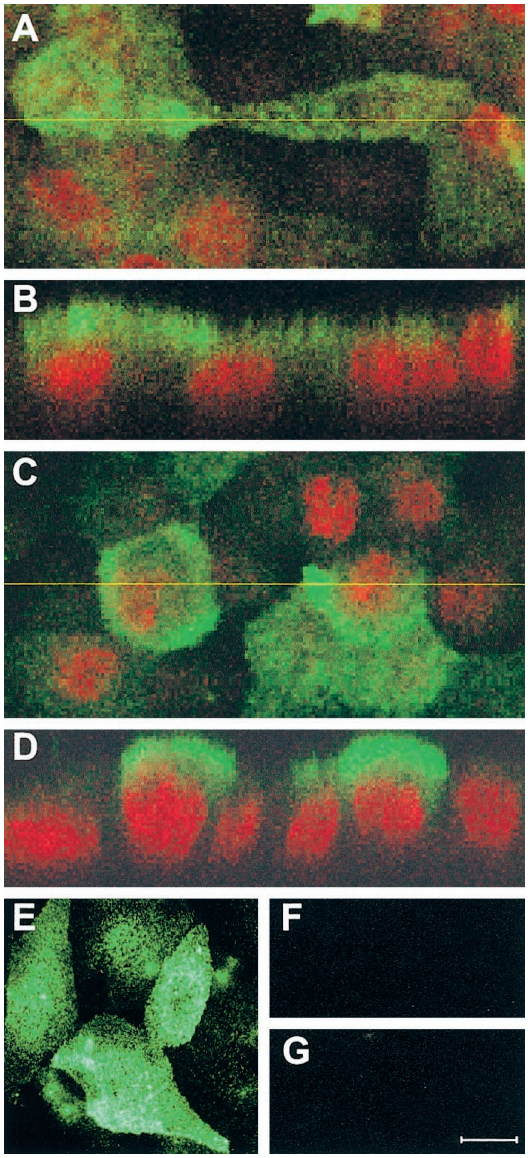

Fig. 3. Immunolocalization of rat and human MRP2 in stably transfected MDCK cells. A and B, MDCK cells expressing human MRP2 were stained with the EAG5 antibody. C and D, rat Mrp2 in transfected MDCK cells was detected by the EAG15 antibody. Nuclei were stained with propidium iodide (red fluorescence). A and C show 0.8-μm optical sections in the *xy*-plane; B and D are vertical sections (*xz*-plane as indicated by yellow lines in A and C). Note that both human MRP2 and rat Mrp2 (green fluorescence) are exclusively localized to the apical membrane of transfected MDCK cells. E, nonpermeabilized MDCK II cells expressing rat Mrp2 were stained with the MDK antibody directed against the amino terminus of Mrp2. F, control vector-transfected MDCK cells were not stained under the same conditions. G, no staining was observed when the EAG15 antibody, directed against the carboxyl terminus of Mrp2, was applied to nonpermeabilized Mrp2-expressing MDCK cells. Same magnification for all panels; bar in G corresponds to 5 μm.

Transport Studies with Membrane Vesicles. Membrane vesicles were prepared from HEK-293 and MDCK II transfectants cultured for 24 h in the presence of 5 mM and 10 mM butyrate, respectively. These relatively high concentrations of butyrate induced maximum levels of MRP2 in these cells. Because of the higher expression level of human MRP2 and rat Mrp2 in HEK-293 transfectants as compared with MDCK transfectants, membrane vesicles from the former were preferred for studies on transport function. These MRP2-containing membrane vesicles of HEK-293 transfectants, which are derived, in part, from intracellular membrane vesicles, were most useful for transport studies. [^{3}H]LTC_4 was transported ATP-dependently into membrane vesicles from HEK-MRP2 cells at a rate of 48 $\pm$ 9 pmol $\cdot$ min^{-1} $\cdot$ mg $protein^{-1}$ at 100 nM LTC_4 (mean $\pm$ S.D.; n = 5; Fig. 4). In membrane vesicles from rat *mrp2*-transfected HEK-293 and MDCK cells, transport rates at this concentration of [^{3}H]LTC_4 were 31 $\pm$ 3 and 24 $\pm$ 2 pmol $\cdot$ min^{-1} $\cdot$ mg $protein^{-1}$, respectively. For the determination of kinetic constants, human MRP2-mediated transport was calculated by subtracting ATP-dependent transport into membrane vesicles from HEK-Co cells from those measured with membrane vesicles from HEK-MRP2 cells. An apparent K_m of 1.0 $\pm$ 0.1 μM (n = 4) was determined for human MRP2 by double-reciprocal plots (Table 1). ATP-dependent transport of [^{3}H]LTC_4 was also detectable in membrane vesicles from HEK-Co cells (Fig. 4B), but the transport rate (11 $\pm$ 4 pmol $\cdot$ min^{-1} $\cdot$ mg $protein^{-1}$; n = 3) at 100 nM LTC_4 was only 22% of that measured with membrane vesicles from HEK-MRP2. The endogenous transport system has a K_m of 0.28 $\pm$ 0.09 μM (n = 4). Membrane vesicles from HEK-MRP2 were also active in the ATP-dependent transport of 17β-glucuronosyl [^{3}H]estradiol (Fig. 5). An apparent K_m of 7.2 $\pm$ 0.7 μM (n = 4) was obtained (Table 1). The transport of 17β-glucuronosyl [^{3}H]estradiol into membrane vesicles from HEK-Co cells was below

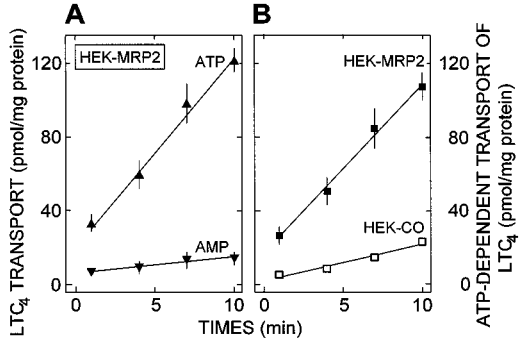

Fig. 4. Transport of [^{3}H]LTC_4 into membrane vesicles from HEK-293 cells transfected with human *MRP2* (HEK-MRP2) or vector pcDNA3.1(+) (HEK-Co). A, membrane vesicles were incubated with 100 nM [^{3}H]LTC_4 in the presence of 4 mM ATP (▲) or 4 mM 5′-AMP (▼). Vesicle-associated radioactivity was determined by rapid filtration (Keppler et al., 1998). B, net ATP-dependent transport into the vesicles from HEK-MRP2 cells (■) and from HEK-Co (□) was calculated by subtracting values obtained in the presence of 5′-AMP from those in the presence of ATP. Each point represents the mean $\pm$ S. D. (n = 4).

10% of that measured with membrane vesicles from HEK-MRP2 cells (Fig. 5B). The K_m value for 17β-glucuronosyl [^{3}H]estradiol in these control transfectants was 2.8 ± 0.3 μM (n = 4). The kinetic constants were also determined for rat Mrp2 and compared with those for human MRP2 and with the constants determined earlier for human MRP1 (Table 1).

Drug Resistance Conferred by MRP2. MDCK-transfectants were studied at confluency for MTT assays when a polarized monolayer had been formed, which is a prerequisite for the apical localization of MRP2. Under this condition, both rat and human MRP2 conferred significant resistance to etoposide and vincristine (Fig. 6; Table 2). It was reported that MDCK cells express endogenous MDR1 P-glycoprotein (Horio et al., 1989). Our immunoblot analysis with the monoclonal antibody C219 showed that the expression level of a 170-kDa protein is similar in all transfectants (not shown). The interference of MDR1 P-glycoprotein with the cytotoxicity assays was, therefore, suppressed by the specific MDR1 modulator LY335979 (Dantzig et al., 1996; Shepard et al., 1998). LY335979, at a concentration of 0.25 μM, sensitized all MDCK-transfectants to etoposide and vincristine without lowering the relative resistance conferred by MRP2 (Fig. 6A; Table 2), indicating that resistance was actually conferred by MRP2 transfected into these cells. The effect of 20 μM BSO, an inhibitor of glutathione synthesis that has been shown to inhibit MRP1-mediated multidrug resistance (Zaman et al., 1995), was also determined. This concentration of BSO did not inhibit MRP1- or MRP2-mediated ATP-dependent transport of LTC_4. Coincubation with 20 μM BSO in the cytotoxicity assays reduced the level of etoposide resistance conferred by MRP2 from 5-fold to 2-fold (Fig. 6; Table 2). Human recombinant MRP2 conferred low-level resistance to epirubicin (1.9-fold; $P < .01$) and to doxorubicin (1.5-fold; $P < .01$) in these confluent MDCK cells. In these cells, MRP2-mediated resistance to cisplatin could not be detected.

In HEK-293 cells, human MRP2 was expressed at a sufficiently high level to allow for cytotoxicity assays in the absence of trichostatin A. Although recombinant MRP2 was localized to a higher proportion to intracellular membranes, it conferred significant levels of drug resistance (Table 3). Resistance to etoposide, cisplatin, doxorubicin, and epirubicin was enhanced 4-fold, 10-fold, 7.8-fold, and 5-fold, respectively (Table 3).

TABLE 1

Kinetic constants for ATP-dependent transport of LTC_4 and 17β-glucuronosyl estradiol by recombinant multidrug resistance proteins Mrp2 from rat, human MRP2, and human MRP1

ATP-dependent transport into membrane vesicles from HEK-293 cells expressing recombinant MRP2 was determined as described under Experimental Procedures. Data for recombinant MRP1 expressed in HeLa cells (Grant et al., 1994) are from our previous work (Leier et al., 1994; Jedlitschky et al., 1996). K_m values were determined as the substrate concentration at half-maximal velocity of ATP-dependent transport. Data represent the mean from four determinations ± S.D.

Recombinant Transporter	K_m	V_{max}/K_m
	μM	$\mu l \cdot mg\ protein^{-1} \times min^{-1}$
Leukotriene C_4		
Mrp2 Rat	1.1 ± 0.2	429
MRP2 Human	1.0 ± 0.1	351
MRP1 Human	0.1 ± 0.02	1000
17β-Glucuronosyl estradiol		
Mrp2 Rat	6.9 ± 1.8	16
MRP2 Human	7.2 ± 0.7	20
MRP1 Human	1.5 ± 0.3	42

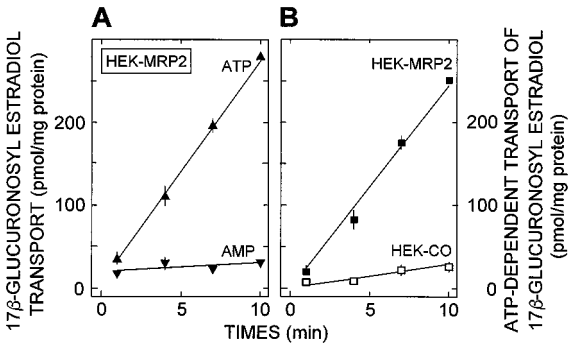

Fig. 5. Transport of 17β-glucuronosyl [^{3}H]estradiol into membrane vesicles from HEK-MRP2 and HEK-Co cells. A, membrane vesicles were incubated with 1.5 μM 17β-glucuronosyl [^{3}H]estradiol in the presence of 4 mM ATP (▲) or 4 mM 5′-AMP (▼). Vesicle-associated radioactivity was determined by rapid filtration. B, net ATP-dependent transport into the vesicles from HEK-MRP2 cells (■) and into those from HEK-Co (□) was calculated by subtracting values obtained in the presence of 5′-AMP from those in the presence of ATP. Mean values ± S. D. (n = 4).

Discussion

Human MRP2 and its orthologs from rat and rabbit were cloned and sequenced recently (Mayer et al., 1995; Büchler et

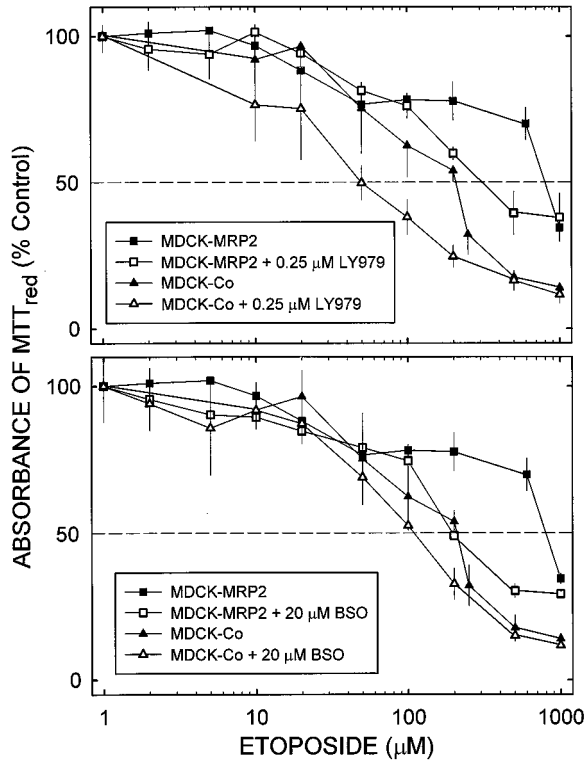

Fig. 6. Etoposide sensitivity of MDCK cells transfected with human *MRP2* (MDCK-MRP2) or vector alone (MDCK-Co). Cytotoxicity was determined using MTT cell viability assays as described under *Experimental Procedures*. Each point represents the mean ± SEM (n = 6). Data from the same control experiments were used for both the top and bottom panels. Top, effect of LY335979 (abbreviated LY979) on etoposide sensitivity of the transfectants. LY335979, at a concentration of 0.25 μM, sensitized both MDCK-MRP2 and MDCK-Co to etoposide without changing the relative resistance conferred by MRP2. Bottom, effect of 20 μM BSO on etoposide sensitivity of the transfectants, with a reduction of the relative resistance of the MDCK-MRP2 transfectants from 5-fold to about 2-fold.

al., 1996; Paulusma et al., 1996, 1997; Taniguchi et al., 1996; Ito et al., 1997; Madon et al., 1997; van Aubel et al., 1998; Evers et al., 1998). The function, particularly of rat Mrp2, as a conjugate-transporting ATPase has been deduced predominantly from studies with hepatocyte canalicular membrane vesicles from normal and Mrp2-deficient animals (Ishikawa et al., 1990; Oude Elferink et al., 1995; Jedlitschky et al., 1997). Stable transfection of MRP2 and its expression at a high level are prerequisites for a detailed functional characterization of this transport protein, which plays an important role in hepatobiliary and renal excretion (Keppler and König, 1997). Stable transfectants expressing human MRP2 (Figs. 1–5) are also of considerable interest for studies on multidrug resistance conferred by this isoform of MRP1. In this study, we established transfected HEK-293 and MDCK cell lines permanently expressing recombinant human MRP2 or rat Mrp2 (Figs. 1 and 2). Expression of recombinant MRP2, driven by the cytomegalovirus promoter, was markedly enhanced by the addition of inhibitors of histone deacetylase, such as butyrate and trichostatin A, to the cell culture (Fig. 1A).

Our previous immunofluorescence studies have demonstrated that human and rat MRP2 are localized under physiological conditions to the apical membrane domain of polarized cells, including hepatocytes and proximal tubule epithelia of the kidney (Büchler et al., 1996; Keppler and Kartenbeck, 1996; Schaub et al., 1997). In contrast, the major portion of recombinant rat and human MRP2 expressed in this study in HEK-293 cells was not sorted to the plasma membrane but retained in a fully glycosylated and functionally active form on intracellular membranes (Fig. 1B; Table 1). HEK-293 cells were reported to be epithelial in nature (Chan et al., 1997); however, these cells did not form a distinct polarized monolayer under our culture conditions. It is unclear whether the incomplete sorting of rat and human MRP2 to the plasma membrane of HEK-293 cells is due to missing proteins required for sorting or a result of an incomplete polarity of the HEK-293 cells in culture. Importantly, the intracellular membranes containing recombinant MRP2 formed vesicles with a high specific activity of ATP-dependent transport of labeled substrates (Table 1; Figs. 4 and 5). However, extensive sorting of human and rat MRP2 to the apical plasma membrane domain was observed in polarized MDCK cells stably transfected with the respective vector constructs (Fig. 3). This observation is consistent with a recent study on MRP2 in this cell line (Evers et al., 1998). Cultures of MDCK cells were shown to form polarized epithelial monolayers with the apical membrane oriented to the medium (Richardson et al., 1981). The apical localization of recombinant rat and human MRP2 demonstrated by confocal laser scanning microscopy (Fig. 3) differs from the sorting and localization of recombinant human MRP1 to the lateral membrane of transfected kidney cells (Evers et al., 1996). It will be of interest to define the amino acid sequences determining the differential sorting of MRP1 and its isoform, MRP2, in polarized cells.

The membrane topology of rat Mrp2, as predicted by the TMAP program (Persson and Argos, 1994), indicated an extracellular localization of the amino terminus (Büchler et al., 1996; Keppler and König, 1997). An extracellular amino terminus of MRP1 was recognized recently on the basis of site-directed mutagenesis studies (Hipfner et al., 1997; Kast and Gros, 1997). In the present study, we used a polyclonal antibody raised against the amino-terminal 25 amino acids of rat Mrp2 to test the predicted membrane topology in nonpermeabilized MDCK cells expressing recombinant Mrp2 (Fig. 3E). Staining of the apical membrane was only observed with the MDK antibody directed against the amino terminus and not with the EAG15 antibody directed against the carboxyl terminus of Mrp2 (Fig. 3G). This result provides direct evidence for the extracellular localization of the amino terminus of Mrp2. Together with the recent analysis of the topology of MRP1 (Hipfner et al., 1997; Kast and Gros, 1997), these analyses suggest that an extracellular amino terminus may be a common feature of the members of the MRP family.

The substrate specificity of human MRP1 and MRP2 has been considered to be quite similar (Keppler et al., 1998), although some kinetic differences were detected recently (Jedlitschky et al., 1997). The availability of membrane vesicles rich in recombinant human MRP2 (Fig. 1B) now has enabled a comparison with recombinant human MRP1 with respect to the substrate specificity and to the kinetic properties of ATP-dependent transport (Table 1). We studied two established endogenous substrates for ATP-dependent trans-

TABLE 2

Resistance to etoposide and vincristine conferred by rat and human MRP2

Sensitivity to etoposide and vincristine was determined using MTT cell viability assays as described in Experimental Procedures. Trichostatin A (125 nM) was added to enhance the expression of recombinant MRP2 in the transfected cells. Data represent the mean ± S.D. ($n \geq 6$). The significance of relative resistance factors (RR) was calculated by Student's t test.

Cell Line	Control		LY335979 0.25 μM		BSO 20 μM	
	IC_{50}[a]	RR[b]	IC_{50}[a]	RR[b]	IC_{50}[a]	RR[b]
Etoposide						
MDCK-Co	163 ± 35	1.0	56 ± 20	1.0	113 ± 9	1.0
MDCK-Mrp2	612 ± 164	3.8[c]	189 ± 6	3.4[c]	235 ± 22	2.1[c]
MDCK-MRP2	809 ± 63	5.0[c]	366 ± 66	6.5[c]	197 ± 6	1.7[c]
Vincristine						
MDCK-Co	8.2 ± 0.1	1.0	4.7 ± 0.1	1.0	9.2 ± 0.2	1.0
MDCK-Mrp2	49.3 ± 1.9	6.0[c]	36.7 ± 0.6	7.8[c]	25.9 ± 0.7	2.8[c]
MDCK-MRP2	18.7 ± 0.1	2.3[c]	16.9 ± 0.1	3.6[c]	19.0 ± 0.7	2.1[c]

[a] IC_{50} values are given in μM

[b] The relative resistance factor (RR) was calculated by dividing the IC_{50} value of cells transfected with MRP2 expression vectors by the IC_{50} value of cells transfected with the control vector (MDCK-Co).

[c] $P \leq .01$ compared to MDCK-Co.

port by members of the MRP family, the glutathione *S*-conjugate, LTC_4, and the glucuronate conjugate, 17β-glucuronosyl estradiol. With both substrates, a high maximal velocity of transport into membrane vesicles from *MRP2*-transfected HEK-293 cells relative to the membrane vesicles from *MRP1*-transfected HeLa cells was observed. As indicated by the higher V_{max}/K_m ratio in the same membrane vesicle preparation, LTC_4 was a better substrate than 17β-glucuronosyl estradiol, both for MRP2 and for MRP1 (Table 1). The concentration of LTC_4 required for half-maximal transport velocity was 10-fold higher for recombinant human MRP2 than for MRP1. The concentration of 17β-glucuronosyl estradiol at half-maximal transport velocity was 4.8-fold higher for recombinant MRP2 than for MRP1. The kinetic constants for recombinant rat Mrp2 were not significantly different from those for human MRP2 (Table 1) although both transporter orthologs share only 78% identical amino acids (Keppler and König, 1997). The K_m values of rat and human MRP2 for LTC_4 (Table 1) were also very similar to the K_m in rat hepatocyte canalicular membrane vesicles (i.e., 1.3 μM) when the same methodology for measurement of ATP-dependent membrane transport was used (Leier et al., 1994; Jedlitschky et al., 1996). This indicates that differences in membrane composition between the HEK-293 cells and rat hepatocytes did not result in a detectable effect on the kinetic properties of Mrp2. We conclude that the intracellular membrane vesicles from our stably transfected HEK-293 cells are well suited to characterize the functional properties of rat and human MRP2, although they lack the complete sorting of MRP2 to the apical membrane domain that is observed in the transfected MDCK cells.

MRP1 has been shown to confer multidrug resistance in transfected cell lines (Grant et al., 1994; Zaman et al., 1995). MRP2 differs markedly from MRP1 by its apical localization and its expression in polarized cells (Büchler et al., 1996; Evers et al., 1996, 1998; Keppler and Kartenbeck, 1996; Schaub et al., 1997), but not so much in its substrate specificity (Table 1). This suggests that drug conjugates (Jedlitschky et al., 1996) and drug complexes with glutathione (Loe et al., 1996) may be substrates for both MRP isoforms. It is in line with this view that a reduction of the MRP2 protein by expression of an antisense construct increased the sensitivity of hepatic cancer cells to several anticancer agents and increased the cellular content of reduced glutathione (Koike et al., 1997). The results of cytotoxicity assays shown in this work provide the first direct evidence for the ability of MRP2 to confer drug resistance. Despite the relatively low sensitivity of MDCK control cells to etoposide (IC_{50} = 163 μM) and vincristine (IC_{50} = 8.2 μM), transfection with human or rat *MRP2* genes conferred additional resistance to these cytotoxic drugs (Fig. 6; Table 2). Immunoblot analysis and sensitization of the cells using the MDR1 modulator LY335979 indicated that the endogenous canine MDR1 P-glycoprotein in MDCK cells is one of the reasons for the low sensitivity of these cells to these cytotoxic agents. The resistance factor conferred by rat or human MRP2 was not significantly affected by LY335979 (Table 2). Sensitization of *MRP2*-transfected MDCK cells by BSO indicates that the drug resistance conferred by MRP2 is related to the intracellular GSH level. Vincristine has been shown to be a substrate for MRP1 only in the presence of GSH (Loe et al., 1996). However, it is unknown at present whether etoposide and vincristine are transported by MRP2 as a GSH complex.

Resistance of tumor cells to cisplatin has been associated with MRP2 overexpression (Taniguchi et al., 1996; Koike et al., 1997; Kool et al., 1997). In our present study, we provide direct evidence that MRP2 overexpression can confer resistance to cisplatin in transfected HEK-293 cells (Table 3). Interestingly, coincubation with 50 μM BSO in the cytotoxicity assays reduced resistance to cisplatin from 10.0-fold to 2.9-fold (data not shown). As the major proportion of recombinant MRP2 is localized to intracellular membranes in HEK-293 cells, rather than to the plasma membrane, one may consider the possibility that cisplatin or its glutathione conjugate is first sequestered in intracellular vesicles and subsequently released into the extracellular space. Alternatively or additionally, the fraction of recombinant MRP2 in the plasma membrane is sufficient to pump out the cisplatin glutathione conjugate. These possibilities are currently under investigation in our laboratory.

TABLE 3

Resistance to antitumor drugs conferred by human MRP2 in transfected HEK-293 cells

Sensitivity to drugs was determined using MTT cell viability assays as described in Experimental Procedures. All values were obtained in the presence of MDR1 *P*-glycoprotein inhibitor LY335979 (0.25 μM). Trichostatin A was omitted in this experiment. Data represent the mean ± S.D. ($n \geq 6$). The significance of relative resistance factors (RR) was calculated by Student's *t* test.

Drug	HEK-Co Cells	HEK-MRP2 Cells	
	IC_{50}	IC_{50}	RR[a]
Etoposide (μM)	0.3 ± 0.1	1.2 ± 0.5	4.0[b]
Cisplatin (μM)	2.4 ± 0.6	24.2 ± 4.4	10.0[b]
Doxorubicin (nM)	44.3 ± 0.3	346.3 ± 48.1	7.8[b]
Epirubicin (nM)	3.8 ± 1.5	18.8 ± 2.0	5.0[b]

[a] The relative resistance factor (RR) was calculated by dividing the IC_{50} value of cells transfected with MRP2 expression vector (HEK-MRP2) by the IC_{50} value of cells transfected with the control vector (HEK-Co).

[b] $P \leq .01$ compared to HEK-Co.

Acknowledgments

We thank Dr. Wolfgang Hagmann and Dr. H.-R. Rackwitz for their support in the generation of the MDK antibody, Dr. Roger Deeley and Dr. Susan Cole for providing their MRP1-transfected HeLa cells (Grant et al., 1994), and Johanna Hummel-Eisenbeiss for her contributions to the measurements of ATP-dependent transport.

References

Büchler M, König J, Brom M, Kartenbeck J, Spring H, Horie T and Keppler D (1996) cDNA cloning of the hepatocyte canalicular isoform of the multidrug resistance protein, cMrp, reveals a novel conjugate export pump deficient in hyperbilirubinemic mutant rats. *J Biol Chem* **271**:15091–15098.

Chan CW, Song Y, Ailenberg M, Wheeler M, Pang SF, Brown GM and Silverman M (1997) Studies of melatonin effects on epithelia using the human embryonic kidney-293 (HEK-293) cell line. *Endocrinology* **138:**4732–4739.

Chang XB, Hou YX and Riordan JR (1997) ATPase activity of purified multidrug resistance-associated protein. *J Biol Chem* **272:**30962–30968.

Chen WY, Bailey EC, McCune SL, Dong JY and Townes TM (1997) Reactivation of silenced, virally transduced genes by inhibitors of histone deacetylase. *Proc Natl Acad Sci USA* **94:**5798–5803.

Cole SPC, Bhardwaj G, Gerlach JH, Mackie JE, Grant CE, Almquist KC, Stewart AJ, Kurz EU, Duncan AM and Deeley RG (1992) Overexpression of a transporter gene in a multidrug-resistant human lung cancer cell line. *Science (Wash DC)* **258:** 1650–1654.

Dantzig AH, Shepard RL, Cao J, Law KL, Ehlhardt WJ, Baughman TM, Bumol TF and Starling JJ (1996) Reversal of *P*-glycoprotein-mediated multidrug resistance by a potent cyclopropyldibenzosuberane modulator, LY335979. *Cancer Res* **56:** 4171–4179.

Evers R, Kool M, van Deemter L, Janssen H, Calafat J, Oomen LCJM, Paulusma CC, Oude Elferink RPJ, Baas F, Schinkel AH and Borst P (1998) Drug export activity of the human canalicular multispecific organic anion transporter in polarized kidney MDCK cells expressing *cMOAT* (*MRP2*) cDNA. *J Clin Invest* **101:**1310–1319.

Evers R, Zaman GJ, van Deemter L, Jansen H, Calafat J, Oomen LC, Oude Elferink

RP, Borst P and Schinkel AH (1996) Basolateral localization and export activity of the human multidrug resistance-associated protein in polarized pig kidney cells. *J Clin Invest* **97:**1211–1218.
Grant CE, Valdimarsson G, Hipfner DR, Almquist KC, Cole SPC and Deeley RG (1994) Overexpression of multidrug resistance-associated protein (MRP) increases resistance to natural product drugs. *Cancer Res* **54:**357–361.
Granzow C, Kopun M, and Krober T (1995) Riboflavin-mediated photosensitization of *Vinca* alkaloids distorts drug sensitivity assays. *Cancer Res* **55:**4837–4843.
Hipfner DR, Almquist KC, Leslie EM, Gerlach JH, Grant CE, Deeley RG and Cole SPC (1997) Membrane topology of the multidrug resistance protein (MRP). A study of glycosylation-site mutants reveals an extracytosolic NH_2 terminus. *J Biol Chem* **272:**23623–23630.
Horio M, Chin KV, Currier SJ, Goldenberg S, Williams C, Pastan I, Gottesman MM and Handler J (1989) Transepithelial transport of drugs by the multidrug transporter in cultured Madin-Darby canine kidney cell epithelia. *J Biol Chem* **264:** 14880–14884.
Ishikawa T, Müller M, Klünemann C, Schaub T and Keppler D (1990) ATP-dependent primary active transport of cysteinyl leukotrienes across liver canalicular membrane. Role of the ATP-dependent transport system for glutathione *S*-conjugates. *J Biol Chem* **265:**19279–19286.
Ito K, Suzuki H, Hirohashi T, Kume K, Shimizu T and Sugiyama Y (1997) Molecular cloning of canalicular multispecific organic anion transporter defective in EHBR. *Am J Physiol* **272:**G16–G22.
Ito K, Suzuki H, Hirohashi T, Kume K, Shimizu T and Sugiyama Y (1998) Functional analysis of a canalicular multispecific organic anion transporter cloned from rat liver. *J Biol Chem* **273:**1684–1688.
Jedlitschky G, Leier I, Buchholz U, Barnouin K, Kurz G and Keppler D (1996) Transport of glutathione, glucuronate, and sulfate conjugates by the *MRP* gene-encoded conjugate export pump. *Cancer Res* **56:**988–994.
Jedlitschky G, Leier I, Buchholz U, Center M and Keppler D (1994) ATP-dependent transport of glutathione *S*-conjugates by the multidrug resistance-associated protein. *Cancer Res* **54:**4833–4836.
Jedlitschky G, Leier I, Buchholz U, Hummel-Eisenbeiss J, Burchell B and Keppler D (1997) ATP-dependent transport of bilirubin glucuronides by the multidrug resistance protein MRP1 and its hepatocyte canalicular isoform MRP2. *Biochem J* **327:**305–310.
Kartenbeck J, Leuschner U, Mayer R and Keppler D (1996) Absence of the canalicular isoform of the *MRP* gene-encoded conjugate export pump from the hepatocytes in Dubin-Johnson syndrome. *Hepatology* **23:**1061–1066.
Kast C and Gros P (1997) Topology mapping of the amino-terminal half of multidrug resistance-associated protein by epitope insertion and immunofluorescence. *J Biol Chem* **272:**26479–26487.
Kast C and Gros P (1998) Epitope insertion favors a six-transmembrane domain model for the carboxy-terminal portion of the multidrug resistance-associated protein. *Biochemistry* **37:**2305–2313.
Keppler D, Jedlitschky G and Leier I (1998) Transport function and substrate specificity of multidrug resistance protein. *Methods Enzymol* **292:**607–616.
Keppler D and Kartenbeck J (1996) The canalicular conjugate export pump encoded by the *cmrp/cmoat* gene, in *Progress in Liver Diseases* (Boyer JL and Ockner RK eds) vol 14, pp 55–67, Saunders, Philadelphia.
Keppler D and König J (1997) Expression and localization of the conjugate export pump encoded by the *MRP2* (*cMRP/cMOAT*) gene in liver. *FASEB J* **11:**509–516.
Koike K, Kawabe T, Tanaka T, Toh S, Uchiumi T, Wada M, Akiyama S, Ono M and Kuwano M (1997) A canalicular multispecific organic anion transporter (cMOAT) antisense cDNA enhances drug sensitivity in human hepatic cancer cells. *Cancer Res* **57:**5475–5479.
Kool M, de Haas M, Scheffer GL, Scheper RJ, van Eijk MJ, Juijn JA, Baas F and Borst P (1997) Analysis of expression of cMOAT (MRP2), MRP3, MRP4, and MRP5, homologues of the multidrug resistance-associated protein gene (MRP1), in human cancer cell lines. *Cancer Res* **57:**3537–3547.
Leier I, Jedlitschky G, Buchholz U, Cole SPC, Deeley RG and Keppler D (1994) The *MRP* gene encodes an ATP-dependent export pump for leukotriene C_4 and structurally related conjugates. *J Biol Chem* **269:**27807–27810.
Lineweaver H and Burk D (1934) The determination of enzyme dissociation constants. *J Am Soc* **56:**658–666.
Loe DW, Almquist KC, Deeley RG and Cole SPC (1996) Multidrug resistance protein (MRP)-mediated transport of leukotriene C_4 and chemotherapeutic agents in membrane vesicles. Demonstration of glutathione-dependent vincristine transport. *J Biol Chem* **271:**9675–9682.
Madon J, Eckhardt U, Gerloff T, Stieger B and Meier PJ (1997) Functional expression of the rat liver canalicular isoform of the multidrug resistance-associated protein. *FEBS Lett* **406:**75–78.
Mayer R, Kartenbeck J, Büchler M, Jedlitschky G, Leier I and Keppler D (1995) Expression of the *MRP* gene-encoded conjugate export pump in liver and its selective absence from the canalicular membrane in transport-deficient mutant hepatocytes. *J Cell Biol* **131:**137–150.
Mosmann T (1983) Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *J Immunol Methods* **65:**55–63.
Müller M, Meijer C, Zaman GZ, Borst P, Scheper RJ, Mulder NH, de Vries EG and Jansen PL (1994) Overexpression of the gene encoding the multidrug resistance-associated protein results in increased ATP-dependent glutathione *S*-conjugate transport. *Proc Natl Acad Sci USA* **91:**13033–13037.
Oude Elferink RP, Meijer DK, Kuipers F, Jansen PL, Groen AK and Groothuis GM (1995) Hepatobiliary secretion of organic compounds: Molecular mechanisms of membrane transport. *Biochim Biophys Acta* **1241:**215–268.
Paulusma CC, Bosma PJ, Zaman GJ, Bakker CT, Otter M, Scheffer GL, Scheper RJ, Borst P and Oude Elferink RP (1996) Congenital jaundice in rats with a mutation in a multidrug resistance- associated protein gene. *Science (Wash DC)* **271:**1126–1128.
Paulusma CC, Kool M, Bosma PJ, Scheffer GL, ter Borg F, Scheper RJ, Tytgat GN, Borst P, Baas F and Oude Elferink RP (1997) A mutation in the human canalicular multispecific organic anion transporter gene causes the Dubin-Johnson syndrome. *Hepatology* **25:**1539–1542.
Persson B and Argos P (1994) Prediction of transmembrane segments in proteins utilising multiple sequence alignments. *J Mol Biol* **237:**182–192.
Richardson JC, Scalera V and Simmons NL (1981) Identification of two strains of MDCK cells which resemble separate nephron tubule segments. *Biochim Biophys Acta* **673:**26–36.
Sanger F, Coulson AR, Barrell BG, Smith AJ, and Roe BA (1980) Cloning in single-stranded bacteriophage as an aid to rapid DNA sequencing. *J Mol Biol* **143:**161–178.
Schaub TP, Kartenbeck J, König J, Vogel O, Witzgall R, Kriz W and Keppler D (1997) Expression of the conjugate export pump encoded by the *mrp2* gene in the apical membrane of kidney proximal tubules. *J Am Soc Nephrol* **8:**1213–1221.
Shepard RL, Tabas L, Gillepsie J, Binkley S, Wrighton S and Dantzig AH (1998) Selectivity of the potent *P*-glycoprotein modulator, LY335979 (Abstract). *Proc Am Assoc Cancer Res* **39:**362.
Taniguchi K, Wada M, Kohno K, Nakamura T, Kawabe T, Kawakami M, Kagotani K, Okumura K, Akiyama S and Kuwano M (1996) A human canalicular multispecific organic anion transporter (cMOAT) gene is overexpressed in cisplatin-resistant human cancer cell lines with decreased drug accumulation. *Cancer Res* **56:**4124–4129.
van Aubel RA, van Kuijck MA, Koenderink JB, Deen PM, van Os CH, and Russel FG (1998) Adenosine triphosphate-dependent transport of anionic conjugates by the rabbit multidrug resistance-associated protein Mrp2 expressed in insect cells. *Mol Pharmacol* **53:**1062–1067.
Wada M, Toh S, Taniguchi K, Nakamura T, Uchiumi T, Kohno K, Yoshida I, Kimura A, Sakisaka S, Adachi S and Kuwano M (1998) Mutations in the canalicular multispecific organic anion transporter (*cMOAT*) gene, a novel ABC transporter, in patients with hyperbilirubinemia II/Dubin-Johnson syndrome. *Hum Mol Genet* **7:**203–207.
Zaman GJ, Lankelma J, van Tellingen O, Beijnen J, Dekker H, Paulusma C, Oude Elferink RP, Baas F and Borst P (1995) Role of glutathione in the export of compounds from cells by the multidrug-resistance-associated protein. *Proc Natl Acad Sci USA* **92:**7690–7694.

Send reprint requests to: Dr. Dietrich Keppler, Division of Tumor Biochemistry, Deutsches Krebsforschungszentrum, D-69120 Heidelberg, Germany. E-mail: d.keppler@dkfz-heidelberg.de