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Cloning and Characterization of the Rat Multidrug Resistance-Associated Protein 1

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ABSTRACT Multidrug resistance-associated protein 1 (MRP1) was originally shown to confer resistance of human tumor cells to a broad range of natural product anticancer drugs. MRP1 has also been shown to mediate efflux transport of glutathione and glucuronide conjugates of drugs and endogenous substrates. An ortholog of MRP1 in the mouse has been cloned and characterized. Significant functional differences between murine and human MRP1 have been noted. Since drug disposition and pharmacology studies often are conducted in rats, there is a need to clone and characterize the rat ortholog of MRP1. We isolated a rat MRP1 (rMRP1) cDNA from rat brain astrocytes, characterized its coding sequences, and verified the transport activity of the protein expressed in MRP1 cDNA-transfected Madin-Darby canine kidney (MDCK) cells. Our results showed that rMRP1 has a coding sequence of 4599 bp, which predicts a polypeptide of 1533 amino acids with an apparent molecular weight of 190 kd by Western immunoblot analysis. rMRP1-transfected MDCK cells are capable of efflux transport of a fluorescent MRP1 marker—calcein—that is inhibitable by known MRP1 inhibitors, indomethacin, and MK571. Sequence analysis indicates that rMRP1 is more closely related to mouse MRP1 than human MRP1.

KEYWORDS: multidrug resistance gene, ABC transporter, MRP1, cloning, functional characterization

INTRODUCTION Multidrug resistance-associated protein 1 (MRP1) is a member of the ATP-binding cassette (ABC) protein superfamily that was originally discovered as the plasma membrane efflux transporter responsible for the multidrug-resistance phenotype in a human small-cell lung carcinoma cell line.[1] In addition to natural product anticancer agents, human MRP1 has since been shown to mediate the efflux transport of glutathione and glucuronide conjugates, notably cysteinyl leukotriene C4 (LTC4) and 17-ß-estradiol 17-(ßD-glucuronide). Moreover, constitutive expression of MRP1 in human tissues is widespread, with high expression in the lungs, kidneys, bladder, spleen, testes, and thyroid.[2] Hence, in addition to its involvement in multidrug resistance to anticancer drugs, MRP1 may have an important physiological function and play a critical role in drug disposition.

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Mouse MRP1 has been cloned and characterized as part of an effort to develop appropriate animal models for investigating the physiological function of MRP1 in vivo.[3,4] Analysis of the predicted amino acid sequence suggested that murine MRP is 88% identical to human MRP1. Stride et al[5] have directly compared the drug resistance profile and transport kinetics of murine and human MRP1 following transfection of their respective complementary DNAs (cDNAs) into human embryonic kidney cells. Significant interspecies differences in MRP1 function were observed. Unlike human MRP1, murine MRP1 failed to confer measurable resistance to anthracyclines and had a much lower maximum transport velocity (V_{max}) for 17-ßestradiol 17-(ßD-glucuronide). More recent structure-activity studies through site-directed mutagenesis showed that the functional differences between murine and human MRP1 are attributed to unconserved amino acid residues located in the third carboxyl-terminal of the polypeptide.[6,7] Since many of the drug disposition and toxicology studies are conducted in rats, interspecies comparison of MRP1 structure and function should be extended to rats.

To date, only partial sequence of a cDNA clone of rat MRP1 (rMRP1) has been reported in the GenBank database (AJ277881).[8] Our laboratory has been interested in the role of MRP1 in the delivery of anionic drugs to the various neural cell types within the brain. We have conducted most of our studies in the rat, or in primary cultures of neurons and astrocytes derived from the rat brain. To ascertain the functional characteristics of rMRP1, we isolated and cloned the MRP1 cDNA clone from rat brain astrocytes, determined its coding sequence, and verified the transport activity of the protein expressed in a transiently transfected Madin-Darby canine kidney (MDCK) cell line.

MATERIALS AND METHODS

Materials and Reagents

Near-term pregnant female Sprague-Dawley rats were supplied by Charles River Laboratory (Wilmington, MA). MDCK and T98G cells were purchased from ATCC (Manassas, VA). Human MRP1-transfected MDCK cells were provided by Dr Joanne Wang (University of Washington, Seattle, WA). Tissue culture media, trypsin-ethylenediaminetetraacetic acid (EDTA), Dulbecco's modified eagle medium (DMEM), and antibiotic solutions were supplied by GIBCO Invitrogen (Carlsbad, CA). Fetal bovine serum (FBS) was obtained from Hyclone

(Logan, UT). dNTPs (dATP, dCTP, dGTP, and dTTP), RNase inhibitor, and 1 kb DNA ladder were obtained from Promega Corporation (Madison, WI). T4 DNA ligase was purchased from GIBCO Invitrogen. Calcein acetoxymethyl ester (calcein-AM) was purchased from Molecular Probes (Eugene, OR). Dr A.W. Ford-Hutchinson at Merck-Frosst Center Canada & Co (Quebec, Canada) kindly provided MK571. Indomethacin was purchased from Sigma-Aldrich (Chicago, IL). Other reagents were of analytical grades or higher.

Isolation of rMRP1 cDNA from Astrocytes

Astrocytes were isolated from neonatal rat brains and cultured according to a modified procedure described by Catlin et al.[9] Briefly, brain cortices of newborn (1- to 2-day-old) Sprague-Dawley rats were dissected, cut into ~1-mm ^{3} pieces, and incubated for 10 minutes with 0.125% trypsin-EDTA at 37°C to obtain single cell suspension. After terminating the reaction by replacing the medium with 10% FBS-DMEM growth medium, the cells were washed 3 times with tissue culture medium. The cells were resuspended, filtered through a nylon mesh of 100-mm pore size, seeded onto a poly-D-lysine-coated 75 cm^2 culture flask, and allowed to grow for 24 hours at 37°C 5% CO_2 The culture medium was replaced every 2 to 3 days thereafter. On day 9, oligodendrocytes and microglia were removed from the culture by vigorously shaking the flasks on an orbital shaker overnight. The astrocytes that remained adherent to the plates were trypsinized, washed with phosphate-buffered saline (PBS), pelleted, and stored at -80°C for RNA extraction.

Total RNA was isolated from cultured astrocytes with a SNAP total RNA purification kit (GIBCO Invitrogen) according to manufacturer specifications. Briefly, cell pellets were solubilized and RNA was eluted from the spin column following the supplier's instruction. Contaminating DNA was removed by digestion with RNase-free DNase I, followed by another round of column purification. Eluted RNA was quantitated by UV spectrophotometer measurement at 260-nm absorbance, aliquoted, and stored at -80°C until use. When needed, a similar procedure was used to isolate RNA from homogenates of rat liver and other cells.

To prepare cDNA of rMRP1 by reverse-transcription polymerase chain reaction (RT-PCR), we designed 2 sets of primers: one for transcription of the first 2871 nucleotides and the other for the remaining 1692 nucleotides at the 3'-end. The primer sequences were based on consensus sequences deduced from the mouse MRP1 (AF022908) and the known partial sequence of rMRP1 (AJ277881) in the NCBI GenBank database. The 2 sets of primer sequences are as follows.

First primer set targeted for the 5'-end of rMRP1-cDNA:

Forward (1) 5' ATGGCGCTGCGCAGCTTCTGC 3' (20)

Reverse (2894) 5' GCCTTCATGTAGTTCCAGTACACG 3' (2871)

Second primer set targeted for the remaining rMRP1-cDNA:

Forward (2860) 5' GTGAAGCTTTCCGTGTACTGGA 3' (2881)

Reverse (4589) 5' TCACACCAAGCCAGCATCCTTGGC 3' (4563)

With total RNA isolated from rat brain astrocytes, and the 2 sets of primers, rMRP1 cDNA was isolated as 2 overlapping fragments using the Titan One-Tube RT-PCR kit (Roche Molecular Biochemicals, Indianapolis, IN) containing Expand high-fidelity enzyme blend: avian myeloblastis virus (AMV) reverse transcriptase, Taq DNA polymerase, and a proofreading polymerase. A typical reaction was initiated by combining 2 reagent mixtures. Mixture 1 consisted of 0.2 mM deoxynucleotide triphosphates (dNTPs), 0.4 µM MRP-specific primers, 5 mM dithiothreitol (DTT), 5 units of RNase inhibitor, 50-200 ng total cellular RNA, and RNase-free water in a final volume of 25 µL. Mixture 2 consisted of 10-µL 5x RT-PCR buffer, 1.5-2.5 mM $MgCl_2$ 1 µL Expand enzyme mixture, and RNase-free water in a final volume of 25 µL for each reaction. Twenty-five microliters each of mixtures 1 and 2 was added to a 0.2-mL thin-walled PCR tube with attached dome cap. The reaction was performed in a GeneAmp PCR system with either a Model 2400 or a Model 9600 thermocycler (PE Biosystems, Foster City, CA). Reverse transcription was performed at 47°C for 30 minutes, followed by denaturing at 94°C for 3 minutes. PCR amplification was started with an initial denature at 94°C for 30 seconds, annealing at 56°C for 30 seconds, and extension at 68°C for 3 minutes, for a total of 35 cycles, followed by the final extension step at 68°C for 10 minutes. Ten microliters of the RT-PCR products were separated on a 1% agarose gel in 1× Tris-borate-EDTA (TBE) buffer (pH 8.0), along with 1 kb DNA ladder, and stained with ethidium bromide for fluorography. The gel was photographed with a Kodak DC120 digital camera. The 2 overlapping fragments of rMRP1 cDNA were isolated as 2.8- and 1.7-KB DNA bands. The 2 DNA fragments were separated and recovered from the 1% gel using Qiagen DNA gel extraction kit (Valencia, CA). Subsequently, each cDNA fragment was digested with Hind III enzyme, and the products were isolated with a Qiagen PCR purification kit. An aliquot of each digested cDNA fragment was mixed together with T4 DNA ligase and allowed to anneal at 15°C overnight. The final full-length rMRP1 cDNA was used for cloning into expression vector.

Cloning of rMRP1 cDNA in a Mammalian Expression Vector

A linearized pCR3.1 mammalian cell expression vector (Invitrogen), which has an overhanging thymine at each 5'-end to facilitate cDNA insertion, was chosen as our cloning vector. An adenine base was added to the 3'-end of the ligated double-stranded MRP1 cDNA as described above by incubating the ligated cDNA with Taq DNA polymerase for 15 minutes at 72°C in a thermal cycler. Subsequently, ligation of cDNA with the linearized pCR3.1 was performed at 15°C overnight. Top 10F' competent cells were transformed with the ligated mixture following the supplier's instruction. DNA-transfected cells were grown in Luria-Bertani medium (LB) broth agar plates containing 25 µg/mL kanamycin

as a selection agent. Positive clones were selected from the plates and expanded in 5 mL of LB broth containing kanamycin in a shaker bath at 37°C. Plasmids from kanamycin resistance clones were extracted using the Qiagen plasmid minipreparation kit. Clones carrying full-length inserts were screened first by size exclusion. The orientation of cDNA insert was further verified with 2 restriction enzymes (EcoR I and Hind III) that produced distinct digestion patterns for proper orientation of the cDNA insert. This is followed by DNA sequence verification of both cDNA insert and the adjacent plasmid sequences.

DNA Sequence Analysis

To fully sequence the rMRP1 cDNA, we used the nested primer design strategy for sequencing the isolated cDNA and cloned cDNA plasmid. The purified pCR3.1-rMRP1 plasmid was used as the sequence template. Sequencing was performed in both the forward and reverse directions. The full-length cDNA was divided into several regions of about 500-700 bp in length. The primers for each region were designed to have a short overlap with adjacent regions. For the 3'-half of rMRP1, the primers were designed based on the known partial sequence of rMRP1 (AJ277881).[8] For the unknown 5'-half region of the rMRP1 clone, we used the T7 promoter sequence on the cloning vector just upstream of the cloning site as the first forward primer, and we chose the known 3'-half sequence in the middle of the cDNA as reverse primer. After obtaining the first pairs of sequence, the second pairs of primers were designed based on the first determined set of sequence. This process was iterated until the entire unknown region of rMRP1 was sequenced. Big dye termination reaction was performed according to the manufacturer's specification for fluorescence labeling (ABI, Foster City, CA). Sequences were separated on an ABI-377 DNA sequencer. DNA sequence data were analyzed and assembled using DNA software Sequencher (Gene Codes, Ann Arbor, MI). From the nucleotide sequence data, the amino acid sequences were deduced. Sequence alignments were performed with human and mouse MRP1s using sequences available in the GenBank database. Percent identities of nucleotide and amino acid sequences among mouse MRP1, human MRP1, and rMRP1 were obtained.

Transfection of rMRP1 cDNA Expression Plasmids into MDCK Cells

To assess the function of the cloned rMRP1, MDCK cells were transfected with the pCR3.1 vector or pCR3.1-rMRP1 plasmid (herein referred to as p-rMRP1) using the lipofectamine2000 reagent (Invitrogen, Carlsbad, CA). Briefly, MDCK cells were allowed to grow for 24 hours at 80%-90% confluence on 12-well plates. The growth medium of the cells was replaced with serum-free medium for 30 minutes. Two micrograms of plasmid DNA and 6 mL of Lipofectamine2000 were each placed in 100 mL of Opti-MEM medium, mixed, and incubated at 20°C for 20 minutes. The DNA-Lipofectamine mixture was added into each well of MDCK cells and incubated at 37°C 5% CO2 incubator for 6 to 8 hours. The cells were then allowed to grow in growth medium containing serum. At 24 hours after transfection, cells from 1 or 2 wells were trypsinized and plated into a T75 flask or a 12-well culture plate at a dilution of about 1:20 in DMEM growth medium. At 48 hours posttransfection, Geneticin (G418) at 500 µg/mL was added. Medium containing G418 was refreshed every 3 to 4 days for 3 weeks. Cells that survived under G418 selection pressure were used subsequently for functional characterization studies.

Calcein Retention and Efflux in p-rMRP1-Transfected MDCK cells

Calcein-AM, a fluorescence substrate for MRP1, was used to assess the efflux transport activity of p-rMRP1-transfected MDCK cells. The pCR3.1 vector-transfected MDCK cells served as a background control. Steady-state calcein retention studies were conducted in the presence and absence of MRP1 inhibitors. Culture medium was first removed from the cells and washed with 2 mL of prewarmed Dulbecco's phosphate-buffered saline containing 1mM glucose (DPBSG) at pH 7.3. For the experiments of inhibition, cells were preincubated for 2 minutes with 1 mL of DPBSG buffer containing either 20µM MK571 or 100µM indomethacin at 37°C. For the corresponding control incubations, the cells were preincubated with DPBSG. The cells were then incubated with 1 mL of 0.5µM calcein-AM in DPBSG with or without the MRP1 inhibitor. After 20 minutes of incubation with calcein-AM, the cells were washed twice with 3 mL of cold DPBSG and lysed with 1.25 mL 1% Triton X-100. The amount of intracellular calcein was measured with a Hatachi-4500 fluorescence spectrophotometer (Hatachi, Ltd, Tokyo, Japan) set at λ_{ex} = 485 nm and λ_{em} = 517 nm.

The distribution of calcein fluorescence intensity (λ_{em} = 517 nm) in the vector- and p-MRP1-transfected cell populations were compared using a fluorescence-activated cell sorter (FACSAN II, Becton Dickinson, Franklin Lakes, NJ). Cells were incubated with calcein-AM in the absence or presence of inhibitors, detached with 0.25% trypsin-1mM EDTA at 4°C. The cells were then washed 3 times by pelleting and resuspending in 3 mL of cold DPBSG. Finally, they were resuspended in 1 mL of PBS at 4°C and subjected to fluorescence activated cell sorting (FACS) analysis. For all experiments described above, cellular protein concentrations were measured using a BCA protein assay kit (Pierce, Rockford, IL). Results of calcein fluorescence measurements were normalized to protein concentration.

RESULTS AND DISCUSSION

Recently, we succeeded in developing an RT-PCR procedure for isolating a full-length cDNA from the 3.8 kb mutidrug resistance protein (MDR1) RNA in cells and

tissues that express the ABC protein.[10] We attempted to adapt the same procedure for isolating the nearly 4.6 KB rMRP1 cDNA from primary rat astrocytes. However, when the rMRP1 cDNA was inserted into the plasmid vector, we consistently observed internal deletion that resulted in cDNA fragments that were much shorter than the predicted 4.6kb length. We were able to overcome the problem by isolating 2 overlapping halves of the rMRP1 cDNA using 2 sets of primers. The 2 half-fragments were purified and ligated, then inserted into a mammalian expression vector, pCR3.1. The proper orientation and the insertion points were verified by restriction endonucleases and sequencing of the inserted cDNA. DNA sequencing was done on both positive and negative strands to ensure its accuracy.

As shown in **Table 1** , we found that the coding sequence of rMRP1 is 4599 nucleotides in length, which should translate into a 1532 amino acid polypeptide. The rMRP1 exhibits a higher nucleotide sequence identity to mouse MRP1 (94.3%) than to human MRP1 (83.0%) (Appendix A). The overall length of the deduced amino acids of rMRP1 (1532 amino acids) is longer than human (1531 amino acids) and mouse (1528 amino acids) MRP1. Nucleotide and deduced amino acid sequence of rMRP1 are presented in Appendix B. Preliminary topological analysis of the amino acid sequence predicted that the rMRP1, as had been reported for human and mouse MRP1, contains 17 transmembrane (TM) domains that are grouped into 3 hydrophobic, polytrophic membrane-spanning regions. The protein also contains 2 hydrophilic, intracellular nucleotide-binding domains (NBD) that feature the characteristic Walker A' and Walker B' signature sequences of ABC proteins.[1,11] Like the human and mouse orthologs, rMRP1 contains a missing 13-amino acid sequence between the Walker A and Walker B motifs in the NH2-proximal NBD, compared to the same region in the COOH-proximal NBD.[12] Stride et al[3] have noted that the highest variability between the human and mouse MRP1 amino acid sequences lies in the linker region following the NH_2-proximal NBD (defined as amino acids 793-969 in human MRP1 and amino acids 793-965 in mouse MRP1). The identity between human and mouse for this variable stretch of amino acid sequence is only 78% compared to 88% for the entire polypeptide. Amino acid sequences in the same region of rMRP1 revealed a high degree of identity with mouse MRP1 (90.7% identity) and a relatively greater difference compared to human MRP1 (76.2% identity).

Significant differences in drug resistance phenotype and drug transport kinetics have been noted between the human and mouse MRP1. Mouse MRP1 expressed in cDNA-transfected human embryonic kidney cells lacks resistance to anthracyclines.[5] Also, it has lower transport efficiency (V_{max}) for 17-ßestradiol 17-(ßD-glucuronide).

Table 1. Comparison of rMRP1 cDNA to Reported Human and Mouse MRP1 and Deduced Amino Acid Sequences*

Characteristics	RMRP1	Human MRP1†	Mouse MRP1‡
cDNA length (bp)	4599	4596	4587
Amino acid length	1532	1531	1528
DNA sequence comparison (relative to rat), %	__	83.0	94.3
Amino acid sequence comparison (relative to rat), %	__	86.8	94.9

*MRP1 indicates multidrug resistance-associated protein 1; rMRP1 = rat MRP
†Data for human MRP1 is derived from Genebank data: Accession L05628.
‡Data for mouse MRP1 is derived from Genebank data: Accession AF02290

Zhang et al[6,7] recently identified 2 nonconserved amino acid residues in the predicted TM helices 14 and 17 of human and mouse MRP1 that are critical for the observed interspecies differences. By converting glutamine 1086 in TM14 of mouse MRP1 to glutamate, as is present in the corresponding position (1089) of human MRP1, these investigators were able to confer anthracycline resistance to the mouse MRP1. Conversion of alanine 1239 to threonine in TM17, as in the corresponding position of human MRP1, increased 17-ßestradiol 17-(ßD-glucuronide) transport activity of mouse MRP1 to a level comparable to that observed with human MRP1. Inspection of our predicted amino acid sequence for rMRP1 showed that rMRP1 has the same critical amino acid residues and is identical to mouse MRP1 (ie, alanine at amino acid 1090 of TM14 and alanine at amino acid 1243 of TM17 of rMRP1). Hence, a similar difference in drug resistance and transport kinetics may exist for rMRP1 as compared to human MRP1.

To assess the transport function of transcript from our rMRP1, MDCK cells were transfected with the p-rMRP1 mammalian plasmid. RT-PCR analysis of total RNA extracted from p-rMRP1-transfected MDCK cells, and homogenates of rat astrocytes and liver by species-specific MRP1 primer set, indicated that successful amplification was achieved with only rat-specific primers, not human-specific primers. As a control, RT-PCR using primer sequence specific using primer sequence specific for human MRP1 was shown to amplify RNA isolated from T98G glioma cells that have previously been shown to express human MRP1,[13] and from human MRP1-transfected MDCK cells obtained from Dr Joanne Wang at the University of Washington (**Figure 1**). These data indicate that the mammalian expression plasmid, p-rMRP1, can be transcribed in the transfected cells and that there are sequence differences between rats and humans such that rat-unique primers cannot be used to amplify human sequences.

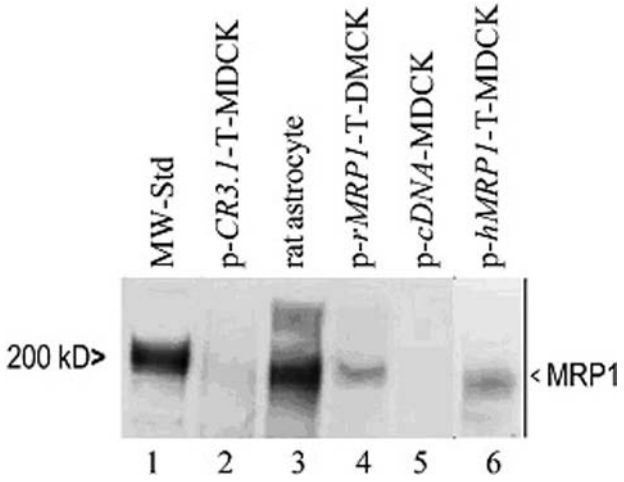

Figure 1. Transcription of MRP1 RNA in p-rMRP1-transfected MDCK cells. Total RNA extracted from rat liver (lanes 2 and 10), rat astrocytes (lanes 5 and 13), human T98G glioma cells (lanes 6 and 14), rMRP1 (lanes 4 and 12) or control plasmid pCR3.1-transfected MDCK cells (lanes 3 and 11), and human MRP1 or control plasmid pcDNA-transfected MDCK cells (lanes 8, 16 and 7, 15 respectively) were subjected to RT-PCR analysis using primer sequences specific for rat (lanes 2-8) or human (lanes 10-16) MRP1. rMRP1-specific primer sequences: 5' primer-CTACAAGGCGGTGATGGAG and 3' primer-CATTCTTACTGTCATCCTCTGAAG at positions 2211-2656. hMRP1-specific primer sequences: 5' primer-CCATATTACAGGTCCGTGATAC and 3' primer-TTGTGGTGCCTGCTGATG at positions 2204-2788. The RT-PCR products, 445 bp for the rat sequences and 584 bp for the human sequences, were separated by electrophoresis on 1% agarose gel, stained with ethidium bromide, and visualized as fluorophotographs. Lanes 1 and 9 show a 100-bp DNA ladder.

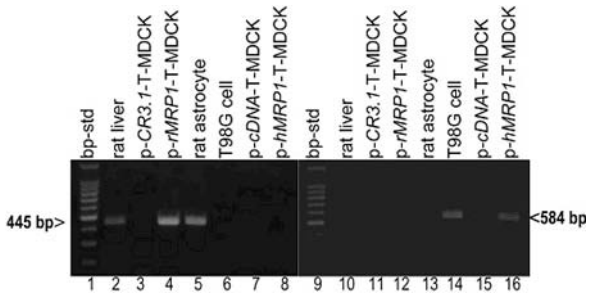

Figure 2. Immunoblot analysis of cDNA-expressed rMRP1 in MDCK cells. Solubilized cell-membrane fractions were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (DS-PAGE), transferred onto Immobilon™-P Polyvinylidene fluoride (PVDF) membrane, and allowed to react with monoclonal antibody directed against human MRP1. Presence of the MRP protein band was visualized on x-ray films using an enzyme-linked chemiluminescence's antibody detection system. Data obtained with membrane fractions from control vector (lane 2) or p-rMRP1 (lane 4) transfected MDCK cells, control plasmid (lane 5) or human MRP1 cDNA (lane 6) transfected MDCK cells, along with that from rat astrocytes (lane 3), were presented. Lane 1 contains prestained MW standard.

The plasma membrane fractions of p-rMRP1-transfected MDCK cells were analyzed for MRP1 protein content by immunoblot using a monoclonal antibody specific against human MRP1 (MRPr1, Alexis Biochemicals, San Diego, CA).[14] We found that the rMRP1 protein expressed in p-MRP1-transfected cells has electrophoretic mobility similar to that expressed in rat astrocytes and human MRP1-transfected MDCK cells (**Figure 2**). The apparent molecular weight of the protein is approximately 180-190 kd, a value consistent with a glycosylated protein of 1530 amino acids in length (**Table 1**).

The transporter function of cloned rMRP1 expressed in p-rMRP1-transfected MDCK cells was assessed by its ability to lower intracellular accumulation of a fluorescence MRP1 substrate—calcein—following its delivery in the form of its acetoxymethyl ester (calcein-AM). The FACS comparison of vector- and p-rMRP1-transfected cells that were preloaded with calcein-AM showed a left-ward shift in the fluorescence distribution of rMRP1-transfected cell population, a definite indication of increased efficiency in exporting calcein (**Figure 3**). This shift in fluorescence intensity was reversible by the addition of MRP-specific inhibitors such as indomethacin or MK571 (data not shown). Comparable results were obtained when total bulk fluorescence of transfected cells in suspension was measured (**Table 2**). **Figure 4** shows the time course of calcein efflux (expressed as a percentage of total preloaded calcein) in vector- and p-rMRP1-transfected MDCK cells; the results clearly demonstrated a much more efficient calcein efflux in the p-MRP1-transfected cells. In addition, MRP1-specific inhibitors—indomethacin and MK571—were able to inhibit the p-rMRP1-mediated calcein transport. In the presence of either 20μM MK571 or 100μM indomethacin, the p-rMRP1-transfected cell-mediated transport activity of calcein can be completely inhibited (**Table 3**).

Table 2. Effects of MRP1 Expression on the Ability of MDCK Cells to Export Calcein, an MRP1 Substrate

Experiment	Ratio of Intracellular Calcein-Fluorescence Between Control Plasmid and p-rMRP1 Plasmid-Transfected MDCK Cells[†]		
	Mean	SD	CV (%)
1	1.80	0.168	9.33
2	1.72	0.291	16.9
3	1.45	0.190	13.1

[*] CV indicates; MDCK, Madin-Darby canine kidney; MRP1, multidrug resistance-associated protein 1; p-r-MRP1, pCR3.1-rMRP1 plasmid; rMRP1, rat MRP1; SD, standard deviation.
[†] Intracellular calcein fluorescence of cloned rMRP1 cDNA (p-rMRP1) and control (pCR3.1) plasmid-transfected cells was measured after 20 minutes incubation in 0.5μM calcein-AM. Calcein fluorescence of control and rMRP1 plasmid-transfected cells was normalized by their respective protein content, and the difference was expressed as the ratio between control and rMRP1-transfected cells. The experiments were replicated on 3 occasions. Data expressed were mean ± SD of 6 replicates for each experiment.

Table 3. Effect of MRP1 Inhibitors to Reverse the Ability of p-rMRP1-Transfected MDCK Cells to Reduce Intracellular Calcein Concentrations*

MRP1 Inhibitor	% Reversal of p-rMRP1 Mediated Reduction in Intracellular Calcein Concentration[†]
Blank buffer	0.0 ± 13.4
+ Indomethacin (100 μM)	87.5 ± 16.8
+ MK571 (20 μM)	93.1 ± 8.6

*MRP inhibitors at indicated concentrations were coincubated with 0.5 μM calcein-AM for 20 minutes. The ability of the respective inhibitors to reverse the p-rMRP1-transfected MDCK cells was analyzed as % of reversal, after correcting for the effects of inhibitors on the control plasmid pCR3.1-transfected cells. MDCK indicates Madin-Darby canine kidney; MK; MRP1, multidrug resistance-associated protein 1; p-r-MRP1, pCR3.1-rMRP1 plasmid.
[†] Data expressed were mean ± standard deviation of quadruplicates.

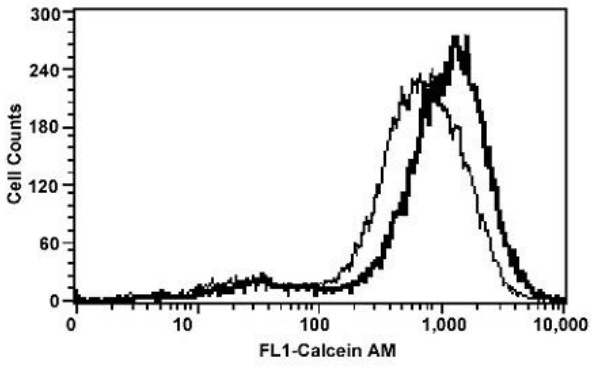

Figure 3. The ability of p-MRP1-transfected MDCK cells to reduce intracellular calcein retention. rMRP1 cDNA plasmid (p-rMRP1) and control plasmid (p-CR3.1) transfected cells were incubated with 0.5 mM calcein-AM for 20 minutes. The distribution of cell counts at varying intracellular calcein fluorescence (FL-1) was analyzed with a fluorescence-activated cell sorter and presented as a semilog histogram.

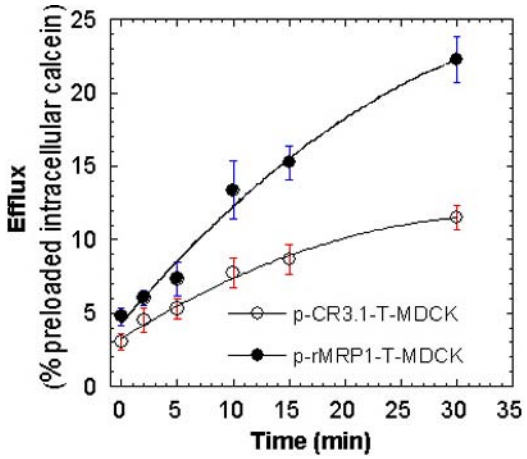

Figure 4. Time-dependent efflux transport of intracellular calcein from vector plasmid-transfected (p-CR3.1) or rMRP1 cDNA plasmid-transfected (p-rMRP1) MDCK cells. The cells were equilibrated in 0.5 mM calcein-AM for 20 minutes. The calcein-AM-containing medium was then removed, and the cells were washed with DPBSG buffer. The cells were resuspended in fresh DPBSG, and intracellular calcein was allowed to efflux. The intracellular calcein fluorescence at indicated time points was measured and expressed as a percentage of preloaded intracellular fluorescence. Data presented were mean ± standard deviation of quadruplicate samples at each time point.

In summary, we have successfully cloned, sequenced, and analyzed the entire coding regions of rMRP1 and demonstrated the efflux transport activity of the cDNA-expressed rMRP1 protein. Our results showed that rMRP1 exhibited an 83% nucleotide sequence identity to human MRP1 and a closer identity to mouse MRP1. The exact functional significance of the difference in the amino acid sequence between rat and human MRP1 needs to be defined. We are currently undertaking a comprehensive comparative study of rat and human MRP1 substrate selectivity and kinetics.

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