Identification of Domains Participating in the Substrate Specificity and Subcellular Localization of the Multidrug Resistance Proteins MRP1 and MRP2

The human multidrug resistance protein MRP1 and its homolog, MRP2, are both thought to be involved in cancer drug resistance and the transport of a wide variety of organic anions, including the cysteinyl leukotriene C4 (LTC4) (K_m = 0.1 and 1 μM). To determine which domain of these proteins is associated with substrate specificity and subcellular localization, we constructed various chimeric MRP1/MRP2 molecules and expressed them in polarized mammalian LLC-PK1 cells. We examined the kinetic properties of each chimeric protein by measuring LTC4 and methotrexate transport in inside-out membrane vesicles, sensitivity to an anticancer agent, etoposide, and subcellular localization by indirect immunofluorescence methods. The following results were determined in these studies: (i) when the NH2-proximal 108 amino acids of MRP2, including transmembrane (TM) helices 1–3, were exchanged with the corresponding region of MRP1, K_{m, LTC4} values of the chimera decreased ~5-fold and K_{m, methotrexate} values increased ~5-fold relative to those of wild-type MRP2 and MRP1, respectively, whereas resistance to etoposide increased ~3-fold; (ii) when the NH2-proximal region up to TM9 of MRP2 was exchanged with the corresponding region of MRP1, a further increase in etoposide resistance was observed, and subcellular localization moved from the apical to the lateral membrane; (iii) when two-thirds of MRP2 at the NH2 terminus were exchanged with the corresponding region of MRP1, the chimeric protein transported LTC4 with an efficiency comparable with that achieved by the wild-type MRP1; and (iv) exchange of the COOH-terminal 51 amino acids between MRP1 and MRP2 did not affect the localization of either of the proteins. These results provide a strong framework for further studies aimed at determining the precise domains of MRP1 and MRP2 with affinity for LTC4 and anticancer agents.

Two representative genes for the ATP binding cassette (ABC) transporter superfamily proteins, P-gp/MDR1 and MRP1, mediate acquisition of a multidrug resistance phenotype through altered membrane transport of various anticancer agents in tumor cells (1, 2). MRP1 confers resistance to a number of relatively hydrophobic natural product drugs including certain anthracyclines, epipodophyllotoxins, methotrexate, and vinca alkaloids (3–7). However, unlike P-gp, MRP1 can also transport a wide range of relatively hydrophilic anionic compounds including potential physiological substrates such as LTC4 and E217G (8–15). Topology studies of MRP1 have demonstrated that MRP1 and P-gp share a similar core structure consisting of two membrane-spanning domains (MDS2 and MDS3) and two nucleotide-binding domains (NBD1 and NBD2), referred to as the MDR-like core (16). The primary distinguishing characteristic of MRP1 and its related proteins, MRP2, -3, -6, and -7, is an additional NH2-terminal region forming a membrane-spanning domain (MSD1) with five transmembrane (TM) helices; however, the function of MSD1 remains to be clarified (1, 16–19). This region is linked to the MDR-like core by a cytoplasmic loop (CL) of ~130 amino acids, which is sometimes referred to as CL3 (17).

The MRP1 homolog, MRP2/cMOAT, shows only a 49% amino acid identity to human MRP1; however, its secondary structure is similar to that of MRP1 (19, 20). Mutations in MRP2 have been identified in patients with Dubin-Johnson syndrome (19, 21–23), indicating an important role for MRP2 in the export of bilirubin into hepatic bile ducts. In addition, MRP2 is involved in the transmembrane transport of some anticancer agents and confers a drug resistance pattern that is similar but not identical to that of MRP1. Resistance to vincristine and etoposide is markedly enhanced in MRP1 cDNA-transfected cells and is diminished in mpr1<–/– cells (5, 11, 24). In contrast, our previous study showed MRP2 cDNA-transfected cells display resistance to vincristine but only low level resistance to etoposide (25, 26). Furthermore, MRP2 transported the β-glucuronide

# The abbreviations used are: ABC, ATP-binding cassette; MRP, human multidrug resistance protein; P-gp, P-glycoprotein; MTX, methotrexate; MDS, membrane-spanning domain; NBD, nucleotide binding domain; TM, transmembrane; mAb, monoclonal antibody; E1,17βG, 17β-estradiol 17-(β-glucuronide); LTC4, leukotriene C4; CL, cytoplasmic loop; NBD, nucleotide-binding domain.
conjugate of the tobacco-specific carcinogen, 4-(methylimidazolino)-1-(3-pyridyl)-1-butanol, with higher efficiency than MRP1, and GSH inhibited rather than stimulated uptake of this conjugate (27). Thus, MRP1 and MRP2 share several substrates in common, but the relative affinities of the two proteins for some of these substrates appear to vary markedly, and the substrate binding pockets of the two proteins must have some significant differences (28, 29). Although recent studies have identified regions and specific amino acids of MRP1 that are important for substrate binding (30–35), the structural basis for the differences between MRP1 and MRP2 as regards substrate affinities and specificities remains unknown.

The most well characterized substrate of MRP1 is the cysteinyl leukotriene, leukotriene C₄ (LTC₄). Knock-out mice lacking mrlp₁ show an impaired response to a leukotriene-mediated inflammatory stimulus (36). Other studies have shown that the mrlp₁-mediated efflux of LTC₄ is involved in regulating dendritic cell migration to lymph nodes (37). At present, LTC₄ is the substrate with the highest affinity to MRP1 and mrlp₁ (Kₘ ~ 100 nM). MRP2 also shows relatively high affinity for this substrate; however, the Kₘ/(LTC₄) of MRP2 is ~10-fold higher than for MRP1 (38, 39).

Many MRP1 structure-function studies have been based on LTC₄ transport activity. Gao et al. (30) suggested that the CL3 region between the amino acids 204 and 281 is essential for LTC₄ transport activity. A recent study indicated that the deletion of all transmembrane helices of MSD1 (MRP1-(204–1531)) had no effect on MRP1-mediated LTC₄ transport (40), suggesting that MSD1 may not participate in LTC₄ transport. However, an LTC₄ photolabeling study revealed that the 281 NH₂-proximal amino acids of MRP1 were essential in the binding of LTC₄ to the NH₂-terminal half of the protein, and CL3 contained a region that is critical for the correct folding of MRP1 (33). Cys⁵ (TM1) mutation of MRP1 changed the NH₂-terminal conformation and LTC₄ transport activity (35). In addition, TM6 (MSD2) may contribute to LTC₄ transport activity (34). Taken together, these data demonstrate that several regions of MRP1 play an important role in the recognition and/or transport of LTC₄. In contrast to MRP1, little is known about regions of specific amino acids responsible for MRP2 substrate specificity and affinity.

Despite the close structural similarity between MRP1 and MRP2 proteins, their subcellular localization differs. MRP1 is present in the basolateral membrane, whereas MRP2 is localized to the apical membrane of polarized cells. MRP1 is involved in the integration into the membrane of the endoplasmic reticulum, intracellular trafficking, and/or recycling by endocytosis. However, additional signals responsible for the differential targeting of the multidrug resistance proteins to particular membrane domains have not been determined.

To identify the regions involved in determining substrate specificity, substrate affinity, and the subcellular localization of MRP1 and MRP2, we created a series of MRP1/MRP2 chimeras and tested them for their ability to transport LTC₄, confer resistance to etoposide, and localize to a distinct membrane region.

**EXPERIMENTAL PROCEDURES**

**Materials**—Vincristine, LTC₄, and MTX were purchased from Sigma. Etoposide was obtained from Nippon Kayaku Co. (Tokyo, Japan). LipofectAMINE 2000 was purchased from Invitrogen. [³H]LTC₄ (182.8 Ci/mmol) and [³H]MTX (5.90 Ci/mmol) were obtained from PerkinElmer Life Sciences.

**Cell Lines and Culture**—LLC-PK₁ (polarized pig kidney epithelial cells) were purchased from the Health Science Research Resources Bank (Osaka, Japan). These cell lines were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 60 μg/ml kanamycin, as described previously (26).

**Western Blot Analysis and Determination of Protein Levels in Transfected Cells**—Immunoblotting was performed as described previously (26). In brief, aliquots of membrane vesicles (40 μg) were electrophoresed on 7.5% SDS-polyacrylamide gels, and transferred onto Immobilon-P membranes. The MRP proteins were identified using the monoclonal antibody (mAb) MRP66, which reacts with an epitope close to the C-terminal end of MRP1 (amino acids 1311–1340) (51). The MRP2 proteins were detected with the mAb M₃I-6, which reacts with an epitope close to the COOH terminus of MRP2 (amino acids 1339–1541) (52). Membranes were incubated with the mAbs for 2 h at room temperature and then with horseradish peroxidase-linked secondary antibody for 40 min at room temperature. Membranes were developed by chemiluminescence following the ECL protocol (Amersham Biosciences). Relative levels of protein expression were estimated by densitometric analysis using an Image Gauge V3.1 software (Fuji film, Tokyo, Japan). The relative protein expression levels were calculated by dividing the densitometry value obtained for the total membrane protein from transfectants expressing the chimeric proteins by the densitometry value obtained for comparable amounts of total membrane protein from transfectants expressing wild-type MRP2 proteins. Each comparison was performed at least three times in independent experiments, and the mean values were used for normalization purposes.

**Construction of Vectors Encoding MRP1/MRP2 Chimera Proteins**—To generate the MRP1-(1–116)/MRP2 cDNA, an MRP1-(1–116) fragment was generated with 5’-3’-restriction enzyme sites, MluI and BsiWI, respectively, by site-directed mutagenesis. PCR amplification was performed using the forward primer (5’-GCC GCG ggc gCC ACC GTC ATT GCG-3’) and the reverse primer (3’-CTC TCA ACC GCG gtc cTT TTC TTT GCG ACC CGG CTC C-5’) with pJ3H-MRP1 (gift from Dr. P. Borst, The Netherlands Cancer Institute) as a template (substituted nucleotides are underlined; letters in italic type indicate restriction enzyme recognition sequences). The PCR product was isolated and subcloned into the EcoRV site (blunt-end ligation) of pT7 Blue-3 plasmid (Novagen). The pcDNA (Promega)-MRP2 construct was digested with BsiWI and NotI to yield a fragment composed of nucleotides 322–1520 of MRP2. This 4.4-kb pcDNA/MPR2 fragment was PCR amplified, which encoded nucleotides 1–369 of MRP1, were ligated to the MluI/NotI-digested pcDNA mammalian expression vector, obtained by multiple ligation, to give the construct pcDNA-MRP1/(1–116)/MRP2. MRP1-(1–1239)/MRP2, MRP1-(1–480)/MRP2, MRP1-(1–555)/MRP2, and MRP1-(1–546)/MRP2 were generated using the same method and the triple ligation procedure. To generate the MRP2 fragment, forward primer was used to generate an MluI restriction site at the 5’-end, with MRP1 as template, the same as those used above. To engineer the other 3’-end, restriction sites of MRP1 fragments were used, with the following reverse primers (the corresponding changes in the restriction
To generate MRPs-(1–1480)/MRP1, an MRP1-(1481–1531) fragment was generated by site-directed mutagenesis using the 5’-restriction enzyme site BclI. PCR amplification was performed using the forward primer 5’TGG ACC Ggt gat cAC TCC GGT CCT AGG GTC TGG-3’ and the reverse primer 3’-CCC AAT TAC TAG GAA CCT GGT CTG-5’ with p32I-MRP1 as template. Subsequent steps were performed by the same method for the ligation procedure described above. The MRP1-(1–1480)/MRP2 vector was generated by PCR amplification of nucleotides 3881–4442 of MRP1 using a forward primer, 5’-GAG CCT CAA GGA GTA TTC AGG-3’, and a reverse primer, 3’-CTG CCT GGG CAC tag TGG TAG-5’. The PCR product was digested with EcoRI and BclI, and the fragment was isolated. The MRP2 cDNA clone was digested with BclI and NotI, leaving nucleotides 4413–4638 of MRP2 attached to the vector pT7 Blue-3. The digested vector and attached fragment were then ligated to the EcoRI-BclI PCR product. The p32I-MRP1 construct was digested with NotI and EcoRI, yielding a fragment composed of nucleotides 1–3580 of MRP1 with a portion of the vector polylinker at its 5’ end. This 3.9-kb NotI-EcoRI fragment was ligated into the HindIII-digested construct. The linear insert was excised using NotI and transferred into a NotI-digested pCIneo expression vector to give the construct pCIneo-MRP1-(1–1480)/MRP2. The integrity of the hybrid constructs was confirmed by restriction analysis and by sequencing full-length constructs.

**Transient and Stable Transfection with Various Chimeric MRP1/ MRP2 Expression Vectors**—For transient transfections, wild-type and chimeric pCIneo-MRP1/MP2 expression vectors were transfected into LLC-PK1 cells. Cells were seeded into two-well culture slides (Falcon) 48–72 h prior to transfection, and DNA (2 μg/well) was added using LipofectAMINE 2000 (Invitrogen), according to the manufacturer’s instructions. After incubation for 48 h, the cells were washed with fresh medium, and 72 h prior to transfection, the LLC-PK1 cells were tested. For stable transfections, exponentially growing LLC-PK1 cells in 24-well plates (1.5 × 10⁴) were washed with phosphate-buffered saline and placed in serum-free medium. Cells were incubated in the presence of 2 μg of LipofectAMINE 2000 and 0.8 μg of expression vector DNA for 24 h and washed with fresh medium. Cells were then incubated in selection medium containing 550 μg/ml G418 for 3–4 weeks. Stable transfectants were selected from the G418-resistant transfectants. We also isolated a G418-resistant mock transfected, LLC-PK1/Vec, produced by transfection of the vector alone.

**Chemosensitivity Testing by Colony Formation Assay**—Cell survival was determined by plating 3 × 10⁴ cells from LLC-PK1 cells transfected with chimeras MRP1/MP2 cDNAs in 35-mm dishes in the absence of drug treatment, which was added 24 h later. After incubation for 7 days at 37 °C, the colonies were counted following the Giemsa staining procedure (25, 26). All drugs were freshly prepared in physiologic saline or dimethyl sulfoxide. Equivalent volumes of saline or dimethyl sulfoxide were added in all control experiments. The 50% lethal dose (IC₅₀) for each cell line was determined from the doseresponse curves. IC₅₀ values and S.D. values were obtained from the best fit of the data to a sigmoidal curve using GraphPad Prism™ software. Relative resistance was obtained by dividing the IC₅₀ values of cells transfected with vectors encoding either wild-type or MRP1/MP2 hybrid proteins by the IC₅₀ of cells transfected with the pCIneo vector alone.

**Immunofluorescence Study**—The localization patterns of the immunoreactive MRP1/MP2 chimeras in transiently transfected LLC-PK1 cells were determined by indirect fluorescent immunostaining. Cells were examined with an MRC-1024 confocal laser-scanning microscope (Bio-Rad) equipped with a ×100 objective. Cells were fixed by incubation with cold methanol/acetone (1:1) for 20 min. Preincubation was performed with rabbit anti-MRP1 (MRP m6) and MRP2 (M, III-6) mAb, followed by fluorescein isothiocyanate-conjugated mouse IgG, as described previously (23). For each transient transfection, at least 30 transfected (as observed by fluorescein isothiocyanate fluorescence) cells were counted on a confocal laser-scanning microscope.

**Membrane Vesicle Preparation**—All steps were performed at 0–4 °C. Stably transfected LLC-PK1 cell monolayers were washed and scraped into phosphate-buffered saline and washed by centrifugation (4000 × g for 10 min). The cell pellet was stored at −80 °C until required. Membrane vesicles were prepared from defrosted LLC-PK1 cells according to a recently described method (23). Vesicles were frozen in liquid nitrogen and stored at −80 °C until required for use. Protein concentrations were determined by the Lowry method, using bovine serum albumin as a standard (53). The orientation of membrane vesicles was determined by examining the nucleotide pyrophosphatase activity in the presence and absence of 1% Triton X-100 with p-nitrophenyl-thymidine 5-monophosphate as the substrate (54), and it was determined that 49% of LLC-PK1 vesicles were inside-out.

**Membrane Vesicle Transport Studies**—ATP-dependent transport of LTC₄ or MTX into the membrane vesicles was measured by filtration, essentially as described by Ishikawa and Ali-Osman (55). The standard incubation medium contained membrane vesicles (60 μg of protein), [3H]LTC₄ (0.01–6 μM) or [3H]MTX (0.004–6 mM, 0.25 μM sucrose, 10 mM Tris-HCl, pH 7.4, 10 mM MgCl₂, 1 mM ATP, 10 mM sodium phosphate and 100 μM creatine phosphokinase with or without unlabeled LTC₄ or MTX in a final volume of 25 μl. The reactions were carried out at 37 °C and stopped with 1 ml of ice-cold stop solution (0.25 μM sucrose, 100 mM NaCl, and 10 mM Tris-HCl, pH 7.4). The diluted samples were passed through Millipore filters (GVWP, 0.22-μm pore size; Millipore Corp.) and counted using liquid scintillation fluid, and the level of radioactivity was measured with a liquid scintillation counter. In the control experiments, ATP was replaced by an equal concentration of 5′-AMP. Rates of net ATP-dependant transport were calculated by subtracting the values obtained in the presence of 5′-AMP from those obtained in the presence of ATP. To examine whether [3H]LTC₄ or [3H]MTX was actually transported into the intravesicular spaces or was bound to the vesicle membranes, the uptake of [3H]LTC₄ or [3H]MTX was measured in the presence of different concentrations of sucrose in the assay mixtures. Data were analyzed using Graph Pad Prism™ software, and kinetic parameters were determined by nonlinear regression analyses and Michaelis-Menten analysis.

**RESULTS**

**Construction of Chimeric MRP1/MP2 Proteins and Their Expression in Polarized Kidney Cells**—To identify regions of MRP1 and MRP2 proteins responsible for their respective substrate specificities, we generated a series of chimeric MRP1/MP2 molecules (Fig. 1). Initially, the NH₂-terminal region of MRP2 was replaced with the corresponding MRP1 sequence (sequence MRP1-(1–484)/MRP2). In this case, because the cytoplasmic region linking the NBD1 to the MSD3 (linker region) is not required for the LTC₄ transport activity of MRP1 (30), this location was used to connect the segments of the chimeric protein. In the other chimeric molecules, either the NH₂-terminal or the COOH-terminal of MRP2 were replaced with the corresponding MRP1 sequence at positions 116 (MSD1; MRP1-(1–116)/MRP2), 239 (CL3; MRP1-(1–239)/MRP2), 480 (MSD2; MRP1-(1–480)/MRP2), 555 (MSD2; MRP1-(1–555)/MRP2), and 1480 (NB2D; MRP1-(1–1480)/MRP2) of MRP1. MRP2/MP2-MRP1-(1–1480–1531) consisted of the NH₂ terminus of MRP2 (amino acids 1–1480) and the COOH terminus of MRP1 (amino acids 1480–1531). Because a single amino acid substitution has been shown to be capable of inducing instability of proteins through a protein quality control system (23, 56), attempts were made, using the design of the chimeras constructs, to prevent structural distortion leading to degradation. Specifically, we chose nonidentical amino acids as the sites for combining MRP1 and MRP2, because identical and conserved amino acid residues could be arranged to form a functional unit, and reorganization of them might disrupt proper protein configurations. As expected, all chimaera proteins expressed in the LLC-PK1 cell line exhibited the same electrophoretic mobility as wild-type MRP2. Fig. 2 shows an immunoblot of the chimeric MRP1/MP2 variants expressed in LLC-PK1 cells by an anti-MRP2 mAb. For these chimeric molecules, we obtained expression of proteins of similar size to those of the wild-type MRP2 (Fig. 2), suggesting that these variants might be processed to mature form with a molecular mass of 190–200 kDa. On the other
The COOH-proximal Half, Including MSD3 and NBD2, Is Exchangeable between MRP1 and MRP2 for High Affinity (MRP1-type) Binding of LTC4—In order to identify regions that might participate in the determination of substrate specificity and affinity, we examined the kinetic character of each chimeric protein for LTC4 transport in isolated LLC-PK1 cell inside-out membrane vesicles. In the control experiments, membranes from LLC-PK1 cells transfected with empty expression vector showed that ATP-dependent tracer uptake was negligible. In all of the experiments, the uptake of [3H]LTC4 by membrane vesicles in the presence of ATP fell as the medium osmolarity increased (0.2 M sucrose). For the characterization of the function of the wild-type and chimeric proteins, in each case the linear phase of the tracer uptake (5 min) was used. ATP-dependent tracer uptake was calculated by subtracting the values measured in the presence of AMP.

We found that both MRP1 and MRP2 efficiently transported LTC4, but the \( K_m \) values were \( \sim 10 \)-fold higher for MRP2 than for MRP1, as described previously (39, 44). The \( K_m \) for the LTC4 uptake of MRP1 was 175.3 nM, whereas that of MRP2 was 1660 nM. The transport data for LTC4 are shown in detail in Fig. 4, and the \( K_m \) and \( V_{\text{max}} \) values for the wild-type and chimeric proteins are summarized in Table I, as are the \( V_{\text{max}} \) values normalized for differences in protein expression levels.

When the NH2-proximal half of MRP2 was replaced with the corresponding region of MRP1 (MRP1-(1–846)/MRP2), the chimeric protein showed very similar \( K_m \) (LTC4, 192.6 nM) values to

hand, the expression levels of chimeric proteins were low compared with those of wild-type MRP2, except in the case of MRP1-(1–239)/MRP2 and MRP1-(1–846)/MRP2. Reproducible results were obtained from the analysis of at least three independent transfected clones.

**Cellular Localization of Chimeric MRP1/MRP2 Proteins**—We next examined the membrane localization of various chimeric proteins in these polarized LLC-PK1 cells by indirect immunofluorescence with the respective specific antibodies (Fig. 3). We took advantage of the larger amount of proteins expressed and stronger signals in transiently transfected cells than were observed in the case of the stable transfectants. All of the transiently expressed chimeric proteins exhibited the same electrophoretic mobility as those of the wild type, as was the case with the stable transfection (data not shown). Antibodies against MRP1 (MRPm6) and MRP2 (M\( _{\text{III}-6} \)) were used for the immunofluorescence study, performed in order to identify each ABC transporter. Wild-type MRP1 and MRP2 were localized in the lateral membrane and the apical membrane of LLC-PK1 cells, respectively, as expected (Fig. 3, A and B). MRP1-(1–116)/MRP2 and MRP1-(1–239)/MRP2 proteins were predominantly localized in the apical membrane of LLC-PK1 cells, although some punctuated staining fluorescence of MRP2 in the cytoplasm was observed (Fig. 3, C and D). In contrast, MRP1-(1–480)/MRP2 was mainly present in the lateral membrane (Fig. 3E). MRP1-(1–555)/MRP2, MRP1-(1–846)/MRP2, and MRP1-(1–1480)/MRP2 were almost exclusively present in the lateral membrane, with some punctuated staining also present in intracellular compartments (Fig. 3, F–H). Taken together, these data indicate that the sorting information indispensable for the apical localization of MRP2 and/or the lateral localization of MRP1 lies between amino acids 239 and 480 of MRP1 and/or MRP2. Furthermore, the reciprocal chimer, MRP2/MPR1-(1480–1531), was localized in the apical membrane (Fig. 3I), suggesting that the COOH terminus 51 and 65 amino acids of the two proteins may be necessary but were exchangeable between MRP1 and MRP2 to achieve the proper membrane localization. Although the staining signals were weaker than those of the transient transfectants, similar results were obtained using the stable transfectants (data not shown).

**Fig. 1. Proposed topology of MRP1 and MRP2 and schema of chimera MRP1/MRP2 protein constructs.** A schematic representation is shown of a proposed secondary structure of MRP1 generated from computer predictions from several algorithms and experimental data (adapted from Hipfner et al. (17)). In this model, the protein has 17 predicted TMs, which are organized into three membrane-spanning domains (NBD1 to -2). The positions of the two nucleotide binding domains (MSD1 to -3). The predicted TMs, which are organized into three membrane-spanning domains (MSD1 to -3). The amino acid numbers refer to the domains (NBD1 and -2) are also indicated. B, schema of chimeric MRP1/MRP2 molecules generated. The amino acid numbers refer to the segment of MRP1 present in each construct.
those of the wild-type MRP1 (175.3 nM). Further exchange of MRP2 with the corresponding region of MRP1 up to amino acid residue 1480 (MRP1-(1–1480)/MRP2) also did not alter the $K_m$ (180.8 nM). Consistent with these observations, the reciprocal chimera, MRP2/MRP1-(1480–1531), showed a $K_m$ and $V_{max}$ value (LTC$_4$) of 1832 nM and 32.3 pmol/5 min/mg, respectively; the $K_m$ here was very similar to that of the wild-type MRP2 (1660 nM). These results indicate that the COOH-proximal half including MSD3 and NBD2 is basically exchangeable between MRP1 and MRP2 in terms of high affinity (MRP1-type) binding of LTC$_4$.

**Fig. 3.** Confocal microscopy of LLC-PK1 transfectants expressing wild-type and MRP1/MRP2 chimera proteins. Confocal analysis with fluorescent immunostaining was followed by the use of MRPm6 for MRP1 and M2III-6 for MRP2, followed by application of fluorescein isothiocyanate-conjugated mouse IgG, as described under “Experimental Procedures.” The locations of MRP1 and MRP2 are indicated in green. Nuclei were stained with propidium iodide and are shown in red. The upper section shows a top view of the monolayer, and the lower section shows a vertical x/z section. The arrowheads indicate the positions where the sections were made. Controls with no first mAb (fluorescein isothiocyanate-conjugated only) were invisible signals in each transfectant.
MSD1 of MRP1 Can Confer High Affinity Binding of LTC4 to MRP2—We next examined the transport kinetics of chimera proteins that had been generated by sequential exchange of small portions of the NH2-proximal half of MRP2 with the corresponding regions of MRP1. Surprisingly, when the NH2-proximal 116 amino acids of MRP2 (the region that includes TM1–3) was replaced with the first 116 amino acids of MRP1 (MRP1-(1–116)/MRP2), the $K_m$ value for LTC4 transport decreased 4-fold from 1660 to 383.7 nM. This result indicates the involvement of MSD1 (i.e. at least TM1–3) in the high affinity recognition of LTC4.

CL3, MSD2, and NBD1 Further Participate in the Determination of Affinity of LTC4 to MRP1 and MRP2—We further examined whether other parts of the NH2-proximal half of MRP1 were involved in high affinity recognition of LTC4. As shown in Table I, chimeric proteins MRP1-(1–239)/MRP2, MRP1-(1–480)/MRP2, MRP1-(1–555)/MRP2, and MRP1-(1–846)/MRP2 showed $K_m$ values for LTC4 transport of 446.6, 489.5, 296.5, and 192.6 nM, respectively. In other words, $K_m$ decreased progressively as the NH2-proximal region contained increasingly longer portions of MRP1. These observations suggest that CL3, MSD2, and NBD1 of MRP1 are also involved in determining the high affinity of MRP1 for LTC4 relative to MRP2, together with MSD1. In particular, it should be noted that the $K_m$ value of MRP1-(1–846)/MRP2 was approximately half of the $K_m$ value of MRP1-(1–480)/MRP2, suggesting that amino acid residues 480–846 might be also involved in binding specificity.

All chimeras showed $V_{max}$ values of 10–20 pmol/5 min/mg when the values were normalized by the relative expression level of chimera proteins, with the exception of chimera MRP1-(1–480)/MRP2, which had a $V_{max}$ value of 64.5 pmol/5 min/mg (Table I). The reason for this decrease in $V_{max}$ values among the chimeric proteins (except for MRP1-(1–480)/MRP2) remains unclear. Low expression or punctuate localization of some chimeras may have affected transport efficiency, but that phenotype was not directly associated with lower $V_{max}$ values.

The Role of MSD1 and the COOH-proximal Half, Including MSD3 and NBD2 of MRP1 and MRP2, in LTC4 Transport Can Be Generalized to MTX Transport—We next examined the generality of the role of MSD1 and COOH-proximal half in the transport of a substrate other than LTC4, namely MTX. In contrast to the LTC4 $K_m$ values, the $K_m$ value for MTX was ~14-fold higher in the case of MRP1 (3.52 mM) than in the case of MRP2 (0.25 mM), indicating a significantly more efficient MTX transport by MRP2 (Fig. 5, Table II). MRP1-(1–846)/
**Table I**

Kinetic parameters of [3H]LTC4 uptake by vesicles prepared from wild-type and MRP1/MRP2 chimeric transfectants

The kinetic parameters of [3H]LTC4 uptake were determined as described in the legend to Fig. 4. The normalized $V_{\text{max}}$ values were obtained by adjusting determined $V_{\text{max}}$ values to compensate for differences in the relative levels of the wild type and chimeric proteins. The relative levels of protein in the various transfectants were estimated to be LLC-MRP2 (1.0), LLC-MRP1-(1-116)/MRP2 (0.2), LLC-MRP1-(1-239)/MRP2 (0.8), LLC-MRP1-(1-480)/MRP2 (0.3), LLC-MRP1-(1-555)/MRP2 (0.3), LLC-MRP1-(1-846)/MRP2 (0.6), LLC-MRP1-(1-1480)/MRP2 (0.2) (shown in Fig. 2). ND, not determined.

| Construct       | $K_m$ (mM) | $V_{\text{max}}$ (pmol/min/mg) | Normalized $V_{\text{max}}$ | $V_{\text{max}}/K_m$ |
|-----------------|------------|--------------------------------|-----------------------------|----------------------|
| MRP1            | 175.3 ± 38.1 | 17.5 ± 1.5                     | ND                          | 99.9                 |
| MRP2            | 1660.0 ± 543.8 | 51.7 ± 6.8                      | 56.6                        | 31.1                 |
| MRP1-(1-116)/MRP2 | 383.7 ± 144.7  | 4.5 ± 0.6                        | 21.6                        | 13.4                 |
| MRP1-(1-239)/MRP2 | 446.8 ± 78.1   | 7.16 ± 0.4                       | 9.6                         | 14.9                 |
| MRP1-(1-480)/MRP2 | 489.5 ± 241.9  | 17.55 ± 3.1                      | 64.5                        | 40.5                 |
| MRP1-(1-555)/MRP2 | 296.5 ± 135.7  | 2.1 ± 0.4                        | 6.7                         | 5.3                  |
| MRP1-(1-846)/MRP2 | 192.6 ± 71.27  | 3.8 ± 0.6                        | 6.9                         | 18.7                 |
| MRP1-(1-1480)/MRP2 | 180.8 ± 66.72  | 1.7 ± 0.2                        | 11.2                        | 9.3                  |

**Table II**

Kinetic parameters of [3H]MTX uptake by vesicles prepared from wild-type and MRP1/MRP2 chimera transfectants

The kinetic parameters of [3H]MTX uptake were determined as described in the legend to Fig. 5. The normalized $V_{\text{max}}$ values were obtained by adjusting determined $V_{\text{max}}$ values to compensate for differences in the relative levels of the wild type and chimeric proteins. The relative levels of protein in the various transfectants were estimated to be LLC-MRP2 (1.0), LLC-MRP1-(1-116)/MRP2 (0.2), LLC-MRP1-(1-480)/MRP2 (0.6), ND, not determined.

| Construct       | $K_m$ (mM) | $V_{\text{max}}$ (pmol/min) | Normalized $V_{\text{max}}$ | $V_{\text{max}}/K_m$ |
|-----------------|------------|-----------------------------|-----------------------------|----------------------|
| MRP1            | 3.52 ± 1.14 | 3.00 ± 0.61                  | ND                          | 851.70               |
| MRP2            | 0.25 ± 0.12 | 0.31 ± 0.04                  | 0.31                        | 1239.60              |
| MRP1-(1-116)/MRP2 | 1.27 ± 0.23   | 2.58 ± 0.16                  | 12.26                       | 2029.92              |
| MRP1-(1-846)/MRP2 | 3.02 ± 1.61   | 2.19 ± 0.55                  | 3.97                        | 723.51               |

**Fig. 5.** Transport studies of [3H]MTX by membrane vesicles. The initial rate of ATP-dependent [3H]MTX uptake by membrane vesicles prepared from transfected LLC-PK1 cells was measured at various MTX concentrations (0.002–6 mM) for 5 min at 37°C, as described under “Experimental Procedures.” The kinetic parameters for MTX transport were determined by Michaelis-Menten analysis of the combined data using GraphPad Prism™ software and are shown in Table I. A, MRP1 (○); B, MRP2 (■); C, MRP1-(1-116)/MRP2 (□); D, MRP1-(1-846)/MRP2 (○). The results shown are the means of triplicate determinations ± S.E. in a single experiment. We obtained similar results from at least two independent experiments.

MRP2 showed very similar $K_m$ (MTX, 3.02 mM) values compared with those of the wild-type MRP1, indicating that the COOH-proximal half that includes MSD3 and NBD2 is basically exchangeable between MRP1 and MRP2 in terms of MRP1-type low affinity binding of MTX. On the other hand, the examination of MRP1-(1-116)/MRP2 and MRP2 in terms of MRP1-type low affinity binding of MTX. On the other hand, the examination of MRP1-(1-116)/MRP2 demonstrated that the $K_m$ value for MTX transport increased 5-fold from 0.25 to 1.27 mM (Fig. 4, Table II). This result indicates the involvement of MSD1 of MRP2, at least the TM1–3 region, in the high affinity recognition of MTX.

Exchange of CL3 and TM6–9 of MRP2 by the Corresponding

MRP1 Region Altered Sensitivity to Etoposide—In order to obtain insight into regions affecting the specificity of transport for substrates other than LTC4, we next examined whether the MRP1/MRP2 chimeric proteins showed altered drug sensitivity to an anticancer agent, etoposide, by a colony formation assay. The results are summarized as relative resistance factors in Table III. The LLC/MRP1 cell population showed an increase in resistance to etoposide relative to the control transfectants (17-fold resistance), whereas the LLC/MRP2 cell population displayed only a 2-fold resistance to etoposide (Table III). Consequently, we took advantage of the apparent differences in the
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Table III

Relative drug resistance of LLC-PK1 cells transfected with wild-type and MRP1/MRP2 chimeras

| Construct | Relative resistance | Normalized by expression level |
|-----------|---------------------|-------------------------------|
| MRP1      | 16.5 ± 1.3          | ND                            |
| MRP2      | 2.2 ± 1.3           | 2.2                           |
| MRP1–116/MPR2 | 1.6 ± 1.1     | 7.5                           |
| MRP1–239/MPR2 | 4.6 ± 1.4     | 6.1                           |
| MRP1–480/MPR2 | 3.7 ± 1.0     | 13.6                          |
| MRP1–555/MPR2 | 9.1 ± 1.4     | 29.8                          |
| MRP1–846/MPR2 | 7.5 ± 1.5     | 13.7                          |
| MRP1–1460/MPR2 | 3.5 ± 1.1     | 21.6                          |

To search for a region(s) involved in mediating etoposide resistance, cells expressing all of the various chimeric proteins displayed increased resistance to etoposide when compared with cells transfected with vector alone (Table III). When normalized for differences in expression levels, the chimeric proteins MRP1–116/MRP2 and MRP1–239/MRP2 showed an increase in the resistance to etoposide that was ~3-fold that of the wild-type MRP2. MRP1–1480/MRP2, MRP1–1–555/MRP2, MRP1–1–846/MRP2, and MRP1–1–1480/MRP2 showed a further ~7–15-fold increase in resistance to the etoposide relative to that of the wild-type MRP2 (Table III). Thus, we concluded that the NH2-terminal 116 amino acids and amino acids 239–480 of the MRP1 protein were involved in conferring higher levels of resistance to etoposide compared with those achieved by MRP2.

DISCUSSION

In order to identify regions determining the substrate specificity of ABC transporters, several studies have been performed using deletion constructs, amino acid substitution, and photoaffinity labeling. However, the regions involved in the establishment of substrate specificity have remained unclear, as have the various kinetic parameters shared among closely related family members such as MRP1 and MRP2. In this paper, we sought to identify the regions responsible for the 10-fold difference in affinity for LTC4 observed between MRP1 and MRP2; we also aimed to account for (i) differences in the ability of the two proteins to confer resistance to etoposide and (ii) differences in their distinct subcellular localizations.

We generated chimeric MRP1/MRP2 cDNA constructs and successfully expressed them in transiently transfected LLC-PK1 cells. For all of these molecules, we obtained the expression of proteins similar in size to that of wild-type MRP2 (Fig. 2), and the proteins were correctly routed to the plasma membrane in the stable transfectants (Fig. 3). However, there were some differences in the plasma membrane expression of different MRP1/MRP2 chimeras. Previous studies using the chimeric proteins murine and human MRP1 (57) and MDR1 and MDR2 (58) have revealed that chimeric proteins were expressed in relatively low levels compared with the levels of wild-type protein; our results agreed in this regard with the previous findings. One possible explanation for this low level of expression may be the slow protein-folding process or an unstable protein structure.

We then used transiently transfected cells in experiments conducted in order to determine subcellular localization. All of the chimeras examined were capable of being enzymatically active. Hence, the sorting behaviors observed in these studies were probably not due to the misfolding of the chimeric proteins but instead are more likely to have reflected the presence of an active apical localization signal in the MRP1 and/or MRP2 sequences. A recent study reported that a COOH-terminal deletion of at least 15 amino acids prevents efficient delivery of the MRP2 protein to the apical membrane, because part of a motif required for apical sorting is lost in polarized HepG2 cells (49). Our results clearly indicate that amino acids 239–480 of the MRP1 and/or the corresponding region of MRP2, including a part of CL3, are responsible for the distinct distribution. A recent study revealed that co-expression of a P-gp-like core with an isolated L0 region (amino acids 204–281) yielded routing to the lateral membrane in MDCKII cells; however, the P-gp-like core, when expressed alone, resulted in a loss of polarized distribution (40). Similarly, co-expression in MDCKII cells of the NH2-terminal fragments MSD1-L0 and MSD1 of the MRP2 protein, with the core fragments A-MRP2 and L0–A-MRP2, respectively, restored the altered cytoplasmic expression of the core alone to the apical plasma membrane (50). Photolabeling studies using [3H]LTC4 have suggested that CL3 might contain a region that is critical for the correct folding of MRP1 (33). Such results, when taken together, suggest that this cytoplasmic region is not only necessary for membrane routing, but that it also may play an additional role in creating a polarized distribution signal by cooperating with MSD2. Our results demonstrated that the 51 and 45 COOH terminus amino acids in MRP1 and MRP2, respectively, are exchangeable between the proteins, without inducing an alteration in their respective localizations. These results furthermore suggest that although the COOH terminus may be necessary for membrane anchoring, it does not appear to be responsible for distinctive localization processes. Certain differences in the amino acid residues of MRP1 and MRP2 in the region between 239 and 480 could be responsible for the apical targeting of MRP2 and/or the basolateral targeting of MRP1, depending on the signals by which the proteins would be sorted or stably anchored into distinct post-Golgi carriers in the trans-Golgi network, leading to distinct membranes (59). Future studies will determine the specific residues of the region 239–480 of the MRP1 protein and/or the corresponding parts of MRP2 that are responsible for proper localization.

We observed that the Km values for LTC4 transport decreased sequentially as the putative active region of MRP2 was exchanged by the corresponding region of MRP1; this replacement proceeded sequentially from the NH2-terminal to the COOH-terminal direction, suggesting that multiple regions located throughout MRP1 and MRP2 might be involved in the determination of specific affinity for LTC4. This result is consistent with a previous report (40) indicating that the addition of MSD1 and CL3 of MRP1 to P-gp is not sufficient to achieve transport of the glutathione conjugate; it is also thought that the rest of the MRP1 molecule is also required for substrate recognition and transport. Koike et al. (60) reported that amino acid substitution of conserved tryptophan residues at amino acid 361 (TM7), 445 (TM8), 459 (TM9), and 553 (TM10) eliminated or selectively reduced transport activity of organic substrates including LTC4, also consistent with our results.

Particularly surprising was our observation that the exchange of the NH2-proximal 116 amino acids of MRP2 by the
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corresponding MRP1 region (MRP1-(1-116)/MRP2) dramatically increased affinity for LTC\(_4\), suggesting the involvement of MSD1, at least the TM1–3 sequence, in determining the high affinity of MRP1 for LTC\(_4\). In addition, MRP1-(1-116)/MRP2 reduced affinity for MTX, which was recognized with 14-fold higher affinity by MRP2 than by MRP1, such that the MSD1 (including TM1–3) region of MRP2 appears to contain the region necessary for high affinity recognition of MTX. This result provides further evidence for the putative role played by TM1–3 in the modulation of an affinity to the substrate. Gao et al. (30) reported that the deletion of amino acids 1–66 of MRP1 markedly decreased LTC\(_4\) transport activity and that substitution of the first MSD of MRP1 (the region 1–228) with the comparable region of the MRP2 also markedly reduced LTC\(_4\) transport activity; that result was consistent with the present findings. Also, previous study has demonstrated that Cys\(_7\) in TM1 is critical for the MRP1 function through maintaining proper structure (35). Similarly, it was reported that substitution of conserved lysine and asparagine residues at amino acids 332 and 336, respectively, in TM6 diminished the ability to transport LTC\(_4\), and GSH transport was reduced (34). Because the cysteine, lysine, and asparagine residues are conserved between MRP1 and MRP2, it is not likely that those cysteine, lysine, and asparagine would be responsible for the \(K_m\) difference between the two proteins, although mutational analysis of the residues did alter the transport activity. Overall, these data suggest that MSD1 and the adjacent region of MRP1 contains several important residues forming a high affinity LTC\(_4\) transport site. Alternatively, Bakos et al. (61) reported that a truncated MRP1 mutant lacking the entire MSD1 region but still containing CL3 (amino acids 204–1531) behaved like wild-type MRP1 in terms of LTC\(_4\) uptake. The reason for this discrepancy is unclear at present, but the following explanations should be considered: (i) MSD1 may carry out a regulatory function to achieve the proper affinity of MRP1 and MRP2 for the substrate, LTC\(_4\), in mammalian cells but not in insect cells; (ii) the chimera protein assumes an unusual configuration to enhance transport activity; or (iii) one function of MSD1 may be to participate in some manner in a dimer configuration. As regards the last possibility, it has been reported that MRP1 appears to be dimer upon reconstitution into crystalline protein/lipid arrays (62). MSD1 may be functionally dispensable in a dimer configuration, provided the dimer complex were stable without MSD1, or the alteration of a portion of a monomer may functionally compensate by a particular region of an accompanying MRP1 counterpart in a homodimeric complex. Along these lines, it is of interest that two polypeptides, CL3 and the remaining distal region of MRP1, when expressed separately in insect cells, can assemble into a functional LTC\(_4\) transporter (30, 40).

We also found in the present study that the COOH-proximal half including TM 12–17 was exchangeable between MRP1 and MRP2, leading to functional expression and high affinity for LTC\(_4\). Recent photolabeling studies using \(N\)-(hydrocinchindin-8'-yl)-4-azido-2-hydroxybenzamide (63), iodoaryl azidorhodamine 123 (64), LTC\(_4\) (33), agostero A (65), and LY475776 (66) have suggested that the COOH-proximal half, especially TM16 and TM17, is the substrate binding site but that the NH\(_2\)-proximal half is also required. Although the COOH-proximal half could be the site for substrate binding itself, the region is unlikely to be fully responsible for the major differences in LTC\(_4\) affinity observed between MRP1 and MRP2, because our results clearly demonstrated that the COOH-proximal half was exchangeable between MRP1 and MRP2. In other words, our results revealing the exchangeability of the COOH-proximal half of MRP1 and MRP2 suggest that identical or conserved amino acids in this COOH-proximal half of the proteins could be necessary for binding and/or subsequent transport processes, whereas nonidentical or nonconserved amino acids are less likely to participate in generating the high affinity site for LTC\(_4\). It has been reported that basic residues in TM6, -9, -16, and -17 of MRP2 that are identical with MRP1 are important for the transport of glutathione-methylfluorescein (67), and a tryptophan residue in TM17 is important for the transport of E17(G by MRP1 and for the transport of MTX, LTC\(_4\) and E17(G by MRP2 (31, 68); these results all support our finding. The exchangeability of the COOH-proximal half of MRP1 and MRP2 in the case of both LTC\(_4\) and MTX transport is consistent with this hypothesis.

We demonstrated that the residues at positions 1–116 (TM1–3) and 239–480 (MSD2) of MRP1 were important for the protein’s ability to confer high levels of resistance to etoposide (Table III). Combined with the LTC\(_4\) transport data showing that amino acids 1–116 and 480–846 may play an important role in forming the high affinity LTC\(_4\) transport site, these observations regarding etoposide suggest that the structural determinants in MRP1 necessary for recognition and transport differ for each substrate and exist in multiple sites of the protein. In general, the present results appear to be consistent with those of previous studies.

In conclusion, we have identified the substrate specificity domains and regions containing the localization signal of MRP1 and MRP2. In MRP1, several sites mediating the recognition and transport of the anionic physiological substrate LTC\(_4\) were found to overlap with, but were not identical to, those for chemotherapeutic agents. It may in the future be possible to design agents capable of inhibiting the ability of MRP1 to confer resistance to some chemotherapeutic agents without interfering with its ability to transport anionic physiological substrates. Future studies will be necessary to investigate which regions of MRPs are critical for recognizing and transporting a variety of anionic physiological substrates and chemotherapeutic agents.

Acknowledgments—We thank Dr. Piet Borst (The Netherlands Cancer Institute) for the human MRP1 cDNA. We are also grateful to Dr. Susan P. C. Cole (Queen’s University) for critical reading of this manuscript and for helpful discussion of the study. We also thank our colleagues, Takashi Kawai and Sei Haga, for helpful advice and conversations. In addition, we thank Drs. Masataka Oitate, Fumihiko Ushigome, Noriko Koyabu, and Yasufumi Sawada (Kyushu University) for helpful input and technical advice.

REFERENCES

1. Loe, D. W., Deely, R. G., and Cole, S. P. (1996) *J. Cancer. Sci.* 32, 945–957
2. Gottesman, M., and Pastan, I. (1988) *Annu. Rev. Biochem.* 57, 377–427
3. Cole, S. P., Bhardwaj, G., Gerlach, J. H., Mackie, J. E., Grant, C. E., Almquist, K. C., Stewart, A. J., Kurz, E. R., Duncan, A. M., and Deely, R. G. (1992) *Science* 258, 1650–1654
4. Grant, C. E., Valdimarsson, G., Hippler, D. R., Almquist, K. C., Cole, S. P. C., and Deely, R. G. (1994) *Cancer Res.* 54, 557–561
5. Cole, S. P. C., Sparks, K. E., Fraser, K., Loo, D. W., Grant, C. E., Wilson, G. M., and Deely, R. G. (1994) *Cancer Res.* 54, 5902–5910
6. Zaman, G. J. R., Flens, M. J. V., Van Leusden, M. R., de Haas, M., Mulder, H. S., Lankelma, J., Pinedo, H. M., Scheper, R. J., Baas, F., Broxterman, H. J., and Borst, P. (1994) *Proc. Natl. Acad. Sci. U.S.A.* 91, 8822–8826
7. Kowano, M., Tob, S., Uchiyama, T., Takano, H., Kohno, K., and Wada, M. (1999) *Anticancer Drug Des.* 14, 123–131
8. Hooijberg, J. H., Broxterman, H. J. K., Koel, M., Assaraf, Y. G., Peters, G. J., Noordhuizen, P., Scheper, J. E., Borst, P., Pinedo, H. M., and Jansen, G. (1999) *Cancer Res.* 59, 2532–2535
9. Muller, M., Meijer, C., Zaman, G. J. R., Borst, P., Scheper, R. J., Mulder, N. H., de Vries, E. G. E., and Jansen, P. L. M. (1999) *Proc. Natl. Acad. Sci. U.S.A.* 96, 13033–13037
10. Jedlitschky, G., Leier, I., Buchholz, U., Barnouni, K., Kurz, G., and Keppler, D. (1996) *Cancer Res.* 56, 988–994
11. Loe, D. W., Almquist, K. C., Deely, R. G., and Cole, S. P. C. (1996) *J. Biol. Chem.* 271, 9675–9682
12. Loe, D. W., Almquist, K. C., Cole, S. P. C., and Deely, R. G. (1996) *J. Biol. Chem.* 271, 9683–9689
13. Jedlitschky, G., Leier, I., Buchholz, U., Hummel-Eisenbeiss, J., Burchell, B.,
Identification of Domains Participating in the Substrate Specificity and Subcellular Localization of the Multidrug Resistance Proteins MRP1 and MRP2
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J. Biol. Chem. 2003, 278:22908-22917.
doi: 10.1074/jbc.M302868200 originally published online April 7, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M302868200

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