A Positively Charged Amino Acid Proximal to the C-Terminus of TM17 of MRP1 Is Indispensable for GSH-Dependent Binding of Substrates and for Transport of LTC_4[†]

Xiao-Qin Ren,[‡] Tatsuhiko Furukawa,[‡] Shunji Aoki,[§] Tomoyuki Sumizawa,[‡] Misako Haraguchi,[‡] Yuichi Nakajima,[‡] Ryuji Ikeda,[‡] Motomasa Kobayashi,[§] and Shin-ichi Akiyama*[,‡]

Department of Cancer Chemotherapy, Institute for Cancer Research, Faculty of Medicine, Kagoshima University, Sakuragaoka 8-35-1, Kagoshima 890-8520, Japan, and Graduate School of Pharmaceutical Sciences, Osaka University, Yamada-oka 1-6, Suita, Osaka 565-0871, Japan

Received July 11, 2002

ABSTRACT: MRP1 is a 190 kDa membrane glycoprotein that confers multidrug resistance (MDR) to tumor cells. Our recent study demonstrated that GSH is required for the labeling of $MRP1_{932-1531}$ with a photoanalogue of agosterol A (AG-A) and suggested that GSH interacts with the L_0 region of MRP1. In this study, we further characterized the GSH-dependent binding site of azido AG-A on MRP1. Coexpression of the N- and C-terminal halves of MRP1 (residues 1−1222, TM1−16, and 1223−1531, TM17, respectively) in Sf21 insect cells reconstituted a functional drug transporter with a K_m for LTC_4 (97 nM) similar to that of intact MRP1. In membrane vesicles from those cells, GSH-dependent photolabeling of the MRP1 fragment (1−1222) required the coexpression of the C-terminal MRP1 fragment (1223−1531). An MRP1 fragment extending from residue 1 to 1295 however could be photolabeled by azido AG-A in a GSH-dependent manner. These data indicate that amino acids 1223−1295 of MRP1 are required for AG-A binding to MRP1 in a GSH-dependent manner. However, cross-linking of the photolabel to MRP1 occurs at a more upstream site. An arginine residue at position 1249 of MRP1 was shown to be important for the GSH-dependent binding of AG-A to MRP1. Mutation of this arginine to alanine (R1249A) resulted in a decreased level of GSH-dependent azido AG-A photolabeling of MRP1. Furthermore, this mutant attenuated MRP1 function by decreasing the level of LTC_4 substrate transport and impairing resistance to the drug vincristine (VCR). In summary, this study demonstrates that a region of MRP1 (amino acids 1223−1295), which includes TM helix 17, is required for azido AG-A binding to MRP1 in a GSH-dependent manner. A GSH-dependent drug binding site may exist in this region. Furthermore, our findings suggest that the charged amino acid Arg^{1249} proximal to the C-terminus of TM helix 17 is indispensable for MRP1−substrate interaction and the function of MRP1.

Multidrug resistance (MDR)[1] is a major obstacle to successful cancer chemotherapy (*1*). Two membrane proteins, P-glycoprotein (P-gp) and the human multidrug resistance protein (MRP1), are frequently overexpressed in MDR cells following drug selection in vitro or in vivo (*2*−*4*). Both the 190 kDa MRP1 and the 170 kDa P-gp are members of the family of ATP-binding cassette (ABC) transporters (*5*, *6*); however, the amino acid identity between these two proteins is restricted to the Walker consensus motifs in the nucleotide binding domains (NBDs). MRP1 is characterized by an extra transmembrane domain (TMD_0) composed of five transmembrane (TM) segments, which is connected to the P-gp-like core region by a cytoplasmic linker region (L_0) (*6*).

Overexpression of either P-gp or MRP1 in cultured cells resulted in a reduced level of drug accumulation and an increase in the rate of ATP-dependent drug efflux (*7*−*9*). However, there is compelling evidence that the two transporters function very differently in drug transport (*3*, *4*). It has been widely accepted that P-gp confers MDR by binding and transporting unconjugated drugs (*1*, *2*). As an organic anion transporter, MRP1 actively transports a wide variety of diverse anionic compounds. LTC_4 is an endogenous substrate of MRP1 with the highest known affinity for MRP1 ($K_m \sim 100$ nM) (*10*, *11*). By using the in vitro inside-out membrane vesicle system, it was found that GSH at physiological concentrations stimulated the ATP-dependent

[†] This work was supported by a Grant-in-Aid from the ministry of Education, Culture, Sports, Science and Technology. X.-Q.R. appreciates the research fellowship and grant from the Japan Society for the Promotion of Science.

* To whom correspondence should be addressed: Department of Cancer Chemotherapy, Institute for Cancer Research, Faculty of Medicine, Kagoshima University, Sakuragaoka 8-35-1, Kagoshima 890-8520, Japan. Phone: +81-99-275-5490. Fax: +81-99-265-9687. E-mail: akiyamas@m3.kufm.kagoshima-u.ac.jp.

[‡] Kagoshima University.

[§] Osaka University.

[1] Abbreviations: MDR, multidrug resistance; P-gp, P-glycoprotein; MRP1, human multidrug resistance protein; ABC transporter, ATP-binding cassette transporter; TM, transmembrane segment; TMD, transmembrane domain; NBD, nucleotide-binding domain; GSH, glutathione; VCR, vincristine; ADM, adriamycin; LTC_4, leukotriene C_4; NNAL, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol; $E_2 17\beta G$, 17-β-estradiol-17 (β-D-glucuronate); AG-A, agosterol A; IACI, *N*-(hydrocinchonidin-8-yl)-4-azido-2-hydroxybenzamide; IAARh123, iodoaryl azidorhodamine 123; [^{125}I]azido AG-A, [^{125}I]-11-azidophenyl agosterol A; HA, hemagglutinin A; TRITC, tetramethylrhodamine isothiocyanate; FITC, fluorescein isothiocyanate; mAb, monoclonal antibody.

10.1021/bi026443s CCC: $22.00
Published on Web 11/05/2002

transport of certain drugs such as vincristine (VCR) (*10*, *12*, *13*), adriamycin (ADM) (*13*, *14*), and aflatoxin B_1 (*15*) as well as certain endogenous hydrophilic anionic conjugates such as estrone 3-sulfate (*16*) and a tobacco-specific carcinogen, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) (*17*).

To clarify the role of GSH in MRP1 drug transport, we synthesized a photoaffinity analogue of AG-A that could reverse MRP1-mediated MDR (*18*). We recently reported that GSH was required for the binding of AG-A to MRP1 (*19*). It was found that GSH-dependent photolabeling of azdio AG-A was inhibited by LTC_4 as well as by several anticancer agents, reversing agents, and conjugated organic anions, suggesting that the binding site of AG-A is a common drug binding site on MRP1 (*19*). Identification of the GSH-dependent drug binding site on MRP1 will be helpful in elucidating the mechanism by which GSH stimulates the drug transporting activity of MRP1.

Previous photolabeling studies of MRP1 have utilized two photoactive drugs, iodoaryl azidorhodamine 123 (IAARh123) and a quinoline-based drug, *N*-(hydrocinchonidin-8′-yl)-4-azido-2-hydroxybenzamide (IACI) (*20*−*22*). These photoactive agents photolabeled MRP1 in the absence of GSH. The labeling sites of the drugs were restricted to TM segments 10, 11, 16, and 17 of MRP1 (*22*). However, it is not known whether other regions of MRP1 are required for binding of these agents. Recently, the labeling sites of LTC_4 on MRP1 have also been identified. LTC_4 labeled two sites in MRP1, one in the N-terminal half and one in the C-terminal half of MRP1. The L_0 region was required for labeling of the N-terminal half of MRP1, although the L_0 region was not the site labeled by LTC_4 (*23*).

Using a method involving photolabeling of coexpressed N- and C-terminal approximate halves of MRP1 and photolabeling of intact MRP1 followed by trypsinization, we determined that the GSH-dependent photolabeling site of azido AG-A lies within the C-terminal half of MRP1 (residues 932−1531) (*19*). However, the exact binding and photolabeling sites of azido AG-A on MRP1 are still unknown. The determination of the GSH-dependent drug binding site of MRP1 will be an important first step in elucidating the machanism of the GSH-dependent transport of some anticancer agents by MRP1.

In the study presented here, we investigated the region that is indispensable for GSH-dependent azido AG-A binding in the C-terminal half of MRP1.

EXPERIMENTAL PROCEDURES

Materials. [^{125}I]NaI [3.7 GBq (100 mCi)/mL] and [14,15,19,20-^{3}H(N)]LTC_4 (146 Ci/mmol) were purchased from Perkin-Elmer Life Sciences (Boston, MA). Unlabeled LTC_4 was from Calbiochem (La Jolla, CA). A monoclonal antibody (mAb) (12CA5) against the HA epitope (YPYDVPDYAS) was obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan). The synthesis and use of [^{125}I]-11-azidophenyl agosterol A ([^{125}I]azido AG-A) was described in a previous paper (*19*). Mouse IgG and protein G−Sepharose 4B were obtained from ZYMED (San Francisco, CA). CELLFECTIN, competent DH10Bac *Escherichia coli* cells, and Lipofectamine were purchased from Life Technologies (Rockville, MD). G418 was purchased from Nacalai Tesque Inc. (Kyoto, Japan). MRPr1 and MRPm6, mAbs against MRP1, were purchased from Progen Biotechnick (Heidelberg, Germany). Other drugs and chemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

Cell Culture, Transfections, Membrane Vesicle Preparation, and Cytotoxicity Assay. LLC-PK1 pig kidney cells were cultured in Dulbecco's modified Eagle's Medium (DMEM) (Nissui Seiyaku Co., Tokyo, Japan) containing 10% fetal calf serum. pCIneo−*MRP1* constructs (described below) were transfected into LLC-PK1 cells with Lipofectamine according to the manufacturer's protocol. Forty-eight hours following transfection, the cells were subcultured at either a 1:20 or a 1:500 dilution, and selected in G418 (1 mg/mL). When subcultured at a 1:500 dilution, G418 resistant colonies were selected and amplified, and the MRP1 expression levels of the resultant colonies were examined by Western blotting. When subcultured at a 1:20 dilution, G418 resistant mass populations were further selected in 40 nM VCR.

Sf21 insect cells were cultured in serum-free Sf-900 II SFM Medium (Life Technologies). Membrane vesicles and crude membranes were prepared as previously described (*24*). Protein concentrations were determined by the method of Bradford (*25*).

The cytotoxicity assay was conducted as previously described (*26*).

Generation of Constructs and Viral Infection. pFastBac *MRP1-His* containing the His-tagged MRP1 coding region was constructed as previously described (*24*). The strategies employed for site-directed mutagenesis of the MRP1 cDNA were described previously (*19*).

MRP1 constructs encoding R1222M and R1249A were generated in a PCR using the forward primers 5′-CA**T**G-CACAGCCTCAGTGCTG-3′ and 5′-**GC**GATGTCATCT-GAAATGGAAACC-3′, respectively (bold denotes mismatched bases encoding the mutations). An *Eco*RV site (underlined) was created by silent mutation (bold) in the R1222M construct using the reverse primer 5′-GA**T**ATC-ACCGCAAACAGGGCAGC-3′.

Insertion of one hemagglutinin A (HA) epitope following amino acid 1222 in MRP1 was carried out as follows. The primers 5′-**CCAGATTACGCTAGC**CACAGCCTCAGT-GCTGGCTTG-3′ (forward) and 5′-**GACGTCATATG-GATA**CCTGGAGATGACCGCAAACAG-3′ (reverse) were used in a PCR to insert a HA epitope (YPYDVPDYAS, bold). The unique *Nhe*I site in pFastBac*MRP1222HA* at the end of the sequence encoding the HA epitope was blunted by T4 polymerase and then self-ligated. The blunting resulted in a stop codon (underlined) behind HA (GCTAGCTAGC). The *Eco*RV−*Kpn*I fragment from the construct encoding R1222M was cloned into the multiple cloning site II of the pFastBac DUAL plasmid between the *Sma*I and *Kpn*I sites. The construct encoding $MRP1_{1-1222}$ and $MRP1_{1223-1531}$ resulted from ligation of the two large *Bss*HII−*Pvu*I fragments of the two plasmids.

Baculoviruses encoding the wild-type and mutant MRP1s described above were generated using the Bac to Bac expression system (Life Technologies) as described previously (*24*). The pCIneo-*MRP1s* mammalian expression constructs were generated by inserting *Bss*HII−*Not*I fragments derived from pFastBac*MRP1s* between the *Mlu*I and *Not*I sites of the pCIneo expression vector.

Photoaffinity Labeling of MRP1 with [^{125}I]Azido AG-A. [^{125}I]Azido AG-A (7.2 μCi/nmol) was used for the experiment. Photolabeling studies were carried out as previously described (*19*). Autoradiograms were exposed for times ranging from 10 h to 3 days. The radioactivities (counts per minute) of the gel slices corresponding to the bands on the autoradiogram were measured with an Auto Well Gamma System. The background radioactivity in equal areas of the gel in each lane was measured and subtracted from the radioactivity of the band.

[^{3}H]LTC$_4$ Uptake by Membrane Vesicles. The extent of [^{3}H]LTC$_4$ uptake was measured using a rapid filtration technique as previously described (*24*). Briefly, isolated membrane vesicles (25 μg of protein) were incubated in the presence or absence of 4 mM ATP in 50 μL of transport buffer [1.37 nM [^{3}H]LTC$_4$, 0.25 M sucrose, 10 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 10 mM phosphocreatine, and 100 μg/mL creatine phosphokinase] or with a range of [^{3}H]LTC$_4$ concentrations (25−800 nM) for 1 min at 37 °C. The reaction was stopped at the indicated times with 3 mL of ice-cold stop solution [0.25 M sucrose, 0.1 M NaCl, and 10 mM Tris-HCl (pH 7.5)]. The samples were passed through Millipore filters (GVWP, 0.22 μM pore size) under a light vacuum. Following three rinses with 3 mL of cold stop solution, the filters were dissolved in liquid scintillation fluid and their radioactivities measured.

Immunoblotting and Immunoprecipitation of MRP1. Immunoblotting was performed as previously described (*24*). Anti-MRP1 monoclonal antibodies MRPr1 (epitope amino acids 229−281) (*27*, *28*) and MRPm6 (epitope amino acids 1389−1531) (*27*, *28*) and the anti-HA mAb 12CA5 were used for Western blotting. Membrane vesicles (300 μg) prepared from Sf21 insect cells infected with a virus encoding both MRP1$_{1-1222}$ and MRP1$_{1223-1531}$ were photolabeled with [^{125}I]azido AG-A in the presence of 5 mM GSH. Membrane vesicles were then solubilized in 2 mL of buffer A [50 mM Tris-HCl (pH 8.0), 150 mM NH_4Cl, and 2 mM $MgCl_2$] containing 1% CHAPS and incubated for 2 h at 4 °C. The solubilized membranes were centrifuged at 12000*g*, and the supernatant was incubated at 4 °C overnight with 8 μg of anti-HA mAb and 8 μg of MRPm6 mAbs. A suspension of 200 μg of 20% protein G−Sepharose in buffer A was then added, and the mixture was rotated for 2 h at 4 °C. The precipitates were washed four times with buffer A containing 1% CHAPS and then used for SDS−PAGE.

Immunofluorescent Cell Staining. To confirm the coexpression and membrane localization of MRP1$_{1-1222}$ and MRP1$_{1223-1531}$ in insect cells, the cells were stained with both a rabbit polyclonal antibody termed MRPP (1:1000) (*29*) which recognizes a unique sequence (KEDTSEQWPV-LVKN) in the L_0 region of MRP1 and the mouse mAb, MRPm6 (1:1000), described above (*27*, *28*). Bound antibodies were detected with a tetramethylrhodamine isothiocyanate (TRITC)-conjugated goat anti-rabbit IgG (EY Laboratories, Inc., San Mateo, CA; 1:200) and a fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Medical Biological Laboratories Co. Ltd., Nagoya, Japan; 1:200). To detect the expression of MRP1 in LLC-PK1 cells, the cells were stained with MRPm6 (1:1000) and FITC-conjugated goat anti-mouse IgG (1:200). Immunofluorescent staining was carried out as previously described with the following modifications (*24*). Briefly, following two gentle rinses with PBS, the cells were fixed with 3.7% formaldehyde for 1 h at 4 °C. Following two washes with PBS, the cells were permeabilized with 100% methanol for 30 min at −20 °C. Following blocking with blocking buffer (3% BSA and 7% normal goat serum in PBS) for 1 h at 4 °C, the cells were incubated overnight at 4 °C with MRPP and MRPm6 in blocking buffer. Following three gentle washes with PBS, the cells were incubated for 1 h with FITC-conjugated rabbit anti-mouse IgG either alone or together with TRITC-conjugated goat anti-rabbit IgG at room temperature. The cells were then washed five times in PBS and once in distilled water. The coverslips with stained cells were mounted with a fluorescence preserver (Immunon, Shandon, Pittsburgh, PA), and the samples were examined with a confocal microscopy (Leica True Confocal Scanner 4D).

RESULTS

Coexpression of MRP1$_{1-1222}$ and MRP1 $_{1223-1531}$ Fragments in Sf21 Insect Cells Reconstitutes a Functional LTC$_4$ Transporter in Sf21 Insect Cells. Agosterol A (AG-A) can completely reverse MRP1-mediated MDR (*18*) and is thus a useful agent for the analysis of the drug binding site(s) on MRP1. We therefore synthesized [^{125}I]azido AG-A, a photoaffinity analogue of AG-A, to characterize the drug binding site(s) in MRP1 (*19*). It has previously been shown that coexpression of the half-molecules MRP1$_{1-932}$ and MRP1$_{932-1531}$ or MRP1$_{1-280}$ and MRP1$_{281-1531}$ of MRP1 in insect cells results in a functional MRP1 transporter (*19*, *30*, *31*). Thus, dual expression of MRP1 fragments in insect cells is therefore a useful model for the analysis of MRP1 drug binding sites. Using this dual expression system, we have previously shown that GSH stimulates azido AG-A binding to a region of MRP1 extending from amino acid 932 to 1531. This region of MRP1 includes the TMD_2 and NBD_2 domains of MRP1 as well as the C-terminal cytoplasmic tail of MRP1.

To further localize and characterize the AG-A binding site on MRP1, we therefore coexpressed half-molecules of MRP1, which were separated at the extracellular loop between TM16 and TM17 in TMD_2, in insect cells for use in binding studies. The predicted domains of the two coexpressed fragments are shown in Figure 1. We first determined the feasibility of coexpressing these half-molecules of MRP1 for use as a model system for MRP1 drug transport. For coexpression of MRP1$_{1-1222}$ and MRP1$_{1223-1531}$, the respective cDNAs were subcloned into a baculovirus dual expression vector. An HA tag was introduced into the C-terminus of MRP1$_{1-1222}$ to allow protein precipitation with the anti-HA mAb. Sf21 insect cells were infected with a recombinant baculovirus encoding either wild-type MRP1, both MRP1$_{1-1222}$ and MRP1$_{1223-1531}$, or MPR1$_{1-1222}$ alone. Western blotting with the MRPr1, MRPm6, and anti-HA mAbs was used to determine the expression levels of the MRP1 proteins in membrane vesicles prepared from the infected cells. As shown in Figure 2A, MRP1$_{1-1222}$ was expressed and was detected as a 130 kDa protein by both the MRPr1 and anti-HA mAbs. As expected, MRP1$_{1223-1531}$ could only be detected by MRPm6 and migrated as a 35 kDa protein. Since MRP1$_{1223-1531}$ contains only one predicted transmembrane segment of MRP1 (TM17, amino acids 1228−1248), indirect double immunofluorescent staining was used to investigate whether MRP1$_{1223-1531}$ was correctly localized in the membrane and was colocalized with

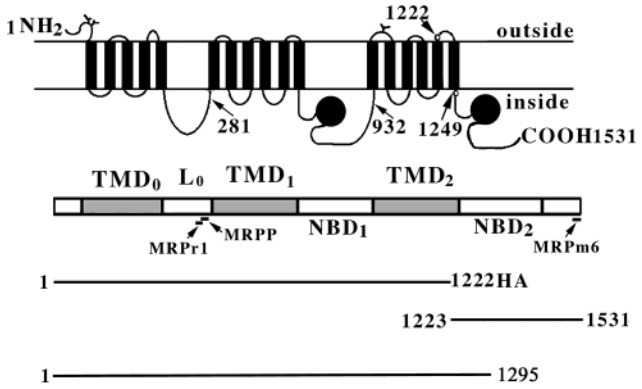

FIGURE 1: Predicted membrane topology model of MRP1, indicating the position of the Arg^{1222} at which the protein was divided. Filled dots represent the approximate location of arginine residues (1222 and 1249) that were altered by site-directed mutagenesis. The predicted 17 transmembrane α-helices, the cytoplasmic linker region L_0, and the two nucleotide binding domains, three TMDs, and two NBDs of MRP1 are shown, and the locations of epitopes for antibodies are indicated. The schematic representations of MRP1 fragments expressed in Sf21 insect cells are also shown.

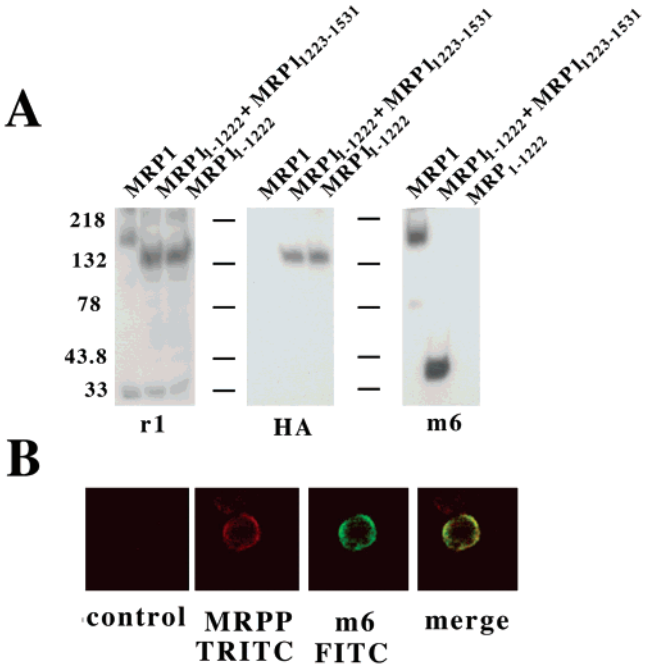

FIGURE 2: Expression of MRP1, $MRP1_{1-1222}$, and $MRP1_{1-1222}$ cotransfected with $MRP1_{1223-1531}$ in Sf21 insect cells. (A) Membrane proteins (10 μg) prepared from the cells expressing MRP1 or the MRP1 fragment(s) were subjected to 8.5% SDS−PAGE and immunoblotted with MRPr1 (r1), anti-HA (HA), and MRPm6 (m6) mAbs as indicated. MRPr1 and anti-HA antibody detect $MRP1_{1-1222}$ as shown in the left and middle panels, whereas $MRP1_{1223-1531}$ is only detected by MRPm6 as shown in the right panel. The sizes of protein standards are indicated in kilodaltons. (B) Immunofluorescent labeling of $MRP1_{1-1222}$ and $MRP1_{1223-1531}$ coexpressed in insect cells. Insect cells were infected with a recombinant baculovirus encoding both $MRP1_{1-1222}$ and $MRP1_{1223-1531}$. Immunofluorescent staining was performed as described in Experimental Procedures using confocal laser scanning microscopy. Staining of $MRP1_{1-1222}$ with the polyclonal antibody, MRPP, is shown in red and staining of $MRP1_{1223-1531}$ with the MRPm6 mAb (m6) in green following detection with TRITC- and FITC-conjugated secondary antibodies, respectively. Colocalization of the proteins is detected as bright yellow in the merged image. No fluorescence was detected in noninfected insect cell membranes (control).

$MRP1_{1-1222}$. For staining of the MRP1 constructs, both an N-terminal polyclonal antibody against MRP1 (MRPP) (*29*) and a C-terminal mAb (MRPm6) were used for the detection of $MPR1_{1-1222}$ and $MRP1_{1223-1531}$, respectively. The fluorescent secondary antibodies that were used were red, TRITC-conjugated anti-rabbit antibody for the detection of the anti-N-terminal MRPP polyclonal antibody and green, FITC-conjugated anti-mouse antibody for the detection of the anti-C-terminal MRPm6 mAb. As shown in Figure 2B, both proteins were expressed at the membrane and colocalization of the proteins was observed as a bright yellow color in the plasma membrane of the infected cells following merging of the signals. No fluorescence was detected in insect cells without infection (Figure 2B). To investigate whether the coexpression of $MRP1_{1-1222}$ and $MRP1_{1223-1531}$ reconstitutes a functional transporter, we examined $[^3H]LTC_4$ uptake in membrane vesicles prepared from these infected cells. As shown in Figure 3A, membrane vesicles coexpressing the two MRP1 fragments did take up $[^3H]LTC_4$ in an ATP-dependent manner. The level of ATP-dependent $[^3H]LTC_4$ uptake by membrane vesicles coexpressing both of the MRP1 fragments decreased in proportion to the increasing osmolarity of the medium, indicating that a considerable amount of $[^3H]LTC_4$ was actually transported into the intravesicular space of the membrane vesicles (Figure 3B). The apparent K_m value of the reconstituted transporter was 97 nM, which was comparable to the K_m value of wild-type MRP1 (103 nM), in the same experiment (Figure 3C). Thus, this system is a good model for further studies aimed at localizing the GSH-dependent binding site of azido AG-A on MRP1.

$MRP1_{1223-1295}$ Is Required for the Binding of Azido AG-A on MRP1. Using the coexpression system described above, membrane vesicles expressing either $MRP1_{1-1222}$ alone or $MRP1_{1-1222}$ together with $MRP1_{1223-1531}$ were photolabeled with $[^{125}I]$azido AG-A in the absence or presence of the indicated concentrations of GSH. Labeled bands were detected following gel electrophoresis and autoradiography. In membrane vesicles coexpressing both MRP1 fragments, $MRP1_{1-1222}$ was weakly photolabeled by $[^{125}I]$azido AG-A in the absence of GSH, and the labeling was enhanced up to 5-fold in a GSH-dependent manner (Figure 4A,B). However, in membrane vesicles expressing $MRP1_{1-1222}$ alone, $MRP1_{1-1222}$ was weakly photolabeled by $[^{125}I]$azido AG-A and the photolabeled band was not enhanced even in the presence of 10 mM GSH (Figure 4A,B). To investigate whether $MRP1_{1222-1531}$ is also photolabeled by $[^{125}I]$azido AG-A in the presence of GSH, membrane vesicles coexpressing both $MRP1_{1-1222}$ and $MRP1_{1223-1531}$ were photolabeled in the presence of 10 mM GSH and solubilized with 1% CHAPS. Both of the MRP1 fragments were precipitated from solubilized membrane vesicles with anti-HA and MRPm6 and analyzed by gel electrophoresis and autoradiography. As shown in Figure 4C, no radioactive band was observed in the precipitated $MRP1_{1223-1531}$ C-terminal fragment. Thus, it appears that $MRP1_{1223-1531}$ is required for the GSH-dependent photolabeling of $MRP1_{1-1222}$ by azido AG-A but $MRP1_{1223-1531}$ itself is not photolabeled by azido AG-A.

To determine which part of $MRP1_{1223-1531}$ is important for GSH-dependent binding of azido AG-A, we expressed a truncated MRP1 that encodes amino acids 1−1295 of MRP1 ($MRP1_{1-1295}$). This truncated MRP1 is lacking the NBD_2 domain and the entire C-terminal cytoplasmic tail. As shown

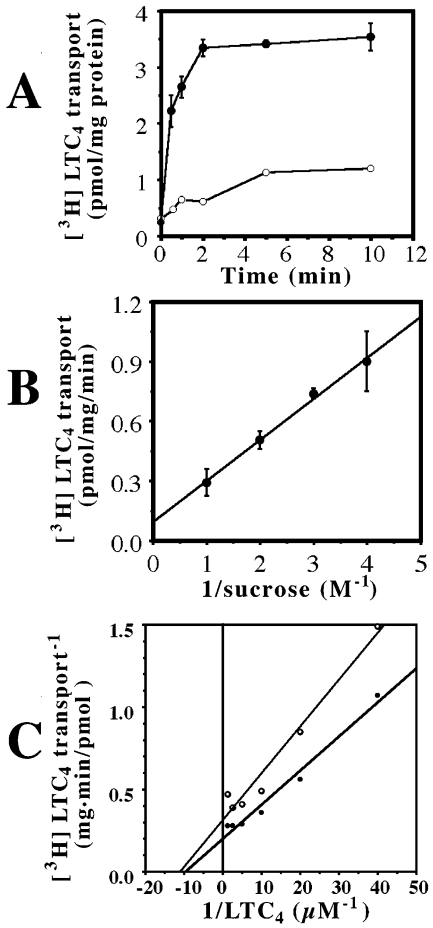

FIGURE 3: Coexpression of $MRP1_{1-1222}$ and $MRP_{1223-1531}$ from a dual expression vector reconstitutes a functional LTC_4 transporter in Sf21 insect cells. (A) Time course of ATP-dependent uptake of [^{3}H]LTC_4 by membrane vesicles coexpressing $MRP1_{1-1222}$ and $MRP_{1223-1531}$. Membrane vesicles (25 μg of protein) were incubated with 50 nM [^{3}H]LTC_4 in 50 μL of transport buffer [0.25 M sucrose, 10 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, 10 mM phosphocreatine, and 100 μg/mL creatine phosphokinase] in the absence (○) and presence (●) of 4 mM ATP at 37 °C for the indicated times. (B) Osmotic sensitivity of ATP-dependent [^{3}H]LTC_4 transport by membrane vesicles coexpressing $MRP1_{1-1222}$ and $MRP_{1223-1531}$. Membrane vesicles (25 μg of protein) were incubated at 37 °C for 3 min in transport buffer containing 50 nM [^{3}H]LTC_4 and the indicated concentrations of sucrose (0.25−1 M) in the presence or absence of 4 mM ATP. (C) Determination of the K_m values of coexpressed MRP1 fragments for [^{3}H]LTC_4. Membrane vesicles coexpressing the MRP1 fragments (25 μg of protein) were incubated at 37 °C for 1 min in transport buffer containing various concentrations of [^{3}H]LTC_4 (25−800 nM) with or without 4 mM ATP. Kinetic parameters of the transport were determined from double-reciprocal plots of the extent of ATP-dependent [^{3}H]LTC_4 uptake by membrane vesicles coexpressing the MRP1 fragments (○) or full-length MRP1 (●). The data represent the means ± standard error from three separate experiments. The K_m value is the mean of two duplicated experiments.

in Figure 5A, $MRP1_{1-1295}$ was expressed and was detected as a 135 kDa band following Western blotting with the N-terminal MRP1-specific antibody MRPr1. As expected, $MRP1_{1-1295}$ was not detected by MRPm6, the epitope of which is within the C-terminal cytoplasmic tail of MRP1. GSH-dependent binding of azido AG-A to this truncated form of MRP1 was observed as shown in Figure 5B. This suggests that NBD_2 and the C-terminal cytoplasmic tail of MRP1 are not required for GSH-dependent binding of AG-A to MRP1. These data indicate that amino acids 1223−1295

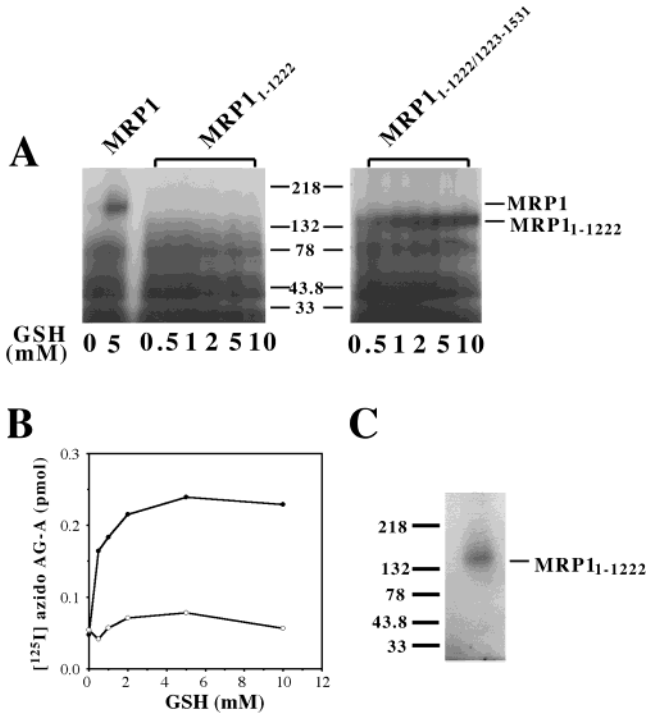

FIGURE 4: Comparison between GSH-dependent photolabeling of $MRP1_{1-1222}$ and of $MRP1_{1-1222}$ coexpressed with $MRP1_{1223-1531}$ in membrane vesicles. (A) Membrane vesicles (100 μg of protein) expressing $MRP1_{1-1222}$ alone or together with $MRP1_{1223-1531}$ were incubated with 5 μM [^{125}I]azido AG-A in the absence or presence of GSH (0.5−10 mM). The samples were then separated via 5 to 20% gradient SDS−PAGE. Autoradiograms were developed after exposure for 12 h at room temperature. (B) Quantification of bound [^{125}I]azido AG-A. Radioactive bands were excised from the gel, and the amount of bound [^{125}I]azido AG-A at various GSH concentrations was measured: (○) $MRP1_{1-1222}$ alone and (●) $MRP1_{1-1222}$ together with $MRP1_{1223-1531}$. (C) Immunoprecipitation of $MRP1_{1-1222}$ and $MRP1_{1223-1531}$ following [^{125}I]azido AG-A photolabeling. Membrane vesicles (300 μg of protein) expressing both $MRP1_{1-1222}$ and $MRP1_{1223-1531}$ were photolabeled as described above in the presence of 10 mM GSH. $MRP1_{1-1222}$ and $MRP1_{1223-1531}$ were precipitated with both the anti-HA and MRPm6 mAbs. The immunoprecipitated proteins were subjected to 5 to 20% gradient SDS−PAGE, and autoradiograms were developed after exposure for 2 days at −70 °C. The 35 kDa $MRP1_{1223-1531}$ fragment was not labeled with [^{125}I]azido AG-A.

of MRP1 are required for AG-A binding to MRP1 in a GSH-dependent manner. However, cross-linking of the photolabel to MRP1 occurs at a more upstream site.

Arg^{1249} in the Region of MRP1 Required for Drug Binding Plays an Important Role in GSH-Dependent Substrate Recognition. It has been demonstrated that charged amino acids in the transmembrane domains of rat and human MRP2 play an important role in the recognition of their substrates (*32*, *33*). The R1257A (TM17) mutant of human MRP2 showed reduced transport activity of GSH-bound methylfluorescein (*33*). There are several basic amino acids in the region of $MRP1_{1223-1295}$. Arg^{1257} in human MRP2 corresponds to Arg^{1249} in human MRP1. To investigate the role of charged amino acids in GSH-dependent photolabeling of azido AG-A to MRP1, we replaced the arginine at position 1249 which is proximal to the C-terminus of TM17 of MRP1 with Ala (R1249A). Arginine at position 1222 in the azido AG-A-photolabeled $MRP1_{1-1222}$ fragment was replaced with Met, and the functions of MRP1 R1222M were compared with those of MRP1 R1249A. The positions of these charged

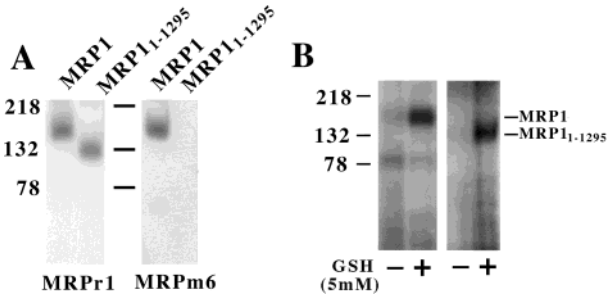

FIGURE 5: [^{125}I]Azido AG-A photoaffinity labeling of MRP1$_{1-1295}$ expressed in insect cells. (A) Expression of MRP1$_{1-1295}$ in insect cells. Membrane vesicles were prepared from Sf21 insect cells infected with recombinant baculovirus encoding wild-type MRP1 or MRP1$_{1-1295}$. The membrane vesicles (10 μg of protein) were subjected to 7.5% SDS−PAGE and immunoblotted with the MRPr1 (left) or MRPm6 (right) mAb. (B) Photolabeling of MRP1$_{1-1295}$. Membrane vesicles from cells expressing MRP1 (left) and MRP1$_{1-1295}$ (right) were photolabeled with 5 μM [^{125}I]azido AG-A in the absence or presence of 5 mM GSH. Samples were subjected to 7.5% SDS−PAGE, and autoradiograms were developed following exposure for 15 h at room temperature.

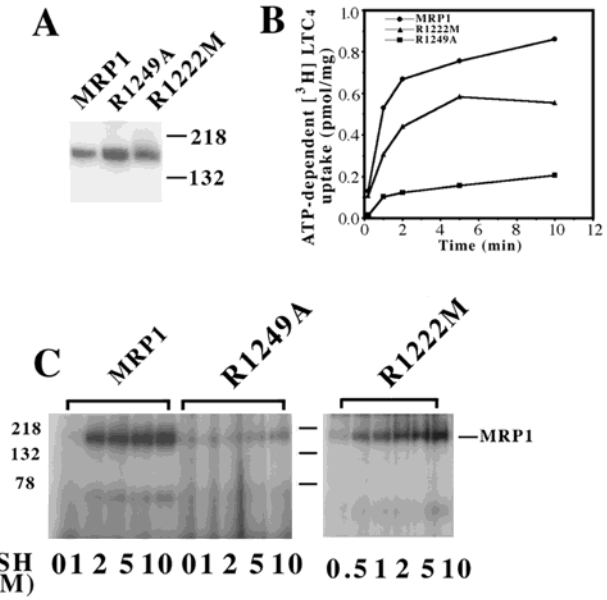

FIGURE 6: Effect of mutation of arginines 1222 and 1249 of MRP1 on ATP-dependent [^{3}H]LTC$_4$ transport and on GSH-dependent [^{125}I]azido AG-A photolabeling. (A) Expression of mutant MRP1s. Wild-type and mutant MRP1s were expressed in insect cells. Membrane proteins (6 μg of each sample) were separated via 7.5% SDS−PAGE and immunoblotted with the MRPr1 mAb. (B) Time course of ATP-dependent uptake of [^{3}H]LTC$_4$ by MRP1 and MRP1 arginine mutants. Membrane vesicles (25 μg of protein) expressing MRP1 R1222M or MRP1 R1249A as indicated were incubated with 1.37 nM [^{3}H]LTC$_4$ at 37 °C in 50 μL of transport buffer as described in the legend of Figure 3 in the presence or absence of 4 mM ATP at the indicated periods. The levels of ATP-dependent uptake shown in the figure were calculated by subtracting the radioactivity obtained in the absence of ATP. The results of one of two duplicate experiments are presented. (C) Effect of specific arginine mutations on the GSH-dependent photolabeling of MRP1 with [^{125}I]azido AG-A. Membrane vesicles (100 μg of protein) expressing MRP1, MRP1 R1249M, and MRP1 R1222M were photolabeled with [^{125}I]azido AG-A in the absence or presence of the indicated concentrations of GSH as described in the legend ofo Figure 2. Photolabeled membrane vesicles were subjected to 7.5% SDS−PAGE, and autoradiograms were exposed for 10−18 h at room temperature.

amino acids are indicated in Figure 1. These mutant MRP1 proteins were first expressed in Sf21 insect cells in an effort to study the effect of the mutations on MRP1−LTC$_4$ transporting activity. Figure 6A shows that the expression level of MRP1 R1222M was comparable to that of wild-type MRP1, whereas the expression level of MRP1 R1249A was higher than that of wild-type MRP1. The membrane vesicles expressing the wild-type and mutant MRP1 proteins were analyzed for their ability to transport LTC$_4$. As shown in Figure 6B, MRP1 R1222M transported LTC$_4$ as efficiently as wild-type MRP1. However, the transport activities of MRP1 R1249A were considerably reduced, suggesting that Arg^{1249} was important for the LTC$_4$ transporting activity of MRP1. To determine if these arginines played a role in MRP1 drug binding, we then tested the abilities of these mutants to be photolabeled by azido AG-A in a GSH-dependent manner. As shown in Figure 6C, GSH stimulated [^{125}I]azido AG-A binding to MRP1 R1222M as efficiently as wild-type MRP1. The level of photolabeling of MRP1 R1249A with [^{125}I]azido AG-A was increased only 1.6-fold in the presence of 10 mM GSH compared to a 10-fold increase observed with wild-type MRP1. These data therefore indicate that Arg^{1249} is important for GSH-dependent photolabeling of MRP1 with [^{125}I]azido AG-A. To test the ability of MRP1 R1249A to confer drug resistance, wild-type *MRP1* and the *MRP1 R1249A* mutant cDNAs were cloned into the mammalian expression vector pCIneo and transfected into pig kidney LLC-PK1 cells. The transfected cells were selected in G418 as described in Experimental Procedures. The mass population of G418 resistant clones was further incubated in 40 nM VCR, a well-characterized substrate of MRP1, to select VCR resistant cells. Transfection of wild-type *MRP1* resulted in the appearance of drug resistant colonies within 2 weeks. Transfection of the pCIneo empty vector or the *MRP1 R1249A* mutant resulted in no VCR resistant clones. However, limited dilution of G418 resistant mass populations transfected with the *MRP1 R1249A* mutant in the medium without VCR resulted in several clones expressing MRP1 R1249A (Figure 7A). The drug resistance of stably transfected clones was further tested in a cytotoxicity assay. As shown in Figure 7B, the MRP1 R1249A mutant impaired the ability to confer resistance to VCR on LLC-PK1 cells. To determine if the inability of the R1249A mutant to confer drug resistance might be due to an inability of the mutant protein to reach the cell membrane, we assessed the cellular localization of MRP1 R1249A by immunofluorescence using MRPm6 mAb. As shown in Figure 7C, the MRP1 R1249A mutant was localized to the plasma membrane in a manner similar to that of wild-type MRP1. Thus, the inability of R1249A to confer drug resistance was not due to impaired trafficking of the protein. These experiments therefore indicated that the arginine at position 1249 proximal to the C-terminus of TM17 of MRP1 is important not only for AG-A but also for LTC$_4$ and VCR binding.

DISCUSSION

P-gp confers MDR by directly binding and transporting substrates (*1*, *2*). It is unclear whether MRP1 mediates MDR by the same mechanism. Elucidation of the mechanism by which MRP1 mediates MDR would clearly be useful for the design of drug strategies for antitumor therapy. There is compelling evidence that GSH is necessary for the transport of certain unconjugated drugs by MRP1 (*10*, *12*−*17*). Our recent study demonstrated that GSH is required for the

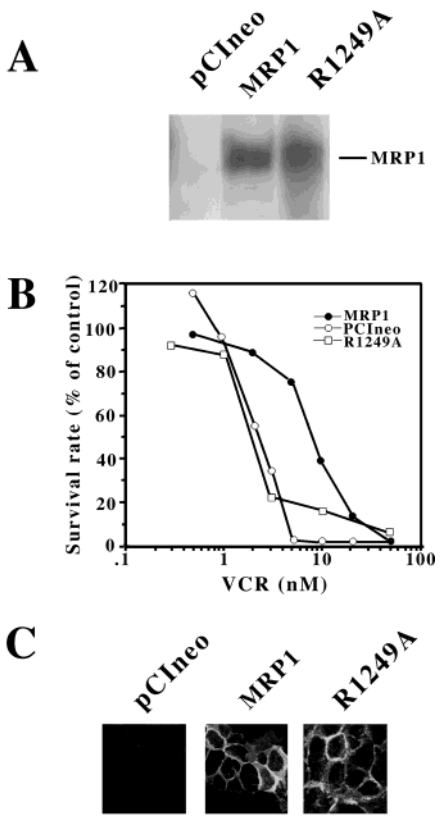

FIGURE 7: Effect of the R1249A mutation in MRP1 on drug resistance in LLC-PK1 cells. (A) Expression of MRP1 and the MRP1 R1249A mutant in LLC-PK1 cells. Crude membranes (50 μg of protein) prepared from LLC-PK1 cells transfected with an expression vector encoding MRP1 or MRP1 R1249A, or with a control empty vector (pCIneo), were analyzed on 7.5% SDS−PAGE. MRP1 constructs were detected by immunoblotting with the MRPm6 mAb. (B) VCR resistance in LLC-PK1 cells stably transfected with wild-type and mutant *MRP1* constructs. LLC-PK1 cells expressing MRP1 (●) or MRP1 R1249A (□), or the cells transfected with an empty vector [pCIneo (○)], were exposed to the indicated concentrations of VCR, and the survival rates were determined. The cytotoxicity assay was conducted as described in Experimental Procedures. The data are presented as mean survival rates of four separate wells in one experiment. (C) Cellular localization of transfected MRP1 constructs. Indirect immunofluorescent staining of MRP1 and MRP1 R1249A expressed in LLC-PK1 cells was carried out with the MRPm6 mAb and an FITC-conjugated secondary antibody. The panels show horizontal sections of cell layers obtained by confocal microscopy. No fluorescence was detected in LLC-PK1 cells transfected with an empty vector (pCIneo).

binding of a photoanalogue of AG-A, [^{125}I]azido AG-A, to the C-terminal half (residues 932−1531) of MRP1 (*19*). Identification of the GSH-dependent drug binding site within MRP1 will help to elucidate the mechanism by which GSH stimulates drug transport. In this study, we further delineated which part in the C-terminal fragment of MRP1 was required for azido AG-A binding to MRP1 in a GSH-dependent manner.

It has been reported that two photoactive agents, IAARh123 and IACI, can photolabel MRP1 in the absence of GSH (*20*−*22*). The detailed mechanism by which they bind to MRP1 and which part of MRP1 is essential for these photoactive probes binding to MRP1 are not known. Both drug-labeled fragments encompass the TM10, TM11, TM16, and TM17 of MRP1 (*22*). Whether the two labeled sites on MRP1 reflect two separate drug binding sites or whether they are brought together by protein folding to form a large drug binding pocket is not known. More recently, the labeling sites of LTC_4, a well-characterized substrate of MRP1, have been identified in the N- and C-proximal halves of MRP1 (*23*). Although the L_0 region was not the labeling site of LTC_4, it was required for the binding of LTC_4 to the N-terminal half of MRP1.

In this study, we further delineated the GSH-dependent AG-A binding site on MRP1 using coexpressed half-molecules of MRP1 in an insect cell expression system. To create these MRP1 fragments ($MRP1_{1-1222}$ and $MRP1_{1223-1531}$), MRP1 was separated at the extracellular loop between TM16 and TM17 in TMD_2. We have shown that coexpression of these molecules reconstitutes a functional LTC_4 tansporter, and thus, this system is a useful model for this study and for the study of the binding of other drugs to MRP1.

Using this system, we have shown that GSH-dependent azido AG-A photolabeling of the $MRP1_{1-1222}$ fragment requires coexpression of the $MRP1_{1223-1531}$ fragment. However, the $MRP1_{1223-1531}$ fragment itself was not photolabeled. We also found that an $MRP1_{1-1295}$, which was lacking the NBD_2 domain and all of the C-terminal cytoplasmic tail, still bound azido AG-A in a GSH-dependent manner. This result is in accordance with the previously reported data of Qian et al. (*23*), who reported that $MRP1_{1-1295}$ as well as intact MRP1 could bind LTC_4. These data indicate that amino acids 1223−1295 of MRP1 are required for binding of AG-A to MRP1 in a GSH-dependent manner. However, cross-linking of the photolabel to MRP1 occurs at a more upstream site (amino acid residues 932−1222).

The photolabeling site of azido AG-A resides within amino acids 932−1222 of MRP1. A region (amino acids 1223−1295) near the photolabeling site was found to be essential for AG-A binding to MRP1, although we do not know whether this region of MRP1 is directly involved in AG-A binding or indirectly influences the azido AG-A binding. It is possible that a GSH-dependent binding site for AG-A may lie between amino acids 1223 and 1295 of MRP1 and the azido group of AG-A photolabels a different site. It thus appears that following drug binding to the transporter molecule, the azido group, which is attached to the drug via a linker region, may then be able to interact with and photolabel a different region of the transporter. These observations are consistent with the finding that the L_0 region was required for the labeling of the N-terminal half of MRP1 with LTC_4, although the L_0 region was not the site labeled by LTC_4.

Charged amino acids in transmembrane segments have been reported to be involved in the determination of the substrate specificity of rat and human MRP2 (*32*, *33*). MRP2 mutants K325M (TM6) and R586L (TM11) that were unable to tansport GSH conjugates retained the ability to transport $E_2 17\beta G$ (*32*). It has also been observed that R1210A (TM16) and R1257A (TM17) mutants of human MRP2 showed decreased transport activity with the GSH−methylfluorescein conjugate (*33*). Thus, charged amino acids appear to play an important role in mediating GSH conjugate transport by MRP2, although it is not known whether they affect binding and/or transport of the substrates. In this study, we have also shown that a positively charged arginine, lying between residues 1223 and 1295, is indispensable for MRP1−substrate interaction and drug transport. Mutation of this

arginine 1249 to alanine almost completely impaired the LTC_4 transport activity of MRP1 and abrogated the ability of MRP1 to confer VCR resistance. VCR was chosen as a substrate for these experiments since VCR is a well-characterized GSH-dependent substrate of MRP1. The inability of R1249A to confer VCR resistance was not due to alterations in MRP1 trafficking since the mutant MRP1 was still localized to the plasma membrane. Thus, this study confirms the importance of charged amino acids for the function of the drug transporters.

The dual expression system in insect cells has proven to be a powerful tool for the study of MRP1 function (*19*, *30*, *31*, *34*). Previous studies indicated that coexpression of $TMD_0L_0TMD_1NBD_1$ and TMD_2NBD_2 or TMD_0L_0 and $TMD_1NBD_1TMD_2NBD_2$ in insect cells reconstitutes a functional transporter (*30*, *31*). However, dual expression of MRP1 fragments separated at cytoplasmic linker sites or inside the transmembrane segment of TMD_2 abrogates the ability of MRP1 to transport LTC_4 (*23*). This study is the first to demonstrate that coexpession of MRP1 fragments separated within the TMD_2 domain can reconstitute a functional transporter and also indicates that a covalent connection between TM16 and TM17 of MRP1 is not needed for the LTC_4 transport function of MRP1.

Our data suggest that the $MRP1_{1223-1531}$ fragment is targeted to and integrated correctly in the plasma membrane. A signal sequence in membrane proteins is responsible for targeting of the protein to the endoplasmic reticulum (ER). Also, topogenic sequences (including the signal sequence) will determine how the protein is folded in the membrane (*35*, *36*). MRP1, a polytopic (spanning the membrane twice or more) protein, is believed to have 17 different transmembrane segments based on various studies of topology determination (*37*−*39*). The $MRP1_{1223-1531}$ fragment is a bitopic membrane protein that contains only one transmembrane segment. The signal sequence required for membrane targeting and orientation may be intrinsic to the 17th transmembrane segment (*40*).

Our previous study suggested that the N-terminal half (residues 1−932) of MRP1 is required for [^{125}I]azido AG-A photolabeling of the C-terminal half of MRP1 (*19*). Other GSH-dependent drug binding sites of AG-A may exist in the N-terminal region of MRP1. Other studies have also shown cooperation between different regions of MRP1 in its function. A nonconserved amino acid (Glu^{1089}) in TM14 was functionally coupled with Thr^{1242} in TM17 to confer resistance to VCR and VP16 (*41*). It is possible that some residue(s) in TM14−16 is also important in mediating AG-A binding. Nevertheless, it is obvious that the entire $MRP1_{1-1222}$ fragment is unable to form a functional binding site for AG-A since no GSH-dependent photolabeling of azido AG-A was found in membrane vesicles expressing $MRP1_{1-1222}$ alone.

Zhang et al. recently suggested that residues with side chain hydrogen bonding potential, clustered in the cytoplasmic half of TM17, participate in the formation of a substrate binding site. However, no residue has been identified in TM17 that is critical for LTC_4 transport. Furthermore, TM17 contains no positively charged residues that could participate in the binding of anionic substrates. Therefore, they suggested that the high-affinity interaction site for LTC_4 probably lies within other TM helices (*42*). We found that Arg^{1249} which is proximal to the C-terminal of TM helix 17 is indispensable for LTC_4 transport. This suggests that the cytoplasmic domain within amino acids 1249−1295 may have an important role in substrate binding.

In conclusion, this study demonstrates that amino acids 1223−1295, a region which includes TM helix 17, are indispensable for binding of azido AG-A to MRP1 in a GSH-dependent manner. Furthermore, the site which is photolabeled following GSH-dependent binding of azido AG-A lies within $MRP1_{932-1222}$. The charged amino acid Arg^{1249} which is proximal to the C-terminal of TM helix 17 is of critical importance for the GSH-dependent binding of AG-A to MRP1 and the transport of LTC_4 and VCR. Now we know that the region of MRP1 between amino acids 1223 and 1295 is essential for azido AG-A binding and R^{1249} is important for GSH-dependent MRP1−substrate interaction and transport of LTC_4. Further study is needed to determine why $MRP1_{1223-1295}$ is indispensable for GSH-dependent azido AG-A binding to MRP1. This knowledge is crucially important for elucidation of the mechanism of GSH-dependent drug transport by MRP1 and for the design of drug strategies for antitumor therapy.

ACKNOWLEDGMENT

We thank Dr. Yukio Ikehara (Fukuoka University, Fukuoka, Japan) for useful discussion and Dr. Kazutake Tsujikawa (Osaka University) for useful advice about the antibody. We thank Dr. Borst for the MRP1 cDNA. We also thank Ms. Hiromi Kakura for her excellent secretarial assistance, Ms. Etsuko Sudo and Ms. Hui Gao for their excellent technical assistance, and Mr. Satoshi Nonaka for technical assistance with experiments using confocal microscopy.

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BI026443S