British Journal of Pharmacology (2003) 138, 1553–1561 © 2003 Nature Publishing Group All rights reserved 0007–1188/03 $25.00
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Localization of the GSH-dependent photolabelling site of an agosterol A analog on human MRP1

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1 Human multidrug resistance protein 1 (MRP1) is a 190 kDa membrane glycoprotein that confers multidrug resistance (MDR) to tumor cells. We recently demonstrated that glutathione (GSH) is required for the labelling of the C-terminal half of MRP1 with a photoanalog of agosterol A (azido AG-A). In this study, we further characterized the GSH-dependent photolabelling site of azido AG-A on MRP1.

2 An epitope-inserted MRP1, MRP1 1222HA, which has two hemagglutinin A (HA) epitopes in the extracellular loop between transmembrane segment (TM) 16 and TM17 of the transporter, could bind azido AG-A in a GSH-dependent manner.

3 Protease digestion of the photolabelled MRP1 1222HA, followed by immunoprecipitation with an anti-HA antibody suggested that the GSH-dependent azido AG-A photolabelling site on MRP1 resides in the region within TM14-17 and the cytoplasmic region proximate to the C-terminus of TM17.

4 Arg^{1210} in human MRP2 that corresponds to Arg^{1202} in human MRP1 has an important role in the transporting activity of MRP2. Therefore, we replaced the Arg residue at position 1202 of MRP1 with Gly. Whereas photolabelling of the mutant MRP1 R1202G was greatly reduced, it retained leukotriene C_4 (LTC_4) transport activity and conferred Vincristine resistance in LLC-PK1 cells.

5 In summary, this study demonstrated that the GSH-dependent azido AG-A photolabelling site on MRP1 resides in the region within TM14-17 and the cytoplasmic region proximate to the C-terminus of TM17. The charged amino acid Arg^{1202} proximate to TM helix 16 is of critical importance for the GSH-dependent photolabelling of MRP1 with azido AG-A. Arg^{1202} itself or the region nearby Arg^{1202} may be involved in azido AG-A photolabelling.

British Journal of Pharmacology (2003) **138,** 1553 – 1561. doi:10.1038/sj.bjp.0705197

Keywords: MDR; MRP1; AG-A; GSH-dependent; photolabelling site; charged amino acid

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

Introduction

Multidrug resistance (MDR) is a major obstacle to successful cancer chemotherapy (Gottesman & Pastan, 1993). Human multidrug resistance protein 1 (MRP1) is frequently overexpressed in non-P-glycoprotein (P-gp)-mediated MDR cells (Gottesman *et al*., 1996; Deeley & Cole, 1997; Hipfner *et al*., 1999). MRP1 is a member of the family of ATP-binding cassette (ABC) transporters (Higgins, 1992). Overexpression of MRP1 in cultured cells resulted in reduced drug accumulation and an increase in the rate of ATP-dependent drug efflux (Cole *et al*., 1994; Zaman *et al*., 1994). As an organic anion transporter, MRP1 actively transports a wide variety of diverse anionic compounds. Leukotriene C_4 (LTC_4) is an endogenous substrate of MRP1 with the highest known affinity for MRP1 (K_m about 100 nM) (Loe *et al*., 1996). By using the *in vitro* inside-out membrane vesicle system, it was also found that glutathione (GSH) at physiological concentrations stimulated the ATP-dependent transports of certain drugs such as vincristine (VCR) (Loe *et al*., 1996; 1998; Renes *et al*., 1999), adriamycin (ADM) (Ding *et al*., 1999; Renes *et al*., 1999) and aflatoxin B_1 (Loe *et al*., 1997) as well as certain endogenous hydrophilic anionic conjugates such as estrone 3-sulfate (Qian *et al*., 2001a) and a tobacco-specific carcinogen, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) (Leslie *et al*., 2001).

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In order to clarify the role of GSH in MRP1 drug transport, we synthesized a photoaffinity analog of agosterol A (AG-A), namely [^{125}I]11-azidophenyl agosterol A ([^{125}I]azido AG-A), which could reverse MRP1-mediated MDR (Chen *et al*., 2001). We found that GSH was required for the binding of azido AG-A to MRP1 (Ren *et al*., 2001), and that GSH-dependent photolabelling of azido 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 (Ren *et al*., 2001).

Using a method involving photolabelling of intact MRP1 followed by trypsinization, we determined that the photolabelling site of azido AG-A lies within the C-terminal half (TMD_2NBD_2) of MRP1. However, the exact photolabelling site(s) of azido AG-A on MRP1 is still unknown.

Previous photolabelling 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) (Daoud *et al*., 2000a, b; 2001). These photoactive agents photolabelled MRP1 in the absence of GSH. The labelling sites of the drugs were restricted to TM segments 10 – 11 and 16 – 17 of MRP1 (Daoud *et al*., 2001). It has also been demonstrated that LTC_4 labels two sites in MRP1, one in the NH_2 (N)- and one in the COOH (C)-terminal half of MRP1 (Qian *et al*., 2001b).

In order to compare the GSH-dependent photolabelling site of azido AG-A on MRP1 with those of other GSH-independent photoactive probes and LTC_4, we further characterized the GSH-dependent photolabelling site of azido AG-A on the C-terminal half of MRP1.

Methods

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, U.S.A.). 1-Chloro-2, 4-dinitrobenzene (CDNB) and a monoclonal antibody (mAb) (12CA5) against the hemagglutinin A (HA) epitope (YPYDVPDYAS) were obtained from Wako Pure Chemical Industries Ltd (Osaka, Japan). 1-Chloro-2, 4-dinitrophenol-*S*-glutathione (DNP-SG) was synthesized as previously described (Ren *et al*., 2000). The synthesis and use of [^{125}I] azido AG-A was described in a previous paper (Ren *et al*., 2001). Mouse IgG and Protein G Sepharose 4B were obtained from ZYMED (San Francisco, CA, U.S.A.). CELLFECTIN, competent DH10Bac *Escherichia coli* cells and Lipofectamine were purchased from Invitrogen corp. (Carlsbad, CA, U.S.A.). 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, U.S.A.).

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. At 48 h following transfection, the cells were subcultured at either a 1 : 20 or a 1 : 500 dilution, and selected in G418 (1 mg ml^{-1}). 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 (Invitrogen corp., Carlsbad, CA, U.S.A.). Membrane vesicles and crude membranes were prepared as previously described (Ren *et al*., 2000). Protein concentrations were determined by the method of Bradford (1976).

MTT assay was performed as previously described (Chen *et al*., 2001).

Generation of constructs and viral infection

pFastBac *MRP1-His* containing the His-tagged MRP1 coding region was constructed as previously described (Ren *et al*., 2000). The strategies employed for site-directed mutagenesis of the MRP1 cDNA were previously described (Ren *et al*., 2001). Insertion of two HA epitopes following amino acid 1222 in MRP1 was carried out as follows. The primers: 5′-**CCAGATTACGCTAGC**CACAGCCTCAGTGCTGGCTTG-3′ (forward) and 5′-**GACGTCATATGGATA**CCTGGAGATGA CCGCAAACAG-3′ (reverse) were used in a PCR reaction in order to insert the first HA epitope (YPYDVPDYAS, bold). These primers also created an *Nhe*I site (underlined) to facilitate the insertion of the second HA epitope. The second HA epitope was generated by the creation of an adaptor molecule encoding the second HA epitope from the following two oligonucleotide DNAs: 5′-CTAGTTACCCTTACGACG TGCCTGATTACG-3′ and 5′-CTAGCGTAATCAGGCACG TCGATAAGGGTAA-3′. This adaptor was then inserted into the prepared *Nhe*I site.

MRP1 constructs encoding R1202G was generated in PCR reaction using the forward primers: 5′-GGGCTCGAGTGT GTGGGCAACTGC-3′ (bold denotes a mismatched base encoding the mutation).

Baculoviruses encoding the wild-type and mutant MRP1s described above were generated using the Bac to Bac expression system (Invitrogen Corp., Carlsbad, CA, U.S.A.) as described previously (Ren *et al*., 2001). 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 labelling of MRP1 with [^{125}I]azido AG-A

[^{125}I]azido AG-A (7.2 μCi nmol^{-1}) was used for the experiment. Photolabelling studies were carried out as previously described (Ren *et al*., 2001). Autoradiograms were exposed in the duration ranging from 10 h to 3 days.

[^{3}H]LTC_4 uptake by membrane vesicles

[^{3}H]LTC_4 uptake was measured using a rapid filtration technique as previously described (Ren *et al*., 2000). Briefly,

isolated membrane vesicles (25 μg) were incubated with or without 4 mM ATP and various concentrations of inhibitors as indicated in 50 μl 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^{-1} creatine phosphokinase) or a range of [^{3}H]LTC_4 concentrations (25 – 800 nM) 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, 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 were measured.

Immunoblotting and immunoprecipitation of MRP1

Immunoblotting was performed as previously described (Ren *et al*., 2000). Anti-MRP1 monoclonal antibodies MRPr1 (epitope amino acids 229 – 281) and MRPm6 (epitope amino acids 1389 – 1351) (Flens *et al*., 1994; Hipfner *et al*., 1994), anti-HA mAb (12CA5) was used for Western blotting. Trypsinization of photolabelled membrane vesicles was carried out as previously described (Ren *et al*., 2001). Membrane vesicles (300 μg) prepared from Sf21 insect cells infected with a virus encoding MRP1 1222HA were photolabelled with [^{125}I]azido AG-A in the presence of 5 mM GSH. Membrane vesicles were then treated with trypsin or V8 protease at the indicated protease/protein ratios for 1 h at 37°C. Both protease treated and untreated membranes were solubilized in 2 ml Buffer A (50 mM Tris-HCl, pH 8.0, 150 mM NH_4Cl, 2 mM $MgCl_2$) containing 1% CHAPS and incubated for 2 h at 4°C. The solubilized membranes were centrifuged at 12,000 × *g*, and the supernatant was incubated at 4°C overnight with 8 μg anti-HA mAb or 8 μg of control mouse IgG. 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 detect the expression of MRP1 in LLC-PK1 cells, the cells were stained with MRPm6 (1 : 1000) and fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (1 : 200). Immunofluorescent staining was carried out as previously described with the following modifications (Ren *et al*., 2000). 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 MRPm6 (1 : 1000) in blocking buffer. Following three gentle washes with PBS, the cells were incubated for 1 h with FITC-conjugated anti-mouse IgG (1 : 200) 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 fluorescence preserver (Immunon™, Shandon), and the samples were examined by a confocal microscopy (Leica True Confocal Scanner 4D Germany).

Results

Localization of the GSH-dependent photolabelling site of [^{125}I]azido AG-A on MRP1 by epitope insertion

We have previously found that a photoanalog of AG-A (azido AG-A) labelled the C-terminal half of MRP1 (TMD_2NBD_2) in a GSH-dependent manner. However, the exact photolabelling site(s) of azido AG-A on MRP1 is still unknown. In the present study, we have used the epitope-inserted MRP1 to determine more exactly the photolabelling site on the C-terminal half of MRP1.

Arg^{1222} is in the central region of C-terminal half of MRP1. It was reported that insertion of one or two copies of the HA epitope following the Arg^{1222} of MRP1 did not alter the capacity of MRP1 to confer drug resistance in mammalian cells. The inserted epitopes were found in an extracellular loop between TM16 and TM17 of MRP1 (Kast & Gros, 1998). In order to determine the site on MRP1 that is photolabelled with the [^{125}I]azido AG-A, we carried out protease digestion of photolabelled MRP1 1222HA, MRP1 with two copies of the HA epitope following the Arg^{1222} of MRP1, in membrane vesicles from Sf21 insect cells. The expression of MRP1 1222HA in insect cells was detected by Western blotting with both anti-HA and MRPr1 mAb. Both antibodies detected a protein of about 170 kDa (Figure 1a). To confirm that the epitope insertion did not alter the transport and photolabelling ability of MRP1, we first examined the ATP-dependent transport of LTC_4 in MRP1 1222HA membrane vesicles and compared with that in wild-type MRP1 membrane vesicles. In accordance with the previous drug-sensitivity study, MRP1 1222HA membrane vesicles showed active LTC_4 uptake. The ATP-dependent uptake in membrane vesicles expressing MRP1 1222HA was about 70% of that expressing wild-type MRP1 (Figure 1b). Secondly, as shown in Figure 2a, MRP1 1222HA bound azido AG-A in a GSH-dependent manner. Thus, insertion of the HA epitope did not affect the transporting activity of MRP1 and GSH-dependent AG-A binding to MRP1. To investigate whether [^{125}I]azido AG-A also photolabelled the C-terminal half of MRP1 1222HA, MRP1 1222HA membrane vesicles were photolabelled with [^{125}I]azido AG-A in the presence of 5 mM GSH and digested with trypsin at the trypsin/protein ratios of 1 : 500 and 1 : 100. The main photolabelled 67 kDa fragment was immunoprecipitated with MRP1 C-terminal-specific antibody MRP m6 (Figure 2b). This result suggested that GSH-dependent photolabelling site of azido AG-A on MRP1 1222HA was in the C-terminal half. We thus used the epitope-inserted MRP1 to further determine the photolabelling site on MRP1.

Figure 3a shows the predicted topology of C-terminal half (TMD_2NBD_2) of MRP1, which has been verified by previous studies (Hipfner *et al*., 1997; Kast & Gros, 1998). MRP1 1222HA membrane vesicles were photolabelled with 5 μM [^{125}I]azido AG-A in the presence of 5 mM GSH and digested with trypsin or V8 protease at a protease/protein ratio of 1 : 25 (w w^{-1}) prior to immunoprecipitation with the anti-HA mAb. The anti-HA mAb specifically immunoprecipitated full-length MRP1 (Figure 3b and c, left lanes). No precipitation was observed with mouse IgG alone (data not shown). Trypsin (1 : 25, w w^{-1}) digestion of azido AG-A photolabelled MRP1 1222HA followed by immunoprecipitation with the anti-HA mAb revealed four photolabelled polypeptides with approx-

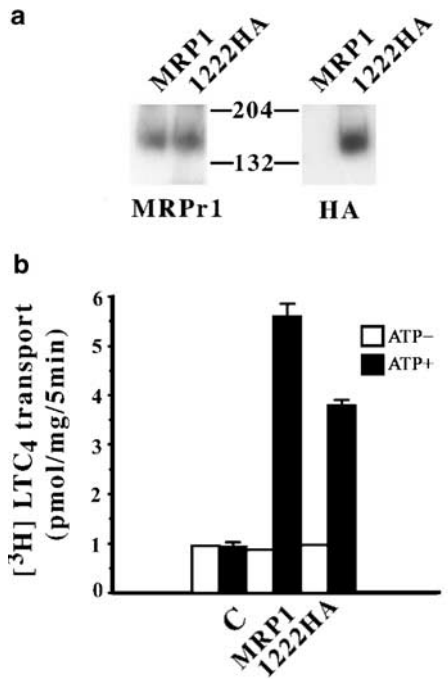

Figure 1 Transport activity of MRP1 1222HA expressed in Sf21 insect cells. (a) Expression of MRP1 1222HA in insect cells. Insect cells were infected with recombinant baculovirus encoding either MRP1 or MRP1 1222HA, a mutant MRP1 with two HA epitopes inserted after Arg^{1222} of MRP1. Membrane vesicles (10 μg) were subjected to 7.5% SDS – PAGE and immunoblotted with the MRPr1 (left), or the anti-HA mAb (right), mAbs. (b) Transport of [^{3}H]LTC_4 by MRP1 1222HA. Membrane vesicles (25 μg of protein) prepared from Sf21 insect cells expressing MRP1 or MRP1 1222HA or from noninfected cells (C), were incubated with 50 nM [^{3}H]LTC_4 in the presence (black columns) or absence (white columns) of 4 mM ATP at 37°C for 5 min in 50 μl of transport buffer (0.25 M sucrose, 10 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, 10 mM phosphocreatine, 100 μg ml^{-1} creatine phosphokinase). The data represent the means ± s.e. of three separate experiments.

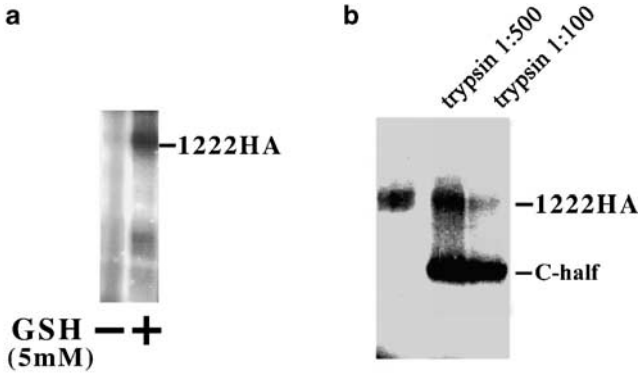

Figure 2 GSH-dependent photolabelling of [^{125}I]azido AG-A to the C-terminal half of MRP1 1222HA. (a) Membrane vesicles expressing MRP1 1222HA were photolabelled with 5 μM [^{125}I]azido AG-A in the absence or presence of 5 mM GSH, and subjected to 7.5% SDS – PAGE. Autoradiograms were developed after 15 h of exposure at room temperature. (b) Photolabelling of MRP1 1222HA was carried out with 5 μM [^{125}I]azido AG-A in the presence of 5 mM GSH followed by treatment with trypsin for 30 min at 37°C at the trypsin/protein ratio of 1 : 100 (right lane), 1 : 500 (middle lane) or untreated (left lane). Solubilized vesicles were immunoprecipitated with C-terminal MRP1 specific mAb m6. The immunoprecipitates were then subjected to 7.5% SDS – PAGE. Autoradiograms were developed after 3 days of exposure at −70°C.

imate molecular masses of 67, 34, 23 and 16 kDa (Figure 3b, right lane). The 67 kDa photolabelled fragment had the same migration rate with $MRP1_{932-1531}$, the C-terminal half of MRP1 in SDS – PAGE (data not shown). Similar results were obtained following digestion with V8 protease (Figure 3c, right lane). Extensive digestion of azido AG-A photolabelled membrane vesicles with higher concentration of trypsin (trypsin/protein 1 : 5), followed by anti-HA mAb immunoprecipitation, did not generate further smaller peptides (Figure 3d). Since the HA epitope was inserted after the amino acid 1222 of MRP1, and the smallest immunoprecipitated photolabelled polypeptide was 16 kDa, a region of MRP1 spanning about 160 amino-acid residues of MRP1 should contain the azido AG-A-labelled site(s) as well as the HA (1222) epitope. Two photoanalogs, IACI and IAArh123, photolabelled MRP1 in the absence of GSH (Daoud *et al*., 2000a, b; 2001). They have been used to photolabel MRP1 1222HA expressed in HeLa cells. Trypsinization of this photolabelled, epitope-inserted MRP1 followed by immunoprecipitation with an anti-HA mAb generated a small photolabelled peptide with a molecular weight of 7 kDa (Daoud *et al*., 2001). We thus investigated whether 7 kDa peptide-containing HA epitope existed in our system by Western blotting using anti-HA antibody. Treatment of MRP1 1222HA membrane vesicles with increasing concentrations of trypsin yielded increasing levels of an about 7 kDa peptide (Figure 3e).

Arg^{1202} is important for GSH-dependent photolabelling of azido AG-A

It has been demonstrated that charged amino acids in the transmembrane domains (TMDs) of rat and human MRP2 play an important role in the recognition of their substrates (Ryu *et al*., 2000; Ito *et al*., 2001). Arg^{1202} is proximate to the N-terminus of TM16 of MRP1 and in the region where azido AG-A-labelling site resides. The position of Arg^{1202} of MRP1 is indicated in Figure 3a. To investigate the role of the charged amino acid in the azido AG-A photolabelling of MRP1, we replaced the Arg^{1202} with Gly (R1202G). The mutant MRP1 protein was first expressed in Sf21 insect cells to study the GSH-dependent photolabelling with [^{125}I]azido AG-A. Figure 4a shows that the expression level of R1202G mutant was comparable to that of wild-type MRP1. The membrane vesicles expressing the wild-type and R1202G MRP1s were analyzed for their abilities to interact with azido AG-A in a GSH-dependent manner. As shown in Figure 4b, wild-type MRP1 was photolabelled with azido AG-A in a GSH-dependent manner; however, the GSH-dependent photolabelling of MRP1 R1202G with [^{125}I]azido AG-A was greatly reduced (Figure 4b). To test whether the mutation affects the function of MRP1, the LTC_4 transport activity of MRP1 R1202G was examined. As shown in Figure 5a, MRP1 R1202G efficiently transported LTC_4. The apparent K_m values of MRP1 R1202G and wild-type MRP1 for LTC_4 were 147 and 117 nM, respectively (Figure 5b).

In order to test the ability of R1202G to confer drug resistance, the wild-type *MRP1* and the *MRP1 R1202G* mutant cDNAs were cloned into the mammalian expression vector pCIneo and transfected into LLC-PK1 cells. The transfected cells were selected in G418 as described in Methods. The mass population of G418 resistant clones was

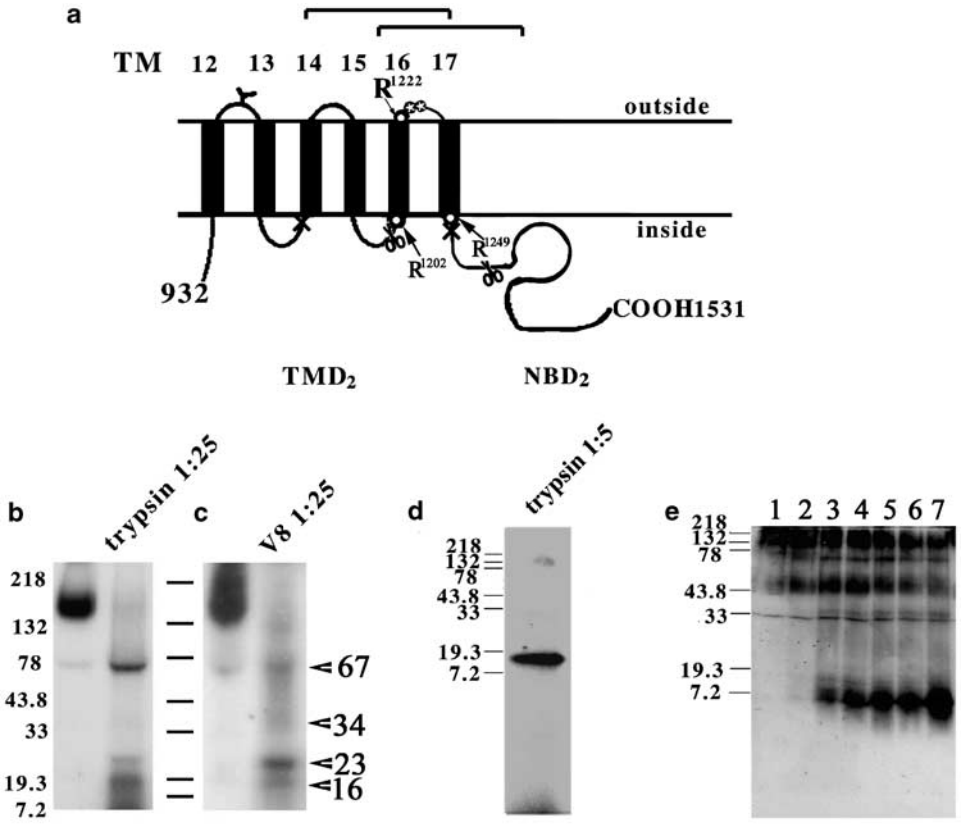

Figure 3 Protease digestion of photolabelled MRP1 1222HA and immuoprecipitated with anti-HA mAb. (a) Predicted topology of the C-terminal half (TMD_2BND_2) of MRP1 indicating two supposed protease digestion sites (cross and scissors pairs). The six transmembrane segments in TMD_2, the positions of the charged amino acids R^{1202}, R^{1222} and R^{1249} (circles) and two inserted HA epitopes (stars) are also shown. (b) Photolabelling of MRP1 1222HA (300 μg) was carried out with 5 μM [^{125}I]azido AG-A in the presence of 5 mM GSH. Photolabelled membrane vesicles were then either untreated (left lane) or treated with trypsin (right lane) at the trypsin/protein ratio of 1 : 25 for 1 h at 37°C. Solubilized vesicles were then immunoprecipitated with anti-HA mAb. The immunoprecipitates were then subjected to 5 – 20% gradient SDS – PAGE. Autoradiograms were developed after 3 days of exposure at −70°C. (c) The photolabelled membrane vesicles were treated with V8 protease instead of trypsin as described in (b). (d) Photolabelled membrane vesicles were treated with trypsin at the trypsin/protein ratio of 1 : 5 for 1 h at 37°C. Solubilized vesicles were immunoprecipitated with anti-HA mAb. The immunoprecipitates were then subjected to 15% SDS – PAGE. Autoradiograms were developed after 3 days of exposure at −70°C. (e) MRP1 1222HA membrane vesicles (50 μg) were digested for 30 min at 37°C with increasing concentrations of trypsin, 12.5, 25, 50, 100, 200 and 400 μg ml^{-1} (lanes 2, 3, 4, 5, 6 and 7, respectively), or without trypsin treatment (lane 1). The trypsin digestions were stopped by adding 2 mM phenylmethylsulfonyl fluoride, and the samples were immediately subjected to 15% gradient SDS – PAGE and immunoblotted with anti-HA mAb.

further incubated in 40 nM VCR, a well-characterized substrate of MRP1, to select VCR-resistant cells. Transfection of wild-type MRP1, or the MRP1 R1202G mutant, but not the pCIneo empty vector, resulted in drug-resistant colonies within 2 weeks. The drug resistance of stably transfected clones that express MRP1 or MRP1 R1202G (Figure 6a) was further tested in a cytotoxicity assay. As shown in Figure 6b, MRP1 and MRP1 R1202G conferred VCR resistance on LLC-PK1 cells. MRP1 R1202G mutant protein was localized to the plasma membrane in a similar manner to the wild-type MRP1, indicating that the R1202G mutation did not affect the trafficking of the mutant protein (Figure 6c).

The fact that the photolabelling of MRP1 with azido AG-A was impaired by the R1202G mutation, but the transport activity and the ability to confer resistance to VCR were retained in this mutant suggests that the R1202G mutation affected only the photolabelling, but not the binding of azido AG-A to MRP1. To investigate whether the MRP1 R1202G mutant can interact with AG-A, the effect of AG-A on [^{3}H]LTC_4 uptake by wild-type MRP1 and the MRP1 R1202G mutant expressed in Sf21 insect cells was examined. In the presence of 2 mM GSH, AG-A inhibited uptake of [^{3}H]LTC_4 in membrane vesicles that express MRP1 R1202G in a dose-dependent manner and the extent of the inhibition was similar to that in membrane vesicles that express wild-type MRP1 (Figure 7). These findings suggest that MRP1 R1202G as well as wild-type MRP1 can interact with AG-A, and Arg^{1202} is not critical to the binding of AG-A to MRP1, although it affects the photolabelling of azido AG-A.

Discussion

MRP1 is frequently overexpressed in non-Pgp-mediated MDR cells (Gottesman *et al*., 1996; Deeley & Cole, 1997; Hipfner *et al*., 1999). Elucidation of the mechanism by which MRP1 mediates MDR would clearly be useful for the design of drug

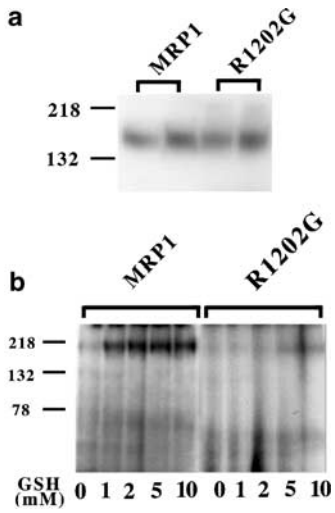

Figure 4 Effect of R1202G mutation on the GSH-dependent photolabelling of MRP1 with [^{125}I]azido AG-A. (a) Wild-type MRP1 and MRP1 R1202G were expressed in insect cells. Membrane proteins (6 and 12 μg of each sample) were separated on a 7.5% SDS – PAGE, and immunoblotted with the MRPr1 mAb. (b) Wild-type MRP1 and MRP1 R1202G membrane vesicles (100 μg of membrane proteins) were photolabelled with [^{125}I]azido AG-A in the absence or presence of the indicated concentrations of GSH as described in the legend to Figure 2a. Photolabelled membrane vesicles were subjected to 7.5% SDS – PAGE and autoradiograms were developed after 10 – 18 h of exposure at room temperature.

strategies for antitumor therapy. There is compelling evidence that GSH is necessary for the transport of certain unconjugated drugs by MRP1 (Loe *et al*., 1996; 1997; 1998; Ding *et al*., 1999; Renes *et al*., 1999; Leslie *et al*., 2001; Qian *et al*., 2001a). Our previous study demonstrated that GSH is required for the labelling of the C-terminal half (TMD_2NBD_2) of MRP1 with a photoanalog of AG-A, [^{125}I]azido AG-A (Ren *et al*., 2001). In the present study, we further investigated the photolabelling site of [^{125}I]azido AG-A within the C-terminal half of MRP1 using an epitope-inserted mutant MRP1.

Two photoactive agents, IAARh123 and IACI, can photolabel MRP1 in the absence of GSH (Daoud *et al*., 2000a, b; 2001). The detailed mechanism by which they bind to MRP1 is not known. Both agents labelled fragments that encompass the TM10 – 11 and TM16 – 17 of MRP1 (Daoud *et al*., 2001). More recently, the labelling sites of LTC_4, a well-characterized substrate of MRP1, have been identified in the N- and C-proximal halves of MRP1 (Qian *et al*., 2001b). It has been shown that the L_0 region was really required for the binding of LTC_4 to the N-terminal half of MRP1, although it was not the labelling site of LTC_4.

We previously ascertained that the labelling site of azido AG-A lies within the C-terminal half (between amino acids 932 and 1531) of MRP1 (Ren *et al*., 2001). Daoud *et al*. devised a useful technique to identify the drug binding site of photoactive agents to MRP1. They used two photoanalogs, IACI and IAArh123, to photolabel a functionally active mutant MRP1 with two inserted HA epitopes following Arg^{1222}. Trypsinization of this photolabelled, epitope-inserted MRP1 followed by immunoprecipitation with an anti-HA mAb generated a small photolabelled peptide with a molecular weight of 7 kDa. Based on the location of the inserted HA epitope and the molecular mass of the peptide, the authors suggested that TM16 and TM17 are involved in the drug binding (Daoud *et al*., 2001). Arg^{1222} is in the central region of C-terminal half of MRP1. We thus used this MRP1 mutant and the method to ascertain the GSH-dependent labelling site of azido AG-A on MRP1 and found that the photoanalog of AG-A labelled the mutant MRP1 1222HA in a GSH-dependent manner. The photolabelling site of azido AG-A on MRP1 1222HA was found within the C-terminal half just as that on wild-type MRP1. We further analyzed the GSH-dependent photolabelling site using this MRP1 mutant. When the membrane vesicles expressing the mutant protein were photolabelled and subsequently digested with trypsin or V8

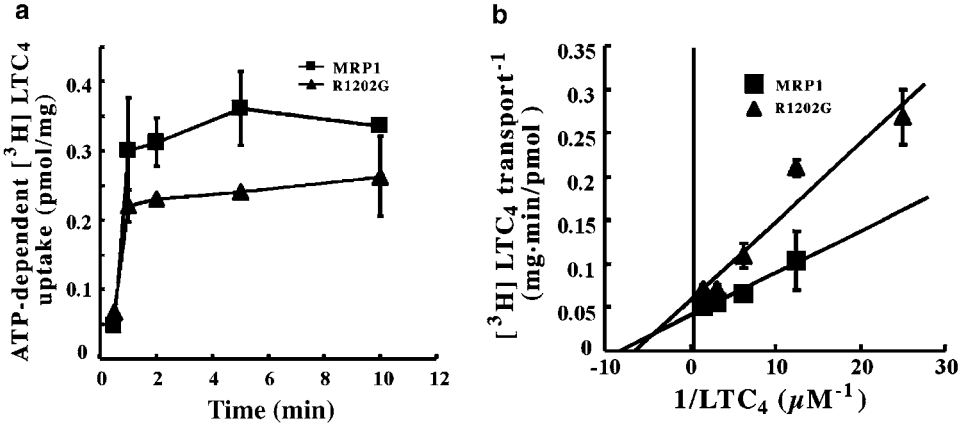

Figure 5 Effect of R1202G mutation on ATP-dependent [^{3}H]LTC_4 transport. (a) Time course of ATP-dependent transport of [^{3}H]LTC_4 by wild-type MRP1 (square) and MRP1 R1202G (triangle) mutant. Membrane vesicles (50 μg) expressing wild-type MRP1 or MRP1 R1202G were incubated with 1.37 nM [^{3}H]LTC_4 at 37°C in 50 μl transport buffer as described in the legend to Figure 1b in the presence or absence of 4 mM ATP at the indicated periods. ATP-dependent uptakes shown in the figure were calculated by subtracting the radioactivity obtained in the absence of ATP. The results represent triplicate experiments (bar: $\pm$s.d.). (b) Determination of the K_m values of MRP1 wild type (square) and MRP1 R1202G (triangle) for [^{3}H]LTC_4. Membrane vesicles were incubated with a range of [^{3}H]LTC_4 concentrations (25 – 800 nM) for 1 min. Kinetic parameters were determined from double reciprocal plots of the ATP-dependent LTC_4 uptake. The results represent triplicate experiments (bar: $\pm$s.d.).

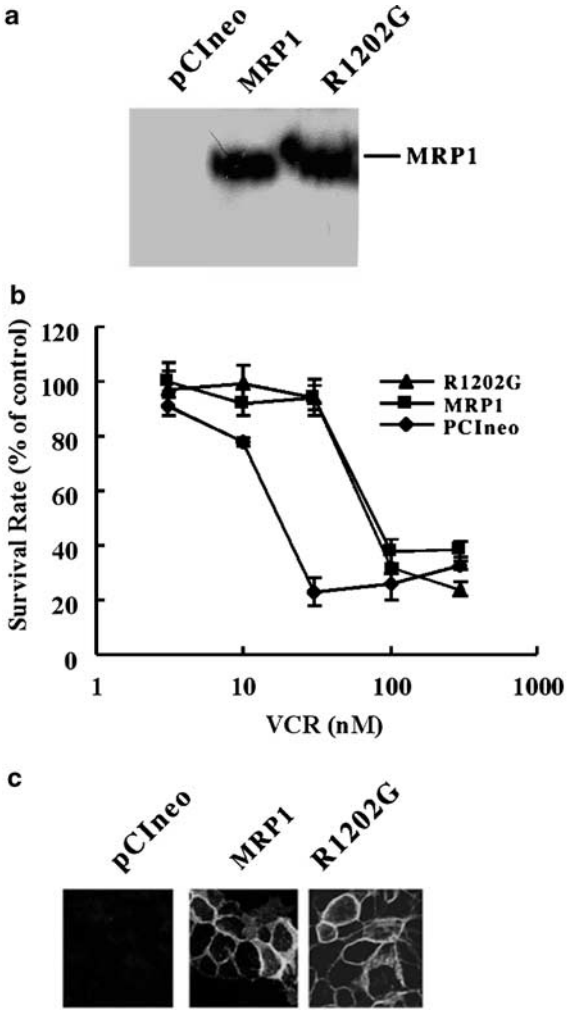

Figure 6 Effect of R1202G mutation on drug resistance in LLC-PK1 cells. (a) Expression of wild-type MRP1 and the MRP1 R1202G mutant in LLC-PK1 cells. Crude membranes (50 μg of protein) prepared from LLC-PK1 cells transfected with either expression vectors encoding wild-type MRP1, MRP1 R1202G, or a control empty vector (pCIneo) were analyzed on 7.5% SDS–PAGE. The expression levels of the MRP1 proteins 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 either wild-type MRP1 (square), MRP1 R1202G (triangle), or transfected with an empty vector (rhombus) were exposed to the indicated concentrations of VCR and the survival rate was determined by MTT assay as described under Materials and methods. The data are presented as mean survival rates of three separate wells in one experiment (bar: $\pm$s.d.). (c) Cellular localization of expressed MRP1 proteins. Indirect immunofluorescent staining of wild-type MRP1 and MRP1 R1202G expressed in LLC-PK1 cells was carried out with the MRPm6 mAb and a FITC-conjugated secondary antibody. The panels show horizontal sections of cell layers obtained by confocal microscopy. Both wild-type MRP1 and MRP1 R1202G were localized in the plasma membrane of LLC-PK1 cells. No fluorescence was detected in LLC-PK1 cells transfected with an empty vector (pCIneo).

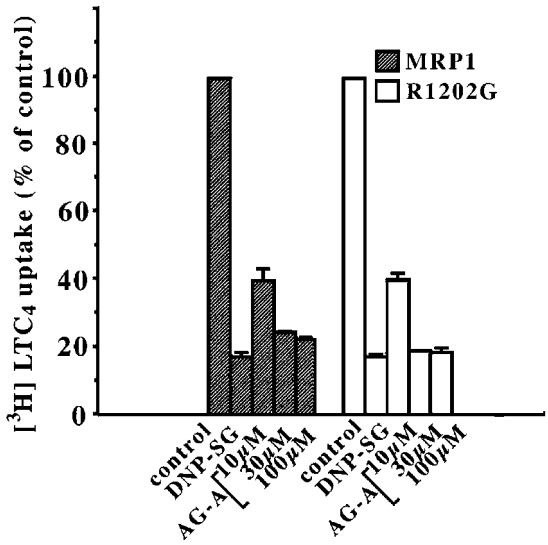

Figure 7 Effect of AG-A on [^{3}H]LTC$_4$ transport by wild-type MRP1 and MRP1 R1202G. ATP-dependent [^{3}H]LTC$_4$ uptake into MRP1 and MRP1 R1202G membrane vesicles from Sf21 insect cells was measured in the presence of 2 mM GSH and 10, 30 or 100 μM of AG-A as indicated. Membrane vesicles (50 μg) were incubated with 1.37 nM [^{3}H]LTC$_4$ at 37°C in 50 μl transport buffer with 4 mM ATP for 3 min as described in the legend to Figure 2. The 100% uptake represents values obtained in the absence of AG-A and DNP-SG (100 μM). The data represent means ($\pm$s.e.) of triplicate determinations in a single experiment.

protease, the smallest photolabelled peptide that could be immunoprecipitated by the anti-HA mAb had a molecular weight of 16 kDa. Extensive digestion of azido AG-A photolabelled membrane vesicles with higher concentration of trypsin followed by anti-HA mAb immunoprecipitation did not generate further smaller peptides. However, we showed the existence of an about 7 kDa peptide by Western blotting using anti-HA mAb.

The difference between IAArh123 and azido AG-A is the requirement of GSH in photolabelling. MRP1 was only slightly photolabelled with azido AG-A in the absence of GSH. The different patterns of tryptic peptides were probably because of the GSH-dependent binding of the photoanalog on MRP1 1222HA. It is possible that GSH or the GSH-dependent azido AG-A photolabelling may have induced a conformational change in MRP1, and hided certain protease digestion site(s). Otherwise, the GSH-dependent photolabelling site of azido AG-A on MRP1 1222HA may be different and more distant from the inserted HA epitope compared with the photolabelling site of IACI. Further studies are required to ascertain these possibilities.

Since the HA epitope was inserted after ammo acid 1222 of MRP1, and the smallest immunoprecipitated photolabelled polypeptide was 16 kDa, a region of MRP1 spanning about 160 amino-acid residues should contain both azido AG-A-labelled site(s) and the HA (1222) epitope. Since the arginine at 1222 of MRP1 in MRP1 1222HA membrane vesicles was reported to be inaccessible to trypsin digestion (Daoud *et al*., 2001), the extracellular loops between TM14 and TM15 or TM16 and TM17 contain no candidate sites for trypsin digestion. The protease digestion sites on MRP1 are probably within the intracellular loop between TM13 and TM14 or TM15 and TM16 or the cytoplasmic region following TM17. These data imply two possibilities. If one of the trypsin digestion sites is proximate to the C-terminus of TM17, the other digestion site is supposed to be in the intracellular loop between TM13 and TM14. On the other hand, if one of the trypsin digestion sites is located in the cytoplasmic portion

between TM17 and NBD_2 and far from TM17, the other digestion site could only be located in the intracellular loop between TM15 and TM16 (Figure 3a). In both cases, TM16 and TM 17 are contained in the region where GSH-dependent azido AG-A photolabelling site resides.

TM16 and TM17 seem to be important for both GSH-dependent and -independent MRP1 – substrates interactions (the present study, Daoud *et al*., 2001). In accordance with this, previous mutagenesis studies have shown that both conserved and nonconserved amino acids in TM17 are important in determining MRP1 substrate specificity (Zhang *et al*., 2002).

It was found that IACI and IAArh123 labelled an N-terminal fragment that encompasses the TM10 – 11 of MRP1 besides the C-terminal TM16 – 17 of MRP1 (Daoud *et al*., 2001). In contrast to these photoactive probes, azido AG-A only photolabelled 16 kDa fragment containing TM16 and TM17 of MRP1 in a GSH-dependent manner. Although no GSH-dependent azido AG-A photolabelling of N-terminal fragment of MRP1 was detected, we found that N-terminal half is required for the GSH-dependent photolabelling of the C-terminal half (Ren *et al*., 2001). Azido AG-A binding site(s) may also reside(s) in the N-terminal half of MRP1, although it was not photolabelled.

Charged amino acids in transmembrane segments have been reported to be involved in the determination of substrate specificity of rat and human MRP2 (Ryu *et al*., 2000; Ito *et al*., 2001). R1210A (TM16) mutant of human MRP2 showed reduced transport activity of GSH methylfluorescein (Ryu *et al*., 2000). Arg^{1210} in human MRP2 correspond to Arg^{1202} in human MRP1. Therefore, we replaced the arginine at 1202 that is proximate to the N-terminus of TM16 of MRP1 with Gly (R1202G) to investigate the role of the charged amino acid in the region where GSH-dependent azido AG-A photolabelling site resides. GSH-dependent photolabelling of the mutant MRP1 R1202G with [^{125}I]azido AG-A was considerably lower than that of wild-type MRP1. The impaired GSH-dependent photolabelling of MRP1 R1202G may be attributable to the impaired binding of MRP1 to AG-A, and thus the GSH-dependent photolabelling with azido AG-A was lowered. The other possibility is that the mutation just affected the process of the photolabelling but not the function of MRP1. We found that this mutant MRP1 could transport LTC_4 and this transport was efficiently inhibited by AG-A. Furthermore, the mutant MRP1 was able to confer VCR resistance on LLC-PK1 cells. These findings imply that Arg^{1202} has an important role in [^{125}I] azido AG-A photolabelling but not binding. This amino-acid residue may interact with the azido group on the side chain of azido AG-A. Alternatively, the azido AG-A photolabelling site may reside in the region nearby Arg^{1202} that is proximate to the N-terminus of TM16, and the replacement of Arg^{1202} with Gly may affect the photolabelling of this site. This finding also suggested that the role of Arg^{1202} in human MRP1 is different from that of Arg^{1210} in human MRP2. However, we need further study to know whether azido AG-A crosslinks to the Arg^{1202} residue.

In conclusion, these results demonstrated that the GSH-dependent azido AG-A photolabelling site on MRP1 resides in the region within TM14 – 17 and the cytoplasmic region proximate to the C-terminus of TM17. The charged amino acid Arg^{1202} proximate to TM helix 16 is of critical importance for the GSH-dependent photolabelling of AG-A of MRP1 with azido AG-A. Arg^{1202} itself or the region nearby Arg^{1202} may be involved in azido AG-A photolabelling.

This work was supported by a Grant-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology. We thank Dr Yukio Ikehara (Fukuoka University) for useful discussions and Dr Kazutake Tujikawa (Osaka University) for useful advice about antibody. We thank Dr Borst for the MRP1 cDNA, Ms Hiromi Kakura for her excellent secretarial assistance, Mses Etsuko Sudo and Hui Gao for their excellent technical assistance, and Mr Satoshi Nonaka for technical assistance for experiments using confocal microscopy. X.-Q. Ren appreciates the research fellowship and grant from the Japan Society for the Promotion of Science.

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(Received September 25, 2002
Revised January 6, 2003
Accepted January 20, 2003)