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Domain Exchangeability between the Multidrug Transporter (MDR1) and Phosphatidylcholine Flippase (MDR2)

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ABSTRACT

Multidrug resistance (MDR) mediated by P-glycoprotein (MDR1) is clinically significant. Understanding how MDR1 substrate specificity is determined will help to overcome MDR to improve cancer treatment. One potential approach to achieve this goal is to study chimeras of MDR1 and its homolog MDR2 (also called MDR3), which has been identified as a phosphatidylcholine flippase. With an approach involving exchanging homologous segments of MDR1 and MDR2 and site-directed mutagenesis, we previously demonstrated MDR1 residues Q330, V331, and L332 in transmembrane domain 6 (TM6) are essential for multidrug transport activity; substituting these residues allows the N-terminal transmembrane region of MDR2 to support MDR1 activity. To further determine the exchangeability between MDR1 and MDR2, we constructed additional MDR1/MDR2 chimeras. We found that the N-terminal half of MDR1 and MDR2 was mostly exchangeable except for a few residues in TM6. However, this degree of exchangeability was not found in the C-terminal half of MDR1 and MDR2. In addition, with substitution of MDR1 residues 318–332 (TM6) and 937–994 (TM11-12), MDR2 had relatively normal affinity for MDR1 substrates, but it did not have multidrug transporter activity. These results suggest that the inability of MDR2 to transport most MDR1 drugs efficiently may be due to failure of those drugs to stimulate ATPase and activate transport as well as to decreased drug binding.

P-glycoprotein (P-gp), encoded by the *MDR*1 gene, is a plasma membrane transporter. Its substrates include a large number of small hydrophobic compounds, some of which are therapeutic drugs used in cancer treatment. Expression of P-gp in cancer cells allows them to become resistant to many different cytotoxic drugs, thus causing failure of cancer chemotherapy (Gottesman et al., 1995). It is not clear why P-gp has such a broad substrate spectrum. Knowledge about the substrate binding domain(s) and residues essential for P-gp substrate specificity is important for developing specific P-gp inhibitors that may prove essential in the clinic.

Although a detailed knowledge about how the structure of P-gp affects function must await high-resolution three-dimensional protein structure analysis, much information can be obtained through mutational studies of MDR1 and its homologous transporter MDR2 (also known as MDR3). MDR2 has a 78% amino acid sequence identity to MDR1 (van der Bliek et al., 1988), but it primarily functions as a phosphatidylcholine flippase (Smit et al., 1993; Ruetz and Gros, 1994). Unlike MDR1, MDR2 does not have a broad substrate spectrum. Although MDR1 and MDR2 have a limited number of common substrates, such as short-chain phospholipids (van Helvoort et al., 1996; Bosch et al., 1997), an antifungal cyclic depsipeptide (Kino et al., 1996), and a common inhibitor, verapamil (Ruetz and Gros, 1994), MDR2 is unable to efflux most MDR1 substrates efficiently (Schinkel et al., 1991). Photoaffinity-labeling experiments have shown that the inability of the mouse mdr2 transporter to confer resistance to MDR1 drugs is associated with reduced binding of drug to the mdr2 protein (Buschman and Gros, 1994). The structural and functional differences between MDR1 and MDR2 provide an opportunity to identify the residues essential for the broad substrate spectrum of MDR1.

Studies of MDR1/MDR2 chimeras show that exchanging ATP binding domains between mdr1 and mdr2 results in little change in the function of mdr1; however, exchanging the homologous segments containing transmembrane regions abrogates the capacity of the mdr1 transporter to confer multidrug resistance (MDR) (Buschman and Gros, 1991; Currier et al., 1992; Zhang et al., 1995). Those studies suggest that the functional differences between MDR1 and MDR2 lie mainly in differences in the transmembrane region. MDR1 consists of two homologous halves; each contains six transmembrane (TM) domains and a cytoplasmic nucleotide-binding domain (Chen et al., 1986). Each half of MDR1 may form a structural subunit because two halves of MDR1 coexpressed in separate polypeptides can still build an active drug transporter (Loo and Clarke, 1994a). In two halves of

ABBREVIATIONS: P-gp, P-glycoprotein; MDR, multidrug resistance; TM, transmembrane; PCR, polymerase chain reaction; MVA, modified recombinant vaccinia virus; FACS, fluorescence-activated cell-sorting analysis; IAAP; iodoarylazidoprazosin.

MDR1, TM6 and TM12 appear to work together, and both appear to be directly involved in the interaction with MDR1 substrates (Bruggemann et al., 1989, 1992; Raviv et al., 1990; Greenberger et al., 1991; Tamai and Safa, 1991; Greenberger, 1993; Morris et al., 1994; Zhang et al., 1995) and they can be cross-linked in intact P-gp (Loo and Clarke, 1996). Substitution of either these two regions in mdr1 with mdr2 residues greatly reduces or eliminates MDR1 activity (Buschman and Gros, 1991; Zhang et al., 1995).

Our strategy to identify the essential MDR1 residues has been to restore the multidrug transporter activity in MDR1/MDR2 chimeras by reintroducing selected MDR1 residues into the MDR2 region of the chimera. Previously, we found that substitutions of Q330, V331, and L332 were sufficient to allow the N-terminal transmembrane region (residue 1–394) of MDR2 to form an active multidrug transporter with the rest of the MDR1 molecule (residues 395-1280). This MDR1/MDR2 chimera was able to transport bisantrene and rhodamine 123 and confer resistance to colchicine and vinblastine (Zhou et al., 1999a). These studies helped define some MDR1 residues in the N-terminal transmembrane region important for multidrug transport. In light of the finding that Q330, V331, and L332 in TM6 allow the N-terminal transmembrane region of MDR2 to support MDR1 activity, we would like to know if these substitutions of MDR1 residues in TM6 allow the entire N-terminal half of the MDR2 molecule, including the MDR2 N-terminal nucleotide-binding domain, to have MDR1-like activity, and if substitutions of MDR1 residues in the TM12 region in the C-terminal half of MDR2 also can produce similar effects.

In this work, we found that MDR1 and MDR2 are largely exchangeable at the N-terminal half except for residues 318–332, but that this structural similarity of MDR1 and MDR2 was not mirrored in the C-terminal half. The substitution of MDR1 residues into TM6 and TM11-12 of MDR2 together enabled MDR2 to interact with MDR1 substrates, but not to transport those MDR1 substrates. These results indicate that the MDR2 sequence is able to support the substrate interaction function of MDR1, but it does not lead to drug transport.

Materials and Methods

Mutagenesis and Vector Construction. A T-to-C point mutation was generated in MDR2 at 1789 bp with polymerase chain reaction (PCR) to create an AatII site. This AatII site in MDR2 and the one at 1786 bp in MDR1 were used as break points for creating chimeras consisting of a half molecule of MDR1 and a half molecule of MDR2. The domain swaps of residues 318–332 and 937–994 were carried out with PCR. Primers overlapping with both MDR1 and MDR2 sequences were used in those reactions. To detect the expression of all the MDR1 and MDR2 chimeras on the cell surface, a FLAG epitope containing the octopeptide DYKDDDDK (Kodak) was inserted in the first extracellular loop between residues F98 and G99 of MDR2. The same FLAG epitope also was previously inserted in wild-type MDR1 between N94 and R95 to generate MDR1(F) (C. Hrycyna, I.P., and M.M.G., unpublished data). MDR1(F) and MDR1/MDR2 chimeras were constructed in a pTM1 vector for expression in a transient vaccinia expression system as previously described (Ramachandra et al., 1996). All PCR products were sequenced to confirm that only the desired mutations were introduced.

Cell Culture and DNA Transfection. A transient protein expression approach based on the method developed by Moss and colleagues (Fuerst et al., 1986; Elroy-Stein et al., 1989; Moss 1991)

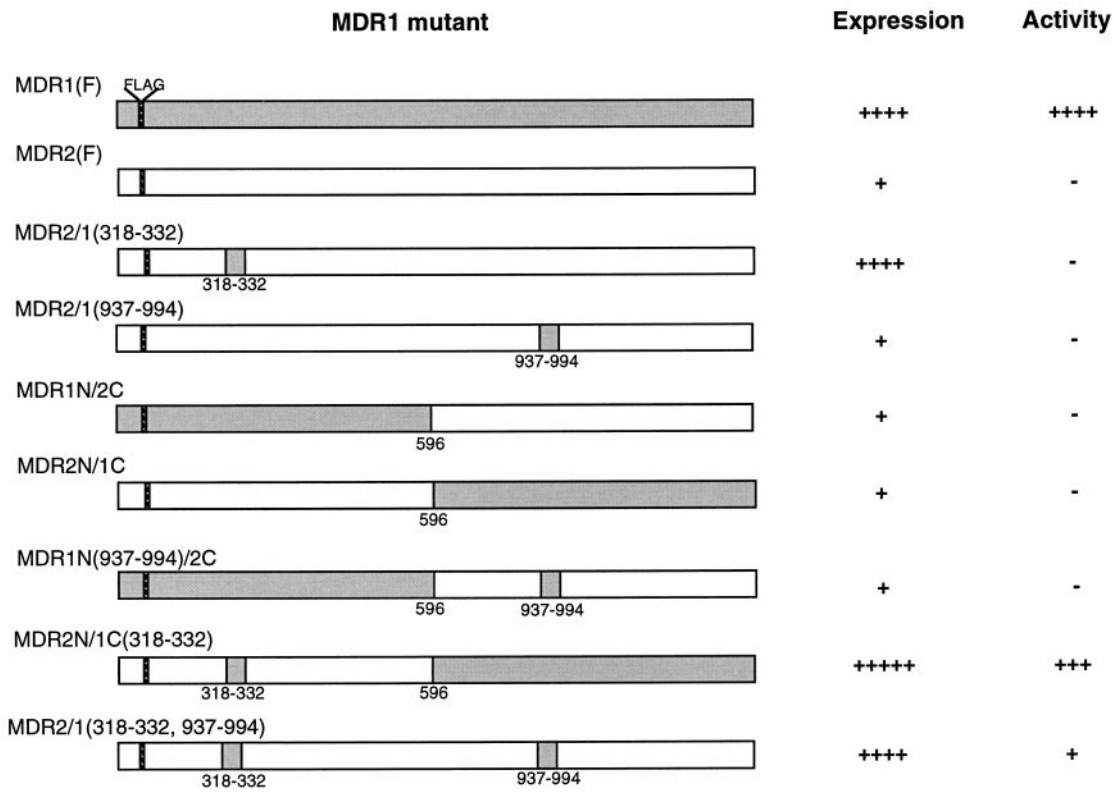

Fig. 1. Summary of the chimeras of MDR1 and MDR2. Shaded boxes, MDR1(F) segments and unshaded boxes, MDR2 segments. The FLAG epitope was inserted in MDR2 between F98 and G99 or in MDR1 between N94 and R95.

was used in this work. With this system, cDNA was transcribed from a T7 promoter by T7 RNA polymerase, which was expressed by a modified recombinant vaccinia virus (MVA). The cotransfection/infection procedure was performed as described previously (Ramachandra et al., 1996). Briefly, 15 μg of DNA was mixed with 45 μl of lipofectin (Life Technologies, Inc., Grand Island, NY) in 3.5 ml of OptiMEM medium and allowed to sit undisturbed at room temperature for 30 min. The mixture was then added to a 75-mm^2 flask preplated with 1.5×10^6 HeLa cells the night before. MVA also was added to the flask at 10^8 plaque-forming units/flask. After a 4-h incubation at 32°C, 12 ml of mimimal essential medium containing 10% fetal bovine serum was added. The cells were cultured at 32°C for another 20 h before analysis of P-gp function.

Fluorescence-Activated Cell-Sorting Analysis (FACS) of P-gp Cell Surface Expression and Fluorescent Drug Accumulation. The cell surface expression of MDR1(F) or its mutants was detected with the M2 monoclonal antibody that recognizes the FLAG epitope (Kodak). Approximately 2 to 3×10^5 cells were incubated in 200 μl of PBS containing 1% BSA and 5 μg of M2 antibody at 4°C for 30 min. After washing twice with ice-cold PBS, the cells were further incubated with fluorescein isothiocyante-conjugated antimouse IgG1 monoclonal antibody (PharMingen, San Diego, CA) at 4°C for 45 min. The cells were then washed and analyzed with a FACSort equipped with a Cellquest program (Becton-Dickinson, San Jose, CA). The fluorescence intensity at FL1 channel was plotted to compare the cell surface expression of MDR1 or its mutants.

In a fluorogenic substrate accumulation assay, 3×10^5 cells were incubated in 2 ml of phenol red-free Iscove's medium (Life Technologies, Inc.) that contained either 2 μM bisantrene, 0.5 μM rhodamine 123, or 0.1 μM calcein AM. After incubation at 37°C for 50 min (15 min for calcein AM), the cells were centrifuged, resuspended in ice-cold PBS, and analyzed by FACS.

[^{3}H]Vinblastine Accumulation Assay. An aliquot of 5×10^5 HeLa cells transfected with MDR1 (F) or the indicated MDR1/MDR2 chimera were washed twice with ice-cold PBS and resuspended in 1 ml of Iscove's medium that contained 15 nM [^{3}H]vinblastine (14.3 Ci/mmol; Amersham Corp., Arlington Heights, IL) with or without 5 μM cyclosporin A. After incubation at 37°C for 40 min, the cells were centrifuged at 4°C for 5 min at 800*g* and washed once in ice-cold PBS. The cell pellet was then resuspended in 0.5 ml of H_2O and transferred into 12 ml of scintillation fluid for determination of radioactivity associated with [^{3}H]vinblastine.

Photoaffinity Labeling with ^{125}Iodoarylazidoprazosin (IAAP). An aliquot of 5×10^5 HeLa cells transiently expressing MDR1(F) or the indicated MDR1/MDR2 chimeras were washed with ice-cold PBS and resuspended in 100 μl of PBS containing 7 nM ^{125}IAAP (Amersham Corp.) with or without 5 μM cyclosporin A or vinblastine at the indicated concentrations. The cell suspensions were incubated in the dark at room temperature for 60 min, followed by cross-linking under UV light at 366 nm for 30 min on ice. The cells were then washed once with ice-cold PBS and the whole-cell lysates were subjected to immunoprecipitation as described previously (Bruggemann et al., 1989, 1992). An anti-P-gp polyclonal antiserum 4007 that is directed against the MDR1 C-terminal ATP binding domain was used in the immunoprecipitation. The immunoprecipitated samples were analyzed by 8% SDS-polyacrylamide gel electrophoriesis and used for detection of protein labeled with ^{125}IAAP.

Results

Construction and Expression of Chimeras of MDR1 and MDR2. To determine whether the sequence differences within TM5-6 and TM11-12 between MDR1 and MDR2 contribute to their substrate preferences, we constructed a series of MDR1/MDR2 chimeras (Fig. 1). In the predicted TM5–6 region (residues 297–346), 10 residues differ between human MDR1 and MDR2, and five of these differences are shared among the MDR1 and MDR2 transporters of human, rat, mouse, and Chinese hamster. All five residues are located between residue 318–332. In the predicted TM11-12 region (residues 937–994), 17 residues differ between MDR1 and

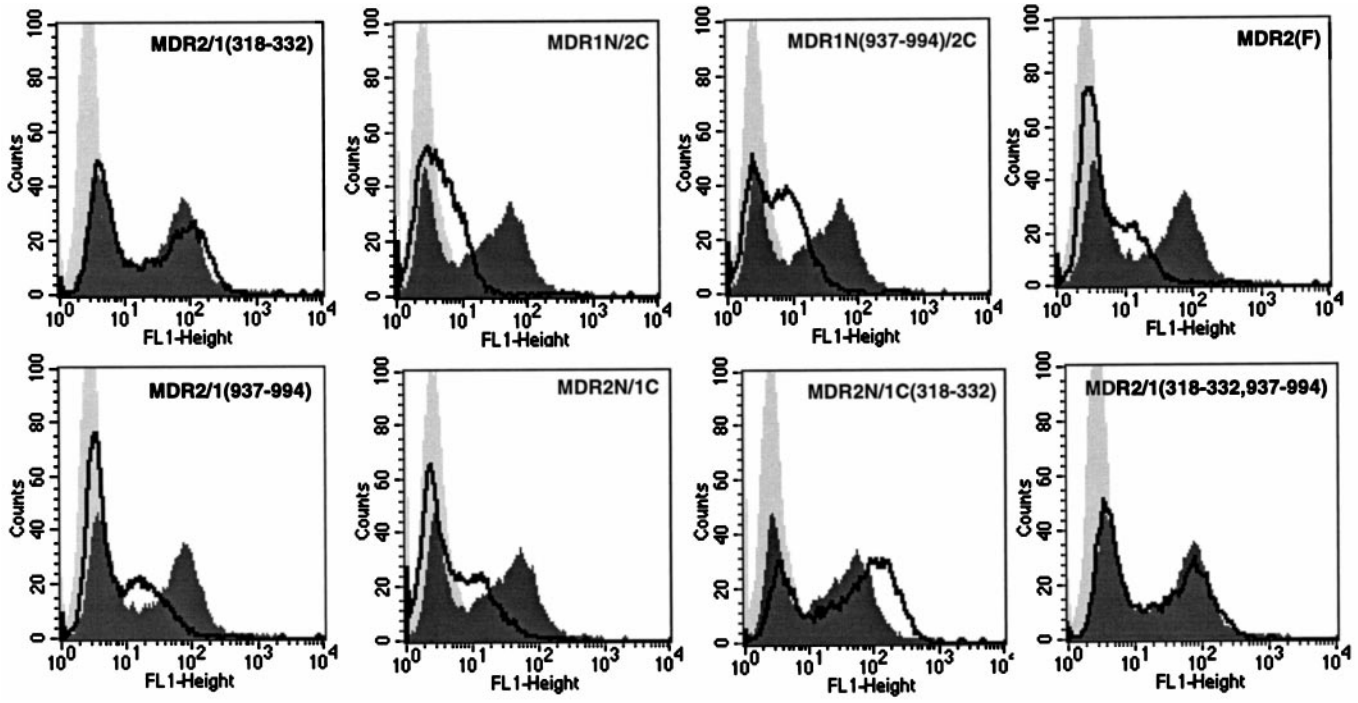

Fig. 2. Cell surface expression of MDR1(F) and other MDR1/MDR2 chimeras. The cell surface expression of MDR1(F) or the indicated MDR1/MDR2 chimeras were determined with the M2 anti-FLAG antibody. The FL 1 height, representing cell surface antibody labeling, was detected with FACS. Light solid, HeLa cells transfected with pTM1 vector; dark solid, HeLa cells transfected with pTM1 MDR1(F); and dark line, HeLa cells transfected with the indicated MDR1/MDR2 chimera.

MDR2, and 15 of these differences are conserved among all the multidrug transporters. To replace TM5-6 and TM11-12 of MDR2 with MDR1 residues, we substituted MDR1 segment 318–332 in the N-terminal half of MDR2(1–596), and MDR1 segment 937–994 in the C-terminal half of MDR2(597–1280). The effect of the substitution of the MDR1 segments in each half of MDR2 was studied in the context of the other half of MDR2, MDR1, and MDR2 containing MDR1 substitutions.

The MDR1/MDR2 chimeras were transiently expressed in HeLa cells with a vaccinia/T7-RNA polymerase system. The cell surface expression of each protein was determined by FACS with a monoclonal antibody against the FLAG epitope. In this assay, the cells subjected to transfection with MDR1(F) or MDR1/MDR2 chimeras were composed of two populations. One population was less fluorescent and overlapped with the cells transfected with the pTM1 vector without insert (Fig. 2, light solid), indicating that this cell population represented the nontransfected population of cells. The other population was more fluorescent and unique to the cells transfected with MDR1(F) (Fig. 2, dark solid) or MDR1/MDR2 chimeras (Fig. 2, dark line), indicating that the increased fluorescence intensity was due to overexpression of MDR1 or MDR1/MDR2 chimeras. As shown in Fig. 2, MDR2(F) and MDR1/MDR2 chimeras consisting of half MDR2 and half MDR1 molecule were expressed on the cell surface, but the amounts of these proteins were only 15 to 25% of the amount of MDR1(F). However, the MDR1/MDR2 chimeras carrying MDR1 residues 318–332 were expressed on the cell surface equivalent to or more than MDR1(F), whereas expression of the chimeras carrying MDR1 residues 937–994 were not markedly improved. A similar result also was obtained with immunoblotting analysis of whole-cell extracts (data not shown). These results suggested that substitution of MDR1 residues 318–332 in the N-terminal half of MDR2 could greatly improve the cell surface expression of MDR2, MDR2N/1C, and MDR2/1(937–994), but substitution

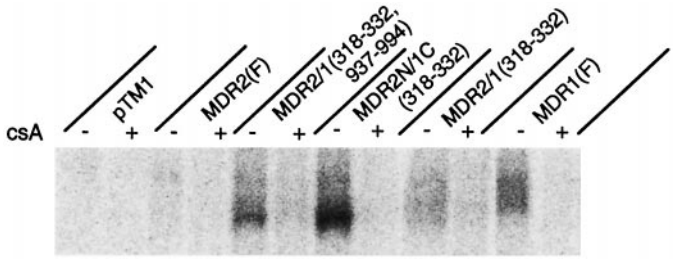

Fig. 3. IAAP photoaffinity labeling of MDR1(F) and MDR1/MDR2 chimeras. HeLa cells (500,000) transiently expressing MDR1(F) or the indicated MDR1/MDR2 chimera were incubated with 7 nM ^{125}IAAP in 100 μl PBS with or without 5 μM cyclosporin A at room temperature for 60 min, followed by cross-linking with UV at 366 nm for 30 min on ice. The P-gps were immunoprecipitated from cell lysates with P-gp polyclonal antibody 4007 and analyzed with 8% SDS-polyacrylamide gel electrophoresis.

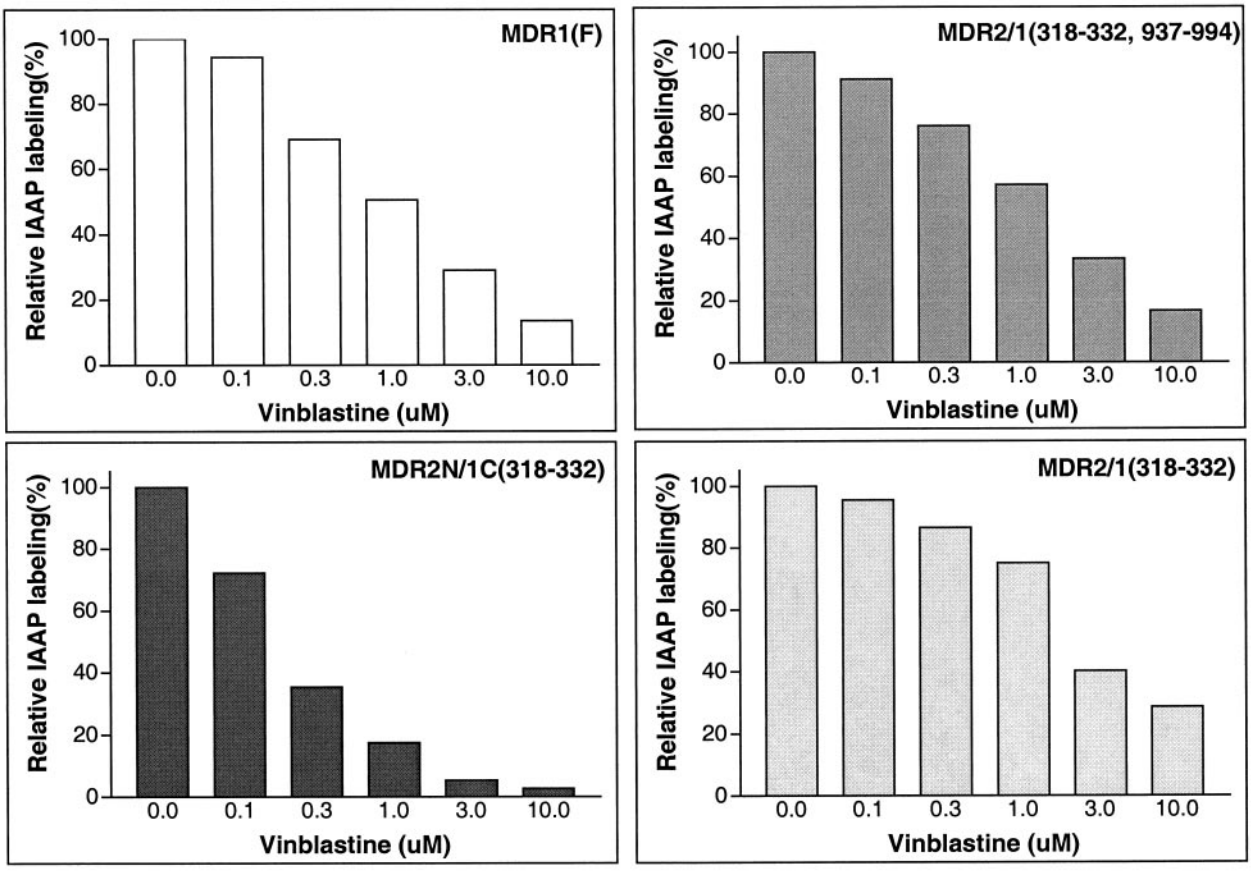

Fig. 4. ^{125}IAAP photoaffinity labeling in the presence of various concentrations of vinblastine. ^{125}IAAP labeling was carried out as described above in the presence of vinblastine at a concentration of 0, 0.1 0.3, 1, 3, and 10 μM. After immunoprecipitation and SDS-polyacrylamide gel electrophoresis separation, the radioactivity associated with P-gp was measured and quantitated with the STORM system. The normalized ^{125}IAAP labeling is shown. The relative ^{125}IAAP labeling in the absence of vinblastine is shown as 100%.

of MDR1 residues 937–994 in the C-terminal half of MDR2 alone did not produce a similar effect.

Study of Substrate Interaction of MDR1/MDR2 Chimeras. Because MDR2/1(318–332), MDR2/1(318–332, 937–994), and MDR2N/1C(318–332) were expressed on the cell surface at a level similar to MDR1(F), we were able to compare their ability to transport or interact with MDR1 substrates. Initially, we performed an ^{125}IAAP photoaffinity-labeling experiment to determine whether these MDR1/MDR2 chimeras could interact with an MDR1 substrate. We found that MDR2/1(318–332, 937–994), MDR2N/1C(318–332), and MDR1(F) photoaffinity-labeled with ^{125}IAAP; MDR2/1(318–332) also bound ^{125}IAAP but at a lower level (Fig. 3). ^{125}IAAP labeling of MDR1(F) and MDR1/MDR2 chimeras was blocked by 5 μM cyclosporin A, indicating that the ^{125}IAAP binding was relatively specific. With ^{125}IAAP labeling as an indicator, we also investigated whether the MDR1/MDR2 chimeras could interact with the P-gp substrate vinblastine by determining if vinblastine would competitively block ^{125}IAAP labeling. We found that vinblastine blocked ^{125}IAAP labeling of MDR1(F) and MDR2/1(318–332, 937–994) in a similar dose-dependent manner, whereas MDR2N/1C(318–332) was even more sensitive to vinblastine, which inhibited ^{125}IAAP labeling at lower concentrations. Vinblastine also blocked ^{125}IAAP labeling of MDR2/1(318–332), but only at higher concentrations (Fig. 4). The results shown in Fig. 4 were obtained from one labeling experiment. Similar results were repeated two or three times in different experiments with overall variations of <10%. The results suggest that MDR2/1(318–332, 937–994) and MDR2N/1C(318–332) had a relatively normal ability to interact with the MDR1 substrates, IAAP, and vinblastine, whereas MDR2/1(318–332) also interacted with MDR1 substrates but with a lower affinity.

Functional Analysis of Chimeras of MDR1 and MDR2. To investigate whether MDR2/1(318–332, 937–994) and MDR2N/1C(318–332) could transport vinblastine, we tested their ability to block [^{3}H]vinblastine uptake. Cells transfected with pTM1, pTM1 MDR1(F), pTM1 MDR2/1(318–332), pTM1 MDR2/1(318–332, 937–994), or pTM1 MDR2N/1C(318–332) were incubated in medium containing 15 nM [^{3}H]vinblastine at 37°C for 40 min, followed by counting accumulated radioactivity with a scintillation counter. We found that cells transfected with MDR1(F) or MDR2N/1C(318–332) accumulated less [^{3}H]vinblastine than cells transfected with the pTM1 vector, and this difference was eliminated when the incubations were carried out in the presence of 5 μM cyclosporin A. Because the [^{3}H]vinblastine accumulation assay was conducted with a mixed cell population containing transfected and nontransfected cells, the nontransfected cell population in each sample was determined by Mab labeling and FACS, so that the accumulation of [^{3}H]vinblastine in nontransfected cells could be subtracted. After normalizing the difference in cell number with [^{3}H]vinblastine accumulation in the presence of cyclosporin A, a P-gp inhibitor, we calculated the relative [^{3}H]vinblastine accumulation in the absence of cyclosporin A. The results indicated that MDR2N/1C(318–332) was the only one of the MDR1/MDR2 chimeras that reduced the accumulation of [^{3}H]vinblastine (Fig. 5).

Because many MDR1 substrates are fluorescent, we performed an FACS to determine the ability of the MDR1/MDR2 chimeras to efflux a few MDR1 substrates. Bisantrene, rhodamine 123, and calcein AM, each having very different chemical structures, were used in the assay. As shown in Fig. 6, the cells transfected with MDR1(F) were composed of two populations. One population of cells, overlapping with the cells transfected with the pTM1 vector, exhibited high fluorescence intensity, indicating a lack of drug-transporter activity. The second cell population was less fluorescent and could be shifted to the high fluorescent population when the MDR1 inhibitor cyclosporin A was added during the incubation (data not shown), which indicated that the reduced fluorescence was associated with MDR1 activity. Consistent with the results of the [^{3}H]vinblastine uptake experiment, we found that MDR2N/1C(318–332) was the only MDR1/MDR2 chimera that was able to efflux all three MDR1 substrates. In addition, it also could transport bodipy-prazosin and bodipy-taxol (data not shown). Compared with MDR2N/1C(318–332), MDR2/1(318–332) had no MDR1-like activity, whereas MDR2/1(318–332, 937–994) was only minimally able to efflux calcein AM and bisantrene, but it was unable to efflux rhodamine 123. With the same assay, we did not detect any efflux activity of MDR2(F), MDR1N/(937–994)/2C, or MDR1N/2C (data not shown).

Discussion

Determination of the residues essential for multidrug transporter activity of MDR1 offers the promise of defining substrate binding domains with the ultimate goal of developing specific inhibitors for different substrates of this clini-

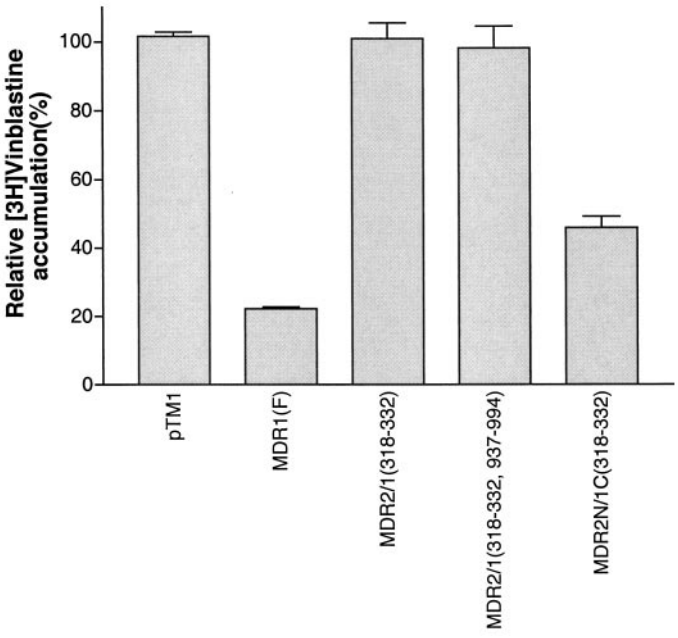

Fig. 5. [^{3}H]vinblastine accumulation assay. An aliquot of 5×10^5 HeLa cells transfected with pTM1 vector, MDR1(F), MDR2/1(318–332), MDR2/1(318–332, 937–994), or MDR2N/1C(318–332) were incubated in medium containing 15 nM [^{3}H]vinblastine at 37°C for 40 min. The amount of [^{3}H]vinblastine accumulated inside cells was determined with a scintillation counter. The relative [^{3}H]vinblastine accumulation was calculated as $100 \times (\text{cpm} - N\% \times P \times \text{cpm}_{\text{pTM1}})/((1-N\%) \times P \times \text{cpm}_{\text{pTM1}})$, where N% represents the percentage of the nontransfected cell population, and $P = \text{cpm}^{\text{(in presence of cyclosporin A)}}/\text{cpm}_{\text{pTM1}}^{\text{(in presence of cyclosporin A)}}$. Because MDR1 activity was fully blocked by 5 μM cyclosporin A, P was used to normalize the difference in cell number. Error bars represent S.D.

cally important transporter. One approach to this goal is to study chimeras of MDR1 and its nonmultidrug transporter-homolog MDR2. With a strategy involving domain interchange, we found that MDR1 residues 318–332 and 937–994 allowed an MDR2 backbone to interact with the MDR1 substrates ^{125}IAAP and vinblastine, but this chimera did not have multidrug transport activity. However, when the N-terminal half of MDR2 contained the MDR1 segment 318–332 together with the C-terminal half of MDR1, it functioned as an active multidrug transporter. In contrast, when the C-terminal half of MDR2 contained MDR1 segment 937–994 together with the N-terminal half of MDR1, the chimera was not well expressed on the cell surface and could not transport MDR1 substrates (Fig. 1). These results suggest that MDR1 and MDR2 have great structural similarity, but this similarity is not symmetrically distributed between the two halves of the molecule.

Expression of MDR1 and MDR2 Chimeras. The difficulty in achieving high-level expression of recombinant MDR2 with stable expression systems has been previously documented (Schinkel et al., 1991; Buschman and Gros, 1994). In this work, we also observed low expression of MDR2 and some of MDR1/MDR2 chimeras, which were detected with antibody labeling of cell surface P-gp and immunoblotting of P-gp with whole-cell lysates. However, substituting the MDR1 segment 318–332 significantly increased the expression of MDR2 and MDR1/MDR2 chimeras. Within this segment, seven residues differ between MDR1 and MDR2. Among them, residues 318, 322, 324, and 327 may be responsible for the increase in protein expression because expres-

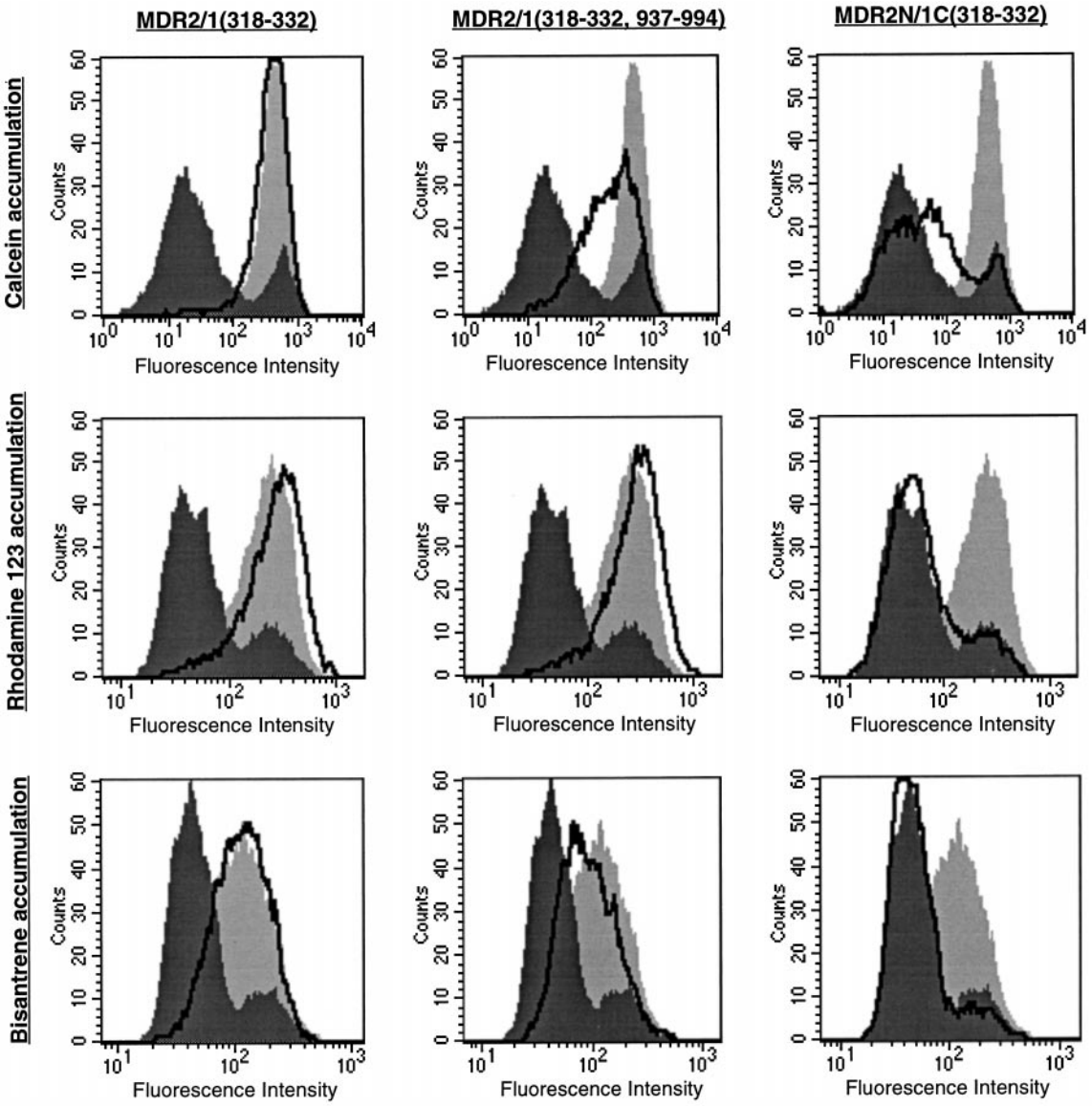

Fig. 6. Functional analysis of transiently expressed MDR1(F) and MDR1/MDR2 chimeras with FACS. HeLa cells transfected with the pTM1 vector, MDR1(F), MDR2/1(318–332), MDR2/1(318–332, 937–994), or MDR2N/MDR1C(318–332 were incubated in medium containing 2 μM bisantrene, 0.5 μM rhodamine 123, or 0.1 μM calcein AM at 37°C for 50 min (15 min for calcein AM). The fluorescence intensity, representing intracellular-accumulated substrate, was detected with FACS. Light solid, HeLa cells transfected with pTM1 vector; dark solid, HeLa cells transfected with TM1 MDR1(F); and dark line, HeLa cells transfected with the indicated MDR1/MDR2 chimera. To better show the difference between two cell populations, enlarged *x*-axes are used for rhodamine 123, calcein, and bisantrene.

sion of MDR2N/1C(330–332) was only 20% of the expression of MDR2N/1C(318–322) (data not shown). Located in the putative extracellular loop connecting TM5 and TM6, residues 318, 322, 324, and 327 are less important for MDR1 activity, but are essential for the recognition of UIC2, a conformation sensitive monoclonal antibody of MDR1 (Mechetner et al., 1997). Substitutions of these residues into MDR2 in a mostly MDR1 backbone did not affect MDR1 activity but eliminated UIC2 binding (Zhou et al., 1999b). MDR1 residues 318–332 increased expression of MDR1/MDR2 chimeras only when they were in the context of an MDR2 N-terminal sequence because the chimera consisting of the N-terminal half of MDR1 and the C-terminal half of MDR2 (MDR1N/2C) was expressed 6-fold less than MDR2/1(318–332).

Because we used a powerful vaccinia virus transient expression system, it is unlikely that the different expression level is due to different rates of transcription or translation. One possibility is that low expression may be caused by a slow protein-folding process or to an unstable protein structure. The differences in the cell surface expression of the different MDR1/MDR2 chimeras make it difficult to compare their drug transporter activities. Therefore, conclusions about relative activity have only been drawn from the analysis of MDR2/1(318–332), MDR2N/1C(318–332), and MDR2/1(318–332, 937–994) because these three chimeras were all expressed at a similar level (Fig. 2).

TM5-6 and TM11-12 of MDR1 Are Essential for Drug Binding Activity. The TM5–6 and TM11–12 region from MDR1 enabled MDR2 to have relatively normal ability to interact with MDR1 substrates ^{125}IAAP and vinblastine. This observation emphasizes the importance of the TM5-6 and TM11-12 region in drug binding, which has been shown previously by several photoaffinity-labeling experiments (Bruggemann et al., 1989, 1992; Raviv et al., 1990; Greenberger et al., 1991; Greenberger, 1993; Tamai and Safa, 1991; Morris et al., 1994; Zhang et al., 1995). In addition, the current result demonstrates that the MDR2 backbone can support the drug-binding activity of the inserted segments of MDR1, indicating that there is great similarity of overall structure between MDR1 and MDR2. However, the lack of full MDR1 activity in MDR2/1(318–332, 937–994) suggests that the overall MDR2 structure in the plasma membrane is not sufficient to support MDR1-like activity, even though it can interact with MDR1 substrates. Because both ATP binding domains of MDR2 support MDR1 activity (Buschman and Gros, 1991; C.A. Hrycyna et al., unpublished data), the lack of MDR1 activity in MDR2/1(318–332, 937–994) could be due to inefficient coupling between drug binding and ATP hydrolysis, or between ATP hydrolysis and drug transport. These events are related to reversible changes in MDR1 conformation (Loo and Clarke, 1997a, b; Mechetner et al., 1997; Wang et al., 1997; Hrycyna et al., 1998; Ramachandra et al., 1998). It is possible that MDR2 conformation, or the conformation of MDR2 in the context of MDR1 drug-interacting sites, may not be able to respond to drug binding or ATP hydrolysis. Alternatively, drug interaction per se may not be an indicator of drug binding to the site that results in drug efflux. Interestingly, uncoupling of drug-binding and transport activity also is observed in an MDR1 mutant carrying a mutation in TM6. In that case, however, the mutated residue S344 is found in both MDR1 and MDR2 (Loo and Clarke, 1994b).

Difference in Conformation May Contribute to Difference in Substrate Specificity of MDR1 and MDR2. Previous mutational studies demonstrate that single mutations at some MDR1 residues can significantly change MDR1 substrate specificity or reduce overall multidrug transporter activity. Most of those residues, including the ones in TM6, TM11, and TM12, are the residues conserved among multidrug transporters (MDR1) and phosphatidylcholine flippases (MDR2). Single mutations occurring at the residues unique to MDR1 consistently produce little or only moderate effects on MDR activity (for review, see Gottesman et al., 1995). Changing multiple residues unique to MDR1 in MDR1, however, results in loss of function or altered substrate specificity, as demonstrated by recent analyses of residues in TM6 and TM12 (Hefkemeyer et al., 1998; Zhou et al., 1999a). The current study emphasizes that the broad substrate specificity of MDR1 is collectively determined by multiple unique MDR1 residues; no single unique MDR1 residue is required for MDR1 activity, but together these unique residues allow binding and transport of MDR-specific substrates, probably by facilitating a specific conformation of MDR1 and MDR1/MDR2 chimera.

In summary, with a domain swapping approach, we found that the MDR1 and MDR2 N-terminal halves were mostly exchangeable except for a few residues in TM6. However, this exchangeability was not seen in the C-terminal halves. In addition, with substitution of MDR1 segments 318–332 and 937–994, MDR2 showed normal ability to interact with two MDR1 substrates but did not have significant transporter activity, which suggests that the inability of MDR2 to transport most MDR1 drugs may not be due simply to a lack of drug-binding ability. In combination with detailed structural studies of MDR1 and MDR2, these results should help define the residues and conformations essential for drug binding and transport.

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