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A single amino acid substitution strongly modulates the activity and substrate specificity of the mouse mdr1 and mdr3 drug efflux pumps

P. GROS*†, R. DHIR*, J. CROOP‡, AND F. TALBOT*

*Department of Biochemistry, McGill University, Montreal, PQ, Canada H3G 1Y6; and ‡Dana–Farber Cancer Institute and The Childrens' Hospital, Boston, MA 02115

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ABSTRACT **Specific protein domains and amino acids responsible for the apparent capacity of P-glycoprotein (mdr) to recognize and transport a large group of structurally unrelated drugs have not been identified. We have introduced a single Ser → Phe substitution within the predicted TM11 domain of mdr1 (position 941) and mdr3 (position 939) and analyzed the effect of these substitutions on the drug-resistance profiles of these two proteins. Mutations at this residue drastically altered the overall degree of drug resistance conveyed by mdr1 and mdr3. The modulating effect of this mutation on mdr1 and mdr3 varied for the drugs tested: it was very strong for colchicine and adriamycin and moderate for vinblastine. For mdr1, the Ser^{941} → Phe^{941} substitution produced a unique mutant protein that retained the capacity to confer vinblastine resistance but lost the ability to confer adriamycin and colchicine resistance. These results strongly suggest that the predicted TM11 domain of proteins encoded by *mdr* and *mdr*-like genes plays an important role in the recognition and transport of their specific substrates.**

Multidrug resistance is caused by the amplification and overexpression of a small gene family, designated *mdr* or *pgp* (1), which is composed of two members in humans, *MDR1* and *MDR2* (2, 3), and three members in rodents, *mdr1* (*mdr1b*), *mdr2*, and *mdr3* (*mdr1a*) (4–7), that code for membrane P-glycoproteins (P-gps). P-gp has been shown to bind ATP (8) and drug analogs (9, 10) and has ATPase activity (11). It is believed to function as an ATP-dependent drug efflux pump to reduce intracellular drug accumulation in resistant cells (1, 12). Sequence analyses of *mdr* gene cDNA clones predict polypeptides composed of 12 transmembrane (TM) domains and two nucleotide binding (NB) sites. P-gps are formed by two symmetrical and sequence homologous halves that share a common ancestral origin with a large group of bacterial transport proteins (13). The *mdr* gene family is part of a larger family of *mdr*-like genes encoding sequence-homologous proteins sharing similar predicted secondary structures and proposed membrane-associated transport functions. The *pfmdr1* gene of *Plasmodium falciparum* (14) associated with chloroquine (CLQ) resistance, the yeast *STE-6* gene responsible for export of the a mating pheromone in *Saccharomyces cerevisiae* (15), and the *CFTR* gene in which mutations cause cystic fibrosis in humans (16) form part of this family. The regions of strongest sequence homology among these proteins overlap the predicted NB sites, which are believed to underlie a common functional aspect of transport (17). The protein segments and residues implicated in substrate binding and transport have not been precisely identified. Despite a very high degree of sequence homology (between 75% and 85% identity), striking functional differences have been detected between individual *mdr* genes. Mouse *mdr1* (18) and *mdr3* (6, 19) and human *MDR1* (20) can confer multidrug resistance in transfection experiments, whereas mouse *mdr2* (5) and human *MDR2* (3) apparently cannot. In addition, mouse *mdr1* and *mdr3* appear to confer distinct drug-resistance phenotypes (6). These observations suggest that the functional differences detected between *mdr* genes may be encoded by a limited number of amino acid residues.

The drug-resistance profiles of transfected cells expressing a cloned cDNA for mouse *mdr3* isolated from a pre-B-cell cDNA library (6) differ qualitatively and quantitatively from that of multidrug-resistant J7-V3-1 mouse macrophages overexpressing the endogenous *mdr3* (*mdr1a*) gene (19). A comparison of the amino acid sequence predicted from the mouse *mdr3* clone (6) with that of *mdr1a* overexpressed in J7-V3-1 cells (7) identifies two differences: one within the first predicted NB site and the other within TM11. In the *pfmdr1* gene of *P. falciparum*, mutations in TM11 have been associated with CLQ resistance (21). Here we show that a single amino acid substitution (Ser → Phe) within the predicted TM11 of *mdr1* (position 941) and *mdr3* (position 939) drastically modulates the activity and substrate specificity of these two drug efflux pumps.

MATERIALS AND METHODS

Site-Directed Mutagenesis. Full-length cDNAs for *mdr1* and *mdr3* cloned in the plasmid pGEM-7Zf (Promega) were digested with *Kpn* I and *Sma* I–*Pst* I, respectively, to generate 1.5-kilobase *Kpn* I and 1.8-kilobase *Sma* I–*Pst* I 3′ end fragments, which were cloned in the corresponding sites of phage M13mp18. Single-stranded DNA templates from these clones were mutagenized *in vitro* with single primers using commercially available reagents (Amersham). Oligonucleotides bearing mismatched bases at the residues to be mutated (see Fig. 1) were 5′-CTGGGTGAAGAA-GAACGT-3′ (*mdr1-F*, Ser^{941} → Phe^{941}) and 5′-GGTGAAG-GAGAACGGTG-3′ (*mdr3-S*, Phe^{939} → Ser^{939}). The integrity of the entire mutated cDNA inserts was verified by DNA sequencing prior to reconstruction into the respective full-length clones.

Transfection Experiments. cDNAs for mutant *mdr* genes were cloned into expression vector pMT2 (1, 6) and introduced into drug-sensitive LR73 hamster cells by cotransfection with pSV2-Neo (22), as described (6). Mass populations of neomycin-resistant colonies were initially selected in Geneticin (G418) at 0.5 mg/ml and independent cell clones stably expressing *mdr1-F* (clones 1F-3, -5, and -9) or *mdr3-S* (clones 3S-2, -5, and -8) were isolated after selection in vinblastine (VBL) (Sigma) at 25 ng/ml and 100 ng/ml,

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Abbreviations: P-gp, P-glycoprotein; TM, transmembrane; NB, nucleotide binding; CLQ, chloroquine; VBL, vinblastine; ACT, actinomycin D; ADM, adriamycin; COL, colchicine.

†To whom reprint requests should be addressed.

respectively. Independent cell clones expressing *mdr1-S* and *mdr3-F* were isolated previously (6). Cell clones 3S-13 and 3S-16 expressing low levels of resistance to VBL (see Fig. 4) were isolated immediately after G418 selection.

Detection of Mutant Proteins. Membrane proteins (20 μg) (23) from control and transfected cells were separated by SDS/polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane by electroblotting, and the blot was incubated with either the mouse monoclonal antibody anti-P-gp C219 (Centocor, Philadelphia) or the isoform-specific rabbit polyclonal antibodies anti-mdr3 B2037 (6) or anti-mdr1 JC61 (J.C., unpublished results), followed by incubation with anti-mouse or anti-rabbit antibodies coupled to alkaline phosphatase.

Drug-Survival Measurements. The drug-survival characteristics of cell clones expressing wild-type or mutant proteins were determined by plating 500 cells in medium containing increasing concentrations of adriamycin (ADM), colchicine (COL), VLB, or actinomycin D (ACT). Colonies containing 50 cells or more were scored 8 days later. Results are expressed as the percentage of cells surviving at a given drug concentration compared with the control dishes containing drug-free medium.

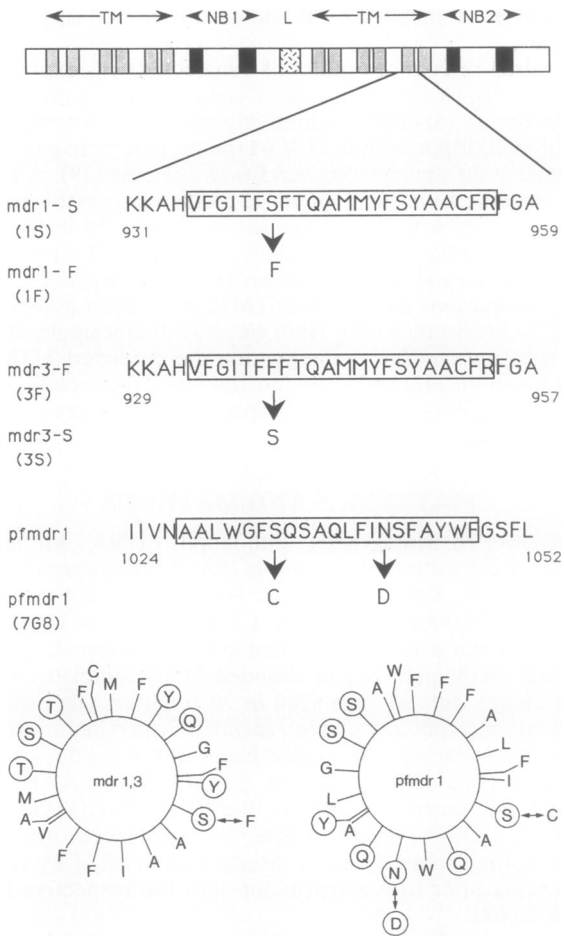

FIG. 1. Site-directed mutagenesis of cDNA clones for *mdr*. A schematic representation of a cDNA for *mdr* is shown at the top. Shaded boxes, TM domains; solid boxes, NB folds (NB1 and NB2); stippled box, linker domain (L). The segment overlapping the predicted TM11 domain (boxed sequence) has been magnified and the amino acid sequence of wild-type and mutant *mdr* clones are shown. The amino acid sequence of the predicted TM11 domain of *pfmdr1* and its mutant 7G8 allele is also shown. Numbers indicate the position of the flanking residues in the predicted sequence of the respective polypeptides. At the bottom, helical wheel projections of the protein segment overlapping TM11 segments for mdr1/mdr3 and pfmdr1 are shown. Hydrophilic residues assigned by the algorithm of Eisenberg *et al.* (24) are circled, and the positions of mutated residues are identified by arrows.

RESULTS

Functional differences have been detected between a cloned cDNA for *mdr3* expressed in transfected cells (6) and the endogenous *mdr3* (*mdr1a*) gene overexpressed in drug-resistant mouse macrophages (19). A comparison of the amino acid sequence predicted for the *mdr3* (6) and *mdr1a* (*mdr3*) (7) clones identifies two differences. Within the 5′ nucleotide binding fold (NB1, Fig. 1), the pair of Gln-Leu residues at positions 526 and 527 of *mdr3* (6) is replaced by His-Val in *mdr1a* (7). The Gln-Leu pair is precisely conserved in all P-gps sequenced to date, in the NB1 site and at the homologous position in the NB2 site. The origin of this sequence difference between *mdr3* and *mdr1a* (GC → CG, nucleotide 1578) is unknown. An additional sequence variation, Ser^{939} (*mdr1a*) vs. Phe^{939} (*mdr3*), is detected within the predicted TM11. The serine residue at this position is conserved in all P-gps sequenced to date except in our reported sequence of *mdr3* (6). cDNA amplification of total RNA from mouse intestine, 70/Z pre-B cells, and P388-VCR cells overexpressing *mdr3*, followed by direct DNA sequencing, identified Ser^{939} in the three RNA preparations (data not shown). This suggests that Phe^{939} in our cDNA for *mdr3* results from a polymerase error during cDNA construction (C → T substitution, nucleotide 2816). Surprisingly, this mutation maps at the homologous position in TM11 of two nonconservative amino acid substitutions present in the mutant 7G8 allele of the *mdr* homolog *pfmdr1* (21) detected in a large

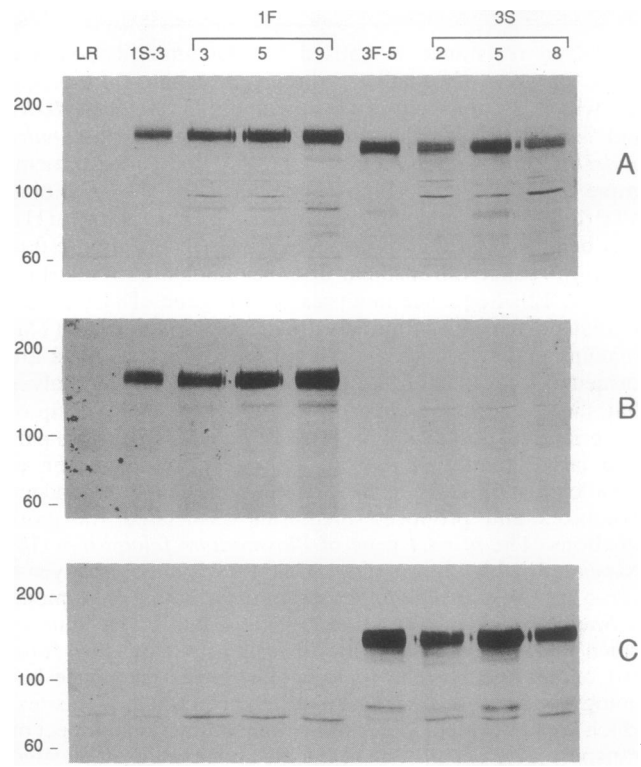

FIG. 2. Identification of mdr1 and mdr3 proteins. Membrane fractions of drug-sensitive LR73 hamster cells (LR) and independent cell clones transfected with *mdr1-F* (1F-3, -5, and -9), *mdr3-S* (3S-2, -5, and -8), *mdr1-S* (1S-3), and *mdr3-F* (3F-5) were analyzed on Western blots with the monoclonal anti-P-gp antibody C219 (*A*) and isoform-specific polyclonal antibodies anti-mdr1 JC-61 (*B*) or anti-mdr3 B2037 (*C*).

proportion of CLQ-resistant isolates of *P. falciparum* (Fig. 1).

To test directly the functional significance of this Ser → Phe substitution for *mdr* function, site-directed mutagenesis was used to convert Phe^{939} (3F) to Ser^{939} (3S) in *mdr3* and to replace the wild-type Ser^{941} (1S) with Phe^{941} (1F) in *mdr1* (Fig. 1). Full-length 1F and 3S cDNAs were cloned in the mammalian expression vector pMT2 and introduced in LR73 cells by cotransfection with a neomycin-resistance marker, and drug-resistant colonies were isolated from mass populations of G418-resistant clones after VBL selection. Membrane fractions were prepared and analyzed on Western blots for presence of wild-type and mutant P-gps. Three cell clones transfected with either the 1F cDNA (1F-3, -5, and -9) or the 3S cDNA (3S-2, -5, and -8) constructs and expressing amounts of P-gp similar to those detected in previously described cell clones expressing 1S and 3F cDNAs (6) were identified and further analyzed with a generic anti-P-gp monoclonal antibody that recognizes mdr1 and mdr3 (C219, Fig. 2*A*) and with isoform-specific anti-mdr1 (Fig. 2*B*) and anti-mdr3 (Fig. 2*C*) polyclonal antibodies. The C219 antibody recognized 180-kDa and 160-kDa proteins overexpressed in the *mdr1* (1F, 1S) and *mdr3* (3F, 3S) clones, respectively (Fig. 2*A*). The 180-kDa and 160-kDa P-gps were also identified by isoform-specific anti-mdr1 (Fig. 2*B*) and anti-mdr3 (Fig. 2*C*) antibodies, respectively. The levels of expression of wild-type and mutant proteins were not identical but were comparable in the clones tested.

The drug-survival characteristics of three independent cell clones stably expressing 3F, 3S, 1F, or 1S proteins were determined and compared for VBL, ACT, ADM, and COL (Fig. 3). The degree of resistance (D10) conferred by wild-type and mutant mdr proteins was expressed as the ratio of drug concentration required to reduce the plating efficiency of individual cell clones by 90%. For mdr3, the replacement of Phe^{939} by Ser^{939} caused a considerable and general increase in the drug-resistance levels conferred by this protein over control levels detected in LR73 cells: resistance to VBL increased from 18 times in 3F clones to 49 times in 3S clones, to ACT increased from 10 times (3F) to 118 times (3S), to ADM increased from 2 times (3F) to 27 times (3S), and to COL increased from 3 times (3F) to 86 times (3S). The increase in resistance detected in 3S over 3F clones was strongest from COL and ADM (increase of 29 times and 14 times, respectively) and smallest for VBL (3 times). These results indicate that the Phe^{939} → Ser^{939} replacement modulates the overall activity of mdr3 and suggest that this modulation may be quantitatively different for individual drugs.

To test this possibility further, we isolated two additional 3S-expressing cell clones (3S-13 and 3S-16) with levels of VBL resistance similar to those detected in cell clones expressing 3F. 3S-13 and 3S-16 were selected immediately after transfection and G418 selection, without further selection in VBL. We then compared the respective resistance profiles of 3S-13 and 3S-16 to that of 3F clones for ACT, ADM, and COL. Should the Phe^{939} → Ser^{939} substitution only affect the overall activity of mdr3, then the drug-resistance profiles of 3S-13 and 3S-16 for ACT, ADM, and COL should be similar to that of 3F clones. Results of this experiment (Fig. 4) indicate that this is not the case. Although the 3S-13 and 3S-16 clones show VBL-resistance profiles similar to that of the 3F clone tested, they display degrees of ADM, COL, and ACT resistance significantly higher than those expressed by the 3F clone. As noted earlier with 3S clones tested in Fig. 3, the enhanced resistance detected in 3S-13 and 3S-16 clones over that of the 3F clone was strongest for COL (9 times) and ADM (7 times).

We then analyzed the effect of the Ser → Phe substitution at the homologous position (residue 941) of mdr1 (Fig. 3). The

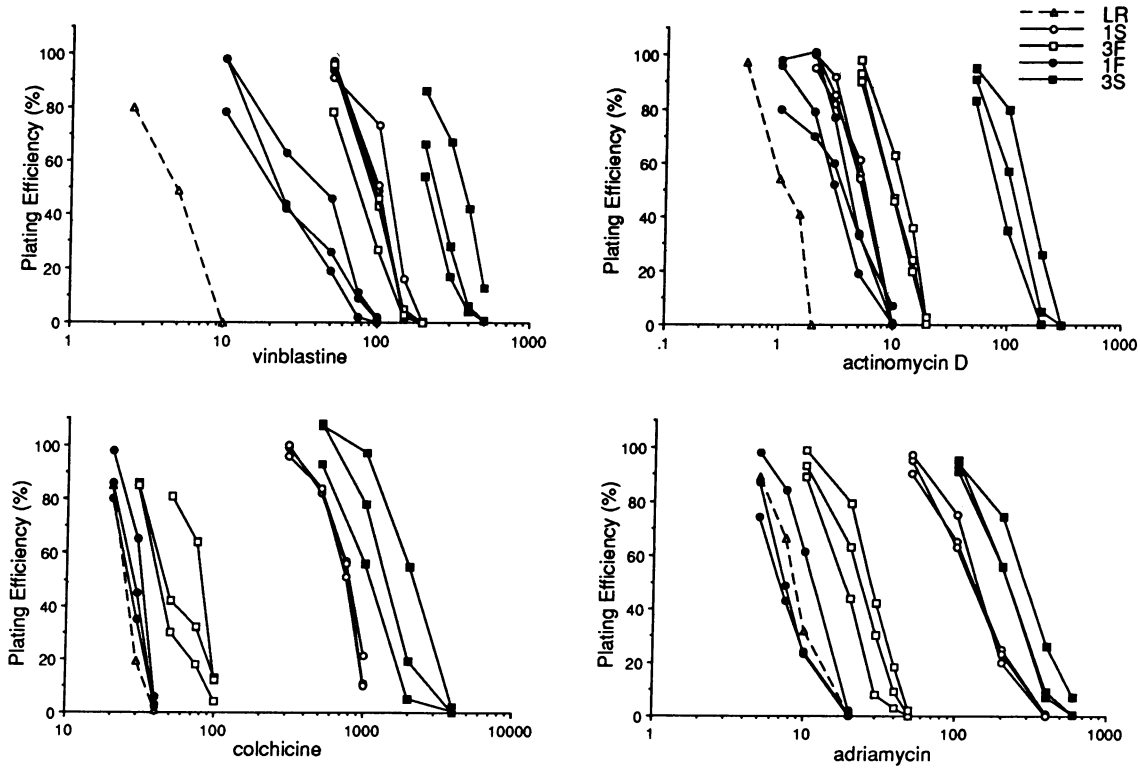

FIG. 3. Drug-survival characteristics of drug-sensitive LR73 hamster cells (LR) and three independent cell clones transfected with *mdr1-S* (1S), *mdr1-F* (1F), *mdr3-S* (3S), or *mdr3-F* (3F). The concentration of the various drugs is in ng/ml. The relative plating efficiency of each clone was calculated by dividing the number of colonies observed at a given drug concentration by the mean number of colonies formed by the same clone in control medium lacking drug and is expressed as a percentage. Each point represents the average of duplicate dishes; each clone was tested twice.

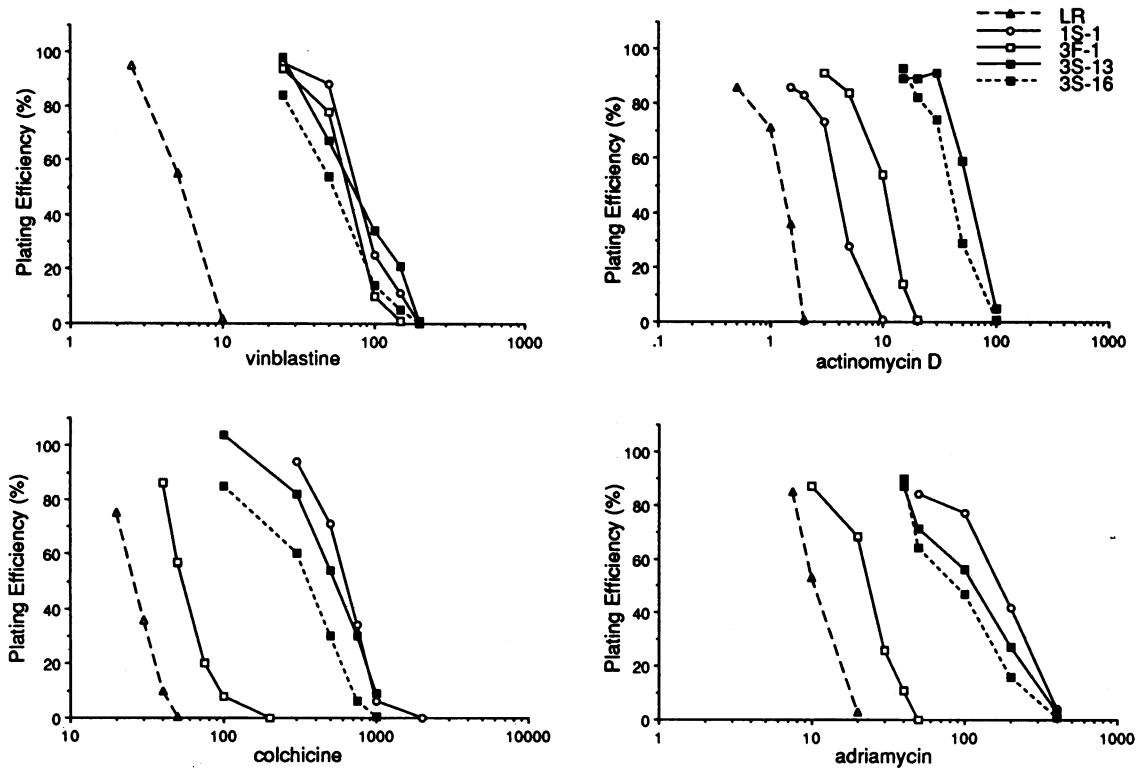

FIG. 4. Drug-survival characteristics of LR73 hamster cells (LR) and cell clones transfected with *mdr1-S* (1S-1), *mdr3-F* (3F-1), or *mdr3-S* (3S-13 and 3S-16). Results are presented as described in Fig. 3.

wild-type Ser^{941} (1S) → Phe^{941} (1F) substitution resulted in a general decrease in the capacity of *mdr1* to convey resistance to VBL, COL, ADM, and ACT. As noted earlier for the homologous mutation in *mdr3*, a comparison of the drug-resistance profiles of 1F- and 1S-expressing clones revealed that the modulating effect of the mutation varied for the four drugs tested: the effect was strongest for COL and ADM where a 33 times and 15 times decrease in resistance, respectively, were noted in 1F clones compared to 1S clones and was more modest for VBL and ACT where 4 and 2 times reductions in resistance were noted, respectively. The 1F clones expressed clearly measurable levels of VBL resistance and to a lower extent ACT resistance but showed levels of ADM and COL resistance indistinguishable from that detected in control drug-sensitive LR73 cells. Therefore, it appears that the P-gp expressed by the 1F mutant has the unique phenotype of conferring VLB but not ADM or COL resistance. The cumulative analysis of *mdr1* and *mdr3* mutants presented here identifies $Ser^{939/941}$ as a critical residue for the overall activity and substrate specificity of the drug efflux pumps encoded by the two genes.

DISCUSSION

Biochemical and genetic analyses suggest that membrane-associated regions of P-gp and P-gp-like polypeptides are likely to participate in substrate recognition and transport. (*i*) Azidopine, a photoactivatable drug analog, labels tryptic fragments identified by epitope mapping as overlapping the TM regions (25, 26). (*ii*) P-gp can be labeled by a membrane-photoactivatable probe 5-[^{125}I]iodonaphthalene-1-azide upon direct energy transfer from doxorubicin or rhodamine, both P-gp substrates (27). (*iii*) A spontaneous Gly → Val mutation at position 185 of human MDR1 protein near TM3 confers preferential COL resistance (28). (*iv*) Analysis of chimeric genes constructed between the biologically active mouse *mdr1* and inactive mouse *mdr2* genes identifies the TM regions as functionally distinct (29). (*v*) Spontaneous mutations in the TM1-2 interval and within TM11 of the *pfmdr1* gene are found associated with CLQ resistance in *P. falciparum* (21). (*vi*) The yeast *mdr* homolog *STE6*, responsible for the export of the mating pheromone **a**, shares with the **a** factor receptor expressed on α cells (*STE3*) two regions of sequence homology near TM7 and TM12, possibly implicated in **a** factor binding (30).

Here, we show that a single amino acid substitution at the homologous position of the predicted TM11 segment of mouse mdr1 and mdr3 proteins profoundly modulates the drug-resistance phenotypes of these two proteins. P-gps encoded by *mdr1* and *mdr3* and bearing a serine at position 939 or 941, respectively, conferred a higher degree of resistance to the four drugs tested than those conveyed by proteins bearing a phenylalanine at that same position. The fact that this substitution affected quantitatively the activity of both proteins and was pleiotropic for the various classes of drugs tested suggests that this residue and/or the TM domain plays a role in the common mechanism of action of the two proteins. Moreover, the modulation of mdr1 and mdr3 activity by this substitution was qualitatively different for the individual drugs tested, suggesting that this residue may also participate in substrate recognition. A comparison of the drug-resistance profiles of mdr1 and mdr3 proteins bearing serine vs. phenylalanine at residues 939 and 941, respectively, shows that the differences in drug-resistance levels are greatest for COL (30 times) and ADM (17 and 14 times) but are much less for VBL (2.5 times). In fact, the *mdr1-F* clone encodes a mutant protein that is unique in that it retains the capacity to confer VBL resistance but has lost the ability to confer COL and ADM resistance. The segregation of the VBL-resistance phenotype from the ADM- and COL-resistance phenotypes within this unusual mutant supports the proposition that VBL may have a binding site on P-gp distinct from that of COL and ADM and that the $Ser^{939/941}$ is critical for the recognition and/or transport of COL and

ADM. Biochemical analysis of P-gp also supports the genetic evidence presented here suggesting that the binding sites for VBL and for COL and ADM may be nonoverlapping on P-gp: photoaffinity labeling of P-gp by analogs of VBL, verapamil, or azidopine can be completely competed by vincristine and VBL but not by COL and ADM (9, 10, 31, 32); in membrane vesicles from MDR cells, VBL competes binding of vincristine at concentrations two orders of magnitude lower than ADM and COL (33, 34). The effect of the Ser → Phe substitution in mdr1 and mdr3 on the level of ACT resistance conferred by these proteins was more complex: a comparison of ACT-resistance profiles of 1F-, 1S-, 3F-, and 3S-expressing clones shows a strong effect on mdr3 and a very weak effect on mdr1. The basis for the differential modulating effect on ACT resistance is unclear but suggests that, in addition to $Ser^{939/941}$, other protein determinants are critical for ACT transport. These determinants may be present in mdr3 but absent in mdr1, and consequently mutations at Ser^{939} would have little effect on an mdr1 background. In agreement with this suggestion is the relatively low level of ACT encoded by both the wild-type (1S) and mutant (1F) mdr1 proteins.

The sequence of *mdr3* reported independently by us (6) and by Hsu *et al.* (*mdr1a*, ref. 7) differs at position 939 (Phe → Ser) and at positions 526 and 527 (Gln-Leu → His-Val) and the drug-resistance phenotypes of cells expressing either versions of the gene are quite different, with mdr1a expressed in J7.V3.1 cells (19) appearing to be a more efficient drug efflux pump than mdr3 (3F). The phenotype of mdr3-S (3S) clones generated here more closely resembles that of J7.V3.1 cells overexpressing mdr1a, suggesting that the $Ser^{939} \rightarrow Phe^{939}$ substitution is responsible for the functional differences initially detected between mdr3 and mdr1a. Interestingly, this substitution also maps at the homologous position (TM11) of two pfmdr1 residues mutated in CLQ-resistant isolates of *P. falciparum*. CLQ resistance in *P. falciparum* is caused by an increased ATP-dependent CLQ efflux (21) and is associated with two allelic variants at the *pfmdr1* gene, one allele (7G8) being Ser^{1034} and $Asn^{1042} \rightarrow Cys^{1034}$ and Asp^{1042} substitutions within TM11 of pfmdr1 (Fig. 1). Since there is as yet no direct assay for pfmdr-1 function, it has not been formally established if both or either of the two TM11 substitutions in pfmdr1 are responsible for CLQ resistance. Nevertheless, these findings and the present results strongly suggest that the predicted TM-11 domain of mdr and pfmdr-1 proteins is critical for substrate recognition and transport. Besides their high degree of hydrophobicity, no significant sequence homology is detected between the TM11 domains of mdr1/3 and pfmdr1 proteins. However, both segments have the potential to form amphipathic helices (Fig. 1). It is interesting to note (*i*) that the mdr $Ser^{939/941}$ residues and the two mutated residues of the 7G8 allele of *pfmdr1* gene fall within the predicted hydrophilic side of this helix and (*ii*) that mdr $Ser^{939/941}$ and pfmdr1 Ser^{1034} map near what appears to be the boundary of the hydrophilic side of this amphipathic helix.

The study presented here has identified TM11, in general, and the unique $Ser^{939/941}$ residue, in particular, as playing a key role in the mechanism of action of mdr1 and mdr3 proteins. These results also suggest that TM11 may be a critical structural determinant of proteins encoded by distantly related members of the *mdr* and *mdr*-like gene family, such as *pfmdr* (14), *CFTR* (16), *STE-6* (15), and *HAM* (35), playing an important role in the mechanism of substrate recognition and transport common to these proteins.

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