Modulatory Effects on Substrate Specificity of Independent Mutations at the Serine$^{939/941}$ Position in Predicted Transmembrane Domain 11 of P-Glycoproteins†

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ABSTRACT: The serine residue located at position 939 and 941 in the predicted transmembrane segment 11 of P-glycoprotein (P-gp) encoded by mouse *mdr3* and *mdr1*, respectively, appears to be important for interaction of chemotherapeutic drugs and reversal agents with P-gp. To further understand the role of this residue in this process and to identify the structural requirements involved, we have replaced this serine residue by alanine, cysteine, threonine, tyrosine, tryptophan, and aspartic acid and tested the effect of these mutations on the overall activity and substrate specificity of *mdr1* and *mdr3*. All mutant proteins could be expressed at high levels in the membrane fractions of LR73 Chinese hamster cells transfected with the corresponding mutant cDNAs. All introduced mutations had limited effect on the capacity of *mdr1* and *mdr3* to confer resistance to vinblastine. The modulatory effect of mutations on resistance to colchicine, adriamycin, and actinomycin D was more dramatic. The hydroxyl group of serine did not seem essential for interaction with these drugs since mutant *mdr1* and *mdr3* bearing alanine or cysteine at that position behaved essentially as wild type, while threonine-bearing mutants showed significantly reduced resistance to these drugs. The insertion at that site of residues with bulkier side chains had more complex effects on P-gp function. While introducing tyrosine, tryptophan, or aspartic acid caused an almost complete loss of colchicine and adriamycin resistance in both *mdr1* and *mdr3*, the replacement to tyrosine or tryptophan had the opposite effect on *mdr1* and *mdr3* for actinomycin D resistance, causing either a 3-fold increase or a 4–8-fold decrease in resistance to this drug, respectively. Drug accumulation and efflux studies indicated that the modulatory effect of the mutations detected in cell survival assays were paralleled by a concomitant modulation of drug transport by mutant proteins. These results indicate that Ser$^{939/941}$ plays a key role in the interaction of colchicine and adriamycin with P-gp and that the size of the carbon side chain is the primary structural determinant at that site. Interaction of P-gp with actinomycin D seems to involve additional nonoverlapping determinants, distinct in *mdr1* and *mdr3*.

Cellular resistance to structurally and functionally unrelated drugs (multidrug resistance, MDR)[1] is caused by the overexpression of a group of membrane proteins known as P-glycoproteins, or P-gps [reviewed in Endicott and Ling (1989) and Roninson (1991)]. P-gps have been shown to bind drugs (Cornwell et al., 1986; Safa et al., 1986, 1989) and ATP analogs (Cornwell et al., 1987; Schur et al., 1989) and to possess ATPase activity (Hamada & Tsuruo, 1988). They are believed to function as membrane-bound, ATP-dependent drug efflux pumps to reduce the intracellular accumulation of drugs in resistant cells. Additional biological activities such as chloride channel (Valverde et al., 1992), ATP carrier (Abraham et al., 1993), pH regulator (Roepe, 1992), and lipid flippase (Higgins & Gottesman, 1992) have recently been proposed for P-gp. P-gps are encoded by a small family of closely related *mdr* genes composed of three members in rodents, *mdr1*, *mdr2*, and *mdr3* (Gros et al., 1986, 1988; Devault & Gros, 1990; Hsu et al., 1990). Predicted amino acid sequence analyses suggest that P-gps are integral membrane proteins formed by twelve putative transmembrane (TM) domains and two nucleotide binding (NB) folds. P-gps form part of a large family of structurally related ABC (ATP binding cassette) transport proteins (Higgins et al., 1990).

Recent studies have suggested that recognition of structurally heterogeneous MDR substrates by P-gp implicates membrane-associated domains of the protein. Energy transfer experiments using daunomycin and a photoactivatable membrane-specific probe suggest that hydrophobic MDR drugs may be recognized by P-gp in association with the membrane lipid bilayer (Raviv et al., 1990). Epitope mapping studies of P-gp tryptic peptides photolabeled with drug analogs such as [^{3}H]azidopine (AZD) and [^{125}I]iodoazidoarylprazosin (IAAP) (Greenberger et al., 1990; Safa et al., 1987, 1990b; Yang et al., 1988) have identified a minor drug binding site (25% of label) within the TM1–6 segment and a major binding site (75%) within a 6-kDa V8 protease digestion fragment overlapping the TM11–12 domain (Bruggemann et al., 1989, 1992; Yoshimura et al., 1989; Greenberger et al., 1990, 1991). Functional analysis of chimeric (Buschman & Gros, 1991; Dhir & Gros, 1992) and mutant P-gps with altered substrate specificities point at TM domains as important determinants for substrate interactions. In human *MDR1*, a Gly^{185} to Val^{185} substitution between TM2 and TM3 was found associated with increased colchicine (COL) resistance and decreased vinblastine (VBL) resistance (Choi et al., 1988). Mutation

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[1] ACT, actinomycin D; ADM, adriamycin; AZD, azidopine; COL, colchicine; FCS, fetal calf serum; IAAP, iodoazidoarylprazosin; MDR, multidrug resistance; MEM, minimal essential medium; NB, nucleotide binding; P-gp, P glycoprotein; PRG, progesterone; pst, position; SRB, sulforhodamine B; TM, transmembrane; VBL, vinblastine; VRP, verapamil.

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of proline residues 233 (TM4), and 866 (TM10) to alanine greatly reduce the capacity of *MDR1* to confer adriamycin (ADM), COL, and actinomycin D (ACT) resistance but not VBL resistance (Loo & Clarke, 1993). In hamster *pgp1*, a Gly^{338}-Ala^{339} to Ala^{338}-Pro^{339} replacement within TM6 was associated with continuous selection and increased resistance to ACT (Devine et al., 1992). Furthermore, mutations within or near TM domains of other members of the ABC family of transport proteins (*CFTR*, *pfmdr*, and *mtp2*) also appear to alter substrate specificity (Foote et al., 1990; Anderson et al., 1991; Powis et al., 1992).

We have recently shown that a single Ser to Phe substitution within TM 11 (pst 941, *mdr1*; pst 939, *mdr3*) strongly modulates the overall activity and the substrate specificity of P-gp (Gros et al., 1991): While this Ser to Phe replacement had little effect on VBL resistance (2–3-fold decrease), it strongly modulated the degree of COL and ADM resistance conferred by mutant P-gps (10–30-fold decrease) (Gros et al., 1991). Biochemical analysis of cell clones expressing either wild-type (1S, 3S) or mutant P-gps (1F, 3F) showed that the mutation affected the capacity of P-gp to reduce cellular drug accumulation and to mediate efflux of preloaded drug (Kajiji et al., 1993). Drug cytotoxicity and drug transport assays carried out in the presence of verapamil (VRP) or progesterone (PRG) suggested that the Ser to Phe substitution also reduced the capacity of these two reversal agents to modulate P-gp activity. Labelling studies with photoactivatable P-gp ligands iodoazidoarylprazosin (IAAP) and azidopine (AZD) indicated that the mutation reduced drug transport by impairing initial drug binding to P-gp (Kajiji et al., 1993). Taken together, these results showed that the $Ser^{939/941}$ residue within TM11 is a key determinant for the binding of drug molecules and known modulators of P-gp.

MATERIALS AND METHODS

Site-Directed Mutagenesis. Site-directed mutagenesis of *mdr1* and *mdr3* cDNA subfragments was carried out using single-stranded M13 phage DNA templates and a commercially available mutagenesis system (Amersham; Arlington Heights, IL). *Sma*I–*Pst*I fragments (1.8 kb) from *mdr1* and *mdr3* overlapping the 3′ half of each cDNA, including $Ser^{939/941}$ in TM11, were subcloned into the corresponding site of bacteriophage vector M13mp19. To facilitate subsequent excision and subcloning of individual mutated fragments, novel restriction sites flanking predicted TM11 on either side were first introduced in each cDNA without alteration of the amino acid sequence of the corresponding protein segment: in *mdr1*, novel *Nru*I (pst 2726) and *Sna*BI (pst 3020) sites were introduced using mutant oligonucleotide primers N1, (5′)CTTCTGCTCGCGAGTAAC(3′), and S1, (5′)CTCAATGATACGTATGATATGA(3′), respectively; in *mdr3*, novel *Nru*I (pst 2722) and *Spe*I (pst 2913) sites were introduced using mutant oligonucleotide primers N1 and S2, (5′)GAGAATACTAGTAGAACATTT(3′), respectively. These mutated fragments were sequenced and reconstructed into the respective full-length *mdr1* and *mdr3* cDNAs, using *Sma*I and *Pst*I. The wild-type Ser^{939} of *mdr3* and Ser^{941} of *mdr1* were independently mutated to novel residues, using the core sequence-specific oligonucleotides (5′)CTGGGTGAAGGAGAACGT(3′) for *mdr1* (pst 2831–2814) and (5′)ATCACGTTCTCCTTCACC(3′) for *mdr3* (pst 2806–2823) modified at the Ser position (underlined) to introduce Ala (GGC, *mdr1*; GCC, *mdr3*), Cys (GCA, *mdr1*; TGC, *mdr3*), Thr (GGT, *mdr1*; ACC, *mdr3*), Trp (CCA, *mdr1*; TGG, *mdr3*), Tyr (GTA, *mdr1*; TAC, *mdr3*), or Asp (GTC, *mdr1*; GAC, *mdr3*) residues. The presence of the introduced mutation and the integrity of the mutagenized segment between the two flanking restriction sites used for excision (*mdr1*, *Nru*I–*Sna*BI; *mdr3*, *Nru*I–*Spe*I) was verified by nucleotide sequencing prior to reconstructing the respective full-length cDNAs. Nucleotide sequencing was by the dideoxy chain-termination method of Sanger (1977), using modified T7 DNA polymerase (Sequenase, USB).

Cell Lines and Tissue Culture. Full-length wild-type and mutant *mdr3* and *mdr1* cDNAs were cloned into the mammalian expression vector pEMC2b and introduced in LR73 Chinese hamster ovary cells by cotransfection with the dominant selectable marker pSV2*neo*, followed by selection in medium containing genetycin (G418), as previously described (Gros et al., 1991). Mass populations of G418-resistant ($G418^R$) colonies were harvested as pools and further selected for P-gp expression by growth in medium containing vinblastine (VBL) at 25 or 50 ng/mL. Mass populations of 50–100 VBL-resistant (VBL^R) colonies were pooled, expended in culture, and frozen at −80 °C in individual aliquots (10% dimethyl sulfoxide and 90% fetal calf serum, FCS). Independent aliquots were thawed out and used for each subsequent experiment. All cell populations were maintained in α minimum essential medium (α-MEM) supplemented with 10% FCS, 2 mM glutamine, penicillin (50 units/mL), and streptomycin (50 μg/mL). Tissue culture medium, serum, and supplements were purchased from Gibco/BRL (Grand Island, NY).

Drug Cytotoxicity Assays. A modification of a cell survival assay (Skehan et al., 1989) based on sulforhodamine B (SRB) staining of cellular proteins was used. Briefly, 5×10^3 cells of either control cells or mass populations of *mdr*-transfected cells expressing either wild-type or mutant P-gps were plated in 96-well titer plates in increasing concentrations of ADM, COL, VBL, and ACT and incubated for 72 h at 37 °C. Cells were washed once in ice-cold PBS and fixed in 17% trichloroacetic acid in phosphate-buffered saline (PBS) for 45 min at 4 °C and then washed extensively in tap water. Total cell proteins were stained with 0.4% SRB in 1% acetic acid for 15 min at room temperature, followed by four washes with 1% acetic acid. The plates were dried, the stain was dissolved in 10 mM Tris (pH 9.0), and quantitation was carried out using an automated ELISA plate reader set at 490 nm. The relative plating efficiency of each mass population was calculated by dividing the absorbance observed at a given drug concentration by the absorbance detected in the same population in medium devoid of drug and is expressed as a percentage. The D_{50} is defined as the drug dose required to reduce the plating efficiency of each population by 50%. ADM was obtained from Adria laboratories, COL and VBL were purchased from Sigma, and ACT was from Merck, Sharp and Dohme.

Drug Transport Assays. For drug transport experiments, drug-sensitive LR control cells and mass populations of *mdr*-transfected clones expressing individual wild type and mutant P-gps were grown to confluency and harvested after trypsin treatment (2 min at 37 °C). Cells were then seeded in 6-well titer plates (1.2×10^6/well) in Dulbecco's minimal essential medium (D-MEM) lacking VBL but supplemented with 10% FCS, glutamine, and antibiotics. Twenty-four hours later, medium was removed and replaced by D-MEM containing 5% FCS, [^{14}C]ADM (specific activity 47.3 μCi/μmol, Amersham), used at a final concentration of 2 μM (specific activity 2.4 μCi/μmol) with (+ATP) or without (−ATP) glucose, glutamine, sodium pyruvate, and 5% FCS or 5% dialyzed FCS, respectively. For ATP depletion, cells were sequentially

incubated at 37 °C with rotenone (20 ng/mL) for 15 min and 2-deoxyglucose (2 mM) for an additional 15 min in D-MEM lacking glucose and glutamine prior to initiation of transport. For drug efflux experiments, cells were loaded with ADM for 1 h under conditions of ATP depletion as above and washed twice with ice-cold PBS, and efflux was initiated by addition of fresh complete medium (+ATP) without ADM. At different times after initiation of transport (incubations at 37 °C), cells were washed twice with ice-cold PBS and removed from the well by trypsin treatment. Cells were washed once with PBS and counted with a hemocytometer, and cell-associated radioactivity was measured directly by adding a sample to liquid scintillation fluid (Beckman Ready Safe), followed by vortexing and scintillation counting. Results are expressed as picomoles of ADM per 10^6 cells. Three independent measurements were obtained for each experimental point.

Detection of P-gps. Wild-type and mutant P-gps were detected by Western blotting in purified membrane fractions of mass populations of *mdr*-transfected clones, as previously described (Gros et al., 1991). Briefly, individual mass populations of VBL^R cell clones were grown to confluency in 175-cm^2 flasks and harvested by trypsinization, and crude membrane extracts were prepared after lysis of the cells in hypotonic medium and homogenization with a Dounce homogenizer (Schurr et al., 1989). Proteins were determined using an amido black-based assay (Bio-Rad), and the preparations were stored at −70 °C in 10 mM Tris, pH 8, and 40% glycerol. Membrane proteins were separated by sodium dodecyl sulfate–polyacrylamide (7.5%) gel electrophoresis (SDS–PAGE) and transferred by Western blotting to nitrocellulose membranes. The blots were treated at 4 °C for 16 h with 1% bovine serum albumin (fraction V) in TBST (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, and 0.02% Tween 20) to reduce nonspecific binding. P-gps were detected by incubating the blots (1 h at 20 °C) with the mouse anti-Pgp monoclonal antibody C219 (Centocor Inc., Philadelphia, PA) used at a dilution of 1:500. After being washed with TBST, the blots were further incubated with a goat anti-mouse IgG antiserum linked to alkaline phosphatase (1:3000 dilution). The blots were washed and developed in alkaline phosphatase buffer (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, and 5 mM $MgCl_2$) containing 5-bromo-4-chloro-3-indolylphosphate p-toluidine (0.17 ng/mL) and nitroblue tetrazolium chloride (0.33 ng/mL) substrates. Blots were dried and photographed.

RESULTS

Epitope mapping experiments using P-gp photolabeled with drug analogs such as AZD and IAAP have identified the TM11–12 segment as a strong drug binding site in the protein (Bruggemann et al., 1989, 1992; Yoshimura et al., 1989; Greenberger, et al., 1990, 1991). In parallel studies, we have demonstrated that a single Ser to Phe substitution within TM11 of P-gps encoded by mouse *mdr1* and *mdr3* strongly modulated both the overall activity and the substrate specificity of the two drug efflux pumps, suggesting that this residue may be involved in drug binding (Gros et al., 1991; Kajiji et al., 1993). In order to further study the key structural and functional determinants of $Ser^{939/941}$ in TM11 that are required for efficient drug interaction at this site and that may be disrupted by the Phe substitution, we have introduced a series of amino acid replacements at that position and have measured the overall activity and substrate specificity of the corresponding mutant P-gps. The importance of the hydroxyl group of serine was tested by independent substitutions to Ala (A), Thr (T), and Cys (C). Although the three residues have comparable size side chains, only Thr and Cys are capable of participating in hydrogen bonding. The importance of the relative size of the side chain within the context of a hydroxyl group was also tested by independent substitutions to Tyr (Y) and Trp (W). Finally, the effect of introducing a charged residue at that position likely to affect the structural and functional properties of TM11 was tested by replacing $Ser^{939/941}$ by an Asp (D). A schematic illustration of the mutagenesis scheme, including the sequence of mutant oligonucleotides, is shown in Figure 1. The effects of these mutations on P-gp function were independently analyzed in two distinct molecular backbones, i.e., in P-gps encoded by mouse *mdr1* and *mdr3*. The mutations were introduced by site-directed mutagenesis of appropriate *mdr* subfragments cloned in M13mp18, followed by reconstruction of the mutations in the respective full-length *mdr* cDNAs. The integrity of the mutagenized fragments and of the restriction sites used for cloning and excision was ascertained by nucleotide sequencing and restriction enzyme fragmentation prior to cloning in the mammalian expression vector pEMC2b.

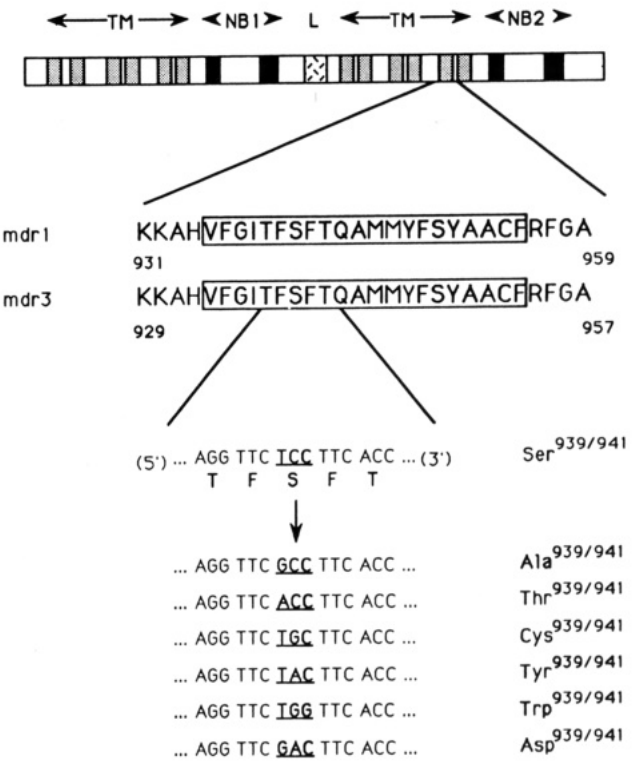

FIGURE 1: Site-directed mutagenesis of *mdr1* and *mdr3*. A schematic representation of the predicted structural domains of P-glycoproteins encoded by mouse *mdr1* and *mdr3* is shown at the top. The predicted transmembrane domains (TM) are numbered from 1 to 12, and the nucleotide binding folds (NB1, NB2) and the linker region (LK) joining the two homologous halves of P-gp are indicated. The nucleotide sequences of the oligonucleotide primers used to introduce discrete mutations at Ser^{941} of *mdr1* and at the corresponding Ser^{939} of *mdr3* are indicated.

Full-length mutant and wild-type *mdr1* and *mdr3* cDNAs cloned into expression vector pEMC2b were introduced into drug-sensitive Chinese hamster ovary cells LR73 by cotransfection of each *mdr* mutant with the dominant selectable marker pSV2*neo*, followed by selection in the drug genetycin (G418). Mass populations of independent $G418^R$ colonies were pooled and expended in culture, and the capacity of wild-type or mutant P-gps to confer drug resistance was tested by plating these mass populations in medium containing two concentrations of VBL (25 and 50 ng/mL). Fourteen days

Table I: Drug Survival Characteristics of Mass Populations of Cell Clones Stably Expressing Wild-Type and Mutant *mdr3* Genes

position 939 residue	D_{50}[a] (ng/mL)			
	VBL	COL	ADM	ACT
Ser	250 ± 40 (23×)[b]	1300 ± 200 (33×)	520 ± 60 (22×)	130 ± 10 (72×)
Ala	220 ± 30 (20×)	680 ± 40 (17×)	300 ± 10 (13×)	120 ± 20 (67×)
Cys	230 ± 30 (21×)	560 ± 60 (14×)	250 ± 40 (10×)	90 ± 20 (50×)
Thr	230 ± 30 (21×)	330 ± 40 (8×)	130 ± 20 (5×)	36 ± 8 (20×)
Tyr	80 ± 8 (7×)	160 ± 10 (4×)	40 ± 4 (2×)	16 ± 3 (9×)
Trp	130 ± 20 (12×)	98 ± 7 (2.5×)	50 ± 10 (2×)	38 ± 7 (21×)
Asp	140 ± 20 (13×)	150 ± 20 (4×)	80 ± 10 (3×)	11 ± 3 (6×)
LR73	11 ± 5	40 ± 10	24 ± 4	1.8 ± 0.6

[a] The drug survival of Chinese hamster LR73 drug-sensitive cells (LR73) and mass populations of cell clones transfected with either wild-type (Ser) or mutant *mdr3* bearing discrete amino acid substitutions at position 939 is expressed as the D_{50} (in nanograms per milliliter), or the dose necessary to reduce the plating efficiency of the control and transfected cells by 50%. Abbreviations: vinblastine (VBL); colchicine (COL); adriamycin (ADM); actinomycin D (ACT). [b] The resistance index is the degree of resistance above background levels expressed in LR73 cells and is shown in parentheses.

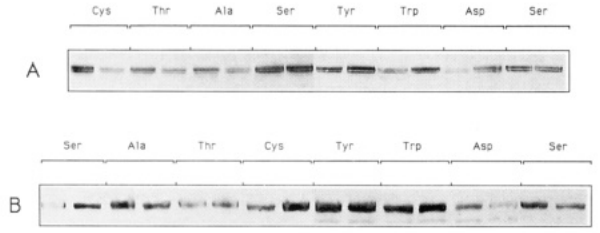

FIGURE 2: Detection of wild-type and mutant P-gps in mass populations of stably transfected cells. Two independent mass populations of G418^R cell clones cotransfected with pSV2neo and either wild-type (Ser) or mutant cDNAs (panel A, *mdr3*; panel B, *mdr1*) were further selected in medium containing vinblastine (25 or 50 ng/mL) and analyzed for the presence of P-gps. Twenty-microgram aliquots of proteins from crude membrane extracts from each mass population were used for Western analysis with the mouse anti-P-gp monoclonal antibody C219. The apparent molecular masses of P-gps encoded by *mdr3* (160 kDa) and *mdr1* (180 kDa) were established using molecular mass markers myosin (200 kDa) and phosphorylase *b* (97 kDa) [not shown]. Membranes from cells expressing the Ser wild-type P-gps were used as internal standard for independent Western blots containing membranes from either Cys/Thr/Ala or Tyr/Trp/Asp mutants, respectively, and are shown twice.

later, mass populations of VBL^R colonies were harvested and frozen as two independent mass populations (25–100 colonies each), and this was repeated for each *mdr1* and *mdr3* mutant. Results from these experiments showed that all *mdr1* and *mdr3* mutants bearing discrete substitutions at position 939/941 conferred drug resistance in this assay, as VBL^R colonies could be obtained in all cases. However, in the case of *mdr1* and *mdr3* mutants bearing either Trp, Tyr, or Asp, fewer colonies than either wild-type or other mutants were obtained at these VBL concentrations. The presence of immunoreactive wild-type and mutant P-gps in membrane fractions from these mass populations was then tested by Western blotting, using the mouse anti-P-gp monoclonal antibody C219 (Figure 2). P-gps of apparent molecular mass 180 kDa (*mdr1*, Figure 2B) and 160 kDa (*mdr3*, Figure 2A) were identified in membrane fractions from transfected cells expressing all mutant forms of *mdr1* and *mdr3*, respectively. The differences in apparent molecular mass of P-gps encoded by *mdr1* and *mdr3* are believed to result from differential posttranslational modification of the two proteins, suggesting that the mutations introduced here did not affect targeting and processing of the respective polypeptides. The amount of specific wild-type and mutant P-gps expressed in these mass populations were not identical but appeared generally similar (Figure 2). Nevertheless, all mutant P-gps on either *mdr1* or *mdr3* background were functional, readily conferred VBL resistance, and appeared properly expressed and posttranslationally modified in the membrane fraction of transfected cells.

The modulatory effect of the introduced mutations on the substrate specificity of P-gps encoded by *mdr1* and *mdr3* was then analyzed. For this, the degree of cellular resistance conferred by each mutant P-gp to ADM, COL, ACT, and VBL was quantitated using a cytotoxicity assay based on a modified sulforhodamine B protein staining assay. These assays were carried out in quadruplicate, using duplicate measurements from two independently derived pools of VBL^R colonies, and results are shown in Table I for *mdr3* and in Table II for *mdr1*. The data are presented as the D_{50}, which is defined as the drug dose required to reduce the plating efficiency of each mass population of transfected clones by 50%. As noted earlier for the Phe^{939} *mdr3* mutant (*mdr3F*; Gros et al., 1991), and as detected in VBL selection of the mass populations of G418^R clones, novel substitutions at Ser^{939} did not have a strong effect on the degree of VBL resistance conferred by *mdr3*. Cell clones expressing either wild-type or mutant *mdr3* showed D_{50}s for VBL that were similar and within a factor of 2 from each other, except for *mdr3Y*, which showed a 3-fold reduction in VBL resistance over the *mdr3S* control (Table I). On the other hand, the effect of these substitutions on *mdr3*-encoded resistance to other drugs was much more pronounced. The conservative substitution of Ser^{939} by small nonpolar residues Ala or Cys in *mdr3A* and *mdr3C*, respectively, had little effect on either the overall activity or the substrate specificity of *mdr3*. Both *mdr3A* and *mdr3C* mutants conferred levels of resistance to VBL, COL, ADM, and ACT that were similar and did not vary by more than 2-fold from those expressed by wild-type *mdr3S* (Table I). On the other hand, the nonconservative replacement of Ser^{939} by either Trp (*mdr3W*), Tyr (*mdr3Y*), or Asp (*mdr3D*) strongly reduced the activity of *mdr3* toward ADM, COL, and ACT. The three mutants gave qualitatively similar results for these drugs and showed D_{50} values generally reduced by a factor of 8–10-fold when compared to those measured for wild-type *mdr3S*. This reduction in activity was most obvious for ADM, where *mdr3W*, *mdr3Y*, and *mdr3D* only showed a small residual activity toward this drug, expressing levels of resistance only 1.5–3-fold above background determined for LR73 drug-sensitive cells. Finally, introducing a threonine at position 939 (*mdr3T*) produced a mutant P-gp expressing levels of resistance to COL, ACT, and ADM that were intermediate between the near wild-type levels measured for *mdr3S*/*mdr3A*/*mdr3C* and the strongly reduced levels expressed by *mdr3W*/*mdr3D*. Overall, these results indicate that none of the five novel residues introduced at position 939 of *mdr3* produced in *mdr3* a major effect on VBL resistance. However, introducing at that position larger residues with bulkier side chains strongly altered the capacity of *mdr3* to

Table II: Drug Survival Characteristics of Mass Populations of Cell Clones Stably Expressing Wild-Type and Mutant *mdr1* Genes

position 941 residue	D_{50}[a] (ng/mL)			
	VBL	COL	ADM	ACT
Ser	120 ± 10 (8×)[b]	600 ± 30 (15×)	200 ± 20 (9×)	7 ± 1 (3.5×)
Ala	139 ± 6 (9×)	580 ± 90 (15×)	190 ± 20 (8×)	8 ± 1 (4×)
Cys	190 ± 90 (13×)	600 ± 100 (15×)	220 ± 8 (10×)	9 ± 1 (4.5×)
Thr	80 ± 20 (5×)	150 ± 30 (4×)	50 ± 10 (2×)	5 ± 1 (2.5×)
Tyr	90 ± 8 (6×)	80 ± 4 (2×)	31 ± 4 (1.5×)	19 ± 1 (9×)
Trp	80 ± 20 (5×)	60 ± 8 (1.5×)	30 ± 1 (1.5×)	20 ± 1 (10×)
Asp	50 ± 10 (4×)	80 ± 10 (2×)	36 ± 2 (1.5×)	5 ± 1 (2.5×)
LR73	15 ± 1	40 ± 2	23 ± 1	2 ± 0.3

[a] The drug survival of Chinese hamster LR73 drug sensitive cells (LR73) and mass populations of cell clones transfected with either wild-type (Ser) or mutant *mdr1* bearing discrete amino acid substitutions at position 941 is expressed as the D_{50} (in nanograms per milliliter), or the dose necessary to reduce the plating efficiency of the control and transfected cells by 50%. Abbreviations: vinblastine (VBL); colchicine (COL); adriamycin (ADM); actinomycin D (ACT). [b] The resistance index is the degree of resistance above background levels expressed in LR73 cells and is shown in parentheses.

confer resistance to other MDR drugs, namely, COL, ACT, and ADM.

Mutational analysis of the equivalent position in *mdr1*, i.e., Ser^{941}, produced results that were similar to those obtained with *mdr3* mutants for drugs VBL, ADM, and COL but were surprisingly different for ACT (Table II). First, the five amino acid substitutions at that position had little effect on the degree of VBL resistance expressed by mutant proteins when compared to their wild-type (*mdr1S*) counterpart, with all D_{50} values for VBL falling within a 2-fold range. Second, conservative substitutions of Ser^{941} to Ala (*mdr1A*) and Cys (*mdr1C*) residues resulted in mutant P-gps with characteristics (D_{50}s) indistinguishable from that of the wild-type protein (*mdr1S*), and this was true for all drugs tested. Third, nonconservative substitutions (Tyr, Trp, and Asp) at position 941 of *mdr1* had a strong modulatory effect on the degree of resistance to COL and ADM expressed by the mutant proteins resulting in 6–8-fold reductions in the levels of resistance to these drugs over those measured in *mdr1S*. As for *mdr3* mutants, this effect was most noticeable in the case of ADM, with mutant proteins (*mdr1Y*/*mdr1W*/*mdr1D*) showing little if any activity toward this drug (1.5× resistance). Fourth, the *mdr1T* mutant, as did its *mdr3* counterpart, conferred levels of resistance that were intermediate between those measured for the *mdr1A*/*mdr1S*/*mdr1C* and the *mdr1Y*/*mdr1W*/*mdr1D* segregating groups. Interestingly, and as opposed to results obtained when the same mutations were introduced in *mdr3*, the replacement of Ser^{941} by either Tyr or Trp in *mdr1Y* and *mdr1W* resulted in mutant proteins conveying ACT resistance levels superior to those conferred by wild-type *mdr1S* by a factor of 3. These results indicate that the modulatory effects of amino acid substitutions at $Ser^{941/939}$ are similar in *mdr1* and *mdr3* P-gp backbones for all drugs tested, except in the case of the Tyr and Trp substitutions, which show opposite effects on the degree of ACT resistance expressed by the mutant proteins, being either severely reduced for *mdr3* or enhanced for *mdr1*.

To determine if variations in activity observed among mutant P-gps in drug survival experiments did indeed reflect parallel modulation of the transport function of these proteins, kinetics of [^{14}C]adriamycin cellular accumulation and efflux were measured in mass populations of cell clones expressing either wild-type or mutant *mdr3* P-gps (Figure 3). The kinetic analysis of [^{14}C]ADM accumulation in these various cell populations demonstrated a very tight correlation between the degree of drug resistance expressed by these cells and the extent of radiolabeled drug accumulation during the 3-h assay. *mdr3S*-expressing clones accumulated little if any [^{14}C]ADM, followed in order of increasing accumulation by *mdr3A*, *mdr3C*, *mdr3T*, *mdr3D*, and finally by *mdr3W* and *mdr3Y*, which showed kinetics and final levels of drug accumulation indistinguishable from those observed in control drug-sensitive LR73 cells (Figure 3A). The differential degree of [^{14}C]-ADM accumulation detected in wild-type and mutant *mdr3*-expressing cell clones was strictly ATP-dependent and was abrogated by treatment of the cells with deoxyglucose and rotenone prior to initiation of the transport reaction (Figure 3B). Finally, the differential capacity of wild-type and mutant *mdr3* P-gps to restrict intracellular [^{14}C]ADM accumulation was paralleled by a concomitant increase in the capacity of these cells to extrude drug preloaded under conditions of ATP depletion (Figure 3C). Taken together, these results demonstrate that the modulatory effect of mutations at position 939 on the degrees of cellular drug resistance expressed by mutant P-gps and measured in cytotoxicity assays is paralleled in these cells by a commensurate impairment of their drug transport function.

DISCUSSION

Identifying the specific P-gp segments and discrete amino acid residues implicated in the recognition and transport of a large number of structurally unrelated compounds that form the MDR spectrum would be a vital prerequisite to the design by medicinal chemistry of more effective antitumor drugs or specific agents capable of blocking P-gp function. The biochemical analysis of P-gp by epitope mapping of photolabeled peptides suggests that the membrane-associated domains of P-gp are primary sites for initial interaction and recognition of drug molecules within the context of the lipid bilayer (Raviv et al., 1990; Bruggemann et al., 1989, 1992; Yoshimura et al., 1989; Greenberger et al., 1990, 1991). On the other hand, genetic analysis of variant P-gps of human (Choi et al., 1988; Loo & Clarke, 1993), hamster (Devine et al., 1992), and murine origins (Gros et al., 1991) conveying altered drug resistance profiles has shown that discrete point mutations within or near TM domains modulate substrate specificity. Of particular interest is the phenotypic characterization of the Gly^{185} to Val^{185} substitution in human *MDR1* which causes decreased resistance to VBL and ACT but increased resistance to COL (Choi et al., 1988). Photolabeling experiments with analogs of VBL, COL, and AZD have indicated that decreased drug resistance in this mutant was linked to increased binding of the photoactivatable ligands (Safa et al., 1990a), leading the authors to propose that the pst 185 mutation did not affect the initial drug binding to P-gp (on rate) but rather the subsequent dissociation of drugs from P-gp (off rate). They further speculated that P-gp-mediated drug transport may involve at least two types of drug/protein interactions, with an initial drug binding site and a second site distinct from the first one but implicated in

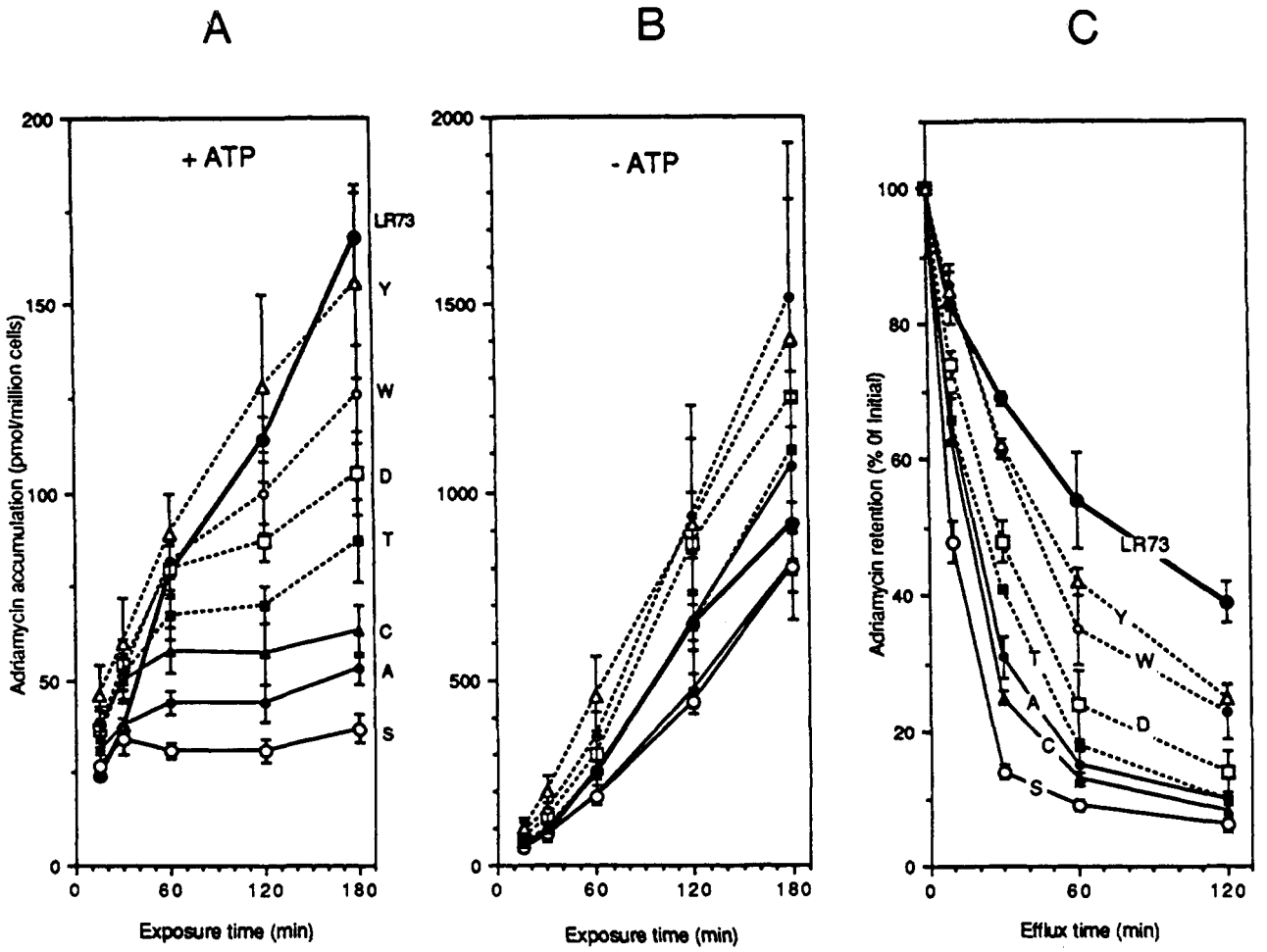

FIGURE 3: [^{14}C]Adrimaycin Accumulation. (A) Monolayer cultures of drug-sensitive LR73 cells (LR) and cell clones transfected with either wild-type (Ser, S) or mutant *mdr3* bearing either Ala (A), Cys (C), Thr (T), Asp (D), Trp (W), or Tyr (Y) at position 939 were incubated at 37 °C with [^{14}C]ADM at a final concentration of 2 μM (specific activity 2.4 μCi/μmol), as described in Materials and Methods. Accumulation of radioactivity was assayed at given times and is expressed as picomoles of incorporated radioactivity/10^6 cells. (B) ATP depletion (-ATP) was achieved by treating the cells with rotenone and deoxyglucose prior to initiation of transport. (C) For efflux, cells were loaded with [^{14}C]ADM for 1 h under conditions at ATP depletion. Cells were then washed with ice-cold PBS, fresh complete medium allowing ATP synthesis was added to initiate efflux, and cell-associated radioactivity was monitored over time. The amount of [^{14}C]ADM retention was determined and is expressed as the percentage of the initial intracellular drug measured at T_0.

drug release and efflux, with ATP hydrolysis required for translocation of drugs both from the first to the second site and from the second site to the extracellular milieu (Safa et al., 1990a). Although speculative, such a mechanism involving ATP-mediated cooperative interactions between distinct P-gp domains is in agreement with the identification of nonoverlapping drug binding sites on the two homologous halves of P-gp (Bruggemann et al., 1989, 1992; Yoshimura et al., 1989; Greenberger et al., 1990, 1991), the functional analysis of discrete P-gp mutants bearing single amino acid substitution in either NB site (Azzaria et al., 1989), and the analysis of chimeric P-gps constructed by exchanging homologous domains of functionally distinct P-gp family members (Buschman & Gros, 1991; Dhir & Gros, 1992).

Independently, we have recently reported that a single Ser to Phe substitution at position 939/941 of mouse *mdr3* and *mdr1* profoundly altered the biological properties of the two encoded P-gps (Gros, et al., 1991) by affecting initial binding of drugs and reversal agents (on rate) to P-gp (Kajiji et al., 1993). Ser$^{939/941}$ was also found to be important for peptide transport by *mdr3*: We have shown that *mdr3* can complement a null allele at the *mdr* homolog *STE6* locus in *Saccharomyces cerevisiae* and can therefore mediate transport of the α peptide pheromone in yeast cells (Raymond et al., 1992). The introduction of the Ser to Phe substitution at pst 939 abrogates the capacity of *mdr3* to complement the biological activity of STE6 (Raymond et al., 1992). Finally, the homologous Ser residue in TM11 of the *Plasmodium falciparum mdr* homolog *pfmdr1* is one of two residues mutated in the 7G8 allele of this gene found in chloroquine-resistant isolates of this parasite (Foote et al., 1990). These findings clearly indicate that TM11 is also important in substrate binding and transport by evolutionary distant *mdr* homologs. Although no sequence homology exists between the predicted TM11 segments of *mdr1/3*, *pfmdr1*, and *STE6*, they can all be arranged easily into amphiphilic helices, with the Ser$^{939/941}$ residues (in *mdr* and *pfmdr1*) mapping the boundary of the hydrophilic and hydrophobic planes of the helix. In the present study, we have further studied the structural and functional requirements of Ser$^{939/941}$ for efficient initial drug binding and transport by P-gp. We have introduced novel amino acids at this site in *mdr1* and *mdr3* and tested the effect of these mutations on overall activity and substrate specificity of the two corresponding P-gps. The effects of the introduced mutations on P-gp function were complex and varied with respect to the specific amino acid introduced, the type of drug tested, and in some instances the parental backbone onto which the mutation was constructed. In the case of VBL, the introduction of novel amino acids at that position had only minor effects on the activity of both *mdr1* and *mdr3* toward that drug,

confirming and extending our previous study of *mdr1* and *mdr3* mutants bearing Phe at that position (Gros et al., 1991). Even the introduction of a negatively charged residue such as Asp within TM11 had only a limited effect on the D_{50} measured for VBL. This is somewhat surprising since none of the human or rodent P-gps harbor such a charged polar residue in the predicted TM domains, suggesting that charged residues at these sites may not have been tolerated to preserve function during evolution of the P-gp family. Nevertheless, these results clearly indicate that $Ser^{939/941}$ plays only a minor role in the interaction of VBL with P-gps encoded by *mdr1* and *mdr3*. In the case of ADM and COL resistance, the modulating effect of the mutations were similar on both *mdr1* and *mdr3* backgrounds, although more dramatic than the effect detected for VBL resistance. While conservative substitutions at $Ser^{939/941}$, such as Ala and Cys, had little effect on the activity of both P-gps toward ADM and COL, the introduction of nonconservative substitutions such as Tyr, Trp, and Asp significantly reduced the activity of both P-gps toward these drugs. These results suggest that $Ser^{939/941}$ is a key determinant for efficient interaction of both *mdr1* and *mdr3* with these two drugs. Taken together, our results suggest that *mdr1* and *mdr3* transport VBL, COL, and ADM by a similar mechanism, which in the case of the two former substrates involves $Ser^{939/941}$. The effect of mutations at position 939/941 on the capacity of *mdr1* and *mdr3* to confer ACT resistance was more complex: Conservative substitutions such as Ala or Cys had little effect on ACT resistance levels, while introduction of an Asp residue strongly decreased the activity of P-gp toward that drug for both *mdr1* and *mdr3*. However, when either a Trp or a Tyr residue was introduced at that position, the modulatory effect was opposite for *mdr1* and *mdr3*, resulting in either a 3-fold increase or a 4–8-fold decrease in D_{50} values for this drug, respectively. These results suggest that ACT transport by P-gp involves independent structural determinants that may map to distinct sites in *mdr1* and *mdr3*. These results are in agreement with our previous analysis on the effect of a Ser to Phe substitution at that site on ACT resistance in *mdr1* and *mdr3* (Gros et al., 1991; Kajiji et al., 1993).

The modulating effects of the introduced mutations on P-gp function appear specific and are unlikely to result from gross alterations in the protein interfering either with appropriate targeting and insertion of P-gp in the plasma membrane compartment or with helix packing within the membrane. This stems from the observations that (1) all *mdr1* and *mdr3* mutants conferred VBL resistance; (2) all mutant proteins were found expressed in the membrane fraction of transfected cells; (3) certain mutations affected the capacity of *mdr1* and *mdr3* to confer resistance to one drug but not another, and (4) some mutations showed effects that were specific and opposite for *mdr1* and *mdr3*.

As detailed above, the mutational analysis presented here shows that $Ser^{939/941}$ is a key structural determinant for efficient interaction of *mdr1* and *mdr3* with ADM and COL. Interactions at that site did not seem to require an intact hydroxyl function perhaps involved in hydrogen bonding, since Ala and Cys could fully substitute Ser at that position while Thr could only partially do so. However, increasing the size of the side chain at that position from Ser to Thr to much bulkier side chains such as those of Tyr, Trp, or the charged Asp profoundly reduced the activity of P-gp toward COL and ADM. Although these results could simply be explained by a local nonspecific effect of the bulkier side chains on discrete helix–helix interactions at a site critical for ADM/COL interaction but less important for VBL interaction, studies from our group have also documented for the drug molecule a minimal size requirement for efficient interactions with P-gp. Structure/activity relationships in three series of COL congeners have suggested two minimal requirements in this molecule for transport by P-gp. These included both an intact nitrogen atom at position C7 of the B ring and a calculated molar refractivity (CMR) value or molar volume superior to 9.7 (Tang Wai et al., 1993). On the other hand, no correlation was detected between the overall lipophilicity of COL analogs and their capacity to interact with P-gp. It is tempting to speculate that the spatial volume requirements for P-gp at position 939/941 necessary for efficient interaction with COL identified herein, and the minimal size requirements for COL (CMR values) identified in the study of COL analogs, are important parameters for efficient and mutual interactions at the same site.

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