Functional Analysis of P-Glycoprotein Mutants Identifies Predicted Transmembrane Domain 11 as a Putative Drug Binding Site†

Shama Kajiji,‡ France Talbot,§ Kart Grizzuti,‡ Valerie Van Dyke-Phillips,‡ Michael Agresti,‖ Ahmad R. Safa,‖ and Philippe Gros*,§

Department of Cancer, Pfizer Central Research, Groton, Connecticut 06340, Department of Biochemistry, McGill University, Montreal, Quebec, Canada H3G 1Y6, and Department of Medicine, University of Chicago, Chicago, Illinois 60637

Received November 18, 1992; Revised Manuscript Received January 28, 1993

ABSTRACT: The substitution of a single serine to phenylalanine residue within the predicted transmembrane domain 11 of P-glycoproteins (P-gps) encoded by mouse *mdr1* (Ser^{941}, 1S; Phe^{941}, 1F) or *mdr3* (Ser^{939}, 3S; Phe^{939}, 3F) strongly modulates both the overall activity and substrate specificity of the two P-gps. In cell clones expressing either wild-type (1S, 3S) or mutant P-gps (1F, 3F), we show that the modulating effect of the mutation on the levels of adriamycin (ADM) resistance detected in drug cytotoxicity assays is paralleled by a similar modulation of the intracellular accumulation and extracellular efflux of radiolabeled adriamycin ([^{14}C]ADM) from preloaded cells. Cytofluorescence studies with ADM on live cells produce similar results and demonstrate strong nuclear ADM accumulation only in drug-sensitive LR cells and in the 1F expressing cells, with little if any accumulation in 1S, 3S, or 3F expressing cells. Drug cytotoxicity and drug transport assays carried out in the presence of verapamil or progesterone suggest that the Ser to Phe substitution also reduces the capacity of these two reversal agents to modulate P-gp activity. Labeling experiments with the photoactivatable P-gp ligands iodoarylazidoprazosin and azidopine indicate a strong reduction in binding of these photoactivatable probes to the mutant P-gps (1F, 3F) as compared to their wild-type counterparts (1S, 3S). These results indicate that the studied mutations in TM11 reduce drug transport by decreasing initial drug binding to P-gp. This phenotype is opposite to that of a mutation near TM3 in human *MDR1* (pst 185), where decreased drug transport is associated with increased drug binding and decreased drug release from P-gp [Safa, A. R., Stern, R. K., Choi, K., Agresti, M., Tamai, I., Metha, N. D., & Roninson, I. B. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 7225–7229].

Multidrug resistance in cultured cells in vitro and tumor cells in vivo is caused by the overexpression of a small group of membrane phosphoglycoproteins called P-glycoproteins (P-gps)[1] (Endicott & Ling, 1989; 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 posess ATPase activity (Hamada & Tsuruo, 1988) and are believed to function as ATP-dependent drug efflux pumps to reduce the intracellular accumulation of drugs in resistant cells. P-gps are encoded by a small family of closely related *mdr* genes, which is composed of three members in rodents (*mdr1*, *mdr2*, *mdr3*) (Gros et al., 1986a, 1988; Devault & Gros, 1990; Hsu et al., 1990) and two members in humans (*MDR1*, *MDR2*) (Chen et al., 1986; Van der Blek et al., 1988). Transfection experiments have demonstrated that human *MDR1* (Ueda et al., 1987) and mouse *mdr1* and *mdr3* (Gros et al., 1986b; Devault & Gros, 1990) can confer mutlidrug resistance to drug-sensitive recipient cells, while human *MDR2* and mouse *mdr2* cannot (Gros et al., 1988; Schinkel et al., 1991). Predicted amino acid sequence analyses of full-length cDNA clones indicate that P-gps are integral membrane proteins formed by 12 transmembrane (TM) domains and 2 nucleotide binding (NB) folds (Gros et al., 1986a; Chen et al., 1986). P-gps form part of a large supergene family of ABC (ATP binding cassette) transport proteins, which include in prokaryotes a large group of bacterial proteins participating in the import or export of amino acids, carbohydrates, and large peptides across the cell membrane (Higgins et al., 1990). In eukaryotes, members of this family include the *Saccharomyces cerevisiae STE6* gene implicated in the transport of the a mating pheromone (McGrath & Varshavsky, 1989), the malarial *Plasmodium falciparum pfmdr1* gene associated with chloroquine resistance (Foote et al., 1989), the *CFTR* chloride channel in which mutations cause cystic fibrosis in humans (Riordan et al., 1989), and the *TAP-1*/*TAP-2* peptide transporters participating in antigen presentation in lymphocytes (Spies & De Mars 1991; Monaco et al., 1990; Deverson et al., 1990; Trowsdale et al., 1990).

Although the mechanism by which P-gp and other members of the ABC superfamily can recognize and transport a large group of structurally unrelated substrates remains unresolved, recent evidence suggests that membrane-associated domains (transmembrane segments, intra- and extracellular loops) participate in substrate recognition and binding. Energy-transfer experiments using photoactivatable ligands 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 identify a major drug binding site near the TM11–12 domain and a minor binding site within the TM1–6 segment (Bruggemann et al., 1989, 1992;

† This work was supported by research grants to A.R.S. from the National Cancer Institute (CA-56078) and to P.G. from the Medical Research Council of Canada. P.G. is a Howard Hughes Medical Institute International Scholar.

* To whom correspondence should be addressed.
‡ Pfizer Central Research.
§ McGill University.
‖ University of Chicago.

[1] Abbreviations: ACT, actinomycin D; ADM, adriamycin; AZD, azidopine; CFTR, cystic fibrosis transmembrane conductance regulator; CLQ, chloroquine; COL, colchicine; FCS, fetal calf serum; GramD, gramicidin D; GramS, gramacidin S; IAAP, iodoarylazidoprazosin; MEM, minimal essential medium; NB, nucleotide binding; P-gp, P-glycoprotein; PRG, progesterone; pst, position; TM, transmembrane; VBL, vinblastine; VRP, verapamil.

0006-2960/93/0432-4185$04.00/0

Yoshimura et al., 1989; Greenberger et al., 1990, 1991). The functional analyses of chimeric (Buschmann & Gros, 1991; Dhir & Gros, 1992) and mutant P-gps also point at TM domains as important determinants for substrate interactions. Human and hamster P-gps bearing mutations within (Devine et al., 1992) or near TM domains (Choi et al., 1988) express drug resistance phenotypes distinct from that of their wild-type counterparts. Furthermore, mutations in the TM1–2 interval and in TM11 of the *P. falciparum mdr* homolog *pfmdr1* are associated with chloroquine (CLQ) resistance (Foote et al., 1990), while mutating charged residues within TM1, TM6, and TM10 of *CFTR* modulate the halide specificity of the channel (Anderson et al., 1991). Finally, natural polymorphic variants within TM domains of *mtp2* (MHC-linked transporter protein 2) may modulate specificity of the peptide transporter (Powis et al., 1992).

The biochemical characterization of mutant P-gps with altered function has started to shed light on the mechanism of drug efflux by P-gp. The substitution of a Gly to Val residue at pst 185 (near TM3) of human *MDR1* causes opposite effects on the degree of drug resistance and transport of colchicine (increase), and vinblastine (decrease) (Choi et al., 1988; Safa et al., 1990a). Labeling experiments using photoactivatable analogs of the two drugs show decreased colchicine binding and increased vinblastine binding to the mutant, suggesting that a decreased rate of drug dissociation from the Val^{185} mutant is responsible for reduced drug transport (Safa et al., 1990a). A two-step mechanism of drug transport was proposed by these authors on the basis of two distinct binding sites: one involved in initial drug binding ("on" rate), and another involved in drug release ("off" rate). On the other hand, we have recently shown that a single Ser to Phe substitution within TM11 (pst 941, *mdr1*; pst 939, *mdr3*) strongly modulates the overall activity and the substrate specificity of P-gps encoded by mouse *mdr1* and *mdr3* (Gros et al., 1991): While this Ser to Phe replacement had little effect on vinblastine (VBL) resistance (2–3-fold decrease in resistance), it strongly modulated the degree of colchicine (COL) and ADM resistance conferred by mutant P-gps (10–30-fold reductions) on both *mdr1* and *mdr3* backgrounds. In the case of *mdr1*, the Ser^{941} to Phe^{941} substitution produced a unique mutant protein that retained the capacity to confer VBL resistance but had lost the capacity to confer either ADM or COL resistance. We have investigated the mechanism by which this mutation perturbs *mdr1* and *mdr3* function and determined that, as opposed to the Val^{185} *MDR1* mutant, mutations in TM11 affect drug transport by reducing drug binding to P-gp.

MATERIALS AND METHODS

Cell Lines and Tissue Culture. Chinese hamster LR73 (LR) ovary cells and their drug-resistant derivatives transfected with wild-type *mdr1* (clone 1S) or *mdr3* (clone 3S) cDNAs or with mutant *mdr1* (clone 1F) or *mdr3* (clone 3F) cDNAs bearing a unique serine to phenylalanine substitution within the predicted TM11 at position 941 (*mdr1*) or 939 (*mdr3*) were obtained as previously described (Gros et al., 1991). All cell lines were maintained in α–MEM supplemented with 10% fetal calf serum, 2 mM glutamine, penicillin (50 units/mL), and streptomycin (50 μg/mL). Drug-resistant transfectants were grown in the same medium supplemented with vinblastine at 25 ng/mL (1F), 50 ng/mL (1S, 3F), and 100 ng/mL (3S).

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 control and *mdr*-transfected cells were plated in 96-well titer plates in increasing concentrations of adriamycin (ADM), colchicine (COL), vinblastine (VBL), actinomycin D (ACT), gramicidin D (GramD), or gramicidin S (GramS) and incubated for 72 h at 37 °C. In some experiments, verapamil (VRP, 5 μM) or progesterone (PRG, 25 μM) were included in the medium to evaluate their reversal effects on wild-type or mutant P-gps. Cells were washed once in ice-cold PBS and fixed in 17% trichloroacetic acid in 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 clone was calculated by dividing the absorbance observed at a given drug concentration by the absorbance detected in the same clone 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 cell clone by 50%. ADM was obtained from Adria laboratories, COL, VBL, GramD, GramS, VRP, and PRG 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 *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 vinblastine 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/mmol, 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 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 them 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. The effect of verapamil on [^{14}C]ADM accumulation was determined as above by including verapamil at increasing concentrations (between 0.05 and 25 μM) in the uptake medium containing [^{14}C]ADM at 2 mM final concentration.

Detection of P-gps. Wild-type and mutant P-gps were detected by Western blotting in purified membrane fractions of independent cell clones, as previously described (Gros et al., 1991). Briefly, individual cell clones were grown to confluency in 175-cm^2 flasks and harvested by trypsinization, and crude membrane extracts were prepared. Purified

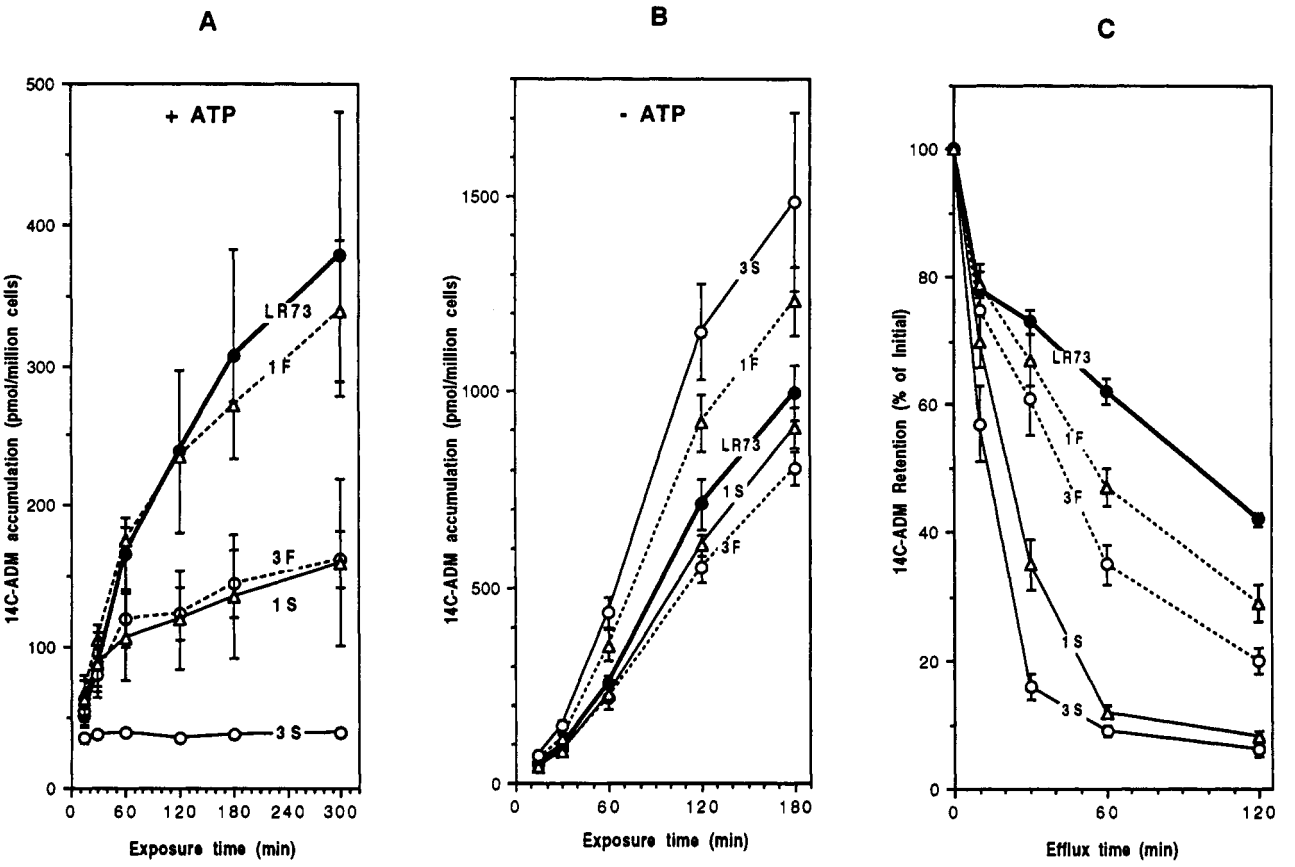

FIGURE 1: [^{14}C]Adriamycin (ADM) accumulation. (A) Monolayer cultures of drug-sensitive LR73 cells (LR) and cell clones transfected with either wild-type *mdr1* (1S), wild-type *mdr3* (3S), or mutant *mdr1* (1F) or *mdr3* (3F) bearing a Ser to Phe substitution within TM11 were incubated at 37 °C with [^{14}C]ADM at a final concentration of 2 μM (specific activity 2.4 μCi/μmol), as described under Materials and Methods. Accumulation of radioactivity was assayed at given times and is expressed as picomoles of incorporated radioactivity per 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 of 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.

introduced. In the case of ACT resistance, the mutation had no effect on the *mdr1* background, while it strongly modulated the capacity of *mdr3* to confer ACT resistance (7.5-fold reduction). Conversely, the mutation caused only a 2-fold reduction in GramD resistance levels in the *mdr3* background, while a 17-fold reduction was noted in the *mdr1* background. Finally, the TM11 mutation had no effect on the D_{50} values determined for all cell clones for GramS, a non-P-gp substrate. Hence, the TM11 mutations appear to affect the overall activity but more importantly the substrate specificity of P-gps encoded by *mdr1* and *mdr3*.

Intracellular Drug Accumulation. The effect of the Ser to Phe mutation within TM11 on the ability of P-gps encoded by *mdr1* and *mdr3* to carry out drug transport (accumulation and efflux) was determined. First, the kinetics of [^{14}C]ADM intracellular accumulation were analyzed over time in drug-sensitive control cells (LR) and in cell clones expressing either wild-type (1S, 3S) or mutant (1F, 3F) P-gps (Figure 1A). In drug-sensitive LR cells and in 1F expressing clones, the kinetics of [^{14}C]ADM accumulation were very similar. Accumulation was linear with time and resulted in comparable overall degrees of accumulation over the 5-h period tested (350 pmol/10^6 cells). 1S and 3F expressing cell clones also showed very similar [^{14}C]ADM accumulation profiles, but the overall drug accumulation was approximately 2.5-fold less (150 pmol/10^6 cells) than that detected in LR controls and 1F expressing clones at 5 h. Finally, 3S expressing clones did not accumulate [^{14}C]ADM during the time course of the experiment (maximum of 30 pmol/10^6 cells), resulting in an 11-fold difference between 3S and LR control cells. The reduced [^{14}C]ADM accumulation detected in 3S, 1S, and 3F expressing cells as compared to 1F expressing cells and control LR cells was ATP-dependent, as deoxyglucose and rotenone treatments to reduce intracellular ATP levels resulted in enhanced but similar degrees of [^{14}C]ADM accumulation in all cells tested (Figure 1B). Finally, the capacity of wild-type and mutant P-gps to efflux preloaded ADM was measured. For this, cells were loaded with [^{14}C]ADM for 1 h under conditions of ATP depletion (–ATP), in order to achieve comparable intracellular drug pools in the various cell populations (see Figure 1B). The drug was then removed, the cells were washed, and efflux was initiated by adding fresh complete medium, containing glucose, glutamine, and sodium pyruvate (+ATP). Cell-associated [^{14}C]ADM radioactivity was then measured over time, and the results are presented in Figure 1C as the percentage of retention of preloaded drug (determined at T_0). In cells expressing wild-type 3S and 1S P-gps, drug efflux was rapid, with only 15% and 35% retained radioactivity after 30 min, respectively. Efflux was essentially completed by 60 min in both 3S and 1S cell clones. In cells expressing 3F and 1F mutant P-gps, drug efflux was much slower than for wild-type P-gps, with only 60% and 65% of preloaded drug retained after 30 min, respectively. Although efflux was faster in 3F than 1F expressing cells, both cell clones still retained about one-third of the preloaded drug even after 120 min. These results indicate a strong correlation between intracellular

Table I: Drug Survival Characteristics of Hamster Cell Clones Stably Expressing Wild-Type and Mutant *mdr1* and *mdr3* Genes

	LR	1S	1F	3S	3F	*n*
VBL[a]	5.2[b] ± 0.7	150 ± 5 (29×)[c]	44 ± 12 (8.5×)	280 ± 85 (54×)	200 ± 40 (38×)	4[d]
ACT	1.8 ± 0.6	8 ± 2 (4.4×)	10 ± 4 (5.6×)	160 ± 40 (89×)	21 ± 5 (12×)	4
ADM	20 ± 5	250 ± 40 (13×)	29 ± 6 (1.5×)	770 ± 70 (39×)	90 ± 10 (4.5×)	4
COL	26 ± 3	780 ± 190 (30×)	44 ± 5 (1.7×)	1400 ± 360 (54×)	160 ± 20 (6.2×)	4
GramD	0.08 ± 0.01	3 ± 1 (38×)	0.2 ± 0.02 (2.3×)	9 ± 2 (112×)	5.3 ± 0.3 (66×)	4
GramS	4.5	4.6	5.3	4.5	5.4	2

[a] Abbreviations: vinblastine (VBL); actinomycin D (ACT); adriamycin (ADM); colchicine (COL); gramicidin D (GramD); gramicidin S (GramS). [b] The drug survival of LR73 drug-sensitive cells (LR) and multidrug resistant clones transfected with either wild-type *mdr1* (1S) or *mdr3* (3S) or mutant *mdr1* (1F) or *mdr3* (3F) is expressed as the D_{50}, or the dose necessary to reduce the plating efficiency of the control and transfected cells by 50%. The D_{50} is expressed in μg/mL for GramD and GramC and in ng/mL for the other cytotoxic drugs. [c] The resistance index is the degree of resistance above background levels expressed in LR73 cells and is shown in parentheses. [d] The number of independent experiments (in duplicate) is indicated.

membrane fractions were enriched by centrifugation on a discontinuous sucrose gradient, as previously described (Schurr et al., 1989). Proteins were determined and the preparations were stored at −70 °C in 10 mM Tris, pH 8, and 40% glycerol. Serial dilutions of 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-P-gp monoclonal antibody C219 (Centocor Inc, Philadelphia, PA) used at a dilution of 1:300. 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-indolyl phosphate–*p*-toluidine (0.17 ng/mL) and nitroblue tetrazolium chloride (0.33 ng/mL) substrates. Blots were dried and photographed.

Photoaffinity Labeling. For photolabeling with the radiolabeled prazosin analog [^{125}I]iodoarylazidoprazosin (IAAP) (specific activity 2000 Ci/mmol; New England Nuclear), purified membrane extracts from control and *mdr*-transfected cells were incubated with IAAP at a final concentration of 30 nM in a reaction buffer containing 0.05 M Tris (pH 7.5) for 1 h at 20 °C, followed by cross-linking under UV for 5 min, as previously described (Greenberger et al., 1990). For serial dilutions of membrane fractions from *mdr*-transfected clones, the final amount of membrane proteins in the reaction was adjusted to 50 μg by adding appropriate amounts of membrane proteins from drug-sensitive LR73 control cells. After photolabeling, the unincorporated radiolabeled IAAP was removed by centrifugation (100000*g*, 20 min), and labeled P-gps were recovered by immunoprecipitation (16 h at 4 °C) with mouse anti-P-gp monoclonal antibody C219 (1 μg/mL) used at a 1:100 dilution, in a final volume of 0.5 mL. Immune complexes were isolated using protein A–Sepharose beads, washed extensively, dissolved in Laemmli sample buffer, and electrophoresed on a 7.5% SDS–polyacrylamide gel. Gels were fixed, dried, and exposed to Kodak XAR films with an intensifying screen (Kronex, E. I., duPont de Nemours) at −70°C. For photolabeling with azidopine (AZD), purified membrane fractions were photolabeled in a buffer containing 100 mM Tris-HCl (pH 7.4), 250 mM sucrose, 10 mM NaCl, 1.5 mM $MgCl_2$, 3 mM ATP, 4% dimethylsulfoxide, and 0.5 μM [^{3}H]azidopine (specific activity 44 Ci/mmol; Amersham Corp.), in a final volume of 0.05 mL. The reaction mixture was preincubated for 30 min at 25 °C in the absence or presence of a nonradioactive competing ligand and then irradiated for 20 min with a UV lamp equipped with two 15-W self-filtering 302-nm lamps, as previously described (Safa et al., 1987). Photolabeled membranes were analyzed directly by SDS–PAGE on a 5–15% gradient gel containing 4.5 M urea, followed by fluorography.

Cytofluorescence Studies in Live Cells. Cells were seeded onto Lab-Tek four-chamber slides (3 × 10^4 cells/0.5 mL per well) in complete D-MEM medium and allowed to grow for 2–3 days. Medium was removed, replaced with the same medium supplemented with ADM (2 mM), and further incubated for 3 h at 37 °C, with or without verapamil at 10 μM. The medium was decanted, the chamber walls were removed, and the slides were washed once with PBS and mounted with cover slips. Cells were examined and photographed within 3 h, under a Zeiss Axiophot phase contrast UV fluorescence microscope equipped with a high-pressure mercury light source (excitation filter 450–490 nm, barrier filter 520 nm). Photomicrographs were taken on Kodak ektachrome films (400 ASA), using equal exposure times for all cell lines.

RESULTS

Drug Survival Characteristics Conferred by Wild-Type and Mutant P-gps. Wild-type P-gps encoded by *mdr1* (Ser^{941}, 1S) and *mdr3* (Ser^{939}, 3S) as well as mutant P-gps encoded by *mdr1* (Phe^{941}, 1F) and *mdr3* (Phe^{939}, 3F) bearing a single Ser to Phe substitution within predicted TM11 were tested for their capacity to confer resistance to vinblastine (VBL), adriamycin (ADM), colchicine (COL), actinomycin D (ACT), gramicidin D (GramD), and gramicidin S (GramS). For this, cell clones stably expressing wild-type (1S, 3S) or mutant P-gps (1F, 3F) were plated in increasing concentrations of the various drugs, and the drug doses necessary to reduce plating efficiency by 50% (D_{50}) were determined (Table I). In the case of VBL, the effect of the mutation on resistance levels was modest, as it decreased the degree of resistance of wild-type vs mutant protein by a factor of 3-fold for *mdr1* and 1.5-fold for *mdr3*. By contrast, the modulating effect of the mutation on ADM resistance levels was very strong as a 10-fold decrease in resistance levels was observed in cell clones expressing mutant proteins (1F, 3F) vs wild-type proteins (1S, 3S). A strong modulating effect was also observed in the case of COL resistance, where 18- and 9-fold decreases in resistance levels were noted in mutant (1F, 3F) vs wild-type expressing clones (1S, 3S), respectively. In the case of *mdr1*, the Ser to Phe substitution completely abrogated resistance to ADM and COL. Therefore, it appears that the Ser to Phe substitution within TM11 had similar modulatory effects on *mdr1* and *mdr3*, which were modest for VBL but very strong for COL and ADM. The consequences of the mutation on ACT and GramD resistance levels were distinct and specific to the parental *mdr* background onto which the mutation was

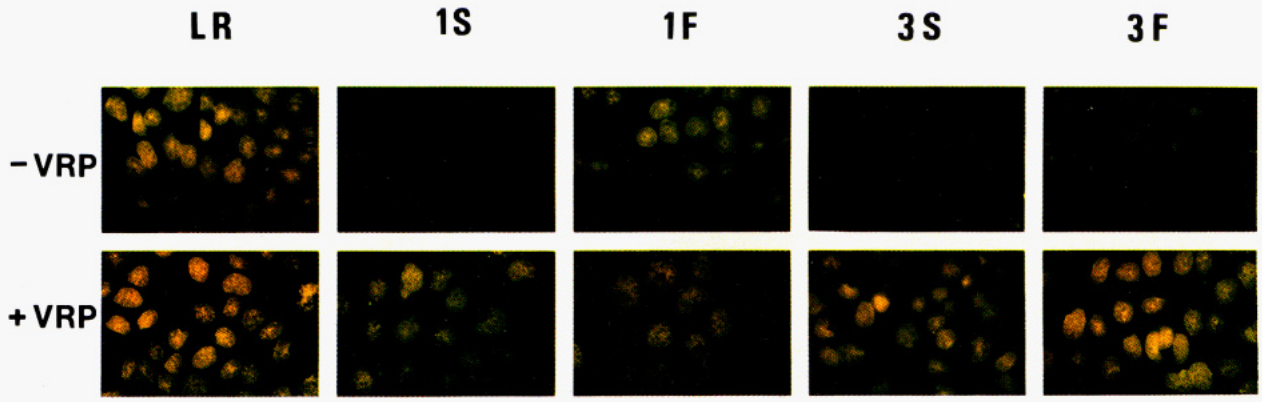

FIGURE 2: Cytofluorescence studies using adriamycin (ADM). Monolayer cultures of drug-sensitive LR73 cells (LR) and cell clones transfected with either wild-type *mdr1* (1S), wild-type *mdr3* (3S), or mutant *mdr1* (1F) or *mdr3* (3F) bearing a Ser to Phe substitution within TM11 were exposed for 3 h to ADM (final concentration of 2 mM) in the presence (bottom panel) or absence (top panel) of verapamil (VRP, final concentration of 10 μM). Cells were visualized under UV light and photographed using identical exposure times for each cell and treatment.

Table II: Effect of Verapamil on Drug Survival Characteristics of Hamster Cell Clones Stably Expressing Mutant and Wild-Type *mdr1* and *mdr3* Genes

	LR		1S		1F		3S		3F	
	–VRP	+VRP	–VRP	+VRP	–VRP	+VRP	–VRP	+VRP	–VRP	+VRP
VBL[a]	4.1[b]	0.6 (6.4×)[c]	150	3 (50×)	27	5.8 (4.7×)	340	7 (49×)	260	24 (11×)
ACT	1.2	0.9 (1.4×)	5.3	1.3 (4×)	4.1	2.3 (1.8×)	91	4.1 (22×)	18	4.5 (4×)
ADM	20	3.3 (6×)	270	15 (18×)	25	2.9 (8.6×)	640	16 (40×)	115	24 (4.8×)
COL	27	6.4 (4.2×)	670	48 (14×)	42	12 (3.5×)	920	380 (2.4×)	160	170 (1×)
GramD	0.068	0.02 (3.4×)	2.3	0.034 (68×)	0.16	0.027 (6×)	6.2	0.12 (80×)	5.1	0.38 (13×)
GramS	4.8	4.8	5.2	4.9	6	6.3	4.9	4.9	6.2	6.2

[a] Abbreviations: vinblastine (VBL); actinomycin D (ACT); adriamycin (ADM); colchicine (COL); gramicidin D (GramD); gramicidin S (GramS). [b] The drug survival of LR73 drug-sensitive cells (LR) and multidrug resistant clones transfected with either wild-type *mdr1* (1S) or *mdr3* (3S) or mutant *mdr1* (1F) or *mdr3* (3F) is expressed as the D_{50}, or the dose necessary to reduce the plating efficiency of the control and transfected cells by 50%. The D_{50} is expressed in μg/mL for GramD and GramS and in ng/mL for the other cytotoxic drugs. [c] The reversal index is the decrease in D_{50} values (fold decrease shown in parentheses) of control and transfected cell clones determined in the absence (–VRP) and presence (+VRP) of verapamil used at 5 μM. Each measurement was in duplicate.

ADM accumulation (Figure 1A), ADM efflux from preloaded cells (Figure 1C), and drug survival characteristics (Table I) of cell clones expressing wild-type or mutant P-gps, suggesting that the TM11 mutation alters the transport activity of P-gps encoded by *mdr1* and *mdr3*.

Cytofluorescence Studies with Live Cells. The subcellular localization of ADM was analyzed in drug-sensitive cells and in cell clones expressing wild-type (1S, 3S) or mutant (1F, 3F) P-gps by taking advantage of the natural fluorescence of this compound. For this, cells were incubated with ADM (final concentration of 2 μM) for 3 h in the absence or presence of VRP and then photographed under UV light (Figure 2). In drug-sensitive LR73 cells exposed to ADM, all cells readily and uniformly accumulated ADM (bottom panel). In these cells, the drug-associated fluorescence was localized exclusively within the nucleus, with no fluorescence detected in the cytoplasm. Likewise, ADM cytofluorescence in 1F expressing cells was identical to that observed in the LR controls, in agreement with the lack of ADM resistance expressed by 1F cells. By contrast, cell clones expressing either 1S or 3S wild-type proteins did not show any detectable degree of ADM accumulation in the 3-h assay. Finally, low levels of drug-associated fluorescence (slightly higher than background levels in 3S cells) could be detected in 3F expressing cells. Similar results were obtained in parallel experiments using higher ADM concentrations (20 μM), except that 3F expressing cells showed significant levels of ADM accumulation similar to those detected in 1F expressing cells (data not shown). In all cases, the inclusion of VRP at 10 μM effectively abrogated the differences detected between cell lines, all cells showing strong nuclear localization of the drug-associated fluorescence (top panel). Taken together, results of ADM cytofluorescence studies are in good agreement with measurements of ADM cytotoxicity and transport carried out on these cell clones. Under the experimental conditions used here, the distribution of intracellular drug was apparently strictly nuclear with neither diffuse nor vesicular staining within the cytoplasm.

Effect of Reversal Agents. The capacity of verapamil (VRP) and progesterone (PRG) to modulate the activity of wild-type and TM11 mutant P-gps was tested. VRP and PRG treatments of P-gp expressing cells reverses their multidrug resistance phenotype by increasing intracellular drug accumulation (Tsuruo et al., 1981; Yang et al., 1989), possibly by competing for drug binding sites on P-gp (Cornwell et al., 1986; Yang et al., 1990; Safa, 1988; Qian & Beck, 1990; Naito et al., 1989). The effect of VRP and PRG on the D_{50}s of drug-sensitive control cells and cell clones expressing wild-type and mutant P-gps was determined in drug cytotoxicity assays and was expressed as a reversal factor (fold) calculated by comparison to the D_{50} value in the absence of modulator (Tables II and III). In drug-sensitive LR cells, VRP and PRG treatments produced a small but reproducible decrease in the D_{50} values for all MDR drugs tested, possibly reflecting a low level of endogenous P-gp expression in these cells. In the case of 1S, VRP (5 μM) treatment abrogated the MDR phenotype of these cells for all drugs tested, with resulting D_{50} values similar to those of LR73 control cells for all drugs tested. Likewise, VRP treatment eliminated in 1F expressing cells VBL and GramD resistance (Table II). Although the reversal effect of VRP appeared greater for 1S than for 1F expressing cells, this could be explained both by the higher degree of resistance expressed by 1S vs 1F cells and by the fact that VRP reverted resistance in both cell clones to control values measured in LR cells. The effect PRG treatment on

Table III: Effect of Progesterone on Drug Survival Characteristics of Hamster Cell Clones Stably Expressing Mutant and Wild-Type *mdr1* and *mdr3* Genes

	LR		1S		1F		3S		3F	
	-PRG	+PRG	-PRG	+PRG	-PRG	+PRG	-PRG	+PRG	-PRG	+PRG
VBL[a]	5.9[b]	1.2 (4.9×)[c]	140	2.5 (56×)	41	1.9 (22×)	305	18 (17×)	180	21 (8.6×)
ACT	2.7	1 (2.7×)	12	3 (4×)	14	1.7 (8.2×)	180	3.2 (56×)	19	2.4 (7.9×)
ADM	20	5.1 (3.9×)	310	33 (9.4×)	29	7.8 (3.7×)	810	210 (3.9×)	80	70 (1.1×)
COL	28	14 (2×)	620	52 (12×)	47	21 (2.2×)	1800	210 (8.6×)	140	160 (0.9×)
GramD	0.102	0.015 (6.8×)	3.9	0.03 (126×)		NA	11	0.18 (61×)	5	0.12 (42×)

[a] Abbreviations: vinblastine (VBL); actinomycin D (ACT); adriamycin (ADM); colchicine (COL); gramicidin D (GramD). [b] The drug survival of LR73 drug-sensitive cells (LR) and multidrug resistant clones transfected with either wild-type *mdr1* (1S) or *mdr3* (3S) or mutant *mdr1* (1F) or *mdr3* (3F) is expressed as the D_{50}, or the dose necessary to reduce the plating efficiency of the control and transfected cells by 50%. The D_{50} is expressed in μg/mL for GramD and GramS and in ng/mL for the other cytotoxic drugs. [c] The reversal index is the decrease in D_{50} values (fold decrease shown in parentheses) of control and transfected cell clones determined in the absence (-PRG) and presence (+PRG) of progesterone used at 25 μM. Each measurement was in duplicate.

Table IV: Effect of Verapamil on Intracellular Accumulation of [^{14}C]Adriamycin in Drug-Sensitive Hamster Cells and in Cell Clones Stably Expressing Mutant and Wild-Type *mdr1* and *mdr3* Genes

	verapamil concentration (μM)				
	0	0.1	0.8	6.2	25
LR	350 ± 33[a]	426 ± 44 (1.2×)[b]	528 ± 46 (1.5×)	607 ± 16 (1.7×)	590 ± 24 (1.7×)
1S	78 ± 22	81 ± 16	163 ± 63 (2.1×)	359 ± 129 (4.6×)	493 ± 111 (6.3×)
1F	178 ± 7	230 ± 21 (1.3×)	356 ± 41 (2×)	438 ± 36 (2.5×)	468 ● 48 (2.6×)
3S	55 ± 6	57 ± 7	100 ± 2 (1.8×)	423 ± 7 (7.7×)	662 ± 32 (12×)
3F	176 ± 4	190 ± 8	283 ± 32 (1.6×)	549 ± 24 (3.1×)	638 ± 16 (3.6×)

[a] The intracellular accumulation of [^{14}C]adriamycin in LR73 drug-sensitive cells (LR) and multidrug resistant clones transfected with either wild-type *mdr1* (1S) or *mdr3* (3S) or mutant *mdr1* (1F) or *mdr3* (3F) is expressed in nanomoles per million cells and is calculated from three independent measurements (mean ± standard error). [b] The reversal index represents the increase in intracellular levels of [^{14}C]adriamycin accumulation caused by exposure to verapamil over control levels measured in the absence (0) of verapamil (fold increase shown in parentheses).

the D_{50} values of 1F and 1S expressing cells was similar to that of VRP, with complete reversal of the MDR phenotype in 1S cells for all drugs and reversal of VBL resistance in 1F cells (Tble III). The effect of the mutation on the capacity of VRP and PRG to modulate P-gp activity was easier to monitor on the *mdr3* background, since both 3S and 3F cells displayed significant resistance levels to all MDR drugs tested. In both 3F and 3S expressing cells, VRP treatment abrogated ADM resistance completely, but did not completely revert resistance to VBL, ACT, COL, and GramD, with residual resistance levels above those of LR control cells (Table II). In general, the reversal effect of VRP was more pronounced on cells expressing wild-type (3S) than mutant (3F) P-gps: This was most evident for VBL (49× vs 11×), ACT (22× vs 4×), and GramD (80× vs 13×). Interestingly, VRP treatment did not affect COL resistance in 3F cells and only slightly decreased the D_{50} value measured in 3S cells. Very similar findings were obtained when PRG was used as a modulator (Table III), PRG showing a stronger modulating effect on 3S than 3F cells. This differential effect of PRG on wild-type and mutant P-gps was most evident for VBL (17× vs 8.6×), ADM (3.9× vs no effect), and COL (8.6× vs no effect). For ADM and COL, PRG showed no reversal activity on 3F expressing cells. Similar but less dramatic effects were also obtained at a lower PRG concentration (10 μM; data not shown).

The capacity of VRP to modulate ADM resistance expressed by wild-type (3S, 1S) and mutant P-gps (3F, 1F) was also analyzed in drug transport experiments (Table IV). For this, the effect of increasing doses of VRP on the accumulation of [^{14}C]ADM was determined. In these experiments, ADM accumulation is expressed as picomoles per 10^6 cells, and the VRP reversal index is expressed as the fold increase in cellular accumulation above control levels measured in the absence of VRP. At concentrations below 1 μM, VRP had little effect on all cell lines. However, at a concentration known to interfere with P-gp function (6.2 μM) VRP appeared to show a stronger effect on cells expressing wild-type (1S, 4.6×; 3S, 7.7X) than mutant P-pgs (1F, 2.5×; 3F, 3.1×). At the highest concentrations tested (25 μM), VRP treatment resulted in levels of ADM accumulation similar in all P-gp expressing cells and comparable to those achieved in LR control cells. Taken together, these results suggest that the TM11 mutation may also effect the capacity of known reversal agents to interfere with P-gp function.

Photoaffinity Labeling. The effect of the Ser to Phe substitution on the ability of P-gps encoded by *mdr1* and *mdr3* to combine radiolabeled P-gp ligands was measured in cross-linking experiments, using two known P-gp ligands, [^{3}H]-azidopine (AZD) and [^{125}I]iodoarylazidoprazosin (IAAP). AZD and IAAP have been shown to bind P-gp (Greenberger et al., 1990; Safa et al., 1987, 1990b; Yang et al., 1988), and their binding site has been tentatively assigned to a 6-kDa tryptic peptide fragment overlapping the TM11–12 region of P-gp (Greenberger et al., 1991). For IAAP photolabeling, serial dilutions of membrane-enriched fractions of 1S, 1F, 3S, and 3F expressing clones (6, 12, 25, and 50 μg, complemented to 50 μg with membranes from LR control cells) were incubated with IAAP (30 nM final concentration) and cross-linked with UV, and photolabeled P-gps were recovered by immunoprecipitation with anti-P-pg monoclonal antibody C219. Immunoprecipitated P-gps were separated by SDS–PAGE and visualized by autoradiography (Figure 3A). A second aliquot of nonlabeled membranes from the same clones was also separated by SDS–PAGE, and wild-type and mutant P-gps were detected by Western blotting, using monoclonal antibody C219 (Figure 3B). In the case of *mdr1*, IAAP photolabeling of 1S produced a stronger signal than that detected for 1F (Figure 3A), even though the amount of 1F protein was greater than that of 1S in the two clones tested (Figure 3B). Similarly, the intensity of the IAAP-photolabeled P-gp in the 3S clone was much stronger (approximately 4-fold)

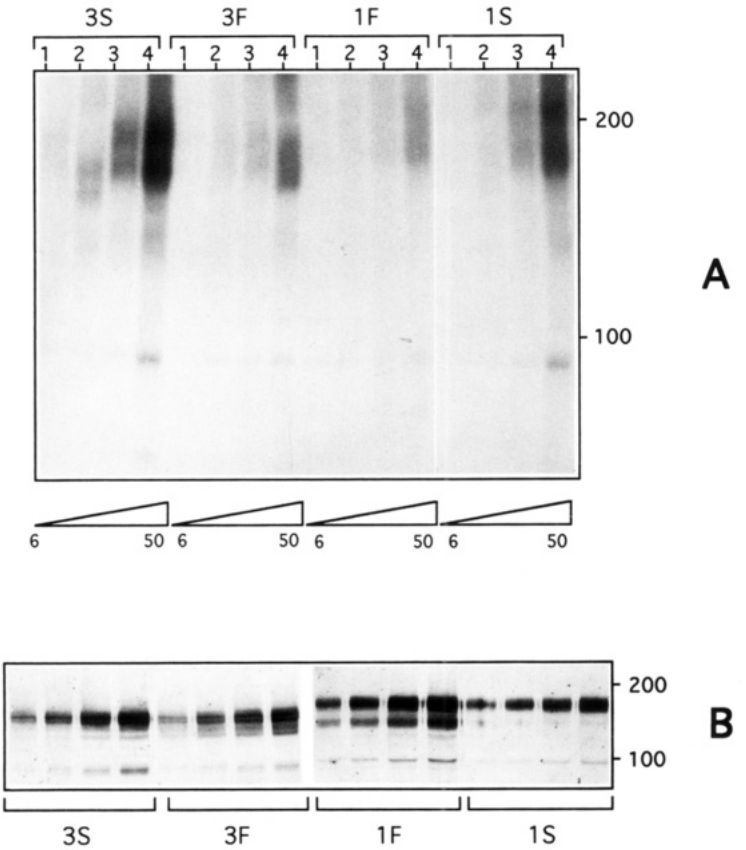

FIGURE 3: Photoaffinity labeling with [^{125}I]iodoarylazidoprazosin (IAAP). (Panel A) Membrane-enriched fractions from cell clones transfected with either wild-type *mdr1* (1S), wild-type (*mdr3*) (3S), or mutant *mdr1* (1F) or *mdr3* (3F) bearing a Ser to Phe substitution within TM11 were exposed to [^{125}]IAAP (final concentration of 30 nM) and cross-linked with UV. For this, either 6 (lane 1), 12 (lane 2), 25 (lane 3), or 50 μg (lane 4) of membrane fractions from each clone was mixed with an appropriate amount of membrane proteins from drug-sensitive LR73 cells to reach a final amount of 50 μg in each tube. After cross-linking, photolabeled wild-type and mutant P-gps were recovered by immunoprecipitation with the mouse anti-P-gp monoclonal antibody C219 and analyzed by SDS–PAGE on a 7.5% gel. Autoradiography was to Kodak XAR films for 4 days with an intensifying screen. (Panel B) Membrane mixtures identical to those described in panel A were analyzed for the presence of wild-type and mutant P-gps by Western blotting using the mouse anti-P-gp monoclonal antibody C219. The P-gp doublet present in the 1F clone was not consistently detected (Gros et al., 1991) and may reflect incomplete posttranslational modification of P-gp. The molecular size markers (in kDa) are the heavy chain of myosin (200) and phosphorylase B (100) in both cases.

than that detected in the 3F clone (Figure 3A), although the relative amounts of 3S and 3F proteins detected in the two clones by Western blotting appeared similar (Figure 3B). For AZD photolabeling, membrane-enriched fractions were incubated with the AZD and cross-linked with UV, and the reaction products were analyzed directly by SDS–PAGE without immunoprecipitation of the specific P-gps (Figure 4). In 1S (lane 2) and 3S (lane 5) expressing clones, proteins of apparent molecular mass 180 and 160 kDa, absent from the control nontransfected LR cells (lane 1), could be detected, respectively. The estimated 180- and 160-kDa molecular masses of the two protein isoforms are in good agreement with previously published estimates (Devault & Gros, 1990; Gros et al., 1991). In 1F (lane 3) and 3F (lane 4) expressing cell clones, proteins of apparent molecular mass similar to their wild-type counterparts were also detected. However, the intensity of the signal detected in the AZD-photolabeled mutant P-gps (1F, 3F) was greatly reduced compared to that produced by their wild-type counterparts. AZD photolabeling of wild-type or mutant proteins was eliminated in all cases by the addition of competing unlabeled azidopine at a final concentration of 50 μM (lanes 6–10). Minor labeled species detected at the bottom of the gel probably represent partial degradation products of the mature photolabeled P-gps. The intensities of these minor species were similar in wild-type and mutant *mdr1* (compare lanes 2 and 3) or *mdr3* (compare lanes 4 and 5) and do not account for reduced binding of IAAP to mature forms of the mutant P-gps. Taken together,

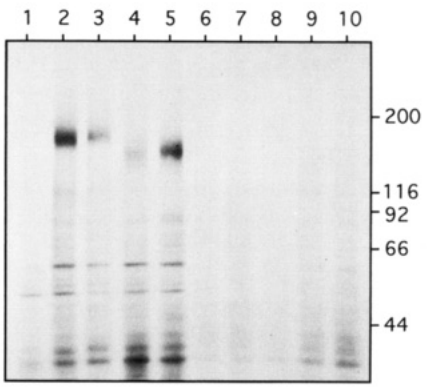

FIGURE 4: Photoaffinity labeling with [^{3}H]azidopine. Membrane-enriched fractions from drug-sensitive LR73 cells (lanes 1 and 6) or cell clones transfected with and containing similar amounts of either wild-type *mdr1* (1S; lanes 2 and 7), wild-type *mdr3* (3S; lanes 5 and 10), or mutant *mdr1* (1F; lanes 3 and 8) or *mdr3* (3F; lanes 4 and 9) bearing a Ser to Phe substitution within TM11 were exposed to [^{3}H]AZD (final concentration of 0.5 μM) in the absence (lanes 1–5) or presence of competing unlabeled AZD (50 μM, lanes 6–10), as described under Materials and Methods. Photolabeled proteins were analyzed directly by SDS–PAGE on a 5–15% gradient gel containing 4.5 M urea and visualized by fluorography. The molecular size markers used (in kDa) were the heavy chain of myosin (200), β-galactosidase (116), phosphorylase B (92), bovine serum albumin (66), and ovalbumin (44).

these photolabeling studies clearly indicate that the Ser to Phe substitution in TM11 of *mdr1* and *mdr3* induces in the

mutant proteins a decreased ability to bind to known photoactivable P-gp ligands such as IAAP and AZD.

DISCUSSION

One of the key unresolved issues in the study of P-glycoprotein-mediated drug resistance is the mechanism by which P-gp can recognize and transport structurally heterogeneous substrates. Recently, progress has been made toward the identification of P-gp domains and amino acid residues implicated in drug binding. The biochemical characterization of P-gp and the genetic analysis of discrete mutations in *mdr* genes and in *mdr* homologs with altered biological activities have suggested that membrane-associated domains play an important role in substrate recognition by the respective transporter. In human *MDR1*, a Gly^{185} to Val^{185} substitution within the interval separating the predicted TM2 and TM3 was found associated with increased COL resistance and decreased VBL resistance in the mutant P-gp (Choi et al., 1988), while in the 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). In the *P. falciparum mdr* homolog *pfmdr1*, the emergence of chloroquine resistance (increased efflux) coincides with the presence of two mutant alleles at the *pfmdr1* locus, either a single amino acid substitution near TM1 (Asn^{86} to Tyr^{86}) or a Ser^{1024}/Asn^{1042} to Cys^{1034}/Asp^{1042} replacement within TM11 (Foote et al., 1990). In the proposed transporters associated with antigen processing in T lymphocytes (*TAP-1*, *TAP-2*), differential transport/loading of distinct populations of peptides into the endoplasmic reticulum for association with rat class I MHC molecules is associated with dramatic sequence variation in two *mtp2* alleles (cim^a, cim^b): Of the 25 amino acid sequence variations between the two alleles, most map in the membrane-asociated portion of the protein, with clusters of two or three residues adjacent to TM domains (Powis et al., 1992). Finally in the CFTR Cl^- channel, the replacement of charged residues within TM1, -6, and -10 alters the anion specificity of the channel (Anderson et al., 1991).

The biochemical characterization of P-gp also points TM domains as important determinants for substrate binding. Energy-transfer experiments with daunomycin have suggested that MDR drugs are recognized in association with the membrane lipid bilayer, most likely by membrane-associated segments of P-gp (Raviv et al., 1990). In cross-linking experiments, P-gp can be labeled with photoactivatable derivatives of VBL (Cornwell et al., 1986; Safa et al., 1986) and COL (Safa et al., 1989) but also with analogs of the calcium channel blocker azidopine (AZD; Safa et al., 1987; Yang et al., 1988) and a photoaffinity probe for the α_1-adrenergic receptor iodoarylazidoprazosin (IAAP; Greenberger et al., 1990; Safa et al., 1990b). Although the two latter compounds are not transported by P-gp, their binding to P-gp is specific and competable by MDR drugs such as VBL, ADM, and ACT and known P-gp reversal agents (Greenberger et al., 1990; Safa et al., 1987; Yang et al., 1988). Epitope mapping of proteolytic P-gp fragments photolabeled with AZD and IAAP using antibodies directed against discrete P-gp segments has been used to characterize the binding site-(s) of these compounds (Bruggemann et al., 1989; Yoshimura et al., 1989; Greenberger et al., 1990). In particular, Greenberger et al. (1991) have shown that AZD and IAAP bind to the same two sites on P-gp, with a weak site (25% of label) within the amino-terminal half and a strong site (75%) in the carboxy-terminal half of the protein. The major binding site has been delineated within a 6-kDa V8 protease digestion fragment which is conserved in *mdr1* and *mdr3* and which maps in close proximity or overlaps the TM11–12 segment. Recently, Bruggemann et al. (1992) have proposed that the two photolabeled sites could cooperate to form a single binding site implicating both homologous halves of P-gp.

We have previously observed that a single Ser to Phe substitution within TM11 of mouse *mdr1* and *mdr3* affects the overall activity and substrate specificity of these two drug efflux pumps (Gros et al., 1991). In the present study, we have further investigated the mechanism by which this mutation affects P-gp function. In drug cytotoxicity assays, the phenotypic expression of this mutation was found to be complex toward individual drugs, resulting in similar modulation of the activity of both pumps but also producing effects specific for each protein: For both *mdr1* and *mdr3*, a mild effect of the mutation on VBL resistance and a strong effect on COL and ADM resistance were detected. On the other hand, the mutation had no effect on the levels of ACT resistance expressed by *mdr1* but strongly modulated *mdr3* activity against the same drug. The opposite situation was noted for resistance to GramD (Table I). These observations suggest that (a) COL, ADM, and VBL transport by *mdr1* and *mdr3* implicate homologous determinants in both proteins, including $Ser^{939/941}$, and (b) ACT and GramD transport by *mdr1* and *mdr3* may involve distinct and nonoverlaping amino acid residues with $Ser^{939/941}$ playing little role if any in ACT transport by *mdr1* and GramD transport by *mdr3*, respectively. Using ADM as a test substrate, we have observed that the reduction (in *mdr3F*) or abrogation (in *mdr1F*) of resistance to this drug caused by the mutation was linked to a proportional increase in intracellular ADM accumulation and concomitant decrease in drug efflux, as measured in transport studies (Figure 1) and as visualized in cytofluorescence experiments (Figure 2). Using two structurally distinct photoaffinity P-gp ligands (AZD, IAAP) in cross-linking experiments, we have shown that partial or complete failure to reduce intracellular drug accumulation by mutant proteins is associated with a reduced capacity to combine the two photoactivatable ligands (Figures 3 and 4). This suggests that reduced drug binding is the mechanism by which the Ser to Phe mutation modulates P-gp activity. The observations that the TM11 mutation (i) alters resistance to some but not all drugs, (ii) modulates the capacity of PRG and VRP to block P-gp, (iii) is located near a photoaffinity-labeled P-gp subfragment (30), and (iv) reduces binding of AZD and IAAP to P-gp together suggest that TM11 in general and $Ser^{939/941}$ in particular play a key role in drug recognition and binding of drugs and reversal compounds. Moreover, $Ser^{939/941}$ is also important for peptide transport by *mdr3*: We have shown that *mdr3* can complement a null allele at the *STE6* locus in *S. cerevisiae* and can therefore transport the **a** 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 *P. falciparum mdr* homolog *pfmdr1* is one of two residues mutated in the 7G8 allele of this gene found in CLQ-resistant isolates of this parasite (Foote et al., 1990). These findings clearly indicate that TM11 is also important in substrate binding and transport by evolutionarily distant *mdr* homologs. Althoug no sequence homology exists between the predicted TM11 segments of *mdr1/3*, *pfmr1*, and *STE6*, they can all be arranged easily into amphiphilic helices, with a $Ser^{939/941}$ residues (in *mdr* and *pfmdr1*) mapping at the boundary of the hydrophilic and hydrophobic planes of the helix. It is tempting to speculate

that the amphiphilic nature of TM11 may be an important functional determinant for possible substrate translocation from a hydrophobic environment (lipid bilayer) to a more hydrophilic milieu, such as a water-filled pore or the extracellular milieu (Reid, 1983; Hait & Aftab, 1992). It is important to note that the effects of substitutions at pst 939/941 on drug resistance profiles, drug transport, and drug binding are specific to this residue and do not reflect a general and random perturbation of the predicted TM11 domain. Mutations of several other residues either within TM11 or within the neighboring TM domains do not result in a similar perturbation of function (P. Gros, unpublished results).

The comparative analysis of discrete P-gp mutants with distinct phenotypes allows additional speculation on the mechanism of action of P-gp. Of particular interest is the biochemical analysis of the Gly^{185} to Val^{185} substitution which causes in human *MDR1* decreased resistance to VBL and ACT but increased resistance to COL (Choi et al., 1988). Photolabeling experiments with analogs of VBL, COL, and AZD indicated that decreased drug resistance in this mutant was linked to increased binding of the photoactivatable ligands (Safa et al., 1990a), the exact opposite of our findings with the TM11 mutant analyzed here. In addition, VRP was found equally active in blocking the Gly^{185} or Val^{185} proteins, also in contrast with the TM11 mutation phenotype which appears to include modulation of VRP reversal effects. The authors proposed that the pst 185 mutation did not affect initial drug binding to P-gp (on rate) but rather the subsequent dissociation of drugs from P-gp (off rate). They speculated that P-gp-mediated drug transport may involve at least two drug binding sites on the protein, an initial drug binding site and a second site distinct from the first one but implicated in drug release and efflux, with ATP hydrolysis required both for translocation of drugs from the first to the second site and for translocation from the second site to the extracellular milieu (Safa et al., 1990a). According to this model, TM11 in general and $Ser^{939/941}$ in particular would form part of the first site and would be implicated in initial binding of drugs, including reversal agents such as VRP. Mutations at this site would affect the on rate of drug binding. An alternative mechanism would be that residues such as Gly^{185} and Ser^{939} may cooperate to form part of a single binding pocket or channel with broad substrate specificity (Devine et al., 1992; Bruggemann et al., 1992). It is conceivable that mutations at different residues within such a pocket (i) may independently affect the on and off rates of transport and (ii) may increase, decrease, or leave intact the binding and transport of individual classes of drugs, a characteristic common to mutants at pst 185 (Choi et al., 1988), at 939/941 (this study), and in TM6 (Devine et al., 1992). The discussion of these theoretical models remains speculative in the absence of three-dimensional structure information on P-gp. Nevertheless, the proposition that amino acid residues from both halves of P-gp (including pst 185, 939/941, and TM6) may be implicated in drug binding and transport is in agreement with the localization of photolabeled P-gp peptides (Bruggemann et al., 1989, 1992; Yoshimura et al., 1989; Greenberger et. al, 1990) and the genetic analysis of chimeric P-gps issued from parents showing distinct biological properties (Buschman & Gros, 1991; Dhir & Gros, 1992).

The identification of specific P-gp segments and amino acid residues implicated in drug binding such as that reported here is a necessary prerequisite to the design of new compounds capable of blocking and/or bypassing the activity of P-gp.

REFERENCES

Anderson, M. P., Gregory, R. J., Thompson, S., Souza, D. W., Sucharita, P., Mulligan, R. C., Smith, A. E., & Welsh, M. J. (1991) *Science 253*, 202–205.

Bruggemann, E. P., German, U. A., Gottesman, M. M., & Pastan, I. (1989) *J. Biol. Chem. 264*, 15483–15488.

Bruggemann, E. P., Currier, S. J., Gottesman, M. M., & Pastan, I. (1992) *J. Biol. Chem. 267*, 21020–21026.

Buschman, E., & Gros, P. (1991) *Mol. Cell. Biol. 11*, 595–603.

Chen, C.-J., Chin, J. E., Ueda, K., Clark, D. P., Pastan, I., Gottesman, M. M., & Roninson, I. B. (1986) *Cell 47*, 381–389.

Choi, K., Chen, C. J., Kriegler, M., & Roninson, I. B. (1988) *Cell 53*, 519–529.

Cornwell, M. M., Safa, A. R., Felsted, R., Gottesman, M. M., & Pastan, I. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 3847–3850.

Cornwell, M. M., Tsuruo, T., Gottesman, M. M., & Pastan, I. (1987) *FASEB J. 1*, 51–54.

Devault, A., & Gros, P. (1990) *Mol. Cell. Biol. 10*, 1652–1663.

Deverson, E. V., Gow, I. R., Coadwell, J., Monaco, J. J., Butcher, G. W., & Howard, J. C. (1990) *Nature 348*, 738–741.

Devine, S. E., Ling, V., & Melera, P. W. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 4564–4568.

Dhir, R., & Gros, P. (1992) *Biochemistry 31*, 6103–6110.

Endicott, J. A., & Ling, V. (1989) *Annu. Rev. Biochem. 58*, 137–171.

Foote, S. J., Thompson, J. K., Cowman, A. F., & Kemp, D. J. (1989) *Cell 57*, 921–930.

Foote, S. J., Kyle, D. E., Martin, R. K., Oduola, A. M. J., Forsyth, K., Kemp, D. J., & Cowman, A. F. (1990) *Nature 345*, 255–258.

Greenberger, L. M., Huang Yang, C. P., Gindin, E., & Horwitz, S. B. (1990) *J. Biol. Chem. 265*, 4394–4401.

Greenberger, L. M., Lisanti, C. J., Silva, J. T., & Horwitz, S. B. (1991) *J. Biol. Chem. 266*, 20744–20751.

Gros, P., Croop, J., & Housman, D. E. (1986a) *Cell 47*, 371–380.

Gros, P., Ben Neriah, Y., Croop, J., & Housman, D. E. (1986b) *Nature 323*, 728–731.

Gros, P., Raymond, M., Bell, J., & Housman, D. E. (1988) *Mol. Cell. Biol. 8*, 2770–2778.

Gros, P., Dhir, R., Croop, J., & Talbot, F. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 7289–7293.

Hait, W. N, & Aftab, D. T. (1992) *Biochem. Pharmacol. 43*, 103–107.

Hamada, H., & Tsuruo, T. (1988) *J. Biol. Chem. 263*, 1454–1458.

Higgins, C. F., Hyde, S. C., Mimmack, M. M., Gileadi, U., Gill, D. R., & Gallagher, M. P. (1990) *J. Bioenerg. Biomembr. 22*, 571–592.

Hsu, S. I. H., Cohen, D., Kirschner, L. S., Lothstein, L., Hartstein, M., & Horwitz, S. B. (1990) *Mol. Cell. Biol. 10*, 3596–3606.

McGrath, J. P., & Varshavsky, A. (1989) *Nature 340*, 400–404.

Monaco, J. J., Cho, S., & Attaya, M. (1990) *Science 250*, 1723–1726.

Naito, M., Yusa, K., & Tsuruo, T. (1989) *Biochem. Biophys. Res. Commun. 158*, 1066–1071.

Powis, S. J., Deverson, E. V., Coadwell, W. J., Ciruela, A., Huskisson, N. S., Smith, H., Butcher, G. W., & Howard, J. C. (1992) *Nature 357*, 211–215.

Qian, X. D., & Beck, W. T. (1990) *J. Biol. Chem. 265*, 18753–18756.

Raviv, Y., Pollard, H. B., Bruggemann, E. P., Pastan, I., & Gottesman, M. M. (1990) *J. Biol. Chem. 265*, 3975–3980.

Raymond, M., Gros, P., Whiteway, M., & Thomas, D. Y. (1992) *Science 256*, 232–234.

Reid, R. E. (1983) *J. Theor. Biol. 105*, 63–76.

Riordan, J. R., Rommens, J. M., Kerem, B. S., Alon, N., Rozmahel, R., Grzelczack, Z., Zielenski, J., Lok, S., Plavsic,

N., Chou, J. L., Drumm, M. L., Iannuzzi, M. C., Collins, F. S., & Tsui, L. C. (1989) *Science 245*, 1066–1073.
Roninson, I. B. (1991) *Molecular and Cellular Biology of Multidrug Resistance in Tumor Cells*, Plenum Press, New York.
Safa, A. R. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 7187–7191.
Safa, A. R., Glover, C. J., Meyers, M. B., Biedler, J. L., & Felsted, R. L. (1986) *J. Biol. Chem. 261*, 6137–6140.
Safa, A. R., Glover, C. J., Sewell, J. L., Meyers, M. B., Biedler, J. L., & Felsted, R. L. (1987) *J. Biol. Chem. 262*, 7884–7888.
Safa, A. R., Metha, N. D., & Agresti, M. (1989) *Biochem. Biophys. Res. Commun. 162*, 1402–1408.
Safa, A. R., Stern, R. K., Choi, K., Agresti, M., Tamai, I., Mehta, N. D., & Roninson, I. B. (1990a) *Proc. Natl. Acad. Sci. U.S.A. 87*, 7225–7229.
Safa, A. R., Agresti, M., Tamai, I., Metha, N. D., & Vahali, S. (1990b) *Biochem. Biophys. Res. Commun. 166*, 259–266.
Schinkel, A. H., Roelofs, M. E. M., & Borst, P. (1991) *Cancer Res. 51*, 2628–2635.
Schurr, E., Raymond, M., Bell, J. C., & Gros, P. (1989) *Cancer Res. 49*, 2729–2734.
Skehan, P., Storeng, R., Scudiero, D., Monks, A., McMahon, J., Vistica, D., Warren, J., Bokesch, H., Kenney, S., & Boyd, M. R. (1989) *Proc. Am. Assoc. Cancer Res. 30*, 612.
Spies, T., & De Mars, R. (1991) *Nature 351*, 323–324.
Trowsdale, J., Hanson, I., Mockridge, I., Beck, S., Townsend, A., & Kelly, A. (1990) *Nature 348*, 741–743.
Tsuruo, T., Ida, H., Tsukagoshi, S., & Sakurai, Y. (1981) *Cancer Res. 41*, 1967–1972.
Ueda, K., Cardarelli, C., Gottesman, M. M., & Pastan, I. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 3004–3008.
Van der Bliek, A. M., Kooiman, P. H., Schneider, C., & Borst, P., (1988) *Gene 71*, 407–411.
Yang, C. P. H., Mellado, W., & Horwitz, S. B. (1988) *Biochem. Pharmacol. 37*, 1417–1421.
Yang, C. P. H., DePinho, S. G., Greenberger, L. M., Arceci, R. J., & Horwitz, S. B. (1989) *J. Biol. Chem. 264*, 782–788.
Yang, C. P. H., Cohen, D., Greenberger, L. M., Hsu, S. I. H., & Horwitz, S. B. (1990) *J. Biol. Chem. 265*, 10282–10288.
Yoshimura, A., Kuwazuru, Y., Sumizawa, T., Ichikawa, M., Ikeda, S. I., Ueda, T., & Akiyama, S. I. (1989) *J. Biol. Chem. 264*, 16282–16291.