Mutations to Amino Acids Located in Predicted Transmembrane Segment 6 (TM6) Modulate the Activity and Substrate Specificity of Human P-glycoprotein†

Tip W. Loo and David M. Clarke*

Medical Research Council Group in Membrane Biology, Departments of Medicine and Biochemistry, University of Toronto, Toronto, Ontario, Canada M5S 1A8

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ABSTRACT: Site-directed mutagenesis was used to investigate whether amino acids located in the predicted transmembrane segment, TM6 (residues 330−351), of human P-glycoprotein play essential roles in drug transport. Mutant cDNAs were expressed in mouse NIH 3T3 cells and analyzed with respect to their ability to confer resistance to cytotoxic drugs. Four mutations were found to strongly alter the drug resistance profile conferred by P-glycoprotein. Mutation of Val338 to Ala resulted in a mutant P-glycoprotein which conferred enhanced resistance to colchicine and reduced relative resistance to vinblastine. By contrast, mutant Gly341 to Val conferred little resistance to colchicine or doxorubicin, while its ability to confer resistance to vinblastine or actinomycin D was retained. A reduction in the ability of P-glycoprotein to confer resistance to all four drugs was observed for mutant Ala342 to Leu. Mutation of Ser344 to Ala, Thr, Cys, or Tyr resulted in mutant P-glycoproteins which were unable to confer drug resistance. Photolabeling of P-glycoprotein with azidopine in the presence of varying amounts of vinblastine showed that mutation of Ser344 to Tyr required approximately 15-fold more vinblastine to inhibit photolabeling when compared to wild-type enzyme. All of the Ser344 mutants were found to have reduced drug-stimulated ATPase activity relative to wild-type enzyme. These results, together with our previous demonstration that changes to Phe335 affected dissociation of vinblastine, suggest that TM6 may play an important role in drug−protein interaction and coupling of drug binding to ATPase activity.

The acquistion by tumor cells of multidrug resistance is a major clinical problem in the treatment of human cancers. Cells exhibiting this phenotype are resistant to a broad spectrum of cytotoxic agents that do not have a common structure or a common intracellular target. The ability of drug-resistant cells to survive is often due to overexpression of a 170 kDa membrane glycoprotein, termed P-glycoprotein [reviewed by Endicott and Ling (1989), Roninson (1991), and Gottesman and Pastan (1993)].

P-glycoprotein, the product of the multidrug resistance gene (MDR1),[1] acts as an energy-dependent pump that extrudes hydrophobic cytotoxic drugs from the cell. Cloning and sequencing of the human MDR1 (Chen et al., 1986) showed that P-glycoprotein contains 1280 amino acids organized in 2 tandem repeats of 610 amino acids joined by a liner region of 60 amino acids. Each repeat consists of an NH_2-terminal hydrophobic domain containing six potential transmembrane helices, followed by a hydrophilic domain containing a nucleotide-binding site.

The substrates of P-glycoprotein, which includes drugs such as vinblastine, colchicine, actinomycin D, and doxorubicin, and peptides such as gramicidin D and *N*-acetylleucylleucylnorleucinal [reviewed by Gottesman and Pastan (1993) and Beck and Qian (1992)], are generally hydrophobic compounds. These compounds enter the cells by passive diffusion through the plasma membrane. P-glycoprotein binds substrates embedded in the membrane (Raviv et al., 1990; Homolya et al., 1993), and their transport out of the cell is coupled to ATP hydrolysis. It has been demonstrated that P-glycoprotein possesses high levels of ATPase activity that is stimulated in the presence of drug substrates (Ambudkar et al., 1992; Sarkadi et al., 1992; Sharom et al., 1993; Al-Shawi & Senior, 1993; Shapiro & Ling, 1994). Coupling of ATPase activity to drug binding appears to involve interactions between both homologous halves of the molecule since no drug transport or stimulation of ATPase activity was observed when each half was expressed as a separate polypeptide (Loo & Clarke, 1994b). Reconstitution of drug-stimulated ATPase activity required coexpression of both half-molecules in the same cell.

One of the unresolved questions in the study of P-glycoprotein is the mechanism by which the enzyme can recognize structurally diverse compounds and couple their removal from the plasma membrane to ATP hydrolysis. One approach to understanding the transport mechanism has been to identify residues and domains critical for drug transport, through structure−function analysis. Using this approach, we and others have identified a number of key amino acids which when changed, alter the substrate specificity of the enzyme (Loo & Clarke, 1993a,b, 1994a; Currier et al., 1992; Kajiji et al., 1994). All of these residues are located within predicted transmembrane segments or cytoplasmic loops connecting these segments. These results indicate that initial

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* To whom correspondence should be addressed at the Department of Medicine, University of Toronto, Room 7342, Medical Sciences Building, 1 King's College Circle, Toronto, Ontario, Canada M5S 1A8. Telephone: 416-978-1105. FAX: 416-978-8765.

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[1] Abbreviations: MDR, multidrug resistance; TBS, Tris-buffered saline; EDTA, ethylenediaminetetraacetic acid; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline.

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interaction of drug molecules occurs within transmembrane domains.

Recent biochemical and genetic evidence suggests that transmembrane segment TM6 may play a particularly important role in drug transport by P-glycoprotein. Devine et al. (1992) detected Gly338 to Ala and Ala339 to Pro mutations in TM6 of P-glycoprotein from Chinese hamster cells that were highly resistant to actinomycin D. It was subsequently shown that the major effect of these mutations was to significantly reduce the ability of the transporter to confer resistance to colchicine and daunorubicin (Devine & Melera, 1994). We have recently shown that mutation of Phe335 in TM6 to Ala or Ser strongly altered the drug resistance phenotype conferred by P-glycoprotein in transfected cells (Loo & Clarke, 1993b). In this case, the mutant protein retained the ability to confer resistance to colchicine but conferred reduced resistance to vinblastine. Photolabeling studies with photoaffinity drug analogues also support the proposition that TM6 plays an important role in drug recognition. Greenberger (1993) showed that one of the major photoaffinity drug-labeling domains in P-glycoprotein for iodoarylazidoproazosin is located within or immediately COOH-terminal to TM6.

In this study, we have utilized site-directed mutagenesis to examine the functional consequences of changes to each of the residues located in TM6 of human P-glycoprotein. We show that TM6 is an important segment since changes to 5 of the 21 residues resulted in mutant P-glycoproteins which lacked the ability to confer drug resistance in transfected cells or conferred altered drug resistance phenotypes in the presence of vinblastine, colchicine, doxorubicin, or actinomycin D.

EXPERIMENTAL PROCEDURES

Oligonucleotide-Directed Mutagenesis. The methods used have been described previously (Loo & Clarke, 1994a). Briefly, a full-length human MDR1 cDNA was modified to encode the epitope for monoclonal antibody A52 (Zubrzycka-Gaarn et al., 1984) at the COOH-terminal end of P-glycoprotein and inserted into vector pMT21 to give pMT21MDR1A52. The sequence at the COOH-terminus of P-glycoprotein that would normally end as TKRQ now became TKR**AISLISNSCSPEFDDLPLAEQREACRR-GDPR**Q. The *Bgl*II fragment (positions 259−1223) from the modified cDNA was then ligated into the polylinker region of Bluescript vector (Stratagene) for site-directed mutagenesis by the method of Kunkel (1985). Fragment *Nsi*I (position 925) to *Bst*BI (position 1135) was then excised and subcloned back into its original position in pMT21MDR1A52. The integrity of the mutated segment was checked by sequencing (Sanger et al., 1977) the entire fragment including the cloning sites of the DNA to be used for transfection.

Cell Culture and DNA Transfection. Procedures for transfection of mouse NIH 3T3 cells, followed by selection in the presence of vinblastine (5 nM) or cholchicine (45 nM), have been described previously (Loo & Clarke, 1993a). In some cases, cells were cotransfected with pWL-neo (Stratagene) and mutant constructs of pMT21MDR1A52 in a 1:10 molar ratio, respectively. Mass populations of neomycin-resistant colonies were selected initially in the presence of Geneticin (G418) at a concentration of 0.72 mM, followed by selection in the presence of vinblastine (5 nM) or colchicine (45 nM). Drug sensitivity was determined by a tetrazolium-based assay for cell viability (Alley et al., 1988), as described previously (Loo & Clarke, 1993a).

[^{3}H]Azidopine Photolabeling. HEK 293 cells were transfected with cDNA by the calcium phosphate precipitation method (Chen & Okayama, 1987). After 48 h, the cells were harvested, washed with PBS, and resuspended in PBS. The cells were then incubated with 250 nM [^{3}H]azidopine (48 Ci/mmol, Amersham) in PBS in the presence or absence of various amounts of vinblastine for 30 min at room temperature in the dark. The cells were then UV-irradiated on ice for 15 min with a 15-W G15T8 germicidal lamp at a distance of 5 cm. Cells were collected by centrifugation, suspended in 100 μL of PBS, and lysed by addition of 9 volumes of buffer I [25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% (v/v) Triton X-100, 0.5% (w/v) sodium deoxycholate, 1% aprotinin, 5 μg/mL leupeptin, and 10 μM *p*-(aminoethyl)benzenesulfonyl fluoride]. The lysate was centrifuged at 13000*g* for 5 min, and the pellet was discarded. Five micrograms of monoclonal antibody A52 was then added to the supernatant. After incubation overnight at 4 °C, the immune complexes were precipitated at 4 °C for 2 h, using protein A−Sepharose CL-4B (Pharmacia). The immunoprecipitated proteins were washed 5 times with buffer I and then subjected to SDS−PAGE, followed by fluorography.

Expression in Sf9 Cells with a Baculovirus Vector. The modified human MDR1 cDNA fragment containing the epitope for monoclonal antibody A52 was subcloned into the multiple cloning site of *Autographa californica* nuclear polyhedrosis virus (AcNPV) transfer vector pBlueBac III (Invitrogen), downstream from the polyhedrin promoter (Smith et al., 1985; Summer & Smith, 1987), to yield pBlueBac-MDR-A52. The cDNAs coding for mutants Ser344 to Ala, Thr, Cys, or Tyr, and also containing the epitope for monoclonal antibody A52, were also subcloned into pBlueBac III. To generate recombinant baculovirus carrying the wild-type or mutant P-glycoprotein cDNAs, the permissive host cell line, Sf9, was cotransfected with the transfer vector containing the desired cDNA and linearized wild-type AcNPV viral DNA using cationic liposomes (Invitrogen). Six days after transfection, recombinant virus was harvested from the culture medium and plaque-purified in the presence of 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside for ease of detection of plaques. Stocks of the purified recombinant virus were prepared, the titer was determined, and the virus was stored at 4 °C.

For preparation of membranes, Sf9 cells grown in spinner flasks were infected with the desired recombinant baculovirus stocks, and membranes were prepared as described previously (Loo & Clarke, 1994b).

ATPase Activity. The ATPase activity was determined by measuring inorganic phosphate liberated. Membranes (50 μg of protein) were incubated in 0.5 mL of buffer containing 100 mM Tris-HCl, pH 8.0, 4 mM EGTA, 4 mM DTT, 100 mM KCl, 10 mM NaN_3, 10 mM $MgCl_2$, 2 mM ouabain, 5 mM ATP, and various amounts of drug substrates, at 37 °C for 20 min as described by Sarkadi et al. (1992). The reaction was terminated by addition of 0.5 mL of 10% (w/v) TCA, and the reaction mixture was centifuged for 5 min at 14000*g*. The amount of inorganic phosphate in the supernatant was determined by a colorimetric reaction at 660 nm (Ames, 1966).

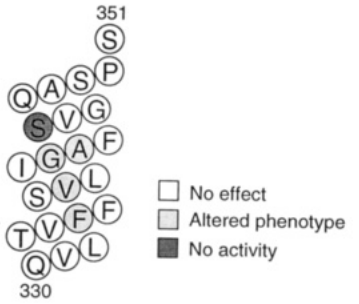

FIGURE 1: Proposed structural model of transmembrane segment TM6 showing the amino acid sequence of the human wild-type enzyme arranged in an α-helical net. The functional consequences of mutation of Phe335, Phe336, and Phe343 (Loo & Clarke, 1993b) and Pro350 (Loo & Clarke, 1993a) were described in earlier papers. No drug-resistant colonies were obtained for Ser344 mutants.

Immunological Procedures. Immunoblotting was performed on proteins separated by SDS−PAGE (Laemmli, 1970) and transferred electrophoretically to nitrocellulose (Towbin et al., 1979). The blots were incubated in 2% (w/v) milk powder in TBS (10 mM Tris-HCl/150 mM NaCl, pH 7.5) for 20 min and then incubated in TBS/1% (w/v) milk powder containing 2 μg/mL A52 antibody for 30 min at room temperature. After being washed twice for 10 min in 0.5% (v/v) Tween 20 in TBS, the blot was incubated in horseradish peroxidase-conjugated anti-mouse secondary antibody (Amersham) at a dilution of 1:1000 in TBS/1% (w/v) milk powder. The blots were washed as described above and developed using the enhanced chemiluminescence technique (Amersham).

Expression levels of P-glycoprotein-A52 were examined using a modified ELISA assay. A sodium dodecyl sulfate-solubilized sample of cells (extract of 3000 cells in 0.4% SDS) was spotted onto 1 cm^2 of nitrocellulose. The nitrocellulose was processed with monoclonal antibody A52 and alkaline phosphatase-conjugated anti-mouse secondary antibody as described above. The nitrocellulose was then placed in 0.3 mL of 1 mg/mL *p*-nitrophenyl phosphate in 10% (v/v) diethanolamine/5 mM $MgCl_2$, pH 9.8, for 15 min at 37 °C. The reaction was stopped by placing samples in an ice bath and color development measured at an absorbance of 540 nm. In these assays, deoxycholate-purified fast-twitch skeletal muscle Ca^{2+}-ATPase (MacLennan, 1970) was used as a standard. Samples containing 0−1000 pg of Ca^{2+}-ATPase were spotted onto nitrocellulose and processed as described above.

RESULTS

Construction and Biological Activity of Mutants. Figure 1 shows an α-helical representation of the predicted transmembrane segment TM6. It is the last transmembrane segment of the first hydrophobic domain and is directly connected to the first nucleotide-binding domain. In a previous study, we showed that mutation of Phe335 altered the substrate specificity of the enzyme (Loo & Clarke, 1993b). Mutation of Phe335 to Ala or Ser resulted in P-glycoproteins which conferred little resistance to vinblastine or actinomycin D, but retained the ability to confer resistance to colchicine and doxorubicin. Mutation of Phe336, Phe343, or Pro350 (Loo & Clarke, 1993a,b) in TM6, however, did not measurably affect the drug resistance profile conferred by P-glycoprotein.

Table 1: Ratio of Number of Vinblastine-Resistant Colonies to Colchicine-Resistant Colonies after Transfection

mutant	vinblastine/colchicine[a]	mutant	vinblastine/colchicine[a]
wild-type	4.8	Gly341→Val	*b*
Gln330→Ala	5.0	Ala342→Leu	*b*
Val331→Ala	7.3	Phe343→Ala	5.3
Leu332→Ala	6.3	Ser344→Ala	*c*
Thr333→Ala	4.5	Val345→Ala	2.0
Val334→Ala	4.0	Gly346→Val	4.6
Phe335→Ala	0.3	Gln347→Ala	1.5
Phe336→Ala	3.3	Ala348→Leu	4.3
Ser337→Ala	5.8	Ser349→Ala	3.7
Val338→Ala	0.2	Pro350→Ala	5.6
Leu339→Ala	3.7	Ser351→Ala	4.8
Ile340→Ser	6.0		

[a] Ratio of number of colonies obtained after transfection and selection in the presence of 5 nM vinblastine and 45 nM colchicine. Each value is an average of three separate transfections done in duplicate. [b] No colchicine-resistant colonies were obtained. [c] No colchicine- or vinblastine-resistant colonies were obtained.

In this study, we tested whether mutation of additional amino acids in this transmembrane segment would alter function. Site-directed mutagenesis was used to change the codon for each amino acid to alanine, except for the codons for Gly, Ile, or Ala. Alanine was chosen as a replacement, since it is a small neutral amino acid that would preserve the α-helical nature of the transmembrane segment as well as minimize alterations in the structural integrity of the protein. Glycines were changed to valine so as to be consistent with our previous study on the glycine residues in the predicted loops of P-glycoprotein (Loo & Clarke, 1994a). Alanine was changed to leucine rather than glycine because a glycine has the potential to introduce a "kink" in the transmembrane segment (Reithmeier & Deber, 1992). Ile340 was changed to serine, since it introduces a change in polarity while maintaining the size of the side chain. The mutant cDNAs were transfected into the drug-sensitive mouse cell line NIH 3T3 followed by selection in the presence of colchicine (45 nM) or vinblastine (5 nM). These two drugs were chosen, since there is evidence to suggest that their binding sites may be nonoverlapping. Mutants have been identified which retain the ability to confer resistance to either one of these drugs while losing the ability to confer resistance to the other. For example, mutant Ser341 to Phe of mouse *mdr1* (Gros et al., 1991 and mutants Pro223 to Ala and Pro866 to Ala of human MDR1 (Loo & Clarke, 1993a) encode for transporters which confer little or no resistance to colchicine, whereas the ability to confer resistance to vinblastine is retained. On the other hand, mutant Phe335 to Ala conferred resistance to colchicine but little resistance to vinblastine (Loo & Clarke, 1993b). Accordingly, cells transfected with the mutant cDNAs resulted in the appearance of drug-resistant colonies in the presence of colchicine or vinblastine (Table 1). The majority of the mutants yielded a greater number of vinblastine-resistant than colchicine-resistant colonies with the exception of mutants Phe335 to Ala or Val338 to Ala. Cells transfected with these two mutants yielded 3−5-fold more colchicine-resistant colonies than vinblastine-resistant colonies. Two other mutant cDNAs, Gly341 to Val and Ala 342 to Leu, yielded vinblastine-resistant colonies but no colchicine drug-resistant colonies.

One mutant, Ser344 to Ala, did not yield any colonies in the presence of either vinblastine or colchicine. Therefore, the side chain of Ser344 may be important for function. Accordingly, we constructed mutants which contained other changes to Ser344. Mutant cDNAs were constructed such that Ser344 was changed to Thr, Cys, or Tyr. Transfection of NIH 3T3 cells with these mutant cDNAs, however, also did not yield any drug-resistant colonies in the presence of colchicine or vinblastine.

Expression of Mutant cDNAs. In previous studies, we identified other mutations such as Pro709 to Ala (Loo & Clarke, 1993a) and Gly to Val changes at positions 251, 268, 269, and 781 (Loo & Clarke, 1994a) which abolished the ability of P-glycoprotein to confer resistance to colchicine or vinblastine. All of these mutants, however, were found to be improperly processed so that they were not targeted correctly to the plasma membrane. It was therefore possible that mutations to Ser344 could also result in improperly processed proteins. To determine whether mutant cDNAs containing changes to Ser344 would yield mature P-glycoproteins, expression was carried out in HEK 293 cells. This transient expression system allows for rapid expression of the mutants and assessment of the structural integrity of the mutant proteins. Figure 2A shows an immunoblot of wild-type P-glycoprotein-A52 and mutants expressed in HEK 293 cells. Cells transfected with the cDNAs of wild-type A52 or mutant P-glycoproteins expressed an immunoreactive product of 170 kDa as the major product, as well as a minor product of 150 kDa. The larger product is mature P-glycoprotein, whereas the 150 kDa product is a core-glycosylated processing intermediate (Loo & Clarke, 1994a). To determine whether the mutant P-glycoproteins reach the plasma membrane, cell-surface labeling was carried out using membrane-impermeable biotin−hydrazide conjugates. Biotin-LC−hydrazide has been used to distinguish cystic fibrosis transmembrane conductance regulator (CFTR) at the surface of epithelial cells from that present in intracellular membranes (Prince et al., 1993). Accordingly, cells expressing wild-type or mutant P-glycoprotein-A52 were treated with periodate to oxidize extracellular sugar moieties to generate aldehydes and then reacted with biotin-LC−hydrazide (Pierce). Detergent extracts of biotinylated cells were immunoprecipitated with monoclonal antibody A52. Figure 2B shows a blot of the immunoprecipitated proteins that was developed with streptavidin-conjugated horseradish peroxide. Only the mature 170 kDa product of the mutant proteins was biotinylated. These results are in agreement with the finding that the mature P-glycoprotein is located in the plasma membrane, whereas the 150 kDa core-glycosylated product is located intracellularly. All of the Ser344 mutants were also labeled with biotin-LC−hydrazide. These results also suggest that none of the mutations to Ser344 had any detectable effect on the biosynthesis or targeting to the plasma membrane of P-glycoprotein. Similarly, mutations (Val338 to Ala, Gly341 to Val, and Ala342 to Leu) which affected the ratio of drug-resistant colonies obtained after transfection and selection in the presence of vinblastine and colchicine did not appear to alter the structure or processing of the enzyme (Figure 2A). Mutation of Phe335 was previously shown not to affect structure or biosynthesis of P-glycoprotein (Loo & Clarke, 1993b).

It was possible that mutations to Ser344 may have affected the ability of the mutant protein to be expressed stably in

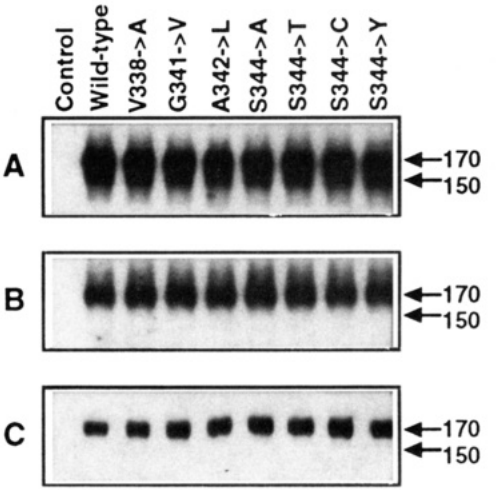

FIGURE 2: Expression and cell-surface labeling of P-glycoprotein mutants with altered drug transport properties. (A) HEK 293 cells were transfected with wild-type and mutant cDNAs. After 48 h, whole cell extracts were subjected to SDS−PAGE on a 6.5% polyacrylamide gel and transferred onto nitrocellulose. The blot was probed with monoclonal antibody A52 at a concentration of 2 μg/mL, followed by horseradish peroxidase-conjugated goat anti-mouse antibody, and developed using the enhanced chemiluminescence detection technique (Amersham). The positions of the mature (170 kDa) and core-glycosylated (150 kDa) P-glycoproteins are indicated. (B) For cell-surface labeling, HEK 293 cells were transfected with wild-type or mutant cDNAs. The cells were harvested 48 h later, chilled on ice, washed twice with ice-cold PBS, and then incubated in PBS containing 10 mM sodium periodate for 30 min in the dark at 4 °C. The cells were then washed with PBS and treated with 2 mM biotin-LC−hydrazide (Pierce) in 100 mM sodium acetate buffer, pH 5.5, for 30 min at room temperature. The cells were washed with 0.1 M Tris-HCl buffer, pH 7.5, and solubilized with detergent, and the labeled P-glycoprotein was immunoprecipitated with monoclonal antibody A52. The immunoprecipitated proteins were separated by SDS−PAGE, transferred onto nitrocellulose, probed with streptavidin-conjugated horseradish peroxidase, and detected by the enhanced chemiluminescence technique. (C) Cell-surface labeling of NIH 3T3 cells stably expressing mutant P-glycoproteins-A52 was carried out as described above. Stable cell lines expressing P-glycoprotein-A52 with changes to Ser344 were generated by cotransfection of the mutant cDNA with plasmid pWL-neo(Stratagene). G418-resistant colonies were isolated and tested for expression of P-glycoprotein-A52 with monoclonal antibody A52. Cell lines expressing the mutant P-glycoprotein-A52 were subjected to cell-surface labeling, and equivalent amounts of mutant P-glycoprotein-A52 were applied in each lane.

mouse NIH 3T3 cells. Accordingly, we cotransfected the mutant cDNAs with pWL-neo plasmid (Stratagene), and G418-resistant colonies were selected. Whole cell extracts of individual clones were tested by immunoblotting with monoclonal antibody A52. It was found that more than 50% of the G418-resistant clones obtained after cotransfection with either wild-type or mutant P-glycoprotein-A52 cDNAs contained the 170 kDa protein as the major product, indicating that mutations to the cDNA encoding Ser344 did not reduce transfection efficiency. Again, the P-glycoproteins in these cell lines could be biotinylated with biotin-LC−hydrazide (Figure 2C), suggesting that the mature P-glycoproteins were present at the cell surface.

Drug Survival Characteristics of Cell Clones Expressing Mutant P-glycoproteins. The functional characteristics of the stable cell lines expressing the mutant P-glycoproteins were further characterized by studying individual drug-resistant colonies. Three to five clones of each mutant were isolated, and detergent extracts from cells of each clone were

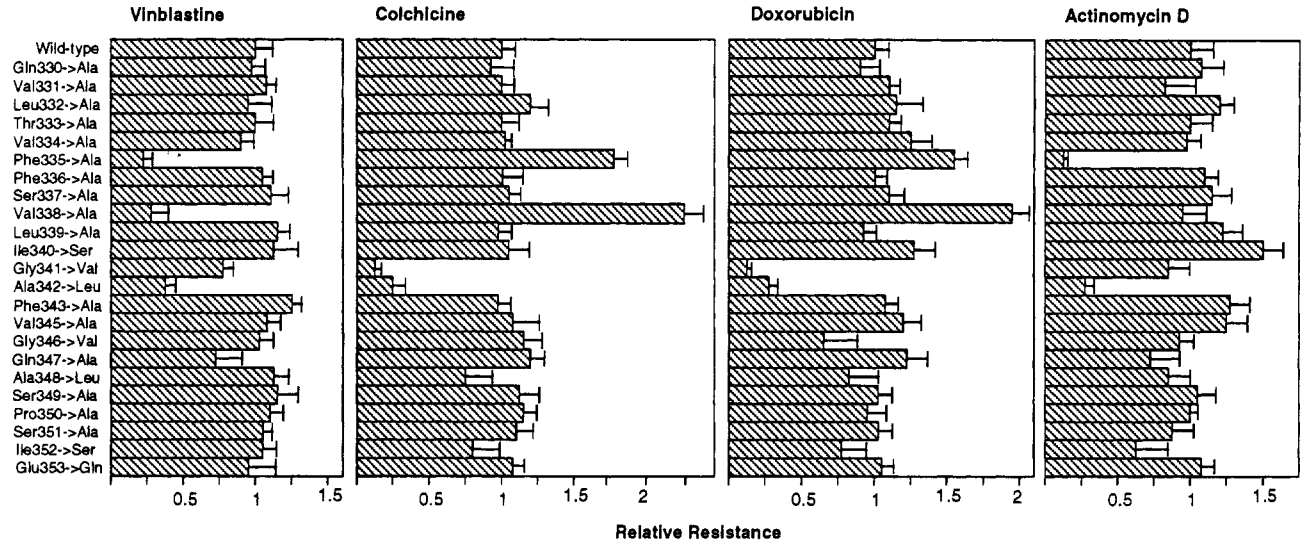

FIGURE 3: Comparison of relative resistances of cell expressing mutant P-glycoproteins. Relative resistances were determined by comparing the ID_{50} (the drug concentration that inhibits plating efficiency by 50%) for each stably transfected cell line and then dividing this value by the amount of P-glycoprotein-A52 per 1×10^5 cells. The amount of P-glycoprotein-A52 in each cell line was measured as described under Experimental Procedures. The results are presented relative to the wild-type, which is arbitrarily assigned a value of 1. Drug-resistant profiles of mutations to Phe335, Phe336, Phe343, and Pro350 have been described previously (Loo & Clarke, 1993a,b). The cDNAs of these mutants were also transfected into NIH 3T3 cells, and the drug-resistant clones were characterized and included for comparison. Each bar represents the average of three to five individual clones which were analyzed at least 4 times.

immunoblotted to test for expression of P-glycoprotein-A52. In each case, an immunoreactive protein product of apparent mass 170 kDa was present as the major product (data not shown), indicating that drug resistance was due to the expression of the mutant MDR1-A52 cDNA.

The drug survival characteristics of each of the mutant clones were analyzed in the presence of various concentrations of vinblastine, colchicine, doxorubicin, and actinomycin D. The ID_{50} (the drug concentration necessary to inhibit cell growth by 50%) for vinblastine was 0.6 ± 0.1 nM for control NIH 3T3 cells and 15.8 ± 2.5 nM for clones expressing similar levels of wild-type P-glycoprotein-A52, representing a 26.3-fold increase in resistance above control cells. The ID_{50} for colchicine, doxorubicin, and actinomycin D for NIH 3T3 cells was 10.0 ± 1.2, 5.2 ± 0.7, and 0.3 ± 0.1 nM, respectively. Cells expressing wild-type P-glycoprotein-A52 demonstrated increased resistance to colchicine (7.7-fold), doxorubicin (7.9-fold), and actinomycin D (19.4-fold) above that of control cells.

Several of the mutants conferred altered drug resistance profiles (Figure 3). Cells expressing mutant Val338 to Ala exhibited preferential resistance to colchicine relative to vinblastine. This phenotype was due to an increase in the ability of mutant Val338 to Ala to confer relative resistance to colchicine (2.20) together with a decrease in the ability to confer relative resistance to vinblastine (0.3). A similar effect was previously observed with another TM6 mutant, namely, Phe335 to Ala (Loo & Clarke, 1993b). The decrease in resistance to vinblastine and increase in resistance to colchicine for these mutants would account for the appearance of a greater number of colonies in the presence of colchicine relative to vinblastine (Table 1). It is interesting to note that the side chains of Phe335 and Val338 would lie close to each other and be on the same face of the α-helix. There was one significant difference, however, in the drug resistance profiles conferred by the two mutants. Mutation of Phe335 to Ala caused a decrease in relative resistance to actinomycin D, whereas the ability of mutant Val338 to Ala to confer resistance to actinomycin D was unchanged.

In contrast to the drug resistance profiles conferred by Phe335 to Ala and Val338 to Ala, mutant Gly341 to Val retained the ability to confer resistance to vinblastine, but showed little ability to confer resistance to colchicine or doxorubicin. Indeed, we were unable to obtain any drug-resistant colonies in the presence of colchicine after transfection of NIH 3T3 cells with the mutant cDNA (Table 1). In all other cases, with the exception of mutants Ser344 to Ala or Ala342 to Leu, it was possible to obtain drug-resistant colonies in the presence of vinblastine or colchicine. The inability to obtain drug-resistant clones in the presence of colchicine with mutant Ala342 to Leu is probably due to the fact that the mutant confers reduced relative resistance to colchicine (0.3). This mutation may alter the overall activity of the transporter since it also confers reduced relative resistance to vinblastine, doxorubicin, and actinomycin D.

Photoaffinity Labeling with Azidopine. Azidopine is a substrate of P-glycoprotein (Tamai & Safa, 1991) and will also label the protein. Photoaffinity probes such as azidopine and iodoarylazidoprazosin label a site which is within or in close proximity to TM6 as well as TM12 (Greenberger et al., 1990; Greenberger, 1993). It has been found that mutation of Ser941 to Phe in mouse *mdr1* or Ser939 to Phe in mouse *mdr3* resulted in a strong reduction in labeling of P-glycoprotein by these compounds (Kajiji et al., 1993). Accordingly, we investigated whether mutations which affected the ability of P-glycoprotein to confer drug resistance also affected the labeling of P-glycoprotein with azidopine. The cDNAs of mutants Val338 to Ala, Gly341 to Val, Ala342 to Leu, and Ser344 to Ala, Thr, Cys, or Tyr were transiently expressed in HEK 293 cells. A transient expression system was utilized since expression levels of MDR1 gene products are very high in this expression system. Whole cells expressing the mutant P-glycoprotein were incubated with [^{3}H]azidopine (250 nM final concentration) in the presence or absence of 100 μM vinblastine and then

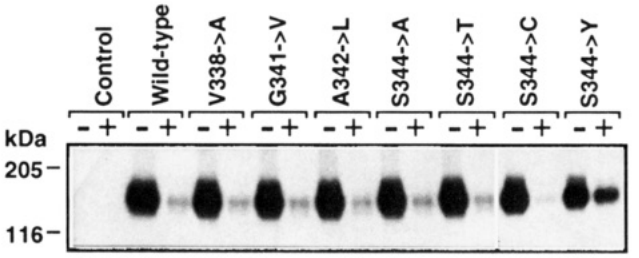

FIGURE 4: Photoaffinity labeling with [^{3}H]azidopine. HEK 293 cells were transfected with wild-type or mutant cDNAs. The cells were harvested 48 h after transfection and photolabeled with 250 nM [^{3}H]azidopine in the absence (−) or present (+) of 100 μM vinblastine. The cells were solubilized with detergent, and labeled P-glycoprotein was immunoprecipitated with monoclonal antibody A52. The immunoprecipitated proteins were separated by SDS−PAGE followed by fluorography.

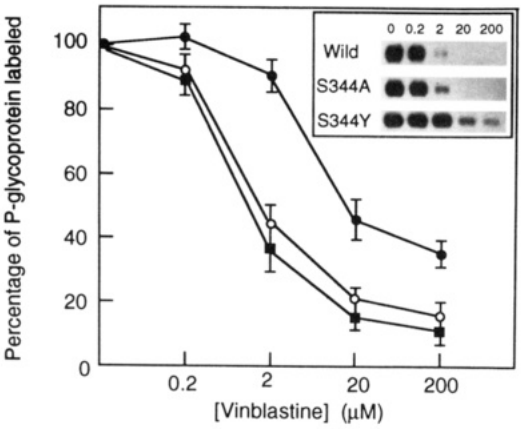

FIGURE 5: Inhibition of [^{3}H]azidopine labeling by vinblastine. HEK 293 cells were transfected with wild−type or mutant cDNAs. The cells were harvested 48 h after transfection and labeled with 250 nM [^{3}H]azidopine in the presence of varying amounts of vinblastine. The cells were solubilized with detergent, and P-glycoprotein-A52 was immunoprecipitated with monoclonal antibody A52 and quantitated by liquid scintillation counting. The amount of azidopine bound to P-glycoprotein-A52 in the presence of vinblastine was expressed as a percent of that which was bound in the absence of vinblastine (100%). Each point is the mean of three separate experiments. Wild-type (■); mutant Ser344 to Ala, (○); mutant Ser344 to Tyr (●). Inset: P-glycoprotein was labeled with azidopine in the presence of different amounts of vinblastine as described above and immunoprecipated with monoclonal antibody A52. The proteins were separated on a 6.5% SDS−PAGE followed by fluorography. The numbers refer to the amount of vinblastine (micromolar) present during labeling with azidopine.

UV-irradiated. The cells were then solubilized with detergent, and the photolabeled P-glycoproteins were immunoprecipitated with monoclonal antibody A52. The immunoprecipitated P-glycoproteins were separated by SDS−PAGE and visualized by fluorography. As shown in Figure 4, each of the mutants would be photolabeled with azidopine. Therefore, none of the mutations greatly reduced azidopine labeling as reported for mouse *mdr1*/*mdr3* Ser939/941 to Phe mutants (Kajiji et al., 1993). Labeling of wild-type and mutant P-glycoproteins by azidopine was inhibited in the presence of vinblastine. Mutant Ser344 to Tyr, however, was labeled to a much greater extent in the presence of 100 μM vinblastine.

To further characterize vinblastine inhibition of azidopine labeling for mutant Ser344 to Tyr, we examined azidopine labeling in the presence of various concentrations of vinblastine. As shown in Figure 5 (inset), photolabeling of the wild-type or mutant Ser344 to Ala P-glycoproteins with [^{3}H]-azidopine was markedly reduced in the presence of 2 μM vinblastine, whereas there is little inhibition of labeling mutant Ser344 to Tyr. We quantitated the amount of label incorporated into P-glycoprotein in the presence of various amounts of vinblastine (Figure 5). The concentration of vinblastine required to reduce azidopine labeling by 50% was approximately 1 μM for wild-type P-glycoprotein-A52. Similar results were obtained with mutants Ser344 to Ala, Cys, or Thr. By contrast, a much higher concentration of vinblastine (approximately 15 μM) was required to reduce azidopine labeling of mutant Ser344 to Tyr. These results suggest that mutation of Ser344 to Tyr causes a large reduction in the apparent affinity of the enzyme for vinblastine.

ATPase Activities of P-glycoprotein Mutants with Changes to Ser344. P-glycoprotein has ATPase activity which is stimulated by drug substrates (Sarkadi et al., 1992; Ambudkar et al., 1992; Al-Shawi & Senior, 1993; Shapiro & Ling, 1994). The calcium channel blocker verapamil has been shown to be the most potent stimulator of ATPase activity, activating the ATPase activity of P-glycoprotein 5−10-fold. Therefore, it was of interest to test whether Ser344 mutants retained drug-stimulatable ATPase activity.

ATPase activity measurements requre a relatively large amount of enzyme. The yield of protein products in mammalian cells, however, was too low to measure ATPase activity. It has been found, however, that expression of P-glycoprotein in insect cells using a baculovirus vector yields large amounts of transporter which has high levels of drug-stimulated ATPase activity (Sarkadi et al., 1992; Loo & Clarke, 1994b). Accordingly, we expressed wild-type and Ser 344 mutants of P-glycoprotein in Sf9 cells using a baculovirus expression system to measure drug-stimulated ATPase activity.

Membranes prepared from cells infected with recombinant baculovirus carrying wild-type or mutant cDNAs were immunoblotted with monoclonal A52 antibody. A large amount of an immunoreactive protein of apparent mass 140 kDa was found to be present in cells infected with virus carrying wild-type or mutant cDNAs but not in cells infected with wild-type baculovirus (data not shown). The 140 kDa protein corresponds to the underglycosylated form of P-glycoprotein as was observed previously (Germann et al., 1990; Sarkadi et al., 1992; Loo & Clarke, 1994b).

Membranes prepared from Sf9 cells infected with recombinant and wild-type baculovirus were then assayed for drug-stimulated ATPase activity. The ATPase activity of wild-type P-glycoprotein was maximally stimulated (5.6-fold) in the presence of 25 μM verapamil (Figure 6A). All four mutants showed significantly less stimulation in the presence of verapamil (2.2−2.9-fold). Half-maximal stimulation of ATPase activity occurred at approximately 1−2 μM in all cases.

Stimulation of ATPase activity by vinblastine was also determined (Figure 6B). The ATPase activity of the wild-type P-glycoprotein was stimulated to a greater extent (3-fold) than that of mutants with changes to Ser344 (1.8−2.1-fold). Half-maximal stimulation of ATPase activity occurred in the presence of approximately 1−2 μM vinblastine for wild-type P-glycoprotein and mutants Ser344 to Ala, Cys, or Thr. By contrast, half-maximal stimulation of mutant Ser344 to Tyr occurred at approximately 6 μM vinblastine. A shift in the apparent affinity for vinblastine in mutant

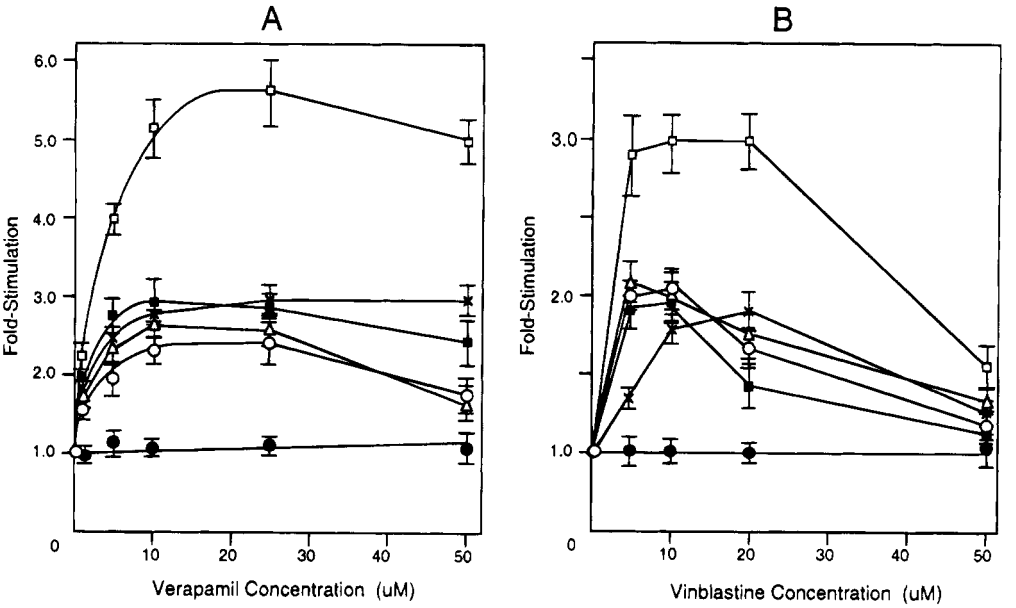

FIGURE 6: Effect of verapamil and vinblastine on the ATPase activity of wild-type and Ser344 mutant P-glycoprotein. Membranes were prepared from Sf9 cells expressing wild-type P-glycoprotein-A52 (□), mutant Ser344 to Ala (○), mutant Ser344 to Thr (■), mutant Ser344 to Cys (△), mutant Ser344 to Tyr (×), or infected with wild-type virus (●). The ATPase activity was measured in the presence of various concentrations of verapamil (A) or vinblastine (B). The fold-stimulation is the ratio of the ATPase activity found in the presence of drug substrate to that found in the absence of drug substrate. Each point is the mean of four separate experiments.

Ser344 to Tyr was also detected in the azidopine labeling assays (Figure 5). This mutation, however, did not alter the affinity of P-glycoprotein for verapamil in the ATPase assay. These results indicate that the major effect of mutations to Ser344 was to make the coupling of ATPase activity to drug binding less efficient.

DISCUSSION

In this study, we found that changes made to certain amino acids within the predicted transmembrane segment, TM6, can modulate the ability of P-glycoprotein to confer resistance to four structurally diverse cytotoxic compounds, namely, vinblastine, colchicine, doxorubicin, and actinomycin D. Of particular interest is the observation that changes to these key residues result in different phenotypes conferred by the mutant P-glycoproteins. Mutations of Phe355 to Ala or Ser (Loo & Clarke, 1993b) or Val338 to Ala result in P-glycoproteins which confer preferential resistance to colchicine rather than vinblastine. This is an intriguing observation since the side chains of these two residues would be located close to one another in an α-helical arrangement, suggesting that this face of the helix participates in drug recognition and/or transport. These residues, however, do not play identical roles since the ability of P-glycoprotein to confer resistance to actinomycin D was reduced in mutant Phe335 to Ala but not in mutant Val338 to Ala.

In contrast to the results observed for mutations to Phe335 and Val338, mutation of Gly341 to Val reduced the capacity of the transporter to confer resistance to colchicine and doxorubicin, whereas the ability to confer resistance to vinblastine or actinomycin D was retained. It is interesting to note that a change at this position in hamster P-glycoprotein was one of the two changes found in a mutant P-glycoprotein which conferred an altered drug-resistance phenotype. Devine et al. (1992) showed the presence of two amino acid changes, Gly338 to Ala (equivalent in position to Gly341 of human P-glycoprotein) and Ala339 to Pro, in TM6 of P-glycoprotein from Chinese hamster cells that had increased resistance to actinomycin D. The major effect of these mutations was an almost 10-fold decrease in the level of colchicine resistance conferred by the mutant protein when compared to wild-type P-glycoprotein (Devine & Melera, 1994). The mutations had a much smaller effect on the ability of the mutant enzyme to confer resistance to vincristine and actinomycin D.

The fact that mutations which alter substrate specificity were mapped to the same residues using two different approach indicates that structure–function analysis will be a useful technique in identifying residues important for function. Another interesting observation is that only a small number of mutations affect activity. Only 14 of the 85 residues in the transmembrane domain that have been modified have been found to modulate substrate specificity or affect the activity of P-glycoprotein (Loo & Clarke 1993a,b, 1994a; this study). Kajiji et al. (1993, 1994) reported that mutations to Ser941 of mouse *mdr1* or Ser943 of mouse *mdr3* alter drug binding to P-glycoprotein, whereas mutations flanking these residues are without measurable consequence. Therefore, it appears that only certain residues are important determinants for substrate specificity and activity.

By mapping the distribution of deleterious mutations, we may learn more about drug–protein interactions. In TM6, for example, it appears that the ability of P-glycoprotein to confer resistance to colchicine correlates with the size of the residue at key sites. Substitution of Phe335 and Val338 by smaller residues increases colchicine resistance while substitution of Gly341 and Ala342 by larger residues decreases resistance to colchicine. Therefore, it appears that the ability of P-glycoprotein to confer resistance to colchicine can be modulated by the size of the residue at these key sites.

It has been postulated that transport of drugs occurs through a single barrel of the transporter (Raviv et al., 1990; Gottesman & Pastan, 1993). The fact that different mutations

to amino acids in the predicted transmembrane segment, TM6, altered the substrate specificity of P-glycoprotein for four structurally diverse compounds suggests that this transmembrane segment may lie close to, or form part of, the transport site. This transmembrane segment is directly connected to the nucleotide-binding site, and it is possible that conformational changes occurring in the nucleotide-binding domain upon ATP hydrolysis could affect TM6. Evidence in support of a role for TM6 in the binding and release of drugs comes from study of the effects of mutant Phe335 to Ala (Loo & Clarke, 1993b). Mutation of Phe335 to Ala resulted in a P-glycoprotein which conferred reduced relative resistance to vinblastine. The apparent affinity of the mutant protein for vinblastine, however, was increased, since a lower concentration of vinblastine was required to inhibit photolabeling by azidopine. Therefore, a plausible explanation for the reduction in the relative resistance of mutant Phe335 to Ala to vinblastine is that the release of the drug during the transport cycle is impaired due to its increased affinity at the binding site.

Evidence that TM6 may also participate in the initial drug−protein interactions was the observations that mutations to Phe355, Val338, and Gly341 resulted in P-glycoproteins which conferred altered drug-resistant phenotypes. In addition, mutant Ser344 to Tyr appeared to reduce the apparent affinity of P-glycoprotein for vinblastine. It required about 15-fold more vinblastine to inhibit photolabeling by azidopine compared to wild-type enzyme. It appears that this effect may be due to increased bulkiness in the side chain since mutation of Ser344 to Ala, Thr, or Cys did not measurably influence vinblastine inhibition of azidopine labeling. It is possible that the increased bulkiness of the side chain may have caused a structural perturbation which affects other parts of the molecule. Residue Ser344, however, appears to be an important residue for activity through its involvement in coupling drug binding to ATPase activity. All of the mutations to Ser344 reduced verapamil- or vinblastine-stimulated ATPase activity. It is possible, however, that the reduction in drug-stimulatable ATPase activity of all the Ser344 mutants could be due to a structural perturbation incurred by the loss of the polar side group.

Transmembrane segment TM6 in CFTR, which is structurally similar to P-glycoprotein, also appears to play an important role in the function of this protein. Mutation of Lys335 or Arg347 to Glu in TM6 altered the permeability and/or conductance ratios for halide ions (Anderson et al., 1991). Mutation of Arg347 to His resulted in the ability of CFTR to eliminate the multiple ion occupancy effects when the pH of the intracellular solution is changed (Tabcharani et al., 1993). This result suggests that some of the residues in TM6 of CFTR are accessible to water and may line the chloride channel.

The results of this study suggest that TM6 plays an important role in determining substrate specificity and the overall activity of P-glycoprotein. It may interact with other transmembrane domains to form a transport channel. Analysis of chimeric P-glycoproteins (Buschman & Gros, 1991; Dhir & Gros, 1992) and half-molecules (Loo & Clarke, 1994b), together with the localization of the azidopine labeling sites (Bruggemann et al., 1989, 1992; Greenberger, 1993), supports the proposition that the two homologous halves of the molecule participate in drug transport. In the carboxyl half of mouse P-glycoprotein, transmembrane segment TM11 has been implicated as a possible drug-binding site (Gros et al., 1991; Kajiji et al., 1993) and may interact with TM6 during drug−protein interaction. The identification of other amino acids critical for P-glycoprotein function would enhance our understanding of the transport mechanism of this intriguing protein.

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