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ORIGINAL ARTICLE

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Further characterization of the sixth transmembrane domain of Pgp1 by site-directed mutagenesis

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Abstract *Purpose*: Several studies have identified amino acid residues located on the hydrophobic side of the helix that forms transmembrane domain 6 (TM6) of the ABC transporter P-glycoprotein (Pgp) as being important for function. The purpose of this study was to determine if alterations to residues on the hydrophilic side could also affect function and to determine the extent to which altering the hydrophobic nature of residues on the hydrophobic side would impair the protein. *Methods*: A full-length cDNA encoding wild-type Pgp1 from CHL cells was used as a template for site-directed mutagenesis. Eight different mutations, three on the hydrophilic side and five on the hydrophobic side, were prepared and transfected into drug-sensitive host cells. Wild-type transfectants served as controls. Drug resistance levels, RD_{50} values for cyclosporin A (CsA) and verapamil, iodoarylazidoprazosin (IAAP) photolabeling and verapamil-stimulated ATPase activity were evaluated. *Results*: Substitution of any one of three amino acid residues on the hydrophilic side of TM6 disrupted function and led to alterations in drug resistance, CsA sensitivity, IAAP photoaffinity labeling, and in one case verapamil-stimulated ATPase activity. Replacement of a hydrophobic residue on the hydrophobic face of the helix with increasingly hydrophilic side-chains led to functional changes, the extent of which did not correlate with the degree of side-chain hydrophilicity. Finally, while the placement of a proline residue along either face of the helix had varying effects on function, in all cases its presence interfered with verapamil-stimulated ATPase activity. *Conclusions*: Taken together these results indicate that both faces of TM6 mediate Pgp1 function and that the expected conformational changes resulting from proline substitutions at a variety of locations within the helix can alter the protein's enzymatic activity.

Keywords P-glycoprotein · TM6 · Site-directed mutagenesis · MDR

Abbreviations *ABC:* ATP binding cassette · *ActD:* actinomycin D · *COLC:* colchicine · *CsA:* cyclosporin A · *DAUN:* daunorubicin · *IAAP:* iodoarylazidoprazosin · *MDR:* multidrug resistance · *Pgp:* P-glycoprotein · *TM:* transmembrane domain · *VCR:* vincristine · *VRP:* verapamil

Introduction

Intrinsic or acquired resistance of tumor cells to a wide variety of anticancer drugs is termed multidrug resistance (MDR), and is recognized as a major obstacle in the treatment of neoplastic disease [10]. One established mechanism for MDR is the overexpression of P-glycoprotein (Pgp) in the plasma membrane of resistant cells [1]. Pgp functions as an ATP-dependent efflux transporter for a variety of structurally and functionally unrelated cytotoxic compounds, including the majority of chemotherapeutic agents used in the treatment of cancers. The efflux action of Pgp is thought to reduce the intracellular drug concentration to below cytotoxic levels, thereby conferring resistance.

Pgp belongs to the superfamily of ATP binding cassette (ABC) transporters and contains two homologous halves each composed of six putative α-helical transmembrane domains (TMs) and one ATP binding site [15]. Despite extensive study, it remains unclear how Pgp recognizes and binds drugs with very dissimilar structures and how it utilizes the energy of ATP hydrolysis for transport. Putative drug-binding regions have been identified on or nearby the 5th, 6th, 11th, and 12th

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transmembrane segments, either by photoaffinity labeling with drug analogs [12] or by studying the effects of site-directed mutagenesis on drug resistance and transport [8, 13, 17, 18, 21]. The results, however, show that single amino acid residues that affect the function of the transporter are found scattered across the entire length of the protein [5, 11, 27], suggesting that high-order structure is involved in drug recognition.

A previous study of a mutant form of Chinese hamster Pgp (Pgp1) obtained from a cell line (DC-3F/ADX) selected for growth in the presence of actinomycin D (ActD) revealed that a double mutation, G338A and A339P, within TM6 increases resistance to the selective agent and also alters cross-resistance to other drugs [8]. An alanine scan of TM6 [18] has confirmed the importance of the analogous residues, G341 and A342, and V338 in MDR1, and additional studies [17] have shown that alteration to F335 is also able to affect drug recognition. Deletion of F335 has been found to drastically reduce the effectiveness of cyclosporin A (CsA) reversal of MDR1-mediated mdr [4], and it has been shown that the A339P mutation in hamster Pgp1 has a similar effect [21]. In addition to these studies, alteration to the TM6 region of the cystic fibrosis transmembrane conductance regulator, another member of the ABC transporter family, has also been found to affect its function [26]. Hence, the importance of TM6 in the interaction of substrates with Pgp and other ABC transporters has been clearly established.

Recently it has been proposed that the drug-binding domain of MDR1 is formed between residues located in TMs 4, 5, and 6, and 10, 11, and 12 [20]. This model is based upon cysteine scanning mutagenesis and disulfide crosslinking studies, and predicts that TM6 residues A342 and L339, both located on the hydrophobic side of the helix, face inward toward the other helixes. While this model would suggest that alterations to residues on the hydrophobic face of the helix would be likely to affect Pgp function, it is not clear what impact, if any, alterations to the hydrophilic side would have. Indeed it has been shown for MDR1 [18] that of the five residues in TM6 found to be sensitive to replacement by alanine, four (V338, F335, G341, A342) are located on the hydrophobic side of TM6 (Fig. 1). While alteration of S344, located on the hydrophilic side, to Ala inactivate the transporter, no other mutations have been found to affect Pgp function. Interestingly, it is the hydrophilic rather than the hydrophobic side of TM11 that has been reported to be more mutation-sensitive and important for drug recognition by mouse mdr3 [14].

Alanine scanning is a powerful tool with which to assess the involvement of amino residues in protein function and structure. However, the lack of an effect does not necessarily rule out the importance of a given residue. Rather it indicates that substitution with alanine is not sufficient to display a phenotype. We therefore used site-directed mutagenesis to introduce several different alterations into the hydrophilic side of Pgp1 to determine if these changes affect function. We also placed a series of amino acids with increasing hydrophilicity into a single location on the hydrophobic side of the helix (see Fig. 1) to determine what effects disruption of the hydrophobic environment may cause. The results showed that substitutions into the hydrophilic side of TM6 altered a wide range of Pgp1 functions and that disruption of the hydrophobic face led to changes that did not correlate with the degree of hydrophilicity of the residue. Finally, we showed that although substitution with proline on either face of the helix led to a variety of effects, consistent among them was a decrease in verapamil (VRP) stimulated ATPase activity. The results indicate that both sides of TM6 are involved in the interaction of Pgp1 with drugs and reversal agents, and with the enzymatic activity of the transporter.

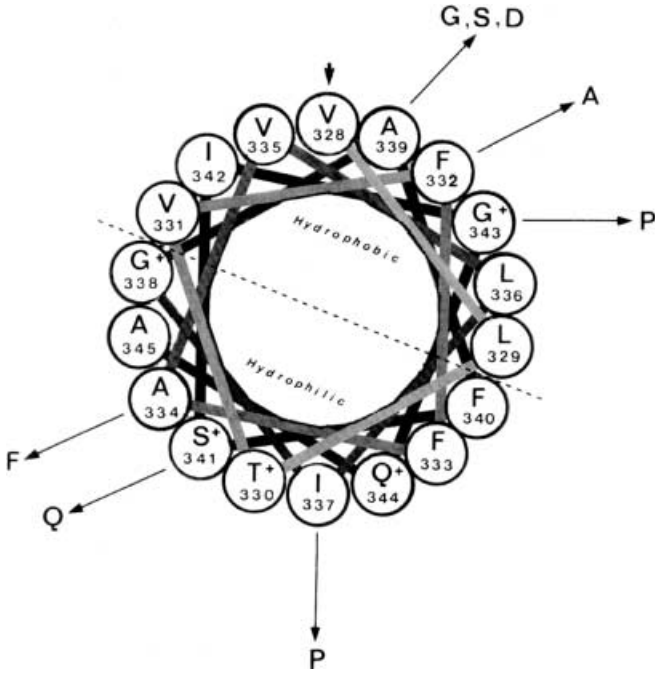


Fig. 1 Helical wheel representation of TM6. Amino acid residues 328 to 345 in the wild-type protein are shown and the perspective is from the external face of the helix toward the cytoplasm. V328 is indicated by the *arrowhead* and is predicted to lie close to or at the external face of the membrane. Residues with hydrophilic sidechains are indicated by a + sign. The numbers indicate the position of the residues in the primary sequence. The *arrows* indicate the various mutations made for this study

Materials and methods

Cell lines

The drug-sensitive Chinese hamster lung fibroblast cell line DC-3F was maintained in minimum Eagle's medium (MEM/F12) containing penicillin/streptomycin, supplemented with 5% fetal calf serum (Life Technologies, Gaithersburg, Md.), and served as the host cell line for all the transfection studies reported here. All chemicals were purchased from Sigma Chemical Company (St. Louis, Mo.), unless otherwise stated. Actinomycin D (ActD), colchicine (COLC), daunorubicin (DAUN), and verapamil (VRP) were also purchased from Sigma. Vincristine (VCR) and cyclo-

sporin A (CsA) were generous gifts from Eli Lilly (Indianapolis, Ind.) and Sandoz (East Hanover, N.J.), respectively. VRP and cyclosporin A (CsA) were dissolved in 15% and 100% ethanol, respectively. Other drugs were dissolved in 0.9% NaCl.

Site-directed mutagenesis and the development of stable transfectants

Site-directed mutagenesis was performed using the QuickChange Site-directed Mutagenesis Kit from Stratagene (La Jolla, Calif.). The plasmid pGA-1 [6] containing a wild-type Pgp1 insert was used as the template for these constructions. Pairs of complementary mutant primers were synthesized by Life Technologies (Gaithersburg, Md.). The sequences of the individual sense strands, with the altered codons underlined, were as follows (the corresponding changes in amino acids are indicated in parentheses):

- 5′-CAAGTGCTCACTGTCGCCTTTGCTGTATTAAT-3′ (F332A)
- 5′-CTCACTGTCTTCTTTTTTGTATTAATTGGGGCA-3′ (A334F)
- 5′-TTCTTTGCTGTATTACCTGGGGCATTCAGTAT-3′ (I337P)
- 5′-GCTGTATTAATTGGGGGATTCAGTATTGGACA-3′ (A339G)
- 5′-GCTGTATTAATTGGGTCATTCAGTATTGGACA-3′ (A339S)
- 5′-GCTGTATTAATTGGGGACTTCAGTATTGGACA-3′ (A339D)
- 5′-GGGGCATTCAGTATTCCACAGGCATCTCCAAA-3′ (G343P)
- 5′-GTATTAATTGGGGCATTCCAGATTGGACAGGC-ATCTCCA-3′ (S341Q)

Mutant cDNAs were synthesized by PCR using the appropriate primers and were confirmed by DNA sequencing. They were then digested with BsiW I and Bgl II to obtain an 885-bp fragment containing the altered TM6 sequence, which was used to replace the corresponding fragment in the wild-type pGA-1 plasmid. After cloning, the inserts were again sequenced to confirm the presence of the desired mutations, and then removed from the plasmids by digestion with BsiW1 and Bgl II. The resulting 2947-bp fragments were directionally cloned into the eukaryotic expression vector, pHBneo, that had been digested with the same enzymes, as described previously [8]. Constructs containing wild-type and mutant cDNAs were transfected into drug-sensitive DC-3F cells using Lipofectin according to the instructions provided by the vendor (Life Technologies, Gaithersburg, Md.). Stable transfectants were obtained by selection with G418 (800 ng/ml; Gibco, Gaithersburg, Md.).

The expression of Pgp1 in stable transfectants was determined by Western blot analysis of total cell lysates as described previously [7]. An anti-hamster Pgp monoclonal antibody, MC-215, obtained from Kamiya Biomedical Company (Thousand Oaks, Calif.) at a dilution of 1:250 was used as the primary antibody. Goat anti-mouse IgG conjugated with peroxidase, at a dilution of 1:50,000 in TBS (Tris-based saline), was used as the secondary antibody. An ECL plus immunoblot kit from Amersham Pharmacia Biotech (Piscataway, N.J.) was used for signal detection. Clones that expressed comparable amounts of Pgp1 were used for further study. To ensure that there was no discrepancy between the relative amounts of Pgp1 found in total cell lysates versus the cell membrane, crude plasma membranes were prepared and analyzed by Western blotting as described previously [7] but using the MC-215 antibody.

Drug resistance and reversal experiments

ED_{50} values, defined as the drug dose required to reduce cell numbers to 50% of control values over a 72-h growth period, were used to measure drug resistance and have been described previously [7]. RD_{50} values, defined as the dose of reversal agent required to reduce drug resistance to 50% of that of controls in 72 h [21], were used to estimate the reversal efficiency of CsA and VRP. All experiments reported here were performed using the same batch of drugs and the results are presented as the mean value and the standard error of the mean obtained from triplicate experiments.

[^{125}I]Iodoarylazidoprazosin labeling

Labeling of Pgp with [^{125}I]iodoarylazidoprazosin ([^{125}I]IAAP) was performed as described by Dey et al, [9] with slight modifications. Crude membrane protein (50 μg) was incubated with 6 μ*M* [^{125}I]IAAP (NEN Life Science Products, Boston, Mass.) in 50 m*M* Tris-HCl, pH 7.5, 50 m*M* NaCl, 50 m*M* KCl, and 10 m*M* $MgCl_2$ at room temperature for 15 min under subdued light and with appropriate shielding. The samples were then illuminated with a UV lamp (366 nm) at room temperature for 15 min and solubilized by mixing with an equal volume of 2× SDS/PAGE sample buffer. Incubation was continued for an additional 30 min at which time the samples were electrophoresed on 7.5% SDS/PAGE gels. The resulting gels were analyzed by autoradiography.

ATPase activity

Crude membranes were prepared by centrifugation of total cell lysates at 100,000 *g* (35,000 rpm) in a Beckman Ti 80 rotor at 4°C for 1 h. Pellets were resuspended in buffer and assayed for ATPase activity as described by Hrycyna et al. [16] with some modifications. Basal activity was determined by incubation of 100 μg crude membrane protein in a reaction mixture containing 50 m*M* Tris-HCl, pH 7.5, 5 m*M* sodium azide, 2 m*M* ethylene-bis(oxyethylenenitrilo) tetraacetic acid, pH 7.0, 2 m*M* ouabain, 2 m*M* dithiothreitol, 50 m*M* KCl, and 10 m*M* $MgCl_2$ at 37°C for 5 min. Drug-stimulated activity was determined by the addition of VRP to a final concentration of 250 n*M* from a stock solution prepared in dimethyl sulfoxide. The final concentration of dimethyl sulfoxide in the reaction mixture was less than 1%, which had been shown not to affect ATPase activity. Parallel experiments were performed in the presence and absence of 300 μ*M* vanadate. After incubation at 37°C for 3 min, the reaction was started by the addition of ATP to a final concentration of 5 m*M* and continued at 37°C for 20 min. The total volume of the reaction was 100 μl and the reaction was terminated by the addition of 100 μl 5% SDS. The amount of inorganic phosphate released was measured by a colorimetric reaction [24]. In brief, 400 μl of reagent A (2.5 *M* sulfonic acid, 1% ammonium molybdate, and 0.014% antimony potassium tartrate), 1 ml distilled water, and 200 μl 1% fresh ascorbic acid were added and the mixture incubated at room temperature for 10 min. The absorbency of the samples was read at 880 nm. A standard concentration curve was generated using potassium phosphate.

Results

Pgp1 expression in stable transfectants

G418-resistant clones from each set of transfections were isolated and total cell lysates prepared and screened by Western blotting for Pgp expression levels. Individual clones expressing similar levels of the various mutant forms of Pgp1 as well as wild-type (Fig. 2) were selected for further study. Additional Western analysis (not shown) of membrane preparations from these clones further ensured that each expressed similar amounts of Pgp in the plasma membrane. Hence, it is unlikely that differences reported here in Pgp function were due to the inability of transfectants to properly process Pgp1 to the surface.

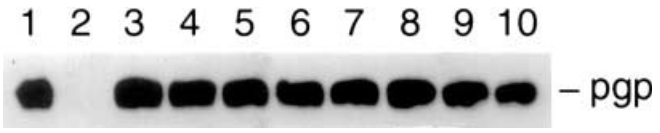


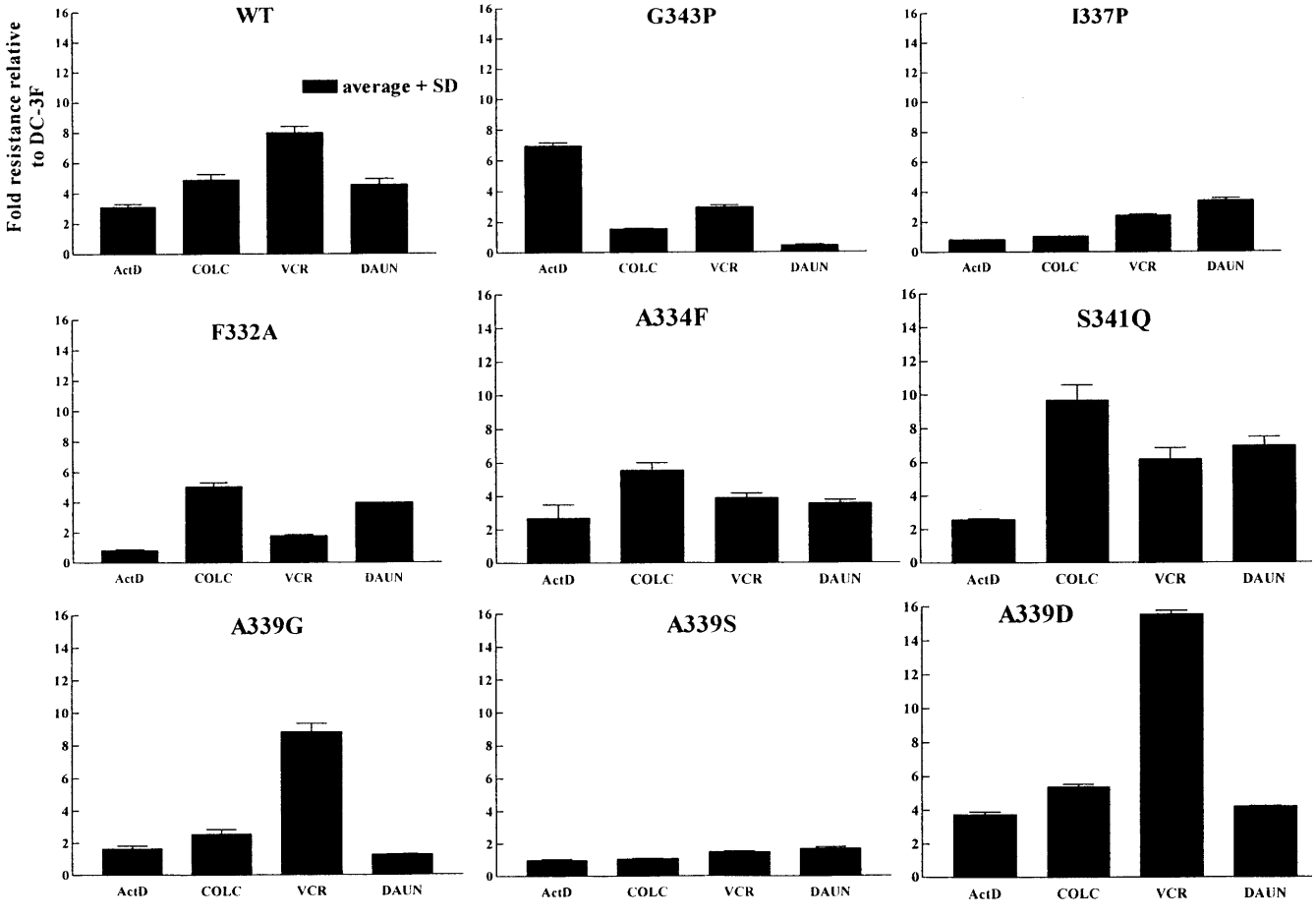
Fig. 2 Expression of Pgp1 in transfectants. Neo-resistant clones from each set of transfections were expanded and analyzed for Pgp expression by Western blotting. A 30-μg aliquot of total cell lysate was used in each case and clones with matching levels of expression selected for further study (*lane 1* wild-type transfectant, *lane 2* drug-sensitive DC-3F control cell line, *lane 3* A339G, *lane 4* A339S, *lane 5* A339D, *lane 6* F332A, *lane 7* G343P, *lane 8* I337P, *lane 9* S341Q, *lane 10* A334F)

Cross-resistance profiles of the transfectants

The ED_{50} values for each of four drugs, ActD, DAUN, VCR, and COLC, were determined for the various transfectants and these values were compared to those for the drug-sensitive parental cell line DC-3F. The results (Fig. 3) are shown as histograms that indicate the fold resistance of each of the transfectants with respect to DC-3F. The cross-resistance profile of the wild-type transfectant is characteristic of that reported previously for hamster Pgp1 in these cells with maximal and minimal resistance to VCR and ActD, respectively, and intermediate resistance to DAUN and COLC [7, 8]. Mutations introduced into the hydrophobic side of the helix, A334F, S341Q, and I337P, all showed different results. A334F slightly reduced resistance to VCR but did not affect the other drugs, whereas S341Q doubled resistance to COLC, slightly increased resistance to DAUN, but did not affect ActD or VCR. The effect of I337P was more dramatic, substantially reducing resistance to all the drugs except DAUN. Hence substitutions into the hydrophilic side of the helix affected resistance presumably by altering the ability of the transporter to recognize drugs.

We next determined the effects of inserting amino acids of increasing hydrophilicity (G < S < D) at a fixed site on the hydrophobic face of the helix at position A339. Alteration of the analogous position in MDR1, F336A, had been shown previously to lower resistance to the four drugs [18], while conversion to Pro in Pgp1 decreased resistance to DAUN and COLC, slightly increased resistance to VCR and doubled resistance to ActD [21]. Substitution with Gly, which is more hydrophilic than Ala (the wild-type residue), reduced resistance to three of the four drugs but did not affect

Fig. 3 Cross-resistance profiles of wild-type and mutant transfectants. The values shown were derived from the ED_{50} values for each of the four drugs in DC-3F as compared to those in the various transfectants, and are expressed as fold increase in resistance relative to DC-3F cell line. The values are the mean and standard error of the mean from three separate determinations



resistance to VCR, while Ser substantially reduced resistance to all of the drugs. The presence of Asp, the most hydrophilic of the substitutions, surprisingly had very little effect on three of the drugs but doubled resistance to VCR. These results clearly support the notion that it is not the hydropathic properties alone of the amino acid side-chains along the hydrophobic face of TM6 that affect drug recognition, but rather their overall biochemical characteristics. Conversion of F332 to Ala confirmed this observation since the small decrease in hydrophobicity between these two residues proportionally lowered resistance to three drugs but had no effect on resistance to VCR, while substitution of G343 with the more hydrophobic Pro, lowered resistance to DAUN, VCR, and COLC, but increased resistance to ActD by more than twofold.

Sensitivity to reversal agents

RD_{50} values of CsA and VRP for the four drugs were determined for all mutations and the results are presented in Table 1. The I337P, A334F, and S341Q mutants had varying effects on the ability of either of these agents to reverse resistance. A334F elevated the RD_{50} of CsA for VCR and DAUN by 13-fold. A 3.5-fold increase in the RD_{50} of CsA for COLC was noted for S341Q, and a more modest 2.5-fold increase was found for I337P for VCR. None of these mutations increased the RD_{50} for VRP, with the exception of A334F which increased it by 7.6-fold for DAUN. Interestingly, the S341Q mutation decreased the RD_{50} for VRP to VCR and DAUN by fivefold and threefold, respectively, and to COLC by threefold as well. Overall these results show that substitutions along the hydrophilic face of TM6 can affect the sensitivity of the transporter to reversal agents.

The A339G, A339S, and A339D mutations also displayed a variety of results. A339G had little effect on the RD_{50} of CsA for ActD, but increased it from five- to sevenfold for the other drugs. A339S had little effect, while A339D increased the RD_{50} for COLC by 3.5-fold. None of the mutations affected the sensitivity of the transporter to VRP for any of the drugs tested with the exception of COLC, and in this case A339G and A339D increased the RD_{50} by 4.4- and 3.7-fold respectively. Additionally, A339G lowered the RD_{50} for ActD tenfold. Hence, as with drug resistance, altering the hydrophobic nature of the hydrophobic face of TM6 at position 339 did not lead to proportional changes in the sensitivity of the transporter to VRP or CsA.

The G343P mutation did not affect the RD_{50} of CsA for ActD or VCR, but did increase it six- and eightfold, respectively, for COLC and DAUN. However, this mutation lowered the RD_{50} of VRP for ActD by tenfold, and increased it for COLC by eightfold. The F332A mutation substantially reduced the RD_{50} of CsA by five- to tenfold for all of the drugs except COLC. No change was noted in the RD_{50} for VRP to three of the drugs, but a tenfold decrease did occur with COLC.

Photoaffinity labeling with [^{125}I]IAAP

The results of the photoaffinity labeling experiments are shown in Fig. 4. 50 μg [^{125}I]IAAP-labeled membrane protein from each of the transfectants was analyzed. As indicated, wild-type Pgp was readily labeled with this photoactive drug, and mutant forms A339D and G343P were labeled with similar intensity (Fig. 4A, lanes 1, 3 and 4, respectively). However, when the A339G, F332A, S341Q, and A334F mutants were analyzed (Fig. 4B, lanes 2, 4, 5, and 7, respectively), no labeling was observed, indicating that these substitutions had interfered with the interaction of Pgp with IAAP, even though none of them severely reduced drug resistance (Fig. 2). When the amount of protein analyzed was increased approximately tenfold, each of the mutant proteins became labeled (Fig. 4B, lanes 3, 6, 8, and 9). Hence, while the affinity for IAAP, when compared to wild-type or to the A339D and G343P mutations, had been reduced, the

Table 1 RD_{50} values of TM6 mutant transfectants. Each of the values shown is the amount (ng/ml) of reversal agent required to reduce resistance to 50% that of controls, and is the mean and standard error of the mean from three separate determinations. Numbers in parentheses represent the ratios of the RD_{50} value for each drug normalized to that of the wild-type transfectant

		ActD	COLC	VCR	DAUN
CsA	WT	416 ± 62 (1.0)	78 ± 4 (1.0)	67 ± 1 (1.0)	86 ± 9 (1.0)
	G343P	850 ± 37 (2.0)	485 ± 28 (6.2)	103 ± 6 (1.5)	731 ± 6 (8.5)
	I337P	376 ± 91 (0.9)	81 ± 1 (1.0)	168 ± 9 (2.5)	93 ± 15 (1.1)
	A339G	590 ± 58 (1.4)	576 ± 45 (7.4)	577 ± 55 (8.6)	453 ± 83 (5.3)
	A339S	149 ± 5 (0.4)	78 ± 5 (1.0)	–	85 ± 3 (1.0)
	A339D	448 ± 87 (1.1)	273 ± 12 (3.5)	113 ± 12 (1.7)	234 ± 13 (2.7)
	F332A	82 ± 5 (0.2)	54 ± 1 (0.7)	9 ± 1 (0.1)	6 ± 1 (0.1)
	A334F	1027 ± 188 (2.5)	81 ± 7 (1.0)	865 ± 45 (12.9)	1165 ± 38 (13.5)
	S341Q	409 ± 16 (1.0)	269 ± 2 (3.5)	70 ± 4 (1.1)	74 ± 1 (0.9)
VRP	WT	587 ± 17 (1.0)	367 ± 42 (1.0)	300 ± 44 (1.0)	133 ± 24 (1.0)
	G343P	75 ± 6 (0.1)	2913 ± 170 (7.9)	563 ± 58 (1.9)	96 ± 2 (0.7)
	I337P	455 ± 69 (0.8)	409 ± 23 (1.1)	505 ± 41 (1.7)	95 ± 2 (0.7)
	A339G	65 ± 1 (0.1)	1616 ± 312 (4.4)	446 ± 25 (1.5)	108 ± 19 (0.8)
	A339S	534 ± 60 (0.9)	464 ± 4 (1.3)	–	221 ± 43 (1.7)
	A339D	674 ± 44 (1.2)	1368 ± 197 (3.7)	268 ± 38 (0.9)	259 ± 6 (1.9)
	F332A	755 ± 29 (1.3)	320 ± 54 (0.9)	525 ± 87 (1.8)	14 ± 1 (0.1)
	A334F	857 ± 100 (1.5)	480 ± 26 (1.3)	400 ± 21 (1.3)	1016 ± 74 (7.6)
	S341Q	720 ± 84 (1.2)	110 ± 22 (0.3)	69 ± 10 (0.2)	38 ± 5 (0.3)

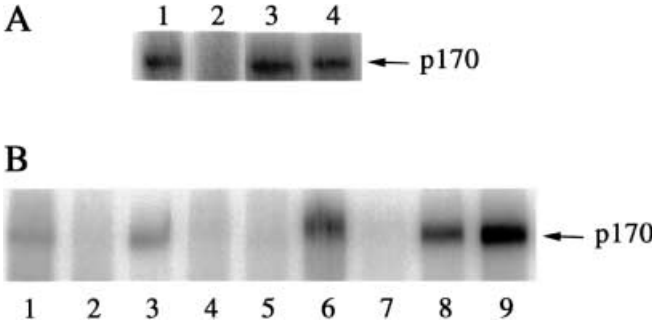


Fig. 4A, B [^{125}I] IAAP labeling of Pgp1. Membrane protein (50 μg) was labeled with [^{125}I] IAAP and fractionated through a 7.5% SDS-PAGE gel. **A** *Lane 1* wild-type transfectant, *lane 2* DC-3F, *lane 3* A339D, *lane 4* G343P. **B** Membrane proteins were analyzed as in **A**. *Lane 1* 50 μg wild-type, *lane 2* 50 μg A339G, *lane 3* 500 μg A339G, *lane 4* 50 μg F332A, *lane 5* 50 μg S341Q, *lane 6* 500 μg F332A, *lane 7* 50 μg A334F, *lane 8* 500 μg S341Q, *lane 9* 500 μg A334F

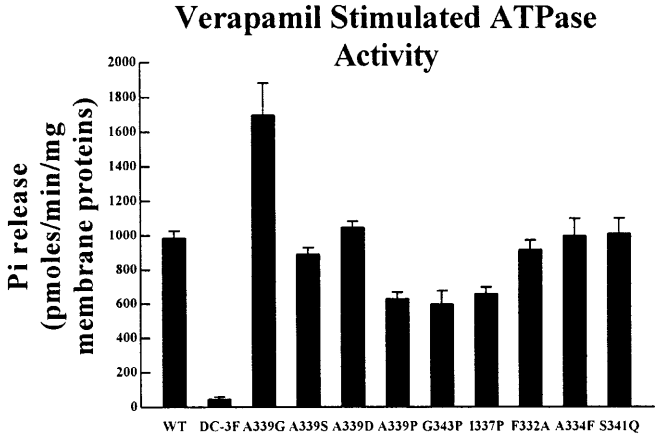


Fig. 5 VRP-stimulated ATPase activity. VRP-stimulated ATPase activity was defined as the difference in phosphate release in the presence and absence of 250 n*M* VRP. Each value shown is the mean and standard error of the mean from three determinations. The A339G mutation enhanced VRP-stimulated ATPase activity by nearly twofold ($P < 0.02$), while A339P, G343P, and I337P reduced it by approximately 60% ($P < 0.01$, $P < 0.02$, and $P < 0.01$, respectively). None of the other mutations had a significant effect

various proteins were still capable of binding this agent, and they did so with different intensities, suggesting that the different mutations altered the affinity for IAAP to different extents. Preliminary results (Song and Melera, unpublished observations) have shown that this labeling can be inhibited in the presence of 0.8 μ*M* CsA, consistent with the known involvement of TM6 in the CsA binding site. Interestingly, two of the mutants that drastically reduced IAAP labeling, A334F and S341Q, reside on the hydrophilic side of TM6.

ATPase activity

Transfection of wild-type Pgp1 into DC-3F cells increased VRP-stimulated ATPase activity by nearly 40-fold (Fig. 5). Two of the mutations located on the hydrophilic side of the helix, A334F and S341Q, had no effect, while the third, I337P, reduced the activity by approximately 40% ($P < 0.01$). Two of the substitutions at position 339, A339S and A339D, had no effect, but A339G increased the activity by 1.7-fold ($P < 0.02$). The F332A mutation had no effect, but G343P and A339P, a naturally selected mutation [8], both decreased VRP-stimulated ATPase activity by approximately 40% ($P < 0.02$).

Discussion

Current models of Pgp structure depict insertion and folding of the protein within the plasma membrane such that the N and C terminal regions approach one another to form a drug-binding domain or channel through which substrates are transported via energy derived from ATP hydrolysis. Although several different renditions of this model have been proposed [3, 12], the most recent, based upon cysteine scanning and crosslinking studies [19, 20], suggests that TMs 4, 5 and 6, and 10, 11 and 12 form this drug-binding domain. The residues within TM6 of MDR1 that have been identified to be within crosslinking distance of TMs 11 and 12 are A342 and L339 [19]. These are analogous with A339 and L336, respectively, in hamster Pgp1, both of which are located on the hydrophobic face of the helix (Fig. 1). Given this alignment, the hydrophilic face of TM6 would be expected to face away from the drug-binding domain toward the lipid protein bilayer of the membrane [20], with its role, if any, in the function of the transporter unclear.

Introduction of different substitutions into three different positions, A334F, S341Q, and I337P, along the hydrophilic face of TM6 affected to varying degrees drug resistance, sensitivity to reversal agents CsA and VRP, IAAP photolabeling and in one case ATPase activity (Table 2). Hence it appears that these residues are important for transporter function. Since, according to the current model, they face away from the drug-binding domain, it is possible that they exert their effects through allosteric interactions, either with components of the plasma membrane [29] or with other portions of the transporter resulting in structural alterations that affect function. Alternatively, they might influence the manner in which substrates enter the transporter from the plasma membrane. It is also possible that during the conformational change that is thought to result from ATP hydrolysis and to accompany drug transport [16, 22, 25], TM6 rotates such that the hydrophilic face turns inward disrupting the high affinity "on site" [9]. Two of the substitutions analyzed, A334F and S341Q, would not be expected to alter the conformation of TM6, yet both have large side-chains, one (A334F) hydrophobic and the other (S341Q) hydrophilic, that might hinder the movement of TM6 during the catalytic cycle [20, 22, 23, 25, 28]. However, because both of these strongly decrease the ability of the protein to be photolabeled with

Table 2 Summary of results. The results from Figures 3, 4, and 5 and Table 1 are summarized (– no change, ↑ an increase, ↓ a decrease; the number of arrows reflects the magnitude of the change). All values are relative to those displayed by the wild-type transfectant (*n.d.* not done)

Mutants	Drug resistance				RD_{50} values								IAAP labeling	ATPase activity
	ActD	COLC	VCR	DAUN	CsA				VRP					
					ActD	COLC	VCR	DAUN	ActD	COLC	VCR	DAUN		
A334F	–	–	↓	–	↑	–	↑↑	↑↑	–	–	–	↑↑	↓↓↓	–
S341Q	–	↑	–	↑	–	↑	–	–	–	↓↓	↓↓	↓↓	↓↓↓	–
I337P	↓↓	↓↓	↓	–	–	–	↑	–	–	–	–	–	n.d.	↓↓
A339G	↓	↓	–	↓	–	↑↑	↑↑	↑↑	↓↓	↑	–	–	↓↓↓	↑
A339S	↓↓	↓↓	↓↓	↓↓	↓	–	n.d.	–	–	–	n.d.	–	n.d.	–
A339D	–	–	↑↑	–	–	↑	–	↑	–	↑	–	–	–	–
*A339P	↑	–	↑	–	↑↑	↑↑	↑↑↑	↑↑	–	↑	–	–	–	↓
F332A	↓↓	–	↓↓	–	↓↓	↓	↓↓↓	↓↓↓	–	–	–	–	↓↓↓	–
G343P	↑	↓	↓	↓↓	–	↑↑	–	↑↑	↓↓↓	↑↑	–	–	–	↓↓

IAAP, it is more likely that they affect drug binding (Table 2), presumably at the "on site".

The I332P mutant is expected to distort the helix and to limit its flexibility [2]. We have reported previously that an A339P mutation on the hydrophobic face of the TM6 helix increases resistance to ActD while decreasing resistance to DAUN and COLC and desensitizing the transporter to reversal by CsA [21]. As shown in Table 2, it also decreases ATPase activity, as does the G343P mutation. Hence, Pro substitutions at three different locations in TM6 spaced approximately 10 Å apart, extending from the center of the helix inward toward the cytoplasm, all significantly reduce VRP-stimulated ATPase activity compared to wild-type, yet basal ATPase activity, although somewhat low in these transfectants due to the relatively low level of Pgp expression, is largely unchanged (data not shown). Although these effects may be due in part to the amino acid side-chain, Pro substitutions are known to cause significant bends in α-helixes [2] and molecular modeling has suggested that the A339P mutation realigns the amino acid side-chains [8]. While similar realignments are likely to be caused by G343P and by I337P, and to be at least partially responsible for the observed changes in drug resistance and reversal agent sensitivity (Table 2), it is the loss of helix flexibility imposed by the Pro residue that is likely to be responsible for loss of VRP-stimulated ATPase activity since the latter is known to be associated with conformational changes in the transporter [23, 25, 28].

Altering the degree of hydrophilicity of the residue at position 339, located essentially in the middle of TM6 and in the center of the hydrophobic face of the helix (see Fig. 1), and shown in MDR1 to lie close to TMs 11 and 12 and therefore thought to form part of the drug-binding domain [19], led to changes consistent with this role (Table 2). Indeed two of these mutations, A339G and A339S, had a strong effect on one or more of the characteristics assessed. However, the most hydrophilic of the substitutions, A339D, had very little effect at all, indicating that the presence of a small negatively charged group within this hydrophobic region does not affect formation of the putative drug-binding domain. Both Ser and Gly are smaller than Ala while Asp is similar in size. Therefore, it is possible that the small side-chains that cannot participate in hydrophobic bonding are not able to stabilize the required interaction with TMs 11 and 12. Interestingly, A339G increased VRP-stimulated ATPase activity by nearly twofold ($P < 0.02$) consistent with the increased flexibility that would be expected by insertion of Gly within an α helix [2].

Deletion of F335 in MDR1 results in loss of CsA sensitivity and IAAP labeling [4], and its conversion to Ala leads to a decrease in resistance to ActD and vinblastine and an increase in resistance to COLC and Adriamycin [17]. Conversion of the analogous residue in Pgp1, F332, to Ala led to very similar results with regard to resistance but in addition strongly decreased the RD_{50}, i.e. sensitized the transporter to CsA, for each of the four drugs and resulted in a large decrease in IAAP labeling (Table 2). Hence, maintaining a residue at this position as opposed to deleting it, imposes vastly different interactions of the transporter with CsA while maintaining similar effects on the interaction of the transporter with IAAP. Conversely, the A339P mutation strongly desensitizes the transporter to CsA, i.e. increases the RD_{50} for all four drugs, but does not affect IAAP labeling (Table 2). Moreover, it requires 20 times more CsA to effectively compete with IAAP for labeling this mutant as opposed to the wild-type (data not shown). Although these results confirm that the sites at which CsA and IAAP interact with Pgp1 overlap, they underscore the differences in those interactions.

Overall, the results presented in this report confirm and extend previous work demonstrating that TM6 plays a key role in mediating the function of ABC transporters. They also provide evidence to establish the effect of the hydrophilic side of the TM6 helix on drug recognition, reversal agent sensitivity, IAAP photolabeling, and VRP-stimulated ATPase activity. While these effects may be due to allosteric interactions within the transporter itself, it is interesting to speculate that

some may reflect the association of the transporter with the plasma membrane. Continuing studies of Pgp structure and function are required to clarify these as well as other possibilities.

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References

1. Beck WT, Danks MK (1991) Characteristics of multidrug resistance in human tumor cells. In: Roninson I (ed) Molecular and cellular biology of multidrug resistance in tumor cells. Plenum Press, New York, pp 3–55
2. Brandon C, Tooze J (1991) Introduction to protein structure. Garland Publishing, New York
3. Bruggeman EP, Currier SJ, Gottesman MM, Pastan I (1992) Characterization of the azidopine and vinblastine binding site of P-glycoprotein. J Biol Chem 276:21020
4. Chan G, Duran GE, Steger KA, Lacayo, NJ, Jaffrezou J-P, Dumontet C, Sickic BI (1997) Multidrug-resistant human sarcoma cells with a mutant P-glycoprotein altered phenotype and resistance to cyclosporins. J Biol Chem 272:5974
5. Demmer AS, Andreae S, Thole H, Tummler B (1999) Iodomycin and iodipine, a structural analogue of azidopine, bind to a common domain in hamster P-glycoprotein. Eur J Biochem 264:800
6. Devine SE, Melera PW (1994) Functional studies with a full length P-glycoprotein cDNA encoded by the Hamster pgp1 gene. Cancer Chemother Pharmacol 33:465
7. Devine SE, Melera PW (1994) Diversity of multidrug resistance in mammalian cells. J Biol Chem 269:6118
8. Devine SE, Ling V, Melera PW (1992) Amino acid substitutions in the sixth transmembrane domain of P-glycoprotein alter multidrug resistance. Proc Natl Acad Sci USA 89:4564
9. Dey S, Ramachandra M, Pastan I, Gottesman MM, Ambudkar SV (1997) Evidence for two nonidentical drug-interaction sites in the human P-glycoprotein. Proc Natl Acad Sci USA 94:10594
10. Endicott JA, Ling V (1989) The biochemistry of P-glycoprotein-mediated multidrug resistance. Annu Rev Biochem 58:137
11. Germann UA (1996) P-glycoprotein – a mediator of multidrug resistance in tumor cells. Eur J Cancer 32A:927
12. Greenberger LM (1993) Major photoaffinity drug labeling sites for iodoaryl azidoprazosin in P-glycoprotein are within, or immediately C-terminal to, transmembrane domains 6 and 12. J Biol Chem 268:11417
13. Hafkemeyer P, Dey S, Ambudkar SV, Hrycyna CA, Pastan I, Gottesman MM (1998) Contribution to substrate specificity and transport of nonconserved residues in transmembrane domain 12 of human P-glycoprotein. Biochemistry 37:16400
14. Hanna M, Brault M, Kwan T, Kast C, Gros P (1996) Mutagenesis of transmembrane domain 11 of P-glycoprotein by alanine scanning. Biochemistry 35:3625
15. Higgins CF (1992) ABC transporters: from microorganisms to man. Annu Rev Cell Biol 8:67
16. Hrycyna CA, Airan LE, Germann UA, Ambudkar SV, Pastan I, Gottesman MM (1998) Structural flexibility of the linker region of human P-glycoprotein permits ATP hydrolysis and drug transport. Biochemistry 37:3660
17. Loo TW, Clark DM (1993) Functional consequences of phenylalanine mutations in the predicted transmembrane domain of P-glycoprotein. J Biol Chem 268:19965
18. Loo TW, Clark DM (1994) Mutations to amino acids located in predicted transmembrane segment 6 (TM6) modulate the activity and substrate specificity of human P-glycoprotein. Biochemistry 33:14049
19. Loo TW, Clark DM (1999) Identification of residues in the drug-binding domain of human P-glycoprotein. J Biol Chem 274:35388
20. Loo TW, Clark DM (2000) The packing of the transmembrane segments of human multidrug resistance P-glycoprotein is revealed by disulfide cross-linking analysis. J Biol Chem 275:5253
21. Ma JF, Grant G, Melera PW (1997) Mutations in the sixth transmembrane domain of P-glycoprotein that alter the pattern of cross-resistance also alter sensitivity to cyclosporin A reversal. Mol Pharmacol 51:922
22. Mechetner EB, Schott B, Morse BS, Stein WD, Druley T, Davis KA, Tsuruo T, Roninson IB (1997) P-glycoprotein function involves conformational transitions detected by differential immunoreactivity. Proc Natl Acad Sci USA 94:12908
23. Ramachandra M, Ambudkar SV, Chen D, Hrycyna CA, Dey S, Gottesman MM, Pastan I (1998) Human P-glycoprotein exhibits reduced affinity for substrates during a catalytic transition state. Biochemistry 37:5010
24. Sarkadi B, Price EM, Boucher RC, Germann UA, Scarborough GA (1992) Expression of the human multidrug resistance cDNA in insect cells generates a high activity drug-stimulated membrane ATPase. J Biol Chem 267:4854
25. Savena ZE, Ambudkar SV (2000) Evidence for a requirement for ATP hydrolysis at two distinct steps during a single turnover of the catalytic cycle of human P-glycoprotein. Proc Natl Acad Sci USA 97:2515
26. Tabcharani JA, Rommens SM, Chang Y-B, Tsul L-C, Riordan JR, Hanrahan JW (1993) Multi-ion pore behavior in the CFTR chloride channel. Nature 336:79
27. Taguchi Y, Kino K, Morishima M, Komano T, Kan SE, Ueda K (1997) Alteration of substrate specificity by mutations at the His61 position in predicted transmembrane domain 1 of human MDR/P-glycoprotein. Biochemistry 36:8883
28. Wang G, Pincheira R, Zhang M, Zhang JT (1997) Conformational changes of p-glycoprotein by nucleotide binding. Biochem J 328:897
29. Zhang J-T, Ling V (1995) Involvement of cytoplasmic factors regulating the membrane orientation of P-glycoprotein sequences. Biochemistry 34:9159