The Drug-Binding Pocket of the Human Multidrug Resistance P-Glycoprotein Is Accessible to the Aqueous Medium†

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ABSTRACT: P-Glycoprotein (P-gp) is an ATP-dependent drug pump that transports a broad range of compounds out of the cell. Cross-linking studies have shown that the drug-binding pocket is at the interface between the transmembrane (TM) domains and can simultaneously bind two different drug substrates. Here, we determined whether cysteine residues within the drug-binding pocket were accessible to the aqueous medium. Cysteine mutants were tested for their reactivity with the charged thiol-reactive compounds sodium (2-sulfonatoethyl)methanethiosulfonate (MTSES) and [2-(trimethylammonium)ethyl)]-methanethiosulfonate (MTSET). Residue Ile-306(TM5) is close to the verapamil-binding site. It was changed to cysteine, reacted with MTSES or MTSET, and assayed for verapamil-stimulated ATPase activity. Reaction of mutant I306C(TM5) with either compound reduced its affinity for verapamil. We confirmed that the reduced affinity for verapamil was indeed due to introduction of a charge at position 306 by demonstrating that similar effects were observed when Ile-306 was replaced with arginine or glutamic acid. Mutant I306R showed a 50-fold reduction in affinity for verapamil and very little change in the affinity for rhodamine B or colchicine. MTSES or MTSET modification also affected the cross-linking pattern between pairs of cysteines in the drug-binding pocket. For example, both MTSES and MTSET inhibited cross-linking between I306C(TM5) and I868C(TM10). Inhibition was enhanced by ATP hydrolysis. By contrast, cross-linking of cysteine residues located outside the drug-binding pocket (such as G300C(TM5)/F770C(TM8)) was not affected by MTSES or MTSET. These results indicate that the drug-binding pocket is accessible to water.

The human multidrug resistance P-glycoprotein (P-gp, ABCB1)[1] transports a wide variety of structurally unrelated compounds of different sizes from the cell. It is a 170 kDa plasma membrane protein the physiological function of which is unknown. It is expressed in relatively high levels in the epithelial cells of some organs such as the intestine and blood−brain/testes barrier and likely protects us from toxic compounds in the diet and environment (*1*−*3*). This protective function is a problem in the clinical setting since overexpression of P-gp in some tumor cells can undermine cancer chemotherapy regimens and some drugs used in the treatment of HIV/AIDS are also substrates of P-gp (*4*, *5*).

P-gp belongs to the large ATP-binding cassette (ABC) family (48 human members) of transporters (*6*). Its 1280 amino acids are organized as two homologous halves that are joined by a linker. Each half has six transmembrane (TM) segments followed by an ATP-binding domain (*7*−*9*). The minimum functional unit is a monomer (*10*), but the two halves of the molecule do not have to be covalently linked for function (*11*, *12*). Both ATP-binding sites are required for activity (*13*−*15*) and likely function in an alternating mechanism (*16*). The nucleotide-binding domains (NBDs) of ABC transporters are highly conserved. The results from disulfide cross-linking studies on P-gp (*17*) and from the studies of several bacterial ABC transporters (*18*−*21*) indicate that ATP binds between the Walker A site of one NBD and the "LSGGQ" signature sequence of the other NBD. In P-gp, drug substrates that stimulate or inhibit ATPase activity cause these sequences to come closer or farther apart, respectively (*22*).

Studies on deletion mutants have shown that the TM domains alone are sufficient to mediate drug binding (*12*). Similar studies on cysteine mutants and their inhibition by different thiol-reactive substrate analogues indicate that residues from multiple TM segments contribute to the common drug-binding pocket (*23*−*27*). Drug substrates bind at distinct regions in a common drug-binding pocket (*28*) that is formed by the interface between the transmembrane domains (TMDs) of both halves of P-gp. Drug binding involves an induced-fit mechanism (*29*), and it appears that only substrates bound in a particular orientation can stimulate ATPase activity (*30*).

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[1] Abbreviations: P-gp, P-glycoprotein; ABC, ATP-binding cassette; TM, transmembrane; HEK, human embryonic kidney; MTS, methanethiosulfonate; M17M, 3,6,9,12,15-pentaoxaheptadecane-1,17-diyl bis-methanethiosulfonate; MTSEA, 2-aminoethyl methanethiosulfonate; MTSES, sodium (2-sulfonatoethyl)methanethiosulfonate; MTSET, [2-(trimethylammonium)ethyl)]methanethiosulfonate; SDS−PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

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The drug-binding pocket appears to be large enough to accommodate at least two structurally different drug substrates simultaneously (*28*). Cross-linking analysis between pairs of cysteines in the drug-binding pocket with thiol-reactive cross-linkers with spacer arms of 2−17 atoms showed that the drug-binding pocket is "funnel-shaped", narrow at the cytoplasmic side, at least 9−25 Å in the middle and wider still at the extracellular side (*31*). Similar dimensions were obtained when stipiamide homodimers of various lengths were used to measure the size of the drug-binding pocket (*32*). Stipiamide dimers greater than 11 Å but less than 35 Å in length could fit into the drug-binding pocket.

P-gp has been postulated to function as "vacuum cleaner" (*33*). Drug substrates enter the lipid bilayer by diffusion and then are extracted by P-gp and transported out of the cell (*34*). An important step in understanding the mechanism of P-gp is how drug substrates in the drug-binding pocket are extruded into the aqueous medium. Since many of the drug substrates of P-gp are quite hydrophobic, hydration of drug substrates in the drug-binding pocket may be an important step during the transport cycle. We would predict from the relatively large nature of the drug-binding pocket that residues within the drug-binding pocket might be accessible to the aqueous medium (*31*).

In this study, we tested whether cysteines within the drug-binding pocket of P-gp were accessible to the aqueous medium by testing whether they could be labeled with charged thiol-reactive compounds.

MATERIALS AND METHODS

Construction of Mutants. Histidine-tagged wild-type P-gp and mutants I306E and I306R were constructed as described previously (*35, 36*). Cysteine-less P-gp was constructed by replacing the seven endogenous cysteines at positions 137, 431, 717, 956, 1074, 1125 and 1227 with alanines (*8*). Cysteine residues were introduced into the TMs of a histidine-tagged cysteine-less P-gp cDNA (*37*). The presence of a histidine tag facilitated purification of the mutant P-gps by nickel-chelate chromatography.

Purification and Measurement of Drug-Stimulated ATPase Activity of P-gp Mutants. Fifty plates (10-cm diameter) of HEK 293 cells were transfected with mutant cDNA. The medium was replaced with fresh medium containing 10 μM cyclosporin A after 24 h at 37 °C. Cyclosporin A is a substrate of P-gp and acts as a potent chemical chaperone in promoting maturation and yield of P-gp (*38*−*40*). After another 24 h at 37 °C, the cells were harvested, washed three times with phosphate-buffered saline (PBS, pH 7.4), and suspended in PBS. The cells were solubilized at 4 °C with one volume (0.75 mL) of PBS containing 2% (w/v) *n*-dodecyl-β-D-maltoside. After 15 min at 4 °C, insoluble material was removed by centrifugation at 16 000 × *g* for 15 min at 4 °C. The supernatant was passed through a DNA miniprep microfuge column (Qiagen) to remove DNA. The flow-through material was subjected to nickel-chelate chromatography as described previously (*36*). The recovery of P-gp was monitored by immunoblot analysis with rabbit anti-P-gp polyclonal antibody (*41*) and enhanced chemiluminescence (Pierce, Rockford, IL) An aliquot of the isolated P-gp$(His)_{10}$ was mixed with an equal volume of 10 mg/mL crude sheep brain lipid (Type II-S; Sigma-Aldrich) that had been washed and suspended in TBS (10 mM Tris-HCl, pH 7.4, and 150 mM NaCl). The P-gp and lipid mixture was sonicated. A sample of the P-gp/lipid mixture was incubated with an equal volume of ATPase buffer containing 100 mM Tris-HCl, pH 7.5, 100 mM NaCl, 20 mM $MgCl_2$, 10 mM ATP, and either no drug substrate, 0.002−20 mM verapamil, 0.002−0.6 mM vinblastine, 0.02−6 mM rhodamine B, or 0.2−60 mM colchicine. The samples were incubated at 37 °C for 30 min, and the amount of inorganic phosphate liberated was determined (*42*).

Disulfide Cross-Linking Analysis. HEK 293 cells were transfected with the mutant cDNAs. After 24 h at 37 °C, the medium was replaced with fresh medium, and the cells were grown for another 48 h at 27 °C. The cells were harvested and washed once with TBS, and then membranes were prepared as described previously (*43*). The membranes were suspended in TBS, and samples were cross-linked by incubation with 0.2 mM 3,6,9,12,15-pentaoxaheptadecane-1,17-diyl bismethanethiosulfonate (M17M) for 15 min at 22 °C. Mutants G300C(TM5)/F770C(TM8) or C137C(TM2)/I935C(TM11) were cross-linked with 1 mM Cu^{2+}(phenanthroline)$_3$ for 15 min at 22 °C as described previously (*44*). The reactions were stopped by addition of SDS sample buffer (125 mM Tris-HCl, pH 6.8, 20% (v/v) glycerol, and 4% (w/v) SDS) containing 50 mM EDTA and no reducing agent.

For labeling of whole cells, the transfected cells were harvested, washed once with TBS, and then treated with 2.5 mM 2-aminoethyl methanesulfonate (MTSEA), 10 mM MTSES, or 1 mM MTSET for 10 min at 22 °C in TBS. Various concentrations of these compounds were used because of their different reactivity with cysteine (*45*). The cells were then washed twice with TBS to remove unreacted compounds. Membranes were then prepared and treated with cross-linking agents as described above.

To test the effect of MTSES, MTSET, or ATP and MTSES on cross-linking, the membranes were suspended in Tris-buffered saline, pH 7.4 (TBS, 10 mM Tris-HCl, pH 7.4, 150 mM NaCl). Samples were incubated for 10 min at 22 °C in the presence various concentrations of MTSES or MTSET, or in the presence or absence of 10 mM ATP, 20 mM $MgCl_2$, and 0−0.5 mM MTSES. The reaction samples were diluted 10-fold with PBS and collected by centrifugation at 34 000 × *g* for 20 min at 4 °C. The membranes were washed once with TBS and suspended in TBS. Samples were then treated with either 0.2 mM M17M or 1 mM Cu^{2+}(phenanthroline)$_3$ for 15 min at 22 °C. The reactions were stopped by addition of SDS sample buffer (125 mM Tris-HCl, pH 6.8, 20% (v/v) glycerol, and 4% (w/v) SDS) containing 50 mM EDTA and no reducing agent. The reaction mixtures were subjected to SDS−PAGE (7.5% polyacrylamide gels) and immunoblot analysis with a rabbit polyclonal antibody against P-gp (*41*) and enhanced chemiluminescence (Pierce, Rockford, IL).

Generation of Stable Cell Lines Expressing P-gp Mutants and Measurement of Drug Resistance. Stable cell lines expressing wild-type P-gp or mutant I306R were generated using the Flp-In system (Invitrogen, Canada). Briefly, the full-length cDNAs of histidine-tagged wild-type P-gp and mutant I306R were subcloned into pcDNA5/FRT vector (Invitrogen, Canada) and cotransfected with the Flp recombinase vector pOG44 into the Flp-In−293 cells. The transfected cells were selected on 500 μg/mL hygromycin B. Hygromycin B-resistant colonies were isolated and tested

for expression of P-gp by immunoblot analysis as described above. Cells expressing wild-type or mutant I306R P-gp were then grown in 24-well plates in the presence of various concentrations (0−2000 nM) of colchicine and in the presence or absence of 25 μM verapamil or 5 μM cyclosporin A. After 5−7 days, the concentration of colchicine that inhibited growth by 50% (D_{50}) was determined by using Alamar Blue (Biosource Inc, Camarillo CA).

Cell-Surface Labeling. Flp-In−293 cells stably expressing wild-type or mutant I306R P-gps were washed twice with PBS, pH 7.4, and incubated with PBS containing 10 mM sodium periodate for 30 min at 4 °C in the dark. The cells were then washed twice with PBS and then incubated with 0.1 M sodium acetate buffer, pH 5.5, containing 2 mM biotin−LC−hydrazide (Pierce, Rockford, IL) for 30 min at 20 °C. The cells were then washed with twice with PBS and then solubilized with PBS containing 1% (w/v) *n*-dodecyl-β-D-maltoside. Insoluble material was removed by centrifugation at 16 000 × *g* for 10 min. The supernatant was transferred to a fresh tube and incubated with Neutravidin beads (Pierce, Rockford, IL) for 2 h at 4 °C. The beads were then washed four times with PBS containing 0.1% (w/v) *n*-dodecyl-β-D-maltoside. SDS sample buffer (0.125 M Tris-HCl, pH 6.8, 20% (v/v) glycerol, 4% (w/v) SDS, and 4% (v/v) 2-mercaptoethanol) was then added to the beads, and equivalent amounts were subjected to SDS−PAGE on 7.5% gels followed by immunoblot analysis with rabbit antibody against P-gp and enhanced chemiluminescence.

RESULTS

We had shown that the common drug-binding pocket in P-gp is shaped like a "funnel", narrow at the cytoplasmic end, at least 9−25 Å in the middle, and wider still at the extracellular end (*31*). From this model, we predicted that the drug-binding pocket might be accessible to the aqueous medium. To test this hypothesis, we determined whether charged thiol compounds could modify a cysteine residue within the drug-binding pocket. A good candidate is mutant I306C in TM5 (Figure 1A). Cys-306 is close to the verapamil-binding site because treatment of mutant I306C with thiol-reactive MTS−verapamil resulted in covalent attachment of the verapamil to Cys-306 (*46*). Covalent attachment of MTS−verapamil to Cys-306 mimics that of verapamil interaction with P-gp because the ATPase activity of the covalently modified mutant was permanently activated. Therefore, Cys-306 lies close to the verapamil-binding site. The TM segments that are predicted to line the drug-binding pocket are shown in Figure 1B.

Since Cys-306 is close to the verapamil-binding site, labeling of this residue with a charged thiol compound should affect verapamil interaction with P-gp. Two useful charged thiol compounds are the negatively charged MTSES and the positively charged MTSET. These compounds have been used to map the aqueous accessibility of several channels and transporters (*47*−*49*). Accordingly, a cysteine was introduced at position 306 in a cysteine-less P-gp. The cysteine-less P-gp is a useful system for cysteine-scanning mutagenesis because it retains the ability to confer drug resistance in transfected cells (*8*). Histidine-tagged mutant I306C was expressed in HEK 293 cells, isolated by nickel-chelate chromatography, and mixed with lipid. Mutant I306C

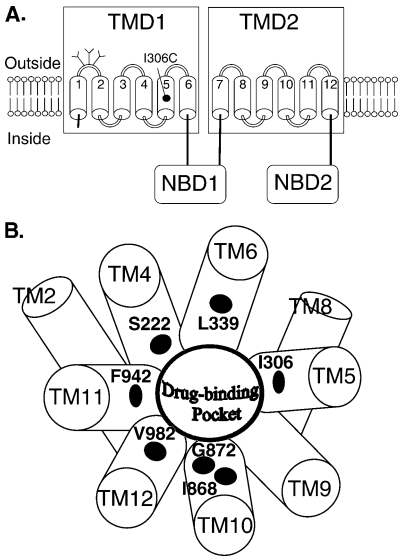

FIGURE 1: Model of P-gp. In panel A, the 12 TMs of full-length P-gp are shown as numbered cylinders, the branched lines represent glycosylation sites, and NBD represents the nucleotide-binding domains. Labeling of residue I306C with MTS-verapamil permanently activated P-gp ATPase activity (*46*). Panel B shows TM segments predicted to contribute to the "funnel"-shaped drug-binding pocket. The residues cross-linked by M17M (*31*) are shown as numbered black dots.

was then treated with 1 mM MTSET or 5 mM MTSES and assayed for verapamil-stimulated ATPase activity after removal of untreated MTSET or MTSES using gel filtration columns. A higher concentration (5-fold) of MTSES was used because MTSES is less reactive than MTSET (*45*). Drug-stimulated ATPase activity is a useful assay because it and the turnover numbers for transport are comparable (*50*). In addition there is good correlation between relative drug resistance and drug-stimulated ATPase activity (*51*). Figure 2 shows that treatment of mutant I306C with either MTSET or MTSES profoundly affected the verapamil-stimulated ATPase activity. Treatment of mutant I306C with MTSET reduced the apparent affinity of P-gp for verapamil from 24 μM to greater than 600 μM. Treatment of mutant I306C with MTSES caused about a 10-fold reduction in apparent affinity (260 μM) for verapamil, as well as about 50% decrease in ATPase activity. The change in activity was apparently due to modification of residue I306C since 1 mM MTSET or 5 mM MTSES had no effect on basal or verapamil-stimulated ATPase activity of cysteine-less P-gp (data not shown).

Since the cysteine-less P-gp shows some differences from wild-type P-gp, such as lower activity with some drug substrates (*8*), it would be useful to test whether introduction of a positive or negative charge at position 306 also affected the affinity of the wild-type P-gp for verapamil. Unfortunately, MTSES or MTSET could not be used to specifically modify a cysteine residue introduced into position 306 in the wild-type P-gp because these compounds would also modify one or more of the endogenous cysteines at positions 137, 431 717, 956 1074, 1125, and 1227. Modification of the endogenous cysteines at positions 431 and 1074 in the Walker A sites by MTSES or MTSET inactivates P-gp (unpublished observations). Therefore, an alternative approach to introduce a charged group at position 306 would be to mutate Ile306 to glutamic acid or arginine. Accordingly, site-directed mutagenesis was used to change residue Ile-

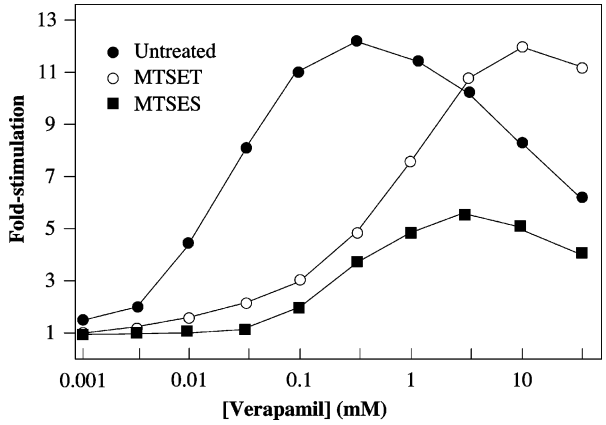

FIGURE 2: Effect of MTSES or MTSET on verapamil-stimulated ATPase activity of mutant I306C. Histidine-tagged mutant I306C P-gp was expressed in HEK 293 cells and isolated by nickel-chelate chromatography. The isolated protein was mixed with lipid, sonicated, and treated for 15 min in the absence or presence of 5 mM MTSES or 1 mM MTSET. Unreacted thiol compounds were then removed by gel filtration (Centri.Spin 20 columns, Princeton Separations, Inc., Adelphia, NJ). Samples were then assayed for drug-stimulated ATPase activity in the presence of various concentrations of verapamil. Fold stimulation is the ratio of the activity in the presence of verapamil to that without verapamil. Each value is the average of three assays.

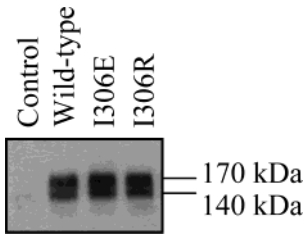

FIGURE 3: Expression of wild-type and mutant P-gps. HEK 293 cells were transfected with wild-type P-gp or with P-gp mutants I306E or I306R (in wild-type background) cDNAs. After 24 h, the medium was replaced with fresh medium. The cells were harvested 24 h later, washed with PBS, and solubilized with SDS sample buffer. Samples were then run on 7.5% SDS−PAGE gels and followed by immunoblot analysis with rabbit anti-P-gp. The positions of the mature (170 kDa) and core-glycosylated (140 kDa) P-gps are indicated.

306 in wild-type P-gp to a negatively (glutamic acid) or positively charged (arginine) residue. Mutation of I306 to glutamic acid or arginine did not affect the maturation of P-gp because both mutants when expressed in HEK 293 cells yielded the 170 kDa mature protein as the major product (Figure 3). Misfolded P-gp mutants are retained in the endoplasmic reticulum as core-glycosylated intermediates (*43*). Introduction of a positive or negative charge at position 306 does not disrupt folding of P-gp suggesting that position 306 (TM5) is located at a site that is not critical for maturation of P-gp. Histidine-tagged wild-type and mutants I306E and I306R P-gps were expressed in HEK 293 cells, isolated by nickel-chelate chromatography and mixed with lipid, and verapamil-stimulated ATPase activity was determined. Figure 4 shows that mutation of Ile306 to glutamic acid or arginine significantly affected the apparent affinity for verapamil. The wild-type P-gp and mutants I306R and I306E showed maximal stimulation of 16.1-, >10.8-, and 7-fold and S_{50} (concentration required for 50% stimulation) of 44, >2200, and 305 μM, respectively.

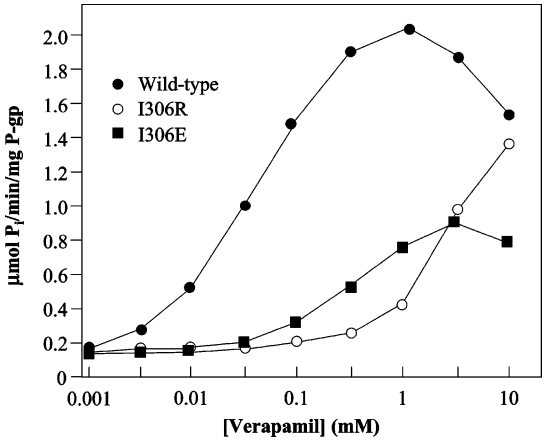

FIGURE 4: Verapamil-stimulated ATPase activity of wild-type and mutant I306R and I306E P-gps. Histidine-tagged wild-type, mutant I306R or mutant I306E P-gps were expressed in HEK 293 cells and isolated by nickel-chelate chromatography. Samples were mixed with lipids, sonicated, and assayed for drug-stimulated ATPase activity in the presence of various concentrations of verapamil. Each value is the average of three assays.

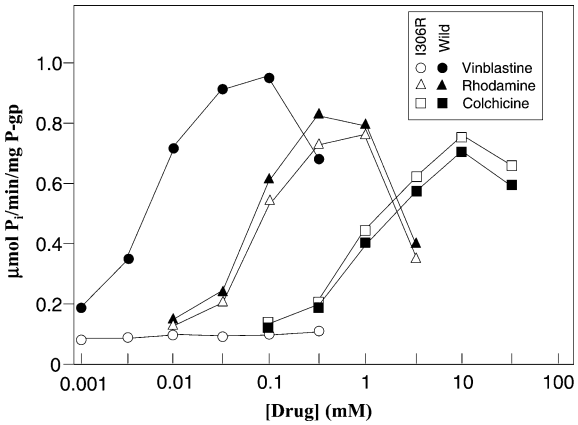

FIGURE 5: Drug-stimulated ATPase activity of wild-type and mutant I306R in the presence of vinblastine, rhodamine B, or colchicine. Histidine-tagged wild-type (filled symbols) and mutant I306R (open symbols) P-gps were expressed in HEK 293 cells and isolated by nickel-chelate chromatography. The isolated proteins were mixed with lipids and sonicated, and drug-stimulated ATPase activity was measured in the presence of various concentrations of vinblastine (△,▲), rhodamine B (○,●), or colchicine (□,■). Each value is the average of three assays.

Since mutant I306R showed the largest change (50-fold) in affinity for verapamil (Figure 4), we tested whether the interaction of this mutant with drug substrates rhodamine B, vinblastine, or colchicine were also changed. These three drug substrates were selected because there was evidence that rhodamine B and verapamil interacted at distinct sites in the common-drug-binding pocket (*30*), while vinblastine and colchicine are classic drug substrates of P-gp (*52*). Figure 5 shows that the ATPase activity of mutant I306R in the presence of rhodamine B and colchicine was similar to that of the wild-type P-gp. Both wild-type and mutant I306R showed a 6−7-fold maximal stimulation in the presence of rhodamine B and S_{50} concentrations of 58 and 67 μM, respectively. Similarly, in the presence of colchicine, both wild-type and mutant I306R had maximal stimulation of about 6−6.5-fold and S_{50} concentrations of about 1 mM. A large difference, however, was observed with vinblastine. Wild-type P-gp showed an 8.2-fold maximal activation and

an S_{50} of 5.4 μM vinblastine. The mutant I306R, however, showed little activation (<2-fold) even at 300 μM vinblastine. These results indicate that mutation of Ile-306 to arginine interferes with its ability to interact with verapamil and vinblastine but not with rhodamine B or colchicine and that Ile-306 must contribute to the verapamil and vinblastine binding sites.

We then determined whether verapamil could interfere with ability of mutant I306R to confer resistance in transfected cells. Mutant I306R was selected because it showed the largest decrease in apparent affinity for verapamil (Figure 4). Since verapamil is relatively nontoxic for cells, verapamil inhibition of colchicine transport was chosen as the functional assay. Colchicine is a substrate of P-gp and is toxic to cells (*53*). The ability of P-gp to confer resistance to colchicine is inhibited by the presence of verapamil (*54*, *55*). We avoided problems associated with generating stable cell lines that involve direct selection with cytotoxic compounds (potential for selecting a clone in which P-gp has additional mutations or problems with integration of P-gp into different chromosomal sites) by generating stable cell lines expressing wild-type or mutant I306R P-gps using the Flp-In system (Invitrogen, Carlsbad, CA). Wild-type and mutant I306R P-gps were subcloned into the pcDNA5/FRT vector and cotransfected with the Flp recombinase expression vector pOG44 into the Flp-In−293 cells that contained a single Flp recombinase target (*56*). The transfected cells were selected on hygromycin B. Individual colonies were expanded and expression of P-gp was confirmed by immunoblot analysis (data not shown). The advantage of this approach for generating stable cell lines is that each cell line expresses the same level of P-gp. To test whether mutation I306R affected folding and trafficking of the mutant protein to the cell surface, we performed cell-surface labeling experiments. HEK 293 cells expressing wild-type and mutant I306R P-gp were treated with sodium periodate to oxidize the carbohydrate groups on proteins and then reacted with biotin hydrazide. The cells were harvested, washed with PBS and solubilized with *n*-dodecyl-β-D-maltoside, and biotinylated proteins were recovered using Neutravidin beads (Pierce, Rockford, IL) and then subjected to immunoblot analysis with rabbit polyclonal antibody against P-gp. The cell-surface labeling experiments showed that equivalent levels of wild-type and mutant I306R P-gps were present at the cell surface (Figure 6A). These results indicate that mutation of Ile-306 to arginine does not affect folding or trafficking of the mutant P-gp to the cell surface. The cells expressing wild-type or mutant I306R P-gps were then incubated with various concentrations of colchicine and verapamil for several days. The concentration of colchicine that caused 50% cell death (D_{50}) was then determined. In the absence of verapamil, cells expressing wild-type or mutant I306R showed about (D_{50} of 200−225 nM) 30-fold increase in resistance to colchicine relative to that of mock-transfected Flp-In−293 cells (D_{50} of 7 nM). In the presence of 25 μM verapamil, however, cells expressing wild-type P-gp were much more sensitive to colchicine than cells expressing mutant I306R (Figure 6B). In the presence of 25 μM verapamil, wild-type P-gp conferred only a 6.7-fold increase in resistance to colchicine. By contrast, the relative resistance of mutant I306R to colchicine in the presence of verapamil was only slightly decreased (27.7- fold). These results are consistent with those obtained with drug-stimulated ATPase activity. Mutation of Ile-306 to arginine had little effect on the ability of P-gp to interact with colchicine but greatly affected the ability of the enzyme to interact with verapamil. To test whether mutant I302R could still interact with the hydrophobic substrate cyclosporin A, we incubated the cells expressing wild-type or mutant I306R P-gp with colchicine and 5 μM cyclosporin A. Figure 6 B shows that cyclosporin A inhibited the ability of the mutant P-gps to confer resistance to colchicine (D_{50}'s of 28 and 35 nM for wild-type and mutant I306R, respectively). These results show that mutant I306R was still able to interact with hydrophobic substrates.

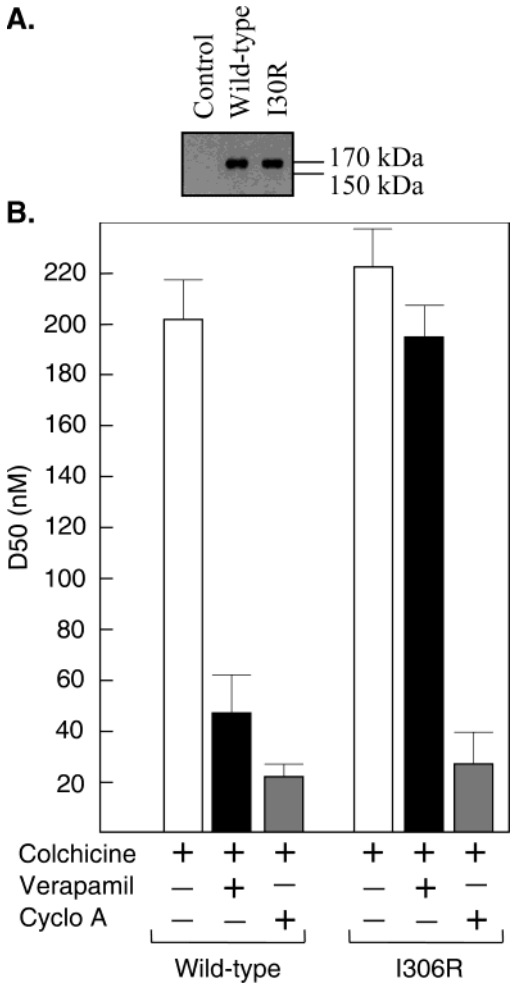

FIGURE 6: Cell-surface labeling and effect of verapamil and cyclosporin A on wild-type and mutant I306R P-gp-mediated colchicine resistance. In panel A, Flp-In−293 cells stably expressing wild-type P-gp (wild-type), mutant I306R P-gp (I306R), or vector only (control) were treated with sodium periodate followed by biotin hydrazide. The treated cells were washed with PBS and solubilized with *n*-dodecyl-β-D-maltoside, and biotinylated proteins were isolated with Neutravidin beads. Equivalent amounts of biotinylated proteins were subjected to SDS−PAGE on 7.5% gels followed by immunoblot analysis with rabbit polyclonal antibody against P-gp. The positions of the mature (170 kDa) and core-glycosylated (140 kDa) P-gps are indicated. In panel B, Flp-In−293 cells lines stably expressing wild-type or mutant I306R P-gp were incubated with various concentrations of colchicine and with (+) or without (−) 25 μM verapamil or with or without 5 μM cyclosporin A (cyclo A). After 5 days, the number of viable cells remaining was determined using Alamar Blue. The concentration of colchicine that inhibited cell growth by 50% (D_{50}) was determined. Each value is the average of three assays.

Another method for determining whether charged thiol compounds have access to the drug-binding pocket of P-gp was to test the effect of MTSES and MTSET labeling on cross-linking between cysteines within the drug-binding pocket. We showed that cysteines in the drug-binding pocket are cross-linked with homobifunctional thiol-reactive cross-linkers (*31*). MTSES and MTSET were used to test for inhibition of cross-linking between cysteines within the drug-binding pocket (mutants L339C(TM6)/F942C(TM11), I306C(TM5)/I868C(TM10), and S222C(TM4)/G872C(TM10)) (*31*). The effect of MTSES and MTSET on cross-linking between cysteines predicted to lie outside the drug-binding pocket (mutants G300C(TM5)/F770C(TM8) and C137C-(TM2)/I935C(TM11), (*44*)) were also determined. Membranes were prepared from HEK 293 cells expressing the mutant P-gps and treated with various concentrations of MTSE,S or MTSET. The membranes were collected by centrifugation, washed with PBS and then treated with various concentrations of the homobifunctional thiol-reactive cross-linker M17M. Figure 7A shows that all three mutants, L339C(TM6)/F942C(TM11), I306C(TM5)/I868C(TM10) and S222C(TM4)/G872C(TM10), of which the cysteines are within the drug-binding pocket were cross-linked with M17M before treatment with MTSES or MTSET. Cross-linking with M17M was inhibited by pretreatment with MTSES or MTSET (Figure 7A). By contrast, cross-linking of mutants G300C(TM5)/F770C(TM8) (Figure 7A) or C137C(TM2)/I935C(TM11) by copper phenanthroline was not inhibited by MTSES or MTSET (data not shown). The cysteines in mutants G300C(TM5)/F770C(TM8) or C137C(TM2)/I935C-(TM11) are in positions in the TM segments that face away from the drug-binding pocket (*44, 57*).

To determine whether cysteine residues are accessible to the external medium, whole cells expressing the cysteine mutants were treated with MTSEA, MTSES, or MTSET. Whole cells were used because membrane preparations contain a mixed population of vesicles in different orientations as well as membrane fragments. Accordingly, cells expressing mutants L531C(NBD1)/C1074C(NBD2), I306C-(TM5)/I868C(TM10), L339C(TM6)/F942C(TM12), and S222C(TM4)/G872C(TM10) were treated with MTSEA, MTSES, or MTSET before cross-linking. Mutant L531C-(NBD1)/C1074C(NBD2) can be used as a control since the cysteines are in the nucleotide-binding domain (NBD) can be cross-linked (*17*) and should be accessible to membrane-permeant MTSEA but not to membrane-impermeant MTSES or MTSET (*58*). Since P-gp has a low affinity for ATP (K_m = 1.2−1.5 mM) (*59*), labeling of the NBD cysteines by thiol-reactive compounds should be possible in the presence of 1−3 mM cellular ATP. Figure 7B,C shows that cross-linking of mutant L531C(NBD1)/C1074C(NBD2) was completely inhibited by MTSEA, whereas MTSES and MTSET caused only minor reduction in cross-linking. These results suggest that the cell membrane is more permeable to MTSEA than to MTSES or MTSET and are consistent with the findings of Holmgren et al. (*58*). Cross-linking of mutants with cysteines within the drug-binding pocket (mutants I306C-(TM5)/I868C(TM10), L339C(TM6)/F942C(TM12), and S222C(TM4)/G872C(TM10)) was also completely inhibited by MTSEA. In contrast to mutant L531C(NBD1)/C1074C-(NBD2), cross-linking of mutants I306C(TM5)/I868C(TM10, L339C(TM6)/F942C(TM12), and S222C(TM4)/G872C-

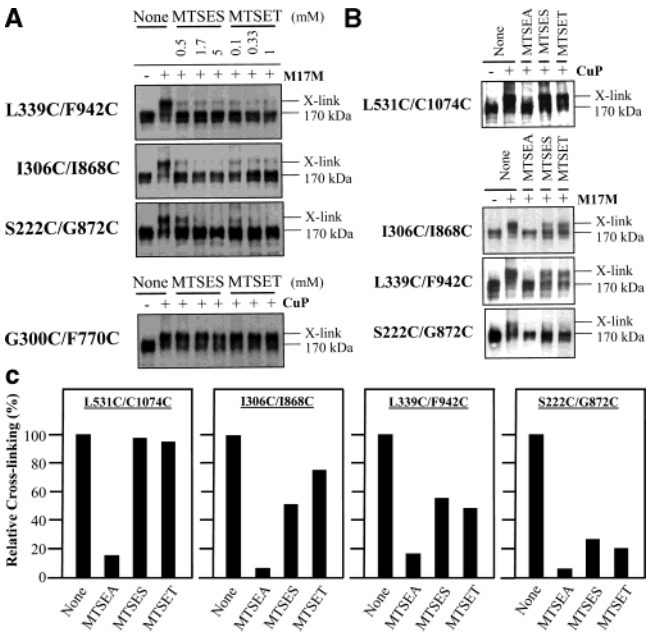

FIGURE 7: Effect of MTSES or MTSET on cross-linking of P-gp mutants containing pairs of cysteines. In panel A, membranes were prepared from HEK 293 cells expressing P-gp mutants L339C(TM6)/F942C(TM11), I306C(TM5)/I868C(TM10), S222C(TM4)/G872C-(TM10), or G300C(TM5)/F770C(TM8) and were preincubated at 22 °C for 10 min in the presence (+) or absence (−) of various concentrations of MTSES or MTSET. The reaction mixtures were diluted 10-fold with TBS, and membranes were collected by centrifugation. The membranes were washed once and suspended in TBS. Samples of mutants L339C/(TM6)/F942C(TM11), I306C-(TM5)/I868C(TM10), and S222C(TM4)/G872C(TM10) were then treated with (+) or without (−) M17M cross-linker for 15 min at 22 °C. Mutant G300C(TM5)/F770C(TM8) was cross-linked with 1 mM copper (phenanthroline)$_3$ (CuP) for 15 min at 22 °C. In panel B, whole cells expressing mutants L531C(NBD1)/C1074C(NBD2), I306C(TM5)/I868C(TM10), L339C(TM6)/F942C(TM11), or S222C-(TM4)/G872C(TM10) were incubated for 10 min at 22 °C in the presence of 2.5 mM MTSEA, 10 mM MTSES, or 1 mM MTSET. The cells were washed with TBS, and membranes were prepared and cross-linked with CuP or M17M. The reactions were stopped by addition of SDS sample buffer containing EDTA and no reducing agent. The mixtures were run on 7.5% SDS−PAGE gels and followed by immunoblot analysis. The positions of the cross-linked (×-link) product and mature (170 kDa) P-gps are indicated. In panel C, cross-linking was quantitated by scanning the gel lanes, followed by analysis using the NIH Image program (available at rsb.info.nih.gov/nih-image/) with a Macintosh computer. The amount of cross-linking is expressed relative to the sample cross-linked in the absence (none) of MTSEA, MTSES, or MTSET.

(TM10) were significantly inhibited by MTSES and MTSET. Therefore, it appears that the drug-binding pocket is accessible to the external aqueous medium.

Finally, we tested whether ATP hydrolysis affected the ability of MTSES to inhibit cross-linking of mutant I306C(TM5)/I868C(TM10) by M17M. This mutant was selected because there is good evidence that Cys-306 faces the drug-binding pocket since labeling of Cys-306 with MTS-verapamil results in permanent activation of ATPase activity. Labeling by MTS-verapamil is inhibited by verapamil (*46*). The rationale for studying the effect of ATP is that ATP hydrolysis can alter the structure of the TM domains (*60, 61*) and is required for extrusion of the drug substrate from the drug-binding pocket into the aqueous medium. Therefore, if hydration of drug substrate in the drug-binding pocket is essential before expulsion of the drug substrate from the

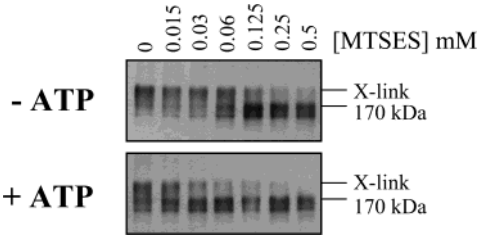

FIGURE 8: Effect of ATP hydrolysis on MTSES modification of mutant I306C(TM5)/I868C(TM10). Membranes were prepared from HEK 293 cells expressing P-gp mutant I306C(TM5)/I868C(TM10) and suspended in TBS containing 10 mM $MgCl_2$. Samples of the membranes were preincubated at 22 °C for 15 min in the presence (+) or absence (−) of various concentrations of MTSES together with (+ATP) or without (−ATP) 10 mM ATP. The reactions were done at 22 °C to slow the rate of ATP hydrolysis by ATPases. The samples were then diluted 10-fold with TBS, and the membranes were then collected by centrifugation. The membranes were washed once and suspended in TBS. Samples were then treated with 0.2 mM M17M cross-linker for 15 min at 22 °C. The reactions were stopped by addition of SDS sample buffer containing EDTA and no reducing agent. The mixtures were run on 7.5% SDS−PAGE gels and followed by immunoblot analysis. The positions of the cross-linked (×-link) product and mature (170 kDa) P-gps are indicated.

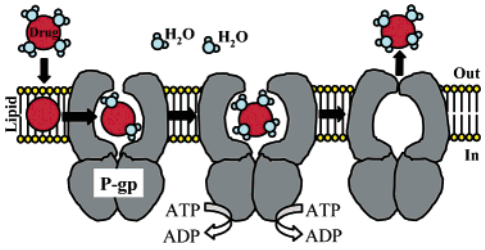

FIGURE 9: Model of P-gp-mediated drug transport. Drug substrate (large ball) is dehydrated as it diffuses from the extracellular aqueous medium into the lipid bilayer (lipid). The drug in the lipid bilayer is extracted by P-gp or diffuses into the drug-binding pocket. While in the drug-binding pocket or during ATP hydrolysis, the drug substrate is rehydrated (little balls attached to large ball). ATP hydrolysis causes conformational changes such as rotation of helices (*60*) in the drug-binding pocket and exposes different residues to the drug-binding pocket (*61*). The change in structure of the drug-binding pocket changes P-gp from a relatively high-affinity to a low-affinity state and results in expulsion of drug substrate into the extracellular medium.

binding pocket, then conformational changes during ATP hydrolysis may increase the opportunity for residues in the drug-binding pocket to be accessible to the aqueous medium. Membranes were prepared from HEK 293 cells expressing mutant I306C(TM5)/I868C(TM10) and treated with various concentrations of MTSES in the presence or absence of ATP. To slow the rate of ATP hydrolysis by other ATPases, the reaction was done at 22 °C. MTSES was selected as the blocking agent because it has a relatively longer half-life in aqueous solutions (*45*). The reaction was cooled to 4 °C, and then the membranes were collected by centrifugation and washed with PBS to remove unreacted MTSES and ATP. The membranes were then treated with the cross-linker, M17M. Figure 8 shows that the presence of ATP promoted modification by MTSES. In the absence of ATP, MTSES concentration of 250 μM was required to substantially reduce (>90%) cross-linking by M17M. In the presence of ATP, however, only 60−125 μM MTSES was sufficient to inhibit cross-linking by M17M.

DISCUSSION

It is not known whether the drug-binding pocket in P-gp is filled with lipid or is accessible to the aqueous medium. In the latter, charged compounds should be able to enter the drug-binding pocket. The ability of the charged thiol-reactive compounds MTSES and MTSET to label cysteine residues in the common drug-binding pocket suggests that the pocket is indeed accessible to the aqueous medium. Modification of mutant I306C by these compounds resulted in a change in affinity for verapamil (Figure 2). Accessibility of residues in the drug-binding pocket to water is further supported by the finding that MTSES and MTSET affected cross-linking of mutants L339C(TM6)/F942C(TM11), I306C(TM5)/I868C(TM10), and S222C(TM4)/G872C(TM10) by the homobifunctional thiol-reactive cross-linker M17M, which is also a substrate of P-gp (Figure 7B). Not all cysteines in the TM domains were accessible for modification by MTSES or MTSET. Cross-linking of mutants G300C(TM5)/F770C-(TM8) or C137C(TM2)/I935C(TM11), the cysteines of which are located outside the drug-binding pocket, was not affected by MTSES or MTSET.

The presence of an aqueous pore in P-gp has been postulated from electron cryomicroscopy of two-dimensional crystals (*62*). Hydrophilic negative stain appeared to accumulate in the chamber within the TM portion of P-gp. The presence of an aqueous chamber has also been demonstrated for the ABC multidrug transporter LmrA from *Lactococcus lactis*. LmrA protein contains only one NBD and one TM domain. The TM domain has six predicted TM segments, and the minimum functional unit is a homodimer (*63*). It shows sequence similarity to P-gp and functionally complements P-gp in human fibroblast cells (*64*). Both P-gp and LmrA show similar drug and modulator specificity. Cysteine-scanning mutagenesis of the TM domain of LmrA and reaction with hydrophilic fluorescein maleimide suggested the presence of a hydrophilic chamber (*65*). It was found that 11 of the 15 membrane-embedded aromatic residues and one face of TM6 were accessible to fluorescein maleimide. Therefore, both P-gp and LmrA appear to contain an aqueous chamber.

Could water play a role in the transport cycle? Hydrophobic drug substrates in the aqueous medium have been shown to partition into the lipid bilayer and are probably dehydrated (*34, 66*). According to the "vacuum cleaner" model for P-gp (*34*), the drug substrate in the lipid bilayer is then extracted by P-gp. The exact mechanism of how P-gp extracts drug substrates from the lipid bilayer is unknown. Hydration of the drug substrate in the binding pocket may prevent the drug substrate from diffusing back into the lipid bilayer. Hydration may also be a mechanism for preparing the drug substrate for subsequent transport to the extracellular aqueous medium (Figure 9). Complete hydration of the bound drug substrate may require ATP hydrolysis. This is suggested by the finding that ATP hydrolysis increased the accessibility of mutant I306C(TM5)/I868C(TM10) to MTSES (Figure 8). This may also explain why P-gp can transport many different hydrophobic compounds into the extracellular aqueous medium. Sharom et al. (*67*) have demonstrated that P-gp can transport drugs into the extracellular aqueous environment because it can generate substantial drug gradients within vesicles. They showed that P-gp proteoliposomes could generate a 5.6-fold colchicine con-

centration gradient with the vesicles. Similarly, transport measurements with spin-labeled verapamil showed that P-gp could generate a 25-fold gradient of verapamil within the proteoliposomes (*68*).

This study also supports the idea that P-gp contains multiple distinct drug-binding sites (*28*, *69*). Mutant I306R affected interaction of P-gp with only some drug substrates (Figures 4 and 5). The mutation caused a large decrease in apparent affinity for verapamil and vinblastine and had little effect on interaction with colchicine and rhodamine B. The mutant, however, could still interact with hydrophobic substrates such as cyclosporin A (Figure 6B) These results are consistent with the labeling studies involving I306C with MTS-rhodamine and MTS-verapamil that show distinct binding sites for the verapamil and rhodamine B (*30*). The results also show that cysteine-less P-gp is a good model system since similar results are obtained when the same mutation is introduced into wild-type P-gp. In addition, there is good correlation between drug-stimulated ATPase activity and drug-resistance assays. The verapamil-stimulated ATPase activity was altered in mutant I306R and was reflected in decreased ability of verapamil to modulate the resistance of the mutant to colchicine.

In summary, the results of this study indicate that regions of the drug-binding pocket are accessible to the aqueous medium. The presence of water in the drug-binding pocket indicates that hydration of drug substrate prior to efflux from the cell may be an important step in the transport cycle.

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