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Mutation of Glycine 185 to Valine Alters the ATPase Function of the Human P-glycoprotein Expressed in Sf9 Cells*

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A single amino acid substitution, $Gly^{185} \rightarrow Val$, in the human P-glycoprotein (Pgp) was previously shown to cause an altered pattern of drug resistance in cell lines transfected with the MDR1 cDNA carrying this mutation. To further define the function of amino acid 185 in the Pgp, the wild-type and the mutant Val^{185} Pgps were expressed in Sf9 insect cells, and their biochemical properties were compared. Verapamil- and colchicine-stimulated ATPase activities were markedly increased with concomitant increase in affinity for these compounds with $Gly^{185} \rightarrow Val$ substitution in the Pgp. However, the vinblastine-stimulated ATPase activities of the wild-type and Val^{185} Pgps were nearly identical. Because transport substrate-induced ATP hydrolysis is generally thought to reflect transport function, these data suggest that colchicine and verapamil are transported at an increased rate with $Gly^{185} \rightarrow Val$ substitution in the Pgp. These results also indicate that amino acid 185 is involved in verapamil and colchicine, but not in vinblastine, binding/transport. Kinetic analyses indicate that cyclosporin A, an inhibitor of Pgp, binds to the verapamil and vinblastine binding/transport site(s) in the Pgp. Taken together, the results presented herein reveal that the verapamil and vinblastine binding/transport site(s) are in close proximity and that the cyclosporin A binding site spans the common region of these two drug binding/transport site(s) in the Pgp molecule.

Tumor cells undergoing chemotherapy often develop resistance to a wide variety of anti-neoplastic drugs in the phenomenon known as multidrug resistance (MDR)[1] (1). Multidrug resistant tumor cells frequently express large quantities of a 130–170-kDa membrane glycoprotein, referred to as P-glycoprotein (Pgp), encoded by the MDR1 gene (2). Evidence suggests that the human Pgp acts as an energy-dependent transporter that extrudes from cells a spectrum of compounds and drugs with diverse structure and function. The human Pgp is a 1280-amino acid polypeptide, with secondary structure predicted to have 12 transmembrane segments and 2 ATP binding domains on the cytoplasmic side of the molecule. The human Pgp shares considerable sequence and structure homology with an ever-growing list of ATP-binding cassette transport proteins (3).

Direct interaction of photoaffinity analogs of chemotherapeutic agents such as colchicine (4), Adriamycin (5), and vinblastine (6) with the Pgp has been reported. Mapping of the drug binding site(s) in Pgp using photoaffinity analogs has indicated that these site(s) are within or in close proximity to the putative transmembrane segments of the molecule (7). However, the transmembrane segments and amino acid residues of the Pgp involved in substrate binding and transport have not been precisely identified. An understanding of the molecular interactions of drugs with the Pgp would greatly facilitate our ability to modulate its function. An approach most commonly employed toward this end by several laboratories is site-directed mutagenesis in combination with analyses of relative drug resistance in cells (8, 9). Although the information obtained from these studies is valuable for understanding the overall process of MDR, these assays do not accurately reflect the transport function of the Pgp.

Recently, Scarborough and co-workers (10) have demonstrated a drug-stimulated ATPase activity of the human Pgp expressed in cultured Sf9 insect cells. Several laboratories have subsequently shown the drug-stimulated ATPase activity in mammalian cells constitutively expressing the Pgp (11–13). Most importantly, the ability of a variety of compounds to stimulate the Pgp ATPase activity has recently been directly correlated with their transport by the Pgp (14). Thus, measurements of the ATPase function of the Pgp may be used to explore the possible molecular mechanism by which the Pgp mediates drug transport.

Cells selected *in vitro* against any single drug from a diverse group of lipophilic cytotoxic compounds usually develop cross-resistance to other drugs in the group. Some multidrug-resistant cell lines are significantly more resistant to the drug used in their selection than to the other drugs (15, 16). Further studies have suggested that a single amino acid substitution, $Gly^{185} \rightarrow Val$, in the human Pgp was responsible for the preferential resistance of KB epidermoid carcinoma cells to colchicine (15, 16, 34). However, it is not known how the ATPase function of the Pgp is modulated by the Val substitution at amino acid 185 in the polypeptide. In this paper, I report the expression of wild-type Pgp and compare its functional properties with that of the Val^{185} Pgp. The results reported here suggest that the amino acid at position 185 in the Pgp is involved in colchicine and verapamil but not in vinblastine binding/transport. The results presented herein also indicate the propinquity of binding/transport site(s) of verapamil, vinblastine, and cyclosporin A in the human Pgp.

EXPERIMENTAL PROCEDURES

Construction of Baculovirus Transfer Vector pVL1393/MDRGa—Standard recombinant DNA procedures were used to construct the baculoviral transfer vector containing human wild-type MDR1 cDNA for expression in Sf9 insect cells (17). A 4.2-kb *Sac*II-*Xho*I fragment of the human MDR1 cDNA containing plasmid, pHaMDRGa, was kindly provided by Dr. M. M. Gottesman (16). For the convenience of subcloning, pHaMDRGa was first digested with *Xho*I and blunt-ended with the

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[1] The abbreviations used are: MDR, multidrug resistance; Pgp, P-glycoprotein; kb, kilobase(s); PAGE, polyacrylamide gel electrophoresis.

Klenow fragment of *Escherichia coli* DNA polymerase I. It was then religated in the presence of *Xba*I linkers. The resulting plasmid was called pHaMDRGa/XbaI. Employing the pHaMDRGa plasmid as a template and *Pfu* DNA polymerase, a fragment of ~1.2 kb was synthesized by polymerase chain reaction with an added *Bam*HI site containing mutagenized forward primer, 5′-GCGGGATCCCGGGATGGATCTTGAAGG-3′, and an *Eco*RI site containing reverse primer, 5′-TCTGAATTCCAAATTTCCC-3′. This polymerase chain reaction-amplified fragment contained a *Bam*HI site at the 5′-end, the ATG start codon of the MDR1 gene, and extended up to the *Eco*RI site at ~1.2 kb. This fragment was digested with *Bam*HI and *Eco*RI and ligated with the ~3.0-kb *Eco*RI-*Xba*I fragment isolated from the above pHaMDRGa/XbaI, in the presence of pVL1393 transfer vector previously digested with *Bam*HI and *Xba*I. The resulting construct, pVL1393/MDRGa, contained the entire coding region of the human MDR1 cDNA and was sequenced by the dideoxynucleotide chain termination sequencing procedure (18) with Sequenase 2.0 from U. S. Biochemical Corp. With the exception of two silent mutations, one at codon 412 (GGC (Gly) to GGT (Gly)) and another at codon 547 (CGC (Arg) to CGT (Arg)), the entire coding sequence was identical to the previously reported sequence (14, 15).

Production of Recombinant Baculovirus Carrying Wild-type MDR1 cDNA—Sf9 insect cells were cotransfected with 0.5 μg of BaculoGold DNA and 5 μg of pVL1393/MDRGa by the calcium phosphate coprecipitation method according to the protocol provided by the manufacturer (PharMingen, San Diego, CA). 5 days after cotransfection, the culture medium was collected and used to infect 30 million Sf9 insect cells in a 162-cm^2 flask. After 3 days of incubation at 27 °C, the culture medium was collected, and the virus was concentrated by centrifugation at 40,000 × *g* for 60 min. The virus pellet was resuspended in fresh Grace's complete medium (19) and used to infect 5 million Sf9 insect cells in a 25-cm^2 flask. After 4 days of incubation at 27 °C, the spent medium was collected, and the recombinant baculovirus was plaque purified using standard procedures (Invitrogen). Two rounds of plaque purification were performed to eliminate the polyhedrin-expressing baculovirus. Two isolates of recombinant baculovirus carrying the wild-type MDR1 cDNA, termed wild-type MDR1 BV 9 and 10 were chosen for the present study.

To ensure that these recombinant baculoviruses contained the wild-type MDR1 cDNA, their DNA was amplified by polymerase chain reaction according to the protocol provided by the manufacturer (Invitrogen), cloned into M13 bacteriophage vector, and sequenced around the region that codes for the amino acid 185 in the Pgp. The results clearly indicated that these recombinant baculoviruses were the wild-type MDR1 BVs (data not shown).

Preparation of recombinant baculovirus carrying the human MDR1 cDNA with Val^{185} substitution in the expressed protein, termed in this paper as Val^{185} MDR1 BV, was previously described by Germann *et al.* (20).

Viral Infections of Sf9 Insect Cells and Preparation of Insect Cell Membrane Fraction—Approximately 30 million exponentially growing Sf9 insect cells were seeded in each 162-cm^2 flask and infected with wild-type MDR1 BV 9 and 10, Val^{185} MDR1 BV, or *E. coli* β-galactosidase cDNA containing baculovirus (β-gal BV). The virus was added to a multiplicity of infection of about 6 plaque-forming units per cell. The flasks were incubated at 27 °C for 3 days. The membrane fraction from the infected cells was prepared as previously described (21).

Pgp ATPase Activity Determination—The Pgp ATPase activity of the membranes was determined in the presence of various drugs as previously described (10, 21, 22). Each of the experiments shown in the figures was carried out 4–6 times using membranes prepared from different batches of cells, with essentially the same results. The data points indicate the average of duplicate determinations in the individual experiments. Membranes prepared from Sf9 insect cells infected with β-gal BV were used as control. These membranes did not contain any drug-stimulated ATPase activity.

SDS-PAGE, Immunoblotting, and Quantitation of Pgp in Membranes—Membrane suspensions were mixed with SDS-PAGE disaggregation buffer, incubated for 20 min at room temperature, and then electrophoresed on 15 × 15 × 0.075-cm 7.5% acrylamide gels as previously described (23, 24). Transfer of proteins from the acrylamide gels onto polyvinylidene difluoride membranes and immunodetection were carried out as described by Sarkadi *et al.* (10). The primary antibody was Pgp-specific C219 antibody, and the secondary antibody was horseradish peroxidase-conjugated anti-mouse antibody from donkey (Amersham Corp). The peroxidase-labeled blots were developed by the enhanced chemiluminescence method, using the Amersham ECL kit. The Pgp bands obtained on the fluorograms were quantitated by volume integration analysis using a Bio-Rad imaging densitometer (GS670), equipped with Molecular Analyst software version 1.1.

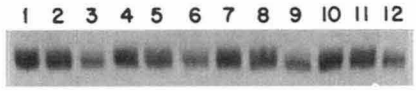

FIG. 1. **Western blot analysis of membranes isolated from Sf9 cells infected with wild-type MDR1 BV and Val^{185} MDR1 BV.** The Sf9 insect cells were infected with wild-type MDR1 BV 9 and 10 and Val^{185} MDR1 BV and incubated at 27 °C for 3 days. The membranes were prepared as described, and equal amounts of membrane protein (20 μg/lane) were subjected to SDS-PAGE and Western blot analysis using C-219 antibody as described under "Experimental Procedures." *Lanes 1, 4, 7, 10*, membranes from wild-type MDR1 BV 9-infected Sf9 cells; *lanes 2, 5, 8, 11*, membranes from wild-type MDR1 BV 10-infected Sf9 cells; *lanes 3, 6, 9, 12*, membranes from Val^{185} MDR1 BV-infected Sf9 cells. Only the ~130-kDa region of the blot is shown. No antibody-reactive material was seen elsewhere in the blots.

Protein Estimation—Protein in the membrane suspensions was determined by the method of Lowry *et al.* (25) as modified by Bensadoun and Weinstein (26) using bovine serum albumin as the standard.

Materials—Tissue culture supplies were obtained from the Lineberger Cancer Center Tissue Culture Facility at the University of North Carolina, Chapel Hill. Molecular biology reagents were from Promega, Boehringer-Mannheim, Stratagene, and Bio 101. BaculoGold transfection kit was from PharMingen (San Diego, CA). The C219 antibody was from Signet (Boston, MA). The horseradish peroxidase-conjugated anti-mouse antibody and ECL detection kit were from Amersham. Colchicine, verapamil, and vinblastine were from Sigma. Cyclosporin A was from Sandoz Pharmaceutical Institute (NJ).

RESULTS

Expression and Quantitation of Pgp in Sf9 Insect Cell Membranes—Expression of human wild-type Pgp in insect cells was examined by Western blot analysis, and the results are shown in Fig. 1. Sf9 insect cells were infected with wild-type MDR1 BV 9 and 10 and Val^{185} MDR1 BV, and the membranes from 3 days post-infection were prepared (21, 22). The proteins in the membranes were separated by SDS-PAGE gels, electrotransferred onto polyvinylidene difluoride membranes, and then probed with the Pgp-specific C219 monoclonal antibody as described under "Experimental Procedures." A single band of approximately 130 kDa was visible in membranes obtained from Sf9 insect cells infected with the wild-type MDR1 BV 9 (Fig. 1, *lanes 1, 4, 7*, and *10*) and 10 (Fig. 1, *lanes 2, 5, 8*, and *11*). These Pgps were termed wild-type Pgp 9 and 10, respectively, both of which comigrated with the Val^{185} Pgp (Fig. 1, *lanes 3, 6, 9*, and *12*). No detectable C219 antibody-reactive proteins were found in control membranes in which Sf9 insect cells were infected with the β-gal BV (data not shown). Thus, Sf9 insect cells infected with wild-type MDR1 BV isolates 9 and 10 produce the wild-type Pgps, which are indistinguishable from the previously characterized Val^{185} Pgp (20).

Analyses of several batches of membrane preparations indicated that the amounts of Pgp expressed were variable. Since the objective of the present study was to measure the functional differences between the wild-type and the Val^{185} Pgps, quantitative estimation of the amount of Pgp protein in these membranes was essential. Although Pgps separated on SDS-PAGE gels were stained with Coomassie Blue and silver, quantitation could not be performed because several non-Pgp protein bands were also observed in this region of the gels. The C219 monoclonal antibody-reactive hexapeptide epitope (27) is identical in the wild-type and Val^{185} Pgps. Thus, immunoblots developed using the C219 antibody as the primary antibody were used for quantitating the Pgps in equal amounts of several membrane preparations (Fig. 1). The amount of Val^{185} Pgp arbitrarily was considered 1 absorbance unit, and the amounts of wild-type Pgps were calculated as multiples of the amount of Val^{185} Pgp. For example, the amount of Val^{185} Pgp in *lane 3*

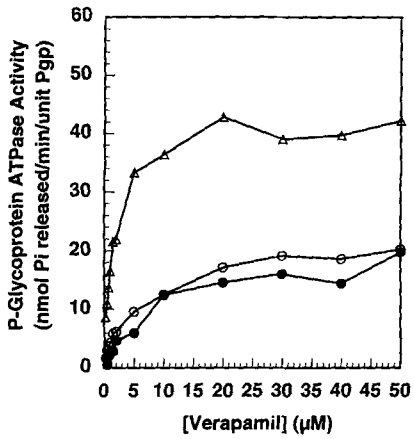

FIG. 2. **Effect of verapamil on the ATPase activity of the wild-type and Val^{185} Pgps.** The wild-type Pgp 9- (○), wild-type Pgp 10- (●), and Val^{185} Pgp- △ containing membranes were incubated with increasing concentrations of verapamil and assayed for ATPase activity as described under "Experimental Procedures." Each data point was the average of duplicate determinations.

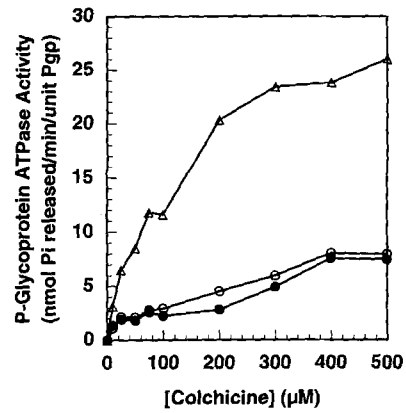

FIG. 3. **Effect of colchicine on the ATPase activity of the wild-type and Val^{185} Pgps.** The wild-type Pgp 9- (○), wild-type Pgp 10- (●), and Val^{185} Pgp- (△) containing membranes were incubated with increasing concentrations of colchicine and assayed for ATPase activity as described under "Experimental Procedures." Each data point was the average of duplicate determinations.

was considered 1 absorbance unit, and the amounts of Pgp in *lanes 1* and *2* were expressed relative to the amounts of Pgp in *lane 3*. Thus, the average values of 4 determinations indicated that the wild-type Pgp 9 and 10 were 1.89 and 1.58 times the amount of Val^{185} Pgp (data not shown). These quantitative measurements would not distinguish between functionally active and inactive forms of the Pgps (see "Discussion").

Drug-stimulated Pgp ATPase Activities—The membrane preparations used for quantitating the Pgp protein as shown in Fig. 1 were used for the following studies. Fig. 2 shows the effects of verapamil on the Pgp ATPase activities in the wild-type Pgp- and Val^{185} Pgp-containing membranes. The drug-stimulated ATPase activity obtained with 1 mg of membrane protein containing Val^{185} Pgp was considered due to 1 unit of Pgp. The drug-stimulated ATPase activities of the wild-type Pgp 9 and 10 were normalized to the amounts of Val^{185} Pgp protein. Verapamil-stimulated wild-type Pgp ATPase activities increased in a concentration-dependent manner. These Pgps exhibited a maximum ATPase activity of approximately 26 nmol of P_i released per min per unit of Pgp at 500 μM verapamil; greater concentrations inhibited ATPase activity (not shown). Control experiments indicated that the stimulation of wild-type Pgp ATPase activity by verapamil was inhibited by vanadate (100 μM) and unaffected by ouabain (1 mM), sodium azide (5 mM), or EGTA (2 mM). The K_a (half-maximal ATPase activating concentration) value for verapamil calculated from the double-reciprocal plots of six different determinations using membranes containing wild-type Pgp 9 and 10 was 4.4 ± 0.8 μM (data not shown). However, the verapamil-stimulated ATPase activity of Val^{185} Pgp reached maximum at about 20 μM, with a K_a value of 1.0 ± 0.2 μM (six determinations). These results suggest that verapamil interacts with Val^{185} Pgp with an affinity about 4 times higher than with the wild-type Pgp.

Colchicine stimulated ATPase activity of the wild-type Pgp 9 and 10, and at 400 μM, these activities reached a maximum of about 8 nmol of P_i released per min per unit of Pgp (Fig. 3). A K_a value of 163 ± 18 μM was calculated from the double-reciprocal plots of five different determinations. The Val^{185} Pgp exhibited a maximal ATPase activity in the presence of colchicine of about 25 nmol of P_i released per min per unit of Pgp, with a K_a of approximately 67 ± 9 μM (five determinations). These results suggest that replacement of Gly^{185} with Val in the Pgp increases the affinity for colchicine by more than 2-fold.

Vinblastine markedly increased the rate of ATP hydrolysis by the wild-type and Val^{185} Pgps (Fig. 4). Both forms of Pgp exhibited nearly identical maximum ATPase activity of approximately 13 nmol of P_i released per min per unit of Pgp in the presence of vinblastine. The K_a for vinblastine calculated from double-reciprocal plots of four determinations was 0.45 ± 0.06 μM. These data suggest that mutation at amino acid 185 in the Pgp polypeptide does not alter the affinity for vinblastine.

Although the above data suggest that the mutation Gly^{185} → Val in the Pgp alters the affinity for verapamil and colchicine, it does not rule out the possibility that the affinities for ATP binding are responsible for these results. However, measurements using increasing concentrations of Mg·ATP on the drug-stimulated Pgp ATPase activities yielded identical K_m values of 0.62 mM for Mg·ATP with both Pgp forms (data not shown). These results indicate that the mutation at amino acid 185 in the Pgp does not affect nucleotide binding.

We have shown recently that cyclosporin A inhibits verapamil- and vinblastine-activated Val^{185} Pgp ATPase activities in a competitive manner, with K_i values of approximately 20 nM (22). In the present study, the interaction of cyclosporin A with the wild-type Pgp in the presence of the above drugs was analyzed (Fig. 5). Verapamil- and vinblastine-stimulated wild-type Pgp ATPase activities were inhibited by cyclosporin A, and the double-reciprocal plots suggested that the inhibition was competitive (Fig. 5, *insets*). The K_i for cyclosporin A in the presence of both of these drugs was 150 ± 10 nM (four determinations). Thus, the affinity for cyclosporin A was increased by more than seven times with Gly^{185} → Val mutation in the Pgp molecule.

DISCUSSION

The colchicine-induced mutation in the MDR1 gene bestows upon transfected cell lines a selective resistance to this drug (15, 16, 34). Although the biochemical properties of the Val^{185} Pgp are known mainly through the work from the laboratories of Gottesman (20) and Scarborough (10, 21, 22), it is not known how the Gly^{185} to Val mutation alters the ATPase function of the Pgp. The results presented here indicate that the wild-type human Pgp expressed in Sf9 cells exhibits drug-stimulated ATPase activity.

To critically examine any functional differences between the wild-type and Val^{185} Pgps, it is necessary to present the drug-stimulated ATPase activity data obtained with equal amounts of Pgp protein in these membranes. Although the densitometric analysis employed to quantitate the Pgps in the membranes is reliable, it does not discriminate functional from nonfunctional Pgp proteins in the membrane preparations. Several functional

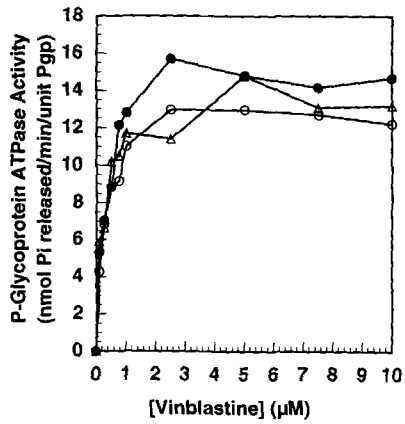

FIG. 4. **Effect of vinblastine on the ATPase activity of the wild-type and Val^{185} Pgps.** The wild-type Pgp 9- (○), wild-type Pgp 10- (●), and Val^{185} Pgp- (△) containing membranes were incubated with increasing concentrations of vinblastine and assayed for ATPase activity as described under "Experimental Procedures." Each data point was the average of duplicate determinations.

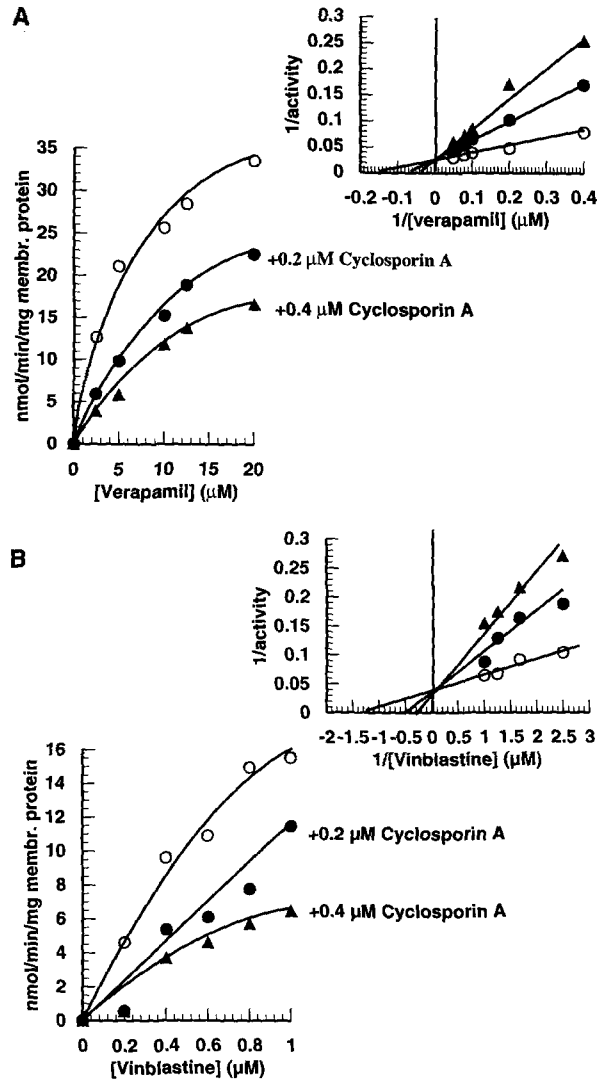

FIG. 5. **Kinetics of inhibitions of the verapamil- and vinblastine-stimulated wild-type Pgp ATPase activity by cyclosporin A.** The verapamil (*panel A*) or vinblastine (*panel B*) concentration was varied, with constant cyclosporin A concentrations of 0 (○), 0.2 (●), and 0.4 μM (▲). *Insets*, double-reciprocal plots of the data.

photoaffinity ATP and drug analogs are available, which may prove to be valuable in quantitating the amounts of Pgp in the membrane preparations (28–30). However, it is not clear how the Gly^{185} → Val mutation regulates the interactions of these photoaffinity analogs with the Pgp. Thus, to minimize this uncertainty, two isolates of wild-type MDR1 BVs, which express different amounts of the wild-type Pgps, were employed in the present study. As shown in Figs. 2–4, the drug-stimulated ATPase activities of the two wild-type Pgps were nearly identical, indicating that the densitometric quantitation of Pgps employed is dependable. In addition, the kinetic parameters K_m, K_a, and K_i, which do not depend on the enzyme concentration, are suitable for describing the functionally different Pgp preparations.

The results presented in Figs. 2 and 3 indicate that the interactions of Pgp with verapamil and colchicine are affected by the mutation at amino acid 185 in the polypeptide. The distinctly different activation profiles of Pgp ATPase and K_a values for these drugs suggest that replacement of Gly^{185} with Val leads to higher affinity interactions with the Pgp. Importantly, the increase in colchicine-induced Val^{185} Pgp ATPase activity compared with that of the wild-type Pgp directly correlates with the increase in drug resistance imparted by the mutation Gly^{185} to Val in the Pgp molecule (15, 16). Homolya *et al.* (14) have recently demonstrated that only those compounds that stimulate ATP hydrolysis are transported by the Pgp. Furthermore, solubilized transport ATPases display ATP hydrolysis-coupled conformational changes, substrate binding, and dissociation, indicating the complete catalytic cycle (31–33). Thus, the Pgp ATPase activity induced by a drug is a direct measure of its transport.

The vinblastine-stimulated ATPase activity profiles and the K_a values obtained with the wild-type and Val^{185} Pgps are nearly identical, indicating that Gly^{185} is not involved in vinblastine binding/transport. In contrast, the relative drug resistance of transfected cell lines suggested that Gly^{185} → Val substitution in the Pgp slightly increases the sensitivity to vinblastine (15, 16). These data would be consistent with the hypothesis that there may be an additional non-Pgp-mediated resistance mechanism for vinblastine action, which could account for these observations (15).

We have shown recently that there are two classes of substrates for Pgp. The first group interacts with the Pgp and elicits ATPase activity, implying that these drugs are transported by the Pgp. The second class of compounds, which include cyclosporins, interact with Pgp with high affinity but fail to elicit ATP hydrolysis. However, the cyclosporins act as competitive inhibitors of transport substrate-induced ATP hydrolysis (22). The data shown in Fig. 5 indicate that cyclosporin A also inhibits verapamil- and vinblastine-stimulated wild-type Pgp ATPase activities in a competitive manner. The simple interpretation of these observations is that cyclosporin A binds to the verapamil and vinblastine binding/transport site(s). Interestingly, the affinity for cyclosporin A increased markedly with Gly^{185} → Val substitution, suggesting that amino acid 185 is involved in the cyclosporin A interactions with the Pgp.

These results may bear in an important way on the issue of number and location of drug binding/transport sites in the Pgp molecule. There may be two drug binding/transport sites in the Pgp that are distinguishable by the presence or absence of amino acid 185, one site to which both verapamil and colchicine bind and a second to which vinblastine alone binds. Because the verapamil- and vinblastine-stimulated Pgp ATPase activities were inhibited by cyclosporin A, the verapamil and vinblastine binding/transport sites are probably in close proximity, and cyclosporin A binds to the common region of both of these sites. Alternatively, amino acid 185 is not in the drug

binding/transport site(s), but mutations at this residue bring about conformational changes that impose different constraints on the Pgp, leading to altered patterns of affinity and ATPase activity with different drugs. Further studies aimed at characterizing the drug binding/transport site(s) of the Pgp should enable us to better define the mechanism of MDR in tumor cells.

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