Coordinate Changes in Drug Resistance and Drug-Induced Conformational Transitions in Altered-Function Mutants of the Multidrug Transporter P-Glycoprotein†

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ABSTRACT: The MDR1 P-glycoprotein (Pgp), responsible for a clinically important form of multidrug resistance in cancer, is an ATPase efflux pump for multiple lipophilic drugs. The G185V mutation near transmembrane domain 3 of human Pgp increases its relative ability to transport several drugs, including etoposide, but decreases the transport of other substrates. MDR1 cDNA with the G185V substitution was used in a function-based selection to identify mutations that would further increase Pgp-mediated resistance to etoposide. This selection yielded the I186N substitution, adjacent to G185V. Pgps with G185V, I186N, or both mutations were compared to the wild-type Pgp for their ability to confer resistance to different drugs in NIH 3T3 cells. In contrast to the differential effects of G185V, I186N mutation increased resistance to all the tested drugs and augmented the effect of G185V on etoposide resistance. The effects of the mutations on conformational transitions of Pgp induced by different drugs were investigated using a conformation-sensitive antibody UIC2. Ligand-binding analysis of the drug-induced increase in UIC2 reactivity was used to determine the K_m value that reflects the apparent affinity of drugs for Pgp, and the Hill number reflecting the apparent number of drug-binding sites. Both mutations altered the magnitude of drug-induced increases in UIC2 immunoreactivity, the K_m values, and the Hill numbers for individual drugs. Mutation-induced changes in the magnitude of UIC2 reactivity shift did not correlate with the effects of the mutations on resistance to the corresponding drugs. In contrast, an increase or a decrease in drug resistance relative to that of the wild type was accompanied by a corresponding increase or decrease in the K_m or in both the K_m and the Hill number. These results suggest that mutations that alter the ability of Pgp to transport individual drugs change the apparent affinity and the apparent number of drug-binding sites in Pgp.

Multidrug resistance is a clinically relevant phenomenon in which tumor cells become cross-resistant to many chemotherapeutic drugs with different mechanisms of action. The best known forms of multidrug resistance are mediated by active transport proteins that belong to the ATP-binding cassette (ABC) superfamily (*1*), including the products of the MDR1 (*2*), MRP1 (*3*), and BCRP1/MXR/ABCP (*4*) genes. The most studied of these genes, MDR1, encodes the P-glycoprotein (Pgp),[1] a 170 kDa integral plasma membrane protein that consists of two homologous halves connected by a linker region. Each half of Pgp includes a hydrophobic domain with six transmembrane segments (TMs) and the nucleotide-binding ABC domain, which is responsible for the ATPase activity of Pgp (*2*). Pgp carries out energy-dependent efflux of a wide range of lipophilic compounds, including many chemotherapeutic drugs, and also acts as a short-chain lipid translocase (*5*).

Molecular determinants that define the spectrum of the substrates for pleiotropic ABC transporters and the relative efficiency of their transport have been extensively investigated. In the case of Pgp, binding of photoactive substrate analogues has implicated TM5, TM6, TM11, and TM12 in drug binding (*6*−*9*). A broader array of functional information has been deduced from the analysis of mutations that alter the relative ability of Pgp to transport different substrates. Altered-function mutations have been found in all parts of the protein, including the intracellular loops, the TM, and even the ABC domains (reviewed in ref *10*). The first altered-function mutation to be identified in ABC transporters was the substitution of glycine to valine at position 185 (G185V), adjacent to TM3 of the human MDR1 Pgp (*11*). This mutation developed in a colchicine-selected cell line and was found to confer an increased resistance to

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[1] Abbreviations: BSA, bovine serum albumin; FACS, fluorescence-activated cell sorter; PBS, phosphate-buffered solution; Pgp, P-glycoprotein.

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colchicine, as well as to etoposide and doxorubicin. At the same time, G185V decreased cellular resistance to vinblastine and Taxol, compared to that of the wild-type Pgp (*11*, *12*), and altered the sensitivity of Pgp to different inhibitors (*13*, *14*). These changes in drug resistance were accompanied by the corresponding changes in drug accumulation (*12*) and altered the kinetics of Pgp-mediated drug transport (*15*). These results have implicated TM3 and its surrounding sequences as an important determinant of Pgp−substrate interactions, a conclusion that has been confirmed and extended in a more recent study involving random mutagenesis of this region (*16*). Biochemical assays showed that G185V enhances the ability of colchicine and inhibits the ability of vinblastine to stimulate the ATPase activity of Pgp (*17*, *18*). On the other hand, G185V was found to decrease the level of Pgp binding of a photoactive analogue of colchicine and to increase the level of binding of a vinblastine analogue, suggesting that this mutation changes the transport efficiency by affecting the release of the substrates from Pgp to the outside of the cell (*12*).

In the study presented here, we have used an unbiased mutation−selection strategy (*19*) to identify new Pgp mutants that would confer increased resistance to etoposide, a topoisomerase II interactive agent, and a relatively poor substrate of Pgp. Etoposide is widely used in the treatment of various solid tumors, and improving the ability of Pgp to transport etoposide may prove helpful in using Pgp for gene therapy aimed at chemoprotection of normal tissues (*20*). The first functional mutation identified by this selection turned out to affect amino acid 186, which is immediately adjacent to the previously identified G185V substitution. We have compared the effects of these two mutations, singly and in combination, with regard to the ability of Pgp to confer resistance to different drugs and drug-induced conformational transitions of Pgp. The conformational effects of the mutations were investigated by quantitative measurements of drug-induced changes in the reactivity of Pgp with the conformation-sensitive monoclonal antibody UIC2 (*21*). This analysis has indicated that the mutations affect the transport of Pgp substrates by altering the apparent substrate affinity and the apparent number of substrate-binding sites in Pgp.

EXPERIMENTAL PROCEDURES

Materials. Etoposide, vinblastine, colchicine, doxorubicin, propidium iodide, oligomycin, and the ATP Bioluminescent Somatic Cell Assay Kit were from Sigma. The UIC2 antibody was purified from the hybridoma supernatant as described previously (*22*). The MRK16 antibody was kindly provided by T. Tsuruo (University of Tokyo, Tokyo, Japan). The UPC10 hybridoma protein (Sigma Immunochemicals) of the same IgG2a isotype was used as a negative control. The goat anti-mouse IgG2a fluorescein isothiocyanate (FITC)-conjugated antibody was from Caltag Laboratories. Primers were obtained from Integrated DNA Technologies, Inc.

Isolation of Altered-Function Mutants. The procedures for the propagation of retroviral plasmid vector pLMDR1 (*23*), carrying MDR1 cDNA with a G185V mutation, in the mutD strain of *Escherichia coli*, transfection of BOSC 23 ecotropic packaging cells (*24*), retroviral transduction of NIH 3T3 cells, etoposide selection, and PCR-based recovery and recloning of the integrated MDR1 cDNA have been previously described (*19*). The recloned library of MDR1 cDNA clones was retransduced into NIH 3T3 cells, which were then subjected to selection using 10 μg/mL etoposide. After selection, 20 randomly picked cell clones were used to recover MDR1 cDNA by Long and Accurate PCR using the XL PCR kit (Perkin-Elmer) and primers flanking the MDR1 cDNA insert of LMDR1. The PCR products were used for sequencing and recloning into the same retroviral vector. Primer sequences and details of the cloning procedure are available upon request.

Sequencing and Site-Directed Mutagenesis. PCR products corresponding to full-length MDR1 cDNA amplified from the selected cells were used directly for sequencing using a set of MDR1-specific sequencing primers (sequences available on request) and the Rhodamine Dye Terminator RR Sequencing Kit (Perkin-Elmer), with the ABI model 373 sequencer. Site-directed mutagenesis was carried out using the Quick-Change Site-Directed Mutagenesis Kit (Stratagene). The primers for site-directed mutagenesis (sequences available on request) consisted of the nucleotide(s) to be mutated and at least 10−15 nucleotides on each side of the mutation.

Drug Resistance Assays. NIH 3T3 cells transduced with different forms of MDR1 or with the insert-free pLXSN vector were maintained in Dulbecco's modified Eagle's medium containing 10% fetal calf serum (Hyclone), 1% glutamate, and a 1% penicillin/streptomycin mixture. For drug resistance clonogenic assays, 250 cells were plated on a 3 cm plate (in triplicate), different drug concentrations were added 24 h later, and the cells were allowed to form colonies for 8 days. Colonies were fixed with methanol, stained with crystal violet (10% w/v in a 10% methanol solution), and counted.

Fluorescence-Activated Cell Sorting Assays. For UIC2 reactivity shift assays, aliquots of the UIC2 antibody were heated at 48 °C for 24 h and stored at 4 °C. As described elsewhere (*26*), such heating maximizes the UIC2 reactivity shift by decreasing the antibody reactivity in the absence but not in the presence of the substrate. For FACS analysis, 1×10^6 cells per sample were trypsinized, washed once with a phosphate-buffered solution (PBS), pelleted, and resuspended in 200 μL of PBS with 1% bovine serum albumin (BSA) and 15 μg/mL primary antibody. When indicated, cells were incubated for 10−15 min with the appropriate drug prior to the addition of the antibody. After 30 min with the primary antibody, cells were washed once with ice-cold PBS, and centrifuged at 4 °C for 5 min at 1500 rpm. Cells were then resuspended in 100 μL of PBS and 1% BSA containing 25 μg/mL FITC-conjugated goat anti-mouse secondary antibody. This reaction mixture was left on ice for 30 min. The cells were then washed once with ice-cold PBS, pelleted, and resuspended in 500 μL of PBS and 1% BSA and 1 μg/mL PI. FACS analysis was performed on the Becton Dickinson FACSort using two-color analysis (to exclude PI-positive dead cells) and acquiring at least 1×10^4 separate events. Cell sorting was performed using Becton Dickinson FACSVantage.

The cellular ATP content was measured by taking 1×10^3 cells from each sample after staining with the primary antibody. Cells were washed twice with PBS at room temperature and pelleted by centrifugation. ATP-dependent

bioluminescence was measured in a Beckman L5500 TD scintillation counter following the protocol provided with the ATP Bioluminescent Somatic Cell Assay Kit.

Data Analysis. Analysis of the FACS results was performed using the SigmaPlot curve-fitting plot program (SISS), to determine the maximum or minimum fluorescence, the concentration of drug that gave half-maximal change in fluorescence (K_m), and the Hill number, n. The best-fit regression through the data points was determined using the binding isotherm given by the following equation:

$$F = F_{min} + [(F_{max} - F_{min})B^n]/(K_m{}^n + B^n)$$

RESULTS

Isolation of the I186N Mutant Conferring Increased Resistance to Etoposide. To isolate Pgp mutants that would further increase the resistance to etoposide conferred by the previously described G185V mutant, we have used the strategy of function-based selection of mutagenized retroviral vectors (*19*). A retroviral plasmid vector carrying the coding sequence of human MDR1 cDNA with the G185V mutation was propagated in a mutator strain of *E. coli*, and the resulting randomly mutagenized retrovirus was transduced into murine NIH 3T3 cells. The infected cells were selected in a high (2 μg/mL) concentration of etoposide. At this concentration, no colonies were formed by cells transduced with the unmutagenized retrovirus, but approximately 0.1% of the cells transduced with the mutagenized virus survived the selection. MDR1 cDNA was recovered by PCR from the surviving cells, recloned into the retroviral vector, and again introduced into NIH 3T3 cells. The newly transduced cells were subjected to selection, using 10 μg/mL etoposide. After selection, 20 randomly picked cell clones were used to recover MDR1 cDNA. The recovered cDNA was sequenced and found to contain an average of 1.2 mutations per 3840 bp of the protein-coding sequence. Twelve of the recovered mutants were individually introduced into NIH 3T3 cells, and the transduced cells were tested for etoposide resistance. One of the selected mutants produced elevated etoposide resistance relative to that of the parental G185V MDR1 cDNA. This mutant was found to carry a single ATT → AAT mutation of codon 186, which resulted in a change of isoleucine to asparagine at the corresponding position (I186N), adjacent to the original G185V substitution.

Effects of G185V and I186N Substitutions on Drug Resistance Profiles. To confirm that the I186N mutation was indeed responsible for elevated etoposide resistance and to investigate how this mutation interacts with G185V, we used site-directed mutagenesis to introduce the I186N substitution into both G185V and the wild-type versions of the MDR1 cDNA. Retroviral vectors carrying the wild-type MDR1, MDR1 carrying the individual G185V or I186N mutation, and MDR1 with both G185V and I186N mutations (G185V/I186N) were individually transduced into NIH 3T3 cells. NIH 3T3 cells transduced with an insert-free retroviral vector were used as a control. MDR1-transduced populations were stained with the MRK16 monoclonal antibody, specific for the human MDR1 Pgp, and then sorted by FACS, to obtain cell populations with equal expression of different forms of Pgp. As shown in Figure 1, MRK16 staining of the sorted populations indicated that all four populations were 100% Pgp-positive and expressed equal amounts of Pgp on the cell surface.

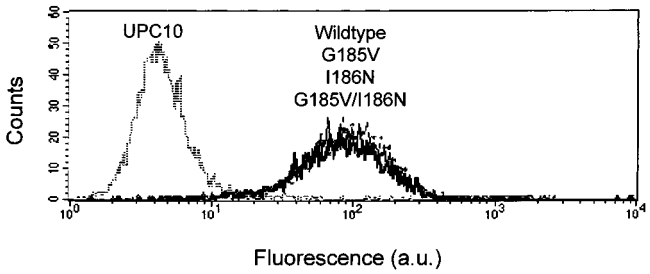

FIGURE 1: Pgp expression in NIH 3T3 cells transduced with wild-type or mutant MDR1. Cells were stained with either the UPC10 isotype control or the MRK16 antibody specific for the human MDR1 Pgp. The overlapping FACS profiles represent four different NIH 3T3 populations expressing either the wild-type MDR1 or MDR1 mutants G185V, I186N, or G185V/I186N (double mutant).

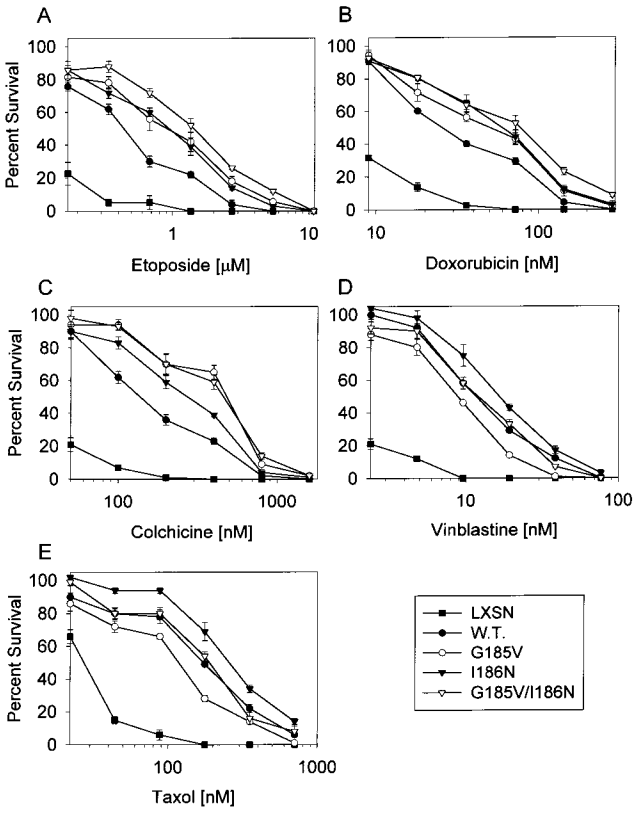

FIGURE 2: Drug resistance conferred by wild-type MDR1 and MDR1 mutants. Clonogenic assays for resistance to the indicated drugs were carried out as described in Experimental Procedures. NIH 3T3 cells were transduced with insert-free LXSN vector (■), with the wild-type MDR1 (●), with G185V (○), with I186N (▼), or with G185V/I186N (▽). Each point is the mean of three samples with the bars representing the standard deviation (often smaller than the size of the symbols). Each experiment was repeated several times with similar results.

Figure 2 shows the results of the clonogenic assays for resistance to different drugs, carried out on the transduced populations. The etoposide assays (Figure 2A) showed that the G185V (○) substitution increased etoposide resistance relative to cells transduced with the wild-type MDR1 (●), in agreement with our previous report (*12*). The etoposide resistance of G185V was further increased by combining it with I186N [G185V/I186N (△)]. I186N (▲) alone produced the same intermediate increase in etoposide resistance as

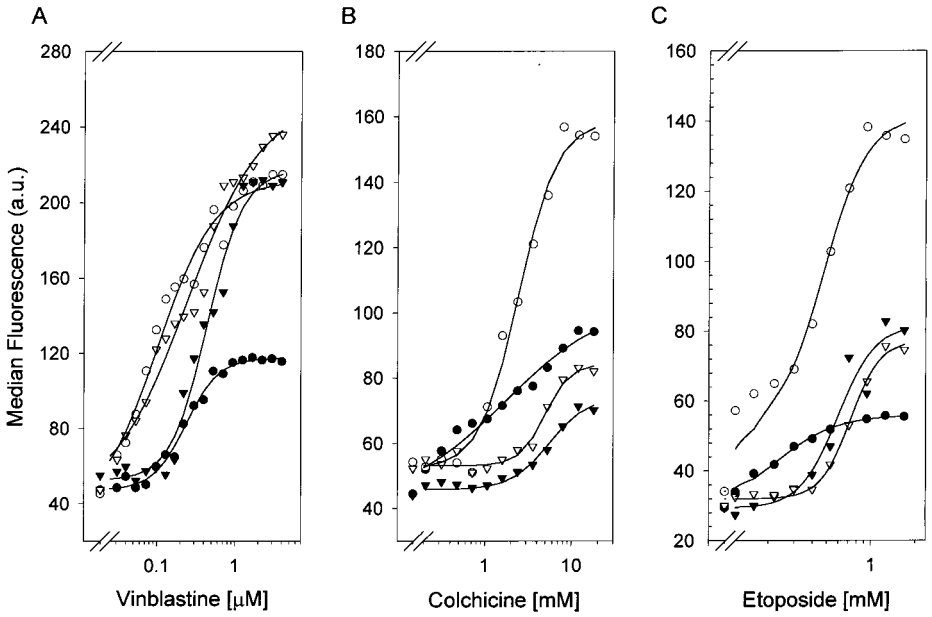

FIGURE 3: UIC2 reactivity of NIH 3T3 cells expressing the wild-type or the indicated mutant forms of MDR1 in the presence of increasing concentrations of vinblastine (A), colchicine (B), or etoposide (C). Median fluorescence values are plotted on the *Y* axis. NIH 3T3 cells were transduced with wild-type MDR1 (●), with G185V (○), with I186N (▼), or with G185V/I186N (▽). Each experiment was repeated several times with similar results.

G185V (Figure 2A). The effects of these mutations on doxorubicin resistance (Figure 2B) were similar to their effects on etoposide resistance. Colchicine assays (Figure 2C) showed that both I186N and G185V increased colchicine resistance relative to the wild-type MDR1, but G185V (a mutation that was originally found in colchicine-selected cells) had a stronger effect. Colchicine resistance conferred by G185V/I186N was indistinguishable from the effect of G185V alone. A major functional difference between G185V and I186N mutations was observed, however, in the vinblastine (Figure 2D) and Taxol (Figure 2E) assays. In agreement with our previous reports (*11*, *12*), G185V decreased the resistance to both of these drugs relative to the wild-type MDR1. In contrast, I186N increased vinblastine and Taxol resistance relative to that of the wild type. These opposite effects of the two mutations were balanced out in the G185V/I186N double mutant, which produced vinblastine and Taxol resistances that were indistinguishable from that of the wild type (Figure 2D,E). These results indicate that I186N increases the ability of MDR1 to confer resistance to all the tested drugs and augments the effect of G185V in providing a high level of etoposide resistance.

Analysis of the Effects of the G185V and I186N Mutations on Drug-Induced Conformational Transitions of Pgp Using UIC2 Immunoreactivity. In the presence of its transport substrates or under the conditions of ATP depletion, Pgp undergoes conformational changes that can be detected by increased immunoreactivity with the monoclonal antibody UIC2 at 37 °C (*21*, *26*, *27*). We have used changes in UIC2 reactivity to investigate how G185V and I186N mutations change the effects of transported drugs on the conformation of Pgp. NIH 3T3 cells carrying the wild-type and different mutant forms of Pgp were incubated with increasing concentrations of vinblastine, colchicine, or etoposide, and stained by indirect immunofluorescence labeling with UIC2 at 37 °C. Representative experiments shown in Figure 3 demonstrate that UIC2 immunoreactivity (plotted as mean fluorescence values determined by FACS analysis) increases in a dose-dependent manner in the presence of each of these drugs. In control experiments, none of the drugs altered the reactivity of any of the tested cell populations with the conformation-insensitive antibody MRK16 (data not shown). Figure 4 shows the values of UIC2 reactivity of all four Pgp-expressing cell populations, which were determined in concurrent assays in the presence or absence of saturating

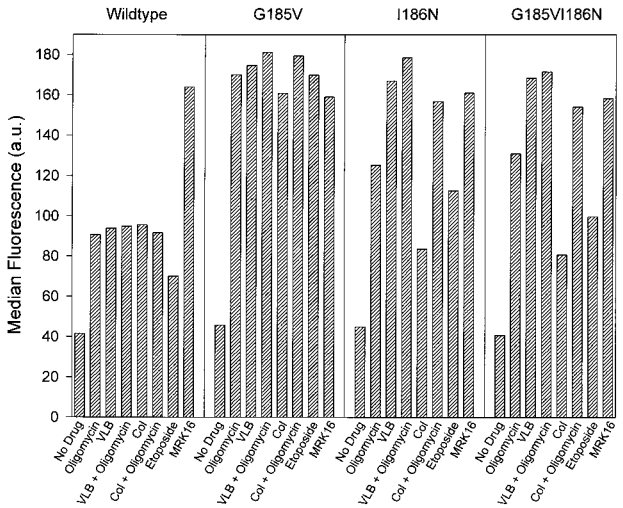

FIGURE 4: UIC2 reactivity of NIH 3T3 cells expressing the wild type or the indicated mutant forms of MDR1 in the presence of saturating concentrations of vinblastine, colchicine, etoposide, or oligomycin. Samples of the indicated cell populations were treated concurrently with 10 μM vinblastine, 5 mM colchicine (Col), 2 mM etoposide, or 2 μM oligomycin or with the indicated drug and oligomycin combinations and then stained with UIC2 as described in Experimental Procedures. Measurement of the level of cellular ATP in oligomycin-treated and control untreated samples showed that oligomycin decreased cellular ATP levels to 3.2, 3.3, 3.4, and 2.9% of the control in the wild type, G185V, I186N, and G185V/I186N, respectively. The experiment was repeated several times with similar results.

concentrations of each of the three drugs. Figure 4 also shows the effects of a metabolic inhibitor oligomycin, at a concentration of 2 μM that we have found to produce a 30−40-fold decrease in the intracellular ATP content of these cells (see the legend of Figure 4).

As described elsewhere (*27*), the relative colchicine resistance provided by the wild-type Pgp in different cellular backgrounds is positively correlated with the fold increase in UIC2 reactivity in the presence of saturating concentrations of colchicine. We have asked therefore whether the relative levels of drug resistance for different Pgp mutants expressed in NIH 3T3 cells would correlate with the fold increase in UIC2 reactivity at the saturating concentrations of the corresponding drugs, but no such correlation was observed. As shown in Figure 4, vinblastine and colchicine increased the UIC2 reactivity of the wild-type Pgp to the same level, matching the level of the cells that were ATP-depleted by oligomycin treatment, while etoposide had a slightly weaker effect. Even in the presence of drugs or oligomycin, however, the UIC2 reactivity of the wild-type protein reached only about one-half of the reactivity observed when the conformation-insensitive MRK16 rather than UIC2 was used as the primary antibody (Figure 4). The level of UIC2 reactivity was not increased further by a combination of oligomycin with either vinblastine or colchicine (Figure 4). The G185V mutation had no effect on UIC2 reactivity in the absence of drugs, but it raised the reactivity in the presence of all the drugs or oligomycin to a level similar to that of MRK16 (Figure 4). Unlike G185V, I186N produced different effects on the fold increase in UIC2 reactivity by different drugs. This mutation increased the effect of vinblastine to the level of MRK16, but it had a weaker effect with etoposide or colchicine (Figure 4). Interestingly, oligomycin reproducibly failed to raise the UIC2 reactivity of I186N Pgp to the maximal level, although a stronger effect was observed when oligomycin was combined with vinblastine or colchicine (Figure 4). The G185V/I186N double mutant showed the same increase in UIC2 reactivity with all three drugs and with oligomycin as I186N alone (Figure 4). These effects of the mutations on the magnitude of drug-induced increase in UIC2 reactivity showed no correlation with their effects on cellular drug resistance (Table 1).

For more detailed analysis of the effects of the mutations on drug-induced conformational transitions of Pgp, we have used the ligand binding equation (see Experimental Procedures) with the curves of Figure 3 to determine the K_m and Hill number parameters, which are phenomenological descriptors of the numerical data for drug-induced increase in UIC2 reactivity. The K_m can be viewed as a measure of the apparent affinity of the ligand for the protein, and the Hill number as the minimum number of ligand molecules that appear to be bound to the protein during the process that brings about the conformation change. These values, together with the fold increase in UIC2 reactivity observed at the saturating concentrations of individual drugs (Figure 4), and with the values of relative resistance of each cell population to the corresponding drugs, are provided in Table 1. This analysis indicated a relationship between the changes in the K_m and Hill number parameters and the effects of the mutations on drug resistance. As can be seen in Table 1, an increase or a decrease in drug resistance relative to that of the wild type was accompanied by a corresponding increase or a decrease in the K_m or in both the K_m and the Hill number. The specific effects of the mutations on the K_m and Hill number changes for different drugs and their potential mechanistic significance are addressed in detail in the Discussion.

Table 1: Effects of G185V and I186N Mutations on the Relative Resistance and Conformational Effects of Vinblastine, Colchicine, and Etoposide[a]

		changes in UIC2 reactivity		
MDR1 mutant	relative resistance	fold increase	K_m (μM)	Hill number
vinblastine				
wild type	7.8	2.2	0.24 ± 0.01	2.1 ± 0.2
G185V	5.7	3.9	0.10 ± 0.01	1.2 ± 0.2
I186N	10.8	3.8	0.44 ± 0.02	1.9 ± 0.3
G185V/I186N	8.1	4.2	0.24 ± 0.04	0.9 ± 0.1
colchicine				
wild type	4.5	2.3	1700 ± 400	0.7 ± 0.1
G185V	15.8	3.6	2400 ± 200	2.0 ± 0.4
I186N	8.6	1.8	5700 ± 1200	1.8 ± 0.2
G185V/I186N	14.6	1.9	4800 ± 500	3.7 ± 1.1
etoposide				
wild type	4.3	1.7	220 ± 10	2.1 ± 0.2
G185V	9.3	3.8	480 ± 30	2.9 ± 0.6
I186N	9.3	2.5	600 ± 70	3.3 ± 0.2
G185V/I186N	13.1	2.4	730 ± 20	4.2 ± 0.3

[a] Relative resistance is the ratio of the LD_{50} of NIH 3T3 cells transduced with the corresponding form of MDR1 to the LD_{50} of cells transduced with the control vector LXSN. Fold increase is the ratio of UIC2 immunoreactivity (mean fluorescence channel) for cells analyzed at the saturating concentrations of different drugs (analyzed in parallel) relative to cells analyzed in the absence of drugs. The K_m and Hill number were determined from the data in Figure 3 using the ligand binding equation (see Experimental Procedures). The data are from one of several experiments that yielded similar results.

DISCUSSION

The I186N Mutation Increases the Pgp Activity. Most of the functional mutations in enzymes eliminate or inhibit their function. Identification of such mutations helps to identify key residues and delineate functional domains of the protein. A much less common class of mutations, however, is made up of mutations that augment some of the functions of an enzyme. Such altered-function mutants are especially useful for understanding the action of multifunctional proteins. Mutations of this type have been previously identified for the multidrug transporter Pgp, which functions simultaneously as an ATPase, as a transporter for a very broad spectrum of substrates, and as a lipid translocase. Pgp mutations that change its relative transport activity for different substrates have already provided valuable information about the mechanism of Pgp-mediated transport. In the study presented here, we have identified a novel mutation, I186N, which improves the ability of Pgp to confer resistance to different cytotoxic drugs. As shown in many previous studies, including those that analyzed the effects of G185V on the accumulation (*12*) and transport (*15*) of different drugs, changes in Pgp-mediated drug resistance reflect the corresponding changes in the outward drug pumping. We also investigated the effects of I186N, together with a previously identified specificity-altering mutation of the adjacent amino acid, G185V, on substrate-induced conformational transitions of the transporter. This analysis revealed that mutations that affect the ability of Pgp to transport

individual substrates also produce coordinate changes in the ability of such substrates to affect the Pgp conformation.

The mutation conferring elevated resistance to etoposide, which we have identified through random mutagenesis and function-based selection, turned out to alter amino acid residue 186 from the hydrophobic isoleucine to the highly polar arginine. This residue abuts TM3 and is immediately adjacent to the G185V mutation, which was present in the original MDR1 cDNA used for mutagenesis and which was already known to increase etoposide resistance. This coincidence raised the possibility that I186N could act by augmenting the effect of G185V and would only be functional in combination with the latter mutation. Testing the effects of G185V and I186N mutations individually and in combination showed that this is not the case. I186N alone increased etoposide resistance to the same extent as G185V (~2-fold), while combining these two mutations enhanced the ability of Pgp to confer etoposide resistance approximately 3-fold relative to that of the wild-type protein. Another possibility suggested by the proximity of the two mutations was that I186N would affect Pgp function in the same way as G185V. This also was not the case, since the two mutations had different effects on resistance to other drugs and on drug-induced conformational transitions. While G185V increased the resistance to some Pgp-transported drugs, it also decreased the activity of Pgp toward other substrates, whereas I186N increased the Pgp activity toward all five of the tested drugs. To the best of our knowledge, I186N is the first reported Pgp mutation with such a uniformly positive effect on the drug transport functions of Pgp. This Pgp mutant may therefore prove to be useful for chemoprotection-oriented gene therapy applications.

G185V and I186N Mutations Change the Parameters of Drug-Induced Conformational Transitions of Pgp. The mechanistic effects of the G185V and I186N mutations were approached by analyzing the effects of drugs on the conformation of Pgp, as measured by increased UIC2 reactivity in the presence of drugs. We have previously found that wild-type Pgp expressed in NIH 3T3 cells shows a considerable increase in UIC2 reactivity in the presence of vinblastine or colchicine, although colchicine did not affect UIC2 reactivity in some other cell lines (*27*). In the study presented here, we have found that etoposide also increases the UIC2 reactivity in NIH 3T3 cells, although it had no effect on the UIC2 reactivity of the wild-type Pgp in the K562 cell line (*21*). We had also observed that NIH 3T3 was the only cell line where the UIC2 reactivity of the wild-type human Pgp, even in the presence of vinblastine or under the conditions of ATP depletion, did not reach the same level as observed with the conformation-insensitive antibody MRK16 (*27*). This limit of UIC2 reactivity in NIH 3T3 cells was overcome by the mutation G185V, which increased the highest levels of UIC2 reactivity in the presence of all three tested transport substrates and oligomycin to the levels obtained with MRK16. Unlike the G185V mutation, the I186N mutation allowed the maximal level of UIC2 reactivity in the presence of vinblastine, but not in the presence of etoposide, colchicine, or oligomycin. The G185V/I186N double mutant was essentially indistinguishable in this respect from I186N alone, indicating that the restrictions on the UIC2 reactivity shift imposed by I186N are dominant over the "releasing" effect of G185V. While the structural basis for the effects of the mutations on the magnitude of drug-induced increase in UIC2 reactivity remains unknown, these effects do not correlate with the changes in drug resistance conferred by the corresponding mutations.

Meaningful correlations with drug resistance were observed, however, for the effects of G185V and I186N mutations on the K_m and Hill number values for this drug-induced increase in UIC2 reactivity. Although the UIC2 reactivity shift is an indirect assay for Pgp−drug interactions, the K_m values determined by the UIC2 reactivity shift assay are similar (for at least some of the substrates) to the values found in other Pgp functional assays (*26*), and may be considered, for the purpose of this discussion, as at least a relative measure of the underlying substrate affinity. This assumption is confirmed by a comparison between the effects of G185V on the K_m values for UIC2 reactivity changes and the previously reported effects of this mutation on drug binding to Pgp. In the study presented here, we observed that G185V increases the K_m for colchicine (i.e., decreases its apparent affinity) and decreases the K_m for vinblastine (i.e., increases the apparent affinity of this drug). These findings are in complete agreement with the earlier work (*12*), where we found that G185V decreases the level of binding of a photoactive colchicine analogue and increases the level of binding of a vinblastine analogue to Pgp. An increased overall level of binding of vinblastine can be due, in mechanistic terms, either to its increased level of binding at the "on" site of Pgp or to a decreased level of debinding from the "off" site of Pgp to the outside of the cell. Since changes in drug binding conferred by G185V were inversely associated with changes in the transport of the corresponding drug, we had suggested that it was debinding at the off site that was affected by this mutation (*12*). The concept of the on and off sites of Pgp was more recently confirmed by independent methods for Pgp (*28*) and for a bacterial protein LmrA, closely related to Pgp (*29*). Despite the results with G185V, we cannot tell a priori whether a higher K_m found in other cases should always point to a higher rate of debinding of the substrate at the off site, or whether it may also reflect drug binding at the on site.

Another phenomenological descriptor of the drug-induced increase in UIC2 reactivity, the Hill number, also shows a good agreement with physical parameters of Pgp−ligand interactions. The Hill numbers for changes in UIC2 reactivity of the wild-type Pgp, induced by ATP, vinblastine, or colchicine (*26*, *27*), correspond to the number of binding sites for the corresponding ligands (2, 2, and 1, respectively), as determined by independent assays (*2*, *6*, *25*). Mutation-induced changes in the Hill number are likely therefore to represent actual changes in the number of available substrate-binding sites (*26*, *27*). How such a change would affect the efficacy of drug transport is not obvious a priori. An increase in the number of drug-binding sites could potentially increase the stoichiometry of pumping so that more drug molecules may be pumped out per each round of ATP hydrolysis, increasing the overall level of transport. Alternatively, a higher number of drug-binding sites could conceivably slow the progress of the drug through Pgp on its way out of the cell, resulting in a decreased level of transport.

Changes in the Transport of Individual Drugs Correlate with the Effects of the Mutations on the Apparent Pgp Affinity and the Apparent Number of Drug Binding Sites. In light of

the considerations described above, we will first consider the effects of the mutations on the transport of vinblastine. G185V, which decreases vinblastine resistance, also decreases the K_m value in the UIC2 shift assay. As discussed above, this observation, together with the previous results (*12*), suggests that G185V decreases the level of vinblastine debinding at the off site. Strikingly, G185V also causes an apparent loss of one vinblastine-binding site, as it decreases the Hill number for vinblastine from 2 to 1. The loss of the binding site could conceivably provide a mechanism for the decreased level of transport of vinblastine by G185V. In contrast to G185V, the I186N mutation increases the vinblastine resistance, and this effect is associated with an increase in the K_m value. On the basis of this observation, we hypothesize that the I186N mutation increases the level of debinding of vinblastine. The increased K_m value of I186N, however, is not associated with a change in the Hill number, which remains at 2, as in the case of the wild type. The Hill number for vinblastine is 1 in the G185V/I186N double mutant, indicating that I186N cannot reverse the loss of a drug-binding site caused by G185V. The double mutant shows an intermediate K_m between those of G185V and I186N, which happens to match the K_m for the wild-type Pgp, and G185V/I186N shows the same level of vinblastine resistance as wild-type Pgp. This analysis suggests that the effects of the mutations on the K_m values for vinblastine are primarily responsible for their effects on the resistance to this drug.

A more complicated situation is observed in the case of colchicine. The G185V mutation, which originally arose in a colchicine-selected cell line and which strongly increases colchicine resistance, produces a moderate increase in the K_m value for colchicine, in agreement with the previous study (*12*). This increase is accompanied by the apparent addition of a colchicine-binding site, as the Hill number increases from 1 to 2. I186N, like G185V, increases resistance to colchicine, and this is accompanied by a higher K_m and an increase in the Hill number from 1 to 2. At the quantitative level, however, I186N provides a stronger increase in the K_m value, but G185V confers a larger increase in resistance. This contradiction raises the question of whether the effect of G185V on colchicine resistance may be due not only to increased debinding but also to some additional factors, or whether an increased colchicine K_m (at least for I186N) may reflect changes in both drug binding and debinding. G185V/I186N produces a higher Hill number than either mutation alone, suggesting an additive effect of the two mutations on the apparent number of drug-binding sites. The effects of the mutations on the K_m value and on drug resistance, however, do not appear to be additive, since the G185V/I186N double mutant has about the same K_m as the I186N mutant but produces the same level of colchicine resistance as the G185V mutant.

In contrast to the complicated colchicine situation, etoposide, the drug that was originally used to select the G185V/I186N double mutant, provides the most straightforward correlation between drug resistance, on one hand, and the K_m and Hill number changes, on the other hand. Both G185V and I186N increase the apparent number of etoposide-binding sites from 2 to 3, while combining these mutations brings this number up to 4. Similarly, the K_m values are increased by either G185V or I186N, and the double mutant exhibits the highest K_m value, i.e., the lowest apparent affinity. The changes in both of these parameters agree with the effects of the mutations on etoposide resistance, which is higher in the G185V or I186N mutant than in the wild type and becomes the highest in the double mutant.

In summary, the observed effects of the G185V and I186N mutations on the transport of individual drugs can be interpreted through the effects of these mutations on the ability of the drugs to alter Pgp conformation, as reflected by the process of the change in UIC2 reactivity. In every case where these mutations increased or decreased drug resistance, this effect was accompanied by a corresponding increase or a decrease in the K_m value, and in some cases in both the K_m and Hill number. In the case of vinblastine and etoposide, the effects of both single and double mutants on drug resistance were positively correlated with the effects of these mutations on the K_m parameter. As we have previously suggested (*12*), this correlation may indicate that mutations can change how easily the drugs are released from Pgp to the outside of the cell. These correlations are less apparent for colchicine, since I186N increases the K_m value out of proportion to the increase in colchicine resistance, and combining the two mutations does not have an additive effect on the K_m for colchicine. We have also found that the changes in the K_m and drug resistance were associated in some cases with the corresponding changes in the Hill number, the apparent number of drug-binding sites on Pgp. This novel finding suggests that an increase in the number of drug-binding sites provides one of the mechanisms for increasing the level of drug transport by Pgp. The structural basis for the drastic effects of the mutations of amino acids 185 and 186 on the apparent affinity and the apparent number of drug-binding sites in Pgp remains the subject for future studies.

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