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Molecular basis of preferential resistance to colchicine in multidrug-resistant human cells conferred by Gly-185 → Val-185 substitution in P-glycoprotein

(*MDR1* gene/membrane transport/photoaffinity labeling/cancer chemotherapy/vinblastine)

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ABSTRACT **Expression of P-glycoprotein, encoded by the human *MDR1* gene, results in cross-resistance to many lipophilic cytotoxic drugs (multidrug resistance). P-glycoprotein is believed to function as an energy-dependent efflux pump that is responsible for decreased drug accumulation in multidrug-resistant cells. Previous work showed that preferential resistance to colchicine in a colchicine-selected multidrug-resistant cell line was caused by spontaneous mutations in the *MDR1* gene that resulted in a Gly-185 → Val-185 substitution in P-glycoprotein. We have now compared transfectant cell lines expressing either the wild-type Gly-185 or the mutant Val-185 P-glycoprotein with regard to their levels of resistance to and accumulation and binding of different drugs. In cells expressing the mutant protein, increased resistance to colchicine and decreased resistance to vinblastine correlated with a decreased accumulation of colchicine and increased accumulation of vinblastine. Expression of the mutant P-glycoprotein also resulted in significantly increased resistance to epipodophyllotoxin and decreased resistance to vincristine and actinomycin D; smaller changes in resistance were observed for several other drugs. Unexpectedly, the mutant P-glycoprotein showed increased binding of photoactive analogs of vinblastine and verapamil and the photoactive compound azidopine and decreased binding of a photoactive colchicine analog. These results suggest that the Gly-185 → Val-185 substitution affects not the initial drug-binding site of P-glycoprotein but another site, associated with the release of P-glycoprotein-bound drugs to the outside of the cell.**

The product of the human *MDR1* gene, P-glycoprotein, has been implicated in an important type of tumor cell resistance to chemotherapeutic drugs known as multidrug resistance (1–3). Cells that express P-glycoprotein show decreased accumulation of and increased resistance to many structurally dissimilar lipophilic compounds that act at different intracellular targets. This group of compounds includes some of the most commonly used antitumor agents, such as *Vinca* alkaloids, anthracyclines, and epipodophyllotoxins, as well as colchicine, actinomycin D, taxol, and some other drugs. P-glycoprotein consists of 1280 amino acids organized into two similar halves, each of which includes a long hydrophobic region with six predicted transmembrane segments and a nucleotide-binding region, which apparently functions as an ATPase (4–7). P-glycoprotein appears to act as an ATP-dependent membrane efflux pump, responsible for decreased drug accumulation in multidrug-resistant cells, as suggested by the observations of increased energy-dependent drug transport in multidrug-resistant cells (8) and membrane vesicles from these cells (9). Biochemical studies showed that P-glycoprotein can bind photoactive drug analogs (10–16) and hydrolyze ATP (7). A number of agents capable of reversing multidrug resistance have been found to serve as P-glycoprotein substrates and inhibitors of P-glycoprotein-mediated drug efflux (10–15). Since P-glycoprotein is frequently expressed in clinically resistant human tumors (17, 18), there is considerable interest in developing new and more efficient P-glycoprotein inhibitors for clinical applications. Understanding the structural determinants of P-glycoprotein–drug interactions may provide the basis for rational design of such compounds.

Biochemical studies have not yet identified any specific amino acid residues involved in P-glycoprotein–drug interactions. A genetic approach to the identification of such residues is suggested by the analysis of P-glycoprotein mutations that alter the pattern of cross-resistance to different drugs. Although multidrug-resistant human cell lines selected with different drugs overexpress the same *MDR1* gene and become cross-resistant to the same group of drugs, some of these cell lines show a preferential increase in resistance to the drug that was used in their selection. In a colchicine-selected series of multidrug-resistant KB carcinoma cells, preferential resistance to colchicine was found to be a result of mutations in the *MDR1* gene that led to a single amino acid substitution in P-glycoprotein, Gly-185 → Val-185 (19). Cells that express P-glycoprotein with a mutant valine residue at position 185 showed a strong increase in their resistance to colchicine and a significant decrease in their resistance to vinblastine, compared to cells that express P-glycoprotein with the wild-type glycine residue at the same position (19). These results imply a role for residue 185, located within the first hydrophobic region of the protein (4), in determining the specificity of P-glycoprotein–drug interactions. It is unknown, however, whether this specificity is conferred at the initial drug binding to P-glycoprotein or in the course of P-glycoprotein-mediated translocation of the drug to the outside of the cell.

In the present study, we have used transfectant cell lines that express P-glycoprotein with either glycine or valine at position 185 to further characterize the influence of this substitution on cross-resistance to different drugs, drug accumulation, and binding of photoactive drug analogs. Our results suggest that the Gly-185 → Val-185 substitution affects not the initial drug binding site of P-glycoprotein but another site, associated with the release of P-glycoprotein-bound drugs to the outside of the cell.

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Abbreviations: ^{125}I-NASV, *N*-(*p*-azido-3-[^{125}I]iodosalicyl)-*N'*-β-aminoethylvindesine; ^{125}I-NASC, *N*-(*p*-azido-3-[^{125}I]iodosalicyl)-aminohexanoyldeacetylcolchicine; ^{125}I-NASVP, *N*-(*p*-azido-3-[^{125}I]-iodosalicyl)aminomethylverapamil.

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MATERIALS AND METHODS

Materials. Actinomycin D, colchicine, doxorubicin, puromycin, verapamil, whole mouse IgG, and fluorescein isothiocyanate-conjugated goat anti-mouse IgG were obtained from Sigma. Vinblastine and vincristine were gifts from Eli Lilly. Taxol and etoposide were obtained from the Natural Products Branch, National Cancer Institute. $Na^{125}I$ (2200 Ci/mmol, 1 Ci = 37 Gbq), [^{3}H]azidopine (44 Ci/mmol), [^{3}H]colchicine (5.4 Ci/mmol), and [^{3}H]vinblastine (12.4 Ci/mmol) were purchased from Amersham. The photoaffinity drug analogs *N*-(*p*-azido-3-[^{125}I]iodosalicyl)-*N'*-β-aminoethylvindesine (^{125}I-NASV), *N*-(*p*-azido-3-[^{125}I]iodosalicyl)aminohexanoyldeacetylcolchicine (^{125}I-NASC), and *N*-(*p*-azido-3-[^{125}I]iodosalicyl)aminomethylverapamil (^{125}I-NASVP) were synthesized as described (13, 16, 20). Anti-P-glycoprotein monoclonal antibody C219 (21) was from Centocor (Malvern, PA). Monoclonal antibody MRK16 (22) was generously provided by Takashi Tsuruo (Japanese Foundation for Cancer Research, Tokyo).

Cell Culture Conditions and Drug Cytotoxicity Assays. Generation of multidrug-resistant derivatives of the KB-3-1 human epidermoid carcinoma cell line by transfection with expression vectors containing *MDR1* cDNA that encodes either the wild-type glycine or the mutant valine residue at position 185 has been described (19). Both the cell lines used in the present study, KB-GSV2 (Gly-185) and KB-VSV1 (Val-185), express P-glycoprotein with an Arg-933 → Ser-933 substitution relative to the wild-type sequence. This substitution was found to have no detectable effect on drug resistance and accumulation (ref. 19 and unpublished data). Cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. Drug sensitivity was determined by colony-formation assays (19).

P-Glycoprotein Assays. Immunoblotting of P-glycoprotein with ^{125}I-labeled monoclonal antibody C219 (21) (specific activity, 1.6×10^5 cpm/ml) was carried out essentially as described (23). For immunofluorescence measurements, 1–2 $\times 10^6$ cells, suspended by treatment with 20 mM EDTA (pH 7.4), were incubated in 100 μl with the mouse monoclonal antibody MRK16 (22) at 50 μg/ml for 1 hr on ice. Cells were then washed and incubated for 1 hr on ice in the dark with 100 μl of fluorescein isothiocyanate-conjugated goat anti-mouse IgG (final concentration, 20 μg/ml). Cells were washed, resuspended in 500 μl of medium, and analyzed on an Epics V fluorescence-activated cell sorter (Coulter). Propidium iodide (133 μg/ml) was added to gate out the dead cells. All the cell lines used in this study were similar in cell size and granularity. The mean fluorescence intensity, after subtraction of background determined by using whole mouse IgG as a primary antibody, was used as a measure of P-glycoprotein expression on the cell surface.

Drug Accumulation Studies. For [^{3}H]vinblastine and [^{3}H]colchicine accumulation studies, cells were grown in 24-well plates to a density of 2×10^4 cells per well. Monolayer cells were then treated with either 45.8 nM [^{3}H]vinblastine (1.25 Ci/mmol) or 79.8 nM [^{3}H]colchicine (5.4 Ci/mmol) in the growth medium at 37°C. At various time points, cells were washed rapidly with cold phosphate-buffered saline and trypsinized, and radioactivity associated with cells was determined.

Photoaffinity Labeling. Exponentially growing cells were harvested with a cell scraper. Cell viability was >90% as determined by trypan blue staining. Suspensions of 5×10^5 cells per assay in Ca^{2+}- and Mg^{2+}-free Dulbecco's phosphate-buffered saline containing 4% dimethyl sulfoxide and 22.7 nM ^{125}I-NASV, ^{125}I-NASC, or ^{125}I-NASVP (220 Ci/mmol) or 0.5 μM [^{3}H]azidopine (44 Ci/mmol) in a final volume of 50 μl were photolabeled after preincubation for 30 min at 25°C in the absence or presence of competing drugs as described (12, 13, 16, 20). After photolabeling, equal amounts of total cellular protein were electrophoresed in $NaDodSO_4$/5–15% polyacrylamide gels containing 4.5 M urea (13). After autoradiography, the amount of radioactivity corresponding to the 170-kDa P-glycoprotein band was determined using an LKB Ultroscan XL laser densitometer.

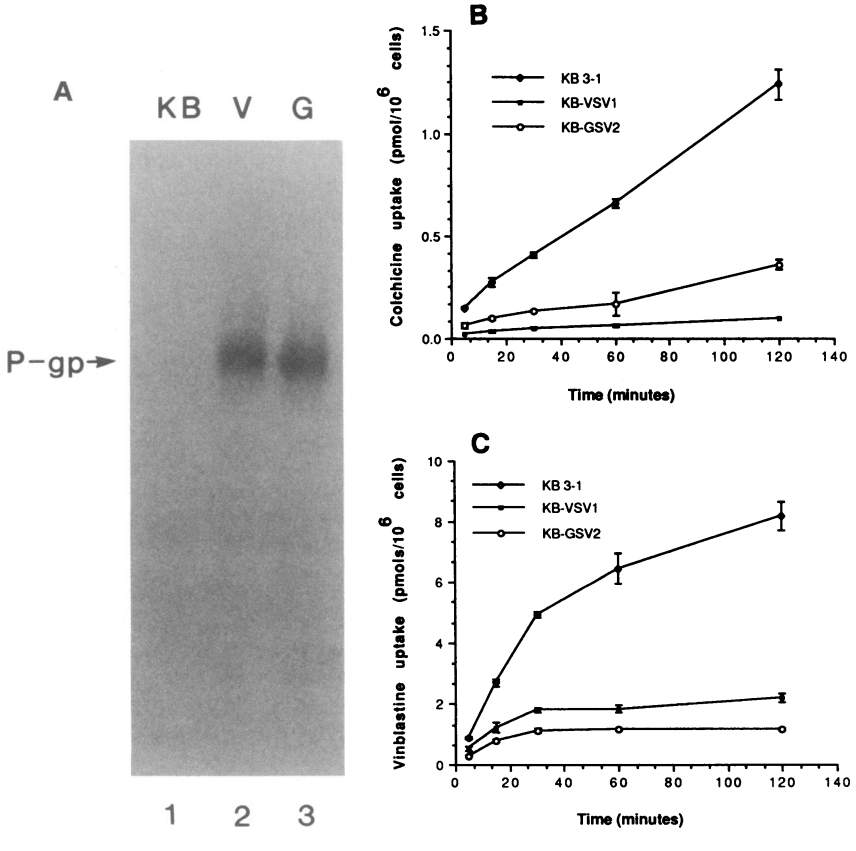

FIG. 1. P-glycoprotein (P-gp) expression and drug accumulation in KB-3-1, KB-VSV1, and KB-GSV2 cell lines. (*A*) Protein extracts from 10^6 cells were fractionated by $NaDodSO_4$/PAGE. The immunoblot was probed with ^{125}I-labeled anti-P-glycoprotein antibody C219. (*B*) [^{3}H]Colchicine accumulation kinetics. (*C*) [^{3}H]-Vinblastine accumulation kinetics.

Table 1. Effect of Gly-185 → Val-185 substitution on drug accumulation and P-glycoprotein binding

Property	KB-VSV1/KB-GSV2
Surface P-glycoprotein (MRK16 immunofluorescence)	1.144 ± 0.16
Vinblastine accumulation	1.872
Colchicine accumulation	0.273
Vinblastine analog binding	3.8
Verapamil analog binding	5.5
Azidopine binding	3.8
Colchicine analog binding	0.28

Except for drug analog binding, each ratio represents the mean of three experiments. See *Materials and Methods* for description of the assays.

RESULTS

To compare the properties of the mutant and wild-type P-glycoproteins, we used multidrug-resistant derivatives of human KB carcinoma cells, KB-GSV2 and KB-VSV1, obtained by transfection with *MDR1*-expressing vectors that encode P-glycoprotein with either the wild-type glycine or the mutant valine residue at position 185, respectively (19). Both cell lines were found to contain a single integrated copy of the transfected *MDR1* cDNA plasmid and to express similar amounts of *MDR1* mRNA, transcribed exclusively from the transfected plasmid (19). The amounts of P-glycoprotein produced in these cell lines were compared by immunoblotting total cellular protein with ^{125}I-labeled anti-P-glycoprotein monoclonal antibody C219, which recognizes a conserved cytoplasmic domain in the C-terminal region of P-glycoprotein (21), and by indirect immunofluorescence measurement of P-glycoprotein on the cell surface by flow cytometry using monoclonal antibody MRK16, which recognizes an extracellular domain of P-glycoprotein (22). The amounts of P-glycoprotein in KB-VSV1 and KB-GSV2 transfectants were very similar by both techniques (Fig. 1*A*; Table 1). No P-glycoprotein was detected in the untransfected recipient KB-3-1 cells by either assay.

Previous work (19) showed that KB-VSV1 cells, expressing the mutant P-glycoprotein, show strongly increased resistance to colchicine, moderately increased resistance to doxorubicin (adriamycin), and significantly decreased resistance to vinblastine, compared to KB-GSV2 cells that express the wild-type P-glycoprotein (19). We have now tested these cell lines for resistance to several other drugs. As summarized in Table 2, KB-VSV1 cells show a significant increase in their resistance to VP16 (epipodophyllotoxin) and a marginal increase in resistance to puromycin, relative to KB-GSV2 cells. KB-VSV1 cells also show a significant decrease in resistance to vincristine, actinomycin D, and taxol. We also tested the ability of verapamil to reverse the resistance to vinblastine in these transfectants; in both cell lines vinblastine resistance was decreased ≈4-fold in the presence of 1 μM verapamil (data not shown).

Table 2. Effect of the Gly-185 → Val-185 substitution on resistance to different drugs

Drug	Relative resistance* KB-GSV2	Relative resistance* KB-VSV1	KB-VSV1/KB-GSV2
Colchicine	7.3†	28.0†	3.8
Etoposide	2.9	8.8	3.1
Doxorubicin	6.1†	12.3†	2.0
Puromycin	5.8	7.4	1.3
Taxol	80.0	37.0	0.5
Vinblastine	62.5†	24.3†	0.4
Actinomycin D	9.6	2.5	0.3
Vincristine	108.0	25.7	0.2

*Relative resistance is expressed as the ratio of LD_{50} (drug concentration that inhibits plating efficiency by 50%) for the given cell line relative to the KB-3-1 recipient cells. The LD_{50} values for KB-3-1 cells were as follows: colchicine, 2.7 nM; etoposide, 200 nM; doxorubicin, 3.1 nM; puromycin, 210 nM; taxol, 1.0 nM; vinblastine, 0.28 nM; actinomycin D, 0.29 nM; vincristine, 0.6 nM.
†From ref. 19.

To determine whether the changes in drug resistance associated with the mutation were due to altered drug accumulation in KB-VSV1 cells, we compared the uptake of [^{3}H]vinblastine and [^{3}H]colchicine in the untransfected KB-3-1 recipient cells and in the two transfected cell lines (Fig. 1 *B* and *C*; Table 1). KB-3-1 cells accumulated 8.712 ± 0.47 pmol of [^{3}H]vinblastine and 1.244 ± 0.075 pmol of [^{3}H]-colchicine per 10^6 cells. The rate of net uptake and the steady-state accumulation of both drugs were significantly higher in the recipient cell line than in either transfectant. KB-VSV1 cells, however, accumulated 3.6 times less [^{3}H]colchicine than KB-GSV2 cells (0.099 ± 0.001 pmol and 0.362 ± 0.026 pmol per 10^6 cells, respectively). At the same time, KB-VSV1 cells accumulated 1.9 times more [^{3}H]-vinblastine than KB-GSV2 cells (2.215 ± 0.13 pmol and 1.183 ± 0.05 pmol per 10^6 cells, respectively). These changes in drug accumulation parallel the changes in drug sensitivity in KB-VSV1 cell line relative to KB-GSV2.

To determine whether the Gly-185 → Val-185 substitution influences binding of drugs to P-glycoprotein, we carried out a series of photoaffinity labeling experiments using photoactive analogs of vinblastine (^{125}I-NASV) (20), colchicine (^{125}I-NASC) (16), and verapamil (^{125}I-NASVP) (13) and the photoactive compound [^{3}H]azidopine (12). Verapamil and azidopine are known to be efficient inhibitors of P-glycoprotein-mediated resistance and of drug binding to P-glycoprotein (10–13, 24). After incubation of intact cells with the photoactive drug analogs and UV crosslinking, total protein extracts were subjected to NaDodSO$_4$/PAGE. Autoradiography (Figs. 2 and 3) showed that each of the drug analogs bound to the 170-kDa P-glycoprotein band, present in both transfectant cell lines but not in the recipient KB-3-1 cells. The mutant P-glycoprotein, however, bound 3.8–5.5 times more of the vinblastine, verapamil, and azidopine analogs but

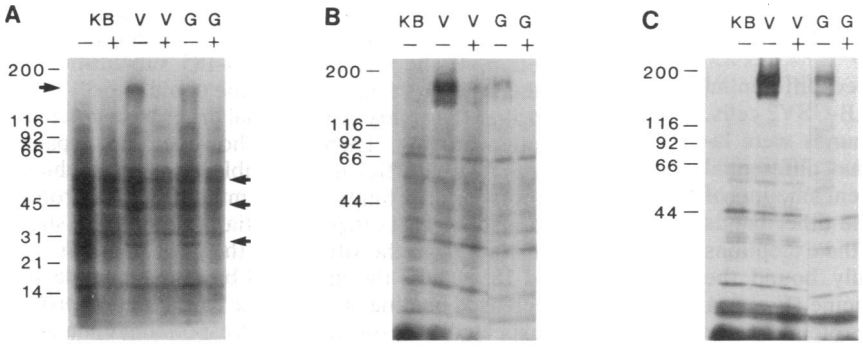

FIG. 2. Photoaffinity labeling of cellular proteins with vinblastine analog ^{125}I-NASV (*A*), verapamil analog ^{125}I-NASVP (*B*), and [^{3}H]azidopine (*C*). Cell lines KB-3-1 (KB), KB-VSV1 (V), and KB-GSV2 (G) were labeled in the absence (−) or presence (+) of 100 μM unlabeled vinblastine, verapamil, or azidopine. Proteins were separated by NaDodSO$_4$/PAGE and autoradiographed. Arrows (top to bottom) indicate the positions of P-glycoprotein, tubulin, and the 44- and 28-kDa proteins. Positions of molecular mass standards (kDa) are indicated at left.

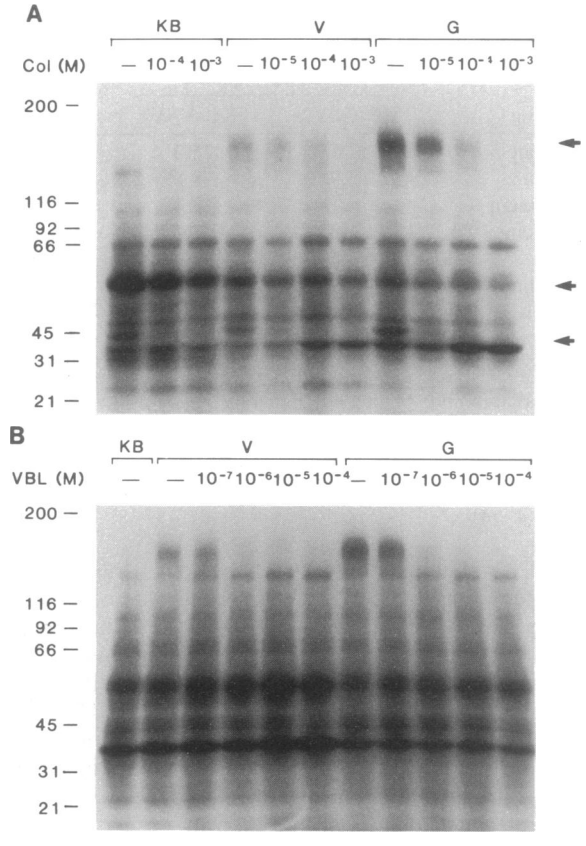

FIG. 3. Photoaffinity labeling of cellular proteins with colchicine analog ^{125}I-NASC. KB-3-1 (KB), KB-VSV1 (V), and KB-GSV2 (G) cells were labeled in the absence (−) or presence of 10^{-5}–10^{-3} M colchicine (Col) (*A*) or 10^{-7}–10^{-4} M vinblastine (VBL) (*B*). Arrows (top to bottom) indicate the positions of P-glycoprotein, tubulin, and the 45-kDa protein. Positions of molecular mass standards (kDa) are indicated at left.

3.6 times less of the photoactive colchicine analog than did the wild-type P-glycoprotein (Table 1).

The binding of photoactive drug analogs to P-glycoprotein was specific, as judged from its inhibition by the analogous nonradioactive drugs (100 μM) in both cell lines. Inhibition was >80% for the photoactive analogs of vinblastine (Fig. 2*A*) and verapamil (Fig. 2*B*) and for azidopine (Fig. 2*C*). Binding of the photoactive colchicine analog was further characterized by incubating KB-VSV1 and KB-GSV2 cells with increasing concentrations of colchicine (Fig. 3*A*) or vinblastine (Fig. 3*B*) for 15 min prior to photoaffinity labeling. At 100 μM colchicine, the amount of P-glycoprotein-bound compound decreased by about 50%, and at 1000 μM its binding was almost completely inhibited in both transfectants (Fig. 3*A*). Further, the addition of as little as 1 μM vinblastine totally inhibited the binding of the photoactive colchicine analog in both transfectants (Fig. 3*B*). In contrast, colchicine at 100 μM did not affect photolabeling of P-glycoprotein with the photoactive analog of vinblastine (data not shown), in agreement with previous studies (10, 11).

While the photoactive drug analogs showed differential labeling of P-glycoprotein in KB-VSV1 and KB-GSV2 cells, other protein bands that bound these compounds were labeled equally in both cell lines, indicating that differential binding of P-glycoprotein was not due to different amounts of drugs accumulated by these cells. Most of the other bands bound the drug analogs nonspecifically, with the exceptions of tubulin, a 44-kDa protein that specifically bound the photoactive analogs of vinblastine and colchicine (Fig. 3 *A* and *B*), and a 28-kDa vinblastine-binding protein. These polypeptides were labeled to the same extent in the recipient and transfected cell lines. The binding of the vinblastine analog to tubulin and to the 44-kDa protein in P388 murine leukemia cells was reported previously (25).

DISCUSSION

We have characterized the effects of the Gly-185 → Val-185 substitution, found in P-glycoprotein from colchicine-selected human KB cells (19), on cellular resistance to different drugs, intracellular drug accumulation, and binding of photoactive drug analogs to P-glycoprotein. We have shown that this amino acid substitution alters the specificity of P-glycoprotein interactions with different drugs by all of the above criteria. As suggested by Brandt-Rauf *et al.* (26), the wild-type P-glycoprotein adopts a unique left-handed conformation at position 185 that is energetically unfavorable for the protein with L amino acids (including valine) at this position. The essential question is whether this inferred conformational change alters the specificity of the initial drug binding to P-glycoprotein or a subsequent step of the translocation of the drug to the outside of the cell. We will discuss this question on the basis of the observed changes in P-glycoprotein interactions with the analogs of colchicine and vinblastine. The analogs of verapamil and azidopine behave similarly to vinblastine in P-glycoprotein binding assays; this may be a consequence of conformational similarities between these compounds (27).

The changes in drug resistance and accumulation in cell lines transfected with the wild-type or mutant *MDR1* genes indicate that the Gly-185 → Val-185 substitution increases the efficiency of P-glycoprotein in the efflux of colchicine and decreases its efficiency in the efflux of vinblastine. If this change were due to increased affinity of the initial drug-binding site of P-glycoprotein for colchicine and its decreased affinity for vinblastine, one could expect that the mutant P-glycoprotein would show increased photolabeling with a colchicine analog and decreased photolabeling with a vinblastine analog. The observed effect is just the opposite: the mutant protein binds much less colchicine and much more vinblastine than the wild-type P-glycoprotein. This result suggests that the observed changes are primarily determined not by the initial association of P-glycoprotein with the drugs, but rather by the subsequent disassociation of drugs from P-glycoprotein, necessary for their release to the outside of the cell. The changes in drug binding, mediated by the Gly-185 → Val-185 substitution, are in contrast to the results of all previous studies on photoaffinity labeling of wild-type P-glycoprotein molecules, where increased binding of photoactive drug analogs reflected increased amounts of P-glycoprotein and therefore directly correlated with the cellular levels of drug resistance.

Another argument against a change in the specificity of the initial drug binding by the mutant protein comes from the competition studies. It was shown previously that vinblastine is much more efficient than colchicine in competing for P-glycoprotein binding by photoactive analogs of vinblastine, colchicine, and doxorubicin (10, 11, 16, 28), suggesting that these drugs bind with different affinity to a common site in P-glycoprotein. If the Gly-185 → Val-185 substitution resulted in altered initial drug binding, one could expect that it would also alter the ability of individual drugs to compete for P-glycoprotein binding. There was, however, no apparent change in the relative efficiency of vinblastine and colchicine as competitors for drug binding to the mutant P-glycoprotein. This result also suggests that the initial drug binding site is spatially distinct from the site where the drug interacts with amino acid 185. Thus, the increased binding of vinblastine and the decreased binding of colchicine to the mutant protein could be expected to result in the corresponding changes in

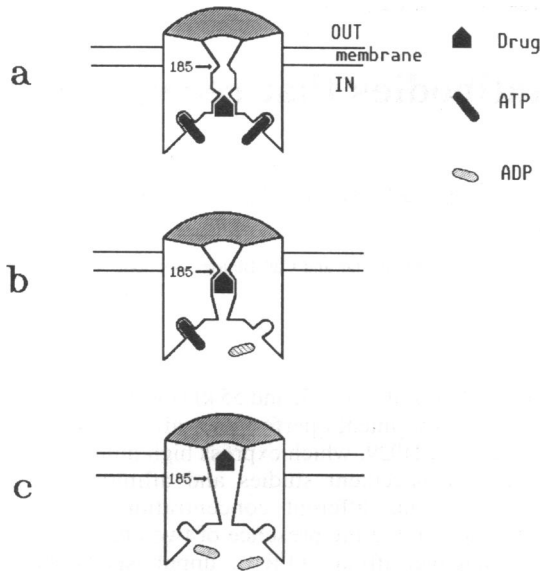

FIG. 4. Model of P-glycoprotein-mediated drug transport [modified from ref. 2 with permission (copyright American Society for Biochemistry and Molecular Biology)]. See text for details.

their ability to compete for each other's binding to P-glycoprotein, provided that the bound substrate accumulated at a site where it could interfere with the initial binding of drugs to P-glycoprotein. Since no such effect was observed, it appears that the critical site in the P-glycoprotein-formed channel, defined by amino acid 185, does not overlap with the initial drug-binding site.

The presence of two critical points in P-glycoprotein-mediated translocation of drugs across the cell membrane is independently suggested by studies on site-directed mutagenesis of the nucleotide-binding sites, which indicated that the function of each of the two sites is required for normal drug efflux by P-glycoprotein (ref. 29; B. Morse and I.B.R., unpublished data). This requirement can be most easily explained by the presence of two "bottlenecks" in the P-glycoprotein-formed channel, each of which can be traversed by the drug with the help of conformational changes in the protein that require the function of a specific ATPase domain. Based on the results of the present study, we speculate that the first "bottleneck" may be associated with the initial drug-binding site, and the second may correspond to the part of the channel that includes amino acid 185 (Fig. 4).

The results of the present study demonstrate that the Gly-185 → Val-185 substitution in human P-glycoprotein directly alters the specificity of P-glycoprotein–drug interactions. Our results suggest that amino acid 185 is located within a critical region, distinct from the initial drug-binding site, where P-glycoprotein-transported drugs accumulate prior to their release to the outside of the cell. Stereochemical modeling and mutational analysis of this region may provide a useful approach to rational design of specific and efficient inhibitors of P-glycoprotein-mediated drug transport.

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