Characterization of the Azidopine and Vinblastine Binding Site of P-glycoprotein*

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To determine the number of drug binding sites that exist on the multidrug transporter, P-glycoprotein, we used azidopine, a dihydropyridine photoaffinity compound that reverses multidrug resistance and labels P-
glycoprotein. Azidopine labels P-glycoprotein in two distinct locations: one labeled site is within the amino half of P-glycoprotein between amino acid residues 198 and 440, and the other site is within the carboxy half of the protein. Vinblastine is a cytotoxic drug that is used in cancer chemotherapy and is a substrate for transport by P-glycoprotein. We found that vinblastine inhibits azidopine labeling to approximately the same extent as each labeled site on P-glycoprotein. Because several studies have shown that amino acid residue 185 of P-glycoprotein plays a critical role in some aspects of drug binding and transport, we also studied the effect that amino acid residue 185 has on azidopine labeling. These studies show that azidopine labels both sites equivalently in both wild-type (G185) and mutant (V185) P-glycoproteins. We conclude from our results that the two halves of P-glycoprotein approach each other to form a single binding site for these drugs.

P-glycoprotein is a 170-kDa plasma membrane protein that is responsible for the phenomenon of multidrug resistance in mammalian cells (reviewed in Refs. 1-3). The human protein, encoded by the MDRI gene, comprises 1280 amino acid residues and 30 kDa of carbohydrate (4). Analysis of the amino acid sequence suggests that the protein has 12 transmembrane domains and two nucleotide binding sites (4-6). Each half of the protein contains six transmembrane domains and one nucleotide binding site, and the two halves share regions of extensive sequence homology, especially in the nucleotide binding regions. P-glycoprotein shares both sequence and structural homology with a large family of proteins (4-15). All of the proteins in this family contain nucleotide binding sites and almost all are involved with the transport of molecules or ions across membranes.

Cells that express P-glycoprotein are protected from a large group of cytotoxic drugs that are used in cancer chemother-

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apy. This group includes Vinca alkaloids, epipodophyllotoxins, colchicine, anthracyclines, actinomycin D, and others, most of which are hydrophobic compounds with a positive charge at physiological pH (16, 17). These drugs enter cells by passive diffusion through the plasma membrane. P-glycoprotein presumably hydrolyzes ATP and pumps the drugs back out of the cells. Recent genetic and biochemical evidence supports this model for the action of P-glycoprotein (18-28). Several agents that reverse multidrug resistance are also known. These include verapamil, quinidine, reserpine, the dihydropyridine calcium channel blockers, and others (29-33). These reversing agents probably interact directly with P-glycoprotein at the same sites as those that are involved in drug transport and thereby inhibit active transport of other cytotoxic drugs (25-27, 31, 32). Not surprisingly, the search for clinically useful agents that reverse multidrug resistance is an active area of research.

Considerable effort has been devoted to the identification of drug binding sites on P-glycoprotein. Several groups have labeled P-glycoprotein with photoaffinity drug analogues, digested the labeled P-glycoprotein into small fragments, and then attempted to identify the labeled fragments (34-39). Azidopine, a dihydropyridine photoaffinity calcium channel blocker, labels P-glycoprotein in two separate locations (35, 36). One labeled site is in the amino half of the protein, and the other labeled site is in the carboxy half of the protein. These results are consistent with two alternative hypotheses (35). (1) There are two binding sites on P-glycoprotein, one on each half of the protein. The photoaffinity drug analogue binds to each site independently and labels each site. (2) There is only one binding site on P-glycoprotein, and this site is formed by the two homologous halves of the protein. After the photoaffinity analogue binds to this site, it labels both halves of the protein. Unfortunately, these experiments have not identified the labeled sites with any degree of precision and there is no evidence that the labeled sites are coincident with the binding site.

A spontaneous mutant of P-glycoprotein may provide more precise information (40). P-glycoprotein from human KB cells selected for multidrug resistance in high concentrations of vinblastine or adriamycin retains a wild-type glycine at position 185. But P-glycoprotein from cells selected for multidrug resistance in high concentrations of vinblastine or adriamycin contains a mutant valine residue at position 185, and P-glycoprotein from these cells labels the wild-type glycine site in the wild-type protein. These two cell lines show different patterns of resistance to vinblastine and colchicine, and these patterns are retained when the mutant and wild-type cDNAs are transfected into a drug-sensitive cell line. Different patterns of drug binding and transport have also been distinguished (40, 41). These results demonstrate that amino acid residue 185 is an important residue for drug binding or transport, but they do not help to distinguish between the binding site hypotheses described above.
The objective of our work was to determine whether there is one drug binding site on P-glycoprotein or two. To do this, we compared azidopine labeling in each half of both the wild-type and the mutant P-glycoproteins described above. We also studied the ability of other drugs, particularly vinblastine, to inhibit azidopine labeling of the wild-type and mutant P-glycoproteins. Our results strongly support the one binding site hypothesis. Consequently, we believe that there is only one drug binding site on P-glycoprotein for azidopine, and that this site is formed by the two homologous halves of P-glycoprotein.

MATERIALS AND METHODS

Cell Lines—The human multidrug resistant KB cell lines have been described (16, 17). KB-C1 cells were selected with colchicine and contain a mutant valine residue at position 185 (39). KB-V1 cells were selected with vinblastine and contain a wild-type glycine residue at position 185 (40). NIH 3T3 cells were transfected with human MDR1 DNA that encoded either a valine or a glycine at position 185. NIH MDR1-V185 cells express a P-glycoprotein that contains a mutant valine at position 185 (4, 40, 41). NIH MDR1-G185 cells express a P-glycoprotein that contains a wild-type glycine residue at position 185. The cDNA used to make the NIH MDR1-G185 cell line was kindly provided by Dr. Kazumitsu Ueda (42).

Immunoprecipitation—Procedures for azidopine labeling and immunoprecipitation of P-glycoprotein have been described previously (35). Results were quantified by the following method. The antigen was eluted from the Protein A-Sepharose with 400 μl of elution buffer, and 80–200 μl was removed, added to 5 ml of scintillation mixture, and counted. The remainder was concentrated by precipitation with acetone (35). The anti-P-glycoprotein antisera have been described before (43–45) and will only be summarized here. Antiserum 4007, antibody C219 (data not shown), which should recognize the 25-kDa CNBr fragment from the carboxy terminus of P-glycoprotein. This negative result suggested that the labeled 25-kDa fragment is from the amino half of P-glycoprotein (see Fig. 1B). Immunoprecipitations with antisera 4328, 4331, 4402, and 4408, which confirm this result, are shown in Fig. 3A. Precipitation with 4328 and 4402 demonstrates that the 25-kDa fragment is from the amino half, as indicated in Fig. 1D. Negative results with antisera 4331 and 4408 eliminate the possibility that fragments from homologous regions of the carboxy half of P-glycoprotein are cross-reacting with antisera 4328 and 4402. Preimmune sera from these rabbits do not immunoprecipitate P-glycoprotein or any of its fragments.

RESULTS

If sufficient vinblastine is present during the azidopine labeling reaction, it prevents azidopine from labeling P-glycoprotein in KB-C1 cells (35). An example of this is shown in Fig. 2. Presumably vinblastine binds to the same site as azidopine and prevents azidopine from labeling P-glycoprotein by competitive inhibition. We wanted to know whether vinblastine inhibited azidopine labeling in each half of P-glycoprotein equally or not. Because at high concentrations vinblastine completely inhibits azidopine labeling in each half of P-glycoprotein, we knew that both labeling sites are susceptible to vinblastine competition.

To obtain quantitative results, we cleaved P-glycoprotein with CNBr. When azidopine-labeled P-glycoprotein from KB-C1 cells is cleaved with CNBr, one prominent band of approximately 25 kDa is obtained. This is shown in Fig. 3A. From the methionine map of P-glycoprotein, presented in Fig. 1D, it is clear that a complete reaction with CNBr may result in two fragments of approximately 25 kDa, one of which is from the amino half of P-glycoprotein (amino acid residues 198–440) and the other of which is from the carboxy half (amino acid residues 1028–1270). We were not able to immunoprecipitate the azidopine-labeled 25-kDa fragment with antiserum 4007, antiserum 4414, or with the monoclonal antibody C219 (data not shown), which should recognize the 25-kDa CNBr fragment from the carboxy terminus of P-glycoprotein. This negative result suggested that the labeled 25-kDa fragment is from the amino half of P-glycoprotein (see Fig. 1B). Immunoprecipitations with antisera 4328, 4331, 4402, and 4408, which confirm this result, are shown in Fig. 3A. Precipitation with 4328 and 4402 demonstrates that the 25-kDa fragment is from the amino half, as indicated in Fig. 1D. Negative results with antisera 4331 and 4408 eliminate the possibility that fragments from homologous regions of the carboxy half of P-glycoprotein are cross-reacting with antisera 4328 and 4402. Preimmune sera from these rabbits do not immunoprecipitate P-glycoprotein or any of its fragments (data not shown).

Fig. 1. Linear map of P-glycoprotein. Amino terminus is on the left. A, the 12 transmembrane domains of P-glycoprotein are numbered 1–12, and the two nucleotide binding sites are circled (NB). The predicted sites of glycosylation (CHO) and the location of amino acid residue 185 are indicated. B, the regions of P-glycoprotein that were used to raise the rabbit antisera are indicated with bars. The epitopes of monoclonal antibody C219 are also shown. C, the locations and sizes of the three tryptic fragments are indicated with bars. D, the 25-kDa azidopine-labeled CNBr fragment is indicated with a bar, and the positions of the methionine residues that produce this fragment are shown. The location of every methionine residue in P-glycoprotein is marked.
The azidopine-labeled 25-kDa CNBr fragment represents a limit digestion of P-glycoprotein and can be used as the basis of a quantitative assay. We labeled P-glycoprotein from KB-C1 cells with azidopine in the presence of different concentrations of vinblastine. We then cleaved an aliquot of this labeled P-glycoprotein with CNBr, immunoprecipitated both cleaved and uncleaved aliquots with antisera 4328, and measured the amount of tritium in the immunoprecipitate. Because there is little or no background from the immunoprecipitation (Fig. 3A), all of the precipitated tritium is from P-glycoprotein, or its fragments, that are labeled with azidopine. We then calculated the total amount of azidopine bound to P-glycoprotein at each concentration of vinblastine, and we calculated the fraction of the total bound azidopine that was bound to the amino half of P-glycoprotein at each concentration of vinblastine.

The results from this experiment are shown in Fig. 4. As the concentration of vinblastine in the labeling reaction is increased, the total amount of azidopine bound to P-glycoprotein decreases dramatically from 100% (defined as the amount of azidopine bound to P-glycoprotein in the absence of vinblastine) to 4% at the highest concentration of vinblastine used. However, at each concentration of vinblastine, the percentage of azidopine bound to the amino half of P-glycoprotein (the 25-kDa CNBr fragment) remains constant at about 60%. From these results we infer that the amount of azidopine incorporated into the carboxy half of P-glycoprotein remains constant at about 40%. Thus vinblastine inhibits the azidopine labeling in both halves of P-glycoprotein equally well with no preference given to either labeling site.

An interesting observation is that vinblastine, when present during the labeling reaction, efficiently inhibits azidopine labeling in KB-C1 cells, in which P-glycoprotein contains a valine residue at position 185, but not in KB-V1 cells, in which P-glycoprotein contains the wild-type glycine residue at position 185. An example of this observation is shown in Fig. 2. Because amino acid residue 185 of P-glycoprotein differs in these two cell lines, we wanted to determine if there is any difference in azidopine labeling of the two halves of P-glycoprotein between the two cell lines.

We first attempted to determine if both halves of P-glycoprotein are labeled by azidopine in KB-V1 cells, as in KB-C1 cells. We labeled P-glycoprotein from both cell lines and digested it with trypsin. Trypsin digestion produces three azidopine-labeled fragments of P-glycoprotein, shown in Fig. 1C (35). The labeled fragment from the amino half of P-glycoprotein is 70 kDa, but after the carbohydrate has been removed with Endo F its molecular mass is 38 kDa. Two labeled fragments are obtained from the carboxy half, one of 55 kDa and another of 40 kDa. The results are shown in Fig. 5. Trypsin digestion of azidopine labeled P-glycoprotein from both cell lines resulted in identical 55- and 40-kDa fragments from the carboxy half of the protein. The 70-kDa glycosylated fragment from the amino half of P-glycoprotein was present in the sample from KB-C1 cells, but a 60-kDa glycosylated fragment was present in the sample from KB-V1 cells. Unfortunately, after digestion with Endo F in the experiment shown here it was difficult to see the 38-kDa fragment in either cell line. These data show that there is no apparent difference in labeling the carboxy half of P-glycoprotein in KB-C1 cells and KB-V1 cells.

To verify that the amino half of P-glycoprotein is labeled

![FIG. 2. Vinblastine inhibition of azidopine labeling.](image)

![FIG. 3. CNBr cleavage of azidopine labeled P-glycoprotein.](image)
was labeled with azidopine in the presence of different amounts of vinblastine, and then immunoprecipitated with 16 μl of antiserum 4007. Each sample was divided into two aliquots, one of the two aliquots was cleaved with CNBr, and then all aliquots were immunoprecipitated with 5 μl of antiserum 4328 and quantitated by liquid scintillation counting. Total azidopine bound to P-glycoprotein was determined from both cleaved and uncleaved aliquots by comparing the amount of azidopine bound to P-glycoprotein in the presence of vinblastine to the amount of azidopine bound to P-glycoprotein in the absence of vinblastine. 100% is defined as the amount of labeling in the absence of vinblastine. Azidopine bound to the amino half of P-glycoprotein was determined from both cleaved and uncleaved aliquots by comparing the amount of azidopine bound to the 25-kDa CNBr fragment at a particular vinblastine concentration to the total amount of azidopine bound to P-glycoprotein at that concentration of vinblastine.

For each point, P-glycoprotein from 4 x 10⁶ KB-C1 cells was labeled with 4 μCi of azidopine in the presence or absence of various concentrations of vinblastine, and then immunoprecipitated with 16 μl of antiserum 4007. Each sample was divided into two aliquots, one of the two aliquots was cleaved with CNBr, and then all aliquots were immunoprecipitated with 5 μl of antiserum 4328 and quantitated by liquid scintillation counting. Total azidopine bound to P-glycoprotein was determined from both cleaved and uncleaved aliquots by comparing the amount of azidopine bound to P-glycoprotein in the presence of vinblastine to the amount of azidopine bound to P-glycoprotein in the absence of vinblastine. 100% is defined as the amount of labeling in the absence of vinblastine. Azidopine bound to the amino half of P-glycoprotein was determined from both cleaved and uncleaved aliquots by comparing the amount of azidopine bound to the 25-kDa CNBr fragment at a particular vinblastine concentration to the total amount of azidopine bound to P-glycoprotein at that concentration of vinblastine.

**FIG. 5. Trypsin digestion of azidopine labeled P-glycoprotein.** For each lane, P-glycoprotein from 4 x 10⁶ cells was labeled with 4 μCi of azidopine and immunoprecipitated with 16 μl of antiserum 4007. Labeled P-glycoprotein from KB-C1 cells or KB-V1 cells was digested with Endo F as indicated, then digested with trypsin. The fragments were run on a 8% gel. Molecular mass markers have been omitted for clarity, but the sizes of the fragments are shown in kDa.

![Trypsin digestion of azidopine labeled P-glycoprotein](image)

by azidopine in KB-V1 cells, we used CNBr cleavage and immunoprecipitation. These results are shown in Fig. 3B. It is clear from this figure that the amino half of P-glycoprotein from KB-V1 cells is labeled. Thus there appears to be no difference in the labeling of the two halves of P-glycoprotein by azidopine between these two cell lines. Immunoprecipitation of this azidopine-labeled 25-kDa CNBr fragment and quantification of the results reveals that approximately 60% of total azidopine is incorporated into the amino half of P-glycoprotein from KB-V1 cells. We believe that the difference seen in the trypsin digestion is the result of differential glycosylation between the two cell lines.

In interpreting the experiments described above we assumed that any differences in labeling or competition between KB-C1 (V185) cells and KB-V1 (G185) cells arose from the identity of the amino acid residue at position 185 of P-glycoprotein. These cell lines were selected independently, however, and it is possible that a second mutation, or some uncharacterized difference between the clones isolated, could be responsible for the difference in azidopine labeling and vinblastine competition. To demonstrate that this was not the case, we repeated some of our experiments with NIH-3T3 cells that had been transfected with MDR1 cDNAs encoding either the wild-type or the mutant P-glycoprotein.

NIH MDR1-V185 cells, like KB-C1 cells, express P-glycoprotein that contains a mutant valine at position 185. NIH MDR1-G185 cells, like KB-V1 cells, express P-glycoprotein that contains a wild-type glycine at position 185. These cell lines contain approximately equal amounts of P-glycoprotein on their cell surfaces (data not shown). We labeled P-glycoprotein from these transfected NIH lines with azidopine in the presence and absence of vinblastine. The results are shown in Fig. 2. We quantified the results from this experiment by immunoprecipitation and measured the amount of tritium incorporated into P-glycoprotein. In the presence of vinblastine, azidopine labeling in KB-C1 cells and NIH MDR-V185 cells was 14 and 24% of the controls, respectively, whereas labeling in KB-V1 cells and NIH MDR-G185 cells was 83 and 72% of the controls. These results clearly demonstrate that the ability of vinblastine to inhibit azidopine labeling depends entirely on the amino acid residue present at position 185. There is inhibition if there is a valine at position 185, but not if there is a glycine at this position. The genetic and biochemical background in the transfected NIH cells is identical, unlike the situation with the KB cell lines, and therefore the inhibition of labeling must be due to residue 185.

To fully characterize the differences in azidopine labeling competition between the wild-type and mutant P-glycoproteins, we examined the abilities of several other drugs that are substrates for transport by P-glycoprotein to inhibit azidopine labeling in the transfected NIH cell lines. The results from the use of vinblastine, vincristine, colchicine, and verapamil are shown in Fig. 6. Actinomycin D, daunomycin, puromycin, and cyclosporin A had no effect on the ability of azidopine to label P-glycoprotein regardless of the residue at position 185 (data not shown). We found that only vincristine inhibited azidopine labeling in one cell line but not the other. Just as in the case with vinblastine, vincristine inhibited azidopine labeling only if a valine was at position 185, but this effect occurred at 10-fold higher concentrations of the competing drug than with vinblastine. We also observed that at high concentrations vinblastine would inhibit azidopine labeling in both NIH MDR1-V185 cells and NIH MDR1-G185 cells. Colchicine did not compete at all, which is consistent with results reported by others (41). Verapamil inhibited azidopine labeling, but the inhibition was identical in the two cell lines.

**DISCUSSION**

Azidopine photoaffinity labels two sites on P-glycoprotein, one in the amino half and one in the carboxy half (35, 36),
consistent with either one or two azidopine binding sites (35). By using a CNBr digestion analysis, which is quantitative, unlike the previous partial tryptic proteolytic analyses (35, 36), we show in this paper that 60% of the azidopine label is in the amino half and 40% in the carboxy half. Both labeling sites are equivalently inhibited by vinblastine, an important substrate for transport by P-glycoprotein. Furthermore, alternate forms of P-glycoprotein with either glycine or valine at residue 185 have equivalent distributions of azidopine label in both amino and carboxy halves, despite the fact that substitution of valine for glycine at position 185 has profound effects on drug binding, substrate specificity (40-42), and ability of vinblastine to inhibit azidopine labeling at these sites (this work). The simplest interpretation of these results is that both azidopine-labeled sites come together in the tertiary structure of the transporter to form a single site.

The CNBr analysis also reveals more precise information than has been previously available on the azidopine-labeling site from the amino half of P-glycoprotein. The exact location of the 25-kDa CNBr fragment is known from the methionine map of P-glycoprotein (Fig. 1) and from the immunoprecipitations with site-specific antisera (Fig. 4). We previously used a partial trypsin digestion to obtain a 38-kDa azidopine-labeled fragment from the amino half of P-glycoprotein (35). The 38-kDa trypsin fragment and the 25-kDa CNBr fragment overlap (Fig. 1) in an 18-kDa region of P-glycoprotein that extends from methionine residue 198, which is in the third transmembrane domain, through the sixth transmembrane domain, which is defined by the carboxy end of the 38-kDa trypsin fragment. These data do not tell us if the binding site is in the hydrophobic transmembrane segments or in the connecting cytoplasmic or extracellular loops.

Recently, Greenberger et al. (39) showed that the photoaffinity label azidoprazosin labels both halves of mouse P-glycoprotein and that the labeled site in the carboxy half is probably located near transmembrane domains 11 and 12. From our results, we believe that the labeled site in the amino half of human P-glycoprotein is near transmembrane domains 5 and 6. Taken together, these results suggest that homologous regions in the two halves of P-glycoprotein, transmembrane domains 5, 6, 11, and 12, are involved in drug binding.

Although azidopine labels both halves of P-glycoprotein in both KB-V1 (wild-type glycine at residue 185) and KB-C1 (mutant valine at residue 185), vinblastine and vincristine inhibit this labeling much more efficiently in KB-C1 cells. By using NIH-3T3 cells transfected with the two different forms of MDR1, we have confirmed that the differences in azidopine labeling and vinblastine inhibition of azidopine labeling depend entirely on the amino acid at residue 185. Azidopine labeling of P-glycoprotein is more efficient in both KB and transfected NIH cells if a valine residue is present at position 185 rather than a glycine (Ref. 41 and data not shown). If there were two azidopine binding sites, then the lower efficiency of azidopine labeling in KB-V1 cells could be explained as a decrease in binding affinity at one site, namely the site in the amino half of P-glycoprotein, which seems most likely to be affected by a mutation at position 185. However, no decrease in labeling in one-half of P-glycoprotein over the other half was observed. In addition, the single mutation at position 185 is responsible for the ability of vinblastine to inhibit azidopine labeling of P-glycoprotein, and, as stated above, this inhibition of azidopine labeling is identical in the two halves of P-glycoprotein.

Because a mutation at amino acid residue 185 is responsible for the inability of vinblastine to inhibit azidopine labeling of P-glycoprotein, and because vinblastine inhibits azidopine labeling at both sites equally, we believe that there is a single binding site for vinblastine and azidopine on P-glycoprotein. This site is formed by the two halves of P-glycoprotein coming together in some fashion to form a single binding site. After azidopine binds to this site, it labels both halves of the protein. Vinblastine, by binding to this site, prevents azidopine from binding to P-glycoprotein and labeling both halves of the protein. In this model, each half of P-glycoprotein contains part of the binding site for these two drugs, but the full binding site is created only when the two halves of P-glycoprotein come together in the correct conformation.

The fact that amino acid residue 185 is involved in drug
binding is not inconsistent with the fact that azidopine labels regions of P-glycoprotein that do not include amino acid residue 185. It is unlikely that the azidopine binding site coincides exactly with the azidopine labeling site (35, 47, 48). The premise of photoaffinity labeling is, of course, that the labeling site is close to the binding site. We know little about the three-dimensional conformation of P-glycoprotein in the plasma membrane, however, and nothing concerning the identity of the amino acid residues that are labeled by azidopine. In our model, amino acid residue 185, if it contributes directly to azidopine binding, should lie within approximately 12 Å of the labeled sites (46, 47).

The results from the experiments in which we used other drugs to inhibit azidopine labeling showed that only the Vinca alkaloids have a pattern of competition that distinguishes between a glycine or a valine at position 185 of P-glycoprotein. There are two obvious explanations for these results, although there might be others. 1) The affinities of these other drugs for the azidopine/Vinca alkaloid binding site are too low to prevent azidopine from binding to P-glycoprotein and labeling the protein. Indeed, none of these drugs showed dramatic inhibition of azidopine labeling, compared to both vinblastine and vincristine. 2) There are different binding sites for different classes of drugs, and it just so happens that azidopine and the Vinca alkaloids bind to the same site. This second possibility is not supported by existing data using other photoaffinity labels showing inhibition by a wide range of P-glycoprotein substrates and inhibitors (18, 38, 41, 48) or by transport experiments which also show competition for vinblastine transport by a wide range of compounds (25–27). Recent data, however, suggest that azidopine and vinblastine may not bind to precisely the same site (49), raising the possibility that allosteric effects could be responsible for the inhibition of labeling seen in the previous studies and our current work.

Recently Safa et al. (41) studied relative drug resistance, drug accumulation, and drug binding in transfected cell lines expressing P-glycoprotein with either a mutant valine or a wild-type glycine at position 185. Despite the fact that it is more efficient at colchicine transport, the mutant P-glycoprotein binds less colchicine and more vinblastine than the wild type protein (41). Furthermore, both colchicine and vinblastine inhibit labeling of P-glycoprotein by photoaffinity analogues of colchicine and vinblastine equally well in both the mutant and the wild-type P-glycoprotein (41). These results were interpreted with a model in which the mutation at residue 185 does not contribute directly to drug binding, but is involved in translocation of the drug from its initial binding site or release from the transporter. Our cartoon version of this model of Safa et al. (41) is shown in Fig. 7B.

In contrast to these previous results (41), our data clearly show that it is possible to observe differences between the mutant and wild-type P-glycoprotein in the ability of some drugs to compete with a photoaffinity analogue. The Vinca alkaloids prevent azidopine from labeling P-glycoprotein with a mutant valine much more effectively than they prevent it from labeling P-glycoprotein with a wild-type glycine. Our results, therefore, are not consistent with the model of Safa et al. (41). We would argue on the basis of our results that amino acid residue 185 might be directly involved in the initial drug binding site, shown schematically in Fig. 7A. However, in this model it is difficult to understand why the mutant protein, which was selected for colchicine resistance, appears to have a lower affinity for colchicine than for vinblastine (41). A possible explanation is that a premise that underlies the use of photoaffinity analogues is incorrect in this case. It is generally assumed that photoaffinity analogues bind to a high affinity site on P-glycoprotein, which is the initial drug binding site, and label that site. However, if these compounds are not labeling an initial high affinity binding site, perhaps because such a site does not exist, they might be recognizing an interior site of the P-glycoprotein channel that is not involved in initial drug recognition. This idea is consistent with previous data suggesting that substrates for P-glycoprotein are presented to the transporter through the plasma membrane, rather than through the cytoplasm (50).

Our analysis and that of Safa et al. (41) indicates that the mechanism of active transport of drugs through the plasma membrane by P-glycoprotein is quite complex. Further experiments and more sophisticated ideas will be required to elucidate this mechanism. The combination of transport studies, photoaffinity analysis, and mutational dissection constitute a powerful tool to understand the mechanism of action of P-glycoprotein and the ATP-dependent superfamily of transporters to which it belongs.

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