Functional Consequences of Proline Mutations in the Predicted Transmembrane Domain of P-glycoprotein*

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Site-directed mutagenesis was used to investigate whether prolines in the predicted transmembrane domains play essential roles in the function of human P-glycoprotein. Mutant cDNAs in which codons for each of the 13 prolines were changed to alanine were expressed in mouse NIH 3T3 cells and analyzed with respect to their ability to confer resistance to various drugs. Mutations of either Pro in transmembrane segment 4 or Pro in transmembrane segment 10, drastically reduced the ability of the mutant proteins to confer resistance to colchicine, adriamycin, or actinomycin D, whereas the capacity to confer resistance to vinblastine was retained. These results strongly suggest that residues in putative transmembrane segments 4 and 10, which are found in identical positions when homologous, presumably duplicated, halves of the transporter are aligned, play important roles in recognition of colchicine, adriamycin, and actinomycin D. They may either interact to form a single drug-binding site or form part of two equivalent, but independent, drug-binding sites. The lack of detectable effect of either mutation on vinblastine transport, however, indicates that there are differences in the requirements for binding of various substrates to P-glycoprotein. Mutation of Pro in transmembrane segment 7 resulted in a protein unable to confer drug resistance. A change at this position was found to induce a structural alteration, since the major protein product observed in transfected cells had an apparent molecular weight of 150,000, whereas the wild-type enzyme had an apparent molecular weight of approximately 170,000. Mutation of the other 10 prolines yielded protein products with structural and functional characteristics indistinguishable from wild-type P-glycoprotein.

P-glycoprotein, the product of the multidrug resistance gene, MDR, is an integral membrane protein that is overexpressed in multidrug resistant cells. The protein acts as an energy-dependent pump that extrudes hydrophobic cytotoxic drugs (reviewed by Endicott and Ling (1989), Kane et al. (1989), Pastan and Gottesman (1991)). Cloning and sequencing of the multidrug resistance gene (MDR1) from human (Chen et al., 1988), hamster (Gerlach et al., 1986), and mouse (Gros et al., 1986) showed that P-glycoprotein contains approximately 1280 amino acids organized in two tandem repeats of about 640 amino acids. Each repeat consists of an NH2-terminal hydrophobic domain containing six potential transmembrane helices, followed by a hydrophilic domain containing a classic ATP-binding site. Intriguingly, P-glycoprotein shares extensive sequence homology with a variety of bacterial and eukaryotic proteins that transport a wide variety of substrates ranging in size from ions to large proteins (reviewed by Higgins et al., (1990), Hyde et al. (1990)). The number of proteins identified as members of this superfamily of transporters has increased rapidly in recent years and includes the cystic fibrosis transmembrane conductance regulator (Riordan et al., 1989), the Plasmodium falciparum P-glycoprotein homolog expressed in chloroquine-resistant parasites (Wilson et al., 1989; Foote et al., 1989), and the proposed peptide transporters involved in antigen presentation (Parmham, 1990). Each member of this superfamily of transport proteins possesses at least one hydrophobic domain consisting of six potential transmembrane helices associated with one or two hydrophilic domains containing a nucleotide-binding consensus sequence. The ATP-binding domains of each member share about 30% sequence identity over a domain of about 200 amino acids.

The segments and amino acid residues of P-glycoprotein involved in substrate binding and transport have not been precisely identified. It has been demonstrated, however, that drugs interact directly with the transporter. Photoaffinity analogues of chemotherapeutic agents such as colchicine (Safa et al., 1989), adriamycin (Busche et al., 1989), and vinblastine (Cornwell et al., 1986) have been shown to bind P-glycoprotein covalently. The binding of these photoactive probes is specifically inhibited by substrates of P-glycoprotein in a preferential order of vinblastine > actinomycin D > adriamycin > colchicine (Cornwell et al., 1986; Safa et al., 1987, 1989; Safa, 1988; Yang et al., 1988; Yusa and Tsumu, 1989). The same order of inhibition is observed in studies on Vinca alkaloid binding and transport by P-glycoprotein (Horio et al., 1988; Nito et al., 1988). These data suggest that the photoaffinity probes and chemotherapeutic drugs share common binding domains in P-glycoprotein. Mapping of the photoaffinity drug-binding sites of P-glycoprotein from human and mouse suggest that there are two drug-binding sites within the predicted transmembrane domains (Greenberger et al., 1990, 1991; Bruggeman et al., 1989); one in each of the NH2- and COOH-terminal halves of the molecule. One of the drug-binding sites is postulated to reside within or be in close proximity to putative transmembrane (TM) segments TM11 and TM12 (Greenberger et al., 1991).

A natural mutation in close proximity to the transmembrane domain of P-glycoprotein was found to alter the activity and substrate specificity of the transporter. Cells expressing
a mutant MDR1 gene that encodes a glycine to valine mutation at position 185 showed increased resistance to colchicine and decreased resistance to vinblastine when compared with cells that express wild-type P-glycoprotein (Choi et al., 1988). Another natural mutation within the predicted TM11 segment of mouse P-glycoprotein (serine 941 to phenylalanine) was still resistant to vinblastine but had lost the ability to confer resistance to adriamyacin and colchicine (Gros et al., 1991). Further support for a role of transmembrane regions in substrate recognition and transport has come from analysis of chimeric genes. Replacement of transmembrane segments of P-glycoprotein with homologous segments from their counterparts which do not confer multidrug resistance yields inactive mutants.

An approach to improving our understanding of the molecular details of the transport mechanism of P-glycoprotein is to identify the residues critical for drug transport through site-directed mutagenesis. The proline residues in P-glycoprotein are of great interest because of their unique structural and functional properties. The structural destabilization that would be induced by replacing the proline residues located in these positions and the predicted loss of cis-conformation of peptide bonds between proline and the preceding amino acid residues makes the former important for studying conformational changes in the protein (Schulz and Schirmer, 1979; Brandl and Deber, 1986; Barlow and Thornton, 1988).

It has been found that the putative transmembrane segments of several membrane-bound transport proteins have a higher number of proline residues than the transmembrane segments of nontransporting membrane-bound proteins, suggesting some functional role for membrane-bound prolines in the transport reaction (Brandl and Deber, 1986).

In this study, we have mutated 13 proline residues in P-glycoprotein. Five of the prolines are located in putative transmembrane helices (TM1, TM4, TM6, TM7, and TM10). 1 is located in the extracellular loop connecting putative transmembrane helices TM7 and TM8, and 7 are located within intracellular loops. We have found that 2 proline residues, located in putative transmembrane segments TM4 and TM10, respectively, are important for function. Mutation of either Pro223 or Pro236 dramatically altered the drug resistance profile of cells expressing the mutant protein relative to colchicine, adriamyacin, or actinomycin D and was significant reduced relative to cells expressing wild-type P-glycoprotein, whereas resistance to vinblastine was retained. These results suggest that the putative transmembrane segments TM4 and TM10 play important roles in the recognition and transport of a subclass of substrates of P-glycoprotein and that the binding sites for vinblastine and for colchicine, adriamyacin, and actinomycin D may be nonoverlapping.

**EXPERIMENTAL PROCEDURES**

**Molecular Cloning—**A cDNA library constructed in λgt10 from human kidney cortex mRNA was a generous gift from Dr. Graeme Bell, University of Chicago (Bell et al., 1986). An oligonucleotide, 5'-CAGTCGCATGGGAGAGCAGC-3', corresponding to nucleotides 1284–1301 and 3213–3233, respectively, of human MDR1 from human KB cells (Chen et al., 1986), was used for screening the cDNA library. Approximately 400,000 plaques were screened with the 5'–labelled probe. Hybridization was carried out at 37 °C for 12–16 h in the presence of 6 × SSC and 10 × Denhardt’s solution. The filters were washed at 56 °C in 6 × SSC. Plaques that gave a positive signal were picked and rescreened with the oligonucleotide probe. Clones that were still positive after the secondary screen were grown and the EcoRI fragments were isolated, subcloned into Bluescript vector (Stratagene), and sequenced by the dyeoxynucleotide chain termination method (Sanger et al., 1977). The full-length MDR1 cDNA (4 kilobase pairs) from human kidney was subsequently modified to facilitate molecular manipulations. The 3'-end EcoRI site of MDR1 cDNA was filled in with the Klenow fragment of DNA polymerase I and an XhoI linker added. An internal EcoRI site was removed from the MDR1 cDNA by changing nucleotide 1181 from a C residue to a T residue by site-directed mutagenesis with oligonucleotide 5'-AATTTGGAATTTAGAAATGT-3'. The full-length MDR1 cDNA was subcloned as an EcoRI to XhoI fragment into the multiple-cloning site of pBSKS (Stratagene) and used as template for site-directed mutagenesis. Deletion of the internal EcoRI site also allowed the full-length MDR1 cDNA to be excised as an EcoRI to XhoI fragment and subcloned after mutagenesis into the EcoRI and XhoI sites of the mammalian expression vector pMT21 (a gift of Dr. R. Kaufman, Genetics Institute, Boston). In a second modification of the full-length cDNA, the epitope for monoclonal antibody A52 (Zubrycka-Gaarn et al., 1984) was inserted at the COOH-terminal of the protein between amino acids 1278 and 1279 in order to act as a specific label for the protein in expression studies. Briefly, a 64-base pair 3'Not fragment encoding the epitope (Clarke et al., 1989) was inserted into the Smal site of vector pBSKS. A 102-base pair BamHI to ClaI fragment containing the cDNA for epitope A52 was then excised and the ends of the linearized molecules were filled in using the Klenow fragment of DNA polymerase I. In the second step, a single HinPI site at the 5'-end of MDR1 cDNA in pBSMDR1 was digested with HinPI and filled in with Klenow fragment. Religation of the two ends generated a BshHI site. The resulting construct was then digested with BshHI, and the ends of the linearized molecules were filled in with Klenow fragment. This modified fragment was then ligated to the blunt-ended 102-base pair ClaI to BshHI fragment of pMT21MDR1. This construct containing a single HinPI site at the COOH-terminal of P-glycoprotein that would normally end as TKRQ now became TKRRAISLNSCSPEFDDLPLAENREACR RDGRPRQ.

**Oligonucleotide-directed Mutagenesis—**Fragments, less than 500 base pairs long, were removed from pBSMDR1 and ligated into the polylinker region of the Bluescript vector for site-specific mutagenesis by the method of Kunkel (1985). The integrity of the mutated segment of the cDNA insert was checked by sequencing the entire insert by the dyeoxynucleotide chain termination method (Sanger et al., 1977). Fragments containing the mutation were subcloned back into their original position in the pMT21MDR1A52 vector for expression in NIH 3T3 cells.

**Cell Culture and DNA Transfection—**Mouse NIH 3T3 cells were maintained in Dulbecco’s modified Eagle’s medium fortified with 0.1 mM α-minimum Eagle’s medium nonessential amino acids, 2 mM L-glutamine, 100 units of penicillin/ml, 100 μg of streptomycin/ml, and 10% (v/v) calf serum, under 5% CO2 at 37 °C. DNA transfection was carried out by the calcium phosphate precipitation method as described by Chen and Okayama (1987) or with lipofectin (GIBCO) according to the manufacturer’s directions. Ten μg of plasmid DNA was added to 2 transfect 2 × 106 NIH 3T3 cells in 5 ml of 10% fetal calf serum, 2 mM glutamine, 0.1% (v/v) calf serum, under 5% CO2 at 37 °C. In some cases cells were co-transfected with pWL-neo (Stratagene) and mutant constructs of pMT21MDR1A52 in a 1:10 molar ratio, respectively. Mass populations of neomycin-resistant colonies were selected initially in the presence of Geneticin (G418) at a concentration of 0.72 μg/ml, followed by selection in the presence of vinblastine (8.7 nM) or colchicine (37.5 nM).

**Drug Survival Measurements—**This assay is a measure of the ability of P-glycoprotein to confer resistance to various drugs and is an indirect measure of the drug transport capacity of P-glycoprotein. One-thousand cells in 0.1 ml were added to wells of a 96-well microtiter plate containing 0.1 ml of media with a series of increasing concentration of colchicine (0–250 nM), adriamycin (0–170 nm), vinblastine (0–87 nM), or actinomycin D (0–50 nm). After 48 h, the medium was removed and replaced with 0.05 ml of Opti-MEM medium (GIBCO) containing 0.2 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). After 2 h, 0.2 ml of dimethyl sulfoxide was added to each well, and the amount of MTT formazan produced was measured at 540 nm. The amount of MTT-formazan produced was a measure of the number of cells still viable in a particular drug concentration.

**Immunological Procedures—**Immunoblotting was performed on proteins separated by sodium dodecyl sulfate-slab gel electrophoresis (Laemmli, 1970) and transferred electrophoretically to nitrocellulose (Towbin et al., 1979). The nitrocellulose blots were incubated in rabbit antibody in TBS (10 mM Tris-HCl, 150 mM NaCl, pH 7.5) for 20 min and then incubated in TBS, 1% (w/v) milk powder containing 2 μg/ml of A52 antibody for 30 min at room temperature. After washing
Expression levels of P-glycoprotein-A52 were examined using a modified enzyme-linked immunosorbent assay. A sodium dodecyl sulfate-solubilized sample of cells (extract of 1000-5000 cells in 0.4% SDS) was spotted onto 1 cm² of nitrocellulose. The nitrocellulose was processed with monoclonal antibody A52 and alkaline phosphatase-conjugated anti-mouse secondary antibody as described above. The nitrocellulose was then washed and processed with 5-bromo-4-chloro-3-indoylphosphate conjugated anti-mouse secondary antibody as described above. The reaction was stopped by placing samples in an ice bath and color development measured at an absorbance of 540 nm. In these assays, deoxycholate-purified fast-twitch skeletal muscle Ca²⁺-ATPase (MacLennan, 1970) was used as a standard. Samples containing 0-1000 pg of Ca²⁺-ATPase were spotted onto nitrocellulose and processed as described above.

**Drug Accumulation Studies**—Monolayer cells in six-well plates were incubated with either 25 nM [³H]vinblastine or 75 nM [³H]colchicine in Opti-MEM medium (GIBCO) at 37 °C. At various intervals, the cells were washed by rapid submersion of the plate into ice-cold PBS (10 mM NaPO₄, 140 mM NaCl, and 2 mM KCl, pH 7.4). The washed cell were trypsinized and the radioactivity present in the cells measured. Monolayer cells were also trypsinized and the number of cells determined.

**[³H]Haldane Photolabeling**—Cells were collected by scraping into PBS containing 5 mM EDTA, washed twice with PBS, and resuspended in hypotonic lysis buffer (10 mM Tris-HCl, pH 7.5, 0.5 mM MgCl₂). The cells were then homogenized and then washed twice with PBS. The sample was then UV-irradiated on ice for 10 min at 150 W. It was then centrifuged at 100,000 g for 10 min at 4 °C to remove nuclei and mitochondria. The pellet obtained by subsequent centrifugation at 10,000 g for 60 min was used as the crude membrane fraction. The final pellet was suspended in 50 mM Tris-HCl, pH 7.5, at a protein concentration of 1-2 mg/ml.

A membrane suspension containing 0.5 µM [³H]Haldane (48 Ci/mmol), 5 mM MgCl₂, and 3 mM ATP was incubated at room temperature for 30 min. The sample was then UV-irradiated on ice for 15 min with a 150-Watt G15T8 germicidal lamp (General Electric) at a distance of 5 cm. Samples were then dissolved by the addition of sodium dodecyl sulfate to 1% (w/v) followed by addition of 3 volumes of immunoprecipitation buffer (25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.5% sodium deoxycholate) and 1 mg/ml bovine serum albumin to give a total volume of 1-1.5 ml. Five µg of monoclonal antibody A52 was then added. After incubation for 14-16 h at 4 °C the antigen-antibody complexes were precipitated at 25 °C for 2 h using protein A-Sepharose (Pharmacia LKB Biotechnology Inc.). The immune complexes were solubilized with 50 µl of sample buffer at 37 °C for 2 min and analyzed by SDS-polyacrylamide gel electrophoresis, followed by fluorography.

**RESULTS**

**Construction of Mutants**—The cDNA for human P-glycoprotein was obtained by screening a human kidney cDNA library. The kidney was chosen for two reasons: the organ contains relatively high amounts of P-glycoprotein, and three partial cDNAs (2-3 kilobase pairs) after sequencing, and the deduced amino acid sequence of MDR1 closely matched the sequence deduced from the mRNAs encoding P-glycoprotein. Therefore, the 150-kDa product may represent a mutant form of the expressed protein. This modification did not alter the ability of P-glycoprotein to bind and transport drugs. In addition, its drug survival characteristics were identical to the wild-type enzyme.

**Expression of Mutants**—Mutants were first transiently expressed in the human HEK 293 cell line. This transient expression system allows for rapid (48 h) expression of the mutants and assessment of the structural integrity of the mutant proteins. Fig. 2 shows an immunoblot of wild-type P-glycoprotein and the mutant proteins expressed in HEK 293 cells. The A52 antibody reacts specifically with the epitope introduced at the COOH terminus of the protein and there is no cross-reactivity with endogenous proteins. We have observed that HEK 293 cells do express low levels of endogenous P-glycoprotein when immunoblots are developed with a rabbit polyclonal antibody raised against the first cytoplasmic domain. A major structural perturbation was observed for mutant Pro185 to Ala. Cells transfected with this mutant expressed an immunoreactive protein of approximately 150 kDa as the major product, whereas the majority of wild-type product corresponded to a mass of about 170 kDa. It was apparent, however, that a minor amount of a 150-kDa immunoreactive product was also present in cells transfected with wild-type MDR1. Therefore, the 150-kDa product may represent a partially processed intermediate in synthesis and transport to the plasma membrane, rather than a degradation product. In support of this hypothesis, we have found that both the mutant and wild-type P-glycoprotein have an apparent mass

<ref>FIG. 1. Simplified representation of location of mutated proline residues based on structural models for P-glycoprotein proposed by Duranena et al. (1988) and Gottesman and Pastan (1988). TM1-TM12 correspond to transmembrane α-helices.</ref>
of 140 kDa when tunicamycin is added to the medium after transfection (Fig. 3). For each of the other mutants, the major product in HEK 293 cells was a protein of 170 kDa (Fig. 2). Each mutant expressed approximately the same amount of P-glycoprotein as that of wild-type in the transient expression system, suggesting that no major structural perturbation occurred by substitution of any of the other proline residues.

**Effect of Mutations on Biological Activity**—The ability of wild-type and mutated MDR1 cDNAs to confer drug resistance was tested by transfecting the drug-sensitive mouse cell line NIH 3T3, followed by selection in the presence of vinblastine or colchicine. Using this assay, NIH 3T3 cells grown in the presence of tunicamycin or vinblastine. All of the mutants, except Pro79Q to Ala yielded colonies which were resistant to vinblastine. Transfection of cells with mutants Pro22Q to Ala, Pro225 to Ala and Pro79Q to Ala did not yield any colonies in the presence of colchicine. Similar results were obtained when these mutants were first introduced into drug-sensitive NIH 3T3 cells by co-transfection with plasmid pWL-neo (Stratagene), and then G418-resistant colonies selected in media containing either colchicine or vinblastine. Results of Western blot analysis of G418-resistant colonies were similar to those shown in Fig. 2. These observations suggest that the Pro79Q-Ala mutation disrupts both structure and transport activity of MDR1, whereas changes to Pro22Q and Pro225 affect colchicine transport.

**Drug Survival Characteristics of Cell Clones Expressing Mutant P-glycoproteins**—Structural and functional characteristics of the stable mutant cell lines were further investigated by studying individual clones which were resistant to vinblastine. Four clones of each mutant were isolated, and detergent extracts from cells of each clone were immunoblotted to test for expression of the mutant P-glycoprotein-A52 protein. In each case, an A52 immunoreactive protein product of 170 kDa was present (data not shown), indicating that drug resistance was due to expression of human P-glycoprotein and not endogenous P-glycoprotein. The drug survival characteristics of each of the mutant clones were analyzed in the presence of colchicine, vinblastine, adriamycin, or actinomycin D. The ID$_50$ (the drug concentration necessary to inhibit cell growth by 50%) for vinblastine was 1.3 ± 0.2 nM for control NIH 3T3 cells and 18.3 ± 1.6 nM for clones with wild-type P-glycoprotein-A52, representing a 14.1-fold resistance above that of the control cells. Clones expressing wild-type P-glycoprotein-A52 also demonstrated increased relative resistance to colchicine (3.8-fold), adriamycin (2.7-fold), and actinomycin D (7.0-fold). The mutant proteins, with the exception of mutants Pro22Q to Ala and Pro225 to Ala, also gave rise to clones with a similar pattern of resistance to these drugs. Mutant Pro22Q to Ala gave the highest resistance to vinblastine (27.6-fold above control) but conferred little or no detectable resistance to actinomycin D (2.2-fold) or colchicine and adriamycin (1.0-fold). Similar results were observed for mutant Pro22Q to Ala, which retained resistance to vinblastine (11.2-fold) but had low resistance to actinomycin (7.2-fold) or colchicine and adriamycin (1.0-fold). We have determined the drug resistance profile of clones from different transfections with these mutants and have consistently observed resistance to vinblastine, decreased resistance to actinomycin D, and little or no resistance to colchicine or adriamycin. The other 10 proline mutants yielded drug resistance profiles to all four drugs which were very similar to clones expressing wild-type P-glycoprotein.

There was, however, considerable variability in the level of resistance of the different clones to a given drug. This variability in the resistance levels of each mutant may be a reflection of different levels of P-glycoprotein-A52 expression in each cell line. Accordingly, we measured the amount of P-glycoprotein present in each mutant cell line by applying detergent extracts from a known number of cells onto nitrocellulose and assaying for reactivity with antibody A52. The relative amount of expression was estimated using purified Ca$^{2+}$-ATPase (which contains a single epitope for monoclonal antibody A52) from rabbit fast-twitch muscle as a standard. We have found that the expression level of P-glycoprotein-A52 is quite variable in each stable cell line. In wild-type cells, the amount of P-glycoprotein-A52 expressed was determined to be 63 ± 6 ng/1 x 10^6 cells. Expression in the mutant cell lines ranged from 48 to 131 ng/1 x 10^6 cells. When the relative resistances are standardized to the level of P-glycoprotein-A52 expression (Fig. 4), it was found that all the proline mutants retained resistance to vinblastine (0.8-1.6) with the highest relative resistance being present in mutant Pro22Q to Ala (1.60). By contrast, mutant Pro22Q to Ala and mutant Pro225 to Ala showed significantly decreased relative resistance to colchicine (0.25-0.50), adriamycin (0.25-0.55), and actinomycin D (0.25-0.35), whereas the other proline mutants...
retained relative resistances which were similar to wild-type enzyme in the presence of these drugs (0.9-1.1).

**Drug Accumulation Characteristics of Cell Clones Expressing Mutant P-glycoproteins**—To determine whether changes in drug resistance associated with mutants Pro²²₃ to Ala and Pro⁶⁶ to Ala were due to altered accumulation in NIH 3T3 cells, we compared the uptake of [³H]vinblastine and [³H]colchicine in the untransfected NIH 3T3 cells and in the two mutant cell lines (Fig. 5). NIH 3T3 cells accumulated 5.61 ± 0.31 pmol of [³H]vinblastine/10⁶ cells after 2 h (Fig. 5A). By contrast, wild-type, mutant Pro²²₃ to Ala, and mutant Pro⁶⁶ to Ala accumulated 7.8-, 8.4-, and 6.1-fold, respectively, less vinblastine than control cells. In the presence of 1 μM verapamil, we observed that both Pro²²₃ to Ala and Pro⁶⁶ to Ala mutant cells had colchicine uptake rates similar to that of control NIH 3T3 cells. These changes in accumulation of vinblastine or colchicine parallel the changes in drug sensitivity.

We also tested the ability of verapamil to reverse vinblastine uptake in the mutant cell lines (Fig. 5B). Verapamil is reported to inhibit the binding of vinblastine to P-glycoprotein (Cornwell et al., 1986). The presence of 28 μM verapamil in the incubation medium increased vinblastine accumulation in wild-type, Pro²²₃ to Ala, and Pro⁶⁶ to Ala mutant cells to levels similar to that of control cells (94, 87, and 97% of control cells, respectively). This reversal by verapamil was also observed for these mutants in drug survival experiments. The presence of 1 μM verapamil in the drug survival experiments decreased the relative resistance to vinblastine by approximately 3-fold for each mutant cell line (data not shown).

**Photofinity Labeling with Azidopine**—Safa et al. (1990) demonstrated that a Gly⁶⁶ to Val change increased the binding of azidopine to P-glycoprotein about 4-fold. To determine whether the Pro²²₃ to Ala and Pro⁶⁶ to Ala mutations influenced binding of azidopine to P-glycoprotein, we carried out photofinity labeling experiments with [³H]azidopine. Membrane vesicles prepared from mutant stable cell lines containing equivalent amounts of P-glycoprotein-A52 were incubated with [³H]azidopine. Following UV cross-linking, the vesicles were subjected to SDS-polyacrylamide gel electrophoresis. Autoradiography (Fig. 6) showed that each mutant protein bound azidopine at levels similar to that observed for wild-type P-glycoprotein. In order to assess the sensitivity of the assay, we also carried out azidopine labeling studies on vesicles prepared from mutant Gly⁶⁶ to Val cell lines (cDNA was a gift from Dr. M. M. Gottesman, National Institutes Health). A large increase in azidopine binding was observed for this mutant (Fig. 6) in agreement with published reports (Safa et al., 1990).

**DISCUSSION**

We have characterized the structural and functional consequences of substitution of alanine for each of the 13 proline residues predicted to reside in the transmembrane domain of P-glycoprotein. Mutation of Pro⁶⁶, predicted to be located on the cytoplasmic side of TM7, resulted in a major structural perturbation. The major product of the cDNA of this mutant was a protein product of apparent mass 150 kDa. This product may represent a partially glycosylated intermediate, since a protein of similar size is present as a minor component in immunoblots of cells transfected with wild-type MDR1 cDNA. The major immunoreactive product of wild-type P-glycoprotein-A52 cDNA is a protein of about 170 kDa. In the presence of tunicamycin, however, both wild-type and mutant MDR1 yield proteins of 140 kDa as the major product. Therefore, the 150-kDa protein synthesized in mutant Pro⁶⁶ to Ala is unlikely to be a degradation product of the 170-kDa protein. A possible explanation for the structural aberration introduced by the substitution of alanine for proline is that the mutation alters the orientation of TM7 in the membrane.
which, in turn, prevents the protein from being fully glyco-
sylated. It is important to note that Pro99 is conserved in all
of the isoforms of P-glycoprotein identified in human, mouse,
and hamster (Devine et al., 1991).

Two mutations, Pro23 to Ala and Pro66 to Ala, had pro-
found effects on the drug resistance phenotypes exhibited by
cells expressing the mutant proteins. Resistance to colchicine,
adriamycin, and actinomycin D was greatly decreased relative
to wild-type, whereas resistance to vinblastine was retained.
The relative resistance to vinblastine conferred by the two
mutations was at least equal to or slightly higher than wild-
type when comparisons were made on the basis of level of
expression. Accordingly, a plausible explanation for the re-
sistance phenotypes is that residues in TM4 and TM10 form
part of the binding site for the drugs colchicine, adriamycin,
and actinomycin D, but not for vinblastine. Prolines 223
(TM4) or 866 (TM10) may be important in drug binding by
kinking the helices in such a way as to bring residues involved
in drug binding into optimal positions for interaction with
colchicine, adriamycin, and actinomycin D.

The results of this study add to a growing body of biochem-
ical and genetic evidence that membrane-associated regions
of P-glycoprotein participate in substrate binding and trans-
port. Mutations have been identified within TM6 (Devine et
al., 1992), TM11 (Gros et al., 1991), TM4 and TM10 (this
study), and regions close to TM3 (Choi et al., 1988) that
confer altered drug resistance profiles. Analysis of chimeric
genes constructed between mouse MDR1 and MDR2 genes
has implicated TM5–6 and TM7–8 as essential components in
the transport process (Buschmann and Gros, 1991). Over-
all, these results suggest that the majority of the transmem-
brane segments may participate in the transport process.

A particularly striking observation is that Pro233 and Pro866
overlap when the NH2- and COOH-terminal halves of P-
glycoprotein are aligned on the basis of internal duplication
(van der Blik et al., 1988). This observation implies that
transmembrane segments TM4 and TM10 either interact as
part of a single binding site or form part of two equivalent,
but independent, drug-binding sites in P-glycoprotein. Do-
main mapping studies of the binding sites with photoaffinity
analogs suggest the presence of a binding site in each half
of the molecule. Bruggeman et al. (1988) labeled human P-
glycoprotein with azidopine and subjected the labeled protein
to protease digestion. Using antibodies to specific regions of
the molecule, it was shown that azidopine labeled two differ-
ent region of P-glycoprotein; one in the NH2-terminal and
the other in the COOH terminus. The same conclusion was
reached with mouse P-glycoprotein (Greenberger et al., 1991).
It is not known, however, whether these two regions come
together to form a single binding site or whether there are
two independent binding sites.

The results of this study also suggest that there may be two
different classes of drug-binding sites. Mutants Pro23 to Ala
and Pro866 to Ala reduced transport of colchicine, adriamycin,
and actinomycin D, but not of vinblastine. A separation of
the vinblastine-resistant phenotype from the adriamycin- and
colchicine-resistant phenotypes was also reported for a serine
941 to phenylalanine substitution in TM11 of mouse P-
glycoprotein (Gros et al., 1991). This mutation in mouse
MDR1 resulted in a protein which did not confer resistance
to adriamycin or colchicine but retained the capacity to confer
vinblastine resistance. The opposite effect was reported for a
spontaneous Gly986 to Val mutation. The mutant P-glycopro-
tein conferred increased resistance to colchicine and adria-
mycin but decreased resistance to vinblastine. Evidence for
the presence of two classes of binding sites has also been
observed in photolabeling studies. Labeling of P-glycoprotein
by azidopine was strongly inhibited by vinblastine and ver-
apamil and only weakly by colchicine or adriamycin (Safa
et al., 1987, 1988; Cornwell et al., 1986). Therefore, these results
and those of this study suggest that the binding sites for
vinblastine and colchicine or adriamycin are nonoverlapping.

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REFERENCES
Bell, G. I., Fong, N. M., Stempken, M. H., Wormstedt, M. A., Caput, D., Wu, L.,
14, 8427–8446
921
J. Biol. Chem. 264, 15483–15488
Biochem. 183, 189–197
Chen, C., Chin, J. E., Ueda, K., Clark, D. P., Pastan, I., Gottesman, M. M.,
529
Clarke, D. M., Murayama, K., Luo, T. W., Leberer, E., Inesi, G., and
403–409
Gerlach, J. M., Endicott, J. A., Juranka, P. F., Henderson, G., Sanangi, F.,
7414–7424
Biol. Chem. 265, 4384–4401
Chem. 266, 20744–20753
88, 7289–7293
U. S. A. 85, 3580–3584
Hyde, S. C., Emery, E., Harshorn, M. J., Mimmack, M. L., Gileadi, U., Pearce,
Nature 346, 362–365