Domain Mapping of the Photoaffinity Drug-binding Sites in P-glycoprotein Encoded by Mouse mdr1b*

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P-glycoprotein is an energy-dependent drug efflux pump with broad specificity for hydrophobic antitumor agents such as vinblastine, doxorubicin, and taxol. We have previously shown that [3H]azidopine and [125I]iodoaryl azidoprazosin, which are photoaffinity probes for the a1-adrenergic receptor, respectively, specifically interact with P-glycoprotein, partially reverse multidrug resistance, and bind to a 6-kDa common domain in the 140-kDa P-glycoprotein molecule (Greenberger, L., Yang, C-P., H., Gindin, E., and Horwitz, S. B. (1990) J. Biol. Chem. 265, 4394-4401). An immunological approach was used to identify the position of photoaffinity drug-binding domains in P-glycoprotein. Analysis was done with a series of site-specific rabbit polyclonal antibodies to peptides that mimic domains in the mouse mdr1b gene product. The antibodies were made against amino acid residues 269-284, 356-373, 665-682, 740-750, 907-924, and 1203-1222. Upon trypsin digestion, cleavage products of 95 and 55 kDa were obtained, which after further digestion migrated at 60 and 40 kDa, respectively. The 40-kDa fragment was recognized by the antibodies to residues 1203-1222 and 919-1276, while the 55-kDa fragment was recognized by these antibodies plus antibodies to residues 740-750 and 907-924. In contrast, the 95- and 60-kDa pepptide fragments were recognized only by the antibody to residues 269-284. The 55- and 40-kDa fragments, as well as the 95- and 60-kDa fragments, were major photolabeled species after digestion of P-glycoprotein. The previously identified 6-kDa photolabeled P-glycoprotein fragment was within the 40-kDa trypsin fragment. These data suggest that there are two photoaffinity drug-binding domains in P-glycoprotein encoded by mouse mdr1b. The C-terminal site most likely resides within or in close proximity to putative transmembrane domains 11-12.

Among the 500,000 deaths per year due to cancer in the United States, it is estimated that 90% of them were influenced by the problem of drug resistance to chemotherapeutic agents (Young, 1989). While there are individual mechanisms that may mediate resistance to a single agent (see Pratt and Taylor (1990)), a common mechanism can account for resistance to multiple antitumor drugs of diverse activities and structures such as doxorubicin, vincristine, and, in some cases, etoposide (see Endicott and Ling (1989) and Gottesman and Pastan (1988)). Such multidrug resistance (MDR) can be due to the overexpression of a membrane protein, P-glycoprotein, which is believed to act as an energy-dependent drug efflux pump with broad substrate specificity. While P-glycoprotein can be greatly overproduced in highly resistant tumor cells that are placed under intense selective pressure in tissue culture, much lower levels of P-glycoprotein are found in normal tissue as well as in tumors that have, or acquire, drug resistance (Fojo et al., 1987; Goldstein et al., 1989). Reports indicate that elevated levels of P-glycoprotein in human tumors can be correlated with a poor clinical prognosis (Chan et al., 1990; Sato et al., 1990; Verrelle et al., 1991).

P-glycoprotein (140-170 kDa, approximately 1280 residues) consists of a small family of highly related molecules. Human MDR1, mouse mdr1a, and mouse mdr1b encode molecules capable of mediating MDR (Ueda et al., 1987; Hsu et al., 1989; DeVault and Gros et al., 1990). However, P-glycoprotein encoded by human MDR2 and mouse mdr2 most likely does not mediate MDR (Roninon et al., 1986; Gros et al., 1988; Bushman and Gros, 1991). (Note that mdr1b, mdr1a, and MDR2 are also known as mdr1, mdr3 (DeVault and Gros et al., 1990), and MDR3 (Van der Blik et al., 1987), respectively.) The protein belongs to a superfamily of transport proteins that have six transmembrane-spanning regions followed by a nucleotide binding fold (Chen et al., 1986; Gros et al., 1986). This motif is repeated twice in P-glycoprotein as well as other structurally related molecules that include the cystic fibrosis transmembrane conductance regulator (Riordan et al., 1989), the Plasmidium falciparum P-glycoprotein homolog expressed in chloroquine-resistant parasites (Wilson et al., 1989; Foote et al., 1989), the yeast STE6 gene product (McGrath and Varshavsky, 1989), adenylylcyclase (Krupinski et al., 1989), and the proposed peptide transporters involved in antigen presentation (see Parham (1990)).

Little is known about the molecular mechanisms governing the transport of chemotherapeutic drugs by P-glycoprotein. However, photoaffinity analogs of chemotherapeutic agents such as vinblastine (Cornwell et al., 1986), doxorubicin (Busche et al., 1989), and colchicine (Safa et al., 1989) have been shown to covalently bind P-glycoprotein. In addition, photoaffinity analogs of agents that reverse multidrug resistance, such as phenylalkylamines (e.g. verapamil), 1,4-dihydropyridines (e.g. azidopin), and prazosin bind P-glycoprotein (Safa, 1988; Yang et al., 1988; Greenberger et al., 1990). Among all photoaffinity analogs examined so far, a preferential order of inhibition among P-glycoprotein substrates has been shown to be by order of their affinity for binding to P-glycoprotein (Greenberger et al., 1990).

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1 The abbreviations used are: MDR, multidrug resistance; TM, transmembrane domain(s).
been found. At equal molar concentrations, vinblastine causes profound inhibition of photoaffinity labeling, doxorubicin is less inhibitory, and colchicine is as inhibitory, or less inhibitory, than doxorubicin (see Greenberger et al., 1990). Kinetic analysis suggests that P-glycoprotein substrates competitively inhibit the interaction of chemotherapeutic drugs selected P-glycoprotein (Horio et al., 1988; Naito and Taura, 1989). These data suggest that reversal agents and chemotherapeutic drugs share common binding domains in P-glycoprotein. Consistent with this deduction, azidopine and isoaryl azidoprazosin share a common 6-kDa binding fragment in P-glycoprotein (Greenberger et al., 1990).

The purpose of this study was to localize the covalent drug-binding site(s) in P-glycoprotein. An immunological approach with a battery of newly generated site-directed polyclonal antibodies has been used to map the binding site of isoaryl azidoprazosin in P-glycoprotein. It is concluded that, like P-glycoprotein encoded by MDRI in human cells (Bruggemann et al., 1988; Yoshimura et al., 1989), P-glycoprotein encoded by mdr1b has one photoaffinity drug-binding site in each half of P-glycoprotein. The domain in the C-terminal half contains only the two last putative membrane domains (TM 11 and 12). This binding domain is in a homologous region compared with a binding domain of a verapamil analog (LU 49888) found in the α1 subunit of the L-type calcium channel (Streisang et al., 1990) and may help explain how verapamil can interact with both P-glycoprotein and the calcium channel.

MATERIALS AND METHODS

Antibody Production—Site-directed polyclonal antibodies to synthetic peptides were produced in New Zealand White rabbits. Peptides were manufactured on an Applied Biosystems peptide synthesizer at the Laboratory for Molecular Analysis at Albert Einstein College of Medicine and purified by high pressure liquid chromatography on a C8 Aquapore Prep-10 reverse phase column. Purified peptides were conjugated to keyhole limpet hemocyanin using glutaraldehyde according to published methods (Jennis and Stumpf, 1983). Rabbits were injected with 150–250 μg of the peptide conjugate in the presence of Freund's complete adjuvant according to published procedures (Harlow and Lane, 1988). A subsequent boost with 200 μg of the peptide-keyhole limpet hemocyanin conjugate in Freund's incomplete adjuvant was done at approximately 3-week intervals 2–4 times. Rabbits were bled approximately every 3 weeks. Bleeds were screened by Western blot immunosay using membranes from cells known to express high levels of P-glycoprotein encoded by mdr1b. In some experiments, antibody to residues 289–294 was purified on an affinity column containing the appropriate peptide linked to Affi-Gel 10 (Bio-Rad).

Three existing site-directed polyclonal antibodies to P-glycoprotein were also used in this study. Antibody to amino acid residues 665–682 has been described by Hsu et al., 1989. Antibody to residues 1203–1222 was obtained from Oncogene Sciences Inc. (Manhasset, NY). Antibody known as "4037," which was made against a bacterial polypeptide composed of amino acid residues 919–1280 of human MDRI, was a generous gift from Dr. M. Gottesman and collaborators (Takata et al., 1990).

Cell—Mouse multidrug-resistant cells were derived from J774.2 macrophage-like cells (Greenberger et al., 1988a). J7, V1–1 and J7, V3–1 cells are approximately 1000-fold resistant to vinblastine and produce P-glycoprotein encoded by mdr1b and mdr1a, respectively (Greenberger et al., 1988a; Hsu et al., 1989). A clone derived from a subpopulation of small size (S1) human ovarian carcinoma cells (OVCAR-3) was made multidrug-resistant by stepwise selection in bisantrene. The latter cells, designated OVC/S1–S1–20, are maintained in 20 μM bisantrene and are >600, 450, and 250-fold resistant to bisantrene, vinblastine, and doxorubicin, respectively.

Membrane and Vesicle Preparation—Membranes were prepared by differential centrifugation and sucrose gradient separation according to previously published methods (Greenberger et al., 1987). Vesicles were prepared by nitrogen cavitation and sucrose gradient centrifugation according to Lever (1977). The final pellet of vesicles was resuspended and stored in 10 mM Tris, pH 7.4, containing 250 mM sucrose (Solution D) in the absence of protease inhibitors at −70°C.

Photoaffinity Labeling of P-glycoprotein—Photo-labeling with [125I]iodoaryl azidoprazosin (2200 Ci/mmol, Du Pont-New England Nuclear) was done as previously described (Greenberger et al., 1990). Briefly, 50 μg/50 μl vesicles were placed in Solution D and incubated with 4–20 μM [125I]iodoaryl azidoprazosin in the dark for 30 min at room temperature. Samples were then irradiated with UV light for 5 min at 4°C. After centrifugation (100,000 × g) for 15 min at 4°C in a Beckman Airfuge, the vesicle pellets were resuspended in 10 μl of Solution D.

Differential centrifugation and sucrose gradient separation according to Lever (1977). The final pellet of vesicles (5 mg/ml) was incubated in 0–250 μg/ml tosylphenylalanine chloromethyl ketone-treated trypsin (Worthington, 248 units/mg) for 1 h at 37°C in a total volume of 20 μl. Digestion was stopped by the addition of aprotinin, phenylmethylsulfonyl fluoride, and soybean trypsin inhibitor to attain the final concentrations of 0.25 μg/ml, 5.5 mM, and 1.0 μg/ml, respectively. After a 5-min incubation at 4°C, samples were either solubilized in Laemmli sample buffer, resolved on 10% gels, transferred to nitrocellulose, and probed according to previously described methods (Greenberger et al., 1987) or prepared for immunoprecipitation by solubilizing the material in an equal volume of 2% sodium dodecyl sulfate containing 2 mg/ml bovine serum albumin in 50 mM Tris, pH 7.4 (lysis buffer). All material in Laemmli sample buffer was intentionally not boiled prior to gel electrophoresis since P-glycoprotein and its fragments migrate aberrantly if boiled (Greenberger et al., 1988b).

Immunoprecipitation and Deglycosylation—Samples in lysis buffer were diluted 5-fold in 1.25% Triton X-100, 190 mM NaCl in 50 mM Tris, pH 7.4, and immunoprecipitated as previously described (Greenberger et al., 1987). Briefly, after incubation with antisera overnight at 4°C, protein A-Sepharose (Pharmacia LKB Biotechnology Inc.) was added. Samples were incubated for 2 h, washed, and bound material was eluted into Laemmli sample buffer or 0.5% sodium dodecyl sulfate containing 0.1 M 2-mercaptoethanol in 0.2 M phosphate buffer, pH 8.6. The latter material was subjected to enzymatic removal of N-linked carbohydrate by N-Glycanase (Genzyme, Boston, MA) according to the manufacturer's suggestions. Digestion was stopped by the addition of 5 × concentrated Laemmli sample buffer and resolved in 10% gels. Radiolabeled polypeptides were detected by autoradiography with an intensifier screen.

Cleveland Mapping—Peptide mapping with Staphylococcus aureus V8 protease (Sigma, 720 units/mg) was done as described by Cleveland et al. (1977).

RESULTS

Site-directed Peptide Antibodies—An attempt was made to raise seven new site-directed antibodies to P-glycoprotein encoded by mdr1b (Table I, numbers 1, 2, 4, 5, and 7–9) (Fig. 1). Each of these proposed regions was chosen because antibodies to such peptides were predicted to reside in an extracellular or cytoplasmic, hydrophilic domain that would share little similarity with the homologous region in the other half of P-glycoprotein (Table I, "Percent homology" column). Each peptide-keyhole limpet hemocyanin conjugate was injected into a pair of rabbits. Antibodies of sufficient quality were successfully obtained to four of these synthetic peptides (numbers 1, 2, 4, and 5). In general, high titer serum, if obtained, was present in both animals. Relatively high titer sera were obtained against synthetic peptides 1, 4, and 5. Moderate titer antisera was obtained to synthetic peptide 2. Low titer serum was obtained to synthetic peptide 8 and was not usable for this study. Essentially no titer was obtained against synthetic peptide 7 or 9.

Three existing site-directed polyclonal antibodies were used in this study. Two antibodies were made against synthetic peptides that mimicked amino acids in the mdr1b gene product (Table I, numbers 3 and 6) (Fig. 1). Antibody 3 was directed to residues 665–682 and is known to selectively recognize P-glycoprotein encoded only by mouse mdrlb but not mouse mdrla (Hsu et al., 1989). Antibody 6 was directed to residues 1203–1222 (obtained from Oncogene Sciences, Inc.). This epitope is found immediately C-terminal to the...
tein but had considerable nonspecific binding to multiple polypeptides of 45-60 kDa. The use of the latter antibody unequivocally distinguishes different halves of P-glycoprotein.

The binding regions for the site-directed antibodies used in this study are shown on a model of P-glycoprotein. Numbers correspond to position of site-directed antibodies and peptides described in Table I. Dots indicate differences in amino acids between the mouse mdrla and mdrlb gene products. NC2 indicates nucleotide-binding site. Not shown is antibody 4007, which was made against amino acids 919-1280 of human MDR1 expressed in bacteria (see Tanaka et al., 1990).

Fig. 1. Location of site-directed antibodies to P-glycoprotein. The binding regions for the site-directed antibodies used in this study are shown on a model of P-glycoprotein. Numbers correspond to position of site-directed antibodies and peptides described in Table I. Dots indicate differences in amino acids between the mouse mdrla and mdrlb gene products. NC2 indicates nucleotide-binding site. Not shown is antibody 4007, which was made against amino acids 919-1280 of human MDR1 expressed in bacteria (see Tanaka et al., 1990).

second dinucleotide fold consensus site within P-glycoprotein. It has 90% conservation with the homologous region in the N-terminal half of P-glycoprotein. The third site-directed antibody, known as 4007, was directed against a fragment from the C-terminal P-glycoprotein that spans residues 919-1280 and is encoded by human MDR1 (Bruggemann et al., 1989; Tanaka et al., 1990). Since this region included the entire highly conserved nucleotide binding fold that is found in both halves of P-glycoprotein, antibody 4007 cannot unequivocally distinguish distinct halves of P-glycoprotein.

The binding of the antibody from the newly made site-directed antibodies to P-glycoprotein was specific since antibody binding to P-glycoprotein was blocked by the peptide that was used as an antigen (Fig. 2). Note that antibodies 1 and 5, which were made against amino acid residues 269-284 and 907-924, respectively, were directed against exactly homologous regions in each half of P-glycoprotein. However, there was no cross-reactivity between the antisera, since the synthetic peptides which mimic the homologous region did not block P-glycoprotein recognition. Hence, these antisera unequivocally distinguish different halves of P-glycoprotein. Both antibodies 4 and 5 specifically detected a minor band at 55 kDa; this is likely to be a proteolytic fragment of P-glycoprotein (see below). Antibody 2 recognized P-glycoprotein but had considerable nonspecific binding to multiple polypeptides of 45-60 kDa. The use of the latter antibody was restricted since it was not possible to affinity purify the antibody.

The specificity of the different antibodies for different P-glycoprotein family members was explored (Fig. 3). Mouse J7.V1-1 and J7.V3-1 cell lines express P-glycoprotein encoded by mdrlb and mdrla, respectively (Hsu et al., 1989). A human ovarian carcinoma cell line derived from OVCAR-3 cells, which was selected with bisantrane, overexpresses P-glycoprotein encoded by MDR1. Antibodies 1, 5, and 6 cross-reacted with P-glycoprotein encoded by mouse mdrla and mdrlb as well as human MDR1. This was expected, since these antibodies were directed against regions of P-glycoprotein family members that share 89-100% homology (Table I). Antibodies 3 and 4 were made against peptides whose amino acid sequences share 55 and 22% homology with the coding sequence in murine mdrla, respectively. These antisera reacted only with P-glycoprotein encoded by mdrlb (in J7.V1-1 cells). Antibody 4, despite being directed to a region that shares 75% homology to the sequence found in human P-glycoprotein encoded by MDR1, detected P-glycoprotein encoded only by murine mdrlb.

Identification of Domains of Tryptic Fragments from P-glycoprotein—Digestion of P-glycoprotein in vesicles was done, and the positioning of the subsequently generated frag-

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### TABLE I

Antibodies to synthetic peptides that mimic domains in P-glycoprotein encoded by mdrlb

<table>
<thead>
<tr>
<th>Synthetic peptide</th>
<th>Amino acid location</th>
<th>No. of amino acids</th>
<th>Percent homology with</th>
<th>Titer</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. QQKELERYNKNL</td>
<td>269-284</td>
<td>16</td>
<td>12</td>
<td>91</td>
</tr>
<tr>
<td>2. NARGAEEFPKID</td>
<td>356-373</td>
<td>16</td>
<td>11</td>
<td>84</td>
</tr>
<tr>
<td>3. SLYRSHKRDQER</td>
<td>665-682</td>
<td>17</td>
<td>6</td>
<td>22</td>
</tr>
<tr>
<td>4. yDDHETKRNQ</td>
<td>740-750</td>
<td>12</td>
<td>11</td>
<td>94</td>
</tr>
<tr>
<td>5. SLTREQFETMYAQLV</td>
<td>907-924</td>
<td>11</td>
<td>11</td>
<td>94</td>
</tr>
<tr>
<td>6. cALTDSEKVQ</td>
<td>1203-1222</td>
<td>20</td>
<td>90</td>
<td>100</td>
</tr>
</tbody>
</table>

*Upper and lower case letters indicate amino acids found and not found in mdrlb, respectively.

Comparison with the homologous half in mdrlb, and the same half in mouse mdrla and human MDR1.

Relative titer on a scale based on visual inspection of immunoreactivity with P-glycoprotein in Western blot immunoassay.

Antigen mdrlb peptide from the linker region (Hsu et al., 1989).

Peptide and antiserum obtained from Oncogene Sciences, Inc.

Antisera 7-9 were not used in this study.

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![Diagram](image-url)
ments was assigned by probing the resultant tryptic fragments with site-specific antibodies (Fig. 4, A and B). When low amounts of trypsin were used (0.5 μg/ml), antibody 1 was the only antibody that detected a broad, faint band at 95 kDa. Antibodies 3, 4, 5, 6, and 4007 detected one or more of the set of fragments at 56–54 (triplet) kDa. Antibody 3 was the only antibody that strongly detected the 56-kDa species but not the 55- or 54-kDa species. If 5.0 μg/ml trypsin was used, the 56-kDa fragment was not detected by any antibody. However, the ability of antibodies 4, 5, 6, and 4007 to detect the 55- and 54-kDa fragments increased. When 10-fold more trypsin was used (50 μg/ml), the intensity of the 55–54-kDa bands was reduced, and a 40-kDa band was detected by antibodies 6 and 4007, but not antibody 4 or 5. The intensity of the 40-kDa band, as detected by antibody 6, increased when 500 or 1250 μg/ml trypsin was used (Fig. 4B). Such an increase in the 40-kDa species appeared to be correlated with reduction in the intensity of the 55–54-kDa band. Furthermore, antibody 4 consistently identified a species at approximately 18 kDa which was likely to be a fragment of the 55–54-kDa species since detection of an 18-kDa fragment coincided with the disappearance and appearance of the 55–54- and 40-kDa fragments, respectively (Fig. 4B).

The analysis described above suggested that P-glycoprotein is initially digested into 95- and 56–54-kDa fragments. These fragments are located in the N-terminal and C-terminal half of the molecule, respectively. The N-terminal of the 56–54-kDa fragment is likely to be between residues 665 and 740 since antibody 3 (directed to residues 665–682), but not antibody 4 (directed to residues 740–750), detected only the 56-kDa fragment and not the 55-kDa fragment. The C-terminal end of the 56-kDa fragment extends at least to residue 1203 since it is detected by antibody 6 (directed to residues 1203–1222). This fragment most likely extends to the C terminus of P-glycoprotein since Bruggemann et al. (1989) have identified a photolabeled 55-kDa tryptic fragment from human MDR1 gene that extends to the C terminus. The 40-kDa fragment probably begins after 919–924 since it was not detected by antibody 5 (directed to residues 907–924) but was detected by antibody 4007 (directed to residues 919–1280). The 40-kDa fragment probably also extends to the C terminus since it was detected by antibody 6, and the predicted 15-kDa fragment from the 55-kDa precursor was identified as a band migrating at 18 kDa by antibody 4. Assuming cleavage occurs between Arg919 and Arg1203 and the 40-kDa fragment extends to the C terminus of P-glycoprotein, the predicted molecular mass is 39–41 kDa. This is in good agreement with the observed molecular weight. In the case of the 55-kDa fragment, cleavage could occur between Arg919 and Lys1203. The predicted molecular mass of the 55-kDa fragment is 61–67 kDa. The 6–14-kDa discrepancy between the observed and predicted size of the 55-kDa fragment may be due to aberrant mobility of this fragment on gels (Greenberger et al., 1988b). This could help explain why the deglycosylated precursor for P-glycoprotein migrates at 120 kDa (Greenberger et al., 1987) while the predicted mass is 140,000 (Gros et al., 1986).

Bruggemann et al. (1989) have reported that, in the human MDR1 gene product, the 55-kDa photolabeled fragment is digested to 40 kDa. They conclude that the 40-kDa fragment begins near amino acid 740 and extends beyond putative transmembrane domain 12 (residue 986) to approximately residue 1070. Since the positioning of the 40-kDa tryptic fragment from mdr1b P-glycoprotein was strikingly different compared with MDR1, further studies were done to confirm the positioning of the 40-kDa species described here. In this study, the 40-kDa fragment was detected by antibodies 6 and 4007. Since both of these antibodies may cross-react with the first half of P-glycoprotein (see Table I), it was possible that the 40-kDa fragment could be derived from an unidentified fragment that could extend from residue 284 to 662. Such a fragment would not be detected by antibody 1 or 3 and would have a predicted molecular mass of approximately 42 kDa. Therefore, further analysis was done to prove that the 40-kDa fragment was derived from the 55-kDa fragment and to confirm the positioning of the 40-kDa fragment (Fig. 5). When more carefully titrated amounts of trypsin were used, it was clear that antibody 6 detected a precursor/product relationship between the 55- and 40-kDa fragments. Antibody 2 (directed to residues 356–373) was useful for positioning the 40-kDa fragment. Despite the high nonspecific binding in the 45–60-kDa region of the blot, antibody 2 did not detect the 40-kDa fragment. Therefore, the 40-kDa fragment does not reside in the first half of P-glycoprotein and must be C-terminal to residues 907–924. Fragments at 95 and 30 kDa were also detected with antibody 2. However, binding to the 30-kDa fragment was not competed with an excess of peptide 2 (data not shown).
Fig. 4. Trypsin digestion profile of the mdr1b gene product. Vesicles (50 μg) containing P-glycoprotein from V1 cells were digested with trypsin for 1 h at 37 °C. Polypeptides were resolved in gels and transferred to nitrocellulose. Blots were probed with the indicated antibody. Antibody dilutions were as in Fig. 3. Arrows from top to bottom are: panel A, 95-, 56-, 54-, and 40-kDa species; panel B, 55-, 54-, 40-, and 18-kDa species.

Fig. 5. Relationship between and positioning of the 55- and 40-kDa fragments. Vesicles were digested with the indicated amounts of trypsin as in Fig. 4. Blots were probed with the the concentrations of antibodies stated in Fig. 3. Analysis and Positioning of Photoaffinity-labeled Fragments from P-glycoprotein—The identification of the photolabeled tryptic fragments of P-glycoprotein was done by first photo-labeling vesicles prior to digestion. The resultant radioactive fragments were then identified by immunoprecipitation followed by gel electrophoresis and autoradiography (Fig. 6). Antibody 1 immunoprecipitated a photolabeled trypsin fragment with a broad smear of 95–65 kDa while antibodies 6 and 4007 immunoprecipitated the 55- and 40-kDa trypsin fragments. Antibody 5 immunoprecipitated only the 55-kDa trypsin fragment; identical results were obtained if antibody 4 was used (data not shown).

The 40-kDa Tryptic Fragment Contains a Small Common Binding Domain for Structurally Diverse Photoaffinity Probes—It has previously been shown that two structurally dissimilar photoaffinity probes for P-glycoprotein bind to a common domain in P-glycoprotein (Greenberger et al., 1990). The smallest size of this domain that has been obtained is 6 kDa. Cleveland mapping was used to determine if the 6-kDa fragment resides in the 40-kDa fragment, and thereby, in the C terminus of P-glycoprotein (Fig. 7). To do this, intact P-glycoprotein was photolabeled, and the 55- and 40-kDa tryptic fragments were identified in the first gel. These bands were then comigrated with S. aureus V8 protease in a second
Phy.

procedure. Radiolabeled fragments were identified by autoradiography. Fragments of 140 kDa (intact P-glycoprotein), 55 kDa, 48 kDa, and 40 kDa were subjected to a second digestion by the Cleveland mapping procedure. Radiolabeled fragments were identified by autoradiography.

Iodoaryl azidoprazosin

ers are known not to be derived from P-glycoprotein (see Fig. 6). It was found that the 140-, 55-, and 40-kDa fragments were digested to a 6-kDa species. In contrast, the 48-kDa fragment gave a distinct digestion profile in which the minimal fragment sizes were 7 kDa and less than 5.5 kDa; this species in gels. The 40-kDa fragment spans transmembrane domains 11 and 12. If it is assumed that the photoaffinity labeling site is within the membrane segment, TM 11-12 is a likely photoaffinity drug-binding domain in P-glycoprotein (Fig. 9).

Drug-binding Domains in P-glycoprotein—The data reported here demonstrate that mouse P-glycoprotein encoded by mdr1b contains two photoaffinity binding domains distributed in the N- and C-terminal halves of the molecule (Fig. 9). The C-terminal fragment must reside C-terminal to amino acid residue 924 since the fragment was not detected by an antibody directed to residues 907–924 but was detected by antibody directed to 919–1280 and 1203–1222. Consistent with this prediction, we have recently obtained an antibody directed to residues 1008–1019 that binds to the 55- and 40-kDa fragments. The size of the photolabeled trypsin fragments (95 and 55 kDa) for P-glycoprotein encoded by mdr1b has the same mobility in gels. Such fragments are probably identical to previous studies using vesicles (Yoshimura et al., 1989) or purified and detergent-solubilized P-glycoprotein (Bruggemann et al., 1989). It is possible that the 40-kDa fragment identified in detergent-solubilized P-glycoprotein is distinct from the 40-kDa fragment identified in vesicle digestion experiments. An advantage to digesting P-glycoprotein in vesicles is that the native conformation of P-glycoprotein is maintained and thereby provides structural information about the protein with respect to the membrane.

The 95-kDa Trypsin Fragment Contains N-Linked Carbohydrate—P-glycoprotein encoded by mdr1b is known to contain approximately 15 kDa of N-linked carbohydrate (Greenberger et al., 1987). Such carbohydrate is likely to be attached to Asp35, Asp91, Asp96, and Asp100 within any of the four external N-linked glycosylation consensus sites. Therefore, it was predicted that the 95-kDa fragment contains carbohydrate. This was verified by examining the effects of endoglycosidase F on the mobility of P-glycoprotein tryptic fragments that were photoaffinity-labeled (Fig. 8). When 0.5–25 µg/ml trypsin were used, antibody 1 immunoprecipitated broad bands at approximately 95 and 50–60 kDa. There was a precursor/product relationship between these bands, such that the 95-kDa fragment was converted to the 50–60-kDa species. When the tryptic fragments were subjected to endoglycosidase F treatment, the 95- and 50–60-kDa bands migrated as sharp bands at 60 and 38 kDa, respectively. In contrast to the fragments obtained with antibody 1, the mobility of the 55- and 40-kDa fragments, which were detected with antibody 6, was not altered after treatment with endoglycosidase F.

**FIG. 8.** Carbohydrate digestion of photolabeled fragments in P-glycoprotein. The photolabeled trypsin fragments from P-glycoprotein were immunoprecipitated as described in Fig. 6 and subjected to N-linked carbohydrate digestion. Molecular mass markers are 200, 116, 97, 68, 43, and 30 kDa.

**FIG. 9.** Location of photoaffinity drug-binding domains in P-glycoprotein. The location of the tryptic fragments from P-glycoprotein is shown. The first and second sets of tryptic fragments are obtained with 5.0 and 50 µg/ml trypsin. The two major fragments that covalently bind drug are in gray and black. Antibody numbers are as in Fig. 1. The model of P-glycoprotein is based on a hydrophathy plot (Chen et al., 1986; Gros et al., 1986); boxes indicate nucleotide-binding domain.

**DISCUSSION**

Drug-binding Domains in P-glycoprotein—The data reported here demonstrate that mouse P-glycoprotein encoded by mdr1b contains two photoaffinity binding domains distributed in the N- and C-terminal halves of the molecule (Fig. 9). The C-terminal fragment must reside C-terminal to amino acid residue 924 since the fragment was not detected by an antibody directed to residues 907–924 but was detected by antibody directed to 919–1280 and 1203–1222. Consistent with this prediction, we have recently obtained an antibody directed to residues 1008–1019 that binds to the 55- and 40-kDa fragments. The size of the photolabeled trypsin fragments (95 and 55 kDa) for P-glycoprotein encoded by mdr1b has the same mobility in gels. Such fragments are probably identical to previous studies using vesicles (Yoshimura et al., 1989) or purified and detergent-solubilized P-glycoprotein (Bruggemann et al., 1989). It is possible that the 40-kDa fragment identified in detergent-solubilized P-glycoprotein is distinct from the 40-kDa fragment identified in vesicle digestion experiments. An advantage to digesting P-glycoprotein in vesicles is that the native conformation of P-glycoprotein is maintained and thereby provides structural information about the protein with respect to the membrane.

The 95-kDa fragment spans transmembrane domains 11 and 12. If it is assumed that the photoaffinity labeling site is within the membrane segment, TM 11–12 is a likely photoaffinity drug-binding domain in P-glycoprotein (Fig. 9). By analogy, TM 5 and 6 would be the drug-binding domain in the N-terminal half of P-glycoprotein. As components of the drug-binding sites in P-glycoprotein are further resolved, this information will be useful for developing models to understand how P-glycoprotein transports chemotherapeutic agents.

Independent evidence supports the conclusion that a photoaffinity drug-binding domain resides in TM 11–12. First, since P-glycoprotein has an affinity for hydrophobic agents (such as iodoaryl azidoprazosin), the binding site within the C-terminal fragment is most likely to reside in a hydrophobic region.

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*2 L. M. Greenberger, personal observation.*
drug-binding pocket or channel that mediates P-glycoprotein with photoaffinity probes has limitations. First, it cannot be
voltage-gated ion channels contain hydrophobic, highly conserved amino acid residues, and these residues are localized within transmembrane domains. In addition, other lipophilic photoaffinity probes for other heavily embedded membrane proteins have been localized within transmembrane domains (Wadzinski et al., 1990; Matsui et al., 1989; Giraudat et al., 1987). Second, based on the work of Bruggemann et al. (1989), the region of overlap between the C-terminal binding domain in human MDR1 and the work reported here is in TM 11–12. Third, TM 11 is perfectly conserved between two mouse genes known to mediate MDR (Hsu et al., 1989; Gros et al., 1986; DeVault and Gros, 1990) (see Fig. 1 for a comparison of mdr-1 and mdr-2). When all other transmembrane domains between mdr-1 and mdr-2 are compared, 1–10 different amino acids are found. Finally, it is interesting to note that mouse mdr-2, which encodes a P-glycoprotein homolog that does not mediate MDR (Hsu et al., 1989), has 5/21, 6/21, and 9/16 divergent amino acid residues compared with mdr-1 within TM 11, TM 12, and the intracellular loop between these putative transmembrane domains, respectively.

Characterization of drug-binding sites in P-glycoprotein with photoaffinity probes has limitations. First, it cannot be certain if the site of covalent attachment of iodoaryl azidoprazosin is within or behaves like a reporter site for drug-binding domains in P-glycoprotein (see Glossman et al. (1987)). Furthermore, additional work is needed to determine if the covalent binding sites identified in each half of P-glycoprotein actually constitute a single binding site or represent two independent drug-binding sites. Finally, it is likely that the covalent drug-binding sites are only part of a three-dimensional drug-binding pocket or channel that mediates P-glycoprotein-dependent efflux of hydrophobic agents.

Relationship between Drug-binding Domains in P-glycoprotein and Other Membrane Proteins—Recently, a photoaffinity binding domain of a verapamil analog, LU-49888, has been identified in the α-subunit of the L-type calcium channel (Striessnig et al., 1990). The α-subunit consists of four linked cassettes; each cassette is composed of six transmembrane domains (S1–S6). The covalent binding domain for LU-49888 is in, and immediately surrounds, the sixth transmembrane domain of the fourth cassette. This agent also covalently binds to P-glycoprotein (Qian and Beck, 1990).

The principal subunits of the Na⁺, K⁺, and Ca²⁺ voltage-gated ion channels contain hydrophobic, highly conserved, S6 segments (Catterall, 1988). Experiments employing site-directed mutagenesis and biochemical analyses of drug-binding domains implicated the S6 region in the formation of the walls of the transmembrane pore of these channels. We hypothesize that the covalent binding domain for LU-49888 within P-glycoprotein and the α-subunit of the calcium channel may occur at homologous sites despite the apparent lack of primary structure conservation within their putative transmembrane regions. Similar proposals have been suggested to account for the action of forskolin on adenylate cyclase, the glucose transporter, and voltage-gated ion channels (Laurensa et al., 1989). If true, this may help explain why verapamil can both reverse P-glycoprotein-mediated MDR and block the calcurop channel. In support of this prediction, molecular modeling with favorable energy conformations of verapamil and azidoprazosin demonstrate that these molecules share highly related three-dimensional spaces. Since azido-

pine and iodoaryl azidoprazosin share a common drug-binding domain in the 40-kDa fragment of P-glycoprotein, and azidoprazosin and verapamil share structural homology, LU-49888 is likely to bind to TM 11–12 in P-glycoprotein (the equivalent of S5–S6 in the α-subunit of the L-type calcium channel).

A P-glycoprotein homolog, designated pfmdr1 (1419 amino acids), has been described as a gene associated with chloroquine-resistant strains of Plasmodium falciparum (Foote et al. (1989) but cf. Wellems et al. (1990)). Such parasites are found in drug-resistant cases of malaria. Among the 34 chloroquine-resistant strains of P. falciparum examined, two resistant alleles, defined by Tyr¹⁰⁴/Asp¹⁰⁴/Tyr¹⁰⁴, respectively, were predictive for chloroquine resistance (Foote et al., 1990). Within these alleles, two resistant strains (BT3 and Pf121) had a mutation only at one residue (Asp¹⁰⁴). Residues 1034 and 1042 reside in putative TM 11 of pfmdr1 and thereby suggest that this domain may participate in the transport of chloroquine.

In conclusion, the data presented suggests that P-glycoprotein encoded by murine mdr-1, like the human form of P-glycoprotein encoded by MDR1, has two photoaffinity drug-binding sites in P-glycoprotein. One binding site is found in each half of P-glycoprotein. The most likely region of the C-terminal binding site is within putative transmembrane domains 11–12. The functional significance of this prediction awaits further investigation.

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