Identification of the Domains of Photoincorporation of the 3′- and 7-Benzophenone Analogues of Taxol in the Carboxyl-Terminal Half of Murine *mdr*1b P-glycoprotein†

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ABSTRACT: P-glycoprotein is an ATP-dependent drug-efflux pump that can transport a diverse range of structurally and functionally unrelated hydrophobic compounds across the plasma membrane. The transporter is composed of two homologous halves, each containing a nucleotide binding fold and six putative transmembrane spanning segments. The contact domains between the murine *mdr*1b P-glycoprotein and two photoreactive Taxol analogues have been mapped by a combination of CNBr digestion and immunoprecipitation studies. We had demonstrated previously that the 3′-*p*-benzoyldihydrocinnamoyl (BzDC) analogue of Taxol specifically photolabeled *mdr*1b P-glycoprotein and now show that the corresponding C-7 analogue likewise specifically photoincorporates into the transporter. CNBr digestion of both photolabeled P-glycoproteins gave rise to an approximate 10 kDa tritium-labeled peptide, each of which was a distinct polypeptide. The CNBr fragment generated from the 3′-BzDC-Taxol-photolabeled P-glycoprotein was immunoprecipitated by a polyclonal antibody (Ab7) raised against amino acid residues 1008−1019 of the *mdr*1b isoform. In contrast, the CNBr fragment generated from the 7-BzDC-Taxol-photolabeled P-glycoprotein was immunoprecipitated by a polyclonal antibody (Ab4) raised against amino acid residues 740−750. The specificity of these reactions was demonstrated by showing that the presence of the appropriate synthetic peptide blocked the immunoprecipitation. Moreover when the antibodies were reversed, no immunoprecipitation occurred. Based on the deduced amino acid sequence of *mdr*1b P-glycoprotein, and its hydropathy plot analysis, our data indicated that the 3′-BzDC group photoincorporates into amino acid residues 985−1088, a region of the transporter that includes half of TM 12 and terminates just after the Walker A motif in the second nucleotide binding fold. The 7-BzDC group photoincorporates into amino acid residues 683−760, a region of the transporter that includes all of TM 7 and half of TM 8 plus the intervening extracellular loop.

The characteristics of multidrug resistant (MDR) cells[1] (see *1*−*3*) include (a) decreased intracellular drug accumulation as a consequence of increased drug efflux, (b) overproduction of a plasma membrane phosphoglycoprotein termed P-glycoprotein that is responsible for reduced drug accumulation, and (c) amplification and/or mRNA overexpression of the gene encoding P-glycoprotein. P-glycoprotein is encoded by two classes of *mdr* genes, I and II. In the human, there is one member of each class, whereas in the mouse there are two members (*mdr*1a and *mdr*1b) in class I and one member in class II (*2*, *3*). Studies have indicated that the overproduction of P-glycoprotein is a major factor in mediating MDR. For example, drug-sensitive cells acquire the MDR phenotype after transfection of the gene encoding P-glycoprotein (see *2*, *3*). However, only class I genes, but not class II, can confer resistance. It is generally believed that class I P-glycoprotein is an energy-driven drug-efflux pump with a broad specificity for hydrophobic agents (*4*−*9*). Recent studies have shown that the protein encoded by the class II *mdr* gene is a phosphatidylcholine-specific translocase (*10*).

P-glycoprotein is a member of the ABC (ATP-binding cassette) superfamily of membrane proteins. Sequence analysis of the *mdr* genes indicates that P-glycoproteins are composed of two homologous halves, each containing a nucleotide binding consensus sequence. Hydropathy plot analysis predicts that each half-domain of P-glycoprotein has six α-helical transmembrane (TM) spanning segments with both the N- and C-termini having a cytoplasmic orientation. The two homologous half-domains are separated by an intracellular "linker" region of approximately 60 amino acid residues.

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[1] Abbreviations: BzDC, *p*-benzoyldihydrocinnamoyl; CNBr, cyanogen bromide; HPLC, high-pressure liquid chromatography; MDR, multidrug resistance; TM, transmembrane; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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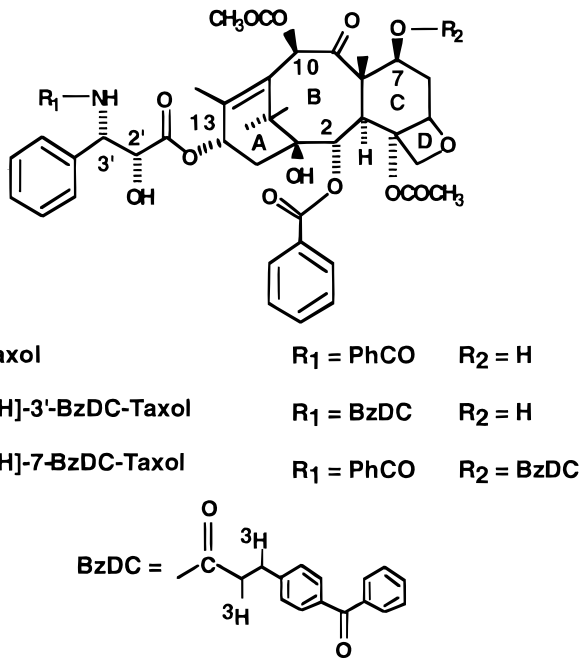

FIGURE 1: Structures of Taxol and the [^{3}H]-C-3′- and [^{3}H]-C-7-BzDC analogues of Taxol.

Our understanding of how P-glycoprotein interacts with and transports such a diverse range of chemotherapeutic drugs, steroids, hydrophobic peptides, and reversal agents is rudimentary. Important questions include the precise number of binding sites on the transporter, whether substrates and reversal agents bind to the same, overlapping, or distinct sites, and what polypeptide domains of the transporter are involved in forming the framework for these sites. Photoaffinity labeling studies using both substrate and reversal agent analogues have been used to gain insight into some of these questions (*11*−*25*). Photoreactive analogues of *vinca alkaloids*, prazosin, verapamil, daunomycin, calcium channel blockers, forskolin, and cyclosporin have all been shown to photolabel P-glycoprotein. In some cases the domains of photoincorporation have been determined. Photoreactive calcium channel blockers such as iodoarylazidoprazosin were shown to label two regions, one in each half of the transporter, that included domains within or immediately C-terminal to TM 6 and TM 12 (*18*). It has been suggested that P-glycoprotein contains two nonidentical drug interaction sites for this prazosin analogue (*24*). In contrast, iodomycin, a photoreactive analogue of daunomycin, incorporated into residues 230−312, which include parts of TM 4 and TM 5 plus the intervening cytoplasmic loop (*25*).

We report in this study the identification of the sites of incorporation in the C-terminal half of P-glycoprotein of two photoreactive Taxol analogues containing a BzDC[1] substituent at either the C-3′ or the C-7 position (Figure 1). Both analogues are substrates for the transporter. Our data show that the C-3′ analogue photoincorporates into residues 985−1088, a region of the *mdr*1b P-glycoprotein which includes the cytoplasmic half of TM 12 and extends into the final cytoplasmic segment. The C-7 analogue, in contrast, labels residues 683−760, a region of the transporter which includes all of TM 7 and half of TM 8 plus the intervening exoplasmic loop.

MATERIALS AND METHODS

Materials and Cells. Tricine, SDS, formic acid, cyanogen bromide (CNBr), poly(ethylene glycol), verapamil, and vinblastine were purchased from Sigma. EN^{3}HANCE was purchased from DuPont NEN. [^{3}H]-3′-BzDC-Taxol (∼50 Ci/mmol) was prepared as described previously (*22*). [^{3}H]-7′-BzDC-Taxol (∼50 Ci/mmol) was synthesized through modification of the hydroxyl group at C-7 with a 4-benzoylcinnamoyl group via coupling with 4-benzoylcinnamic acid in the presence of dicyclohexylcarbodiimide and 4-(dimethylamino)pyridine, followed by deprotection (HF/pyridine) and tritiation of the double bond with tritium gas in the presence of a catalytic amount of $RhCl(PPh_3)_3$ in toluene. Taxol and baccatin III were obtained from the Drug Development Branch, National Cancer Institute, Bethesda, MD. The site-directed antibodies used in this study have been described previously. The preparation and characterization of antibody Ab3 (residues 665−682) were described by Hsu et al. (*26*) and antibodies Ab1 (residues 269−284), Ab4 (residues 740−750), and Ab5 (residues 907−924) by Greenberger et al. (*13*). The antibody Ab7 (residues 1008−1019) was a generous gift from Dr L. M. Greenberger (*18*), while the antibody Ab6 was obtained from Oncogene Science.

The vinblastine-resistant cell line, J7.V1-1, which is ∼1000-fold resistant to vinblastine, was isolated by stepwise selection from the drug-sensitive macrophage-like cell line J774.2 and maintained in 1 μM vinblastine (*27*). Membrane fractions from both cell lines were isolated as described previously (*27*).

Growth Inhibition Assays. The ID_{50} of each cell line was determined using CellTiter 96 AQ_{ueous} nonradioactive cell proliferation assay (Promega Corp.). Cells were resuspended in drug-free medium, and 10^3 cells/well were added to 96-well tissue culture plates and allowed to attach overnight. Following the addition of 50 μL of medium, a serial dilution of the drug to be tested was carried out. When verapamil was present, it was added prior to the drug at a final concentration of 50 μM. The cells were incubated for 72 h, and then 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium and phenazine methosulfate solutions were freshly mixed and added to the cells. After 1−2 h at 37 °C, plates were read at 490 nm on a Bio-Tek Instruments microplate reader, Model EL320.

Photoaffinity Labeling of Membranes. Plasma membranes (50 μg) were resuspended in 10 mM Tris-HCl (pH 7.4) containing 250 mM sucrose and incubated with either 1 μM [^{3}H]-BzDC or [^{3}H]-7-BzDC-taxol for 2 h at room temperature. Samples were irradiated for time periods up to 3 h on ice with a 350 nm ultraviolet light (Model RPR-3500A°; Southern New England Ultraviolet Co., Branford, CT). Radiolabeled membrane proteins were resolved by SDS−PAGE on a 7% gel. For fluorography, the gels were treated with EN^{3}HANCE, then with 50% poly(ethylene glycol), and exposed to Kodak X-Omat AR film at −70 °C. The amount of ^{3}H incorporated into P-glycoprotein was quantitated by excising the gel slice containing P-glycoprotein and solubilizing it in 90% Beckman tissue solubilizer (BTS-450), followed by liquid scintillation counting. For competition studies, 50 μM samples of the various competitors were

included in the reaction mixture, and a 1 h period of photolysis was employed.

CNBr Digestion and Immunoprecipitation. CNBr digestion of P-glycoprotein was performed as described previously (*28*, *29*). Briefly, radiolabeled P-glycoprotein was resolved by SDS−PAGE (7% gel) and transferred to nitrocellulose. The region of the blot corresponding to P-glycoprotein was excised and digested with CNBr (250 μL of a 100 mg/mL solution in 70% formic acid) at room temperature for 2 h. The supernatant was taken to dryness, and the residue was dissolved and reevaporated twice with H_2O to remove residual formic acid. Samples were separated by SDS−PAGE (9.8% gel) with 0.1 M Tris, 0.1M Tricine, and 0.1% SDS as the cathode buffer (*30*), and visualized by fluorography. Immunoprecipitation of CNBr-released fragments was performed as described previously (*31*). Briefly, 1 volume of immunoprecipitation lysis buffer (50 mM Tris-HCl, pH 7.4, containing 2% SDS and 2 mg/mL bovine serum albumin) and 4 volumes of dilution buffer (50 mM Tris-HCl, pH 7.4, containing 1.25% Triton X-100, 1 mg/mL bovine serum albumin, and 190 mM NaCl) were added to each sample. Antibodies were added to a final dilution of 1:10 (v/v) and incubated at 4 °C for 15 h. When used, competing peptides were added at a final concentration of 30 μg/mL. Immune complexes were precipitated with protein A−Sepharose CL-4B (Sigma), washed with 50 mM Tris-HCl, pH 7.4, containing 150 mM NaCl, 0.03% SDS, and 0.1% Triton X-100, and eluted into 1× Laemmli sample buffer. Samples were resolved on a 9.8% Tricine gel and subjected to fluorography.

Tryptic Digestion of P-glycoprotein. After photolabeling, membranes were suspended in 10 mM Tris-HCl (pH 7.4) containing 250 mM sucrose and digested with 100 μg of *N*-tosyl-L-phenylalanine chloromethyl ketone(TPCK)-treated trypsin (Worthington) for 1 h at 37 °C as described previously (*13*). Proteolysis was terminated by the addition of aprotinin (0.25 μg/mL), phenylmethanesulfonyl fluoride (3.5 mM), and soybean trypsin inhibitor (200 μg/mL), and the samples were analyzed by SDS−PAGE (10% gel) and fluorography.

RESULTS

The 3′- and 7-BzDC Analogues of Taxol Are Substrates for Murine mdr1b P-glycoprotein. To determine whether the presence of the BzDC group influences the ability of P-glycoprotein to transport these Taxol analogues, their cytotoxic potentials against a P-glycoprotein-expressing cell line and its parental drug-sensitive cell line have been evaluated. The J7.V1-1 cell line was derived from J774.2 by selection in increasing concentrations of vinblastine and expresses predominantly a P-glycoprotein that is the product of the *mdr*1b gene (*26*). Both the 7-BzDC and the 3′-BzDC analogues of Taxol were cytotoxic to J774.2 cells with IC_{50} values of 490 and 2600 nM, respectively (Table 1). The IC_{50} for Taxol was 67 nM. The IC_{50} values for the 3′- and 7-BzDC analogues against the P-glycoprotein-expressing J7.V1-1 cell line could not be determined due to the limited solubility of these analogues in H_2O. There was little effect on J7.V1-1 cell growth in the presence of 10 μM 7-BzDC-Taxol or 100 μM 3′-BzDC-Taxol. The presence of 50 μM verapamil, a known MDR reversal agent, significantly decreased the IC_{50} for Taxol and each analogue, thereby indicating that the presence of the BzDC substituent does not affect the ability of these analogues to be transported by P-glycoprotein.

Table 1: Drug Sensitivity of J774.2 and J7.V1-1 Cell Lines for Taxol and the BzDC Analogues of Taxol

	ID_{50} (nM)[a]		
cell line	Taxol	7-BzDC	3′-BzDC
J774.2	67	490	2600
J7.V1-1			
−verapamil	8200	>10000	>100000
+verapamil	36	120	420

[a] ID_{50}, drug concentration that inhibits cell division by 50% after 72 h.

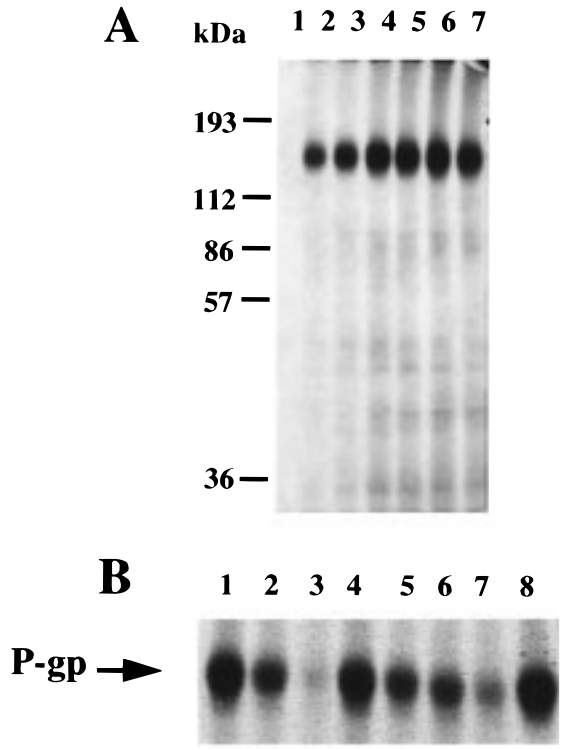

FIGURE 2: [^{3}H]-7-BzDC-Taxol specifically photolabels murine *mdr*1b P-glycoprotein. In panel A, membrane fractions from J7.V1-1 were incubated with 1 μM analogue for 1 h at 25 °C, irradiated at 350 nm on ice for the indicated time periods, and analyzed by SDS−PAGE (7% gel) and fluorography (lane 1, 0 min; lane 2, 15 min; lane 3, 30 min; lane 4, 60 min; lane 5, 120 min; lane 6, 180 min; and lane 7, 240 min). In panel B, membranes were incubated with 1 μM analogue in the presence of 50 μM competitor for 1 h at 25 °C, followed by irradiation for 1 h. Samples were analyzed as described in panel A (lane 1, no competitor; lane 2, 7-BzDC-Taxol; lane 3, 3′-BzDC-Taxol; lane 4, baccatin; lane 5, Taxol; lane 6, verapamil; lane 7,vinblastine; and lane 8, bleomycin).

We had previously reported that the [^{3}H]-3′-BzDC analogue specifically photoincorporated into both *mdr*1a and *mdr*1b P-glycoprotein (*22*). We now demonstrate that the [^{3}H]-7-BzDC analogue of Taxol also specifically photolabels both *mdr*1a (data not shown) and *mdr*1b P-glycoproteins (Figure 2, panels A and B) present in membranes isolated from J7.V3-1 and J7.V1-1 cells, respectively (*21*). A 170 kDa protein was the only protein labeled significantly by the 7-BzDC analogue in these membrane preparations. The identity of this ^{3}H-labeled protein as P-glycoprotein was established by immunoprecipitation with the

P-glycoprotein-specific polyclonal antibody R3 (data not shown). No labeling of any protein was observed in membranes isolated from the parental drug-sensitive cell line, J774.2 (data not shown). There was a progressive increase in the incorporation of the 7-BzDc analogue into *mdr*1b P-glycoprotein over the 3 h time course of irradiation at 350 nm (Figure 2, panel A). To reduce the possibility of nonspecific labeling of the transporter, all subsequent experiments were performed with 1 h of photolysis. Photoincorporation of the 7-BzDC analogue into the transporter was significantly reduced (>90%) by the inclusion of a 50-fold excess of either vinblastine or 3′-BzDC-Taxol and to a lesser extent (60−80%) by Taxol, 7-BzDC-Taxol, and verapamil (Figure 2, panel B). Interestingly, baccatin III, a taxane which lacks the side chain attached to the C-13 of the A ring, did not compete. Baccatin III also did not inhibit photolabeling of P-glycoprotein by the 3′-BzDC analogue (data not shown). Bleomycin, a water-soluble compound that is not an MDR substrate, also did not compete.

CNBr Cleavage of Photolabeled mdr1b P-glycoprotein. CNBr digestion was performed on *mdr*1b P-glycoprotein photolabeled with either the [^{3}H]-3′- or the [^{3}H]-7-BzDC analogues of Taxol. In these experiments, the photolabeled P-glycoproteins were resolved initially by SDS−PAGE, and transferred to nitrocellulose, and the region of the blot containing the transporter was digested with CNBr in 70% formic acid for 2 h. These conditions were shown previously to release >90% of the CNBr fragment containing the linker region of *mdr*1b P-glycoprotein (*28*, *29*). Approximately 60% of the ^{3}H in P-glycoprotein labeled with the [^{3}H]-3′-BzDC analogue was released from the nitrocellulose after CNBr treatment. The corresponding value for the [^{3}H]-7-BzDC-Taxol-labeled P-glycoprotein was ∼65%. Extending the period of digestion or increasing the concentration of CNBr did not result in the release of additional radioactivity. SDS−PAGE/fluorography of the CNBr-released peptides from both photolabeled P-glycoproteins revealed the presence of an ∼10 kDa tritium-labeled peptide (Figure 3). Minor labeling of 29, ∼5.5, and 3 kDa peptides was also observed in the CNBr digest of 3′-BzDC-Taxol-labeled P-glycoprotein.

Inspection of the deduced amino acid sequence of murine *mdr*1b P-glycoprotein revealed that there were only three predicted CNBr fragments with masses in the 10 kDa range. We have previously shown, however, that the CNBr fragment containing the linker region peptide ran anomalously on SDS−PAGE and also migrated in this mass range (*28*, *32*). Based on this information, the possible identities of the ∼10 kDa tritium-labeled CNBr fragments from the [^{3}H]-3′- and [^{3}H]-7-BzDC-labeled-P-glycoproteins are listed in Table 2. To aid in the identification of these two radiolabeled CNBr fragments, we performed immunoprecipitation studies with Abs3, -4, and -7 (see Table 2). Ab3 is specific for the linker region of *mdr*1b P-glycoprotein (*26*). Although P-glycoprotein is composed of two homologous halves, it is known that Ab4 and Ab7 do not cross-react with the corresponding segments in the N-terminal half of the transporter (*13*, *18*). Ab3 was unable to immunoprecipitate the ∼10 kDa CNBr fragment from either sample (data not shown). In contrast, Ab4 could immunoprecipitate the labeled CNBr fragment from P-glycoprotein containing the [^{3}H]-7-BzDC derivative (Figure 4, panel A), whereas Ab7 immunoprecipitated the corresponding fragment from the [^{3}H]-3′-BzDC-Taxol-labeled protein (Figure 4, panel B). To document the specificity of these immunoprecipitations, the synthetic peptide against which the antibodies were raised was included in the incubation. In both cases, the inclusion of the appropriate competing peptide inhibited immunoprecipitation (Figure 4, lane 2, panels A and B). Additionally, when the antibodies were switched, no labeled fragments were immunoprecipitated (Figure 4, lane 3, panels A and B). Although the CNBr fragments appear to be similar in mass, the immunoprecipitation results indicated that they were distinct peptides. These data suggest that the [^{3}H]-3′-BzDC group photoincorporates into residues 985−1088, a region of the transporter which includes the last half of TM 12 and extends into the final cytoplasmic segment. The [^{3}H]-7-BzDC group, in contrast, photoincorporates into residues 683−760, a region of the transporter which includes all of TM 7 and the first half of TM 8.

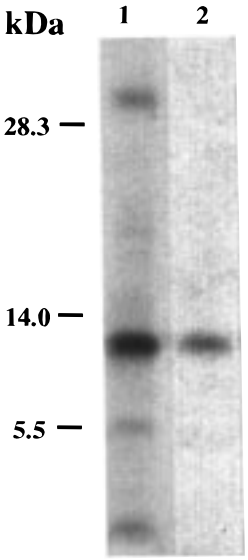

FIGURE 3: CNBr digestion of P-glycoprotein photolabeled with either the [^{3}H]-3′- or the [^{3}H]-7-BzDC analogues of Taxol. After photolabeling, P-glycoprotein was resolved by SDS−PAGE (7%), transferred to nitrocellulose, and digested with CNBr as described under Materials and Methods. The released peptides were subjected to SDS−PAGE and fluorography. Lane 1, [^{3}H]-3′-BzDC-Taxol-photolabeled P-glycoprotein; lane 2, [^{3}H]-7-BzDC-Taxol-photolabeled P-glycoprotein.

Table 2: CNBr-Generated Peptides from Murine *mdr*1b P-glycoprotein with Masses in the 10 kDa Range

residues	predicted mass (Da)	epitope	domain
514−615	10953	−	cytoplasmic
627−682[a]	6410	Ab3	linker region
683−760	8961	Ab4	TM 7 and part of TM 8
985−1088	11200	Ab7	part of TM 12 plus C-terminal Walker A motif

[a] This peptide migrates at a position corresponding to approximately twice its predicted mass on SDS−PAGE (*28*, *32*).

Mild Trypsinization of Photolabeled mdr1b P-glycoprotein. To confirm these findings, we have performed limited proteolysis on the photolabeled P-glycoproteins. Previous studies from our group have shown that tryptic digestion of membranes containing *mdr*1b P-glycoprotein initially gave rise to 55, 56, and 95 kDa fragments (*13*). The 55 and 56 kDa polypeptides were derived from the C-terminal half of

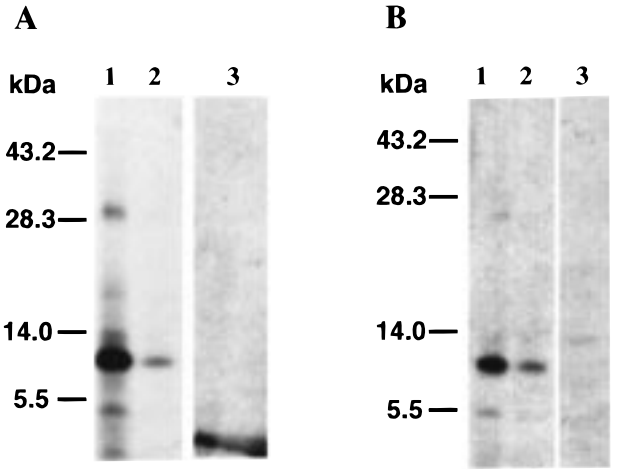

FIGURE 4: Immunoprecipitation of the CNBr fragments from [^{3}H]-3′- and [^{3}H]-7-BzDC-Taxol-labeled P-glycoproteins. Panel A: CNBr digest of the [^{3}H]-3′-BzDC-Taxol-labeled P-glycoprotein immunoprecipitated with either Ab7, in the absence (lane 1) or presence (lane 2) of competing peptide, or Ab4 (lane 3). Panel B: CNBr digest of the [^{3}H]-7-BzDC-Taxol-labeled P-glycoprotein immunoprecipitated with either Ab4, in the absence (lane 1) or presence (lane 2) of competing peptide, or Ab7 (lane 3) (see Materials and Methods for experimental details).

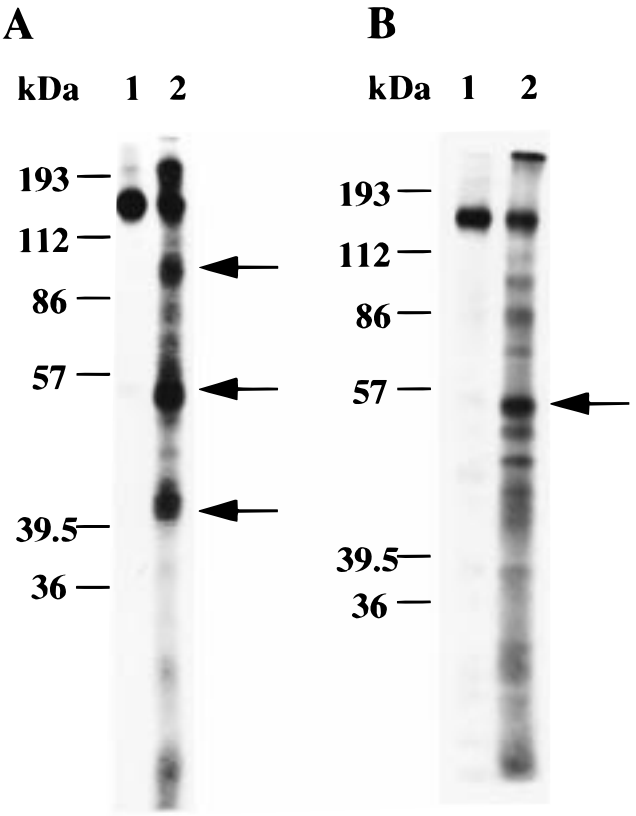

FIGURE 5: Tryptic digestion of [^{3}H]-3′- and [^{3}H]-7-BzDC-Taxol-labeled *mdr*1b P-glycoprotein. After photolabeling, membranes (100 μg) were digested with trypsin (100 μg) for 1 h at room temperature and analyzed by SDS−PAGE (8% gel) and fluography as described under Materials and Methods. Panel A, 3′-BzDC-Taxol; panel B, 7-BzDC-Taxol. In each panel, lane 1, control; lane 2, trypsin. The arrows in panels A and B indicate the major radiolabeled fragments.

the transporter. The 56 kDa fragment was recognized by Abs3, -4, -5, -6, and -7 (see Figure 6), whereas the 55 kDa fragment was recognized by the same panel of antibodies with the exception of Ab3. This latter antibody was raised against a peptide corresponding to amino acids 665−682. The 55 kDa fragment therefore extends from just N-terminal to TM 7 to at least amino acid 1203. Both fragments could be further digested to a 40 kDa fragment which was recognized by Ab6 and Ab7, but not by Ab3, -4, or -5. This fragment is believed to extend from just N-terminal to TM 11 to amino acid 1203 at a minimum. The 95 kDa tryptic fragment is known to be glycosylated in the surface loop between TM 1 and TM 2 and, since it was not recognized by Ab3, was the result of cleavage in the linker region immediately N-terminal to residue 665.

Membranes containing *mdr*1b P-glycoprotein, labeled with either the [^{3}H]-3′- or the [^{3}H]-7-BzDC derivatives of Taxol, were digested with trypsin as previously described, and subjected to SDS−PAGE/fluorography. As can be seen from Figure 5 (panel A), P-glycoprotein labeled with the [^{3}H]-3′-BzDC analogue gave rise to both 56/55 and 40 kDa fragments. In contrast, under the same conditions, P-glycoprotein labeled with the [^{3}H]-7-BzDC analogue gave rise only to a 56/55 kDa polypeptide (Figure 5, panel B). No 40 kDa labeled tryptic fragment was observed. As mentioned above, the 40 kDa fragment contains TM 11 and TM 12, but not TM 7 or TM 8. These proteolysis studies are, therefore, in excellent agreement with the CNBr/immunoprecipitation data.

DISCUSSION

In the absence of a detailed knowledge of the tertiary structure of P-glycoprotein, photoaffinity labeling can be a powerful tool for identifying structural domains of the transporter involved in drug binding. We have initiated a research program designed to map the sites of interaction between Taxol and murine *mdr*1b p-glycoprotein. Taxol is an important antitumor agent approved for the treatment of breast and ovarian carcinomas. It is also an ideal molecule for the proposed mapping studies since photoreactive groups can be introduced at several positions around the taxane nucleus. We have been successful in using this approach to map the contact sites for Taxol in β-tubulin (*33*−*35*). In the present study, we describe the mapping of the contact domains between P-glycoprotein and the [^{3}H]-3′- and [^{3}H]-7-BzDC analogues of Taxol. As can be seen from Figure 1, the BzDC groups in these two analogues are positioned at opposite ends of the molecule. In the C-3′ analogue, it replaces the benzamido group of the A ring side chain, whereas in the C-7 analogue, the 7-OH group of the C ring is substituted. The BzDC group has several excellent features for the proposed studies (*36*). First, photolysis occurs at 350 nm, reducing the potential of UV-induced damage to proteins. Second, the BzDC group has a propensity to photoincorporate into unreactive C−H bonds. This may be important since the drug binding site in P-glycoprotein is likely to contain apolar side chains necessary for interaction with hydrophobic drugs. It could be argued, however, that the bulkiness of the BzDC group could compromise the ability of P-glycoprotein to transport these Taxol analogues. Comparison of the IC_{50} values (Table 2), determined in the absence and presence of verapamil, with the P-glycoprotein-expressing cell line J7.V1-1 and the parental drug-sensitive cell line J774.2 clearly indicates that both BzDC analogues are transported by P-glycoprotein. In addition, the 3′- and 7-BzDC analogues of Taxol have been

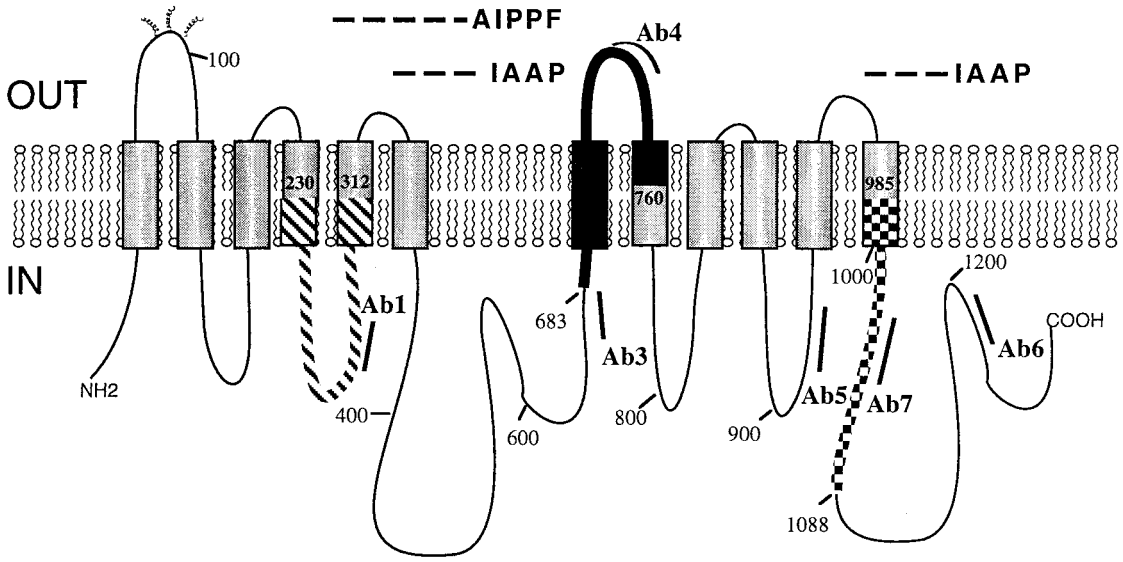

FIGURE 6: Predicted topology of P-glycoprotein in a lipid bilayer. The model is based on hydropathy plot analysis of the deduced amino acid sequence of P-glycoprotein.The epitopes for the antibodies used in this study are indicated, as are the specific domains identified as the sites of photoincorporation of [^{3}H]-3′-BzDC-Taxol (checked), [^{3}H]-7-BzDC-Taxol (solid), and [^{125}I]iodomycin (striped). The approximate sites of photoincorporation of [^{125}I]iodoarylazidoprazosin (IAAP) and 6-*O*-[[2-[3-(4-azido-3-[^{125}I]iodophenyl)propionamido]ethyl]carbamyl]-forskolin (AIPPF) are indicated by dashed lines.

shown to be similar to Taxol in their ability to stabilize microtubules to cold-induced depolymerization in vitro and in vivo to cause both microtubule bundle formation and mitotic arrest in cells.[2]

We had reported previously that the [^{3}H]-3′-BzDC-Taxol can specifically photolabel *mdr*1b P-glycoprotein present in membranes isolated from drug-resistant J7.V1-1 cells (*22*). In this study, we demonstrate that the [^{3}H]-7-BzDC analogue also specifically photoincorporates into *mdr*1b P-glycoprotein. Both analogues exhibit a remarkable degree of specificity for the transporter. Even though a total membrane preparation from the drug-resistant cell line was used in these experiments, only P-glycoprotein was labeled to any significant extent by both analogues. Since Taxol is a hydrophobic drug, which presumably enters the cell by passive diffusion across the lipid bilayer, it might have been expected that these BzDC analogues of Taxol would act as general labeling agents for integral membrane proteins. Obviously, this is not the case. Photoincorporation of the [^{3}H]-7-BzDC analogue into P-glycoprotein was competed best (>90%) by 3′-BzDC-Taxol and vinblastine, the latter not being structurally related to Taxol. This result is not surprising, since P-glycoprotein is a transporter with an exceedingly broad specificity for hydrophobic drugs. In other studies, involving a diverse range of photoprobes, vinblastine also has been observed to be one of the best competitors of photoincorporation, even though it was structurally dissimilar to the reagents under investigation (see *19*).

To identify the domains of P-glycoprotein labeled by these two Taxol analogues, CNBr has been employed to cleave the protein at methionine residues, followed by immunoprecipitation of the labeled fragments using a collection of polyclonal antibodies raised against defined regions of the *mdr*1b P-glycoprotein. Chemical, rather than enzymatic, fragmentation was used since cleavage occurs at well-defined positions in the former. With enzymatic digestion, it is often difficult, without additional analysis, to assign the precise cleavage sites. In addition, all of our site-directed polyclonal antibodies contain cleavage sites for the enzymes commonly used for protein digestion, e.g., trypsin and V8 protease.

CNBr digestion of the two photolabeled P-glycoproteins gave similar CNBr fragmentation patterns, each containing a major ~10 kDa tritium-labeled peptide. Immunoprecipitation studies revealed, however, that although these peptides were of similar mass, they were distinct peptides. The CNBr fragment generated from the [^{3}H]-3′-BzDC-Taxol-photolabeled P-glycoprotein was immunoprecipitated by a polyclonal antibody (Ab7) raised against amino acid residues 1008−1019 of the *mdr*1b isoform. In contrast, the CNBr fragment generated from the [^{3}H]-7-BzDC-Taxol-photolabeled P-glycoprotein was immunoprecipitated by a polyclonal antibody (Ab4) raised against amino acid residues 740−750. Based on the deduced amino acid sequence of *mdr*1b P-glycoprotein, and its hydropathy plot analysis, our data indicate that the [^{3}H]-3′-BzDC photoincorporated into amino acid residues 985−1088, a region of the transporter which includes half of TM 12 and terminates just after the Walker A motif in the second nucleotide binding fold. The [^{3}H]-7-BzDC group, on the other hand, photoincorporated into amino acid residues 683−760, a region of the transporter that begins immediately N-terminal to TM 7 and includes all of TM 7, the extracellular loop 4, and half of TM 8.

Important questions regarding P-glycoprotein structure concern the number of drug binding sites and the domains of the transporter involved in forming these sites. The two domains of P-glycoprotein identified in these current experiments reside in the C-terminal half of the transporter, and both include putative TM spanning segments, the presumed sites of photoincorporation. However, at this stage it is unclear whether both domains are part of the same or distinct binding pockets. Labeling of the N-terminal half of P-glycoprotein was observed with the [^{3}H]-3′-BzDC-Taxol. Trypsinization of the [^{3}H]-3′-BzDC-Taxol-labeled-P-glycoprotein generates a 95 kDa labeled fragment (Figure 5, panel

[2] S. B. Horwitz and G. A. Orr, manuscript in preparation.

A), and it has been demonstrated previously that this polypeptide includes the six N-terminal transmembrane spanning domains of the transporter (*14*). Moreover, after CNBr digestion of the photolabeled P-glycoproteins, 35−40% of the tritium is not released from the nitrocellulose and may represent radioactivity associated with the N-terminal half of the transporter. CNBr digestion of *mdr*1b P-glycoprotein is predicted to generate a 24 kDa fragment containing TM 3−TM 6 from the N-terminal half of the transporter. This peptide apparently remains bound to nitrocellulose after CNBr treatment since it is not detected by Ab1 in the released fragments (data not shown). This is not surprising since the peptide is extremely hydrophobic, containing 15 phenylalanines, 5 tryptophans, and 5 tyrosines. The corresponding CNBr fragment containing TM 3−TM 6 from human P-glycoprotein was observed when CNBr digestion was performed in solution (*15*). The minor 29 kDa CNBr fragment generated from the 3′-BzDC-Taxol-labeled P-glycoprotein (Figure 3, lane 1) is believed to be a partial digestion product consisting of residues 985−1268. A fragment of this size was recognized on immunoblots of CNBr-digested *mdr*1b P-glycoprotein by both Ab6 and Ab7 (data not shown). Ab6 was raised against a synthetic peptide corresponding to residues 1203−1222 (*14*).

Photoaffinity labeling of the drug binding site(s) in P-glycoprotein has been performed previously. In some of these studies, a photoreactive group was attached to a substrate/reversal agent (*11*−*15*, *17*−*24*); in others, the compound used was inherently photoreactive, e.g., progesterone (*16*) and iodomycin (*25*). However, in only a limited number of cases have the actual domains of photoincorporation been identified (Figure 6). The principal domains of P-glycoprotein labeled by [^{125}I]iodoarylazidoprazosin were within or immediately C-terminal to both TM 6 and TM 12 (*13*, *18*), and recent studies have suggested that the transporter contains two nonidentical drug binding sites for this compound (*24*). It is likely that similar domains were labeled by an aryl azide analogue of forskolin (*20*). Labeling of P-glycoprotein by iodomycin, an analogue of daunomycin, was restricted to residues 230−312, a region of the transporter consisting of part of TM 4, the second cytoplasmic loop, and part of TM 5 (*25*). Although TM 7/TM 8 of P-glycoprotein have not been identified previously as sites of incorporation for photoreactive MDR substrates/reversal agents, mutagenesis studies have clearly established the importance of this region of the protein for transporter function. Replacement of TM 7 and TM 8 in human MDR1 by the corresponding region of MDR2 destroyed transporter activity (*37*). Likewise, single mutations at specific positions within TM 7 also resulted in mutant P-glycoproteins that were nonfunctional (*38*, *39*).

No labeling of the C-terminal half of the transporter was observed with iodomycin (*25*). It is important to stress, however, that the lack of labeling in this region of the transporter does not necessarily imply that a binding site for iodomycin does not exist in the C-terminal half of P-glycoprotein. For productive photolabeling to occur, a potential site of incorporation has to be in close proximity to the photogenerated reactive species. For example, it has been estimated that the reactive volume of a BzDC analogue approximated a sphere with a radius of 3.1 Å centered on the ketone oxygen (*36*). If a potential site of incorporation is not within this sphere of reactivity, no photoincorporation will occur even though binding has taken place.

It is also not surprising that different domains of the transporter can be labeled by different photoreactive analogues, even though the analogues may bind to the same site in P-glycoprotein. Mutagenesis studies have revealed that the amino acid residues which affect the cross-resistance phenotype are not restricted to any one area of the transporter, but occur throughout the protein (*38*−*42*). One possible interpretation of these findings is that different domains of the protein participate in the overall three-dimensional arrangement of the drug binding site(s). If true, then the orientation of the photoreactive group in the drug binding site will dictate which domain of the transporter is labeled. For example, in our studies on mapping the Taxol binding site in β-tubulin, the two domains so far identified lie far apart in the primary sequence (*33*−*35*). The 3′-benzamido and the 2-benzoyl groups of Taxol were shown to interact with amino acid residues 1−31 and 217−231 of β-tubulin, respectively.

We believe that the approach described in this paper, of using Taxol as a "drug platform", around which photoreactive groups can be attached, will help us to delineate the molecular architecture of the drug binding pocket(s) for Taxol in P-glycoprotein. Experiments are currently in progress to determine whether the domains labeled by the two Taxol analogues employed in this study are part of the same, or distinct, binding site(s).

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