Identification of Residues in the Drug-binding Site of Human P-glycoprotein Using a Thiol-reactive Substrate*

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Communication

The human multidrug resistance P-glycoprotein (product of the MDR1 gene) is an ATP-dependent transporter located in the plasma membrane of many cells and is able to extrude a wide variety of hydrophobic compounds and drugs (reviewed in Ref. 1). Its physiological role is unknown but studies on “knock-out” mice suggest that it protects the organism from endogenous and exogenous cytotoxic compounds (2, 3). More recently, we have shown that these segments lie close to each other in the tertiary structure (20, 21). In this study, we identified a thiol-reactive compound, dibromobimane (dBBn), that was a potent stimulator of the ATPase activity of Cy5-less P-glycoprotein. We previously showed that residues Leu272, Leu297, Val302, and Ala305 lie along the point of contact between helices TM6 and TM12 when both are aligned in a left-handed helix (Loo, T. W., and Clarke, D. M. (1997) J. Biol. Chem. 272, 20896–20898). Taken together, these results suggest that the interface between TM6 and TM12 likely forms part of the potential drug-binding pocket in P-glycoprotein.

The human multidrug resistance P-glycoprotein is a member of the ATP-binding cassette family of transport proteins. Its 1280 amino acids are organized in two tandem repeats, each repeat consisting of a hydrophobic domain followed by an ATP-binding domain (4). Many different approaches have been used to study the mechanism of ATP-dependent drug efflux. It is known that the minimum functional unit is a monomer (5) and that both tandem repeats are required to couple drug binding to ATPase activity (6). Both ATP-binding sites are important because inactivation of either site by mutagenesis or chemical modification inhibits drug-stimulated ATPase activity (7–10). The transmembrane domains appear to contain the drug-binding site(s) and likely form the translocation pathway through the membrane. Labeling studies with photoactive analogs of drug substrates and results of mutational analysis suggest that TM6 and TM12 may be involved in drug-protein interactions (11–19). More recently, we have shown that these two segments lie close to each other in the tertiary structure (20, 21).

In this study, we identified a thiol-reactive compound, dibromobimane (dBBn),† that was a potent stimulator of the ATPase activity of Cy5-less P-glycoprotein. We combined it with cysteine-scanning mutagenesis to examine the contribution of TM6 and TM12 to coupling of drug binding to ATPase activity. We introduced a cysteine residue at each position in TM6 or TM12 in a Cy5-less P-glycoprotein and then probed these cysteine mutants with dBBn. Our rationale was that a thiol-reactive substrate should also occupy the drug-binding site of P-glycoprotein, covalently bind to a nearby cysteine residue, and inhibit drug-stimulated ATPase activity. We show that the compound dBBn is a particularly useful thiol-reactive probe for such an approach, because it was a relatively potent stimulator of the ATPase activity of Cy5-less P-glycoprotein and both its reactivity and its ability to act as a substrate could be quenched with cysteine. We show that the drug-stimulated ATPase activities of mutants L339C and A342C (TM6) and L975C, V982C, and A985C (TM12) were particularly sensitive to inhibition by dBBn and that the inhibition was prevented by various drug substrates. These results suggest that the interface between TM6 and TM12 is critical for P-glycoprotein-drug interactions and likely forms part of the potential drug-binding pocket.

**EXPERIMENTAL PROCEDURES**

Construction of Mutants—Cysteine residues were introduced into a Cy5-less mutant of P-glycoprotein containing a histidine tag at the COOH terminus as described previously (21). The presence of a histidine tag facilitated purification of the mutant P-glycoprotein by nickel-chelate chromatography (8).

Expression and Purification of P-glycoprotein Cys Mutants—Expression and purification of P-glycoprotein were carried out as described previously (21). Briefly, forty 10-cm diameter culture plates of HEK 293 cells were transfected with the mutant cDNA. After 24 h, the medium was replaced with fresh medium containing 10 μM cyclosporin A. The transfected cells were then harvested 24 h later and solubilized with 1% (w/v) n-dodecyl-β-D-maltoside, and the mutant P-glycoproteins were isolated by nickel-chelate chromatography (8).

Measurement of Drug-stimulated ATPase Activity—P-glycoprotein recovered by nickel-chelate chromatography was diluted with an equal volume of 100 mg/ml crude sheep brain phosphatidylethanolamine (Sigma, Type II, commercial grade) that had been washed with Tris-buffered saline to remove traces of phosphate and then sonicated. ATPase activity was initiated by addition of an equal volume of buffer contain-

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† The abbreviations used are: dBBn, dibromobimane; TM, transmembrane segment.
ing 100 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10 mM MgCl₂, 10 mM ATP, and the desired drug to a sample containing 100 ng of P-glycoprotein-lipid mixture. The concentration of the drugs in the ATPase assays was 1 mM verapamil, 0.1 mM vinblastine, 5 mM colchicine, and 1 mM for others. The samples were incubated at 37 °C, and the amount of inorganic phosphate liberated was determined by the method of Chifflet et al. (22).

For dBBn inhibition, the mutant P-glycoprotein-lipid mixture was preincubated with 1 mM dBBn (Molecular Probes Inc.) for 5 min at 37 °C, followed by addition of cysteine, pH 7.5, to a concentration of 40 mM and incubation at 37 °C for another 5 min. ATPase activity was then determined as described above.

Immunoblotting—Whole cell lysates of transfected HEK 293 cells were subjected to SDS-polyacrylamide gel electrophoresis, transferred onto a sheet of nitrocellulose, and probed with a rabbit polyclonal antibody against P-glycoprotein and enhanced chemiluminescence (21).

RESULTS

Identification of a Thiol-reactive Compound—We previously showed that a Cys-less P-glycoprotein has near wild-type levels of drug-stimulated ATPase activity (23). This Cys-less P-glycoprotein was then used to identify thiol-reactive compounds that would be potent stimulators of the Cys-less P-glycoprotein ATPase activity. The compound could then be used with cysteine-scanning mutagenesis to identify residues in the transmembrane domain that are important for function. The rationale for this approach was that for a compound to stimulate P-glycoprotein ATPase activity, it must be able to interact at the binding site(s) of P-glycoprotein and that reaction of the compound with a thiol group should inhibit ATPase activity. Because P-glycoprotein transports hydrophobic compounds, hydrophobic thiol-specific compounds were tested. Fig. 1 shows the ability of various thiol-reactive compounds to stimulate the ATPase activity of Cys-less P-glycoprotein compared with that of verapamil. There was little or no stimulation of ATPase activity by N-ethylmaleimide, p-chloromercuriphenylsulfonic acid, p-chloromercuribenzoic acid; NEM, N-ethylmaleimide.

![Stimulation of Cys-less P-glycoprotein ATPase Activity by verapamil and thiol-reactive compounds](image1)

**FIG. 1.** Stimulation of Cys-less P-glycoprotein ATPase Activity by verapamil and thiol-reactive compounds. Equivalent amounts of purified Cys-less P-glycoprotein (His)₁₀ were mixed with lipid, and the ATPase activity was measured in the presence or the absence of the indicated compound (1 mM, except for acrylodan (0.25 mM)). In the case of dBBn plus Cys (dBBn + Cys), the enzyme was preincubated with 1 mM dBBn and 40 mM cysteine, pH 7.5, for 5 min at 37 °C. Fold stimulation is the ratio of the activity with drug to that found without drug substrate. CMPS, p-chloromercuriphenylsulfonic acid; CMPS, p-chloromercuribenzoic acid; NEM, N-ethylmaleimide.

![Verapamil-stimulated ATPase activities of P-glycoprotein Cys mutants](image2)

**FIG. 2.** Verapamil-stimulated ATPase activities of P-glycoprotein Cys mutants. A, equivalent amounts of purified mutant P-glycoprotein (His)₁₀ were added to lipid and assayed for verapamil-stimulated ATPase activity as described under “Experimental Procedures.” The activities are expressed relative to that of Cys-less P-glycoprotein, and each is the average of two different experiments. The activity of mutant S344C was not determined (ND) due to our inability to express enough enzyme. Asterisks identify mutant proteins that were not assayed due to proteolytic degradation. The upper and lower panels show TM6 and TM12 Cys mutants, respectively. B, whole cell extracts of HEK 293 cells expressing wild-type, Cys-less, glycosylation-deficient N91A/N94A/N99A and single Cys mutants that exhibited little or no verapamil-stimulated ATPase activity were subjected to immunoblot analysis as described under “Experimental Procedures.” The transfected cells were grown for 24 h in the absence (+) or the presence (∗) of 10 μM cyclosporin A. For mutant S344C, three times the normal amount of lysate was loaded onto the gel. The positions of the mature (170 kDa), core glycosylated (150 kDa) and unglycosylated (140 kDa) forms of P-glycoprotein are indicated.

its ability to stimulate ATPase activity. Therefore, dBBn was considered to be a useful thiol-reactive substrate, because it was a relatively potent stimulator of the Cys-less P-glycopro-
The inhibition of mutants G346C and G989C were not determined (ND) due to low expression, and mutants indicated by asterisks were not assayed due to proteolytic digestion. The inhibition of mutants G346C and G989C were not determined (ND) due to their low activities. The upper and lower panels show TM6 and TM12 Cys mutants, respectively.

Construction and Measurement of Verapamil-stimulated ATPase Activities of Single Cys Mutants—We constructed 42 different mutants that contained a single cysteine at each position of TM6 and TM12. Each mutant also contained a polyhistidine tag at the COOH terminus to facilitate recovery by nickel-chelate chromatography (8). Each mutant P-glycoprotein was expressed in HEK 293 cells in the presence of cyclosporin A (Fig. 2B, lanes 3 and 4). A similar pattern was observed for mutants G346C, A985C, G984C, and Q990C, suggesting that the low ATPase activity in these mutants was not due to a processing defect. Mutants G341C and G984C, however, appeared to be degraded quite rapidly. Both mutants contained immunoreactive products of apparent masses 120 and 95 kDa, respectively, as the major products. Mutants A342C and Q347C also showed decreased ATPase activity in the presence of cyclosporin A, with the 120-kDa protein as the major product. This appeared to be a degradation product rather than a nonglycosylated product because it had a higher mobility than the glycosylation-deficient P-glycoprotein (N91A/N94A/N99A) (Fig. 2B, lanes 23 and 24). For both mutants, the amount of mature protein increased in the presence of cyclosporin A. Mutant S344C consistently yielded very low levels of immunoreactive P-glycoprotein in the presence or the absence of cyclosporin A (Fig. 2B, lanes 13 and 14).

Inhibition by dBBn—To test for inhibition of ATPase activity by dBBn, each of the 37 active Cys mutants was treated with 1 mM dBBn for 5 min at 37 °C, quenched with cysteine, and then assayed for verapamil-stimulated ATPase activity (Fig. 3). Verapamil was used because it is the most potent stimulator of P-glycoprotein ATPase activity. The activities of the dBBn-treated samples were expressed relative to their mock-treated controls. Mutants G341C, S344C, G346C, G984C, and G989C were not assayed because of their low or defective expression (Fig. 2B). 31 of the 37 mutants retained more than 80% of their activity when treated with dBBn, whereas V981C retained 59% of its activity. In contrast, mutants L339C, A342C, L975C, V982C, and A985C were significantly inhibited by dBBn, because they retained only 10, 40, 13, 25, and 32% of their activities, respectively. The concentration of dBBn required to give 50% inhibition of ATPase activity for mutants L339C, L975C, V982C, A985C, and A342C were 90, 112, 320, 480, and 700 μM, respectively.

Inhibition of dBBn Inactivation by Drug Substrates—We then tested whether the drug substrates, verapamil, vinblastine, and colchicine, could protect the mutant P-glycoproteins against inactivation by dBBn. Each mutant P-glycoprotein was preincubated with verapamil, vinblastine, or colchicine, treated with dBBn, and then quenched with cysteine. The amount of ATPase activity was then measured and compared
with a sample that was not treated with dBBn. Due to the low ATPase activities of mutants A342C and A985C, their protection assays were done only in the presence of verapamil. Verapamil-stimulated ATPase activity of Cys-less P-glycoprotein is more than twice that obtained with vinblastine or colchicine. As shown in Fig. 4, mutants A342C and A985C were protected from dBBn inactivation by verapamil. Similarly, mutants L339C, L975C, and V982C were also protected from dBBn inactivation by various drug substrates. All three mutants retained more than 80% of their vinblastine-stimulated ATPase activity after treatment with dBBn. Colchicine was also very effective in protecting mutant L339C from dBBn inactivation because it retained about 80% of its colchicine-stimulated ATPase activity. More modest protection by colchicine was seen for mutants L975C and V982C. By contrast, verapamil was the least effective of the substrates. It offered little or no protection for mutant V982C and only moderately protected mutants L339C and L975C. The drug-stimulated ATPase activities of the Cys-less P-glycoprotein were not affected by dBBn.

**DISCUSSION**

The use of cysteine-scanning mutagenesis in combination with a thiol-specific substrate to identify important residues in P-glycoprotein-drug interactions has several advantages over approaches that use only mutagenesis (13–15) or that involve photo labeling with a radioactive analog of drug substrates. A major advantage is that the use of dBBn is a direct approach for probing the active site of P-glycoprotein. It is a relatively good stimulator of Cys-less P-glycoprotein ATPase activity, and its activity can be abolished with cysteine. Covalent attachment of dBBn to a cysteine residue also introduces a large bulky group into the protein, and the presence of such a large covalently bound group in the drug-binding site(s) would disrupt activity. Significant inhibition of activity suggests that the majority of the protein was modified with dBBn. A problem with using only mutagenesis, such as alanine-scanning mutagenesis, is that drug-protein interactions likely involve a large number of residues, so that a single change may not have a measurable effect. In addition, when a change in substrate specificity is observed, it is often difficult to determine if this is due to local or global structural changes. A difficulty in photolabeling with radioactive analogs of drug substrates is that the concentration of photolabel required for stoichiometric labeling of the protein makes it economically unfeasible.

In this study we showed that modification of five residues (Leu339, Ala342, Leu975, Val982, and Ala985) in TM6 and TM12 by dBBn inhibited ATPase activity of the mutants. In a recent cross-linking study (24), we showed that TM6 and TM12 helices are likely to be arranged in a left-handed coiled coil (Ref. 24 and Fig. 5). Cross-linking between residues F343C/M986C, G346C/G989C, and P350C/S993C was prevented by the presence of drug substrates. Residues Leu339, Ala342, Leu975, Val982, and Ala985 are next to or lie close to these cross-linked residues and along the TM6/TM12 interface. As with the cross-linked residues, inhibition of the activities of these mutants by dBBn was prevented by drug substrates (verapamil, vinblastine, and colchicine). Taken together, the results suggest that these residues may form part of a potential drug-binding pocket in P-glycoprotein (Fig. 5).

TM6 of other ATP-binding cassette transporters such as the cystic fibrosis transmembrane conductance regulator also appears to be important for function. Cheung and Akabas (25, 26) mutated each residue in TM6 of cystic fibrosis transmembrane conductance regulator to cysteine and measured the reactivity of the water-accessible residues to charged, hydrophilic sulfhydryl-specific methanethiosulfonate reagents. They showed that

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![FIG. 5. Location of dBBn-sensitive residues in TM6 and TM12 arranged as a left-handed coiled coil (large circles) and TM12 (small circles) residues arranged as α-helical nets were superimposed in a left-handed coiled coil as described previously (24). The residues from each helix that face each other are shown along the i+7 axis. The arrows point toward the cytoplasmic surface. Residues that were inhibited by dBBn are shaded.](image-url)