Simultaneous Binding of Two Different Drugs in the Binding Pocket of the Human Multidrug Resistance P-glycoprotein*

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‡ The abbreviations used are: P-gp, P-glycoprotein; TM, transmembrane; HEK, human embryonic kidney; AM, acetoxymethyl ester; TMEA, tris-(2-maleimidoethyl)amine; ABC, ATP-binding cassette.

The human multidrug resistance P-glycoprotein (P-gp, ABCB1) transports a wide variety of structurally diverse compounds out of the cell. The drug-binding pocket of P-gp is located in the transmembrane domains. Although occupation of the drug-binding pocket by one molecule is sufficient to activate the ATPase activity of P-gp, the drug-binding pocket may be large enough to accommodate two different substrates at the same time. In this study, we used cysteine-scanning mutagenesis to test whether P-gp could simultaneously interact with the thiol-reactive drug substrate, Tris-(2-maleimidoethyl)amine (TMEA) and a second drug substrate. TMEA is a cross-linker substrate of P-gp that allowed us to test for stimulation of cross-linking by a second substrate such as calcein-acetoxymethyl ester, daunomycin, cyclosporin A, rhodamine B, progesterone, and verapamil. We report that verapamil induced TMEA cross-linking of mutant F343C(TM6)/V982C(TM12). By contrast, no cross-linked product was detected in mutants F343C(TM6), V982C(TM12), or F343C(TM6)/V982C(TM12) in the presence of TMEA alone. The verapamil-stimulated ATPase activity of mutant F343C(TM6)/V982C(TM12) in the presence of TMEA decreased with increased cross-linking of the mutant protein. These results show that binding of verapamil must induce changes in the drug-binding pocket (induced-fit mechanism) resulting in exposure of residues F343C(TM6)/V982C(TM12) to TMEA. The results also indicate that the common drug-binding pocket in P-gp is large enough to accommodate both verapamil and TMEA simultaneously and suggests that the substrates must occupy different regions in the common drug-binding pocket.

The multidrug resistance P-glycoprotein (P-gp); MDR1 or ABCB1 gene product) uses ATP to transport structurally diverse compounds out of the cell (recently reviewed in Refs. 1 and 2). Overexpression of P-gp in tumors and in organs such as the liver, kidney, and the blood-brain barrier can undermine cancer and AIDS chemotherapy regimens because many of the therapeutic drugs are substrates of P-gp (3, 4).

P-gp is one of 48 ATP-binding cassette (ABC) transporters in humans (5). The 1280 amino acids of P-gp are arranged as two repeating units of 610 amino acids that are joined by a linker region of about 60 amino acids (6). Each repeat has six transmembrane (TM) segments and a hydrophilic domain containing an ATP-binding site (7–9). The minimum functional unit is a monomer (10). Both halves of the molecule are essential for activity but do not have to be covalently linked (11, 12). Both ATP-binding sites are required for activity (8, 13–15), but the TM domains alone are sufficient to mediate drug binding (12).

An important goal in understanding the mechanism of P-gp is to determine the location and number of drug-binding sites. Studies with thiol-reactive substrate analogs of P-gp and cysteine mutants have shown that residues from multiple TM segments contribute to a common drug-binding pocket (16–21).

P-gp in the resting state is in the “closed” conformation where the cytoplasmic ends of the TM segments are close to each other but far apart at the extracellular end of the molecule (20, 22, 23). Covalent binding of a single molecule of the drug substrate verapamil in the drug-binding pocket was sufficient to permanently activate P-gp (24). The dimensions of the P-gp drug-binding pocket as determined with thiol-reactive cross-linker substrates, however, indicated the drug-binding pocket may accommodate more than one substrate at the same time (20).

To determine whether more than one substrate could bind to P-gp at the same time, we used the drug substrate Tris-(2-maleimidoethyl)amine (TMEA). TMEA is a useful compound for analyzing the P-gp drug-binding pocket because it will cross-link cysteine residues if they are close to the binding site of TMEA. Binding of a second drug substrate will inhibit TMEA cross-linking if there is significant overlap of the binding sites. Similarly, cross-linking could be unaffected or enhanced if the binding site of the second drug substrate does not overlap that of TMEA. In this study, we used cysteine-scanning mutagenesis and reaction with TMEA in the presence of other drug substrates to determine whether P-gp could simultaneously bind to two different drug substrates in the common drug-binding pocket.

MATERIALS AND METHODS

Construction of Mutants—Cysteine residues were introduced into a histidine-tagged Cys-less P-gp cDNA (25). Cys-less P-gp was constructed by replacing the seven endogenous cysteines at positions 137, 431, 717, 956, 1074, 1125, and 1227 with alanines (7). The Cys-less P-gp retained the ability to confer multidrug resistance and exhibited drug-stimulated ATPase activity (7, 26). The presence of the histidine tag facilitated purification of the mutant P-gps by nickel-chelate chromatography (26).

Treatment of Mutants with TMEA—HEK 293 cells (10 10-cm diam-
the presence of 0, 0.1, or 1 mM TMEA. The reactions were stopped by addition of 300 mM cysteine, pH 7.0, to a final concentration of 30 mM. After 10 min at 21 °C, SDS sample buffer (125 mM Tris-Cl, pH 6.8, 20% (v/v) glycerol, 4% (w/v) SDS, and 4% (v/v) 2-mercaptoethanol) was added. The mixtures were subjected to immunoblot analysis with rabbit polyclonal anti-P-gp antibody (28) and enhanced chemiluminescence (Pierce).

To monitor the time course of verapamil-stimulated TMEA cross-linking, the membranes were pre-incubated with 1 mM verapamil for 10 min at 21 °C, the membranes were then incubated for 0–32 min in the presence of 0.1 mM TMEA. The reactions were stopped by addition of SDS sample buffer containing 30 mM cysteine.

To monitor the dependence of cross-linking on TMEA concentration, membranes were pre-incubated with 1 mM verapamil for 10 min at 21 °C and then treated with various concentrations (0–1 mM) of TMEA for 15 min at 21 °C. The reactions were stopped by addition of SDS sample buffer containing 30 mM cysteine. The dependence of TMEA cross-linking on verapamil concentration was determined by pre-incubating the membranes in the presence of various concentrations (0–1 mM) of verapamil for 10 min at 21 °C followed by treatment with 0.1 mM TMEA for 15 min at 21 °C. The reactions were stopped by addition of SDS sample buffer containing 30 mM cysteine.

Expression, Purification, and Measurement of Drug-stimulated ATPase Activity of P-gp Mutants—HEK 293 cells were transfected with the mutant cDNAs. The medium was replaced after 24 h with fresh medium containing 10 μM cyclosporin A. Cyclosporin A is a substrate of P-gp and acts as a powerful chemical chaperone in promoting maturation of P-gp and increases the yield of mature P-gp (29–32). After another 24 h, the transfected cells were harvested, and the mutants were isolated by nickel-chelate chromatography. The isolated mutant P-gps were mixed with lipid (sheep brain lipid, type IIs, Sigma) and sonicated as described previously (26). An aliquot of the P-gp/lipid mixture was incubated with 1 mM verapamil for 10 min at 21 °C and then treated with various concentrations (0–1 mM) of TMEA for 15 min at 21 °C. The reactions were stopped by addition of cysteine, pH 7.0, to a final concentration of 30 mM. After another 10 min at 21 °C, the samples were mixed with an equal volume of ATPase buffer containing 100 mM Tris-Cl, pH 7.4, 100 mM NaCl, 20 mM MgCl₂, 10 mM ATP and incubated slowly at low temperature (36), whereas incubation at a higher temperature (37 °C) results in increased thermal motion in P-gp. The reactions were stopped by addition of cysteine and the samples subjected to immunoblot analysis. One mutant F343C(TM6)/V982C(TM12) showed substrate-stimulated cross-linking with TMEA. Verapamil, R(+)-verapamil, and S(-)-verapamil promoted cross-linking of P-gp with TMEA (Fig. 1A). By contrast, none of the drug substrates promoted cross-linking in mutant F343C(TM6) or mutant V982C(TM12) (Fig. 1, B and C). Similarly, the drug substrates did not promote cross-linking in mutant F343C(TM6)/V982C(TM12) in the absence of TMEA (Fig. 1D). Similar results were obtained when cross-linking was carried out with 1 mM TMEA (data not shown).

Both stereoisomers of verapamil, R(+)-verapamil and S(-)-verapamil, promoted TMEA cross-linking of mutant F343C(TM6)/V982C(TM12) (Fig. 1A). This is consistent with our observation that both isomers were equal in their ability to stimulate the ATPase activity of wild-type P-gp (about 15-fold stimulation; K₉ about 30 μM).

Mutant F343C(TM6)/V982C(TM12) showed a time-dependent increase in the level of cross-linking (Fig. 2A). In the presence of 0.1 mM TMEA and 1 mM verapamil, cross-linking prod-

**FIG. 1. Effect of drugs on cross-linking of P-gp mutants.** Membranes were prepared from HEK 293 cells expressing P-gp mutants F343C(TM6)/V982C(TM12) (A and D), F343C(TM6) (B), or V982C(TM12) (C). The membranes were pre-incubated at 21 °C for 10 min with no drug (No Drug), calcein-AM (CAM), colchicine (Colch), demecolcine (Deme), cyclosporin A (Cyclo), rhodamine B (Rhod), progesterone (Prog), verapamil (Ver), R(+)-verapamil (R-Ver), or S(-)-verapamil (S-Ver). The reaction mixtures were then treated with (+) or without (-) 0.1 mM TMEA for 15 min at 21 °C. The reactions were stopped by addition of cysteine and SDS sample buffer. The mixtures were subjected to immunoblot analysis. The positions of the cross-linked (X-link) product and mature (170 kDa) P-gps are indicated.

![Diagram of cross-linking](image-url)
The samples were then treated for 15 min at 21°C for the presence of various concentrations (0–1 mM) of TMEA. The activities were determined. Mutant F343C was also treated with various concentrations of TMEA in the absence of verapamil (+Ver). The activities are expressed relative to sample that was not treated with TMEA. The structure of TMEA is shown (inset).

Fig. 2. Characteristics of verapamil-stimulated TMEA cross-linking of mutant F343C(TM6)/V982C(TM12). Membranes were prepared from HEK 293 cells expressing mutant F343C(TM6)/V982C(TM12). A, membranes were pre-incubated with 1 mM verapamil for 10 min at 21°C and then treated with 0.1 mM TMEA at 21°C for the indicated times (min). B, membranes were pre-incubated with 1 mM verapamil for 10 min at 21°C and then treated with various concentrations of TMEA for 15 min at 21°C. C, membranes were pre-incubated with the indicated concentrations of verapamil for 10 min at 21°C and then treated with 0.1 mM TMEA for 15 min at 21°C. The reactions were stopped by addition of cysteine and SDS sample buffer. Samples were subjected to immunoblot analysis. The positions of the cross-linked (X-link) product and mature (170 kDa) P-gps are shown.

The dependence of cross-linking on TMEA concentration was also determined. Membranes from mutant F343C(TM6)/V982C(TM12) were pre-incubated with 1 mM verapamil and then reacted with various concentrations (0–1 mM) of TMEA. Fig. 2B shows cross-linked product was readily detected in the presence of 0.1–1 mM TMEA. Similarly, the dependence of TMEA cross-linking on the verapamil concentration was determined by pre-incubating membranes with various concentrations of verapamil (0 to 1 mM) followed by treatment with 0.1 mM TMEA for 15 min at 21°C. Cross-linked product was detected at 3 μM verapamil. The amount of cross-linked product increased with increasing concentrations of verapamil, with >50% of the mutant cross-linked in the presence of 0.3 or 1 mM verapamil.

To determine whether the active form of the mutant F343C(TM6)/V982C(TM12) was reacting with TMEA, we tested for inhibition of drug-stimulated ATPase activity. The rationale was that cross-linking should cause inhibition of activity because activity is dependent on rearrangement of TM segments within P-gp (37, 38). Accordingly, histidine-tagged Cys-less, F343C(TM6), V982C(TM12), and F343C(TM6)/V982C(TM12) mutant P-gps were isolated by nickel-chelate chromatography, mixed with lipid, sonicated, and incubated for 10 min at 21°C in the presence of 1 mM verapamil. The samples were then treated for 15 min at 21°C in the presence of various concentrations (0–1 mM) of TMEA. The reactions were quenched by addition of cysteine, and verapamil-stimulated ATPase activities were determined. The activity of the Cys-less P-gp was not inhibited by TMEA (data not shown) (25). There was good correlation, however, between the concentration of TMEA required for substantial cross-linking (0.1–1 mM TMEA; Fig. 2B) and the concentration (0.1 to 1 mM) required for more than 50% inhibition of the activity of mutant F343C(TM6)/V982C(TM12). Half-maximal inhibition occurred at 84 μM TMEA. The mutants F343C(TM6) and V982C(TM12) were also tested for inhibition by TMEA. We previously reported that the activity of mutant V982C(TM12) showed half-maximal inhibition at 420 μM TMEA when incubated with TMEA in the absence of verapamil (25). In the present study, incubation of mutant V982C(TM12) with TMEA in the presence of 1 mM verapamil gave a similar pattern of inhibition, with half-maximal inhibition occurring at 410 μM TMEA (Fig. 3).

Fig. 3. Effect of TMEA on verapamil-stimulated ATPase activities of P-gp mutants. Histidine-tagged P-gp mutants F343C(TM6), V982C(TM12), or F343C(TM6)/V982C(TM12) were expressed in HEK 293 cells and isolated by nickel-chelate chromatography, mixed with lipid, and sonicated. Samples were incubated with 1 mM verapamil (+Ver) for 10 min at 21°C and then treated with various concentrations of TMEA for 15 min at 21°C. The reactions were quenched by addition of cysteine, pH 7.0, and verapamil-stimulated ATPase activities were determined. Mutant F343C was also treated with various concentrations of TMEA in the absence of verapamil (−Ver). The activities are expressed relative to sample that was not treated with TMEA. The structure of TMEA is shown (inset).

DISCUSSION

In a previous study (25), we showed that TMEA alone could cross-link mutant L339C(TM6)/V982C(TM12). In this mutant cross-linking was inhibited by the presence of verapamil. By contrast, cross-linking in mutant F343C(TM6)/V982C(TM12) by TMEA occurred only in the presence of verapamil (Figs. 1 and 2). An explanation for these differences is that binding of verapamil causes conformational changes in TM6 resulting in changes in the positions of residues 339 and 343. Such substrate-induced conformational changes add further support for the substrate-induced fit mechanism of binding. We had shown previously (39) that drug substrates such as colchicine, cyclo-
sporin A, demecolcine, and progesterone can promote direct cross-linking between cysteine residues in the TM segments lining the putative drug-binding pocket with a zero length cross-linker (copper phenanthroline). It was found that progesterone promoted cross-linking in mutants P350C(TM6)/G939C(TM11), P350C(TM6)/V991C(TM12), and P350C(TM6)/A935C(TM11); cyclosporin A promoted cross-linking in mutant P350C(TM6)/G939C(TM11), and colchicine and demecolcine promoted cross-linking in mutant P350C(TM6)/V991C(TM12). When TM6 is modeled as an α-helical wheel, residues 339 and 343 are on the same face of the helix but separated by a turn of the helix (Fig. 4A). Binding of verapamil would cause a 5 Å displacement of TM6 toward the extracellular side. This would cause Cys-343 to move to a more favored position to react with TMEA. Movements of such magnitude have been reported with TM segments that line the calcium-binding site in the sarcoplasmic reticulum (SERCA1) ATPase (40). The crystal structures of SERCA1 with and without bound calcium shows movements in TMs 3 and 4 of about a 5 Å toward the outside of the membrane.

The ability of drug substrates to alter the structure of a protein has also been reported for other multidrug-binding regulatory proteins. The multidrug-binding protein QacR is a soluble regulatory protein from Staphylococcus aureus whose expression can be induced by structurally diverse cationic lipophilic drugs. The crystal structures of QacR complexed with six different drugs were reported (41). QacR forms a dimer and binds one ligand. A stoichiometry of 1 ligand per dimer has also been reported for other multidrug-binding proteins such as EmrR and BmrR (42, 43). Ligand binding induced large conformational changes in the drug-binding pocket of QacR and indicated the presence of two separate potentially overlapping binding sites within a single pocket that was surrounded by five α-helices.

Analysis of the drug-binding pocket of P-gp indicates that it may share many features with QacR. In the absence of drug substrates, the six TM segments (TMs 4–6 and 10–12) that contribute residues to the drug-binding site are close to each other because cysteines in TMs 4–6 can be directly cross-linked to cysteines in TMs 10–12 with copper phenanthroline (zero length cross-linker) (22, 34). Binding of drug substrates increases the distance between TMs 4–6 and TMs 10–12 to at least 10–15 Å (20). It is interesting that these distances are consistent with those reported recently between opposing TM6 helices (14 Å) of the ABC transporter MsbA from Vibrio cholerae (44). The crystal structure of MsbA was in the closed conformation. MsbA has some homology to P-gp and requires two monomers to form the minimum functional unit.

The ability of verapamil to promote cross-linking of mutant F343C(TM6)/V982C(TM12) with TMEA indicates that both verapamil and TMEA can simultaneously occupy the drug-binding pocket. The results also suggest that binding of one rather than two TMEA molecules occurred because cross-linking by TMEA was almost complete in mutant F343C(TM6)/V982C(TM12) in the presence of verapamil. Binding of two TMEA molecules would have resulted in little cross-linking. The cross-linking pattern of mutant F343C(TM6)/V982C(TM12) in the presence of verapamil plus TMEA suggests that single molecules of verapamil and TMEA are bound to sites that are close to each other in the drug-binding pocket. Kinetic and labeling studies suggest that P-gp has up to three different drug-binding sites (45–47). One way of reconciling these results is through a model. Fig. 4B shows that the drug-binding pocket is located at the interface between the TM domains from both halves of P-gp. Within this binding pocket are regions that bind to specific drug substrates whose sites could potentially overlap. Our results support the presence of two distinct sites within the drug-binding pocket.

TMEA is a substrate of P-gp because it can stimulate the ATPase activity of Cys-less P-gp (25). Although TMEA cross-linked C343(TM6)/C982(TM12), this position may not be the exact TMEA drug-binding site that leads to ATPase activation and transport. The exact location could not be determined because cross-linking of mutant C343(TM6)/C982(TM12) produced an inactive protein. The TMEA-binding site is probably close by, but because verapamil caused confor-
mational changes in P-gp, the TMEA-binding site may also have been altered in a way that allows the TMEA molecule to act as a “reporter” of any conformational change.

Fig. 4C shows a model of verapamil-induced conformational change promoting cross-linking between C949(TM6) and C982(TM12). Binding of verapamil causes TM6 to move toward the extracellular surface through a turn of the helix. This results in exposure of Cys-949 so that it can be cross-linked to C982 with TMEA.

In summary, the results from this study provide further evidence for the substrate-induced fit mechanism for drug binding and that two different substrates can bind simultaneously in the same drug-binding pocket.

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