Substrate-induced Conformational Changes in the Transmembrane Segments of Human P-glycoprotein

DIRECT EVIDENCE FOR THE SUBSTRATE-INDUCED FIT MECHANISM FOR DRUG BINDING*

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The human multidrug resistance P-glycoprotein (P-gp, ABCB1) is quite promiscuous in that it can transport a broad range of structurally diverse compounds out of the cell. We hypothesized that the transmembrane (TM) segments that constitute the drug-binding site are quite mobile such that drug binding occurs through a “substrate-induced fit” mechanism. Here, we used cysteine-scanning mutagenesis and oxidative cross-linking to test for substrate-induced changes in the TM segments. Pairs of cysteines were introduced into a Cys-less P-gp and the mutants treated with oxidant (copper phenanthroline) in the presence or absence of various drug substrates. We show that cyclosporin A promotes cross-linking between residues P350C(TM6)/G939C(TM11), while colchicine and demecolcine promoted cross-linking between residues P350C(TM6)/V991C(TM12). Progesterone promoted cross-linking between residues P350C(TM6)/A935C(TM11), P350C(TM6)/G939C(TM11), as well as between residues P350C(TM6)/V991C(TM12). Other substrates such as vinblastine, verapamil, cis-(Z)-flupenthixol or trans-(E)-flupenthixol did not induce cross-linking at these sites. These results provide direct evidence that the packing of the TM segments in the drug-binding site is changed when P-gp binds to a particular substrate. The induced-fit mechanism explains how P-gp can accommodate a broad range of compounds.

P-glycoprotein (P-gp)1 is an ATP-dependent drug pump that is capable of transporting structurally diverse compounds out of the cell (recently reviewed in Refs. 1 and 2). P-gp is a member of the ATP-Binding Cassette (ABC) family of transporters. 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 (3). Each repeat has six transmembrane (TM) segments and a hydrophilic domain containing an ATP-binding site (4–6). P-gp functions as a monomer (7), but the two halves of the molecule do not have to be covalently linked for function (8, 9). Studies on deletion mutants have shown that the TM domains alone are sufficient to mediate drug binding (9). Drug binding requires the contribution of the six NH2-terminal TM segments and the six COOH-terminal TM segments (10).

An important goal in understanding the mechanism of P-gp is to determine how P-gp recognizes so many structurally diverse compounds. Studies on cysteine mutants and their reactivity with different thiol-reactive substrate analogs indicate that the drug-binding site is lined with residues from multiple TM segments (11–16). Therefore, it has been proposed that P-gp binds these substrates through an induced-fit mechanism where the size and shape of the substrate changes packing of the TM segments.

In this study we used cysteine-scanning mutagenesis and oxidative cross-linking to test for rearrangement of the TM segments in the presence of different substrates.

MATERIALS AND METHODS

Construction of Mutants—A histidine-tagged Cys-less P-gp cDNA was constructed (14, 17). Pairs of cysteines were then introduced into the Cys-less P-gp (18). The presence of the histidine tag facilitated purification of the mutant P-gps by nickel-chelate chromatography (19). A cDNA coding for the TM domains only (TMD1 +2, residues 1–379 + residues 681–1025) and tagged with the epitope for monoclonal antibody A52 was constructed as described previously (9, 20).

Expression and Disulfide Cross-linking Analysis—HEK 293 cells were transfected with the mutant cDNAs. After 24 h, the medium was replaced with fresh media and the cells grown for 72 h at 27 °C. Membranes were prepared as described previously (17, 19) and used for disulfide cross-linking analysis (21) by incubation with Cu(II)(phenanthroline)3 (0.2 mM final concentration) under the conditions described in the figure legends.

Expression, Purification, and Measurement of Drug-stimulated ATPase Activity of P-gp Mutants—The histidine-tagged P-gp mutants were expressed in HEK 293 cells, purified, and mixed with lipid as described previously (19). An aliquot of the P-gp/lipid mixture was assayed for drug-stimulated ATPase activity by addition of an equal volume of buffer containing 100 mM Tris-HCl, pH 7.5, 100 mM NaCl, 20 mM MgCl2, 10 mM ATP and 10 mM colchicine, 2 mM dodecylmaltose, 0.4 mM progesterone, 0.2 mM cis-(Z)-flupenthixol, 1 mM verapamil, or 20 μM vinblastine. These concentrations gave maximal stimulation of the mutant P-gp mutant P350C. The samples were incubated for 30 min at 37 °C, and the amount of inorganic phosphate liberated was determined (22).

RESULTS

We had previously shown that substrate binding occurred through a “substrate-induced fit” mechanism (14). The TM segments contributing residues to the drug-binding site are predicted to be quite mobile so that drug binding causes rearrangement of the TM segments. Cysteine-scanning mutagenesis and oxidative cross-linking studies are ideal techniques for detecting altered packing of the TM segments in the presence of drug substrates. The TM segments in the resting state are arranged in a “funnel

brane; HEK, human embryonic kidney; TMD1+2, residues 1–379 + residues 681–1025 of P-gp.

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1 The abbreviations used are: P-gp, P-glycoprotein; TM, transmembrane; HEK, human embryonic kidney; TMD1+2, residues 1–379 + residues 681–1025 of P-gp.
mature P-gp migrates with an apparent mass of 170 kDa. noblot analysis. Fig. 1 shows that in the absence of oxidant, EDTA and the samples subjected to SDS-PAGE and immu-

membranes were incubated at 21 °C for 10 min with 5 mM colchicine (Colch), 1 mM demecolcine (Dem), 1 mM verapamil (Ver), 0.1 mM vinblastine (Vin), 0.1 mM cyclosporin A (Cyc), 2 mM cis-(Z)-flupenthixol (Cis-Flu), 2 mM trans-(E)-flupenthixol (Trans-Flu), 2 mM progesterone (Prog), or no drug substrate. The reaction mixtures were then treated with oxidant for 10 min at 21 °C with 0.2 mM copper phenanthroline (oxidant). The reactions were stopped by addition of SDS sample buffer containing 50 mM EDTA and no reducing agent. The mixtures were subjected to immunoblot analysis. The positions of the cross-linked (X-link) product and mature (170 kDa) P-gp are indicated.

shape” in which the cytoplasmic ends are close together (15, 21). The cytoplasmic side of TM6 and TM12 is relatively close together, because mutant P350C(TM6)/S993C(TM12) could be cross-linked. We showed that cross-linking of this mutant inhibited drug-stimulated ATPase activity, but activity was restored after addition of a thiol reducing agent (23). Therefore, it may be possible to detect changes in the cross-linking pattern if P350C(TM6) or S993C(TM12) is paired with another cysteine at the cytoplasmic end of other TM segments.

Accordingly, 143 double cysteine mutants that were con-
structed previously were used in this study (21). These mu-
tants contained P350C(TM6) and another cysteine on the cytoplasmic half of TMs 7–12 (i.e. P350C + another cysteine at positions: 711–723 (TM7), 770–783 (TM5), 828–840 (TM9), 867–879 (TM10), 935–947 (TM11), and 986–994 (TM12) or S993C(TM12) with another cysteine in the cytoplasmic half of TMs 1–6 (i.e. S993C + another cysteine at positions: 51–61 (TM1), 130–141 (TM2), 185–196 (TM3), 226–237 (TM4), 293–304 (TM5), and 343–351 (TM6)). We had shown that most of the double cysteine mutants retained at least 70% of the verapamil-stimulated ATPase activity of the Cys-less P-gp (21). We then tested compounds of different shapes and sizes (colchicine, demecolcine, verapamil, vinblastine, cyclosporin A, progesterone, cis-(Z)-flupenthixol and trans-(E)-flupenthixol) for their ability to promote cross-linking. Vinblastine and colchicine are classic transport substrates of P-gp (24, 25). Demecolcine and verapamil induce large increases in ATPase activity of P-gp (26). Cyclosporin A is a large cyclic peptide substrate (27), while the flupenthixol isomers have opposite effects on the ATPase activity of P-gp (28). Progesterone is a steroid substrate of P-gp (29, 30). The mutants were expressed transiently in HEK 293 cells. Membranes were prepared and preincubated at 21 °C for 10 min with or without saturating levels of colchicine (5 mM), demecolcine (1 mM), verapamil (1 mM), vinblastine (0.1 mM), cyclosporin A (0.1 mM), cis-(Z)-flupenthixol (2 mM), trans-(E)-flupenthixol (2 mM), or progesterone (2 mM). The membranes were treated with 0.2 mM copper phenanthroline (oxidant) for 10 min at 21 °C. The reactions were stopped by addition of EDTA and the samples subjected to SDS-PAGE and immu-

Cross-linked P-gp, however, migrates in SDS-PAGE with a slower mobility than mature P-gp. Moreover, we treated the membranes several times with tris-buffered saline if they were prepared from cells that had been exposed to a drug substrate. This is because residual substrate in the membrane could potentially influence the cross-linking pattern. When the membrane was washed with tris-buffered saline, it showed no cross-linking at 21 °C (Fig. 1), but some cross-linking was detected when membranes prepared from cyclosporin A-treated cells were treated with oxidant (21). Therefore, in this study, membranes were prepared from transfected cells that were not treated with any substrate.

To compare the effectiveness of the drug substrates in promoting cross-linking, time-dependent cross-linking studies were done on P350C(TM6)/A935C(TM11), P350C(TM6)/G939C(TM11), and P350C(TM6)/V991C(TM12). Fig. 2 shows that in the absence of drug substrate, no cross-linked product was detected in any of the mutants. Progesterone, however, promoted cross-linking in all three mutants and was the only drug substrate that promoted cross-linking of mutant

Fig. 1. Effect of drugs on disulfide cross-linking of P-gp mutants. Membranes were prepared from HEK 293 cells expressing mutants P350C(TM6)/A935C(TM11), P350C(TM6)/G939C(TM11), P350C(TM6)/V991C(TM12), and P350C(TM6)/A869C(TM10). The membranes were incubated at 21 °C for 10 min with 5 mM colchicine (Colch), 1 mM demecolcine (Dem), 1 mM verapamil (Ver), 0.1 mM vinblastine (Vin), 0.1 mM cyclosporin A (Cyc), 2 mM cis-(Z)-flupenthixol (Cis-Flu), 2 mM trans-(E)-flupenthixol (Trans-Flu), 2 mM progesterone (Prog), or no drug substrate. The reaction mixtures were then treated with oxidant for 10 min at 21 °C with 0.2 mM copper phenanthroline (oxidant). The reactions were stopped by addition of SDS sample buffer containing 50 mM EDTA and no reducing agent. The mixtures were subjected to immunoblot analysis. The positions of the cross-linked (X-link) product and mature (170 kDa) P-gp are indicated.

Fig. 2. Time-dependent cross-linking of P-gp mutants. Membranes prepared from HEK 293 cells expressing mutants P350C(TM6)/A935C(TM11), P350C(TM6)/G939C(TM11), or P350C(TM6)/V991C(TM12) were preincubated for 10 min at 21 °C with no drug, 1 mM progesterone, 0.1 mM cyclosporin A, 5 mM colchicine, or 1 mM demecolcine. The reaction mixtures were then treated with 0.2 mM copper phenanthroline at 21 °C for the indicated times. The reaction was stopped by addition of SDS sample buffer containing 50 mM EDTA and no reducing agent. The mixtures were subjected to immunoblot analysis. The positions of the cross-linked (X-link) product and mature (170 kDa) P-gp are indicated.
P350C(TM6)/A935C(TM11). In mutant P350C(TM6)/G939C(TM11), progesterone was more efficient in promoting cross-linking than cyclosporin A. In mutant P350C(TM6)/V991C(TM12), the drug substrates colchicine, demecolcine, and progesterone were equally effective in promoting cross-linking.

Cross-linking promoted by the presence of drug substrates cyclosporin A, colchicine, demecolcine, or progesterone could be explained on the basis that drug binding to the TM domain alters TM segment packing. It has been reported, however, that some drug substrates such as progesterone bind to mouse P-gp in a region of the nucleotide-binding domain that is in close proximity to the ATP site (33). To test whether cyclosporin A, colchicine, demecolcine, or progesterone can bind to the TM domains, we used a “drug rescue” assay involving a P-gp mutant that lacked the NBDs (TMD1–2, residues 1–379 + residues 681–1025). The rationale for the drug rescue assay is that the TMD1–2 mutant is misfolded when transiently expressed in the absence of drug substrate and is retained within the cell as a 80-kDa core-glycosylated protein (34). Expression of the mutant TMD1–2 in the presence of drug substrate, however, induces the mutant protein to fold properly into a 100-kDa protein endoglycosidase H-resistant form that is transported to the cell surface (9). It appears that the drug substrate diffuses into the cell and acts as a specific chemical chaperone to bind and stabilize the newly synthesized misfolded P-gp that is present transiently and thereby induce proper folding and trafficking of the protein (10, 35).

The TMD1–2 deletion mutant was expressed in HEK 293 cells with or without demecolcine, colchicine, or progesterone. The cells were solubilized with SDS buffer and subjected to immunoblot analysis. Fig. 3 shows that mutant TMD1–2 is expressed as an 80-kDa protein in the absence of substrate. In the presence of demecolcine, colchicine, or progesterone, however, the presence of a 100-kDa protein is detected. Cyclosporin A also induced proper folding of mutant TMD1–2 (9). Therefore, these drug substrates can interact with only the TM domains.

We then tested whether the mutants could interact with colchicine, demecolcine, progesterone, cis-(Z)-flupenthixol, verapamil, and vinblastine, because only some of the drugs affected the cross-linking pattern of the mutants. It was important to determine that the drugs that had no effect on cross-linking could still bind to the mutant P-gps. There is good correlation between drug-stimulated ATPase activity and drug transport (36) (37). Accordingly, we tested whether the drug substrates colchicine, demecolcine, progesterone, cis-(Z)-flupenthixol, verapamil, or vinblastine stimulated the ATPase activities of histidine-tagged mutants P350C(TM6)/A935C(TM11), P350C(TM6)/G939C(TM11), and P350C(TM6)/V991C(TM12). Their activities were compared with that of mutant P350C (parent). We previously showed that mutant P350C exhibited high levels of drug-stimulated ATPase activity (11). Fig. 4 shows that the ATPase activities of mutants P350C(TM6)/A935C(TM11), P350C(TM6)/G939C(TM11), and P350C(TM6)/V991C(TM12) were stimulated by colchicine, demecolcine, progesterone, cis-(Z)-flupenthixol, verapamil, or vinblastine. The ATPase activities of the mutants were inhibited by cyclosporin A and trans-(Z)-flupenthixol (data not shown). This is consistent with previous observations that saturating concentrations of cyclosporin A inhibits P-gp ATPase activity, while the cis-(Z)- and trans-(Z)-isomers of flupenthixol stimulate and inhibit, respectively, the ATPase activity of P-gp (26, 38, 39). These results indicate that the mutant P-gps can still bind drug substrates.

**DISCUSSION**

Cross-linking of mutant P350C(TM6)/S993C(TM12) is inhibited by drug substrates (23). In the present study, we show that
substrates such as progesterone are effective in promoting new cross-links with P350C (Fig. 2). In the absence of drug substrate, residue P350C in TM6 can be cross-linked to S993C in TM12. The presence of progesterone, however, promoted cross-linking of residue P350C(TM6) with two residues in TM 11 (A935C and G939C) and to residue V991C in TM12. One way to explain these results is through a model (Fig. 5). Based on the “funnel-shape” model of the TM segments (15), the cytoplasmic ends of TMs 6, 11, and 12 are placed closer together than the extracellular ends (Fig. 5A). In the absence of substrate, it is possible to cross-link P350C(TM6)/S993(TM12). Therefore, the presence of drug substrate (progesterone) likely causes a slight rotation/rearrangement of TM12 relative to TM6 such that residue V991C comes closer to P350C. Colchicine and demecolcine likely induced similar conformational changes, since both drug substrates also promoted cross-linking of mutant P350C(TM6)/V991C(TM12). Since TM12 is directly connected to TM11, any (rotational or lateral) movement in TM12 would likely involve similar movement in TM11. When the residues in TM11 are modeled as an α-helical wheel, residues A935 and G939C are found on the same face of the TM segment (Fig. 5B). In the presence of progesterone both residues must come close to P350C(TM6) to be cross-linked (Fig. 1).

Cyclosporin A also promoted cross-linking between TM6 and TM11, but only between P350C and G939C. It is possible that cyclosporin A altered the tilt or distance between TM6 and TM11 such that only A939C but not A935C were close enough to P350C to be cross-linked.

The ability of a substrate to change the cross-linking pattern suggests that the TM segments can change their shape to accommodate structurally different compounds. Slight rotational and/or lateral movement in any TM segment could result in numerous permutations of residues contributing to the drug-binding site. A substrate with one structure would cause specific shifts in the different TM segments responsible for its binding (induced-fit). Therefore, it follows that common residues could be involved in the binding of different substrates. This would account for the ability of P-gp to bind structurally diverse compounds.

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