Disulfide Cross-linking Analysis Shows That Transmembrane Segments 5 and 8 of Human P-glycoprotein Are Close Together on the Cytoplasmic Side of the Membrane*

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Human P-glycoprotein (P-gp) transports a wide variety of structurally diverse compounds out of the cell. Knowledge about the packing of the transmembrane (TM) segments is essential for understanding the mechanism of drug recognition and transport. We used cysteine-scanning mutagenesis and disulfide cross-linking analysis to determine which TM segment in the COOH half of P-gp was close to TM5 and 6 since these segments in the NH2 half are important for drug binding. An active Cys-less P-gp mutant cDNA was used to generate 240 double cysteine mutants that contained 1 cysteine in TM5 or 6 and another in TM7 or 8. The mutants were subjected to oxidative cross-linking analysis. No disulfide cross-linking was observed in the 140 TM5/TM7 or TM5/TM8 mutants. By contrast, cross-linking was detected in several P-gp TM5/TM8 mutants. At 4 °C, when thermal motion is low, P-gp mutants I299C(TM5)/G774C(TM8), I299C(TM5)/F770C(TM8), and G300C(TM5)/F770C(TM8) showed extensive cross-linking with oxidant. These mutants retained drug-stimulated ATPase activity, but their activities were inhibited after treatment with oxidant. Similarly, disulfide cross-linking was inhibited by vanadate trapping of nucleotide. These results indicate that significant conformational changes must occur between TMs 5 and 8 during ATP hydrolysis. We revised the rotational symmetry model for TM packing based on our results and by comparison to the crystal structure of MsbA (Chang, G. (2003) J. Mol. Biol. 330, 419–430) such that TM5 is adjacent to TM8, TM2 is adjacent to TM11, and TM5 and 7 are next to TMs 6 and 12, respectively.

The human multidrug resistance P-glycoprotein (P-gp,1 ABCB1) is a 170-kDa plasma membrane protein that actively extrudes a wide variety of structurally unrelated compounds of different sizes from the cell. The physiological function of P-gp is unknown, but it likely protects the organism from toxic compounds in the diet and environment (1–3). P-gp is present in relatively higher levels in some organs such as the intestine and blood-brain/testes barrier, and overexpression of P-gp in some tumor cells can undermine AIDS and cancer chemotherapy regimens (4, 5).

P-gp is one of 48 members of the ATP binding cassette (ABC) family of transporters (6). It has 1280 amino acids that are organized in two repeating units of 610 amino acids that are joined by a linker region of about 60 amino acids (7). Each repeat has six transmembrane (TM) segments and a hydrophilic domain containing an ATP-binding site. Mapping studies on the full-length protein have confirmed the predicted topology (8, 9). Both ATP-binding sites are required for activity (10–12). The minimum functional unit is a monomer (13), but the two halves of the molecule do not have to be covalently linked for function (14, 15).

An important aspect in determining the mechanism of P-gp is to understand the arrangement of the two halves of the protein. This is because binding of ATP (16, 17) and drug substrates (18–22) occurs at the interface between the two halves. Drug substrates bind at distinct regions in a common drug binding pocket (29) that is formed by the interface between the transmembrane domains (TMDs) of both halves of P-gp and involves an induced-fit mechanism (24, 25).

Much is known about how the nucleotide binding domains (NBDs) of ABC transporters interact because they are highly conserved. The results from disulfide cross-linking studies on P-gp (16) and from the crystal structures of several bacterial ABC transporters (26–28) indicate that ATP binds between the Walker A site of one NBD and the LSGGQ signature sequence of the other NBD. In P-gp, drug substrates that stimulate or inhibit ATPase activity cause these sequences to come closer or farther apart, respectively (29).

Little is known about the interactions between the TMDs of ABC transporters because these regions are less conserved. In P-gp, studies on deletion mutants show that the TMDs alone are sufficient for drug binding (15). This is consistent with the observation that P-gp interacts with drug substrates when they are in the lipid bilayer (30–32). Disulfide cross-linking studies and labeling of cysteine mutants with thiol-reactive drug substrates indicate that TMs 4–6 of TMD1 and TMs 9–12 of TMD2 form the drug binding pocket (21, 22, 33, 34). Therefore, determining how the TM segments of TMD1 interact with the TM segments of TMD2 will provide new insight for understanding the mechanism of drug transport. In this study we used cysteine-scanning mutagenesis and disulfide cross-linking analysis to determine the position of the cytoplasmic side of TMD1 relative to that of TMs 5 and 6 in TMD1.
MATERIALS AND METHODS

Construction of Mutants—Cysteine residues were introduced into a histidine-tagged Cys-less P-gp cDNA (35). Cys-less P-gp was constructed by replacing the seven endogenous cysteines at positions 137, 431, 717, 956, 1074, 1125, and 1227 with alanines (8). The presence of a histidine tag facilitated purification of the mutant P-gps by nickel-chelate chromatography (36). Cysteines were re-introduced into the Cys-less P-gp cDNA near the cytoplasmic ends of TMs 5, 6, and 8.

Disulfide Cross-linking Analysis—HEK 293 cells were transfected with the mutant cDNAs. After 24 h, the medium was replaced with fresh medium, and the cells were grown for another 48 h at 27 °C. The cells were harvested and washed once with phosphate-buffered saline (PBS; 10 mM sodium phosphate, 150 mM NaCl), pH 7.4, and then membranes were prepared as described previously (37). The membranes were suspended in PBS, pH 7.4, and samples were cross-linked by incubation with 1 mM Cu(H2O)2(SH)2 (phenanthroline), as the oxidant for various times at 4, 22, or 37 °C as described in the legends to Figs. 2, 3, and 5. To test for the effect of drug substrates on cross-linking, the membranes were preincubated for 10 min at 22 °C in the presence of no drug, 0.1 mM cyclosporin A, 1 mM demecolcine, or 1 mM verapamil before the addition of oxidant.

To test for the effect of vanadate trapping of nucleotide on cross-linking, the membranes were suspended in Tris-buffered saline, pH 7.4 (10 mM Tris-HCl, pH 7.4, 150 mM NaCl). Samples were incubated for 10 min at 37 °C in the presence of 10 mM ATP, 20 mM MgCl2, and 0.2 mM sodium vanadate. The samples were then cooled in an ice-bath for 5 min before cross-linking at 4 °C. Sodium vanadate was boiled for 3 min to break polymeric species (38) and chilled in an ice-bath before use. The reactions were stopped by the addition of SDS sample buffer (125 mM Tris-HCl, pH 6.8, 20% (v/v) glycerol, and 4% (w/v) SDS) containing 50 mM EDTA and no reducing agent. The reaction mixtures were subjected to SDS-PAGE (7.5% polyacrylamide gels) and immunoblot analysis with a rabbit polyclonal antibody against P-gp (39).

Purification and Measurement of Drug-stimulated ATPase Activity of P-gp Mutants—Fifty plates (10-cm diameter) of HEK 293 cells were transfected with mutant cDNA. After 24 h at 37 °C, the medium was replaced with fresh medium containing 10 μM cyclosporin A. Cyclosporin A, a substrate of P-gp, acts as a potent chemical chaperone in promoting maturation and yield of P-gp (40, 41). These concentrations caused maximal stimulation of ATPase activity of P-gp (42, 43). The ATPase assays were then performed as described above.

RESULTS

We have used cysteine-scanning mutagenesis and reaction with thiol reactive substrates to show that TMs 4 to 6 of TMD1 and TMs 9 to 12 of TMD2 contribute residues to the common drug binding pocket of P-gp (see Figs. 1, A and B) (20–22, 34). Transmembrane segments 5 and 6 are particularly interesting because labeling of mutants I306C (TM5) and F343C(TM6) with methanethiosulfonate (MTS)-verapamil or MTS-rhodamine, respectively, caused permanent activation of P-gp ATPase activity (23, 44). These results indicated that TMs 5 and 6 do indeed line the drug binding pocket. Therefore, we were interested in determining the TM segment in TMD2 that was close to TMs 5 or 6.

In previous cross-linking studies, we showed that the drug binding pocket was funnel-shaped, closed at the cytoplasmic end and wide at the extracellular side (34). We then developed a model of P-gp TM packing based on cross-linking studies (Fig. 1B) (33). In this model, the cytoplasmic end of TM6 is predicted to be close to that of TMs 7 or 8. Because these ends of the TM segments are close together, it would be the most appropriate area to test for proximity with a zero-length (copper phenanthroline) cross-linker. It has been estimated that the α-carbons of cross-linked cysteine residues can only be a maximum distance of 7 Å from each other with the average 5–6 Å (45). Accordingly, we used cysteine-scanning mutagenesis and disulfide cross-linking analysis to test for the presence of cross-linked product. We first tested for evidence of cross-linking between TM6 and TMs 7 or 8. We constructed 140 double cysteine mutants that contained one cysteine at positions 344–350 in TM6 and another cysteine at positions 711–720 in TM7 or positions 770–779 in TM8. The mutant cDNAs were expressed in HEK 293 cells. Membranes were then prepared, and samples were treated with oxidant (copper phenanthroline) for 10 min at 37 °C. The cross-linked samples were subjected to SDS-PAGE and immunoblot analysis. Cross-linking between cysteines located in TMD1 with another located in TMD2 can be readily detected because the cross-linked product migrates with slower mobility than the mature full-length protein in SDS-PAGE gels (46, 47). Cross-linked product was not detected...
in any of the mutants (data not shown). It appears that the cytoplasmic end of TM6 does not come close enough to the cytoplasmic ends of TMs 7 or 8 for cross-linking to occur.

An alternative arrangement is that the positions of TMs 5 and 6 are reversed. To test for this possibility we did disulfide cross-linking analysis of TM5/TM8 double cysteine mutants. A hundred double cysteine mutants were constructed that contained one cysteine at positions 293–302 of TM5 and another at positions 767–776 in TM8. HEK 293 cells were transfected with the mutant cDNAs, and membranes were prepared and subjected to cross-linking at 37 °C for 10 min. Eleven mutants (I293C/F775C, N296C/F770C, N296C/G774C, I297C/F771C, I299C/G774C, I299C/F770C, I299C/G774C, G300C/F767C, G300C/F770C, G300C/F771C, and G300C/G774C) showed relatively strong (>50%) cross-linking (Table I). An example is shown in Fig. 2A. Mutant I297C(TM5)/F771C(TM8) shows that the majority of the mutant migrates with slower mobility in SDS-PAGE gels after treatment with oxidant. Fig. 2A shows that cross-linked product was not detected with the single cysteine mutants I297C(TM5) or F771C(TM8). Six mutants (A295C/I297C, N296C/F770C, N296C/G774C, I297C/F771C, I299C/F770C, and G300C/F775C) showed relatively weak (<50%) cross-linking (Table I). An example of relatively strong and weak cross-linking is shown for mutants N296C(TM5)/F770C(TM8) and I297C(TM5)/F770C(TM8), respectively (Fig. 2B). The remaining 83 mutants showed no cross-linking (Table I).

Proteins can undergo considerable thermal motion at higher temperatures. One way to reduce thermal motion is to do cross-linking at lower temperatures. The eleven mutants (I293C/F775C, N296C/F770C, N296C/G774C, I297C/F771C, I299C/G774C, I299C/F770C, I299C/G774C, G300C/F767C, G300C/F770C, G300C/F771C, and G300C/G774C) that showed relatively strong cross-linking at 37 °C were subjected to cross-linking at 22 and 4 °C. Only four mutants (N296C/G774C, I299C/F770C, I299C/G774C, and G300C/F770C) still showed cross-linking at 22 and 4 °C. Mutants I293C/F775C, N296C/F770C, I297C/F771C, G300C/F771C, and G300C/F774C were cross-linked only at 22 °C, whereas mutants I297C/F771C and G300C/F767C showed no cross-linking at either 22 or 4 °C. Fig. 3 shows the temperature-dependent cross-linking of mutants N296C/G774C, I299C/F770C, I299C/G774C, and G300C/F770C. Cross-linked product was detected very rapidly at 22 °C in the four mutants, with the majority of the cross-linked product detected by 1 min. All four mutants were cross-linked at a slower rate at 4 °C, with most of the cross-linked product detected by 8 min. Fig. 3 also shows that in mutant N296C/F770C about 50% of the cross-linked product was detected by 8 min at 22 °C, and cross-linked product was barely detectable after 32 min at 4 °C.


detected by 8 min. Fig. 3 also shows that in mutant N296C/F770C about 50% of the cross-linked product was detected by 8 min at 22 °C, and cross-linked product was barely detectable after 32 min at 4 °C.

To test if the mutants N296C/G774C, I299C/F770C, I299C/G774C, and G300C/F770C retained the ability to interact with drug substrates, they were expressed in HEK 293 cells, isolated by nickel-chelate chromatography, mixed with lipid, and assayed for drug-stimulated ATPase activity. Drug-stimulated ATPase activity is a useful assay because it has been shown that the turnover numbers for transport and ATPase activity are comparable (48). In addition there is good correlation between drug resistance and ATPase assays (49). Accordingly, the substrates verapamil and demecolcine were used to measure drug-stimulated ATPase activities since these compounds highly stimulate (8–10-fold) the ATPase activity of Cys-less P-gp (29). At saturating concentrations of drug substrate (1 mM verapamil or 2 mM demecolcine) all four mutants showed drug-stimulated ATPase activity. Mutant I297C/F770C had about the same amount of ATPase activity as Cys-less P-gp. The verapamil- and demecolcine-stimulated ATPase activities rel-

| TABLE I | Cross-linking between residues in TMs 5 and 8 |
|---------|--------------------------------------------|
| TM5     | F767C | I768C | T769C | F770C | F771C | L772C | G773C | G774C | F775C | T776C |
| I293C   | –     | –     | –     | –     | –     | –     | –     | –     | –     | –     |
| T294C   | –     | –     | –     | –     | –     | –     | –     | –     | –     | –     |
| A295C   | –     | –     | –     | –     | + +  | –     | –     | –     | –     | –     |
| N296C   | –     | –     | –     | –     | –     | + +  | –     | –     | –     | –     |
| I297C   | –     | –     | –     | –     | –     | + +  | –     | –     | –     | –     |
| S298C   | –     | –     | –     | –     | –     | + +  | –     | –     | –     | –     |
| I299C   | –     | –     | –     | –     | + +  | + +  | –     | –     | –     | –     |
| G300C   | + +  | + +  | + +  | + +  | –     | –     | –     | –     | –     | –     |
| A301C   | –     | –     | –     | –     | –     | –     | –     | –     | –     | –     |
| A302C   | –     | –     | –     | –     | –     | –     | –     | –     | –     | –     |

- No cross-linked product detected in SDS-PAGE gels at 37 °C.
- Cross-linked product was also detected at 22 °C.
- Cross-linked product was also detected at 22 °C and at 4 °C.
- Relatively weak cross-linking (<50% of P-gp cross-linked) at 37 °C.
- Relatively strong cross-linking (>50% of P-gp cross-linked) at 37 °C.
F770C, I299C/G774C, and G300C/F770C were inhibited by the verapamil-stimulated ATPase activities of mutants N296C/G774C, I299C/G774C, or G300C/F770C. The membranes were then treated with 1 mM oxidant (CuP) at 22 °C and 4 °C for the indicated times (min). The reactions were stopped by addition of SDS sample buffer containing 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-gps are indicated.

We then tested whether cross-linking affected the verapamil-stimulated ATPase activities of mutants N296C/G774C, I299C/F770C, I299C/G774C, and G300C/F770C. Cys-less P-gp and the mutants were treated with oxidant at 22 °C for 10 min, and the reactions were stopped by the addition of EDTA followed by measurement of verapamil-stimulated ATPase activity. Fig. 4 shows that Cys-less P-gp retained more than 90% of its activity after treatment with oxidant. By contrast, the verapamil-stimulated ATPase activities of mutants N296C/G774C, I299C/F770C, I299C/G774C, and G300C/F770C were inhibited by 60–95% after treatment with oxidant. These results suggest that cross-linking inhibits conformational changes in P-gp during ATP hydrolysis (47, 50).

Because there is evidence that TM5 lines the drug binding pocket of P-gp (44), we tested whether drug substrates that stimulate (demecolcine and verapamil) or inhibit (cyclosporin A) (29) ATPase activity would affect the cross-linking pattern observed in mutants N296C/G774C, I299C/F770C, I299C/G774C, and G300C/F770C. Membranes were preincubated with saturating levels of cyclosporin A (0.1 mM), demecolcine (1 mM), verapamil (1 mM), or no drug substrate (None) for 10 min. The samples were then cooled to 4 °C and treated with oxidant for 10 min. Fig. 5 shows that the drug substrates had little or no effect on the cross-linking pattern of mutant N296C/G774C. Mutant I299C/F770C showed a small decrease in cross-linking with cyclosporin A and demecolcine, whereas mutant I299C/G774C was only slightly affected by the drug substrates. Mutants G300C/F770C showed the largest decrease in cross-linking with cyclosporin A. It appears that drug substrates had little effect on the cross-linking of the mutants. By comparison, we have shown that these drug substrates can abolish cross-linking between TMs 6 and 12 (47). This would be expected if cross-linking occurred within the drug binding pocket of P-gp.

We then tested the effect of vanadate trapping on cross-linking. Vanadate trapping of nucleotide at the ATP-binding sites can cause conformational changes in the TM segments (51). Vanadate traps ADP at one of the two NBDs by mimicking the transition state of the γ-phosphate of ATP during ATP hydrolysis. Vanadate trapping at one site inhibits ATP hydrolysis at the second site (12). Accordingly, the effect of vanadate trapping on cross-linking of mutants N296C/G774C, I299C/F770C, I299C/G774C, and G300C/F770C was examined. Membranes were preincubated with or without ATP, MgCl2, and sodium vanadate at 37 °C for 10 min. The membranes were cooled to 4 °C and cross-linked with oxidant for 10 min at 4 °C. Fig. 6 shows that vanadate trapping of nucleotide almost completely abolished cross-linking in all four mutants. These re-
Close Proximity of TM5 and TM8 Residues of P-glycoprotein

FIG. 6. Effect of ATP plus vanadate on cross-linking. Membranes from HEK 293 cells expressing P-gp mutants N296C/G774C, I299C/F770C, I299C/G774C, or G300C/F770C were preincubated at 37 °C for 10 min in the presence (+) or absence (−) of ATP and MgCl2 plus sodium vanadate (ATP/VO4). The samples were then cooled to 4 °C and then treated with (+) or without (−) oxidant (CuP) for 15 min at 4 °C. The reactions were stopped by the addition of SDS sample buffer containing 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-gps are indicated.

FIG. 7. Model showing cross-linking results and organization of the TM helices. A, the predicted TM segments 5, 6, and 8 are shown as α-helices, with the amino acid positions shown as white circles. The lines between the residues in TM5 and 8 represent cysteines that are cross-linked when treated with oxidant at 4 °C. The filled circle represents an MTS-verapamil molecule that can label Cys-306(TM5) and lies close to residues 335, 339, and 342 in TM6. B, revised model of TM packing in P-gp. The positions of TM5 and 6 have been reversed from that shown in Fig. 1B as a result of the present study. TMs 11 and 12 have also been switched because they are equivalent to TMs 5 and 6 in the homologous halves of P-gp. The positions of TMs 1 and 7 have also been changed based on comparison to the crystal structure of MsbA (52) and on the observation that mutations to TM6 can affect proteolytic sensitivity of the extracellular loop connecting TM1 and 2 (62) and that TMs 1 and 7 can be substituted to yield mutants that still confer drug resistance (61).

is also interesting to note that residue Ile-306 is located on the opposite face of TM5. Position 306 of TM5 has been shown to face the drug binding pocket because Cys-306 can be cross-linked to cysteines in TMs 10, 11, and 12 with thiol-reactive cross-linkers that are also substrates of P-gp (34). Cross-linking was completely blocked in the presence of drug substrates. In addition, MTS-verapamil labeled Cys-306 caused permanent activation of P-gp ATPase activity (44). Labeling of Cys-306 was blocked by drug substrates. In Fig. 7A, verapamil is shown to be interacting with the opposite side of TM5 and facing residues Phe-335, Leu-339, and Ala-342 of TM6. We previously showed through mutational and labeling studies with MTS-verapamil that residues Phe-335, Leu-339, and Ala-342 lie close to the verapamil-binding site (21, 59, 60). The model may explain why the presence of drug substrates had relatively little effect on cross-linking of mutants N296C/G774C, I299C/F770C, I299C/G774C, and G300C/F770C. The cross-linkable residues appear to be on the opposite side of the drug binding face of TM5. A large decrease in cross-linking was observed only in mutant G300C/F770C with the cyclosporin A.

Cyclosporin A is a relatively large compound compared with demecolcine and verapamil.

DISCUSSION

Disulfide cross-linking was observed between TM5/TM8 residues and not between TM6/TM7 or TM6/TM8 residues, suggesting that TM5 is closer to TM8. This is consistent with the latest structure from MsbA from V. cholera (52). MsbA is a bacterial ABC half-transporter of lipids that has six TM segments and one NBD. It likely functions as a dimer. Although the TM segments in MsbA are only about 20% homologous to that of P-gp, it has biochemical characteristics similar to P-gp such as substrate stimulation of ATPase activity and interaction with substrates in the lipid bilayer (53, 54). The initial crystal structure of MsbA from Escherichia coli (55) showed it to be a dimer where the TMDs were closer together on the extracellular side than on the cytoplasmic side. In addition, the NBDs were oriented such that the Walker A and LSGGQ signature sequences faced opposite directions. The recent structure of MsbA from V. cholera showed that the TMDs were closer together on the cytoplasmic side than on the extracellular side, and the NBDs were close to each other (52). The cross-linking data on P-gp (16, 33, 34, 47) could not be reconciled with the initial crystal structure of MsbA but is consistent with the latest structure (52) in that the Walker A and LSGGQ sequences face each other and the positions of TM5 and TM6 are arranged such that TM5 (TMD1) would be closer to TM8 (TMD2).

Cross-linking studies must be interpreted with caution since cross-link formation detects dynamic collisions and chemical reactions between residues and not simply their proximities (56). In support of the cross-linking approach to determine proximity, however, it has been shown that there is strong correlation between collision rates and proximity (57, 58). Further support is the finding that cross-linking between the Walker A sites and LSGGQ signature sequences in P-gp are consistent with crystal structure information about the NBDs of bacterial ABC transporters (16).

A model summarizing the results of this study is shown in Fig. 7A. The predicted TM5 and TM8 segments are modeled as α-helices. In this arrangement it is found that residues that are cross-linked at 4 °C are located on one face of TMs 5 and 8. It results suggest that vanadate trapping of nucleotide caused conformational changes between TM5 and TM8.

170 kDa product and mature (±) P-gps are indicated.

N296C/G774C
I299C/F770C
I299C/G774C
G300C/F770C

- - + ATP/VO4
- - + CuP
X-link 170 kDa

Drug
ATP
ATP

Membrane
In
Out

Fig. 7. Model showing cross-linking results and organization of the TM helices. A, the predicted TM segments 5, 6, and 8 are shown as α-helices, with the amino acid positions shown as white circles. The lines between the residues in TM5 and 8 represent cysteines that are cross-linked when treated with oxidant at 4 °C. The filled circle represents an MTS-verapamil molecule that can label Cys-306(TM5) and lies close to residues 335, 339, and 342 in TM6. B, revised model of TM packing in P-gp. The positions of TM5 and 6 have been reversed from that shown in Fig. 1B as a result of the present study. TMs 11 and 12 have also been switched because they are equivalent to TMs 5 and 6 in the homologous halves of P-gp. The positions of TMs 1 and 7 have also been changed based on comparison to the crystal structure of MsbA (52) and on the observation that mutations to TM6 can affect proteolytic sensitivity of the extracellular loop connecting TM1 and 2 (62) and that TMs 1 and 7 can be substituted to yield mutants that still confer drug resistance (61).
Conformational changes between TMs 5 and 8, however, are important for activity. For example, vanadate trapping of nucleotide nearly abolished cross-linking in mutants N296C/G774C, I299C/F770C, I299C/G774C, and G300C/F770C (Fig. 6), although cross-linking inhibited drug-stimulated ATPase activity.

The cross-linking results from this study indicate that our model of the packing of the TM segments in P-gp (Fig. 1B) should be modified such that the positions of TMs 5 and 6 are switched. This would be more consistent with the arrangement of these helices in MshA (52). Because P-gp consists of two homologous halves that have 43% amino acid identity (7), then the relative positions of the equivalent TM segments in TM22 (TMs 11 and 12) should also be reversed. Finally, in comparing P-gp to the structure of MshA it appears that TMs 1 and 7 should be placed between TMs 4 and 5, and TMs 10 and 12, respectively. TMs 1 and 7 seem to play similar roles in P-gp because each can be substituted with the other to yield functional proteins, although only 9 of the 21 residues between them are identical (61). The TM1 and TM7 replacement mutants TM1/TM1 and TM7/TM7 were both able to confer resistance to colchicine and vinblastine. By contrast replacement of TM12 with TM6 (i.e. TM6/TM6) resulted in an inactive protein (61). These results are consistent with the model showing TMs 5 and 6 to be cross-linked to cysteines at the cytoplasmic ends of TMs 4, 5, and 6 (33). The model in Fig. 7B is more consistent with these results as it places TM6 closer to TMs 10, 11, and 12 and TM12 closer to TMs 4, 5, and 6. For verapamil interactions TM12 would be predicted to be close to TMs 5 and 6 because we previously showed that mutant G984C(TM12) was inhibited by reaction with MTS-verapamil. Verapamil also protected G984C(TM12) from inhibition by MTS-verapamil (21). The cytoplasmic end of TM12 can come close to residues 1299 in TM5 because the mutant I299C(TM5)/S993C(TM12) can be cross-linked at 4°C with oxidant (33). The model also offers a possible explanation as to how mutations in TM6 can alter the protease sensitivity of the extracellular loop (ECL1) connecting TM segments 1 and 2. We had shown that mutations in TM6 such as G341C promoted proteolytic cleavage of P-gp between residues Arg-113 and Tyr-114 in ECL1 in the endoplasmic reticulum (62). Mutant G341C was particularly sensitive to cleavage at R113 since no full-length protein was detected in cells expressing the mutant. Residue Gly-341 is also on the opposite side of TM6 helix that faces the drug binding pocket and may be important for interacting with residues in TM1.

In summary, the results from this study indicate that TM5 and 8 are close together at their cytoplasmic ends and requires a revision of our earlier model of TM packing. The revised model of TM packing in P-gp resembles that of the recent structure of MshA (52). Therefore, homology modeling of P-gp based on the crystal structure of MshA may identify other close contact regions within P-gp. Further cross-linking studies would allow us to “walk” along the protein and find close contacts/bridges between residues.

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