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Drug Rescue Distinguishes between Different Structural Models of Human P-Glycoprotein

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Supporting Information

ABSTRACT: There is no high-resolution crystal structure of the human P-glycoprotein (P-gp) drug pump. Homology models of human P-gp based on the crystal structures of mouse or *Caenorhabditis elegans* P-gps show large differences in the orientation of transmembrane segment 5 (TM5). TM5 is one of the most important transmembrane segments involved in drug−substrate interactions. Drug rescue of P-gp processing mutants containing an arginine at each position in TM5 was used to identify positions facing the lipid or internal aqueous chamber. Only the model based on the *C. elegans* P-gp structure was compatible with the drug rescue results.

The P-glycoprotein drug pump (P-gp, ABCB1) catalyzes the ATP-dependent efflux of a wide range of lipophillic compounds such as hydrophobic drugs, steroids, peptides, and detergents out of the cell.[1−3] It is expressed in the epithelium of liver, kidney, and gastrointestinal tract and at the blood−brain or blood−testes barrier where it functions to protect us from cytotoxic compounds. It is clinically important because it affects the absorption, distribution, and clearance of a wide range of drugs and contributes to multidrug resistance in diseases such as cancer and AIDS. Because of its clinical importance, intensive efforts have been made to understand how it works and develop specific inhibitors to improve chemotherapy.

An accurate model of human P-gp is important for understanding its mechanism and for *in silico* docking studies for the discovery of novel inhibitors and identification of the drug-binding sites.[4−6] The 1280 amino acids of human P-gp[7] are organized as two tandem repeats that are joined by a linker region. Each repeat consists of an NH_2-terminal transmembrane domain (TMD) containing six transmembrane (TM) segments followed by a nucleotide-binding domain (NBD) (Figure 1A). The drug-binding pocket consists of 12 TM segments and has multiple and overlapping drug-binding sites.[8,9] Studies of P-gp truncation mutants show that the TMDs alone are sufficient to mediate binding of drug substrates.[10]

Homology models of human P-gp based on the mouse and *Caenorhabditis elegans* crystal structures generally yielded similar structures.[6,11] There were, however, significant differences in the orientation of TM3−TM5 in the two models. Accurate knowledge of the orientation of TM5 is particularly critical for understanding P-gp−drug interactions because residues in TM5 have been shown to play critical roles in binding of drug substrates and coupling of drug binding to

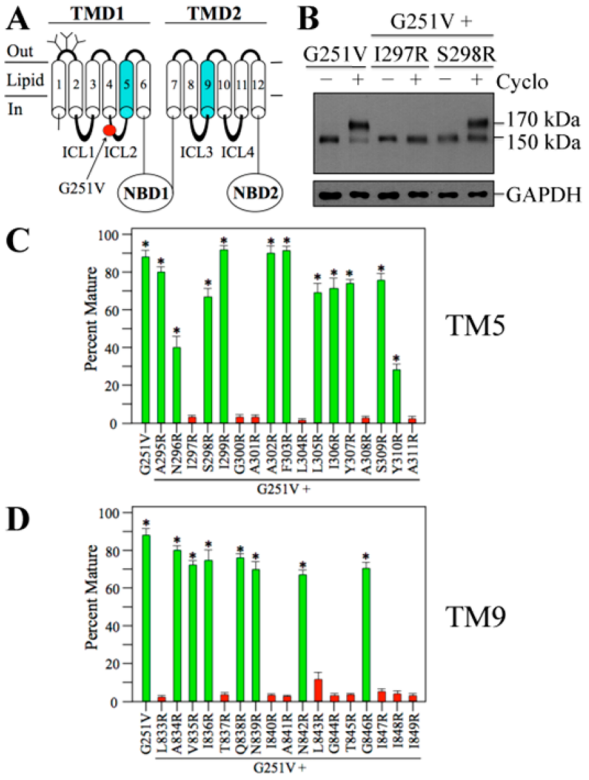

Figure 1. Drug rescue of TM5 and TM9 G251V P-gp arginine mutants. (A) Schematic model of human P-gp. (B) Immunoblot analysis of P-gp mutants expressed in the absence (−) or presence (+) of cyclosporine A (Cyclo). (C and D) Amounts of mature protein in TM5 (C) or TM9 (D) arginine mutants after expression in the presence of cyclosporine A. An asterisk indicates a significant difference from the amount of the mature form observed when the G251V parent was expressed without cyclosporine A (~5% mature).

activation of ATPase activity. For example, there is biochemical evidence that Ile306 in TM5 forms part of the drug translocation pathway. It was found that labeling of the I306C mutant with a thiol-reactive derivative of the substrate verapamil activated ATPase activity ~8-fold[12] and labeling was blocked by verapamil. In addition, it was found that the I306R mutation inhibited binding of a subset of P-gp drug substrates.[13] These results suggest that residue Ile306 is important for binding of drug substrates and activation of

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ATPase activity. Models of human P-gp based on the mouse or *C. elegans* structures, however, predict very different locations for Ile306. The model based on the mouse crystal structure (mouse model) shows that Ile306 lies on the lipid face, while that based on the *C. elegans* structure shows that it faces the internal aqueous chamber (*C. elegans* model). Therefore, we developed a drug rescue method to differentiate between the two competing models.

Accordingly, the ability of drug substrates to promote maturation of a processing mutant (G251V) containing an arginine at each position in TM5 was used to map the locations of residues that faced the lipid bilayer (would prevent rescue) or the aqueous channel (would be rescued). The rationale for using this assay was that drug substrates such as cyclosporine A can promote maturation of a P-gp processing mutant (G251V).[14] The G251V mutation is located in the second intracellular loop (ICL2) (Figure 1A) and appears to trap P-gp in a partially folded conformation as a 150 kDa core-glycosylated protein. Expression in the presence of a drug substrate induces G251V to complete the folding process to yield an active mature 170 kDa protein.[15] Introduction of an arginine onto the lipid face of TM5 would inhibit drug rescue. Arginine has a large free energy barrier (17 kcal/mol) for insertion into the lipid bilayer.[16] Insertion of an arginine on the aqueous face would not inhibit drug rescue.

Examples of drug rescue of G251V and TM5 mutants G251V/I297R and G251V/S298R are shown in Figure 1B. When processing mutant G251V is expressed in the absence of cyclosporine A, the major product was the immature 150 kDa protein (~95% of total P-gp). Expression in the presence of cyclosporine A promoted maturation such that mature 170 kDa P-gp became the major product (~90% of total P-gp). Mutant S298R but not I297R could be rescued by cyclosporine A.

Arginine mutations were then introduced into each position of TM5 or TM9 in the G251V background. TM9 was selected as a control because the models based on the mouse and *C. elegans* structures predicted that the residues had similar orientations in the membrane. The mutants were expressed in HEK 293 cells in the presence of 5 μM cyclosporine A, and P-gp was detected following immunoblot analysis of whole cell extracts. Expression was essentially the same for all mutants, but they differed in the relative amounts of mature or immature P-gp. The amount of mature 170 kDa P-gp relative to total P-gp (170 + 150 kDa proteins) was determined. Figure 1C shows that cyclosporine A promoted maturation of 11 of the 17 mutants tested (positions 295, 296, 298, 299, 302, 303, 305–307, 309, and 310) by 6–18-fold. In TM9, cyclosporine A promoted maturation of seven (positions 834–836, 838, 839, 842, and 846) of the 17 arginine mutants to yield mature 170 kDa P-gp as the major product (Figure 1D).

Arginines introduced at positions 297, 300, 301, 304, 308, and 311 in TM5 and positions 833, 837, 840, 841, 843–845, and 847–849 in TM9 could not be rescued with cyclosporine A. The locations of some of these residues that could not be rescued are shown in the homology models of human P-gp based on the mouse (Figure 2A) or *C. elegans* (Figure 2B) crystal structures.

There is agreement in both models that TM9 mutants that could not be rescued are consistently found to be facing the lipid environment (Figure 2A,B). Mutants that could be rescued faced the internal aqueous chamber.

There were considerable differences, however, when the TM5 results were interpreted in the two models. Arginines at positions 297, 300, 301, 304, 308, and 311 that could not be rescued with cyclosporine A would be expected to face the lipid environment. In the model based on the mouse structure (Figure 2A), these residues are found to be facing the drug-binding pocket while residues that could be rescued with cyclosporine A (positions 295, 298, 302, 305, and 309) face the lipid environment. By contrast, the results are consistent with the model based on the *C. elegans* structure (Figure 2B). The residues that were rescued with cyclosporine A face the drug-binding pocket, while those not recued with substrate (positions 297, 300, 301, 304, 308, and 311) face the lipid environment (Figure 2B).

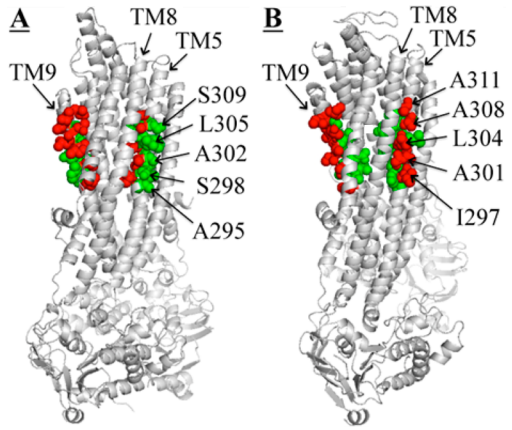

Figure 2. Homology models of human P-gp based on the crystal structures of mouse (A)[17] or *C. elegans* (B)[11] showing the results of TM5 or TM9 arginine mutagenesis viewed from the lipid side. Some rescued (green) and nonrescued (red) residues are labeled.

These results suggest that the orientation of TM5 is more consistent with that based on the *C. elegans* crystal structure. The recent report that the presence of a Glu256–Arg276 salt bridge in ICL2 is critical for folding[18] is also more consistent with the model based on the *C. elegans* crystal structure.

The results show that drug rescue of arginine mutants can be a useful approach for mapping the orientation of P-gp TM segments. Arginine mutagenesis alone can be useful for mapping because insertion of a charged residue at a lipid interface would be expected to inhibit maturation or would not be rescued with drug substrates. Arginine is particularly useful because it differs from other charged amino acids because it will remain charged in a lipid environment.[16] P-gp contains an aqueous translocation pathway,[19] so arginines inserted onto an aqueous face would not affect maturation. In some cases, insertion of an arginine into a P-gp TM segment will promote maturation of a processing mutant by forming hydrogen bonds with an amino acid in an adjacent TM segment.[20]

In conclusion, the biochemical data are more consistent with the human P-gp model based on the *C. elegans* crystal structure. In addition, the results demonstrate that arginine mutagenesis and drug rescue are valuable tools for learning about the structure of P-gp and other membrane proteins. For example, arginine mutagenesis was successfully used to map the orientation of TM6 in CFTR.[21]

■ ASSOCIATED CONTENT

Supporting Information

Details of experimental procedures. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

The manuscript was written through contributions of both authors. Both authors approve the final version of the manuscript.

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Notes

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NOTE ADDED IN PROOF

Three new structures of mouse P-gp were recently published [Ward, A. B., et al. (2013) *Proc. Natl. Acad. Sci. U.S.A. 110*, 13386−13391] that are consistent with our biochemical results and the *C. elegans* structure.