Arginines in the First Transmembrane Segment Promote Maturation of a P-glycoprotein Processing Mutant by Hydrogen Bond Interactions with Tyrosines in Transmembrane Segment 11

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A key goal is to correct defective folding of mutant ATP binding cassette (ABC) transporters, as they cause diseases such as cystic fibrosis. P-glycoprotein (ABCB1) is a useful model system because introduction of an arginine at position 65 of the first transmembrane (TM) segment could repair folding defects. To determine the mechanism of arginine rescue, we first tested the effects of introducing arginines at other positions in TM1 (residues 52–72) of a P-glycoprotein processing mutant (G251V) that is defective in folding and trafficking to the cell surface (20% maturation efficiency). We found that arginines introduced into one face of the TM1 helix (positions 52, 55, 56, 59, 60, 62, 63, 66, and 67) inhibited maturation, whereas arginines on the opposite face of the helix promoted (positions 64, 65, 68, and 71) or had little effect (positions 61, and 69) on maturation. Arginines at positions 61, 64, 65, and 68 appeared to lie close to the drug binding sites as they reduced the apparent affinity for drug substrates such as vinblastine and verapamil. Therefore, arginines that promoted maturation may face an aqueous drug translocation pathway, whereas those that inhibited maturation may face the lipid bilayer. The highest maturation efficiencies (60–85%) were observed with the Arg-65 and Arg-68 mutants. Mutations that removed hydrogen bond acceptors (Y950F/Y950A or Y953F/Y953A) in TM11 predicted to lie close to Arg-65 or Arg-68 inhibited maturation but did not affect maturation of the G251V parent. Therefore, arginine may rescue defective folding by promoting packing of the TM segments through hydrogen bond interactions.

Mutations that cause an amino acid change in the corresponding protein are responsible for about half the genetic changes known to cause disease (1). A common defect is that mutant proteins synthesized in the endoplasmic reticulum are defective in folding and trafficking (processing mutants), leading to reduced levels or absence of functional protein at a required location (2).

A classic example of a disease caused by a processing mutation is cystic fibrosis. About 90% of cystic fibrosis patients express a mutant of the cystic fibrosis transmembrane conductance regulator (CFTR) chloride channel that is missing amino acid Phe-508 (ΔF508 CFTR). The lack of functional CFTR at the surface of epithelial cells lining the airways leads to lung failure due to chronic inflammation (3).

The P-glycoprotein (P-gp) drug pump is a useful model system for studying defective folding and trafficking of processing mutants such as ΔF508 CFTR. Like CFTR, P-gp is an ABC (ATP binding cassette) transporter whose maturation is inhibited (retained in the endoplasmic reticulum) by deletion of the amino acid equivalent to Phe-508 (Tyr-590) (4). ABC transporters are characterized by the presence of two transmembrane (TM) domains (TMDs) containing six TM segments and two nucleotide binding domains (NBDs). ΔY490 P-gp can be rescued by carrying out expression in the presence of drug substrates (5). The mutant protein is delivered to the cell surface in a functional form. Processing mutations appear to inhibit folding by disrupting packing of TM segments at the interface between the TMDs (6), a defect that can be repaired by expression in the presence of drug substrates (6, 7). Drug substrates appear to rescue P-gp processing mutants through direct interactions with the TM segments because their effects can be mimicked by introducing arginine mutations into positions predicted to line the drug binding domain such as position 65 in TM segment 1 (8, 9). It was found that the L65R mutation promoted maturation of P-gp processing mutants such as ΔY490 and G251V (9).

The mechanism of arginine rescue is unknown. The arginine side chain is positively charged and has the potential to form hydrogen bond interactions with the TM segments because their effects can be mimicked by introducing arginine mutations into positions predicted to line the drug binding domain such as position 65 in TM segment 1 (8, 9). It was found that the L65R mutation promoted maturation of P-gp processing mutants such as ΔY490 and G251V (9).

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2 The abbreviations used are: CFTR, cystic fibrosis transmembrane conductance regulator; ABC, ATP binding cassette; P-gp, P-glycoprotein; NBD, nucleotide binding domain; HEK, human embryonic kidney; TM, transmembrane; TMD, TM domain.
with a L65R substitution was previously found to be particularly efficient (9). One goal was to test whether it was possible to rescue P-gp processing mutants by introducing arginines at other positions in TM1.

Another reason to examine the effects of arginines at other positions in TM1 is that there is evidence that the TM segments of P-gp surround an aqueous drug binding channel open to the extracellular environment (11, 12). For example, electron cryomicroscopy of purified hamster P-gp revealed a potential aqueous pore between the TMDs because it accumulated hydrophilic stain (11). The ability to label a cysteine (I306C(TM5)) deep within the predicted drug binding pocket of P-gp with charged thiol-reactive compounds such as (2-sulfonatoethyl) methanethiosulfonate or [2-(trimethylammonium)ethyl] methanethiosulfonate also suggested the presence of an aqueous pore (12). The presence of an aqueous chamber is controversial, however, as fluorescence spectroscopy studies on hamster P-gp suggested that the drug binding sites were located in a very hydrophobic environment with a polarity lower than cholesterol. If TM1 lined a hydrophobic channel then we would expect that most introduced arginines would inhibit maturation. If TM1 lines an aqueous channel then we would expect that arginines introduced onto the aqueous face would not inhibit maturation.

Accordingly, we first performed arginine-scanning mutagenesis on TM1 of a P-gp processing mutant (G251V) to test their effects on maturation of the mutant. We found evidence that the extracellular end of TM1 appears to line an aqueous channel, as most arginines introduced on one face did not inhibit maturation. It was also found that arginines introduced into a cluster of positions on one face of the extracellular end of TM1 could promote maturation. The most efficient rescue was observed with the M68R mutant. We present evidence which suggests that Arg-68 promotes maturation through hydrogen bond interactions with tyrosines in the extracellular half of TM1.

**EXPERIMENTAL PROCEDURES**

Construction of Mutants—Two tagged versions of human P-gp were used in this study. A P-gp cDNA containing the epitope for monoclonal antibody A52 at the COOH-terminal end was used to distinguish the expressed protein from any endogenous P-gp (13). The second P-gp cDNA was modified to contain a 10-histidine tag at the COOH-terminal end to facilitate purification of the expressed protein by nickel-chelate chromatography (14). Mutations were introduced into wild-type or processing mutant G251V (13) P-gp cDNAs as described previously (6, 15). To perform arginine-scanning mutagenesis of TM1, the cDNA of mutant G251V P-gp was modified to create a set of mutants that contained one arginine at positions 52–72. For disulfide cross-linking analysis, the cDNA of mutant L339C(TM6)/F728C(TM7) (16) was modified to also encode the G64R, M68R, or V71R mutations. The integrity of all the mutant cDNAs was confirmed by sequencing the entire cDNA (17).

Expression and Detection of Mutants—The mutant P-gps were transiently expressed in human embryonic kidney (HEK) 293 cells in Dulbecco’s modified Eagle’s medium with 10% (v/v) calf serum as described previously (14). For drug-rescue assays, the mutants were expressed in the presence of no drug, 5 μM cyclosporin A, 30 μM verapamil, 5 μM vinblastine, or 30 μM rhodamine B for 24 h. Whole cell SDS extracts were subjected to SDS-PAGE (6% (w/v) polyacrylamide gels) and immunoblot analysis with monoclonal antibody A52 (18). Digestion with endoglycosidase H (Endo H) or peptide N-glycosidase F (New England Biolabs, Mississauga, ON) were performed as described previously (19, 20).

**Purification of P-gp and Measurement of ATPase Activity—**Histidine-tagged P-gps were expressed in HEK 293 cells and then isolated by nickel-chelate chromatography as described previously (14). Recovery of P-gp was monitored by immunoblot analysis with rabbit anti-P-gp polyclonal antibody (14). A sample of the isolated histidine-tagged P-gp was mixed with an equal volume of 10 mg/ml sheep brain phosphorylcholin- amine (Type II-S, Sigma) that had been washed and suspended in Tris-buffered saline. The sample was sonicated, and ATPase activity measured in the presence of various concentrations of verapamil.

Disulfide Cross-linking Analysis—The double cysteine mutants L339C(TM6)/F728C(TM7), G64R(TM1)/L339C(TM6)/ F728C(TM7), M68R(TM1)/L339C(TM6)/F728C(TM7), or V71R(TM1)/L339C(TM6)/F728C(TM7) were transiently expressed in HEK 293 cells. Membranes were prepared as described previously (21) and suspended in Tris-buffered saline, pH 7.4. A sample of the membrane was then incubated in the presence or absence of various concentrations of vinblastine, cyclosporin A, or rhodamine B for 15 min at 20°C. The samples were then cooled on ice for 10 min and treated with 0.2 mM concentrations of the homobifunctional methanethiosulfonate cross-linker 3,6,9,12-tetraoxatetradecane-1,14-diylbis- methanethiosulfonate (M14M, 20.8 Å) (Toronto Research Chemicals, Toronto, ON) for 4 min on ice (9, 22). The reactions were stopped by the addition of 2× 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 then subjected to SDS-PAGE (7.5% (w/v) polyacrylamide gels) and immunoblot analysis with a rabbit polyclonal antibody against P-gp. Intramolecular disulfide cross-linking between TMD1 and TMD2 can be detected because the cross-linked product migrates with a slower mobility on SDS-PAGE gels (23). The gel lanes were scanned, and the amount of cross-linked protein was quantitated using the NIH Image program (available at rsb.info.nih.gov/.nih-image1) and an Apple computer.

**RESULTS**

Effect of TM1 Arginines on Maturation of Processing Mutant G251V—The crystal structure of the bacterial ABC drug transporter Sav1866 showed that the 12 TM segments of the transport complex surrounded a funnel-shaped chamber exposed to the extracellular environment (24). The structure of Sav1866 likely resembles P-gp because they both can interact with similar drug substrates such as doxorubicin, vinblastine, and Hoechst 33342, and P-gp also contains 12 TM segments (Fig. 1A). Cross-linking analyses of P-gp also suggest that the structures are similar. For example, cross-linking analyses showed
that P-gp was similar to Sav1866 because cysteines in NBD1 could be cross-linked to cysteines introduced into the fourth intracellular loop in TMD2 (25). Another structural similarity obtained by cross-linking analysis was that TM segments 2 and 5 in P-gp were found to lie next to TMs 11 and 8, respectively (26, 27), an arrangement consistent with the structure of Sav1866. Cross-linking analysis of P-gps 12 TM segments also suggested that they also surround a funnel-shaped chamber exposed to the extracellular environment (28). At least part of the chamber may be filled with (12) or is accessible to water (12). Therefore, we have changed the predicted organization of the TM segments for P-gp (9) to resemble Sav1866 (Fig. 1B). The first two TM segments of each TMD are now closely associated with the last four TM segments of the opposite TMD. The recent crystal structure of another bacterial ABC transporter showing homology to P-gp, MsbA, showed a similar arrangement of the TM segments (29).

We previously observed that introduction of arginines into TM segments such as L65R (TM1), T199R (TM3), I306R (TM5), and F343R (TM6) promoted maturation of P-gp processing mutants (9). Arginines were introduced into these positions because cysteine-scanning mutagenesis and modification with thiol-reactive drug substrates suggested that they lined the drug binding domain. Covalent modification of cysteines at positions 65(TM1), 199(TM3), 306(TM5), or 343(TM6) caused activation of P-gp ATPase activity (30–32). In this study we tested whether arginines can be accommodated at other positions in a TM segment of P-gp. The first TM segment (TM1) was selected for arginine-scanning mutagenesis because the extracellular loop connecting TM1 and TM2 contains three glycosylation sites (Fig. 1A) that can act as reporters for monitoring correct insertion of the TM segment into the membrane. TM1 also lines the drug binding domain (31), and introduction of an arginine at position 65 efficiently promoted maturation of a processing mutant (9). If an arginine mutation prevents insertion of the TM segment into the lipid bilayer, then no core glycosylation would take place. The glycosylation sites can also act as reporter molecules for monitoring subsequent folding of the protein as the addition of complex carbohydrates occurs in the Golgi. Maturation of P-gp can easily be monitored by immunoblot analysis of whole cell extracts as unglycosylated, immature, and mature forms of P-gp migrate as 140, 150, and 170 kDa proteins, respectively.

If the arginines were introduced into TM1 of a processing mutant that shows partial maturation (both 150 and 170 kDa detectable in cells), then it would be possible to detect whether the introduced arginine inhibits maturation (decrease the relative level of 170 kDa P-gp), promotes maturation (increase the relative level of 170 kDa P-gp), or has no effect. Therefore, processing mutant G251V was selected for analysis as it shows inefficient maturation relative to wild-type P-gp (33). The G251V mutation is located in the first intracellular loop (see Fig. 1A).

Accordingly, single arginine mutations were introduced at positions 52–72 of TM1. Residues 52–72 of TM1 likely spans the membrane based on the hydropathy plots of the amino acid sequence (34) and alignment of the sequences with those from the crystal structures of the homologous bacterial ABC transporters Sav1866 and MsbA (29).

HEK 293 cells were transfected with the mutant cDNAs, and whole cell extracts were subjected to immunoblot analysis (Fig. 2A). In wild-type P-gp, the major product was the 170-kDa mature protein. Immunoblot analysis of the G251V processing mutant, however, showed that the major product was the 150-kDa immature protein along with a minor amount (about 20%) of mature 170-kDa product (Fig. 2). The most common effect of introducing arginines into mutant G251V was to reduce the level of mature P-gp (12 of 21 mutants). Most of the arginines in the cytoplasmic end of TM1 (residues 53–60, 62, and 63) inhibited maturation of the mutant. Fig. 2 shows that the presence of an arginine at these positions reduced the level of mature product in mutant G251V from about 20% to less than 5% (Fig. 2B). Introduction of arginines at positions 52 or 61 had little effect on maturation of G251V P-gp. Introduction of arginines at the extracellular half of TM1 (positions 64, 65, 68, and 71), however, increased maturation of mutant G251V. Mutants M68R and L65R showed the highest increase in maturation efficiency (85 and 60% mature 170-kDa mature, respectively) (Fig. 2). Mutants G64R and V71R showed maturation efficiencies of

**FIGURE 1. Schematic models of P-gp.** A, the 12 TMs of P-gp are shown as numbered cylinders. The locations of the processing mutations are shown as gray balls. P-gp was truncated at residue Thr-1023 (indicated by a double line) to make the ΔNBD2 processing mutant. The position of the L65R mutation that promotes maturation of P-gp processing mutants is shown as a black ball. Cysteines that show direct cross-linking in the presence of oxidant in the presence of ATP (Cys-68 to Cys-950 or Cys-953) are shown as white balls. Residues that show cross-linking with M14M cross-linker and can be protected with drug substrates (Cys-339—Cys-728) are shown as speckled white balls. The branched lines represent the locations of glycosylated sites. B, model of relative positions of the TM segments that surround the drug binding pocket (oval labeled as DRUG). The amino acid sequence of TM1 predicted to span the lipid bilayer (residues 52–72) is shown as an α-helical net. The more hydrophobic residues at the NH2 and COOH termini of TM1 are also shown. The position of Leu-65 (L65R mutation promotes maturation of processing mutants) is shaded in black.
about 45%. Introduction of arginines at other positions at the extracellular end of TM1 had little effect (10–20% mature P-gp in mutants L67R, M69R, and L70R) or reduced the level of mature product to less than 10% of total P-gp (mutants P66R and F72R).

Mutants G251V, V53R/G251V (inhibited maturation) and M68R/G251V (enhanced maturation) were subjected endoglycosidase H or F digestion to determine whether the alterations in mobility of the mutant P-gp were indeed due to changes in the glycosylation state of the protein. Endoglycosidase H only cleaves core-glycosylated proteins, whereas endoglycosidase F removes core or complex carbohydrate. Fig. 3 shows that the 150-kDa immature protein was sensitive to both endoglycosidases, whereas the 170-kDa protein was only sensitive to endoglycosidase F. These results indicate that the 150-kDa immature proteins were only core-glycosylated, whereas the 170-kDa mature proteins were processed in the Golgi. Cell-surface labeling studies showed that only the mature 170-kDa protein was labeled (data not shown) and confirmed the previous observation that the 170-kDa protein was at the cell surface (8, 9). Similar results were obtained with the other arginine mutants (data not shown). These results suggest that the introduced arginines did not prevent insertion of TM1 into the lipid bilayer.

Because the M68R mutation was most efficient in promoting maturation of the G251V processing mutant, we tested whether it could also promote maturation of a different processing mutant. Accordingly, the M68R mutation was introduced into the ΔNBD2-P-gp processing mutant (20). The ΔNBD2-P-gp processing mutant does not mature as it lacks the last 257 amino acids that form NBD2 (see Fig. 1A). Mutants ΔNBD2-P-gp, M68R/ΔNBD2-P-gp, and ΔNBD2-P-gp lacking the three glycosylation sites (N91A, N94A, N99A) were expressed in HEK 293 cells, and whole cell SDS extracts were subjected to immunoblot analysis. Fig. 4 shows that the M68R mutant yielded an extra P-gp protein that corresponded in size to the mature protein (Fig. 4). Treatment with endoglycosidases H and F showed that the immature form (100 kDa) was sensitive to endoglycosidases H and F, whereas the slower migrating M68R protein (115 kDa) was sensitive to endoglycosidase F but not endoglycosidase H (data not shown).

**Drug Rescue of P-gp G251V Arginine Mutants That Do Not Show Enhanced Maturation**—If the arginine mutants that showed reduced maturation relative to the G251V (such as
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FIGURE 4. Effect of the M68R mutation on maturation of the ∆NBD2 processing mutant. Whole cell extracts of HEK 293 cells expressing mutants ∆NBD2-P-gp with no changes (None), the M68R mutation, or N91A, N94A and N99A changes to the glycosylation sites (Unglycos) were subjected to immunoblot analysis. The positions of mature, immature, and unglycosylated (Unglycos) forms of ∆NBD2 P-gp are indicated.

FIGURE 5. Drug-rescue of P-gp processing mutants. HEK 293 cells expressing mutants G251V, T55R/G251V, H61R/G251V, or M69R/G251V were treated with no drug (None), 5 μM cyclosporin A (Cyclo), 30 μM verapamil (Ver), 5 μM vinblastine (Vin), or 30 μM rhodamine B (Rhod) for 24 h. Whole cell extracts were then subjected to immunoblot analysis. The positions of mature (170 kDa) and immature (150 kDa) forms of P-gp are indicated.

V53R, Fig. 2A) were not grossly misfolded, then it should be possible to promote their maturation by carrying out expression in the presence of drug substrates. Expression of P-gp processing mutants in the presence of drug substrates such as cyclosporin A, verapamil, vinblastine, or rhodamine B promotes maturation of the protein (5, 35). Therefore, mutants showing reduced (T55R/G251V) or similar maturation efficiencies (H61R/G251V, M69R/G251V) to the G251V parent were expressed in the presence of 5 μM cyclosporin A, 30 μM verapamil, 5 μM vinblastine, or 30 μM rhodamine B. These drug concentrations promoted rescue of the G251V parent so that mature (170 kDa) protein was the major product in the treated cells (Fig. 5). By contrast, a mutant where the introduced arginine inhibited maturation of G251V (mutant T55R) could not be rescued with any of the drug substrates (Fig. 5). Some mutants were rescued only with some of the drug substrates. For example, only cyclosporin A rescued mutant H61R/G251V (Fig. 5). By contrast, all drugs except verapamil rescued mutant M69R/G251V (Fig. 5).

The effects of drug substrates on the expression of the other arginine/G251V mutants are summarized in Table 1. Mutants L65R and M68R yielded mature 170-kDa P-gp as the major product in the absence of drug substrates. Mutants V52R, L67R, and L70R resembled the parent (G251V) P-gp, as expression in any of the drug substrates promoted efficient maturation (mature P-gp became the major product) of the protein. By contrast, 12 of the mutants (V53R, G54R, T55R, L56R, A57R, A58R, I59R, A63R, P66R, and F72R) showed little or no rescue in the presence of drug substrates. It is possible that the arginine mutations may have altered their structures to disrupt all P-gp-drug interactions. Mutant G64R only showed significant rescue with cyclosporin A and rhodamine B. It appears that introduction of an arginine at positions 61, 64, and 69 perturbed only some P-gp-drug interactions.

Effect of Suppressor Arginine Mutations on P-gp-Drug Interactions—Because mutant M68R/G251V showed efficient maturation (about 85%) when expressed in the absence of drug substrates (Fig. 2), we tested whether Arg-68 affected P-gp-drug interactions in disulfide protection assays using mutant L339C(TM6)/F728C(TM7) as described previously (9). Because the G64R and V71R changes also promoted maturation of the G251V processing mutant (Fig. 2), we also introduced these mutations into the L339C(TM6)/F728C(TM7) mutant. The L339C(TM6)/F728C(TM7) is a useful mutant because the cysteines at positions 339 and 728 will cross-link when treated with the M14M thiol-reactive cross-linker. Cross-

| Mutation          | No drug | Cyclosporin A | Verapamil | Vinblastine | Rhodamine |
|-------------------|---------|---------------|-----------|-------------|-----------|
| None              | -       | -             | -         | -           | -         |
| V52R              | -       | -             | -         | -           | -         |
| V53R              | -       | -             | -         | -           | -         |
| G54R              | -       | -             | -         | -           | -         |
| T55R              | -       | -             | -         | -           | -         |
| L56R              | -       | -             | -         | -           | -         |
| A57R              | -       | -             | -         | -           | -         |
| A58R              | -       | -             | -         | -           | -         |
| I59R              | -       | -             | -         | -           | -         |
| A63R              | -       | -             | -         | -           | -         |
| P66R              | -       | -             | -         | -           | -         |
| M68R              | -       | -             | -         | -           | -         |
| L65R              | -       | -             | -         | -           | -         |
| M69R              | -       | -             | -         | -           | -         |
| L70R              | -       | -             | -         | -           | -         |
| G64R              | -       | -             | -         | -           | -         |
| V71R              | -       | -             | -         | -           | -         |
| F72R              | -       | -             | -         | -           | -         |

* Change in the amount of mature (170 kDa) protein relative to that in the absence of drug substrate.

* Change in the amount of mature (170 kDa) protein relative to the mutant G251V expressed in the absence of drug substrate.
linking can readily be detected because cross-linked P-gp migrates at a higher molecular mass than the mature 170-kDa protein on SDS-PAGE gels. Drug substrates such as cyclosporin A, vinblastine, and rhodamine B protect the mutant from cross-linking. Drug substrates may inhibit cross-linking because they occupy a site between Cys339(TM6) and Cys728(TM7) (see Fig. 1B). Verapamil may bind to a different site as it does not inhibit cross-linking of mutant L339C(TM6)/F728C(TM7). In a previous cross-linking study we found that introduction of L65R change into mutant L339C(TM6)/F728C(TM7) reduced its apparent affinity for vinblastine by about 60-fold but had little effect on P-gp interactions with cyclosporin A or rhodamine B (9).

Membranes were prepared from HEK 293 cells expressing mutants L339C(TM6)/F728C(TM7), G64R/L339C(TM6)/F728C(TM7), M68R/L339C(TM6)/F728C(TM7), and V71R/L339C(TM6)/F728C(TM7). The membranes were preincubated in the presence of various concentrations cyclosporin A, vinblastine, or rhodamine B followed by treatment with M14M cross-linker for 4 min on ice. Cross-linking was performed at 0 °C to minimize thermal motion of the proteins. Samples were then subjected to immunoblot analysis. A representative example of the effects of various concentrations of vinblastine on cross-linking of the mutants are summarized in Table 2. The G64R/L339C(TM6)/F728C(TM7) mutant also showed a large reduction in apparent affinity for vinblastine (>100-fold), whereas the V71R change had little effect. None of the arginine mutations caused large changes (less than a 2-fold change) in the level of cyclosporin A or rhodamine B required for protecting the mutant from cross-linking (Table 2).

The G64R, L65R, and M68R mutations preferentially affected P-gp-vinblastine interactions. We previously reported that residue Leu-65 appeared to lie close to the verapamil binding site (31). It was shown that mutant L65C could be labeled with a thiol-reactive derivative of verapamil to cause permanent activation of ATPase activity. Activation of P-gp ATPase activity in the presence of drug substrates appears to be a measure of P-gp-drug interactions, as there is a good correlation with drug transport (36). It was not possible to use the cross-
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![Graph showing Verapamil-stimulated ATPase activities of wild-type and mutant G64R and M68R P-gps.](image)

Verapamil-stimulated ATPase activities of wild-type and mutant G64R and M68R P-gps. Histidine-tagged P-gps were expressed in HEK 293 cells and isolated by nickel-chelate chromatography. The isolated P-gps were mixed with lipid, and ATPase activities were measured in the presence of various concentrations of verapamil. Each value is the average of triplicate experiments.

Linking protection assay with mutant L339C(TM6)/F728C(TM7) to test for alterations in verapamil interactions as it does not block cross-linking of these cysteines (9). Therefore, we measured drug stimulation of ATPase activity of mutants G64R and M68R using various concentrations of verapamil to test whether the apparent affinity for verapamil of the P-gp mutants was altered. Verapamil is a useful compound to test P-gp-drug interactions, as it is one of the most potent activators of P-gp ATPase activity. It can stimulate P-gp ATPase activity by more than 15-fold (37).

Accordingly, mutations G64R or M68R were introduced into a wild-type P-gp that contained a histidine tag at the COOH-terminal end. Histidine-tagged wild-type and mutants G64R and M68R P-gps were expressed in HEK 293 cells, and the proteins were isolated by nickel-chelate chromatography. The isolated P-gps were mixed with lipid and assayed for ATPase activity in the presence of various concentrations of verapamil.

Maximal stimulation of wild-type P-gp ATPase activity by verapamil (2.0 µmol P_i/min/mg of protein) occurred in the presence of 0.3 mM drug (Fig. 7). Half-maximal activation of wild-type ATPase activity occurred in the presence of 29 µmol of verapamil. Verapamil also stimulated the ATPase activities of mutants G64R and M68R (1.6 and 1.8 µmol P_i/min/mg of protein, respectively). Although both mutants G64R and M68R showed relatively high activation of ATPase activity with verapamil, their apparent affinities for verapamil were reduced by about 19-fold and 4-fold, respectively. Half-maximal activation of the ATPase activities of mutants G64R and M68R required 540 µM and 120 µM verapamil, respectively, compared with 29 µM for wild-type P-gp.

Effect of TM11 Mutations on Rescue of Mutant G251V Containing the L65R and M68R Changes—One reason that Arg-68 caused the largest increase in maturation of mutant G251V is that the arginine residue may promote packing of the TM segments. We previously showed that processing mutations appear to inhibit maturation of the protein by disrupting packing of the TM segments between the two TMDs (6). One type of interaction between the arginines and tyrosines that may promote folding of processing mutants is hydrogen bonding. The side chain of arginine contains multiple hydrogen bond donors, whereas the hydroxyl group of tyrosine can act as a hydrogen bond donor or acceptor. Because arginines have the highest capacity to form (up to three) hydrogen bonds, it is possible that they promote maturation by forming hydrogen bonds with residues in other TM segments. Previous cysteine cross-linking studies suggested that residue 68 in TM1 might lie close to the hydrogen bond acceptors Tyr-950 or Tyr-953 in TM11 (38). To test if Tyr-950 or Tyr-953 influences the ability of the M68R mutation to promote maturation of the G251V mutant, we introduced the Y950A or Y953A mutations into mutant M68R/G251V mutant. The mutants were expressed in HEK 293 cells, and whole cell SDS extracts were subjected to immunoblot analysis. Fig. 8 shows the maturation efficiencies compared with the M68R/G251V parent. The presence of the Y950A or Y953A mutation reduced the maturation efficiency to less than 50% (Fig. 8A, lanes 6 and 9). Not all mutations in TM11, however, affected maturation of the M68R/G251V mutant. The presence of mutations Q946A, M948A, M949A, F951A, or Y953A did not appear to affect the maturation of the M68R/G251V mutant (Fig. 8A, lanes 2–5, 7, and 8).

Because the L65R mutation also promoted maturation of mutant G251V, we tested whether the Y950A or Y953A mutations would have any effect on maturation of the L65R/G251V mutant. Fig. 8B shows that introduction of the Y950A mutation caused a relatively large reduction in the maturation efficiency of the mutant (Fig. 8B, lane 3), whereas the Y953A mutation had little effect (Fig. 8B, lane 4). In contrast, both Y950A and Y953A affected the maturation efficiency of the M68R/G251V mutant. The Y950A and Y953A mutations did not affect maturation of the (G251V) parent (Fig. 8A, lanes 13 and 14) or wild-type P-gp (data not shown). These results suggest that interactions between Arg-65(TM1) and Tyr-950(TM11), Arg-68(TM1) and Tyr-950(TM11), or Arg-68(TM1) and Tyr-953(TM11) may be responsible for the enhanced maturation of the G251V mutant.

Another potential type of interaction between the arginines and tyrosines is cation-π interactions. Cation-π interactions can occur between the cationic side chains of Lys or Arg with the aromatic side chains of Phe, Tyr, or Trp (39). To determine whether hydrogen bonding or cation-π interactions were responsible for the effects observed with the Arg-65 or Arg-68 mutations, we tested the effects of Tyr to Phe changes at positions 950 and 953 in TM11. Conservative replacement of Tyr-950 or Tyr-953 with Phe would only disrupt hydrogen bond interactions. Mutants L65R/G251V/Y950F, M68R/G251V/Y950A/Y953A, and M68R/G251V/Y950F/Y953F were constructed, and the cDNAs were expressed in HEK 293 cells. Only the Y950F change was introduced into the L65R/G251V mutant since the Y953A change did not affect maturation of the mutant (Fig. 8B, lane 4). Immunoblot analysis shows the Y950A/Y953A and Y950F/Y953F changes in M68R/G251V (Fig. 8A, lanes 11 and 12) or Y950F change in L65R/G251V (Fig. 8B, lane 5) reduced maturation to levels similar to that observed in the G251V parent. Introduction of the Y950F and Y953F
changes into the G251V parent did not affect its maturation efficiency (Fig. 8, A, lane 15, and B, lane 6). The results suggest that Arg-65 or Arg-68 interact with tyrosines in TM11 through hydrogen bond interactions.

**DISCUSSION**

It was found that maturation of mutant G251V was not inhibited when arginines were introduced at many positions on the extracellular half of TM1. Indeed, introduction of arginines at four locations (Gly64, Leu-65, Met-68, Val-71) promoted maturation of the mutant. When TM1 is modeled as an α-helix (Fig. 9A, Chamber view), most of the residues that promoted maturation or had little effect when replaced with arginine were located on one face. Examination of the crystal structure of Sav1866 after alignment of the TM1s of Sav1866 and P-gp (Fig. 9A, Chamber view) (29) revealed that arginines introduced at positions 64, 65, 68, and 71 face the central chamber that is predicted to form the drug translocation pathway (24). Most arginines introduced into the face of TM1 predicted to face the lipid bilayer (Fig. 9A, Lipid view) caused inhibition of maturation (Fig. 2).

An explanation for the effects of arginines introduced into TM1 on maturation of P-gp is that one side of TM1 lines an aqueous inner chamber, whereas the opposite face lies in a hydrophobic environment. Introduction of positively charged groups into TM1 would not inhibit maturation if they were located in an aqueous environment. There is, however, a high free energy barrier for introducing arginines into the lipid phase (10, 40). Therefore, it would be expected that arginines introduced into the lipid face of TM1 would inhibit maturation. Although the side chains of charged amino acids can undergo large pKₐ shifts in nonpolar regions of membrane proteins (41), arginine is a unique amino acid because it remains charged in nonpolar environments (40).

Introduction of an arginine at position 70 did not inhibit maturation although it was predicted to lie on the lipid face of TM1 (Fig. 9A, Lipid view). Because position 70 is close to the interface between the lipid and outer aqueous environment, the arginine side chain could undergo snorkeling (42) to point away from the membrane core.

The extracellular and intracellular halves of TM1 showed quite different effects upon introduction of arginines. Maturation of P-gp was not inhibited when arginines were introduced into the predicted aqueous face of the extracellular half of TM1, whereas nearly all arginines introduced into the intracellular half inhibited maturation. It appears that the cytoplasmic half of TM1 may reside in a hydrophobic environment. In the crystal structure of Sav1866, only the outer-leaflet half of TM1 lines the predicted drug translocation pathway. The inner-leaflet portion of TM1 protrudes away from the predicted chamber (24).

Evidence that TM1 lined the putative drug translocation pathway at the level of the outer leaflet were the observations that arginines introduced at positions 64, 68 (this study), and 65 (9) caused a reduction in the apparent affinity for vinblastine in cross-linking protection assays or for verapamil in ATPase assays (Table 3). There was a good correlation between the drug-rescue results (Table 1) and the cross-linking or ATPase assays (Table 3). For example, in mutant G64R the apparent affinities for vinblastine and verapamil were reduced. By contrast, its apparent affinity for cyclosporin A or rhodamine B was unaffected (Table 3). A similar pattern was observed in the drug rescue assay. Cyclosporin A and rhodamine B but not vinblastine or verapamil promoted maturation of the mutant G64R/G251V (Table 1).

The H61R mutation appeared to perturb vinblastine interactions as expression in the presence of vinblastine did not promote maturation of the mutant H61R/G251V (Fig. 5). These

**FIGURE 8. Effect of TM11 mutations on maturation of M68R/G251V and L65R/G251V mutants.** A, HEK 293 cells were transfected with mutants M68R/G251V (lanes 2–12) or G251V (lanes 1, 13–15) containing the indicated TM11 mutations. Whole cell extracts were subjected to immunoblot analysis. B, HEK 293 cells were transfected with mutants M65R/G251V (lanes 2–5) or G251V (lanes 1, 6) containing the indicated TM11 mutations. Whole cell extracts were subjected to immunoblot analysis. The positions of mature (170 kDa) or immature (150 kDa) P-gps are indicated.
results are consistent with a previous study that examined the effects of arginine substitutions to residues Ala-57 to Leu-65 in TM1 on drug transport by P-gp (43). It was found that the H61R, G64R, and L65R mutations altered the substrate specificity for P-gp substrates colchicine, doxorubicin, and etoposide compared with cells expressing the wild-type transporter. We also observed that arginine mutations to some residues in TM1 could alter P-gp interactions with drug substrates other than vinblastine. For example, several of the TM1 arginine mutants showed reduced interactions with verapamil. Mutants H61R/G251V or M69R/G251V showed little drug rescue with verapamil (Fig. 5), and mutants G64R and M68R in a wild-type background showed reductions in apparent affinity for verapamil in ATPase assays (Fig. 7). Therefore, these results are consistent with the prediction that residues in the extracellular half of TM1 line the drug translocation pathway of P-gp. Residue Val-71, however, appeared to lie outside the drug binding pocket since the V71R mutation did not affect P-gp-drug interactions (Table 3).

Residue Met-68 is predicted to be located in a segment of TM1 that lies in the extracellular leaflet close to surface (Fig. 1B). The observation that the M68R mutation can reduce the apparent affinities for the drug substrates verapamil and vinblastine would be unexpected because it appears that P-gp initially interacts with drug substrates in the inner leaflet of the bilayer (44). One explanation is that the M68R mutation causes an indirect effect on the vinblastine and verapamil binding sites. The structural alterations may be small because the M68R change did not appear to affect P-gp interactions with cyclosporin A or rhodamine B (Table 2). Another explanation is that the M68R mutation has altered the predicted “OFF-sites” of P-gp. Studies with cis-flupenthixol and [125I]iodoarylazidoprazosin suggested that drug substrates could interact with two sites, an initial binding site (ON-site) and a site involved in drug release (OFF-site) (45). Because the OFF-sites would be involved in drug release, they may be located in the outer portions of P-gp and would be sensitive to mutations such as M68R.
Arginine Mutagenesis of P-gp TM1

Some mutations such as G64R, L65R, M68R, and V71R actually promoted maturation of the G251V mutant. The L65R and M68R mutations were particularly effective in promoting maturation as they increased maturation efficiency of the G251V mutant from about 20 to 60–85%. One explanation is that introduction of arginine at positions 64, 65, 68, and 71 promotes folding of P-gp by affecting the orientation of TM1 in the lipid bilayer. We previously showed that packing of the TM segments between the two TMDs is a rate-limiting state in maturation of ABC transporters such as P-gp and CFTR (6, 46). Processing mutations such as G251V may alter the orientation of one or more TM segments in the membrane to interfere with subsequent packing of the TM segments at the interface between TMD1 and TMD2. Introduction of an arginine into TM1 may promote maturation by helping to orientate TM1 into a conformation where one face aligns with the aqueous channel.

An alternative possibility to explain why the L65R and M68R mutations were particularly effective in promoting maturation of the G251V mutant is that they promoted packing of the TM segments by forming hydrogen bonds with Tyr-950 and/or Tyr-953 in TM11. Therefore, hydrogen bonds between the TM segments between TMD1 and TMD2. Introduction of an arginine into TM1 may promote maturation by helping to orientate TM1 into a conformation where one face aligns with the aqueous channel.

| TM1 arginines that promote maturation | Does arginine reduce the apparent drug affinity? |
|---------------------------------------|-----------------------------------------------|
| G64R                                  | Yes                                           |
| L65R                                  | No                                            |
| M68R                                  | No                                            |
| V71R                                  | No                                            |

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### REFERENCES
1. Ng, P. C., and Henikoff, S. (2006) *Annu. Rev. Genomics Hum. Genet.* 7, 61–80.
2. Sanders, C. R., and Myers, J. K. (2004) *Annu. Rev. Biophys. Biomol. Struct.* 33, 25–51.
3. Rowe, S. M., Miller, S., and Sorscher, E. J. (2005) *N. Engl. J. Med.* 352, 1992–2001.
4. Hoof, T., Demmer, A., Hadam, M. R., Riordan, J. R., and Tummler, B. (1994) *J. Biol. Chem.* 269, 20575–20583.
5. Loo, T. W., and Clarke, D. M. (1997) *J. Biol. Chem.* 272, 709–712.
6. Loo, T. W., Bartlett, M. C., and Clarke, D. M. (2002) *J. Biol. Chem.* 277, 27585–27588.
7. Loo, T. W., and Clarke, D. M. (1998) *J. Biol. Chem.* 273, 14671–14674.
8. Loo, T. W., Bartlett, M. C., and Clarke, D. M. (2006) *J. Biol. Chem.* 281, 29436–29440.
9. Loo, T. W., Bartlett, M. C., and Clarke, D. M. (2007) *J. Biol. Chem.* 282, 32043–32052.
10. Dorairaj, S., and Allen, T. W. (2007) *Proc. Natl. Acad. Sci. U. S. A.* 104, 4943–4948.
11. Rosenberg, M. F., Velarde, G., Ford, R. C., Martin, C., Berridge, G., Kerr, I. D., Callaghan, R., Schmidlin, A., Wooding, C., Linton, K. J., and Higgins, C. F. (2001) *EMBO J.* 20, 5615–5625.
12. Loo, T. W., Bartlett, M. C., and Clarke, D. M. (2004) *Biochemistry* 43, 12081–12089.
13. Loo, T. W., and Clarke, D. M. (1994) *J. Biol. Chem.* 269, 7243–7248.
14. Loo, T. W., Bartlett, M. C., and Clarke, D. M. (1995) *J. Biol. Chem.* 270, 21449–21452.
15. Loo, T. W., and Clarke, D. M. (1994) *Biochemistry* 33, 14049–14057.
16. Wang, Y., Loo, T. W., Bartlett, M. C., and Clarke, D. M. (2007) *Mol. Pharmacol.* 71, 751–758.
17. Sanger, F., Nicklen, S., and Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U. S. A.* 74, 5463–5467.
18. Loo, T. W., and Clarke, D. M. (1995) *J. Biol. Chem.* 270, 21839–21844.
19. Loo, T. W., and Clarke, D. M. (1994) *J. Biol. Chem.* 269, 28683–28689.
20. Loo, T. W., and Clarke, D. M. (1999) *J. Biol. Chem.* 274, 24759–24765.
21. Loo, T. W., and Clarke, D. M. (2000) *J. Biol. Chem.* 275, 5253–5256.
22. Loo, T. W., and Clarke, D. M. (2002) *Proc. Natl. Acad. Sci. U. S. A.* 99, 3511–3516.
23. Loo, T. W., Bartlett, M. C., and Clarke, D. M. (2006) *Biochem. J.* 399, 351–359.
24. Dawson, R. J., and Locher, K. P. (2006) *Nature* 443, 180–185.
25. Zohnerciks, J. K., Wooding, C., and Linton, K. J. (2007) *FASEB J.* 21, 3937–3948.
26. Loo, T. W., Bartlett, M. C., and Clarke, D. M. (2004) *J. Biol. Chem.* 279, 7692–7697.
27. Loo, T. W., Bartlett, M. C., and Clarke, D. M. (2004) *J. Biol. Chem.* 279, 18232–18238.
28. Loo, T. W., and Clarke, D. M. (2001) *J. Biol. Chem.* 276, 36877–36880.
29. Ward, A., Reyes, C. L., Yu, J., Roth, C. B., and Chang, G. (2007) *Proc. Natl. Acad. Sci. U. S. A.* 104, 19005–19010.
30. Loo, T. W., Bartlett, M. C., and Clarke, D. M. (2003) *J. Biol. Chem.* 278, 50136–50141.
31. Loo, T. W., Bartlett, M. C., and Clarke, D. M. (2006) *Biochem. J.* 396, 537–545.
Arginines Mutagenesis of P-gp TM1

32. Loo, T. W., Bartlett, M. C., and Clarke, D. M. (2003) J. Biol. Chem. 278, 20449–20452
33. Loo, T. W., and Clarke, D. M. (1999) FASEB J. 13, 1724–1732
34. Chen, C. I., Chin, J. E., Ueda, K., Clark, D. P., Pastan, I., Gottesman, M. M., and Roninson, I. B. (1986) Cell 47, 381–389
35. Loo, T. W., and Clarke, D. M. (2002) J. Biol. Chem. 277, 44332–44338
36. Ambudkar, S. V., Cardarelli, C. O., Pashinsky, I., and Stein, W. D. (1997) J. Biol. Chem. 272, 21160–21166
37. Loo, T. W., and Clarke, D. M. (1999) J. Biol. Chem. 274, 35388–35392
38. Loo, T. W., Bartlett, M. C., and Clarke, D. M. (2005) Biochemistry 44, 10250–10258
39. Gallivan, J. P., and Dougherty, D. A. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 9459–9464
40. Li, L., Vorobyov, I., MacKerell, A. D., Jr., and Allen, T. W. (2008) Biophys. J. 94, 11–13
41. Cymes, G. D., Ni, Y., and Grosman, C. (2005) Nature 438, 975–980
42. Chamberlain, A. K., Lee, Y., Kim, S., and Bowie, J. U. (2004) J. Mol. Biol. 339, 471–479
43. Taguchi, Y., Morishima, M., Komano, T., and Ueda, K. (1997) FEBS Lett. 413, 142–146
44. Chen, Y., Pant, A. C., and Simon, S. M. (2001) Cancer Res. 61, 7763–7769
45. Dey, S., Ramachandra, M., Pastan, I., Gottesman, M. M., and Ambudkar, S. V. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 10594–10599
46. Chen, E. Y., Bartlett, M. C., Loo, T. W., and Clarke, D. M. (2004) J. Biol. Chem. 279, 39620–39627
47. Therien, A. G., Grant, F. E., and Deber, C. M. (2001) Nat. Struct. Biol. 8, 597–601
48. Choi, M. Y., Cardarelli, L., Therien, A. G., and Deber, C. M. (2004) Biochemistry 43, 8077–8083
49. Wang, Y., Bartlett, M. C., Loo, T. W., and Clarke, D. M. (2006) Mol. Pharmacol. 70, 297–302
50. Van Goor, F., Straley, K. S., Cao, D., Gonzalez, J., Hadida, S., Hazlewood, A., Joubran, J., Knapp, T., Makings, L. R., Miller, M., Neuberger, T., Olson, E., Panchenko, V., Rader, J., Singh, A., Stack, J. H., Tung, R., Grootenhuis, P. D., and Negulescu, P. (2006) Am. J. Physiol. Lung Cell. Mol. Physiol. 290, 1117–1130
51. Pedemonte, N., Lukacs, G. L., Du, K., Caci, E., Zegarra-Moran, O., Galietta, L. J., and Verkman, A. S. (2005) J. Clin. Investig. 115, 2564–2571