Suppressor Mutations in the Transmembrane Segments of P-glycoprotein Promote Maturation of Processing Mutants and Disrupt a Subset of Drug-binding Sites*

Tip W. Loo, M. Claire Bartlett, and David M. Clarke

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From the Department of Medicine and Department of Biochemistry, University of Toronto, Toronto, Ontario M5S 1A8, Canada

Defective folding of cystic fibrosis transmembrane conductance regulator protein missing Phe508 (ΔF508) is the major cause of cystic fibrosis. The folding defect in ΔF508 cystic fibrosis transmembrane conductance regulator might be correctable because misfolding of a P-glycoprotein (P-gp; ABCB1) mutant lacking the equivalent residue (ΔY490) could be corrected with drug substrates or by introduction of an arginine residue into transmembrane (TM) segments 5 (I306R) or 6 (F343R). Possible mechanisms of arginine rescue were that they mimicked some of the effects of drug substrate interactions with P-gp or that they affected global folding such that all drug substrate/modulator interactions with P-gp were altered. To distinguish between these mechanisms, we tested whether arginines introduced into other TMs predicted to line the drug-binding pocket (TM1 or TM3) would affect folding. It was found that mutation of L65R(TM1) or T199R(TM3) promoted maturation of processing mutants. We then tested whether arginine suppressor mutations had local or global effects on P-gp interactions with drug substrates and modulators. The L65R(TM1), T199R(TM3), I306R(TM5), or F343R(TM6) mutations were introduced into the P-gp mutant L339C(TM6)/F728C(TM7), and thiol cross-linking was carried out in the presence of various concentrations of vinblastine, cyclosporin A, or rhodamine B. The presence of arginine residues reduced the apparent affinity of P-gp for vinblastine, cyclosporin, A, or rhodamine B. The presence of arginine residues reduced the apparent affinity of P-gp for vinblastine (L65R, T199R, and I306R), cyclosporin (I306R and F343R), or rhodamine B (F343R) by 4–60-fold. These results show that the arginine mutations affect a subset of drug-binding sites and suggest that they rescue processing mutants by mimicking drug substrate interactions with P-gp.

Deleterious folding of a mutant membrane protein in the endoplasmic reticulum and/or trafficking to its normal cellular destination is responsible for many inherited diseases. The protein usually has a deletion or substitution of an amino acid (1) leading to their retention in the endoplasmic reticulum and subsequent degradation by the endoplasmic reticulum-associated degradation system (2).

A classic example of a genetic disease caused by an amino acid change in a membrane protein is cystic fibrosis. The most common cause of cystic fibrosis is deletion of Phe508 (ΔF508) in the cystic fibrosis transmembrane conductance regulator (CFTR; ABCB7) protein. The ΔF508 mutation causes misfolding of CFTR such that the amount of mature CFTR at the cell surface is reduced from about 25% (wild type) to less than 1% (3, 4). An important goal in developing a treatment for protein-folding diseases is to correct folding/trafficking defects in the mutant proteins.

The human multidrug resistance P-glycoprotein (P-gp, ABCB1) is a useful model system for studying protein misfolding because processing mutations can be rescued with drug substrates (reviewed in Ref. 5). P-gp is an ABC (ATP-binding cassette) protein that uses ATP to transport a variety of cytotoxic compounds out of the cell (6, 7). It has two homologous halves that are joined by a linker region (8). Each half has a transmembrane domain (TMD) containing six transmembrane (TM) segments and a nucleotide-binding domain (NBD). The drug-binding pocket is at the interface between the two TMDs (9, 10), and a P-gp mutant lacking both NBDs retained the ability to bind drug substrates (11). The drug-binding pocket is relatively large and can simultaneously bind different drug substrates (12, 13). Drug substrate binding likely occurs by an “induced fit” mechanism (14).

Deletion of residue Tyr490 (ΔY490) in P-gp, which is equivalent to Phe508 in CFTR, also results in protein misfolding (15). The ΔY490 P-gp mutant protein is retained in the endoplasmic reticulum by molecular chaperones (16). The mutant could be rescued, however, by carrying out expression in the presence of drug substrates or modulators (pharmacological chaperones) (17). It was not known whether drug substrates promoted maturation of P-gp processing mutants through direct interaction with the TMDs of the protein (11) or indirectly by affecting protein folding pathways such as stress-induced changes in the levels of molecular chaperones (18–20). Recent evidence suggests that drug substrates promoted maturation of P-gp proc-

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† Recipient of the Canada Research Chair in Membrane Biology. To whom correspondence should be addressed: Dept. of Medicine, University of Toronto, Rm. 7342, Medical Sciences Bldg., 1 King’s College Circle, Toronto, Ontario M5S 1A8, Canada. Tel. or Fax: 416-978-1105; E-mail: david.clarke@utoronto.ca.

2 The abbreviations used are: CFTR, cystic fibrosis transmembrane conductance regulator; P-gp, P-glycoprotein; MTS, methanethiosulfonate; M14M, 3,6,9,12-tetraoxatetradecane-1,14-diyi bismethanethiosulfonate; TM, transmembrane; TMD, transmembrane domain containing either the six NH2- or COOH-terminal transmembrane segments; NBD, nucleotide-binding domain; HEK, human embryonic kidney; TPCK, L-1-tosylamido-2-phe-nylethyl chloromethyl ketone.
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essing mutants by a direct mechanism because suppressor mutations (arginines) introduced into TM segments 5 (I306R) and 6 (F343R) also promoted maturation of the protein (21). One explanation for this observation was that the arginine residues mimicked the effects of drug substrates by promoting interactions among the TM segments. A recent study on the effect of silent polymorphisms suggested that an alternative mechanism for arginine rescue was that they affected global folding events during synthesis (22). Silent single-nucleotide polymorphisms in a gene that change codon usage would not be expected to alter structure or function of a protein because they do not change the amino acid sequence. An unexpected finding was the observation that silent polymorphisms that introduced rare codons into the MDR1 gene yielded a P-gp that was more protease-resistant than wild-type enzyme and, more importantly, exhibited altered interactions with all substrates and modulators. The likely mechanism of the rare codons was to slow the timing of co-translational folding and insertion into the membrane to change the conformation of P-gp and the structure of all its drug substrate-binding sites (22). Arginines introduced into TMs 5 or 6 could have had a similar effect because the TM5–TM6 hairpin is critical for folding because mutations in these segments could influence distal folding events (23). In addition, packing of the TM segments appears to be the rate-limiting step in folding of ABC transporters because it is the only step that occurs post-translationally (24).

To distinguish between these mechanisms, we tested whether arginines introduced into other TM segments predicted to be less important for folding (TMs 1 and 3) would still promote maturation of processing mutants. TM1 does not appear to be critical for post-translational folding because P-gp in which TM1 is replaced by TM7 (60% of the amino acids are different) was properly folded and exhibited activity (25). Also, in cysteine-scanning mutagenesis studies involving the NH₂-terminal half of P-gp, it was found that changes to some residues in TMs 2 and 4 but not in TM3 inhibited maturation of P-gp (26). Therefore, TM3 also appeared to be relatively less important for folding.

Because TMs 1 and 3 appear to be less important for folding of P-gp, we wanted to insert arginine residues at sites predicted to line the drug-binding pocket and test whether they promoted maturation of processing mutants and had local or global effects on the drug-binding sites. We previously showed using cysteine-scanning mutagenesis and labeling with a thiol-reactive drug substrate, methanethiosulfonate-verapamil (MTS-verapamil), that residue Leu⁶⁵ lined the drug-binding pocket of P-gp (27).

No amino acid in TM3 has yet been identified to line the drug-binding pocket. It is likely, however, that TM3 is important for drug binding because it is highly labeled with photoaffinity analogs of the drug substrate, propafenone (10). Therefore, another goal of this study was to identify a specific residue in TM3 that lined the drug-binding pocket. Interaction of drug substrates such as verapamil or rhodamine B with P-gp can readily be detected because they stimulate its ATPase activity. Our initial approach to identify residues within the TM segments that line the drug-binding pocket was to utilize cysteine-scanning mutagenesis and test for inhibition of ATPase activity after reaction with thiol-reactive MTS derivatives of verapamil (9) or rhodamine (28). The ATPase activity of residues predicted to line the drug-binding pocket was inhibited after labeling, and labeling could be protected with drug substrates. A consistent feature of the labeling and protection studies with thiol-reactive analogs of drug substrates was that equivalent TM segments in the two halves of the protein appeared to line the drug-binding pocket. Examples include TMs 4 and 10 (28), TMs 6 and 12 (28), and TMs 1 and 7 (27, 29). It was shown that TM9 in the COOH-terminal half of P-gp contributes to the drug-binding pocket (28, 30), but no evidence was found that TM3 also contributed to drug binding (i.e. inhibition of activity was not protectable by drug substrate). It was then noted that P-gp was an unusual transporter because covalent attachment of a drug substrate to the protein could also cause permanent activation of its ATPase activity (29, 31). Therefore, it was possible that labeling of a cysteine in TM3 might result in activation rather than inhibition of ATPase activity. Accordingly, we tested whether labeling of any cysteine residues introduced into TM3 would activate P-gp ATPase activity and be a candidate for testing whether replacement of the residue with an arginine would promote maturation of a processing mutant.

MATERIALS AND METHODS

Construction of Mutants—Cysteines at positions 137, 431, 717, 956, 1074, 1125, and 1227 in wild-type P-gp were changed to alanine to create cysteine-less P-gp (32). Single cysteine residues were introduced at each position (residues 190–209) in TM3 in Cys-less P-gp. All of the mutants also contained a 10-histidine tag at the COOH-end to facilitate purification of P-gp by nickel-chelate chromatography (33). For disulfide cross-linking analysis, the cDNA of mutant L339C(TM6)/F728C(TM7) (34) was modified to also encode the L65R, T199R, I306R, or F343R mutations.

Expression of Mutants, Purification, and Measurement of ATPase Activity—Human embryonic kidney (HEK) 293 cells were transiently transfected with the mutant P-gp cDNAs as described previously (33). The transfected cells were incubated at 30 °C for 24 h before harvest to promote maturation of the protein. The cysteine mutants were incubated at low temperature to promote maturation because mutation of the seven endogenous cysteines in P-gp to alanines reduces the efficiency of maturation of Cys-less P-gp relative to wild-type enzyme (32).

Histidine-tagged P-gp was isolated by nickel-chelate chromatography as described previously (33). A sample of the isolated histidine-tagged P-gp was mixed with an equal volume of 10 mg/ml sheep brain phosphatidylethanolamine (Type II-S; Sigma) that had been washed and suspended in Tris-buffered saline (10 mM Tris-HCl, pH 7.4, and 150 mM NaCl). The sample was sonicated, and ATPase activity was measured in the absence of drug substrate or in the presence of various concentrations of rhodamine B (0.01–3 μM), verapamil (0.001–3 μM), vinblastine (0.6–60 μM), or colchicine (0.1–10 μM). The samples were incubated for 30 min at 37 °C, and the amount of inorganic phosphate released was determined (35).

Reaction of P-gp Mutants with MTS-Rhodamine—Histidine-tagged TM3 single cysteine mutants were expressed in thirty
plates (10-cm diameter) of HEK 293 cells. The cells were then incubated at 30 °C for 24 h. The cells were washed three times with phosphate-buffered saline (10 mM sodium phosphate, pH 7.4, 150 mM NaCl) and then suspended in a total volume of 1.5 ml of nitrilotriacetic acid-phosphate-buffered saline buffer (10 mM Tris-HCl, pH 8.0, 100 mM sodium phosphate and 150 mM NaCl). The cells were solubilized by the addition of an equal volume of nitrilotriacetic acid-phosphate-buffered saline buffer containing 2% (w/v) n-dodecyl-β-D-maltoside. Insoluble material was removed by centrifugation at 16,000 × g for 15 min at 4 °C. DNA was removed from the supernatant by passage through a miniprep plasmid DNA spin column (Qiagen). Half of the supernatant (1.3 ml) was then incubated with the desired concentration of MTS-rhodamine (0.01–3 mM) for 10 min at 20 °C, whereas the remaining sample served as an untreated control. In the drug protection studies, the solubilized material was preincubated with 5 mM rhodamine B (saturating concentrations) for 10 min at 20 °C prior to labeling with 1 mM MTS-rhodamine. The samples were then cooled in an ice bath, followed by the addition of 0.15 ml of 3 mM NaCl and 0.05 ml of 1 mM imidazole, pH 7.0. Histidine-tagged P-gp was then isolated by nickel-chelate chromatography as described previously (33). The recovery of P-gp was monitored by immunoblot analysis with rabbit anti-P-gp polyclonal antibody (33).

**Disulfide Cross-linking Analysis**—The double cysteine mutants L339C(TM6)/F728C(TM7), L65R(TM1)/L339C(TM6)/F728C(TM7), T199R(TM3)/L339C(TM6)/F728C(TM7), I306R(TM5)/L339C(TM6)/F728C(TM7), or F343R(TM6)/L339C(TM6)/F728C(TM7) were transiently expressed in HEK 293 cells (32). The membranes were prepared and suspended in Tris-buffered saline. A sample of the membrane was then incubated in the presence or absence of various concentrations of vinblastine, cyclosporin A, or rhodamine B for 5 min at 20 °C.

Intramolecular disulfide cross-linking between TMD1 and TMD2 can be detected because the cross-linked product (21). An example of the effects of Arg and Tyr changes to P-gp processing mutant ΔY490, the 12 TM segments are shown as numbered cylinders. The branched lines represent glycosylated sites, and the linker region connecting the two halves of the molecule is represented with zigzag lines. NBD1 and NBD2 represent the NH2- and COOH-terminal nucleotide-binding domains, respectively. TMD1 and TMD2 contain TMs 1–6 and TMs 7–12, respectively. The locations of residues Leu65(TM1), Thr199(TM3), Ile306(TM5), and Phe443(TM6) are shown as filled circles. Residues L339C(TM6) and F728C(TM7) could be cross-linked with M14M cross-linker and are shown as open circles. The positions of ΔY490 and G251V-processing mutations are shown as gray circles. A, whole cell SDS extracts of HEK 293 cells expressing mutant G251V, F343R(TM6)/ΔY490 or F343Y(TM6)/ΔY490 were subjected to SDS-PAGE on 5.5% gels and immunoblot analysis. The positions of mature (170 kDa), immature (150 kDa), and degraded (130 kDa) forms of P-gp are indicated.

**RESULTS**

**Screening TM3 Single Cysteine Mutants for Activation of ATPase Activity by Covalent Attachment of a Thiol-reactive Drug Substrate**—The TM5-TM6 hairpin of P-gp (Fig. 1A) appears to play an important role in the co- and/or post-translational folding steps during synthesis because mutations introduced into these TM segments of wild-type P-gp could promote proteolytic cleavage at the extracellular side of TM2 (23). Therefore, changes to the TM5-TM6 hairpin can affect distal folding events. Some changes in TM5 (I306A or G) or in TM6 (F343Y) in the ΔY490 P-gp mutant also caused the mutant protein to be unstable because cells expressing these mutants contained relatively large amounts of a 130-kDa degradation product (21). An example of the effects of Arg and Tyr changes to Phe443 in mutant ΔY490 P-gp is shown in Fig. 1B. HEK 293 cells expressing wild-type P-gp yield the mature 170-kDa protein as the major product, whereas the major product in processing mutant ΔY490 is the immature 150-kDa protein. In cells expressing mutant F343Y/ΔY490, the 130-kDa protein was the major product. By contrast, mutant F343R/ΔY490 yielded about equivalent amounts of the mature 170-kDa and immature 150-kDa proteins and very little 130-kDa product. Therefore, the presence of arginine mutations in TM5 (I306R) or TM6 (F343R) might have promoted maturation of ΔY490 P-gp by influencing distal folding events. Alterations in the timing of
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cotranslational folding such as slowing the speed of folding can alter the structure and insertion of P-gp into the membrane. For example, it was recently shown that slowing synthesis of P-gp by introducing rare codons into the MDR1 gene yielded a protein that was more protease-resistant than that synthesized from the normal template (22).

Arginine rescue may be a unique feature of the TM5-TM6 hairpin because this segment is critical for folding. Therefore, rescue by arginine should not be observed in TM segments predicted to be less important for folding. Two TM segments that do not appear to be critical for folding are TMs 1 and 3. When TM1 was replaced with TM7 (60% of the amino acids are different), the (TM7/TM7) mutant P-gp matured like wild-type enzyme (25). Evidence that TM3 might be less important for folding comes from the results of cysteine mutagenesis studies. It was found that changes to residues in TMs 2 and 5, but not in TM3, inhibited maturation of some of the mutants (26). The next step was then to identify residues in TM1 or TM3 that line the drug-binding pocket, for replacement with arginine and to test whether they could promote maturation of a processing mutant.

To identify residues that line the drug-binding pocket of P-gp, we used cysteine-scanning mutagenesis and reaction with thiol-reactive analogs of drug substrates (9, 28). Cysteines that line the drug-binding pocket are covalently modified with the drug analog and cause activation of P-gp ATPase activity. In TMs 5 and 6, residues Ile106(TM5) and Phe343(TM6) were selected for substitution with arginine because cysteines introduced at these sites caused activation of P-gp ATPase activity after covalent modification with a thiol-reactive substrate (31, 38). Residue Leu65 in TM1 also showed similar properties as covalent modification of Cys65 with MTS-verapamil activated P-gp ATPase activity (27). Therefore position 65 in TM1 was selected for introduction of an arginine residue.

We then tested whether cysteines introduced at positions 190–209 of TM3 could react with MTS-verapamil to cause permanent activation of ATPase activity. We previously showed that all of the TM3 single cysteine mutants exhibited at least 85% of the activity of the parent Cys-less P-gp (9). We first tested whether any of the TM3 cysteine mutants showed any change in the apparent affinity for drug substrates. The histidine-tagged mutants were isolated with lipids, and assayed for drug-stimulated ATPase activity in the presence of various concentrations of rhodamine B (0.01–3 mM), verapamil (0.001–3 mM), vinblastine (0.6–60 mM), or colchicine (0.1–10 mM). None of the mutants showed more than a 50% change in apparent affinity for any of the drug substrates when compared with Cys-less P-gp (data not shown).

The next step was to treat the TM3 single cysteine mutants with MTS-verapamil. HEK 293 cells expressing histidine-tagged single cysteine mutants were solubilized with n-dodecyl-β-D-maltoside, and insoluble was material removed by centrifugation. The solubilized extracts were then treated for 10 min at 20 °C with or without 3 mM MTS-verapamil. This concentration of MTS-verapamil was sufficient to completely modify Cys65(TM1) (27), Cys728(TM7) (29), and Cys306(TM5) (31). P-gp was then isolated by nickel-chelate chromatography that also removed unreacted MTS-verapamil. The isolated P-gps were mixed with lipids and assayed for ATPase activity in the absence of drug substrate. None of the single cysteine mutants showed enhanced ATPase activity after treatment with MTS-verapamil (data not shown).

The TM3 cysteine mutants may not have reacted with MTS-verapamil because TM3 may not be involved in the binding of verapamil. We then tested whether the single cysteine TM3 mutants could be modified by MTS-rhodamine because verapamil and rhodamine were previously reported to bind at different sites in the drug-binding pocket (38). MTS-rhodamine is a substrate of Cys-less P-gp because it can stimulate its ATPase activity up to 6.2-fold with half-maximal stimulation occurring at 110 μM (28). Accordingly, the single TM3 cysteine mutants were expressed in HEK 293 cells and solubilized with detergent, and insoluble material was removed by centrifugation. The solubilized extract was treated with or without 2 mM MTS-rhodamine. The solubilized ATPase activity of Cys-less P-gp was not affected after treatment with MTS-rhodamine and isolation by nickel-chelate chromatography. Because MTS-rhodamine was shown to be a substrate of Cys-less P-gp (28), the results indicate that the unreacted compound was effectively removed during nickel-chelate chromatography.

The ATPase activities of 18 of the 20 mutants after treatment with MTS-rhodamine were not increased after treatment with MTS-rhodamine (data not shown) and were similar to that of Cys-less P-gp (Fig. 2B). After treatment with MTS-rhodamine, their activities could still be stimulated 6–7-fold with rhodamine B. The mutants Q195C and T199C, however, exhibited different properties than Cys-less P-gp after treatment with MTS-rhodamine. Whereas mutant Q195C showed a 6.4-fold stimulation of ATPase activity with rhodamine B before treatment with MTS-rhodamine, its activity could only be stimulated 1.3-fold after treatment with MTS-rhodamine. Apparently, covalent modification inhibits its rhodamine B-stimulated ATPase activity. By contrast, covalent modification of mutant T199C with MTS-rhodamine fully activated its ATPase activity such that rhodamine B had no further effect on its activity.

If MTS-rhodamine activates P-gp ATPase activity because it occupies the rhodamine-binding site when it is attached to Cys199, then labeling of mutant T199C should be protectable with rhodamine B. We first determined the concentration of MTS-rhodamine required to cause half-maximal activation of ATPase activity. Histidine-tagged mutant T199C was expressed in HEK 293 cells, solubilized with detergent, and reacted with various concentrations of MTS-rhodamine. P-gp
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FIGURE 2. Effect of MTS-rhodamine on ATPase activity of TM3 cysteine mutants. A, histidine-tagged Cys-less or TM3 single cysteine P-gp mutants (residues 190–209) were expressed in HEK 293 cells. The cells were solubilized with n-dodecyl-β-d-maltoside, and insoluble material was removed by centrifugation. The supernatants were then treated with or without 2 mM MTS-rhodamine. Histidine-tagged P-gps were then isolated by nickel-chelate chromatography. Equivalent amounts of P-gp were mixed with lipid, sonicated, and assayed for ATPase activity in the absence of drug substrate. B, Cys-less, Q195C and T199C P-gp mutants were treated with (+) or without (−) 2 mM MTS-rhodamine and histidine-tagged P-gp isolated by nickel-chelate chromatography. Equivalent amounts of P-gp were mixed with lipid and ATPase activity was determined in the presence (+) or absence (−) of 2 mM rhodamine B (Rhod B). The fold stimulation is the ratio of activity of a sample treated with MTS-verapamil to that of an untreated sample. Each value is the mean ± S.D. (n = 3).

FIGURE 3. Labeling of mutant T199C with increasing concentrations of MTS-rhodamine and protection by rhodamine B. A, HEK 293 cells expressing histidine-tagged mutant T199C were solubilized with n-dodecyl-β-d-maltoside, and insoluble material was removed by centrifugation. Equivalent amounts of supernatant were incubated with 0–3 mM MTS-rhodamine for 10 min at 20°C. P-gp was then isolated by nickel-chelate chromatography. Equivalent amounts of P-gp were mixed with lipid and assayed for ATPase activity in the absence of drug substrate. The fold stimulation is the ratio of activity of a sample treated with MTS-rhodamine to that of an untreated sample. B, the solubilized supernatant was incubated with 1 mM MTS-rhodamine in the presence (+) or absence (−) of 5 mM rhodamine B (Rhod B). P-gp was then isolated by nickel-chelate chromatography. Equivalent amounts of P-gp were mixed with lipid and assayed for ATPase activity in the absence of drug substrate. The activities are expressed relative to the sample treated with 1 mM MTS-rhodamine in the absence of rhodamine B. Each value is the mean ± S.D. (n = 3).

was then isolated by nickel-chelate chromatography and mixed with lipid, and the ATPase activity was determined. Fig. 3A shows that maximal and half-maximal activation of ATPase activity occurred at 2 and 0.88 mM MTS-rhodamine, respectively. To test whether rhodamine B protected mutant T199C from labeling, HEK 293 cells expressing the histidine-tagged mutant were solubilized with detergent and treated with 1 mM MTS-rhodamine in the presence or absence of 5 mM rhodamine B for 10 min at 20°C. The P-gps were isolated by nickel-chelate chromatography and mixed with lipids, and ATPase activities were determined. Fig. 3B shows that ATP hydrolysis was reduced by 78% when mutant T199C was reacted with MTS-rhodamine in the presence of 5 mM rhodamine B.

Effect of L65R and T199R Mutations on Maturation of a P-gp Processing Mutant—We then tested whether arginines introduced into TM1 (Leu65) (27) or TM3 (Thr199) promoted maturation of a P-gp processing mutant. Processing mutant G251V was chosen because it yields very low levels of mature 170-kDa protein and could be rescued by the presence of arginine in TM5 (I306R) or in TM6 (F343R) (21). Accordingly, mutants L65R(TM1)/G251V, T199R(TM3)/G251V, I306R(TM5)/G251V (positive control), and A342R(TM6)/G251V (negative control) were constructed and expressed in HEK 293 cells. Whole cell SDS extracts were then subjected to immunoblot analysis. Fig. 4A shows that the presence of an arginine residue at positions 65(TM1), 199(TM3), and 306(TM5) promoted maturation of mutant G251V. In cell surface labeling studies, it was found that the 170-kDa protein could be detected at the cell surface (data not shown) as was reported previously with the I306R(TM5)/ΔY490 and F343R(TM6)/ΔY490 mutants (21).
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By contrast, the presence of an arginine at position 342(TM6) did not rescue mutant G251V. Similar results were obtained when the arginines were introduced into processing mutant ΔY490 P-gp (Fig. 4B).

Effect of an Arginine Introduced into TM Segments 1, 3, 5, or 6 on Drug Substrate Interactions with P-gp—We then tested whether arginines introduced at positions 65(TM1), 199(TM3), 306(TM5), or 343(TM6) promoted maturation by mimicking drug interactions with P-gp or by affecting global folding of P-gp. If the arginines mimic drug substrate interactions with P-gp, then it would be expected that they would affect only a subset of drug-binding sites, whereas effects on global folding would alter all drug substrate interactions with P-gp (22).

Cross-linking between cysteine residues located in the two TMDs of P-gp is a useful approach for monitoring P-gp-drug interactions. The cross-linked product could readily be detected as it migrates with lower mobility on SDS-PAGE, and cross-linking could be inhibited by drug substrates (29). For example, mutant L339C(TM6)/F728C(TM7) could be cross-linked with M14M cross-linker at 0 °C, and cross-linking was inhibited by the drug substrates vinblastine, cyclosporin A, and rhodamine B (Fig. 5). The advantages of the cross-linking assay compared with drug stimulation of ATPase activity are that it does not rely on long range conformational changes between the NBDs and TMDs, and high concentrations of drug substrates could be used that would normally inhibit ATPase activity. Accordingly, mutants L65R(TM1)/L339C(TM6)/F728C(TM7), T199R(TM3)/L339C(TM6)/F728C(TM7), I306R(TM5)/L339C(TM6)/F728C(TM7), and F343R(TM6)/L339C(TM6)/F728C(TM7) were constructed and expressed in HEK 293 cells. The membranes were prepared, preincubated with various concentrations of vinblastine, cyclosporin A, or rhodamine B, and then cross-linked with M14M for 4 min on ice. The reactions were stopped by the addition of SDS sample buffer containing 50 mM EDTA and no thiol reducing agent, and the samples were subjected to immunoblot analysis with a rabbit polyclonal antibody to P-gp. The positions of mature (170 kDa) and cross-linked (X-link) P-gp are indicated.

FIGURE 5. Effects of drug substrates on cross-linking of mutant L339C(TM6)/F728C(TM7). Membranes prepared from HEK 293 cells expressing mutant L339C(TM6)/F728C(TM7) were pretreated for 5 min at 20 °C in the absence (None) or presence of 0.02 mM vinblastine (Vin), 0.02 mM cyclosporin A (Cyclo) or 0.2 mM rhodamine B (Rhod B). The samples were chilled on ice for 10 min then treated with (+) or without (−) 0.2 mM M14M for 4 min on ice. The reactions were stopped by the addition of SDS sample buffer containing 50 mM EDTA and no thiol reducing agent. The samples were subjected to immunoblot analysis with a rabbit polyclonal antibody to P-gp. The positions of mature (170 kDa) and cross-linked (X-link) P-gps are indicated.
prepared from cells expressing mutant G251V that were grown in the absence or presence of cyclosporin A, mutant L65R(TM1)/G251V, or wild-type P-gp. The membranes were then treated with various concentrations of TPCK-trypsin. The reactions were stopped by the addition of trypsin inhibitor, and the samples were subjected to SDP-PAGE on 6% gels followed

FIGURE 6. Effect of arginine mutations on drug substrate-P-gp interactions. Membranes were prepared from HEK 293 cells expressing mutants L339C(TM6)/F728C(TM7) or I306R(TM5)/L339C(TM6)/F728C(TM7). The samples were then preincubated with various concentrations of vinblastine (A, Vin), cyclosporin A (C, Cyclo), or rhodamine B (E, Rhod B) for 5 min at 20 °C, chilled on ice for 10 min and then treated with 0.2 mM M14M for 4 min on ice. The reactions were stopped by the addition of SDS sample buffer containing 50 mM EDTA and no thiol reducing agent. The samples were subjected to immunoblot analysis with a rabbit polyclonal antibody to P-gp. The positions of mature (170 kDa) and cross-linked (X-link) P-gps are indicated. B, D, and F, the immunoblots were scanned and quantitated as described under “Materials and Methods.” Percent cross-linked is the amount of cross-linked P-gp observed in the presence of various concentrations of drug substrate relative to that in the absence of drug substrate. Each value is the mean ± S.D. (n = 3).
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**TABLE 1**

Concentrations of drug substrates required to inhibit cross-linking by 50%

| Mutant                           | Vinblastine* | Cyclosporin A | Rhodamine B |
|---------------------------------|--------------|---------------|-------------|
| L339C(TM6)/F728C(TM7)           | 0.7 ± 0.3    | 0.6 ± 0.2     | 88 ± 20     |
| L65R(TM1)/L339C(TM6)/F728C(TM7) | 40 ± 8b      | 0.5 ± 0.3     | 65 ± 17     |
| T199R(TM3)/L339C(TM6)/F728C(TM7)| 4.5 ± 0.9b   | 1.1 ± 0.4     | 77 ± 15     |
| I306R(TM5)/L339C(TM6)/F728C(TM7)| 42 ± 9b      | 15 ± 3b       | 96 ± 11     |
| F343R(TM6)/L339C(TM6)/F728C(TM7)| 1.2 ± 0.4    | 11 ± 2b       | 320 ± 38b   |

* Each value is the mean ± S.D. (n = 3 separate cross-linking experiments).

b The presence of the arginine mutation caused >3-fold decrease in the apparent affinity for the substrate.

by immunoblot analysis. Fig. 7 shows that the mature 170-kDa protein in mutant G251V expressed in the presence of cycloporin A was 100-fold more resistant to trypsin compared with the mutant expressed in the absence of drug substrate. Similarly, the L65R(TM1)/G251V mutant and wild-type mature proteins were 100-fold more resistant to trypsin when compared with the 150-kDa protein of mutant G251V. These results indicate that the L65R mutation converts mutant G251V into a more protease-resistant conformation.

**DISCUSSION**

The observation that arginine suppressor mutations at positions 65(TM1) and 199(TM3) also promoted maturation of P-gp processing mutants indicates that arginine rescue is not a unique property of the TM5-TM6 hairpin and that the likely mechanism is that they mimic drug substrate interaction with P-gp. It is unlikely that the arginine mutations promoted P-gp maturation by nonspecifically altering the timing of co-translational folding and insertion into the membrane because only a subset of drug-binding sites were altered by each arginine. When wild-type P-gp synthesis was slowed by the introduction of rare codons into the MDR1 gene, it was reported that the global structure of the protein was changed (became more protease-resistant) and that it exhibited altered interactions with all substrates and modulators that were tested (22). The observation that the arginine mutations perturbed only a subset of the drug-binding sites (Table 1) indicates that they have local effects on P-gp folding compared with the global effects of silent polymorphisms (22). For example, the L65R mutation only caused a large reduction (57-fold) in the apparent affinity for vinblastine and little change in affinities for cyclosporin A or rhodamine B. The reduction in affinity of mutant L65R(TM1) for vinblastine is consistent with the observation that it had reduced ability to confer resistance to vinblastine (39). The F343R mutation exhibited decreased apparent affinity for rhodamine B (3.6-fold) and cyclosporin A (18-fold) but not for vinblastine (<2-fold).

Therefore, the suppressor arginines appear to mimic the effects of drug substrates during rescue of P-gp processing mutants. A model of the rescue of mutant ΔY490 P-gp by drug substrates and arginines is shown in Fig. 8. In the initial stages of
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protein synthesis, both wild-type or ΔY490 P-gps adopt loosely folded conformations in which packing of the TM segments is incomplete (37). Wild-type P-gp matures to yield a more compact structure, whereas the ΔY490 mutation causes the protein to be trapped in the incompletely folded conformation. A drug substrate promotes maturation of ΔY490 P-gp by promoting packing of the TM segments (11). Arginines at positions Leu65(TM1), Thr199(TM3), Ile306(TM5), or Phe343(TM6) would line the drug-binding pocket and mimic the effects of drug substrates to promote maturation. The effects of arginines resembled that of drug substrates because they also converted P-gp into a more protease-resistant conformation (Fig. 7). The ability of arginines at different locations in the drug-binding pocket to promote folding is consistent with the observation that drugs (such as verapamil and rhodamine B) occupying different sites in the drug-binding pocket could still promote maturation of P-gp processing mutants (12).

The implications of the model (Fig. 8) for rescue of CFTR and P-gp processing mutants are that corrector compounds could bind to different regions in the TM domains to promote maturation of the protein and that simultaneous occupation of different sites could have additive or synergistic effects on maturation. Indeed, it was recently reported that a combination of corrector compounds such as VRT-325 and corr-2b or Hoechst 33342 and rhodamine B had additive effects on maturation of processing mutants of CFTR and P-gp, respectively (40).

Labeling of either Cys199(TM3) (this study) or Cys343(TM6) (38) with MTS-rhodamine caused permanent activation of ATPase activity. Labeling of Cys199(TM3) or Cys343(TM6) by MTS-rhodamine to permanently activate P-gp ATPase activity suggests that P-gp can bind a drug substrate that is in different orientations. This might be possible because the protein shows substrate-induced conformational changes (14). The apparent flexibility of the P-gp drug-binding pocket to accommodate different drug substrates and in different orientations through an induced fit mechanism may be common to other proteins that are able to recognize structurally diverse substrates. For example, the soluble BmR transcription factor (41), human nuclear receptor PXR (42), Staphylococcus aureus repressor QacR (43), and cytochrome P450 3A4 (44) can bind a wide variety of compounds. The crystal structures of these proteins show the presence of a single drug-binding pocket. In the PXR receptor and cytochrome P450 3A4 proteins, the same ligand can assume multiple orientations.

Labeling of Cys199 with MTS-rhodamine suggests that it is within the drug-binding pocket. The labeling of Cys199 also appeared to be specific for MTS-rhodamine because the ATPase activity of mutant T199C was not activated by MTS-verapamil. By contrast, the ATPase activity of mutant L65R was appeared to be specific for MTS-rhodamine because the ATPase activity of mutant L65R was completely abolished because it did not stimulate ATPase activity at vinblastine concentrations of up to 200 μM (45). Higher concentrations of vinblastine could not be used because they inhibit the ATPase activity of wild-type P-gp. The results from the cross-linking protection assay, however, showed that mutant I306R could still interact with vinblastine but with reduced affinity (Fig. 6A). Identification of other arginine suppressor mutations that promote maturation of a P-gp processing mutant might be a useful preliminary screening tool for identifying critical residues that directly contribute to the drug-binding pocket.

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