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The W232R Suppressor Mutation Promotes Maturation of a Truncation Mutant Lacking both Nucleotide-Binding Domains and Restores Interdomain Assembly and Activity of P-glycoprotein Processing Mutants[†]

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ABSTRACT: ATP-binding cassette (ABC) proteins contain two nucleotide-binding domains (NBDs) and two transmembrane (TM) domains (TMDs). Interdomain interactions and packing of the TM segments are critical for function, and disruption by genetic mutations contributes to disease. P-glycoprotein (P-gp) is a useful model to identify mechanisms that repair processing defects because numerous arginine suppressor mutations have been identified in the TM segments. Here, we tested the prediction that a mechanism of arginine rescue was to promote intradomain interactions between TM segments and restore interdomain assembly. We found that suppressor W232R(TM4/TMD1) rescued mutants with processing mutations in any domain and restored defective NBD1−NBD2, NBD1−TMD2, and TMD1−TMD2 interactions. W232R also promoted packing of the TM segments because it rescued a truncation mutant lacking both NBDs. The mechanism of W232R rescue likely involved intradomain hydrogen bond interactions with Asn296(TM5) since only N296A abolished rescue by W232R and rescue was only observed when Trp232 was replaced with hydrogen-bonding residues. In TMD2, suppressor T945R(TM11) also promoted packing of the TM segments because it rescued the truncation mutant lacking the NBDs and suppressed formation of alternative topologies. We propose that T945R rescue was mediated by interactions with Glu875(TM10) since T945E/E875R promoted maturation while T945R/E875A did not.

The human multidrug resistance P-glycoprotein (P-gp,[1] ABCB1) is a 170 kDa membrane protein that uses ATP to pump hydrophobic molecules out of the plasma membrane of the cell (*1*, *2*). The protein is highly expressed in the apical membranes of epithelial cells of intestine, kidney, liver, and blood−brain/testes barriers (*3*). Its physiological role is likely to protect us from cytotoxic compounds in our diets and environment as it will block uptake of these compounds in the intestine and catalyze their efflux in the urine and bile (*4*). P-gp is clinically important because it can interfere in cancer and AIDS/HIV chemotherapies since many of the drugs are substrates (*5*).

P-gp is composed of 1280 amino acids that are organized as two homologous halves (43% amino acid identity) joined by a linker region (*6*). Each half starts with a transmembrane domain (TMD) containing six transmembrane (TM) segments followed by a cytosolic nucleotide-binding domain (NBD) (*7*). Both halves of the protein are required for drug transport although they do not have to be covalently linked for function (*8*). Two ATP molecules bind at the interface between the Walker A sites and LSGGQ sequences between the NBDs (*9*). ATP hydrolysis occurs by an alternating mechanism (*10*), and inhibition at either site inhibits activity of the protein (*11*, *12*).

The TMDs of P-gp are critical for function as they contain the drug-binding sites and translocation pathway across the membrane. Studies on truncation mutants lacking the NBDs showed that the TMDs alone were sufficient to mediate binding of drug substrates (*8*). The drug-binding pocket appears to lie at the interface between the TMDs (*13*−*16*). The drug-binding pocket may contain up to four distinct drug-binding sites (*17*−*21*). Drug substrates may bind through a "substrate-induced fit" mechanism (*15*).

It has also been observed that drug substrates can rescue P-gp processing mutants that are defective in folding and trafficking to the cell surface (*8*, *22*). Although P-gp drug rescue is not useful for developing therapeutic strategies to inhibit P-gp during chemotherapy, understanding the mechanism will aid in the development of approaches to correct folding defects in other ABC transporters. For example, processing defects in mutants (such as ΔF508-CFTR) of the CFTR chloride channel are the main cause of cystic fibrosis (*23*). The equivalent mutation in P-gp (ΔY490) also inhibits maturation of P-gp, but expression of the mutant in the presence of drug substrates yields a functional molecule at the cell surface (*22*).

Compounds (correctors) have been identified that rescue ΔF508-CFTR, but their efficiency of rescue is very low (*24*−*27*). By contrast, drug rescue of P-gp processing mutants results in yields of mature protein that closely resemble that of wild-type

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[1]Abbreviations: P-gp, P-glycoprotein; NBD, nucleotide-binding domain; TMD1, NH_2-terminal transmembrane domain containing TM segments 1−6; TMD2, COOH-terminal transmembrane domain containing TM segments 7−12; TMD1 + 2, truncation mutant consisting of residues 1−379 plus 681−1025; C-half P-gp, truncation mutant consisting of residues 681−1280; TM, transmembrane; ER, endoplasmic reticulum; HEK, human embryonic kidney; GADPH, glyceraldehyde-3-phosphate dehydrogenase; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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enzyme (*22*). Knowledge obtained by studying P-gp drug rescue is directly applicable to rescue of CFTR processing mutants because the effects of processing mutations are similar in both P-gp and CFTR. For example, processing mutations occupying similar positions in CFTR (ΔF508) and P-gp (ΔY490) show defective packing of the TM segments (*28*, *29*) and disruption of interdomain assembly (*30*, *31*).

Disruption of interdomain assembly affects packing of the TM segments because NBD−TMD interactions are critical for assembly. Both deletions (ΔF508 in CFTR and ΔY490 in P-gp) appear to disrupt NBD1 interactions with the fourth intracellular loop of TMD2 (*31*). Studies on truncation mutants show that removal of both NBDs in P-gp or CFTR yields proteins that fail to mature (*32*). An important difference between ΔNBD2−CFTR and ΔNBD2−P-gp is that the former but not the latter can mature (*32*). This could be due to the fact that P-gp contains TM segments in both TMD1 (all except TM5) and TMD2 (all except TMs 8 and 10) that insert poorly in the membrane, whereas the TM segments in CFTR that insert poorly into the membrane are found only in TMD2 (*33*). Therefore, NBD1−TMD2 interactions may be required to stabilize poorly inserting TM segments in TMD2 of both CFTR and P-gp, but only P-gp requires NBD2−TMD1 interactions to stabilize poorly inserting TM segments in TMD1. The large number of poorly inserting TM segments in P-gp may also explain why multiple topologies have been detected during synthesis (*34*−*37*) and why the translation rate could alter its topology and change P-gp substrate specificity (*38*).

Drug substrates appear to rescue P-gp processing mutants by binding at the interface between TMD1 and TMD2 to promote packing of the TM segments (*39*). Arginine suppressors that mimicked the effects of drug rescue were first identified in TMD1 (*40*, *41*). In this study, the G251V processing mutant was used as a reporter molecule because it exhibits partial maturation (about 15% mature). This mutant allows us to detect whether the introduced arginine has a suppressor, neutral, or inhibitory effect as detected by the glycosylation state of the protein. Suppressor arginines in TM1 appear to promote maturation by forming hydrogen bonds with residues in TMD2 within the drug-binding pocket to restore TMD1−TMD2 interactions (*41*). Therefore, it appears that drug substrates or suppressor mutations promote maturation by acting at the interface between TMD1 and TMD2. Unfortunately, targeting the interface between TMD1 and TMD2 in ABC proteins like CFTR to correct folding defects would likely impair function since this interface may form the substrate translocation pathway through the membrane.

A more recent study, however, identified suppressor arginines that were predicted to reside outside the TMD1−TMD2 interface (*42*). For example, the W232R(TM4 in TMD1) change was a very potent suppressor mutation, but TM4 is not predicted to be close to any TM segments in TMD2 (Figure 1A). The W232R mutation may stabilize TMD1 as it promotes maturation of a mutant lacking the critical NBD2−TMD1 interface (ΔNBD2−P-gp) (*42*). Whether the W232R mutation could promote maturation of a TMD1 + 2 mutant lacking both NBDs is unknown. Previously identified suppressor mutations in TMD1 (I306R-(TM5) or F343R(TM6)) (*40*) did not promote maturation of TMD1 + 2 (unpublished data). It is possible that suppressor mutations in both TMD1 and TMD2 are required to promote maturation of the TMD1 + 2 mutant.

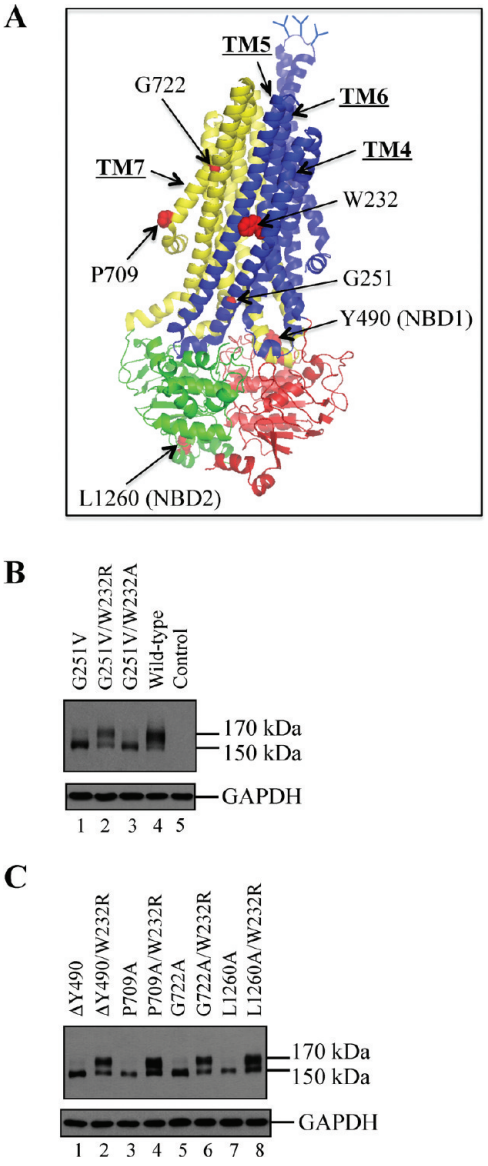

FIGURE 1: Rescue of P-gp mutants containing processing mutations in different domains by W232R. (A) Homology model of human P-gp in the closed conformation (*55*). The branched lines represent glycosylation sites, and the domains are colored as TMD1 (blue), NBD1 (red), TMD2 (yellow), and NBD2 (green). The red balls show the locations of Trp232 and the processing mutations at positions 251 (G251V), 490 (ΔY490), 709 (P709A), 722 (G722A), and 1260 (L1260A). (B) HEK 293 cells were transfected with A52-tagged wild-type P-gp, mutants G251V, G251V/W232R, and G251V/W232A, or plasmid vector (control). Whole cell extracts were subjected to immunoblot analysis with monoclonal antibody against A52 or GAPDH. (C) HEK 293 cells were transfected with A52-tagged mutants containing processing mutations in different domains and with or without the W232R mutation ((ΔY490(NBD1) ± W232R, P709A(linker region) ± W232R, G722A(TMD2) ± W232R, or L1260A(NBD2) ± W232R). Whole cell SDS extracts were subjected to immunoblot analysis with monoclonal antibody against A52 or GAPDH. The positions of mature (170 kDa) or immature (150 kDa) forms of P-gp are shown. Lanes are numbered below the blots.

In this study we provide evidence for a direct mechanism of rescue by suppressor mutations that involves intradomain interactions between adjacent TM segments to correct global folding defects and defects in topology. Although both NBD1−TMD2 and NBD2−TMD1 interactions play critical roles in P-gp maturation, single suppressor mutations in either TMD1 or TMD2 could still rescue the TMD1 + 2 mutant.

MATERIALS AND METHODS

Construction of Mutants. Two tagged versions of human P-gp were used in this study. A P-gp construct containing the epitope tag for monoclonal antibody A52 at the C-terminal end was used to distinguish the protein from any endogenous P-gp (*43*). The second P-gp cDNA was modified to contain a ten-histidine tag at the COOH-terminal end to facilitate purification of the expressed protein by nickel-chelate chromatography (*44*). Mutations were introduced into wild-type P-gp or processing mutants containing processing mutations in different domains (G251V in TMD1, ΔY490 in NBD1, P709A in the linker region, G722A in TMD2, or L1260A in NBD2) as described previously (*28*). The truncation mutants TMD1 + 2 (residues 1−379 plus 681−1025) or C-half P-gp (residues 681−1280) were constructed as described previously (*8*, *45*). Construction of the double cysteine mutants in a Cys-less background was described previously (*31*, *46*, *47*).

Transient Expression of Mutants. The mutant P-gps were transiently expressed in human embryonic kidney (HEK) 293 cells using the calcium phosphate precipitation method described by Chen and Okayama (*48*). Briefly, 6 h after transfection with cDNA, the medium (Dulbecco's modified Eagle's medium containing 0.1 mM α-minimum Eagle's medium nonessential amino acids, 2 mM L-glutamine, 100 units of penicillin/mL, 100 μg of streptomycin/mL, and 10% (v/v) calf serum) was replaced with fresh medium, and the cells were incubated for 24 h at 37 °C. HEK 293 cells expressing mutant P-gps were also grown in the absence or presence of 10 μM cyclosporin A for 24 h because it acts as a pharmacological chaperone to increase the yield of mature enzyme (*22*). Whole cell extracts (corresponding to about 50000 cells) were subjected to immunoblot analysis using monoclonal antibody A52. An equivalent amount of the sample was loaded onto a 10% (v/v) SDS−PAGE gels and subjected to immunoblot analysis with monoclonal antibody against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Santa Cruz Biotechnology, Santa Cruz, CA) and served as an internal control.

Immunoblot Analysis. SDS−PAGE gels (1.5 mm gels) were run using the Hoefer Mighty Small System (Fisher Scientific, Ottawa, Ontario, Canada). The gels were placed onto a piece of nitrocellulose, and transfer was for 1 h at 490 mA at 20 °C. The nitrocellulose was then incubated with blocking buffer (TBS, pH 8.0, containing 2% (w/v) skim milk and 0.5% (w/v) Tween 20) for 30 min at 20 °C and then for 16 h in blocking buffer containing 1 μg/mL (monoclonal antibody A52) or 1:500 dilution of rabbit polyclonal antibody against P-gp (*49*). The blots were then washed (6 × 5 min) with wash buffer (TBS, pH 8.0, containing 0.5% (w/v) Tween 20) and incubated for 45 min at 20 °C with 1:10000 dilution of horseradish peroxidase-conjugated goat anti-mouse or goat anti-rabbit antibodies (KPL Laboratories, Gaithersburg, MD). The blots were then washed with wash buffer (6 × 5 min) and then developed using enhanced chemiluminescence (HyGlo; Denville Scientific, Montreal, Canada). The amount of mature and immature product was quantitated by scanning the gel lanes followed by analysis with the NIH Image program (available at http://rsb.info.nih.gov/nih-image1) using an Apple computer. Results are expressed as mean ± SD of n measurements. The Student's two-tailed t test was used where $P < 0.05$ was considered to be significant.

Drug Resistance Assays. Baby hamster kidney (BHK) cells expressing A52-tagged wild-type P-gp or mutants G251V, G251V/W232R, W232R, W232A, N296A, E875A, or T945A were generated as described previously (*25*). Briefly, BHK cells were cotransfected with P-gp cDNA and pWL-neo (Stratagene, Cedar Creek, TX) at a ratio of 10:1. After 6 h, the medium was replaced with that containing 1 mg/mL G418 (BioShop, Burlington, Ontario, Canada). G418-resistant colonies were isolated after 7 days and expanded, and clones expressing wild-type or P-gp mutants were identified by immunoblot analysis with monoclonal antibody A52. BHK cells expressing wild-type or mutant P-gps were then incubated with various concentrations of colchicine (0−50 μM), vinblastine (0−256 nM), or paclitaxel (0−5 μM) for 6 days at 37 °C. Cell death was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (*50*) to determine the drug concentration that caused 90% cell death (LD_{90}). The relative resistance was obtained by dividing LD_{90} of the P-gp expressing cells by that of control cells.

Disulfide Cross-Linking Analysis. The G251V or L1260A processing mutations were introduced into Cys-less P-gp containing pairs of cysteines in different domains (L443C(NBD1)/S909C(TMD2), L531C(NBD1)/C1074(NBD2), or C137(TMD1)/A935C(TMD2)) with or without the W232R mutation. The L1260A and L1260A/W232R mutations were also introduced into a Cys-less P-gp containing the A266C(TMD1)/F1086C(NBD2) mutations. The mutants were transiently expressed in HEK 293 cells. Membranes were prepared as described previously (*51*). A sample of membrane (in Tris-buffered saline (TBS), pH 7.4, at a protein concentration of 0.4 mg/mL) was then incubated in the presence or absence of 1 mM oxidant (copper phenanthroline) for 15 min at 0 °C. The reactions were stopped by 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. Samples of the reaction mixtures (1 μg of protein) were then subjected to SDS−PAGE (6.5% (w/v) polyacrylamide gels) and immunoblot analysis with rabbit polyclonal antibody against P-gp. Intramolecular disulfide cross-linking between domains can be detected because the cross-linked product migrates with a slower mobility on SDS−PAGE gels (*52*).

Trypsin Digestion. Membranes were prepared from HEK 293 cells expressing A52-tagged TMD1 + 2 or TMD1 + 2(W232R) and suspended in TBS, pH 7.4, at a protein concentration of 5 mg/mL. The membranes were treated for 5 min at 20 °C with various concentrations of TPCK-trypsin (Sigma, Mississauga, Ontario, Canada; 12000 BAEE units/mg). The reactions were stopped by addition of lima bean trypsin inhibitor (Worthington, Freehold, NJ) (*53*). Samples were subjected to immunoblot analysis with monoclonal antibody A52.

Purification of P-gp and Measurement of ATPase Activity. Histidine-tagged wild-type and mutant P-gps were expressed in HEK 293 cells and then isolated by nickel-chelate chromatography as described previously (*44*). A sample of the isolated histidine-tagged P-gp was then mixed with an equal volume of 10 mg/mL sheep brain phosphatidylethanolamine (type II-S; Sigma) that had been washed and suspended in TBS. The sample was sonicated, and aliquots were assayed for drug-stimulated ATPase activity by addition of an equal volume of buffer containing 100 mM Tris-HCl, pH 7.5, 100 mM NaCl, 20 mM $MgCl_2$, 10 mM ATP, and various concentrations of verapamil (0−6 mM) or rhodamine B (0−6 mM). The samples were incubated for 30 min at 37 °C, and the amount of inorganic phosphate liberated was determined using the method of Chifflet et al. (*54*).

Generation of P-gp Models. The homology model of human P-gp structure was generated by Globisch et al. (*55*) and was based on that of Sav1866 (*56*). The models were viewed using PyMol (*57*).

RESULTS

W232R Promotes Maturation of Mutants with Processing Mutations in Any Domain. The W232R suppressor mutation that promotes maturation of the G251V processing mutant is located in TM4 of TMD1 (Figure 1A). In the P-gp model (Figure 1A), TM4 was predicted to be adjacent to TM segments 5 and 6 of TMD1 and away from TM segments in TMD2. We tested whether a W232A change would also promote maturation of the G251V mutant. Maturation of P-gp can be monitored by SDS−PAGE because the three core-glycosylated sites in the extracellular loop connecting TM segments 1 and 2 (Figure 1A) are converted to complex carbohydrate in the Golgi. It was found that only the W232R change promoted maturation of the mutant (Figure 1B, lanes 1−3). The amount of mature P-gp relative to total (mature plus immature) was 70 ± 6% for mutant W232R/G251V and 92 ± 5% for wild-type P-gp. By contrast, immature protein was the major product in mutants G251V (10 ± 4% mature) and G251V/W232A (13 ± 5% mature). The results indicate that introduction of the arginine at 232 rather than removal of the tryptophan was responsible for the increase in maturation in mutant G251V.

Suppressor mutations that promote maturation and trafficking of a mammalian ABC transporter were first identified in CFTR, P-gp's sister protein (*58*−*60*). Suppressor mutations can rescue ΔF508-CFTR by a variety of mechanisms. Examples include removal of the ER retention signals (arginine-framed trafficking motif mutations; R29K, R516K, R555K, and R766K) (*61*, *62*), introduction of a combination of CFTR suppressor mutations (F949/Q637R or F29S/F494N/Q637R) that increase solubility of NBD1 (*63*), or introduction of suppressor mutations such as V510D (TMD1) (*64*) and R1070W(TMD2) (*65*) that restore NBD1−TMD2 interactions. In a recent study of four of the CFTR suppressor mutations located in NBD1 (I539T, G550E, R553M, and R555K), it was found that they only restored maturation of mutants that had processing mutations in NBD1 but not those that had processing mutations in other domains such as NBD2 (N1303K) or TMD2 (L1065P or R1066C) (*66*).

We previously observed that W232R could rescue the ΔY490 (NBD1) and G251V (TMD1) P-gp processing mutants (*42*). We then tested whether W232R could rescue mutants containing processing mutations in other domains. The W232R mutation was introduced into mutants P709A (linker region), G722A (TMD2), or L1260A (NBD2). The mutants were expressed in HEK 293 cells at 37 °C, and whole cell extracts were subjected to immunoblot analysis. The previously reported ΔY490 and ΔY490/W232R mutants (*42*) were included for comparison. In all cases, introduction of the W232R mutation rescued the processing mutants such that the 170 kDa mature form of P-gp was the major product (Figure 1C). The results show that the W232R mutation could rescue mutants with processing mutations in any domain.

Effect of Arg Mutations to Flanking Tryptophans in Other TM Segments. TM segments are often flanked by tryptophans at the lipid−water interface (*67*). P-gp contains six tryptophans at the flanking ends of TM segments (Figure 2A). Recently, the membrane insertion characteristics of the 12 TM segments of P-gp were investigated (*33*). It was shown that only TM segments 5, 8, and 10 inserted stably into the endoplasmic reticulum (ER) membrane (*33*). It is possible that the W232R mutation promotes maturation because the charged arginine helps TM4 to insert stably into the membrane. Therefore, some of the other Trp to Arg changes to flanking tryptophans could also promote maturation of G251V P-gp by helping to anchor other TM segments in the membrane.

Accordingly, mutants G251V/W212R(TM3), G251V/W315R-(TM5), G251V/W708R(TM7), and G251V/W855R(TM10) were constructed. The W136R mutation inhibited maturation of G251V as shown previously (*42*), and the mutant was included in this study as a control (Figure 2B, lanes 8 and 9). The mutants were transiently expressed in HEK 293 cells in the absence or presence of cyclosporin A. Mutants were expressed in the presence of cyclosporin A to test if expression in the presence of a drug substrate could rescue the mutant protein if the introduced Arg did not promote maturation (*22*). Mutant G251V/T55R(TM1) (Figure 2B, lanes 3 and 4) was included as a negative control because its maturation was unaffected by the presence of cyclosporin A (*41*). Whole cell extracts were subjected to immunoblot analysis (Figure 2B). It was found that none of the other Trp to Arg mutations promoted maturation of the G251V processing mutant in the absence of cyclosporin A (Figure 2B, lanes 8, 10, 14, 16, and 18). Indeed, all of the mutations except W232R (Figure 2B, lane 12) inhibited maturation when expression was carried out in the absence of drug substrates (Figure 2C). All of the tryptophan mutants could be rescued, however, when they were expressed in the presence of cyclosporin A (Figure 2B, lanes 9, 11, 13, 15, 17, and 19). The results show that only the W232R mutation promoted maturation in the absence of cyclosporin A.

The effect of the W136R, W212R, W315R, W708R, and W855R mutations on maturation of wild-type P-gp was then tested. The mutations were introduced into wild-type P-gp and the mutants were expressed in the absence or presence of cyclosporin A. All of the mutants except W708R (Figure 2D, lane 9) expressed the mature 170 kDa protein as the major product (Figure 2D, lanes 1, 3, 5, 7, and 11), and the amount of mature protein increased in the presence of cyclosporin A (Figure 2D, lanes 2, 4, 6, 8, and 12). The major product in mutant W708R was the immature 150 kDa protein (Figure 2D, lane 9). In the presence of cyclosporin A, however, the mature 170 kDa protein was the product (Figure 2D, lane 10).

Effect of W232R on Domain−Domain Interactions. It has been observed that processing mutations appear to trap P-gp in the ER as a core-glycosylated protein with incomplete domain−domain contacts. Processing mutations have been observed to disrupt contacts between the TMDs (*28*), between the NBDs (*46*), and between the NBDs and TMDs (*31*) in cysteine cross-linking studies.

To test if the W232R mutation restored domain−domain contacts, it was introduced into the G251V or L1260A processing mutants that also contained pairs of cysteines at the TMD1−TMD2 (C137(TMD1)/A935C(TMD2), NBD1−NBD2 (L531C(NBD1)/C1074(NBD2), or NBD1−TMD2 (L443C(NBD1)/S909C(TMD2) interfaces. The G251V and L1260A parents were used because the G251V mutation is in the same domain as W232R (TMD1) whereas L1260A is in another domain (NBD2). Cross-linking between the domains can be detected because cross-linked P-gp migrates slower on SDS−PAGE gels (*68*).

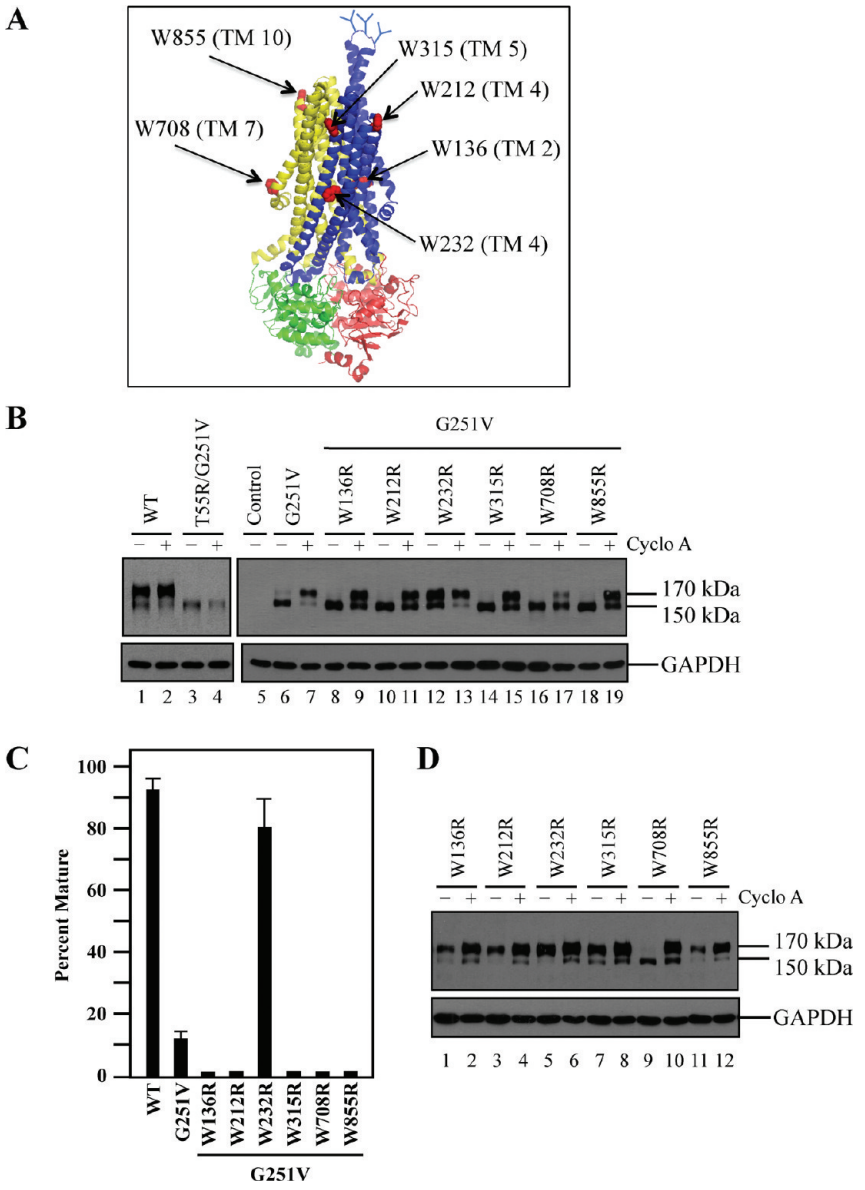

FIGURE 2: Effect of Trp to Arg mutations on maturation of processing mutant G251V or wild-type P-gp. (A) The positions of W136(TM2), W212 (TM4), W232(TM4), W315(TM5), W708(TM7), and W855(TM10) are shown as red balls in the homology model of human P-gp. (B) Cells transfected with vector (control), A52-tagged wild-type P-gp (WT), mutant G251V cDNA containing T55R, or the indicated Trp to Arg mutation were expressed in the absence (−) or presence (+) of 10 μM cyclosporin A (cyclo A). Whole cell SDS extracts were subjected to immunoblot analysis with monoclonal antibody against A52 or GAPDH. (C) The amount of mature P-gp (percent mature) relative to total P-gp (170 plus 150 kDa) from untreated cells (no cyclosporin A) is shown. The results are the average values from three separate transfections ± SD. (D) A52-tagged mutants W136R(TM2), W212R(TM4), W232R(TM4), W315R(TM5), W708R(TM7), or W855R(TM10) in a wild-type background were expressed in HEK 293 cells in the absence (−) or presence (+) of 10 μM cyclosporin A (cyclo A). Samples were subjected to immunoblot analysis with monoclonal antibody against A52 or GAPDH. The positions of mature (170 kDa) and immature (150 kDa) forms of P-gp are indicated. Lanes are numbered below the blots.

Introduction of the W232R mutation into all of the G251V (Figure 3A, lanes 3, 7, and 11) or L1260A (Figure 3B, lanes 3, 7, and 11) double-cysteine processing mutants restored maturation. In the absence of the W232R mutation, all of the double cysteine mutants yielded immature protein that did not show cross-linking when treated with the copper phenanthroline (CuP) oxidant (lanes 2, 6, and 10 in Figure 3A,B). The mature W232R mutants, however, showed cross-linking between the cysteines when they were treated with oxidant (lanes 4, 8, and 12 in Figure 3A,B). The results suggest that the W232R mutation promotes maturation by inducing long-range conformation changes to promote TMD1−TMD2, NBD1−NBD2, and NBD1−TMD2 interactions in P-gp.

Since the L1260A mutation is located in the NBD2 (Figure 1A), we also examined the effects of W232R on NBD2−TMD1 interactions. We previously showed that cysteines introduced at positions Ala266(TMD1) and Phe1086(TMD2) could be directly cross-linked in the mature protein but not if it contains the L1260A mutation (*31*). Accordingly, we introduced the W232R mutation into mutant A266C/F1086C/L1260A. Membranes were prepared from cells expressing the mutant and subjected to cross-linking with oxidant. Figure 3C shows that the W232R mutation promoted maturation of the mutant A266C/F1086C/L1260A (lane 3) and that only the mature protein was cross-linked (lane 4).

The N296A Mutation Abolishes W232R Rescue. We previously demonstrated that arginines introduced into processing

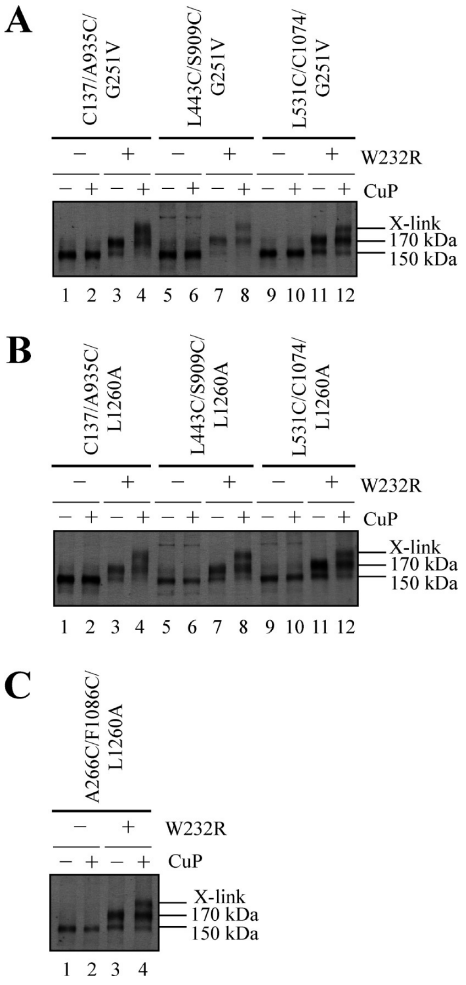

FIGURE 3: Effect of W232R on cross-linking between domains of processing mutants. Membranes were prepared from cells expressing P-gp processing mutants G251V ± W232R (A) or L1260A ± W232R (B) that also contained pairs of cysteines in various domains (L443C(NBD1)/S909C(TMD2), L531C(NBD1)/C1074(NBD2), C137(TMD1)/A935C(TMD2)). Membranes were also prepared from cells expressing the L1260A processing mutant ± W232R containing cysteines in TMD1 and NBD2 (A266C/F1086C) (C). Samples were treated with (+) or without (−) oxidant (copper phenanthroline) (CuP) for 15 min at 0 °C. The reactions were stopped by addition of sample buffer containing 50 mM EDTA. Samples were subjected to immunoblot analysis. The positions of mature (170 kDa), immature (150 kDa), and cross-linked (X-link) forms of P-gp are indicated. Lanes are numbered below the blots.

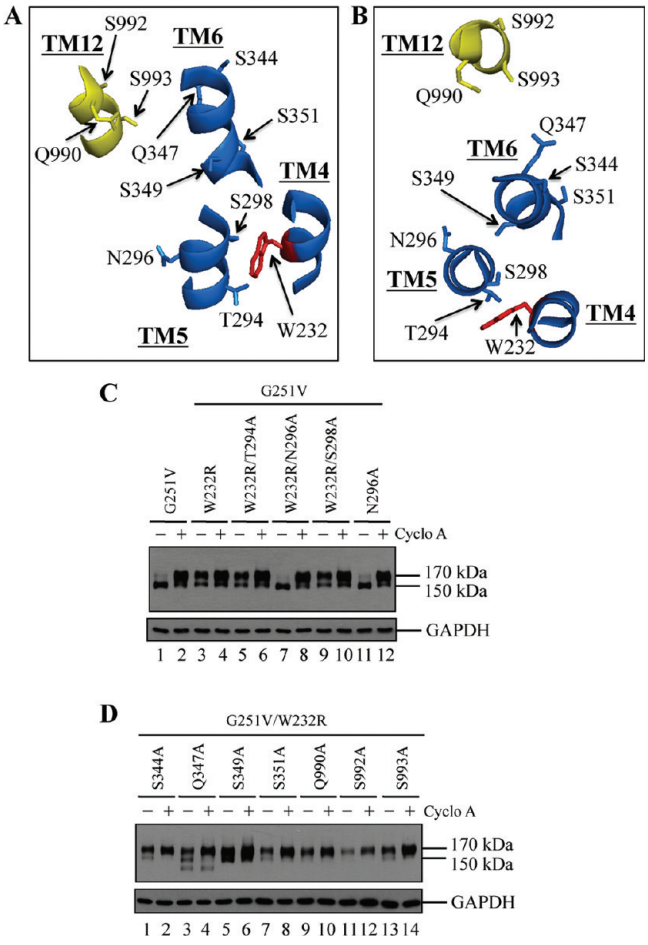

FIGURE 4: Effect of mutating residues capable of forming hydrogen bonds with W232R on maturation of G251V. (A, B) Trp232 (red color) in TM4 with potential hydrogen-bonding residues in TM segments 5, 6, and 12 are viewed from different angles. (C) HEK 293 cells transfected with A52-tagged mutant G251V or G251V/W232R containing alanine mutations in potential hydrogen-bonding residues in TM5 (T294A, N296A, S298A) or G251V/N296A were expressed in the absence (−) or presence (+) of cyclosporin A (cyclo A). Whole cell SDS extracts were subjected to immunoblot analysis with monoclonal antibody against A52 or GAPDH. (D) HEK 293 cells transfected with A52-tagged mutant G251V/W232R(TM4) containing mutations in potential hydrogen-bonding residues in TM6 (S344A, Q347A, S349A, S351A) or TM12 (Q990A, S992A, S993A) were cultured in the absence (−) or presence (+) of cyclosporin A (cyclo A). Whole cell SDS extracts were subjected to immunoblot analysis with monoclonal antibody against A52 or GAPDH. The positions of mature (170 kDa) and immature (150 kDa) forms of P-gp are indicated. Lanes are numbered below the blots.

mutants appeared to enhance maturation by promoting TMD1−TMD2 interdomain hydrogen bond interactions (such as TMD1-(M68R(TM1)) with TMD2(Y950(TM11))) (*41*). The model in Figure 1A, however, suggests that W232R must enhance maturation by a different mechanism since it does not lie close to any TM segments in TMD2. The model predicts TM4 to be adjacent to TM5 and TM6 in TMD1 (Figure 1A). Therefore, W232R may promote maturation through hydrogen bond interactions with residues in TM5 or TM6. There was biochemical evidence, however, suggesting that TM4 could also come into contact with TM12 as cysteines introduced into the intracellular ends of these two TMs could be directly cross-linked when the mutants were treated with oxidant (copper phenanthroline) (*51*). To test for evidence of hydrogen bond interactions of W232R with residues in TM segments 5, 6, or 12, potential hydrogen bond partners (Thr294(TM5), Asn296(TM5), Ser298(TM5), Ser344(TM6), Gln347(TM6), Ser349(TM6), Ser351(TM6)), (Gln990(TM12), Ser992(TM12), or Ser993(TM12)) (see Figure 4A,B) were mutated to alanine in a G251V/W232R background. The rationale was that removal of a hydrogen-bonding partner would abolish the ability of W232R to promote maturation. The mutants were expressed in the presence or absence of cyclosporin A and whole cell extracts subjected to immunoblot analysis. It was observed that only one mutation in TM5 (N296A) inhibited maturation of the G251V/W232R mutant when it was expressed in the absence of cyclosporin A (Figure 4C, lane 7). By contrast, mutation of potential hydrogen-bonding residues in TM6 (Ser344, Q347, S349, or Q351) or TM12 (Gln990, Ser992, or S993) to alanine did not affect maturation of G251V/W232R (Figure 4D). Introduction of the N296A mutation into G251V/W232R(TM4) caused the mutant to behave in a fashion similar to the original

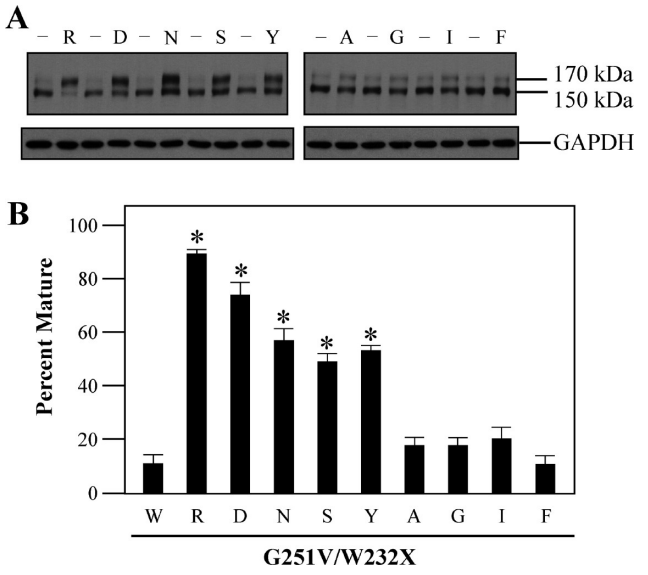

FIGURE 5: Effect of different W232 mutations on maturation of G251V. (A) HEK 293 cells expressing A52-tagged G251V containing mutations in W232 (−, no change) capable (Arg(R), Asp(D), Asn(N), Ser(S), Tyr(Y)) or incapable (Ala(A), Gly(G), Ile(I), Phe(F)) of forming hydrogen bonds were subjected to immunoblot analysis with monoclonal antibody against A52 or GAPDH. The positions of mature (170 kDa) and immature (150 kDa) forms of P-gp are indicated. (B) The amount of mature P-gp (percent mature) relative to total P-gp (170 plus 150 kDa) in mutant G251V/W232X (X = W, R, D, N, S, Y, A, G, I, or F) is shown. The results are the average values from three separate transfections ± SD. An asterisk indicates significant difference ($P < 0.05$) from parent (G251V).

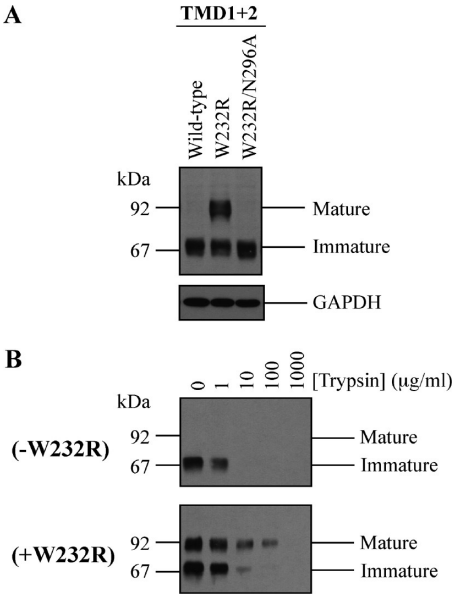

FIGURE 6: Effect of the W232R mutation on maturation and protease sensitivity of a P-gp truncation mutant lacking the NBDs (TMD1 + 2). (A) Whole cell extracts of cells transfected with A52-tagged wild-type, W232R, or W232R/N296A forms of TMD1 + 2 were subjected to immunoblot analysis with monoclonal antibody against A52 or GAPDH. (B) Membranes prepared from cells expressing A52-tagged wild-type (−W232R) or mutant W232R (+W232R) forms of TMD1 + 2 were treated with the indicated concentrations of trypsin and samples subjected to immunoblot analysis. The positions of mature and immature forms of TMD1 + 2 are indicated.

G251V parent. Like the G251V parent (Figure 4C, lanes 1 and 2), the G251V/W232R/N296A mutant (Figure 4C, lane 7) showed about 15% maturation efficiency when expressed in the absence of drug substrates, and the mature 170 kDa P-gp was the major product when expressed in the presence of cyclosporin A (Figure 4C, lane 8). Therefore, the presence of the N296A mutation prevented the ability of W232R to promote maturation of the G251V mutant. Figure 4A (lanes 11 and 12) shows that the N296A change alone had little effect on the maturation characteristics of the G251V parent because it could still be rescued with cyclosporin A.

These results suggest that Arg232(TM4) promotes maturation of the G251V mutant through hydrogen bond interactions with Asn296(TM5) because the N296A mutation inhibited the ability of W232R to promote maturation of G251V (Figure 4C, lane 7). Asn can act as a hydrogen bond acceptor or donor. If W232R rescues by forming a hydrogen bond with Asn296, then we infer from the models (Figure 4A,B) that it would require a slight rotation of TM5 to bring the Asn side chain closer to the side chain of arginine at position 232. It is possible that replacement of the hydrophobic side chain of Trp232 with the bulky charged side chain of arginine may have altered the orientation of TM segments 4 and 5 to allow Arg232 to interact with Asn296.

To test if other mutations to Trp232 would promote maturation, it was changed to other residues capable (Asp, Asn, Ser, Tyr) or incapable (Ala, Gly, Ile, Phe) of forming hydrogen bonds in a G251V background. The mutants were expressed in the absence of drug substrates and whole cell extracts subjected to immunoblot analysis (Figure 5A). It was found (Figure 5B) that the Trp232 changes to Trp, Asp, Asn, Ser, or Tyr increased the maturation efficiency by 6.0-, 5.0-, 3.7-, 3.1-, or 3.5-fold ($P < 0.05$), respectively. No significant increase in maturation efficiency of G251V was observed when Trp232 was changed to amino acids that do not form hydrogen bonds (Ala, Gly, Ile, or Phe). The pattern of rescue of the G251V/W232X mutants was consistent with the prediction that hydrogen bond interactions were responsible for the increase in maturation efficiency.

Rescue by W232R Does Not Require the NBDs. In wild-type P-gp, the NBDs appear to play an important role in maturation since deletion of NBD2 (contains residues 1−1023) or both NBDs (TMD1 + 2; contains residues 1−379 + 681−1025) yields P-gp truncation mutants that fail to mature (*32*). The NBD truncation mutants will mature, however, if they are expressed in the presence of drug substrates (*32*). Alanine-scanning mutagenesis of potential hydrogen bond partners suggested that W232R rescue involved Asn296 in TM5 (Figure 4C, lane 7). Since residues W232R and Asn296 were located in the TMDs, it was possible that the NBDs would not be required for rescue. To test if the NBDs were required for rescue, the W232R mutation was introduced into a truncation mutant lacking both NBDs (TMD1 + 2(W232R)). When TMD1 + 2(W232R) was expressed in HEK 293 cells, it was found that about 50% of the protein was present as mature protein (Figure 6A). No detectable mature protein was observed in cells expressing TMD1 + 2 lacking W232R or with mutant TMD1 + 2(W232R/N296A) (Figure 6A).

Drug substrates can also promote maturation of P-gp TMD1 + 2 (*8*). Drug substrates appear to promote maturation by inducing the protein to change from a loosely folded protease-sensitive conformation to a tightly folded protease-resistant conformation. To test if W232R also converted TMD1 + 2 into a protease-resistant conformation, membranes prepared from cells expressing TMD1 + 2 with or without W232R were treated with various concentrations of trypsin. It was found that the

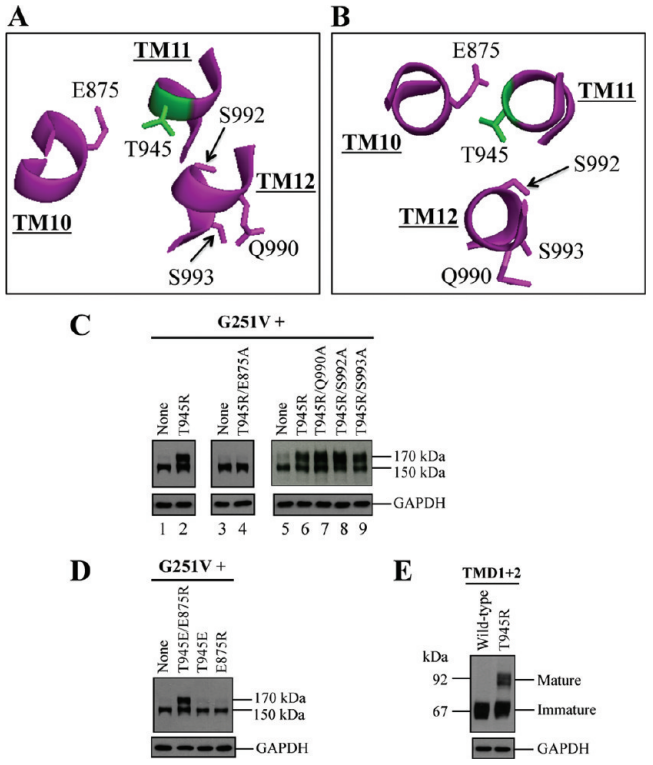

FIGURE 7: Effect of mutations in Thr945 or Glu875 on the rescue of G251V P-gp or mutant TMD1 + 2. (A, B) Two views showing Thr945 (green) in TM11 with potential hydrogen-bonding residues Glu875(TM10), Ser992(TM12), Ser993 (TM12), or Gln990 (TM12). (C) Whole cell extracts from cells expressing A52-tagged mutant G251V (none) or G251V/T945R (T945R) with the indicated changes to potential hydrogen bond partners in TM10 (lane 4) or TM12 (lanes 7−9) were subjected to immunoblot analysis with monoclonal antibody against A52 or GAPDH. Lanes are numbered below the blots. (D) Whole cell extracts of cells expressing A52-tagged G251V (none) or G251V mutants containing opposite charges at positions 945 and 875 (T945E/E875, T954E, or E875R) were subjected to immunoblot analysis with monoclonal antibody against A52 or GAPDH. The positions of mature (170 kDa) and immature (150 kDa) forms of P-gp are indicated. (E) Whole cell extracts of cells transfected with A52-tagged wild-type or T945R forms of TMD1 + 2 were subjected to immunoblot analysis with monoclonal antibody against A52 or GAPDH. The positions of mature and immature forms of TMD1 + 2 are indicated.

mature form of TMD1 + 2(W232R) was 15.5 times more resistant to trypsin relative to the immature forms of the protein (Figure 6B). Rescue by W232R resembled drug rescue as both converted TMD1 + 2 to a mature protease-resistant conformation.

Interactions of T945R(TM11) with Glu875(TM10) Repair Defects in C-Half Topology. To test if intradomain interactions in TMD2 could also act as a rescue mechanism, we examined the T945R suppressor mutation. The T945R mutation was selected as it was one of the most effective suppressor mutations identified in TMD2 (*42*). A potential residue in TM10 that could form a hydrogen bond with T945R(TM11) as shown in a model of packing of the TM segments (Figures 7A and B) is Glu875(TM10). A G251V/T945R/E875A mutant was constructed to test if removal of the negative charge would affect maturation. It was found that T945R no longer promoted maturation when the E875A mutation was present (Figure 7C, lane 4). To determine if it was specific for Glu875(TM10), we tested the effect of alanine mutations on potential hydrogen bond partners in TM12 since residues such as Ser992 (Figure 7A,B) are predicted to be in close proximity. Maturation was not reduced

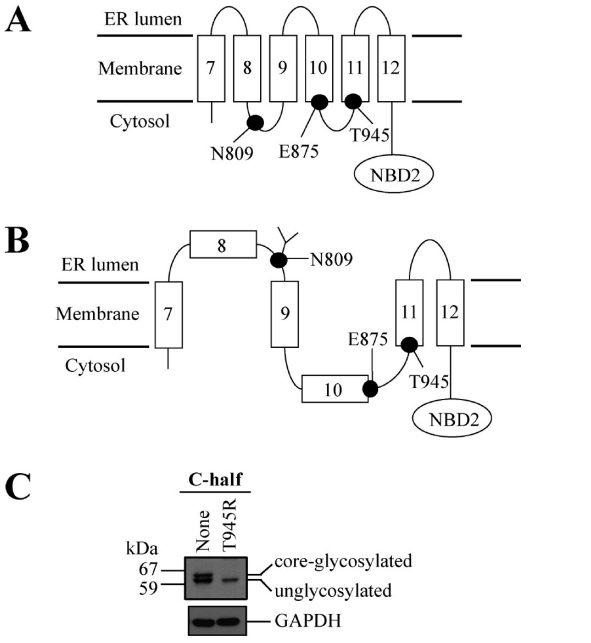

FIGURE 8: Effect of T945R mutation on expression of C-half P-gp. (A) Model of the normal topology of C-half P-gp. (B) Model of an alternative topology found in the ER (glycosylated at Asn809, indicated by branched line). (C) Whole cell extracts of cells expressing A52-tagged wild-type (None) or T945R forms of C-half P-gp were subjected to immunoblot analysis with monoclonal antibody against A52 or GAPDH. The positions of core-glycosylated (alternative topology) and unglycosylated (normal topology) forms of C-half P-gp are indicated.

by the Q990A, S992A, or S993A TM12 mutations (Figure 7C, lanes 7, 8, and 9).

Arg945 and Glu875 have the potential to form a salt bridge between TM segments 10 and 11. Therefore, we tested the effect of reversing the charges by constructing mutant G251V/T945E/E875R. It was found that the T945E/E875R combination but not individual T945E or E875R mutations promoted maturation of G251V (Figure 7D).

Since both Glu875 and Thr945 are located in TMD2, we tested if the T945R mutation would promote maturation of the truncation mutant lacking the NBDs (TMD1 + 2). Immunoblot analysis of whole cell extracts of the transfected cells showed that the T945R mutation promoted maturation of TMD1 + 2 (Figure 7E).

Multiple topologies have been detected when P-gp is synthesized in the ER (*34*, *35*, *37*). For example, expression of C-half P-gp yields a mixture of glycosylated and unglycosylated P-gp proteins (*45*, *69*). Native C-half P-gp should not be glycosylated since the only potential glycosylation site (Asn809-Thr810-Thr811) would be located outside of the lumen of the ER (Figure 8A). An alternative topology (Figure 8B) was detected, however, that had improper insertion of TM segments 8−10 to cause glycosylation at Asn809 (*69*).

To test if T945R would promote or inhibit the alternative topology, it was introduced into C-half P-gp. Whole cell immunoblots showed that C-half P-gp yielded a doublet of immunoreactive P-gp (Figure 8C). We previously showed that the slower migrating P-gp C-half protein was core-glycosylated (*45*). Expression of C-half/T945R yielded a single protein product corresponding to unglycosylated P-gp (Figure 8C). The C-half/T945R protein was not glycosylated as it was not sensitive to endoglycosidases (data not shown).

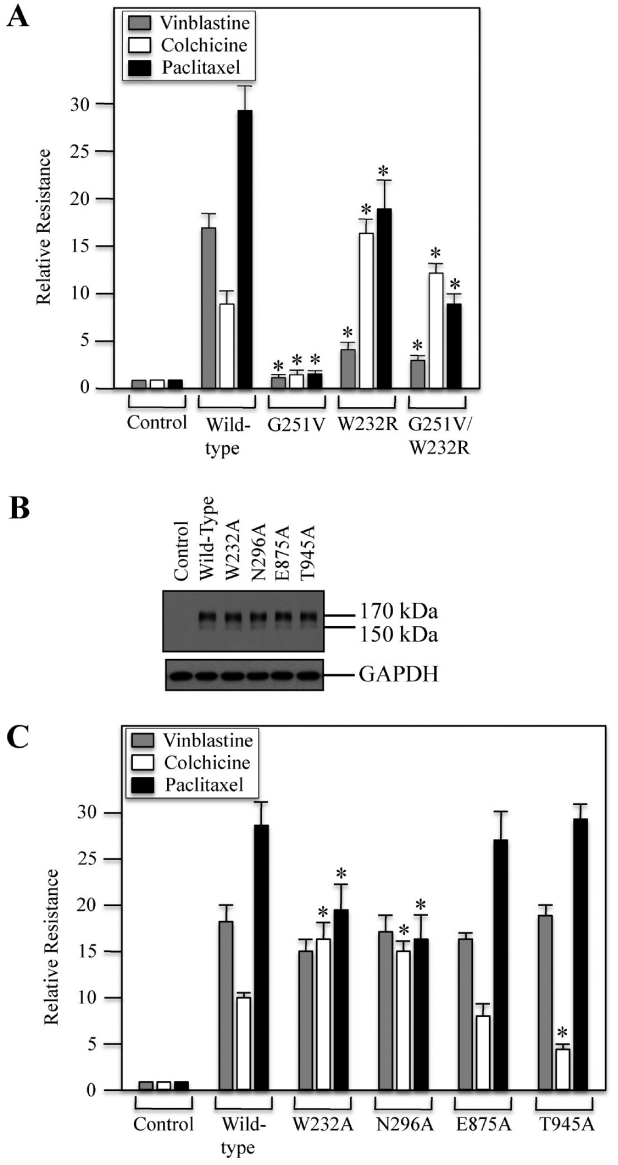

FIGURE 9: Drug resistance profiles of P-gp mutants. (A) Stable BHK cell lines expressing no P-gp (control), equivalent levels of wild type, mutants G251V, W232R, or G251V/W232R P-gp were incubated for 6 days in the presence of various concentrations of drug substrates vinblastine (gray bars), colchicine (white bars), or paclitaxel (black bars). The concentration of drug substrate required to inhibit growth by 90% (LD_{90}) was determined, and the relative resistance was determined as the ratio of this value to that of control cells. Each value is average of triplicate assays ± SD. (B) Whole cell extracts of BHK cells expressing vector (control), A52-tagged wild-type, mutant W232A, N296A, E875A, or T945A P-gp were subjected to immunoblot analysis with monoclonal antibody against A52 or GAPDH. The positions of mature (170 kDa) and immature (150 kDa) forms of P-gp are indicated. (C) The drug resistance profile of each mutant shown in (B) in the presence of vinblastine, colchicine, or paclitaxel was determined. Each value is average of triplicate assays ± SD. An asterisk indicates significant difference ($P < 0.05$) from wild-type P-gp.

Effect of Mutations on Activity. To test if rescue by an arginine suppressor mutation yielded an active transporter, we examined the ability of mutants G251V and G251V/W232R to confer resistance to cytotoxic compounds colchicine, paclitaxel, and vinblastine. Colchicine and vinblastine were selected because they are classic P-gp drug substrates (*70*) while paclitaxel is an important anticancer drug that is also a substrate of P-gp (*2*).

Stable BHK cell lines expressing wild-type P-gp or mutants G251V, W232R, or G251V/W232R were generated by cotransfecting P-gp cDNAs with pWL-neo vector followed by selection with G418. Clones expressing the mutant P-gp were identified by immunoblot analysis. Cell lines expressing similar levels of P-gps were then incubated with various concentrations of colchicine, vinblastine, or paclitaxel. The concentrations of drug substrate required for 90% cell death (LD_{90}) in control BHK cells were 467, 2.5, and 23 nM for colchicine, vinblastine, and paclitaxel, respectively. The LD_{90} values for wild-type P-gp were 4200 nM colchicine (9-fold increase), 43 nM vinblastine (17-fold increase), and 670 nM paclitaxel (29-fold increase) (Figure 9A). The W232R mutation altered the drug resistance profile as it conferred only a 4-fold increase in resistance to vinblastine but showed relatively high levels of resistance to colchicine (16-fold; $P < 0.05$) and paclitaxel (19-fold; $P < 0.05$) (Figure 9A). The decreased resistance to vinblastine and increased resistance to colchicine were consistent with the reported effects of W232R on drug-stimulated ATPase activity (*42*). Mutant W232R showed an increased S_{50} (concentration required for 50% stimulation of activity) for vinblastine and increased maximum stimulation in the presence of colchicine. Cells expressing mutant G251V showed almost no increase ($P < 0.05$) in drug resistance compared to control cells. This is to be expected since mutant G251V is a processing mutant in which the majority of P-gp is misfolded in the cell. Introduction of the W232R mutation into G251V, however, yielded a functional P-gp. Cells expressing mutant G251V/W232R were more resistant to colchicine (12-fold; $P < 0.05$) compared to cells expressing wild-type P-gp (9-fold) but were less resistant to vinblastine (3-fold ($P < 0.05$) versus 17-fold) and paclitaxel (18-fold ($P < 0.05$) versus 29-fold). These results show that the W232R mutation restored maturation of mutant G251V P-gp to yield a functional transporter at the cell surface.

We then examined whether residues Trp232, Asn296, Glu875, or Thr945 played an important role in the activity or maturation of P-gp by testing the effects of alanine mutations. Stable BHK cell lines expressing mutants W232A, N296A, E875A, or T945A were generated. Immunoblot analysis of whole cell extracts showed that none of the mutations inhibited maturation of P-gp (Figure 9B). Cells expressing these mutants were then incubated with various concentrations of colchicine, vinblastine, or paclitaxel. All of the mutants conferred resistance to these drug substrates, but there were changes in substrate specificity (Figure 9C). For example, cells expressing mutants W232A and N296A were more resistant to colchicine ($P < 0.05$) than cells expressing wild-type P-gp, but their resistance to paclitaxel was reduced ($P < 0.05$). Cells expressing mutant T945A were as resistant to vinblastine as cells with wild-type P-gp but showed decreased resistance to colchicine ($P < 0.05$).

Interaction of drug substrates verapamil and rhodamine B with the mutants could not be examined in the drug-resistance assays because these compounds are not toxic to BHK cells. Therefore, another way to test for P-gp interactions with drug substrate is to measure drug-stimulated ATPase activity. It was important to test the effect of mutations on the verapamil and rhodamine B activities because they interact with P-gp at different sites in the drug-binding pocket (*71*), and it would be possible to determine if the mutations only had local or global effects on the structure. Accordingly, drug-stimulated ATPase activities of the mutants were determined. The E875A and T945A mutations altered verapamil interactions with P-gp (Figure 10A).

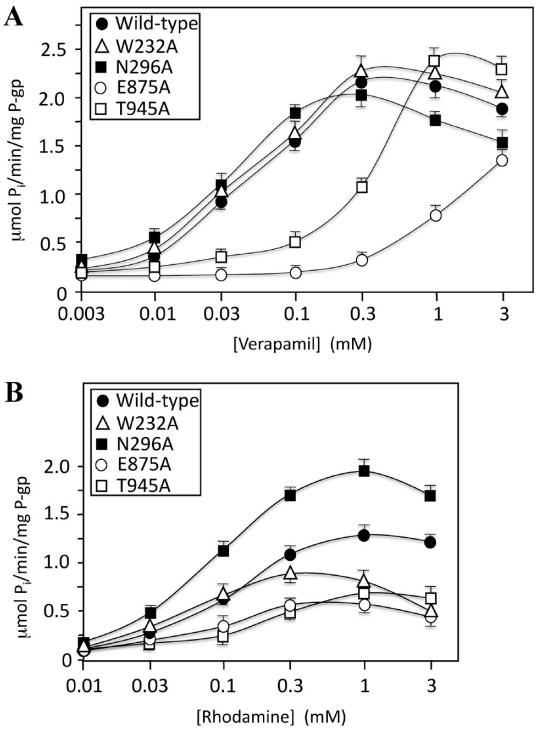

FIGURE 10: Effect of mutations on drug-stimulated ATPase activity. Histidine-tagged wild-type or mutant W232A, N296A, E875A, or T945A P-gp was expressed in HEK 293 cells and isolated by nickel-chelate chromatography. The isolated P-gp proteins were mixed with lipid, and ATPase activities were determined in the presence of various concentrations of verapamil (A) or rhodamine B (B). Each value is the average of triplicate assays ± SD.

Higher concentrations of verapamil were required for half-maximal activation (S_{50}) of ATPase activity for mutants T945A (370 μM) and E875A (> 1 mM) compared to wild-type P-gp (48 μM). The verapamil-stimulated ATPase activities of mutants W232A and N296A, however, resembled that of wild-type enzyme (S_{50} of 30 and 42 μM, respectively).

Figure 10B shows that all four mutations affected rhodamine B-stimulated ATPase activity. The maximum activity was increased by 50% in mutant N296A when compared to wild-type P-gp. By contrast, the maximum activity of mutants W232A, E875A, and T945A were reduced relative to wild-type P-gp. The S_{50} of mutants W232A (45 μM) and T945A (200 μM) differed from wild-type P-gp (102 μM) while those of mutants N296A (78 μM) and E875A (86 μM) were similar. It was not surprising that the mutations affected rhodamine B-stimulated ATPase activity because it was previously shown that treatment of mutants W232C, N296C, and T945C (E875C was not tested) with a thiol-reactive analogue of rhodamine inhibited activity (*15*).

In summary, the E875A and T945A mutations altered both verapamil- and rhodamine B-stimulated ATPase activity while the W232A and N296A mutations only affected rhodamine B-stimulated ATPase activity. The large effect of the T945A mutation on ATPase activity is consistent with the observation that wild-type P-gp containing W232R exhibits verapamil- and rhodamine-stimulated ATPase activity whereas wild-type P-gp containing T945R does not (*42*).

DISCUSSION

Several significant conclusions can be drawn from this study. First, the W232R suppressor mutation located in the first domain (TMD1) can repair defects in maturation caused by processing mutations located in any other domain. It also restored maturation of the G251V processing mutant to yield a functional transporter at the cell surface (Figure 9A). The mechanism of repair was to correct interdomain assembly (TMD1−TMD2, NBD1−NBD2, and NBD−TMD interactions) that had been disrupted by the processing mutations. Second, the NBDs are not required for rescue by W232R or T945R. Third, the topology of P-gp can be modulated by suppressor mutations. Fourth, intradomain interactions between adjacent TM segments can promote maturation. This conclusion is highly significant because we previously concluded that drugs or suppressor arginines could only promote maturation of processing mutants through interactions at domain interfaces (*40*). This finding suggests that it may be possible to rescue other ABC processing mutants implicated in diseases (such as CFTR) by identifying compounds that act outside critical domain interfaces.

Drug substrates and inhibitors rescue P-gp processing mutations by binding at the interface between TMD1 and TMD2 (*8*). This mechanism could potentially be a drawback when rescuing CFTR processing mutants because compounds such as benzbromarone that are predicted to bind at the interface of TMD1 and TMD2 (*72*) inhibit CFTR channel activity (*73*). Therefore, the finding that P-gp processing mutants can be rescued by suppressor mutations that promote intradomain interactions suggests that it may be possible to use structure-based virtual screening of chemical libraries (*74*) to identify CFTR correctors that interact with the TMDs at sites that would not inhibit channel activity.

The effects of processing and suppressor mutations on interdomain assembly of P-gp show many similarities to its sister protein, CFTR. For example, processing mutations appear to trap P-gp in a loosely folded conformation that is about 100 times more sensitive to proteases relative to the mature form of the protein (*39*). The immature mutant appears to be highly sensitive to proteases because contacts between the various domains (TMD1−TMD2 (*28*), NBD1−NBD2 (*46*), NBD−TMD (*31*)) are incomplete. Processing mutations have also been identified throughout CFTR (*75*−*77*). The most common cystic fibrosis mutation (ΔF508 in NBD1) also traps CFTR in a protease-sensitive conformation with defective domain−domain contacts (*29*, *31*, *78*, *79*). It appears that the ΔF508 mutation inhibits folding of NBD1 and its ability to stably associate with other domains resulting in altered CFTR−chaperone interactions, ER retention, and enhanced degradation (*65*). Second-site suppressor mutations in NBD1 (such as I539T/G550E/R553M/R555K) can restore interdomain assembly (*65*, *66*) to yield a more stable ΔF508-CFTR molecule (*64*, *66*). Maturation of ΔF508-CFTR can also be promoted by introduction of suppressor mutations at the NBD1−TMD2 (intracellular loop 4) interface. For example, the R1070W mutation was postulated to promote folding through hydrophobic interactions with NBD1 in ΔF508-CFTR (*65*). Similarly, it was suggested that the V510D suppressor mutation in NBD1 promoted folding of ΔF508-CFTR by forming a salt bridge with Arg1070 (*64*). Other suppressor mutations in NBD1 such as G550E appear to rescue ΔF508-CFTR by altering the conformation of NBD1 (*62*).

Why do processing mutations that are dispersed throughout P-gp or CFTR impact global folding of the molecules? It is likely that point mutations throughout P-gp or CFTR cause global misfolding because both proteins require cooperative posttranslational folding between domains to yield a stable

molecule (*80*, *81*). It was postulated (*80*) that folding of ABC transporters is different from other proteins because it occurs posttranslationally while the folding of soluble multidomain proteins occurs by a cotranslational mechanism. The mechanism of P-gp folding also appears to occur by a posttranslational mechanism because point mutations or suppressor mutations dispersed along its entire length can impact global folding.

There is a major difference in the folding of P-gp and CFTR, however, in the requirement for NBD2. A CFTR deletion mutant lacking NBD2 will show a maturation efficiency similar to full-length CFTR (*32*) whereas a ΔNBD2-P-gp mutant does not mature. A possible explanation may be due to differences in the membrane-integration characteristics of the two transporters and the need for stabilization through NBD−TMD interactions. For CFTR, it was found that all of the TM segments of TMD1 were integrated stably in the membrane as well as four of six TM segments in TMD2 (*33*). For P-gp, only one TM segment in TMD1 and two in TMD2 were found to stably integrate in the membrane. The instability of the P-gp TM segments may explain why alternative topologies have been detected in the ER (*34*−*37*). Cysteine cross-linking assays have demonstrated NBD1−TMD2 and NBD2−TMD1 interactions (*30*, *31*, *82*) in P-gp and CFTR. NBD−TMD interactions may be important for packing of the TM segments into a native stable structure because truncation mutants lacking the NBDs do not mature (*8*, *32*). Since all of the TM segments of CFTR TMD1 stably integrate into the membrane, NBD2−TMD1 interactions may not be required for maturation. By contrast, NBD2−TMD1 interactions may be important for maturation of P-gp since five of six TM segments are not stably inserted in the membrane.

P-gp TMD1 + 2 could be rescued to yield mature protein in a protease-resistant conformation by the W232R mutation. Tryptophans are commonly found at the aqueous-lipid boundaries of TM segments due to their interfacial anchor properties (*67*). Therefore, one possible explanation for the ability of the W232R-(TM4) mutation to promote maturation of the P-gp processing mutants was that the positively charged side chain of arginine could serve as a better anchor for TM4. Our results, however, suggest that the W232R(TM4) mutation likely promotes maturation of processing mutations through intradomain hydrogen bond interactions with Asn296(TM5) rather than through its anchoring properties. This is because mutation of Asn296(TM5) to alanine abolished the ability of the W232R(TM4) mutation to promote maturation (Figure 4C, lane 7). The maturation characteristics of other W232 mutations also support the prediction that hydrogen bond interactions were important for maturation (Figure 5).

Evidence for intradomain interactions between TM segments was also obtained for T945R(TM11) with Glu875(TM10) in TMD2 (Figure 7C). Strong support for the prediction that intradomain interactions between TM segments can modulate topology was the observation that T945R inhibited the formation of an alternative topology in C-half P-gp when it was expressed alone (Figure 8C).

The ability of suppressor mutations in the TMDs to promote folding of processing mutants has also been observed in the yeast ABC drug transporter, Yor1p (*83*). Suppressor mutations in TM2 (F270S) and TM12 (R1168M) restored trafficking and activity of a processing mutant. It was concluded that the suppressor mutations restored folding in the TMDs.

The TMDs of P-gp appear to show considerable plasticity during synthesis. Multiple topologies have been detected (*34*−*37*), and drug substrates (*8*) or suppressor mutations like W232R (this study) can convert the domains from protease-sensitive to protease-resistant conformations. The plasticity of the TMDs may contribute to alterations in the substrate specificity of P-gp since they contain the drug-binding sites. For example, it has been proposed that silent polymorphisms may change P-gp substrate specificity by altering the timing of insertion of the protein into the membrane (*38*). Perhaps the presence of drug substrates during synthesis may alter the structure of the TMDs and change substrate specificity.

Do the residues in this study that are implicated in rescue (Trp232, Asn296, Glu875, or Thr945) normally play an important role in the maturation/function of P-gp or are they gain-of-function mutations? It was previously reported that residue Glu875 could contribute to folding of TM segments because the E875C mutation altered the topology of C-half Pgp (*45*). Residues Trp232, Asn296, and Thr945 appear to play important roles in the structure and/or function of P-gp because alanine mutants had altered drug resistance or ATPase activity profiles (Figures 9C and 10). These results are consistent with those from cysteine mutagenesis studies where modification of mutant T945C by thiol-reactive analogues of verapamil or rhodamine or mutants W232C and N296C by a thiol reactive analogue of rhodamine inhibited their activity (*13*, *15*).

The importance of residues Trp232, Asn296, Glu875, and Thr945 in the structure and/or function of P-gp suggests that these residues could be conserved at similar positions in related ABC transporters. Residues Trp232 and Glu875 are conserved in the human (ABCB1 and ABCB4 (phosphatidylcholine transporter)), hamster (P-gp1, P-gp2, and P-gp3), and mouse (ABCB1a, ABCB1b, and ABCB4). Residue Asn296 is conserved in all ABC proteins mentioned above except mouse ABCB1b (has Ser) while Ser replaces Thr945 in human ABCB4, mouse ABCB4, and hamster P-gp3 (*84*, *85*). Residues Trp232, Asn296, Glu875, and Thr945 are not conserved, however, when the P-gp sequence was aligned with those from CFTR or putative bacterial drug pumps (MsbA and Sav1866) (*86*). These observations indicate that the residues are conserved only in closely related mammalian drug pumps and phosphatidylcholine transporters.

The observation that intradomain suppressor mutations can promote maturation suggests that the arginine-rescue approach may be useful to map packing of TM segments in polytopic membrane proteins. The requirements for performing arginine rescue are that a processing mutation has been identified and that the protein contains carbohydrate so folding can be monitored by modification of the core-glycosylated protein in the ER. Many methods have been developed to determine the boundaries of TM segments in polytopic membrane proteins such as reaction of cysteine mutants with membrane-permeant or -impermeant probes (*7*) or monitoring the orientation of inserted epitope or glycosylation tags (*87*, *88*). It has been more difficult to determine packing of the TM segments. Packing of P-gp TM segments has been studied by cysteine mutagenesis and disulfide cross-linking analysis (*47*, *89*, *90*), but this approach can only be used to map interdomain contacts. Cross-linking between cysteines in different domains causes the cross-linked P-gp to migrate slower on SDS−PAGE gels. Intradomain cross-linking, however, is difficult to detect as it does not cause a detectable mobility change on SDS−PAGE gels. Another potential disadvantage of the cross-linking approach is that thermal motion of the protein can lead to cross-linking between distant cysteines. The arginine-rescue approach to map packing of TM segments probably only detects contacts in the native structure. Mutations that introduce

non-native hydrogen bonds into proteins would likely cause misfolding (*91*, *92*).

Arginine rescue works well with P-gp as over 17% of arginines (37 of 216 changes) introduced into TM segments promoted maturation of a processing mutant (*42*). The large number of suppressor arginine suggests that the TM segments of P-gp appear to be quite mobile. Evidence for mobility of the TM segments is suggested by the large number of cross-links observed between TM segments in a cysteine mutagenesis study (*51*), that drug binding occurs through an induced-fit mechanism (*93*), that ATP hydrolysis can cause rotation of one or more helices (*94*), and that other residues at position 232 whose side chains are of different sizes and capable of forming hydrogen bonds (Asp, Ser, Tyr) could rescue G251V (Figure 5). Therefore, mobility of the TM segments could explain how an arginine introduced at position 232 in TM4 could interact with Asn296 in TM5 when the homology model (Figure 4A,B) shows these two residues would be too far apart for any interaction. Slight rotation of TM5 during folding would enable Asn296 to interact with Arg232.

Interactions between Arg232 and Asn296 (or Arg945 with Glu875) could occur during folding in the ER before P-gp adopts its native structure (Figure 1A). It has been postulated that the structure of early intermediates of P-gp or CFTR may differ from the final native structure (*53*, *95*, *96*). Finally, the predicted structure of human P-gp (Figure 1A) is a homology model (*55*) based on the structure of bacterial ABC transporter Sav1866 (*97*) that was crystallized in the absence of lipid or nucleotide. Both lipid and ATP can alter the structure of P-gp (*98*, *99*).

In summary, we report that suppressor mutations can rescue an ABC protein by promoting local intradomain interactions between TM segments to restore global folding of the protein and correct defects in topology. A suppressor mutation in either TMD was capable of promoting maturation of P-gp even when both NBDs were removed.

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