Processing Mutations Located throughout the Human Multidrug Resistance P-glycoprotein Disrupt Interactions between the Nucleotide Binding Domains

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

From the Canadian Institutes of Health Research Group in Membrane Biology, Departments of Medicine and Biochemistry, University of Toronto, Toronto, Ontario M5S 1A8, Canada

The most common cause of cystic fibrosis is misfolding of the cystic fibrosis transmembrane conductance regulator (CFTR) protein because of deletion of residue Phe-508 (ΔF508). P-glycoprotein (P-gp) is an ideal model protein for studying how mutations disrupt folding of ATP-binding cassette proteins such as CFTR because specific chemical chaperones can be used to correct folding defects. Interactions between the nucleotide binding domains (NBDs) are critical because ATP binds at the interface between the NBDs. Here, we used disulfide cross-linking between cysteines in the Walker A sites and the LSGGQ signature sequences to test whether processing mutations located throughout P-gp disrupted interactions between the NBDs. We found that mutations present in the cytoplasmic loops, transmembrane segments, and linker regions or deletion of Tyr-490 (equivalent to Phe-508 in CFTR) inhibited cross-linking between the NBDs. Deletion of Phe-508 in the P-gp/CFTR chimera also inhibited cross-linking between the NBDs. Cross-linking was restored, however, when the mutants were expressed in the presence of the chemical chaperone cyclosporin A. The “rescued” mutants exhibited drug-stimulated ATPase activity, and cross-linking between the NBDs was inhibited by vanadate trapping of nucleotide. These results together with our previous findings (Loo, T. W., Bartlett, M. C., and Clarke, D. M. (2002) J. Biol. Chem. 277, 27585–27588) indicate that processing mutations disrupt interactions among all four domains. It appears that cross-talk between the cytoplasmic and the transmembrane domains is required for establishment of proper domain-domain interactions that occur during folding of ATP-binding cassette protein transporters.

Cystic fibrosis (CF) is a lethal inherited disorder caused by mutations in the gene coding for the cystic fibrosis transmembrane conductance regulator (CFTR) protein. Human CFTR has 1480 amino acids and acts as a cAMP-regulated chloride channel. It is located at the surface of epithelial cells where it regulates salt and fluid transport across the cell membrane. The most common CF-associated mutation (found on at least one chromosome of 90% of affected individuals) is deletion of phenylalanine 508 (ΔF508) in the first nucleotide binding domain (NBD1). The ΔF508 mutation causes misfolding of the CFTR protein. The misfolded protein is not targeted correctly to the apical membrane and is retained in the endoplasmic reticulum in an inactive form and is degraded rapidly. The ΔF508 CFTR mutant, however, is temperature-sensitive because expression at lower temperatures promotes maturation and targeting to the cell surface in an active form.

Therefore, it is important to understand how mutations such as ΔF508 interfere with the normal folding and maturation of CFTR.

A useful model system for studying folding and maturation of CFTR is the multidrug resistance P-glycoprotein (P-gp). P-gp is an ATP-dependent drug pump located in the plasma membrane. It transports a wide variety of structurally diverse compounds out of the cell (for review, see Ref. 10). P-gp and CFTR are members of the ATP-binding cassette (ABC) family of transporters and are predicted to have similar structures. Both proteins have two NBDs and two transmembrane domains (TMDs), each of which contains six predicted transmembrane (TM) segments. Mutations in P-gp at positions equivalent to those found in CFTRs of CF patients such as deletion of Tyr-490 (equivalent to Phe-508 in CFTR) also cause the mutant P-gps to be retained in the endoplasmic reticulum. The advantage of using P-gp for studying protein misfolding is that the processing defects in P-gp can be corrected by expressing the protein in the presence of drug substrates (chemical chaperones). The “rescued” mutant P-gps are transported to the cell surface in a functional form. Also, the contact points between the various domains have been mapped through disulfide cross-linking analysis. Knowledge about these contact points is essential because they can serve as “reporters” for providing important information about domain-domain interactions.

In this study, we tested whether processing mutations affected interactions between the NBDs.
**EXPERIMENTAL PROCEDURES**

Construction of Mutants—Cys-less P-gp was constructed by changing the endogenous cysteines at postions 137, 431, 517, 956, 1074, 1125, and 1227 to alanines (12) and inserting the cDNA into the mamalian expression vector pMT21 as described previously (21). None of the cysteines is needed for activity because the Cys-less P-gp retained activity (12). The Cys-less P-gp cDNA was also modified to code for 10 histidine residues at the COOH end of the molecule (Cys-less P-gp-His10). The mutants containing an endogenous cysteine in the Walker A sites (Cys-431 in NBD1 or Cys-1074 in NBD2) and a second cysteine in the LSGGQ signature sequence to yield mutant C431(NBD1-Walker A)/L1176C(NBD2-signature) and L531C(NBD1-signature)/C1074Y(NBD2Walker A) were described previously (18). The processing mutation ΔY490 was introducted into mutant C431(NBD1-Walker A)/L1176C(NBD2-signature), whereas processing mutations G268V, P709G, G722A, and A841L were introducted into mutant L531C(NBD1-signature)/C1074Y(NBD2Walker A). The oligonucleotide 5'-GCCACACCCGATAAA-GAAAAATCATCTTPTTGGCGCTGAATAATGTG-3' was used to replace residues 383-396 in P-gp with residues 385-TIKENIFPG-396 of CFTR (P-gp/CFTR chimera) (22). Phe-508 in the P-gp/CFTR chimera was then deleted with oligonucleotide 5'-ATAAAAGAAATATCATCGGCCGTGAAAATGTC-3' (23). The integrity of the mutated cDNA was confirmed by sequencing the entire cDNA (23).

Expression, Purification, and Measurement of Drug-stimulated ATPase Activity—Expression of the cDNAs in HEK 293 cells and purification of histidine-tagged P-gp mutants have been described previously (24). Briefly, 50 10-cm diameter culture plates of HEK 293 cells were transfected with the mutant cDNA, and the medium was replaced 24 h later with fresh medium containing 10 μM cyclosporin A. Cyclosporin A is a substrate of P-gp and acts as a powerful chemical chaperone for promoting maturation of P-gp (14, 25). The transfected cells were then harvested 24 h later, solubilized with 1% (w/v) n-dodecyl β-maltoside, and the mutant P-gp isolated by nickel-chelate chromatography (nicket-nitritriactric acid columns, Qiagen, Inc., Mississauga, ON, Canada). The P-gp-His6 tag mutants were eluted from the nickel column with buffer containing 10 mM Tris-HCl, pH 7.5, 500 mM NaCl, 300 mM imidazole, pH 7.0, 0.1% (w/v) n-dodecyl β-maltoside, and 10% (v/v) glycerol, and mixed with an equal volume of 10 mg/ml crude sheep brain histone (Sigma) that was washed and suspended in 10 mM Tris-HCl, pH 7.5, and 150 mM NaCl. The P-gp-lipid mixture was then sonicated for 45 s at 4°C (bath type probe, maximum setting, Branson Sonifier 450, Branson Ultrasonic, Danbury, CT). An aliquot of the sonicated P-gp-lipid mixture was assayed for drug-stimulated ATPase activity by the addition of an equal volume of buffer containing 100 mM Tris-HCl, pH 7.5, 100 mM NaCl, 20 mM MgCl2, 10 mM ATP, and saturating concentrations of verapamil (1 mM) or colchicine (10 mM). The samples were incubated for 30 min at 37°C, and the amount of inorganic phosphate liberated was determined (26).

Cross-linking Analysis—For cross-linking analysis, HEK 293 cells expressing the mutant P-gp were grown in the presence of 10 μM cyclosporin A. Membranes were prepared from transfected and suspended in Tri-buffered saline (10 mM Tris-HCl, pH 7.4, 150 mM NaCl). Samples were treated with 0.5 mM copper phenanthroline for 15 min at 20°C. The reactions were stopped by the addition of EDTA to a final concentration of 50 mM in SDS sample buffer (0.125% Tris-HCl, pH 6.8, 20% (v/v) glycerol, and 4% (w/v) SDS) that contains no dithiole-reducing agent.

To test for the effect of ATP, membranes were prepared from cells expressing P-gp mutants that were grown in the presence of cyclosporin A and incubated for 10 min at 4°C in the presence or absence of 10 μM ATP and 15 mM MgCl2. The membranes were then subjected to oxidative cross-linking with 0.5 mM copper (phenanthroline), for 5 min at 20°C. The reactions were stopped by the addition of SDS sample buffer containing 50 mM EDTA, and the samples were subjected to immunoblot analysis.

To test for the effect of ATP plus vanadate, orthovanadate was prepared from Na2VO4 and boiled for 2 min to break down polymeric species (27). The mutant membranes were incubated with 0.2 mM vanadate and 10 μM MgCl2, for 10 min at 37°C. Under these conditions, P-gp ATPase activity is inhibited by more than 90% (28). The cross-linked samples were subjected to SDS-PAGE on 7.5% acrylamide gels and immunoblot analysis with rabbit polyclonal antibody (29) followed by enhanced chemiluminescence (Pierce).

**RESULTS**

Effect of the ΔY490 Mutation on Maturation and Activity of P-gp—P-gp is a typical ABC transporter because it is composed of four domains, two TMDs and NBDs that are joined together by a linker region (Fig. 1A). The two halves of the protein show 48% amino acid identity (11). The two halves of P-gp interact specifically and will associate with each other to form a functional complex when the two halves are expressed as separate polypeptides (30). The TMD1-TMD2 and NBD1-NBD2 interfaces are important because they form the common drug binding pocket (31) and ATP binding sites, respectively (18).

Crystal structure studies of bacterial ABC transporters (32, 33) and cross-linking analysis of P-gp (18) show that the two ATP molecules bind at sites located between the Walker A site of one NBD and the LSGGQ signature sequence of the second NBD. In P-gp, the Walker A sites and the LSGGQ signature sequences of the two NBDs are quite close together because cysteines introduced into these sites can be directly cross-linked with oxidant (Fig. 1B).

The ability to cross-link a cysteine in the Walker A site of one NBD with a cysteine in the LSGGQ signature sequence of the other NBD serves as a useful reporter molecule for monitoring the interactions between the NBDs. Accordingly, we tested whether the presence of processing mutations affected cross-linking between NBD1 and NBD2. Processing mutations cause the protein to be retained in the endoplasmic reticulum as a core-glycosylated 150-kDa protein that is degraded rapidly (34). The rationale for this approach was that by knowing the contact points in the functional P-gp, we could determine whether mutations that cause protein misfolding interfere with the establishment of proper domain-domain interactions. The first processing mutant tested was ΔY490 in NBD1 (Fig. 1A). Residue Tyr-490 of P-gp is equivalent to Phe-508 in CFTR (13). Therefore, residue Tyr-490 was deleted in mutant C431(NBD1-Walker A)/L1176C(NBD2-signature). The mutant was expressed in HEK 293 cells and expression detected by immunoblot analysis. Fig. 2A shows that in the absence of drug substrate, the deleted Tyr-490 residue inhibited maturation of P-gp such that the major product was a 150-kDa protein. Mat-
were incubated for 24 h in the presence of E. Walker A/L1176C(NBD2-signature) cDNAs. F508)/C431(NBD1-Walker A)/L1176C(NBD2-signature) – cells were transfected with mutant P-gp/H9004 cross-linking between C431(NBD1) and L1176C(NBD2).

50 mM EDTA. The samples were then treated with endoglycosidase F reactions were stopped by the addition of SDS sample buffer containing 0.5 mM cyclosporin A and then reacted with were prepared from transfected cells treated with (or without (Cyclo) or without (Cyclo) cyclosporin A) and then reacted with (+) or without (−). The reactions were stopped by the addition of 50 mM EDTA, and the samples were subjected to immunoblot analysis. B, membranes were prepared from transfected cells grown in the presence of cyclosporin A. Equivalent amounts of whole cell SDS extracts were subjected to immunoblot analysis with rabbit polyclonal antibody against P-gp followed by enhanced chemiluminescence. B, membranes were prepared from transfected cells treated with (+ CyClo) or without (−) cyclosporin A and then reacted with (+) or without (−) 0.5 mM copper phenanthroline (CuP) for 10 min at 20 ºC. The reactions were stopped by the addition of SDS sample buffer containing 50 mM EDTA, and the samples were subjected to immunoblot analysis. C, membranes from transfected cells grown in the presence of cyclosporin A were preincubated for 10 min at 37 ºC with (or without (−) ATP plus vanadate. The samples were then treated with (+) or without (−) 0.5 mM copper phenanthroline for 10 min at 20 ºC. The reactions were stopped by the addition of SDS sample buffer containing 50 mM EDTA. D, membranes were prepared from transfected cells treated with (+ CyClo) or without (− CyClo) cyclosporin A and then reacted with (+) or without (−) 0.5 mM copper phenanthroline for 10 min at 20 ºC. The reactions were stopped by the addition of SDS sample buffer containing 50 mM EDTA. The samples were then treated with endoglycosidase F (Endo F) (New England Biolabs) for 15 min at 37 ºC and subjected to immunoblot analysis. E, membranes prepared from transfected cells grown with (+ CyClo) or without (− CyClo) cyclosporin A were reacted with (+) or without (−) 0.5 mM copper phenanthroline for 10 min at 20 ºC. The reactions were stopped by the addition of SDS sample buffer containing 50 mM EDTA, and the samples were subjected to immunoblot analysis. D and E, the positions of cross-linked (X-link), mature (170 kDa), immature (150 kDa), cross-linked deglycosylated (X-link, CHO), and deglycosylated (140 kDa) P-gp are shown.

urination of the mutant 150-kDa protein to the mature 170-kDa protein was restored when expression was carried out in the presence of the drug substrate cyclosporin A (Fig. 2A). We then determined whether mutant Y490/C431(NBD1-Walker A)/L1176C(NBD2-signature) retained drug-stimulated ATPase activity. The histidine-tagged mutant was grown in the presence of cyclosporin A, isolated by nickel-chelate chromatography, mixed with lipid, and assayed for verapamil-stimulated ATPase activity. The mutant retained 82 ± 8% of the verapamil-stimulated ATPase activity of that of mutant C431(NBD1-Walker A)/L1176C(NBD2-signature) (data not shown). Cross-linking analysis was then done on the misfolded and rescued forms of mutant P-gp. Membranes were prepared from cells expressing mutant ΔY490/C431(NBD1-Walker A)/L1176C(NBD2-signature) that had been grown with or without 10 µM cyclosporin A and subjected to cross-linking with copper phenanthroline. Fig. 2B, right panel, shows that cross-linked product was detected only after expression of the mutant in the presence of cyclosporin A. By contrast, no cross-linked product was detected in membranes from cells that were not treated with drug substrate (Fig. 2B, left panel). To determine whether the mutant could still trap nucleotide in the presence of vanadate, the membranes were treated with ATP plus vanadate and then subjected to oxidative cross-linking. Fig. 2C shows that vanadate trapping of nucleotide inhibited cross-linking of the mutant. It was possible, however, that the immature P-gp may have been cross-linked but did not show a shift in electrophoretic mobility because of limited glycosylation of the immature product. To rule out this possibility, membranes were prepared from cells expressing mutant C431(NBD1-Walker A)/L1176C(NBD2-signature) that were grown in the presence of cyclosporin A, cross-linked, and then treated with endoglycosidase F to remove the carbohydrate groups. Treatment of mature cross-linked mutant ΔY490/C431(NBD1-Walker A)/L1176C(NBD2-signature) with endoglycosidase F still resulted in a relatively slow migrating cross-linked product (Fig. 2D, right panel). Treatment of the cross-linked, deglycosylated mutant P-gp with dithiothreitol caused the cross-linked product to disappear and increased the amount of the 140-kDa protein (data not shown). Therefore, the inability to detect a slower migrating form of P-gp after treatment of the immature 150-kDa protein with oxidant was not caused by differences in the amount of glycosylation. These results show that the interface between the two NBDs in the misfolded mutant ΔY490/C431(NBD1-Walker A)/L1176C(NBD2-signature) is structurally different from the mature 170-kDa P-gp.

The crystal structure of NBD1 of CFTR (35) predicts that Tyr-490 in P-gp or Phe-508 in CFTR can be aligned in the third α-helix. Accordingly, we constructed a P-gp/CFTR chimera where the segment forming the third α-helical structure in P-gp (383-399) in NBD1 of P-gp was replaced with the corresponding α-helical region (501-517) from CFTR. Residue Phe-508 in the P-gp/CFTR chimera was then deleted and the resulting mutation introduced into the C431(NBD1-Walker A)/L1176C(NBD2-signature) mutant to give mutant P-gp/CFTR(ΔF508)/C431(NBD1-Walker A)/L1176C(NBD2-signature). Mutant P-gp/CFTR(ΔF508) was expressed in HEK 293 cells in the presence or absence of cyclosporin A. Membranes were then prepared, treated with or without oxidant (copper phenanthroline), and subjected to immunoblot analysis. Fig. 2E shows that cross-linked product was detected only when the P-gp/CFTR chimeric protein was expressed in the presence of cyclosporin A.

Effect of Other Processing Mutations on Cross-linking of NBD Cysteine Mutants—It is possible that disruption of NBD1-NBD2 interactions by ΔY490 may be a local effect because it is in the same domain (NBD1) as one of the reporter cysteines (Cys-431).

Therefore, we tested whether other processing mutations that are located in other domains affected NBD1-NBD2 interactions. We have shown that mutations G268V in the second cytoplasmic loop (ICL2) connecting TMs 4 and 5 (36), P709G in the linker region connecting the two halves of the molecule (37), G722A in TM7 (38) and A841L in TM9 (this study) caused misprocessing of wild-type P-gp. These mutations were then
introduced into a mutant with different contact points at the NBD1-NBD2 interface (mutant L531C(NBD1-signature)/C1074(NBD2-Walker A)) (18). The mutants were expressed in HEK 293 cells in the presence or absence of cyclosporin A. Equivalent amounts of whole cell SDS extracts were subjected to SDS-PAGE and immunoblot analysis. Fig. 3 (upper panel) shows that introduction of the processing mutations, G268V (ICL2), P709G(linker), G722A(TM7), or A841L(TM9) into P-gp mutant L531C(NBD1-signature)/C1074(NBD2-Walker A) resulted in the expression of only immature core-glycosylated (150-kDa) protein when expressed in plain media (Fig. 3, upper panel). Expression of the mutant proteins in the presence of drug substrate cyclosporin A, however, resulted in the appearance of the mature 170-kDa P-gp (Fig. 3, lower panel).

We then tested whether mutants G268VICL2/L531C(NBD1-signature)/C1074(NBD2-Walker A), P709G(linker)/L531C(NBD1-signature)/C1074(NBD2-Walker A), G722A(TM7)/L531C(NBD1-signature)/C1074(NBD2-Walker A), and A841L(TM9)/L531C(NBD1-signature)/C1074(NBD2-Walker A), which were rescued with cyclosporin A, were active by measuring drug-stimulated ATPase activity. The histidine-tagged mutants were expressed in HEK 293 cells and isolated by nickel-chelate chromatography (24). Equivalent amounts of the isolated proteins were mixed with lipid, sonicated, and ATPase activity measured in the presence of a saturating concentration of verapamil (1 mM) or colchicine (10 mM). The activities are expressed relative to mutant L531C(NBD1-signature)/C1074(NBD2-Walker A) (None), which contained no processing mutation. Each value is the average of two separate measurements.

Another method to determine whether there are proper interactions between the NBDs is to test for the effect of vanadate trapping of nucleotide at the interface between the NBDs. Vanadate traps ADP at one of the two NBDs by occupying the position of the γ-phosphate adjacent to ADP. Vanadate trapping at one site inhibits ATP hydrolysis at the second site (41). Accordingly, we examined the effect of vanadate trapping of nucleotide on cross-linking of mutants G268VICL2/L531C(NBD1-signature)/C1074(NBD2-Walker A), P709G(linker)/L531C(NBD1-signature)/C1074(NBD2-Walker A), and A841L(TM9)/L531C(NBD1-signature)/C1074(NBD2-Walker A). Membranes were prepared from cells expressing the mutants that had been grown in the presence or absence of cyclosporin A. The membranes were subjected to oxidative cross-linking with copper phenanthroline followed by immunoblot analysis. Fig. 5A, lower panel, shows that cross-linked product was detected in all of the mutants that were grown with cyclosporin A. Treatment of the cross-linked sample with the reducing agent dithiothreitol resulted in the disappearance of the cross-linked product (data not shown). No cross-linked product was detected in these mutants when grown in the presence of plain media (Fig. 5A, upper panel).
linking of the mature (cyclosporin A-treated) mutants. Vanadate trapping of nucleotide had no effect on the immature form of P-gp (expressed without cyclosporin A) because no cross-linked product was detected (Fig. 5A, upper panel). To determine whether the mutants retained the ability to interact with ATP, the membranes were preincubated with ATP and MgCl₂ and then subjected to oxidative cross-linking. Fig. 5B shows that amount of cross-linked product in each mutant was reduced in the presence of ATP, indicating that the mutants could still interact with ATP.

Cross-linking between thiol groups by copper phenanthroline is very efficient if the β-carbons are a maximal distance of 7 Å apart, with the average being 5–6 Å (42). It was possible that the cysteines in the misprocessed mutants were not cross-linked with copper phenanthroline because the cysteines were too far apart or were buried within the altered NBDs such that they were not exposed to the aqueous environment. To test whether they were far apart but accessible, we tested for cross-linking with homobifunctional thiol-reactive cross-linkers. Alkylthiosulfonate cross-linkers are useful because they are generally more reactive with cysteine than other thiol-reactive compounds such as maleimides (43). Mutant ΔY490/C431(NBD1-Walker A)/Y490/C431(NBD1-Walker A) was expressed in HEK 293 cells in the absence of the chemical chaperone cyclosporin A. Membranes were prepared, and samples were treated with relatively short (1,3-propanediyl bis-methanethiosulfonate (M3M, 6.5 Å) and long cross-linkers (3,6-dioxaoctane-1,8-diyl bismethanethiosulfonate (M8M, 13 Å)) (16). The samples were subjected to immunoblot analysis. There was no shift in the electrophoretic mobility of the 150-kDa core-glycosylated protein when the mutant was treated with M3M (data not shown). Treatment of the mutant with 25–100 μM M8M, however, resulted in the appearance of a protein that migrated with lower electrophoretic mobility than the 150-kDa protein in SDS-PAGE (Fig. 6A). To test whether cross-linking by M8M was inhibited by vanadate trapping of nucleotide, the membranes were preincubated with ATP plus vanadate for 10 min at 37 °C, cross-linked with 25 μM M8M, followed by immunoblot analysis. Fig. 6B shows that the amount of cross-linked product was not decreased after treatment with ATP plus vanadate. Similar results were observed when mutants G268V(ICL2)/L531C(NBD1-signature)/C1074(NBD2-Walker A), P709G(linker)/L531C(NBD1-signature)/C1074(NBD2-Walker A), G268V(ICL2)/L531C(NBD1-signature)/C1074(NBD2-Walker A), and G268V(ICL2)/L531C(NBD1-signature)/C1074(NBD2-Walker A) were grown without cyclosporin A and treated with either M3M or M8M (data not shown). These results suggest that the cysteines are still exposed in the misprocessed mutants but that the NBDs show inefficient vanadate trapping of nucleotide.

**DISCUSSION**

The inability to cross-link cysteines in the Walker A of one NBD with another cysteine in the LSGGQ signature sequence of the second NBD in the ΔY490 mutant (Fig. 2) suggests that the orientation at the NBD interface is altered. The misalignment between the NBDs appeared to persist even when mutations outside of the NBDs (G268V(ICL2), P709G(linker), G722A(TM7), and A841L(TM9)) were introduced into P-gp (Fig. 5). The incorrect orientation of the NBDs may be one reason why the immature 150-kDa form of P-gp does not exhibit any detectable drug-stimulated ATPase activity (44). It has been shown that the orientations of the Walker A in one NBD relative to the LSGGQ signature sequence in the second NBD are critical for coupling of drug binding to ATP hydrolysis. Substrates such as calcein-AM, demecolcine, cis(2)-flupenthixol, and verapamil which stimulate ATPase activity of P-gp increase the rate of cross-linking between cysteines in the Walker A and LSGGQ signature sequences (39). By contrast, substrates such as trans-(E)-flupenthixol and Hoechst 33342, etc.
which inhibit ATPase activity, prevent cross-linking between the these sites (39). Attempts to restore cross-linking of the immature 150-kDa mutant P-gp by including stimulatory substrates (verapamil) during cross-linking did not result in the appearance of cross-linked product in SDS-PAGE (data not shown). The lack of cross-linking even in the presence of verapamil is consistent with the finding that introduction of processing mutations into P-gp also disrupts packing of the TM segments, thereby disrupting the common drug binding pocket at the TMD interface (17).

The activities of misprocessed P-gp mutants G268V(ICL2), P709G(linker), G722A(TM7), and A841L(TM9) were restored when the mutants were grown in the presence of the drug substrate cyclosporin A (Fig. 5). All mutants, except for mutant A841L(TM9) retained more than 90% of their verapamil- and colchicine-stimulated ATPase activities compared with that of mutant L531C/NBD1-signature)/C1074(NBD2-Walker A). The verapamil-stimulated ATPase activity of mutant A841L(TM9) was unaffected, but it only retained about 30% of the colchicine-stimulated ATPase activity. A mutation in TM9 affecting drug-stimulated ATPase activity would be consistent with the findings that this residue contributes to the common drug binding pocket (31) and may be important for the binding of colchicine but not verapamil.

Rescuing misprocessed mutants with cyclosporin A also restored the ability of cysteines in the Walker A and LSGGQ signature sequences to be cross-linked with oxidant. The drug binding pocket between the TMDs likely forms cotranslationally and exists transiently during synthesis of the misprocessed mutants. The presence of drug substrate (specific chemical chaperone) during synthesis likely acts as a scaffold to stabilize the TMDs. This may allow the mutant protein to overcome folding barriers such as inducing long range conformational changes in the NBDs for the formation of proper contacts during the folding. These barriers may not be present in the folding of wild-type enzyme. In the absence of drug substrate, the drug binding pocket may collapse as a result of improper contacts among the TMD and NBD domains. This may explain why drug substrates cannot rescue the misfolded mutants once they have been synthesized (data not shown). Interactions among the four domains of wild-type P-gp are shown as a model in Fig. 7A, in which they interact correctly to allow the protein to mature and form an active enzyme. It appears that the individual domains of ABC transporters can fold properly because the structure of NBD1 of CFTR resembles the structure of a complete ABC transporter such as MsA (45) and BtuCD (46). The individual domains of ABC transporters may undergo cotranslational protein folding that is postulated to occur during folding of eukaryotic protein (47, 48). The next step would involve superfolding of the individual domains to form a functional protein (25). The presence of a processing mutation, however, interferes with the ability of the various domains to form correct contacts (Fig. 7B) resulting in a “loosely folded” immature 150-kDa P-gp that does not undergo further processing. This conformation is about 100-fold more sensitive to proteases than the “compact” conformation observed with wild-type P-gp (25). The presence of drug substrate during synthesis of the misprocessed mutant stabilizes the drug binding pocket and increases the chance for proper contacts to form between the TMDs and NBDs and between the NBDs, resulting in a properly folded molecule (Fig. 7C).

![Image](https://i.imgur.com/352x496-528x737.png)

**Fig. 7. Model of domain interactions in P-gp.** A, wild-type P-gp shows efficient maturation because the four domains interact properly during protein folding. B, the presence of a processing mutation (X) prevents protein folding by inhibiting proper interactions between the TMDs (17) and the NBDs. C, the presence of a drug substrate (chemical chaperone) (gray rectangles) during folding, however, promoted correct interactions among the domains. The letters A and S (in white) represent positions of the Walker A and signature sequences, respectively.

In summary, the presence of processing mutations interferes with interactions between the TMDs, between TMDs and NBDs (17), and between the NBDs. The TMDs are the most feasible target for inducing proper folding of ABC transporters because drug substrates rather than the presence of ATP are important for promoting proper folding and maturation of P-gp mutants. This is consistent with the finding that interaction of drug substrates with the TMDs alone was sufficient to promote proper folding and trafficking of the protein (50). Therefore, the results show that the TMDs are the most feasible targets for the search of compounds that can induce proper folding of misprocessed ABC transporters such as $\Delta F508$-CFTR.

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