Introduction of the Most Common Cystic Fibrosis Mutation (ΔF508) into Human P-glycoprotein Disrupts Packing of the Transmembrane Segments*

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The most common mutation in cystic fibrosis (deletion of phenylalanine 508 (ΔF508) in the cystic fibrosis conductance transmembrane regulator (CFTR) gene) causes defective synthesis of CFTR protein. To understand how this deletion interferes with protein folding, we made the equivalent deletion (ΔY490) in P-glycoprotein (P-gp). A Cys-less P-gp with cysteines in transmembrane (TM) 4 or TM5 can be cross-linked with a cysteine in TM12. Deleting Tyr490 in P-gp resulted in an inactive and defectively processed mutant in which no cross-linking between TM4 or TM5 and TM12 was detected. Expression of the ΔY490 mutant in the presence of a chemical chaperone corrected the processing defect and yielded active P-gp mutants that could be cross-linked between TM4 or TM5 and TM12. Cross-linking between TM4 or TM5 and TM12 was also detected when residues 483TIAENIRYG491 in P-gp were replaced with residues 501TIKENIIFG509 from CFTR (P-gp/CFT). Deleting Phe508 in the P-gp/CFT chimera, however, caused defective processing of the mutant protein and no detectable cross-linking between TM4 or TM5 and TM12. The processing defect was corrected with a chemical chaperone and yielded active P-gp/CFT mutant proteins that could be cross-linked. These results show that deletion at residue 490 disrupts packing of the TM segments possibly by affecting interaction between the first nucleotide-binding domain (Tyr490) and the first cytoplasmic loop (Glu184).

Cystic fibrosis (CF)1 is a lethal inherited disorder caused by mutations in the gene coding for the cystic fibrosis transmembrane conductance regulator (CFTR) protein. CFTR is a cAMP-dependent chloride channel located at the surface of epithelial cells and regulates salt and fluid transport across the cell membrane (1, 2). The most common CF-associated mutation (found on at least one chromosome of 90% of affected individuals) is deletion of phenylalanine 508 (ΔF508) (3). The defective ΔF508 CFTR mutant protein is not targeted correctly to the apical membrane and is retained in the endoplasmic reticulum in an inactive form (4) and rapidly degraded (5–7).

Folding and trafficking defects also occur in the sister protein of CFTR, P-glycoprotein (P-gp). P-gp and CFTR have similar structures, and both are members of the ATP-binding cassette (ABC) superfamily of transporters (8). P-gp is an ATP-dependent drug pump in the plasma membrane and transports a wide variety of structurally diverse compounds out of the cell (for review, see Ref. 9). The 1280 amino acids of P-gp are organized in two repeating units of 610 amino acids that are joined by a linker region of about 60 amino acids (10). Each repeat has six transmembrane (TM) segments and a hydrophilic domain containing an ATP-binding site (11, 12). Mutations in P-gp at positions equivalent to those found in CFTRs of CF patients (such as deletion of Tyr490 or Phe508 in CFTR) also cause the mutant P-gps to be retained in the endoplasmic reticulum (13).

The observation that folding defects in misprocessed P-gp mutants can be corrected by drug substrates (14) and that the transmembrane domains (TMDs) can bind substrates in the absence of the nucleotide-binding domains (NBDs) (15) indicates that mutations in the NBDs may disrupt packing of the TMDs. In this study, we used disulfide cross-linking analysis to determine whether the “ΔF508-CFTR-like” mutations affect TM packing.

EXPERIMENTAL PROCEDURES

Construction of Mutants—Histidine-tagged wild-type, ΔY490 P-gp, and Cys-less P-gp cDNAs were inserted into the mammalian expression vector pMT21 as described previously (11, 14, 16). Cysteine residues were put back into the Cys-less P-gp/His, as needed. Construction of mutants Δ235C(TM4)/S993C(TM12), Δ225C(TM4)/S993C(TM12), L236C(TM4)/S993C(TM12), and S999C(TM5)/S999C(TM12) were described previously (17). The oligonucleotide 5′-GCCACACGACATAAAAGAAAATATCATCGGCCGTGAAAATGTC-3′ was used to replace residues 483TIAENIRYG491 in P-gp with residues 501TIKENIIFG509 from CFTR (P-gp/CFT). Deleting Phe508 in the P-gp/CFT chimera, however, caused defective processing of the mutant protein and no detectable cross-linking between TM4 or TM5 and TM12. The processing defect was corrected with a chemical chaperone and yielded active P-gp/CFT mutant proteins that could be cross-linked. These results show that deletion at residue 490 disrupts packing of the TM segments possibly by affecting interaction between the first nucleotide-binding domain (Tyr490) and the first cytoplasmic loop (Glu184).

RESULTS

Residue Tyr490 of P-gp is located in NBD1, and its position is equivalent to Phe508 in CFTR (13). Therefore, the ΔY490 P-gp (P-gp(ΔY490)) mutant was constructed. The crystal structures

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‡ The abbreviations used are: CF, cystic fibrosis; CFTR, cystic fibrosis conductance transmembrane regulator; TM, transmembrane; TMD, transmembrane domain; TMD1, NH2-terminal TMD; TMD2, COOH-terminal TMD; NBD, nucleotide-binding domain; NBD1, NH2-terminal NBD; NBD2, COOH-terminal NBD; P-gp, P-glycoprotein; ABC, ATP-binding cassette; HEK, human embryonic kidney.

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of ABC transporters HisP, MalK, TAP1, MJ1267, MJ0796, BtuCD, and Rad50 show that the residues that can be aligned with Tyr490 in P-gp or Phe508 in CFTR are within the third α-helix of the NBD (21–27). Accordingly, we also constructed a P-gp/CFTR chimera in which the segment forming the α-helical structure in P-gp (458TIAENIRYG491) in NBD1 of P-gp was replaced with the corresponding α-helical region (501TIKENI-FIG509) in CFTR (28, 29). The Phe508 in this chimera was then deleted to generate a P-gp/CFTR(AF508) chimera. The mutants were transiently expressed in HEK 293 cells in the presence or absence of the drug substrate cyclosporin A. Cyclosporin A acts a potent chemical chaperone and induces maturation of any protein that can be made to fold into an active conformation if synthesized in the presence of a drug substrate. Since the drug-binding domain is located in the TMDs (15, 31, 32), it is possible that the presence of the deletion mutations affects the proper packing of the TM segments. To test this possibility, we examined TM packing by cross-linking analysis. We had previously shown that four pairs of cysteine residues in TM4, TM5, and TM12, A233C(TM4)/S993C(TM12), L236C(TM4)/S993C(TM12), and I299C(TM5)/S993C(TM12), were cross-linked at 4 °C with copper phenanthroline (17). These four pairs of cysteines could then serve as markers for detecting the proper folding of the TM segments in the deletion mutants. Accordingly, the P-gp(AF490) and the P-gp/CFTR(AF508) mutations were introduced into the cDNAs of these cysteine marker mutants. The mutants were expressed in the presence or absence of 10 μM cyclosporin A, and the membranes prepared from the transfected cells were treated with 0.2 mM copper phenanthroline at 4 °C for 0–16 min. The cross-linked membranes were subjected to SDS-PAGE and immunoblot analysis to detect the presence of cross-linked product. The cross-linked product migrates with lower mobility on SDS-PAGE (33, 34). Fig. 2 shows that when these mutants were expressed in the absence of substrate, the major product was the immature 150-kDa protein in which no cross-linking was detected (Fig. 2, No Cyclo A). By contrast, when these mutants were expressed in the presence of cyclosporin A, the major product was the mature 170-kDa protein, which could be cross-linked in a time-dependent manner (Fig. 2, +Cyclo A). Similar results were obtained with the P-gp(AF490) cysteine mutants (data not shown). It is interesting to note that cross-linked product was detected with all four markers, indicating that cyclosporin A was able to induce folding of the TM segments into a native conformation. How the deletion mutations in the NBD affect packing of the TM segments is not clear. It is possible that Tyr490 in P-gp (or Phe508 in CFTR) is within or close to a region in the NBD that can interact with the intracellular loops connecting the TM segments. One possibility is that NBD1 interacts with TMD1 since it was shown that these two domains inter-
act when they are expressed as separate polypeptides (35). The first intracellular loop connecting TM2 and TM3 is a potential site of interaction since it is a relatively large loop (49 amino acids, residues 140–188), and mutations within this loop are known to affect activity of P-gp (36, 37). Accordingly, we made 49 double cysteine mutants containing a cysteine at position 490 and another at each of the 49 positions predicted to be the first intracellular loop. The mutants were then expressed in HEK 293 cells in the presence of cyclosporin A, and the membranes were treated with oxidant before or after treatment with ATP or ATP plus vanadate. Cross-linking in the absence of vanadate was done at 37 °C rather than 4 °C because molecular motion might make it easier to form cross-links. Vanadate trapping of nucleotide locks the protein in the transition state (38) and can alter the conformational state of P-gp (39). For most of the mutants (e.g. N183C/Y490C), no cross-linked product was detected when cross-linking was done at 37 °C (Fig. 3). Cross-linked product was observed, however, in mutant E184C/Y490C (Fig. 3). Cross-linking was enhanced with vanadate trapping. No cross-linked product was detected with either Y409C or E184C (data not shown). These results indicate that Tyr490 is in a region that is close or comes close to the first intracellular loop during the catalytic cycle.

**DISCUSSION**

The P-gp(ΔY490) and P-gp(CFTR)(ΔF508) mutants are expressed only as the immature proteins and do not exhibit any detectable activity. Expression in the presence of drug substrate cyclosporin A (chemical chaperone), however, induced maturation of the P-gp(ΔY490) and P-gp(CFTR)(ΔF508) mutants. The mature mutant proteins had activity characteristics similar to the parent protein. The characteristics of mutants P-gp(ΔY490) and P-gp(CFTR)(ΔF508) are quite similar to those seen in CFTR with deletion of Phe 508 (CFTR(ΔF508)). Immature CFTR also exhibits no activity because of its altered structure (4). Inducing maturation of CFTR(ΔF508) by growth at lower temperatures (40) or in the presence of glycerol (41), however, correctly targets the protein to the membrane and restores chloride channel activity.

Studies on short peptides containing Phe508 show this region has a propensity to form an α-helix (42, 43). Recent crystal structure studies on bacterial ABC transporters HisP and BtuCD have aligned regions corresponding to Phe508 in CFTR or Tyr490 in P-gp and predicted them to be within a short α-helix. Similarly, the "nnpredict" program developed by Kneller et al. (Ref. 44, and available at www.cmpharm.ucsf.edu/~nomi/nnpredict-instrucs.html) predicts Tyr490 in P-gp to be at the end of a short helical (7–9 residues) region. Deletion of a residue in a short α-helix or truncations may cause destabilization of the helix (45). Also, helical regions containing phenylalanine or tyrosine may be stabilized by side-chain interactions (46). Indeed, solution structure studies on peptides based on the Phe508 region of CFTR show that deletion of this residue destabilizes the helix (43).

**TABLE I**

**Drug-stimulated ATPase activities of mutants**

| Mutant                  | $K_{0.5}$ for ATP | Verapamil stimulation | Vinblastine stimulation |
|-------------------------|------------------|-----------------------|------------------------|
|                         | mM               | μM                    | μM                     |
| P-gp                    | 1.1              | 24                    | 5.6                    |
| P-gp(CFTR)              | 1.1              | 26                    | 6.0                    |
| P-gp(ΔY490)             | 1.2              | 26                    | 5.9                    |
| P-gp(CFTR)(ΔF508)       | 1.1              | 27                    | 5.8                    |

*a* ATPase activity measured using 0.8 mM verapamil.

*b* Drug-stimulated ATPase activity obtained with 10 mM ATP.

*c* -Fold stimulation is the ratio of activity with 0.8 mM verapamil or 30 μM vinblastine to that without drug.
While the deletions in the NBDs can cause local disruption of structures, the results in this study show that these localized perturbations can have distal effects such as disruption of packing of the TM domains. It is likely that domain-domain interactions in ABC transporters are critical during folding of the native molecule. Two observations support this idea. First, we show in this study with P-gp that deletion of a residue in NBD1 disrupted TM packing. No cross-product was observed between TM4 or TM5 and TM12 when the mutants were expressed in the absence of drug substrate. Interactions between TM4 and TM5 with TM12 appear to be especially important since these three TM segments form part of the drug-binding site (31, 32, 47, 48). Second, a P-gp deletion mutant missing the NBDs also does not fold properly (15). In both cases, however, the correct TM packing was restored when the mutants were expressed in the presence of a drug substrate (this study and Ref. 15).

The presence of drug substrate during folding of a processing mutant allows the mutant protein to overcome a folding barrier (“hurdle”) that is not present during folding of the native enzyme. The hurdle in the processing mutants appears to be the interaction between TM segments in TMD1 and TMD2. When the TM segments in TMD1 and TMD2 are allowed to interact, the mutant protein proceeds to fold normally (15). These interactions are outlined in a model in Fig. 4. In the folding of native enzyme (Fig. 4A), there are interactions between the NBDs, between the TM segments, and between the NBDs and the TM domains. The presence of a deletion mutation in NBD1 disrupts its structure and causes the TM domains to be far apart and also prevents NBD1 from interacting with TMD1 (Fig. 4B). NBD1 may interact with TMD1 through the first intracellular loop. This is supported by some evidence from the crystal structure of the bacterial ABC transporter BtuCD in which the region predicted to correspond to Phe508 in CFTR or Tyr490 in P-gp may interact with the first intracellular loop (27). In our study we show that the first intracellular loop must be close to NBD1 especially after vanadate trapping (Fig. 3). Vanadate trapping must lock the molecule in a more rigid conformation thereby allowing residues Tyr490/Glu184 to be in contact for a longer period. In Fig. 4C, the presence of drug substrate (chemical chaperone) allows the TM domains to pack properly and likely forces the NBD1 to fold properly. It is also possible that correct packing of the TM domains may restrict the perturbation caused by deletion of Tyr490 in P-gp (or Phe508 in CFTR) to be localized within NBD1. The fact that proper packing of the TM segments can correct the folding defect due to a mutation in NBD1 shows that there is “cross-talk” between the TM domains and the NBDs. In wild-type P-gp, the NBDs must be proper for proper folding of the TM segments since a P-gp deletion mutant (lacking the NBDs) is not folded properly and is not trafficked to the cell surface (15). The requirement for the NBDs for proper folding, however, is bypassed when the deletion mutant is expressed in the presence of drug substrate (15).

In summary, there is significant cross-talk between the cytoplasmic (NBD) and transmembrane domains during folding of ABC transporters. Deletion of residue 490 disrupts packing of the TM segments. A similar mechanism for disrupting pack-