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Modulating the Folding of P-Glycoprotein and Cystic Fibrosis Transmembrane Conductance Regulator Truncation Mutants with Pharmacological Chaperones

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ABSTRACT

Cystic fibrosis transmembrane conductance regulator (CFTR) and P-glycoprotein (P-gp) are ATP-binding cassette (ABC) transporters that have two transmembrane domains (TMDs) and two nucleotide-binding domains (NBDs). Defective folding of CFTR lacking phenylalanine 508 (ΔPhe508) in NBD1 is the most common cause of cystic fibrosis. The Phe508 position seems to be universally important in ABC transporters because deletion of the equivalent residue (Tyr490) in P-gp also inhibits maturation of the protein. The pharmacological chaperone VRT-325 can repair the ΔPhe508-type folding defects in P-gp or CFTR. VRT-325 may repair the folding defects by promoting dimerization of the two NBDs or by promoting folding of the TMDs. To distinguish between these two mechanisms, we tested the ability of VRT-325 to promote folding of truncation mutants lacking one or both NBDs. Sensitivity to glycosidases was used as an indirect indicator of folding. It was found that VRT-325 could promote maturation of truncation mutants lacking NBD2. Truncation mutants of CFTR or P-gp lacking both NBDs showed deficiencies in core-glycosylation that could be partially reversed by carrying out expression in the presence of VRT-325. The results show that dimerization of the two NBDs to form a "nucleotide-sandwich" structure or NBD interactions with the TMDs are not essential for VRT-325 enhancement of folding. Instead, VRT-325 can promote folding of the TMDs alone. The ability of VRT-325 to promote core-glycosylation of the NBD-less truncation mutants suggests that one mechanism whereby the compound enhances folding is by promoting proper insertion of TM segments attached to the glycosylated loops so that they adopt an orientation favorable for glycosylation.

Cystic fibrosis (CF) is a lethal inherited disorder caused by mutations in the gene coding for the cystic fibrosis transmembrane conductance regulator (CFTR) (Riordan et al., 1989). CFTR is a cAMP-activated chloride channel located on the apical surface of epithelial cells, where it helps to regulate salt and fluid transport. The lack of functional CFTR in the lung is currently the most serious problem for patients with CF because it contributes to lung failure as a result of long-term inflammation. The most common CF-associated mutation that is found on at least one chromosome of approximately 90% of affected individuals is the deletion of phenylalanine 508 in the first nucleotide-binding domain (NBD1) of CFTR (Bobadilla et al., 2002). Little of the ΔPhe508 CFTR protein is properly delivered to the cell surface because the protein is defective in folding (Chen et al., 2000) and the protein is rapidly degraded (Cheng et al., 1990). The Phe508 position is also important for folding of other ATP-binding cassette transporters as deletion of the equivalent residue in human P-glycoprotein (P-gp) (ΔTyr490) also causes defective folding of P-gp and rapid degradation (Hoof et al., 1994).

A clinical goal in CF research is to develop a drug rescue approach to specifically rescue CFTR mutants with folding defects such as ΔPhe508 CFTR. The feasibility of a drug-rescue approach was first demonstrated using the ΔTyr490 P-gp model system (Loo and Clarke, 1997). Expression of ΔTyr490 P-gp in the presence of drug substrates was found to promote maturation of the mutant to yield a functional transporter at the cell surface. Therefore, high-throughput screening of chemical libraries was performed to identify correctors (pharmacological chaperones) that could promote folding of ΔPhe508 CFTR (Pedemonte et al., 2005; Van Goor et al.,

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ABBREVIATIONS: CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; P-gp, P-glycoprotein; TM, transmembrane; TMD, transmembrane domain; NBD, nucleotide-binding domain; HEK, human embryonic kidney; DMSO, dimethyl sulfoxide; PAGE, polyacrylamide gel electrophoresis; E-64, *N*-(*trans*-epoxysuccinyl)-L-leucine 4-guanidinobutylamide; PNGase F, peptide *N*-glycosidase F; M14M, 3,6,9,12-tetraoxatetradecane-1,14-diyl bismethanethiosulfonate; VRT-532, 4-methyl-2-(5-phenyl-1*H*-pyrazol-3-yl)-phenol.

2006). Some correctors were relatively specific for rescue of ΔPhe508 CFTR. For example, corr-4a is a bisaminomethyl bithiazole compound that promotes maturation of ΔPhe508 CFTR but shows little rescue of a mutant dopamine receptor 4 (Pedemonte et al., 2005), whereas the thiazole derivative corr-2b and the pyrazole derivative VRT-532 also promote maturation of ΔPhe508 CFTR but show little or no rescue of a P-gp processing mutant (Wang et al., 2006). An interesting corrector is the quinazoline derivative VRT-325 that can promote maturation of both P-gp and CFTR processing mutants (Loo et al., 2005).

The mechanism of how correctors like VRT-325 promote folding of CFTR and P-gp processing mutants is unknown. One possibility is that correctors interact with the NBDs to promote native interactions between NBD1 and NBD2 so that they form the characteristic "nucleotide-sandwich dimer" structure of ATP-binding cassette transporters (Smith et al., 2002). Although the ΔPhe508 mutation has little effect on the structure, stability, or folding of NBD1 (Lewis et al., 2005), it impairs NBD1–NBD2 interactions and vectorial folding of NBD2 (Du et al., 2005). A second possible mechanism of correctors is that they promote folding of the transmembrane domains (TMDs). It has been found that both the ΔPhe508-type CFTR (Chen et al., 2004) and P-gp (Loo et al., 2002) mutants have altered packing of the transmembrane (TM) segments. To distinguish between these two mechanisms of corrector action, we constructed P-gp and CFTR deletion mutants lacking one or both of the NBDs. The truncation mutants were then tested to determine whether the removal of NBD2 would inhibit corrector-induced folding (favor the NBD1-NBD2 interaction model) or whether the folding of mutants lacking one or both NBDs could still be modulated by correctors (favor the TMD folding mechanism).

Materials and Methods

Construction of Mutants. The construction of ΔPhe508 CFTR and G268V P-gp cDNAs was described previously (Loo et al., 2005). The CFTR cDNAs coding for ΔNBD2 CFTR (residues 1–1196), the NH_2-terminal half-molecule (N-half CFTR) (residues 1–836), COOH-terminal half-molecule (C-half CFTR) (residues 837-1480), or TMD1 + 2 (TMD1 + 2 CFTR) (residues 1–438 plus 838-1178) were modified to contain the epitope tag for monoclonal antibody A52 at the COOH-terminal end of the protein and inserted into the pMT21 expression vector. The P-gp truncation mutants ΔNBD2 P-gp (residues 1–1023), TMD1 + 2 P-gp (residues 1–379 plus 681-1025), TMD1 P-gp (residues 1–379), N-half P-gp (residues 1–682), and C-half P-gp (residues 681-1278) containing the E875C mutation were constructed as described previously (Loo and Clarke, 1999a,b). The truncation mutants contained the A52 epitope at their COOH-terminal ends.

Expression of Mutants. HEK 293 cells were transfected with the cDNAs as described previously (Loo et al., 2006c). To test for the effect of compounds on expression of CFTR or P-gp processing mutants, cells were grown for 24 h in Dulbecco's modification of Eagle's medium with 2% (v/v) calf serum and 0.1 mM nonessential amino acids at 37°C in a 5% CO_2 incubator in the presence of 10 μM VRT-325, corr-4a, curcumin, thapsigargin, cyclosporin A, or 250 μM 7,8-benzoquinoline or 100 μM miglustat. Compounds were prepared as 1000× stocks in DMSO. Control cells were incubated in the presence of 0.1% (v/v) DMSO. Cells were harvested and solubilized with 2× SDS sample buffer [125 mM Tris-HCl, pH 6.8, 20% (v/v) glycerol, 4% (w/v) SDS, and 4% (v/v) 2-mercaptoethanol] containing 50 mM EDTA, 1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, 10 μM E-64, 2 mM benzamidine, 2 μg/ml aprotinin, and 20 μM leupeptin. Samples were then subjected to SDS-PAGE (5.5% acrylamide gels for full-length P-gp or CFTR) and immunoblot analysis with rabbit polyclonal antibody against CFTR or P-gp or with mouse monoclonal antibody A52.

For treatment with endoglycosidase H, a one-tenth volume of 0.5 M sodium citrate, pH 5.5, was added to the solubilized cells followed by addition of 20,000 U/ml endoglycosidase H (New England Biolabs, Mississauga, ON, Canada). The sample was treated for 15 min at 20°C. For treatment with peptide *N*-glycosidase F (PNGase F), a one-tenth volume of 0.5 M sodium phosphate, pH 7.5, and one-tenth volume of 10% (v/v) Nonidet P-40 were added to cell extract followed by addition of 10,000 U/ml PNGase F (New England Biolabs). Samples were incubated for 15 min at 37°C and then subjected to immunoblot analysis as described above.

Cell Surface Labeling. Cell surface labeling of ΔNBD2/Q1071P CFTR was performed as described previously (Loo et al., 2006c). In brief, cell surface carbohydrate was oxidized with sodium periodate and then labeled with biotin-LC-hydrazide. The cells were solubilized with 1% (w/v) Triton X-100 and biotinylated CFTR recovered with monoclonal antibody A52. Samples were subjected to immunoblot analysis using streptavidin-conjugated horseradish peroxidase.

Disulfide Cross-Linking Analysis. P-gp mutant L339C (TM6)/F728C (TM7) was transiently expressed in HEK 293 cells (25 10-cm plates). The cells were harvested, washed three times with phosphate-buffered saline, and membranes were prepared. The membranes were suspended in 0.3 ml of Tris-buffered saline, pH 7.4, and preincubated for 15 min at 22°C in the presence of 10 or 100 μM corr-2b, corr-3a, corr-4a, VRT-532, or VRT-325 or no drug. Samples were then cross-linked by incubation with 0.2 mM 3,6,9,12-tetraoxatetradecane-1,14-diyl bismethanethiosulfonate (M14M, 2.08-nm spacer arm), a methanethiosulfonate cross-linker, (Toronto Research Chemicals, Downsview, ON, Canada) for 3 min on ice. The cross-linking reactions were stopped by the addition of 2× SDS sample buffer [125 mM Tris-HCl, pH 6.8, 20% (v/v) glycerol, 4% (w/v) SDS containing 50 mM EDTA, and no reducing agent]. The reaction mixtures were then subjected to SDS-PAGE (7.5% polyacrylamide gels) and immunoblot analysis with a rabbit polyclonal antibody against P-gp. Intramolecular disulfide cross-linking between TMD1 and TMD2 can be detected as a result of slower mobility of cross-linked product on SDS-PAGE gels.

Results

The first step was to compare various compounds for their efficiency in promoting maturation of P-gp and CFTR processing mutants. Compounds such as thapsigargin, curcumin (Egan et al., 2004), miglustat (Norez et al., 2006b), and benzo(*c*)quinolizinium compounds (Dormer et al., 2001) are predicted to promote maturation of membrane proteins such as ΔPhe508 CFTR by disrupting protein-chaperone interactions in the endoplasmic reticulum. Cyclosporin A promotes maturation of P-gp processing mutants through direct interactions with the protein (Loo and Clarke, 1997), whereas an NH_2-terminal wild-type CFTR half-molecule was postulated to promote maturation of ΔPhe508 CFTR through a direct transcomplementation interaction (Cormet-Boyaka et al., 2004). The ability of the various compounds and N-half molecules to promote maturation of ΔPhe508 CFTR and the G268V P-gp processing mutants were compared with VRT-325 and corr-4a. VRT-325 was selected because it can promote maturation of CFTR or P-gp processing mutants whereas corr-4a is more specific for CFTR. The G268V P-gp processing mutant was used in the study as the protein shows a very low efficiency of maturation (Loo et al., 2005). Expression of P-gp and CFTR half-molecules was confirmed by immunoblot analysis (data not shown).

The G268V P-gp and ΔPhe508 CFTR mutants were expressed in the presence of the various compounds for 24 h, and whole-cell extracts were subjected to immunoblot analysis. The mature and immature forms can be distinguished on SDS-PAGE gels because CFTR (Fig. 1A) and P-gp (Fig. 1B) contain 2 and 3 *N*-glycosylation sites, respectively, that are core-glycosylated in the endoplasmic reticulum but are modified with complex carbohydrates if they are delivered to the Golgi. Figure 2A shows that expression of ΔPhe508 CFTR in HEK 293 cells yields little mature protein unless it is expressed in the presence of 10 μM VRT-325 or 10 μM corr-4a (Fig. 2B). Maturation of the P-gp processing mutant G268V was readily promoted by expression in the presence of VRT-325 and cyclosporin A (Fig. 2A). Therefore, VRT-325 was a particularly useful compound because it could promote maturation of both CFTR and P-gp. The next step was to examine which parts of the molecules were important for maturation (conversion of immature core-glycosylated protein to mature endoglycosidase H- resistant product).

CFTR and P-gp are members of the ATP-binding cassette family of transporters. These transporters have two TMDs and two NBDs (Fig. 1). CFTR also has a regulatory domain (Fig. 1A). Structural studies suggest that pharmacological chaperones may promote folding of CFTR or P-gp processing mutants by facilitating NBD1–NBD2 interactions and vectorial folding of NBD2 (Du et al., 2005) or by promoting folding of the TMDs (Loo and Clarke, 1999b; Chen et al., 2004). To test the importance of the NBDs for maturation, P-gp truncation mutants lacking NBD2 (ΔNBD2 P-gp) or both NBDs (TMD1 + 2 P-gp) are useful because both proteins are normally expressed as immature proteins (Fig. 3, A and B, − lanes). Expression of ΔNBD2 P-gp (Fig. 3A) or TMD1 + 2 (P-gp) (Fig. 3B) in the presence of 10 μM VRT-325 however, promoted maturation of the protein. We have demonstrated previously that the mature 120-kDa ΔNBD2 and 90-kDa TMD1 + 2 P-gp proteins were resistant to endoglycosidase H but sensitive to PNGase F and were delivered to the cell surface (Loo and Clarke, 1999b).

To determine the minimum domain structure required for maturation, we tested the effect of VRT-325 on maturation of TMD1 P-gp or N-half P-gp because these two proteins are normally expressed as core-glycosylated proteins (Loo and Clarke, 1995). Maturation was not observed when TMD1 or N-half P-gps were expressed in the presence of 10 μM VRT-325 (Fig. 3C). These results indicate that the minimum structure required for maturation of P-gp with VRT-325 was the

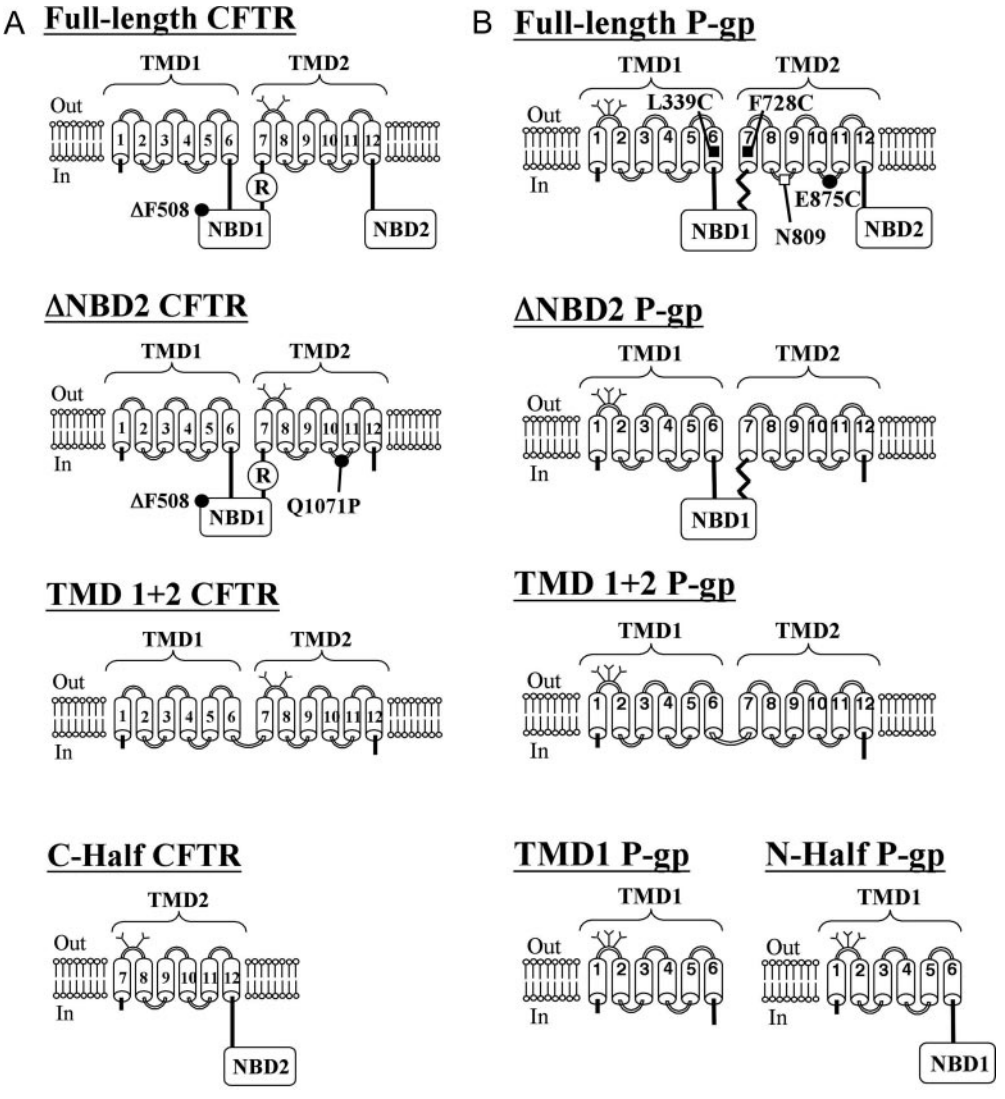

Fig. 1. Models of CFTR and P-gp. The cylinders represent TM segments, the glycosylation sites are represented by branched lines, and R represents the R domain. The CFTR models (A) of full-length CFTR (residues 1–1480), ΔNBD2 CFTR (residues 1–1023), TMD1 + 2 CFTR (residues 1–438 plus 838-1178), and C-half CFTR (residues 837-1480) are shown. The position of the Q1071P mutation in ΔNBD2 CFTR is indicated. For P-gp (B), ● in full-length P-gp (residues 1–1280) represent the locations of processing mutations examined in this study. □ represents an *N*-glycosylation site that is not modified in wild-type P-gp. ■ represents sites in the TMDs that were used in disulfide cross-linking analysis. The models of ΔNBD2 P-gp (residues 1–1023), TMD1 + 2 (residues 1–379 plus 681-1025), TMD1 P-gp (residues 1–379), and N-half P-gp (residues 1–682) truncation mutants are shown.

either two TMDs or one TMD and at least part of the second TMD. Interactions between the NBDs was not required to promote maturation of P-gp with VRT-325.

Some P-gp mutants are synthesized with an altered topology. For example, expression of P-gp C-half (COOH-terminal P-gp half-molecule) mutant E875C yielded a protein that becomes glycosylated at Asn809 (Loo and Clarke, 1999a). Residue Asn809 is not normally glycosylated because it is located on the cytoplasmic side of the membrane in the loop connecting TMs 8 and 9 (Fig. 1B). The presence of the E875C mutation seems to disrupt folding so that TMs 8 and /or 9 are not properly inserted into the membrane. To test whether VRT-325 could correct the topology, C-half E875C P-gp was expressed with or without N-half P-gp (NH_2-terminal P-gp half-molecule) in the presence or absence of VRT-325. The C-half molecule contained an A52 epitope tag, and glycosylation at Asn809 was monitored by testing for sensitivity to endoglycosidase H. Figure 3D shows that core-glycosylated C-half E875C P-gp could be detected when the protein was expressed with or without N-half P-gp or expressed by itself in the presence or absence of VRT-325. When C-half Glu875 P-gp was expressed in the presence of both N-half P-gp and VRT-325, however, core-glycosylated C-half P-gp expression was suppressed. These results suggest that expression of C-half E875C and N-half P-gps in the presence of VRT-325 promoted interactions between the two proteins to enhance the yield of C-half polypeptide that is not glycosylated.

VRT-325 may promote interactions between the two half-molecules of P-gp by interacting at the drug-binding domain. The drug-binding domain is located at the interface between the two TMDs (Loo et al., 2006a,b). An approach to test whether VRT-325 can occupy the drug-binding domain is to determine whether it will inhibit cross-linking between a cysteine in TMD2 (F728C in TM7) (Loo et al., 2006b) and a cysteine in TMD1 (L339C in TM6) (Loo and Clarke, 2001) (see Fig. 1B) that is predicted to lie on each side of the drug-binding domain. Disulfide cross-linking between cysteines in the TMDs can readily be detected because the cross-linked protein migrates slower on SDS-PAGE gels. Cross-linking can be inhibited in the presence of drug sub-

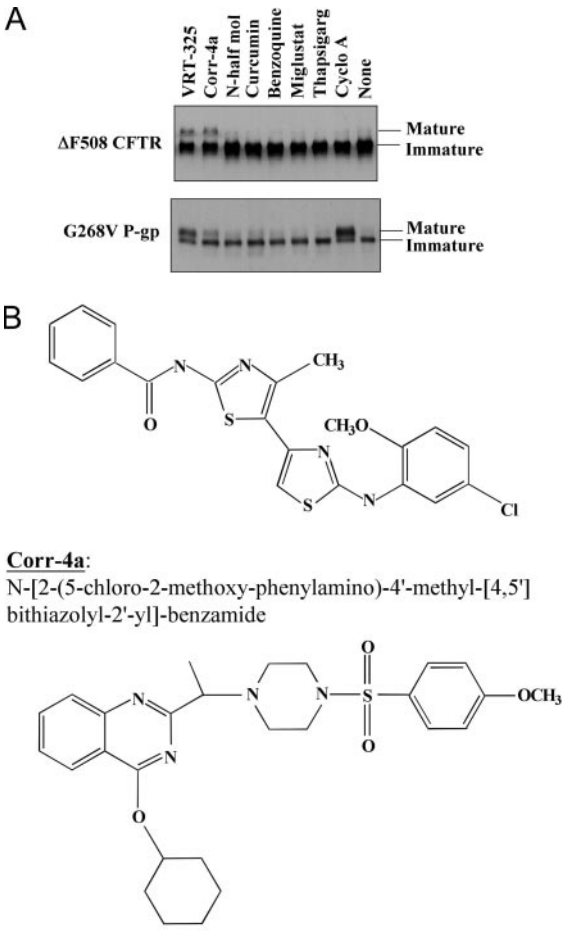

Fig. 2. Effects of compounds or transcomplementation on maturation of P-gp and CFTR processing mutants. A, HEK 293 cells were transfected with cDNAs for ΔPhe508 CFTR or G268V P-gp, cotransfected with NH_2-terminal half-molecule (N-half mol) derived from wild-type CFTR or P-gp. Cells were then incubated for 24 h in the presence of 10 μM VRT-325, 10 μM corr-4a, 10 μM curcumin, 250 μM 7,8-benzoquinoline (benzoquine), 100 μM miglustat, 10 μM thapsigargin (thapsigarg), 10 μM cyclosporin A (Cyclo A), or 0.1% (v/v) DMSO (None). Whole-cell extracts were then subjected to immunoblot analysis. The positions of mature and immature CFTR or P-gp are indicated. The structures of VRT-325 and corr-4a are also shown (B).

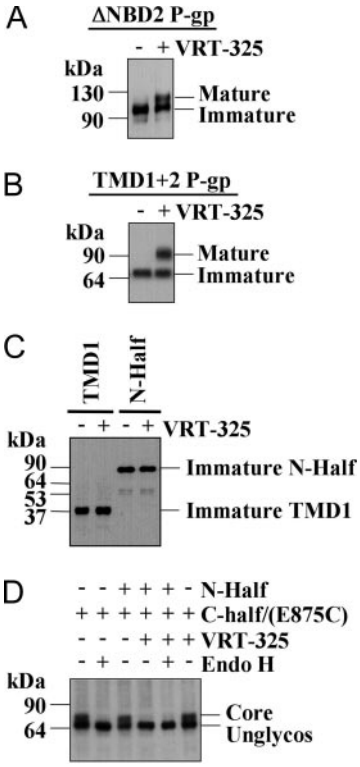

Fig. 3. Effect of VRT-325 on maturation of P-gp truncation mutants. A52-tagged P-gp mutants lacking NBD2 (ΔNBD2 P-gp) (A), missing both NBDs (TMD1 + 2) (B), missing the back half (N-Half), or missing the last three quarters of the molecule (TMD1) (C) were expressed for 24 h in the presence (+) or absence (−) of 10 μM VRT-325. Equivalent amounts of whole-cell SDS extracts were subjected to immunoblot analysis. The positions of mature and immature proteins are indicated. D, HEK 293 cells were transfected with C-half/E875C P-gp with (+) or without (−) N-half P-gp and then incubated for 24 h in the presence (+) or absence (−) of 10 μM VRT-325. Whole-cell extracts were treated with (+) or without (−) endoglycosidase H and subjected to immunoblot analysis with monoclonal antibody A52. The positions of the core-glycosylated (Core) and unglycosylated (Unglycos) C-half proteins are indicated.

strate (Loo et al., 2006b). Mutant L339C (TM6)/F728C (TM7) shows cross-linking when it is treated with the cross-linker M14M (Fig. 4, "None" lane). We tested different classes of corrector molecules such as corr-2b, corr-3a, corr-4a, corr-4b (Pedemonte et al., 2005), VRT-532 (Wang et al., 2006), and VRT-325 (Loo et al., 2005) for their abilities to inhibit cross-linking of L339C (TM6)/F728C (TM7). We found that VRT-325 was the most potent inhibitor because reduced cross-linking was observed in the presence of 10 or 100 μM VRT-325 (Fig. 4). Inhibition of cross-linking was also observed with 100 μM corr-3a and corr-4b. No inhibition of cross-linking was observed with corr-2b, corr-4a, or VRT-532. The results suggest that compounds corr-3a, corr-4b, and VRT-325 can directly interact with P-gp. Although the correctors may block cross-linking through the occupation of the drug-binding domain of P-gp, it cannot be ruled out that they bind at another site and cause distant conformational changes. It was shown previously that VRT-325, corr-3a, and corr-4b, but not corr-2b, corr-4a, or VRT-532, promoted maturation of the G268V P-gp processing mutant (Wang et al., 2006).

The next step was to test whether NBD1–NBD2 interactions were essential for the promotion of CFTR maturation with correctors. A CFTR cDNA lacking NBD2 (ΔNBD2 CFTR) was constructed and expressed in HEK 293 cells. CFTR was found to be different from P-gp because ΔNBD2 CFTR matured in the absence of correctors to yield an endoglycosidase H-resistant protein as the major product (Fig. 5A).

If correctors interact with NBD2 of CFTR to promote maturation, then they have no effect on a ΔNBD2 processing mutant. To test whether correctors could still promote maturation of a CFTR mutant lacking NBD2, we first needed to introduce a processing mutation into ΔNBD2 CFTR. Therefore, the Q1071P processing mutation was introduced into the ΔNBD2 CFTR. The Q1071P processing mutation was selected because it is located in the fourth cytoplasmic loop (see Fig. 1B), and it was postulated to inhibit maturation because it disrupted NBD2–TMD2 interactions (Seibert et al., 1996). Because NBD2 is no longer present in ΔNBD2 CFTR, we can now test whether the Q1071P mutation would still inhibit maturation. It was found that the processing mutant Q1071P could still inhibit maturation of CFTR lacking NBD2 (ΔNBD2/Q1071P, Fig. 5B). Therefore, the Q1071P mutation seems to interfere in the folding of TMD2. Maturation of the ΔNBD2/Q1071P CFTR mutant could be promoted, however, when expression was carried out in the presence of VRT-325 or corr-4a (Fig. 5B). The increase in maturation occurred in a concentration-dependent manner (Fig. 5C).

The conversion of ΔNBD2/Q1071P CFTR to an endoglycosidase H-resistant protein after expression in the presence of correctors indicates that it reached the Golgi. To determine whether expression in the presence of correctors would increase the level of ΔNBD2/Q1071P at the plasma membrane, cell surface labeling was carried out. HEK 293 cells transfected with ΔNBD2/Q1071P cDNA containing an A52-epitope tag were expressed in the presence or absence of 10 μM VRT-325 or corr-4a. The cells were treated with sodium periodate to oxidize carbohydrate groups, and the oxidized car-

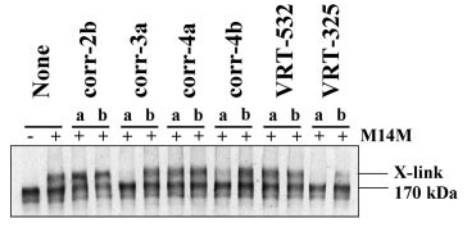

Fig. 4. Effect of corrector molecules on disulfide cross-linking of P-gp mutant L339C (TM 6)/F728C (TM7). Membranes were prepared from HEK 293 cells transfected with P-gp L339C (TM6)/ F728C (TM7) cDNA and then incubated at 22°C for 15 min in the presence of 0.1 mM (a) or 0.01 mM (b) corr-2b, corr-3a, corr-4a, corr-4b, VRT-532, VRT-325, or no corrector (None). Samples were then cooled on ice and treated with (+) or without (−) M14M cross-linker on ice for 3 min. The reactions were stopped by addition of SDS sample buffer, and samples were subjected to immunoblot analysis. The positions of mature (170 kDa) and cross-linked (X-link) P-gps are indicated.

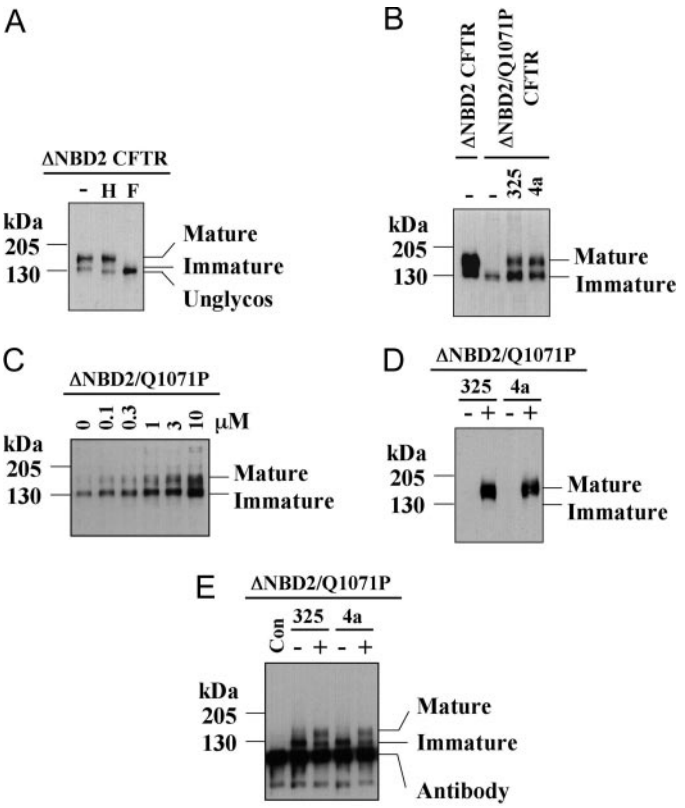

Fig. 5. Effects of VRT-325 and corr-4a on maturation of CFTR truncation mutants. A, whole-cell extracts of HEK 293 cells transfected with A52-tagged ΔNBD2 CFTR cDNA were treated with endoglycosidase H (H), PNGase F (F), or untreated (−). Samples were then subjected to immunoblot analysis with monoclonal antibody A52. B, CFTR truncation mutants ΔNBD2 CFTR or ΔNBD2/Q1071P containing C-terminal A52 tags were expressed in the presence of 10 μM VRT-325 (325) or 10 μM corr-4a or no corrector (−) for 24 h. Whole-cell extracts were then subjected to immunoblot analysis with monoclonal antibody A52. C, the concentration-dependence of maturation of ΔNBD2/Q1071P CFTR was determined by expressing the mutant in the indicated concentrations of corr-4a (4a) for 24 h followed by immunoblot analysis of whole-cell extracts. D, HEK 293 cells transfected with A52-tagged ΔNBD2/Q1071P CFTR cDNA were grown in the presence (+) or absence (−) of 10 μM VRT-325 (325) or 10 μM corr-4a (4a) for 24 h. Cell-surface labeling was then performed on whole cells using biotin-LC-hydrazide after oxidation of surface carbohydrates. Biotinylated CFTR was immunoprecipitated using monoclonal antibody A52, and samples were subjected to immunoblot analysis using streptavidin-conjugated horseradish peroxidase. The positions of mature (Mature) and immature (Immature) CFTR proteins are indicated. E, samples from the immunoprecipitation experiments and that from untransfected cells (Con) were subjected to immunoblot analysis with A52 monoclonal antibody.

bohydrates were labeled with biotin-LC-hydrazide. CFTR was isolated by immunoprecipitation with A52 antibody and subjected to immunoblot analysis with horseradish peroxidase conjugated to streptavidin. Figure 5D shows that cell surface expression of ΔNBD2/Q1071P was increased after expression in the presence of VRT-325 or corr-4a. A control immunoblot developed with monoclonal antibody A52 shows that similar levels of ΔNBD2/Q1071P protein were recovered after immunoprecipitation (Fig. 5E). CFTR protein expression of ΔNBD2 CFTR in stable BHK cells yielded functional cAMP-activated chloride channel activity in whole-cell iodide efflux assays (data not shown). It has been reported previously that ΔNBD2 CFTR can form functional channels at the cell surface when expressed in a low-temperature system (Chan et al., 1999).

To test whether a CFTR lacking both NBDs could undergo maturation, a TMD1 + 2 CFTR mutant was constructed and then expressed in the presence or absence of 10 μM VRT-325 or corr-4a. Whole-cell extracts were treated with or without endoglycosidase H and then subjected to immunoblot analysis. In contrast to P-gp TMD1 + 2, CFTR TMD1 + 2 was inefficiently core-glycosylated (Fig. 6A, − lanes). Expression of CFTR TMD1 + 2 in the presence of VRT-325 or corr-4a, however, enhanced core-glycosylation to yield approximately equal amounts of core-glycosylated and unglycosylated proteins (Fig. 6A, + lanes). The correctors seem to promote folding of the glycosylated loop between TM segments 7 and 8 in CFTR TMD1 + 2 to enhance the efficiency of glycosylation.

A C-half CFTR was then constructed to test the effects of correctors on glycosylation of CFTR missing the front half of the protein. When C-half CFTR was expressed in the absence of correctors, it was found that the protein was also inefficiently glycosylated (Fig. 6B). The C-half CFTR product yielded a mix of unglycosylated and core-glycosylated protein. Expression of C-half CFTR was then carried out in the presence or absence of 10 μM VRT-325 or corr-4a. Immunoblot analysis showed that expression of C-half CFTR in the presence of correctors did not significantly enhance glycosylation of the protein (Fig. 6C).

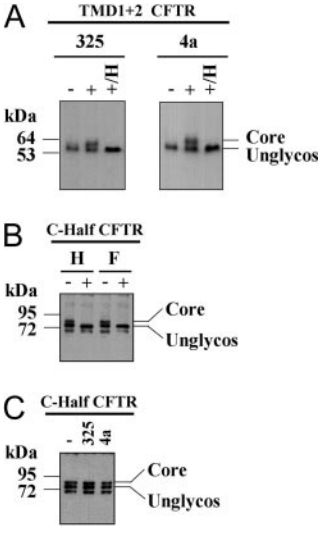

Fig. 6. Effect of VRT-325 or corr-4a on glycosylation of C-half or TMD1 + 2 CFTRs. A, A52-tagged TMD1 + 2 CFTR was expressed in the presence of 10 μM VRT-325 (325, +), 10 μM corr-4a (4a, +), or no corrector (−) for 24 h. Whole-cell extracts were subjected to immunoblot analysis with monoclonal antibody A52. Some samples from cells treated with corrector were treated with endoglycosidase H (+/H). B, whole-cell extracts of HEK 293 cells transfected with C-half CFTR cDNA and incubated in the absence of correctors were treated with (+) or without (−) endoglycosidase H (H) or PNGase F (F) followed by immunoblot analysis with monoclonal antibody A52. C, HEK 293 cells transfected with C-half CFTR were incubated in the presence of 10 μM VRT-325 (325) or corr-4a (4a) for 24 h. Whole-cell extracts were then subjected to immunoblot analysis with monoclonal antibody A52. The positions of core-glycosylated (Core) and unglycosylated (Unglycos) CFTR proteins are indicated.

Discussion

This study shows that establishment of native interactions between the NBDs of CFTR or P-gp is not required for corrector-induced maturation of the proteins. Instead, we found that the correctors could still influence the folding of P-gp or CFTR truncation mutants lacking both NBDs. Both TMDs were required because P-gp constructs containing only TMD1 (TMD1 or N-half P-gps) (Fig. 3) or CFTR constructs containing only TMD2 (C-half CFTR) (Fig. 6) showed no increase in maturation or increased efficiency in glycosylation when expressed in the presence of correctors. The observation that VRT-325 could block disulfide cross-linking between cysteines located in TM6 (L339C) and TM7 (F728C) (Fig. 4) suggested that there was direct interaction of the compound with P-gp. Because cysteines at positions 339 (TM6) and 728 (TM7) are predicted to line the drug-binding pocket, the ability of VRT-325 to block cross-linking suggests that the compound occupies the drug-binding domain. It is possible, however, that VRT-325 binds to a more distal site but affects cross-linking through long-range conformational changes.

The ability of VRT-325 to promote glycosylation of CFTR TMD1 + 2 and suppress aberrant glycosylation of E875C C-half P-gp provides a clue as to how correctors can aid in the folding process. They seem to be able to modulate the insertion of TM segments into the bilayer during synthesis. In the case of TMD1 + 2 CFTR, core-glycosylation was inefficient in the absence of corrector. Inefficient core-glycosylation of TMD1 + 2 CFTR suggests that the extracellular loop containing the two N-glycosylation sites (between TM segments 7 an 8) was not positioned correctly during synthesis of the protein in the absence of corrector. The acceptor sites for N-linked glycosylation must be 12 to 14 residues from the lumenal ends of the TM segments (Nilsson and von Heijne, 1993). It seems that expression of TMD1 + 2 CFTR in the presence of VRT-325 or corr-4a promoted the insertion of TM segments 7 and 8 in an orientation so that the high-mannose oligosaccharide acceptor sites were in the lumen at a distance of 12 to 14 residues above the membrane. The problems with proper orientation of the glycosylated loop may involve TM8. TM8 of CFTR is unusual because it has poor stop translocation properties, and removal of a negatively charged residue in TM8 (Asp 924) altered the topology of the extracellular loop between TM segments 7 and 8 (Carveth et al., 2002). Asp924 normally forms a salt bridge with Arg347 in TM6 of TMD1 (Cotten and Welsh, 1999). Core-glycosylation of P-gp TMD1 + 2 was much more efficient than CFTR TMD1 + 2. In addition, the TMD1 + 2 P-gp protein could undergo maturation when expressed in the presence of VRT-325. Therefore, insertion and orientation of P-gp TM segments may be more efficient than CFTR. Insertion and orientation of CFTR TM segments may be prone to errors because it has been shown that many of the TM segments have poor signal and stop-

transfer determinants (Sadlish and Skach, 2004). This may be due in part to the large number (11) of charged residues in the TM segments that affects the stability of the TM segments in the bilayer. For example, in a study that used a truncation mutant consisting of the first quarter of the molecule (included TMs 1–6), it was shown that TM6 of CFTR, which has three positively charged residues, is very unstable in the lipid bilayer (Tector and Hartl, 1999). Insertion of P-gp TM segments into a proper orientation may be more efficient than CFTR because they do not contain any charged residues. Although P-gp may be more efficiently synthesized than CFTR to yield a native topology, it is not without problems. The C-half of P-gp can be synthesized with alternative topologies (Zhang and Ling, 1991; Skach et al., 1993; Loo and Clarke, 1999a). In addition, the E875C P-gp C-half mutant showed aberrant glycosylation that could be corrected upon expression in the presence of VRT-325.

A hydrophobic pharmacological chaperone may promote folding of CFTR and P-gp by interacting with the hydrophobic face of one or more TM segments to act as "pharmacological anchor" to retain the unstable TM segments in the lipid bilayer. A model showing the alteration of the topologies of the P-gp E875C C-half mutant and CFTR TMD1 + 2 is shown in Fig. 7. Expression in the presence of a corrector enhances the expression of a truncation mutant with a native topology.

CFTR was also found to be different from P-gp because ΔNBD2 CFTR matured in the absence of a pharmacological chaperone (Fig. 5). In contrast, ΔNBD2 P-gp is only synthesized as immature protein unless it is expressed in the presence of a drug substrate such as cyclosporin A (Loo and Clarke, 1999b). In agreement with Chan et al. (1999), we found that ΔNBD2 CFTR formed functional channels at the cell surface (data not shown). It has been found, however, that the number of amino acids deleted in NBD2 and their locations can greatly influence maturation and activity of the protein. For example, a ΔNBD2 CFTR mutant lacking amino acids 1185 to 1349 did not mature in HEK 293 cells (Zerhusen and Ma, 1999). Channel measurements on microsomal membranes, however, showed that the mutant was active. In contrast, deletion of residues 1165 to 1450 of NBD2 yielded mature protein when expressed in COS-1 cells, but no channel activity could be detected (Pollet et al., 2000). A small deletion of only the last 65 amino acids of CFTR will inhibit maturation, whereas CFTR containing a deletion of 200 amino acids could mature and yield functional channels at the cell surface (Gentzsch and Riordan, 2001). It is possible that some of the COOH-terminal ends of the NBD2 CFTR truncation mutants are unstructured and may interfere with folding of the remainder of the protein. It is apparent, however, that NBD1-NBD2 or NBD2-TMD2 interactions are not essential for rescue with VRT-325 or corr-4a because both compounds could promote maturation of ΔNBD2/Q1071P CFTR.

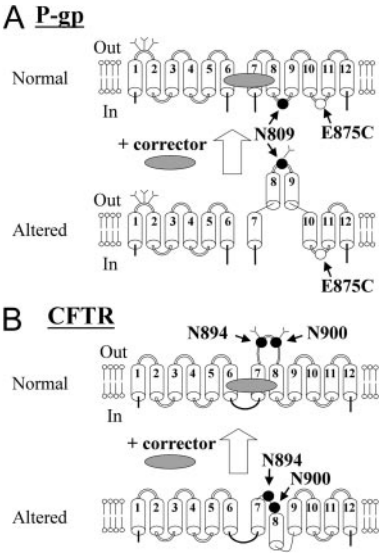

Fig. 7. Models of altered topologies of P-gp and CFTR. The cylinders represent TM segments, whereas the branched lines represent glycosylation sites. A, P-gp mutant E875C shows increased expression of a C-half polypeptide that is glycosylated at Asn809 (N809) (Altered). Therefore, TM segments 8 and/or 9 must not be properly orientated in the membrane. Expression of C-half E875C in the presence of wild-type N-half P-gp and VRT-325 (+ corrector) enhances the relative level of C-half polypeptide with a native topology (Normal). Disulfide cross-linking analysis suggests that VRT-325 (gray oval) can directly interact with TM segments. It may act as a pharmacological anchor to select for a relative topology. B, TMD1 + 2 CFTR is not efficiently glycosylated at the native Asn894 (N894) and Asn900 (N900) sites when expressed in the absence of correctors, which suggests that TMs 8 and/or 9 are not correctly orientated in the membrane (Altered). Expression of TM1 + 2 in the presence of correctors (gray oval) reorients TM 8 and/or 9 into a native topology that can be core-glycosylated (Normal). Correctors may interact with one or more TM segments.

A second difference between P-gp and CFTR was that TMD1 + 2 P-gp can leave the endoplasmic reticulum for the addition of complex carbohydrate, but CFTR TMD1 + 2 did not. One reason may be that CFTR requires a coat protein complex II signal that is located in NBD1 to leave the endoplasmic reticulum (Wang et al., 2004). This signal is missing in TMD1 + 2 CFTR.

We have now observed that VRT-325 can affect folding of CFTR processing mutants in two ways. First, it can induce TMD1–TMD2 interactions in partially folded CFTR processing mutants to promote TM packing to yield a native conformation (Loo et al., 2006c). Second, VRT-325 can modulate insertion of TM segments to yield segments that are properly oriented in the lipid bilayer. Compounds such as VRT-325 and corr-4a were more efficient than other approaches such as transcomplementation or disruption of CFTR-chaperone interactions with compounds such as thapsigargin, curcumin, or miglustat in promoting maturation of ΔPhe508 CFTR. Compounds such as thapsigargin, curcumin, and miglustat that are postulated to modulate CFTR-chaperone interactions, however, have been reported to be most effective with short incubation periods of 2 h (Norez et al., 2006a). Transcomplementation of ΔPhe508 CFTR or ΔTyr490 P-gp with their respective N-half wild-type polypeptides did not yield detectable levels of full-length protein (Fig. 2A). In the original transcomplementation studies (Cormet-Boyaka et al., 2004), it was reported that different cell lines and expression systems influenced the yield of mature CFTR. The efficiency of maturation is very low when transcomplementation studies with ΔPhe508 CFTR were performed in HEK 293 cells but very efficient in vaccine-infected COS-7 cells (Cormet-Boyaka et al., 2004).

In conclusion, VRT-325 is a useful compound to promote maturation of CFTR because it promotes packing of the TM segments between TMD1 and TMD2 of CFTR and can en-

hance the insertion of TM segments in the correct orientation in the lipid bilayer.

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