Correctors Promote Maturation of Cystic Fibrosis Transmembrane Conductance Regulator (CFTR)-processing Mutants by Binding to the Protein*\textsuperscript{[S]}

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The most common cause of cystic fibrosis (CF) is defective folding of a cystic fibrosis transmembrane conductance regulator (CFTR) mutant lacking Phe\textsuperscript{508} (ΔF508). The ΔF508 protein appears to be trapped in a prefolded state with incomplete packing of the transmembrane (TM) segments, a defect that can be repaired by expression in the presence of correctors such as corr-4a, VRT-325, and VRT-532. To determine whether the mechanism of correctors involves direct interactions with CFTR, our approach was to test whether correctors blocked disulfide cross-linking between cysteines introduced into the two halves of a Cys-less CFTR. Although replacement of the 18 endogenous cysteines of CFTR with Ser or Ala yields a Cys-less mutant that does not mature at 37 °C, we found that maturation could be restored if Val\textsuperscript{510} was changed to Ala, Cys, Ser, Thr, Gly, Ala, or Asp. The V510D mutation also promoted maturation of ΔF508 CFTR. The Cys-less/V510A mutant was used for subsequent cross-linking analysis as it yielded relatively high levels of mature protein that was functional in iodide efflux assays. We tested for cross-linking between cysteines introduced into TM6 and TM7 of Cys-less CFTR/V510A because cross-linking between TM6 and TM7 of P-glycoprotein, the sister protein of CFTR, was inhibited with the corrector VRT-325. Cys-less CFTR/V510A mutant containing cysteines at I340C(TM6) and S877C(TM7) could be cross-linked with a homobifunctional cross-linker. Correctors and the CFTR channel blocker benz bromarone, but not P-glycoprotein substrates, inhibited cross-linking of mutant I340C(TM6)/S877C(TM7). These results suggest that corrector molecules such as corr-4a interact directly with CFTR.

Cystic fibrosis (CF)\textsuperscript{2} is a lethal inherited disorder caused by mutations in the gene coding for the cystic fibrosis transmembrane conductance regulator (CFTR) protein (1). The most common genetic lesion is deletion of Phe\textsuperscript{508} (ΔF508). The ΔF508 CFTR protein undergoes rapid degradation because it is defective in folding. The Phe\textsuperscript{508} position is also important for folding of other ATP-binding cassette transporters, such as the sister protein of CFTR, P-glycoprotein (P-gp). Deletion of the equivalent residue in human P-gp (ΔY490) also causes defective folding of P-gp and rapid degradation (2).

Studies on P-gp showed that a drug-rescue approach with drug substrates could be used to correct folding defects in processing mutants such as ΔY490 P-gp (3). Therefore, several groups have used high throughput screening to search for small molecule correctors that would promote maturation of CFTR-processing mutants (4, 5). The quinazoline derivative VRT-325 was first identified that can promote maturation of both P-gp and CFTR-processing mutants (3, 5). Some correctors, such as corr-4a, corr-2b, and VRT-532, however, appeared to specifically promote maturation of ΔF508 CFTR (6). Further study showed that correctors could have an additive effect on maturation of CFTR-processing mutants (7). It is not known, however, whether correctors directly bind to CFTR or have indirect effects on the cellular folding pathways. In this study, we tested whether correctors directly interacted with CFTR by assaying for inhibition of disulfide cross-linking between the two halves of the protein.

EXPERIMENTAL PROCEDURES

Construction of Mutants—Mutations were introduced into CFTR cDNAs by the method of Kunkel (8). Cys-less CFTR was constructed by replacing Cys\textsuperscript{509} and Cys\textsuperscript{592} with leucine (9) and the other 16 endogenous cysteines at positions 76, 126, 225, 276, 343, 491, 524, 657, 832, 866, 1344, 1355, 1395, 1400, 1410, and 1458 with alanine. A total of 16 double cysteine mutants were constructed using the Cys-less CFTR molecule by introducing one cysteine into the Thr\textsuperscript{338}–Ser\textsuperscript{341} segment of TM6 and one cysteine into the Ala\textsuperscript{875}–Leu\textsuperscript{878} segment of TM7. The Cys-less CFTR cDNA was also modified to include the V510X mutations. Mutations I340C(TM6) and S877C(TM7) were inserted into Cys-less CFTR/V510A singly or together. The V510X mutations were then introduced into ΔF508 CFTR as described above. The ΔF508 mutation was also introduced into the Cys-less/V510A and I340C(TM6)/S877C(TM7)/Cys-less/V510A mutants. The cDNAs were sequenced to confirm the presence of mutations (10).

Expression of Mutants and Measurement of Iodide Efflux—CFTR mutant cDNAs were transiently transfected in human

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\textsuperscript{2} The abbreviations used are: CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; HEK 293 cells, human embryonic kidney cells; BHK, baby hamster kidney; NBD, nucleotide-binding domain; P-gp, P-glycoprotein; TM, transmembrane; TMD, transmembrane domain; M5M, 1,5-pentanediyl-bismethanethiosulfonate; M11M, 3,6,9-trioxaundecane-1,11-diyl-bismethanethiosulfonate; M17M, 3,6,9,12,15-pentaaxaheptadecane-1,17-diyl-bismethanethiosulfonate.
embryonic kidney (HEK) 293 cells as described previously (11). Whole cell SDS extracts were then subjected to SDS-PAGE (5.5% acrylamide gels) after expression for 48 h at 37 °C followed by immunoblot analysis with rabbit polyclonal antibody against CFTR (11). Measurement of cAMP-stimulated iodide efflux was performed on baby hamster kidney (BHK) cells stably expressing CFTR protein as described previously (6) (11). The forskolin mix that was added at time 0 to stimulate CFTR contained 10 μM forskolin, 200 nM isobutylmethylxanthine, 50 nM CPT-cAMP, and 10 μM VRT-532.

Disulfide Cross-linking Analysis—CFTR mutant I340C(TM6)/S877C(TM7) Cys-less/V510A CFTR was expressed in HEK 293 cells. The cells were harvested, washed three times with phosphate-buffered saline, and suspended in Tris-buffered saline (pH 8.0). To study the effect of drugs on cross-linking, cell samples were preincubated for 30 min at 22 °C in the absence or presence of various compounds. The final concentrations were 0.003, 0.03, or 0.33 mM corr-4a, VRT-325, VRT-532, or verapamil and 0.007, 0.07, or 0.7 mM benz bromarone or demecolcine. Samples were then incubated with 0.1 mM cross-linker (1,5-pentanediyl-bismethanethiosulfonate (M5M) (9.1 Å), 3,6-dioxaoctane-1,8-diyI-bismethanethiosulfonate (M8M) (13 Å), 3,6,9-trioxadecane-1,14-diyI-bismethanethiosulfonate (M11M) (16.9 Å), 3,6,9,12-tetraoxatetradecane-1,14-diyI-bismethanethiosulfonate (M14M) (20.8 Å), or 3,6,9,12,15-pentaoxaheptadecane-1,17-diyI-bismethanethiosulfonate (M17M) (24.7 Å) (Toronto Research Chemicals) for 16 min at 22 °C in the presence of 0.01% n-dodecyl-β-D-maltoside to enhance the solubility of the cross-linkers. The reactions were stopped by the addition of 2× SDS sample buffer containing 50 mM EDTA and no reducing agent, and samples were subjected to SDS-PAGE (7.5% polyacrylamide gels) and immunoblot analysis as described previously (12). All transfections and assays were repeated at least in triplicate.

RESULTS

We had previously shown that the corrector VRT-325 directly interacts with P-gp because it inhibited cross-linking between cysteines located in TM6 and TM7 (12). Our goal was to test whether correctors that promoted maturation of ΔF508 CFTR such as corr-4a, VRT-325, and VRT-532 directly interacted with the protein. Since P-gp and CFTR are both ATP-binding cassette transporters, it was possible that the correctors would also inhibit cross-linking between TM6 and TM7 of CFTR. To use a cross-linking approach with CFTR, the first step would be to construct a Cys-less CFTR that matures and is functional at the cell surface.

A Cys-less CFTR in which all 18 endogenous cysteines were changed to serines did not mature at 27 or 37 °C, so it was unsuitable for cross-linking analysis (11). We then constructed another Cys-less CFTR in which Cys590 and Cys592 were changed to leucine and the remaining 16 cysteines were changed to alanine. These changes have been reported to yield mature protein when expression is carried out at 27 °C (13, 14). The yield of mature Cys-less CFTR is often limiting even after expression at 27 °C. Therefore, it was important to use a Cys-less CFTR that yielded relatively high amounts of mature protein when expressed at 37 °C. Thibodeau et al. (15) had shown that mutating residue Phe508 to most other amino acids (except cysteine) inhibited maturation of CFTR. Therefore, we predicted that introduction of a cysteine close to Phe508 may also promote maturation of Cys-less CFTR at 37 °C. Accordingly, we replaced the residues at positions 508, 509, 510, 511, or 512 of Cys-less CFTR with cysteine. Residues occupying equivalent positions in TM6 and TM7 of P-gp and CFTR are aligned (TM6 P-gp, Val331-Ser351; TM6 CFTR, Ile332-Arg352; TM7 P-gp, Val712-Ser733; TM7 CFTR, Phe760-Trp782). The underlined residues were mutated to cysteine. Residues occupying equivalent positions in TM6 and TM7 of P-gp (L339C(TM6)/F508C(TM7)) and CFTR (I340C(TM6)/S877C(TM7)) are boxed.

We then tested whether replacing Val510 of Cys-less CFTR with other amino acids would promote maturation. Cys-less mutants in which residue Val510 was changed to Pro, Thr, Tyr, Gly, Ala, Ser, Leu, Asp, Phe, or Trp were constructed. The mutants were expressed in HEK 293 cells transfected with the mutant cDNAs were subjected to immunoblot analysis. The mature and immature forms of CFTR can be distinguished on SDS-PAGE gels because CFTR contains two N-glycosylation sites (Fig. 1A) that are core-glycosylated in the endoplasmic reticulum but are modified with complex carbohydrates in the Golgi. Fig. 2A shows that only the V510C mutation promoted maturation of Cys-less CFTR at 37 °C.

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We then tested whether the changes to residue Val510 that promoted maturation of Cys-less CFTR yielded a functional protein. Stable BHK cell lines expressing mutants V510A, V510C, V510S, V510G, or V510D were generated for use in iodide efflux assays. It was found that all the mutants except V510D were active in the iodide efflux assays. The results of the most active (V510A) and least active (V510D) mutants are shown in Fig. 2C. The results of mutants V510C, V510S, or V510G are not shown for clarity.
To test whether the Val510 changes could also promote maturation of F508 CFTR (in a wild-type background), we introduced the Val510 mutations that promoted maturation of Cys-less CFTR (Val510 to Cys, Gly, Ala, Ser, Asp, or Thr) into F508 CFTR. The F508 CFTR/V510X mutants were expressed in HEK 293 cells at 37 °C, and whole cell extracts were subjected to immunoblot analysis. Fig. 2D shows that introduction of an Asp mutation at position 510 was most effective in promoting maturation of F508 CFTR.

The Cys-less/V510A CFTR mutant was then used for disulfide cross-linking analysis to test whether correctors directly interacted with the protein. Since corrector VRT-325 inhibited cross-linking of P-gp mutant L339C(TM6)/F728C(TM7), we aligned TM segments (6 and 7) of P-gp and CFTR to identify the equivalent residues in CFTR. The segments Thr338 to Ser341 (TM6) and Ala875 to Leu878 (TM7) in CFTR (Fig. 1B) were predicted to overlap residues L339C(TM6) and F728C(TM7) of P-gp. Accordingly, 16 double cysteine mutants were constructed to contain one cysteine in segment 338–341 (TM6) and another in segment 875–878 (TM7). The 16 double cysteine mutants were initially constructed in a Cys-less CFTR lacking V510A, so they required expression at 27 °C for maturation of CFTR (data not shown). The cells were then subjected to cross-linking analysis with M11M cross-linker. Relatively strong cross-linking was observed in mutant I340C(TM6)/S877C(TM7), whereas the other mutants showed little or no cross-linking (data not shown). The I340C(TM6)/S877C(TM7) mutations were then introduced into the Cys-less/V510A CFTR background. The I340C(TM6)/S877C(TM7) Cys-less/V510A CFTR mutant was expressed in HEK 293 cells, and samples were treated with methanethiosulfonate cross-linkers of various sizes and subjected to immunoblot analysis. Cross-linking between the TMDs causes CFTR to migrate slower on SDS-PAGE gels (11). Fig. 3A shows that mutant I340C(TM6)/S877C(TM7) Cys-less/V510A CFTR could be cross-linked with various methanethiosulfonate cross-linkers. The magnitude of the change in the mobility of cross-linked CFTR was similar to that observed when cysteines introduced into TM6 and TM12 were cross-linked (11). The smallest (M5M) and largest (M17M) cross-linkers were less efficient in promoting cross-linking of P-gp mutant L339C(TM6)/F728C(TM7), we aligned TM segments (6 and 7) of P-gp and CFTR to identify the equivalent residues in CFTR. The segments Thr338 to Ser341 (TM6) and Ala875 to Leu878 (TM7) in CFTR (Fig. 1B) were predicted to overlap residues L339C(TM6) and F728C(TM7) of P-gp. Accordingly, 16 double cysteine mutants were constructed to contain one cysteine in segment 338–341 (TM6) and another in segment 875–878 (TM7). The 16 double cysteine mutants were initially constructed in a Cys-less CFTR lacking V510A, so they required expression at 27 °C for maturation of CFTR (data not shown). The cells were then subjected to cross-linking analysis with M11M cross-linker. Relatively strong cross-linking was observed in mutant I340C(TM6)/S877C(TM7), whereas the other mutants showed little or no cross-linking (data not shown). The I340C(TM6)/S877C(TM7) mutations were then introduced into the Cys-less/V510A CFTR background. The I340C(TM6)/S877C(TM7) Cys-less/V510A CFTR mutant was expressed in HEK 293 cells, and samples were treated with methanethiosulfonate cross-linkers of various sizes and subjected to immunoblot analysis. Cross-linking between the TMDs causes CFTR to migrate slower on SDS-PAGE gels (11). Fig. 3A shows that mutant I340C(TM6)/S877C(TM7) Cys-less/V510A CFTR could be cross-linked with various methanethiosulfonate cross-linkers. The magnitude of the change in the mobility of cross-linked CFTR was similar to that observed when cysteines introduced into TM6 and TM12 were cross-linked (11). The smallest (M5M) and largest (M17M) cross-linkers were less efficient in promoting cross-
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FIGURE 4. Effect of CFTR modulators and correctors on disulfide cross-linking of CFTR mutant I340C(TM6)/S877C(TM7) Cys-less/V510A CFTR. HEK 293 cells expressing mutant I340C(TM6)/S877C(TM7) Cys-less/V510A CFTR were incubated at 22 °C for 30 min in the absence (None) or presence of the indicated compounds. The samples were then treated with (+) or without (−) 0.1 mM M11M at 22 °C for 16 min. The final concentrations of the compounds were 0.007 mM (lane a), 0.07 mM (lane b), and 0.7 mM (lane c) of benzbrromarone (Benz), or demecolcine (Deme), or 0.003 mM (lane a), 0.033 mM (lane b), 0.33 mM (lane c) of corr-4a (4b, 8), VRT-325 (325, C), VRT-532 (325, D), or verapamil (Ver, E). Immunoblot analysis was also performed on mutants Cys-less/V510A (G) or I340C(TM6)/S877C(TM7)/Cys-less/V510A (H) with ΔF508 or without (−) the ΔF508 mutation that were transiently expressed in HEK 293 cells for 24 h at 27 °C. The reactions were stopped by the addition of SDS sample buffer, and samples were subjected to immunoblot analysis. The positions of immature, mature and cross-linked (X-link) CFTRs are indicated.

linking when compared with M11M (Fig. 3A). Therefore, M11M was used in subsequent cross-linking assays. Cross-linking was not detected in mutants containing either I340C(TM6) or S877C(TM7) alone (Fig. 3B).

A method to test whether a compound can occupy the drug-binding pocket is to determine whether it will inhibit cross-linking between cysteines located in the predicted binding region (12, 16). The I340C(TM6)/S877C(TM7) Cys-less/V510A CFTR mutant was used as a reporter molecule to study CFTR interactions with CFTR correctors such as corr-4a (4), VRT-325 (5, 17), and VRT-532 (5, 6). We first tested the system by using a compound that binds within the CFTR channel pore (at the interface between the two TMDs) and to see whether it could block cross-linking of mutant I340C(TM6)/S877C(TM7) Cys-less/V510A CFTR. Benzbrromarone is a CFTR inhibitor that blocks channel activity by occupying the pore (18). Accordingly, HEK 293 cells expressing mutant I340C(TM6)/S877C(TM7) Cys-less/V510A CFTR were preincubated with various concentrations of benzbrromarone and then treated with M11M cross-linker. The reactions were stopped by the addition of SDS sample buffer containing no reducing agent and samples subjected to immunoblot analysis. Fig. 4A shows that benzbrromarone at a concentration of 0.7 mM blocked cross-linking. Similarly, the correctors corr-4a (Fig. 4B), VRT-325 (Fig. 4C), or VRT-532 (Fig. 4D) nearly abolished cross-linking at concentrations of 0.33 mM. To test whether inhibition of cross-linking was specific, we tested the hydrophobic compounds verapamil and demecolcine that are substrates of P-gp (19). Verapamil (Fig. 4E) and demecolcine (Fig. 4F) did not inhibit cross-linking of mutant I340C(TM6)/S877C(TM7) Cys-less/V510A CFTR. These results suggest that benzbrromarone, corr-4a, VRT-325, and VRT-532 inhibited cross-linking through direct interactions with CFTR. We could not test whether correctors blocked cross-linking in the ΔF508/I340C(TM6)/S877C(TM7)/Cys-less/V510A mutant because mature CFTR was not detected in mutants ΔF508/Cys-less/V510A or ΔF508/I340C(TM6)/S877C(TM7)/Cys-less/V510A even when expressed at 27 °C (Fig. 4, G and H).

DISCUSSION

Mutation of residue Val510 to other amino acids such as Cys, Ser, Ala, Gly, Thr, and Asp could suppress folding defects of Cys-less CFTR (Fig. 2B). Position 510 appears to be particularly important for CFTR maturation as introduction of the suppressor mutation V510D into ΔF508 CFTR also promoted maturation of the protein (Fig. 2D). NBD1 of CFTR appears to be particularly important for CFTR maturation as studies by Teem et al. (20, 21) showed that mutations within or close to the LSGGG signature sequence promoted maturation of CFTR-processing mutants.

The segment of amino acids encompassing Phe508 to Val510 appears to be a critical component for CFTR folding because mutations to Phe508 inhibit maturation (15), whereas some mutations to Val510 promote maturation (this study). Residues Phe508 and Val510 may be important for NBD1-TMD1 interactions during folding of CFTR (22). The V510D mutation may counter the effects of ΔF508 by acting as a suppressor mutation, perhaps by forming a salt bridge with a positive amino acid located in one of the intracellular loops. The first intracellular loop contains 6 positively charged amino acids. The region surrounding Val510 might also be involved in a regulation pathway for CFTR trafficking as a protein kinase CK2 site (Ser511) has been identified in this segment (23).

Although V510D was the most efficient suppressor mutation because it promoted maturation of both Cys-less and ΔF508 CFTRs, it was less useful than the V510A change in Cys-less CFTR because it showed reduced iodide efflux activity. Some changes to amino acids close to Phe508 could be detrimental to function because it has been shown that the Phe508 aromatic side chain is important for ion channel function (14).

The ability of VRT-325, VRT-532, and corr-4a (Fig. 4) to block cross-linking of I340C(TM6)/S877C(TM7) Cys-less/V510A CFTR suggests that they interact directly with CFTR. Compounds VRT-532 and corr-4a may be particularly interesting because they show specificity for rescue of CFTR as they did not promote maturation of P-gp-processing mutants (6). In addition, rescue of ΔF508 CFTR with corr-4a was found to be superior to less specific approaches that utilize curcumin, miglustat, and thapsigargin (12).

The proposed effects of the V510D mutation and correctors on maturation of ΔF508 CFTR are shown in the models in supplemental Fig. 1. The ΔF508 CFTR is predicted to be trapped in
a “loosely folded” immature conformation since the presence of the mutation interferes with formation of the NBD1-TMD1 contacts, which in turn would disrupt proper packing of the TM segments (supplemental Fig. 1A). The V510D suppressor mutation is predicted to overcome the effects of the ΔF508 CFTR mutation by promoting interactions between TMD1 and NBD1 (supplemental Fig. 1B). When ΔF508 CFTR is expressed in the presence of the corrector corr-4a, it likely interacts with and promotes packing of the TM segments to yield mature CFTR (supplemental Fig. 1C). Packing of the TM segments in CFTR appears to be a critical step in folding as interactions between the TMDs are monitored by proteins such as derlin-1 and RMA-1 (24, 25) and appear to be the only folding step that occurs post-translationally (26). It is possible, however, that correctors such as corr-4a bind outside the TMDs and promote maturation (and inhibit cross-linking) through long range conformational effects. It should be pointed out that although we have demonstrated that correctors corr-4a, VRT-325, and VRT-532 can directly interact with CFTR, it is still possible that they could promote maturation of CFTR by interactions with other targets.

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