The chemical chaperone CFcor-325 repairs folding defects in the transmembrane domains of CFTR-processing mutants

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Most patients with CF (cystic fibrosis) express a CFTR [CF TM (transmembrane) conductance regulator] processing mutant that is not trafficked to the cell surface because it is retained in the endoplasmic reticulum due to altered packing of the TM segments. CL4 (cytoplasmic loop 4) connecting TMs 10 and 11 is a ‘hot-spot’ for CFTR processing mutations. The chemical chaperone CFcor-325 (4-cyclohexyloxy-2-{[4-(4-methoxy-benzene-sulphonyl)piperazin-1-yl]-ethyl}-quinazoline) rescued most CL4 mutants. To test if CFcor-325 promoted correct folding of the TMDs (TM domains), we selected two of the CL4 mutants (Q1071P and H1085R) for disulphide cross-linking analysis. Pairs of cysteine residues that were cross-linked in mature wild-type CFTR were introduced into mutants Q1071P and H1085R. The cross-linking patterns of the Q1071P or H1085R double cysteine mutants rescued with CFcor-325 were similar to those observed with mature wild-type double cysteine proteins. These results show that CFcor-325 rescued CFTR mutants by repairing the folding defects in the TMDs.

Key words: chemical chaperone, cystic fibrosis transmembrane conductance regulator (CFTR), disulphide cross-linking, misprocessed mutant, transmembrane domain, transmembrane segment packing.

INTRODUCTION

Cystic fibrosis (CF) is caused by mutations in the gene coding for the CFTR [CF TM (transmembrane) conductance regulator] protein. It is a cAMP-dependent chloride channel located in the apical membrane of many epithelia where it plays a key role in regulating salt and water homeostasis ([1], but see [1a]). The most common mutation in CF is deletion of residue Phe508 in regulating salt and water homoeostasis ([1], but see [1a]). Early studies on the ΔF508-CFTR mutant showed that growth at low temperatures (27°C) [4] or in the presence of non-specific osmolytes such as glycerol or TMAO (trimethylamine N-oxide) [5,6] induced the protein to fold properly and proceed to the cell surface where it exhibited significant chloride channel activity. The observations that processing mutants could still mediate channel activity offered hope that a pharmacological therapy could be developed for CF.

Studies on the human multidrug resistance P-gp (P-glycoprotein), a sister protein of CFTR, support the feasibility that a drug-rescue method could be used to correct CFTR processing defects [7]. High throughput screening of chemical libraries (Vertex Corp., San Diego, CA, U.S.A.) has identified such a compound termed CFcor-325 (4-cyclohexyloxy-2-{[4-(4-methoxy-benzene-sulphonyl)piperazin-1-yl]-ethyl}-quinazoline) [8]. It is not clear, however, whether CFcor-325 simply caused the release of misprocessed CFTR mutant protein from molecular chaperones in the ER as suggested for SERCA1 (sarcoplasmic/endoplasmic reticulum Ca2+-ATPase 1) inhibitors [9] or whether it affects folding of CFTR.

In the present study, we tested whether CFcor-325 could rescue the processing mutations in CL4. We then used disulphide cross-linking analysis to study whether expression of CFTR processing mutants in the presence of CFcor-325 affected the folding of the mutants.

EXPERIMENTAL

Construction and expression of mutants

The cDNAs of wild-type and CL4 mutants (H1054D, G1061R, L1065P, R1066H, Q1071P, L1077P, H1085R and W1098R) were inserted into pcDNA3 vector (Invitrogen, Oakville, ON, Canada) as described previously [2]. Construction of CFTR double cysteine mutants M348C(TM6)/T1142C(TM12), T351C(TM6)/T1142C(TM12) and W356C(TM6)/W1145C(TM12) was described previously [10]. Site-directed mutagenesis was used to introduce the Q1071P or H1085R mutations into the double cysteine mutants as described previously [11].

Transient expression of the mutants in HEK-293 cells (human embryonic kidney 293 cells) was done as described previously [12]. BHK cells (baby hamster kidney cells) stably expressing mutant CFTRs were generated by co-transfection of the mutant cDNA with pWL-neo (Stratagene, La Jolla, CA, U.S.A.). G418-resistant colonies were then selected. Clones expressing CFTR were identified by subjecting the cell lysates to immunoblot analysis with rabbit anti-CFTR polyclonal antibody or monoclonal antibody M3A7 and enhanced chemiluminescence [10].

Abbreviations used: BHK cells, baby hamster kidney cells; CF, cystic fibrosis; CFcor-325, 4-cyclohexyloxy-2-{[4-(4-methoxy-benzene-sulphonyl)piperazin-1-yl]-ethyl}-quinazoline; CL4, cytoplasmic loop 4; TM, transmembrane; CFTR, CF TM conductance regulator; CL4, cytoplasmic loop 4; endo H, endoglycosidase H; ER, endoplasmic reticulum; HEK-293 cells, human embryonic kidney 293 cells; M5M, 1,5-pentanediyl-bismethanthiosulphonate; M8M, 3,6-dioxaoctane-1,8-diyl-bismethanethiosulphonate; M17M, 3,6,9,12,15-pentaoxaheptadecane-1,17-diyl-bismethanethiosulphonate; M3A7, monoclonal antibody; M8M, 1,5-pentanediyl-bismethanthiosulphonate; M17M, 3,6,9,12,15-pentaoxaheptadecane-1,17-diyl-bismethanethiosulphonate; M5M, 1,5-pentanediyl-bismethanthiosulphonate; M8M, 3,6-dioxaoctane-1,8-diyl-bismethanethiosulphonate; M17M, 3,6,9,12,15-pentaoxaheptadecane-1,17-diyl-bismethanethiosulphonate; PNGase F, peptide N-glycosidase F; TMD, TM domain.

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Cells expressing CFTR processing mutants were grown in Opti-MEM medium (Invitrogen) containing 2% (v/v) calf serum and with or without 3 µM CFcor-325.

Disulphide cross-linking analysis

The cDNAs of double cysteine mutants M348C(TM6)/T1142C(TM12), T351C(TM6)/T1142C(TM12) and W356C(TM6)/W1145C(TM12) constructed in wild-type, mutant Q1071P or mutant H1085R backgrounds were expressed in HEK-293 cells in the presence or absence of 3 µM CFcor-325. The cells were harvested, washed twice with PBS (10 mM sodium phosphate buffer, pH 7.4, and 150 mM NaCl) and suspended in PBS. The cells were then incubated with 0.2 mM M5M (1,5-pentanediyl-bismethanethiosulphonate), M5S (3,6-dioxaoctane-1,8-diylibismethanethiosulphonate) or M17M (3,6,9,12,15-pentoaxaheptadecane-1,17-diylibismethanethiosulphonate) for 15 min at 20°C. The reactions were stopped by addition of an equal volume of 2% SDS sample buffer [125 mM Tris/HCl (pH 6.8) 20% (v/v) glycerol, 4% (v/v) SDS and 0.004% (v/v) 2-mercaptoethanol] containing 50 mM EDTA and no thiol reducing agent. The samples were then subjected to immunoblot analysis by SDS/PAGE (7.5% gel).

Endoglycosidase digestion

Whole cell SDS extracts were treated with endo H (endoglycosidase H; New England Biolabs, Mississauga, ON, Canada) or PNGase F (peptide N-glycosidase F; New England Biolabs) and samples were subjected to immunoblot analysis with anti-CFTR monoclonal antibody M3A7 and enhanced chemiluminescence as described previously [13].

Cell surface labelling

Cell surface labelling of CFTR was performed as described previously [14]. Briefly, expressing wild-type, mutant Q1071P or H1085R CFTR were grown with or without 3 µM CFcor-325. The cells were harvested, washed twice with PBS (pH 7.4) and then treated in the dark with PBS containing 10 mM NaIO4 for 10 min at 20°C. The cells were then washed with PBS and treated for 10 min at 20°C with 100 mM sodium acetate buffer (pH 5.5) containing 2 mM biotin-LC-hydrazide (Pierce, Rockford, IL, U.S.A.). The cells were then washed twice with 100 mM sodium acetate buffer (pH 5.5) and solubilized with Tris-buffered saline (10 mM Tris/HCl, pH 8.0, and 150 mM NaCl) (pH 7.4) containing 1% (w/v) Triton X-100. Biotinylated CFTR was recovered by immunoprecipitation with monoclonal antibody M3A7. The immunoprecipitated proteins were subjected to SDS/PAGE on 5.5% gels and biotinylated CFTRs were detected with streptavidin-conjugated horseradish peroxidase and enhanced chemiluminescence.

Iodide efflux assays

Measurement of cAMP-stimulated iodide efflux was done on BHK cells, stably expressing the mutant CFTR, grown in the presence or absence of 3 µM CFcor-325 as described previously [8].

RESULTS

CL4 of CFTR is a hot-spot for CF-associated mutations because mutations to about one-third of the residues in CL4 (between residues 1052 and 1098) have been linked to patients diagnosed with CF [2]. In general, patients with severe CF symptoms expressed a CL4 processing mutant (Figure 1) [2]. We had previously shown that CFcor-325 could induce maturation of AF508-CFTR [8]. We then tested whether CFcor-325 could promote maturation of CL4 processing mutations. Accordingly, HEK-293 cells were transfected with mutants H1054D, G1061R, L1065P, R1066H, Q1071P, L1077P, H1085R or W1098R cDNAs. After 24 h, the medium was replaced with fresh Opti-MEM medium with or without 3 µM CFcor-325. After 48 h, the cells were harvested and subjected to immunoblot analysis. Figure 2(A) shows that the processing mutants expressed the 160 kDa core-glycosylated protein as the major product in the absence of CFcor-325, while wild-type CFTR expressed the 190 kDa protein as the major product. Approximately 65% of the total CFTR protein detected in wild-type CFTR when expressed without CFcor-325 was present as the mature product. In contrast, the amount of mature protein in all the processing mutants except mutant R1066H was less than 5% (Figure 2B). The amount of mature CFTR in mutant R1066H was 10%. Expression of mutants H1054D, G1061R, R1066H, Q1071P, L1077P, H1085R and W1098R in the presence of 3 µM CFcor-325, however, induced expression of the 190 kDa mature protein. The type of mutation influenced the efficiency of rescue by CFcor-325 (Figure 2B). The chemical chaperone most efficiently rescued mutants R1066H, Q1071P, H1085R and W1098R as the amount of mature CFTR was approx. 30–40% of the total CFTR protein (Figure 2B). Lower levels of rescue were observed with mutants H1054D, G1061R and L1077P (5–15% mature CFTR protein). Mutant L1065P was the only mutant that could not be rescued with CFcor-325 (Figure 2B). The proline at position 1065 may have caused severe structural alterations because of its ability to destabilize α-helices [15]. We had proposed that processing mutations inhibit maturation of ABC transporters (ATP-binding-cassette transporters), such as P-gp [16] or CFTR [10], by trapping the proteins in incompletely folded states because of incomplete or defective packing of the TM segments. This was based on the results of disulphide cross-linking studies of the contact points between TMD1 (TM domain 1) and TMD2. It was shown that wild-type CFTR containing a cysteine residue in TM6 and another in TM12 [M348C(TM6)/T1142C(TM12), T351C(TM6)/T1142C(TM12) or W356C(TM6)/W1145C(TM12)] were cross-linked with homobifunctional cross-linkers of various lengths. Introduction of the
Correction of CFTR folding defects

Figure 2 Effect of CFcor-325 on CFTR CL4 processing mutations

(A) HEK-293 cells expressing wild-type or CL4 processing mutants were incubated with (+) or without (−) 3 µM CFcor-325 for 48 h. Whole cell extracts were subjected to immunoblot analysis with rabbit polyclonal antibody against CFTR and enhanced chemiluminescence. The positions of the mature and immature CFTRs are indicated. (B) The amount of mature CFTR relative to total (mature plus immature) was quantified by scanning the gel lanes followed by analysis with the NIH Image program (available at http://rsb.info.nih.gov/nih-image/) using a Macintosh computer.

ΔF508 mutation into these double cysteine mutants, however, inhibited maturation of the mutant protein and abolished cross-linking between the introduced cysteine residues [10]. Similar results were observed with P-gp [16]. Therefore we wanted to test whether expression of a CL4 processing mutant in the presence of CFcor-325 restored correct packing of the TM segments. Two CL4 mutants, Q1071P and H1085R, were selected for cross-linking analysis because they were efficiently rescued with CFcor-325. Expression of mutants Q1071P or H1085R in the presence of CFcor-325 increased the amount of mature CFTR from less than 5% (in the absence of corrector) to more than 30% (Figures 2A and 2B). We first confirmed whether the 190 kDa mature CFTR protein was at the cell surface. CFTR that is retained in the ER is only core-glycosylated and is sensitive to digestion with endo H. Mature 190 kDa CFTR, however, contains complex carbohydrate groups that are resistant to endo H but not to PNGase F. Accordingly, cells expressing wild-type, mutant Q1071P or mutant H1085R CFTR and grown in the presence or absence of CFcor-325 were extracted with SDS and then treated with endoglycosidases. Figure 3 shows that the 170 kDa immature CFTRs but not the 190 kDa mature CFTRs of wild-type, mutant Q1071P or mutant H1085R were sensitive to digestion with endo H. Both the 190 kDa mature and 170 kDa immature CFTRs were sensitive to digestion with PNGase F. Therefore expression of mutant Q1071P and H1085R in the presence of CFcor-325 promoted maturation and trafficking of the proteins from the ER to the Golgi where complex carbohydrates are added to the protein.

We then tested whether the 190 kDa mature CFTRs were present at the cell surface by cell surface labelling. Cells expressing wild-type, mutant Q1071P or mutant H1085R CFTRs were grown in the presence or absence of 3 µM CFcor-325. The cells were then treated with sodium periodate to oxidize the carbohydrate groups, followed by treatment with biotin-LC-hydrazide as described in the Experimental section. Biotinylated CFTRs were immunoprecipitated with anti-CFTR monoclonal antibody M3A7 and the immunoprecipitated proteins were subjected to SDS/PAGE and Western-blot analysis with streptavidin-conjugated horseradish peroxidase. The positions of mature (Mature) and immature (Im mature) CFTRs are indicated.

Figure 3 Endoglycosidase digestion

Whole cell SDS extracts of HEK-293 cells expressing wild-type, mutant Q1071P or mutant H1085R in the absence (−) or presence (+) of 3 µM CFcor-325 were treated with endo H (H), PNGase F (F) or no endoglycosidase (−). Samples were subjected to immunoblot analysis and enhanced chemiluminescence. The positions of mature, immature and deglycosylated (Unglycos) CFTRs are indicated.

Figure 4 Cell surface labelling of CFTR

(A) BHK cells (Control) or BHK cells stably expressing wild-type (Wild-type), mutant Q1071P or mutant H1085R CFTRs were grown in the presence (+) or absence (−) of 3 µM CFcor-325. The cells were treated with sodium periodate followed by biotin-LC-hydrazide as described in the Experimental section. Biotinylated CFTRs were immunoprecipitated with anti-CFTR monoclonal antibody M3A7 and the immunoprecipitated proteins were subjected to SDS/PAGE and Western-blot analysis with streptavidin-conjugated horseradish peroxidase. The positions of mature (Mature) and immature (Immature) CFTRs are indicated. (B) The amount of biotinylated CFTRs was quantified using the NIH Image program and is expressed relative to wild-type CFTR.
Figure 5 Measurement of cAMP-stimulated iodide efflux activities

Iodide efflux assays were performed on BHK cells stably expressing wild-type, mutant Q1071P or mutant H1085R CFTRs that were grown in the presence (+ CFcor-325) or absence (untreated) of CFcor-325 as described in the Experimental section. The activity of mutant H1085R grown without CFcor-325 was similar to that of mutant Q1071P (untreated) and is omitted for clarity. Time 0 is the start of stimulation through addition of 10 μM forskolin.

shows that the 190 kDa mature but not the 170 kDa immature wild-type CFTR (grown in the absence of CFcor-325) was biotinylated. Similarly, labelled mature 190 kDa CFTR proteins were detected in mutants Q1071P and H1085R only after rescue with CFcor-325. Figure 4(B) shows that there was good correlation between the amount of biotinylated CFTR proteins in mutants Q1071P and H1085R with the amount of mature CFTRs detected in Figure 2(B) (63 and 50% of wild-type respectively).

Next, we tested whether mutant Q1071P or H1085R was active at the cell surface after rescue with CFcor-325. Mutants Q1071P and H1085R CFTRs were stably expressed in BHK cells because adherent cells are essential for the iodide efflux assays. Also, BHK cells do not express endogenous CFTR protein. The BHK cells stably expressing mutants Q1071P or H1085R were treated for 48 h with or without 3 μM CFcor-325 and then used in iodide efflux assays. Iodide efflux assays were used rather than chloride channel measurements because very few channels other than CFTR can conduct iodide ions [17]. The cells were then loaded with sodium iodide and cAMP-stimulated iodide efflux was measured. Figure 5 shows that wild-type CFTR exhibited iodide efflux activity in the absence of CFcor-325. In contrast, BHK cells stably expressing CFTR mutants Q1071P or H1085R demonstrated iodide efflux activity only when grown in the presence of CFcor-325 (Figure 5). These results suggest that CFcor-325 induced maturation and trafficking of the misprocessed mutant proteins to the cell surface in an active form. Therefore mutants Q1071P and H1085R were used for cross-linking studies.

Accordingly, M348C(TM6)/T1142C(TM12), T351C(TM6)/T1142C(TM12) or W356C(TM6)/W1145C(TM12) mutations were introduced into a Q1071P or H1085R CFTR background. Mutants Q1071P or H1085R containing M348C(TM6)/T1142C(TM12), T351C(TM6)/T1142C(TM12) or W356C(TM6)/W1145C(TM12) mutations were then transiently expressed in HEK-293 cells in the presence or absence of 3 μM CFcor-325 for 48 h and then treated with the homobifunctional cross-linkers M5M, M8M or M17M. The reactions were stopped by addition of SDS sample buffer and samples were subjected to immunoblot analysis. Intramolecular cross-linking between cysteines located in the two TMDs can be readily detected because the cross-linked CFTR migrates with slower mobility in SDS/PAGE gels [10]. Figure 6 shows that all of the mutants expressed the mature 190 kDa protein in the presence of CFcor-325. Each mutant showed a distinct cross-linking pattern. Mature mutant Q1071P/M348C(TM6)/T1142C(TM12) protein was cross-linked with M5M and M8M (Figure 6A). Mature mutant Q1071P/T351C(TM6)/T1142C(TM12) protein was cross-linked with M8M and to a lesser extent with M17M, while the mature mutant Q1071P/W356C(TM6)/W1145C(TM12) protein was cross-linked with M5M, M8M and M17M or with no cross-linker (None) for 15 min at 20 °C. The cells were then lysed with SDS sample buffer (no reducing agent) and samples were subjected to immunoblot analysis. The positions of the mature, immature and cross-linked (X-link) CFTRs are indicated.

M5M and M8M (Figure 6A). Mature mutant Q1071P/T351C(TM6)/T1142C(TM12) protein was cross-linked with M8M and to a lesser extent with M17M, while the mature mutant Q1071P/W356C(TM6)/W1145C(TM12) protein was cross-linked with M5M, M8M and M17M or with no cross-linker (None) for 15 min at 20 °C. The cells were then lysed with SDS sample buffer (no reducing agent) and samples were subjected to immunoblot analysis. The positions of the mature, immature and cross-linked (X-link) CFTRs are indicated.
show any cross-linked product and their disappearance after cross-linking is probably due to aggregation [10]. An example of aggregation is shown for mutant Q1071P/M348C(TM6)/T1142C(TM12) (Figure 7). The mutant was expressed in the absence of CFcor-325 and the membranes were subjected to cross-linking with M5M, M8M or M17M and immunoblot analysis. Figure 7 shows that treatment of the membranes with the cross-linkers enhanced aggregation of CFTR so that more immunoreactive material was present in the stacking gel compared with membranes not treated with cross-linker. The cross-linked aggregates could be reduced with dithiothreitol to yield the 170 kDa immature protein. Similar results were observed with mutant H1085R/M348C(TM6)/T1142C(TM12) (results not shown). Therefore these results indicate that CFcor-325 promotes proper folding of the misprocessed CFTR to yield a native-like conformation.

**DISCUSSION**

In the present study, we showed that expression of a CFTR processing mutant in the presence of the chemical chaperone CFcor-325 induced the molecule to fold into a conformation that resembles wild-type CFTR (Figure 8). It has been shown that CFTR has an altered structure when its maturation is inhibited. The incompletely folded CFTR is inactive and is more sensitive to protease digestion than the mature CFTR [18].

A drug-rescue approach to correct folding defects in membrane proteins was first observed in P-gp [7]. It was found that processing mutations in P-gp resulted in a loosely folded molecule that was relatively more sensitive to protease digestion than the mature wild-type protein. The misfolded molecule was inactive because of incomplete packing of the TM segments and altered contacts between the NBDs (nucleotide-binding domains) [16,19,20]. Expression of P-gp processing mutants in the presence of drug substrates induced the protein to fold into a native conformation by promoting superfolding between the TMDs [21].

It is unlikely that CFcor-325 simply caused release of the misprocessed mutants from molecular chaperones in the ER as proposed for curcumin [9]. This would imply that the structure of the rescued CFTR and that found in the ER would be similar. Our results, however, show that this is unlikely to be the mechanism for CFcor-325. The altered cross-linking pattern observed between misprocessed mutants that were grown in the presence or absence of CFcor-325 (Figure 6) indicates that CFcor-325 induces superfolding of CFTR.

**Figure 7** Effect of dithiothreitol on immature CFTR treated with cross-linker

HEK-293 cells expressing mutant Q1071P/M348C(TM6)/T1142C(TM12) in the absence of CFcor-325 were treated with 0.2 mM M5M, M8M or M17M cross-linker for 15 min at 20°C. Samples were then incubated with (-DTT) or without (+DTT) 30 mM dithiothreitol for 5 min at 20°C. Following SDS/PAGE on a 5.5 % gel, the stacking and separating gels were transferred onto a sheet of nitrocellulose and CFTR protein was detected by immunoblot analysis. The positions of the mature, immature and cross-linked (X-link) CFTRs are indicated.

Since both CFTR and P-gp processing mutations can be rescued with CFcor-325, we propose that the mechanism of rescue by CFcor-325 is similar to that proposed for P-gp [16] (Figure 8). The model in Figure 8 shows the two TMDs and two NBDs of CFTR. The regulatory domain (R domain) is not shown since it is not essential for maturation [22]. Maturation of wild-type CFTR protein from a loosely folded state to a more compact structure requires conformational changes that allow proper interactions between the NBDs and TMDs (Figure 8A). The presence of a processing mutation such as Q1071P introduces a thermodynamic hurdle during folding so that the protein is trapped in an immature conformation (Figure 8B). The presence of CFcor-325 during the folding process acts as a scaffold between the TMDs and allows proper interactions between the TMDs resulting in a protein with a native-like conformation (Figure 8C).

It is not clear how point mutation in CFTR causes misprocessing of the protein. A contributing factor is that wild-type CFTR is not efficiently synthesized. It has been shown that the synthesis of wild-type CFTR is relatively inefficient because 50–70% of the protein is rapidly degraded [23]. Another problem is that many TM segments in CFTR do not possess efficient signal and stop-transfer sequences or contain charged residues that interfere with insertion or retention of the TMs in the lipid bilayer during biosynthesis [24,25]. Therefore mutations in the cytoplasmic loops could further lower the efficiency of insertion of the TM segments. Binding of CFcor-325 to one or more of the TM segments during synthesis may help anchor and stabilize these TM segments until proper contacts can be made with the remaining TM segments. Further work will be needed to establish whether CFcor-325 does indeed interact with the TMDs of CFTR.

In summary, CFcor-325 is a very effective chemical/pharmacological chaperone for rescuing most CL4 processing mutants by repairing folding defects in the TMDs.
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