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Enhanced Cell Surface Stability of Rescued $\Delta F508$ Cystic Fibrosis Transmembrane Conductance Regulator by Pharmacological Chaperones

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Synopsis

Misfolded proteins destined for the cell surface are recognized and degraded by the ER-associated degradative (ERAD) pathway. Temperature-sensitive (TS) mutants at permissive temperature escape ERAD and reach the cell surface. Here, we examined a TS mutant of the cystic fibrosis transmembrane conductance regulator, $\Delta F508$ CFTR, and analyzed its cell surface trafficking after rescue (r $\Delta F508$). We show that r $\Delta F508$ endocytosis is 6-fold more rapid ($\sim 30\%/2.5$ min) than wild-type (WT, $\sim 5\%/2.5$ min) CFTR at 37°C in polarized airway epithelial cells (CFBE41o-). We also investigated r $\Delta F508$ endocytosis under two other conditions: at culture at permissive temperature (27°C) and following treatment with pharmacological chaperones. At low temperature, r $\Delta F508$ endocytosis slowed to WT rates ($20\%/10$ min), indicating that the cell surface trafficking defect of r $\Delta F508$ is TS. Furthermore, r $\Delta F508$ is stabilized at low temperature; its half-life increases from <2 h at 37°C to >8 h at 27°C . Pharmacological chaperone treatment at 37°C corrected the r $\Delta F508$ internalization defect, slowing endocytosis from $\sim 30\%/2.5$ min to $\sim 5\%/2.5$ min, and doubled $\Delta F508$ surface half-life from 2 to 4 hours. These effects are $\Delta F508$ CFTR-specific, as pharmacological chaperones did not affect WT CFTR or transferrin receptor internalization rates. The results indicate that small molecular correctors may recapitulate the effect of permissive temperature, not only by rescuing $\Delta F508$ CFTR from ERAD, but also by enhancing its cell surface stability.

Keywords

CFTR; $\Delta F508$ rescue; temperature-sensitive; endocytosis; cell surface trafficking

Introduction

The cystic fibrosis transmembrane conductance regulator (CFTR) is a cyclic AMP (cAMP)-activated chloride channel expressed in a variety of tissues [1–4]. CFTR functions in the apical membrane of polarized epithelial cells and undergoes endocytosis via clathrin-coated

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vesicles [5–11]. Mutations in the *CFTR* gene lead to cystic fibrosis (CF), the most common hereditary lethal disease among Caucasians [12, 13]. There are more than 1500 mutations listed in the CFTR mutation database (<http://www.genet.sickkids.on.ca/cftr>), but a three base pair deletion resulting in loss of a phenylalanine at position 508 ($\Delta F508$) is the most prevalent disease-causing mutation.

$\Delta F508$ CFTR is a well-known example of a clinically relevant temperature-sensitive (TS) processing mutant. At 37°C, the restrictive temperature, the $\Delta F508$ protein is rapidly degraded by endoplasmic reticulum associated degradation (ERAD), preventing $\Delta F508$ expression at the cell surface and resulting in the CF phenotype [14–16]. At 27°C, the permissive temperature, some of the $\Delta F508$ protein escapes ERAD and is delivered to the cell membrane, where it is called “rescued” $\Delta F508$ (r $\Delta F508$) CFTR. Because r $\Delta F508$ CFTR partially retains its chloride channel activity [17], several methods have been introduced to promote $\Delta F508$ CFTR escape from ERAD and deliver it to the cell membrane [18–24], but to date, the most efficient method to rescue $\Delta F508$ CFTR is permissive temperature cell culture [25]. Although first observed 15 years ago, it remains unclear how 27°C culture facilitates $\Delta F508$ CFTR escape from ER quality control. It is well established, however, that returning cells to the restrictive temperature after low temperature rescue results in rapid internalization and degradation of r $\Delta F508$ CFTR [26, 27]. It is not known whether r $\Delta F508$ CFTR displays the same cell surface instability, or how function is affected, if left at the permissive temperature.

In addition to low temperature culture, chemical compounds such as glycerol [28], DMSO [29], and organic solutes [24] have also been shown to facilitate $\Delta F508$ CFTR escape from ERAD. These compounds exert their effects by enhancing the efficiency of $\Delta F508$ CFTR folding or increasing the host cells’ differentiation and polarity [29]. Recently, a handful of small molecular correctors were identified by high-throughput screening based on their ability to promote r $\Delta F508$ CFTR expression [20, 22, 30, 31]. In most cases, the mechanism by which these compounds facilitate ERAD escape is not known. Furthermore, their effects at the cell surface have not been tested.

Although the effects of low temperature culture on $\Delta F508$ folding in the ER has been studied for years, it is not known whether the surface defects exhibited by r $\Delta F508$ CFTR are also TS, and therefore possibly related to the ER folding defect. Additionally, it has not been determined whether treatment of r $\Delta F508$ CFTR with chemical compounds known to promote rescue can affect $\Delta F508$ cell surface properties, such as surface stability. Answers to these questions are essential to understand the altered trafficking, decreased stability and compromised function of the r $\Delta F508$ CFTR protein. In the present study, we provide more detailed information on the effects of permissive temperature culture and of two small molecule correctors on r $\Delta F508$ CFTR cell surface trafficking. We used two different CFTR-expressing model cell lines, HeLa and CFBE41o-, in order to identify cell type and polarization specific differences in the cell surface trafficking of wild-type (WT) and r $\Delta F508$ CFTR, and to determine if methods known to facilitate $\Delta F508$ exit from the ER also stabilize r $\Delta F508$ at the cell surface.

Experimental Procedures

Cell lines and cell culture

Cells were maintained in a 37°C humidified incubator in 5% CO₂. HeLa ΔF , HeLaWT, CFBE41o- ΔF and CFBE41o-WT cells were developed and cultured as previously described [26]. HeLa cells were grown in Eagle’s modification of MEM medium (Invitrogen, Carlsbad, CA) supplemented with 10% FBS. CFBE14o- cell cultures were maintained in DMEM/F12 medium (Invitrogen) with 10% FBS. For experiments requiring polarized cells,

CFBE41o-ΔF and CFBE41o-WT cells were seeded on 12-mm diameter Transwell filters (Corning-Costar, Corning, NY). Under these conditions, the cells formed polarized monolayers with trans-epithelial resistances of $>1000 \Omega \cdot \text{cm}^2$, as measured by a Millicell Electrical Resistance System (Millipore, Billerica, MA).

Small molecular correctors (pharmacological chaperones)

Small molecular correctors were provided by Cystic Fibrosis Foundation Therapeutics, Inc. Compounds tested were CFcor-325 (VRT-325, 4-Cyclohexyloxy-2-[1[4-(4-methoxy-benzensulfonyl)-piperazin-1-yl]-ethyl]-quinazoline) [20, 32] and Corr-4a ([2-(5-Chloro-2-methoxy-phenylamino)-4'-methyl-[4,5']bithiazolyl-2'-yl]-phenyl-methanone [22]. Both compounds were utilized at a 10 mM stock concentration in DMSO and a 10 μM working concentration in OPTI-MEM medium supplemented with 2% calf serum (v/v). The presence of the DMSO vehicle (0.1%) in the media did not mediate ER escape of ΔF508 CFTR in control samples, or facilitate changes in internalization. In all experiments, control samples contained the DMSO vehicle.

Immunoprecipitation

Cells were lysed in RIPA buffer (1% Nonidet P-40, 0.5% sodium deoxycholate, 150 mM NaCl, 50 mM Tris, pH 8.0) containing Complete Mini protease inhibitor (Roche, NJ). CFTR was immunoprecipitated using a mouse monoclonal anti-CFTR C-terminal antibody (24-1, ATCC number HB-11947) and protein A-agarose (Roche, NJ) [8]. Transferrin receptor (TR) was immunoprecipitated using mouse anti-TR (B/325) and Protein A-agarose [33].

Internalization assays

CFTR internalization assays were performed as described previously [8, 9]. Briefly, surface carbohydrate (CHO) groups on the cells were oxidized with sodium periodate (NaIO_4 , 10mM), washed on ice with Mg^{2+} - and Ca^{2+} -supplemented PBS buffer (PBS m/c) and warmed to 37°C or 27°C for 2.5 or 10 minutes. Oxidized CHO groups remaining on the cell surface after a warm-up period were labeled with biotin-LC-hydrazide (1 mg/mL, Pierce, Rockford, IL), followed by cell lysis in RIPA buffer. CFTR was then immunoprecipitated as described above. CFTR internalization was identified as percentage of loss of biotinylated CFTR during the warm-up period compared to the control samples (no warm-up period).

CFTR cell surface half-life measurements

Cells were metabolically labeled [8] and biotinylated as described [9]. Initial (0 time point) samples were immediately lysed in RIPA, and the rest of the samples were transferred back to a 37°C incubator for 2, 4 and 8 hours. At each time point, cells were lysed in RIPA buffer, CFTR was immunoprecipitated and analyzed by SDS-PAGE and autoradiography (PhosphorImager, Amersham Biosciences, Piscataway, NJ). Half-lives of the proteins were calculated as previously described [8, 34].

Metabolic pulse-chase experiments

For experiments at 37°C, cells were metabolically labeled as described [8]. For metabolic labeling during permissive temperature (27°C) culture, cells were transferred to 27°C and their medium replaced with cysteine- and methionine-free minimal essential medium (Specialty Media; Phillipsburg, NJ) for one hour. Cells were subsequently given 300 $\mu\text{Ci}/\text{mL}$ [^{35}S]-methionine/cysteine (EasyTagTM; PerkinElmer Life Sciences; Waltham, MA) for 18 hours at 27°C. This extended metabolic labeling pulse was necessary to produce a labeled CFTR signal at 27°C that formed sufficient amounts of maturely glycosylated CFTR (C band) to follow during the chase period. Upon completion of the labeling, the medium was

replaced with complete medium without radioisotopes; cells were cultured at either 37°C or 27°C for the time-points indicated.

Western Blot

Total and biotinylated CFTR or TR were detected as previously described [8]. Briefly, CFTR and TR were immunoprecipitated and analyzed by SDS-PAGE and Western blot. Total CFTR was detected with polyclonal anti-CFTR NBD-2 antibody (H-182, Santa Cruz Biotechnology, Santa Cruz, CA). Total TR was detected with polyclonal anti-TR external domain antibody. Biotinylated CFTR and TR were detected with HRP-labeled avidin. Chemiluminescence was induced with High Sensitivity Immobilon-Western Substrate (Millipore). The membranes were exposed for different time periods, up to 3 minutes, and a linear range for a standard set of diluted samples was calibrated. Western blots were analyzed and densities measured using Image J, ScionImage, or IPLab software. Results are means of at least three independent experiments.

Ussing chamber analyses

Measurements of short-circuit current (I_{sc}) and electrical resistance (R_t) were performed as previously described [35]. Briefly, filters containing monolayers of either CFBE41-ΔF or CFBE41o-WT cells were mounted in Ussing chambers (Jim's Instruments, Iowa City, IA) and bathed on both sides with solutions containing (mM) 120 NaCl, 25 NaHCO₃, 3.3 KH₂PO₄, 0.83 K₂HPO₄, 1.2 CaCl₂, 1.2 MgCl₂, 10 HEPES (Na⁺-free), 10 mannitol (apical compartment), and 10 glucose (basolateral compartment). Osmolarity of all solutions, as measured by a freezing depression osmometer (Wescor, Inc., Logan, UT), was between 290 and 300 mOsm. Bath solutions were stirred vigorously by bubbling continuously with 95% O₂, 5% CO₂ at 37°C (pH 7.4). Monolayers were short-circuited to 0 mV and I_{sc} (μA/cm²) was measured with an epithelial voltage clamp (VCC-600, Physiologic Instruments, San Diego, CA). A 10-mV pulse of 1-sec duration was imposed every 10 sec to monitor R_t , which was calculated using Ohm's law. Data were collected using the Acquire and Analyze program, version 1.45 (Physiologic Instruments). Results are plotted as Δ I_{sc} forskolin (response to forskolin) and Δ I_{sc} glybenclamide (response to glybenclamide). During the Ussing chamber analysis, the temperatures (27°C or 37°C) were maintained using a temperature-controlled water bath for at least 30 min before analysis. Following establishment of steady state values of I_{sc} and R_t , forskolin (10 μM), and/or the indicated inhibitors were added to the apical compartments, and I_{sc} and R_t were measured continuously until a new steady state was reached.

Statistical analysis

Results were plotted as means ± standard deviation (S.D.). Statistical significances among means were expressed as p values and were calculated using two-tailed Student's *t* test.

Results

ΔF508 CFTR rescue by permissive temperature in HeLaΔF cells

In order to study the cell surface trafficking of rΔF508 CFTR, we first established whether permissive temperature culture of HeLaΔF cells generates sufficient amounts of fully glycosylated ΔF508 CFTR at the cell surface to measure endocytosis rates. Following culture at 27°C for 48 hours, CFTR was immunoprecipitated from whole cell lysates and analyzed for the presence of maturely glycosylated Band C (rΔF508). The results in Fig. 1A demonstrate that a significant amount of fully glycosylated CFTR is formed during 27°C culture. To confirm that the fully glycosylated rΔF508 CFTR was expressed at the cell surface, cells were surface biotinylated using biotin-LC-hydrazide [9]. Biotinylated CFTR

was detected in HeLa Δ F cells after 27°C culture, but not in cells maintained at 37°C (Fig. 1A). Control cells expressing WT CFTR served as a comparison to illustrate the level of Δ F508 CFTR rescue.

To determine the stability of r Δ F508 CFTR after permissive temperature rescue, WT- or r Δ F508-expressing HeLa cells were raised to the restrictive temperature and the protein half-lives of WT and r Δ F508 CFTR were determined by metabolic pulse-chase analysis. For these experiments, cells were cultured at 27°C for 24 hours and metabolically labeled with ³⁵S-methionine for an additional 18 hours at 27°C (see Experimental Procedures). The cells were then returned to 37°C, and the labeled proteins were chased for the time periods specified. The results indicate that the half-life of WT CFTR is 12 ± 1.5 h, whereas the half-life of r Δ F508 CFTR is 4 ± 1 h (Fig. 1B). The r Δ F508 CFTR protein half-life is much shorter than that of WT CFTR ($p < 0.001$); these results are in agreement with previously published experiments performed in BHK and CHO cells [7, 36].

Extended half-life of r Δ F508 CFTR at permissive temperature in HeLa Δ F cells

Next, we tested whether extended permissive temperature culture would affect the half-lives of WT and r Δ F508 CFTR in HeLa cells. We followed the protocol described above, but instead of transferring the cells to 37°C after metabolic labeling, they were kept at 27°C for the time periods indicated. The results show that at 27°C, the half-lives of WT and r Δ F508 CFTR were 60 ± 11 h and 63 ± 9 h, respectively (Fig. 1C), demonstrating that the instability of r Δ F508 CFTR compared to WT observed at the restrictive temperature is not apparent at the permissive temperature; in other words, that the r Δ F508 half-life defect is a TS defect.

We next sought to determine why r Δ F508 has a short half-life at restrictive temperature by examining the internalization properties of WT and r Δ F508 CFTR in HeLa cells and epithelial cells (CFBE41o- and Calu-3) under both non-polarized and polarized conditions. These experiments served to test cell-type and polarization-specific differences in the trafficking of the two proteins.

r Δ F508 CFTR endocytosis is accelerated in airway epithelial cells

To determine the cell surface stability of WT and r Δ F508 CFTR in HeLa cells at 37°C, we measured their internalization rates using a two-step biotinylation protocol [8]. To measure CFTR endocytosis rates, we oxidized the CHO groups of cell surface glycoproteins at the initial (zero) timepoint (see Experimental Procedures) and then allowed the proteins to internalize for 2.5 minutes, at which time the oxidized glycoproteins remaining at the cell surface were labeled with biotin. An internalization time of 2.5 minutes was chosen for all assays conducted at 37°C because, at this temperature, previous internalization timecourses in multiple cell lines indicated that the periodate-oxidized CFTR modified at the initial timepoint did not recycle back to the cell surface (data not shown). Since the biotin-LC hydrazide is membrane impermeable, the only biotin-accessible CFTR is that which remains on the cell surface during the warm-up period (see Experimental Procedures). Therefore, changes in the surface pool of CFTR after a 2.5 min warm-up period were reflected in a loss of “biotinylatable” CFTR, and this loss corresponds to the amount of CFTR that had internalized from the cell surface.

Following biotin labeling, cells were lysed and total CFTR was immunoprecipitated as described above. At each timepoint, biotinylated CFTR was detected by western blot with avidin-HRP. With this protocol, we do not observe biotin labeling of Band B CFTR unless cells are first permeabilized [9], suggesting that under non-permeabilizing conditions such as those presented herein, our biotinylation method does not label intracellular proteins. The results of these experiments indicate that both WT and r Δ F508 CFTR endocytosis rates are

rapid, with $25 \pm 5\%$ and $27 \pm 5\%$ of the surface pool internalized in 2.5 min, respectively (Fig. 2A). This result is consistent with a previous comparison of WT and r Δ F508 CFTR internalization rates in BHK cells [27].

Since *in vivo* CFTR is expressed on the apical surface in epithelial cells, we next compared the surface stability of WT and r Δ F508 CFTR in both polarized and non-polarized human airway epithelial cells at 37°C. In CFBE41o-WT cells grown on plastic dishes (non-polarized), CFTR endocytosis slowed down to $14 \pm 4\%$ internalized in 2.5 min ($p = 0.02$ compared to HeLa). In contrast, $29 \pm 5\%$ of r Δ F508 CFTR was internalized during the same time period in CFBE41o- Δ F (Fig. 2B), a rate similar to that seen in the HeLa cells ($p = \text{NS}$). Furthermore, the difference between WT and r Δ F508 CFTR internalization rates was more pronounced when the cells were grown as polarized monolayers. In polarized cells, WT CFTR internalization dropped significantly to $3.2 \pm 2\%/2.5$ min ($p < 0.0001$ compared to HeLa), whereas r Δ F508 internalization remained at $30 \pm 3\%/2.5$ min. As a further control, we measured WT CFTR internalization in polarized Calu-3 cells and found a similar result ($5 \pm 1\%/2.5$ min; $p = \text{NS}$), indicating that the surface pool of WT CFTR is very stable in polarized epithelia (Fig. 2C), whereas r Δ F508 CFTR is not. Because it was clear that WT CFTR trafficking did not appear to be faithfully represented in HeLa cells, and our goal was to identify the defect in r Δ F508 CFTR compared to the control WT protein, we focused our efforts on analyzing the r Δ F508 CFTR trafficking defect in human airway epithelial cells.

Shortened cell surface half-life of r Δ F508 CFTR in CFBE41o- Δ F cells

Because polarization affected WT but not r Δ F508 internalization, we next tested the cell surface half-lives of both WT and r Δ F508 CFTR in CFBE41o- cells using a cell surface biotinylation-based assay (Fig. 3). The results indicate that the surface half-life of biotinylated WT CFTR was 8.5 ± 1 h in non-polarized cells (Fig. 3, upper left panel) and 8.3 ± 0.3 h in polarized cells (Fig. 3 upper right panel, $p = \text{NS}$), demonstrating that the stability of the WT protein was not affected by cell polarization. Examination of r Δ F508 CFTR indicated that the surface half-life of the rescued protein was very short, less than 2 h, under both non-polarized and polarized conditions (1.8 ± 0.1 versus 2.0 ± 0.2 h, respectively; $p = \text{NS}$) (Fig. 3, middle panels). Thus, r Δ F508 CFTR surface stability is decreased compared to WT CFTR, and polarization did not affect surface stability of either protein (Fig. 3, lower panel).

Permissive temperature culture stabilizes r Δ F508 CFTR in polarized epithelial cells

Since the r Δ F508 trafficking defect in epithelial cells is due to enhanced endocytosis, we tested whether permissive temperature culture corrected this defect. To answer this question, we compared the effects of 27°C treatment on WT and r Δ F508 CFTR cell surface trafficking in polarized CFBE41o- cells using cell surface half-life and internalization experiments. The results show that both WT and r Δ F508 CFTR are extremely stable at the cell surface at 27°C, with cell surface half-lives much greater than 8 hours (Fig. 4, upper panel). Because CFTR levels were not monitored beyond 8 hours, we cannot calculate to what extent permissive temperature culture stabilized WT or r Δ F508 CFTR. However, since WT and r Δ F508 CFTR surface half-life measurements were similar at 27°C, these results reveal that 27°C treatment eliminated the drastic difference in surface half-life between WT and r Δ F508 CFTR seen at 37°C. Measurement of WT and r Δ F508 CFTR internalization rates at 27°C revealed a dramatic decrease in both cell lines. In fact, CFTR internalization was immeasurable after the 2.5-minute warm-up period. After a 10-minute warm-up period, WT and r Δ F508 CFTR internalization rates were $20 \pm 3\%/10$ min ($p = 0.05$) and $22 \pm 4\%/10$ min ($p = 0.0004$), respectively (Fig. 4, middle and lower panels), which indicated that 27°C treatment eliminated the difference in internalization rates between WT and r Δ F508 CFTR.

Importantly, the results of these studies indicate that both the short surface half-life and the rapid internalization rate of rΔF508 are TS defects.

Permissive temperature culture corrects the functional defect associated with rΔF508

In addition to the trafficking defect, rΔF508 fails to respond to cAMP after forskolin stimulation in CFBE41o-ΔF cells [26]. Since we observed that permissive temperature culture corrects the endocytosis defect in rΔF508 and restores the protein half-life to levels equivalent to WT, we next tested whether it might also correct the functional defect [26]. In these experiments, polarized CFTR-expressing CFBE41o-ΔF monolayers were cultured at 27°C for 48 hours to facilitate rΔF508 expression on the cell surface. These monolayers were then mounted in Ussing chambers, where cAMP-activated I_{sc} was measured at either 37°C or 27°C. 10 μM forskolin was added to the apical compartment to enhance intracellular cAMP levels and activate I_{sc} . When the current reached a maximum and stabilized, glybenclamide was added at increasing concentrations to block I_{sc} . In some experiments, a CFTR-specific inhibitor was used to block currents [23], as previously described [35]. Forskolin was used consistently to activate I_{sc} because we found that, in both WT- and ΔF508-expressing cells, it was sufficient to stimulate cAMP-mediated chloride currents. A cocktail designed to maximally stimulate cAMP responses (forskolin, 3-isobutyl-1-methylxanthine [IBMX], and bromoadenosine-cAMP) did not increase the current beyond the forskolin-induced current. Likewise, glybenclamide was used consistently because the CFTR-specific inhibitor did not further inhibit the currents, indicating that glybenclamide provided maximal CFTR channel inhibition (data not shown). The magnitude of the resulting I_{sc} was compared to parallel CFBE41o-WT controls (see Experimental Procedures). The results show that at 37°C, the cells expressing WT CFTR channels produce an anion current that is readily stimulated by forskolin and completely inhibitable by glybenclamide (Fig. 5, upper left panel), while the cells expressing rΔF508 exhibit very weak responses to both treatments (Fig. 5, middle left panel), in agreement with our previous findings [26]. When the temperature is maintained at 27°C, however, rΔF508 CFTR expressing cells exhibit an anion current similar to cells expressing WT CFTR (Fig. 5, upper right and middle right panels). Direct channel stimulation by genistein further enhanced the rΔF508 CFTR current at 27°C (data not shown), in agreement with a previous observation that at 37°C, rΔF508 CFTR produced a current in response to genistein [29]. This restoration of the functional defect suggests that the loss of cAMP response has been regained at 27°C. Taken together with the previous studies, these experiments reveal that both the surface trafficking and functional activity defects of rΔF508 CFTR are TS.

Pharmacological chaperones correct the internalization defect and increase the surface stability of rΔF508 CFTR

As shown above, maintenance of the TS ΔF508 protein at the permissive temperature corrects multiple trafficking and functional defects, in that 27°C treatment not only rescues ΔF508 CFTR from ERAD, but stabilizes the endocytosis and surface stability defects as long as cells are maintained at 27°C. Based on these results, we asked whether two pharmacological chaperones which facilitate ΔF508 CFTR exit from the ER, a quinazoline compound (CFcor-325) and a bisaminomethylthiazole compound (Corr-4a) [20, 31], have any effect on rΔF508 or WT CFTR cell surface trafficking at 37°C.

First, we studied the role of the compounds on WT and rΔF508 CFTR endocytosis in CFBE41o- cells. For these experiments, CFBE41o-WT or -ΔF cells were cultured for 48 hours at 27°C, followed by a 1-h pre-treatment with 10 μM CFcor-325, Corr-4a, or a vehicle control at 37°C. Internalization assays were then performed at 37°C. The results indicate that both CFcor-325 and Corr-4a decreased the internalization rate of rΔF508 CFTR from 30% to ~5% and ~1%, respectively ($p < 0.005$) (Fig. 6, upper and middle panels).

Interestingly, the compounds had no effect on WT CFTR or transferrin receptor (TR) endocytosis (from the apical surface; Fig. 6, lower panels), suggesting that the effect was specific for rΔF508 CFTR.

Pharmacological chaperones extend the cell surface half-life of rΔF508 CFTR

Next, we monitored the effects of CFcor-325 or Corr-4a on the cell surface half-life of WT and rΔF508 CFTR. For these experiments, CFBE41o-WT or -ΔF cells were cultured for 48 hours at 27°C, followed by treatment with 10 μM CFcor-325, Corr-4a, or a vehicle control at 37°C. CFTR cell surface half-lives were then evaluated in the presence of correctors or the vehicle using the surface biotinylation-based assay. The results indicate that treatment with either small molecule corrector stabilized rΔF508 CFTR compared to untreated controls (Fig. 7): CFcor-325 extended the half-life of rΔF508 CFTR from 2.5 ± 0.4 h to 4.6 ± 0.9 h ($p = 0.004$), and Corr-4a extended the half-life from 2.6 ± 0.6 h to 4.5 ± 1.2 h ($p = 0.03$), indicating that both compounds stabilized the rΔF508 CFTR half-life. Significantly, neither compound affected the half-lives of WT CFTR or TR (Fig. 7, lower panels), suggesting the effects are specific to rΔF508 CFTR.

Discussion

The fate of rΔF508 CFTR at the cell surface after low temperature rescue is one of the first examples of how the cellular quality control mechanisms operate at the plasma membrane/early endosome [27]. Although culture at its permissive temperature allows some of the TS ΔF508 CFTR protein to escape from ERAD, this maturely glycosylated rΔF508 CFTR is rapidly degraded once the temperature is raised to the restrictive temperature, 37°C. The goal of this study was to follow the cell surface fate of rΔF508 at the permissive and restrictive temperatures, 27°C and 37°C, and compare the results to the WT protein. Since CFTR is normally expressed in epithelial cells, we also examined the fate of both proteins in polarized epithelia to determine whether any epithelial-specific differences exist between WT and rΔF508 CFTR surface trafficking. Additionally, we performed functional studies to measure transepithelial chloride currents in response to physiological stimuli, such as cAMP. And finally, upon observing that permissive temperature treatment stabilizes and functionally corrects rΔF508 CFTR at the cell surface, we tested whether pharmacological chaperones that permit escape from ERAD stabilize the rΔF508 surface pool.

A number of studies have shown that culturing cells at 27°C is an efficient method to facilitate ΔF508 CFTR delivery to the cell surface [7, 10, 18–20, 22, 24–27, 31, 36]. However, the fate of the low temperature rescued ΔF508 CFTR at the cell surface has only been followed at 37°C [10, 26, 27]. Herein we show that when the cells are kept at 27°C, the stability of rΔF508 CFTR is enhanced, and the differences between WT and rΔF508 CFTR trafficking and half-life that are seen at 37°C disappear. One potential mechanism for this observation is that protein degradation slows down at the permissive temperature, resulting in accumulation of both the WT and ΔF508 CFTR. This hypothesis is supported by the report that ubiquitination of rΔF508 CFTR is inhibited at 28°C [27]. However, another possibility is that at 27°C, ΔF508 CFTR folds properly and remains in a properly folded state, resulting not only in exit from the ER but also in a more stable surface phenotype at this temperature. When cells are returned to 37°C, the conformation of rΔF508 CFTR reverts to a misfolded stage, as proposed previously [36]. In the misfolded conformation, proteins are more likely to be accessible to ubiquitination and subsequent degradation by cell surface associated mechanisms.

The endocytosis defect of rΔF508 CFTR is eliminated at 27°C, but our data indicate that endocytosis still occurs, albeit more slowly. In a number of cell types, temperatures between 16°C and 22°C block degradation of endocytosed proteins by preventing their transport to

lysosomes [37–39]. $\Delta F508$ CFTR has been shown to accumulate in endocytic-like structures similar to the WT protein when the cells were shifted to 16°C [7], consistent with the idea that the early part of the endocytic pathway still operates at this temperature, but delivery to the later stages is blocked. It remains unclear whether transport to the lysosome, lysosomal processing, or the initial steps of ubiquitin-dependent endocytosis are still functional at 27°C, but it is clear from our results here that internalization of r $\Delta F508$ CFTR is dramatically slowed down to WT levels at 27°C, which is consistent with the latter possibility. Further support comes from proteasomal inhibition studies using lactacystin in BHK cells expressing r $\Delta F508$ CFTR [7]. In these studies, lactacystin treatment dramatically stabilized the surface pool of r $\Delta F508$ CFTR [7], consistent with the idea that the free ubiquitin pool is limiting during proteasomal inhibition.

One important result from our study is that the surface defect of r $\Delta F508$ CFTR is due to an enhanced endocytosis rate compared to WT CFTR. Interestingly, we have also shown here that this defect is only present in polarized epithelial cells, such as the CFBE41o- cells tested here, and not in HeLa cells. Our results demonstrate that WT CFTR endocytosis is dependent both on cellular background and polarization. In non-polarizing HeLa cells, more than 30% of WT CFTR internalizes within 2.5 minutes. In CFBE41o- WT and Calu-3 cells grown on plastic dishes (non-polarized), CFTR internalization slows down to ~15%. When the cells are grown on permeable supports (polarized), CFTR internalization is only 2–5% in 2.5 minutes.

It is tempting to speculate that WT CFTR internalization rates are so low in polarized epithelia because the majority of the WT CFTR is anchored to the cytoskeleton and does not participate in the internalization process. This notion is consistent with the results of Haggie *et al.*, (2006) in which single particle tracking was used to demonstrate that CFTR is coupled to the actin cytoskeleton via EBP50/ezrin and immobilized at the plasma membrane [40].

The difference we observed in WT CFTR internalization rates in polarized epithelia differs from our results in HeLa cells and from the work of Lukacs and coworkers in BHK cells [27]. One explanation for these discordant results is that in non-polarized cells, the cell membrane and the cytoskeleton are less organized compared to polarized cells, resulting in inefficient tethering of WT CFTR and an extended mobile pool available for endocytosis.

Importantly, we observed that the trafficking of r $\Delta F508$ CFTR did not follow the same kinetics as WT CFTR in polarized epithelial cells, at least at 37°C. At this temperature, regardless of cell line or polarization, r $\Delta F508$ CFTR endocytosis rates remain consistently high at about 30% in 2.5 minutes. One possible explanation for this observation is that, in contrast to our model for WT CFTR, the majority of r $\Delta F508$ CFTR is not tethered properly to the cytoskeleton, resulting in a more mobile surface pool. This inefficient cytoskeletal tethering could result from the mutant protein's failure to interact with one or more of the cytosolic factors responsible for stabilization of the WT protein. The idea that r $\Delta F508$ CFTR is not in a large macromolecular complex is supported by the observation that r $\Delta F508$ CFTR is poorly responsive to cAMP-mediated stimuli in CFBE41o- ΔF cells at 37°C [26], suggesting that r $\Delta F508$ CFTR has lost the association with the previously identified apical signaling complex [7, 26, 41]. Alternatively, it is possible that, after rescue from ERAD at permissive temperature, r $\Delta F508$ reverts to a misfolded state when cells are returned to the restrictive temperature. The resulting conformational alterations in the protein may enhance its ability to interact with the clathrin-based endocytic machinery and/or cell surface associated ubiquitination machinery. The consequence is that r $\Delta F508$ CFTR may be endocytosed and degraded in the lysosome more actively than the WT protein at 37°C.

Because improved surface half-life does not necessarily translate to improved channel activity, it is essential to note that the biochemical rescue mediated by permissive temperature culture is accompanied by a functional correction. We showed that after permissive temperature rescue, continued culture at 27°C results in rΔF508 CFTR channels that are functionally similar to WT CFTR channels in their ability to respond to physiological stimuli such as forskolin. This functional correction also supports the idea that under permissive temperature, the rescued protein is associated with the functional complex [26] that allows activation by cAMP. The idea that the channel activity is TS is further supported by a recent report on ΔF508 CFTR with two altered RXR motifs that demonstrated that the single channel activity of the rescued mutant decreased as the temperature increased from 30 to 37°C [42].

Since extended culture at the permissive temperature slowed endocytosis rates of rΔF508 CFTR to WT levels in addition to mediating its escape from ERAD, an obvious question was whether chemical correctors that facilitate ΔF508 CFTR exit from the ER would also provide rΔF508 cell surface stability at 37°C. The results illustrated that two small molecules known to rescue ΔF508 CFTR from ERAD, CFcor-325 and Corr-4a, also stabilized the mutant protein at the cell surface. Importantly, these compounds had no effect on endocytosis or protein half-life of two other cell surface molecules, WT CFTR and TR, suggesting that the effects are rΔF508 CFTR specific. Further support for the idea that the compounds are specific comes from a recent report by Clarke and coworkers demonstrating that these compounds interact directly with CFTR [43].

A significant result of this study is that both compounds corrected the rΔF508 internalization defect to WT rates. Despite this effect, although the surface half-life of the rescued protein increased with these compounds, the corrected half-life remained significantly shorter than WT. This result indicates that correcting the internalization defect of the rΔF508 protein may not be sufficient for optimal stabilization, and supports previous findings that impaired recycling is a critical component in the compromised surface stability of rΔF508 CFTR [27].

In summary, in polarized epithelial cells, permissive temperature culture of a TS mutant, ΔF508 CFTR, not only rescues it from ERAD but also stabilizes it at the cell surface and restores its cAMP responsiveness, suggesting that both the stability and the functional defects at the cell surface are TS, not just the maturation defect. Two pharmacological chaperones, CFcor-325 and Corr-4a, mediate an effect similar to permissive temperature treatment, but the rΔF508 correction of cell surface half-life is only partial. Our results indicate that permissive temperature culture of a clinically relevant TS ER processing mutant can facilitate correction of both cell surface trafficking and functional defects, and offer hope that the treatments that enhance the release of misfolded proteins from the ER may also benefit protein stability and function at the cell surface. Understanding this process in more detail will bring a broader based knowledge of how pharmacological chaperones and different classes of compounds can be used to promote protein function and stability of this and other TS mutations.

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Abbreviations

cAMP

cyclic AMP

CF	cystic fibrosis
CFTR	cystic fibrosis transmembrane conductance regulator
ΔF508 CFTR	CFTR with deleted phenylalanine at position 508
ERAD	endoplasmic reticulum-associated degradation
I_{sc}	short-circuit current
PKA	protein kinase A
rΔF508	rescued Δ F508
R_t	electrical resistance
TR	transferrin receptor
TS	temperature sensitive
WT	wild-type

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Fig. 1. $\Delta F508$ CFTR rescue and stability in HeLa cells at. A. Low-temperature (27°C) rescue of $\Delta F508$ CFTR in HeLa ΔF cells

CFTR was immunoprecipitated from HeLa ΔF cells that had been cultured at 27°C or 37°C for 48 h or HeLaWT cells cultured at 37°C, analyzed by SDS-PAGE, Western blotted, and detected with anti-CFTR NBD2 polyclonal antibody (left panel). Arrows indicate ER (Band B) and post-ER forms (Band C) of CFTR. The presence of r $\Delta F508$ CFTR at the cell surface (Band C) was confirmed by cell surface biotinylation, immunoprecipitation of CFTR and Western blotting with HRP-avidin (right panel). **B. WT and r $\Delta F508$ half-lives at 37°C.** CFTR half-lives were measured in metabolic pulse-chase experiments (see Experimental Procedures). HeLa ΔF and HeLaWT cells were cultured at 27°C for 24 hours, followed by metabolic labeling for 18 hours and chase at 37°C for 18 hours. At the time points indicated, cells were subjected to lysis, CFTR immunoprecipitation, SDS PAGE and phosphorimaging. Representative gels of pulse-chase experiments for WT and r $\Delta F508$ CFTR (upper and middle panels) and calculated half-lives (lower panel) are shown, n=4. **C. Extended half-lives of WT and r $\Delta F508$ CFTR at 27°C.** Pulse-chase experiments were performed in HeLa ΔF and HeLaWT cells as described in B except that the chase was maintained at 27°C (see Experimental Procedures). Representative images (upper and middle panels) and calculated half-lives (lower panel) are shown, n=4.

Fig. 2. rΔF508 CFTR endocytosis in HeLa and airway epithelial cells. A. Internalization of WT and rΔF508 CFTR in HeLa cells
 CFTR endocytosis was measured using a modified biotinylation assay (see methods). Representative gels of immunoprecipitated (total) and cell surface biotinylated WT and ΔF508 CFTR (surface) at 0 and 2.5 minutes of internalization are shown. Internalization rates of WT and rΔF508 CFTR are plotted as % decrease in CFTR Band C density after a 2.5-minute internalization (lower panels, n=5, mean ± S.D). **B. WT and rΔF508 CFTR internalization in non-polarized and polarized epithelial cells.** CFBE41o- ΔF and CFBE41o- WT cells grown under non-polarized (plastic dishes, left panels) or polarized (permeable supports, right panels) conditions were tested for CFTR internalization. Representative gels and summary of 5 individual experiments are shown. Internalization rates are plotted as % density decrease of cell surface Band C CFTR after a 2.5-minute internalization compared to 0 min (n=5, mean ± S.D). **C. WT CFTR internalization in polarized Calu-3 cells.** Calu-3 cells were grown under polarized (permeable supports) conditions were tested for CFTR

internalization. A representative gel showing cell-surface biotinylated WT CFTR (surface) at 0 and 2.5 minutes of internalization are shown, n=4.

Fig. 3. Cell surface half-lives of WT and r Δ F508 CFTR in CFBE41o- cells

Representative gels of WT (upper panels) and r Δ F508 CFTR (middle panels) half-life measurements performed at 37°C, under non-polarized and polarized conditions, are shown. Half-lives were calculated using densitometry followed by analysis as described previously (34), and values were plotted in the lower panel (n=4, mean $\pm$ S.D.).

Fig. 4. Surface stability of r $\Delta F508$ CFTR at 27°C in airway epithelial cells

Cell surface stability (upper panels) and internalization rates (middle panels) of CFTR were measured at 27°C (see Experimental Procedures). In polarized CFBE41o- cells, both WT and r $\Delta F508$ CFTR are extremely stable at 27°C. There was no detectable decrease of biotinylated band C CFTR during the 8 hours of chase. Representative gels from CFBE41o-WT and CFBE41o- ΔF are shown (upper panels). For internalization rates, total and cell surface CFTR were detected. Representative gels (middle panels) and summary of four individual experiments (lower panel) are shown. Internalization rates are plotted as % of density decrease of Band C CFTR at 10 min, n=4.

Fig. 5. Ussing chamber analysis of rΔF508 CFTR after low temperature correction
Polarized CFBE41o-WT and CFBE41o-ΔF508 monolayers were cultured at 27°C for 48 hours. Cells were then mounted in Ussing chambers and temperature-equilibrated (37°C or 27°C, as indicated) for 30 minutes, followed by measurement of baseline steady state I_{sc} and R_t values. Forskolin (FSK, 10 μM) was added to the apical chambers, and I_{sc} and R_t values were monitored until a new baseline was obtained. The indicated concentrations of glybenclamide (GLYB) were then added apically, and I_{sc} and R_t values were monitored. Representative traces (upper and middle panels) and graphical summaries (lower panels) of $n = 5$ are shown. Results are plotted as ΔI_{sc} (μA/cm²) of FSK, which represents a positive current following FSK activation, or ΔI_{sc} (μA/cm²) of Glyb, which represents a negative current following administration of GLYB to block channel activity. ΔF508 CFTR-expressing monolayers exhibited significantly blunted responses to FSK and GLYB compared to WT CFTR-expressing monolayers at 37°C (left panel). These responses were significantly enhanced when monolayers were maintained at permissive temperature (27°C; right panel; * $p < 0.005$, + $p < 0.01$ (bottom panel)), so that there were no significant differences between WT and rΔF508 CFTR responses at this temperature (bottom panel).

Fig. 6. CFcor-325 and Corr-4a increase the stability of WT and rΔF508 CFTR

Internalization assays for WT and rΔF508 CFTR were performed at 37°C in CFBE41o-ΔF and CFBE41o-WT cells following low temperature rescue in the presence or absence of CFcor-325 or Corr-4a. Representative gels (upper panels) and summary of four experiments (middle panels) are shown for each corrector. TR internalization rates, measured under identical conditions, are plotted in the lower panels. WT CFTR and TR internalization rates were tested as controls. About 5% of WT CFTR and ~30% of rescued ΔF508 CFTR internalized in 2.5 minutes in untreated cells. Both CFcor-325 and Corr-4a treatment decreased rΔF508 CFTR internalization in CFBE41o-ΔF cells significantly. No changes in WT and TR internalization rates were measured, n=4 (p<005).

Fig. 7. CFcor-325 and Corr-4a extend the cell surface half-life of rΔF508 CFTR

Polarized CFBE41o-ΔF and CFBE41o-WT cells were cultured at 27°C for 48 hours, returned to 37°C, and treated with CFcor-325 or Corr-4a for 8 hours. Cell surface CFTR half-lives were measured following cell surface biotinylation (see Experimental Procedures). Representative gels and a summary of four experiments are shown (upper and middle panels). TR half-life was also measured in the presence or absence of correctors as additional control (lower panel), n=4.