Conformational and Temperature-sensitive Stability Defects of the ΔF508 Cystic Fibrosis Transmembrane Conductance Regulator in Post-endoplasmic Reticulum Compartments*

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Deletion of phenylalanine at position 508 (ΔF508) is the most common cystic fibrosis (CF)-associated mutation in the CF transmembrane conductance regulator (CFTR), a cAMP-regulated chloride channel. The consensus notion is that ΔF508 imposes a temperature-sensitive folding defect and targets newly synthesized CFTR for degradation at endoplasmic reticulum (ER). A limited amount of CFTR activity, however, appears at the cell surface in the epithelia of homozygous ΔF508 CFTR mice and patients, suggesting that the ER retention is not absolute in native tissues. To further elucidate the reasons behind the inability of ΔF508 CFTR to accumulate at the plasma membrane, its stability was determined subsequent to escape from the ER, induced by reduced temperature and glycerol. Biochemical and functional measurements show that rescued ΔF508 CFTR has a temperature-sensitive stability defect in post-ER compartments, including the cell surface. The more than 4–20-fold accelerated degradation rate between 37 and 40 °C is, most likely, due to decreased conformational stability of the rescued ΔF508 CFTR, demonstrated by in situ protease susceptibility and SDS-resistant thermoaggregation assays. We propose that the decreased stability of the spontaneously or pharmacologically rescued mutant may contribute to its inability to accumulate at the cell surface. Thus, therapeutic efforts to correct the folding defect should be combined with stabilization of the native ΔF508 CFTR.

Cystic fibrosis (CF) is one of the most prevalent lethal genetic disorders among Caucasian populations (1). The CF gene encodes the cystic fibrosis transmembrane conductance regulator (CFTR), a cAMP-regulated Cl⁻ channel and conductance regulator, expressed at the apical membrane of secretory epithelia (2, 3). CFTR, a member of the ABC transporter family, consists of two structurally homologous halves, each comprised of six transmembrane (TM) helices and a nucleotide binding domain (NBD). The most common cystic fibrosis (CF)-associated mutation, deletion of phenylalanine at position 508 (ΔF508) in the NBD1, is found in >90% of the patients and detected in ~70% of CF chromosomes (1, 7). It is believed that deletion of Phe-508 interrupts the posttranslational folding of CFTR (4, 5, 9–11) and targets the core-glycosylated folding intermediate for degradation, predominantly via the ubiquitin-proteasome pathway at the ER (12, 13). Exposure of ER-retention signals may contribute to the inability of folding intermediate(s) to exit the ER (14). Accordingly, negligible expression of ΔF508 CFTR could be detected at the cell surface by immunochemical techniques in recombinant cells, CF primary airway cells, and CF tissues (9, 15, 16).

The recognition that the ΔF508 CFTR channel is functional both in vivo (17–19) and after its reconstitution into the phospholipid bilayer (20) suggested that the CF phenotype could be alleviated by relocating the mutant CFTR from the ER to the plasma membrane. Reduced temperature (10, 21, 22), chemical chaperones (23–25), and down-regulation of Hsp70 (26, 27) activity are thought to partially revert the folding defect of ΔF508 CFTR and promote the accumulation of the functional channel at the cell surface. Importantly, using more sensitive electrophysiological techniques, constitutive accumulation of ΔF508 CFTR was documented in the plasma membrane of primary epithelia from ΔF508 homozygous mice (21, 28) and in the intestinal and gallbladder epithelia of homozygous ΔF508 patients (29, 30). These studies parallel the results of recent immunolocalization reports to some extent and suggest that the processing defect of the ΔF508 CFTR is tissue-specific (30–33). If the ER retention of the mutant is not complete, accelerated disposal from the post-ER compartments could contribute to its inability to express at the physiological level in certain tissues.

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1 The abbreviations used are: CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; ER, endoplasmic reticulum; BFA, brefeldin A; CHX, cycloheximide; eno-H, endoglycosidase H; PNGase F, peptide N-glycanase; HA, hemagglutinin; mAb, monoclonal antibody; TM, transmembrane; NBD, nucleotide binding domain; wt, wild type; ECL, enhanced chemiluminescence; NHS-SS-biotin, sulfo-succinimidyl-2-(biotinamido)ethyl-1,2-dithiopropionate.
Indeed, based on indirect evidence, we proposed that the rescued ΔF508 CFTR has a short residence time at the cell surface of Chinese hamster ovary cells (CHO), expressing ΔF508 CFTR heterologously (34). However, neither this nor any other study has provided direct biochemical or structural information regarding the behavior of ΔF508 CFTR following its escape from ER.

Here we provide direct biochemical and functional evidence for the biological instability of the complex-glycosylated ΔF508 CFTR. Furthermore, we show that the stability defect is temperature-sensitive, which is likely due to an attenuated conformational stability of the native state, demonstrated by increased protease susceptibility and the thermoaggregation tendency of the rescued mutant relative to its wt counterpart. The implications of these observations are fundamental with respect to understanding the cellular phenotype and designing more efficient therapeutic strategies in CF.

EXPERIMENTAL PROCEDURES

Cell Lines—A mixture of stably transfected baby hamster kidney (BHK) cells, expressing human wt and ΔF508 CFTR with a carboxy-terminal hemagglutinin (HA) epitope, was generated and maintained as described (4). Characterization of the HA-tagged CFTR variants will be described elsewhere.

Isolation of Microsomes—Isolation of ER, Golgi, and plasma membrane-enriched microsomes from BHK cells was performed using nitrogen cavitation and differential centrifugation as described (11). Where specified, the core-glycosylated wt or mutant CFTR was eliminated from the cells during a 3-h incubation in the presence of cycloheximide (CHX, 100 μg/ml). The microsomal pellet was resuspended in HSE medium (10 mM sodium HEPES, 0.25 M sucrose, pH 7.6) and used either immediately or after being snap-frozen in liquid nitrogen.

Limited Proteolysis and Glycosidase Digestion—Microsomes were isolated from ΔF508 and wt CFTR-expressing BHK cells and incubated at a protein concentration of 1.5–1.5 and 0.6–1.0 mg/ml, respectively, in the presence of trypsin or proteinase K for 15 min at 4 °C in digestion buffer (phosphate-buffered saline) as described. Proteolysis was terminated by the addition of phenylmethylsulfonyl fluoride to 1 mM, and samples were immediately denatured in 2× Laemmli sample buffer at 37 °C for 20 min.

To distinguish between high mannose and complex-type N-linked oligosaccharide modification of CFTR, cell lysates were incubated with endoglycosidase H (endo-H) and peptide N-glycosidase F (PN-Gase-F)-sensitive, core-glycosylated polypeptide with an apparent molecular mass ~140–150 kDa (Fig. 1a, empty arrowhead) at 37 °C (9). The processing defect of the ΔF508 CFTR could be partially overcome by the combination of glycerol and low temperature treatment, similarly to its nontagged counterpart, as reported in a number of cultured cells (10, 23, 24). Following the optimization of the rescue conditions, accumulation of the complex-glycosylated ΔF508 CFTR was indicated by the appearance of endo-H-resistant immunoreactive polypeptide with an apparent molecular mass ~170 kDa (Fig. 1a, black arrowhead) (9). The N-linked oligosaccharide modification and the apparent molecular mass of the rescued ΔF508 CFTR are virtually identical to that of the HA-tagged complex-glycosylated wt CFTR (Fig. 1).

To verify that the deletion of Phe-508 destabilizes the complex-glycosylated wt and rescued ΔF508 CFTR was determined upon inhibition of protein biosynthesis with CHX or vesicular transport from ER to Golgi with brefeldin A (BFA). After the accumulation of the complex-glycosylated ΔF508 CFTR at 26 °C, cells were incubated in the presence of CHX or BFA at 37 °C, and the remaining CFTR was measured with quantitative immunoblotting, using anti-HA mAb as a function of incubation time (Fig. 1, b and c). Densitometry revealed that the half-life of the complex-glycosylated ΔF508 CFTR (τ1/2 ~ 5 h) is at least four times shorter than that of the wt CFTR (τ1/2 ~ 22 h) at 37 °C, regardless of the inhibitor (Fig. 1d). Similarly fast disposal of the ΔF508 CFTR (τ1/2 ~ 5 h) could be observed in the absence of CHX or BFA, after shifting the temperature from 26 to 37 °C during the chase (Fig. 1c).

To verify that the deletion of Phe-508 destabilizes the complex-glycosylated CFTR, metabolic pulse-chase experiments were performed on transfectants expressing wt or rescued ΔF508 CFTR (Fig. 2a). Phosphorimage analysis confirmed that the complex-glycosylated ΔF508 was eliminated four times faster (τ1/2 ~ 4.5 h) than wt CFTR (τ1/2 ~ 18 h) at 37 °C (Fig. 2b). Similar turnover rates were obtained upon rescuing the mutant Pierce) three times for 15 min at 37 °C. Following the solubilization of the cells in RIPA buffer, biotinylated CFTRs were affinity-isolated on streptavidin-Sepharose (Sigma), separated with SDS-polyacrylamide gel electrophoresis, and visualized with anti-HA mAb and ECL.

RESULTS

Deletion of Phe-508 Compromises the Stability of CFTR in the Post-ER Compartments—To attain high sensitivity immunodetection, the influenza HA epitope-tagged ΔF508 and wt CFTR were expressed stably in BHK cells. Immunoblot analysis showed that the HA-tagged ΔF508 CFTR appears as an endoglycosidase H (endo-H)- and peptide N-glycosidase F (PN-Gase-F)-sensitive, core-glycosylated polypeptide with an apparent molecular mass ~140–150 kDa (Fig. 1a, empty arrowhead) at 37 °C (9). The processing defect of the ΔF508 CFTR could be partially overcome by the combination of glycerol and low temperature treatment, similarly to its nontagged counterpart, as reported in a number of cultured cells (10, 23, 24). Following the optimization of the rescue conditions, accumulation of the complex-glycosylated ΔF508 CFTR was indicated by the appearance of endo-H-resistant immunoreactive polypeptide with an apparent molecular mass ~170 kDa (Fig. 1a, black arrowhead) (9). The N-linked oligosaccharide modification and the apparent molecular mass of the rescued ΔF508 CFTR are virtually identical to that of the HA-tagged complex-glycosylated wt CFTR (Fig. 1).

The biological stability of the complex-glycosylated wt and rescued ΔF508 CFTR was determined upon inhibition of protein biosynthesis with CHX or vesicular transport from ER to Golgi with brefeldin A (BFA). After the accumulation of the complex-glycosylated ΔF508 CFTR at 26 °C, cells were incubated in the presence of CHX or BFA at 37 °C, and the remaining CFTR was measured with quantitative immunoblotting, using anti-HA mAb as a function of incubation time (Fig. 1, b and c). Densitometry revealed that the half-life of the complex-glycosylated ΔF508 CFTR (τ1/2 ~ 5 h) is at least four times shorter than that of the wt CFTR (τ1/2 ~ 22 h) at 37 °C, regardless of the inhibitor (Fig. 1d). Similarly fast disposal of the ΔF508 CFTR (τ1/2 ~ 5 h) could be observed in the absence of CHX or BFA, after shifting the temperature from 26 to 37 °C during the chase (Fig. 1c).

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FIG. 1. Stability of the complex-glycosylated ΔF508 and wt CFTR cellular pool. The expression of HA-tagged wt and ΔF508 CFTR in stably transfected BHK cells was monitored with immunoblotting, using anti-HA mAb and ECL. Black arrowhead, complex-glycosylated (band C); empty arrowhead, core-glycosylated (band B); gray arrowhead, unglycosylated CFTR (band A). a, the processing defect of the ΔF508 CFTR was partially reverted by incubating the cells in medium supplemented with 10% glycerol at 26 °C for overnight. Endo-H and PNGase F digestions were performed as described under "experimental Procedures." To facilitate immunodetection of the rescued mutant, cells were incubated for 1 h with cycloheximide. 50, 100, and 20 μg of protein were separated from ΔF508, rescued ΔF508, and wt CFTR expressors, respectively. b, the disappearance of complex-glycosylated wt CFTR was monitored in the presence of BFA (5 μg/ml) or CHX (100 μg/ml). Subsequent to the indicated chase, cells were solubilized and equal amounts of protein (20 μg) were immunoblotted with anti-HA mAb. c, a similar approach was used to determine the in vivo stability of the complex-glycosylated ΔF508 CFTR, after a 24-h rescue treatment (see a). Expression of Na⁺/K⁺-ATPase was monitored with anti-Na⁺/K⁺-ATPase mAb. For reference, total protein from untreated wt (20 μg) and ΔF508 CFTR expressors (100 μg) were loaded (two lanes on the far right). d, half-life determination of the wt and mutant CFTR. The complex-glycosylated wt and ΔF508 CFTR persisting in the cell was calculated from densitometry of immunoblots shown on b and c. The chase was performed in the absence or presence of CHX or BFA, as indicated at 37 °C. Data are expressed as percentage of the initial complex-glycosylated wt or ΔF508 CFTR. Mean ± S.E. (n = 3–8).
Tant with either glycerol or reduced temperature alone, and on mock-treated wt CFTR, ruling out that osmotic or cold stress can account for the difference (data not shown). The accelerated disappearance of $\Delta F508$ CFTR also cannot be attributed to the loss of the carboxyl-terminal epitope, because similar half-lives were measured with antibodies to epitopes located in NBD1, NBD2, or at the amino-terminal tail (data not shown). Finally, preliminary results obtained on pancreatic ductal epithelia expressing the $\Delta F508$ CFTR constitutively showed that the mutation impairs the stability of CFTR in polarized epithelia to a degree comparable with that found in nonpolarized cells (data not shown). These results collectively support the notion that the instability is an intrinsic property of the rescued $\Delta F508$ CFTR rather than epitope- or cell-specific.

Biochemical and Functional Stability of $\Delta F508$ CFTR at the Cell Surface—To examine whether the turnover of the plasma membrane-associated mutant CFTR is similar to that of the complex-glycosylated $\Delta F508$ CFTR pool, which comprises the trans-Golgi network, secretory vesicles, and endosomes as well, the fate of the rescued $\Delta F508$ CFTR was followed with cell surface biotinylation and iodide efflux measurements.

The plasma membrane proteins of BHK cells, expressing $\Delta F508$, rescued $\Delta F508$, or wt CFTR, were covalently tagged with NHS-SS-biotin, affinity-isolated on streptavidin beads, and immunoblotted with anti-HA mAb. Both the rescued $\Delta F508$ and the wt CFTR are amenable to biotinylation, in contrast to the ER-resident, core-glycosylated $\Delta F508$ CFTR (Fig. 3a). Densitometric analysis revealed that $\sim50\%$ of the biotinylated $\Delta F508$ CFTR disappeared after 4 h and became undetectable by 10 h of chase at 37 °C (Fig. 3b). In contrast, the turnover of biotinylated wt CFTR was more than 4-fold slower ($t_{1/2} \sim 18$ h, data not shown) than the rescued $\Delta F508$ CFTR but comparable with the complex-glycosylated wt CFTR pool (Fig. 1d and 25).

The lack of endogenous cAMP-dependent anion conductance of BHK cells permitted us to monitor the arrival of functional $\Delta F508$ CFTR to the plasma membrane by the iodide efflux assay. At permissive temperatures, the cAMP-stimulated iodide release was proportional with the length of the rescue period up to 8 h (Fig. 4a, inset). After allowing $\Delta F508$ CFTR to accumulate at the cell surface for 5 h, raising the temperature to 37 °C evoked a rapid disappearance of the mutant. The amount of iodide released by cAMP-dependent protein kinase stimulation decreased by 50% after 4 h and became undetectable after 10 h of chase at 37 °C (Fig. 4, a and b) in cells expressing rescued $\Delta F508$, whereas no decrease was apparent over 10 h in wt CFTR expressors. Because the cAMP-activated iodide release could not be detected in rescued mock-transfected and parental BHK cells (data not shown), the functional and the biotinylation studies jointly indicate that the $\Delta F508$ CFTR channels are as unstable at the cell surface as in the post-ER compartments.

Taken together, the immunoblot, metabolic pulse-chase, biotinylation, and iodide measurements provide the first direct evidence that the functional and biochemical half-life of the complex-glycosylated $\Delta F508$ CFTR is 4–5-fold shorter than its wt counterpart at 37 °C, suggesting that structural differences may persist between the rescued $\Delta F508$ and wt CFTR at the plasma membrane.

The Thermostability of Rescued $\Delta F508$ CFTR in Vivo—To test whether the stability defect of the complex-glycosylated $\Delta F508$ CFTR at 37 °C is related to its thermolability, pulse-labeled cells were chased at temperatures ranging from 28 to 40 °C. Although the $t_{1/2}$ of wt and rescued $\Delta F508$ CFTR converged at 28 °C (data not shown), a striking difference became apparent at temperatures above 30 °C. Rescued $\Delta F508$ CFTR was at least 20-fold more unstable ($t_{1/2} \sim 0.8$ h) than its wt counterpart ($t_{1/2} \sim 18$ h) at 40 °C (Fig. 5a and b). In sharp contrast, no difference could be resolved in the relative turnover rates of the core-glycosylated (ER-resident) wt and $\Delta F508$ CFTR between 28 and 40 °C (Fig. 5b), consistent with the notion that the core-glycosylated form represents a common folding intermediate, which is less susceptible to thermal denaturation (4, 5, 11). Considering that unfolding of soluble and membrane pro-
teins upon heat shock accelerates their cellular degradation (36), our results suggest that the thermal resistance of the complex-glycosylated ΔF508 CFTR toward unfolding is substantially lower than its wt counterpart. Because large quantities of purified CFTR are not available to test this hypothesis directly, the protease susceptibility of native and the thermoaggregation propensity of solubilized CFTR variants were measured as indirect indicators of their structural stability.

The Impact of ΔF508 Mutation on the Thermoaggregation and Protease Susceptibility of CFTR—SDS-solubilized cell lysates, obtained from BHK cells expressing ΔF508, rescued ΔF508, or wt CFTR, were heat-denatured at temperatures ranging between 37 and 100 °C. Insoluble aggregates were sedimented by centrifugation, and monomeric CFTR remaining in the supernatant was quantified by immunoblotting. The thermostability of solubilized CFTR was characterized by measuring the aggregation temperature ($T_a$), at which 50% of monomeric CFTR is converted into SDS-resistant aggregates (Fig. 6a).

The $T_a$ of rescued ΔF508 CFTR was 10 °C lower ($T_a \sim 65 ^\circ C$) than its wt counterpart ($T_a \sim 75 ^\circ C$) but 10 °C higher than the core-glycosylated ΔF508 CFTR ($T_a \sim 55 ^\circ C$) (Fig. 6b). The following observations suggest that the progressively decreasing thermostability of the rescued and core-glycosylated ΔF508 CFTR is likely the consequence of structural differences. Firstly, distinct aggregation pattern was also measured on immunoprecipitated wt and mutant CFTR (data not shown), implying that their aggregation propensity is independent of other polypeptides. Secondly, no difference could be documented in the thermoaggregation of the polytopic Na$^+$/K$^+$-ATPase, in parental BHK cells, or in cells expressing wt, ΔF508, or rescued ΔF508 CFTR (Fig. 6c). Conversely, ERP72
heat-denaturation was performed in Laemmli sample buffer for 2 h at 37 °C. Subsequent chase was performed at the indicated temperatures. For comparison, both wt and rescued ΔF508 CFTR were loaded. The turnover of the core- and complex-glycosylated ΔF508 and wt CFTR was determined with phosphorimaging analysis from experiments shown on a (means  ±  S.E., n = 3–4, inset and data not shown). The ratio of complex-glycosylated wt and ΔF508 CFTR half-lives (filled circle) is plotted as a function of temperature. In contrast, the relative turnover rate of the core-glycosylated wt and mutant CFTR (empty circles) is insensitive to the same temperature range.

FIG. 5. The stability defect of the rescued ΔF508 CFTR is temperature-sensitive. a, after the pulse labeling of wt (20 min at 37 °C) and ΔF508 CFTR (3 h at 26 °C in the presence of 10% glycerol) expressors, the conversion of core- to complex-glycosylated form was allowed to occur for 2 h at 37 °C. Subsequent chase was performed at the indicated temperatures. For comparison, both wt and rescued ΔF508 CFTR were loaded. b, the turnover of the core- and complex-glycosylated ΔF508 and wt CFTR was determined with phosphorimage analysis from experiments shown on a (means  ±  S.E., n = 3–4, inset and data not shown). The ratio of complex-glycosylated wt and ΔF508 CFTR half-lives (filled circle) is plotted as a function of temperature. In contrast, the relative turnover rate of the core-glycosylated wt and mutant CFTR (empty circles) is insensitive to the same temperature range.

and GRP78, soluble ER proteins, were resistant to aggregation, presumably due to their fully denatured state in SDS, in contrast to polytopic membrane proteins, which tend to preserve their aggregation tendency following detergent solubilization (37) (Fig. 6c). Finally and most importantly, no significant difference between the thermoaggregation tendency of ΔF508, rescued ΔF508, and wt CFTR was detected (Tₐ ≈ 75 °C) when heat-denaturation was performed in Laemmli sample buffer supplemented with 8 M urea (Fig. 6, a and b); suggesting that urea denaturation of residual structural elements, prevailing in SDS micelles (37), abolishes the differences in the thermostability of CFTR variants.

Limited proteolysis in conjunction with immunoblot analysis was used as an alternative and more direct method to demonstrate that the rescued ΔF508 CFTR is structurally distinct from its wt counterpart. This approach was instrumental in revealing the distinct conformation of the cytosolic domains (representing more than 70% of the CFTR polypeptide) in the complex-glycosylated wt and the core-glycosylated ΔF508 CFTR and to monitor the folding of wt CFTR (11). Microsomes were isolated with differential centrifugation, and the cleavage patterns of wt, ΔF508, and rescued mutant CFTR, obtained with limited trypsin and proteinase K digestions, were visualized with immunoblotting. CHX treatment of the cells ensured that the core-glycosylated CFTR was degraded prior to the isolation of microsomes, enriched in the complex-glycosylated wt or rescued ΔF508 CFTR (Fig. 7a).

The in situ protease resistance of rescued ΔF508 CFTR to trypsin and proteinase K was consistently 2-fold lower than that of the wt CFTR, regardless of whether the NBD2- or the NBD1-specific mouse monoclonal M3A7 and L12B4 anti-CFTR antibody was used (Fig. 7, b and c, and data not shown). Differences in the banding patterns between the rescued ΔF508 and wt CFTR could also be recognized, which was more obvious with the NBD2-specific M3A7 than with the NBD1-specific L12B4 mAb. This suggests that the altered accessibility of the proteolytic cleavage sites are not restricted to the NBD1, but encompasses the NBD2 in the rescued ΔF508 CFTR (Fig. 7, b and c). A more substantial difference was observable in the protease resistance and proteolytic digestion patterns of the rescued and core-glycosylated ΔF508 CFTR (Fig. 7, b and c). Because neither the location, N-linked glycosylation, nor the association with peripheral membrane proteins alters the protease susceptibility of the wt CFTR (11), these results imply that the conformation and/or conformational stability of the rescued ΔF508 CFTR lies between that of the core-glycosylated ΔF508 CFTR and the fully mature wt CFTR.

DISCUSSION

While “saturation” of the ER quality control cannot be precluded in heterologous systems overexpressing ΔF508 CFTR, this is not the case in the homozygous knock-in transgenic mouse, expressing ΔF508 CFTR under its endogenous promoter (21, 28). Short circuit current measurements have shown that at least 4% of the wt CFTR activity is present in the apical membrane of the intestinal epithelium and approximately 1–2% in cultured gall bladder epithelium of the homozygous ΔF508 mouse, which could be augmented by 16–18-fold at the reduced temperature (21). Extrapolating from our results, stabilization of the ΔF508 CFTR could be responsible for a 4-fold increase of the plasma membrane chloride conductance, whereas the rest of the difference could be attributed to increased folding efficiency. Conversely, at physiological temperature, the short residence time of the spontaneously or pharmacologically rescued ΔF508 CFTR compromises its ability to accumulate at the plasma membrane, thus providing additional explanation for the difficulties in detecting spontaneously escaped ΔF508 CFTR both in native tissues and in cultured cells (9, 15, 16).

A number of mechanisms, or the combination thereof, could explain the biological instability of the complex-glycosylated ΔF508 CFTR. First, structural alterations may accelerate the endocytosis and inhibit the recycling of the ΔF508 CFTR from the endosomes to the cell surface, promoting the proteolytic degradation in the endolysosome. Second, preferential delivery of the mutant from post-ER compartments to the lysosomes may occur, similarly to the progressive aggregation of furin in the trans-Golgi network (38). Because repeated attempts failed to detect aggregated ΔF508 CFTR in the detergent-insoluble fractions and the cell surface appearance of the mutant was
verified with both biochemical and functional assays, this scenario is unlikely to be the case. Finally, deletion of Phe-508 may promote the exposure of a dispersed degradation signal, comprised of hydrophobic patches and flexible loops in the rescued mutant. These motifs, similar to those described in the hydroxymethylglutaryl-CoA reductase (39) and conceivably exposed in the structurally destabilized G-protein-coupled receptor (40), can be recognized by the cellular proteolytic mechanisms and are perhaps responsible for the degradation.

Whether a global structural destabilization, affecting the cytosolic and the transmembrane domains, is exclusively responsible for the accelerated degradation of the complex-glycosylated ΔF508 CFTR or altered targeting mechanisms are also involved remains to be established. Based on the observations that structural destabilization of carboxyl-terminally truncated CFTR and G-protein-coupled receptors coincides with their accelerated disposal, and in the case of the mutant CFTR this process depends upon the activity of the ubiquitin-proteasome degradation pathway, we favor the third scenario.

The indistinguishable in vivo and in vitro protease susceptibility profile of the core-glycosylated ΔF508 and the early folding intermediate of wt CFTR, with the absence of aggregated ΔF508 CFTR in the detergent-insoluble fraction, suggested that ΔF508 favors the formation of folding intermediate(s) (11). This may occur by imposing a kinetic block on the folding reaction of CFTR, by energetically destabilizing the native form, or a combination of these. Whereas previous data obtained on recombinant NBD1 domain are compatible with a kinetic block (41, 42), the present results and data derived using synthetic peptides (43) suggest that deletion of Phe-508 has multiple effects. The mutation not only interferes with the posttranslational folding in a temperature-dependent manner, as reported by a number of laboratories (4, 5, 10, 11, 21), but

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Fig. 6. Thermoaggregation of ΔF508 and wt CFTR. a, wt, ΔF508, and rescued ΔF508 CFTR-expressing cells were solubilized in 2× Laemmli sample buffer, and samples were incubated at the indicated temperature for 5 min. Aggregates were sedimented, and wt, ΔF508, and rescued ΔF508 CFTR remaining in the supernatant were visualized with anti-HA mAb and ECL. To eliminate the core-glycosylated forms in wt and rescued ΔF508 CFTR expressors, cells were treated with CHX (100 μg/ml) for 2 h before solubilization. ERP72 and GRP78 were detected with antibodies described under "Experimental Procedures." A significant fraction of the SDS-resistant aggregates, containing CFTR variants, could be recovered in Laemmli sample buffer comprising 8 M urea at room temperature (data not shown). Where indicated, heat denaturation was performed in 2× Laemmli sample buffer supplemented with 8 M urea. b and c, quantitative assessment of the thermoaggregation tendencies. b, the monomeric complex-glycosylated wt and ΔF508 CFTR (rescued ΔF) and the core-glycosylated ΔF508 CFTR (ΔF) were quantified with densitometry on immunoblots shown on a and expressed as the percentage detected without heat denaturation (means ± S.E., n = 3–7). c, the thermoaggregation of ERP72 (solid symbols) and Na+/K+-ATPase (open symbols) was determined as described for CFTR in untransfected BHK cells (triangles) and wt (diamonds), rescued ΔF508 (squares), or core-glycosylated ΔF508 CFTR (circles) expressors (mean ± S.E., n = 3–7).

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2 M. Benharouga, M. Haardt, and G. L. Lukacs, unpublished observation.
also renders temperature-sensitive stability defect to the complex-glycosylated (or native) ΔF508 CFTR. According to the classical terminology the distinctive feature of the temperature-sensitive folding mutants is the absence of detectable defects in the protein formed at the permissive temperature (44). Therefore, ΔF508 CFTR appears not to belong to this category of mutations. Intriguingly, while glycerol could partially rescue the folding defect of the ΔF508 CFTR at 37 °C, it was unable to restore the impaired stability of the rescued form, 3 suggesting that distinct structural alterations are responsible for the folding and the stability defect.

In summary, the in vivo turnover measurements together with the protease susceptibility and thermaaggregation results indicate that whereas the ΔF508-imposed folding defect can be partially overcome, the biological and structural characteristics of the rescued ΔF508 CFTR, generated at permissive temperature, remain distinct from its wt counterpart. Development of novel strategies to stabilize the native ΔF508 CFTR would complement the present therapeutic approaches aiming to correct the folding defect at the ER, in tissue culture models (10, 22–25, 45), in CF mice (21), and in human trials (46, 47).

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