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CFTR: folding, misfolding and correcting the $\Delta F508$ conformational defect

Gergely L. Lukacs¹ and A.S. Verkman²

¹Department of Physiology and GRASP, McGill University, Montréal, Quebec H3E 1Y6, Canada

²Departments of Medicine and Physiology, University of California, San Francisco, CA 94143-0521, USA

Abstract

Cystic fibrosis (CF), the most common lethal genetic disease in the Caucasian population, is caused by loss-of-function mutations of the CF transmembrane conductance regulator (CFTR), a cyclic AMP-regulated plasma membrane chloride channel. The most common mutation, deletion of phenylalanine 508 ($\Delta F508$), impairs CFTR folding and, consequently, its biosynthetic and endocytic processing as well as chloride channel function. Pharmacological treatments may target the $\Delta F508$ CFTR structural defect directly by binding to the mutant protein and/or indirectly by altering cellular protein homeostasis (proteostasis) to promote $\Delta F508$ CFTR plasma membrane targeting and stability. This review discusses recent basic research aimed at elucidating the structural and trafficking defects of $\Delta F508$ CFTR, a prerequisite for the rational design of CF therapy to correct the loss-of-function phenotype.

Cystic fibrosis (CF) and approaches to alleviate the CF clinical phenotype

The cystic fibrosis transmembrane conductance regulator (CFTR) protein is a cyclic AMP (cAMP)-regulated chloride channel expressed in the plasma membrane (PM) of secretory epithelia in the airways, intestine, pancreas, testis and exocrine glands, as well in some non-epithelial cell types. Each CFTR molecule contains two membrane-spanning domains (MSD1 and MSD2), two nucleotide binding domains that participate in ATP binding and hydrolysis (NBD1 and NBD2), and a regulatory domain (R) whose phosphorylation regulates channel gating (Figure 1a) [1]. Loss-of-function mutations in CFTR cause the autosomal recessive disease cystic fibrosis (CF), which has an incidence of approximately 1 in 2000 Caucasians [2]. The most common CF-causing CFTR mutation, deletion of the phenylalanine residue at position 508 ($\Delta F508$), is present in one or both alleles in approximately 90% of CF patients [2]. Nearly 2000 other mutations have been identified (<http://www.genet.sickkids.on.ca/cfr>) that produce the loss-of-function phenotype by preventing full-length translation or by impairing some combination of CFTR folding, stability and channel gating [3–6]. The clinical features of CF include chronic lung infection with progressive deterioration of lung function, pancreatic insufficiency, male infertility, and meconium ileus in the newborn [7]. The median life expectancy for individuals with CF is currently 39 years in the US, with lung disease as the principal cause of morbidity and mortality.

Current therapies treat the symptoms of CF disease, including antibiotics, anti-inflammatory agents, mucolytics, nebulized hypertonic saline, pancreatic enzyme replacement, and lung

transplantation [8, 9]. There is great interest in therapies that treat the pathogenic mechanisms of CF disease or correct the underlying basic defects responsible for CFTR loss-of-function. A challenge in the development of disease mechanism-based therapies is the incomplete understanding of how CFTR loss-of-function produces CF disease, particularly CF lung disease. There is evidence, albeit controversial, supporting diverse pathogenic mechanisms, including airway surface liquid (ASL) dehydration caused by reduced chloride/bicarbonate secretion or sodium hyperabsorption, impaired submucosal gland fluid secretion, intrinsic hyperinflammation, defective granulocyte and macrophage function, as well as other mechanisms [8]. Notwithstanding our incomplete knowledge of CF disease mechanisms, it is widely assumed that CF organ pathology could be alleviated by restoring functional expression of $\Delta F508$ CFTR at the PM by correcting its folding defect [10]. In addition, therapeutic efforts are under development to increase non-CFTR-mediated fluid secretion (by activating calcium-activated chloride channels) and reduce sodium channel-mediated fluid absorption (by inhibiting ENaC, the epithelial Na^+ channel), and introduce functional CFTR with gene replacement therapy [8].

In general, defects in protein conformation, as occurs for mutant CFTRs, could be rescued by the following: (i) stabilizing the protein native state with pharmacological chaperones (PCs) that bind directly to the mutant protein; and/or (ii) enhancing the protein folding efficiency within target cells using proteostasis regulators (PRs) [11–13]. Modulation by PRs involves, but is not limited to, altering the activity of transcriptional, folding and/or membrane trafficking machinery, as well as impeding the degradation of partially folded, but functional, conformers at the endoplasmic reticulum (ER) or PM [12, 13]. This review covers recent progress in our understanding of $\Delta F508$ CFTR folding, processing and functional defects to highlight potential sites of intervention by PC- and PR-based drug therapy. We also discuss high-throughput screening (HTS) efforts to identify small molecules to correct the basic mechanistic defect in CF.

CFTR domain folding, assembly and misassembly

CFTR domain assembly

In most heterologously expressing cells only 20–40% of newly synthesized CFTR nascent chains attain their native conformation, exit the ER, and undergo complex glycosylation in the Golgi compartment [14, 15]. Interestingly, however, the maturation efficiency of CFTR endogenously expressed in two epithelial cell lines was reported to be nearly 100% [16]. Partially folded channels are disposed of by ER-associated degradation (ERAD) via the ubiquitin–proteasome system (UPS) [17]. Although the underlying cause of the inefficient folding of CFTR remains enigmatic, it is probable that the energetic instability of individual NBDs, the slow domain assembly, and the relatively fast ERAD kinetics all contribute to inefficient folding (see below) and sensitize CFTR to structural perturbations by mutations.

Molecular modeling, using bacterial ABC transporters as templates, has provided insights into the three dimensional domain-swapped architecture of CFTR (Figure 1a), which has been substantiated by cysteine (Cys) crosslinking experiments [18–20] (see Glossary). Accordingly, in native CFTR the F508 residue interfaces with the coupling helix of cytoplasmic loops 4 (CL4) and 1 (CL1) in MSD2 and MSD1, respectively (Figure 1a). NBD2 associates with CL2 and CL3 of MSD1 and MSD2, respectively [18–20]. These interfaces not only serve to relay ATP-dependent conformational changes of the NBDs to the MSDs, which are involved in chloride channel gating, but also appear to have a crucial role in CFTR biogenesis [18–25].

Based on studies of CFTR protease susceptibility and intramolecular FRET, it was concluded that whereas NBD1 folds largely cotranslationally, the native structure of NBD2

and, hence, of the full channel are attained post-translationally [26–28] (Figure 1b). Consistent with the slow post-translational conformational maturation, assisted by prolonged interaction with molecular chaperones [29], NBD–MSD interfaces were detected only in mature, complex glycosylated wild type (WT) CFTR, but not in immature, core glycosylated CFTR [18, 19].

Analysis of the ER processing and PM expression of split and truncated CFTRs and multiple domain combinations revealed that the minimal folding unit of CFTR comprises MSD1, NBD1, R and MSD2 in mammalian cells [30]. Taken together with the known CFTR domain-swapped architecture, a cooperative domain folding model has been proposed that invokes domain–domain interactions and energetic coupling as essential in CFTR co- and post-translational conformational maturation [30] (Figure 1b), similar to that proposed for other soluble multi-domain proteins [31]. The four-domain, minimal folding unit of CFTR attains its native tertiary structure post-translationally with slow kinetics similar to that of WT CFTR [30].

Linking NBD1 misfolding in Δ F508 CFTR to domain misassembly

The mechanistic link between the folding defects observed for Δ F508 NBD1 and the full Δ F508 CFTR protein remains only partly understood due to lack of detailed structural information on both WT and mutant CFTRs. Initial studies suggested that Δ F508 NBD1 refolding is impaired, but that its backbone structure and thermodynamic stability are similar to those of WT CFTR [4]. Minimal differences between WT and Δ F508 NBD1 were found by analysis of equilibrium denaturation profiles and crystal structures [32–34], with structural data showing limited alterations that were restricted to the flexible surface loop at residues 507–511 [32, 33, 35]. Whereas molecular dynamics simulations revealed little difference in the folding thermodynamics of Δ F508 versus WT NBD1, the folding kinetics of Δ F508 NBD1 was delayed [36], in line with its decreased refolding yield at room temperature [33], suggesting that the folding activation energy of Δ F508 NBD1 was increased compared with that of WT NBD1 (Figure 2a, b). Recent thermal aggregation studies, however, showed that the Δ F508 mutation decreases the midpoint aggregation temperature from 41 °C to 33 °C, indicating conformational destabilization of the rescued Δ F508 NBD1 native-like state (N*) [37]. Isothermal calorimetry revealed that the melting temperature (T_m) of NBD1 mutants containing three solubilizing mutations, or deletion of the regulatory insertion (Δ RI, residues 407–433), was decreased by the Δ F508 mutation from 58.7 °C to 51.5 °C and from 49.8 °C to 43.6 °C [38, 39]. These data, together with the reduction of T_m from 41 °C to 33 °C of NBD1 containing one versus no solubilizing mutations [40], suggest that the Δ F508 mutation produces both kinetic and thermodynamic folding defects in the isolated NBD1 domain. The kinetic and thermodynamic folding defect, in principle, can be corrected by PRs and PCs, respectively (Figure 2d) [11, 13]. These observations provide a possible explanation for the temperature-sensitive conformational defect of Δ F508 NBD1 at 37 °C and the suppression of this defect at reduced temperature [33, 41, 42]. These observations are also consistent with predictions of molecular dynamics computations [43, 44] and hydrogen–deuterium exchange measurements [35] showing that the increased conformational freedom of Δ F508 CFTR at 22–24 °C is restricted to the linker region (amino acids 492–499) and the vicinity of the F508 residue. Although the mechanism of global destabilization of Δ F508 NBD1 at 37 °C remains to be understood, temperature-dependent energetic stabilization probably accounts for the native-like X-ray crystal structure of Δ F508 NBD1 at the permissive temperature (4–16 °C) [32].

The absolute magnitude of the Δ F508 NBD1 energetic defect and its contribution to the global misfolding of Δ F508 CFTR are unknown. Given the cooperative folding mechanism of CFTR, it is reasonable to assume that although the folding energetics of WT NBD1 is

further stabilized by coupled domain folding, this process is compromised for Δ F508 NBD1. Consistent with this idea is the several-fold increase in protease susceptibility of Δ F508 NBD1 [27, 30, 45] and the increased reactivity of Cys491 residue of NBD1 in Δ F508 CFTR as compared with WT CFTR [25].

As a consequence of impaired assembly of the interface between NBD1 and MSD2/MSD1 (Figure 1a), Δ F508 destabilizes the conformations of MSD1, MSD2 and NBD2 [21, 27, 29, 30, 45], as supported by Cys crosslinking data [25, 46]. The cooperative misassembly of the first four CFTR domains culminates in impairment in folding of NBD2 [27, 45] (Figure 1b) and the MSD1/MSD2 and NBD1/NBD2 interfaces [25, 47, 48]. Conversely, CF-associated point mutations in MSD1, MSD2 or NBD2 destabilize the conformation of the other three domains, similar to what is reported for Δ F508 and consistent with cooperative and global domain misassembly [30]. Although cooperative misfolding offers multiple interfaces that could be targeted by a synergistic combination of PCs or a PC in combination with a second site suppressor mutation [24, 49], it imposes additional hurdles to decipher the mechanism of PC action and for development of single drug corrective therapy.

Notably, second site suppressor mutations in Δ F508 NBD1 or at the NBD1–CL4 interface partially restore the folding and processing defects as well as the NBD1–CL4 interface [21–25, 37, 50]. Intriguingly, complete energetic correction of Δ F508 NBD1 failed to efficiently restore Δ F508 CFTR biosynthetic processing [40], suggesting that correction of both NBD1 energetics and interface instability is required to robustly normalize Δ F508 CFTR processing, which presents a significant challenge for efficient corrector therapy.

Membrane trafficking and functional defects of Δ F508 CFTR

The activity of cellular protein homeostasis networks limits the escape of non-native Δ F508 CFTR from the ER and reduces its metabolic stability at the PM. Modulation of these protein quality control systems by PRs may thus alleviate the Δ F508 CFTR loss-of-function phenotype and be exploited therapeutically [13].

Δ F508 CFTR retention and degradation at the ER

A significant fraction of newly synthesized, non-native or inefficiently folding polypeptides, including Δ F508 CFTR, is degraded by the UPS following p97/Cde48-dependent retrograde translocation from the ER into the cytoplasm [51]. Molecular chaperone and co-chaperone complexes can sense the CFTR folding state and, depending on the details and dwell-time of the chaperone–CFTR interaction, assist either folding or ubiquitination-dependent ERAD. Whereas the folding of the cytosolic domains of CFTR requires both Hsp70 and Hsp90, in concert with a subset of co-chaperones [e.g. Hdj2 (DNAJ1), HsBp1, Hop, and p23] and small heat shock proteins, the luminal chaperones calnexin and calreticulin promote the ER retention of core glycosylated CFTR folding intermediates until their folding cycle or degradation is completed [2]. In addition, N-linked glycosylation stabilizes the CFTR fold directly [52].

Ubiquitination, a prerequisite for CFTR ERAD that facilitates retrotranslocation and proteasome targeting, is defined by the rate of CFTR Ub conjugation and deubiquitination, as well as the configuration of the Ub chain. Distinct E3 Ub ligases are responsible for the co- and post-translational ubiquitination of non-native CFTRs that recognize misfolding of the N- and C-terminal CFTR regions in a chaperone-dependent or -independent manner. For example, whereas the J-domain protein DNAJB12 facilitates Hsc70 and Rma1-dependent ubiquitination of the non-native N-terminus cotranslationally [53–55], CHIP (C-terminal Hsc70 interacting protein) preferentially recognizes the misfolded full-length CFTR. Gp78, an E4 ER Ub ligase [55], assists in poly-Ub chain extension, whereas UCH-L1 (ubiquitin C-

terminal hydrolyse-L1) and Usp19 (ubiquitin specific protease 19) stabilize the Δ F508 CFTR nascent chain by catalyzing Ub cleavage [56, 57]. The cytosolic Nedd4-2 and Fbs1 E3 ligases have also been implicated in the ERAD of Δ F508 CFTR [58, 59]. Although ablation of an E3 ligase or overexpression of a deubiquitinating enzyme were unable to rescue Δ F508 CFTR processing, perhaps due to the redundancy of the ER quality control machinery, the combination of a second site suppressor mutation (Val510Asp) with inhibition of ubiquitination and exposure to a corrector molecule (Corr-4a) led to the robust maturation of the mutant protein in cell culture models [24, 60]. These observations support the notion that a combination of a PR and a PC can synergistically rescue the Δ F508 CFTR folding/processing defect, similar to that described for mutant enzymes in lysosomal storage diseases, a paradigm that may be applicable to CF therapy [11].

Peripheral protein quality control recognizes and eliminates non-native Δ F508 CFTR from the PM

The metabolic and functional instability of low-temperature or corrector-rescued Δ F508 CFTR (r Δ F508 CFTR) was recognized in non-polarized cells and validated in both primary and immortalized epithelia [5, 61–63]. This instability may be a significant limitation for corrector therapies that only partially normalize the channel conformational defect [63, 64]. The rapid, ubiquitination-dependent elimination pathway of r Δ F508 CFTR is similar to that of several other conformationally defective PM proteins [65]. Phenotype-based, small interfering RNA (siRNA) screens identified CHIP and gp78 as E3 Ub ligases responsible for the removal of r Δ F508 CFTR from the PM in HeLa and IB3 respiratory cells [66] (Figure 3b). Hsc70 and Hsp90, acting in concert with a subset of co-chaperones [e.g. DNAJ1 (Hdj2), Bag1, HOP, and Aha1], were required for r Δ F508 CFTR ubiquitination, rapid endocytosis and lysosomal degradation [66]. Ubiquitinated r Δ F508 CFTR is redirected from endosomal recycling towards lysosomal delivery by the endosomal sorting complex required for transport (ESCRT0-III) [61, 66]. c-Cbl has been identified as an endocytic adaptor and endosomal E3 ligase of WT CFTR in CF bronchial epithelia [67]. Conversely, activation of the deubiquitinating Usp10 enzyme can stabilize WT CFTR by facilitating its recycling [68]. The roles of c-Cbl and Usp10 in the r Δ F508 CFTR PM stability remain to be elucidated. Although components of the peripheral quality control machinery represent potential drug targets for PRs, their ubiquitous expression and function in all cells presents a challenge for CF therapies based on modulating their activity.

Δ F508 CFTR gating defect

CFTR functions as an ATP-gated ion channel following R-domain phosphorylation by cAMP-dependent protein kinase (PKA). ATP binding at two composite sites (site 1 comprising the NBD1 Walker motifs and the signature motif of NBD2 and site 2 comprising the NBD2 Walker motifs and the NBD1 signature motif) induces NBD dimer formation followed by a conformational change of the MSD domains and channel pore opening [69]. Subsequent ATP hydrolysis, primarily at the NBD2 site, facilitates channel closing, which can proceed by preserving the ATP-binding site 1 and dissociation of binding site 2, without complete separation of the NBD dimer [70]. ATP-independent channel isomerization may also mediate gating, albeit with slower kinetics [70]. Point mutations interfering with the composite ATP binding site can interfere with ATP binding and function without causing significant trafficking defects, as exemplified by the G551D mutation [3, 71]. Conversely, trafficking mutants may have no defect in gating function, as illustrated by the N287Y mutation within the second intracellular loop of CFTR in which channel endocytosis from the PM is increased without impairment in channel gating [72].

Electrophysiological characterization of Δ F508 CFTR at the PM is challenging because of its low density and rapid channel inactivation at 37 °C [73]. The open probability (P_o) of

$\Delta F508$ CFTR at the PM is very low even with maximal phosphorylation [6]. The macroscopic inactivation and metabolic disposal of $\Delta F508$ CFTR at the PM is more than 4- to 6-fold faster than that of WT CFTR, which may be due to thermal unfolding [42, 61]. The gating of reconstituted $\Delta F508$ CFTR inactivates even faster in phospholipid bilayers at 37 °C [73]. The underlying mechanism of the gating defect is poorly understood, but may involve impairments in NBD dimerization, dimer stability and/or conformational coupling between the NBDs and MSDs. Consistent with the conformational destabilization hypothesis, stabilization of NBD1 by solubilizing or the ΔRI mutations partially corrected the $\Delta F508$ CFTR gating defect [48], a mechanism that could be mimicked by PCs.

Implications for CF therapy

Correction of defective $\Delta F508$ CFTR folding at the ER by a small molecule is an attractive approach to treat CF because it targets the underlying defect in the appropriate CFTR-expressing cells. In principle, if a PC or PR can modify the $\Delta F508$ CFTR structure to resemble that of WT CFTR, then downstream consequences of defective folding may also be corrected. However, compounds identified thus far have limited efficacy. Because of the complexity of $\Delta F508$ CFTR folding defects discussed above, it may not be possible to develop a single PC or PR that efficiently rescues all of the conformational, trafficking and functional defects, necessitating the use of combinations of PCs, PRs or both a PC and PR [10, 13]. Modulation of protein quality control systems and/or the folding and degradation machinery activity of $\Delta F508$ CFTR represent complementary approaches to rescue the functional channel. This is exemplified by modulating the acetylation level of histones, transcription factors and other cytosolic factors (e.g. Hsp90) with inhibitors of histone deacetylase 7 (HDAC7) [74] or using the combination of Corr-4a and inhibition of DNAJB12 [24].

The practical drug discovery strategy used to date utilizes separate functional assays to screen for $\Delta F508$ CFTR ‘potentiators’, which normalize defective $\Delta F508$ CFTR chloride channel gating, and ‘correctors’, which promote $\Delta F508$ CFTR exit from the ER and accumulation in the PM (Box 1). We point out, however, that the distinction between potentiators and correctors is somewhat artificial, as normalization of channel gating and cellular processing both depend on $\Delta F508$ CFTR folding.

Box 1

High throughput screening (HTS) to identify correctors and potentiators of $\Delta F508$ CFTR

To monitor the plasma membrane halide conductance, many assays utilize a cytoplasmic, genetically encoded yellow fluorescent protein (YFP)-based indicator whose fluorescence is quenched by iodide. As diagrammed in Figure I, potentiator activity is assayed in $\Delta F508$ CFTR-transfected epithelial cells following low-temperature rescue in which $\Delta F508$ CFTR is targeted to the plasma membrane by 18–24 h incubation at reduced temperature, with the test compound (together with the cAMP agonist) added just before assay [76]. $\Delta F508$ CFTR function is deduced from the kinetics of decreasing fluorescence in response to extracellular iodide addition, which is a quantitative measure of iodide influx into the cells. By contrast, corrector activity, which is defined as efficacy in increasing $\Delta F508$ CFTR cell surface expression, is assayed in $\Delta F508$ CFTR-expressing cells by a > 12 h incubation with test compound at 37 °C, followed by washout and addition of both a potentiator, such as genistein, and a cAMP agonist just before assay of iodide influx [64]. Alternative HTS assays have been developed based on the readout of membrane potential using fluorescent probes [75] and on the readout of

$\Delta F508$ CFTR cell surface expression using antibodies against engineered extracellular epitopes [64, 98].

Figure 1.

(a) YFP potentiator assay. Fisher rat thyroid (FRT) cells co-expressing human $\Delta F508$ CFTR and a halide-sensing YFP are incubated at a reduced temperature (27 °C) for 18–24 h before assay to target $\Delta F508$ CFTR to the plasma membrane. Test compounds are added for 10 min in the presence of forskolin before iodide (I^-) addition. $\Delta F508$ CFTR function is assayed in a plate reader by quantifying YFP fluorescence quenching in response to iodide addition, (b) Corrector assays. YFP-based assay: cells are incubated with test compounds at 37 °C for 24 h $\Delta F508$ CFTR function is assayed by iodide addition in the presence of forskolin and the potentiator genistein. Membrane potential based assay (V_m): $\Delta F508$ CFTR activation is monitored by potential-sensitive fluorescent dyes, using a FRET-based assay in low chloride medium. Immunodetection assay: the plasma membrane density of $\Delta F508$ CFTR exposing a 3xHA epitope tag at its exofacial surface is detected by cell surface ELISA.

Primary compound screening has been done using $\Delta F508$ CFTR transfected cell lines, such as Fischer rat thyroid cells or fibroblasts, with follow-on testing done in primary cultures of human bronchial epithelial cells from CF patients homozygous for the $\Delta F508$ mutation [64, 75]. The activity of potentiators is, in general, cell type-independent and instantaneous, which are probably the consequence of potentiators binding directly to the mutant CFTR as a PC. By contrast, based on the strong cell type dependent activity of several correctors, and a lack of evidence for their direct binding to $\Delta F508$ CFTR, some correctors may act as PRs that target cell-specific proteostasis networks or factors that interact with corrector-bound $\Delta F508$ CFTR. A concern in corrector discovery is the use of $\Delta F508$ CFTR transfected, overexpressing cell lines, in which compounds with potentially high efficacy in native human CF cells may escape detection. Another general concern is the separate identification

and development of potentiators and correctors, in which a single compound having multiple, albeit weak, activities might escape detection.

Prior to HTS, several classes of potent and efficacious $\Delta F508$ CFTR potentiators were known, including flavones, xanthines, benzimidazoles and dihydropyridines. HTS efforts by multiple laboratories [75–78] have yielded additional small molecule potentiators (Figure 4, top). For example, the phenylglycine potentiator PG-01, when added together with a cAMP agonist, activates $\Delta F508$ CFTR chloride conductance with low nanomolar potency, with maximum efficacy comparable to that of the flavone genistein (Figure 4, bottom), restoring $\Delta F508$ CFTR open probability to approximately that of WT CFTR. PG-01, as well as the Vertex Pharmaceuticals compound VX-770, also corrects defective channel gating in the CF-causing mutant Gly551Asp CFTR, a CFTR ‘gating’ mutant that is targeted to the cell PM but has low chloride conductance even after maximal cAMP stimulation [78]. VX-770 has shown preliminary efficacy in Phase II clinical trials in CF patients carrying the Gly551Asp mutation [79]. Although potentiators may be clinically useful in the relatively small group of CF patients having the Gly551 Asp substitution, a potentiator alone is unlikely to have clinical benefit in CF caused by the $\Delta F508$ mutation both because little $\Delta F508$ CFTR is expressed at the PM and its conformational defect could be distinct from molecules that have constitutively escaped from the ER or are rescued by correctors. Another concern with available potentiators is that superphysiological concentrations of cAMP agonists are generally needed for potentiator efficacy following low temperature or corrector rescue, indicating that the rescued $\Delta F508$ CFTR remains in a non-native conformation at the PM. In contrast to the rescued $\Delta F508$ CFTR, WT CFTR is activated by low concentrations of cAMP agonists without the need for a potentiator.

Correctors have been identified that promote $\Delta F508$ CFTR cell surface expression. Some nutraceuticals and drugs approved for other indications have been reported to have $\Delta F508$ CFTR corrector activity, including curcumin, miglustat and sildenafil [80–82]. However, follow-on studies have failed to confirm *bona fide* corrector action of these compounds. The compound 4-phenylbutyrate (Buphenyl), an approved drug used for an erythrocyte urea cycle disorder, increases $\Delta F508$ CFTR PM expression in cell culture models, perhaps as a PR. However, a clinical trial showed only small increases in chloride conductance, measured by nasal potential difference of $\Delta F508$ CF patients following large doses of 4-phenylbutyrate [83]. Downregulation of Aha1, a co-chaperone of Hsp90 [84], and inhibition of HDAC7 [74] have been shown to rescue the $\Delta F508$ CFTR mutation in both cultured and primary respiratory epithelia by modestly facilitating folding at the ER and stability at the PM. Whereas Aha1 inhibition improves $\Delta F508$ CFTR folding by modulating Hsp90 activity, the underlying mechanism of HDAC7 inhibition, which may involve targeting multiple facets of $\Delta F508$ CFTR folding, trafficking and degradation at the ER and PM via PRs, remains to be elucidated.

HTS has produced several classes of small molecule $\Delta F508$ CFTR correctors [64]. The bithiazole corr-4a (Figure 4, bottom) increased $\Delta F508$ CFTR cell surface expression after a 12–24 h incubation at 37 °C, resulting in increased chloride conductance following channel activation by both a potentiator and cAMP agonist. Follow-on medicinal chemistry yielded bithiazole analogs with improved potency and pharmacological properties [85, 86]; although the EC₅₀ for the best bithiazoles is approximately 300 nM, their maximal efficacy in restoring chloride conductance in primary cultures of $\Delta F508$ human bronchial cells is only approximately 8% of non-CF human primary epithelia [64, 75]. The corrector efficacy needed to confer clinical improvement in CF is unclear, as is the validity of translating efficacy data from cell culture models to humans. Screening by Vertex Pharmaceuticals Inc. has yielded other classes of correctors, with the most promising compound, VX-809, in Phase II clinical trials [75, 87]. Although indirect evidence suggests that VX-809 promotes

$\Delta F508$ CFTR conformational maturation at the ER and can restore 15% non-CF channel activity in primary respiratory epithelia [87, 88], definitive proof for this mechanism of action remains to be determined. Other small-scale screening efforts have yielded additional candidate correctors, including the approved drug glafanine and the phenylhydrazone RDR1, as well as a series of compounds from computational screening [49, 89, 90]; however, each of these compounds appear to have low efficacy. Although *in silico* drug discovery efforts involving docking computations have significant potential, success hinges on reliable 3D structural information of the drug target, which unfortunately is not available for $\Delta F508$ CFTR.

The molecular mechanisms of corrector action remain largely unknown. Initial studies of Corr-4a supported a mechanism of direct bithiazole- $\Delta F508$ CFTR interaction and with consequent improved $\Delta F508$ CFTR folding efficiency, ER escape and PM targeting [64]; selectivity studies showed that Corr-4a did not rescue other mutant membrane proteins for which pharmacological or low-temperature rescue is possible. Evidence has also been reported that Corr-4a interacts with $\Delta F508$ CFTR in the ER to promote its folding, stabilizes NBD2, and/or interferes with the channel ubiquitination [91–93]. There is evidence suggesting a direct interaction between corrector VRT 325 and $\Delta F508$ CFTR, as it alters CFTR ATPase activity [94]. Notwithstanding these and other lines of suggestive evidence, definitive data are lacking concerning the biologically relevant target(s) of available correctors. The one exception is RDR1, which can conformationally stabilize the mouse $\Delta F508$ NBD1 [90] according to differential scanning fluorimetry and probably binds to $\Delta F508$ CFTR, a hallmark of a PC. Similar studies performed on isolated $\Delta F508$ CFTR may be able assess whether corrector molecules can directly bind to and conformationally stabilize the mutant channel.

Of the available correctors, the bithiazoles and aminoarylthiazoles appear to at least partially normalize $\Delta F508$ CFTR folding, as their prolonged incubation results in greater chloride conductance in response to a cAMP agonist (without potentiator) [77, 95]. In a proof-of-concept study, a hybrid bithiazole–phenylglycine corrector–potentiator was synthesized that, when cleaved by intestinal enzymes, yields an active bithiazole corrector and phenylglycine potentiator [96]. Recently, a cyanoquinoline class of $\Delta F508$ CFTR correctors was identified with independent potentiator action [97]. Although their corrector and potentiator efficacies are comparable to those of Corr-4a and genistein, respectively, their EC_{50} is at micromolar rather than nanomolar concentrations, and their mechanism of action remains unknown.

Future perspectives

From the basic science perspective, although there is now a substantial body of data on the biophysics and cellular mechanisms of CFTR folding and $\Delta F508$ CFTR misfolding *in vitro*, there remain major gaps in our knowledge of how critical components of the quality control machinery recognize the folding defect in tissues. Correctors of $\Delta F508$ CFTR misprocessing have rapidly moved to clinical trials despite even a rudimentary understanding of their mechanism of action. From the translational medicine perspective, there remains a need to identify correctors with improved potency and efficacy, ideally a single compound that efficiently rescues the $\Delta F508$ CFTR structural and functional defects as a PC (Box 2). Recent insights reviewed herein on the structure and cell biology of $\Delta F508$ CFTR will unquestionably foster future drug discovery efforts. Understanding the molecular mechanism of the conformational destabilization of $\Delta F508$ NBD1 and its role in the cooperative domain misassembly process may facilitate the design of novel, structure-targeted HTS efforts to isolate corrector molecules with preferential binding to CFTR. Whether single drug therapy for CF caused by the $\Delta F508$ mutation is possible or not remains to be determined. The alternative, as suggested by the data and analyses described

in this review, is multi-drug therapy using more than one corrector or potentiator as PC, perhaps in combination with a PR. Current efforts to identify correctors, based largely on phenotype screens, have not been successful in identifying highly efficient molecules. Perhaps the next generation of corrector screens should be more mechanism-based and targeted to specific, well-characterized defects in $\Delta F508$ CFTR folding and structure.

Box 2

Outstanding questions

What are the major, correctable structural defects of $\Delta F508$ CFTR?

To what extent can the $\Delta F508$ CFTR structural defects be corrected by a single compound, or a combination of PCs and PRs?

How should new HTS assays be designed to isolate structure-targeted PCs *in vitro* and *in vivo*?

Will efficient correction of the $\Delta F508$ CFTR folding defect necessitate combination therapy using both PCs and PRs? Can efficient PRs with few off-target effects be selected?

What preclinical cellular model(s) for testing PCs and PRs will best predict clinical efficacy?

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Glossary

Complex glycosylated CFTR	N-glycan chain containing fucose, galactose and sialic acid instead of terminal mannose residues, a modification that occurs in Golgi compartments
Computational screening	virtual screening of compounds by molecular docking computations in which the energetics of compound interaction with a target are calculated using high-resolution crystal structure data for the target
Core glycosylated CFTR	containing N-glycan chains with terminal mannose residues
Cysteine (Cys) crosslinking experiment	introduction of Cys pairs at rationally selected locations in the Cys-less CFTR background and covalent, bifunctional thiol (SH) reactive reagents to probe structural predictions about the proximity of residue pairs by monitoring the channel electrophoretic mobility shift upon crosslinking efficiency
Energetic coupling of domain folding	domain-domain interactions that confer kinetic and/or thermodynamic stabilization of one or a pair of interacting domains

Hydrogen–deuterium exchange	quantification of the amide hydrogen atoms exchange with deuterium in the NBD1, used as indicator of conformational stability
Intramolecular fluorescence resonance energy transfer (FRET)	a spectroscopic method to measure the proximity of a donor and acceptor fluorophore linked covalently to Specific residues in CFTR in which acceptor fluorescence is excited by fluorescence from a nearby donor
Isothermal calorimetry	measurement of the heat energy that is required to unfold a protein
Midpoint aggregation temperature	the temperature that induces 50% aggregation of a protein
Molecular dynamics computations	mathematical model of the kinetics of protein conformational changes based on high-resolution protein structure data
Nutraceutical	food or other natural product that contains biologically active compound(s) that prevent and/or treat disease
Open probability (P_o)	the fraction of time a channel is in its open state
Thermal aggregation studies	determination of the NBD1 oligomerization propensity as a function of temperature by light scattering or fluorescence

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Figure 1.

CFTR predicted structure and folding model, **(a)** Homology model of human CFTR in the outward-facing configuration. CFTR structure was visualized with MacPyMOL based on the model of Mornon et al. [99]. The nucleotide binding domains (NBDs) 1 and 2, the regulatory domain (R) and the membrane spanning domains (MSDs) 1 and 2 of CFTR are color coded, and the F508 amino acid residue is indicated. The interface between the NBDs and the MSDs formed by the cytoplasmic loops (CLs) 1–4 are shown in the insert, **(b)** Cooperative folding of WT CFTR (upper panel) and misassembly of $\Delta F508$ CFTR (lower panel). Whereas individual domains can achieve loosely folded conformations and domain assembly cotranslationally, the compactly folded, native tertiary structure with native NBDs–MSDs interfaces are formed post translationally and minimally requires assembly of MSD1–NBD1–R–MSD2 [30]. Thermodynamic and kinetic destabilization of $\Delta F508$ NBD1 and disruption of NBD1–CL4 and CL1 interface compromises the cooperative domain assembly with conformational destabilization of the four domains to variable extents [30, 37, 39]. The protease susceptibility of NBD1 and NBD2 increases nearly 2- to 5-fold and 60-fold, respectively, in $\Delta F508$ CFTR [27, 30, 45]. The estimated folding free energy of individual domains is color-coded based on inference from *in vitro* folding energetic studies and *in vivo* processing of domain combinations [38–40].

Figure 2.

Possible impact of the $\Delta F508$ mutation and corrector molecules on the energetics of folding of the isolated NBD1 domain. Kinetic and thermodynamic destabilization of the isolated NBD1 by the $\Delta F508$ mutation, as compared with WT (a) may lead to increased folding activation energy $\Delta G_t^\ddagger$ (b), decreased folding free energy ΔG^0 (c) or a combination of these (d). The NBD1 folding scheme assumes a simplified two state folding mechanism with a single transition state (T) between the native (N) and unfolded (U) states, (e) Whereas pharmacological chaperones (PCs) could preferentially normalize the native state (N) folding energetics (right), proteostasis regulators (PRs) may preferentially accelerate domain folding by suppressing the transition state (T) free energy (center). The altered N and T states of the mutant are indicated by a star.

Figure 3.

Selected constituents of the ER and peripheral quality control machinery that limit $\Delta F508$ CFTR accumulation at the plasma membrane, **(a)** Endoplasmic reticulum (ER) quality control (QC). E3 (CHIP, Rma1, Nedd4-2 and Fbs1) and E4 (gp78) ubiquitin (Ub) ligases that contribute to the ubiquitination of non-native nascent CFTR chains are numbered. COMMD1 inhibits peripheral ubiquitination with an unknown mechanism [100]. Co-chaperones that participate in the regulation of Hsc70/Hsp70 and Hsp90 activity are not included, except for DNAJB12, a J-domain protein that facilitates Rma1- and Hsc70-dependent ubiquitination of $\Delta F508$ CFTR. Deubiquitination by the ER-anchored USP19 or soluble UCH-L1 can metabolically stabilize $\Delta F508$ CFTR. **(b)** Peripheral QC. Chaperone-dependent or chaperone-independent ubiquitination contribute to the rapid internalization and recycling defect of non-native CFTR from the PM. Following endocytosis, ubiquitinated channels are recognized by the ESCRT0-I components (e.g. Hrs, STAM-1 and Tsg101) at early endosomes and rerouted from the constitutive recycling pathway towards lysosomal degradation. Inhibition of E3 ligase and chaperone-dependent recruitment or overexpression of Usp10 can metabolically stabilize CFTR at the PM by facilitating recycling and delaying lysosomal delivery.

Figure 4. Small molecule potentiators and correctors of $\Delta F508$ CFTR. Chemical structures of indicated $\Delta F508$ CFTR potentiators and correctors.