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FOLDING BIOLOGY OF CYSTIC FIBROSIS: A CONSORTIUM-BASED APPROACH TO DISEASE

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INTRODUCTION

Cystic fibrosis (CF) is a complex disease that has a variety of confounding features that lead to the symptoms found in the clinic. It is largely a protein misfolding disease of the cystic fibrosis transmembrane conductance regulator (CFTR) [1,169,170], but numerous components in the cell contribute to the manifestation of misfolding phenotype, including translational events, chaperone systems, and degradative pathways as well as trafficking and regulatory protein interactions that dictate its function(s) as a channel and master regulator of sodium, chloride, and bicarbonate transport at the apical cell surface [2]. Such a complex array of interactions (the CFTR interactome [3]) [4–6] emphasize the need for a concerted, consortium-based approach to encourage interaction of investigators at all levels of the disease etiology to accelerate the pace of tool, assay, and target discovery that would lead to more effective treatments. Below is a brief overview of the folding and biology of CFTR by members of the *CF*olding *C*onsortium (CFC), a group effort founded by the Cystic Fibrosis Foundation that selectively addresses contemporary issues in our understanding of the protein (mis)folding problem in this disease [171].

CYSTIC FIBROSIS AND THE CYSTIC FIBROSIS CONDUCTANCE REGULATOR: GENETICS AND CLINICAL MANIFESTATIONS DEFINING THE DEPTH OF THE PROBLEM

CFTR is an epithelial ion channel expressed in exocrine glands that conducts chloride and bicarbonate across the plasma membrane and regulates transepithelial transport of sodium [2,7]. Absence of functional CFTR in CF

engenders gross dysfunction of multiple organs, including the lungs, pancreas, liver, intestine, vas deferens, and sweat glands. CF pulmonary ramifications involve deranged maintenance of the airway surface liquid, diminished mucociliary clearance, and death due to respiratory failure. Median life expectancy of individuals with cystic fibrosis is presently approximately 37 years (27 years in ΔF/ΔF homozygous patients) [8].

Based on its primary amino acid and domain structure, CFTR is a member of the ATP-binding cassette (ABC) gene family. Like other ABC proteins, CFTR includes two membrane spanning regions [transmembrane (TM) domains 1 and 2], and two ATP-binding cassettes [also known as nucleotide-binding domains (NBDs)]. The regulatory domain (RD) of CFTR is not typically observed in other ABC proteins and contains a number of protein kinase A phosphorylation sites that contribute to anion conductance activation [9–15].

CFTR is synthesized as a single 150-kDa polypeptide chain. As with other ABC proteins, when CFTR NBDs and TMs are expressed as "half-molecules," the domains find each other, reassociating into functional ion channels at the cell surface [9,10]. Domain interactions are critical for CFTR channel activity. For example, both TM1 and TM2 are postulated to contribute α-helices to the transmembrane chloride conductive pore [11,12] and to interact with the NBDs [16]. Cyclic AMP (cAMP)–dependent phosphorylation by protein kinase A (PKA) is thought to change the orientation of the R-region to allow access to the pore [13,14]. Mutant CFTR lacking an R-region no longer requires phosphorylation for activity [15]. NBDs 1 and 2 dimerize and jointly hydrolyze ATP to serve as a switch that permits ion-channel gating.

The biochemical and structural basis underlying CFTR domain interactions, role in channel activity, and interaction with other proteins comprising the CFTR interactome are an area of intense interest in a number of laboratories. Such an understanding is essential for discovering small molecules (biologics and chemicals) capable of restoring CFTR function in disease. The CFTR gene comprises approximately 250,000 base pairs on chromosome 7 that encode the 1480 amino acids of the intact protein. Over 1500 disease-associated mutations have been identified and characterized based on their underlying pathogenic mechanism. The most common ΔF508 mutation is found in approximately 70% of defective *CFTR* alleles and is defined as a class II defect, leading to an ion channel with substantial residual function, but which is degraded by the proteasome early in biogenesis and before it can arrive at the cell surface [2]. Premature truncation alleles in CFTR are noted by the convention "X" (e.g., the G542X defect) and constitute mutations in class I. Some premature truncation mutants arise from frameshift mutations (e.g., 621 + 1G→T) and 1717-1G→A) and are not denoted as "X" mutations. Certain of these have been observed with a particularly high incidence in Ashkenazi Jews. Mutant CFTR proteins that are synthesized full length but fail to function normally as chloride channels are well described (class III defects), including the G551D mutation, for example.

Additional CFTR mutations exhibit partial activity of the ion channel (class IV), lower levels of CFTR mRNA (class V) or plasma membrane, and surface instability of the protein (class VI). Each of the mutants provides unique windows into the disease process that need to be addressed to achieve resolution of the initial biogenesis problem that triggers CF.

TRANSLOCATION INTO THE ER MEMBRANE

CFTR folding can be viewed as a series of sequential steps in which the nascent polypeptide is targeted to the endoplasmic reticulum (ER), oriented and inserted into the lipid bilayer, folded within the membrane, cytosol, and ER lumen, and finally assembled into its mature tertiary multidomain structure. Disruption at any one of these steps could potentially lead to misfolding of CFTR and failure to exit the ER or function properly at the cell surface. A major challenge in understanding and correcting CFTR misprocessing is therefore to define the precise point where the mutant protein deviates, either structurally or kinetically, from the normal folding pathway.

The earliest recognized stage of CFTR folding involves formation and insertion of hydrophobic helical transmembrane segments (TMs) into the lipid bilayer of the ER membrane [17,18]. In vitro reconstitution experiments reveal that ER targeting is mediated by a signal recognition particle (SRP) at a nascent chain length of about 100 amino acids as TM1 first emerges from the ribosome [19]. TM domain folding therefore begins during protein synthesis at specialized sites in the ER that contain a complex set of membrane proteins, including the Sec61 α,β,γ heterotrimer, oligosaccharyltransferase, signal peptidase, TRAM, and TRAP [20,21]. Collectively, these proteins form the translocon, a protein-conducting channel through which the nascent CFTR polypeptide chain translocates into the ER lumen and is transferred into the ER membrane. Although the precise details of membrane insertion remain a mystery, studies examining early stages of CFTR topogenesis have begun to address how and when each of its 12 TMs are oriented in the membrane and how this process can be affected by disease-related mutations [22,23], yet much remains to be done [2,24].

Models derived from relatively simple, engineered polytopic proteins demonstrate that complex topology can be generated by the action of sequential, independent topogenic determinants contained within the nascent polypeptide [25]. As these determinants pass through the ribosome exit tunnel and translocon, they control the pathway for peptide movement by opening the translocon protein-conducting pore into the ER lumen (i.e., signal sequences) or closing the pore to allow nascent chain egress into the cytosol (i.e., stop transfer sequences) [26,27]. In this manner, each TM can be properly oriented within the translocon co-translationally as it exits the ribosome. Surprisingly, CFTR and other native proteins display variations on this model in which some TM segments do not direct the expected topology independently but, rather,

control the translocation pathway by nonsequential and/or cooperative mechanisms [17,23,28–30]. For example, CFTR TM1 signal sequence activity is depressed by two native polar residues, Glu92 and Lys95, in its hydrophobic core [22]. Translocation of the first extracellular loop (ECL2) is therefore facilitated by coordinated activities of both TM1 and TM2. Corresponding TMs in the second membrane spanning domain, TM7 and TM8, use a different topogenesis mechanism. Whereas TM7 functions as an efficient signal sequence that initiates translocation of ECL4 [23], another polar residue, Asp924, within TM8 decreases stop transfer activity such that TM8 extends transiently into the ER lumen and requires TM7 to terminate translocation effectively and span the membrane [23]. Furthermore, most CFTR TMs are separated by connecting loops that contain only a few residues (TM3–4, 5–6, 9–10, and 11–12) and are therefore probably inserted into the membrane as helical pairs via poorly understood mechanisms [17].

How, then, do disease-causing mutations disrupt early stages of CFTR TM folding and insertion? Although only a small subset of TM mutations has been examined to date, studies indicate that structural defects that disrupt translocation and folding are complex. Topological analysis of two class II (trafficking) mutations, G85E and G91R, demonstrate that introducing a third polar residue into the hydrophobic core of TM1 depresses signal sequence activity further but does not disrupt transmembrane topology [31]. Rather, the redundant activity of TM2 inserts the mutant polar residue into the plane of the membrane, and this in turn perturbs other aspects of CFTR folding (e.g., helical packing, domain–domain interactions, or both) that are recognized by ER sensing mechanisms [32]. CFTR folding is disrupted in a different manner by the P205S mutation in TM3. In this case, Pro205 is required to prevent CFTR misfolding by disfavoring nonnative β-structure within the helical segment [33]. Finally, a number of studies have suggested that tertiary folding of CFTR TMs can also be altered by mutations in cytosolic domains such as NBD1 (ΔF508 and short intracellular loops [12,16,34–39]). Thus, normal and pathological mechanisms of CFTR TM domain folding involve both early events of co-translational TM orientation/integration and subsequent helical packing/domain assembly that begin in association with ER biosynthetic machinery [40,41] and are completed after the nascent protein has been released into the membrane [36,37,42–45]. Understanding the rules that govern how transmembrane domain insertion is coordinated and/or potentially disrupted by mutation in these domains and cytosolic domains will shed important new insight into CF and related membrane protein folding disorders.

CO-TRANSLATIONAL FOLDING OF CFTR

Of all newly synthesized proteins that fold in the ER, those with transmembrane domains, such as CFTR, face an additional challenge: Such patients must fold domains in up to three topologically distinct spaces—the ER lumen,

the membrane, and the cytosol. The domains in CFTR cannot be considered independent: They must affect each other's conformations, even across the membrane. As an example, changes in the cytosolic tails of the single transmembrane fusion proteins of HIV and influenza virus affect structural features of their ectodomains [46,47]. How mutation affects folding within a particular space and how this event is communicated across the bilayer remains a major challenge for understanding CFTR maturation.

Each compartment carries its own set of molecular chaperones that may assist the protein concurrently during its folding. Classical chaperone families of the Hsp70 and Hsp90 classes and their co-chaperones reside in both ER and cytosol [48,49], but the lectin chaperones calnexin and calreticulin are specific to the ER [50]. A wealth of folding enzymes such as oxidoreductases and proline *cis–trans* isomerases catalytically drive folding in the lumen of the ER. Given its multimembrane spanning topology, CFTR has indeed been suggested to use both lumenal chaperones (calnexin) [51] and cytosolic chaperones (extended Hsp90/70 machineries) [3,52–55]. The role of most of these components remains to be elucidated. Folding assistance within the membrane may be controlled through the transmembrane domain of calnexin or the translocon-associated J-proteins that are membrane anchored (co-chaperones of Hsp70s). The polytopic membrane protein Derlin-1, involved in degradation of misfolded CFTR (see below), associates with CFTR in a protein complex during synthesis [56,57], suggesting that folding is a challenge for both wild-type and variant protein. The increase in membrane proteins with identified chaperone function [58] will undoubtedly expand the number of putative chaperones beyond our current level of anticipation.

Communication between folding machineries in the cytosol and the ER lumen is probably disrupted in disease. This could again require folding assistants with a transmembrane domain such as Derlin-1. Evidence for this possibility came from the fact that the Hsp70 co-chaperone ERJ1 was found to have such a coordinating function in translocation. It associates with the ribosome on the cytosolic face of the ER membrane while recruiting BiP in the ER lumen to drive translocation [59]. Considering the various chaperonelike activities of the ribosome, and considering the need for co-translational folding (see above), these same J-proteins may be involved in the cross-membrane coordination of protein folding. Whereas cytosolic J proteins are important for CFTR maturation [54,60], to date none of the transmembrane ER J proteins have been implicated, but are intriguing candidates.

Proteins start to fold during synthesis and complete folding and assembly after translation, although this remains a modest controversy for CFTR, with a large amount of evidence favoring posttranslational folding in most circumstances [36,37,39,42–44]. Aside from the obvious hierarchy in time (posttranslational folding can never occur before co-translational folding), essential differences do exist between the two processes. During synthesis the C-terminus of a protein or domain is tethered to the ER membrane. This limits conformational freedom and is likely to affect folding pathways and may

contribute to the inability of mutants to achieve early folding events. Freedom may be even more limited when the N-terminus is tethered to the membrane, either because an ER targeting signal peptide is not (yet) cleaved off and functions as a (transient) transmembrane domain, or because the folding domain is the C-terminal of a transmembrane domain. CFTR, with its 12 transmembrane helices, needs to fold its domains in the vicinity of the membrane, and to a large extent with N- and C-termini tethered.

During synthesis the ribosome is attached to the membrane, and for proteins such as CFTR it alternates between a sealed connection with the translocon and a partial attachment, the latter during synthesis of the cytosolic parts, perhaps a critical event disrupted in some mutants. The ribosome may not only offer chaperone activity (through its RNA or associated chaperones), but may also limit conformational freedom of the nascent chain because of steric hindrance. This occurs because the ribosome has a width of about 25 nm [61], whereas the translocon pore is an order of magnitude smaller [62]. Thus, location of mutant chain in the pore may alter the sequential links between kinetics and translation, and those of translocation/folding.

Other factors to take into account are co- and posttranslational modifications. CFTR has two N-linked glycans in the fourth extracellular loop, which is the first extracellular loop of the second transmembrane domain and the first after a long stretch of cytosolic sequence. Many other ABC-transporters also have N-linked glycans in this position, indicative of a general function for a glycan. Because N-linked glycosylation for most glycoproteins is crucial for their proper folding [50], their co-translational addition is well timed. Still, early co-translational folding can be faster than glycosylation, as illustrated by the inhibition of glycan addition by early disulfide bond formation during folding of a protein in the ER [63], although an increasing number of proteins are found to add glycans after synthesis. This competition and mutual dependence of folding and glycosylation may well determine or even regulate the outcome of the folding process, leading potentially to multiple conformations of the same protein, even if the number of glycans eventually is identical. One possibility is that because transporters such as CFTR contain suboptimal transmembrane domains, with charged and hydrophilic residues, addition of this large polar sugar moiety may prevent back-sliding of the translocated and inserted transmembrane domains. CFTR was shown to indeed need its glycans for stability, suggesting that they affect conformation [64]. How mutations affect the link between translation and N-glycan modification has not been investigated.

CFTR does not contain disulfide bonds or any other known modifications in the small lumenal parts of its sequence, but its cytosolic R region can be phosphorylated and its NBD1 and NBD2 domains can bind ATP. Whereas ATP binding is not a covalent modification, it does influence protein conformation. Phosphorylation of the R region and ATP hydrolysis have a regulatory role for channel activity, and ATP binding and phosphorylation affect R-NBD1 interaction [65] and NBD dimerization [66]. The effect of CFTR

phosphorylation on its conformation and activity are unknown and probably extends to the folding stage, but this remains to be explored, particularly in mutants. We cannot exclude the fact that phosphorylation affects the folding process of CFTR or even that particular sites need to be modified to allow proper folding of the protein. The effect of ATP is less clear, although it is clear that ATP is required for CFTR folding, in particular for its release from the large ribosome–translocon-complex CFTR residues, probably in response to ATP-dependent chaperones [40]. Whereas the structure of purified NBD1 is not affected by ATP binding [67], in the context of the complete protein a direct role for ATP on CFTR conformation still cannot be excluded and indeed is likely.

Perhaps the most important determinant of co-translational folding is translation itself. Because in the absence of denaturant a protein will not remain unfolded, all newly synthesized proteins will start folding as nascent chain. As the average translation rate in a mammalian cell is four or five amino acid residues per second, a protein of about 1500 residues needs about 5.5 min to be made, although the average translation rate measured for CFTR is even lower: 2.7 residues per second implying a synthesis time of about 9 minutes for the average CFTR molecule [68]. On average, this is slow compared to the formation rate of secondary structure, but fast compared to the time it usually takes for CFTR to fold and exit the ER, which indicates that most of the protein folding occurs posttranslationally. Confounding the issue of translation rate is the fact that the ribosome has variable speed on the mRNA, due to RNA secondary structure and rare codons, which cause ribosome pausing and the piling up of ribosomes behind the pausing ribosome [69]. Pausing provides time for protein folding in the absence of downstream domains, which may decrease misfolding. More speculative but attractive is the notion that pausing sites may be present in specific positions in the mRNA to regulate the outcome of folding, possibility having an important influence on the biogenesis of CFTR.

Initial evidence for an effect of codon use and pausing on the conformation of the translated protein was found for MDR1, where a silent mutation did lead to a conformational difference and hence disease [70]. Indeed, well-timed pausing may be needed to ensure both proper insertion and proper folding. CFTR's ATP-dependent release from the ribosome–translocon complex does not happen immediately upon chain termination [40], and association with cytosolic Hdj2 and Hsp70, which started during synthesis, persists [40,54,56]. Although folding studies on CFTR followed for up to 3 hours after synthesis did not show conformational changes any longer [43], the protein did continue to mature after translation [44]. This maturation involves domain assembly [9,16], which is crucial for CFTR's function and may be the main reason for the slow exit of CFTR from the ER. Misfolding of CFTR or its mutants leads to efficient degradation by the cytosolic proteasome [71,72] (see below). The initiating event for degradation may well be co-translational, because the deletion of Phe 508 can be detected when only about one-third of the protein has been synthesized. Ubiquitination of CFTR starts during synthesis [73], the E3 ubiquitin ligase associates with CFTR nascent chains [56], but the E3 CHIP

associates only after synthesis, implying steps in which both wild-type and, particularly, select mutants affect recognition for degradation of CFTR folding intermediates [56,64,74].

In summary, folding of CFTR starts during, and continues for up to an hour after, synthesis. All throughout the process, the protein associates with a series of chaperone complexes, and CFTR (mutants) can be recognized by the chaperone and degradation machineries as permanently misfolded, resulting in ubiquitination and degradation, a condition that is enhanced in many cases by disease mutations. Our understanding of these events is in its infancy.

RECOGNITION AND DEGRADATION OF MUTANT CFTR

While successful folding and maturation in the ER is a prerequisite for protein transport through the secretory pathway, it is estimated that a high percentage of translated proteins fail to be exported [75]. Retention leads to their ubiquitination and degradation by the 26S proteasome, a process denoted as ER-associated degradation (ERAD) [76,77]. The presence of a degradative pathway assures that proteins recognized as displaying highly evolutionary conserved features of "unfoldedness" are removed from the cell to avoid accumulation within the ER or the other compartments. Accumulation can lead to cell stress and the formation of toxic protein aggregates—events that trigger both lumenal and cytosolic stress responses, although neither response appears relevant to early disease, given the efficiency by which CFTR is degraded.

The selection of protein substrates for ERAD, such as CFTR, is mediated by molecular chaperones, which facilitate protein folding or degradation, depending on the conformational state of the target protein [78,79]. As noted above, the most common disease mutation that is rapidly degraded is ΔF508 [80], a class II mutation [81]. The evidence that ΔF508 is indeed a CFTR folding/processing mutation destined for ERAD comes from several fronts, and includes its temperature sensitivity, its exaggerated interactions with molecular chaperones, and the presence of intermediate conformations of the protein trapped in folding complexes, conditions under which ΔF508 CFTR appears to gain access to degradative pathways [18,82]. Although the issue of its folding in relation to translation is not settled for wild-type CFTR [43], several lines of evidence favor the concept that failure of the ΔF508 mutant to achieve appropriate domain–domain interfaces is the limiting factor in mutant protein progression [2,16,35,37,44,83,84].

Because CFTR folding is facilitated by ER-based core chaperone machinery that includes Hsp70 [53,85,86], Hsp90 [52,87], the Hsp40 co-chaperones [54,60,88,89–90], a nucleotide exchange factor [88], and calnexin [51,64,91], it is thought that these constitute a metabolic pathway to organize the polypeptide chain chemistry into an organized structure, that, in principle would be protected from degradation [3]. Indeed, many of the known chaperones have been shown to decrease NBD aggregation and improve productive CFTR folding [92]. In

contrast, unstable conformations of CFTR remain bound to chaperones, resulting in their polyubiquitination and degradation [55–57,71,93,94]. Thus, intersections between the biosynthetic and degradation pathways are monitored by a complement of chaperone and co-chaperone interactions that determine the fate of CFTR. In this manner, the interactions of CFTR with folding/degradation components are determined by conformation and likely energetics of the fold [95–97]. This can be assessed indirectly by comparing the proteolytic cleavage patterns of wild-type and ΔF508 CFTR. Such studies have indicated that protease cleavage patterns are similar for the immature wild-type and ΔF508 proteins, whereas the digestion pattern of wild-type CFTR is different, reflecting a more compact, folded conformation [37,39,43]. These data support the concept that ER-retained mutants achieve intermediate conformations in the normal CFTR folding pathway but that their variant structures and interactions with chaperone or degradative systems impair passage beyond one or more critical folding steps.

One early step in detection of the mutant fold leading to degradation involves derlin-1, which initiates CFTR extraction from the ER in cooperation with the AAA-ATPase, p97 [56,57,98]. One possibility is that derlin-1 interacts with ΔF508 CFTR and some other class II mutations due to instability within their transmembrane domains. This initial step is followed by CFTR ubiquitinylation by ER resident E3 ubiquitin ligases (e.g., RMA1 and gp78) [56,99], followed by proteasome-mediated degradation. A later step involves CHIP, an Hsp70/Hsp90-interacting E3 ubiquitin ligase, which is recruited to chaperone-bound CFTR, initiating its ubiquitinylation and degradation [55]. A third step by which CFTR can leave the productive folding pathway involves the calnexin cycle and recognition by a putative lectin in the ER, EDEM [64,100], which also links Derlin and p97 to glycoprotein extraction and proteolysis [101]. Thus, CFTR faces many steps that contribute to its degradation and these contribute to disease.

Given the complexity of CFTR folding and degradation pathways, it is not surprising that novel components will continue to be discovered. A yeast expression system, coupled with a microarray analysis, has been used to select candidates for involvement in CFTR degradation. Yeast cells handle wild-type CFTR similarly to the manner in which mammalian cells degrade the ΔF508 mutant. Yeast that express CFTR are not under stress, as they grow as well as cells lacking the CFTR expression vector, and an unfolded protein response (UPR) is not evoked [102]. Therefore, the expression of specific factors required for the ERAD of CFTR might be induced in this system. Indeed, a number of factors have now been identified using this approach. More than 150 transcripts were increased more than 1.6-fold in yeast expressing CFTR, and these included genes known to be involved in CFTR biogenesis in yeast or mammalian cells. Examples include *HSP82*, which encodes the yeast Hsp90 homolog, and *FES1*, which is a nucleotide exchange factor for cytosolic Hsp70 [52,88,102,103]. Of interest is that the message encoding the small heat-shock protein (sHsp), Hsp26, increased

significantly and the expression of CFTR was completely stabilized in yeast deleted for the two sHsps, Hsp26 and Hsp42. Intriguingly, the stability of other yeast ERAD substrates was not altered in strains deleted for the sHsp, and therefore the sHsps show some selectivity for CFTR.

In extending this work to mammalian cells expressing wild-type and ΔF508 CFTR, the overexpression of a mammalian sHsp, αA-crystallin, selectively accelerated the degradation of ΔF508 CFTR [74], and similar findings have now also emerged for mammalian Hsp27 (Ahner and Frizzell, unpublished observations). These findings add CFTR to a growing list of protein folding/aggregation diseases that involve interactions with sHsps [57], where the sHsps have been implicated primarily as factors that stabilize proteins during cell stress [104], and associate with partially unfolded proteins to maintain their solubility until more favorable conditions return [105–111]. It is currently thought that the sHsps tend not to interact with either native or completely denatured proteins, but with proteins of an intermediate, foldable conformation [112–114], and they can distinguish between structurally identical wild-type and mutant proteins based on small differences in unfolding free energy [115–118]. While the association of sHsps with their substrates is ATP-independent, substrate re-folding often involves the action of ATP-dependent chaperones (e.g., Hsp70, Hsp90, and Hsp104) [106,111,119–122]. This property of sHsps may contribute to their association with immature ΔF508 CFTR, which adopts an intermediate conformation that has the potential for rescue, as discussed above. Accordingly, the interaction of sHsps with CFTR may represent a branch point between degradation and folding where mutant CFTR could be recovered to the productive folding pathway [96,97]. Of more concern is the fact that these machineries are highly variable between cell types; hence, what rescues CFTR from degradation and promotes trafficking of variant CFTR in one cell or tissue type may not suffice in another [3,123,124].

CFTR STRUCTURE

An understanding of the structure of folded and misfolded variants will undoubtedly contribute significantly to our understanding of disease. Although a high-resolution structure of full-length CFTR remains elusive, recently published biochemical and structural analyses of full-length CFTR using low-resolution cryo-EM approaches [125] have illustrated key features that reflect the spatial relationship of domains probably observed in ABC transporters MsbA, BtuCD, Sal177, and Sav1866 [126–129].

The most progress has been made with understanding the fold of soluble, isolated ABC transporter NBDs and have begun to reveal the domain arrangement and mechanochemistry of this feature of CFTR structure and function. The findings indicate that nucleotide-dependent association–dissociation of the two NBDs plays a central role in harvesting the energy of ATP binding and hydrolysis [130,131]. These studies show that mutant, catalytically

inactive NBDs can form stable symmetrical dimers in the presence of ATP. This dimer places two ATPs at the dimer interface, between the Walker A and B consensus sequences of one domain and the Signature sequence (LSGGQ) of the opposing domain with the two ATPs forming the bulk of the interface [131]. The Hill coefficient of 1.7 measured for the activity of the homodimeric ABC transporters is consistent with a cooperative two-site mechanism [132]. Upon hydrolysis, electrostatic mismatch at the active site and α-helical subdomain rotation within the NBDs [133,134] leads to a rapid dissociation event. This arrangement neatly solves the problem of the positive cooperativity of ATPase activity catalyzed by the isolated NBDs and the open active site and structurally remote transporter signature sequence in the monomeric NBD structures [131–135]. Recent structures of intact ABC transporters MsbA, BtuCD, Sav1866, and P-glycoproteins [171] reveal that the TMDs interact with one surface of this dimer [136–138], and thus suggest that NBD interactions can be coupled efficiently to a scissoring motion of the integral membrane solute pathway of the transporters [139].

By contrast to the homodimeric NBDs, the two NBDs of CFTR are distinct from each other in important ways. Hydrolytically active NBDs have a catalytic glutamate residue at the end of the Walker B consensus. Whereas NBD2 (the C-terminal NBD of CFTR) has a glutamate, NBD1 (the N-terminal NBD) has a serine at this position. In addition, NBD1 also lacks a critical histidine residue in the H-loop. Recent studies with the murine CFTR NBD1 indicate that it does not catalyze significant hydrolysis of ATP [67]. NBD1 contains the canonical LSGGQ in the highly conserved Signature sequence, whereas NBD2 has a more unusual LSHGH sequence. Thus, if CFTR NBD1 and NBD2 dimerize to form an ATP sandwich dimer, like the homodimeric NBDs, one of the two nucleotide sites (Walker A and B from NBD1 and the Signature from NBD2) is very unusual, perhaps consistent with CFTR's identity as a channel within a family of transporters.

Recently, structures of murine CFTR-NBD1, both wild-type and ΔF508, which is 78% identical to the human CFTR-NBD1, have been solved at high resolution [67,84]. The structure of NBD1 with AMP-PNP bound highlights both the similarities and differences between CFTR-NBD1 and the other ABC transporter NBDs solved to date and discussed above. No significant conformational changes between the six murine NBD1 structures were observed, regardless of the nucleotide bound finding. Moreover, ΔF508 showed only minor difference from wild-type. However, this has recently been found to likely result from suppressor mutations embedded in the ΔF508 sequence necessary to generate crystals [140]. The fold of the new CFTR-NBD1 structures is very similar to that of the other ATP-binding cassettes with a β-sheet subdomain, a core containing the Walker A and B sequences (similar to the RecA/F1 fold), and an α-helical subdomain that contains the signature sequence and is the site of the ΔF508 mutation. This residue is located on the surface of NBD1 at a position remote from the NBD dimer interface, but presumed to be at or near the interface for interaction with the TMDs. The location of F508 at the putative NBD–TMD interface is consistent with previous studies indicating that deletion of F508 did not affect the stability of the isolated domain significantly but destabilized the intact CFTR structure [39,141–143] and analyses of missense mutations at this position.

CFTR NBD1 contains an insertion between the first and second β-strands (residues 406 to 434) which is largely disordered in the crystals. However, when NBD1 is phosphorylated, a partial ordering of this insertion is observed which may partially occlude the nucleotide [67,84] and perhaps accounts for the very unusual nucleotide binding kinetics observed [144] (see below). Thus, by contrast to earlier predictions of the boundaries of NBD1, the first strand is composed of residues 393 to 400, and the phenylalanine at position 400 is in position to interact potentially with the nucleotide base. In all of the CFTR–NBD1 structures, the nucleotide is bound in an unusual conformation. NBD1 also has additional secondary structural elements that serve to further alter the putative dimer interface, including a helix at the extreme C-terminus that has previously been assigned to the regulatory (R) domain. Thus, formation of a canonical ATP sandwich dimer will either require reorientation of these elements or compensatory changes at the NBD2 interface to accommodate them. These initial structural characterizations of CFTR and its homologs provide new insight about the function and dysfunction of this important protein. The NBD1 domain is thought to "talk" to both the C1 and C4 cytoplasmic loops linking the various transmembrane domains. Thus, NBD1 plays a pivotal role in assembly of the full-length polypeptide, a process assisted largely by chaperones [3,16,127–129].

TRAFFICKING TO THE CELL SURFACE

The capacity of the ER to export cargo protein necessarily encompasses critical energetic relationships between protein translation, folding, degradation, and trafficking pathways [95–97,169,170,172]. Such an approach reveals an *export landscape*, defined as the "minimal export threshold" which embraces the key fundamental properties of the protein fold-folding kinetics, misfolding kinetics, and thermodynamic stability [96]. This three-dimensional landscape reveals that CFTR escapes via a highly adaptable ER environment through a diverse combination of folding-and trafficking-related pathways. Recognition that a network of interacting components may be interlinked to achieve folding and trafficking from an energetic standpoint may be the achilles heel of CF and of misfolding disease in general, allowing a broad range of both direct and indirect chemical and biological approaches to promote recovering of misfolded CFTR variants for function at the cell surface [145].

The first step in delivery of CFTR to the cell surface from the ER is its interaction with the COPII machinery [123,146]. This machinery consists largely of a small group of cytoplasmic components, including the small GTPase Sar1 and the Sec23/24 adaptor complex, which together recognize a

diacidic code on the folded NBD1 domain of CFTR [147]. This interaction between CFTR and the adaptor complex results in its collection into vesicles budding from the ER surface, a process driven by the self-assembly of the cytosolic protein complex Sec13/31 [148]. The overall process can be defined as a set of linked kinetic equilibria in which the kinetics of folding, the strength of the adaptor recognition, and the kinetics of cage self-assembly, possibly catalyzed by the cargo–adaptor complexes, dictate the steady-state rate of incorporation of CFTR into COPII vesicles [123]. Moreover, CFTR exiting the ER is in competition with a large number of other cargo poised for exit. Therefore, numerous factors contribute in an as yet to be defined manner to the overall success or failure of either wild-type and mutant CFTR to evade the ERAD metabolic pathway and interact with the COPII export pathway. Like degradation and chaperone components, the levels of the COPII machinery vary widely between cell types, suggesting that CFTR wild-type and variants may face unique conditions in different cell types comprising various tissues that either robustly support or provide more limited support for export.

Following the exit from the ER, CFTR is transported through the Golgi, where its two N-linked oligosaccharides are further processed to more complex structures, followed by delivery to the cell surface. Almost nothing is known about trafficking of CFTR through the Golgi, although the small Rho-like GTPase TM10 and the protein CAL have been implicated in selection for exit from the trans Golgi network, for delivery to the cell surface, and/or for recycling from early endocytic compartments to the late compartments or the Golgi [149–151]. The physiological impact of these events on mutant function remains to be more fully clarified.

STABILITY AND TRAFFICKING AT THE CELL SURFACE

Compelling evidence indicates that ΔF508 CFTR molecules reaching the post-Golgi compartments are metabolically and functionally unstable in nonpolarized [37,45,152] and polarized [37,153–155] heterologous expression systems, as well as in primary culture of human respiratory epithelia [37]. The cell surface resident ΔF508 CFTR retains some channel activity [156]. Moreover, recent morphological and functional observations suggest that small amounts of mutant may constitutively escape the ER in primary culture, freshly isolated human and animal cells, and native tissues [157–159]. Thus, the fate of the ΔF508 CFTR in post-Golgi compartments probably influences the severity of the disease and the efficacy of therapeutic interventions in rescuing the ΔF508 CFTR as well as other variants whose cell surface activities and stability remain largely unexplored.

Reduced temperature can overcome ΔF508 failure to exit the ER by facilitating folding and/or increasing protein stability [35,45]. Intriguingly, the quasi-native conformation of the rescued ΔF508 CFTR is partially lost upon elevating the ambient temperature to 37°C, in accord with the mutant

metabolic destabilization and profoundly increased ubiquitination [37]. The temperature-sensitive stability defect is associated with accelerated internalization and impaired recycling of the rescued ΔF508 CFTR back to the cell surface. This is in sharp contrast with the slow internalization and highly efficient recycling of wild-type CFTR, a prerequisite to maintaining the channel and slow metabolic turnover at the plasma membrane [37,152,153,155].

While the underlying mechanism of accelerated internalization of the rescued ΔF508 CFTR at physiological temperature remains elusive, it appears that the ubiquitination pathway plays the central role in rerouting a variety of nonnative, internalized CFTRs from recycling for lysosomal proteolysis in BHK cells [37,71], although a recycling defect could not be observed in respiratory epithelia [153]. Rescuing the peripheral folding defect attenuated the relative ubiquitination level of the mutant. Inactivation of the E1 ubiquitin-activating enzyme also stabilized ΔF508 and the C-terminal truncation Δ70 CFTR at the cell surface. These and other observations, in concert with the preferential association of ΔF508 CFTR with Ub-binding proteins (e.g., Hrs, STAM-2, and TSG101) and components of the Ub-dependent endosomal sorting machinery (ESCRT II and ESCRT III) suggest a link between ubiquitination-modification and lysosomal degradation of misfolded CFTR from the cell surface [37] and highlight potential drug targets for mutant stabilization.

A large number of additional factors have been found to associate with CFTR at the cell surface. These involve multiple components of the endocytic and recycling machineries [160], although their exact roles remained to be assessed. Moreover, the capacity of CFTR to interact with other proteins through its C-terminal PDZ domain, which, in turn, link the protein to the amiloride-sensitive epithelial Na^+ channel (ENAC) and G-protein coupled receptors and other signaling pathways [160,161], suggests that we have much to learn about CFTR as a "hub" protein in human physiology and how these linked pathways are disrupted at the onset of disease and during aging in response to CFTR misfolding.

CURRENT EFFORTS AND FUTURE OPPORTUNITIES TO CORRECT CFTR FOLDING: THE WAY FORWARD

Despite extensive past and ongoing efforts to date in the CF field, many challenges remain to fully characterize the basic biochemical and biophysical properties that direct CFTR folding and function, and to more fully understand the networks that direct both folding and function in different cell types. The concept that ΔF508 and possibly other mutants are trapped in folding intermediates implies that the ΔF508 can be rescued from intermediate conformations if the limiting steps are appropriately manipulated by adjusting the folding, trafficking, and stabilization environment associated with the ER and/or the cell surface. That this can occur is demonstrated by the fact that ΔF508 CFTR can be rescued biochemically and functionally not only by low

temperature as discussed above [162] but by intragenic suppressor mutations [163,164], alteration of putative ER retention motifs [165], and/or altered chaperone activities [3,102]. In the latter case, demonstration that manipulation of the folding pathway managed by the Hsp90 chaperone through its co-chaperone Aha1 can lead to cell surface conductance supports the idea that a novel class of compounds, referred to as *proteostasis regulators* [145], can be used to adjust mutant protein folds to modify disease progression. When used independently or combined with chemical chaperones [166], or more likely, pharmacological chaperones that could bind to and stabilize the fold directly, including compounds referred to as correctors [167,168], or that activate cell surface–localized CFTR (potentiators), unanticipated avenues for disease intervention at early and late stages may result.

Interaction of mutant CFTR with local folding environment challenges the protein homeostasis or "proteostasis" program of the cell [169,170,172]. It is now apparent that an understanding of the individual contribution(s) of translation, folding, degradation, trafficking, and regulation of channel activity at the cell surface will be important in deciphering the basis for disease onset and its progression. Of central concern is that disease progression is also associated with alterations not only in surface anion and cation balance, but also inflammatory responses, alteration of carbohydrate processing, and glycan (mucin) deposition and predisposes, for example, the lung to pathogenic environments that contribute significantly to pulmonary failure. Our knowledge base of how the CFTR fold and its function contribute to these processes normally and in response to mutation is very limited. In this regard, a recent effort by the CF Foundation has led to the construction of a comprehensive "Roadmap" of current known CFTR interaction pathways using the GeneGo platform. These maps provide a baseline to begin to organize our current knowledge base, including what contributes to and/or dictates CFTR function in health and disease. Moreover, these pathways provide a starting point to integrate CFTR folding with function and therapeutics, and perhaps drive a multiple pathway-based approach to correcting this chronic early-onset disease. To accomplish these goals, it will take the combined efforts not only of the CF Consortium, but the CF community in general, to develop new tools, identify new targets, and provide new assays that are amenable to drug screening efforts that could be used to move our understanding of basic science of the folding problem into the clinic.

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