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Defects in processing and trafficking of the cystic fibrosis transmembrane conductance regulator

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Defects in processing and trafficking of cystic fibrosis transmembrane conductance regulator. Cystic fibrosis (CF) is caused by inherited mutations in the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR), a cAMP-regulated chloride channel expressed in epithelial tissues. Most mutations in CF patients result in rapid intracellular degradation of the CFTR protein. While this defect is thought to result from abnormal protein folding, it is unclear how mutant and wild-type (WT) proteins differ in structure, how the cell is able to distinguish these differences, and how the fate of the mutant protein is determined. By examining the initial steps of CFTR assembly into the endoplasmic reticulum (ER) membrane, it has recently been shown that CFTR utilizes two redundant translocation pathways to direct N-terminus folding events. Mutations that block one pathway therefore do not alter transmembrane topology, but rather appear to disrupt intracellular trafficking through perturbations in higher order tertiary structure. These studies suggest that cellular quality control machinery acts at least in part, by monitoring proper interactions between CFTR subdomains. The end result of this process is the conversion of misfolded CFTR into a membrane-bound, polyubiquitinated complex. This complex recruits cytosolic degradation machinery to the endoplasmic reticulum membrane where CFTR is degraded as it is extracted from the lipid bilayer. Understanding how cellular machinery mediates this process will be an important step in designing strategies to modify protein folding and degradation in CF and related ion channelopathies.

The cystic fibrosis transmembrane conductance regulator (CFTR) is a complex, polytopic membrane protein expressed in the apical membrane of selected epithelial cells. CFTR functions directly as a cAMP regulated chloride channel [1] and also regulates the activity of other membrane proteins including the epithelial sodium channel (ENaC) [2] and the outwardly rectifying chloride channel (ORCC) [3]. It thus plays a key role in the movement of ions and water across epithelial tissues. Not surprisingly, CFTR disruption results in a pleiotropic phenotype [4]. The most profound effects are insufficiency of the exocrine pancreas, increase in sweat chloride concentration, male infertility and recurrent pulmonary infections. In most cystic fibrosis (CF) patients, chronic airway inflammation results in progressive pulmonary scarring, reduced lung function and ultimately, death. While CFTR is also expressed in the kidney (proximal and distal tubules, cortical collecting duct and inner medullary collecting duct [5]), only mild renal abnormalities are observed in CF patients [reviewed in 6]. These include decreased ability to excrete a salt load, mild urinary concentrating defects, increased proximal sodium reabsorption and altered drug excretion. CFTR is also expressed in the apical membrane of renal cysts in patients with polycystic kidney disease, and may play a role in chloride and fluid secretion into the cyst lumen [7, 8].

More than 800 mutations in the *CFTR* gene have been identified in CF patients [9]. These are broadly grouped into four classes: (I) defective protein synthesis; (II) defective protein processing; (III) defective ion conduction; and (IV) defective regulation of channel gating [10]. Defective processing is by far the most common mechanism of protein disruption, accounting for more than 2/3 of clinical CF cases [11]. While these latter patients synthesize adequate amounts of functional CFTR protein, the mutant protein is rapidly degraded prior to reaching the plasma membrane. To understand the molecular basis of CF it will therefore be necessary to define the molecular mechanism(s) by which CFTR is folded, assembled and packaged into cellular membranes and trafficked through cells.

CFTR is a member of the ATP binding cassette (ABC) transporter superfamily [12]. It contains two hydrophobic domains (each with six predicted transmembrane (TM) segments), two cytosolic nucleotide binding domains (NBDs) and a cytosolic regulatory (R) domain. Like most eukaryotic membrane proteins, CFTR is synthesized and assembled in the endoplasmic reticulum (ER). During the earliest steps in this process, nascent chain-ribosome complexes are targeted to the ER membrane, and TM segments are precisely oriented and inte-

Key words: cystic fibrosis, CFTR, biogenesis, endoplasmic reticulum, topology, ion channel.

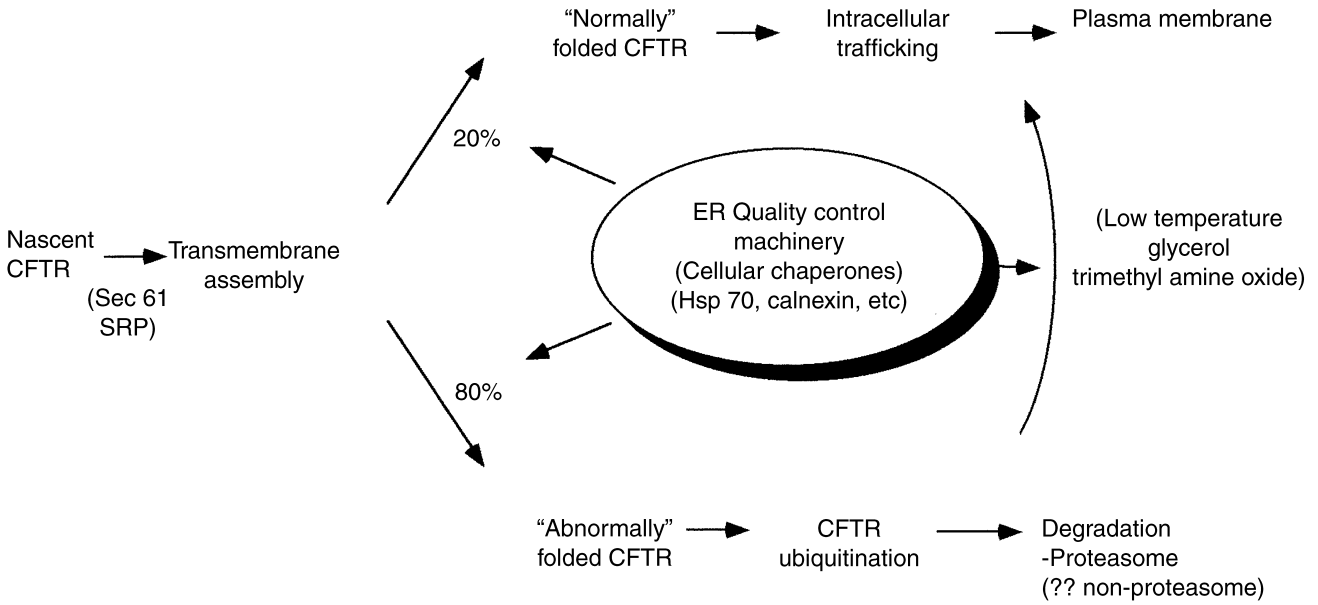

Fig. 1. Cystic fibrosis transmembrane conductance regulator (CFTR) biogenesis and degradation. Synthesis of nascent CFTR begins on cytosolic ribosomes that are targeted via SRP to the Sec61 complex in the endoplasmic reticulum (ER) membrane. As translation continues, transmembrane topology is established in the ER membrane and folding and assembly of membranous, lumenal and cytosolic domains is facilitated by cellular chaperones. For unclear reasons, only 20% of WT CFTR is normally trafficked out of the ER compartment where it then undergoes gradual turnover [20]. Most CFTR protein (~80%) fails to exit the ER, undergoes polyubiquitination and is rapidly degraded by the 26S proteasome. For many CFTR mutants (such as ΔF508), ER degradation accounts for nearly 100% of newly synthesized protein despite the fact that the mutant protein forms functional chloride channels in the ER membrane. A major challenge in CF is therefore to devise methods to rescue CFTR into productive folding pathways. This strategy has been proved in principle using reduced temperature [21], and molecular chaperones such as glycerol, and trimethylamine oxide [22, 23].

grated into the lipid bilayer [13]. Additional biogenesis events involve the packing of transmembrane helices, folding of cytosolic domains, and finally, assembly of these domains into a mature tertiary structure [14]. This process is mediated by specialized cellular machinery that includes the Sec61 translocation complex and cytosolic (hsp70, hsp40) as well as ER (calnexin) chaperones that assist folding and prevent aggregation of folding intermediates [15, 16]. CFTR maturation is thus a stepwise and compartmentalized process that coordinates folding of different protein domains in the lipid environment of the ER membrane, the oxidizing environment of the ER lumen and the reducing environment of the cytosol (Fig. 1). At the center of this process is a stringent quality control mechanism capable of discriminating normally folded from abnormally folded proteins [17]. Quality control machinery thus prevents misfolded CFTR from exiting the ER compartment and is responsible for its degradation via the cytosolic ubiquitin/proteasome pathway [18, 19].

This article reviews recent studies that address three questions related to the underlying defect in CFTR processing and trafficking. How is WT CFTR assembled into the ER membrane, and how do inherited mutations affect this process? What general structural features in mutant CFTR might serve to alert cellular quality control machinery? Finally, how is misfolded CFTR delivered to cytosolic proteases?

MECHANISM OF CFTR ASSEMBLY INTO THE ER MEMBRANE

Conventional models predict that the transmembrane topology of polytopic proteins is established through the action of sequential signal and stop transfer sequences as the nascent chain emerges from the ribosome [24]. Signal sequences function to target nascent chains to the ER, facilitate ribosome binding to the membrane, and open a large aqueous translocation channel through which the elongating nascent chain moves to reach the ER lumen [25–29]. Stop transfer sequences terminate ongoing translocation, disrupt the ribosome-membrane junction, close the translocon, and direct the hydrophobic TM helix laterally out of the translocon and into the lipid bilayer [29–31]. A large number of molecular events must therefore be precisely coordinated as polytopic proteins such as CFTR are "stitched" into the ER membrane.

If CFTR followed a conventional biogenesis model, then the first TM segment (TM1) should encode signal sequence activity capable of orienting the N-terminus in the cytosol and the first extracellular loop (ECL1) in

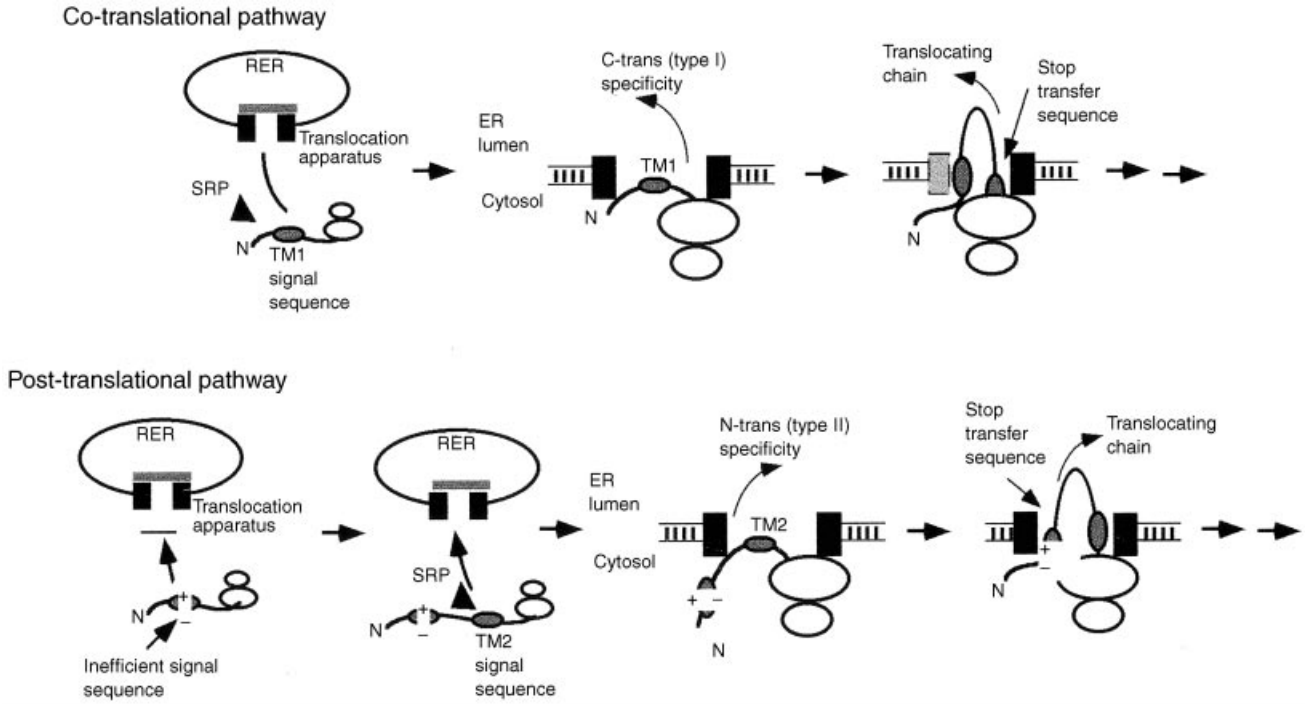

Fig. 2. Alternate pathways for CFTR N-terminus transmembrane assembly. In the conventional or cotranslational pathway, CFTR topology is established through sequential action of TM1 signal sequence activity and TM2 stop transfer activity. During this process the TM1 gates the translocon open and the nascent chain translocates into the ER lumen in an N→C terminus direction as it emerges from the ribosome. The post-translational pathway is utilized by most (>60%) of WT chains and essentially all G85E and G91R mutant chains. Here, TM2 acts as the initial signal sequence to start translocation which proceeds in a C→N terminus direction (designated by arrow). In both pathways, the final topology of the nascent chain is equivalent; TM1 and TM2 each span the membrane and the intervening peptide loop, ECL1, resides in the ER lumen. Ribosomes (open circles), TM segments (shaded ovals), translocon channel (black rectangles), and lipid bilayer are indicated (modified from [13], used with permission from *Journal of Biological Chemistry*).

the ER lumen. Surprisingly, when TM1 signal sequence activity was tested in a defined heterologous cassette, TM1 was unable to efficiently initiate translocation or span the membrane [13]. This suggested either that CFTR assembly into the ER membrane was inefficient, or that topogenic information in addition to TM1 was required for N-terminus transmembrane assembly. Subsequent analysis confirmed the latter prediction by demonstrating that TM2 also functioned as a signal sequence with translocation specificity complimentary to that of TM1. Moreover, by simultaneously disrupting signal sequence activities of TM1 and TM2, it was shown that TM2 was able to independently orient TM1 and ECL1 in the ER membrane after this region had been synthesized in the cytosol. This post-translational translocation activity of TM2 was ribosome dependent, indicating that TM2, like TM1, utilized established translocation machinery (for example, signal recognition particle) for ER targeting [32].

Mutagenesis studies of TM1 and TM2 thus define two alternate translocation pathways by which CFTR acquires its proper N-terminus transmembrane topology (diagrammed in Fig. 2). For a minority of nascent chains, TM1 functions as a signal sequence to initiate translocation into the ER lumen. In these chains, TM2 stop transfer activity terminates ongoing translocation and establishes the membrane boundaries of TM1, TM2 and ECL1. In chains where TM1 fails to start translocation, however, TM1 and TM2 emerge from the ribosome into the cytosol where TM2 initiates translocation of its N-terminus flanking residues. Here, TM1 functions in the capacity of a stop transfer sequence and is positioned post-translationally into its proper orientation. In this manner, TM2 provides a backup mechanism for ensuring proper topology in chains where TM1-mediated translocation has failed.

Further analysis indicated that two charged residues located within the hydrophobic membrane spanning core of TM1 (E92 and K95) were responsible for the weak TM1 signal sequence activity. Mutating these residues to alanine markedly improved the ability of TM1 to direct translocation [13] but completely disrupted CFTR chloride channel activity in *Xenopus* oocytes (unpublished observations). Thus, for CFTR, structural features required for protein function (such as residues E92 and K95) directly conflict with structural features necessary to direct CFTR topology via the cotranslational pathway (Fig. 2). The presence of TM2 signal sequence activity,

by providing an alternate mechanism to ensure CFTR topology, therefore enables TM1 to contain the necessary charged residues. This increased sequence diversity within TM1 would not have been possible if CFTR biogenesis were restricted solely to the conventional mode of biogenesis. While CFTR provides the first example of this type of redundancy in topogenic pathways, it seems likely that other polytopic proteins, particularly those with specialized structural requirements, will exhibit additional variations in transmembrane assembly.

EFFECTS OF INHERITED MUTATIONS ON CFTR TRANSMEMBRANE ASSEMBLY

Two CF mutations, G85E and G91R, each introduce an additional charged residue within the hydrophobic core of TM1. These mutations also disrupted CFTR chloride efflux in microinjected *Xenopus* oocytes by preventing newly synthesized protein from exiting the ER [33]. This suggested that G85E and G91R CFTR mutants failed to fold properly and were recognized by ER quality control machinery similar to the common ΔF508 mutant [11]. To understand how charged residues within TM1 influenced CFTR folding, we compared N-terminus transmembrane assembly and topology in WT and mutant chains. Topologic analysis revealed that each mutation completely eliminated TM1 signal sequence activity but had no effect on CFTR topology [13, 33]. Thus, in these mutant chains, TM2 was entirely responsible for directing translocation of ECL1. More importantly, because mutant TM1 and TM2 each spanned the membrane in their native orientations, ER quality control machinery must have been able to detect the presence of the aberrant charged residues localized within the plane of the lipid bilayer. To determine how this might occur, WT and mutant CFTR constructs were truncated after the second transmembrane segment, the first transmembrane domain (TM6), NBD1 or the R domain. Expression of these constructs expressed in *Xenopus* oocytes demonstrated that cellular quality control machinery was effectively able to distinguish WT from mutant chains only after synthesis of the R domain had been completed [34].

These studies demonstrated that in order for CFTR to acquire a stable structure in the ER membrane, multiple protein domains must be synthesized and properly assembled. In addition, they indicated that G85E and G91R mutations likely interfered with late, rather than early, assembly events required for CFTR tertiary structure. A subtle alteration in the first transmembrane domain such as the insertion of a charged residue may thus indirectly influence folding interactions at distant sites in the molecule. This provides an intriguing model as to how mutant proteins might be recognized by ER quality control machinery, and how mutations in diverse regions of CFTR could give rise to similar trafficking phenotypes. If ER quality control machinery recognized structural interfaces between CFTR subdomains, then subtle structural changes that influence the strength and/or kinetics of these interactions could be recognized by quality control machinery in much the same manner as unassembled oligomeric subunits. ER quality control machinery would therefore not be required to recognize each local structural perturbation, but rather it might serve to monitor more global aspects of protein compaction.

Finally, it should be noted that "abnormal" protein folding in terms of ER quality control is operational and entirely based on a cellular response, namely ER associated degradation [17]. While it is often tempting to view the acquisition of protein function as a criteria for "normal" folding, in the case of CFTR this is not necessarily correct. CFTR protein containing the ΔF508 mutation is clearly capable of forming cAMP gated chloride channels with nearly normal conduction properties [35]. Yet essentially 100% of ΔF508 CFTR is degraded in the ER. Conversely, WT CFTR protein truncated after the R domain at residue #836 is nearly as stable as full length protein in *Xenopus* oocytes [33], yet its chloride channel activity is $<5\%$ of wild-type (unpublished observations). Thus, protein maturation in a functional sense may be distinct from structural maturation as determined by ER quality control machinery. This process is further complicated by observations that the efficiency of intracellular trafficking differs markedly between cell systems. In mammalian cells, 80% of WT and $\sim$99% of ΔF508 CFTR is degraded in the ER, while in *Xenopus* oocytes, $<10\%$ of WT and $\sim$80% of ΔF508 is degraded in the ER [34]. It is unknown whether these differences in intracellular trafficking reflect different folding efficiencies or alternatively, different stringencies in quality control systems. In either case, understanding the relationship between CFTR quality control and CFTR functional maturation will likely require detailed structural studies and the identification of cellular components responsible for discriminating subtle structural differences.

CFTR DEGRADATION BY THE UBIQUITIN/ PROTEASOME PATHWAY

The hallmark of abnormal CFTR processing and trafficking is rapid degradation of CFTR protein in a pre-Golgi and lysosome-independent compartment. Surprisingly, several studies have now demonstrated that the cytosolic ubiquitin-proteasome pathway plays a key role in ER associated degradation not only of CFTR, but also of a wide variety of misfolded secretory, bitopic and polytopic protein substrates [17, 36]. In the ubiquitin proteasome pathway, substrates are first modified by covalent addition of multiple ubiquitin moieties through the action of cytosolic (and/or membrane bound) ubiqui-

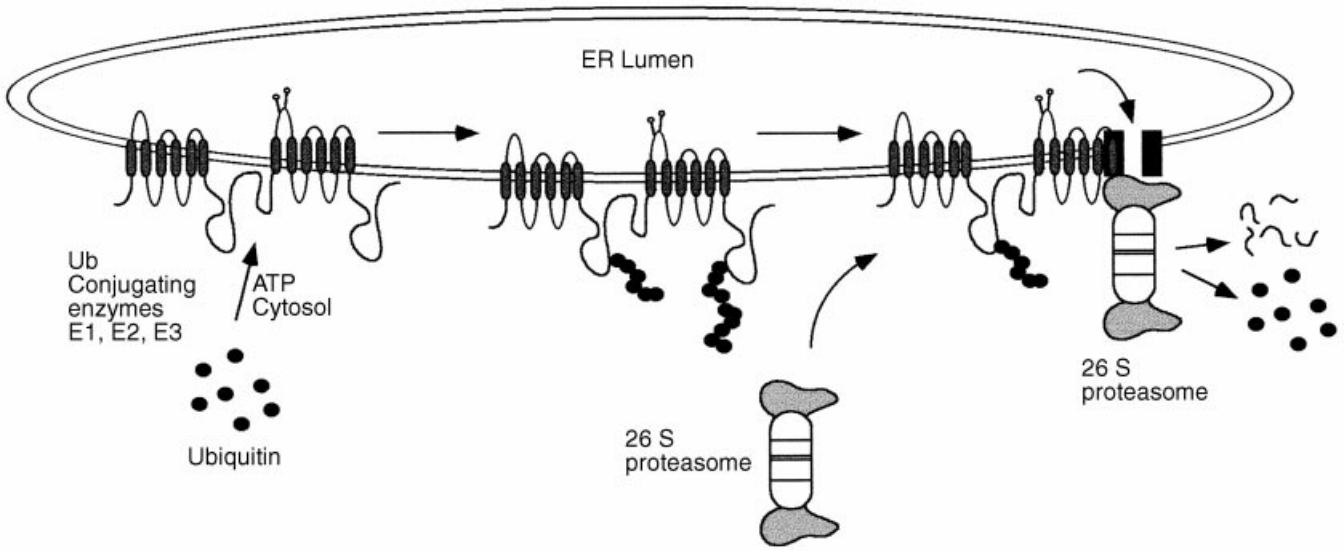

Fig. 3. Proteasome-mediated CFTR degradation. Full length, membrane-integrated and glycosylated CFTR undergoes polyubiquitination at the ER membrane in an ATP- and cytosol-dependent manner [40]. This complex remains tightly bound to the ER membrane and recruits cytosolic proteolytic machinery that includes the 26S proteasome. During degradation, ubiquitin moieties are removed and CFTR is cleaved into small (TCA soluble) peptide fragments as the protein is extracted from the ER membrane. One possibility is that ATPase activity within the 19S proteasome regulatory subunit facilitates CFTR unfolding and/or membrane extraction.

tin activating (E1), conjugating (E2), and ligating (E3) enzymes [reviewed in 37]. Polyubiquitinated proteins are then recognized by the cytosolic 26S proteasome complex; ubiquitin chains are removed; and the substrate is digested into small peptide fragments. These observations require that cytosolic degradation machinery gains access to proteins in the lumen (or membrane) of the ER, and suggest that translocation across the ER membrane is a bidirectional process that is regulated in part by the folded state of a given protein [33, 38].

The degradation of polytopic proteins by cytosolic proteases poses an additional topologic challenge in that multiple transmembrane helices must be removed from the bilayer. Then, at what stage of biogenesis is CFTR recognized for degradation, and once recognized, how is CFTR delivered to the cytosolic proteolytic complex? Recently, Sato, Ward and Kopito used an in vitro expression system to demonstrate that CFTR ubiquitination might actually begin prior to the completion of protein synthesis [39]. Using a similar rabbit reticulocyte lysate-based expression system, we showed that full length and membrane integrated CFTR is also a substrate for polyubiquitination (Fig. 3) [40]. CFTR ubiquitination required cytosolic components as well as ATP. By allowing ubiquitination to occur in the presence of the proteasome inhibitor hemin, we demonstrated that ubiquitinated CFTR remained tightly associated with the ER membrane until it was degraded into trichloroacetic acid-soluble fragments. Furthermore, pre-ubiquitinated, membrane-bound CFTR could be degraded only in the presence of additional cytosol [40].

These results suggest that polyubiquitinated CFTR is involved in recruiting cytosolic degradation machinery directly to the ER membrane, consistent with studies by Rivett, Palmer and Knecht, which demonstrate that proteasomes are bound to the ER in living cells [41]. Because hemin inhibits the proteasome by blocking ATPase activities within the 19 S subunit (PA700) [42], it is also possible that the unfolding activity of the proteasome itself might be involved in extracting CFTR from the membrane. This would explain our observation that CFTR degradation was tightly coupled to extraction of TM helices from the lipid bilayer. It would also explain why proteasome inhibitors such as peptide aldehydes and/or lactacystin that directly inactivate the catalytic active site in the proteasome, but do not effect ATPase activity [42], might give rise to cytosolic intermediates of ER degradation substrates.

CFTR PROCESSING AND TRAFFICKING AS A PARADIGM FOR ION CHANNELOPATHIES

In the past decade medical research has uncovered the genetic basis for an expanding group of ion channelopathies. A significant challenge in the next decade will be to decipher the underlying cellular and metabolic pathways that are influenced by these mutations. This will involve: (*1*) identifying the molecular components and steps that regulate normal biosynthetic processes; (*2*) defining how specific mutations influence these pathways; and (*3*) devising strategies for controlled manipulation of these pathways in human disease. In this regard,

CF has served as a key example for understanding fundamental mechanisms of protein biogenesis, folding and degradation. It seems highly likely that these studies will have far reaching implications, and that future efforts to understand and correct the CF defect at the cellular level will impact an ever growing variety of ion channelopathies.

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APPENDIX

Abbreviations used in this article are: ABC transporter superfamily, ATP binding cassette transporter superfamily; ATP, adenosine 5'-triphosphate; CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; E1, ubiquitin activating enzyme; E2, ubiquitin conjugating enzyme; E3, ubiquitin ligating enzyme; ECL, extracellular loop; ECL1, first ECL in the ER lumin; ENaC, epithelial sodium channel; ER, endoplasmic reticulum; hsp, heat shock protein; NBD, nucleotide binding domain; ORCC, outwardly rectifying chloride channel; TM, transmembrane; TM1, first TM segment; WT, wild-type.

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