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Biogenesis, Trafficing, and Function of Wild-Type and Mutant Cystic Fibrosis Transmembrane Conductance Regulator (CFTR)

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BIOGENESIS, TRAFFICKING, AND FUNCTION OF WILD-TYPE AND MUTANT CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR (CFTR)

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ABSTRACT

Cystic fibrosis (CF) is a genetic disease resulting from mutations in the cystic fibrosis transmembrane conductance regulator (CFTR), a cAMP-activated chloride channel that functions at the apical surface of epithelial cells and plays a critical role in mucociliary clearance. The most common mutation in the *CFTR* gene involves a deletion of phenylalanine at position 508 in the protein (ΔF508 CFTR). ΔF508 CFTR is recognized as misfolded in the endoplasmic reticulum (ER) and is degraded. *In vitro* manipulations such as culturing cells at 27˚C can "rescue" ΔF508 CFTR to the plasma membrane (rΔF508 CFTR), but no pharmacological agent has produced comparable results. Compared to the wild type (WT) protein, rΔF508 CFTR is very rapidly cleared from the plasma membrane and has channel-gating defects. Some other disease-causing CFTR mutations also exhibit altered cell surface properties. Herein, we characterized how two small molecules, which help with proper protein folding (pharmacological chaperones corr-4a and VRT-325) improved the cell surface stability and function of rΔF508CFTR, and we examined the cell surface trafficking and function of two previously uncharacterized CFTR mutations, R31C and R31L. Corr-4a ([2-(5-Chloro-2-methoxy-phenylamino)-4'-methyl-[4,5']bithiazolyl-2'-yl]-phenyl-methanone) promotes CFTR rescue and Vertex-532 (4-Methyl-2-(5-phenyl-1H-pyrazol-3-yl)-phenol) affects its channel gating. Our analysis of ΔF508 CFTR-expressing polarized epithelial cells treated

with corr-4a revealed that rΔF508 CFTR was stabilized at the cell surface, but the cAMP responsiveness was still abnormal. Cell surface biotinylation and Ussing chamber analyses revealed that in the presence of corr-4a, there was enhanced channel activity at 6 h. The addition of the VRT-532 dramatically increased the cAMP responsiveness of the channel, thus the combination of compounds was necessary to correct two major defects associated with this mutation. We also determined that, like ΔF508, the R31C and the R31L mutations compromised the biogenesis and enhanced the clearance of CFTR from the cell surface, which synergistically decreased surface expression to cause disease. In summary, our studies on the trafficking and functional defects of 3 naturally occurring mutations in CFTR contribute to understanding the molecular basis for the altered trafficking of the CFTR protein; this is an important step in developing therapies for CF.

DEDICATION

This work is dedicated to my parents and family in Lithuania. It is dedicated to my father, who raised me to reach goals and never stop. And it is dedicated my mother who gave me her gentle love always and was a much stronger supporter of me than she could imagine. I also dedicate this work to my sister and her family for just loving me for who I am. Were it not for all of them, I would never have been able to survive so far from home for so long and achieve my highest educational goal.

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LIST OF ABBREVIATIONS

ABC	ATP-binding cassette
AP-2	adaptor protein complex II
ASL	airway surface liquid
ATP	adenosine triphosphate
A_{2B} AR	A_{2B} adenosine receptor
Band B	core glycosylated CFTR
Band C	complex glycosylated CFTR
BHK	baby hamster kidney
β_2 AR	β_2 adrenergic receptor
CAL	CFTR associated ligand
cAMP	cyclic adenosine monophosphate
Calu-3	human lung adenocarcinoma
CAP70	CFTR associated protein of 70kDa
CF	cystic fibrosis
CFBE41o-	CF bronchial epithelium
CFTR	cystic fibrosis transmembrane conductance regulator
CHIP	cytosolic U-box Hsc70-interacting protein
CHO	Chinese hamster ovary
CMV	cytomegalovirus
COP	coat protein complex

Corr-4a	[2-(5-Chloro-2-methoxy-phenylamino)-4'-methyl-[4,5']bithiazolyl-2'-yl]-phenyl-methanone
COS-7	kidney cells of the African green monkey
C-tail	carboxyl-terminal tail
ΔF508	CFTR mutation with phenylalanine missing at position 508
DMSO	dimethyl sulfoxide
DTT	dithiothreitol
EBP 50	ezrin/radixin/moesin binding phosphoprotein 50
ENaC	epithelial sodium channel
ER	endoplasmic reticulum
ERAD	endoplasmic reticulum associated degradation
FRT	Fisher rat thyroid
GTPase	guanosine trihosphatase
HeLa	immortalized human cervical cancer cells
HBE	human bronchial epithelium
HEK	human epithelial kidney
Hsc	heat-shock cognate
Hsp	heat-shock protein
HTS	high throughput screening
I_{sc}	short-circuit current
mRNA	messenger ribonucleic acid

MSD	membrane-spanning domain
NBD	nucleotide-binding domain
MDCK	epithelial-like Madin darby canine kidney cells
MRP1	multidrug resistance protein 1
NHERF	Na^+/H^+ exchanger regulatory factor
N-tail	aminoterminal tail
ORCC	outwardly-rectifying chloride channel
pI	isoelectric point
PKA	protein kinase A
PKC	protein kinase C
P_o	open probability
R domain	regulatory domain
rΔF508	ΔF508 mutant CFTR which escaped ERAD
RIPA	radioimmune precipitation buffer
RNAi	RNA interference
ROMK	outer medullary potassium channels
R_t	electrical resistance
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SNARE	soluble N-ethylmaleimide-sensitive factor attachment receptor
SPQ	6-methoxy-*N*-(3-sulfopropyl)quinolinium
T84	human colon carcinoma cell line

TR	transferrin receptor
TS	temperature-sensitive
UPR	unfolded protein response
VRT-532	4-Methyl-2-(5-phenyl-1H-pyrazol-3-yl)-phenol
WT	wild-type

INTRODUCTION

Cystic Fibrosis and the CFTR Protein

Cystic fibrosis (CF) is one of the most common chronic genetic diseases. It is a progressive and frequently fatal autosomal recessive genetic disease that affects the body's exocrine glands. In the United States, ~30,000 individuals have CF. Approximately 1 in 25 people of European descent, 1 in 22 people of Ashkenazi Jewish descent, 1 in 46 Hispanics, 1 in 65 Africans, and 1 in 90 Asians carry at least one abnormal CFTR allele (91).

CF is caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene, located on the long (q) arm of chromosome 7 at position 31.2. Functional insufficiency of the mutated gene product, the aberrant CFTR protein, gives rise to symptoms of CF such as exocrine pancreatic insufficiency; high salt concentration in sweat; obstructive airway disease characterized by thick, dehydrated mucus in airway surface liquid (ASL), and chronic bacterial infections which lead to respiratory failure. Male infertility, caused by obstruction or absence of the vas deferens, (138), as well as reduced fertility in females, are also frequent complications of CF (93).

The CFTR protein is a 168-kDa chloride channel that belongs to the adenosine triphosphate (ATP)-binding cassette (ABC) transporter super-family. ABC transporters are the largest class of transporters in humans (33) and consist of two membrane-spanning domains (MSDs) and two nucleotide binding domains (NBDs). These transporters use ATP to move specific molecules (lipids, ions, or polypeptides) across plasma membranes. They can also function to pump substrates into membrane-bound

organelles. CFTR (also referred to as ABC transporter ABCC7) is a symmetrical, polytopic glycoprotein comprised of two hydrophobic transmembrane domains, TMD1 and TMD2, which respectively connect to two cytosolic hydrophilic nucleotide binding domains, NBD1 and NBD2. A unique feature of CFTR is that it contains a cytoplasmic regulatory domain with multiple phosphorylation sites (R) that regulates channel function by stimulating ATPase activity and channel gating upon phosphorylation (79).

CFTR Expression and Function

CFTR is expressed at the apical surface of a number of epithelial cell types in functionally diverse organs such as the kidney, exocrine pancreas, intestine, vas deferens, sweat duct glands, salivary glands, and bronchial submucosal glands (18, 66). Its main roles in these tissues are to regulate electrolyte and fluid movement across membranes and to maintain ionic and water homeostasis (28, 65). Interestingly, CFTR mRNA has been found in immune cells, such as alveolar macrophages and neutrophils (90). However, it is not fully understood what role CFTR plays in these cells.

The CFTR protein primarily functions as a chloride (Cl^-) ion channel and it secretes or reabsorbs salt and water (4, 6). The function of CFTR in airway epithelial cells is summarized in figure 1. The direction of Cl^- movement through the CFTR channel depends on the function of the epithelia in which CFTR is expressed.

For example, sweat glands can absorb electrolytes independent of fluid secretion because the gland can be divided into the secretory coil and the reabsorptive duct. In this context, the secretory coil actively secretes an isoosmotic fluid into the lumen of the water-impermeable distal half (86). Chloride ions are reabsorbed through CFTR in the

reabsorptive ducts of the sweat glands, and in the absence of CFTR, Cl^- can be secreted but not reabsorbed. That is why the sweat is salty in patients with CF (summarized in (9)).

In contrast, in apical membranes of airway epithelial cells and serous secretory cells where CFTR secretes and reabsorbs Cl^-, intracellular ion balance is maintained through the basolateral sodium-potassium pump and other ion transporters. Chloride and sodium (Na^+) are reabsorbed through CFTR and an epithelial sodium channel (ENaC). In the absence of CFTR, ENaC becomes overactive, which causes water to follow into the cell, resulting in a dehydrated airway surface (summarized in (9)). Therefore, the direction of chloride ion secretion mediated by CFTR is cell and tissue type specific, and the failure to control the ion flow has devastating consequences and leads to the disease.

In addition to its role as a Cl^- channel, CFTR regulates other channels and transport proteins. However, these regulations are very complex and appear to be cell type specific. In all cell types studied so far, however, CFTR is a part of a large macromolecular signaling complex at the plasma membrane with approximately 200 proteins from different cell lines interacting with wild-type (WT) or mutant forms of this protein (47, 66, 120). A schematic diagram, shown in figure 2, depicts CFTR and some of its macromolecular associates. As is illustrated, both the amino and carboxyl terminal tails (N- and C-tails, respectively) of the CFTR protein are located on the portion of the protein that faces the cytoplasm, and these tails interact with multiple proteins. At the C-tail, proteins such as adaptor complex II (AP-2), ezrin-radixin-moesin-binding phosphoprotein (EBP50), CFTR-associated ligand (CAL) and CFTR-associated protein of 70kDa (CAP70) interact, while the N-tail interacts with SNAREs (syntaxin 1A,

SNAP23) and numerous folding-, trafficking-, and recycling-regulating chaperones (CHIP, Hsc/Hsp70, Hsc/Hsp40 and others). In addition to protein interactants at the tails, CFTR also exhibits protein-protein associations at the R, NBDs, and TMDs.

At a single-channel level, CFTR is regulated by cyclic adenosine monophosphate (cAMP) dependent protein kinase A (PKA) and protein kinase C (PKC) phosphorylation, ATP binding, and hydrolysis (71). Upon signaling, a local increase in cAMP leads to the phosphorylation of the CFTR Cl^- channel by PKA, resulting in rapid Cl^- efflux from the channel (66). Phosphorylation occurs at multiple sites in the R domain, which alters CFTR's conformation and its contacts with other parts of the protein (90). CFTR that has been activated by the cAMP agonist forskolin or cAMP analogs undergoes less endocytosis from the plasma membrane, and more functional CFTR chloride channels are detected (71, 85), suggesting that activated CFTR is stabilized at the cell surface.

Even though highly controversial and not generally well accepted, CFTR-facilitated ATP release is thought to positively regulate many other channels, such as the outwardly-rectifying Cl^- channels (ORCC) (100), Ca^{2+}-activated outwardly-rectifying anion channels, anion exchangers, sodium-bicarbonate transporters, renal outer medullary potassium channels (ROMK1 and ROMK2), and aquaporin channels (25, 60, 61, 101, 105, 132). The WT CFTR is known to negatively regulate epithelial Na^+ channels (ENaC). The WT CFTR is also known to regulate protein processing and intracellular compartment acidification (summarized in (100)). It had been demonstrated that alveolar macrophages expressing CFTR participates in phagosomal pH control and has bacterial killing capacity. Alveolar macrophages from *Cftr*$^{-/-}$ mice exhibited defective killing of internalized bacteria and lysosomes from *CFTR*-null macrophages failed to acidify,

although they retained normal fusogenic capacity (38). Overall, WT CFTR regulation of many channels and processes in the cell occurs by molecular mechanisms that are not well understood and most likely involve macromolecular signaling complexes.

CFTR Trafficking

When in the endoplasmic reticulum (ER), nascent CFTR is cotranslationally glycosylated on two asparagine (N) residues (N-linked glycosylation), and this core-glycosylated, immature form of the protein is referred to as band B. Properly-folded CFTR leaves ER and is delivered to the Golgi compartment with the help of coat protein complex (COP) COPII-vesicles (119). In the Golgi, CFTR undergoes a final glycosylation modification, where mannoses are trimmed and glycan side chains are added, CFTR becomes insensitive to endoglycosidase H (Endo-H). The mature, complex-glycosylated protein is designated as band C. Mature CFTR leaves the Golgi in vesicles that travel directly to the apical plasma membrane or to the recycling endosomes, whereby CFTR may also eventually reach the plasma membrane (12). At least for a short while, CFTR resides in most compartments of the protein secretory pathway when on its way to the cell plasma membrane. A schematic diagram of CFTR trafficking is shown in figure 3.

Because during trafficking from the ER to the cell surface CFTR protein undergoes glycosylation modifications, within any pool of CFTR expressed in cells, there is always a mixture of core glycosylated form, band B (ER form) and complex glycosylated, band C, (post-Golgi) CFTR. Differentially glycosylated forms of CFTR can be distinguished by the difference in their molecular weights, when subjected to

denaturing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE; see figure 4).

The CFTR localization in the cells is also cell type dependent. In the first reported WT CFTR cellular localization studies performed in Cos-7 cells transiently transfected with WT CFTR, most of the CFTR staining was intracellular, and only a very weak signal was observed at the plasma membrane (24). The results were replicated in HeLa cells transfected with WT CFTR and therefore exogenously expressing this protein (36). In contrast, in polarized epithelial cells such as intestinal (T84 cells) and airway epithelial cells endogenously expressing WT CFTR, most of the CFTR signal was localized around the apical membrane with very little perinuclear staining (12). This reinforces the idea that most of WT CFTR expressed endogenously in epithelial cells is located near the plasma membrane, whereas this pattern does not hold true for cells that grossly overexpress CFTR.

Furthermore, immunofluorescence and immunoelectron microscopy of striated duct epithelial cells from rat salivary glands demonstrated CFTR association with membranes of subapical vesicles (125). Other CFTR trafficking studies found this protein in the endosomes (13, 34, 44, 69).

There are several important players in CFTR vesicular trafficking, including Rab guanosine triphosphatases (GTPases). Rab family of proteins is known to regulate various steps of membrane traffic, including vesicle formation, vesicle movement along actin and tubulin networks, and membrane fusion. Rab 5 facilitates the trafficking of proteins from the cell surface to the early endosomes. Rab 9 and Rab 11 promote trafficking from the early endosomes to the *trans*-Golgi network and back to the cell

surface. Rab7 is responsible for CFTR movement from the early endosomes to the late endosomes and lysosomes (3, 112).

CFTR Biogenesis

Normally, endogenous CFTR expression levels are very low in the cell, a common limiting factor for biochemical experiments. Therefore, many CFTR studies addressed this issue by transducing cells with various vectors carrying cDNA of CFTR. Cells expressing the transgene either stably or transiently produce abundant amounts of CFTR for biochemical experiments. CFTR expression from these vectors is usually driven by viral promoters like the cytomegalovirus (CMV) promoter. Taking this approach, early studies determined that only a very small fraction of WT CFTR reaches the mature stage (band C), while the majority of nascent CFTR (~75%) is rapidly degraded by the proteasome in human embryonic kidney (HEK) 293 cells (123, 124). More recent studies confirmed that biogenesis was inefficient in cells heterologously expressing WT CFTR (only ~20% of the protein became maturely glycosylated in COS-7 cells and ~35% in HeLa cells) (116). Inefficient processing of WT CFTR observed in those studies made scientists wonder whether the inefficient biogenesis could be an artifact of using cells transfected with CFTR cDNA. To test this hypothesis, biogenesis studies were performed in human epithelial cells endogenously expressing WT CFTR (Calu-3 and T84 cells). These studies revealed that CFTR biogenesis in these epithelial cells was ~100% efficient (116). These results suggest that the differences in CFTR maturation observed between transduced cells (recombinant CFTR) and epithelial cells endogenously expressing CFTR could result from the differences in the regulatory

mechanisms governing endogenous and recombinant CFTR biogenesis. Moreover, it has been shown that Calu-3 cells (endogenously expressing CFTR) express similar amount of CFTR as transduced HeLa cells, in spite that they contain only one-fourth the amount of CFTR messenger RNA (mRNA) (116). It is now understood that cells regulate endogenous CFTR expression at the transcriptional, translational and maturational levels, while recombinant CFTR expression is regulated only through maturation efficiency (87).

A number of chaperones are known to interact with and assist in the folding of CFTR during its biogenesis (2). These include the ER luminal chaperone calnexin (82, 120), cytosolic Hsp70, Hsp90, and Hsp40 chaperones, and their co-chaperones (67, 109, 120, 131, 134, 135). To date, approximately 31 chaperones and co-chaperones of CFTR have been identified (107, 120). This fact only reinforces the complexity of the CFTR biogenesis process.

Clathrin-coated Endocytosis of CFTR

A number of reports suggest that CFTR undergoes regulated trafficking between intracellular compartments and the cell surface as summarized in a review by Bertrand and Frizzell (12). Mutations can influence CFTR trafficking both in the secretory and recycling compartments, supporting the concept that regulated CFTR trafficking is vital to normal epithelial function.

It is becoming clear that the endocytosis of proteins is not only a process by which nutrients are taken up from the extracellular environment, but it is also a key element in regulating the distribution of cell surface proteins. Mutations that affect the

endocytic traffic of ion channels may have very severe clinical consequences. In Barter's syndrome, the Renal Outer Medullary Potassium channel, ROMK1, responsible for potassium transport out of the cells is mutated (37). In Liddle's syndrome, leading to sodium hyperabsorption, ENaC endocytosis is reduced, caused by a mutation (108). CFTR is cleared from the apical surface (71, 85) through clathrin-coated vesicles (18, 19, 71) and CF may originate from *CFTR* mutations that alter the cell surface trafficking of the CFTR protein.

Integral membrane proteins such as CFTR undergo clathrin-dependent endocytosis after being concentrated in pits coated with clathrin. Soluble clathrin molecules are locally recruited and self-associate at the plasma membrane to generate a polygonal clathrin lattice, which quickly forms a deeply-invaginated bud before detaching into the cytosol (29). Invaginated buds are released from plasma membrane with the help of a GTPase called dynamin, which functions as an enzyme to physically drive membrane vesiculation or as a regulator of vesicle formation (29, 54). This process is driven by the association of the plasma membrane with adaptor protein (AP-2) (29, 54).

Cell surface proteins undergo endocytosis via their endocytic motifs that interact with the clathrin-based machinery (3). Endocytic motifs consist of short linear arrays of amino acids in the cytoplasmic domains of integral membrane proteins. These motifs usually have about two or three conserved amino acids critical for function. These amino acids are usually hydrophobic and bulky (3).

Co-immunoprecipitation, yeast two hybrid screens, assays with dominant-negative cell lines, and pull-down experiments have all revealed associations between

tyrosine-based endocytosis signals and clathrin adaptor complexes (77). The most notable, physiologically relevant interaction occurs between the CFTR endocytic motif and the μ2 subunit of AP-2 (127). In the study by Wiexel *et al,* cross-linking experiments revealed that an YDSI sequence in CFTR interacted with the μ2 subunit, but a dominant-negative μ2 was not able to interact with CFTR. As a consequence, endocytosis of CFTR in the mutant cells was greatly diminished compared to WT cells (127). These results indicate that the μ2 AP-2 subunit selectively interacts with the YDSI sequence of CFTR, and abolishing this interaction interferes with CFTR's ability to enter clathrin-coated pits.

The actin cytoskeleton, which is composed of F-actin and its interacting proteins, is organized under the plasma membrane of polarized epithelial cells (50). It has been demonstrated that myosin VI, an actin-dependent, minus-end directed mechanoenzyme, plays important role in clathrin-mediated endocytosis in epithelial cells (22). Interestingly, myosin VI was also found to interact with cell surface CFTR in polarized human airway epithelial cells (50). Although, myosin VI had no affect on CFTR recycling to the plasma membrane (111), it was determined that it regulates CFTR endocytosis by interacting with the myosin VI adaptor protein referred to as Disabled 2 (Dab2) and clathrin. To support the role of the actin cytoskeleton in CFTR cell surface trafficking, it has been shown that disruption of actin filaments decreases CFTR endocytosis (78). Therefore, the actin cytoskeleton plays an important role in CFTR endocytosis in mammalian cells.

Endocytic Signals in CFTR

Tyrosine based endocytic (internalization) signals can be divided into two groups: NPXY, where X is a variable amino acid, and YXXΦ, where Φ is a bulky hydrophobic amino acid. Membrane proteins with the more common YXXΦ motif contain dileucine sequences (D/EXXXLL/I and DXXLL) (3). In some cases, one of the leucines may be substituted with isoleucine, valine, alanine, or methionine (126).

A chimeric approach, designed to identify the necessary motifs for CFTR endocytosis, determined that CFTR endocytosis may be mediated by a tyrosine-based signal, $Y^{1424}SDI^{1427}$, as well as a dileucine sequence in the carboxyl-terminal tail (C-tail) (84). In these studies, our laboratory generated CFTR-human transferrin chimeric proteins and determined that only one mutation (Y1424A) inhibited the endocytosis of the chimera by approximately 40%, suggesting that it was the critical signal for CFTR endocytosis (84).

Lukacs and colleagues used a second chimeric approach, CFTR and the interleukin 2 receptor α-chain (Tac) to determine the cell surface trafficking signals in CFTR. Morphological and functional assays revealed the presence of multiple internalization motifs at the C-terminus of CFTR: a phenylalanine-based motif (Phe^{1413}) and a bipartite endocytic signal comprised of a tyrosine (Y^{1424}) and a di-Leu-based (Leu^{1430}-Leu) motif. Replacing any one of the three internalization motifs with alanine prevented the endocytosis of the chimera, suggesting that internalization of CFTR is regulated by multiple endocytic sorting signals at the C-tail (55). Subsequently, it was determined that the isoleucine residue at position 1427 is also important for CFTR endocytosis (81). Ablating an internalization signal YXXI by substituting Tyr^{1424} and

Ile[1427] with alanines doubled the surface expression of CFTR and the single mutation Y1424A showed an intermediate phenotype, as monitored by surface biotinylation (81).

Additionally, Neil A. Bradbury's group reported a naturally-occurring mutation, N187Y, found within an intracellular loop of CFTR that introduced a non-canonical tyrosine-based endocytic signal (106). This new endocytic signal caused a two-fold increase in the removal of CFTR from the cell surface compared to the WT protein, without affecting the protein's biosynthesis or channel function (106). These observations suggest that CFTR mutations might introduce other non-canonical internalization signals, not necessarily in the C-tail, that could have an effect on CFTR clearance from the plasma membrane.

It has been suggested that CFTR clearance from the cell surface may provide a mechanism for controlling the cAMP-stimulated chloride channel function at the cell surface (66, 84). It has been demonstrated that elevating intracellular cAMP levels and subsequently activating CFTR resulted in reduced WT CFTR endocytosis rates. Because the endocytosis rate of the cAMP irresponsive G551D CFTR mutant was not affected the study concluded that inhibition of endocytosis requires physiological CFTR function (85).

CFTR endocytosis rates depend on more than just the presence of endocytic signals. CFTR clearance from the cell surface is regulated by the protein distribution within the cell, cell type, differentiation stage, CFTR phosphorylation conditions and polarization of the cells (115). Generally, CFTR endocytosis is rapid in both epithelial and non-epithelial cells, as monitored by cell surface biotinylation assays (20, 71, 85). In T84 cells, about 16% of surface CFTR was internalized in a one minute (85); in Chinese

hamster ovary (CHO) cells, internalization was about 5% per minute (71). From these endocytosis studies, it seems likely that internalization of CFTR is different in different cell lines. The state of cell polarity also plays an important role in this process (115).

CFTR Recycling

Recycling allows cells to regulate the steady-state levels of cell surface proteins and also protects proteins from degradation. Some proteins, such as receptors, may undergo multiple rounds of endocytosis and recycling (3). CFTR has also been shown to be recycled from intracellular vesicles back to the plasma membrane (19).

Lukacs and coworkers demonstrated that CFTR is recycled back to the cell surface in CFTR-transfected CHO cells (70). They used cyclohexamide or brefeldin A that block CFTR synthesis or ER to cell surface trafficking respectively, to block newly synthesized CFTR delivery to the cell surface. Under these conditions they showed that forskolin-stimulated CFTR Cl^- currents remained unchanged for up to 24 hours. This suggests that CFTR was not degraded, but rather recycled to the plasma membrane. The fact that endocytosed CFTR is very rapidly recycled was also shown by pulse-chase studies in combination with domain-selective cell surface biotinylation in stably-transfected Madin-Darby canine kidney cells (MDCK) (113). This group used C-terminally truncated human CFTR (CFTR-ΔTRL) missing PDZ domain and tested whether the PDZ-interacting domain of CFTR regulated its sorting from Golgi to the plasma membrane. Deletion of the PDZ-interacting domain reduced the CFTR surface half-life from about 24 to 13 hours by reducing the apical recycling of CFTR (113).

Similarly, in another study, using an extracellular epitope tagged WT CFTR construct, Sharma and colleagues demonstrated that ~65% of the endocytosed CFTR was recycled to the plasma membrane in a matter of 10 min (103). Overall, there is growing evidence that CFTR undergoes endocytosis and recycling, but the exact reason why it does so is not completely understood.

CFTR Mutations and Cystic Fibrosis

Mutations in *CFTR* may affect the number of CFTR molecules produced in the cells, their trafficking and/or channel activity. When CFTR is mutated, it may result in absent or decreased function or absence of CFTR and Cl^- anion function conductance at the apical membranes of epithelial cells. Consequently, CFTR dysfunction causes increased airway Na^+ absorption and up-regulation of ENaC (5, 72). It also leads to aberrant concentrations of other ions and altered fluid homeostasis. Further, as a consequence, direct and indirect interactions between CFTR and its binding partners change.

Over 1500 mutations have been identified in the CFTR gene (CF Genetic Analysis Consortium, http://www.genet.sickkids.on.ca/cftr). CFTR mutations are scattered throughout the entire coding region of the gene, although mutations are more common in the NBD and R domains presumably because of their mass (93). CFTR mutations can be classified into groups according to how they disrupt CFTR function (reviewed in (93). Furthermore, it is becoming clear that certain mutations can exhibit multiple defects that collectively contribute to the CF phenotype. For example, one

mutation may promote processing, folding, biogenesis and surface stability defects. An overview of the types of CFTR mutations is provided in figure 5.

Class I mutations affect the biosynthesis of the protein and result in no or reduced CFTR mRNA levels. These mutations include nonsense, frameshift, or aberrant splicing of mRNA (138). The most severe CF mutations belong to this group of mutations, because there is no protein being synthesized. The most common mutation in this group, G542X, introduces a premature termination codon, resulting in the transcription of a severely truncated, unstable CFTR mRNA.

Class II mutations are the most prevalent and result in a misfolded CFTR protein. Misfolded CFTR fails to exit the ER and is degraded by the proteasome. Failure of misfolded CFTR to exit the ER results in a lack of functional protein at the plasma membrane. A classic example of this class of *CFTR* mutation is ΔF508, the deletion of phenylalanine at the 508 position.

Class III CFTR mutants are exported from ER and are delivered to the cell plasma membrane, but have altered channel activity, regulation or gating properties. The most common mutation in this group is the missense mutation where glycine is mutated to aspartic acid at codon position 551 (G551D). G551D is the third commonest CF mutation with a worldwide frequency of ~2% on CF chromosomes (129). It is an interesting mutation because its trafficking is normal but the Cl^- function seems to be defected. It was found that G551D mutation responds only to genistein, known to potentiate the CFTR channel but not to activate it (58). To date, all mutations belonging to this group have been found in the NBD domain encoding regions of *CFTR*.

Class IV CFTR mutations affect Cl^- conductance. In this class of mutations, CFTR is synthesized normally, trafficked to the plasma membrane, and is responsive to activation by physiological stimuli, however, the Cl^- currents are altered. Some of these mutations have reduced single channel Cl^- conductance (G622D, R792G and E822K), whereas others have elevated Cl^- conductance (H620Q, A800G).

CFTR mutations that belong to class V result in reduced CFTR levels. These mutations result in the least severe phenotype. Class V mutations alter the function of the spicing machinery. As a result, both aberrantly and correctly spliced transcripts are being produced. The phenotype depends on the levels correctly and incorrectly spliced transcripts. These vary among patients and organs of the same patient. More recently, another class of mutation has been proposed (class VI; (136). Class VI mutations may cause truncation or frame shift. The resulting protein maintains normal biosynthetic processing and channel function, but the biological stability and/or recycling of the fully processed protein is dramatically reduced. Class VI mutants may lack amino acids at C-terminal tail. Examples of these mutants include (Q1412X) or frameshift mutations (4326delTC, 4279insA).

The ΔF508 Mutation

The most common CF-causing mutation is a three-nucleotide deletion (CTT) that results in the absence of a single phenylalanine (F) in exon 10 at position 508 within the protein (ΔF508). This class II mutation accounts for two-thirds of the CF cases worldwide (14). In over 90% of all CF cases, at least one defective allele contains ΔF508 (14). This mutation is found in NBD1 domain and results in CFTR misfolding and

disruption of NBD2 and NBD1 domain interactions (39). Furthermore, it is thought to disrupt the proper folding of transmembrane domains TMD1 (23) and TMD2 (133). The misfolded ΔF508 CFTR protein is recognized by the ER quality control machinery and undergoes ERAD (24, 59).

Because the majority of ΔF508 CFTR fails to leave the ER, ΔF508 CFTR appears as band B (with no band C) on SDS-PAGE gels, after isolation from sweat duct cells of fresh skin (63), intestine (73) or from airway biopsies of CF patients homozygous for this mutation (65). Nevertheless, very little amount of ΔF508 CFTR is detected at the cell surface by immunochemical techniques in recombinant cells, CF primary airway cells, or CF tissues (24, 36, 62). Contrary to most reports, a comprehensive study reported the presence of some ΔF508 CFTR in post-Golgi compartments and at the plasma membrane (62). It is likely that at least at a minimal level of ΔF508 CFTR can escape from the ER quality control in patients. Furthermore, it was noted that in patients with greater ΔF508 CFTR surface expression, the CF disease is less severe compared to patients with less surface CFTR (128). Therefore, there is a great interest in directing CF research towards ΔF508 CFTR rescue and in understanding the molecular basis for CF pathogenesis.

Certain biochemical manipulations such as reduced temperature (35, 40), chemical chaperones (8, 98) and manipulation of certain folding chaperones (97) allow ΔF508 CFTR to escape from ERAD and traffic to the plasma membrane. Under these conditions, it functions as Cl^- channel, but its channel activity is compromised (7, 32, 34, 35).

ΔF508 CFTR is Temperature Sensitive

ΔF508 CFTR is subjected to ERAD at 37˚C (restrictive temperature) (35, 89). However, Cl^- channel activity was detected in ΔF508 CFTR-expressing Xenopus oocytes, Vero cells, and Sf9 insect cells (35). Because Xenopus oocytes and Sf9 cells require lower culture temperature than mammalian cells, it was suggested that the processing of ΔF508 CFTR might be temperature sensitive (TS) (35). Denning and colleagues started with 30˚C and lowered the cell culture temperature to 23˚C, whereby they determined that the optimal culture temperature for ΔF508 CFTR to reach the plasma membrane was 26˚C (35, 44, 80, 89). Surface biotinylation assays and Ussing chamber experiments confirmed that when ΔF508 CFTR-expressing cells were cultured at low temperature (27˚C; permissive temperature) for about 24-72 hours, ΔF508 CFTR trafficked to the plasma membrane (7, 35, 102).

CFTR is not the only protein that has been recognized as TS mutant. There are several other clinically important proteins that exhibit a TS phenotype. These include, p53, the main player involved in soft-tissue sarcoma; the multidrug resistance protein 1 (MRP1, another ABC transporter super-family member), associated with drug resistance in human cancer cells (45, 83). Like ΔF508 CFTR, other TS mutants tend to carry mutations that affect the protein structure and result in protein misfolding. As is the case in ΔF508 CFTR, TS mutants are recognized as misfolded only at the restrictive culture temperature, but if temperature is shifted to the permissive temperature, the defect can be minimized. Although the permissive or restrictive culture temperatures vary with each protein, all of these defects can be partially corrected *in vitro* using similar interventions.

Even though the observation that the trafficking of ΔF508 CFTR is temperature sensitive is 15 years old, the mechanism underlying low temperature rescue is still not completely understood (112, 115). Most likely, permissive temperature rescue of ΔF508 CFTR is due to a combination of factors that include ERAD inhibition and facilitated folding by altering chaperone interactions (35). Though the mechanism is complicated, permissive temperature culture is currently the most efficient way to facilitate ΔF508 CFTR escape from ERAD and trafficking to the cell surface.

Surface Trafficking of rΔF508 CFTR

After permissive temperature rescue, if ΔF508 CFTR expressing cells are returned to the restrictive temperature, rΔF508 CFTR is very rapidly cleared from the plasma membrane (7, 103). It has been demonstrated that rΔF508 CFTR has a much shorter surface half-life than WT CFTR (70). Indeed, quantitative studies showed that the surface half-life of rΔF508 CFTR is about 10-fold shorter than the WT protein in BHK cells (103). However, when rΔF508 CFTR expressing cells are kept at permissive temperature, the surface instability of this protein increases similar to WT CFTR (115).

In addition to surface stability defect, rΔF508 CFTR fails to respond to physiological stimuli known to activate WT CFTR, such as elevation in cAMP levels. Previous reports indicate that low temperature rΔF508 responded to rising cAMP levels by producing increased Cl^- current upon stimulation in Xenopus oocytes and Sf9 cells (21, 35). The same results were observed in HeLa cells transiently expressing ΔF508 CFTR protein, whereupon rΔF508 CFTR efficiently responded to forskolin (a direct activator of adenyl cyclase) and promoted Cl^- transport (7). Importantly, when studies

were carried out in human airway epithelial cells that were polarized, CFBE41o$^-$, rΔF508 failed to respond to cAMP–mediated signaling pathways. These cells were responsive only to genistein, which has been shown to activate CFTR by a cAMP-independent process (7). There are other studies supporting this finding that rΔF508 CFTR poorly responds to cAMP–mediated pathways compared to WT CFTR, even in heterologous system (117).

On the other hand, when Ussing chamber experiments were performed at the permissive temperature after low temperature rescue of ΔF508 CFTR, the response to cAMP was partially restored (115). These observations suggest that the surface defects exhibited by rΔF508 CFTR are temperature-sensitive (115). Besides the functional defects, the rΔF508 CFTR also has trafficking defects associated with rapid removal from the cell surface and rapid degradation (115).

Sharma and colleagues showed that the endocytosis rate of rΔF508 CFTR was not significantly different from WT CFTR (103). In contrast, others have demonstrated that the endocytosis rate of rΔF508 CFTR is much faster than the WT protein (103, 112, 115). This disparity could be explained by the difference in cell lines and polarity of the cells used for these studies. Other studies suggested that the rΔF508 CFTR mutation affects post-endocytic trafficking of the mutant protein, diverting it from the recycling pathway to the late endosomes, multivesicular bodies, and lysosomes (44, 103). These observations suggest rΔF508 instability could be a combination of endocytosis and recycling defects associated with this mutant protein. To support this, a study showed that rΔF508 CFTR is recognized as misfolded at the cell surface and is consequently degraded

through the ubiquitin-dependent endosomal sorting machinery, whereas the WT protein is not (103).

Cl^- Channel Activity of rΔ508 CFTR

In addition to the surface stability and trafficking defects, the single channel properties of rΔF508 CFTR are also altered (118). By 1993 it was determined that WT CFTR is regulated by cAMP-dependent protein kinase (PKA) (56). More recent studies on isolated membrane patches from cells expressing rΔF508 CFTR suggest that the rescued mutant protein does not retain all functions of WT CFTR. Although the channel is functional, it does not respond normally to activation by PKA and ATP (1, 7, 57). Additionally, there are studies showing that rΔF508 CFTR also exhibits a three-fold reduction in the single Cl^- channel open probability (P_o) after cAMP stimulation compared to WT protein (32, 40). This defect could be caused by the mutant's inability to interact with cAMP associated kinases, since cAMP dependant phosphorylation of the R domain has been shown to increase CFTR single channel P_o (79).

It is established that regulation of CFTR is accomplished through activation of surface G protein–coupled receptors that stimulate adenyl cyclase and raise cAMP levels, which in return activates PKA. PKA phosphorylates the R domain of CFTR, and Cl^- transport occurs (11, 17, 26, 27, 53, 74). There are two G protein coupled signaling cascades known to activate WT CFTR: A_{2B} adenosine receptors (A_{2B} ARs) and $ß_2$ adrenergic receptors ($ß_2$ ARs). Both of these pathways are very complex and only partially understood. The regulation is likely to occur through multiple interactions between CFTR and its binding partners near or at the plasma membrane (49, 74, 110). These interactions could be different for WT and rΔF508 CFTR, since the mutant protein

is misfolded. A number of studies point out that interacting proteins may be very different for WT and mutant CFTR (47, 66, 120). Thus, it is not surprising that rΔF508 CFTR could respond differently to stimuli known to activate WT CFTR.

Indeed, these studies confirm that the ΔF508 CFTR mutation may cause significant conformational changes in the protein. Even though rΔF508 CFTR reaches the plasma membrane, its interactions at the cell surface may be altered, and this could at least partially account for abolished cAMP regulation.

Correctors and Potentiators of ΔF508 CFTR

Even though the ΔF508 mutation interferes with CFTR folding, trafficking, membrane stability, and channel properties, resulting in impaired epithelial function (24), *in vivo* and *in vitro* studies on human nasal cells of CF patients have indicated that boosting ΔF508 CFTR functional activity from less than 1% to 5% of normal levels may greatly reduce disease severity or even eliminate the principal disease manifestations (64, 88). Because the majority of CF patients would benefit from the restoration of ΔF508 CFTR function, a number of attempts have been made to identify pharmacological agents that could replicate the effects of low temperature rescue and force the mutant protein to the cell surface and correct its function. Glycerol (96, 98), the epithelial differentiating agent dimethyl sulfoxide (DMSO) (8), sodium butyrate (52), and other compounds have been shown to rescue the ΔF508 CFTR mutant, but these compounds have limited clinical potential, and none could mimic the effects of low temperature culture. Therefore, recently, several groups have carried out high throughput screening (HTS) to search for novel small molecule correctors (promote biogenesis and folding) and

potentiators (enhance channel activity or surface stability of the rescued protein) to correct ΔF508 CFTR function (114, 130).

Several structural classes of potentiators that restore the function of ΔF508 CFTR have been identified, such as phenylglycine, sulfonamides, tetrahydrobenzothiophenes (114). In contrast, relatively few small-molecule correctors of ΔF508 CFTR have been identified. The end point of the drug selection process in these studies was functional CFTR channel activity in the plasma membrane of Fisher rat thyroid (FRT) cells stably transduced with ΔF508 CFTR. The limited success of screening efforts may be attributed to the lack of more relevant secondary screens, since the applied models are neither predictive nor disease relevant. Therefore, testing the previously-identified compounds on CF relevant cell lines is necessary. Even some of the studies managed to do some of their screens in epithelial cell lines, but they did not focus on the events that occurred after the rescue of ΔF508 CFTR.

A recent report from our laboratory focused on the cell surface stability of rΔF508 CFTR in the presence of two recently identified correctors (corr-4a and CFpot-325). We showed that the endocytosis and half-life defects of the mutant protein can be partially corrected with these compounds (115). Herein, our studies provide further characterization of two compounds, both of which mediate their effects at micromolar (μM) concentrations. The first compound, corr-4a ([2-(5-Chloro-2-methoxy-phenylamino)-4'-methyl-[4,5']bithiazolyl-2'-yl]-phenyl-methanone), belongs to the bisaminomethylbithiazole class. Corr-4a has been shown to facilitate ΔF508 CFTR trafficking from the ER to the plasma membrane in several cell lines, such as BHK (80, 122), human embryonic kidney cells (HEK) (122), and CFBE41o$^-$ (115). ΔF508 CFTR

folding was promoted by corr-4a binding to CFTR. Corr-4a blocked disulfide cross-linking between cysteines introduced into the two halves of Cys-less CFTR suggesting that CFTR and corr-4a interact (122). Also it was demonstrated that corr-4a was specific to CFTR and did not promote trafficking of other proteins (80). Even though this corrector has been studied extensively in the last few years, its effects on the rΔF508 CFTR Cl^- channel function in physiological systems have not yet been determined.

The second compound, VRT-532 or CFpot-532 (4-Methyl-2-(5-phenyl-1H-pyrazol-3-yl)-phenol), was among the most potent and efficacious potentiators identified in a high-throughput screen (114). This group observed the response to VRT-532 in ΔF508-CFTR-NIH/3T3 cells only after forskolin addition, indicating that this compound was potentiating and not activating ΔF508 CFTR under their experimental conditions (114). Patch-clamp recording and cell signaling analysis in FRT cells monolayers showed that this compound could potentiate forskolin–stimulated Cl^- secretion in other CFTR mutants, particularly G551D-CFTR, although this potentiation was ~5 fold weaker than that observed for ΔF508 CFTR (114). Furthermore, VRT-532 could increase the P_o of ΔF508 CFTR to the levels seen in WT CFTR by increasing its burst duration (rather than by increasing single channel conductance) (114). In human bronchial epithelial cells (HBE), VRT-532 increased the forskolin–stimulated short circuit current (I_{sc}) even in non-CF tissues, suggesting that this potentiator also affects WT CFTR (114).

Other studies indicated that VRT-532 could specifically rescue CFTR processing mutants without promoting maturation of a P-glycoprotein processing mutant (121). Although VRT-532 was not as efficient a corrector of ΔF508 CFTR maturation as corr-4a, it had the ability to act as both a corrector and potentiator of CFTR processing mutants

(121). Therefore, it is considered a good lead compound to study chemical chaperones with corrector and potentiator characteristics.

Together, these data suggest that two different compounds with different mechanisms of action might be able to function synergistically. Therefore, we asked whether these compounds could be used in combination to achieve correct protein folding, trafficking, and function of the mutant protein. Consequently, there is a need for a careful analysis of potent and efficacious correction agents that act either alone or in combination to restore the ion channel function of ΔF508 CFTR, as well as the trafficking of ΔF508 CFTR in biologically-relevant models. Our studies are designed to investigate the mechanism of action of these previously-identified small molecule correctors and potentiators in physiologically relevant cell models, and to focus on the restoration of CFTR Cl^- channel function.

Arg31 Mutations

Although the ΔF508 mutation has been extensively studied, less is known about the molecular basis of other mutations detected in CF patients. R31C and R31L are two naturally-occurring missense mutations found in the N-terminal tail of CFTR and they result in a mild CF phenotype (137) and (www.genet.sickkids.on.ca/cftr/). Both of these mutations are rare. The R31C mutation, 223C/T variation in exon 2, was found in a 45-year-old French male CF patient diagnosed in childhood who was pancreatic sufficient with moderate pulmonary symptoms and had an abnormal sweat test. As a compound heterozygote, he had another substitution, I556V, located in exon 11. Interestingly, the man's child was found to be R31C homozygous without CF phenotype, suggesting that

R31C is probably a polymorphism (46). The missense R31L mutation, 224G/T variation in exon 2, was found in a 24-year-old female CF patient who was pancreatic sufficient with normal lung function, but who had a positive sweat test. The other allele was not identified. Although R31C mutation was described only for one patient with symptoms, it does not minimize the importance of this mutation because there can be plenty of CF patients who carry at least one of the alleles with this mutation. R31L was found in 284 CF chromosomes and 144 normal chromosomes (137), suggesting that these mutations may be easily missed if not specifically looked for.

R31C and R31L are particularly interesting, because potentially they create internalization signals, $Y^{28}RQC^{31}$ and $Y^{28}RQL^{31}$, by introducing a hydrophobic residue at the Y + 3 position. This is particularly exciting, given that CFTR internalization signals were found only in the C-terminal domain previously (55, 81, 84). Furthermore, another mutation was recently identified, N287Y, that enhances endocytosis over WT levels (106). This suggests that tyrosine-based internalization signals can be recognized at a number of sites in the CFTR molecule. These results underline the importance of analyzing R31L and R31C, and determining how they alter transport of CFTR to and at the cell surface.

Significance of the Study

Much of the work on CFTR has focused on understanding protein biogenesis and function based on the most common ΔF508 mutation. However, only a few studies have concentrated on mutations that have milder disease phenotypes to see how they affect the surface stability of the mutant protein. The focus of the first publication herein was to

determine the trafficking properties of two N-terminal mutations of CFTR, R31C and R31L, respectively that are associated with mild disease phenotype. Studying these mutations will provide valuable information regarding the nature of the defects associated with two naturally-occurring CFTR mutations and emphasize the importance of the N-terminus of CFTR molecule. By determining the biogenesis, protein stability, surface expression, internalization rate, and functional activity of the Arg31 mutants, we will develop a better understanding of the role of the Arg31 and its immediate environment in the CF phenotype. These studies in turn will help us to deepen our understanding regarding the molecular basis of regulation of cell surface expression of WT CFTR.

Since the majority of CF patients would benefit from the restoration of normal CFTR function, there has been a substantial effort in the CF field to identify small molecules that promote the proper folding and function of ΔF508 CFTR. In the second manuscript, we studied the effects of the small molecule corrector corr-4a and potentiator VRT-532 on rΔF508 CFTR Cl^- channel function. Our studies in CFTR-expressing polarized epithelial cells will hopefully not only provide important information regarding the stability defect of the surface pool of rΔF508 CFTR over time, but also shed light on how the rΔF508 CFTR Cl^- channel functions and is regulated. The studies reported here indicate that it is likely that the combination of a corrector, a compound that facilitates proper folding, and a potentiator, a compound that enhances the properties of a single channel of rΔF508 CFTR, will be necessary to fully correct the two major defects of this mutant protein.

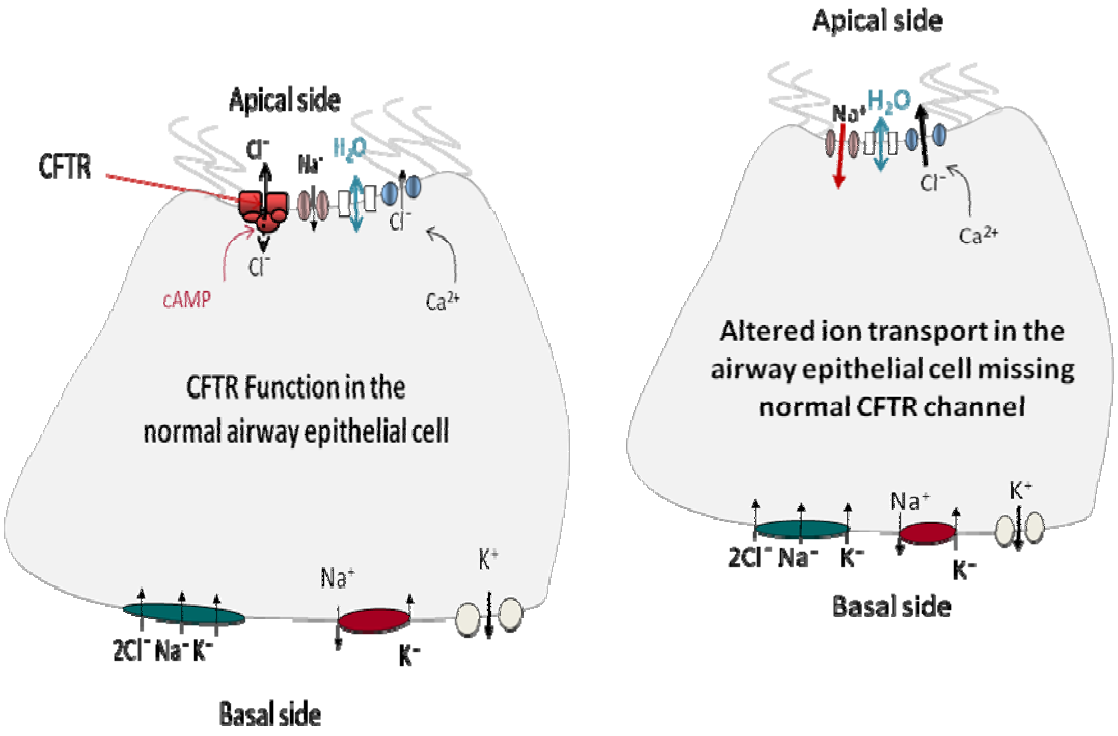

Figure 1. CFTR function in airway epithelial cells. On the left: CFTR functions as a channel which secretes or reabsorbs chloride (Cl^-). Intracellular ion balance is maintained through the ion transporters found in the basolateral membrane of the cell. Under physiological conditions, epithelial cells in airway secrete not only Cl^-, but also reabsorb sodium (Na+) and regulate water (H_2O) movement to hydrate the airway surface liquid (ASL). On the right: In the absence of CFTR, Cl^-secretion is abolished and epithelial sodium channels (ENaC) become overactive. More Na^+ is reabsorbed from the lumen compared to physiological conditions; H_2O follows Na^+ leading to dehydrated ASL. Furthermore, in the absence of CFTR Cl^- cannot be reabsorbed, however other Cl^-channels can still secrete Cl^-. As a result sodium chloride (NaCl) accumulates in the ASL.

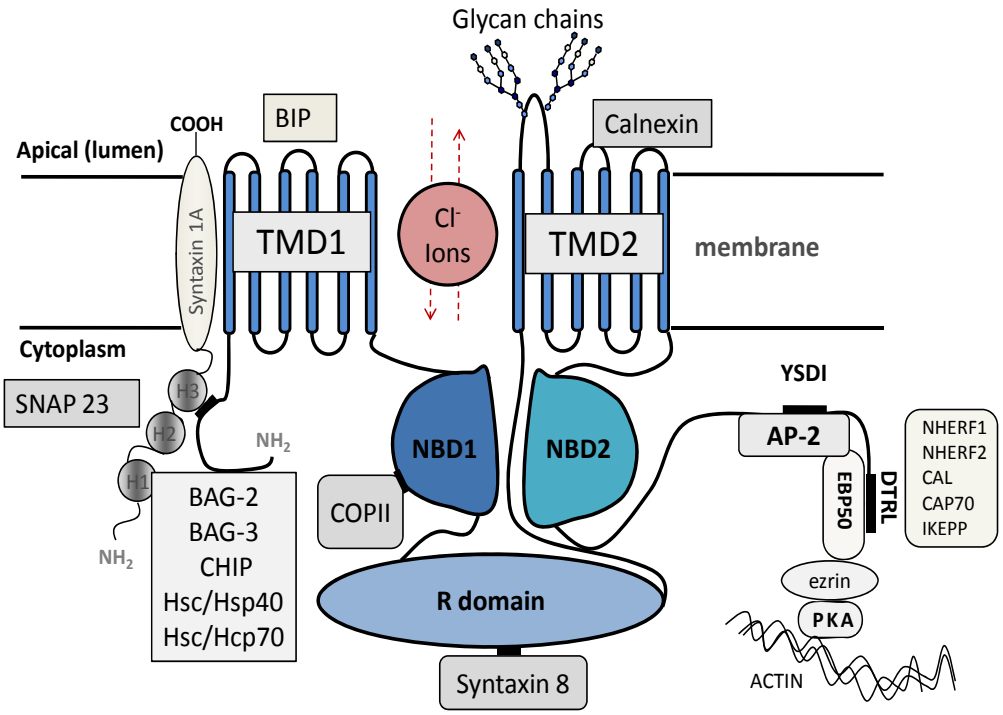

Figure 2. Some of the known CFTR binding partners. CFTR is comprised of two transmembrane domains (TMD1 and TMD2), each connected to a nucleotide binding domain (NBD1 and NBD2). It also has a large regulatory domain (R) which upon phosphorylation regulates the channel's function by stimulating ATPase activity and gating (79). The CFTR protein contains two N linked glycosylation sites, located on the fourth extracellular loop of TMD2. CFTR interacts with a number of proteins on its way to the plasma membrane, beginning in the ER; only a very few are depicted here. Syntaxin 1A and SNAP-23 bind CFTR and down-regulate its function. COP-II helps CFTR to travel from the ER to the Golgi, and syntaxin 8 regulates CFTR recycling. The carboxyl terminal tail (DTRL) sequence is important for binding proteins possessing PDZ domains: ezrin-radixin-moesin binding protein-50 (EBP50), CFTR associated protein-70 (CAP-70), Na/H exchanger regulatory factor 1 (NHERF1), Na/H exchanger regulatory factor 2 (NHERF2), CFTR- associated ligand (CAL), intestinal and kidney-enriched PDZ protein (IKEPP). These interactions modulate trafficking, transport, and intermolecular signaling of CFTR. The main chaperone components affecting CFTR ER associated folding are calnexin, found in the lumen of the ER. The cytosolic chaperones Hsc-Hsp40/70 play critical role in WT and mutant CFTR folding. The CFTR endocytosis signal YSDI is also depicted, it interacts with adaptor complex II (APII).

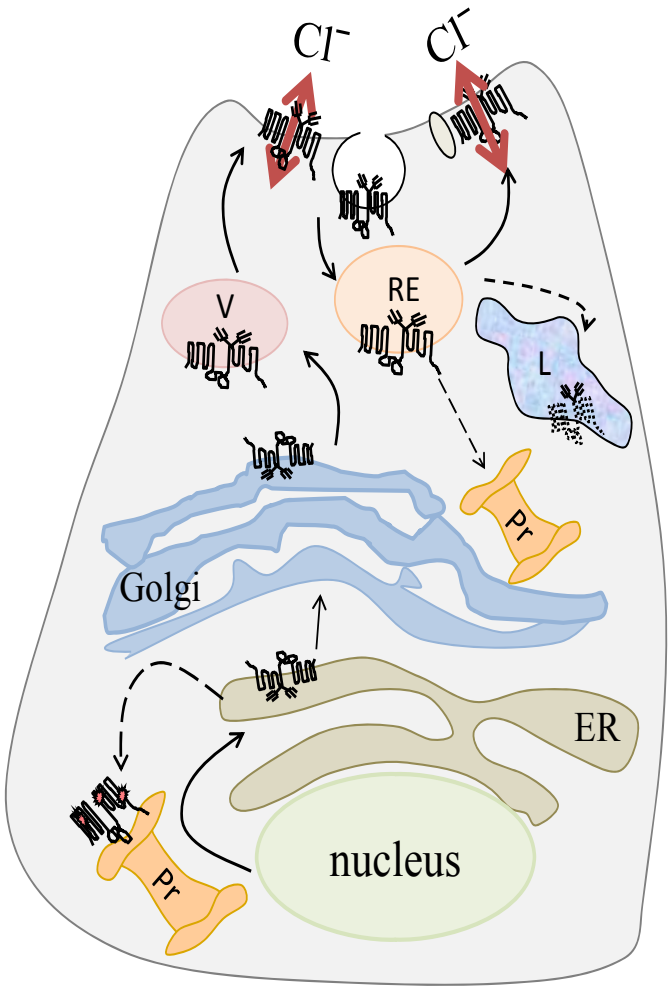

Figure 3. CFTR biogenesis and trafficking. Nascent CFTR is synthesized and inserted into the ER membrane cotranslationally. Misfolded CFTR is degraded by proteasomes (Pr). From the ER, CFTR trafficks to the Golgi, and undergoes final glycosylation modifications. From the Golgi secretory vesicles are formed (V) and CFTR traffics to the plasma membrane, where it functions as Cl^- channel and regulator of other ion channels as well. It is endocytosed from the plasma membrane into recycling endosomes (RE). Those endosomes can return to the plasma membrane or be diverted to the late endosome–lysosome (L) pathway for degradation.

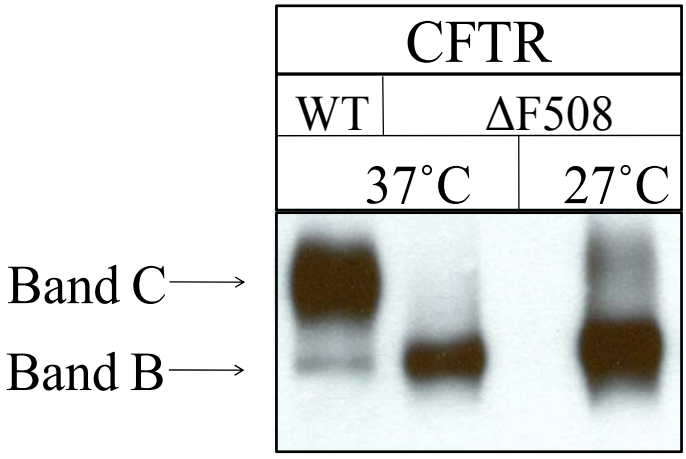

Figure 4. CFTR migration patterns on an SDS-PAGE gel. Human bronchial epithelial cells (CFBE41o$^-$) expressing either WT CFTR (WT) or ΔF508 CFTR (ΔF508) were lysed in RIPA buffer. CFTR was immunoprecipitated (IP) using mouse monoclonal anti-CFTR C-terminal antibody (24-1, A.T.C.C. no. HB-11947) coupled to Protein A–agarose beads (Roche) CFTR was separated on 6% SDS-PAGE followed by western blot analysis. CFTR was visualized using HRP-conjugated secondary antibodies and a chemiluminescent HRP substrate. WT CFTR migrates as two bands, representing core-glycosylated form of the protein (band B) and the complex-glycosylated form of the protein (Band C). ΔF508 CFTR migrates only as Band B when cultured at 37˚C, because at this temperature none of the ΔF508 CFTR protein exits the ER. When ΔF508 CFTR cells are cultured at 27˚C, some of the CFTR escapes the ER and traffics to the post-Golgi compartments.

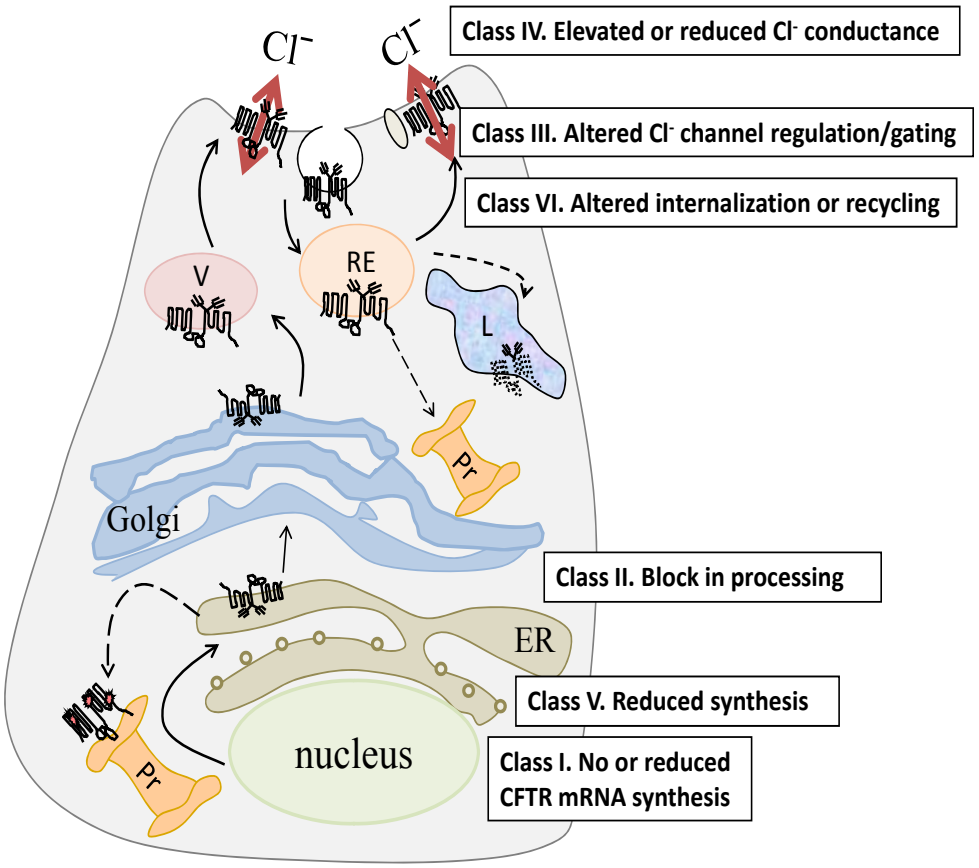

Figure 5. Classes of CFTR mutations. Class I: no or minimal mRNA (G542X); Class II: misprocessed protein (ΔF508); Class III: Defective Cl^- channel activity (G551D); Class IV: altered conductance (G622D, R792G, H620Q, A800G); Class V: low levels of functional protein (A455E); Class VI: altered cell surface stability (Q1412X, 4326delTC).

MUTATIONS IN THE AMINO-TERMINUS OF THE CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR ENHANCE ENDOCYTOSIS

by

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ABSTRACT

Efficient endocytosis of the cystic fibrosis transmembrane conductance regulator (CFTR) is mediated by a tyrosine-based internalization signal in CFTR's carboxy-terminal tail, $Y^{1424}SDI^{1427}$. In the present studies, two naturally-occurring cystic fibrosis mutations in the amino-terminus of CFTR, R31C and R31L were examined. To determine the defect that these mutations cause, the Arg31 mutants were expressed in COS-7 cells and their biogenesis and trafficking to the cell surface tested in metabolic pulse chase and surface biotinylation assays, respectively. The results indicated that both R31 mutants were processed to band C at ~50% the efficiency of the wild type protein. However, once processed and delivered to the cell surface, their half-lives were the same as wild type protein. Interestingly, indirect immunofluorescence and cell surface biotinylation indicated that the surface pool was much smaller than could be accounted for based on the biogenesis defect alone. Therefore, the Arg31 mutants were tested in internalization assays and found to be internalized at two times the rate of the wild type protein. Patch clamp and SPQ analysis confirmed reduced amounts of functional Arg31 channels at the cell surface. Together, the results suggest that both R31C and R31L mutations compromise biogenesis and enhance internalization of CFTR. These two additive effects contribute to the loss of surface expression and the associated defect in chloride conductance that is consistent with a disease phenotype.

Introduction

The cystic fibrosis transmembrane conductance regulator (CFTR) is the chloride channel that is defective in cystic fibrosis. CFTR is found at the apical surface of a

number of epithelial cell types, and is known to undergo endocytosis (1,2) through clathrin-coated vesicles (2,3). Further, it has been suggested that CFTR internalization may provide a mechanism for controlling the cAMP-stimulated chloride channel activity at the cell surface (2). The internalization and recycling pathways of cell surface CFTR suggest that significant amounts of CFTR are found within the endocytic pathway, and this is consistent with a number of studies in different cell types (4-9).

CFTR endocytosis is mediated by a tyrosine-based signal, $Y^{1424}SDI^{1427}$, in the carboxyl-terminal cytoplasmic tail of CFTR (10-12). This sequence conforms to a consensus internalization signal that consists of *YXXΦ*, where *Φ* is a large hydrophobic residue and *X* is any residue (13). This sequence, YSDI, has been shown to interact with the μ2 subunit of the AP-2 adaptor complex (14-16). Alanine substitutions of the tyrosine and isoleucine residues result in an accumulation of the double alanine mutant CFTR at the cell surface with a matching increase in chloride channel function (12). Interestingly, loss of the signal has no effect on the protein half-life, suggesting that internalization is not the rate-limiting step for CFTR degradation.

Over 1400 mutations have been identified in the CFTR gene and these are classified into six classes (reviewed in (17)). Class II mutations that affect protein maturation are the most prevalent, and the prototype of this class, ΔF508, is the most common disease causing mutation. This mutation confers a temperature-sensitive folding defect (18): when cell lines expressing this protein are cultured at 27°C for 2 days, ΔF508 CFTR is released to the cell surface (19) where it reduces single channel activity (20). Interestingly, even after delivery to the cell surface, ΔF508 is rapidly down-regulated (21), suggesting multiple defects that need to be corrected to restore normal chloride

channel function. Therefore, identification of the structural features in CFTR responsible for proper cell surface trafficking are of great interest.

In the present studies, we examined two naturally-occurring mutations R31C and R31L, which cause mild CF (CF Genetic Analysis Consortium, http://www.genet.sickkids.on.ca/cftr). To determine the defects caused by these missense mutations, we expressed them in COS-7 cells and analyzed their biogenesis and trafficking. Our results indicate that both R31C and R31L have compromised biogenesis and enhanced endocytosis compared with wild-type CFTR. These two additive defects contribute to the low surface expression of the mutants. Patch clamp studies confirmed the biochemical data and showed a dramatic decrease in the functional surface pool of both Arg31 CFTR mutants.

Materials and Methods

Construction of CFTR Mutants

The R31C and R31L mutants were prepared by PCR mutagenesis. Cloning vectors, oligos, enzymes and reagents were from Invitrogen Corp. PCR reactions were performed using "Platinum Pfx" polymerase according to the manufacturer's suggested protocol. Using the CFTR gene contained in plasmid pCDNA3.1+ as template, the R31L and R31C mutants were constructed by PCR-mutagenesis using a pair of internal primers for each mutant, annealing at the R31 coding site, and a pair of external primers, one annealing at the Nhe-I site of the multilinker of the vector, and one annealing at the BspE-I site in the CFTR gene. The oligos used were (5' to 3'): CFTR_R31L(+), GAGGAAAGGATACAGACAGCTCCTGGAATTGTCAGACATATAC

CFTR_R31L(-), GTATATGTCTGACAATTCCAGGAGCTGTCTGTATCCTTTCCTC

CFTR_R31C(+),

GAGGAAAGGATACAGACAGTGCCTGGAATTGTCAGACATATAC

CFTR_R31C(-), GTATATGTCTGACAATTCCAGGCACTGTCTGTATCCTTTCCTC

PCDNA-Nhe-N, GGCTAGCGTTTAAACTTAAGC

CFTR-BspEI-C, GAATATTTTCCGGAGGATGATTC

For each mutation, a pair of PCR reactions was performed, one using the external N primer and the internal (-) primer, another using the external C primer and the internal (+) primer. An aliquot from each reaction was then combined in a second reaction with both external primers. Annealing at, and extension from, the homologous termini of the first reaction products provided the template for the second reaction. The products of the second reactions were cloned using the PCR cloning vector pCR-Blunt. Mutant clones were verified by DNA sequence analysis (UAB CFAR/CCC Sequencing Core) and sub-cloned back into the CFTR gene in pCDNA3.1+ at the Nhe-I and BspE-I sites.

Cell Culture and Transient Transfection of COS-7 Cells

COS-7 cells were obtained from the ATCC (www.atcc.org). The cells were cultured as described previously (10) and transiently transfected using LipofectAMINE Plus reagent (Invitrogen) according to the manufacturer's directions. Transfection efficiencies were monitored by co-transfection with GFP and generally approximated 60%. The cells were incubated at 37°C in a humidified incubator for 48 h before analysis.

Immunoprecipitation of CFTR

One T75 flask of confluent transfected COS-7 cells was used 48 h post-transfection. The cells were lysed in RIPA buffer containing a protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). CFTR was immunoprecipitated using a mouse monoclonal anti-C terminal tail 24-1 antibody (ATCC number HB-11947) coupled to protein A agarose (Roche Diagnostics, Mannheim, Germany) (22).

Metabolic Pulse-Chase Assays

These experiments were performed as described previously (22) and analyzed by SDS-PAGE and autoradiography (PhosphorImager, Amersham Biosciences). Calculation of protein half-lives was performed as described by Straley et al. (23). Maturation efficiency was measured by comparing the density of the labeled immature, core-glycosylated form (B band) to the density of the fully glycosylated protein (C band) using IPLab software (Scanalytics, Inc.) (24).

Cell Surface Biotinylation

Cell surface glycoproteins were biotinylated using previously established methods (12) with the following modifications. Briefly, after surface biotinylation with biotin LC hydrazide, the cells were lysed in RIPA buffer with a protease inhibitor cocktail (30 min on ice), and CFTR was immunoprecipitated with a monoclonal antibody to CFTR (24-1) coupled to protein A agarose. CFTR was eluted from the beads with 2X Laemmli sample buffer (SB), run on SDS-PAGE gels, and transferred to PVDF membranes (BioRad). Biotinylated CFTR was detected with HRP-labeled avidin. Chemiluminescence was

induced with Pico Super Signal Peroxide solution (Pierce). The membranes were exposed for different time periods up to 1 min and a linear range for a standard set of diluted samples was calibrated.

Endocytosis Assays

Internalization assays were performed as described previously with minor modifications (10,12). Biotinylated CFTR was detected using Western blot analysis after calibration of a standard curve of diluted samples. CFTR internalization was detected as percentage loss of biotinylated CFTR in the 2.5-minute warm-up period compared to the control samples (no warm-up period).

Microscopy

Indirect immunofluorescence was performed as described previously (24).

Patch clamp analysis

Macroscopic currents were recorded in the excised, inside-out configuration. Patch pipettes were pulled from Corning 8161 glass to tip resistances of 3 mOhm. CFTR channels were activated following patch excision by exposure of the cytoplasmic face of the patch to catalytic subunit of protein kinase A (PKA; 110 U/ml; Promega) and MgATP (1 mM). Currents were recorded in symmetrical solutions containing (in mM): 140 N-methyl-D-glucamine-Cl, 3 $MgCl_2$, 1 EGTA and 10 TES (pH 7.3). Macroscopic currents were evoked using a ramp protocol from +80 to -80 mV with a 4 sec time period. Signals from macroscopic recording were filtered at 20 Hz. Data acquisition and analysis were

performed using pCLAMP 9.1 software (Axon Instruments). All patch clamp experiments were performed at 21-23°C.

SPQ Fluorescence Assays

CFTR function in individual cells was assayed using the halide-quenched dye SPQ (25). Briefly, cells were loaded for 10 min with SPQ (10 mM) by hypotonic shock and then mounted in a specially designed perfusion chamber for fluorescence measurements. Fluorescence (*F*) of single cells was measured with a Zeiss inverted microscope, a PTI imaging system, and Hamamatsu camera. Excitation was at 340 nm, and emission was >410 nm. All functional studies were at 23°C. Baseline fluorescence was measured in isotonic NaI buffer (NaI buffer; 130 mM NaI, 5 mM KNO_3, 2.5 mM $Ca(NO_3)_2$, 2.5 mM $Mg(NO_3)_2$, 10 mM D-glucose, 10 mM HEPES), followed by perfusion with isotonic dequench buffer ($NaNO_3$ replaced NaI) to measure unregulated efflux, and then $NaNO_3$ buffer with 20 μM forskolin as indicated. At the end of each experiment, cells were returned to the NaI buffer for requench (1100 sec). Increase in fluorescence above the basal (NaI quenched) level is shown (% increase F > basal). The data are cumulative from three coverslips in each condition studied in a paired fashion on three separate days (n = 120 cells/curve). The bottom 10% of cells in all conditions (attributable to inadequate SPQ loading, cell detachment, etc) were discarded and the data obtained from the top 90% of cells in each condition were analyzed as previously described (26,27). WT CFTR-expressing cells had an increased dequench in NO_3 buffer and NO_3 buffer + forskolin as previously described, presumably due to varying levels of WT CFTR activation.

Statistical Analysis

Results were expressed as means ± S. D. Statistical significance among means was determined using the Student's t test (two samples) unless otherwise indicated.

Results

The Arg31 mutations inhibit processing

Because missense mutations in the amino-terminus of CFTR often result in processing defects (28), and the Arg31 mutations were nonconservative substitutions, we first examined the effects of these two changes on the biogenesis of CFTR. Figure 1 shows the location of the mutations in the context of the CFTR molecule. To examine the maturation efficiency of the Arg31 mutants, they were expressed in COS-7 cells, and biogenesis tested in metabolic pulse-chase experiments (Figure 2). For wild type CFTR, 21.0 ± 1.0% (mean ± S.D.) of the protein was converted from the immature, core-glycosylated form (Band B) to the maturely glycosylated protein (Band C). This result is consistent with previous reports on the processing of wild type CFTR in COS-7 cells (29) (22). A significant proportion of Band B was converted to Band C (17.7 ± 1.5%) by 2 h and was maximal at 4 h after the labeling. With the R31C and R31L mutants, however, very little processing occurred during the first two h of chase (0% and 4.7 ± 1.5%, respectively). By 4 h, 10.3 ± 3.0% (R31C) and 11.3 ± 2.1% (R31L) of CFTR was converted to the mature form, indicating that, although there is a processing defect, it does not cause complete inhibition.

Transfected cells were next cultured at 37 °C or 27 °C to determine whether maturation of the Arg31 mutants improved at a lower temperature. The results (Fig. 2C)

indicate that there is no improvement in processing at 27°C. Overall, the maturation experiments suggest that in contrast to ΔF508 CFTR, compromised processing of the Arg31 mutations is not the result of a temperature sensitive folding defect.

The Arg31 mutations have no effect on protein half-life

To determine whether these substitutions affected protein stability, half lives of the fully processed mutant proteins were measured in metabolic pulse-chase experiments (Fig.3). The results indicate that the protein half-life of the wild-type protein is 13.3 ± 1.2 h, and the R31C and R31L half-lives are 12.7 ± 0.6 h and 14.3 ± 3.1 h, respectively, indicating that although the processing of the mutants was decreased by 50%, the stability of the processed proteins was comparable to wild type CFTR.

Reduced surface expression of *R31C and R31L*

Since some mutant protein was processed correctly and the protein half-life appeared normal, the surface pool of the Arg31 mutants was examined next. Wild type and Arg31 mutant CFTR intracellular distributions were monitored using indirect immunofluorescence (Fig. 4A). For wild type CFTR, perimeter staining was clearly seen as indicated by the arrows. For the R31C and R31L mutants, however, staining was much more restricted to an intracellular, reticular pattern. Perimeter staining was difficult to see in the Arg31 mutants. To confirm this observation, cells expressing wild type, R31C and R31L CFTR were surface biotinylated (Fig. 4B), CFTR was immunoprecipitated, and the biotinylated fraction was detected by Western blot analysis. The results indicate that the surface pool of the R31C and R31L mutants is 20.3 ± 7.5% and 33.6 ± 11.7% of the wild

type protein, respectively (*n=4, p<0.005*, and *p<0.05,* respectively). This suggests that the surface pool is smaller than would be predicted based on the biogenesis and half-life experiments.

R31C and R31L are internalized more rapidly than the wild type CFTR

Because the surface pool was smaller than predicted, we next tested whether the mutations affected CFTR clearance from the cell surface. To test this, a two-step biotinylation assay was used to monitor CFTR endocytosis during a 2.5 min warm-up period. This period of time is within the linear phase of CFTR endocytosis in these cells (10). The results indicated that 24.8 ± 5.9% of the wild type protein was internalized in 2.5 min (Fig.5). This rate of endocytosis was consistent with previous results (12). The R31C and R31L mutants, however, have dramatically altered internalization kinetics with 54 ± 4% and 55 ± 13.9% of the R31C and R31L mutants, respectively (internalized during the same warm-up period). This result suggests these substitutions affect the surface stability of CFTR and are consistent with the lowered surface expression seen with immunofluorescence and surface biotinylation.

The functional activity of the R31C and R31L mutants is severely compromised

As a final measure of the total CFTR chloride channels at the cell surface, we tested the functional activity of the R31C and R31L mutants in two complementary functional assays, macroscopic patch clamp experiments and SPQ assays.

In the first studies, inside-out membrane patches were excised from transfected COS-7 cells using large tip pipettes (3 mOhm). CFTR channels were activated with

normally saturating concentrations of protein kinase A and MgATP. For cells transfected with wild type CFTR, the majority of patches (4 of 6) contained many CFTR channels (> 30-50) as deduced from the magnitude of the peak currents recorded at +/- 80 mV (assuming single channel currents of about 0.5 pA; Fig. 6A). The recorded currents were determined to be CFTR-mediated on the basis of three criteria: (i) dependence on MgATP; (ii) inhibited by the voltage-dependent pore blocker glibenclamide (30) and (iii) absence from untransfected cells (data not shown). Cells transfected with the R31L and R31C mutants exhibited two differences as compared to wild-type transfected cells. First, for each mutant the CFTR-mediated currents were much smaller across those patches that exhibited detectable channel activity (Fig. 6B and C). Second, cells that were transfected with the mutants exhibited far more silent patches (i.e., patches that exhibited no detectable channel activity; see Fig. 6 legend). The silent patches might represent untransfected cells, although the fact that wild type-transfected cells exhibited very few silent patches makes this less likely. These macroscopic patch clamp results are consistent with the notion that the Arg31 mutations reduce the steady-state surface expression of CFTR. As with any macroscopic functional assay, however, these findings do not exclude the possibility that the Arg31 mutations also affect the single channel properties of CFTR.

In the second functional analyses, the Arg31 mutants were tested using an SPQ halide efflux protocol to measure anion transport through the cell surface membrane. Fig. 6E shows that in wild type CFTR expressing cells, halide transport was activated after stimulation with cAMP agonists (20 μM forskolin and 100 μM 3-isobutyl-1-methlyxanthine). Analysis of the Arg31 mutants, however, revealed that the halide

transport activities were reduced compared to the wild type protein. This result, in combination with the patch clamp analysis, supports the biochemical data and indicates that there is a decrease in the functional chloride channels at the surface in cells expressing the Arg31 mutants compared to the wild type controls.

Discussion

The R31C and R31L are naturally-occurring missense CF mutations that appear to have a mild phenotype (31) (www.genet.sickkids.on.ca/cftr/). Both of these mutations are rare (identified once in 284 CF chromosomes, (for the R31L mutation). The R31C mutation was found in a 45-year-old male CF patient diagnosed in childhood who was pancreatic sufficient with moderate pulmonary symptoms and a positive sweat test. His other allele is the I556V mutation. The R31L was found in a 24-year-old female CF patient who was pancreatic sufficient with normal lung function, but a positive sweat test. The other allele was not identified. Based on these descriptions, both mutations appear to be associated with mild disease and our biochemical and functional data support this idea since there is some surface expressed and functional chloride channel activity associated with these mutants.

The analysis of the Arg31 CFTR suggests that replacement of the arginine with either cysteine or leucine at position 31 affects protein biogenesis. The biogenesis defect is not complete, however, since some CFTR is processed properly and reaches the cell surface. Interestingly, not only was biogenesis compromised by ~50%, the kinetics of transport from endoplasmic reticulum to the Golgi as assessed by glycosylation changes appeared slower for the two mutants, particularly at the 2 h time point. By 6 h, however,

there was a decrease in the amount of labeled band C for both of the mutants compared with earlier time points. Surprisingly, once the protein was fully processed, the stability of the Arg31 mutants was comparable with the wild type protein.

The fact that these substitutions affect biogenesis is not surprising given that in the Cystic Fibrosis Mutation Database there are 27 missense mutations that have been identified within the 80-residue amino-terminal tail (www.genet.sickkids.on.ca/cftr/). Furthermore, our own studies using deletion analysis have shown that the amino-terminus is essential for CFTR protein processing (10, 32). One possible reason for the critical nature of the amino-terminus is that this region has been shown to control protein kinase A-dependent channel gating through a physical interaction with the distal region of nucleotide binding domain 1 and the R domain (residues 595-823) (33). Furthermore, alanine scanning of this region shows that a number of sites are critical for processing (32), suggesting that point mutations in this region of the molecule are poorly tolerated even when the substitutions are alanine residues. In the case of the Arg31 mutations, the substitutions are nonconservative and based on the data; these nonconservative changes affect CFTR processing.

The reason these two mutations affect endocytosis is unclear. One intriguing possibility, however, is that they introduce potential internalization signals in the amino-terminus of CFTR, $Y^{28}xxC^{31}$ and $Y^{28}xxL^{31}$. With the introduction of the hydrophobic residue at position 4, they conform to a consensus internalization signal that consists of *YXXΦ*, where Φ is a large hydrophobic residue and *X* is any residue (13). Whether these mutations are recognized by the cell's endocytic machinery, however, cannot be determined from the present studies. In fact, it is possible that these mutations somehow

affect the recognition of the tyrosine-based signal in the carboxy-terminus, and promote enhanced endocytosis. A third possibility is that the mutations alter the tertiary structure of CFTR and this influences CFTR's rate of clearance from the cell surface. However, if the structure were dramatically altered, then the protein half-life might be affected, and this is certainly not the case with these particular mutations.

Another CFTR mutation was recently identified in the second intracellular loop, N287Y, which affects endocytosis over wild type levels (34). Interestingly, the N287Y mutation does not introduce a consensus tyrosine-based signal and did not affect biogenesis of CFTR. This suggests that overactive internalization alone is sufficient to promote a disease phenotype (34). In the case of the Arg31 mutants, a combination of a biogenesis defect, coupled with enhanced endocytosis, results in a decrease in the functional amount of CFTR at the cell surface. The mild phenotype of patients harboring these mutations is consistent with the residual amount of activity of the mutants found in this study.

The altered surface stability defect in the Arg31 mutants without a corresponding defect in protein stability is in contrast to the ΔF508 mutation. ΔF508 is a class II mutation that misfolds during biogenesis and is rapidly degraded by the ER-associated degradation pathway (35). ΔF508, however, is a temperature-sensitive mutation (19), and some CFTR escapes endoplasmic reticulum associated degradation when cells are cultured at 27°C. Recent studies on rescued ΔF508 CFTR in airway epithelia indicate that like the Arg31 mutants, ΔF508 CFTR is also more rapidly internalized and degraded than the wild type protein (36). The Arg31 mutants do have a partial biogenesis defect,

indicating their status as class II mutations. However, culturing at 27°C has no effect on ArgA31 mutant biogenesis, indicating that they are not temperature-sensitive. Analysis of the ΔF508, N287Y, and R31L and R31C indicate that alterations in the transport of CFTR at the cell surface, whether it is enhanced internalization or compromised recycling, can result in a disease phenotype.

In summary, two naturally-occurring mutations in the amino-terminal cytoplasmic tail of CFTR at position 31 dramatically enhance endocytosis. This, in combination with a partial biogenesis defect, leads to diminished chloride channel activity at the cell surface and explains two of the cellular defects observed in these mutations.

Footnotes

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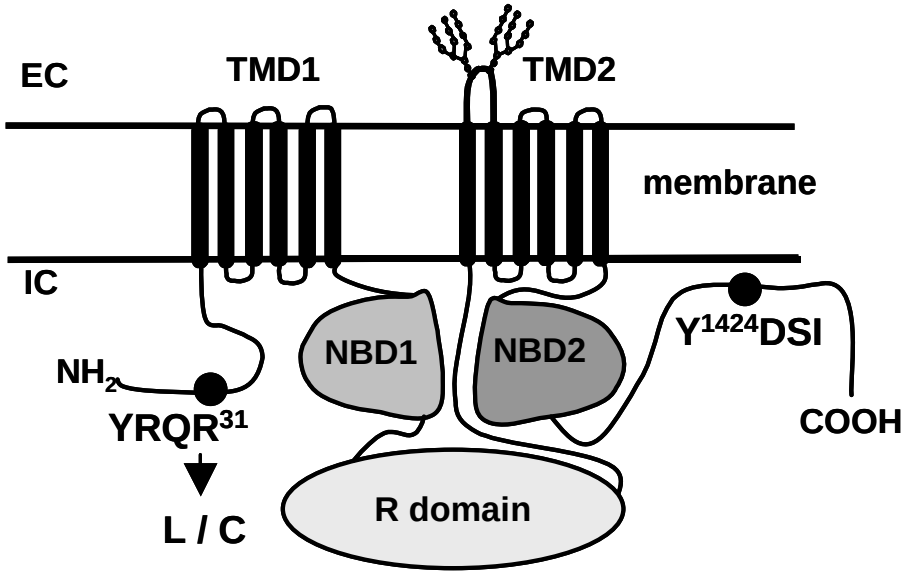


Fig 1. Schematic diagram of CFTR. The cystic fibrosis transmembrane conductance regulator (CFTR) molecule consists of 12 transmembrane spanning domains (TMD1, TMD2), two cytoplasmic nucleotide-binding domains (NBD1 and NBD2), a regulatory domain (R), and amino- and carboxy-terminal cytoplasmic tails. CFTR has an internalization signal in its carboxy-terminal tail that consists of $Y^{1424}DSI^{1427}$ (Peter et al., 2002). This motif conforms to the YXXΦ motif first identified for tyrosine-based internalization signals, where *Φ* is a large hydrophobic residue and *X* is any residue (13).

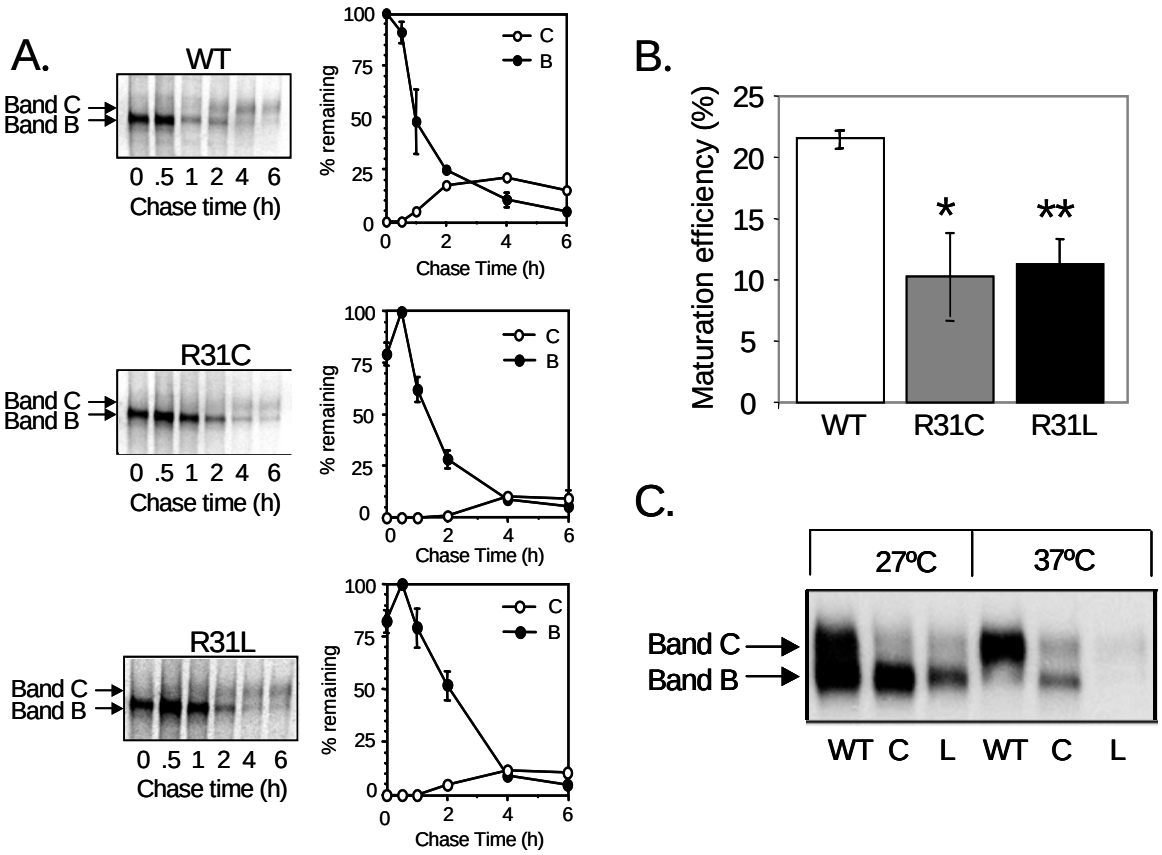
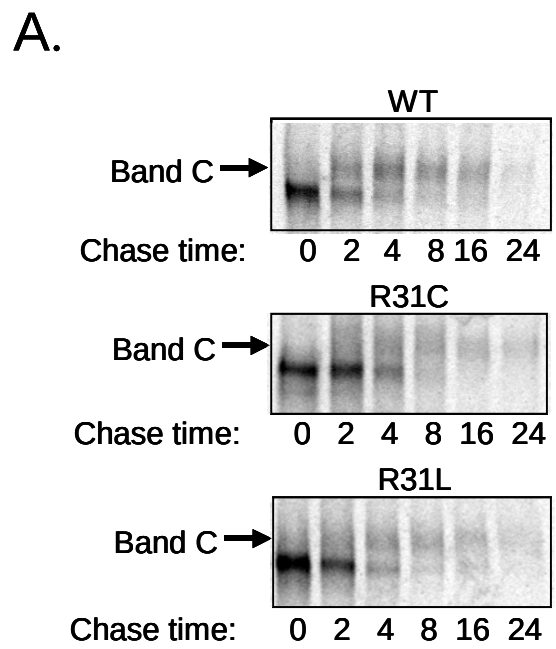


Fig 2. Protein maturation of R31C and R31L CFTR is inefficient compared to wild type CFTR. COS-7 cells were pulse-labeled for 30 min. at 37ºC with 300 μCi/μl ^{35}S methionine. After the pulse, radioactive media was replaced by complete medium, and cells were lysed at time points indicated. CFTR was immunoprecipitated with 24-1 antibody, and analyzed by SDS-PAGE on 6% gels and Phosphorimager analysis (Amersham Biosciences). CFTR maturation efficiency was measured by comparing the maximum density of labeled band B after a 30-min pulse (100%) to the density of band C after 4 h of chase using IPLab software. *A*, representative metabolic labeling experiments are shown for wild type (WT), R31C, and R31L (left panels). Band B represents the core glycosylated form of CFTR and band C represents fully glycosylated protein. The average disappearance of band B (maturation and/or degradation) and formation of band C at each time point are shown (right panels). The disappearance of band B (closed circles) and formation of band C (open circles) were calculated based on densitometry at each time point. Results are plotted as percent band B at the 0 time point. B, summaries of the protein maturation efficiencies for each of the constructs. Maturation efficiencies of WT, R31C, and R31L were calculated after 4 h of chase (average ± S.D., n = 3; *$p < 0.005$, **$p < 0.001$). C, R31C (C) and R31L (L) are not temperature sensitive. WT, R31C and R31L CFTR were immunoprecipitated from COS-7 cells 48 h after transfection. 24 h post-transfection, the cells were cultured at either 37°C or 27°C for 24 h before analysis. CFTR was *in vitro* phosphorylated and analyzed by SDS-PAGE and Phosphorimager analysis. 27°C incubation increased the amount of B band, but did not influence C band production for R31C or R31L CFTR.



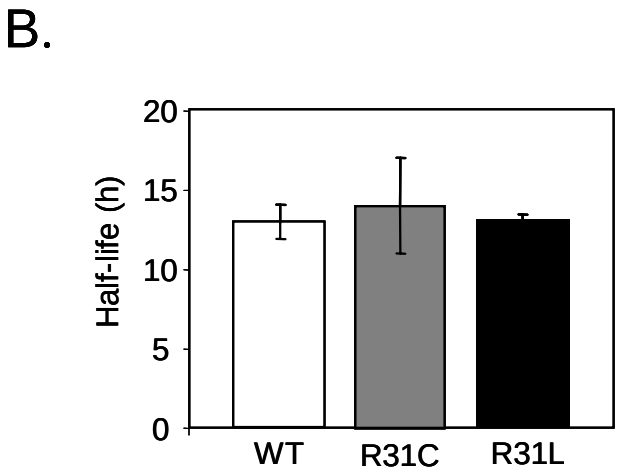


Fig 3. The R31 mutations have a normal protein half-life. Wild type, R31C and R31L CFTR half-lives were determined in COS-7 cells 24 h after transfection. After a 1-h pulse with 300 μCi/μl ^{35}S methionine-containing media and the indicated chase periods in complete media, the cells were lysed in RIPA buffer and CFTR was immunoprecipitated using 24-1 antibody. Samples were analyzed by SDS-PAGE and Phosphorimager analysis. *A*, representative gels of wild type (WT), R31C, and R31L CFTR half lives are shown. The rate of disappearance of band C over time indicates the half-life of the maturely glycosylated protein. *B*, average CFTR protein half-lives were measured by densitometry (n = 3).

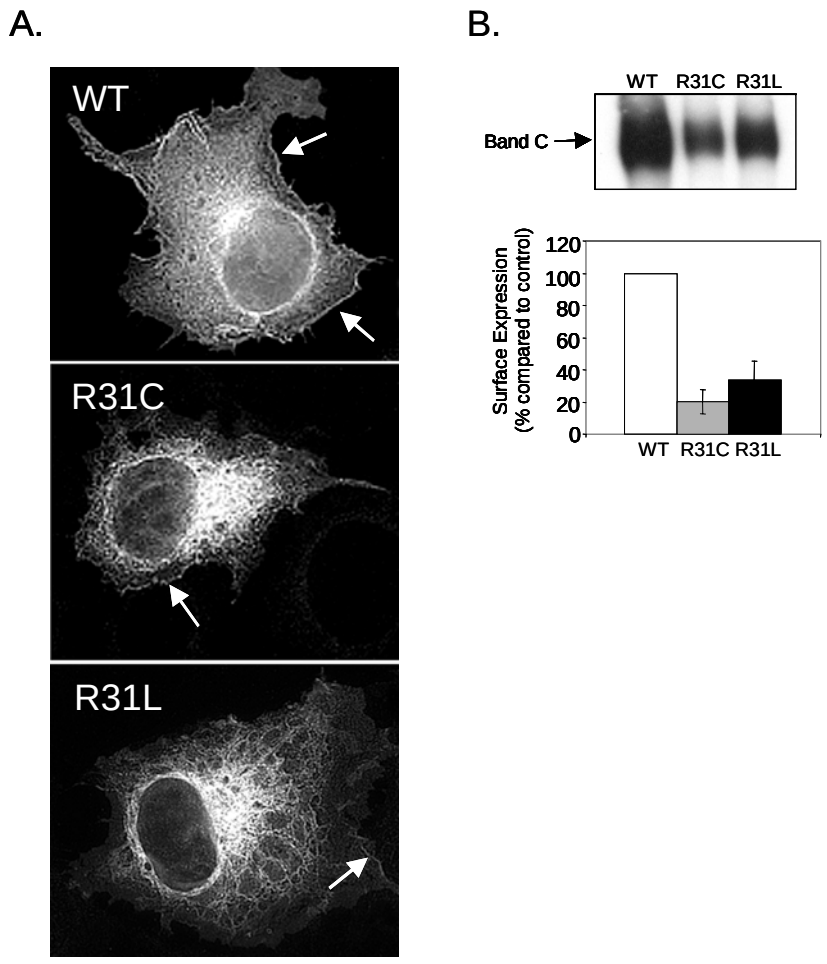


Fig 4. R31C and R31L surface expression is lower than wild type CFTR. *A*, Wild type, R31C, and R31L CFTR distributions were examined in COS-7 cells 48 h after transfection using indirect immunofluorescence. Cells were fixed with methanol (-20°C for 10 min), and CFTR was stained with 24-1 antibody and an anti-mouse Alexa fluor 596 secondary antibody. Arrows indicate the prominant cell surface expression of the wild type protein CFTR and the diminished amount of surface staining in the R31C and R31L CFTR expressing cells. *B*, Surface expression of CFTR was measured using a surface biotinylation assay. Cell surface proteins were labeled with biotin LC hydrazide. The cells were lysed, CFTR was immunoprecipitated with 24-1, and the samples were analyzed by SDS-PAGE and Western blot. Biotinylated CFTR was detected with HRP-avidin. The average surface expression for each of the constructs is shown below (n = 4; *$p < 0.005$, **$p < 0.05$).

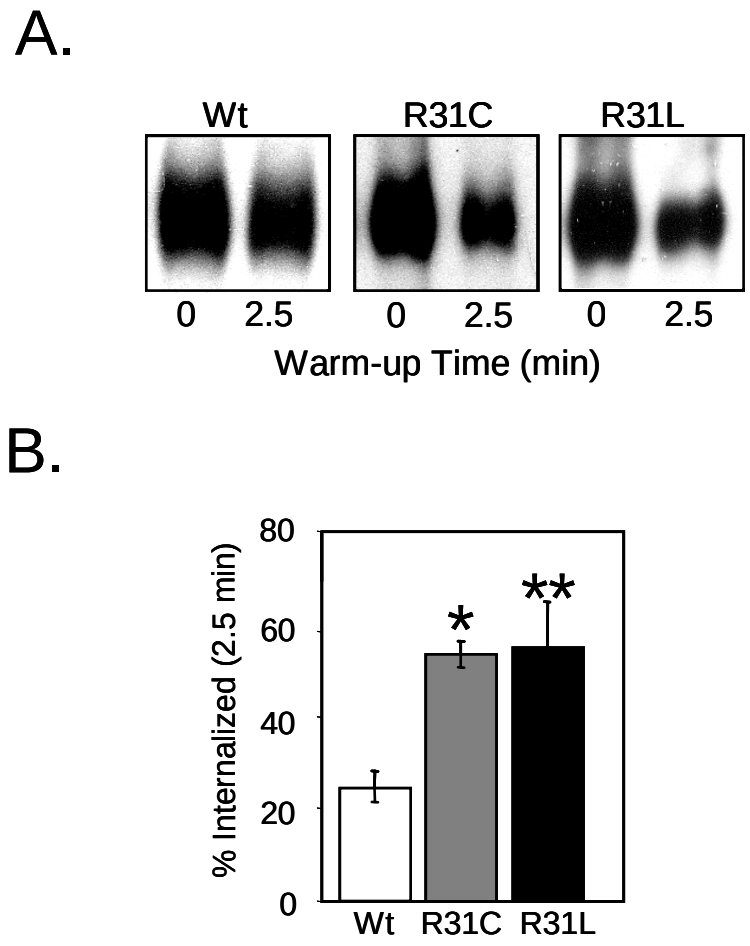


Fig 5. Internalization of the R31C and R31L CFTR mutants is dramatically enhanced compared to the wild type protein. *A*, Wild type, R31C and R31L CFTR internalization in COS-7 cells. COS-7 cells were analyzed 48 h post-transfection for each of the constructs. CFTR internalization was monitored using a two-step cell surface periodate/LC-hydrazide biotinylation procedure (see Materials and Methods). At the zero time points, both steps were conducted at 4°C to label the entire surface pool of CFTR. Internalization is monitored by a loss of biotinylation of the surface pool by including a 37°C incubation period (2.5 min) between periodate and biotin LC-hydrazide treatments. After biotinylation, the cells were lysed, and CFTR was immunoprecipitated using the 24-1 antibody. The samples were then analyzed by SDS-PAGE and Western blot. Biotinylated CFTR was detected using HRP-avidin and the 0 and 2.5 min time points for each construct were compared. Internalization was more than 2-fold faster for both R31 mutants compared to the wild type protein ($n = 3$, $*p < 0.001$, $**p < 0.05$).

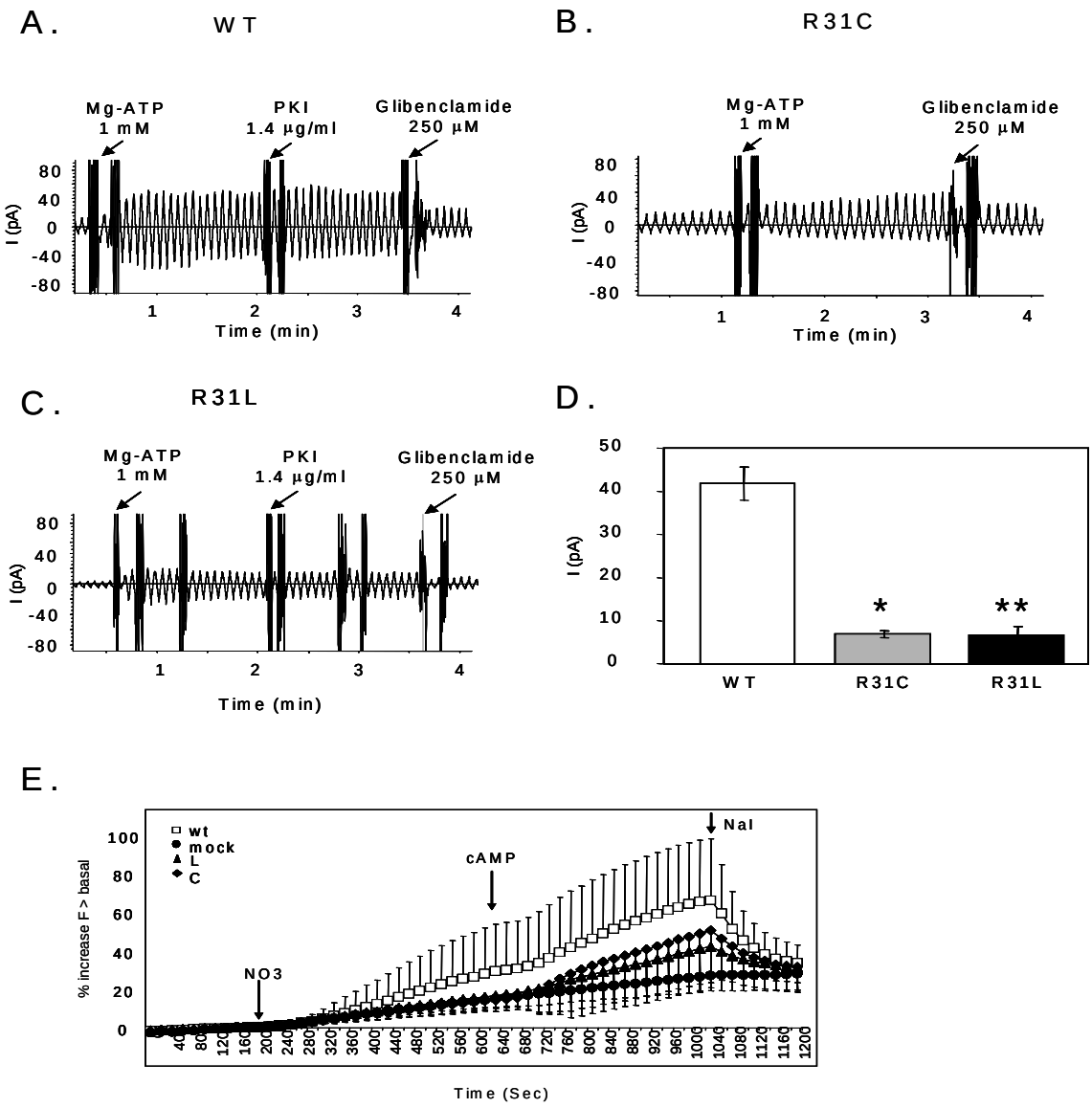


Fig 6. R31C and R31L mutants have diminished channel activity compared to the wild type protein. A-C. Representative current traces for WT, L-mutant and C-mutant. D. Mean glibenclamide-sensitive currents ($\overline{X}$ ±SEM) recorded at –80 mV for each construct (n = 4 patches each). E. Functional analysis of wild type and R31C and R31L CFTR using SPQ fluorescence. The change in SPQ fluorescence is shown for COS-7 cells expressing wild type, R31C and R31L CFTR. Cells were stimulated with 20 µM forskolin (at *arrow*, cAMP). The change in SPQ fluorescence was significantly more than for the negative control (mock-transfected cells), but substantially lower than for the wild type protein. Curves were generated from the mean ± S.D. of cells studied over three days. The wild type CFTR sample had significantly higher iodide flux (in the presence of forskolin) from the R31 mutants or mock sample ($p < 0.001$ by χ^2 test for each condition compared with wild type CFTR).

FUNCTIONAL STABILITY OF RESCUED ΔF508 CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR (CFTR) IN AIRWAY EPITHELIAL CELLS

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SUMMARY

The most common disease-causing mutation in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene, ΔF508, results in the production of a misfolded protein that is rapidly degraded by the endoplasmic reticulum-associated degradation (ERAD) pathway. The mutant protein is temperature sensitive; although prior studies in human airway epithelial cells indicate that after ΔF508 CFTR is delivered to the cell surface at the permissive temperature, the channel is poorly responsive to physiological stimuli and is rapidly degraded from the cell surface at 37˚C. In the present studies, we tested the effect of a recently characterized pharmacological corrector, corr-4a, on cell surface stability and function of the low-temperature rescued protein (rΔF508 CFTR). We demonstrated that corr-4a significantly enhanced the stability of rΔF508 CFTR. Upon using firefly luciferase-based reporters to investigate the mechanisms by which low temperature and corr-4a enhance rΔF508 CFTR stability, we found that both interventions inhibited various stages of ubiquitin-dependent ΔF508 CFTR degradation. Ussing chamber studies to measure channel function revealed that while corr-4a improved the cAMP-mediated rΔF508 CFTR response, the functional activity of the latter did not correlate with protein stability. However, the CFTR channel activator VRT-532, added after corr-4a treatment, significantly augmented cAMP-stimulated currents, revealing that once it reaches the cell surface, rΔF508 CFTR function could be further enhanced using CFTR potentiator. Our studies demonstrated that stabilizing ΔF508 CFTRon the plasma membrane alone was not sufficient to obtain adequate ΔF508 CFTR function in airway epithelial cells. These results strongly support the idea that sufficient

correction of ΔF508 CFTR requires a chemical corrector that (1) promotes folding and exit from the ER; (2) enhances surface stability; and (3) improves channel activity.

INTRODUCTION

Cystic fibrosis (CF) is caused by defects in the CF transmembrane conductance regulator (CFTR), a cAMP-regulated chloride channel that regulates ion and water balance across epithelia (1, 2). CFTR defects lead to altered exocrine secretion in a number of organ systems, including the airways, the gastrointestinal track, pancreas, and sweat glands (1). Dehydrated mucus in the airways leads to chronic infections and eventual decline of lung function, and this phenotype is the leading cause of morbidity and mortality associated with CF (2).

Although more than 1500 mutations have been identified in the *CFTR* gene (available at http://www.genet.sickkids.on.ca), the most common is the ΔF508 mutation. The misfolded gene product is recognized by the endoplasmic reticulum (ER) quality control machinery, retrotranslocated into the cytosol, and degraded through ERAD by the proteasome (3-7). Because of the severe nature of the processing defect, little if any ΔF508 CFTR reaches the apical cell surface, resulting in defective cAMP-dependent chloride conductance in the affected tissues.

Given that rΔF508 CFTR retains some biological activity (8), and that more than 90% of the patients with CF have at least one allele of ΔF508 CFTR (9), there is considerable interest in understanding the molecular mechanisms controlling ΔF508 CFTR biogenesis and degradation. A number of interventions, including cell culture at low temperature (~27˚C) or with chemical chaperones such as glycerol, can rescue the

mutant protein from ERAD and facilitate ΔF508 CFTR surface expression to some degree (10, 11). Therefore, an ongoing concerted effort seeks to identify novel compounds that facilitate ΔF508 CFTR rescue from ERAD, increase surface stability, and improve channel function.

Other strategies for rescuing ΔF508 CFTR from ERAD have included sarcoplasmic endoplasmic-reticulum Ca^{2+}-ATPase (SERCA) inhibitors such as curcumin and thapsigargin (12, 13), sodium 4-phenylbutyrate (14), and several small molecules identified through high throughput screens (15-17).

It has been reported that two of the most effective corrector reagents, corr-4a ([2-(5-Chloro-2-methoxy-phenylamino)-4'-methyl-[4,5'bithiazolyl-2'-yl]-phenyl-methanone) and Vertex-325 (VRT-325; 4-Cyclohexyloxy-2-{1-[4-(4-methoxy-benzensulfonyl)-piperazin-1-yl]-ethyl}-quinazoline) may directly interact with ΔF508 CFTR (19, 20). Recent studies from our laboratory demonstrated that these compounds ablated the rapid endocytosis of low temperature rescued ΔF508 CFTR in polarized human airway epithelial cells (21), but the mechanism of action and the effects of these compounds on the long-term stability and chloride channel activity of ΔF508 CFTR are unclear.

In addition to compounds that facilitate ΔF508 CFTR biogenesis (rescue) and/or surface stability (e.g., correctors such corr-4a (15)), another group of compounds enhances ΔF508 CFTR channel activity (e.g., potentiators such as VRT-532, which directly activates the channel (16)). Interestingly, VRT-532 has recently been shown to act as a corrector as well (19).

In order to develop efficient therapies for CF caused by the ΔF508 mutation, it will be necessary to identify compounds that rescue ΔF508 CFTR from ERAD and correct the function of the mutant protein. Whether surface expression correlates with channel function is not clear; i.e., corrected protein that escaped ERAD may have compromised channel function. Therefore, in the present study, we investigated how corr-4a alters the cell surface stability and function of ΔF508 CFTR in polarized CFBE41o-ΔF cells. Using firefly luciferase-based reporters of the ubiquitin-dependent proteasome system (UPS), we investigated the mechanism by which corr-4a enhances ΔF508 CFTR stability. We also followed the activity of rescued ΔF508 CFTR in the presence or absence of corr-4a for up to 12 h. Because protein stability and function did not fully correlate, we investigated if the addition of a potentiator could complement the effect of corr-4a. Our results suggest that corr-4a is not sufficient for adequate correction of the rescued protein. In other words, the ΔF508 CFTR on the cell surface is functionally compromised, and treating CF resulting from the ΔF508 mutation requires a combination of rescue, stabilization, and correction of the channel defect.

MATERIALS AND METHODS

Cell lines and cell culture

Cells were maintained in a 37°C humidified incubator at 5% CO_2 concentration. CFBE41o-ΔF and CFBE41o-WT cell lines were developed and cultured as described previously (22). The non-transduced parental CFBE41o-cell line is homozygous for the ΔF508 mutation (23), but the endogenous ΔF508 CFTR expression is below detection as monitored by RT-PCR or Ussing chamber analysis (parental cells, present studies).

CFBE41o- cell cultures were maintained in DMEM (Dulbecco's modified Eagle's medium) and Ham's F12 medium (50:50, v/v) (Invitrogen) with 10% (v/v) FBS. For experiments requiring polarized cells, CFBE41o-ΔF and CFBE41o-WT cells were seeded onto 6.5 mm Transwell filters (Costar, Corning) for the Ussing chamber experiments or on 24 mm Transwell filters for the surface biotinylation experiments. Cells were grown at a liquid-liquid interface, and media was changed every 24 h. Under these conditions, the cells formed polarized monolayers with transepithelial resistances of >1000 Ω-cm^2 after 4 days of culture.

Correctors and potentiators of ΔF508 CFTR

Corr-4a ({2-(5-chloro-2-methoxy-phenylamino)-4′-methyl-[4,5′]-bithiazolyl-2′-yl}-phenyl-methanone) (15) and VRT-532 (4-methyl-2-(5-phenyl-1H-pyrazol-3-yl)-phenol (16) were provided by Cystic Fibrosis Foundation Therapeutics (Bethesda, MD, U.S.A.). Both compounds were dissolved in DMSO at a 10 mM stock concentration and diluted in OPTIMEM medium (Invitrogen) supplemented with 2% (v/v) FBS at 10 μM working concentration, as described (21). In all control experiments, DMSO was added to the same final concentration as a vehicle control.

Immunoprecipitation

Immunoprecipitations were performed as described previously (21). Briefly, CFTR was immunoprecipitated from 500 μg (CFBE41o-WT cells) or 1000 μg (CFBE41o-ΔF cells) of total protein using 1 μg (final concentration) mouse monoclonal anti-CFTR C-terminal antibody (24-1, ATCC # HB-11947) coupled to 36 μl of Protein G agarose

beads (Roche). Immunoprecipitation reactions were carried out for 2 h at 4°C. Immunoprecipitated proteins were *in vitro* phosphorylated and visualized by SDS-PAGE followed by autoradiography and phosphorimaging, or by SDS-PAGE followed by Western blotting (24).

Real-Time RT-PCR

Isolation of total cellular RNA and real-time RT-PCR were performed as described previously (25). Briefly, total RNA was isolated using the RNeasy mini kit (Qiagen) according to the manufacturer's protocol. Quantitative real-time PCR was performed using the ABI PRISM 7500 sequence detection system as described in the ABI User bulletin #2 (Applied Biosystems [ABI], Foster City, CA). Total CFTR mRNA levels were evaluated using Assay-On-Demand primer mix (assay ID: Hs00357011_m1). Glyceraldehyde 3-phosphate dehydrogenase mRNA levels (GAPDH; assay ID: Hs99999905_m1) were studied in parallel as an internal control. Results were expressed as CFTR mRNA levels relative to GAPDH mRNA (means ± SDERR). Primer efficiencies for GAPDH and CFTR assays were 101% and 95%, respectively.

CFTR cell-surface half-life experiments

Cells were cultured at 27°C for 48 hours to facilitate ΔF508 CFTR rescue and delivery to the cell surface (21). The cells were then transferred to a 37°C incubator and cultured for 0 to 12 h in the presence or absence of 10 μM corr-4a. Cell surface glycoproteins were biotinylated as described previously (24). CFTR was immunoprecipitated with a monoclonal antibody to CFTR (24-1) coupled to protein G-

agarose, run on 8% SDS-PAGE gels, and transferred to polyvinylidene difluoride membranes (BioRad). Biotinylated CFTR was detected with horseradish peroxidase-labeled avidin. Chemiluminescence was induced with Pico Super Signal peroxide solution (Pierce). The membranes were exposed for time periods up to 1 min, and a linear range for a standard set of diluted samples was calibrated as described previously (24). Western blots were analyzed and densities measured using IPLab (BD Biosciences) software. The results represent n>3 experiments.

Firefly luciferase based reporter assays of the ubiquitin-dependent degradation pathways

HeLa ΔF cells grown on 6-well plates were transfected with 0.5 μg plasmid DNA encoding one of the UPS reporters in triplicates (see **Table 1**). At 30 hours post-transfection, cells were transferred to 27°C culture and left untreated, or kept at 37°C and treated with 10 μM Corr-4a for 12 hours in serum-free Opti-MEM. Untreated, transfected cells cultured at 37°C for 12 hours served as controls. All wells received fresh media (with or without corr-4a) every two hours. Firefly luciferase activities in cell lysates were measured using the Promega Luciferase Assay System, according to the manufacturer's protocol on a TD-20/20 manual luminometer. For each condition, luciferase activity was expressed as the percent increase in luciferase activity compared to controls cultured at 37°C without corrector, and *p* values were determined using a paired two-tailed Student's t-test.

Ussing chamber studies

Tissue culture inserts were mounted in Ussing chambers (Jim's Instruments, Iowa

City, IA) and bathed with Normal Ringer (NR) solutions containing (in mM) 120 NaCl, 25 $NaHCO_3$, 3.3 KH_2PO_4, 0.83 K_2HPO_4, 1.2 $CaCl_2$, 1.2 $MgCl_2$, 10 HEPES (Na^+-free), 10 mannitol (apical compartment), and 10 glucose (basolateral compartment), as described previously (26). Bath solutions were vigorously stirred by bubbling continuously with 95% O_2, 5% CO_2 at 37°C (pH 7.4). Monolayers were short-circuited (clamped) to 0 mV, and short-circuit currents (I_{sc}) were measured using a voltage clamp (VCC-600, Physiologic Instruments, San Diego, CA). A 10 mV pulse of 1 s duration was imposed every 10 s to monitor resistance (R_t), which was calculated using Ohm's law. Data were collected using the Acquire and Analyze program (version 1.45; Physiologic Instruments). Following establishment of steady-state values of I_{sc} and R_t (usually within 5-10 min from mounting the filters to the Ussing chambers), normal Ringer's solution in the apical side of chambers was changed for low chloride (5 mM Cl^- solution containing 120 mM $C_6H_{11}O_7Na$, 1.2 mM $C_{12}H_{22}CaO_{14}$, and 1.2 mM C_{12} H $_{22}MgO_{14}$). The basolateral side was maintained at 125 mM Cl^-. Amiloride 0.1 mM was added to the apical side, and DMSO or corr-4a (10 μM), were added into the apical compartments. I_{sc} and R_t were measured continuously until a new steady state was reached. Basolateral membranes were then permeabilized by the addition of amphotericin B (100 μM). Permeabilization was followed by a drop in R_t. CFTR was stimulated as described for each experiment. Forskolin (FSK, Calbiochem, Gibbstown, NJ) was used at 10 μM, genistein (GEN) (Sigma) at 50μM, and VRT-532 at 10 μM final concentration in the apical chambers. Currents were blocked using CFTR-172_{inh} (2-thioxo-4-thiazolidinone analog; Calbiochem).

Statistical analyses

Results are mean ± S.E. and statistical significances were calculated by using the two-tailed Student's *t* test. P values of < 0.05 were considered significant.

RESULTS

Corr-4a mediated rescue of ΔF508 CFTR biogenesis in human airway epithelial cells.

In order to follow the trafficking of rΔF508 CFTR at the cell surface, we first determined the effects of corr-4a on ΔF508 CFTR biogenesis in human airway epithelial cells (CFBE41o-ΔF). We treated polarized CFBE41o-ΔF cells with varying concentrations of corr-4a for 12 h at 37°C. Following treatment, CFTR was immunoprecipitated from whole-cell lysates and analyzed for the presence of mature, fully glycosylated protein, or band C (rΔF508 CFTR). Cell surface ΔF508 CFTR levels were determined following cell surface biotinylation. The results in Figure 1 demonstrate that although we tested a wide concentration range for corr-4a, the efficiency of ΔF508 CFTR rescue was rather minimal. Longer treatment resulted in significant cellular toxicity mediated by the compound (data not shown). The most effective corr-4a concentration range was 2.5 to 10 μM.

Low temperature culture (27°C), applied as a positive control, produced a significant amount of fully glycosylated band C. Compared to low temperature culture, corr-4a treatment resulted in significantly less biotinylated CFTR (Figure 1, right panel). Therefore, for all subsequent studies, we utilized low temperature correction to deliver ΔF508 CFTR to the cell surface, because this method consistently produced levels of rescued protein that were sufficient to study cell surface trafficking and function. To

analyze the effects of corr-4a on cell surface trafficking and function, we returned cells expressing low temperature rescued protein to 37°C and added the compound to the culture media for the time periods specified.

Reduced activity of low temperature-rescued ΔF508 CFTR compared to wild type CFTR.

Previous reports indicated that low temperature rΔF508 CFTR was poorly responsive to cAMP mediated stimuli (23). In the present studies, we used Ussing chambers to compare the functional activity of rΔF508 with WT CFTR in the presence or absence of corr-4a at 37°C. To begin testing the effects of corr-4a on ΔF508 CFTR function at 37°C, we included a 1 hour pretreatment with corr-4a during the last hour at 27°C. This pretreatment was sufficient to stabilize the surface pool of rΔF508 CFTR in our previous studies (21). To insure that the monitored short circuit currents (I_{sc}) could be attributed to CFTR, we applied the following conditions: 1) I_{sc} were measured after permeabilization of the basolateral membranes with amphotericin B; 2) tracings were recorded in the presence of amiloride, which blocks epithelial sodium channels (ENaC); 3) the effects of forskolin, genistein, and a specific CFTR inhibitor (CFTRinh-172) were all tested (27, 28); 4) I_{sc} were also monitored in the parental CFBE41o- cells, which do not express detectable levels of CFTR mRNA.

Ussing chamber assays were prepared and executed at 37°C, and the analysis was done as quickly as possible since ΔF508 CFTR is rapidly degraded from the cell surface under these conditions (21, 29-31). The cells were placed at 37°C (0 time point) and spent 30 to 40 min at this temperature before the forskolin stimulation. Under these conditions, the forskolin-stimulated I_{sc} for ΔF508 CFTR was only ~5% of WT (Figure

2A-D). Although both the forskolin- and forskolin+genistein- activated I_{sc}s were slightly greater in the presence of corr-4a, these trends toward improvement did not reach statistical significance. In the CFBE41o- cells (CFBE parental), there was no forskolin-stimulated activity, supporting the view that the forskolin-stimulated currents in CFBE41o-ΔF cells were due to rΔF508 CFTR. The significant I_{sc} response to UTP, which activates P2Y receptors, indicates that the cells were viable under our experimental conditions (Figure 2, panels E and F).

To confirm the presence of CFTR in each of the cell lines, we determined mRNA and protein levels. CFTR was immunoprecipitated from CFBE41o-WT, CFBE41o-parental and CFBE41o-ΔF cells and compared to HeLa-parental and HeLa-ΔF (Figure 2, panel G). WT CFTR appeared as core glycosylated Band B and fully glycosylated Band C (Figure 2, panel G, lane 1). There was no detectable CFTR in CFBE41o- parental cells (panel G, lane 2). ΔF508 CFTR was seen as the immature band B form in CFBE41o-ΔF cells (panel G, lane 3), identical to ΔF508 CFTR expressed in HeLa-ΔF cells (panel G, lane 4).

To confirm the lack of CFTR in CFBE41o- parental cells, CFTR mRNA levels were measured using real time RT-PCR. The results shown in Figure 1H indicate that ΔF508 CFTR mRNA levels in CFBE41o-ΔF cells were higher than WT CFTR message levels in CFBE41o-WT cells, and therefore, the dramatic differences in the current profiles could not be attributed to enhanced WT CFTR mRNA levels. Further, ΔF508 mRNA was undetectable in the CFBE41o- parental cell line, supporting the idea that these cells lack endogenous ΔF508 CFTR, and therefore the currents demonstrated in panels A and C can be directly attributed to CFTR function at the cell surface.

Corr-4a extends the activity of rΔF508 CFTR at 37°C

To test the effects of corr-4a on the channel activity of rΔF508 CFTR, cells were cultured at 37°C for 6 and 10 h in the presence or absence of corr-4a (10 μM). Ussing chamber analysis of these cultures revealed a significant increase in the forskolin-stimulated currents of corr-4a treated monolayers at 6 h (Figure 3A), but by 10 h, the difference between the corr-4a treated and untreated monolayers was gone (Figure 3B). Neither the forskolin+genistein activated currents nor the $CFTR_{inh}$-172 inhibitor effects were different in control and corr-4a treated cells at the 10 h time point. The baseline currents, e.g., pre-stimulation currents, were dramatically lower in corr-4a treated monolayers at 6 h than they were at the 0 time point (10.4 ± 2.5% (control) versus 19.8 ± 4% (corr-4a treated)) (Figure 3C). After 10 h of corr-4a treatment, the baseline currents had returned to control levels (6.4 ± 4.6% compared to 6.4 ± 3.3%, respectively). To determine if the differences in baseline currents could be attributed to differences in CFTR activity, we applied $CFTR_{inh}$-172. The baseline currents at 6 h decreased compare to 0 time point in both the control and corr-4a treated monolayers, with a greater decrease in current in the corr-4a treated samples (data not shown). This supports the hypothesis that CFTR participates in basal ion transport and that the elevated baseline currents in corr-4a treated cells were due to enhanced levels of functional CFTR. Since both the forskolin-stimulated and the baseline currents returned to control levels in corr-4a treated monolayers by 10 h, these data suggest that the effect of the corrector was no longer present at the 10-h time point.

Corr-4a increases the stability of rΔF508 CFTR at 37°C

To determine the effects of corr-4a on rΔF508 CFTR protein levels, we monitored total CFTR levels (Band B and C) and also followed the surface pool of ΔF508 CFTR using surface biotinylation. These studies revealed that corr-4a stabilized both the immature and the maturely glycosylated forms (bands B and C) for up to 12 h at 37°C (Figure 4A and B). Analysis of the surface CFTR pool (biotinylated fraction) indicated a significant increase in corr-4a treated cells at 6 and 8 h (A and C). Taken together, the functional and biochemical studies confirm the hypothesis that corr-4a stabilizes rΔF508 CFTR. Importantly, even in the presence of corr-4a, the surface pool of rΔF508 CFTR decreased to <10% after 12 h. of levels at time 0. This suggests that corr-4a mediated rescue/correction of ΔF508 CFTR is short-lived.

We also observed that corr-4a had the most dramatic effect on the stability of the band B of CFTR. The amount of Band B was 34.6 ± 7.6% of the 0 time point after 12 h in the corrector-4a treated samples, compared to 9 ± 2% in the control samples. Although the fully processed band C decreased to 13.3% ± 5.5% by 12 hours, the effect of corr-4a on the cell surface stability of the rescued ΔF508 CFTR was still significant.

Low temperature and corr-4a inhibit ubiquitin mediated degradative pathways.

The increases in CFTR B and C band levels in the presence of corr-4a suggested that corr-4a inhibited protein degradation. Previous studies suggested that ΔF508 CFTR in the ER and ΔF508 CFTR at the cell surface are degraded by different pathways, both of which are ubiquitin dependent: rΔF508 CFTR is degraded from the cell surface through an ubiquitination dependent lysosomal pathway, and ubiquitin-dependent ERAD

is responsible for band B degradation. Therefore, we designed studies to test which distinct steps of the ubiquitin-dependent degradative pathway were involved in ΔF508 CFTR degradation using three firefly luciferase based reporters of the UPS, as described previously (Table 1) (32). A fourth firefly luciferase construct that did not contain a UPS reporter tag (FL-luc) was also tested as a control. The product of the CL-1-FL construct containing a sixteen amino acid degron sequence was designed to monitor the E1-E3 ubiquitination machinery (33, 34). The 4-Ub-FL construct contains four in-frame ubiquitin sequences. Therefore, the product of this construct is suitable to monitor the degradation of a protein that has already been modified by ubiquitin (32). The p21-FL construct produces a UPS substrate that undergoes rapid degradation by the 20S proteasome without being ubiquitinated (35-40). Therefore, the latter two constructs should give information about the activity of the 26S and 20S proteasomes, respectively, independent of the ubiquitin conjugation activity of the cell.

To confirm the functionality of the constructs, we tested the effects of two proteasome inhibitors on luciferase activity in cells expressing each of the constructs (Figure 5). The results demonstrate that culturing cells expressing the constructs in the presence of ALLN (50 μM) or epoxomycin (500 nM) increased the luciferase activity increased more than 5-fold for the CL-1-FL, 4-Ub-FL, and p-21-FL constructs compared to untreated controls. Interestingly, culturing the cells at 27°C also dramatically increased the luciferase activities from all three constructs, indicating that low temperature inhibits proteasome function. In contrast, corr-4a treatment increased luciferase activity only in cells transfected with CL-1-FL, the construct that requires ubiquitination before degradation. Corr-4a did not inhibit the degradation of the constructs that do not require

ubiquitination for proteasomal degradation, indicating that corr-4a has no direct inhibitory effect on the proteasome. These results strongly support the idea that corr-4a inhibits the E1-E3 ubiquitination pathway and explains the increased stability of the B band form of ΔF508 CFTR in the corr-4a treated samples.

WT CFTR is unaffected by corr-4a treatment.

Because corr-4a inhibits rΔF508 CFTR and CL-1-Fl degradation, we tested whether corr-4a has any effect on WT CFTR that is degraded from the cell surface through a ubiquitin-independent pathway. Since ΔF508 CFTR expressing cells were subjected to low temperature culture to deliver ΔF508 CFTR to the cell surface, we treated the WT CFTR expressing cells to identical low temperature culture conditions as a control. During the last hour of low temperature culture, we treated both cell lines with DMSO or 10 μM corr-4 for up to 12 hours. We then examined the surface pool of CFTR over time, as well as the total amounts of band B and band C during this time course. The results indicate that corr-4a treatment produced no significant changes in band B or band C levels. We measured slightly more Band B at 6 hours in the corr-4a treated samples than in the control samples in some experiments, suggesting that corr-4a treatment might affect degradation from the ER (Figure 6).

The presence of a CFTR potentiator enhances the effect of corr-4a.

Corr-4a stabilized both the mature and immature forms of ΔF508 CFTR for up to 12 hours, but we measured significant changes in the forskolin-stimulated activity at the 6-hour time point only. These results suggest that the increased protein stability does not

necessarily reflect corrected channel function. To test this hypothesis, we repeated the 6-hour corr-4a exposure experiments, this time conducting the Ussing chamber analysis in the presence of a known channel potentiator, VRT 532. In agreement with our previous results (Figure 3), both the baseline and the forskolin-stimulated currents increased significantly in corr-4a treated cells. More importantly, the presence of VRT 532 in the bathing fluid further enhanced the forskolin-stimulated currents (Figure 7). We noted no additional effects in the presence of genistein, supporting the idea that in the presence of VRT 532, rΔF508 CFTR could be maximally activated by cAMP. We could block the forskolin+VRT 532-activated currents with $CFTR_{inh}$-172, indicating that the currents were CFTR-mediated. These results confirm our previous finding that although corr-4a enhances both the stability and function of rΔF508 CFTR, the channel is still compromised and requires a potentiator for maximum channel activity.

DISCUSSION

In vivo and *in vitro* studies have suggested that enhancing ΔF508 CFTR activity by as little as 5% may diminish the symptoms of the disease (2, 41, 42). Therefore, a major thrust of CF research has been directed at identifying and characterizing small molecules that facilitate ΔF508 CFTR rescue from ERAD. Previously, we established that two of these correctors, corr-4a and VRT 325, stabilized the surface pool of ΔF508 CFTR by inhibiting endocytosis (21). In the present study, we focused on the mechanism by which corr-4a rescues and stabilizes ΔF508 CFTR and the functionality of the rescued protein in the presence and absence of corr-4a. We asked the following questions: 1) Do low temperature culture and corr-4a utilize the same mechanism to rescue ΔF508 CFTR? 2) How long after delivery to the cell surface can ΔF508 CFTR be activated by

physiological stimuli, such as forskolin, in the presence or absence of corr-4a? 3) Do cell surface ΔF508 CFTR levels correlate with physiological activity in the presence of corr-4a?

Although the effects of low temperature on ΔF508 CFTR have been established for quite some time (10, 21, 31, 43, 44), the mechanism by which low temperature facilitates ΔF508 CFTR exit from the ER is not clear. The simplest interpretation is that at lower temperature, the channel folds in a conformation that is closer to that of the wild-type protein and is competent to exit the ER. However, if that is true, why is the majority of protein retained in the ER and why is the function of the rescued, cell surface ΔF508 CFTR compromised? One possibility is that only a small percentage of the protein is properly folded and rescued. However, if this was true, the functionality of the rescued protein should be comparable to wild type. The other hypothesis is that some partially folded protein escapes ERAD because the components or the pathways of the quality control mechanism are altered. To support this hypothesis, our results indicate that both low temperature culture and corr-4a increase the level of ΔF508 CFTR band B. Furthermore, both conditions alter the function of the ubiquitin-dependent degradative pathways. Using luciferase-based reporter constructs, we found that low temperature culture inhibited both the E1-E3 cascade and the function of the proteasome. In contrast, corr-4a only inhibited the E1-E3 cascade without altering the function of the proteasome. Therefore, it is likely that inhibition of the E1-E3 cascade by corr-4a is responsible for the stabilization ΔF508 CFTR. Interestingly, attempts by us and others to rescue ΔF508 CFTR using proteasome inhibitors have not been successful, although inhibition of the proteasome does stabilize the B band [4, 6, 46] and cell surface CFTR (31). More recent

results have indirectly suggested that selective inhibition of ERAD may rescue ΔF508 CFTR in CF tracheal epithelial cells transfected with ΔF508 CFTR (45). Considering these data and that CFTR has to be extracted from the ER membrane before degradation in the proteasome (4, 46), it is tempting to speculate that inhibition of ERAD steps prior to extraction, such as would be the case for the E1-E3 cascade, may indirectly help the protein escape from the ER.

Regarding the cell surface stability of rΔF508 CFTR, it has been established that rΔF508 CFTR is rapidly cleared from the cell surface at 37ºC (21, 29-31, 47). Chemical chaperones like corr-4a seem to stabilize rΔF508 CFTR, but their mechanisms of actions are not clear. Our previous studies showed that the correctors corr-4a and VRT-325 inhibit ΔF508 CFTR endocytosis (21). Interestingly, the results of the ubiquitin-dependent degradation assays presented herein suggest that corr-4a stabilizes cell surface ΔF508 CFTR by inhibiting ubiquitination and subsequent degradation from the cell surface. Considering that corr-4a treatment had no effect on WT CFTR cell surface stability, these results complement a previous report demonstrating that low temperature rescued ΔF508 CFTR is still folded incorrectly at the cell surface and directed to the lysosome through a ubiquitin-dependent pathway, while the degradation of the WT protein from the cell surface is not ubiquitin-dependent (48). Although the studies by Dr Lukacs's group show ubiquitin-dependent, lysosomal degradation of rΔF508 CFTR, the studies by Dr Gentch's lab indicate that the cell surface stability of rΔF508 CFTR increased following proteasome inhibition (31). Whether rΔF508 CFTR degradation from the cell surface is mediated by the lysosome, the proteasome or both remains an open

question. Importantly, both proposed pathways seem to require ubiquitin, and our data clearly demonstrate that corr-4a interferes with ubiquitination.

These results also agree with our previous findings indicating that corr-4a had no effect on the cell surface stability or endocytosis of WT CFTR or transferrin receptor, since cell surface clearance of these proteins does not require ubiquitination [21]. We therefore hypothesize that corr-4a treatment may affect the cell surface clearance of all proteins that require ubiquitination. Considering that the optimal "corrector" for CF would be ΔF508 CFTR-specific, our results also point to the importance of studies that investigate the complex mechanisms by which ΔF508 CFTR correctors work.

A recent study suggests that ΔF508 CFTR export requires a local folding environment that is sensitive to heat/stress inducible factors found in some cell types, and these results suggest that low temperature correction is necessary but not sufficient for ΔF508 export (49). In this intriguing study, it was proposed that folding and rescue efficiency of ΔF508 CFTR depend on the cell type, and that the chaperone environment is a critical component for successful ΔF508 CFTR folding at reduced temperatures. Moreover, different chaperone activities or balance of activities may be responsible for the folding of ΔF508 CFTR at reduced temperature, and these may be different from WT. As it relates to our study, it is possible that E1-E3 inhibition also alters the chaperone environment, possibly through inducing ER stress and activating the UPR (25, 50).

Corr-4a-mediated enhancement of rΔF508 CFTR responsiveness to cAMP was most evident at 6-8 hours of treatment. While this result represents a significant functional improvement over untreated controls, cell surface biotinylation studies indicated that rΔF508 CFTR was also present at the membrane at the later (12 h) time

points, but no longer responded to the physiological cAMP mediated stimuli. This observation suggests that protein stability and functional integrity are separate features of rΔF508 CFTR. In terms of repairing the ΔF508 CFTR channel defect that is responsible for CF, forskolin-stimulated channel activity is a better readout than the presence of protein at the cell surface. Therefore, our results point to a clear unmet need for additional measures (e.g. potentiators) beyond stabilizing the protein, measures that functionally correct channel activity, in order to activate all rescued channels at maximum capacity and repair the CF defect.

Based on previous results, it is clear that in human airway epithelial cells, rΔF508 CFTR channel activity is compromised at 37°C but not at 27°C (21,22). Furthermore, we have demonstrated that returning the cells to physiological conditions (37°C) after low temperature rescue effects a rapid loss of functional activity (21). This observation differentiates the act of stabilizing the protein at the cell surface, or for that matter promoting ER exit, from promoting proper protein folding that corrects channel function. Our studies also establish that understanding how low temperature rescue works will be a critical feature for developing therapies designed to promote rescue, protein stability, and channel activity of ΔF508 CFTR. It may well be that identifying a single compound that promotes all of these aspects for correction of ΔF508 CFTR may prove to be difficult. In addition to examining the mechanism and the effects of low temperature rescue and corr-4a on ΔF508 CFTR, our studies also establish a protocol for the evaluation of newly identified chemical chaperones.

Keywords: cell-surface trafficking, cystic fibrosis transmembrane conductance regulator (CFTR), ΔF508 rescue, short-circuit current

Abbreviations used: CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; ERAD, ER-associated degradation; FBS, fetal bovine serum; HRP, horseradish peroxidase; I_{sc}, short-circuit current; DMEM, Dulbecco's modified Eagle's medium; NS, not significant; rΔF508, rescued ΔF508; R_t, transepithelial resistance; TS, temperature-sensitive; WT, wild-type.

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Table 1.

Reporters of ubiquitination dependent degradation

Reporter	Targeting Description
FL	Slow UPS degradation
CL-1-FL	Targeted to E1-E3 by CL1-degron, rapid degradation
4-Ub-FL	Ubiquitinated, targeted to 26S, rapid degradation
p21-FL	Does not require ubiquitination, targeted to proteasome core by p21

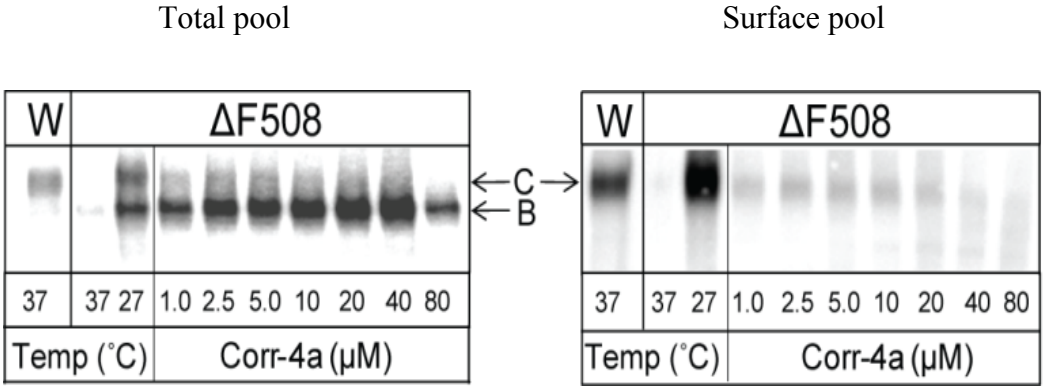

Figure 1. Chemical chaperone rescue of ΔF508 CFTR in human airway epithelial cells. Polarized CFBE41o-ΔF cell monolayers were treated with various concentrations of corr-4a for 12 h at 37°C. The cells were then surface biotinylated, and CFTR was immunoprecipitated from 500 μg of cell lysate. The lysate was split into two fractions and analyzed by SDS-PAGE and Western blotting for total CFTR (left panel) and surface CFTR (right panel) using anti-CFTR antibodies or avidin-HRP, respectively. Control samples included CFBE41o$^-$ WT (WT CFTR) grown at 37°C, CFBE41o$^-$ ΔF cells maintained at 37°C without treatment, and CFBE41o ΔF grown at 27°C for 48 h (low temperature correction). A representative gel of 3 is shown.

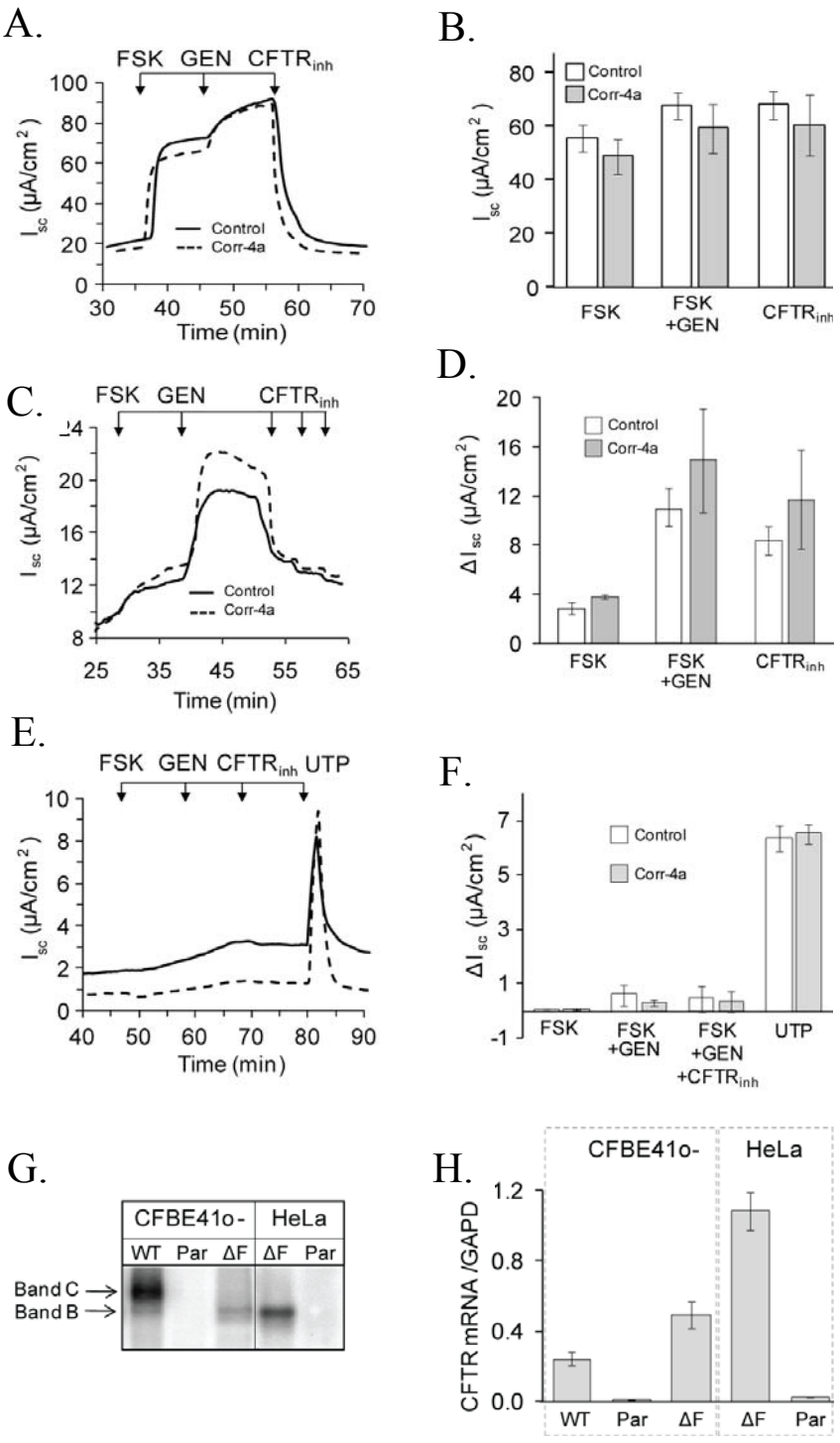

Figure 2. Ussing chamber analysis of WT and ΔF508 CFTR after low temperature correction. (A and B) CFBE41o$^-$ΔF, (C and D) Polarized CFBE41o$^-$WT, and (E and F) CFBE41o$^-$ parental cell monolayers were cultured at 27°C for 48 h and studied in Ussing chambers at 37°C as described in the Experimental section. Under low chloride (1.2 mM

NaCl, 115 mM Na gluconate), and 100 μM amiloride in the apical chamber, the basolateral membranes of the monolayers were permeabilized with amphotericin B. After reaching a baseline steady state I_{sc}, the monolayers were treated sequentially with forskolin (FSK, 10 μM), genistein (GEN, 50μM), and CFTR-172_{inh} (20+20+60 μM). Representative tracings are shown on the left, and the summaries of the experiments are plotted on the right. Pretreatment of the cell monolayers during the last 1 h at 27°C with corr-4a had no significant effect on the ΔI_{SC} compared to control (treated with 0.01% DMSO). The FSK responses of control ΔF508 CFTR-expressing monolayers were ~5% of the WT CFTR expressing responses (Panels B and D (white bars), n = 11; 2.9 ± 0.46 versus 55.2 ± 5.12 mA/cm^2, respectively), whereas the FSK responses of the corr-4a treated ΔF508 CFTR expressing monolayers were 7% of the WT responses (Panels B and D (grey bars), n = 8-10; 3.66 ± 0.19 versus 48.49 ± 6.6, respectively). The CFBE41o- parental cell line (Panels E and F) did not respond to FSK treatment, but UTP (100 μM) produced a response, indicating that alternative channels had been activated (n = 4). Relative CFTR protein levels (Panel G) and message levels (Panel H) are shown for the CFBE41o-WT, CFBE41o- parental, CFBE41o-ΔF, HeLa-ΔF, and HeLa parental cells. CFTR protein was immunoprecipitated from 500 μg total cellular proteins using 24-1 (an anti-C terminal antibody), *in vitro* phosphorylated using γ^{32}P ATP, PKA (protein kinase A), separated on 6% PAGE and detected using Phosphorimager analysis. CFTR mRNA levels are plotted relative to GAPDH. CFTR mRNA levels in HeLa-ΔF and HeLa parental cells were measured as positive and negative controls, respectively. The results are given as mean and S.D. of n = 4 samples amplified under the same conditions.

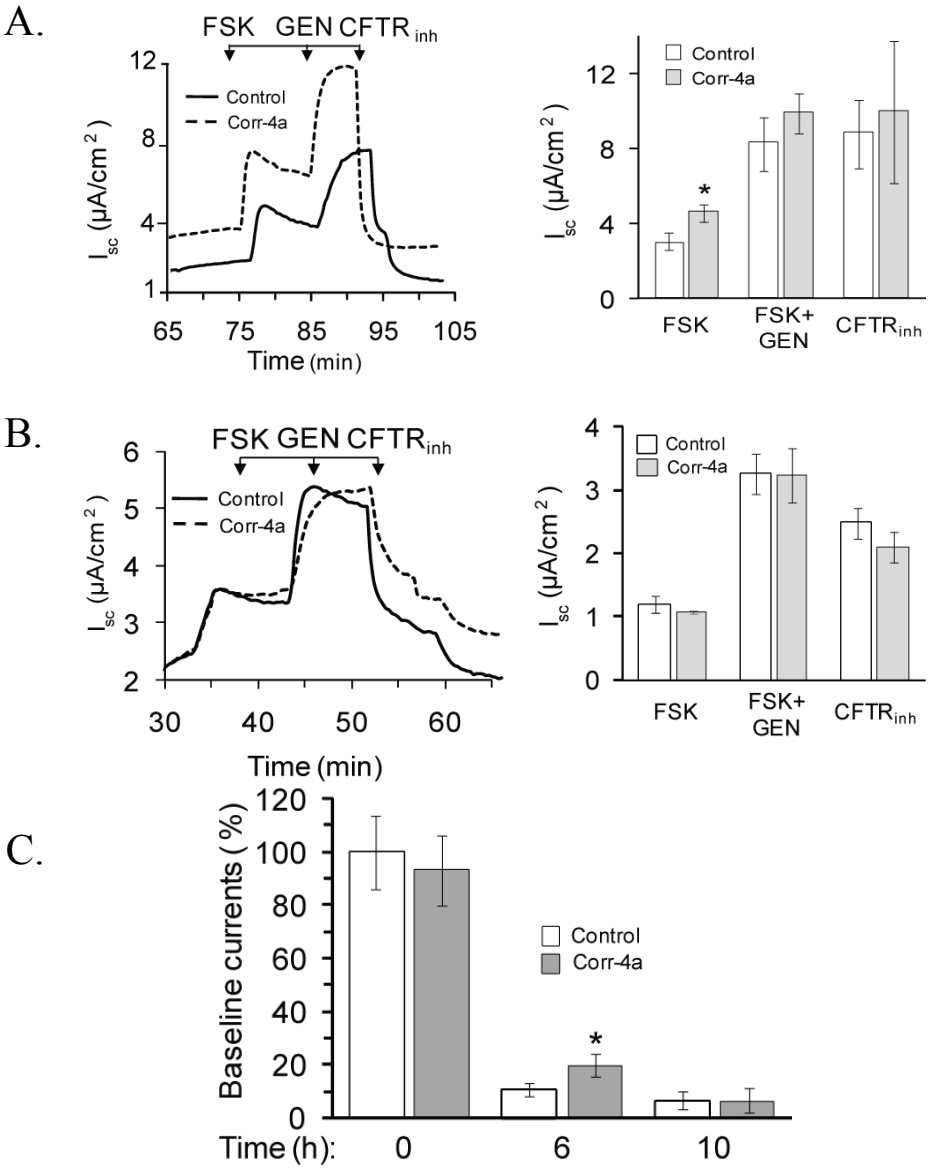

Figure 3. Functional stability of low temperature rescued ΔF508 CFTR at 37°C in the presence of corr-4a. CFBE41o$^-$ΔF cells were treated as described in Figure 1, but were cultured for additional 6 h (Panel A) or 10 h (Panel B) at 37°C in the presence or absence (Control) of corr-4a prior to Ussing chamber analysis. The experiments were performed as described in Figure 1. Panel C: The baseline currents were compared to controls (0 time point after 27 °C rescue =100%). In panels A and B, representative tracings are shown at the left and the summaries are plotted on the right. At 6 hours, the FSK-stimulated short-circuit current (Panel A, right side, n = 7) and the baseline currents (Panel C, n = 12) of the corr-4a treated CFBE41o-ΔF508 cells were significantly different from the controls ($p < 0.05$). At 10 h of 37°C culture, there was no significant difference between the treated and nontreated samples.

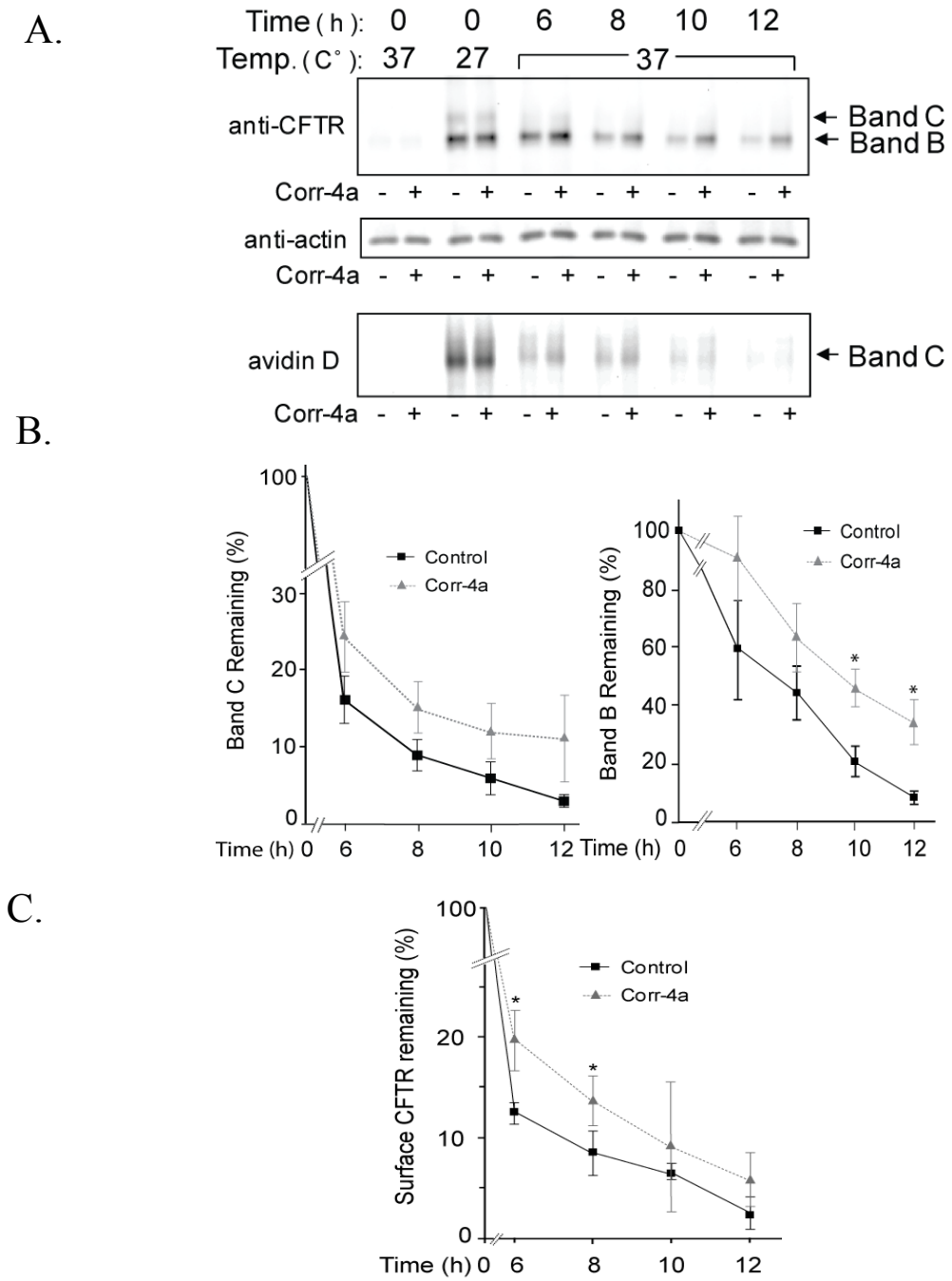

Figure 4. Corr-4a stabilizes rΔF508 CFTR. Polarized CFBE41o-ΔF cells were cultured at 27°C for 48 h. Cells were then transferred to 37°C and treated with corr-4a or vehicle for various times up to 12 h. Cells cultured at 37°C were tested as control (0 time, 37°C). Total CFTR levels were analyzed following immunoprecipitation and Western blotting. Cell surface CFTR levels were measured following cell surface biotinylation. A: A representative gel illustrates total CFTR levels (A, top panel). Band B and Band C are

marked with arrows. Actin levels are shown as a loading control (middle panel). Cell surface CFTR levels are shown in the lower panel at the same time points as total CFTR levels. Representative gels of 3 or more experiments are shown. B: Quantification of total CFTR. Band C and band B levels were calculated by densitometry (n=5). Band C expression was significantly higher in corr-4a treated samples at 6 h ($p=0.03$; 16.36 ± 3 vs $24.6 \pm .5$), 8 h ($p=0.02$; 9 ± 1.9 vs 15.5 ± 3.3), and 10 h ($p=0.02$; 6 ± 2.2 vs 12.3 ± 3.6). Band B expression was higher at 10 and 12 h (* $p < 0.05$). C: Quantification of cell surface CFTR. Surface ΔF508 CFTR expression was significantly higher in corr-4a treated cells at 6 h ($p=0.04$; 12.5 ± 1 and 19.8 ± 2.9).

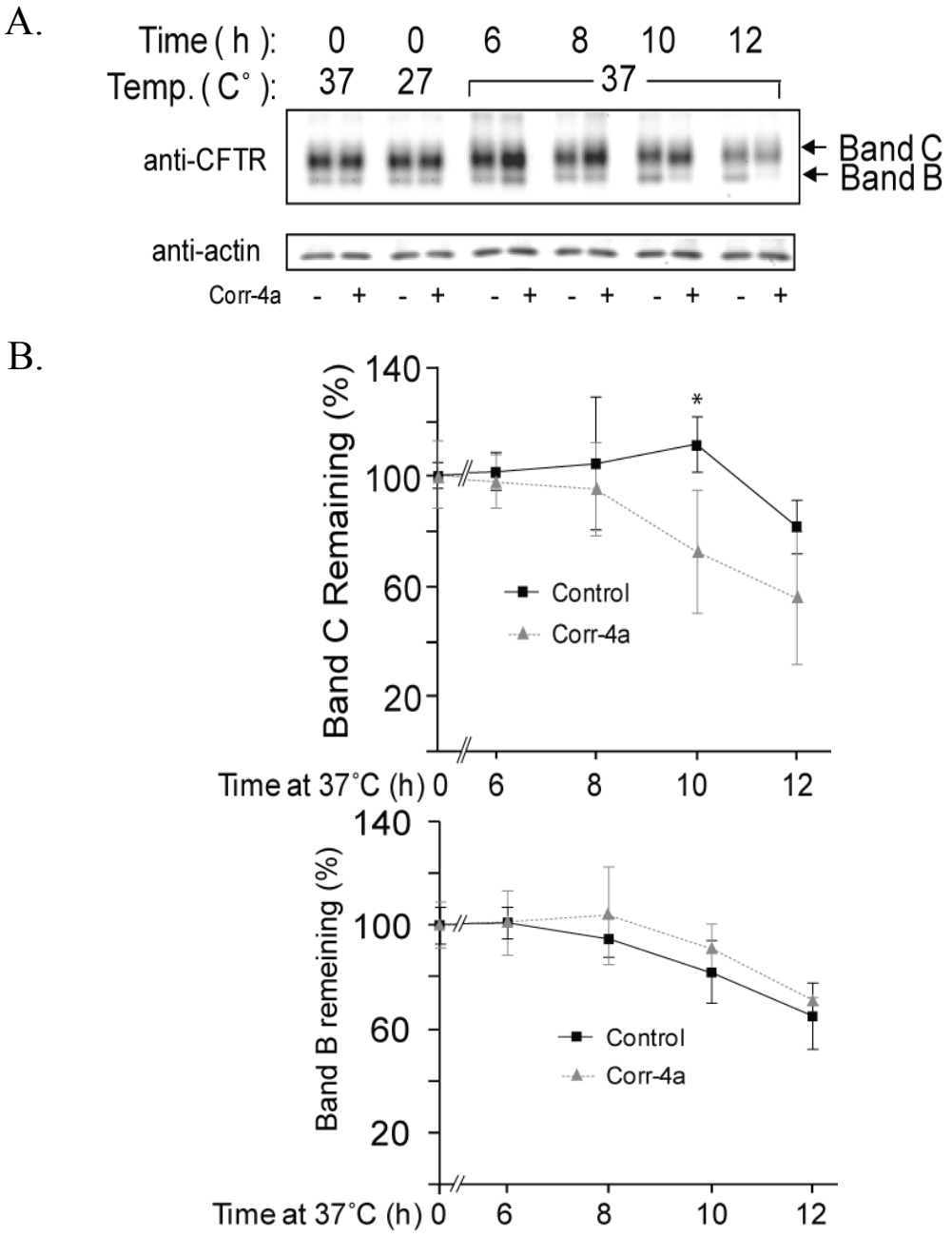

Figure 5. Stability of low temperature rescued WT CFTR at 37°C in the presence of corr-4a. CFBE41o$^-$ WT cells were treated and analyzed as the CFBE41o$^-$ ΔF508 cells shown in Fig. 4. A: Total CFTR (anti-CFTR) Representative gels of 3 are shown. B: Quantification of WT CFTR band C and band B levels.

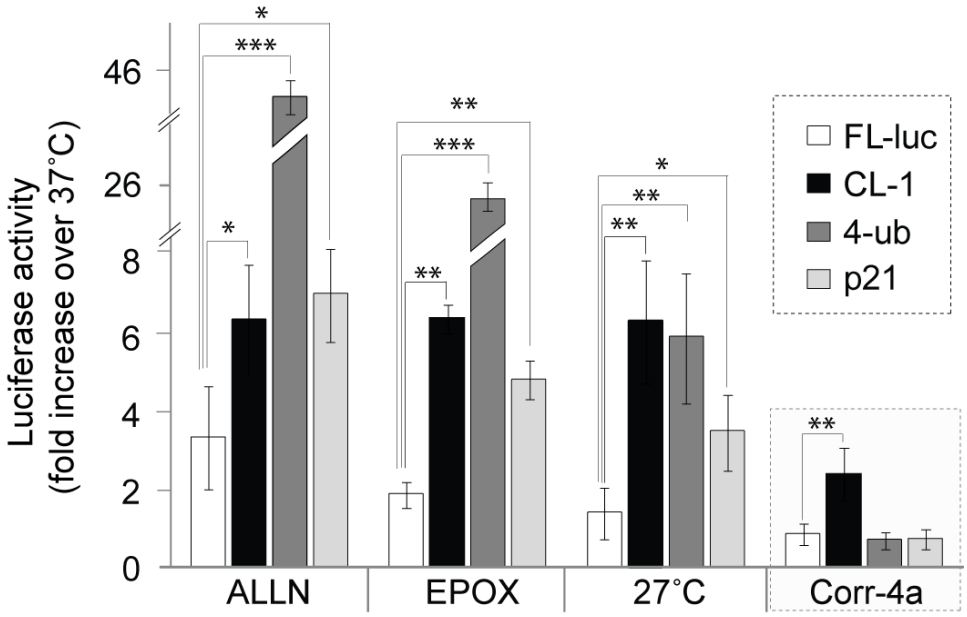

Figure 6. Low temperature and corr-4a effects on the ubiquitin-proteasome system. HeLa cells were transfected with luciferase (FL-luc), a luciferase construct with a degron sequence added (CL-1), a luciferase construct with 4 in frame ubiquitin sequences at the N-terminus (4-ub), or with a luciferase construct containing a C-terminal sequence of p21 that targets proteins to the proteasome without ubiquitin modification (see Table 1). Luciferase activity for each construct was normalized to light units measured in control (untreated) cells cultured at 37°C (1 fold). The increases in luciferase activity are fold increases (n = 3; * $p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

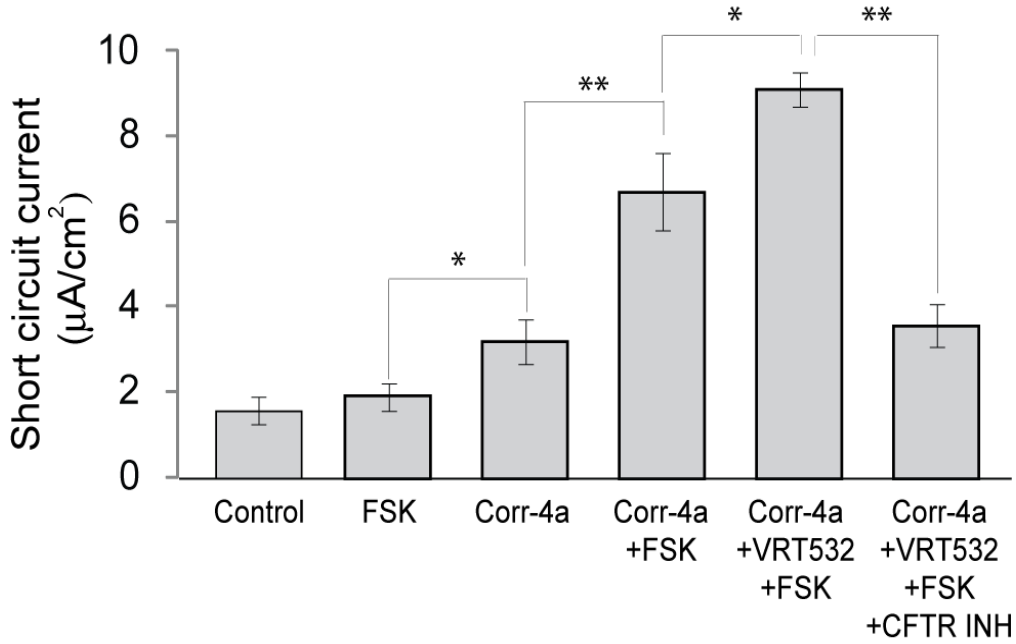

Figure 7. Increases in the functional activity of corr-4 treated ΔF508 CFTR-expressing CFBE41o-cells with VRT-532. CFBE41o-ΔF508 CFTR expressing cells were temperature corrected (48 h at 27˚C) and cultured at 37˚C for 6 h in the presence or absence (control) of corr-4a. The baseline currents were higher in the corr-4a treated cells (corr-4a) than the control. Short-circuit currents in the presence of the potentiator, VRT-532, were enhanced in during forskolin activation (Corr-4a, VRT-532, FSK) ($n > 6$; * $p < .05$; **$p < .01$).

SUMMARY

The results of the studies presented in this dissertation provide novel insights into the biological and functional differences between WT and mutant CFTR. The majority of CF related research focuses on the most common ΔF508 mutation that results in protein misfolding (24, 36, 59), premature stop codons causing mRNA instability (92), or mutants that lack channel activity (G551D) (15, 16). Only, few studies have focused on mutations that influence the cell surface stability of the protein causing mild CF. While these mutations are rare, understanding their role on CFTR trafficking and function provides valuable information regarding the complexity of symptoms associated with different types of CF. The focus of the first study was to determine the trafficking properties of two amino-terminal mutants (R31C and R31L) of CFTR. In these mutants, arginine at the 31 position of the human WT CFTR is substituted with cysteine or leucine respectively. These amino acid substitutions create novel potential endocytic signals that enhance the clearance of the mutant proteins from the cell surface. The enhanced endocytosis rates provide a mechanism by which these two mutations lead to CF phenotype. However, our studies also revealed that in addition to decreased cell surface stability, the maturation efficiencies of the mutants were also compromised. Therefore we conclude that biogenesis and cell surface trafficking defects in concert contribute to significantly decreased cell surface CFTR expression and chloride channel function and lead to mild CF.

The second study focused on the ΔF508 CFTR mutant. Our results provide valuable information regarding the functional defects associated with rΔF508 CFTR at the cell surface following small molecule corrector, corr-4a, and potentiator, VRT-532, treatments. It has been shown that while low temperature correction enhances the level of rΔF508 CFTR at the plasma membrane, the rescued protein is functionally compromised (7, 103, 115). However, our results indicate that in addition to its effect on biogenesis, corr-4a partially corrects the functional defect of rΔF508 CFTR. Furthermore, treatment with a potentiator, VRT-532, further enhances the function of the rescued mutant, providing support to the hypothesis that a combination of correctors and potentiators will be necessary to develop efficient therapies for this form of CF.

To date there are 52 missense mutations identified within the first 90-residues of CFTR in the Cystic Fibrosis Database (www.genet.sickkids.on.ca/cftr/). The 90 residue amino-terminal tail (N-tail) has been shown to be essential for CFTR processing in many ways. An analysis of CFTR's N-tail in a chimeric molecule with the extracellular domain of the transferrin receptor revealed that this domain was capable of mediating the endocytosis of a reporter molecule (84). Furthermore, different deletion mutants in the CFTR N-tail (Δ2-79, Δ2-59, Δ35-45 and Δ60-72) expressed in Cos-7 cells produced processing mutants that failed to exit the ER, suggesting that this domain is important for proper folding and/or trafficking of CFTR. Support for this idea comes from the fact that no biotinylated protein was found on the cell surface following expression of the deletion mutants and that the protein was sensitive to endoglycoside H indicating ER, core glycosylated form only. Finally, SPQ analysis revealed that cells expressing the mutants did not show forskolin-stimulated Cl^- channel activity (84). The N-tail of CFTR is

predicted to form an α-helix by secondary structure algorithms, therefore it is presumable that any mutations in this region of the CFTR could affect its conformation. However, in the mutational analysis of this region where single residues between amino acids 46 and 62 where substituted with alanine, only three residues (L53, W57 and E60) diminished CFTR processing. These mutants displayed only the immature form of the protein when expressed in Cos-7 cells (30).

The N-tail of CFTR has been demonstrated to control PKA dependant CFTR Cl^- channel gating. The most important amino acids for this activity are found among the cluster of amino acids at the positions of 46-60 residues (75). Particularly the replacement of the charged residues with alanine at positions E51, E54 and D58 revealed abnormal Cl^- channel function and dramatically reduced cAMP activated macroscopic currents compared to the WT CFTR when expressed in Xenopus oocytes (75). Sequential elimination of negative charges in this region of CFTR led to a greater inhibition of CFTR Cl^- channel activity underlining the importance of N-tail in normal CFTR function (75). To verify that the N-tail positively regulates CFTR activity, Kirk and colleagues tested Δ2-79 CFTR, an N-tail deletional mutant together with a glutathione-S-transferase (GST) fusion protein containing the N-terminus of CFTR. Following injection of the two constructs into Xenopus oocytes, they observed a cAMP-dependent current increase due to of the association of N-tail with the deletion mutant of CFTR. If they mutated acidic residues at positions E54 and D58 in the GST fusion protein and eliminated the negative charge, the positive regulation of CFTR function was abolished. This data confirm that removing acidic residues from this region of the protein has detrimental effects on CFTR function. Importantly, the region from residues 1 to 46 in the N-tail of CFTR was not

examined in the similar fashion. So one can only speculate what kind of effect the addition or removal of negative charges by naturally occurring mutations could have. A recent report on several naturally occurring mutations in the N-tail of CFTR found that the S50P, E60K, G85E and G85V mutations failed to produce a mature and functional protein at the plasma membrane (43). They also reported that while P5L and Y89C were processed normally, they both had compromised channel gating properties (43).

Our Arg31 mutants represent the removal of positive charges from the N-tail www.genet.sickkids.on.ca/cftr/. Although there are not many acidic residues in this region, there are 2 other naturally occurring mutations in this region, D36N and D44G. The D36N mutation was found in a 9 month old female, with borderline sweat chloride, carrying ΔF508 on the other allele as reported in the Cystic Fibrosis Data Base. The D44G mutation confers a mild phenotype with pulmonary symptoms and the patient carries ΔF508 on the other allele (42). Therefore, it is not surprising that point mutations in the N-tail of CFTR are not well tolerated.

The importance of the N-tail of CFTR is also illustrated by the fact that this region of the protein has been shown to associate with other domains of CFTR and these intramolecular interactions are essential for proper CFTR protein folding, maturation and function. It has been demonstrated that the N-tail of CFTR physically interacts with NBD1 and the distal part of the R domain (residues 595-823) controlling PKA-dependant CFTR Cl^- channel gating (75). Certain mutations in the N-tail of CFTR at positions D47, E51, E54 and D58 were shown to disrupt this interaction (75), suggesting that that this particular part of the protein may have a much more important role than anticipated in both CFTR trafficking and channel gating.

Given the critical role that this domain plays in CFTR folding, it is not surprising that Arg31 mutations cause mild CF disease. Data provided in the first study supports the idea that these mutations are not severe since the Arg31 proteins are made, folded and trafficked from the ER to the plasma membrane, where they both have functional Cl^- channel activity. Nevertheless, a closer look at Arg31 mutations suggests that substitution of arginine with either cysteine or leucine at position 31 affects CFTR's biogenesis. Arg31 missense substitutions cause partial biogenesis defects resulting in about 50% less maturation efficiency compared to the WT CFTR. Furthermore, the transport of these two mutants from the ER to the Golgi was slower as monitored by the temporal changes in the glycosylation patterns.

Interestingly, there are no point mutations in the consensus endocytic signals of CFTR that have ever been described. There are, however, premature stop codon insertions (Q1412X, S1455X, and L1399X) or frameshift mutations (4326delTC, 4279insA and 4271delC) (48). These mutants do not contain the carboxyl-tail of CFTR in which the tyrosine-based endocytic signal is located. Furthermore, these mutants do not possess biogenesis defects, since they manage to be properly folded and traffic to the cell surface. However, from the cell surface these mutants are cleared very rapidly (5-6 fold faster compared to WT protein) and are substrates for ubiquitin dependent, lysosomal degradation (10). Although these mutations appear to lack the endocytic signal found in the WT protein, they still display a CF phenotype that is similar to those observed in our Arg31 mutants. This suggests that other features, such as PDZ interactions of the protein can mediate endocytosis besides the known internalization signals.

Recently, a patient with the mutation 991A→T resulting in N287Y substitution was identified. The N287Y substitution does not affect the biosynthesis of CFTR, nor does it affect the single channel kinetics. The only defect that this particular mutation has is that it is internalized 50% faster than WT CFTR, therefore reduces the steady-state levels of CFTR in the plasma membrane (106). These observations suggest that overactive endocytosis alone can result in a disease phenotype. Overall, the N287Y mutation appeared to generate a novel, non-canonical, tyrosine-based endocytic signal that is present within the body of the protein rather than at the termini of CFTR. Non-canonical endocytic motifs have recently been described for other proteins as well, one of them being the ligand-gated ion channel $P2X_4$ (94). In this study, targeted mutation in the tyrosine binding motif site binding μ2 subunit of AP-2 caused an increase in surface receptor number and function. Thus it is possible that some naturally occurring mutations could affect modulation of the surface number of certain ion channels by altering the rates of insertion and/or endocytosis.

Both Arg31 mutations generate a CFTR protein with dramatically enhanced endocytosis rates. Exactly why these two mutations affect the endocytosis of CFTR is not clear. Being non-conservative substitutions, both of these mutations introduce a hydrophobic residue close to an upstream tyrosine (Y), creating potential internalization signals in the N-terminus of CFTR, $Y^{28}XXC^{31}$ and $Y^{28}XXL^{31}$. Non-canonical tyrosine-based endocytic motifs (YXXGΦ) have been found in other transmembrane proteins such as the β_2-adrenergic receptor, glutamate receptor 2, glutamate receptor 3 (GluR2 and GluR3) and cannabinoid receptor-2. These signals are important for regulation of their surface levels and therefore have an effect on function (95). It also could be that N-tail

mutations somehow affect the recognition of the tyrosine-based internalization signal found in the carboxyl terminus, and therefore enhance endocytosis rates. Because mutational analyses of the carboxyl terminus were not performed in these studies, it is hard to determine if these particular mutations enhance CFTR endocytosis by acting as signals themselves. It is also possible that the Arg31 mutations influence the tertiary structure of CFTR and therefore the effect is indirect. However, if the structure of CFTR is dramatically altered, one might predict to see a change in the protein half-life, but the protein half-lives of these mutants are comparable to WT CFTR. Furthermore, Arg31 mutations could enhance endocytosis by interfering with protein-protein interactions that are important for CFTR to function as a macromolecular complex at the plasma membrane. Loss of these interactions may facilitate endocytosis by other mechanisms. Previous studies indicate that the N-tail of CFTR interacts with syntaxin 1A that inhibits CFTR activity (76). If syntaxin 1A binds to CFTR, one question that comes to mind is whether the N-tail would be available for interaction with the clathrin-associated endocytic machinery at the cell surface. One possibility is that Arg31 mutations influence the loss of interactions that are important to keep CFTR at the cell surface and therefore enhance its clearance from the plasma membrane. In the case of Arg31 mutations, a combination of reduced biogenesis and enhanced endocytosis results in the disease phenotype. Interestingly, a child found to be R31C homozygous did not present any CF symptoms, suggesting that R31C is asymptomatic unless coupled with a more severe phenotype allele (46).

Even though there are multiple classes of CF-causing mutations, they can be roughly grouped into two main categories (129, 138). The first group of mutations affects

channel density at the cell surface by various mechanisms. The second group of mutations generates CFTR that reaches plasma membrane, but has Cl^- transport defects. Several mutations, including the most common mutation in CF, ΔF508, display features that belong to both groups of mutations. Protein misfolding and ERAD result in very little if any is exported from ER. This results in minimal or no CFTR at the cell surface. However, if any of the ΔF508 CFTR reaches the cell surface it also presents Cl^- transport defects.

The maturation and localization of ΔF508 CFTR in tissues have been controversial topics in the CF field. The localization of ΔF508 CFTR in native human tissues has been difficult to assess because of the limited specificity and sensitivity of CFTR antibodies. However, some studies indicated ΔF508 CFTR expression differences between species. For example, it was observed that in mouse RIII mammary epithelial cells (C127I) grown at 37°C, some of fully glycosylated ΔF508 CFTR reached the plasma membrane (51). Furthermore, immunohistochemical studies detected ΔF508 CFTR at the plasma membrane in airway and intestinal epithelia from ΔF508 CFTR homozygous patients also (62). In contrast, no ΔF508 CFTR could be detected at the cell surface by immunochemical techniques in recombinant ΔF508 CFTR expressing cells, CF primary airway cells, and CF tissues (24, 36). More recently using reportedly more sensitive antibodies, Boucher and colleagues very thoroughly examined the cell type and region-specific expression of CFTR in normal and ΔF508 CFTR expressing airways. They reported that no ΔF508 CFTR could be detected at the cell surface from cells isolated from ΔF508 CFTR homozygous patients (65).

ΔF508 CFTR comprises a number of defects, and therefore displays a combination of features belonging to different groups of mutations. Importantly, the Arg31 mutations we analyzed also possess features that are similar to ΔF508 CFTR and could be classified to the same group of mutations based on their compromised biogenesis. However, in contrast to ΔF508 CFTR, the Arg31 mutants do not exhibit any protein stability defects. Therefore, it is difficult to define the particular class of mutations to which the Arg31 mutations belong. It is clear though that analyzing naturally-occurring R31C, R31L, N287Y and ΔF508 mutations with partial biogenesis defects, enhanced endocytosis or accelerated degradation can in combination or separately lead to CF phenotype.

In summary, in the case of Arg31 mutations, the consequences are: partial biogenesis defect, which leads to reduced Cl^- channel activity on the cell surface and dramatically increased clearance from the plasma membrane. These cellular defects are sufficient to cause mild CF when coupled with a severe mutation in the other allele.

The second study focused on the surface stability defects of rΔF508CFTR. Previous studies established that there are cell and expression type specific differences in CFTR biogenesis and trafficking (116). For example, it is known that WT CFTR internalization rates differ dramatically between HeLa cells and CFBE41o- airway epithelial cells. Specifically, endocytosis rates are lowest in CFBE41o- cells when cells are polarized (115). It is known that polarized expression of CFTR in the apical membrane requires specific CFTR interactions with a PDZ interacting proteins (113), therefore slower endocytosis could be partially contributed to these interactions. Whereas, rΔF508 CFTR endocytosis rates remain very rapid, about 30% in 2.5 minutes,

regardless of cell type or state of polarization. It was also noted that rΔF508 CFTR has much shorter surface protein half-life compared to WT CFTR (115). These observations suggest that rΔF508 CFTR has a surface stability defect, and it is important to study its trafficking in a relevant environment. Therefore, we performed all of our studies in polarized CFBE41o- airway epithelial cells.

ΔF508 CFTR has a processing defect and this defect is TS (35). Furthermore, a recent study revealed that the surface instability and normal Cl^- channel function defects are also TS, and the channel function defects could be partially corrected at the permissive temperature (115). Therefore, in the studies presented here, we used a combination of low temperature and chemical chaperone treatment to study the rΔF508 CFTR Cl^- channel defect. Interestingly, the chemical chaperone corr-4a, improved the surface stability of the low temperature corrected rΔF508 CFTR at 37˚C. Several mechanisms could account for this effect. First, clathrin coated endocytosis is a very complex process that involves multiple steps and components. One of the main players is the actin cytoskeleton, which in general is responsible for membrane dynamics (41). Localized recruitment and polymerization of actin has been detected in endocytic sites for several proteins (99). As mentioned in the introduction, WT CFTR is known to interact with a variety of proteins such as NHERF1, CAL, CAP70 and others (summarized in (66)). It has been suggested that the NHERF1 interaction with WT CFTR anchors CFTR to the actin cytoskeleton at the cell surface (104). Therefore, enhanced rΔF508 CFTR internalization rates and shortened surface half-life could be due to the absence of CFTR interacting proteins that tether CFTR to the actin cytoskeleton. It is possible that the ΔF508 mutation in CFTR alters protein conformation. In fact, it has been demonstrated that ΔF508 CFTR disrupts

the folding of NBD2 and its native interaction with NBD1 using protease susceptibility assays (39). The ΔF508 mutation has also been found to increase the protease sensitivity of MSD1 (31). And finally, it was proposed that this mutation also disrupts the interaction between NBD1 and MSD1 (summarized in (90)).

The chemical chaperone, corr-4a, used in our study could act by partially correcting rΔF508 CFTR conformation and thereby prolonging rΔF508 CFTR surface residence. This is significant since even partial correction of rΔF508 CFTR could allow it to function more like WT CFTR and regain its physiological, cAMP-mediated responsiveness. We observed that although corr-4a stabilizes the surface pool of rΔF508 CFTR to some degree, the cAMP-mediated responses were only restored for a short period after 6 hours of treatment. These results suggest that facilitation of ΔF508 CFTR delivery to the plasma membrane is not sufficient if the "rescued" protein does not have physiological channel activity. Furthermore, when we used the combination of the corr-4a, which stabilized the protein at plasma membrane, and then stimulated the cells with the potentiator, VRT-532, which enhanced channel activity by increasing the open probability, cAMP-mediated responses dramatically increased making the channel function more like WT CFTR. Interestingly, VRT-532 had no effect on WT CFTR activity. Presumably, because WT CFTR functions physiologically and therefore, stimulation of WT CFTR expressing cells with potentiator has no additive effect.

Recently, it has been reported that corr-4a interacts with CFTR in the ER and promotes its folding (68). Therefore, corr-4a could help ΔF508 CFTR to escape the ER in a more physiological conformation and have a longer residence time at the plasma membrane. At the plasma membrane, rΔF508 CFTR in the presence of corr-4a enhances

the cAMP-activated channel activity when compared to rΔF508 CFTR without corr-4a treatment. However, the effect of corr-4a is short lived and lasts only for a couple of hours. The reason for the limited activity is unclear, but one of the explanations could be that corr-4a metabolism in the cell is very quick.

In our firefly luciferase based proteasome reporter assays, we observed that corr-4a treatment caused a minimal amount of proteasomal inhibition. Supporting the proteasome inhibitory effect of corr-4a, our biochemical studies suggest that corr-4a increases the stability of the core glycosylated form of the ΔF508 CFTR. However, the proteasome inhibitory effect is not CFTR specific as indicated by increased luciferase activity from the reporter constructs. While proteasomal inhibition causes the core glycosylated form of the protein to accumulate, it does not promote complex glycosylation and therefore functional protein at the plasma membrane (59). This leads to the speculation that it is not enough to promote delivery to the cell surface, but rather indicates that stabilizing it in the plasma membrane and correcting its channel function are both required for effective ΔF508 CFTR rescue.

Overall, our studies indicate that correction of the multiple defects associated with ΔF508 CFTR will require a combination of treatments that include correction of misfolding in the ER, trafficking to plasma membrane, short surface residence, and defective Cl^- channel function. However, the obvious difficulty of this task should not falter our interest to develop efficient therapies for CF.

FUTURE DIRECTIONS

Studying the Arg31 mutations

As mentioned in the introduction, more than one thousand five hundred other mutations have been identified in the *CFTR* gene. No point mutations have been identified in the endocytic signal of CFTR, but in our studies, we examined two naturally occurring R31C and R31L mutations that appeared to introduce a potential internalization signal in the N-terminus of CFTR. By studying these two particular mutations, we learned that even minor substitutions can have an effect on the cell surface stability of CFTR by affecting biogenesis and increasing internalization. It would be interesting to analyze the effect of these mutations on single channel activities. One possibility is that the open probability of either R31 mutant is not affected, and that the whole cell patch clamp and SPQ assays will show reduced amounts of current on the cell surface because there are fewer channels there. On the other hand, it is possible that the function of a single Cl^- channel is compromised. Only single channel analysis will be able to differentiate between these two possibilities. Given the mild nature of the disease in patients that carry mutations at R31, however, it is unlikely that the channel activity is severely compromised.

Knowing that the mechanism of action and regulation of CFTR is still far from completely resolved, understanding how mutations in each domain affect structure and function is necessary for understanding how this channel works. Studying the N-terminal mutations of CFTR is important because this region of CFTR is believed to associate with other domains of CFTR as well as associate with a large number of other proteins such as syntaxin 1A. The syntaxin 1A binding site

does not include the Arg31 residue; the latter could still influence syntaxin 1A-CFTR interaction. One way to study this would be to examine and compare CFTR channel activities plus and minus syntaxin 1A in oocytes, and compare the activities of the wild type protein with the two Arg31 mutants in whole cell patch clamp analysis.

Studying the ΔF508 mutation

Since rescued ΔF508 CFTR has low activity on the cell surface and is rapidly degraded (Varga et al., 2008), any studies aimed at examining the mechanism of these defects and potential therapies are of great importance. For example, incorrect folding of mutant protein can be corrected by altering chaperone composition within the cell (Balch, 2008). Studies aimed at understanding this process could identify chaperone compositions that facilitate ΔF508 CFTR folding. Alternatively, the degradation pathways that remove wild type and mutant CFTR from the plasma membrane could be different and this difference may be a site of potential intervention. For example, while endocytosis of the wild-type protein is mediated by the adaptor complex AP-2, ΔF508 CFTR may be cleared from the cell surface via ubiquitination and a different adaptor complex; it would be important to distinguish this differences involved in clearing ΔF508 from the surface could serve as a target. This could include a different adaptor complex, such a Dab2, or targeting the E3 ligase at the cell surface that mediates ΔF508 CFTR ubiquitination. Another possibility is to study the trafficking differences between wild type and mutant CFTR (endocytosis and/or recycling) to learn how to improve the instability associated with ΔF508 CFTR defect. A final

possibility, and the most promising in the CF field, is to identify and use correctors and/ or potentiators to correct the folding of ΔF508 CFTR.

All of the studies on corr-4a after low temperature rescue provide important new information. In spite of the fact that there has been a substantial effort in the CF field to identify compounds that facilitate ΔF508 CFTR folding and biogenesis, there has been a problem with the way the screens were performed. The model often used, Fisher rat thyroid cells (FRT), is neither predictive nor relevant. These cells were stably transfected with ΔF08 CFTR and have extremely high levels of protein, making their analysis superficial. The problem, however, is that significant amounts of ΔF508 CFTR are able to escape ERAD and are delivered to the cell surface without any sort of intervention. This does not happen *in vivo*. Using this cell line in the screens means that the screens may not identify compounds that promote biogenesis, but they may identify compounds that stabilize the protein at the cell surface. Corr-4a was identified in this screen, and as was shown in my studies, was fairly ineffective in promoting ΔF508 CFTR biogenesis in the human bronchial epithelial cell line (CFBE41o-). It did, however, stabilize the surface pool as monitored in an endocytosis assay (Varga et al., 2008). This indicates that corr-4a is partially effective in stabilizing ΔF508, possibly in promoting initial protein escape from ERAD and in stabilizing the surface pool. However there are a number of features of the wild type protein that are not corrected by corr-4a in ΔF508 CFTR, the most important of which is the chloride channel activity. Thus identifying additional compound(s) that work in concert with corr-4a, or in place of corr-4a will be necessary, so that more efficacious therapies for CF can be developed.

With regard to future directions of this work, studies reported here indicate that we must identify a corrector that will promote more efficient ΔF508 CFTR rescue from ERAD than corr-4a. One way of doing this is to screen compounds in the CFBE41o- parental cell line that endogenously expresses ΔF508 CFTR. This line is derived from a CF patient that is homozygous for the ΔF508 mutation. No detectable ΔF508 CFTR reaches the cell surface without some form of intervention. Therefore, identifying compounds in a screen based on surface chloride channel function that is activated by elevations in cAMP more relevant screen than those currently employed. Upon the identification of a corrector with this type of screen, we can analyze the corrector in the same manner as we did with corr-4a in the second manuscript. We are in fact doing this type screen now for new corrector molecules. We will use assays such as cell surface biotinylation and internalization to monitor the changes of the surface pool of CFTR at the plasma membrane in the absence or presence of each particular compound identified. Aside from identifying a more efficient corrector than corr-4a, we also need to use the knowledge learned from Ussing chamber experiments to look for compounds that correct the forskolin-stimulated responsiveness of rescued mutant CFTR. Luciferase assays will continue to be very important in determining if the compounds work through inhibition of the ubiquitin-proteasome system. This would allow us to determine if the stabilized protein is due to inhibition of proteasomal function. These extended studies would allow us to test for compound specificity. This would satisfy our main goal of identifying compounds that are CFTR specific and therefore act on ΔF508 CFTR without affecting other proteins. Our studies on the corr-4a show that a general inhibition of the ubiquitin-

proteasomal system is not what we want since there could be a number of nonspecific effects that could be counterproductive. Therefore, an appropriate future direction is to identify a compound or compounds that specifically facilitate the folding of ΔF508 rather than a compound that interferes with ΔF508 CFTR degradation such as corr-4a.

Each of the experimental assays performed to test the effects of corr-4a on ΔF508CFTR will be applied to test any future compound. Once the compound of interest is found through large screens, we will then determine the optimal concentration and treatment times, and then use the previously described assays to verify the surface expression and function of the rescued protein. Utilizing protocols developed through the present studies will enable the dissection of the mechanism of action of the newly identified corrector molecules. Performing the relevant screens and analyzing the compounds identified, including understanding their mechanism of action, will open doors to development of new CF therapies and will provide important benefits to a large number of CF patients that have this mutation.

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