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Pharmacologic restoration of ΔF508 CFTR-mediated chloride current

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Pharmacologic restoration of ΔF508 CFTR-mediated chloride current. Cystic fibrosis (CF) is an autosomal inherited disorder caused by over 800 different mutations in the *CFTR* gene. The most common mutation, ΔF508, causes a trafficking arrest in the endoplasmic reticulum and the CFTR protein is degraded. Restoration of CFTR trafficking in vitro restores cAMP-mediated chloride transport at the cell surface. The hypothesis of this discussion is that the short chain fatty acids, butyrate and 4-phenylbutyrate, up-regulate mature CFTR at the plasma membrane. Evidence that these compounds regulate CFTR production and maturation in part through effects on molecular chaperones in CF cells in culture is discussed. The oral drug, 4-phenylbutyrate, was tested in a Phase I clinical trial in CF subjects and further trials are underway. Other new therapeutic approaches directed at different classes of mutations in CFTR are also discussed. Chemical and pharmacologic agents that regulate endogenous gene expression at different steps in the biosynthetic processing pathway of a membrane glycoprotein will be needed to comprehensively treat a complex inherited disorder like cystic fibrosis.

Despite 10 years of intensive investigation of the genetics, pathophysiology, and clinical phenotypes of cystic fibrosis (CF), the disease remains one of the most common lethal inherited autosomal recessive disorders in the Caucasian population worldwide. The gene encodes the cystic fibrosis transmembrane conductance regulator (CFTR) protein, a 1480 amino acid, glycosylated, membrane glycoprotein that functions as a cAMP-regulated chloride channel in exocrine glands or secretory epithelia [1]. Seventy percent of patients carry one or two copies of the ΔF508 mutation, and over 800 additional mutations account for the remaining disease-causing alleles. The ΔF508 allele is a trafficking mutation, meaning that the mutant protein is blocked from maturation in the endoplasmic reticulum and is targeted for premature proteolysis [2]. Although chloride channel conductance is affected to some degree [3–6], if the ΔF508 protein is redirected to the cell surface, cAMP-mediated chloride transport can be restored (abstract; Rubenstein et al, *Pediatr Pulmon* S17:211, 1998) [7]. Thus, therapies directed at aberrant CFTR protein trafficking would be desirable.

Key words: cystic fibrosis, protein trafficking, butyrates.

TRAFFICKING DEFECTS IN CFTR CAN BE CORRECTED

Cystic fibrosis transmembrane conductance regulator (CFTR) biosynthesis is a balance between normal processing and proteolysis (Fig. 1A). Membrane glycoproteins typically assemble in the endoplasmic reticulum where they are core-glycosylated, then move to the Golgi apparatus to complete glycosylation, and then arrive at the plasma membrane, via vesicles that bud off from the Golgi. In vitro studies in heterologous cell culture systems suggest that at best, only 25% of newly synthesized wild-type CFTR escapes the endoplasmic reticulum to be glycosylated in the Golgi and inserted onto the plasma membrane attaining a final mobility of about 180 kDa on SDS-PAGE [8–10]. The other 75% of apparently normal protein is ubiquitinated and degraded [11]. ΔF508 is even less successful than wild type CFTR. ΔF508 CFTR fails to progress to the Golgi apparatus and the cell surface (Fig. 1C), and runs as a core-glycosylated protein of approximately 150 kD on an SDS-PAGE gel. Failure to pass through the Golgi can be recognized by failure to become fully glycosylated and attain a mobility of 170 to 180 kD on an SDS-PAGE gel (Band C, Fig. 1A). The deletion of the phenylalanine at position 508 is hypothesized to result in misfolding of the nascent chain as it emerges from the ribosome, where it is recognized by the molecular chaperones that target it for early proteolysis. There is much evidence for misfolding of CFTR: a reduced stability of ΔF508 at the cell membrane [12], slightly altered circular dichroism spectra of a peptide containing this site [13], restoration of processing by incubation of cells at temperatures between 25 and 29°C [7, 8], and restoration of processing by chemical chaperones [9]. Similar misfolding and impairment of

protein trafficking is seen for mutations in the alpha$_1$-antitrypsin gene, aquaporin-2 in nephrogenic diabetes insipidus [14], Tay-Sachs disease, familial hypercholesterolemia, and for accumulation of insoluble protein aggregates in Alzheimer's and prion-associated disorders.

It was initially hypothesized that by blocking ER-associated proteolytic degradation, a greater amount of mature ΔF508-CFTR would be produced. However, inhibition of CFTR proteolysis by lactacystin or ALLN does not force CFTR through the normal pathway [11]. Thus, therapeutic interventions designed to restore normal trafficking must be targeted proximal to ubiquitination and proteolysis in the endoplasmic reticulum. Detailed studies of nascent CFTR polypeptide chain interactions with molecular chaperones would be informative.

TEMPERATURE–SENSITIVE CFTR TRAFFICKING

Growth of ΔF508 CFTR-expressing cells at reduced temperature (25 to 30°C) dramatically corrects the trafficking defect and restores cAMP-mediated chloride transport at the plasma membrane [12] This effect can be mimicked at 37°C by chemical "chaperones" such as glycerol, dimethylsulfoxide (DMSO), trimethylamine-N-oxide (TMAO), and deuterated water [15]. Extremely high concentrations are required, and thus, direct in vivo application is impractical. High throughput screening and combinatorial chemistry approaches may accelerate the identification of a clinically applicable compound.

Studies of organ-specific adaptations to stress may also provide clues to alternative chemical chaperones. The kidney has adapted local environments to protect itself against hyperosmotic stress. By accumulation of "cellular osmolytes," urea-mediated protein denaturation is avoided. TMAO is synthesized by the renal medulla to protect the intracellular environment against urea. Carbohydrates or myoinositol, free amino acids such as glycine or alanine, and methylamines such as betaine, are either transported in or synthesized by other tissues in hyperosmotic stress [15]. It might be possible to adapt these principles locally within the lung, liver, or intestine.

BUTYRATE-MEDIATED CORRECTION OF CFTR PROCESSING

Gene expression (fetal hemoglobin is one example) can be modulated by small chain fatty acids such as butyric acid and its derivatives. The effect can be reproduced by inhibitors of histone deacetylase [16]. Genes active during fetal development are usually silenced in postnatal life. Inhibition of histone deacetylase derepresses fetal hemoglobin and many other genes. Given that CFTR is also more abundant in fetal than adult tissue [17, 18], it is not surprising that the butyrates can up-regulate CFTR expression. However, it is not yet clear that up-regulation of ΔF508 mRNA expression would be sufficient to overwhelm the cellular stress mechanisms leading to premature destruction, or whether additional genes in the trafficking pathway must be modulated. Low molecular weight fatty acids, such as butyrate and phenylbutyrate, restore ΔF508 to the cell surface at 37°C in culture [19]. Treatment of CF cell cultures expressing endogenous levels of ΔF508 with sodium butyrate or sodium 4-phenylbutyrate at nontoxic concentrations increases total CFTR protein and normal CFTR processing and leads to cAMP-mediated chloride conduction at the cell surface [20]. Butyrate-mediated effects on CFTR may be at least twofold—an induction of CFTR expression and modulation of molecular chaperone expression.

Wild-type CFTR "folding" and progression to a protease-resistant state is assisted by endoplasmic reticulum (ER)-associated chaperone proteins. Heat shock protein 70 (Hsp70) [21] and calnexin [22], but not BiP and Grp94 [2] appear to associate with CFTR transiently. Prolongation of the complex of Hsp70 and ΔF508 CFTR has been observed, suggesting that Hsp70 may recognize CFTR for early removal. Hsc70, a member of the Hsp70 family, is constitutively expressed, and has also been implicated as a signal for removal to the proteolytic pathway [23]. Hdj-2/Hsc70 complexes associate with wild-type and ΔF508 CFTR [24]. Preliminary data implicate a phenylbutyrate-mediated specific reduction in Hsc70 to explain the shift of mutant CFTR onto the trafficking pathway [8]. Because ΔF508 retains some chloride channel activity [4, 5], modulation of these chaperone associations or additions of a chemical chaperone are reasonable therapeutic avenues.

In a randomized, double-blind, placebo-controlled trial in CF patients homozygous for ΔF508, one week of phenylbutyrate, at 20 g/day, was associated with a modest induction of chloride transport based on nasal potential difference measurements [25]. Importantly, no safety issues arose in the CF trial, suggesting that a moderate, but selective, reduction in Hsc70 was not associated with widespread disruption of the quality control mechanism for folding or degrading proteins. 4-Phenylbutyrate (Buphenyl) is a newly approved drug for the rare inherited urea cycle disorders, where it functions to provide an alternative vehicle for waste nitrogen excretion by conjugation with glutamine. 4-Phenylbutyrate is under study in the hemoglobinopathies [26, 27], adrenoleukodystrophy [28], and in cancer trials [29]. Dose escalation and pharmacokinetic studies are continuing in adults with CF.

There may be other pharmaceuticals capable of regulating the molecular chaperones. Deoxyspergualin (DSG) is a stable synthetic analog of the natural product spergualin, and is known to compete effectively for binding with Hsp70 and Hsp90. DSG has potent immunosuppressive

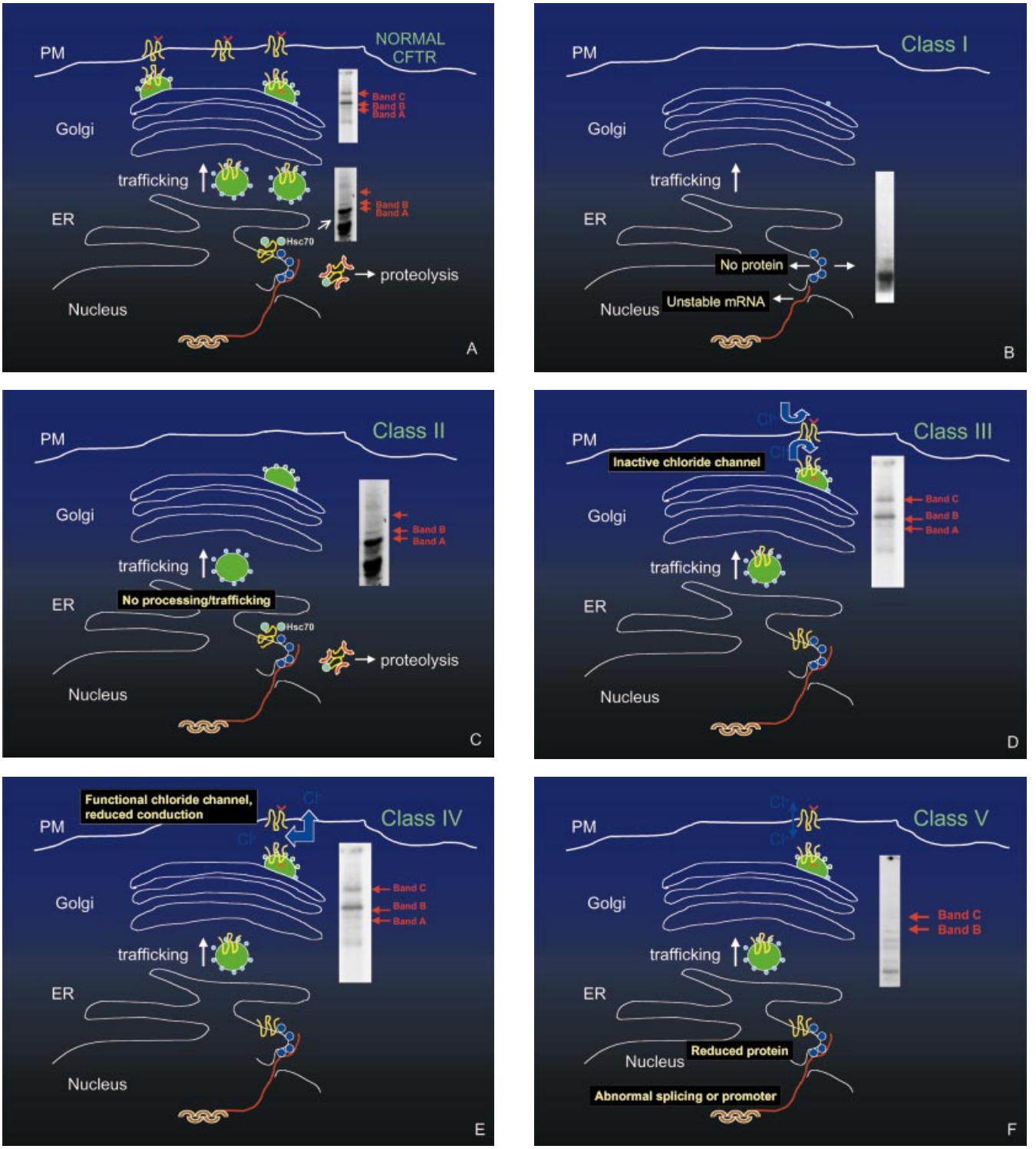

Fig. 1. CFTR biogenesis and trafficking. (*A*) Normal or wild-type CFTR biosynthesis is a balance between early proteolysis from the endoplasmic reticulum (75%, bands A and B on SDS-PAGE of immunoprecipitated and phosphorylated CFTR) and processing (25%, bands A, B, and C on SDS-PAGE). (*B*) Class I mutations, such as W1282X, produce an early stop codon, unstable mRNA, and absence of CFTR protein. (*C*) Class II mutations are trafficking mutations. The ΔF508 example results in early degradation (99%, bands A and B only). (*D*) Class III mutations produce fully processed CFTR resident on the plasma membrane (bands A, B, C). (*E*) Class IV mutations produce fully processed protein that trafficks normally (bands A, B, C). (*F*) Class V mutations involve promoter and splicing abnormalities and typically result in very low levels of normal CFTR (bands A, B, C). Publication of this figure in color was provided by a grant from Ucyclyd Pharmaceuticals, Inc., Phoenix, AZ, USA.

activity mediated in part through interactions with Hsc70 and Hsp90. One research group has studied DSG and reported appearance of functional cAMP-stimulated CFTR chloride channel activity at the cell surface in both immortalized human CF airway and biliary epithelial cells [30].

CFTR CHLORIDE CHANNEL ACTIVATORS

The previous discussion has been confined to modulators of trafficking; however, the ΔF508 CFTR is still less active as a chloride channel than normal CFTR. Both trafficking and chloride channel properties appear, at least in vitro, to be favorably altered by CPX (8-cyclopentyl-1,3-dipropylxanthine) [31, 32], an adenosine receptor antagonist that directly binds to ΔF508 CFTR in a region that structurally resembles the adenosine receptor. A Phase I dose escalation and safety trial was recently completed in CF, and a Phase II trial is in progress. Other analogs are undergoing laboratory assessment and look very promising [33].

Genistein, a member of the flavonoid class of molecules, activates CFTR through a highly debated mechanism [34]. Genistein is a tyrosine kinase inhibitor; however, not all tyrosine kinase inhibitors activate CFTR. Genistein does not increase cAMP levels [35], and more recent data implicate direct binding to the nucleotide binding domains of phosphorylated CFTR [36]. Genistein and related flavonoids are ubiquitous in the human diet in plant foods such as soybeans and legumes. Their pharmacology and toxicology have been intensely investigated. It is known that flavonoid intake from a normal diet can result in a blood concentration of 1 to 2 μmol/L in humans [37]. Small pilot studies of genistein and related flavonoids in nasal epithelia of normal volunteers demonstrated augmentation of the nasal potential difference response to isoproterenol [38].

Genistein and other flavonoid compounds effectively activate chloride conduction through the ΔF508 CFTR [39], although they do not appear to favorably restore trafficking. One group has demonstrated that pretreatment of CF cells with 4-phenylbutyrate or low temperature to induce ΔF508 trafficking to the plasma membrane, allowed genistein to activate chloride transport (abstract; Fischer et al, *Pediatr Pulmon* S17:239, 1998). Results from a pilot clinical trial of topical genistein in CF nasal epithelia alone or in combination with isoproterenol demonstrated a modest hyperpolarization in the nasal potential difference response to low chloride in normal volunteers, but not in CF patients homozygous for ΔF508. Restoration of ΔF508 CFTR trafficking will be necessary for the flavonoids to be effective (abstract; Gondor et al, *Pediatr Pulmon* S17:253, 1998). On the other hand, genistein alone induced a small chloride response in CF patients carrying one copy of the G551D mutation. G551D is a Class III mutation that is synthesized, resides in the plasma membrane, but is not activated with isoproterenol (Fig. 1D). The ability of genistein to activate G551D in the intestine of the G551D CF mouse has also been observed [40].

NEW APPROACHES TO CLASS I DEFECTS IN CFTR

Pharmacologic correction of CFTR defects must take under consideration the full range of observed abnormalities. Beginning with the Class I defects, it is clear that a variety of approaches will be required. Class I mutations generate premature stop codons that result in truncated mRNA transcripts that are inherently unstable and fail to be translated into proteins (Fig. 1B). The efficiency of translation termination tends to vary as a function of the surrounding amino acid, that is, context-sensitivity. Certain aminoglycosides, such as G418 and gentamicin, suppress premature chain termination and restore readthrough to full length CFTR [41]. W1282X and R553X can be rescued in vitro. Given that W1282X mRNA is unusually stable in certain patients [42], topical, inhaled, and intravenous gentamicin is under study in the U.S. and Israel (abstract; Wilschanski et al, *Pediatr Pulmon* S17:168, 1998) [43]. Preliminary data from these small clinical trials is encouraging. Modest nasal potential difference responses to isoproterenol in CF patients homozygous for stop codon mutations have been reported (abstract; Wilshanski et al; ibid).

CLASS III REGULATION OR ACTIVATION MUTATIONS ARE DIFFICULT TO REPAIR

Class III CFTR mutants exhibit reduced responses to regulatory molecules (Fig. 1D). G551D is a common Class III mutation that does not conduct chloride in response to elevated cAMP, but is trafficked to the plasma membrane. Other Class III mutations can interfere with nucleotide binding and hydrolysis, and disrupt CFTR function to a variable degree. These mutants sustain a reduced response to adenosine 5′-triphosphate (ATP); examples include S1255P, G551S, G1244E, and G1349D. It may be possible to find molecules that alter the interaction of the CFTR domains with ATP and restore activity to these mutants.

CLASS IV CONDUCTION MUTATIONS ARE ASSOCIATED WITH A MILD PHENOTYPE

Class IV mutants such as R117H, G314E, R334 W, and R347P are associated with normal trafficking, protein kinase A (PKA)-dependent response to phosphorylation, and ATP binding. Defects instead lie in chloride conductance or channel gating (Fig. 1E). The mutations

tend to occur within the regions of CFTR that form the chloride pore. R347P affects the rate of chloride flow, whereas R117H and P574H reduce the channel open time. The latter mutation is sometimes placed in Class II because of a reduced efficiency of processing. The milder clinical phenotypes observed in patients with a Class IV mutation suggest that treatments aimed at promoting CFTR trafficking and activating or increasing chloride conductance would tend to support a milder course.

One drug that may be capable of stimulating Class IV CFTR chloride conduction is an approved phosphodiesterase inhibitor that sustains elevated cAMP. Milrinone (together with forskolin to initiate elevated cAMP) favorably restores cAMP-mediated chloride secretion to nasal epithelia of the ΔF508 murine model, but in preliminary in vivo studies, does not alter nasal potential difference measurements of patients carrying the ΔF508 mutation [44]. The murine nasal epithelium appears to process and traffic the ΔF508 CFTR more efficiently than human tissue and this is a likely explanation for the different responses of the two species. A second murine model, the R117H mouse, expresses a mutant which is found on the cell surface in humans. Encouraging responses to milrinone and forskolin in the nasal epithelia in this model suggest further studies with human carriers of R117H would be useful [44]. Milrinone itself is used for short-term intravenous therapy of congestive heart failure as a positive inotrope and vasodilator, and thus is unlikely to be practical in CF; however, analogs might be found that are more appropriate for CF.

CLASS V MUTATIONS GENERATE A SMALL AMOUNT OF NORMAL CFTR

Class V mutations typically are associated with a milder phenotype or even the absence of classic CF, as seen in males with infertility due to congenital absence of the vas deferens (CBAVD). Mutations in the CFTR promoter, in introns that regulate mRNA splicing, or that cause inefficient levels of protein to be produced, are grouped together in Class V (Fig. 1F). When splicing efficiency is reduced, variable levels of normal CFTR can escape to provide some function as is seen with 3849 + 10kb C→T. This mutation creates a partially active splice site in intron 19 that leads to insertion of a new 84 bp exon with a stop codon. Patients with this mutation are diagnosed later, attain better nutritional status, exhibit variable male fertility, and can have normal or slightly elevated sweat chlorides. Therapies that would maximally activate the normal CFTR or increase mRNA production for the CFTR already present in these patients might confer clinical benefit.

SUMMARY

Chemical and pharmacologic mediators of protein trafficking are needed in CF and other diseases caused by trafficking mutations. Drugs that regulate endogenous gene expression may also be useful in the induction of alternative enzymatic pathways in diseases such as CF, the hemoglobinopathies, adrenoleukodystrophy, and cancer. CF is one example where multiple approaches will be needed due to the spectrum of abnormalities observed. Certainly, genotyping will become clinically indicated in all the inherited disorders and thus may specifically direct therapeutic intervention.

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