A MECHANISM FOR ALTERED POLYUNSATURATED FATTY ACID BIOSYNTHESIS IN CYSTIC FIBROSIS

By

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To my family

for all that we have been through

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LIST OF ABBREVIATIONS

Δ5D, Δ5-desaturase

Δ6D, Δ6-desaturase

AA, arachidonic acid

ACC, acetyl-CoA carboxylase

AICAR, 5-aminoimidazole-4-carboxamide-1-β-4-ribofuranoside

AMPK, AMP-activated protein kinase

AP-2α, activating enhancer binding protein 2α

CaMKKβ, Ca^{2+}/calmodulin-dependent protein kinase kinase β

CF, cystic fibrosis

CFTR, cystic fibrosis transmembrane conductance regulator

COX, cyclooxygenase

CRAC, calcium release activated channel

DECR, 2,4-dienoyl-CoA reductase

DHA, docosahexaenoic acid

ENaC, epithelial sodium channel

EPA, eicosapentaenoic acid

FEV_1, forced expiratory volume in one second

HDAC, histone deacetylase

HBE, human bronchial epithelial

IL, interleukin

LA, linoleic acid

LNA, α-linolenic acid

LOX, lipoxygenase

LKB1, liver kinase B1

LT, leukotriene

MSD, membrane-spanning domain

MUFA, monounsaturated fatty acid

NBD, nucleotide-binding domain

PFT, pulmonary function test

PG, prostaglandin

PGC-1α, PPARγ coactivator 1α

PKA, protein kinase A

PLA_2, phospholipase A_2

PPAR, peroxisome proliferator-activated receptor

PPRE, peroxisome proliferator response element

PUFA, polyunsaturated fatty acid

qRT-PCR, quantitative real-time polymerase chain reaction

SFA, saturated fatty acid

SRE, sterol regulatory element

SREBP, sterol regulatory element-binding protein

TRPC, transient potential receptor canonical channel

CHAPTER I

INTRODUCTION

Overview

Cystic fibrosis (CF) is one of the most common autosomal recessive genetic disorders. CF is a debilitating multi-organ disease caused by genetic mutations leading to absence of functional cystic fibrosis transmembrane conductance regulator (CFTR) protein in epithelial cells. Progressive pulmonary disease, characterized by excessive inflammation and recurrent infections, is the primary source of morbidity and mortality in CF patients.

Alterations in polyunsaturated fatty acid (PUFA) composition play a key role in CF pathophysiology. CF cells exhibit aberrant PUFA biosynthesis resulting in increased biosynthesis of arachidonic acid (AA), the precursor of important pro-inflammatory mediators. The mechanism by which absence of functional CFTR leads to aberrant PUFA biosynthesis has been elusive and is the topic of this dissertation. This dissertation is the first delineation of a pathway linking PUFA metabolism to CFTR.

This dissertation opens with an overview of *n*-3 and *n*-6 PUFAs, PUFA biosynthesis, and the physiological roles of PUFAs. The subsequent section of the first chapter details the etiology and pathophysiology of CF with an emphasis on the involvement of PUFAs. The final section of Chapter I discusses the AMP-activated protein kinase (AMPK) and its relationship with both lipid metabolism and CFTR.

Chapter II details an AMPK-dependent mechanism resulting in altered PUFA biosynthesis in CF. The findings demonstrate elevated activity of AMPK in CF bronchial epithelial cells and show that it results from increased phosphorylation of AMPK by

Ca^{2+}/calmodulin-dependent protein kinase kinase β (CaMKKβ). The findings further demonstrate that elevated AMPK activity in CF cells stimulates increased expression and activity of Δ6-desaturase and Δ5-desaturase (Δ6D and Δ5D, respectively), key PUFA biosynthetic enzymes. Inhibition of AMPK or CaMKKβ reduces Δ6D and Δ5D expression and activity, thus abolishing the metabolic alterations seen in CF cells. These results signify a novel AMPK-dependent mechanism linking the genetic defect in CF to alterations in PUFA biosynthesis.

Chapter III delves deeper into the mechanism by which AMPK increases Δ6D and Δ5D expression. Chapter III examines transcriptional activity induced by the Δ6D promoter in CF and WT bronchial epithelial cells. Through promoter reporter studies, it is determined that Δ6D promoter activity is elevated in CF cells and is potentiated by AMPK activation. Elevated Δ6D promoter activity and mRNA expression in CF are independent of transcription factors known to stimulate Δ6D expression. These results signify that AMPK induces Δ6D transcription via previously unidentified regulators of Δ6D expression.

The fourth and final chapter discusses the broad implications of the findings described in this dissertation. Chapter IV discusses potential for improvements in CF therapy, including novel therapeutic targets and possible markers for CF disease severity. Chapter IV examines future research approaches that will lead to more complete understanding of CF pathophysiology, with an emphasis on the role of PUFA metabolism.

Polyunsaturated Fatty Acids

n-3 *and n*-6 *Polyunsaturated Fatty Acid Biosynthesis*

Fatty acids are comprised of a carboxylic acid moiety and an aliphatic hydrocarbon chain of varying length and degrees of unsaturation. Saturated fatty acids (SFA) contain no double bonds while monounsaturated fatty acids (MUFA) contain a single double bond. Polyunsaturated fatty acids (PUFA) contain two or more double bonds in the aliphatic chain. PUFAs are categorized according to the number of carbons between the most distal double bond and the methyl end of the molecule (Figure 1). For example, in *n*-3 PUFAs, there are three carbons between the most distal double bond and the methyl end of the molecule. In *n*-6 PUFAs, this distance is six carbons. In addition to systematic scientific names, PUFAs have common names and shorthand nomenclature. The shorthand nomenclature indicates the number of carbons, the number of double bonds and the number of carbons between the most distal double bond to the methyl end of the PUFA (Figure 1, Table 1) (1).

The most physiologically relevant PUFAs are the *n*-3 and *n*-6 PUFAs. Mammals lack the enzymes necessary for *de novo* biosynthesis of *n*-3 and *n*-6 PUFAs. However, mammals are able to synthesize long-chain *n*-6 and *n*-3 PUFAs from linoleic acid (LA) and α-linolenic acid (LNA), respectively, obtained from the diet. Hence, LA and LNA are referred to as essential fatty acids. Long-chain PUFA biosynthesis requires desaturation steps catalyzed by Δ6-desaturase (Δ6D) and Δ5-desaturase (Δ5D), and elongase steps catalyzed by elongase 5 and elongase 2 (Figure 2). The reaction catalyzed by Δ6D is the rate-limiting step. Of the *n*-6 PUFAs, arachidonic acid is the most physiologically important. The most physiologically important *n*-3 PUFAs are

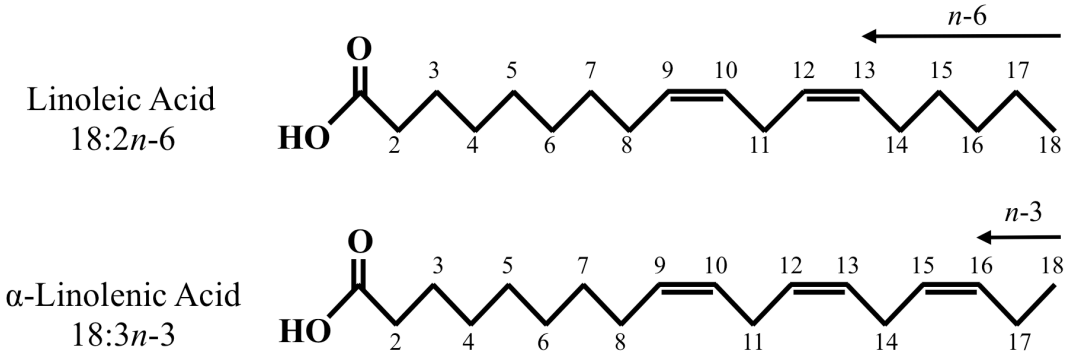

Figure 1 Polyunsaturated fatty acid structure
PUFAs are long chain aliphatic carboxylic acids with two or more double bonds. Linoleic acid has 18 carbons and two double bonds, and is an *n*-6 PUFA because there are six carbons between the most distal double bond and the methyl end of the molecule (indicated by arrow). α-linolenic acid 18 carbons and three double bonds, and is an *n*-3 PUFA because there are three carbons between the most distal double bond and the methyl end of the molecule (indicated by arrow).

Table 1 PUFA nomenclature

***n*-6 PUFAs**		
Common Name	**Abbreviation**	**Shorthand Nomenclature**
Linoleic acid	LA	18:2*n*-6
Arachidonic acid	AA	20:4*n-6*
***n*-3 PUFAs**		
α-linolenic acid	LNA	18:3*n*-3
Eicosapentaenoic acid	EPA	20:5*n*-3
Docosahexaenoic acid	DHA	22:6*n*-3

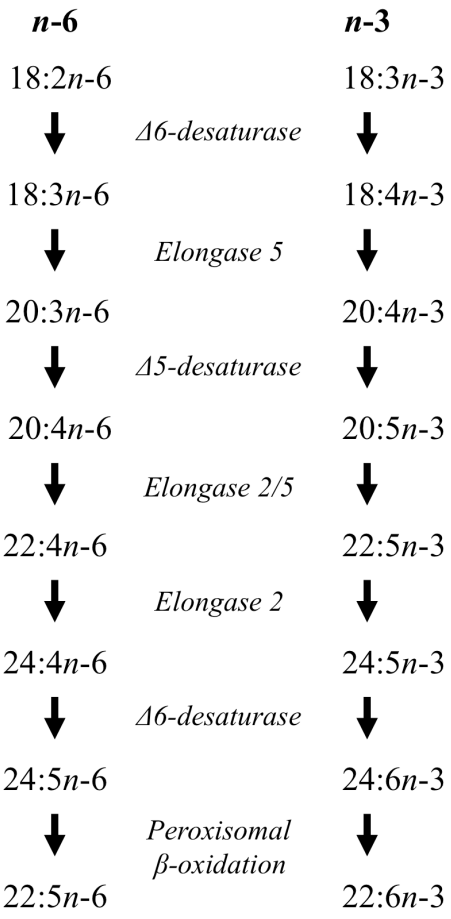

Figure 2 PUFA biosynthesis pathways
n-6 and *n*-3 PUFAs are synthesized in parallel pathways utilizing the same enzymes. The first reaction catalyzed by Δ6-desaturase is the rate-limiting step in these pathways.

eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Since mammals lack a Δ4-desaturase, conversion of EPA to DHA requires two elongations and a round of peroxisomal β-oxidation (Figure 2). Mammals lack the enzymes necessary to convert *n*-3 PUFAs to *n*-6 PUFAs and vice-versa (2, 3).

Δ6D and Δ5D are endoplasmic reticulum membrane-bound enzymes that catalyze the desaturation of polyunsaturated fatty acyl-CoA. Δ6D catalyzes the formation of a double bond six carbons from the carboxyl end, while Δ5D catalyzes the formation of a double bond five carbons from the carboxyl end (4, 5). Δ6D has specificity for LA, LNA, 24:5*n*-6, and 24:6*n*-3. Δ5D has specificity for 20:3*n*-6 and 20:4*n*-3 as shown in Figure 2. The genes encoding Δ6D and Δ5D are arranged in a head-to-head orientation in chromosome 11 separated by 11,000 base pairs, and have 71% nucleotide sequence identity. Δ6D and Δ5D both contain 444 amino acids and have 75% amino acid similarity. Both enzymes contain two membrane-spanning domains and identical amino-terminal cytochrome b_5-like domains that serve as a critical electron carriers for desaturation reactions (4–6). Δ6D and Δ5D have wide tissue distribution, and are expressed at comparable levels in the liver, lung, brain, testis, and heart (4, 5).

The enzymatic activities of Δ6D and Δ5D parallel the expression level of their respective mRNAs, which suggests the absence of post-translational regulation (4, 7). Two transcription factors have been confirmed as transcriptional regulators of Δ6D and Δ5D expression: sterol regulatory element binding protein-1 (SREBP-1) and peroxisome proliferator-activated receptor α (PPARα). SREBP-1 and PPARα are major regulators of fatty acid metabolism and typically induce transcription of mutually exclusive sets of genes. SREBP-1 primarily induces transcription of genes necessary for fatty acid

biosynthesis. PPARα primarily induces transcription of genes necessary for fatty acid catabolism (8, 9). The genes encoding Δ6D and Δ5D are unique in that both SREBP-1 and PPARα induce their transcription (10, 11). Analysis of the Δ6D promoter has revealed two functional sterol regulatory elements (SRE), which are binding sites for SREBP-1 (7). The Δ6D promoter also contains a functional peroxisome proliferator response element (PPRE), which is a binding site for PPARα (12). There are no reported analyses of the Δ5D promoter. However, the Δ5D promoter likely contains at least one SRE and PPRE since Δ5D mRNA is induced by SREBP-1 and PPARα (10, 11).

Elongases catalyze the condensation of long-chain fatty acyl-CoA and malonyl-CoA, thus lengthening the fatty acid chain by two carbons. Elongase 5 has specificity for 18:3*n*-6, 18:4*n*-3, AA, and EPA. Elongase 2 has some overlap with elongase 5, with specificity for AA and EPA, as well as 22:4*n*-6 and 22:5*n*-3 (Figure 2) (3, 13). Analogous to Δ6D and Δ5D, elongases do not undergo post-translational modification, so elongase enzymatic activities parallel their respective mRNA levels. SREBP-1 induces transcription of the genes encoding elongase 5 and elongase 2 (14–16). Elongase 5 and elongase 2 are expressed in liver, testes, lungs, white adipose, and brain tissue (3).

Physiological Roles

PUFAs are key components of phospholipids that constitute biological membranes. PUFAs typically occupy the SN_2 position of membrane phospholipids. The identity of the fatty acid in the SN_2 position influences the structural properties of the phospholipid bilayer. Membrane fluidity is positively correlated with the number of double bonds in the PUFA in the SN_2 position (1, 17, 18). Changes in membrane fluidity

impact the function and localization of integral membrane proteins such as transporters, channels, and receptors (19–21). This is especially true for membranes in neural tissues such as the retina, in which DHA is indispensible for normal function and development (21–24). The phospholipid bilayer also acts as a storage site for PUFAs. Phospholipase A_2 enzymes cleave PUFAs from the SN_2 position in phospholipids for utilization in other biologically important cellular processes (25, 26).

PUFAs modulate the activity of several transcription factors to influence transcription of particular genes. The transcriptionally inactive, full-length form of SREBP-1 is sequestered in the endoplasmic reticulum membrane. Proteolytic processing releases the transcriptionally active SREBP-1 nuclear fragment, which then translocates to the nucleus to induce transcription of target genes (9). PUFAs inhibit proteolytic processing of SREBP-1. Through inhibition of SREBP-1 activity, PUFAs suppress the expression of fatty acid biosynthetic enzymes, including the PUFA biosynthetic enzymes such as Δ6D, Δ5D, and elongases (27–31). Therefore, PUFAs exert feedback inhibition of the enzymes required for PUFA biosynthesis. On the other hand, PUFAs also serve as endogenous ligands for PPARα to stimulate expression of fatty acid catabolic enzymes (32, 33). As noted above, PPARα also induces expression of Δ6D and Δ5D. Thus, depending on the circumstances, PUFAs may suppress or induce expression of PUFA biosynthetic enzymes. One of several mechanisms by which *n*-3 PUFAs exert anti-inflammatory effects is via stabilization of inhibitor of κB (IκB), resulting in diminished expression of pro-inflammatory nuclear factor κB target genes (NF-κB) (34–36). Additionally, PUFAs may influence epigenetic gene regulation. Animal studies have

revealed that maternal PUFA intake influences DNA methylation of PUFA metabolic genes in offspring (37–40).

Cyclooxygenases (COX) and lipoxygenases (LOX) catalyze the conversion of PUFAs to important paracrine and autocrine inflammatory mediators that exert their effects through G protein-coupled receptors (41, 42). These inflammatory mediators participate in the exceedingly complex regulation of initiation and resolution of inflammation. Of the PUFA-derived inflammatory mediators, products derived from AA, EPA, and DHA are the most important physiologically. Inflammatory mediators derived from AA are important initiators of inflammation; however, there is evidence that they are important for the resolution of inflammation as well. Inflammatory mediators derived from *n*-3 PUFAs universally exert anti-inflammatory effects (43). The 20-carbon AA- and EPA-derived inflammatory mediators are collectively known as eicosanoids. COX enzymes catalyze the first step in the metabolism of AA and EPA to prostaglandins (PG) and other prostanoids. LOX enzymes catalyze the first step of metabolism of AA and EPA to leukotrienes (LT). AA-derived prostaglandins, especially PGE_2, have a wide array of pro-inflammatory actions and play important roles in edema, increased pain sensitivity, and increased vascular permeability to facilitate leukocyte extravasation (44). AA-derived LTB_4 is a potent neutrophil chemoattractant, and promotes neutrophil extravasation (45). LOX enzymes also convert AA to lipoxins, which promote resolution of inflammation (46). EPA-derived eicosanoids are significantly less potent than their AA-derived counterparts and compete for binding at receptors, thereby attenuating the pro-inflammatory actions of AA-derived eicosanoids (47). Furthermore, EPA is a competitive substrate with AA in COX and LOX reactions (48). LOX enzymes also

participate in the production of anti-inflammatory resolvins and protectins from EPA and DHA (43).

Another mechanism by which *n*-3 PUFAs exert beneficial physiological effects is by acting as ligands for certain G protein-coupled receptors. Circulating *n*-3 PUFAs are ligands for GPR120, a recently discovered G protein-coupled receptor. DHA and EPA exert anti-inflammatory and insulin-sensitizing effects through GPR120 signaling (49). GPR120 is expressed in intestines, lungs, macrophages, and adipose tissue (50–52). EPA- or DHA-dependent GPR120 signaling attenuates toll-like receptor and tumor-necrosis factor α inflammatory signaling. Furthermore GPR120 is required for the insulin-sensitizing and anti-obesity effects of *n*-3 supplementation in mice on a high fat diet (49, 53). One study demonstrated that persons carrying a dominant negative mutation in GPR120 are more likely to be obese (53).

Cystic Fibrosis and Other Diseases

PUFAs are involved in the pathophysiology of cystic fibrosis (CF), a debilitating autosomal recessive disorder. CF patients exhibit consistent alterations in PUFA composition, including reduced levels of LA and DHA in serum and increased AA levels in certain tissues. Studies in CF cell culture models have revealed that CF cells have increased expression and activity of Δ6D and Δ5D, which results in increased conversion of LA to AA (54–56). Increased production of pro-inflammatory AA-derived prostaglandins and leukotrienes are consistently observed in CF patients (57). Increased production of AA and AA-derived inflammatory eicosanoids contribute to the excessive inflammation observed in CF. In CF patients, excessive pulmonary inflammation is the

primary source of morbidity and mortality (58). The roles of PUFAs in CF will be discussed in further detail in the *Cystic Fibrosis* section of this chapter.

PUFAs have been implicated in a number of other disease states as well (59). The recurrent theme in these disease states is that a high ratio of *n*-6 PUFAs to *n*-3 PUFAs are associated with increased risk of disease. Exceedingly high LA content in Western diets exacerbate this risk (60). *n*-3 PUFAs, especially EPA and DHA, have a protective effect against various forms of cardiovascular disease (61) such as hypertension (62), hyperlipidemia (63), myocardial infarction (64, 65), and arrhythmias (66, 67). One study implicated increased Δ6D activity in the progression of cardiovascular disease (68). There is also evidence that *n*-3 PUFAs have protective effects against cerebrovascular events (69). Studies indicate that *n*-3 PUFAs may improve recovery from stroke and traumatic brain injury (70–75). A high *n*-6:*n*-3 PUFA ratio is associated with increased risk of psychiatric disorders (76–78). High *n*-6:*n*-3 PUFA ratios have also been observed in patients with atopic dermatitis (79). Epidemiologic studies and studies in model systems have suggested that *n*-3 PUFAs have anti-tumorigenic effects in breast cancer (80–82). Reducing AA levels via inhibition of Δ6D suppresses lung and melanoma tumor growth in mice (83).

Cystic Fibrosis

Cystic Fibrosis Transmembrane Conductance Regulator

Major advances in molecular biology made way for the discovery that CF is caused by mutations in the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR) (84–86). CFTR is a cyclic AMP-dependent anion channel located in

the apical membrane of epithelial cells in the lungs, intestines, exocrine glands, and reproductive system (87). While anion conductance is the best-known function of CFTR, it also regulates the function of other ion channels and various proteins.

The *CFTR* gene spans an approximately 250,000 bp region on the long arm of chromosome 7. *CFTR* contains 27 exons that are translated into a single polypeptide of 1480 amino acids that requires extensive post-translational modifications for proper function (85). In the endoplasmic reticulum, chaperones direct the CFTR folding into its final conformation (88). Core glycosylation of CFTR takes place in the endoplasmic reticulum, and subsequent complex glycosylation occurs in the Golgi apparatus. The fully glycosylated CFTR protein is transported to the apical plasma membrane (88, 89).

The CFTR protein contains two membrane-spanning domains (MSD), two cytoplasmic nucleotide-binding domains (NBD), and a cytoplasmic regulatory domain (R domain) organized as depicted in Figure 3 (85). CFTR is folded such that the MSDs form the channel pore. Each MSD contains 6 membrane-spanning α helices. In the three-dimensional structure, the MSDs interact with the NBDs, and the NBDs are in close proximity to the R-domain (Figure 3) (87).

CFTR is member of the ATP-binding cassette (ABC) transporter ATPase superfamily (87, 90). As such, CFTR anion conductance is regulated by ATP hydrolysis in the nucleotide-binding sites. NBD1 and NBD2 each have two halves of nucleotide binding sites that combine to form two ATP-binding sites. When the ATP-binding sites are unoccupied, NBD1 and NBD2 exist in monomeric form, and the CFTR channel is in

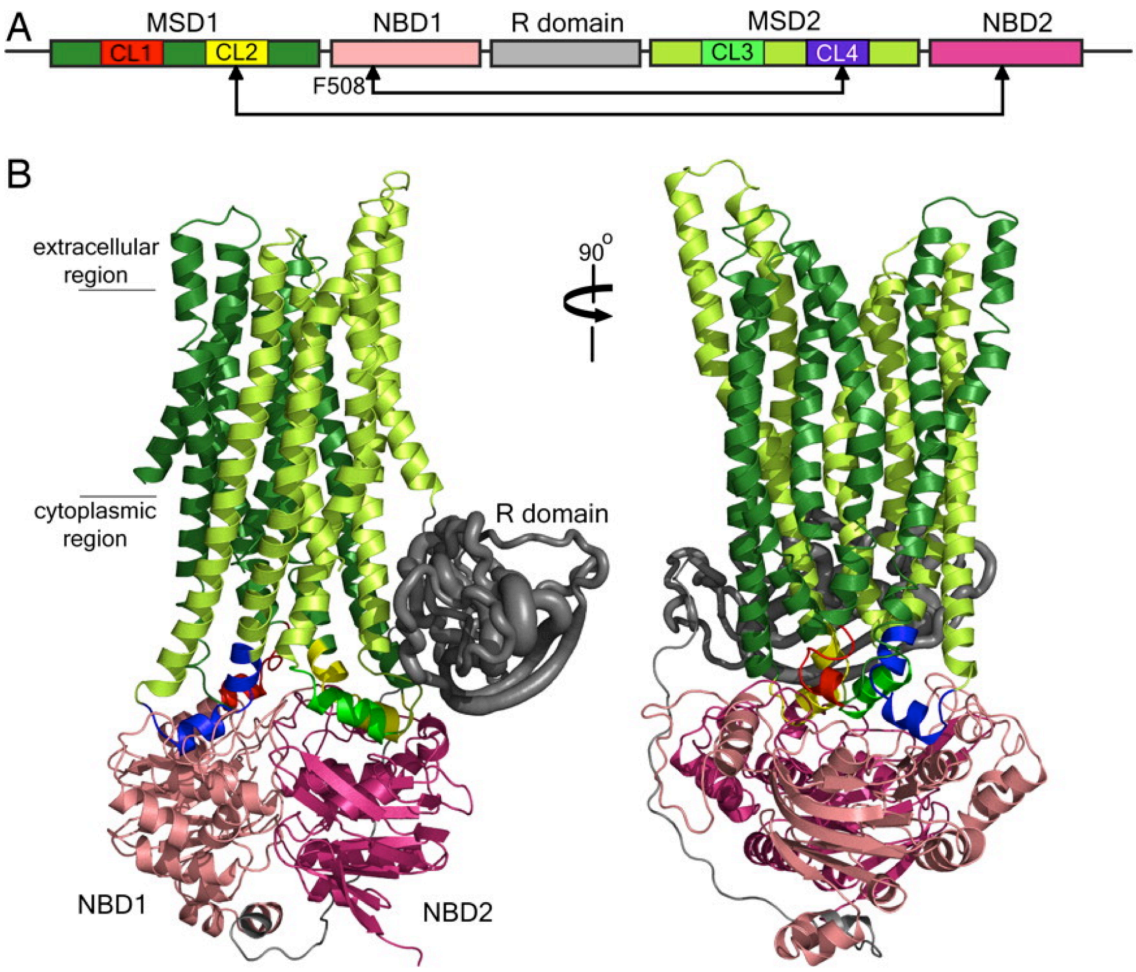

Figure 3 CFTR structure
(**A**) Primary structural organization of CFTR membrane spanning domains (MSD), nucleotide-binding domains (NBD), and regulatory domain (R domain). MSDs contain cytoplasmic loops (CL) that interact with NBDs. (**B**) Three-dimensional representation of CFTR. Adapted from Serohijos *et al*. (91)

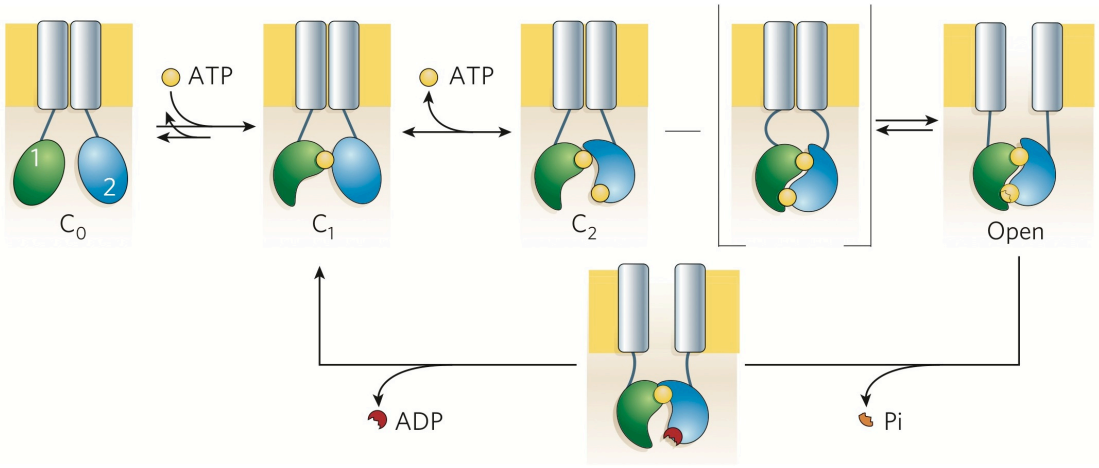

Figure 4 Regulation of CFTR Gating by ATP
Binding of two ATP molecules in the NBDs is required for the CFTR channel to adopt an open conformation. Hydrolysis and dissociation of one or both ATP molecules stabilizes the closed conformation of the CFTR channel. Adapted from Gadsby *et al*. (87)

the closed conformation. The CFTR channel shifts to the open conformation when NBD1 and NBD2 form a heterodimer upon binding two ATP molecules. Hydrolysis and dissociation of one or both ATP molecules causes the channel to shift back to the closed conformation (Figure 4) (87, 90).

In the ABC transporter superfamily, CFTR is unique in that it contains an R domain. The R domain imposes a level of regulation that supersedes ATP cycling in the NBDs. The R domain appears to be unstructured and contains numerous phosphorylation sites (87). Phosphorylation of the R domain by the cyclic AMP-dependent protein kinase (protein kinase A or PKA) is required for CFTR channel activity. PKA-mediated phosphorylation of the R domain induces a conformational change that permits NBD dimerization and ATP cycling. Phosphorylation of the R domain by protein kinase C does not activate CFTR by itself, but potentiates the activating effects of PKA-mediated R domain phosphorylation (92). AMPK opposes the effects of PKA on CFTR. Phosphorylation of the R domain by AMPK at Ser737 and Ser768 stabilizes the R domain in a conformation that prevents CFTR anion conductance (93, 94).

CFTR exhibits cyclic-AMP dependent conductance of several anions of physiological relevance. CFTR is the only ABC transporter that functions in this capacity. Moreover, while other ABC transporters function as active transporters, CFTR functions as a passive transporter (87). CFTR is conducive to halide ions; of these, chloride is the most relevant physiologically (95). CFTR-dependent chloride conduction is an important regulator of the osmolality of cytoplasm and extracellular fluids (96). CFTR also conducts bicarbonate ions, and in doing so, may influence intracellular and extracellular

pH levels (97, 98). CFTR may influence cellular redox status through conductance of glutathione ions (99).

CFTR is termed a conductance regulator because it not only conducts anions, but also regulates the function of other ion channels. CFTR activates the outwardly rectifying chloride channel, possibly through an ATP-dependent mechanism (100). CFTR inhibits the amiloride-sensitive epithelial sodium channel (ENaC), though the mechanism is unclear (101–103). Inhibition of ENaC by CFTR may involve a direct protein-protein interaction between the two ion channels (104). CFTR attenuates calcium influx through inhibition of transient receptor potential canonical channel 6 (TRPC6) (105). Furthermore, CFTR attenuates store-operated calcium entry through the calcium release-activated calcium channel (CRAC) (106).

Etiology and Pathophysiology

Cystic fibrosis (CF) was first characterized in an extensive clinical and pathology study published in 1938 (107). This study included the first description of the clinical manifestations of CF. Since the publication of that seminal report, advances in clinical understanding and treatment of CF have increased the median predicted survival age from less than 1 year of age in 1938 to approximately 41 years of age as of 2012 (108). Today, CF is recognized as one of the most common autosomal recessive diseases (109). Prevalence is highest in Ashkenazi Jews (1 in 2,300 individuals have CF) and Caucasians (1 in 2,500). Carrier rates are approximately 1 in 25 in these populations. CF is less common in Hispanics (1 in ~10,000) and African Americans (1 in ~15,000). CF is very rare in East Asian and African populations (58, 110).

CF is caused by recessive mutations in the *CFTR* gene that result in absent or defective CFTR function. More than 1800 mutations have been identified, which fall into six mutation classes (108). Class I mutations result in early termination codons that cause complete absence of CFTR at the apical plasma membrane. Class II mutations result in improper folding and post-translational modification of CFTR. The improperly folded protein undergoes proteasomal degradation, resulting in severe reduction or complete absence of CFTR at the plasma membrane. Class III mutations result in the expression of nonfunctional CFTR due to defective gating. Class IV mutations result in functional CFTR, but with attenuated channel permeability to ions. Class V mutations result in attenuated transcription of *CFTR* due to altered transcriptional regulation. Class VI mutations result in increased turnover of CFTR at the plasma membrane (111, 112). Class I, Class II, and Class III mutations are associated with more severe clinical symptoms of CF due the absence of functional CFTR (58). Class IV, Class V, and Class VI mutations are relatively rare and are associated with mild disease.

The ΔF508 mutation, a Class II mutation, is the most common disease-causing *CFTR* mutation. The ΔF508 mutation results in an in-frame deletion of the phenylalanine residue at position 508, which is in NBD1 of CFTR (85, 113, 114). ΔF508 accounts for approximately 70% of all disease-causing CFTR alleles worldwide; only a handful of other mutations account for more than 1% each. In the United States, approximately two-thirds of CF patients are ΔF508 homozygotes, and approximately 90% of CF patients have at least one ΔF508 allele (110). As with other Class II mutations, the ΔF508 mutation interferes with CFTR folding and maturation. Misfolded ΔF508 CFTR is rapidly degraded via polyubiquitination and proteosomal degradation (115–117).

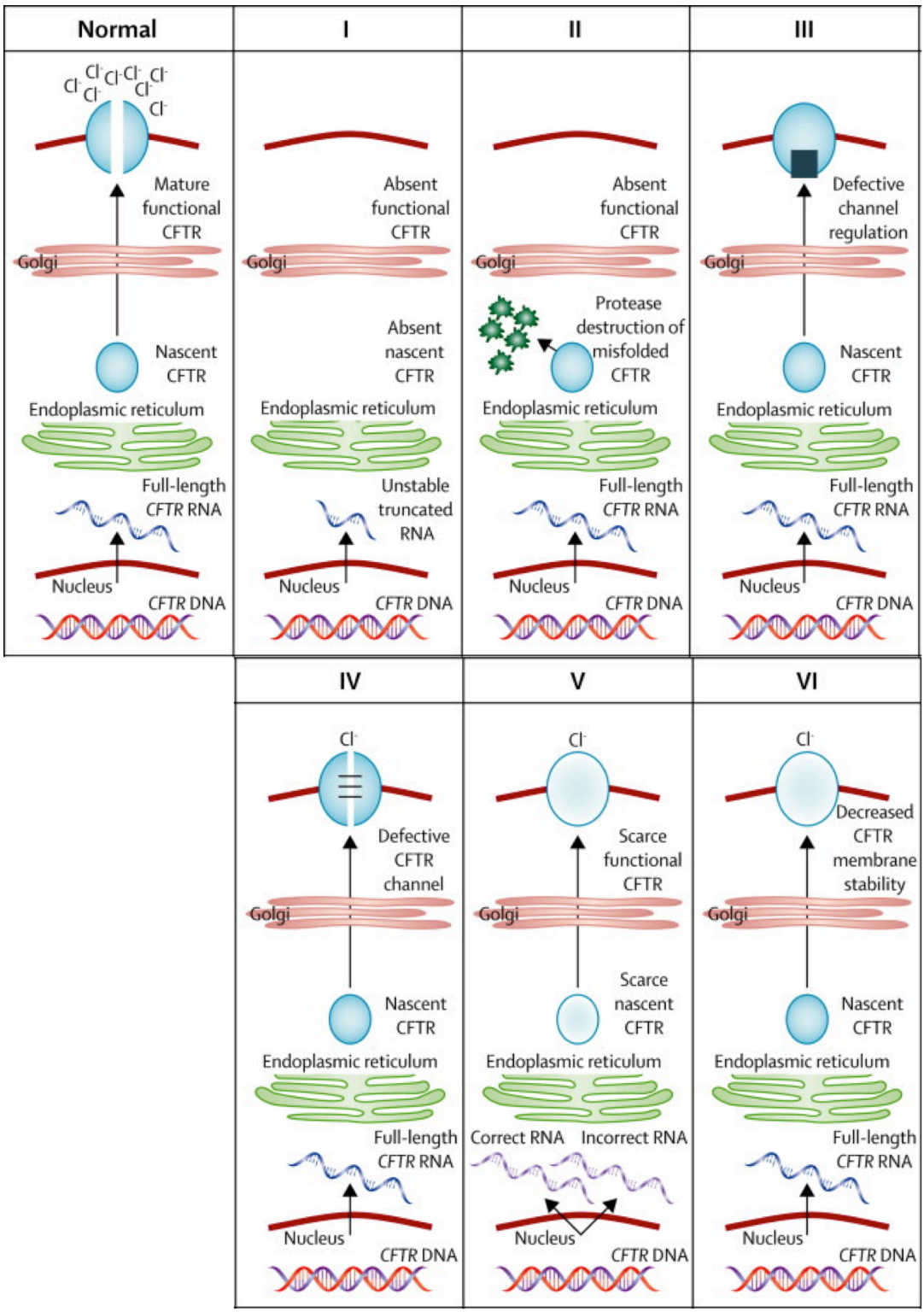

Figure 5 CFTR mutation classes
Adapted from Boyle *et al*. (118)

A negligible amount of ΔF508 CFTR escapes degradation, but exhibits severely defective function at the plasma membrane (119).

CFTR is localized to the apical membrane of epithelial cells in a variety of organs. CFTR is predominantly expressed in epithelial cells of the lungs, the exocrine pancreas, the intestines, the reproductive tracts, and eccrine sweat glands (120). The clinical features of CF reflect the tissue distribution of CFTR. Consequently, the major clinical manifestations of CF are progressive pulmonary disease, exocrine pancreatic dysfunction, intestinal obstruction, infertility, and excessively salty sweat (58).

Progressive pulmonary disease, the primary source of morbidity and mortality in CF patients, is responsible for 80-90% of deaths (58). The pathophysiology of CF pulmonary disease is complex and not entirely understood. Some aspects of CF pulmonary disease are easily attributable to loss of functional CFTR. For other aspects, the connection is less clear. This is evidenced by the lack of a strong correlation between *CFTR* genotype and pulmonary disease severity (121). Inflammation and bacterial infections play prominent roles in causing progressive destruction of the lung parenchyma and deterioration in pulmonary function. Ultimately, most CF patients succumb to respiratory failure and require lung transplantation for survival (58).

CFTR is expressed in epithelial cells of the airways and is a major regulator of mucus viscosity and periciliary layer height. The periciliary layer is a layer of watery fluid that lies directly on top of the airway epithelium. The mucus layer lies on top of the periciliary layer. The physical characteristics of the mucus layer and periciliary layer are important for clearance of pathogens and inhaled materials (122). The upward motion required for clearance of pathogens or particles trapped in the mucus layer is generated

by constant cilia beating within the periciliary layer. Coughing assists in airway clearance when cilia are overwhelmed (122). In healthy lungs, adequate hydration keeps mucus viscosity low, and maintains sufficient periciliary layer height. CFTR is indispensible in maintaining hydration of the mucus and periciliary layers. CFTR-mediated chloride efflux and inhibition of sodium influx prevent excess water reabsorption (123, 124).

In the lungs of CF patients, the absence of functional CFTR impedes chloride efflux and permits excess sodium influx through ENaC. Defective ion transport causes water to follow an osmotic gradient, resulting in dehydration of the mucus and periciliary layers. Dehydration of the mucus layer causes its viscosity to increase. The periciliary layer loses height due to dehydration, which allows the increasingly viscous mucus to impede cilia function (123, 124). As airway clearance declines, thick viscous mucus plugs the airways. CF patients develop dyspnea and a persistent cough as their lungs attempt to compensate for reduced airway clearance (125, 126).

Reduced airway clearance and viscous mucus in CF lungs create a permissive environment for bacterial growth. In the first few years of life, the predominant species that colonize CF lungs are *Staphylococcus aureus* and *Haemophilus influenzae*. In childhood, *Pseudomonas aeruginosa* becomes the predominant bacterial species (127). *Burkholderia cepacia* species are also common in CF patients. Bacterial infection contributes to destruction of lung parenchyma in CF patients. CF patients infected with *P. aeruginosa* or *B. cepacia* typically have worse prognoses (58).

Excessive inflammation is the chief source of destruction of lung parenchyma in CF. Inflammatory responses to infection in CF airways are disproportionate to the level of infection (128, 129). Pro-inflammatory mediators and neutrophils are present to

greater degree in CF airways relative to normal lungs even in the absence of infection (130–134). Compared to healthy airways, infection in CF airways elicits a significantly more robust release of pro-inflammatory interleukins (IL) and eicosanoids, including IL-1β, IL-6, IL-8 and LTB_4 (135, 136). IL-8 and LTB_4 are potent neutrophil chemoattractants, so large quantities of neutrophils are recruited to CF airways. Neutrophils utilize proteases, elastases, and oxidases to kill bacteria. When neutrophils die, these enzymes are released and cause significant damage to lung parenchyma (137). Elevated neutrophil elastase and myeloperoxidase have been detected in bronchoalveolar lavage fluid from CF patients (138, 139). Furthermore, in CF airways, there are diminished levels of lipoxins, resolvins, protectins, and IL-10, all of which promote resolution of inflammation (46, 140, 141). Inflammatory lung damage further increases the susceptibility to infection in CF patients. Consequently, a vicious cycle of excessive inflammation and recurrent infections combine to cause progressive destruction of the lung parenchyma and progressive decline in pulmonary function. While many studies have noted excessive inflammation in CF, mechanisms linking loss of functional CFTR to excessive inflammation have been elusive.

The deterioration of pulmonary function in CF patients is nonlinear. Patients experience periods of relatively stable disease punctuated by occasional pulmonary exacerbations. Pulmonary exacerbations are periods during which CF patients require hospitalization due to worsening disease symptoms. Pulmonary function tests (PFT), such as forced expiratory volume in 1 second (FEV_1), are commonly used to measure lung function in CF patients. Performance on PFTs declines rapidly during a pulmonary

exacerbation. After an exacerbation, pulmonary function does not return the pre-exacerbation level. Consequently, pulmonary function declines in a stepwise manner (58).

Exocrine pancreatic dysfunction is common in CF patients. CFTR genotype-phenotype correlations are strong for pancreatic dysfunction; individuals with severe Class I-III mutations have a higher degree of pancreatic dysfunction. Loss of functional CFTR interferes with pancreatic digestive enzyme secretion, the primary function of the exocrine pancreas. The absence of adequate chloride and bicarbonate efflux leads to dehydration and acidification of exocrine pancreatic secretions. Increased viscosity impairs the flow of pancreatic secretions through pancreatic ducts. The trapped digestive enzymes cause autolysis of pancreatic tissue (142). The pancreas undergoes cystic degeneration and becomes fibrotic, the pathologic description of which is the source of the term "cystic fibrosis" (107). Symptoms of exocrine pancreas dysfunction include steatorrhea, abdominal pain, bloating and flatulence (126). Pancreatic enzyme replacement therapy has considerably reduced morbidity and mortality due to pancreatic dysfunction in CF patients. However, CF-related diabetes as a result of pancreatic autolysis is a common comorbidity as CF patients reach adulthood (126).

CF patients are susceptible to intestinal obstruction and intussusception. CF intestinal disease parallels CF pulmonary disease in that mucosal dehydration, infection, and inflammation are present (143). CFTR-mediated chloride efflux in intestinal epithelial cells helps to maintain hydration of intestinal mucosa and luminal contents. Dehydration due to loss of CFTR-mediated chloride efflux in CF intestines increases the risk of intestinal obstruction (144). Moreover, loss of CFTR-mediated bicarbonate efflux interferes with intestinal mucus production (145). Before the advent of improved therapy

for CF, meconium ileus and malnourishment secondary to pancreatic dysfunction caused significant mortality in the first year of life (107).

CF patients typically have reduced fertility. Infertility is almost universal in males with CF due to congenital bilateral absence of the vas deferens (146). CFTR is expressed in the epithelial cells of the vas deferens. Loss of functional CFTR leads to obstruction and involution of the vas deferens *in utero*. Normal CFTR function is essential for development of the vas deferens; absence of vas deferens is common even in individuals with mild CFTR mutations (126). Females with CF experience reduced fertility due to increased viscosity of fluids in the fallopian tubes (147).

In eccrine sweat glands, functional CFTR is necessary for sodium chloride reabsorption in the production of hypotonic sweat. Thus, loss of functional CFTR results in defective sodium chloride reabsorption and elevated sweat chloride concentration. Pilocarpine iontophoresis, also known as the sweat chloride test, is a commonly used diagnostic tool for CF. A sweat chloride level greater than 60 mmol/L has high positive predictive value for the diagnosis of CF (142, 148, 149).

Polyunsaturated Fatty Acids in CF

Consistent alterations in *n*-6 and *n*-3 PUFA composition have been well documented in CF patients. Evidence suggests that these alterations play an important role in the excessive inflammation observed in CF pathophysiology. PUFAs are precursors for inflammatory mediators that may influence the clinical course of CF. Reduced LA and DHA levels are the most consistently observed PUFA alterations.

Increased production of AA-derived pro-inflammatory eicosanoids is also a consistent observation (150).

Numerous studies have reported significantly reduced levels of LA and DHA in plasma, serum, and erythrocyte membranes from CF patients (151–161). Furthermore, reduced LA and DHA levels have been observed in epithelial cells scraped from the nasal mucosa of CF patients, as well as nasal and rectal biopsy tissue from CF patients (162). Low levels of DHA likely contribute to hyperactive inflammation in CF patients. DHA is a precursor of anti-inflammatory resolvins and protectins, which are present at diminished levels in airways of CF patients (141).

Studies have been less consistent with regard to whether AA levels are increased in blood of CF patients; however, increased AA-derived eicosanoids are consistently observed. Increased AA levels have been detected in bronchoalveolar lavage fluid from CF patients, and the increase was independent of infection status (163). Other studies have found no change in AA levels in plasma (152, 153). In CF cell culture models, AA levels are consistently increased (56, 164). Increased conversion of AA to pro-inflammatory eicosanoids may obfuscate elevations in AA biosynthesis in CF patients. Increased levels of AA-derived prostaglandins and leukotrienes have been detected in bronchoalveolar lavage fluid, saliva, exhaled breath condensates, and urine from CF patients (135, 165–169). Moreover, in CF airways, there is increased expression of enzymes necessary for conversion of AA to pro-inflammatory eicosanoids including phospholipase A_2 (PLA_2), COX, and LOX, enzymes (170–172).

Alterations in PUFA levels were first noted in the 1960s (173). At the time, altered tissue PUFA levels were thought to be a consequence of malabsorption due to

pancreatic insufficiency. However, more recent studies demonstrate that altered PUFA composition is directly linked to loss of functional CFTR and is not secondary to malabsorption. The PUFA alterations are independent of pancreatic function (158, 159). Furthermore, they are present in well-nourished CF patients with sufficient pancreatic function (156). Moreover, malabsorption would lead to reduced prostaglandin and leukotriene production as opposed to the increased production observed in CF patients (166). Additionally, altered PUFA composition is seen in CF cell culture models in which pancreatic function is irrelevant (54–56, 164). One study demonstrated that after lung transplantation, plasma PUFA composition in CF patients approximates PUFA composition seen in healthy individuals. This raises the possibility that the PUFA alterations derive from loss of CFTR in the lung (174). Individuals with other non-CF conditions that feature pulmonary inflammation such as asthma and upper respiratory infections do not have the same degree of PUFA alterations as CF patients, which implies that the PUFA alterations are specific to CF (162). These studies suggest the PUFA alterations are directly related to loss of functional CFTR.

The magnitude of the alterations in PUFA composition correlates with disease severity. *CFTR* mutations associated with more severe disease are also associated with more drastic reductions in serum LA and DHA (153, 154). In fact, the product of LA levels multiplied by DHA levels has shown promise as a biomarker that can differentiate CF individuals from individuals without CF with high sensitivity and specificity (155). CF carriers display an intermediate PUFA phenotype (152). LA levels correlate with FEV_1 values in CF patients; those with lower LA levels tend to have worse FEV_1 values

(157, 161, 175). Additionally, as reductions in DHA-derived resolvins or increases in AA-derived eicosanoids increase in scale, CF disease severity increases (141, 168, 169).

An interesting note in the 1938 clinical and pathologic report on CF was that CF patients who received cod-liver oil were more likely to survive the first year of life (107). Cod-liver oil has high *n*-3 PUFA content. Since the early 1990s, investigators have conducted a number of clinical trials examining the effects of dietary *n*-3 supplementation in CF patients. The clinical trials were limited in scope and duration, but offered some evidence that *n*-3 PUFA supplementation would be of benefit to CF patients. In most of the trials, DHA was given in combination with EPA. Increased serum or plasma *n*-3 PUFA levels were observed after *n*-3 supplementation in all of the trials, which indicates adequate intestinal absorption. In a few trials, *n*-3 PUFA supplementation caused statistically significant improvements in FEV_1 (176–179). In one trial, *n*-3 PUFA supplementation resulted in reductions in pulmonary exacerbation frequency (178). A number of trials noted significant reductions in inflammatory markers such as LTB_4 and TNFα (176, 178–181). Overall, the effect sizes were relatively small in these clinical trials. The small effect sizes may be attributable to weaknesses in study design such as inadequate *n*-3 PUFA doses, improper placebo controls, small sample sizes, and short study durations (182). Better understanding of the relationship between CFTR and PUFAs may aid in development of efficacious PUFA-related therapies for CF.

Results from DHA supplementation studies in CF mouse models underscore the importance of PUFAs in CF pathophysiology. CFTR null and ΔF508 CFTR mouse models exhibit similar PUFA alterations as CF patients; in CFTR-expressing tissues, LA and DHA levels are diminished while AA levels are increased. DHA supplementation

results in significantly increased DHA levels and significantly decreased AA levels (183–185). DHA supplementation also reduces pathologic signs of CF in the CFTR null murine model (183, 184). Importantly, DHA supplementation attenuated the inflammatory response to lipopolysaccharide in CFTR null mouse lungs (183).

Polyunsaturated Fatty Acid Metabolism is Altered in Cystic Fibrosis Cells

CF cell culture models have allowed for more detailed analysis of mechanisms leading to altered PUFA composition in CF. CF cell culture models exhibit similar alterations in PUFA levels as those observed in CF patients (54–56, 164, 186). The CF cell culture model in which PUFA alterations have been studied most extensively is a 16HBEo- bronchial epithelial cell line in which CFTR expression is silenced by stable transfection expressing an antisense oligonucleotide sequence complementary to CFTR. Altered PUFA levels have also been observed in immortalized cell lines derived from patients hetero- or homozygous for the ΔF508 allele.

Studies in CF cell culture models have revealed that metabolism of LA to AA is increased in CF cells (54–56, 164, 186). CF cells exhibit increased expression and activity of Δ6D and Δ5D, and consequently, increased metabolism of LA to AA (56, 164). Furthermore, CF cells produce more AA and AA-derived eicosanoids than control cells following LA supplementation (187, 188). LA supplementation has similar effects in a CFTR null mouse model. Following LA supplementation, CFTR null mice had significantly higher AA levels and neutrophil counts in lung tissue than control mice (188). Furthermore, COX and LOX enzyme expression is elevated in CF cells (56, 189), and CF cell culture models exhibit excessive production of pro-inflammatory mediators

in response to stimuli (190–193). So, in addition to increased LA to AA metabolism, CF cells exhibit increased metabolism of AA to eicosanoids. Chapter II and Chapter III of this dissertation will outline a mechanism connecting CFTR to metabolism of LA to AA.

Studies in CF cell culture models have revealed other alterations in PUFA metabolism as well. EPA to DHA metabolism is diminished in CF cells (55, 56). There is also evidence of increased retroconversion, an abbreviated form of β-oxidation in which DHA is converted to EPA (164, 194). DHA supplementation in CF cells suppresses Δ6D and Δ5D expression and activity, thereby reducing LA to AA metabolism (54, 164).

Current Therapy

In 2012, the Food and Drug Administration and its European counterpart approved ivacaftor for treatment of CF patients with a G551D allele or other less common Class III gating mutations. Ivacaftor is the first CFTR mutation-specific drug approved for use in CF patients. Ivacaftor potentiates the gating of G551D CFTR, but the precise mechanism of action is not fully understood (195, 196). Clinical trials demonstrated that ivacaftor treatment produces significant clinical improvement in this subpopulation of CF patients, including significant improvements in lung function, reduced pulmonary exacerbation frequency, reduced necessity for antibiotics, normalization of sweat chloride, and improvements in qualitative measures of quality of life in CF patients (197–199). While ivacaftor is a remarkable achievement in CF therapeutics, its use is beneficial in less than 5% of CF patients at a cost of $300,000 per patient per year (200, 201). There are no drugs currently available that improve the function of ΔF508 CFTR, of which at least one copy present in up to 90% of CF patients.

For the vast majority of CF patients, mainstays of CF therapy are aimed at managing CF clinical manifestations. Aggressive therapy prolongs life and improves quality of life for CF patients. Pancreatic enzyme replacement therapy has eliminated much of the morbidity due to pancreatic insufficiency. CF patients receive oral, inhaled, or intravenous antibiotics to combat bacterial infection (126). Hypertonic saline or inhaled mannitol increase hydration of the mucus and periciliary layers (126, 197). Mucolytics, such as dornase alpha, reduce the mucus layer viscosity. Postural drainage and chest percussion techniques, along with bronchodilators, aid in airway clearance to reduce breathing difficulties (125). High-dose ibuprofen slows the progression of CF pulmonary disease in clinical trials, but concerns of side effects preclude prolonged use in CF patients (202–204).

The current nutritional recommendation is for CF patients to consume high-calorie, high-fat diets in order to maintain body weight. Since Western diets have especially high *n*-6 PUFA content, particularly LA, and low in *n*-3 PUFA content, this dietary recommendation may require refinement (60). As described above, CF cells have increased uptake of LA, increased conversion of LA to AA, and increased production of AA-derived pro-inflammatory mediators. Consequently, this dietary recommendation may exacerbate inflammation in CF patients. As described above, dietary *n*-3 PUFA supplementation may be beneficial.

AMP-activated Protein Kinase

Structure and Regulation by Adenine Nucleotides

AMP-activated protein kinase (AMPK) is a major metabolic regulator that regulates a wide variety of cellular processes. AMPK is a heterotrimeric protein containing a catalytic α subunit, and regulatory β and γ subunits (205). There are two isoforms each of the α and β subunits, and three isoforms of the γ subunit. Thus, there are twelve possible αβγ configurations with varying tissue distributions. The physiological relevance of the different configurations is not entirely understood (205, 206). The catalytic α subunit contains the kinase domain. Phosphorylation of threonine-172 in the activation loop of the α subunit is necessary for maximum AMPK activity. The β subunit interacts with the α and γ, and serves as a scaffold for the AMPK heterotrimer (207). Glycogen-binding domains in the β subunit may allow AMPK to sense cellular glycogen levels. The γ subunit contains four cystathionine β synthase domains (CBS), which combine to form four nucleotide-binding sites that are indispensible for the ability of AMPK to respond to cellular energy levels (Figure 6) (208).

Each of the four nucleotide-binding sites in the AMPKγ subunit has a different role in the regulation of AMPK activity. AMP perpetually occupies one of the nucleotide-binding sites due to extremely tight binding. Another nucleotide-binding site is perpetually unoccupied because it lacks the conserved aspartate residues necessary for nucleotide binding (209). These first two nucleotide-binding sites are not involved in the regulation of AMPK activity. The remaining two nucleotide-binding sites are the primary

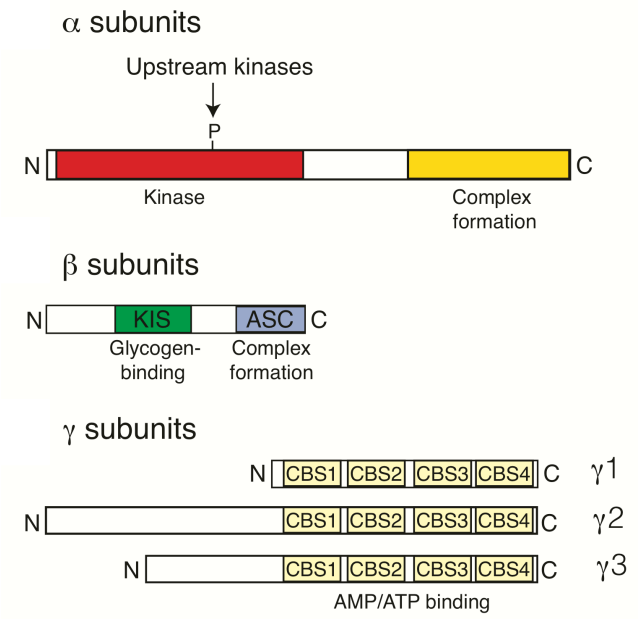

Figure 6 AMPK subunits
AMPKα subunits contain an amino-terminal catalytic domain. LKB1 and CaMKKβ can both phosphorylate AMPK at Thr172 in AMPKα subunits. AMPKβ subunits serve as a scaffold for heterotrimer formation and contain a glycogen-binding domain. AMPKγ subunits contain four cystathionine β synthase that form the nucleotide-binding sites. Image adapted from Hardie *et al*. (210)

effectors of AMPK regulation by adenine nucleotides. These nucleotide-binding sites reversibly bind ATP, ADP, or AMP depending on the relative concentrations of these adenine nucleotides. Binding of AMP to one of the regulatory sites causes allosteric activation of AMPK, leading to an approximately tenfold increase in AMPK activity (211–213). Binding of AMP or ADP to the other regulatory site induces a conformational change that renders Thr172 in the AMPKα subunit resistant to the action of protein phosphatases (209, 214). AMP is approximately tenfold more potent than ADP in inhibiting dephosphorylation of AMPK (213). Inhibition of dephosphorylation has a much larger effect on AMPK activity than allosteric activation by AMP; AMPK activity increases several hundredfold when phosphorylated at Thr172 in the AMPKα subunit (212, 215).

As described above, the relative concentrations of the adenine nucleotides are major determinant of AMPK activity. In a typical cell, ATP concentration ranges from 3-8 mM, ADP concentrations range from 50-200 μM, and AMP concentrations range from 1-50 μM. Nearly all of the ATP molecules coordinate to magnesium ions, which significantly weakens binding of ATP to the AMPKγ subunit. This allows AMP and ADP to compete with ATP for binding, despite their much lower concentrations (209). Hydrolysis of ATP by energy-consuming cellular processes produces ADP. Furthermore, adenylate kinase converts two molecules of ADP to a molecule of AMP and a molecule of ATP for further use by ATP-consuming processes. Therefore, when cells consume ATP at a high rate, relatively small declines in ATP concentrations result in comparatively large increases in concentrations of ADP, and especially AMP (216). This

results in increased binding of AMP and ADP to the AMPKγ subunit. Consequently, AMPK activity is sensitive to small declines in ATP concentrations.

Activation by LKB1 and CaMKKβ

Phosphorylation of Thr172 in the activation loop of the AMPKα subunit is required for maximum AMPK activity. While AMP can increase AMPK activity approximately tenfold, phosphorylation of AMPKα by AMPK kinases increases AMPK activity several hundredfold. Allosteric activation and phosphorylation combine to increase AMPK activity more than 1000-fold (212, 215). The two kinases that can phosphorylate AMPK in mammalian cells are liver kinase B1 (LKB1) and calcium/calmodulin dependent protein kinase kinase β (CaMKKβ).

LKB1 is one of the two AMPK kinases in mammalian cells. LKB1 forms a heterotrimeric complex with STRAD and MO25 (217–219). This complex stabilizes LKB1 in an active conformation (219, 220). LKB1 appears to be constitutively active in phosphorylating AMPKα. However, at basal conditions, protein phosphatases dephosphorylate AMPKα at a rate greater than the rate of AMPKα phosphorylation by LKB1. Thus, at basal conditions, AMPK is predominantly unphosphorylated. As described above, when AMP or ADP bind to the AMPKγ subunit, a conformational change occurs that inhibits AMPK dephosphorylation by protein phosphatases, and allows LKB1-dependent AMPK phosphorylation to predominate. Thus, LKB1-dependent phosphorylation of AMPK is regulated by the intracellular AMP concentration (221, 222).

On the other hand, CaMKKβ-dependent phosphorylation of AMPK is dependent on the intracellular calcium concentration (223, 224). When the intracellular calcium

concentration rises, calcium binds to calmodulin, a ubiquitous calcium-binding protein that transduces calcium signals (225). Upon binding calcium/calmodulin, CaMKKβ undergoes a conformational change that is required for phosphorylation of AMPK (226). Unlike LKB1, CaMKKβ-mediated AMPK phosphorylation is not dependent on the intracellular AMP concentration because CaMKKβ phosphorylates AMPK at a rate greater than the rate of AMPK dephosphorylation by protein phosphatases (224, 226). CaMKKβ phosphorylates AMPK in response to a wide range of signal transduction pathways that utilize calcium as a second messenger. Thus, activation of AMPK is not simply restricted to cells undergoing energetic stress (227).

Roles in Lipid Metabolism

Lipid metabolism is among the myriad cellular processes regulated by AMPK to promote net production of ATP. Through phosphorylation of downstream targets, AMPK has immediate and long-term effects on lipid metabolic pathways. These pathways include β-oxidation, cellular uptake of fatty acids, *de novo* synthesis of SFA and MUFA, sterol biosynthesis, and fatty acid metabolic gene expression.

AMPK stimulates β-oxidation through inhibitory phosphorylation of acetyl-CoA carboxylase 2 (ACC2) (205). ACC2 is localized to the mitochondrial membrane and catalyzes the formation of malonyl-CoA from acetyl-CoA. Malonyl-CoA is a potent inhibitor of carnitine palmitoyltransferase-1 (CPT-1), the enzyme that transports fatty acids into mitochondria for β-oxidation (1). Thus, when AMPK phosphorylates ACC2, synthesis of malonyl-CoA ceases, leading to an increased rate of β-oxidation and subsequent ATP production (228). Furthermore, AMPK stimulates cellular fatty acid

uptake by inducing migration of fatty acid translocase to the plasma membrane (229). Cellular uptake of fatty acids provides more substrate for β-oxidation.

AMPK inhibits *de novo* synthesis of SFAs and MUFAs through inhibitory phosphorylation of acetyl-CoA carboxylase 1 (ACC1) (230). ACC1 catalyzes the same reaction as ACC2, but ACC1 is localized to the cytoplasm. Unlike the *n*-3 and *n*-6 PUFAs, SFAs and MUFAs can be synthesized *de novo* from acetyl-CoA in a reaction catalyzed by fatty acid synthase (FAS). FAS catalyzes the condensation of malonyl-CoA with acetyl-CoA to form a four-carbon fatty acyl-CoA. FAS catalyzes subsequent malonyl-CoA condensations to lengthen the fatty acyl-CoA chain in two-carbon increments. The primary product of FAS is palmitic acid (PA, 16:0), which requires seven rounds of malonyl-CoA condensation (1). Inhibition of ACC1 by AMPK deprives FAS of a necessary substrate for *de novo* fatty acid synthesis (230). Malonyl-CoA is also a necessary substrate for elongation reactions of PUFA biosynthesis pathways (231, 232).

AMPK inhibits sterol biosynthesis through inhibitory phosphorylation of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase (233). HMG-CoA reductase catalyzes the rate-limiting step of sterol biosynthesis (1).

AMPK exerts longer-term changes in lipid metabolism by influencing the transcription of lipid-metabolizing genes. AMPK phosphorylates SREBPs, and in doing so, inhibits proteolytic processing of SREBP, resulting in suppression of lipid biosynthetic gene expression (234). AMPK induces mitochondrial biogenesis and expression of mitochondrial catabolic enzymes by inducing the activity of peroxisome proliferator-activated receptor gamma coactivator alpha (PGC-1α) (235). PGC-1α is a critical co-activator for transcriptional regulation by a number of transcription factors,

including PPARs (236–239). The mechanism by which AMPK induces PGC-1α activity is not clear, but may involve direct phosphorylation (240). AMPK phosphorylates other transcription factors, including stimulatory phosphorylation of activating enhancer binding protein 2α (AP-2α) (241), AICAR response element binding protein (242), and forkhead box O3 (FOXO3) (243); and inhibitory phosphorylation of hepatic nuclear factor 4α (HNF4α) (244) and carbohydrate responsive element binding protein (ChREBP) (245). AMPK influences gene expression via histone modifications as well. AMPK can stimulate transcription of particular genes by phosphorylating and inhibiting a subset of histone deacetylases (HDACs) (54, 55), as well as by directly phosphorylating histone H2B (56).

Interaction with CFTR

As mentioned in the *Cystic Fibrosis Transmembrane Conductance Regulator* subsection, AMPK inhibits CFTR channel activity via inhibitory phosphorylation of the R domain at Ser737 and Ser768 (93, 94). Baseline phosphorylation by AMPK stabilizes the closed state of the CFTR channel (246). The inhibitory effect of AMPK on CFTR is facilitated by a protein-protein interaction between the carboxyl-terminus of the AMPKα subunit and the cytoplasmic carboxyl-terminus of CFTR. Furthermore, AMPK co-localizes with CFTR at the apical plasma membrane in CFTR-expressing cells (93, 247). Since CFTR relies on ATP hydrolysis for channel gating, inhibition of CFTR by AMPK is consistent with the role of AMPK as a promoter of net ATP production. AMPK links the activity of CFTR to cellular metabolic status (248).

A recent report demonstrated that CF cells exhibit elevated AMPK activity relative to control cells (249). This observation was made in primary cells obtained from CF patients and in an immortalized CF cell culture model. Absence of functional CFTR at the plasma membrane resulted in increased AMPK activity, and more diffuse distribution of AMPK distribution in the cell. CFTR reciprocally inhibits AMPK. The study also demonstrated that the presence or absence of CFTR had no effect on intracellular AMP and ATP concentrations (249). However, no mechanism explaining increased AMPK activity in CF cells was reported. Chapter II of this dissertation will discuss the mechanism causing increased AMPK activity in CF.

As described above, AMPK regulates a number of lipid-metabolizing pathways, which raises the possibility that altered PUFA composition in CF is a result of increased AMPK activity. Indeed, some of the observed PUFA metabolic alterations in CF cells are consistent with increased AMPK activity. Retroconversion, an abbreviated form of β-oxidation in which DHA is converted to EPA, is elevated in CF cells (164, 194). CF cells also exhibit increased cellular uptake of LA and DHA (54). However, no prior work has examined the effects of AMPK on PUFA biosynthesis. The work described in Chapter II and Chapter III of this dissertation is the first investigation of the impact of AMPK on PUFA biosynthesis.

Chapter II

ABNORMAL N-6 POLYUNSATURATED FATTY ACID METABOLISM IN CYSTIC FIBROSIS IS CAUSED BY ACTIVATION OF AMP-ACTIVATED PROTEIN KINASE[1]

Introduction

Cystic fibrosis (CF) is a common inherited disease primarily affecting the pulmonary, gastrointestinal, endocrine, and reproductive systems, leading to significant morbidity and mortality (58). CF is caused by mutations in the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR) (84), a cyclic AMP-activated anion channel located in the apical membrane of epithelial cells (87). Among the myriad manifestations of these mutations are consistent alterations in polyunsaturated fatty acid (PUFA) metabolism (57, 150, 250). Consequently, CF patients have characteristic alterations in PUFA composition, including decreased levels of linoleate (LA) and docosahexaenoate (DHA) in blood, which are accompanied by increased arachidonate (AA) in tissues (155, 162). The magnitude of these alterations correlates with disease severity, suggesting a link to pathophysiology (153, 154, 157, 162, 175).

The PUFA alterations associated with CF have been recapitulated in models of CF. Both *CFTR* knockout (183, 251) and ΔF508 (184) mouse models exhibit changes similar to CF patients. A similar pattern is observed in cultured bronchial epithelial cells lacking CFTR (54, 55). These results suggest that PUFA alterations are intrinsically

[1] This chapter has been adapted from the paper: Umunakwe OC and Seegmiller AC. (2014) Abnormal n-6 fatty acid metabolism in cystic fibrosis is caused by activation of AMP-activated protein kinase. *J Lipid Research* **55**(7):1489-1497.

linked to loss of CFTR function. However, until recently, the mechanism of this linkage was largely unknown.

Recent studies have attributed alterations in PUFA levels in CF cells to changes in the activities of PUFA-metabolizing enzymes. This is particularly true for the n-6 PUFA metabolic pathway, which includes conversion of LA to AA through a series of desaturation and elongation reactions. These reactions are catalyzed by Δ6-desaturase (Δ6D), which is rate-limiting, elongase 5 (ELOVL5), and Δ5D-desaturase (Δ5D) (2). Cultured bronchial epithelial cells lacking CFTR exhibit significantly greater expression and activity of both Δ5D and Δ6D, leading to reduced LA levels and increased AA levels, which is typical of CF (56). Furthermore, suppression of Δ5D and Δ6D over-expression by DHA supplementation reverses these PUFA alterations (164).

One potential candidate connecting CFTR mutations with PUFA metabolic enzymes is AMP-activated protein kinase (AMPK). AMPK is a heterotrimeric protein, composed of a catalytic α subunit, and regulatory β and γ subunits, that is sensitive to changes in cellular metabolic status (205). When activated, it promotes net ATP synthesis by regulating a variety of cellular processes, including lipid metabolism. Through phosphorylation of downstream targets, AMPK induces cellular uptake and β-oxidation of fatty acids, and inhibits *de novo* synthesis of saturated and monounsaturated fatty acids (206, 252). While the effect of AMPK on PUFA desaturation and elongation is unknown, there is a clear connection between AMPK and CF. AMPK is part of a macromolecular complex that that interacts with and regulates CFTR activity (253). This complex serves as a scaffold that connects CFTR and other ion channels to a number of signal

transduction networks. Of note, CF bronchial epithelial cells exhibit greater AMPK activity than their wild-type counterparts (249).

Complete activation of AMPK requires phosphorylation of threonine-172 in the α-subunit by upstream kinases. In mammalian cells, the primary AMPK kinases are liver kinase B1 (LKB1) and Ca^{2+}/calmodulin-dependent protein kinase kinase β (CaMKKβ). While LKB1-mediated AMPK phosphorylation is dependent on intracellular AMP concentration, CaMKKβ-mediated AMPK phosphorylation is stimulated by increased intracellular Ca^{2+} concentration (218, 219, 223). AMPK activation in CF bronchial epithelial cells appears to be unrelated to intracellular AMP concentration (249). However, CF bronchial epithelial cells are known to exhibit aberrant calcium homeostasis and increased Ca^{2+} signaling (254, 105, 255, 106), suggesting that CaMKKβ may mediate the observed increase in AMPK activity.

In the present study we investigated the potential role of increased AMPK activity in altered PUFA metabolism in CF bronchial epithelial cells. Specifically, we tested the hypotheses that AMPK activity is enhanced in CF cells due to a Ca^{2+}-dependent increase in phosphorylation of AMPK by CaMKKβ, and that increased AMPK activity leads to increased fatty acid desaturase expression and activity.

Materials and Methods

Materials

STO-609 was obtained from EMD Millipore (Billerica, MA) and dissolved in 100 mM NaOH. BAPTA-AM was obtained from Abcam (Cambridge, MA) and dissolved in DMSO. EDTA was obtained from Mediatech (Manassas, VA). Dorsomorphin

dihydrochloride (Compound C) and 5-aminoimidazole-4-carboxamide-1-β-4-ribofuranoside (AICAR) were obtained from Tocris Bioscience (Minneapolis, MN) and dissolved in water. Fura-2, AM was obtained from Life Technologies (Grand Island, NY). Rabbit monoclonal antibodies for detection of human AMPKα, phospho-AMPKα (T172), ACC, and phospho-ACC (S79) were obtained from Cell Signaling Technology (Beverly, MA). Polyclonal goat anti-rabbit secondary antibody was obtained from Abcam. Mouse monoclonal antibody for detection of human β-actin was obtained from Sigma-Aldrich (St. Louis, MO). Polyclonal sheep anti-mouse secondary antibody was obtained from GE Healthcare Life Sciences (Pittsburgh, PA). Radioactively labeled [1-^{14}C]18:2n-6 (55 mCi/mmol) was purchased from American Radiolabeled Chemicals (St. Louis, MO). Fatty acid methyl ester standards (18:2n-6, 18:3n-6, 20:2n-6, 20:3n-6, 20:4n-6, 22:4n-6, 22:5n-6) were purchased from NuChek Prep (Elysian, MN). HPLC grade solvents were purchased from Fisher Scientific (Pittsburgh, PA) and IN-flow 2:1 liquid scintillation cocktail was purchased from IN/US Systems (Tampa, FL).

Cell Culture

16HBEo$^-$ sense and antisense cells were a gift from Dr. Pamela Davis (Case Western Reserve University School of Medicine, Cleveland, OH). IB3-1 and C38 cells were obtained from ATCC (Manassas, VA). Cells were grown in tissue culture flasks pre-coated with LHC Basal media (Invitrogen, Carlsbad, CA) containing 0.1 mg/mL BSA (Sigma-Aldrich), 10 µg/mL human fibronectin (Sigma-Aldrich), and 3 µg/mL vitrogen (Angiotech Biomaterials, Palo Alto, CA). Complete culture medium consisted of Minimum Essential Medium + Glutamax (Invitrogen) supplemented with 100 µg/mL

streptomycin, 100 U/mL penicillin, and 10% horse serum (Atlanta Biologicals, Lawrenceville, GA). Cells were grown at 37ºC in a 5% CO_2 humidified incubator. Medium was changed three times weekly. Experiments were performed after cells reached 100% confluence.

SDS-PAGE and Immunoblotting

Total protein was isolated from cells using RIPA buffer (Sigma-Aldrich) and 2X Halt Protease and Phosphatase Inhibitor Cocktail (Thermo Scientific, Waltham, MA). Protein concentrations were determined by BCA assay (Thermo Scientific). Protein samples were mixed 1:1 with 2X Laemmli Sample Buffer (Bio-Rad Laboratories, Hercules, CA) and boiled for 5 min. Then, volumes equivalent to 15-25 μg of protein were loaded into pre-cast 4-20% gradient polyacrylamide gels (Bio-Rad). After electrophoresis, protein was transferred onto Immobilon-P PVDF membranes (EMD Millipore). Membranes were blocked using 5% (w/v) Blotting Grade Blocker (Bio-Rad) in TBS-Tween (Sigma-Aldrich). After antibody incubations, protein bands were detected using SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific). Membranes were exposed to Amersham Hyperfilm ECL film (GE Healthcare). Films were scanned and densitometry was performed using Image J analysis software (NIH). β-actin was used as a loading control. For repeat immunoblotting, membranes were stripped using Restore Western Blot Stripping Buffer (Thermo Scientific).

Fura-2 AM Assay

Cells were cultured to confluence in 96-well plates. A 1 mM stock solution of Fura-2 AM in DMSO was diluted to 1 μM in Hank's Balanced Salt Solution (HBSS). To load cells with Fura-2 AM, cells were washed with HBSS then incubated in 1 μM Fura-2 AM for 60 minutes 37°C. After incubation, cells were washed with HBSS then incubated in HBSS for 30 additional minutes. Fluorescence emission at 510 nm was measured with excitation wavelengths of 340 and 380 nm. Untreated cells were used for autofluorescence correction. 340/380 nm fluorescence excitation ratios were determined as a relative measure of intracellular Ca^{2+} concentrations.

Quantitative Real-Time PCR

Specific primers for quantification of mRNA from *FADS1* (Δ5D), *FADS2* (Δ6D), *ELOVL5*, and *RPLP0* genes were described previously (56). Total RNA was isolated from cells using TRIzol (Invitrogen) according to the manufacturer's instructions. Contaminating DNA was removed from the RNA samples using DNA-free (Ambion, Austin, TX) according to manufacturer's instructions. cDNA was synthesized from 1 μg of total RNA using iScript cDNA synthesis kit (Bio-Rad). Quantitative real-time PCR (qRT-PCR) was performed in 10 μL reactions containing 50 ng cDNA, 156 nM forward and reverse primers, and 1X iTaq Universal SYBR Green (Bio-Rad) in 96-well plates. Each reaction was performed in duplicate. C_t values were determined using the CFX96 Real Time PCR Detection System with CFX Manager software (Bio-Rad). Relative mRNA levels were calculated using the comparative C_t method with *RPLP0* as a reference gene.

Desaturase Activity Assay

Confluent cells were incubated in Minimum Essential Medium containing 10% reduced-lipid fetal bovine serum (Hyclone, Logan, UT) and 4.7 μM of [1-^{14}C]LA (18:2n-6) for 4 hours. Cells were then washed and incubated an additional 20 hours in complete medium. Cells were scraped on ice and pelleted by centrifugation, then resuspended in 0.5 mL PBS. Lipids were extracted using a modified method of Folch *et al.* (256). Briefly, lipids were extracted by addition of 3 mL chloroform-methanol (2:1 v/v). After centrifugation, the organic phase was separated and dried under nitrogen. Fatty acids were methylated by adding 0.5 ml of 0.5 N methanolic NaOH (Acros Organics, Geel, Belgium) and then heated at 100ºC for 3 minutes, followed by addition of 0.5 ml BF_3 and heating at 100ºC for 1 minute. The resulting fatty acid methyl esters were extracted into 1 mL of hexane, followed by addition of 6.5 mL of water saturated with NaCl. After centrifugation, the hexane layer was retrieved and dried completely under nitrogen.

For HPLC analysis, fatty acid methyl esters were dissolved in 50 μL of acetonitrile, and 20 μL was injected into an HPLC instrument (Agilent 1200 series; Agilent Technologies, Santa Clara, CA) equipped with an Agilent Zorbax Eclipse XDB-C18 column, 4.6 X 250 mm, 5 μm. A guard column of 4.6 X 12.5 mm, 5 μm was used in conjunction with the analytical column. The fatty acids were separated using a binary solvent system. Solvent A consisted of HPLC grade H_2O with 0.02 wt. % H_2PO_4, and solvent B was 100% HPLC grade acetonitrile. The solvent program started with 42% solvent A and 58% solvent B for 25 min, followed by a linear gradient from 58% to 61% solvent B over 2 min, a hold for 8 min, another linear gradient from 61% to 100% solvent

B over 15 min, and a hold for 20 min, followed by reconstitution of the original conditions. The flow rate was 1 mL/min. Peaks were detected by ultraviolet absorbance at 205 nm and identified by comparison with retention times of unlabeled fatty acid methyl ester standards. Radioactivity from ^{14}C-labeled fatty acid methyl esters was measured with a scintillation detector (β-RAM Model 4, IN/US Systems) coupled to the HPLC. The counting efficiency of this detector is 90% for ^{14}C with 5 CPM background.

Statistical Analysis

Statistical differences between groups were evaluated by the Mann-Whitney test using STATA or by two-way ANOVA followed by Tukey's Honestly Significant Difference post-hoc test for multiple comparisons using R (R Foundation for Statistical Computing, Vienna, Austria).

Results

AMPK activity was studied in two cell culture models known to exhibit CF-related changes in PUFA composition (55, 56). The first was 16HBEo$^-$ bronchial epithelial cells stably transfected with plasmids expressing an oligonucleotide sequence complementary to CFTR in either the sense or antisense orientation (257). Cells transfected with the sense oligonucleotide (S cells) maintain normal CFTR expression, while CFTR expression is silenced in cells transfected with the antisense oligonucleotide (AS cells) (55, 257). The second model, IB3-1, was derived from the bronchial epithelium of a CF patient with a ΔF508/W1282X CFTR genotype (258). The isogenic

control cell line, C38, was generated by stable transfection of normal CFTR cDNA into IB3-1 cells.

Protein levels of phosphorylated AMPK (pAMPK) and total AMPK were measured by immunoblotting to determine relative AMPK activation in CF and control cells. In both cell models, pAMPK levels were significantly greater in CF (AS or IB3-1) cells than in the corresponding controls (S or C38 cells). There was no significant difference in total AMPK protein levels between CF and control cells. Accordingly, the pAMPK/AMPK ratio was significantly greater in CF cells than in control cells (Figure 7). AMPK activity was assessed by measuring phosphorylation of acetyl-CoA carboxylase (ACC), which is phosphorylated and inhibited by AMPK (205). In both cell models, phosphorylated ACC (pACC) levels and pACC/ACC ratios were significantly greater in CF than control cells, indicative of increased pAMPK activity (Figure 7).

To test the hypothesis that increased AMPK activity in CF cells results from increased CaMKKβ activity, cells were treated with STO-609, a specific inhibitor of CaMKKs (259). Treatment with STO-609 at two different concentrations caused a significant decline in pAMPK and pACC levels in CF cells only. There was no significant effect on total AMPK or ACC protein levels. Accordingly, pAMPK/AMPK and pACC/ACC ratios declined considerably in CF cells treated with STO-609, indicating decreased activation of AMPK to the level seen in control cells (Figure 8).

Since CaMKKβ must bind Ca^{2+}/calmodulin in order to phosphorylate AMPK, CaMKKβ-dependent AMPK phosphorylation is influenced by intracellular Ca^{2+} concentration (226). Thus, to investigate the role of Ca^{2+} signaling, we treated cells with a combination of EDTA to chelate extracellular Ca^{2+} and BAPTA-AM to chelate

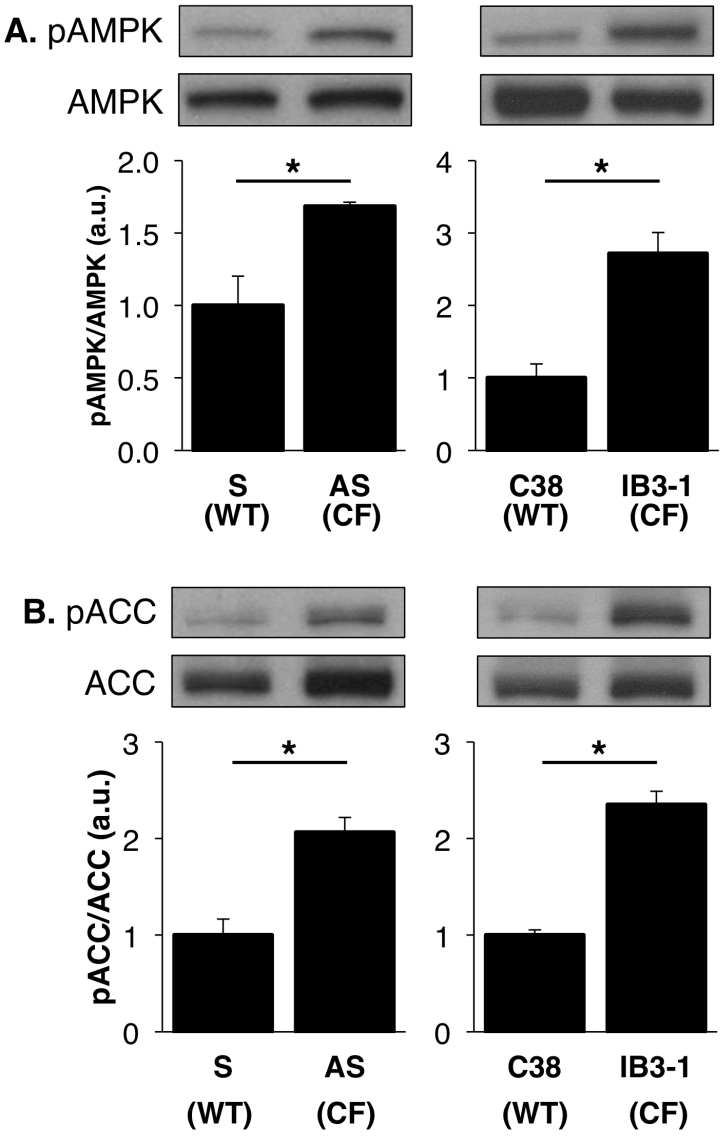

Figure 7 AMPK activity is increased in CF cells
Protein was isolated from CF (AS or IB3-1) and control (S or C38) cells two days post confluence as described in Materials and Methods. Phosphorylated (pAMPK) and total AMPK (**A**) and phosphorylated (pACC) and total ACC (**B**) were detected in all cell types by immunoblotting. Autoradiographs from representative immunoblots are shown. Autoradiographs were scanned and the relative intensity of each band was measured by densitometry. Bar graphs represent the mean ratio of pAMPK/AMPK or pACC/ACC as fold change relative to control cells. Data are presented as mean ± SEM (n=3) and are representative of at least three independent experiments. *, $P<0.05$ by Mann-Whitney test.

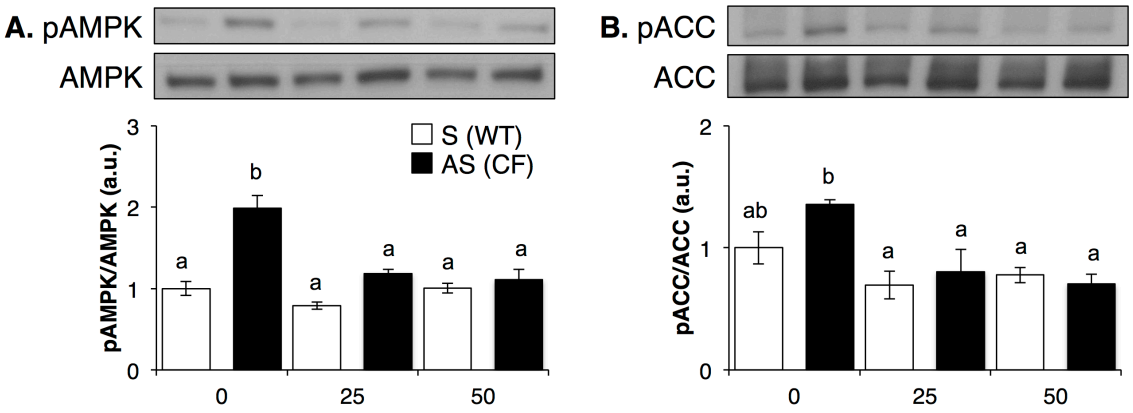

Figure 8 Activation of CaMKKβ causes elevated AMPK activity in CF cells

Post-confluent control (S) and CF (AS) cells were treated with STO-609 at the indicated concentrations for 24 hours. Protein was isolated and immunoblotting performed using antibodies for pAMPK and total AMPK (**A**) and for pACC and total ACC (**B**). Autoradiographs from representative immunoblots are shown. Autoradiographs were scanned and the relative intensity of each band was measured by densitometry. Bar graphs represent the mean ratio of pAMPK/AMPK or pACC/ACC as fold change relative to control cells. Data are presented as mean ± SEM (n=3) and are representative of at least three independent experiments. Statistical significance was determined by two-way ANOVA with Tukey's HSD post-test for pairwise comparisons. Unlike letters denote significant differences (P<0.05) in pairwise comparisons.

intracellular Ca^{2+}. We assayed relative intracellular Ca^{2+} concentrations in CF and WT cells using the cell-permeable ratiometric fluorescent calcium indicator Fura-2, AM. The excitation wavelength causing maximum Fura-2 fluorescence emission at 510 nm shifts from approximately 380 nm to 340 nm as the Ca^{2+} concentration increases (Figure 9). Thus, the 340/380 nm excitation ratio for Fura-2 AM is a relative measure of intracellular Ca^{2+} concentration. CF cells exhibited elevated intracellular Ca^{2+} concentration compared to WT cells, as evidenced by a significantly greater 340/380 nm fluorescence excitation ratio. Ca^{2+}chelation with EDTA and BAPTA-AM reduced intracellular Ca^{2+} concentrations in CF and WT cells, and had a more prominent effect in CF cells (Figure 9). Similar to the effect of STO-609, Ca^{2+} chelation caused reductions in AMPK and ACC phosphorylation, with a more pronounced effect in CF cells (Figure 10).

Modulators of AMPK activity were used to determine the role of AMPK activation of PUFA metabolism. Compound C (dorsomorphin dihydrochloride) is an inhibitor of AMPK that acts by binding directly to the kinase domain of the catalytic AMPKα subunit (260, 261). Accordingly, Compound C treatment reduced pACC levels in CF and control cells, indicative of decreased AMPK activity (Figure 11A). As previously described (56, 164), vehicle-treated CF cells exhibited increased expression of both Δ6D and Δ5D compared with controls, as measured by qRT-PCR. However, CF cells treated with Compound C exhibited a significant dose-dependent decline in Δ6D mRNA levels, such that they were equivalent to control cells at the highest dose tested. There was an even more dramatic decline in Δ5D mRNA levels after Compound C treatment that was seen in CF and control cells alike (Figure 11B,C). Compound C treatment had no effect on ELOVL5 expression (not shown).

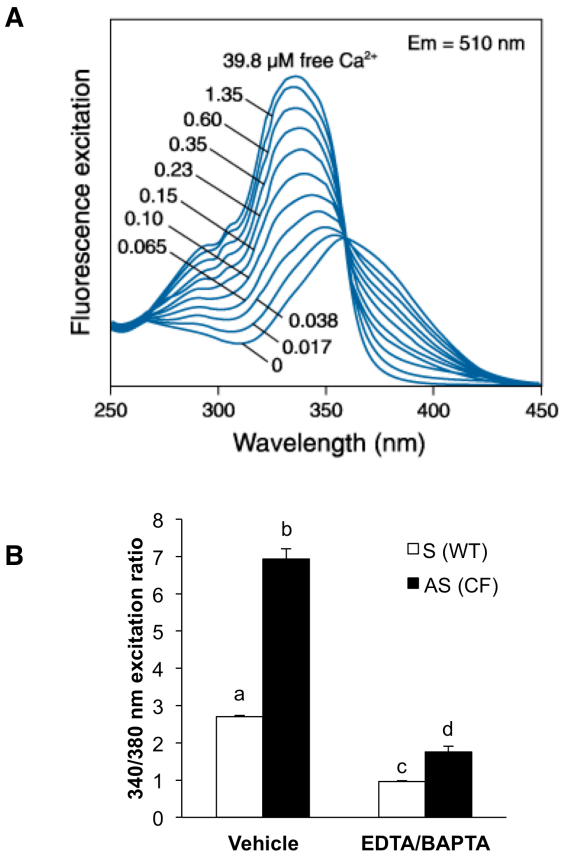

Figure 9 Intracellular Ca^{2+} concentration is elevated in CF cells
Fura-2 AM assay was performed on post-confluent cells as described in Materials and Methods. (**A**) Fura-2 AM fluorescence excitation spectra from *The Molecular Probes Handbook* (262). (**B**) Fluorescence emission at 510 nm was measured with excitation wavelengths of 340 and 380 nm. Bar graphs represent mean 340/380 nm fluorescence excitation ratios. Data are presented as mean ± SEM (n=6) and are representative of two independent experiments. Statistical significance was determined by two-way ANOVA with Tukey's HSD post-test for pairwise comparisons. Unlike letters denote significant differences (P<0.05) in pairwise comparisons.

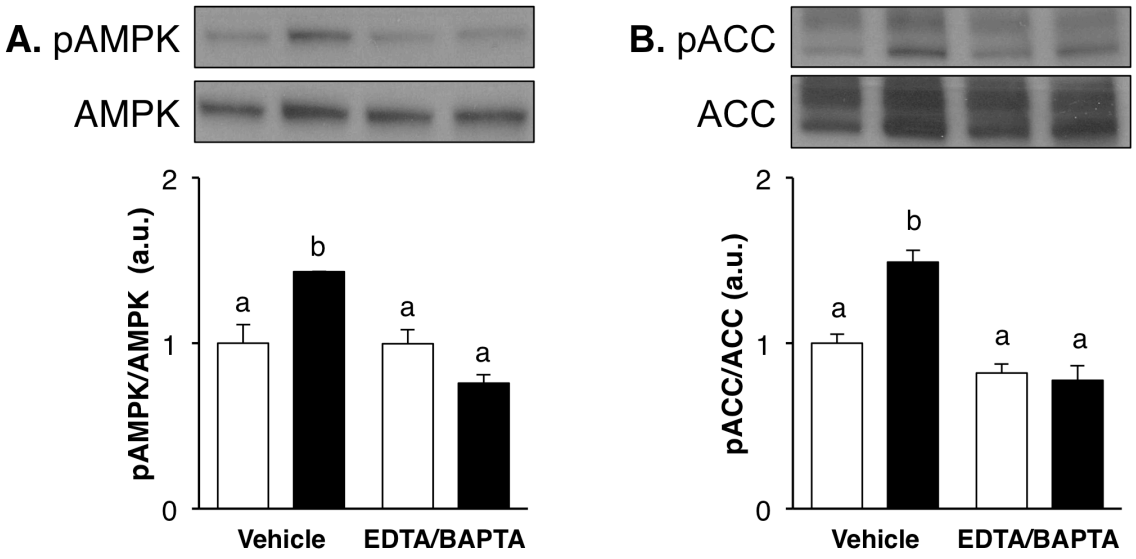

Figure 10 Calcium-dependent activation of CaMKKβ causes elevated AMPK activity in CF cells

Post-confluent control (S) and CF (AS) cells were treated EDTA (1 mM) and BAPTA-AM (100 μM) for 24 hours. Protein was isolated and immunoblotting performed using antibodies for pAMPK and total AMPK (**A**) and for pACC and total ACC (**B**). Autoradiographs from representative immunoblots are shown. Autoradiographs were scanned and the relative intensity of each band was measured by optical densitometry. Bar graphs represent the mean ratio of pAMPK/AMPK or pACC/ACC as fold change relative to control cells. Data are presented as mean ± SEM (n=3) and are representative of at least three independent experiments. Statistical significance was determined by two-way ANOVA with Tukey's HSD post-test for pairwise comparisons. Unlike letters denote significant differences ($P<0.05$) in pairwise comparisons.

Previous studies indicated that increased Δ6D and Δ5D mRNA levels in CF cells correlate with increased desaturase activity (56, 164). Because Δ6D is rate-limiting, the metabolism of [^{14}C]-labeled LA to [^{14}C]-labeled AA can be used as a measure of desaturase activity. As seen in previous studies, vehicle-treated CF cells displayed greater LA to AA metabolism when compared to control cells. This was indicated by increased detection of labeled AA and reduced detection of labeled LA resulting in an elevated AA/LA ratio in CF cells relative to control cells. Treatment with Compound C resulted in increased LA and decreased AA levels, reducing the AA/LA ratio. Importantly, this treatment also eliminated the significant differences observed between vehicle-treated CF and control cells (Figure 11D).

The opposite effect was observed when cells were treated with the AMPK activator 5-aminoimidazole-4-carboxamide-1-β-4-ribofuranoside (AICAR). When phosphorylated within cells, AICAR becomes ZMP, an AMP-analog that increases AMPK phosphorylation and activity (263). Treatment with AICAR increased pAMPK and pACC levels in CF and control cells, indicative of AMPK activation (Figure 12A). As expected, this treatment caused a significant increase in both Δ6D and Δ5D mRNA levels in CF and control cells (Figure 12B,C). Similar to Compound C, there was no effect of ELOVL5 expression (not shown). However, despite these changes, AICAR did not increase the rate of LA to AA metabolism (Figure 12D). This may be due to the inhibitory effect of AICAR on ACC. This would be expected to reduce levels of malonyl CoA, the product of ACC and a necessary substrate for the ELOVL5 step in LA to AA metabolism.

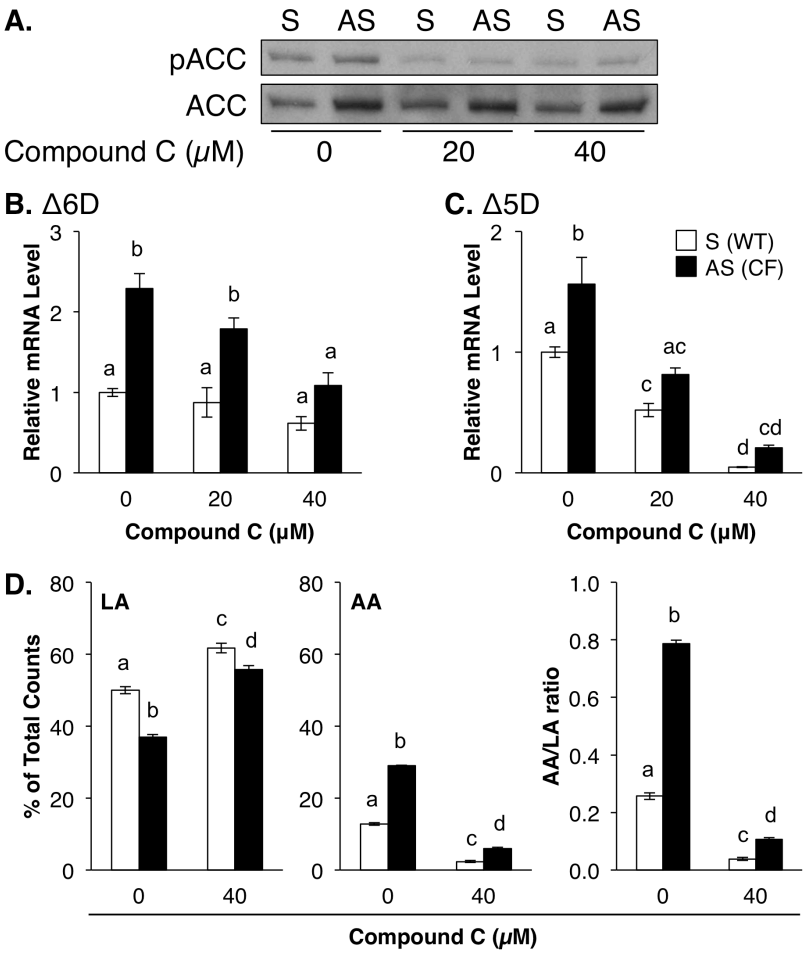

Figure 11 AMPK inhibition with Compound C reduces desaturase mRNA levels and activity

(A) Post-confluent control (S) and CF (AS) cells were treated with Compound C at the indicated concentrations for 30 minutes prior to protein isolation and immunoblotting using antibodies for pACC and total ACC. Autoradiographs from representative immunoblots are shown. **(B, C)** Control and CF cells were treated with Compound C at the indicated concentrations for 24 hours prior to RNA isolation. Relative Δ6D **(B)** and Δ5D **(C)** mRNA levels were determined by qRT-PCR. Data are shown as mean ± SEM (n=4) and are representative of at least three independent experiments. **(D)** Control and CF cells were treated with 40 μM Compound C for 24 hours, then incubated in medium containing [^{14}C]-LA for 4 hours. Cells were then treated with Compound C in medium without [^{14}C]-LA for 20 additional hours. Conversion of [^{14}C]-LA to [^{14}C]-AA was measured by HPLC. Bar graphs indicate the percent of total counts detected in LA or AA peaks and as the ratio of AA/LA. Data are shown as mean ± SEM (n=3) and are representative of at least three independent experiments. Statistical significance was determined by two-way ANOVA with Tukey's HSD post-test for pairwise comparisons. Unlike letters denote significant differences (P<0.05) in pairwise comparisons.

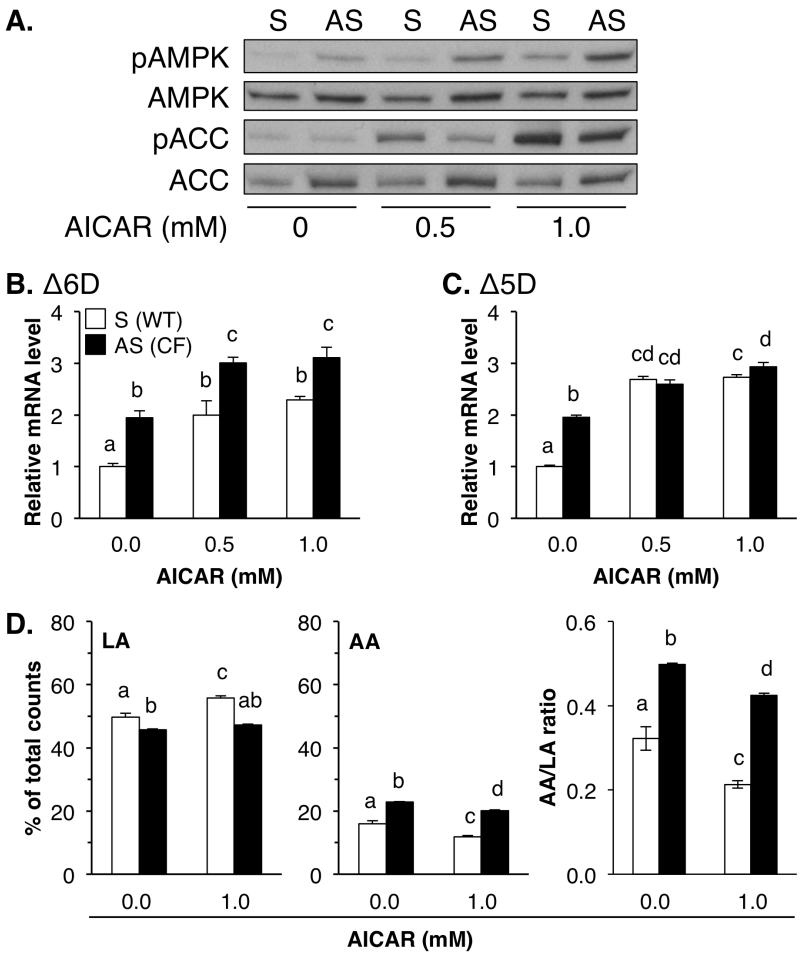

Figure 12 AMPK stimulation with AICAR increases desaturase mRNA levels

(A) Post-confluent control (S) and CF (AS) cells were treated with AICAR at the indicated concentrations for 30 minutes prior to protein isolation and immunoblotting using antibodies for pAMPK, total AMPK, pACC, and total ACC. Autoradiographs from representative immunoblots are shown. **(B, C)** Control and CF cells were treated with AICAR at the indicated concentrations for 24 hours prior to RNA isolation. Relative Δ6D **(B)** and Δ5D **(C)** mRNA levels were determined by qRT-PCR. Data are shown as mean ± SEM (n=3) and are representative of at least three independent experiments. **(D)** Control and CF cells were treated with 1 mM AICAR for 24 hours, then incubated in medium containing [^{14}C]-LA for 4 hours. Cells were then treated with AICAR in medium without [^{14}C]-LA for 20 additional hours. Conversion of [^{14}C]-LA to [^{14}C]-AA was measured by HPLC. Bar graphs indicate the percent of total counts detected in LA or AA peaks and as the ratio of AA/LA. Data are shown as mean ± SEM (n=3) and are representative of at least three independent experiments. Statistical significance was determined by two-way ANOVA with Tukey's HSD post-test for pairwise comparisons. Unlike letters denote significant differences (P<0.05) in pairwise comparisons.

The role of the AMPK pathway in LA to AA metabolism was confirmed by inhibiting CaMKKβ. Treatment with STO-609, which reduced AMPK activity, caused significant declines in both Δ6D and Δ5D mRNA levels in CF cells, which were more pronounced in CF cells (Figure 13A,B). Accordingly, treatment with STO-609 reduced LA to AA metabolism to control cell levels (Figure 13C). Ca^{2+} chelation with EDTA and BAPTA-AM caused similar effects, reducing Δ6D and Δ5D mRNA levels in CF cells to that of control cells (Figure 14A,B). Treatment with EDTA/BAPTA also reduced LA to AA metabolism, but in CF cells only (Figure 14C).

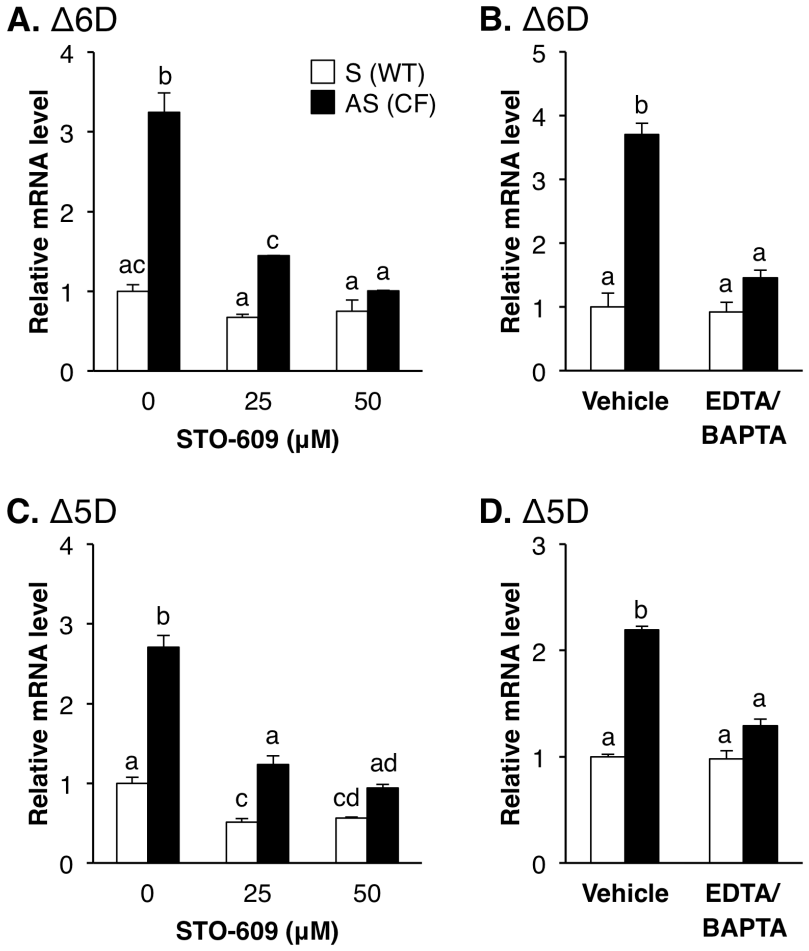

Figure 13 CaMKKβ inhibition reduces desaturase mRNA expression
Post-confluent control (S) and CF (AS) cells were treated with STO-609 at the indicated concentrations **(A, C)** or EDTA (1 mM) and BAPTA-AM (100 μM) **(B, D)** for 24 hours prior to analysis. RNA was isolated and relative Δ6D **(A, B)** and Δ5D **(C, D)** mRNA levels were determined by qRT-PCR. Data are shown as mean ± SEM (n=3) and are representative of at least three independent experiments. Statistical differences were determined by two-way ANOVA with Tukey's HSD post-test for pairwise comparisons. Unlike letters denote significant differences ($p < 0.05$) in pairwise comparisons.

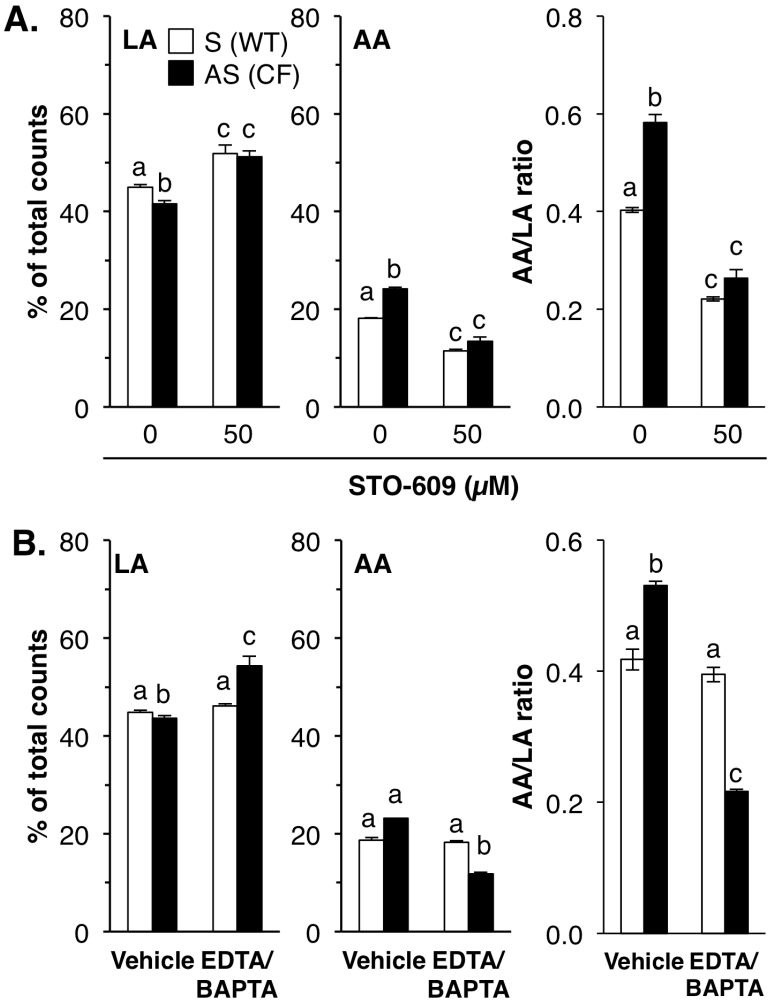

Figure 14 CaMKKβ inhibition reduces desaturase activity
Post-confluent control (S) and CF (AS) cells were treated with **(A)** STO-609 at the indicated concentrations or **(B)** EDTA (1 mM) and BAPTA-AM (100 μM) for 24 hours prior to analysis. After treatment, cells were incubated in medium containing [^{14}C]-LA for 4 hours. Cells were washed and then incubated in medium without [^{14}C]-LA for 20 additional hours with STO-609 or EDTA/BAPTA-AM. Conversion of [^{14}C]-LA to [^{14}C]-AA was measured by HPLC. Bar graphs indicate the percent of total counts detected in LA or AA peaks and as the ratio of AA/LA. Data are shown as mean ± SEM (n=3) and are representative of at least three independent experiments. Statistical significance was determined by two-way ANOVA with Tukey's HSD post-test for pairwise comparisons. Unlike letters denote significant differences (P<0.05) in pairwise comparisons.

Discussion

Many studies have documented the consistent alterations in PUFA levels in the blood and tissues of CF patients and the potential role these alterations play in disease pathophysiology [reviewed in (57, 150, 250)]. However, the connection between mutations in the *CFTR* gene and changes in PUFA metabolism has remained elusive. This is the first study to elucidate a clear mechanistic pathway between these seemingly disparate observations. A schematic overview of these findings is presented in Figure 15.

This study demonstrates increased phosphorylation and activity of AMPK in two different CF bronchial epithelial cell culture models. A number of studies have confirmed alterations in PUFA metabolism in these cell lines (54–56, 164, 187, 264). These cell lines differ in their mechanism of CFTR silencing, one using antisense RNA to block CFTR translation (257), while the other carries the ΔF508 mutation that blocks transit of functional protein to the cell surface (258). That both cell lines exhibit similar activation of AMPK implies that absence of functional CFTR protein at the cell surface is responsible for the AMPK activation. These results confirm those of a prior study indicating increased AMPK phosphorylation and activity in primary bronchial epithelial cells from CF patients (249). However, another study that transiently disrupted CFTR expression using RNA interference in an intestinal epithelial cell line did not observe a difference in AMPK activity (265). This difference may be attributable to the differences in cell type and mechanism of CFTR silencing.

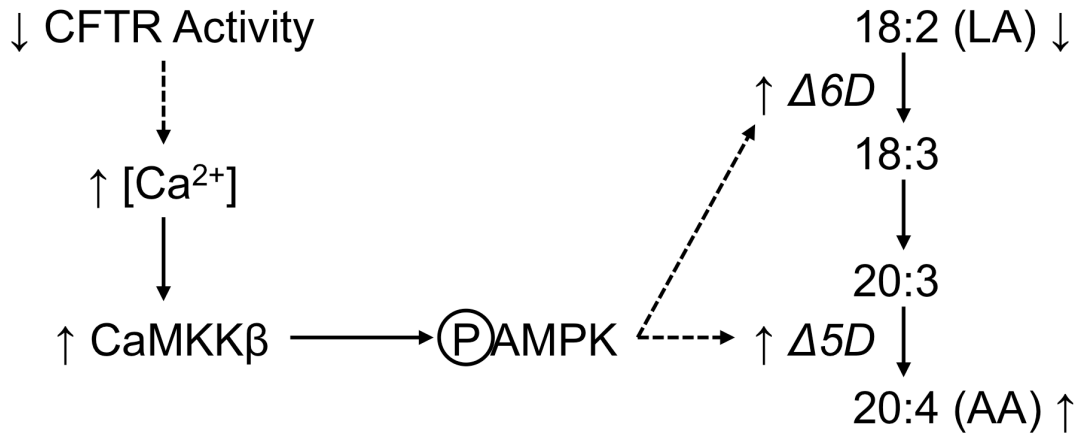

Figure 15 Schematic of mechanism linking loss of CFTR to elevated desaturase activity

Loss of CFTR function due to mutation causes aberrant Ca^{2+} metabolism leads to increased intracellular Ca^{2+} concentrations. This activates CaMKKβ, which phosphorylates and activates AMPK. Increased AMPK activity indirectly stimulates expression and consequently, activity, of Δ6D and Δ5D. This increases LA to AA metabolism, which results in the characteristic PUFA abnormalities observed in CF. Direct effects are indicated by solid lines, while indirect effects are indicated by dashed lines.

AMPK is activated by one of two protein kinases, LKB1 or CaMKKβ (218, 219, 223). LKB1 is constitutively active, but its activity is slower than that of protein phosphatases that dephosphorylate AMPK, maintaining AMPK in an inactive state. Under conditions of energy deprivation, AMP binds to AMPK and induces a conformational change that inhibits dephosphorylation, shifting equilibrium towards active pAMPK (252). A previous study showed that there is no elevation in AMP/ATP ratios in CF bronchial epithelial cells, suggesting that differences in the LKB1 activation pathway are not be responsible for increased pAMPK in CF cells (249). Instead, differences in AMPK activation in CF cells are more likely to arise from differential activation of CaMKKβ. Indeed, the current study demonstrates that inhibition of CaMKKβ using either a small molecule inhibitor STO-609 or by Ca^{2+} chelation reduced activation of AMPK and normalized expression of fatty acid desaturases in CF cells to levels seen in control cells. We presume that the remaining AMPK activity after CaMKKβ inhibition was due to constitutive LKB1-dependent AMPK activation, which did not appear to differ between CF and control cells.

These findings are bolstered by numerous studies showing abnormal Ca^{2+} metabolism in CF cells. Endoplasmic reticulum Ca^{2+} stores are increased in CF (266, 267). Store-operated Ca^{2+} entry is increased in CF cells due to increased plasma membrane expression of Orai1, a Ca^{2+} release-activated calcium channel (106). There is also evidence for elevated TRPC6-mediated calcium influx in CF cells (105), and studies have noted increased Ca^{2+} signaling in response to external stimuli including purine nucleotides, bradykinin, and cytokines (267–269).

While the role of AMPK in lipid metabolism has been studied extensively, to our knowledge, no previous study has connected AMPK with PUFA desaturation and elongation. Previous reports have shown that increased expression and activity of fatty acid desaturases contribute to the alterations in PUFA composition seen in CF cells (56, 164). The current study demonstrates that both direct inhibition of AMPK with Compound C and indirect inhibition by blocking CaMKKβ reduce Δ6D and Δ5D mRNA levels and activity. Notably, diminution of AMPK activity significantly reduced or eliminated differences in desaturase expression and activity between CF and control cells. Conversely, AMPK stimulation with AICAR increased Δ6D and Δ5D mRNA levels. However, AICAR failed to stimulate LA to AA metabolism. As indicated above, this may be due to reduction in levels of malonyl-CoA, a necessary substrate fore elongation reactions (231, 270). Because AMPK phosphorylates and inhibits ACC, which catalyzes the production of malonyl-CoA, supraphysiogical activation of AMPK by AICAR may reduce malonyl-CoA levels to the extent that the ELOVL5 step becomes rate-limiting.

The mechanism by which AMPK induces Δ6D and Δ5D expression and activity is not known, but there are a number of potential mechanisms. AMPK has been shown to phosphorylate and activate PGC-1α, a coactivator of PPARα (237, 239, 240). Activation of PPARα has been shown to stimulate Δ6D expression and activity by binding to a PPAR response element in its promoter (12). AMPK can also alter gene expression by histone modification. AMPK can stimulate transcription by phosphorylating and inhibiting a subset of histone deacetylases (HDACs) (271, 272), as well as by directly phosphorylating histone H2B (273). Interestingly, altered HDAC activity has been

observed in CF cells (274, 275). Whether AMPK induces Δ6D and Δ5D expression and activity through one of these mechanisms will need to be examined experimentally.

The present study focuses on bronchial epithelial cells. However CF-related PUFA alterations have been observed in multiple CFTR-expressing tissues and in plasma of both model organisms and patients (162, 183). This has been connected to increased Δ6D and Δ5D mRNA expression in the lung and intestinal epithelium of CF mice (Njoroge, S., Laposata, M., and Seegmiller, A. C., unpublished observation). Although AMPK activity has not been measured in other CF tissues, it is possible that AMPK activation is responsible for PUFA alterations in other tissues. Alternatively, it is possible that pulmonary epithelium is a major contributor to PUFA alterations in blood and other tissues. For example, Witters *et al*. recently reported that lung transplantation appeared to correct plasma PUFA alterations in CF patents (174).

Finally, the findings in the present study raise the possibility that the AMPK pathway could be a therapeutic target in CF. Studies in a CF mouse model indicate that correction of the PUFA alterations by dietary supplementation which large doses of DHA can ameliorate CF-related pathology (183). However, replicating this result in human studies has been challenging (150). With demonstration that AMPK plays a role in altered PUFA metabolism in CF, it is conceivable that interventions targeting the AMPK signaling pathway either alone or as an adjuvant to PUFA supplementation may have therapeutic benefit in CF patients.

Chapter III

Δ6-DESATURASE PROMOTER ACTIVITY IS ELEVATED IN CYSTIC FIBROSIS CELLS

Introduction

Cystic fibrosis (CF) is one of the most common autosomal recessive diseases. CF affects the respiratory, digestive, integumentary, and reproductive systems. Chronic obstructive pulmonary disease is the leading cause of morbidity and mortality in CF patients (58). CF is caused by mutations in the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR) (84), a cyclic AMP-activated anion channel located in the apical membrane of epithelial cells (87).

An incompletely explained CF manifestation is altered polyunsaturated fatty acid (PUFA) metabolism (57, 150, 250). CF patients have well-described characteristic alterations in PUFA composition that include decreased levels of linoleic acid and docosahexaenoic acid in blood and tissues, and increased levels of arachidonic acid (AA) and AA-derived inflammatory mediators (155, 162). The magnitude of these alterations correlates with disease severity, suggesting a link to pathophysiology (153, 154, 157, 162, 175). Studies in CF cell culture models indicate that increased expression of Δ6-desaturase (Δ6D), the enzyme that catalyzes the rate-limiting step in long-chain PUFA biosynthesis, is a key contributor to these PUFA alterations (56, 164). We recently reported that increased Δ6D expression is downstream of increased AMP-activated protein kinase (AMPK) activity in CF (276). AMPK is a metabolic regulator that regulates a wide variety of cellular processes (208). However, the mechanism by which increased AMPK activity in CF cells leads to increased Δ6D expression remains unclear.

Δ6D is unique in that both sterol regulatory element binding protein-1 (SREBP-1) and peroxisome proliferator-activated receptor α (PPARα) promote Δ6D transcription (10, 11). SREBP-1 and PPARα are major regulators of fatty acid metabolism and typically induce transcription of mutually exclusive sets of genes. SREBP-1 promotes transcription of genes necessary for fatty acid synthesis while PPARα primarily promotes transcription of genes necessary for fatty acid catabolism (8, 9). Analysis of the Δ6D promoter has revealed two functional binding sites for SREBP-1 (SRE) (7) and a functional binding site for PPARα (PPRE) (12). Whether SREBP-1 or PPARα mediate increased Δ6D expression in CF is unknown.

DHA supplementation reduces Δ6D expression and activity in CF cells and corrects the PUFA alterations (164). Congruently, dietary DHA supplementation corrects PUFA alterations and reduces pathologic features of CF in mouse models (183, 185), raising the possibility that DHA exerts beneficial effects in CF via negative regulation of Δ6D expression. DHA inhibits proteolytic processing of SREBP-1, thus inhibiting SREBP transcriptional activity (29, 31).

In the present study, we tested the hypothesis that increased Δ6D expression in CF results from increased Δ6D promoter activity. Through promoter reporter studies, we determined that Δ6D promoter activity is elevated in CF cells and is potentiated by AICAR, an AMPK activator. Elevated Δ6D promoter activity in CF cells is independent of SREBP-1 and PPARα. However, DHA supplementation suppresses Δ6D promoter activity via inhibition of SREBP-1.

Materials and Methods

Cell Culture

16HBEo- bronchial epithelial cells stably transfected with plasmids expressing an oligonucleotide sequence complementary to CFTR in either the sense or antisense orientation were a gift from Dr. Pamela Davis (Case Western Reserve University School of Medicine, Cleveland, OH) and cultured as previously described (276). Cells transfected with the sense oligonucleotide maintain normal CFTR expression (WT cells), while CFTR expression is silenced in cells transfected with the antisense oligonucleotide (CF cells).

RNA interference and Quantitative real-time PCR

Cells were cultured in MEM + Glutamax (Invitrogen, Carlsbad, CA) containing 10% horse serum (Atlanta Biologicals, Lawrenceville, GA) and no antibiotics in 6-well plates. Twenty-four hours after seeding, cells were transfected with 600 pmol of scrambled siRNA or siRNA targeting SREBP-1 or PPARα (Life Technologies, Grand Island, NY) using Lipofectamine RNAiMAX (Life Technologies). The transfection was repeated 3 days later. Seven days post-seeding, total RNA was isolated from confluent cells using TRIzol (Invitrogen) reagent, contaminating DNA was eliminated using DNA-free (Ambion, Austin, TX), followed by cDNA synthesis using iScript cDNA synthesis kit (Bio-Rad Laboratory, Hercules, CA). Specific primers for each gene of interest were designed using Beacon Designer software (PREMIER Biosoft International, Palo Alto, CA). The forward and reverse primer sequences are listed in Table 2. Quantitative real-time PCR was performed in 10 μL reactions containing 50 ng cDNA, 156 nM forward

and reverse primers, and 1X iTaq Universal SYBR Green (Bio-Rad) in 96-well plates. C_t values were determined using the CFX96 Real Time PCR Detection System with CFX Manager software (Bio-Rad). Relative mRNA levels were calculated using the comparative C_t method with *RPLP0* as a reference gene.

Table 2 qRT-PCR Primers

Target Gene	Forward Primer	Reverse Primer
FADS2	GTATTCGGTGCTGGTGATTGTAGGG	GCCAAGCCTAACATCTTCCACAAG
SREBF1a	GCAGATCGCGGAGCCATG	CCAGCATAGGGTGGGTCAAATAGG
SREBF1c	CTGCTGACCGACATCGAAG	GCATAGGTGGGTCAAATAGG
SREBF2	TGACCCTGGGAGAGACATCG	CGCTGCTGCTACCACTAC
PPARA	GGCGAGGATAGTTCTGGAAG	CAGGATAAGTCACCGAGGAG
RPLP0	GACAGACACTGGCAACATTG	ATGGCAGCATCTACAACCC

Δ6D promoter luciferase reporter assay

The Δ6D proximal promoter region -851/-48 (+1 is the translation start site) was amplified from genomic DNA using the primer pair in Table 3. The amplicon was restriction digested with *KpnI and HinDIII* (New England Biolabs, Ipswich MA), then ligated into *KpnI/HinDIII* sites of the pGL4.14 luciferase reporter vector (Promega, Madison, WI) to generate Δ6D promoter luciferase reporter vectors. For promoter truncations, PCR was performed on Δ6D promoter luciferase reporter vectors using the primers pairs listed in Table 3. Restriction digests were performed on the PCR amplicons using *KpnI and HinDIII*, then ligated into *KpnI/HinDIII* sites of the pGL4.14 vector to generate -687/-48, -535/-48, -399/-48, -254/-48, -195/-48, and -119/-48 vectors. SRE mutations were generated using QuikChange II Site-Directed Mutagenesis kit (Agilent Technologies, Santa Clara, CA) and primers pairs listed in Table 3.

Table 3 Primers for synthesis of promoter reporter vectors

Amplification of Δ6D Promoter (-851 to -48 bp)		
Forward Primer		**Reverse Primer**
GAGAGGTACCGGCGCGGACAATGTGGGAT		GAGAAAGCTTCGCGCCGGCTGCTCGCT
Δ6D Promoter Truncations		
	Forward Primer	**Reverse Primer**
-687	GAGAGGTACCGTGCAAACCCCCAGAGCGCC	CGAGTGGGTAGAATGGCGCTGG
-535	GAGAGGTACCGCAGCAGGGCTCGACTCCAC	CGAGTGGGTAGAATGGCGCTGG
-399	GAGAGGTACCTCGCTGTGGAAACTCGGGCG	CGAGTGGGTAGAATGGCGCTGG
-254	GAGAGGTACCGAGGGAGGGGCGGAGAAGG	CGAGTGGGTAGAATGGCGCTGG
-195	GAGAGGTACCCAGGAAGGCAGGGACACTC	CGAGTGGGTAGAATGGCGCTGG
-119	GAGAGGTACCAAAAGCCGAAAGCAAGAG	CGAGTGGGTAGAATGGCGCTGG
Δ6D Promoter Mutations		
	Forward Primer	**Reverse Primer**
Mut1	CAATGGCAGGgaattCGACGCGACCGGATTGGTG	GaattcCCTGCCATTGGCCCAGGAG
Mut2	TGCAGGCGCTgaatTcATCGCTGTGGAAACTCG	gAattcAGCGCCTGCACCAATCCG

Cells were cultured in MEM + Glutamax containing 10% horse serum and no antibiotics in 24-well plates. Twenty-four hours after seeding, cells were co-transfected with 800 ng of the appropriate Δ6D promoter luciferase reporter vector and 200 ng of Renilla luciferase vector pGL4.74 (Promega) using Lipofectamine 2000 (Life Technologies). Where indicated, cells were co- transfected with 100 ng of a vector expressing constitutively active nuclear SREBP-1c (ATCC, Manassas, VA) or a pcDNA3 empty vector control. Seventy-two hours post-transfection, luciferase activity was measured using Dual-Glo Luciferase Assay (Promega), with Renilla luciferase luminescence acting as an internal control.

For AICAR experiments, cells were transfected as described above. Twenty-four hours post-transfection, culture medium was replaced with culture medium containing 1 mM AICAR (Tocris Biosciences, Minneapolis, MN). Dual-Glo Luciferase Assay was performed 48 hours later. Cells were transfected and luciferase activity was assayed as described above.

For DHA supplementation experiments, cells were cultured in DMEM containing 10% reduced lipid fetal calf serum and no antibiotics supplemented with 20 μM DHA for the duration of the experiments. Cells were transfected and luciferase activity was assayed as described above.

PPAR reporter assay

Cells were cultured in MEM + Glutamax containing 10% horse serum and no antibiotics in 96-well plates. PPAR reporter assay kit was obtained from Qiagen (Valencia, CA). The PPRE reporter is a mixture of firefly luciferase under control of PPRE tandem repeats and constitutively expressed Renilla luciferase. The negative control to measure background transcriptional activity consisted of a mixture of non-inducible firefly luciferase under control of a basal promoter element and constitutively expressed Renilla luciferase. The positive control for transfection efficiency consisted of a mixture of constitutively expressed firefly and Renilla luciferase. Cells were transfected with 200 ng of PPRE reporter, negative control, or positive control using Lipofectamine 2000. Twenty-four hours after transfection, cells were treated with fenofibrate, Wy-14643, GW-6471, GW-7467 (Cayman Chemical Company, Ann Arbor, MI), or vehicle. Eighteen hours later, luciferase activity was measured using Dual-Glo Luciferase Assay, with Renilla luciferase luminescence acting as an internal control. PPRE reporter luciferase activity was normalized to negative control luciferase activity.

Statistical analysis

Statistical differences between groups were evaluated one- or two-way ANOVA followed by Tukey's Honestly Significant Difference post-hoc test for multiple comparisons or by unpaired t-tests using R (R Foundation for Statistical Computing, Vienna, Austria).

Results

We sought to determine whether increased Δ6D mRNA levels in CF cells result from increased Δ6D promoter activity. To measure Δ6D promoter activity, we transiently transfected WT and CF cells with Δ6D promoter luciferase reporter vectors. The Δ6D proximal promoter in these studies corresponded to the nucleotide sequence -851 bp from the translation start site. Due to the presence of multiple Δ6D transcription start sites, the translation start site is denoted as +1. Δ6D proximal promoter activity was at least fourfold greater in CF cells than in WT cells (Figure 16).

In an effort to determine factors responsible for increased Δ6D promoter activity in CF cells, we examined promoter activity of truncated forms Δ6D promoter (Figure 16). Deletion of the functional PPRE upon truncation of the Δ6D proximal promoter to -535 bp, caused Δ6D promoter activity to increase by 43% in WT cells, but caused no change in CF cells. This caused the fold change in Δ6D promoter activity in CF cells relative to WT cells to decline significantly. Truncation to -399 bp, which eliminates the two functional SREs, caused a 20% decline in Δ6D promoter activity in WT cells and a 25% decline in CF cells, but did not change the CF/WT Δ6D promoter activity ratio. Further truncations caused additional declines in promoter activity in both CF and WT cells, but

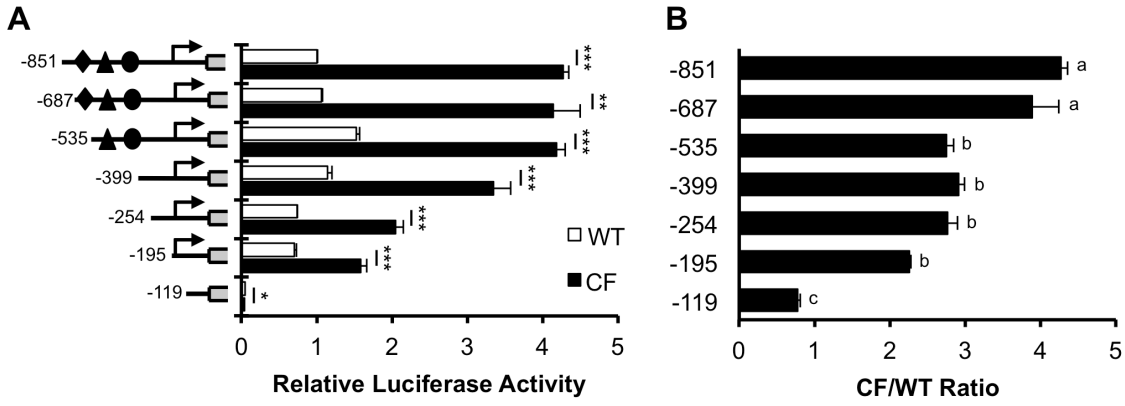

Figure 16 Δ6D promoter activity is elevated in CF cells
(**A**) Relative Δ6D promoter activity in CF and WT cells was measured by luciferase assay. Various Δ6D promoter lengths were tested and are depicted in the diagram. The Δ6D proximal promoter contains a PPRE (diamond), an SRE (triangle), and an E-box like SRE (circle). Data are shown as mean ± SEM (n=3) and are representative of three independent experiments. Unpaired t-tests were performed to compare Δ6D promoter activity between CF and WT cells (*P<0.05, **P<0.01, ***P<0.001). (**B**) Fold change in Δ6D promoter activity in CF cells relative to WT cells. Data are shown as mean ± SEM (n=3) and are representative of three independent experiments. Statistical differences were determined by one-way ANOVA with Tukey's HSD post-test for pairwise comparisons. Unlike letters denote significant differences (P<0.05) in pairwise comparisons amongst all groups.

the CF/WT Δ6D promoter activity ratio remained unchanged. Δ6D promoter activity was equivalent in CF and WT cells only when the promoter was truncated to -119, which virtually eliminated all Δ6D promoter activity in CF and WT cells.

We subsequently assessed the effects of AICAR, an AMPK activator, on Δ6D promoter activity. We have previously shown that AICAR increases AMPK activity and Δ6D mRNA levels and activity in CF and WT cells (276). AICAR significantly increased Δ6D promoter activity in CF and WT cells, with a more pronounced effect in WT cells. Δ6D promoter activity in AICAR-treated WT cells eclipsed that in untreated CF cells. AICAR caused Δ6D promoter activity to increase approximately sevenfold in WT cells and approximately threefold in CF cells. The effect of AICAR on Δ6D promoter activity in CF and WT was not significantly altered by truncations of the Δ6D promoter (Figure 17).

To determine the contribution of SREBP-1 to Δ6D promoter activity in these cells, we mutated the SREs by site-directed mutagenesis. To validate the SRE mutations, we co-transfected cells with a nuclear SREBP-1c expression vector or an empty vector. Overexpression of SREBP-1c increased Δ6D promoter activity in CF and WT cells. Transactivation of the Δ6D promoter by SREBP-1c was abolished when both SREs were mutated (Figure 18A). Mutating either SRE caused baseline Δ6D promoter activity to decline significantly in WT and CF cells (Figure 18B). The extent of the decline was statistically similar in WT and CF cells. There was no additional effect when both sites were mutated.

We further investigated whether SREBP-1 plays a role in increased Δ6D expression in CF cells by measuring the effect of siRNA-mediated SREBP-1 knockdown

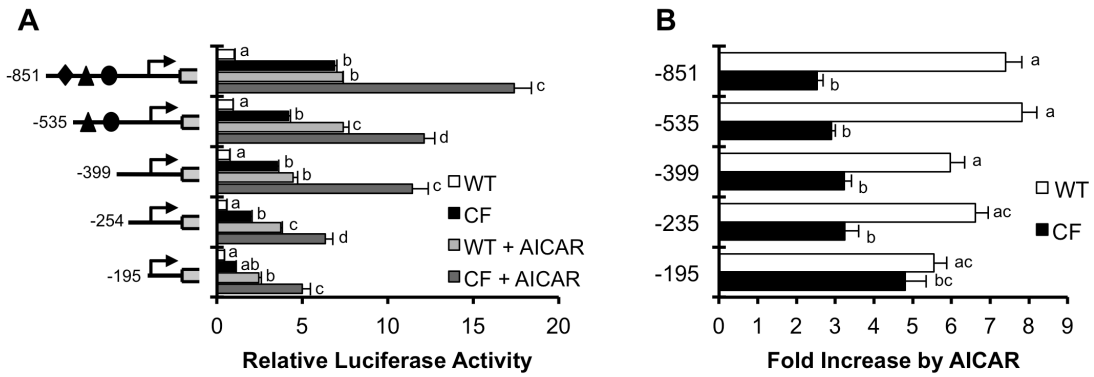

Figure 17 Δ6D promoter activity is potentiated by AICAR
(**A**) Relative Δ6D promoter activity in CF and WT cells in the presence or absence of AICAR was measured by luciferase assay. Various Δ6D promoter lengths were tested. Data are shown as mean ± SEM (n=3) and are representative of three independent experiments. Statistical differences were determined by two-way ANOVA with Tukey's HSD post-test for pairwise comparisons. Unlike letters denote significant differences ($P<0.05$) in pairwise comparisons within each promoter length. (**B**) Fold increase in Δ6D promoter activity by AICAR. Data are shown as mean ± SEM (n=3) and are representative of three independent experiments. Statistical differences were determined by two-way ANOVA with Tukey's HSD post-test for pairwise comparisons. Unlike letters denote significant differences ($P<0.05$) in pairwise comparisons amongst all groups.

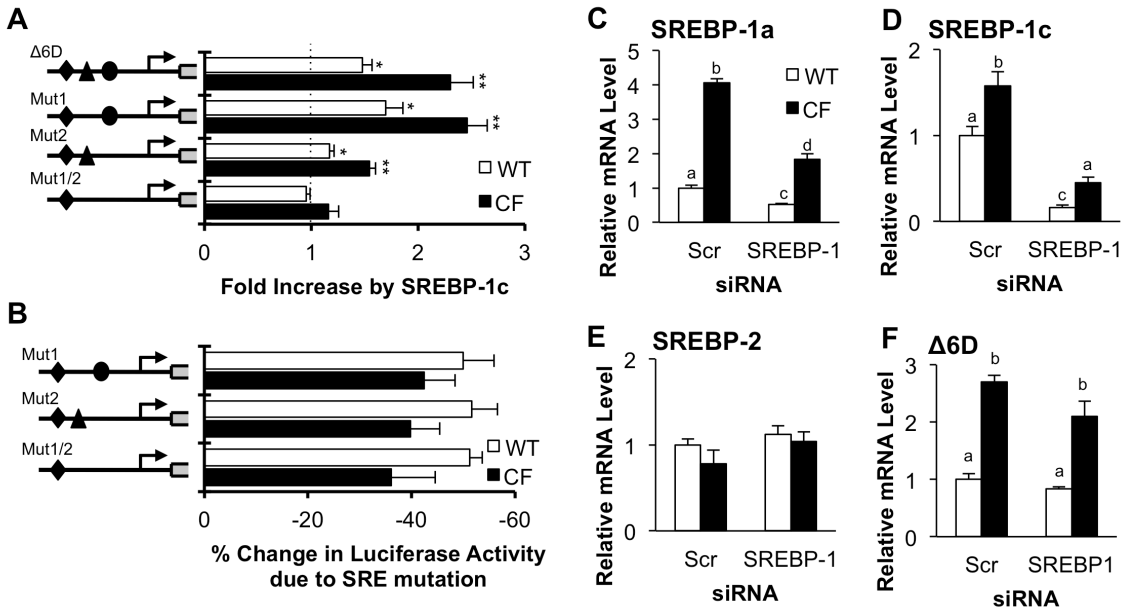

Figure 18 Elevations in Δ6D promoter activity and mRNA expression in CF cells are SREBP-1-independent

(**A**) Fold increase in Δ6D promoter activity induced by SREBP-1c was measured by co-transfecting cells with nuclear SREBP-1c expression vectors and Δ6D promoter luciferase reporter vectors with SRE (triangle) and/or the E-box like SRE (circle) mutated by site-directed mutagenesis. Data are shown as mean ± SEM (n=3) and representative of three independent experiments. One sample t-tests were performed to determine whether mean fold increases were greater than 1 (*P<0.05, **P<0.01). (**B**) Percent change in Δ6D promoter activity due to SRE mutations was determined by comparing promoter activity of Δ6D promoters with SRE mutations versus Δ6D promoters without mutations. No significant differences detected. (**C-F**) Relative mRNA levels of SREBP-1a, SREBP-1c, SREBP-2, and Δ6D mRNA levels in CF and WT cells were measured by qRT-PCR after transfection with scrambled siRNA or siRNA targeting SREBP-1. Data are shown as mean ± SEM (n=3) and representative of three independent experiments. Statistical differences were determined by two-way ANOVA with Tukey's HSD post-test for pairwise comparisons. Unlike letters denote significant differences (P<0.05) in pairwise comparisons.

on Δ6D mRNA levels in CF and WT cells. ΔΔC(t) analysis revealed that SREBP-1c is the predominant SREBP-1 isoform in these cells. SREBP-1c mRNA levels were 34-fold greater than SREBP-1a mRNA levels in WT cells and 13-fold greater in CF cells (not shown). Transfection with siRNA targeting SREBP-1 caused SREBP-1c mRNA levels to decline by 84% in WT cells and 72% in CF cells. SREBP-1a mRNA levels declined approximately 50% in both WT and CF cells, while SREBP-2 mRNA levels were unchanged. Δ6D mRNA levels were significantly greater in CF cells than in WT cells transfected with scrambled siRNA. SREBP-1 knockdown caused Δ6D mRNA levels to decline by approximately 20% in WT and CF cells, but this decline was not statistically significant. Δ6D mRNA levels remained significantly higher in CF cells than in WT cells after SREBP-1 knockdown (Figure 18C-F).

We also investigated the effects of siRNA-mediated PPARα knockdown on Δ6D mRNA levels in CF and WT cells. Transfection with siRNA targeting PPARα resulted in 80% knockdown of PPARα mRNA, but had no effect on Δ6D mRNA levels (Figure 19A,B). To directly measure PPAR activity, we transfected cells with PPRE luciferase reporter plasmids. After transfection, we treated cells with PPARα activators fenofibrate, GW 7647, or Wy 14643, or PPARα inhibitor GW 6471. The luciferase activity detected in WT and CF cells was negligible, and the PPARα activators and inhibitor had no effect (Figure 19C). The minimal detected luciferase activity was likely non-specific background.

We then studied the effects of DHA on Δ6D promoter activity. DHA caused a dose-dependent reduction in Δ6D promoter activity in both WT and CF cells (not shown). We then measured the effect of DHA on various lengths of the Δ6D proximal promoter.

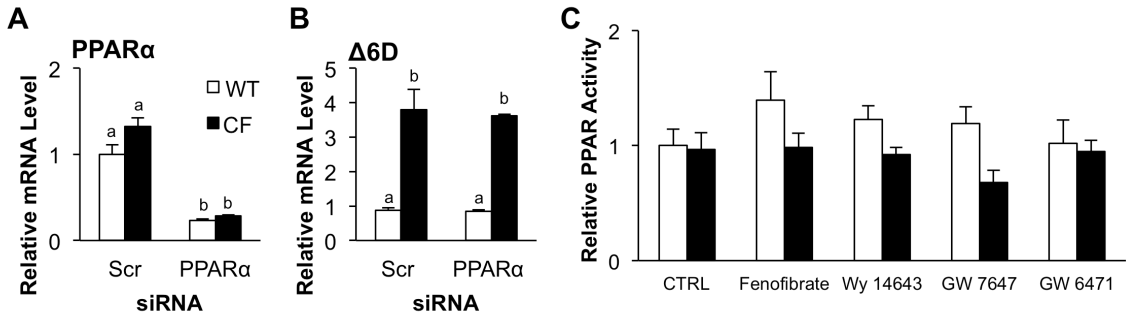

Figure 19 Elevations in Δ6D promoter activity and mRNA expression are PPARα-independent

PPARα and Δ6D mRNA levels in CF and WT cells were measured by qRT-PCR after transfection with scrambled siRNA or siRNA targeting PPARα. Data are shown as mean ± SEM (n=3) and are representative two independent experiments. Statistical differences were determined by two-way ANOVA with Tukey's HSD post-test for pairwise comparisons. Unlike letters denote significant differences ($P<0.05$) in pairwise comparisons. (**C**) Relative PPAR activity was measured by transfecting CF and WT cells with PPRE-luciferase reporter constructs followed by luciferase assay. Cells were treated with PPARα activators fenofibrate, GW 7647, or Wy 14643, or PPARα inhibitor GW 6471. Data are expressed as mean ± SEM (n=3) of fold change relative to negative control and are representative of two independent experiments. No significant differences were detected.

DHA suppressed Δ6D promoter activity approximately 40% in both WT and CF cells. Deletion of the -535 to -399 region significantly attenuated the suppressive effect of DHA (Figure 20A). As this region contains the SREs, we measured the effect of DHA on Δ6D promoter activity when the SREs were mutated. Mutating the SREs abolished the effect of DHA on promoter activity (Figure 20B).

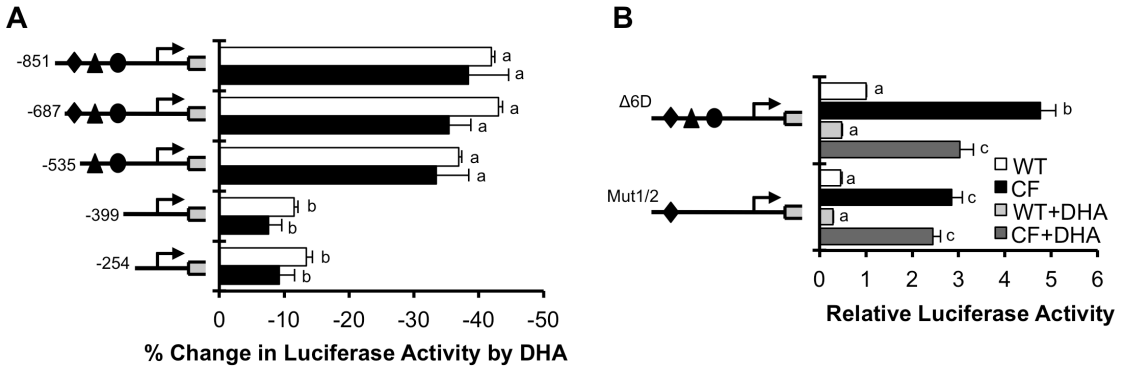

Figure 20 DHA suppresses Δ6D promoter activity
(**A**) Percent change in promoter activity of various lengths of the Δ6D proximal promoter was determined by comparing promoter activity in cells supplemented with 20 μM of DHA versus cells that were not supplemented. (**B**) Relative Δ6D promoter activity in the presence or absence of 20 μM of DHA was measured after mutating the both the SRE (triangle) and the E-box like SRE (circle) by site-directed mutagenesis as described in *Methods*. Data are shown as mean ± SEM (n=3). Unlike letters denote significant differences ($P<0.05$) in pairwise comparisons.

Discussion

Increased Δ6D activity is a major cause of the PUFA alterations that contribute to CF pathophysiology. However, a mechanism explaining increased Δ6D expression and activity has been elusive. We recently reported that increased Δ6D mRNA levels and activity are downstream of increased AMPK activity in CF cells (276). The present study provides clues about the mechanism by which AMPK increases Δ6D expression in CF cells. We demonstrate that Δ6D expression is increased in CF due to increased Δ6D promoter activity. AICAR, an AMPK activator, potentiates Δ6D promoter activity. These results indicate that AMPK up-regulates transcription of Δ6D.

The findings indicate that increased Δ6D transcription in CF cells is independent of SREBP-1 and PPARα. Δ6D promoter luciferase reporter experiments indicate that SREBP-1 activity accounts for a similar portion of transcriptional activity at the Δ6D in CF and WT cells. When we abolished SREBP-1 binding through mutation of the SRE sites, Δ6D promoter activity declined comparably in CF and WT cells, such that the relative baseline difference in Δ6D promoter activity between CF and WT cells was unchanged. Correspondingly, SREBP-1 knockdown reduced Δ6D mRNA levels to a similar extent in CF and WT cells; however, the reductions were not statistically significant. Overall, the data suggests that endogenous SREBP-1 activity is similar in CF and WT cells, and that the elevation of Δ6D promoter activity in CF is SREBP-independent. PPARα activity, on the other hand, was undetectable. No PPARα activity was detected in the PPRE luciferase reporter assay. Thus, PPARα activity does not account for differential Δ6D expression in CF and WT cells. SREBP-1 and PPARα are

the only transcription factors that have been reported to promote Δ6D transcription (10, 11). Thus, ruling out SREBP-1 and PPARα represents an important step in elucidating the mechanism, and suggests that a novel regulator of Δ6D transcription is responsible for increased Δ6D expression in CF.

The Δ6D promoter deletion analysis indicated that Δ6D promoter activity is elevated in CF cells until the promoter was truncated from -195 bp to -119 bp. Analogously, AICAR potentiated Δ6D promoter activity of all promoter lengths beyond -195. The Δ6D promoter is does not contain a TATA box and contains multiple transcription start sites within the -195 to -119 bp promoter region (7, 12). It is possible that an unidentified regulator of Δ6D transcription binds in the -195 to -119 bp region. Another possibility is sequence-independent regulation of Δ6D transcription in CF, such as epigenetic alterations in histone acetylation or DNA methylation. There is precedent for increased gene expression in CF due to altered histone deacetylase activity (274, 275). Studies have revealed that AMPK regulates a subclass of histone deacetylases and can phosphorylate histone H2B to increase expression of a subset of genes (271–273). Further studies will be necessary to determine whether epigenetic modifications play a role in elevating Δ6D transcription in CF cells.

Because DHA supplementation diminished Δ6D mRNA and activity in CF cells in a previous study (164), we tested the hypothesis that DHA exerts this effect through transcriptional mechanisms. We found that DHA caused a dose-dependent suppression of Δ6D promoter activity in WT and CF cells. Further analysis revealed that this effect was dependent on the SREs, a finding that is in agreement with a previous study examining the effect of PUFAs on the Δ6D promoter (7). DHA suppressed Δ6D promoter activity to

a similar extent in CF and WT cells, which further supports the conclusion that SREBP-1 activity is similar in CF and WT cells. Although a previous study showed that 20 μM DHA reduces Δ6D mRNA levels in CF cells to WT levels (164), Δ6D promoter activity remained significantly higher in CF cells at this concentration in the present study. This result signifies that a portion of the suppressive effect of DHA on Δ6D mRNA and activity in CF is attributable to suppression of transcriptional activity at the Δ6D promoter via SREBP-1.

In summary, the present study provides insight into the mechanisms that govern transcriptional regulation of Δ6D in CF. Δ6D upregulation in CF cells occurs at the transcriptional level. Δ6D is a key contributor to the PUFA alterations that play an important role in CF pathophysiology. Because PUFAs are precursors to important pro- and anti-inflammatory mediators, these alterations may contribute to the heightened inflammatory state observed in CF patients (58). AA-derived mediators tend to be pro-inflammatory while DHA-derived mediators are anti-inflammatory. Notably, elevated production of AA-derived pro-inflammatory mediators has been observed in CF patients (135, 165–167). Complete understanding of the mechanisms governing PUFA alterations in CF may provide new therapeutic targets for treatment of CF.

Chapter IV

DISCUSSION

A Mechanism Linking CFTR to PUFA Biosynthesis

Shortly after the recognition of CF as a distinct disease in 1938, investigators made the observation that CF patients have altered PUFA composition (57). Since then, much progress has been made in the understanding of CF, including the discovery that CF is caused by mutations in *CFTR*. However, the source of altered PUFA composition in CF has remained a mystery. Within the last several years, investigators have discovered that aberrant PUFA biosynthesis causes some of the PUFA alteration in CF. In particular, low LA levels result from increased Δ6D- and Δ5D-catalyzed LA to AA metabolism in CF bronchial epithelial cells (54–56). Yet, the connection between CFTR and PUFA biosynthesis has remained unclear. The work described in this dissertation is the first to establish a mechanism linking PUFA biosynthesis to the underlying cause of CF.

In addition to its function as a cyclic AMP-dependent anion channel, CFTR regulates a number of ion channels. Regulation of calcium channels by CFTR is the first step in the mechanism linking CFTR to PUFA biosynthesis. The presence of CFTR attenuates calcium influx through TRPC6, CRAC, and possibly other calcium channels as well (105, 106). Inhibition of TRPC6 by CFTR appears to involve a direct protein-protein interaction. Several studies have reported increased calcium signaling in CF cells (267–269). This dissertation corroborates those reports. The intracellular calcium concentration is elevated in CF cells relative to control cells.

The elevation in intracellular calcium concentration in CF cells leads to CaMKKβ-dependent phosphorylation of AMPK. CaMKKβ is one of two kinases that phosphorylate and activate AMPK in mammals. CaMKKβ phosphorylates AMPK in a calcium/calmodulin-dependent manner. This dissertation demonstrates that AMPK activity is elevated in CF cells due to CaMKKβ-dependent AMPK phosphorylation. Inhibition of CaMKKβ with small molecule STO-609 reduces activation of AMPK in CF cells to the level observed in control cells. Chelation of intracellular calcium similarly reduces activation of AMPK in CF cells to the level observed in control cells. Neither of these interventions significantly affects AMPK activation in in control cells. Taken together, these findings suggest that baseline CaMKKβ-dependent AMPK phosphorylation is minimal in control cells. Loss of functional CFTR causes the intracellular calcium concentration to rise above the threshold level required for CaMKKβ-dependent phosphorylation of AMPK.

Maximal inhibition of CaMKKβ eliminates only the amount of AMPK phosphorylation and activity in CF cells that exceeds the amount AMPK phosphorylation and activity in control cells. This result suggests that LKB1 is responsible for the bulk of baseline AMPK phosphorylation and activity in control cells, and that CaMKKβ is responsible for the excess AMPK phosphorylation and activity in CF cells. There are several lines of evidence suggesting that LKB1-dependent AMPK phosphorylation is equivalent in CF and control cells. First, intracellular adenine nucleotide concentrations do not differ between CF and control cells (249). Increased LKB1 activity would require an increase in intracellular AMP or ADP concentration. Second, phosphorylation of

AMPK is equivalent in CF and control cells after maximal inhibition of CaMKKβ. Third, calcium chelation recapitulates the effects of CaMKKβ inhibition in CF and control cells.

This dissertation establishes a connection between elevated AMPK activity and altered PUFA biosynthesis in CF cells. No prior study had investigated the effect of AMPK on PUFA biosynthesis. The major enzymes in PUFA biosynthesis are Δ6D, Δ5D, elongase 5, and elongase 2. The reaction catalyzed by Δ6D is the rate-limiting step. Pharmacological activation of AMPK with AICAR results in increased Δ6D and Δ5D mRNA levels. Pharmacological inhibition of AMPK with Compound C results in diminished Δ6D and Δ5D mRNA levels. Neither intervention had an effect on elongase expression. These results indicate that AMPK is a positive regulator of Δ6D and Δ5D expression. Additional experiments demonstrate that excess AMPK activity in CF accounts for the elevation in Δ6D and Δ5D mRNA expression. Maximal inhibition of CaMKKβ, which eliminates excess AMPK activity in CF cells, also eliminates excess Δ6D and Δ5D mRNA expression in CF cells. Similarly, calcium chelation reduces Δ6D and Δ5D mRNA expression in CF cells to the level observed in control cells.

Altered PUFA biosynthesis in CF cells is characterized by increased metabolism of LA to AA due to Δ6D and Δ5D activity. Because the reaction catalyzed by Δ6D is the rate-limiting step in PUFA, measurement of the conversion of ^{14}C-labeled LA to AA is a proxy for desaturase activity. AMPK inhibition with Compound C significantly reduces desaturase activity, paralleling its effects on Δ6D and Δ5D mRNA levels. CaMKKβ inhibition with STO-609 or calcium chelation reduces desaturase activity in CF cells to the level seen in control cells, paralleling their effects on Δ6D and Δ5D mRNA. Conversely, the effects of AMPK activation with AICAR on desaturase activity do not

parallel its stimulatory effect on Δ6D and Δ5D mRNA levels. A likely explanation is that AICAR, at the concentrations used in these studies, increases AMPK activity, and thus, inhibitory phosphorylation of ACC to levels far exceeding the physiological levels. Such extensive inhibition of ACC would drastically reduce the malonyl-CoA concentration. Malonyl-CoA is a required substrate for elongase reactions. So, while AMPK activation with AICAR at the concentrations used in these studies increases Δ6D and Δ5D mRNA expression, it likely diminishes the malonyl-CoA concentration to the extent that the elongase reaction becomes the rate-limiting step in PUFA biosynthesis pathways.

Promoter reporter studies were undertaken to determine the mechanism by which AMPK increases Δ6D and Δ5D mRNA expression. Experiments were performed using the proximal promoter element of Δ6D, which has been characterized in previous studies (7, 12). No characterization of the Δ5D promoter has been reported in the literature. However, Δ5D is typically regulated in tandem with Δ6D, so results obtained with the Δ6D proximal promoter are likely applicable to the Δ5D proximal promoter as well. The Δ6D promoter reporter studies reveal that Δ6D promoter activity is significantly elevated in CF cells. Activation of AMPK with AICAR significantly increases Δ6D promoter activity, which indicates that AMPK is a positive regulator of Δ6D transcription.

AMPK regulates transcription of particular genes through several transcriptional mechanisms. AMPK influences the activity of transcription factors PPARα and SREBP-1, and both of these transcription factors have been reported to induce Δ6D expression (11, 234, 239). Given the literature, AMPK-dependent regulation of Δ6D via PPARα or SREBP was an appealing hypothesis. However, promoter reporter analyses and RNA interference experiments reveal that the elevation in Δ6D transcription in CF cells is

independent of both SREBP-1 and PPARα. After mutation or deletion of the SREBP-1 and/or PPARα binding sites, transcriptional activity at the Δ6D promoter remains significantly elevated in CF cells relative to control cells. Furthermore, deletion of the SREBP-1 and PPARα binding sites does not diminish the capacity of AMPK activation with AICAR to stimulate Δ6D promoter activity. No other transcription factors have been reported as regulators of Δ6D transcription. Thus, AMPK must act through an unidentified regulator of Δ6D transcription.

As noted in Chapter I, AMPK inhibits CFTR activity via phosphorylation of Ser737 and Ser768 in the R domain. This raises the question of whether CFTR is upstream or downstream of AMPK in the regulation of Δ6D and Δ5D. The results presented in this dissertation indicate that CFTR is upstream of AMPK in the regulation of Δ6D and Δ5D. If AMPK were acting through inhibition of CFTR, then modulation of AMPK activity would have no effect on Δ6D and Δ5D expression and activity in cells lacking CFTR. Thus, stimulation of Δ6D and Δ5D expression and activity by AMPK in CF cells could not be explained by a mechanism reliant on AMPK-dependent inhibition of CFTR.

In summary, this dissertation establishes a connection between CFTR and PUFA biosynthesis. The absence of functional CFTR in CF bronchial epithelial cells results in aberrant calcium homeostasis. Increased intracellular calcium concentration stimulates CaMKKβ-dependent activation of AMPK. AMPK induces expression of Δ6D and Δ5D and consequently, LA to AA metabolism (Figure 21).

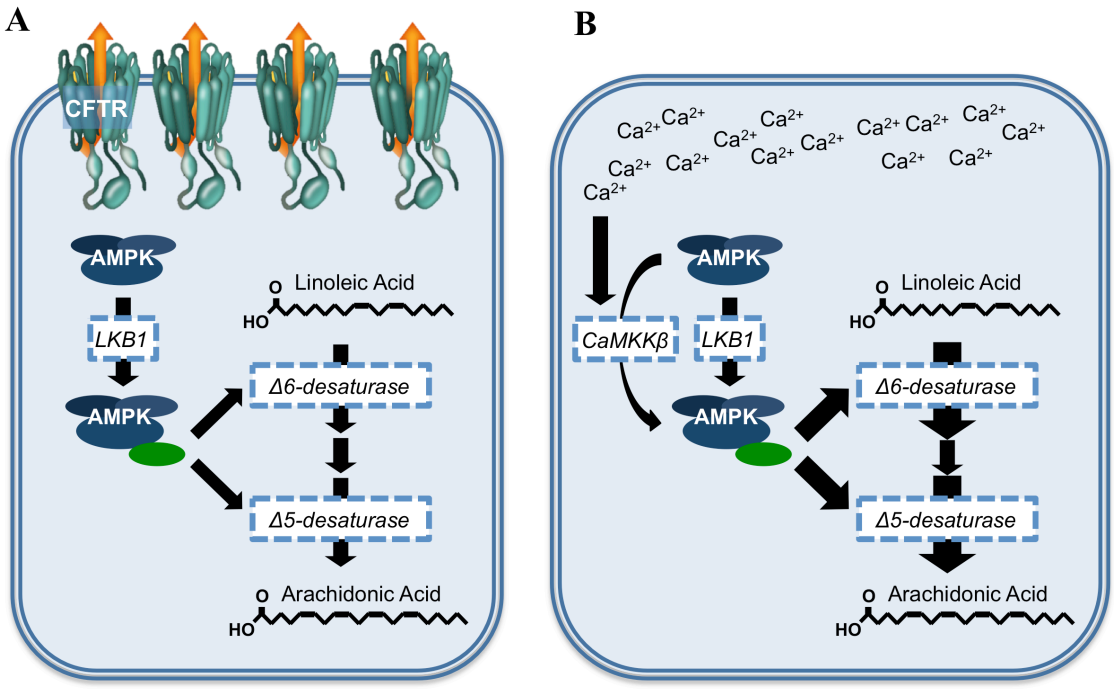

Figure 21 A Mechanism Linking CFTR to PUFA Biosynthesis
(**A**) In bronchial epithelial cells with normal CFTR expression, LKB1-dependent AMPK phosphorylation maintains basal levels of the phosphorylated, active form of AMPK. AMPK stimulates expression and activity of Δ6-desaturase and Δ5-desaturase. (**B**) In CF bronchial epithelial cells, absence of functional CFTR results in excess abundance of the phosphorylated, active form of AMPK due to CaMKKβ-dependent AMPK phosphorylation. Increased intracellular calcium (Ca^{2+}) concentration stimulates CaMKKβ-dependent AMPK phosphorylation. The resulting increase in AMPK activity causes increased expression and activity of Δ6-desaturase and Δ5-desaturase. This results in increased metabolism of linoleic acid to arachidonic acid in cystic fibrosis bronchial epithelial cells relative to control cells.

Future Directions

Mechanism of Δ6D and Δ5D regulation by AMPK

The mechanism by which AMPK regulates transcription of Δ6D and Δ5D remains an open question. The promoter reporter studies offer some clues. The Δ6D proximal promoter segment cloned into the luciferase vectors is comprised of the region -851 bp to -48 bp upstream of the Δ6D translation start site. Because Δ6D has multiple transcription start sites, the Δ6D translation start site is typically designated as +1 to avoid confusion (7, 12). Incremental 5' truncations of the Δ6D proximal promoter sequence from -851 bp to -195 bp do not significantly alter the elevation in Δ6D promoter activity observed in CF cells, nor do they diminish the stimulatory effect of AMPK activator AICAR on Δ6D promoter activity (Figure 16 and Figure 17). Deletion of the -195 to -119 bp segment virtually eliminates Δ6D promoter activity in both CF and control cells. In other words, Δ6D promoter activity is significantly elevated in CF cells relative to control cells until the -195 to -119 bp segment is deleted.

The findings suggest that AMPK stimulates the activity of a transcription factor that binds within the -195 to -119 bp segment of the Δ6D proximal promoter. Putative transcription factor binding sites within this segment can be identified using a bioinformatics approach. A search using PROMO, a "virtual laboratory for the study of transcription factor binding sites in DNA sequences," reveals a number of putative transcription factor binding sites in this segment of the Δ6D promoter (277, 278). When performed without species restrictions, the PROMO search reveals a total of 85 transcription factors predicted to bind within the -195 to -119 segment of the Δ6D promoter. Restricting the PROMO search to transcription factors and putative binding

sites found in humans narrows the list to 13 transcription factors (Figure 22). Of those 13, AP-2α is notable. A recent study identified AP-2α as an AMPK-regulated transcription factor. Three putative AP-2α binding sites are present in the -195 to -119 region of the Δ6D promoter. Of these, the putative site located at -170 to -165 bp is a perfect match for the GCAGGC AP-2α consensus sequence. The other putative AP-2α sites, located at -191 to -186 and -162 to -157, both have sequences of GAAGGC, which is only 3.75% dissimilar from the consensus sequence, accounting for the relative frequencies of each nucleotide in the sequence.

A recent report examining the effects of nicotine on abdominal aortic aneurysm formation demonstrated that AMPK induces matrix metalloproteinase (MMP)-2 expression in vascular smooth muscle cells via stimulatory phosphorylation of AP-2α (241). Nicotine causes the formation of reactive oxygen species, leading to CaMKKβ-dependent AMPK activation (241, 279). The AMPKα2 isoform translocates to the nucleus where it phosphorylates AP-2α at Ser219 (241). It appears that AP-2α must be phosphorylated to induce gene expression (241, 280). Intriguingly, elevated levels of MMP-2, MMP-7, MMP-8, MMP-9, and MMP-12 have been detected in bronchoalveolar lavage fluid, sputum, and serum from CF patients relative to controls (139, 281–284). Epithelial cells are the predominant producers of MMP-2 and MMP-7 in the airways. Though MMP-9 is predominantly produced by neutrophils, epithelial cells produce it as well (285, 286). These findings raise the possibility that AP-2α transcriptional activity is elevated in CF bronchial epithelial cells.

No published reports have linked AP-2α to Δ6D or Δ5D expression. Whether AMPK induces Δ6D and Δ5D expression via AP-2α will need to be determined

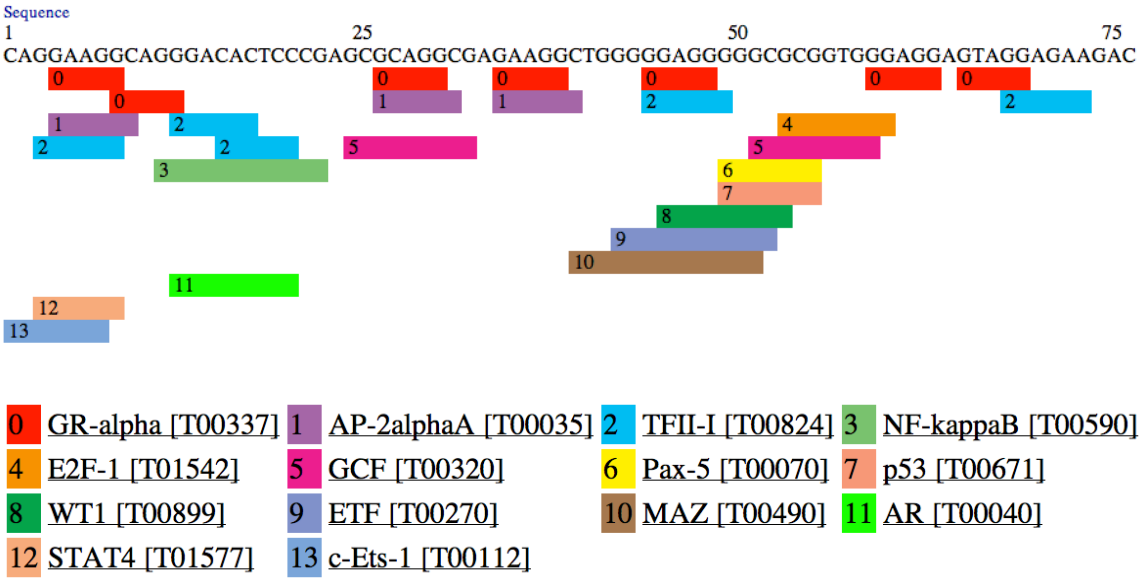

Figure 22 Putative transcription factor binding sites in Δ6D proximal promoter (-195 to -119 bp)

A PROMO search was used to identify putative transcription factor binding sites in the -195 to -119 bp segment of the Δ6D proximal promoter. The first nucleotide in the sequence corresponds to -195. Binding sites are numbered and color-coded. Thirteen transcription factors were identified. The search was restricted to transcription factors and binding sites that are present in humans.

experimentally. Site-directed mutagenesis of the three putative AP-2α binding sites in the -195 to -119 bp segment of the Δ6D promoter would reveal whether one or more of these putative AP-2α binding sites are necessary for the elevation in Δ6D promoter activity in CF cells. Along the same lines, if AMPK induces Δ6D promoter activity via AP-2α, then AMPK activation by AICAR would not have an effect on Δ6D promoter activity after mutation of the putative AP-2α sites. If the data supports a role for AP-2α in the regulation of Δ6D promoter activity, similar analysis of the Δ5D proximal promoter should corroborate the findings. RNA interference targeted against AP-2α would complement the site-directed mutagenesis experiments. Furthermore, relative binding of AP-2α to the Δ6D or Δ5D promoters in CF and control cells could be assessed by chromatin immunoprecipitation. The interaction between AMPK and AP-2α could be analyzed by immunoprecipitation using anti-AMPKα and anti-AP-2α antibodies. There are no antibodies available specific for AP-2α phosphorylated at Ser219; however, relative phosphorylation of AP-2α in CF and control cells may be determined by first immunoprecipitating AP-2α, then immunoblotting with antibodies specific for phosphorylated serine residues. Should AP-2α prove to be the transcription factor that mediates the elevation in Δ6D promoter activity, other genes differentially regulated in CF and control by AMPK via AP-2α could be determined by performing RNA-seq or microarray.

If AP-2α is not involved in the up-regulation of Δ6D and Δ5D expression in CF, a non-biased approach may be the most appropriate approach to identify candidate transcription factor(s) that mediate increased Δ6D and Δ5D expression in CF. DNA affinity chromatography can be used to isolate proteins that interact with Δ6D or Δ5D

promoter elements, followed by mass-spectrometry to identify the transcription factors. Whether one or more of the identified transcription factors mediates increased Δ6D and Δ5D expression in CF cells could then be determined experimentally using the approaches outlined above and in Chapter III.

An alternative possibility is that AMPK induces Δ6D and Δ5D expression via histone modifications. Post-translational modifications of histones, such as methylation, acetylation, and phosphorylation, control the degree of DNA compaction, which affects binding of transcriptional machinery (287–289). Histone acetylation is associated with open chromatin and transcriptional stimulation, while histone deacetylation is associated with closed chromatin and transcriptional repression. A number of different histone deacetylases (HDAC) can modify histone acetylation status (287, 288). Studies have revealed altered histone deacetylase activity in CF cells. Recent studies show that reduced HDAC2 activity in CF cells promotes transcription of particular genes by increasing histone acetylation at their promoters (274, 275). Furthermore, AMPK inhibits the activity of class II HDACs. Phosphorylation of class II HDACs by AMPK prevents their translocation to the nucleus (271, 272). AMPK can directly phosphorylate histone H2B to stimulate gene transcription (273). Whether histone modifications differentially affect Δ6D and Δ5D expression in CF and control cells would need to be determined experimentally. Differences in histone modifications near the promoter elements of Δ6D and Δ5D can be measured by chromatin immunoprecipitation. If differences in histone modifications are observed, further analysis may include RNA interference to determine the specific HDACs involved.

Mechanism of Low DHA in CF

The reason for reduced DHA levels in CF cells remains unclear. As Δ6D and Δ5D are required for DHA biosynthesis, increased Δ6D and Δ5D activity is unlikely to account for low DHA levels in CF cells. Detailed examination of the steps required for DHA biosynthesis may offer testable hypotheses to explain low DHA levels in CF.

As shown in Figure 2, DHA can be synthesized from LNA. EPA biosynthesis from LNA involves alternating desaturation and elongation reactions in the endoplasmic reticulum. However, because mammals lack expression of a Δ4-desaturase, DHA biosynthesis from EPA requires extra steps. EPA is elongated to 22:5*n*-3 by elongase 2 or elongase 5, then further elongated to 24:5*n*-3 by elongase 2. Δ6D converts 24:5*n*-3 to 24:6*n*-3. The desaturation and elongation reactions occur in the endoplasmic reticulum. 24:6*n*-3 is transported to peroxisomes and retroconverted to DHA, a process in which a β-oxidation reaction removes two carbons from 24:6*n*-3 to generate DHA. This β-oxidation reaction requires straight-chain acyl-CoA oxidase, D-bifunctional protein, and 3-ketoacyl-CoA thiolase (290, 291).

DHA is preferentially transported out of peroxisomes to the endoplasmic reticulum, rather than undergo further β-oxidation in peroxisomes. β-oxidation of DHA is inefficient because a Δ2,Δ4-dienoyl-CoA intermediate is formed by the acyl-CoA oxidase reaction, which is the first step of β-oxidation. The conjugated bonds in the Δ2,Δ4-dienoyl-CoA intermediate prevent further β-oxidation. In order for β-oxidation to proceed, 2,4-dienoyl-CoA reductase (DECR) converts the Δ2,Δ4-dienoyl-CoA to a Δ3-enoyl-CoA. Enoyl isomerase isomerizes the Δ3-enoyl-CoA to a Δ2-enoyl-CoA so that β-oxidation can proceed (Figure 23) (292, 293). The rate of peroxisomal DHA β-oxidation

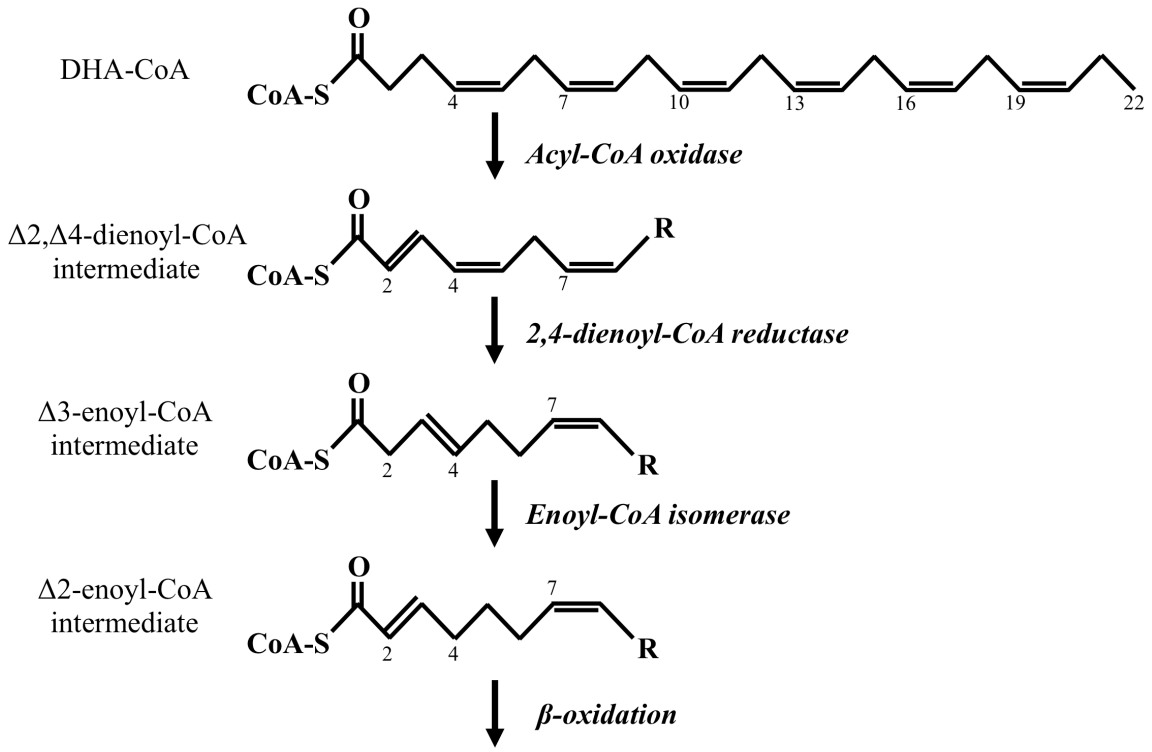

Figure 23 β-oxidation of DHA requires additional steps
2,4-dienoyl-CoA reductase is the rate-limiting step in β-oxidation of DHA.

is low for several reasons. The DECR reaction is rate-limiting and occurs at a relatively slow rate (292). Acyl-CoA esterases rapidly hydrolyze DHA-CoA to its free fatty acid form; only acyl-CoA esters can be β-oxidized (294). Moreover, the DHA is probably transported to the endoplasmic reticulum as a free fatty acid (294). Overall, the rate of DHA transport to the endoplasmic reticulum for utilization in other cellular processes greatly exceeds the rate of its β-oxidation. However, β-oxidation of DHA to EPA, also termed retroconversion, may occur when DHA is present in excess (194).

One or more aspects of DHA metabolism may be altered in CF cells. Increased β-oxidation of DHA in peroxisomes is a possible explanation for low DHA levels in CF cells. Indeed, in CF cells, there is evidence of increased retroconversion of DHA to EPA (164). This suggests that DECR activity is elevated in CF cells. An increase in DECR activity may shift the equilibrium from preferential DHA transport to the endoplasmic reticulum toward preferential peroxisomal β-oxidation of DHA (Figure 24). No published studies have compared β-oxidation rates between CF and control cells. Rates of peroxisomal β-oxidation of DHA could be assessed by incubating CF and control cells with ^{14}C-labeled DHA, then measuring the production of ^{14}C-labeled CO_2. If peroxisomal β-oxidation of DHA is elevated in CF cells, then CF cells will produce more ^{14}C-labeled CO_2 than control cells. Whether AMPK stimulates peroxisomal β-oxidation of DHA will need to be determined experimentally.

DHA biosynthesis is another aspect of DHA metabolism that may be altered in CF cells. DHA biosynthesis from EPA requires two elongase reactions. Three elongase reactions are required for DHA biosynthesis from LNA. Each elongase reaction requires malonyl-CoA as a substrate (231, 232). Thus, DHA biosynthesis may be more sensitive

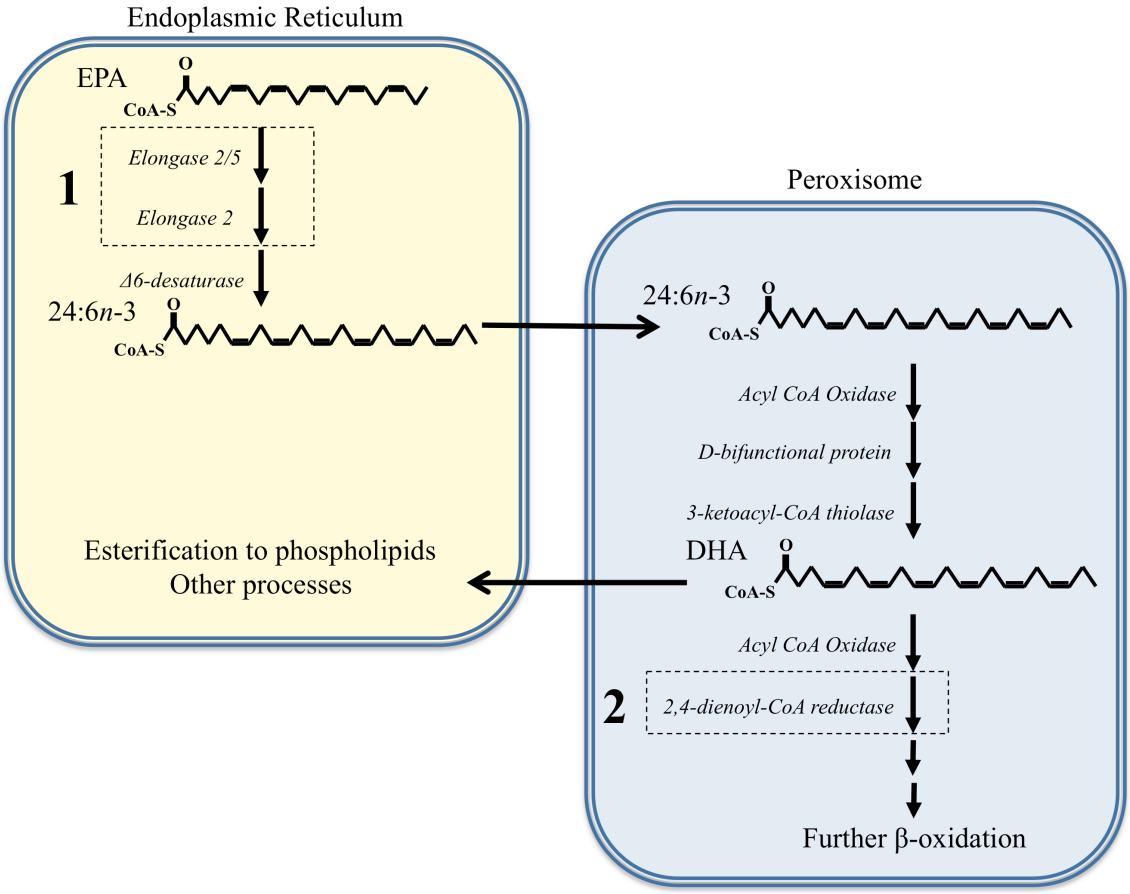

Figure 24 Potential explanations for low DHA levels in CF cells
(**1**) Reduced malonyl-CoA concentration as a result of increased AMPK activity in CF cells may impair biosynthesis of 24:6*n*-3 in the endoplasmic reticulum.
(**2**) 2,4-dienoyl-CoA reductase activity may be elevated in CF cells. Increased 2,4-dienoyl-CoA reductase activity would increase the propensity of DHA to remain in peroxisomes for further β-oxidation, rather than transport to the endoplasmic reticulum for utilization in other cellular processes.

to reduced malonyl-CoA levels as a result of inhibitory phosphorylation of ACC by AMPK (Figure 24). Indeed, in CF cells, EPA to DHA metabolism is markedly diminished relative to control cells (56). Relative malonyl-CoA concentrations in CF and control cells can be measured using high-performance liquid chromatography. The effects of small-molecule modulators of AMPK and ACC on metabolism of ^{14}C-labeled EPA to DHA will need to be determined experimentally.

AMPK and PUFA metabolism in other Cystic Fibrosis Tissues

In order to obtain a complete understanding of the source of PUFA alterations in CF, further studies examining PUFA metabolism in CFTR-expressing tissues are necessary. A starting point would be to recapitulate the studies described in this dissertation in primary cells from airways of CF and non-CF patients. Thus far, PUFA metabolic studies have been limited primarily to human airway epithelial cells (54–56, 164). Similarly, studies examining AMPK activity in CF, including this dissertation, have been performed almost exclusively in human bronchial epithelial (HBE) cells (249). The exceptions are noted below. Although the results of a CF lung transplant study suggest that altered PUFA levels in plasma from in CF patients derive primarily from loss of functional CFTR in lungs (174), abnormal PUFA metabolism in other CFTR-expressing tissues likely contributes as well. This dissertation establishes an AMPK-dependent mechanism for altered PUFA metabolism in CF HBE cells. Whether this mechanism is active in other CFTR-expressing tissues will need to be determined experimentally.

Altered PUFA biosynthesis has been observed in pancreatic epithelial cells. Human pancreatic epithelial ΔF508 CF cells of pancreatic duct origin display similar

PUFA alterations to those observed in CF airway epithelial cells. Furthermore, ΔF508 CF pancreatic epithelial cells display elevated metabolism of LA to AA (186). This study did not examine Δ6D and Δ5D expression, and it preceded the discovery that AMPK activity is increased in CF cells (186). Whether PUFA alterations in CF pancreatic epithelial cells are a result of an AMPK-dependent mechanism will need to be determined experimentally.

One study in a colon carcinoma cell line found that depletion of CFTR by RNA interference had no effects on PUFA levels or on AMPK phosphorylation. However, these results must be interpreted with caution. First, the CFTR-"depleted" cells maintained 40% of the CFTR expression observed in control cells. Second, the culture medium used in this study appears to have been entirely deficient in PUFAs. In addition, cells were serum-starved before analysis. These culture conditions resulted in the detection of miniscule amounts of *n*-3 and *n*-6 PUFAs in CFTR knockdown and mock transfected cells. Third, a carcinoma cell line may not accurately represent PUFA metabolism in intestinal epithelium (265). AMPK activity and PUFA levels should be determined experimentally in an appropriate CF intestinal cell model. However, further development of CF intestinal cell models is necessary since the number of CF intestinal epithelial cell models is limited (295).

Examination of PUFA metabolism in animal models of CF would be of interest. Several studies have demonstrated that mouse CFTR null or ΔF508-CFTR CF models display similar PUFA alterations as CF patients (183, 184, 296). However, no study has examined AMPK activity in CF mouse models. The recently generated pig and ferret CFTR-null CF models exhibit disease phenotypes that bear closer resemblance to human

CF disease than mouse models (297, 298). Thus far, no published studies have examined PUFA composition in pig and ferret models. Investigations of PUFA metabolism in pig, ferret, and mouse models may offer improved insight into the role of AMPK and PUFAs in CF in whole organisms. If these models exhibit AMPK-dependent alterations in PUFA metabolism, interventions that target this pathway could be assessed for therapeutic benefit.

Implications

Variation in Cystic Fibrosis Pulmonary Disease Severity

CF is a debilitating disease in which pulmonary inflammation is the major source of morbidity and mortality. There is a striking lack of correlation between CFTR genotype and CF pulmonary disease severity. The variation in CF pulmonary disease severity amongst individuals with identical CFTR mutations suggests that CF pulmonary disease severity is multifactorial (121). PUFAs play an important role in CF pathophysiology, and the magnitude of PUFA alterations correlates with pulmonary disease severity in CF patients. Thus, genetic variation in the factors that control PUFA levels may explain phenotypic variation in pulmonary disease severity.

This dissertation delineates a pathway linking CFTR to PUFA metabolism. Genetic variations that alter expression or function of any protein in this pathway may alter the course of disease in CF patients. For instance, non-CF studies have demonstrated that individuals carrying single nucleotide polymorphisms resulting in attenuated expression of Δ6D or Δ5D have lower AA levels and lower instances of inflammatory disease (299–302). Hence, CF patients with single-nucleotide polymorphisms (SNP) that

attenuate the function of calcium channels, CaMKKβ, AMPK, Δ6D, and/or Δ5D may have milder inflammation. SNPs in the genes encoding these proteins may have prognostic value in CF patients.

Cystic Fibrosis Nutritional Recommendations

Diet is a major determinant of PUFA composition. For decades, CF patients have been advised to consume high-fat, high-calorie diets to maintain body weight. The lack of specific dietary recommendations regarding the types of fat may be problematic because Western diets have exceedingly high LA content. The high *n*-6 content in Western diets have been implicated in rising incidence of a number of disorders that involve inflammation (60). This is especially important in CF since pulmonary inflammation is the major source of morbidity and mortality in CF patients. As outlined in this dissertation, relative to healthy cells, CF cells exhibit increased metabolism of LA to AA and AA-derived pro-inflammatory eicosanoids. Thus, unless care is taken to limit LA consumption, the high-fat dietary recommendation may cause unintentional harm to CF patients by exacerbating the production of AA-derived pro-inflammatory eicosanoids.

Novel Therapeutic Targets

Pulmonary inflammation is the primary cause of the progressive decline in lung function in CF patients. Thus, considerable efforts have been made to investigate the use of anti-inflammatory pharmacological agents in CF patients. Clinical trials demonstrate that high-dose ibuprofen significantly slows the progressive decline in lung function in CF patients, but concerns of gastrointestinal side effects and potential renal complications

limit long-term use (202, 204). Clinical trials have been inconclusive regarding the anti-inflammatory benefit of corticosteroids in CF patients, and they may impair growth in children with CF (203). Leukotriene receptor antagonism reduced neutrophil infiltration but unexpectedly increased the rate of adverse pulmonary events, resulting in early termination of a recent clinical trial (303). These clinical trials indicate that anti-inflammatory therapy may be beneficial in CF patients, but certainly, there is room for improvement in the treatment of inflammation in CF.

In clinical trials of anti-inflammatory pharmacological agents, ibuprofen offered the greatest clinical benefit. The mechanism of action of ibuprofen is the inhibition of COX enzymes. COX enzymes catalyze the conversion of AA to pro-inflammatory eicosanoids, a process that is dysregulated in CF. The beneficial effects of high-dose ibuprofen indicate that increased production of AA-derived pro-inflammatory eicosanoids plays a key role in the exaggerated inflammation in CF patients.

This dissertation describes a novel AMPK-dependent pathway leading to increased Δ6D- and Δ5D-catalyzed AA biosynthesis in CF cells. Inhibition of this pathway may offer therapeutic benefit by limiting AA production, and thus, limiting substrate availability for the synthesis of AA-derived pro-inflammatory eicosanoids. Inhibition of calcium signaling, CaMKKβ, or AMPK would likely have many side effects, but several lines of evidence indicate that inhibition of Δ6D and/or Δ5D would be a suitable therapeutic target to combat excessive inflammation CF. Individuals carrying single-nucleotide polymorphisms that attenuate Δ6D and Δ5D expression have reduced AA levels and lower incidences of inflammatory diseases (299–302). Furthermore, small-molecule Δ6D and Δ5D inhibitors have been shown to reduce AA levels and reduce

inflammation in mice (83, 304–306). Small-molecule Δ6D and Δ5D inhibitors have not been tested in humans.

Dietary *n*-3 PUFA supplementation can be considered an alternative to small-molecule Δ6D and Δ5D inhibitors in humans. Some of the anti-inflammatory effects of *n*-3 PUFAs are attributed to antagonism of AA metabolism. Not only do *n*-3 PUFAs act as competitive substrates for COX and LOX reactions required for AA metabolism to pro-inflammatory eicosanoids, they also act as competitive substrates for the Δ6D and Δ5D reactions required for AA biosynthesis. Furthermore, *n*-3 PUFAs suppress the expression of Δ6D and Δ5D. In recent years, a number of clinical trials have examined the utility of dietary *n*-3 PUFA supplementation in CF patients, and more trials are in progress (150). In some of the trials, *n*-3 PUFA supplementation reduced inflammatory markers, improved FEV_1 values, and reduced the frequency of pulmonary exacerbations. Overall, the results have demonstrated the therapeutic benefit of dietary *n*-3 supplementation, although the effect sizes were not large enough to conclusively recommend *n*-3 supplementation in CF patients.

It is possible that the pharmacodynamics of *n*-3 PUFA supplementation in CF patients are not sufficient to produce large therapeutic benefits. Direct pharmacological targeting of Δ6D or Δ5D alone or in combination with *n*-3 PUFA supplementation may be of therapeutic benefit. Anti-inflammatory effects of several Δ6D and Δ5D small-molecule inhibitors have been demonstrated in mice (83, 304–306). The most recent mouse study examined the effect of Δ6D inhibition on lung and melanoma tumor growth. Analogous to observations in CF cells, certain tumor microenvironments exhibit increased Δ6D activity, AA biosynthesis, and AA metabolism to pro-inflammatory

eicosanoids. Δ6D inhibition reduced production of AA-derived pro-inflammatory eicosanoids and suppressed tumor growth (83). However, more complete characterization of the pharmacodynamics of these inhibits is necessary before they can be used in humans. Perhaps, Δ6D and Δ5D small-molecule inhibitors will one day provide therapeutic benefit in CF patients.

Closing

Although, altered PUFA composition has been observed in CF patients for decades, little progress had been made in elucidating a mechanism directly linking these changes in PUFA composition to the mutations that cause CF. This dissertation is the first delineation of a mechanism connecting loss of functional CFTR to the PUFA alterations seen in CF patients. Loss of functional CFTR results in increased calcium signaling, which in turn leads to CaMKKβ-dependent activation of AMPK. This dissertation makes the novel finding that AMPK induces expression and activity of Δ6D and Δ5D, leading to increased metabolism of LA to AA. Increased AA biosynthesis and metabolism to pro-inflammatory eicosanoids are major contributors to the excessive pulmonary inflammation that is the primary source of morbidity and mortality in CF patients. Elucidation of the mechanism leading to augmented AA biosynthesis in CF opens the door for novel therapeutic strategies to combat excessive inflammation in CF.

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