Universidade de Lisboa
Faculdade de Ciências
Departamento de Química e Bioquímica

Restoration of cystic fibrosis transmembrane conductance regulator function to CF mutants by enhancing protein processing and channel activity

André Schmidt

Doutoramento em Bioquímica
(ramo Genética Molecular)

2008

Universidade de Lisboa
Faculdade de Ciências
Departamento de Química e Bioquímica

Restoration of cystic fibrosis transmembrane conductance regulator function to CF mutants by enhancing protein processing and channel activity

André Schmidt

Doutoramento em Bioquímica
(ramo Genética Molecular)

Tese orientada por Prof. Doutora Margarida D. Amaral
e Doutor David N. Sheppard

2008

André Schmidt foi bolseiro de doutoramento da Fundação para a Ciência e Tecnologia do Ministério da Ciência, Tecnologia e Ensino Superior SFRH/BD/19415/2004

De acordo com o disposto no artigo 40º do Regulamento de Estudos Pós Graduados da Universidade de Lisboa, Deliberação nº 961/2003, publicada no Diário da Republica – III Série nº 153 - 5 de Julho de 2003, foram utilizados nesta dissertação resultados dos seguintes artigos:

Schmidt, A, Hughes, LK, Cai, Z., Mendes, F., Li, H., Sheppard, D.N., Amaral, M.D (2008): Prolonged treatment of cells with genistein modulates the expression and function of the cystic fibrosis transmembrane conductance Cl^- channel. *Br. J. Physiology* **153** (6); 1311-1323.

Sousa M., Ousingsawat J., Seitz R., Puntheeranurak S., Regalado A., Schmidt A., Grego T., Jansakul C., Amaral M.D., Schreiber R., Kunzelmann K. (2007): An extract from the medicinal plant Phyllanthus acidus and its isolated compounds induce airway chloride secretion: A potential treatment for cystic fibrosis. *Mol Pharmacol* **71** (1); 366-376.

Schmidt, A. & Amaral M.D. (2008): Investigation of the mechanism of action of several small molecule CFTR correctors in rescuing the trafficking defect of F508del-CFTR. (*manuscript in preparation*).

No cumprimento do disposto na referida deliberação, esclarece-se serem da nossa responsabilidade a execução das experiências que permetiram a elaboração dos resultados apresentados (excepto quando referido em contrário), assim como a interpretação e discussão dos mesmos.

Artigos publicados em revistas internacionais contendo resultados obtidos durante o doutoramento:

Scott-Ward TS, Li H, Schmidt A, Cai Z, Sheppard DN. (2004): Direct block of the cystic fibrosis transmembrane conductance regulator Cl^- channel by niflumic acid. *Mol Membr Biol.* **21** (1); 27-38.

Cai Z, Scott-Ward TS, Li H, Schmidt A, Sheppard DN. (2004): Strategies to investigate the mechanism of action of CFTR modulators. *J Cyst Fibros.* **3** (Suppl 2); 141-147. Review

Roxo-Rosa M, Xu Z, Schmidt A, Neto M, Cai Z, Soares CM, Sheppard DN, Amaral MD. (2006): Revertant mutants G550E and 4RK rescue cystic fibrosis mutants in the first nucleotide-binding domain of CFTR by different mechanisms. *Proc Natl Acad Sci U S A.* **103** (47); 17891-17896.

Pissarra L.S., Farinha C.M., Xu Z., Schmidt A., Thibodeau P.H., Cai Z., Thomas P.J., Sheppard D.N. and Amaral M.D. (2008): Solubilizing mutations used to crystallize one CFTR domain attenuate the trafficking and channel defects caused by the major cystic fibrosis mutation. *Chem Biol.* **15** (1); 62-69.

Preface

Cystic Fibrosis (CF) is a genetic disease caused by mutations in the gene encoding the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) protein, an ABC-transporter whose main function is permeation of chloride ions (Cl^-) through the plasma membrane. CFTR is a monomeric, 1480 amino acid (aa) protein, composed of several domains, which undergoes complex processing to be expressed at the apical membrane of epithelia. In healthy tissues, the CFTR Cl^- channel is mainly found at the apical membrane of epithelial cells, where it is activated by cAMP-dependent phosphorylation.

The most common CF-causing mutation is present in 90% of all patients (F508del-CFTR, a single amino acid (aa) deletion of phenylalanine at position 508 of the peptide chain), is mainly retained within the endoplasmic reticulum, but which retains partial Cl^- channel function when it does reach the plasma membrane.

CFTR dysfunction, either by impaired protein function or absence of the protein at the epithelial membrane, causes ion transport across the membrane to be altered, leading to chronic lung disease (the main cause of morbidity and mortality), pancreatic dysfunction, raised electrolyte levels in sweat, and male infertility (Collins, 1992; Rowe *et al*., 2005). Most current therapies are directed at CF symptoms, rather than rescuing the basic defect caused by dysfunction of CFTR. However, more recent drug-discovery efforts have been directed at identifying pharmacological agents, aimed at correcting the basic defect in CF. Most of the pharmacological agents aim to rescue F508del-CFTR and are grouped into two classes, i) “corrector” compounds, aimed at restoring the mutant protein to its correct apical

membrane localisation, and ii) “potentiator” compounds, which restore normal Cl^- channel function of the surface-rescued protein.

The objective of this work was to investigate the mechanism of action of some of these compounds on the CFTR protein, by assessing processing and trafficking of the protein by biochemical methods, and function of the chloride channel by electrophysiological techniques. Although not all compounds tested here will be used in a clinical setting, the approaches undertaken here are valid for future testing of other small molecules. Moreover, the study of the effects caused by different compounds suggests that their point of action is at different steps of CFTR biogenesis, trafficking and Cl^- channel function. These observations support the possibility of therapeutic interventions based on different chemical scaffolds, to ultimately benefit the CF patient.

Acknowledgments

Começo com um agradecimento especial á Prof. Dr. Margarida Amaral pela sua orientação e aconselhamento, o seu entusiasmo e ensinamentos científicos. Agradeço ter-me recebido no seu laboratório, ter acreditado em mim e nas minhas capacidades; o seu constante incentivo foi imprescindível, sem o qual a realização deste trabalho não teria sido possível. Aos membros laboratório agradeço a troca de conhecimentos, a entre-ajuda e a amizade. Ás sucessivas direcções do INSA Dr. Ricardo Jorge agradeço terem-me proporcionado as condições necessárias a realização deste trabalho, ao longo destes anos.

I want to express my gratefulness to Dr. David Sheppard, for your infatigable dedication, your demanding scientific expectations, your excellent teaching and tireless supervision. Moreover, I would like to thank you for welcoming me in your lab in Bristol, for the constant challenge to improve myself scientifically and help me grow as a person. I extend my thanks to the Sheppard lab, for your teachings, help and friendship.

Agradeço á minha querida mamã Silvia a educação e carinho que me deu e o apoio incodicional que sempre disponinibilzou. Meinem Vater Lothar danke Ich für Deine Unterstützung, Fürsorge und für die Kraft die Du mir gegeben hast, meinen Weg zu gehen. Meinen lieben Brüdern Ivo und Vasco danke Ich für Eure Freundschaft und Beistand.

Por fim, quero dedicar esta tese à Joana e à Elena, pelo apoio incondicional que me dão, pela paciênca infinita para me aturar e por me incentivarem a querer ser uma pessoa melhor.

Table of Contents

Preface vii
Acknowledgments ix
Table of Contents xi
Summary xiv
Resumo xvi
Abbreviations xxiv

I. Introduction

1. Cystic Fibrosis 1
2. Cystic Fibrosis Gene and Protein 6
   2.1 *CFTR* Gene 6
   2.2 CFTR Protein 7
      2.2.1 Structure 7
      2.2.2 Biogenesis, Processing & Degradation 9
      2.2.3 CFTR Functions 17
         2.2.3.1 CFTR as a Chloride Channel 17
         2.2.3.2 Bicarbonate and Glutathione Transporter 22
         2.2.3.3 Regulator of Epithelial Ion Transport 23
         2.2.3.4 Mechanism of Epithelial Ion Transport 24
         2.2.3.5 Other Functions 27
3. CF Causing Mutations, Functional Classes and Rescuing Strategies 30
   3.1 CFTR Rescuing Strategies 34
      3.1.1 The “Bypass” Approach 34
      3.1.2 Mutation-specific Approaches 35
      3.1.3 Rescuing F508del-CFTR 38
         3.1.3.1Physical 38
         3.1.3.2 Genetic 38
         3.1.3.3 Chemical & Pharmacological Chaperones 40
4. Objectives of This Work 42

II. Materials and Methods

1. Cells and Culture Conditions 47
2. Biochemical Analysis 49
2.1 Protein Quantification 49
2.2 Western Blot 49
2.3 Pulse-chase and Immunoprecipitation 50
3. Functional Characterisation 52
3.1 Iodide Efflux 52
3.2 Patch-Clamp Technique 52
3.3 Noise Analysis 54
3.4 Statistics 55
4. Preparation of Reagents 56

III. Results and Discussion

Chapter 1 Long-term effects of genistein on CFTR 61
1. Abstract 61
2. Introduction 62
3. Results 63
3.1 Genistein fails to Rescue F508del-, but Modulates wt-CFTR 63
3.2 Genistein Modulates Processing Efficiency of CFTR protein 67
3.3 Effects of Genistein on Localisation of CFTR 76
3.4 Single-channel Properties are Altered by Genistein 77
3.5 Noise Analysis of the Effect of Genistein on CFTR function 80
4. Discussion and Conclusions 83

Chapter 2 Cellular effects of *Phyllanthus acidus* 90
1. Abstract 90
2. Introduction 91
3. Results 92
4. Discussion and Conclusions 94

Chapter 3 Small Molecule CFTR Correctors 96
1. Abstract 96
2. Introduction 97
3. Results 100
3.1 Steady-state analysis to determine concentrations and pre-treatment times to be used in processing studies. 101
3.2 VRT-325 Rescues F508del-, Increases Processing Efficiency of F508del-4RK-, but not of F508del-G550E-CFTR 104
3.3 VRT-532 Rescues F508del-CFTR, but has no Effect on Genetic Revertants 107
3.4 VRT-640 Stabilises immature F508del-, and Rescues F508del-CFTR, but has no Effect on Genetic Revertants 110
3.5 C4a Rescues F508del-CFTR, but has no Effect on Genetic Revertants 112
3.6 Quantitave analysis for the effects of compounds on rescuing F508del-CFTR and on F508del-CFTR genetic revertants 114
4. Discussion and Conclusions 117

IV. General Considerations 125

Future Directions 133

V. References 139

Summary

Cystic Fibrosis (CF) is the most common lethal monogenic autosomal recessive disease in the Caucasian population. The disease affects 1 in 2500-6000 live births, with 1 in 25 individuals carrying a CF-causing mutation. The clinical phenotype is mostly characterised by lung plugging with thick mucus, leading to recurrent lung infections (>95% of patients), pancreatic insufficiency (~85% of patients), meconium ileus (5-10% of patients) and male infertility. While it remains a lethal disease, with symptomatic therapies life expectancy has greatly improved in recent years. It is expected that with a better understanding of the mechanisms of disease more appropriate therapeutics can be discovered towards a cure.

The gene, whose dysfunction gives rise to the disease, was identified in 1989 and encodes a protein called Cystic Fibrosis Transmembrane Conductance Regulator (CFTR), a Cl^- channel regulated by cAMP-dependent phosphorylation, located at the apical membrane of epithelial cells of affected organs.

More than 1500 mutations of the *CFTR* gene have been identified to date, most of them with poorly understood impact on CFTR protein biogenesis and function. The most common mutation, termed F508del, found in ~70% of CF patients worldwide, is a three nucleotide deletion, leading to the loss of a single amino acid (phenylalanine at position 508 of the polypeptide chain). The effect of this mutation is a mislocalisation of the protein, due to its retention at the endoplasmatic reticulum (ER), and consequent degradation.

Since the discovery of the disease-causing gene, great efforts have been undertaken to investigate the underlying mechanisms causing

CF, namely on biogenesis, processing, intracellular localisation, trafficking and function of this ATP-Binding Cassette (ABC) transporter family member.

This work aims to investigate effects of small molecules on CFTR processing, by attempting to evaluate the mechanism of action of these compounds on protein synthesis, trafficking and maturation, and ultimately function at the apical membrane. The compounds studied can be grouped into two classes, based on their known effect on CFTR protein: i) “potentiator” compounds, including components of the *Phyllanthus acidus* extract, genistein and VRT-532, and ii) “corrector” compounds, or “chemical/pharmacological chaperones” such as VRT-325, VRT-640 and C4a.

These small molecules were assessed for their long-term effect on CFTR processing in cultured baby hamster kidney (BHK) cells, stably expressing several CFTR variants, including genetic revertants of the F508del-CFTR trafficking defect. For some compounds, shown to modulate CFTR function directly, such as *Phyllanthus acidus* extracts, we could observe no effect on CFTR processing. Another compound, genistein, which interacts directly with CFTR to modulate Cl^- channel function, while unable to rescue surface expression of F508del-CFTR, impaired wt-CFTR processing in prolonged treatment conditions. Finally, novel small molecule CFTR correctors, such as VRT-325, VRT-532, VRT-640 and C4a, previously shown to partially rescue surface expression of F508del-CFTR, were studied to gain insight into their mechanism of rescue by assessment of their effects on other CFTR variants, namely genetic revertants of F508del-CFTR.

Key words: CFTR, Cystic Fibrosis, CFTR Chloride Channel, Small Molecule CFTR Correctors, Processing.

Resumo

A Fibrose Quística (FQ) é a doença autossómica recessiva letal mais comum na população Caucasiana, com uma incidência de cerca de 1 em 2500-6000 nascimentos, dependendo da região geográfica, e com uma frequência de portadores de 1 em 25 indivíduos. O principal aspecto clínico é a disfunção pulmonar. A desidratação e elevada viscosidade das secreções brônquicas em associação com a sua deficiente remoção através da limpeza ("clearance") mucociliar estão na origem da obstrução pulmonar característica da doença. A elevade viscosidade do muco, leva à obstrucção das glândulas exócrinas e, consequentemente, das vias respiratórias, o que leva a infecções recorrentes (pricipal factor de morbilidade e mortalidade para >95% dos doentes). Para além destes característicos ciclos de infecção, são também frequentemente observadas outros sintomas em doentes com FQ. Entre estes, incluem-se insuficiência pancreática (~85% dos doentes), *meconium ileus* (ileo meconial, 5-10% dos doentes) e diabetes (2-3% dos doentes). Quase a totalidade (98%) dos homens adultos com FQ são infertéis devido à ausêncial dos vasos deferentes ou azoospermia obstructiva e a fertilidade feminina é também reduzida devido à viscosidade das secreções do colo do útero. Antes da causa genética da doença ser conhecida, os pediatras diagnosticavam a doença pela constatação de que os doentes com FQ apresentam elevadas concentrações de saes no suor. Tal caracteristíca da doença constitui a base da pricipal ferramenta de diagnóstico mais comum: o teste da concentração de iões de cloreto no suor. Ainda hoje em dia, na maioria dos casos, este é o método de diagnóstico inicial indicativo de doença.

Desde a identificação do gene *CFTR*, em 1989, mais de 1500 mutações foram já identificadas. No entanto, é ainda desconhecida o

impacto da maioria destas na actividade da proteína CFTR. Torna-se então necessário aprofundar os conhecimentos sobre a biologia célular da proteína CFTR normal e mutada, para se compreender na totalidade a fisiopatologia da doença e assim corrigir o defeitobásico que lhe está subjacente.

A FQ é causada por mutações no gene que codifica para a proteína CFTR (do inglês, *Cystic Fibrosis Transmembrane Conductance Regulator*). A proteína CFTR faz parte da família dos transportadores ABC (do inglês, *ATP Binding Cassette*, cuja função dependente de ATP é o transporte através de membranas celulares de um vasto conjunto de diferentes compostos. Entre estes, contam-se açucares, aminoácidos, aniões, drogas, péptidos, proteínas e lípidos. Tal como outros membros da família de transportadores ABC, a CFTR é uma proteína complexa, com multíplos domínios. A cadeia polipeptídica contém 1480 residuos de amino-ácidos que formam: (i) dois domínios transmembranares, cada com seis hélices, que em conjunto formam o poro pelo qual a passagem dos iões de cloreto ocorre, (ii) dois domínios de ligação a nucleótidos ATP, que formam um dímero e regulam a abertura e fecho da CFTR como canal de iões de cloreto, e (iii) um domínio regulador (único na familia de transportadores ABC), que é o sítio principal da fosforilação necessária para a activação. A CFTR é principalmente expressa na membrana apical de células epiteliais das vias respiratórias e intestinos, células pancreáticas e em células secretoras das glândulas sudoríparas. A função da proteína CFTR como canal de iões de cloreto é regulada por níveis de ATP disponíveis no meio intracelular e pelo estado de fosforilação da proteína. O nível de fosforilação é principalmente mediado pela proteína-cinase A (PKA), cuja actividade depende do cAMP.

A mutação mais comum (abreviadamente designada por F508del), presente em ~70 % dos doentes com FQ, consiste na deleção de três nucleótidos, resultando na perda de um resíduo de fenilalanina na posição 508 da cadeia polipeptídica. A proteína mutada é retida a nível do retículo endoplasmático pelo respectivo mecanismo de controlo de qualidade de *folding* proteico, pois esta mutação impede a cadeia polipeptídica de adquirir a sua conformação nativa. Assim, a CFTR com a mutação F508del não chega a ser expressa na membrana apical da célula, onde normalmente exerce a sua função como canal de iões de cloreto. Esta retenção intracelular no retículo endoplasmático leva à rápida degradação da proteína pelo proteasoma, por uma viadependente da ubiquitina.

Desde que foi identificado ogene codificante para a proteína CFTR, têm sido desenvolvidos grandes esforços de investigação dos mecanismos da sua biogénese, tráfego intracelular, e função deste canal de iões de cloreto. Estes avanços na compreensão da patofisiologia da FQ foram possíveis graças a um grande número de modelos celulares e animais, que contribuiram para um melhor esclarecimento dos mecanismos da FQ com o objectivo final de desenhar estratégias destinadas a corrigir o defeito básico desta doença.

A base dos modelos cellulares utilizados no presente trabalho foi uma linha celular de fibroblastos imortalizados a partir de rim de hamster, chamada *Baby Hamster Kidney* (BHK). Estas células foram escolhidas pela sua elevada taxa de proliferação, fácil manuseamento, por não expressarem CFTR endogenamente e por terem sido amplamente descritas em estudos prévios da proteína CFTR, nomeadamente por abordagens bioquímicas. Para a produção dos novos modelos celulares aqui usados procedeu-se à transformação da linha parental

BHK com plasmídeos contendo o cDNA CFTR onde haviam sido previamente introduzidas por muagènese dirigida as diferentes variantes analisadas neste trabalho.

As abordagens experimentais utilizadas no decurso deste trabalho incluram ensaios bioquímicos para avaliação de níveis proteícos em estados estacionários (por *Western Blot*) e avaliação de taxas de decaimmento, tráfego e de maturação através de marcação radioactiva (*pulse*), seguindo o destino da proteína ao longo do tempo (*chase*), seguido de immunoprecipitação (*pulse-chase*). Foram também utilizadas técnicas de electrofisiologia, nomeadamente effluxo de iodeto e *patch-clamp* em *single-channel*, na modalidade "*excised inside-out*", para avaliar a função da proteína CFTR numa população de células e como um único canal de iões de cloreto, respectivamente

Este trabalho teve como objectivo principal investigar os efeitos de compostos químicos no processamento e função da proteína CFTR normal e mutada, para avaliar os mecanismos de acção que estas moléculas exercem na síntese protéica, tráfego e maturação, e, por fim, função na membrana celular como canal de cloreto. Os compostos seleccionados para e presente trabalho podem ser agrupados em duas classes, tendo em conta os seus efeitos já descritos na proteína CFTR nomeadamente: (i) compostos "potenciadores", cujos efeitos se exercem sobre a actividade de CFTR como canal de iões de cloreto, e (ii) compostos "correctores", que "libertam" a proteína CFTR mutante da sua localização intracelular para a superfície da célula.

Os compostos "potenciadores" são assim designados pelos seus efeitos agonistas na estimulação da função da proteína CFTR como canal de iões de cloreto. Nesta classe de compostos esta incluído o

extracto de *Phyllanthus acidus,* uma planta do sudueste asiático, que é há muito utilizada na medicina tradicional para tratamento duma variedade de doenças, nomeadamente respiratórias. Para além do seu efeito potenciador, este extracto também foi demonstrado ter efeitos correctores em outros modelos celulares, e também ser activador de canais de iões de cloreto dependentes de cálcio. Na classe de potenciadores, encontra-se também a genisteína, um flavonoíde que foi previamente demonstrado poder interagir directamente com a proteina CFTR, modulando a sua função como canal de iões de cloreto. Finalmente, o VRT-532, identificado em pesquisas por métodos de *screening* de alto rendimento de bibliotecas de compostos na busca de estimuladores da função do CFTR.

Na classe de compostos descritos como “correctores”, são agrupados compostos descritos como serem capazes de parcialmente corrigirem a retenção intracelular de F508del-CFTR e promoverem a sua localização na membrana celular. Os estudos aqui apresentados de compostes nesta classe incluiram o VRT-325, o VRT640 e o C4a, todos identificados por pesquisas por métodos de *screening* de alto rendimento em bibliotecas de compostos químicos procurando identificar compostos promotores do tráfego da F508del-CFTR.

Os resultados apresentados no presente trabalho para as experiências com genisteína em células BHK, demonstraram que esta não teve qualquer efeito no tráfego intracelular da F508del-CFTR. No entanto, este composto teve efeitos, após tratamento prolongado, na proteina wt-CFTR, nomeadamente: (i) o tratamento com concentraçãoes altas de genistéina, nos estudos por efluxo de iodeto e por patch-clamp demonstrou uma redução de função como canal de iões de cloreto da wt-CFTR. (ii) o tratamento com concentrações baixas de genisteína, avaliado por técnicas bioquímicas demonstrou

um processamento mais eficiente após 2 h de tratamento, comparado com experiências controlo.

Quanto ao tratmento prolongado (48 h) do extracto de *Phyllanthus acidus*, nas proteínas wt- e F508del-CFTR expressas em células BHK não se observou qualquer efeito na síntese proteíca ou tráfego e maturação. Porém, demonstrou-se que este extracto é um modulador da função da proteína CFTR como canal de iões de cloreto. Nomeadamente, em estudos com traqueias de ratinhos *cftr*$^{F508del/F508del}$ e em células epiteliais humanas F508del-CFTR, demonstrou-se este extracto ter aumentar a corrente transepitelial dependente de CFTR.

Finalmente, testou-se o efeito de tratamento prolongado das pequenas moléculas, identificadas através de pesquisa por métodos de *screening* de alto rendimento em bibliotecas de compostos químicos, nomeadamente VRT-325, VRT-532, VRT-640 e C4a. Para além da CFTR wt e F508del, estes estudos foram também efectuados em células BHK expressando as variantes de CFTR previamente descritas como revertentes genéticos do defeito de tráfego da F508del-CFTR, nomeadamente a 4RK e a G550E. Os mecanismos destas duas variantes em restaurar a loclização da F508del-CFTR á superficie da célula foram caracterizados no nosso laboratório, tendo sugerido vias distintas de reparação daquela proteína mutante. Assim, enquanto a G550E parece corrigir o defeito de *folding* da F508del-CFTR, levando a este mutante a ser reconhecido pelos mecanísmos de contolo de qulidade celulares como conformação natíva e portanto fazer o tráfego normal para a membrana celular. O 4RK na F508del-CFTR leva a este mutante a não ser reconhecida (*bypass*) pelos mecanísmos de contolo de qulidade celulares, permitindo que este mutante “escape” da sua retenção intracelular, e seja localizada na membrane celular.

O objectivo dos presentes estudos foi expor estas variantes a agentes que levam à correcção do defeito de tráfego da F508del-CFTR para além do tratamento prolongado com cada um destes compostos para assim elucidar o seu mecanismo de acção. Nestes estudos, observou-se que apenas o VRT-325 apresentou um efeito aditivo com o provocado por um dos revertentes genéticos (4RK) da F508del-CFTR, aumentando a respectiva eficiência de processamento desta variante da proteína CFTR. Assim, tendo por base que o efeito daquele revertente se situa a nivel de um “bypass” no controlo de qulidade do retículo endoplasmático, este efeito aditivo é sugestivo duma corcção a nível do folding da F508del-CFTR por parte do VRT-325.

Na sua globalidade, estes estudos de tratamentos a longo termo com diferentes compostos permitem uma melhor compreensão dos mecanísmos subjacentos do defeito básico da F508del-CFTR. Assim, prevê-se que que os conhecimentos aqui adquiridos sejam relevantes ao desenvolvimento de estratègias que permitam eventualmente chegar a uma cura para esta doença.

Palavras-chave: CFTR, FQ, Canal de Cloreto CFTR, Pequenas Moleculas Corretoras de CFTR, Processamento.

Abbreviations

4-PB	4-phenylbutyrate
4RK	R29K+R516K+R555K+R766K
aa	Amino acid
ADP	Adenosine 5’ Diphosphate
AFT	Arginine-framed tripeptide
AMP	Adenosine 5’ Monophosphate
ATP	Adenosine 5’-Triphosphate
BHK	Baby Hamster Kidney cells
C4a	N-[2-(5-Chloro-2-methoxy-phenylamino)-4'-methyl-[4,5']bithiazolyl-2'-yl]-benzamide
cAMP	Cyclic Adenosine Monophosphate
CBAVD	Congenital bilateral absence of the vas deferens
CF	Cystic Fibrosis
CFTR	Cystic Fibrosis Transmembrane Conductance Regulator
CFTR	Gene encoding CFTR
CHIP	Carboxy-terminus of Hsc70 interacting protein
CIP	CFTR Interacting Protein
Cl^-	Chloride ion
CUAVD	Congenital unilateral absence of the vas deferens
DAPI	4',6-diamidino-2-phenylindole dihydrochloride
ECL4	Extracellular loop 4
ENaC	Epithelial Na^+ channel
ENaC	Epithelial Na^+ channel
ER	Endoplasmic Reticulum
ERAD	ER-associated degradation
ERQC	Endoplasmic Reticulum Quality Control
f_c	Corner frequency
FITC	Fluorescein isothiocyanate
GERAD	Glycan-moiety dependant ERAD
GSH	γ-Glutamyl-cystenyl-glycine
H^+	Hydrogen ion

Hdj	HumanDnaJ
Hsc	Heat shock cognate
Hsp	Heat shock protein
HTS	High Throughput Screening
i	Single-channel current amplitude
IBI	Interburst interval
IP	Immunoprecipitation
K^+	Potassium ion
MBD	Mean burst duration
MSD	Membrane-Spanning Domain
N	Number of active channels
Na^+	Sodium ion
NBD	Nucleotide-binding domain
NHERF1	Na+/H+ exchanger regulatory factor 1
NMDG	N-methyl-D-glucamine
P-gp	P-glycoprotein
PI	Pancreatic Insufficiency
P_o	Open probability
PTC	Premature Termination Codon
RD	Regulatory domain
RT	Room temperature
SDS	Sodium Dodecyl Sulfate
TES	N-tris[Hydroxymethyl]methyl-2-aminoethanesulphonic acid
Ub	Ubiquitin
UPP	Ubiquitin Proteasome Pathway
UT	Untransfected
VRT-325	4-cyclohexyloxy-2-{1-[4-(4-methoxy-benzenesulfonyl)piperazin-1-yl]ethyl}quinazoline
VRT-532	4-Methyl-2-(5-phenyl-1H-pyrazol-3-yl)-phenol
VRT-640	2-{1-[4-(4-Chloro-benzensulfonyl)-piperazin-1-yl]-ethyl}-4-piperidin-1-yl-quinazoline
WB	Western blot

I. Introduction

I. Introduction

1. Cystic Fibrosis

Cystic Fibrosis (CF) is a life-shortening autosomal recessive disease, mainly present in the Caucasian populations. The rate of incidence in this group is 1 in 2500 to 6000 live births, with a carrier frequency of 1 in 25 to 40 individuals, depending on the geographic region.

In Northern European medieval folklore, there is a reference to CF: "*Woe to that child which when kissed on the forehead tastes salty. He is bewitched and soon must die*." (Welsh *et al*., 1995). This early mention already reflects the association between salty skin (elevated concentration of sodium chloride in the sweat of CF patients, a hallmark of CF) and premature death (Welsh and Smith, 1995). The first references in the medical literature to CF symptoms go back to the mid 17th century, when a number of reports referred to infants and children with *meconium ileus*, a severe form of intestinal obstruction at birth and another hallmark of CF. CF as a disease probably remained unrecognised until the mid 1930's, as several of its clinical features resemble those of other diseases. These include common pneumonia, bronchiectasis, asthma, failure to thrive, and celiac disease. Indeed, in places where such conditions are still common, CF may still remain underdiagnosed.

The first medical descriptions of CF as a disease in its multiple clinical aspects were made in Switzerland by Fanconi in 1936, who termed it as *"mukoviszidose"*, a German term meaning "thickened mucus". The description of CF as "cystic fibrosis of the pancreas" in 1938 by Dorothy Anderson in the United States gave the first complete patho-

physiological description of the disease (Anderson, 1938). A decade later, Farber emphasised the clinical relevance of mucoviscidosis (Farber, 1945), describing “a generalised state of thickened mucus” as the main cause for CF morbidity and mortality. Simultaneously, Anderson and Hodges presented the first indicators of the autosomal recessive inheritance pattern of this ailment (Anderson and Hodges, 1946).

In 1953, Di Sant'Agnese described the excess of sodium chloride in the sweat of CF children. Based on this physiological dysfunction, the sweat chloride test was developed, which remains a reliable, cheap diagnostic test for CF.

In the early 1980’s, knowledge on CF advanced rapidly, due to two independent and complementary observations regarding abnormal salt transport in epithelia. Knowles and colleagues investigated the diminished chloride (Cl^-) and increased sodium (Na^+) transport in the CF respiratory epithelium (Knowles *et al*., 1981). Soon afterwards, Quinton and Bijman provided an explanation for the Cl^- impermeability of the epithelial cells lining the ducts of sweat glands (Quinton and Bijman, 1983). Later in the 1980’s, the basic electrolytic defect was localised in the apical membrane of CF epithelial cells (Boucher *et al*., 1986; Frizzell *et al*., 1986).

Concomitantly in the 1980’s researchers began to pursue the identification and isolation of the gene which when mutated is responsible for CF. This was finally achieved in 1989 by the joint efforts of three laboratories, and provided the basis to tackle the disease at the molecular level (Riordan *et al*., 1989; Rommens *et al*., 1989; Kerem *et al*., 1989). The protein encoded by this gene was called the Cystic fibrosis transmembrane conductance regulator

(CFTR) and later shown to function as a Cl^- channel (Welsh *et al*., 1995). CF is thus caused by a defect that affects the transport of Cl^- across the epithelial tissues. But, as initially shown by Knowles *et al*. (Knowles *et al*., 1986), it also results in enhanced Na^+ absorption in CF respiratory epithelium.

Due to the fact that water follows the direction of salt transport across the epithelia, the CF defect is also described by some as causing enhanced water reabsorption, which according to these authors leads to increased salt concentration in the airway surface liquid (ASL) (Puchelle *et al*., 2002). Altogether, this drastic dehydration causes an increased thickness of mucus in the respiratory epithelium of CF patients, decreased mucus clearance, attracts bacterial pathogens, leading to successive cycles of infection and inflammation, causing airway obstruction and loss of lung function, in the so-called "CF pathogenesis cascade" (See Figure I.1) (Amaral and Kunzelmann, 2007).

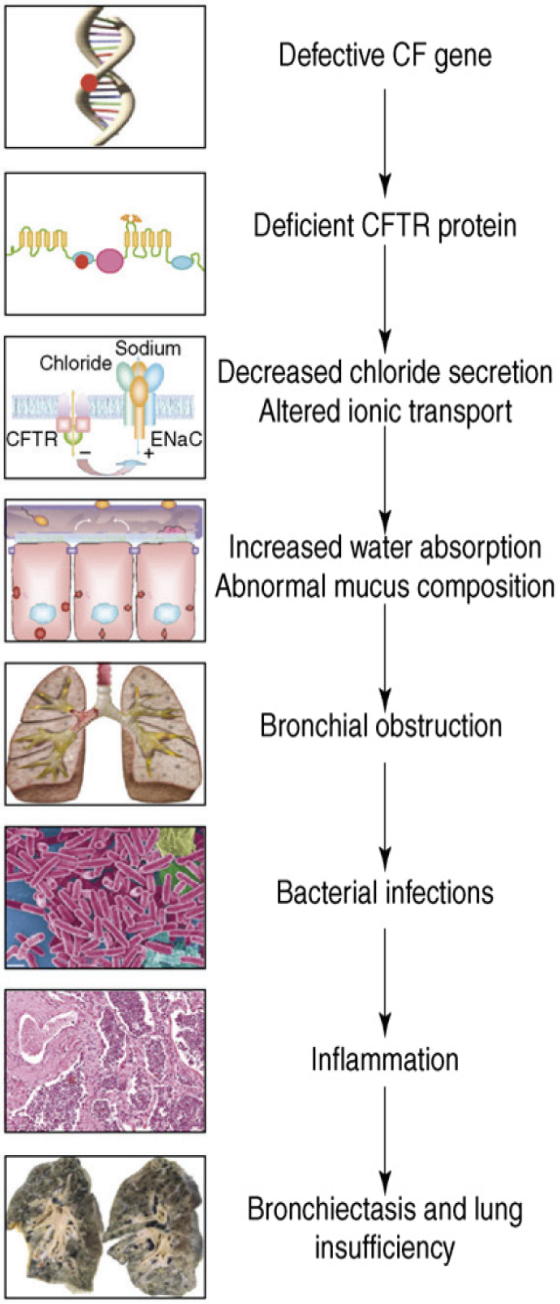

Figure I.1 - CF Pathogenesis Cascade: From primary gene defect to lung disease (Amaral and Kunzelmann, 2007).

Other pathophysiological features in CF tissues include submucosal gland dilation, caused by mucus blockage and reduced Cl^- reabsorption in sweat ducts. Furthermore, a number of factors involved in inflammatory responses are elevated in CF. These include interleukin-8, interleukin-6, tumour necrosis factor α, and leukotriene B_4. In parallel, anti-inflammatory cytokines and proteases are found in reduced levels (Sagel and Accurso, 2002; Koehler *et al*., 2004). Mannose binding protein and α-antitrypsin (Freedman *et al*., 2004), as well as toll-like receptors (Muir *et al*., 2004), shown to be present at increased levels, contributing to the pro-inflammatory state observed in CF tissues (Heeckeren *et al*., 1997). However, the mechanism for the altered levels of inflammatory factors remains unexplained. It is unclear whether these alterations are caused by CFTR dysfunction or only arise as a consequence of disease progression, including microbial challenge (Accurso, 1997).

Another hypothesis to explain the pathophysiology of CF disease is the so-called “low-salt” and “isotonicity” model, where a reduced secretion of ions and consequential decreased salt concentration in the mucus leads to decreased water secretion from the epithelium to the ASL (Rowe *et al*., 2005).

It has been postulated that the altered salt concentration in ASL also causes reduction in the activity of the host anti-microbial peptides, which are inactivated at high salt concentrations (Smith *et al*., 1996; Bals *et al*., 1999; Joo *et al*., 2004).

In the majority of patients, the gastrointestinal tract is also affected, with ~85 % exhibiting pancreatic insufficiency (PI), due to obstruction of the pancreatic ducts and resulting scarring (or fibrosis), leading to loss of exocrine function. *In utero* PI can lead to post-natal steatorrhea

and failure to thrive, the latter a common cause for diagnosis. About 5 % of newborns with CF present *meconium ileus*, its occurrence being almost sufficient for diagnosis (Collins, 1992). In adult CF patients, male infertility is almost universal, mostly due to the congenital bilateral (or unilateral) absence of the vas deferens (CBAVD or CUAVD). Female patients have reduced fertility, but health during pregnancy and impact of treatments on foetus are of concern. Other manifestations include liver cirrhosis and *diabetes mellitus* (Davies *et al*., 2007). The absence of functional CFTR in CF tissues has also been reported to affect the glycoconjugates found on the cell surface of epithelial cells (Dosanjh *et al*., 1994). One pathophysiological hypothesis has suggested (Barasch and Al-Awqati, 1993) that modified surface glyco-antigens may be specifically recognised by two major pathogens (*Pseudomonas aeruginosa* and *Haemophilus influenza*) causing CF lung disease (Saiman and Prince, 1993; Pier *et al*., 1997).

Despite the current number of therapeutics available, the main causes of early death are repeated lung infections, ultimately leading to loss of lung function. Current therapeutic interventions, which are mostly symptomatic and not corrective, combined with early and correct diagnosis, have resulted in a considerable increase in the quality of life and life-expectancy which is nowadays in the mid thirties in the USA (Cystic Fibrosis Genetic Analysis Consortium Report, 2007).

Nevertheless, in order to cure the disease, it is now clear that it is necessary to correct the basic defect underlying CF. Soon after the identification of the CF gene, it was thought that the cure would be easily achievable by gene therapy. However given the major hurdles associated with this strategy, gene therapy is at a standstill and it is currently believed that pharmacological approaches will be a faster route to correct the basic defect in CF (Amaral and Kunzelmann, 2007).

2. Cystic Fibrosis Gene and Protein

After being mapped to the long arm of chromosome 7, band 31 (7q31), by linkage analysis of patients and their families using a large number of polymorphic markers (Knowlton *et al*., 1985; Tsui *et al*., 1985; Wainwright *et al*., 1985), the *CFTR* gene was finally identified by positional cloning in 1989. A combination of chromosome walking and jumping led to the identification, isolation and sequencing of the *CFTR* gene (Kerem *et al*., 1989; Riordan *et al*., 1989; Rommens *et al*., 1989). This gene was found to be expressed at very low levels, and restricted to epithelial tissues (Riordan *et al*., 1989). Strong evidence that this was the gene defective in CF came from detection of a 3 bp deletion in exon 10 of the gene, causing the loss of a single amino acid (aa), phenylalanine 508 in a number of families affected by CF (Cheng *et al*., 1990; Cutting *et al*., 1990). This mutation which was initially termed ΔF508 and nowadays is referred to as F508del, was found to be present in ~70 % of mutant alleles (Kerem *et al*., 1989). Definite confirmation that this was the CF gene came by restoration of ionic transport to CF epithelia by transfection with the CFTR cDNA (Rich *et al*., 1990; Drumm *et al*., 1990). Identification of CFTR as a regulated Cl^- channel came from reconstituting purified, recombinant CFTR protein into lipid bilayers, allowing single-channel analysis (Bear *et al*., 1992). Demonstration that CFTR is a Cl^- channel came from experiments assessing its ion-selectivity (Anderson *et al*., 1991a).

2.1 *CFTR* Gene

The *CFTR* gene spans 189 kb of the human genome (Ellsworth *et al*., 2000) and comprises 27 exons, which encode a 6.2 kb transcript that originates a 1480-amino acid (aa) protein (Riordan *et al*., 1989) (See figure I.2).

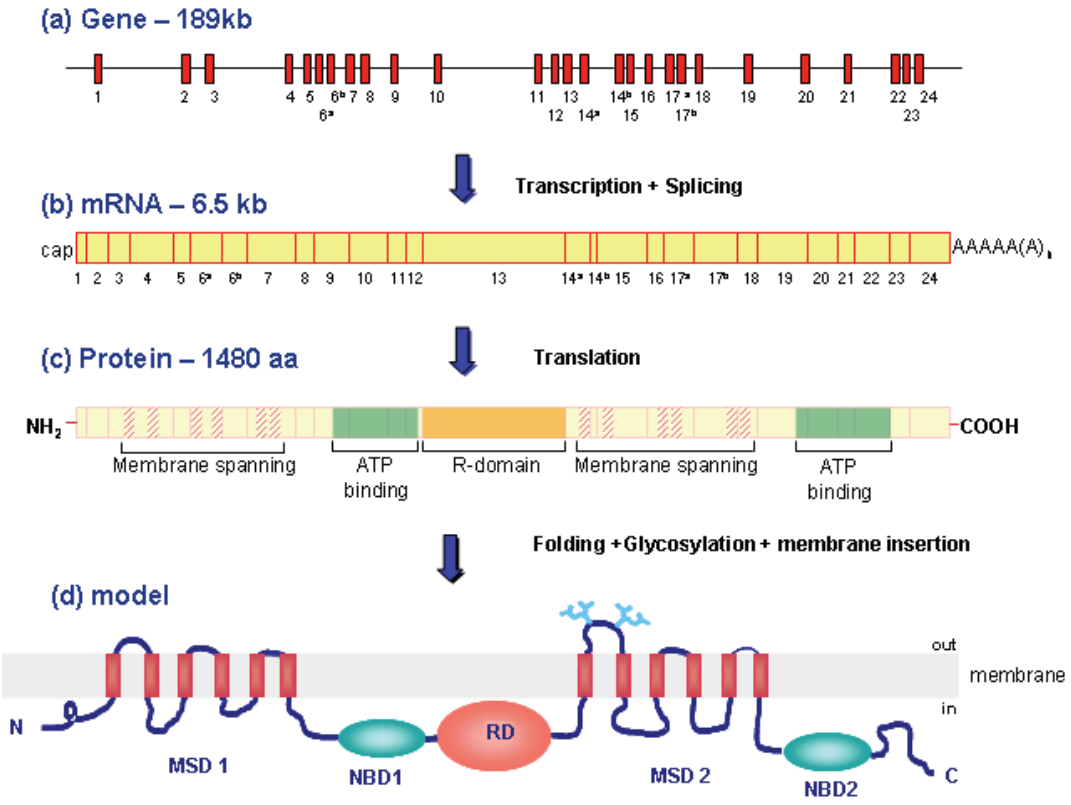

Figure I.2 - Scheme of the *CFTR* gene, CFTR mRNA and CFTR protein. (Abbreviations: N, N-terminus; MSD, membrane-spanning domain; NBD, nucleotide-binding domain; RD, regulatory domain; C, C-terminus; see section 2.2.1 for details).

2.2 CFTR Protein

2.2.1 Structure

After the initial identification of the *CFTR* gene, Riordan and colleagues (Riordan *et al*., 1989) proposed a structure based on the polypeptide chain sequence, that turned out be very similar to the currently accepted model for CFTR protein structure.

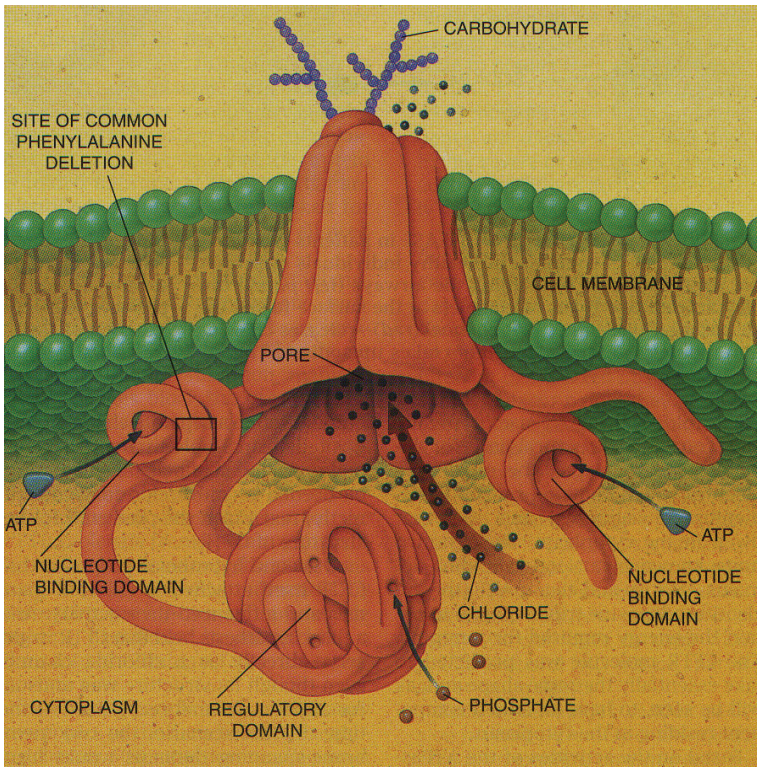

Figure I.3 - Putative structure of CFTR protein (Welsh and Smith, 1995).

The CFTR protein is a member of the ATP-Binding Cassette (ABC) transporter family (Hughes, 1994), which in humans comprises of more than fifty members (one of the largest encoded in the human genome), and is also known as ABCC7. ABC transporters are involved in active transport across cell membranes, and are present in a wide range of organisms, from bacteria to mammals. The mechanism of transport is usually Adenosine 5'-Triphosphate (ATP) driven and substrates of ABC transporters include a vast selection of metabolites and small molecules, comprising amino acids, proteins, sugars, lipids and drugs (Higgins, 1992; Schneider and Hunke, 1998).

ABC transporters have common structural features; namely membrane-spanning domains (MSDs), usually composed of six membrane spanning α-helices, and nucleotide binding domains (NBDs), which bind and hydrolyse ATP. Family members comprise "Half-transporters", comprised of one MSD and one NBD that dimerise

so two MSDs and two NBDs form an active transporter, while “full” ABC transporters, like CFTR, include two MSDs and two NBDs. Uniquely, CFTR also possesses a large regulatory domain (RD) linking the two halves of the protein, which contains multiple phosphorylation sites and many charged amino acids (See figure I.4). The NBDs of ABC transporters possess Walker A and Walker B motifs, found in wide variety of proteins, and particular features that distinguish them from NBDs of other proteins, such as the LSGGQ signature motifs which are critical for NBD1/NBD2 dimerisation. This dimerisation is believed to be essential for the transport activity of most ABC transporters (Davidson and Chen, 2004).

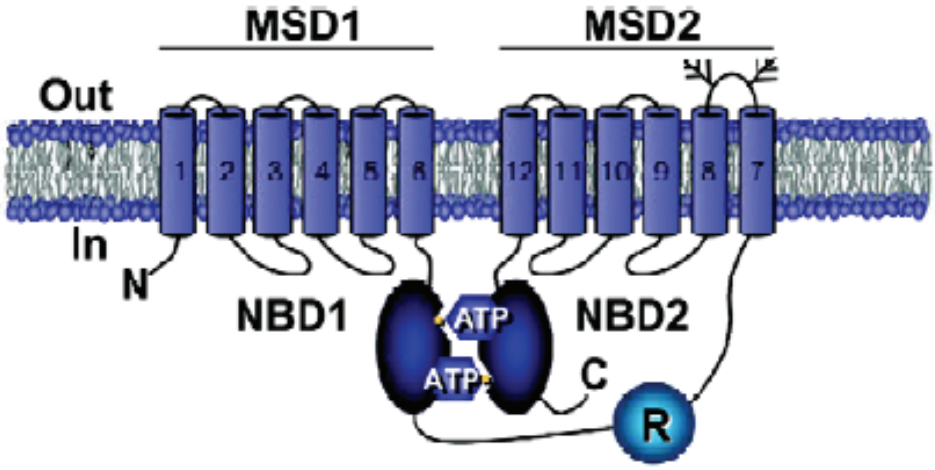

Figure I.4 - Model of the CFTR protein at the plasma membrane. (Abbreviations as in Figure I.2; see text for details). Reproduced from (Li *et al*., 2007).

2.2.2 Biogenesis, Processing & Degradation

Like most membrane proteins, the CFTR protein is cotranslationally inserted into the membrane of the endoplasmatic reticulum (ER) (Lu *et al*., 1998) where it is core-glycosylated (Cheng *et al*., 1990). This immature form, of ~140 kDa, is also known as band B (see figure I.5). The protein exits the ER to the Golgi, on a pathway to its membrane

localisation As the protein proceeds through the Golgi, it undergoes a maturation process, gradually reaching its fully-glycosylated form as it progresses through the Golgi compartments towards the cellular membrane, giving rise to a 150 to 170 kDa protein (See Figure I.5), also known as band C (Cheng *et al*., 1990).

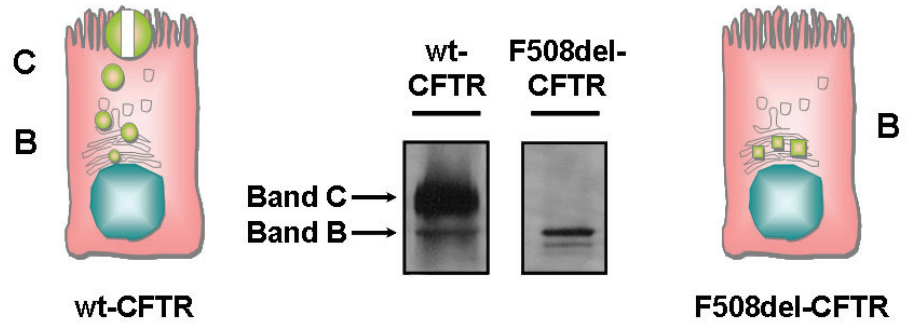

Figure I.5 - Western Blot of BHK cells expressing wt- (left) and F508del-CFTR (right). Cartoons with permission, Amaral M.D., unpublished; own WB figures.

CFTR is thus a large protein, composed of several distinct domains, that undergoes a complex biogenesis and processing. The co-translational insertion of the nascent polypeptide chain into the ER-membrane is mediated via a specific signal sequence at the N-terminus recognised by the signal recognition particle (SRP), which in turn directs the association to a translocon complex, composed of many subunits including Sec61, forming a ribosome-translocon complex (RTC) (Bebok *et al*., 1998a). A number of specific mechanisms coordinate the insertion, orientation and progression of ER-membrane insertion of CFTR, by shielding the hydrophobic regions of the nascent chain, allowing correct folding and eventual domain association (Skach, 2000; Sadlish and Skach, 2004).

Data by some authors (Lu and Pedersen, 2000) indicate that only after successful insertion of MSD2 into the ER-membrane and synthesis of NBD2, is a stable conformation of the protein achieved. The overall biogenesis process includes the participation of the chaperone pair

Heat shock cognate 70 (Hsc70) and human DnaJ 2 (Hdj-2) which probably never release molecules that are unable to achieve a folded conformation (Meacham *et al*., 1999). According to the working model of Farinha and Amaral (2005), this constitutes the first ER quality control checkpoint (Figure I.6): If the Hsc70 chaperone remains associated to its substrate for too long, it recruits the carboxy-terminus of Hsc70 interacting protein (CHIP), an E3 ubiquitin (Ub) ligase, that sends the protein for degradation by the Ub-proteasome pathway or system (UPP/UPS) (Meacham *et al*., 2001). This process is termed ER-associated degradation (ERAD) (Meusser *et al*., 2005). During its biogenesis, CFTR protein is core-glycosylated at two asparginine residues (N804 and N900), located in the extracellular loop 4 (ECL4). This is important for the recruitment of the ER membrane chaperone calnexin. According to the working model of Farinha and Amaral (2005) the calnexin cycle constitutes the second ER quality control checkpoint (Figure I.6). Like Hsc70/Hsp70, calnexin helps the CFTR protein to achieve a native conformation, but if the substrate remains in the calnexin cycle through multiple rounds, it is sent for degradation in a process that is mediated by its glycan moiety and thus termed GERAD (for glycan-moiety dependent ERAD) (Farinha and Amaral, 2005). More recently, Roxo-Rosa *et al*. (2006) postulated that if CFTR successfully passes the two initial ER folding checkpoints, it is assessed for its native conformation at a third ER quality control checkpoint. This is a retention mechanism that recognises arginine-framed tripeptide (AFTs) motifs at the ER exit sites, sorting correctly folded proteins into coat protein complex II (COPII) coated vesicles (Yoo *et al*., 2002) (See Figure I.6).

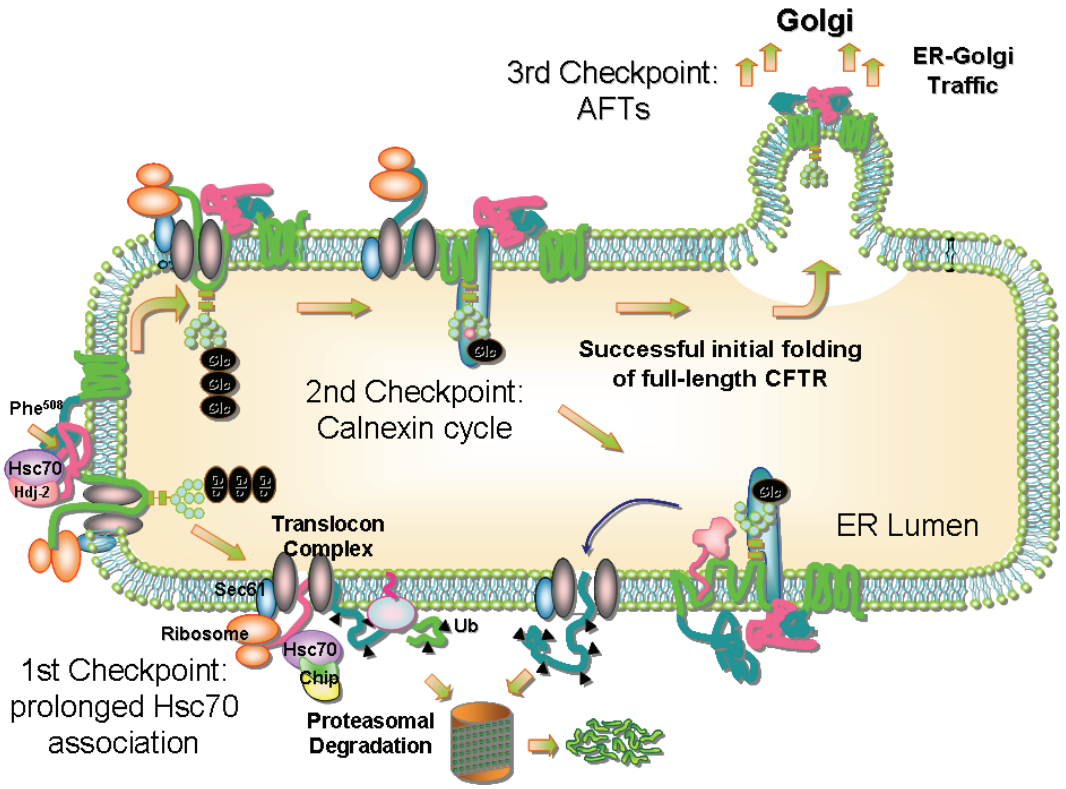

Figure I.6 - Model of CFTR Biogenesis into ER membrane. Legend: CFTR-MSDs, green line; NBDs, dark green line; RD, pink line; other proteins indicated. See text for more details (Adapted from (Farinha and Amaral, 2005)).

Indeed, for proteins that are to be localised to the cellular membrane, a secretory pathway from the ER via the Golgi is used. Exit from the ER is dependent on masking ER retention signals on the CFTR protein to allow its incorporation into the COPII coated vesicles, such as the above mentioned AFTs or diacidic motifs (Wang *et al*., 2004). These form a pre-Golgi complex that in turn fuses to the *cis* Golgi, traversing *medial* and *trans* Golgi, before being targeted to the cellular membrane. Other proteins have signals that participate in their active export from the ER, such as a terminal Valine (Nufer *et al*., 2002). When this was incorporated into the CFTR polypeptide chain, it was found to increase efficiency of export of wt-CFTR, but failed to have an effect on F508del-CFTR (Wendeler *et al*., 2007).

Regarding the early stages of CFTR trafficking to the plasma membrane, different models have been proposed (Figure I.7, (Bannykh *et al*., 2000))

In the first model (Figure I.7, model 1), CFTR is inefficiently selected for ER export to the Golgi via COPII vesicles, and then proceeds to the cell surface as described above. Non-selected CFTR is sent directly for degradation from the ER. In a second model (Figure I.7, model 2), a larger pool of CFTR (folded and misfolded) is included in COPII vesicles, but only CFTR protein that has undergone a degree of maturation will reach the *cis* Golgi, while the rest is recycled back to the ER in COPI vesicles. The third model (Figure I.7, model 3) contemplates a more speculative explanation for the low levels of CFTR in the pre-Golgi and peripheral localisation within the Golgi, by hypothesising direct transport of mature CFTR to the *trans* Golgi and even to the endosomes compartment, from where it is inserted to the plasma membrane (Bannykh *et al*., 2000).

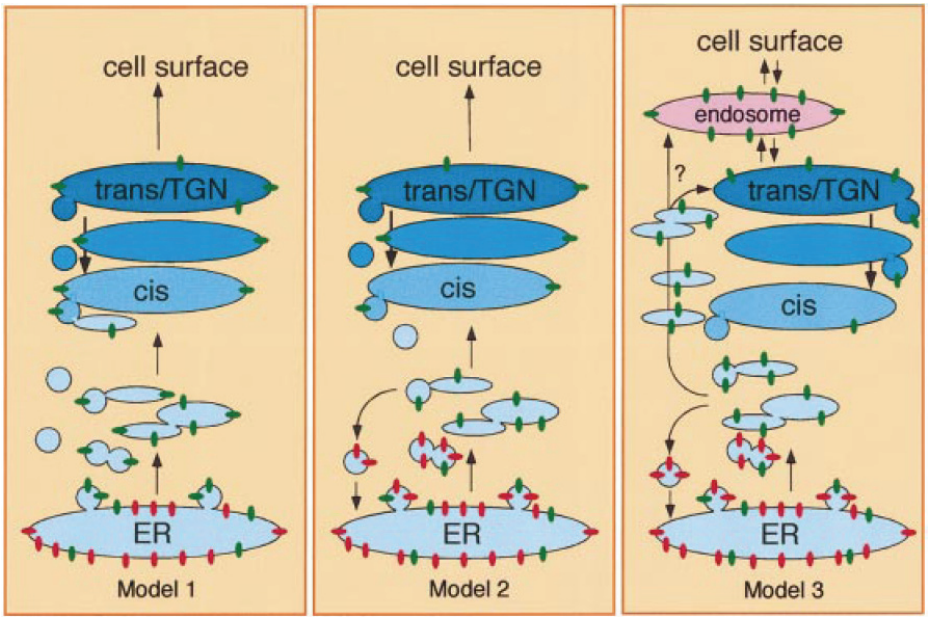

Figure I.7 - Models of CFTR trafficking from the ER to the cell surface. Each of the panels illustrates a possible pathway for movement of CFTR from the ER through the early secretory pathway. Wild-type CFTR is shown by the green (folded, mature form) or red (misfolded form). (Bannykh *et al*., 2000)

This maturation process by which CFTR acquires its fully-glycosylated form, involves a number of Golgi resident glycosyltransferases that sequentially add monosaccharidic residues, including N-acetylglucosamine, galactose, fucose, neuraminic and syalic acid (O'Riordan *et al*., 2000). While these are not required for CFTR Cl^- channel function (Gregory *et al*., 1991; Morris *et al*., 1993), it is generally believed that these moieties confer stability to CFTR at the membrane (Farinha and Amaral, 2005).

Recent studies have elucidated the processes occurring in the late stages of CFTR trafficking to the membrane (exocytosis) and also its subsequent internalisation (endocytosis) as well as recycling. Endocytosis of CFTR from the cellular membrane is important for regulation of CFTR protein density at the cellular membrane. CFTR is thought to be specifically targeted to the clathrin-dependent endocytosis pathway by a tyrosine motif (YDSI) present at the C-terminus of CFTR (Bradbury *et al*., 1999; Prince *et al*., 1999), recognised via the AP-2 protein. Dynamins are required for vesicle fission, myosin VI, a molecular motor that drives cargo to the minus end of F-actin, promoting vesicle movement along actin filament inwardly (Swiatecka-Urban *et al*., 2004) and myosin Vb in the opposite direction, i.e. from recycling endosomes (Rab11a-specific) back to the apical membrane (Swiatecka-Urban *et al*., 2007), all play a role in CFTR membrane recycling. Furthermore, a number of Rab GTPases have been identified as being involved in these endocytic and recycling processes. Rab5 is involved in the initial step in promoting endocytosis of CFTR, Rab7 increases CFTR degradation by enhancing the trafficking of CFTR to late endosomes and lysosomes, while Rab9 mediates CFTR trafficking from late endosomes to the TGN (Gentzsch *et al*., 2004). Recycling of CFTR from early endosomes can be mediated by Rab4 which directly targets CFTR back to the plasma

membrane (Saxena *et al*., 2006). RME-1, another GTPase, facilitates exit of CFTR from the recycling endosome (Picciano *et al*., 2003). PDZ binding proteins can inhibit CFTR endocytosis from the plasma membrane, as well as facilitate recycling of internalised CFTR from early endosomes (See Figure I.8, Left)(Ameen *et al*., 2007).

This PDZ binding motif (DTRL) is found at the C-terminus of CFTR and has been shown to be involved in forming macromolecular complexes, localising and anchoring CFTR to the apical actin network, through recruitment of EBP50 and Ezrin (Short *et al*., 1998)(See figure I.8, Right). The PDZ binding motif also provides a molecular basis for interaction with other apical membrane proteins, such as the epithelial Na^+ channel (ENaC), through recruitment of proteins such as Na+/H+ exchanger regulatory factor 1 (NHERF1) and Yes-associated protein 65 (YAP-65) (Kunzelmann, 2001)(See figure I.8, Right). NHERF1 has also been shown to be important in stabilising CFTR protein at the plasma membrane, its knockdown leading to an enhanced removal of temperature rescued CFTR from the cell membrane (Kwon *et al*., 2007).

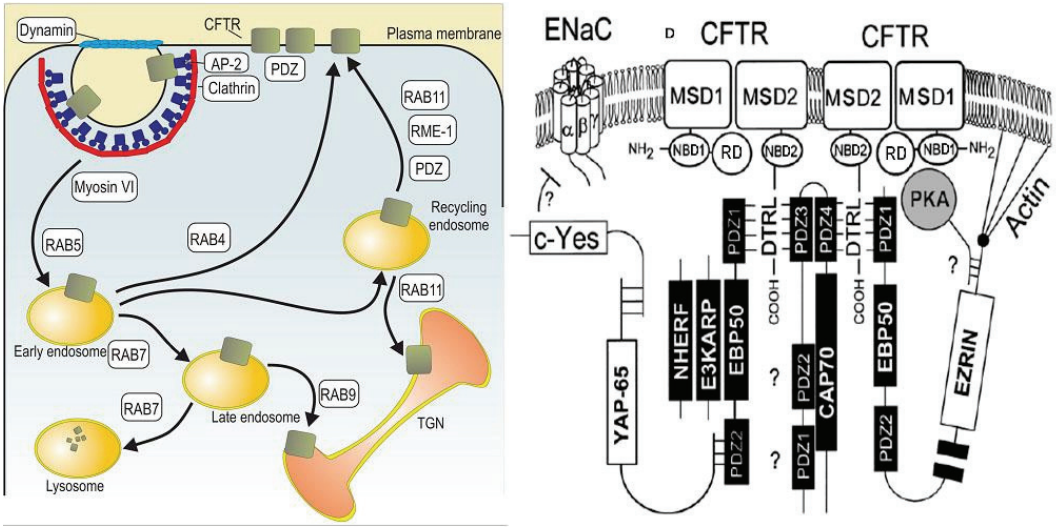

Figure I.8 - Left, Model of Endocytosis and Recycling of CFTR (brown rectangle). TGN, *trans* Golgi network, see text for other details (Ameen and Apodaca, 2007). Right, Model of interaction of CFTR via the PDZ binding motif (Kunzelmann, 2001).

Intracellular trafficking of CFTR can be divided into four major categories (Ameen *et al*., 2007): (i) CFTR is translated in the endoplasmic reticulum (ER) where core sugars are added to the protein. Wild-type CFTR traffics to the *Trans* Golgi network (TGN) where the core sugars are modified into complex carbohydrates, and then trafficked to the apical plasma membrane. (ii) CFTR is efficiently removed from the cell surface by clathrin-mediated endocytosis using trafficking signals embedded in the amino acid sequence of CFTR. (iii) From endosomes, CFTR can recycle back to the cell surface in a direct manner, or via recycling endosomes. (iv) Internalised CFTR can be directed to lysosomes for degradation. (See figure I.9).

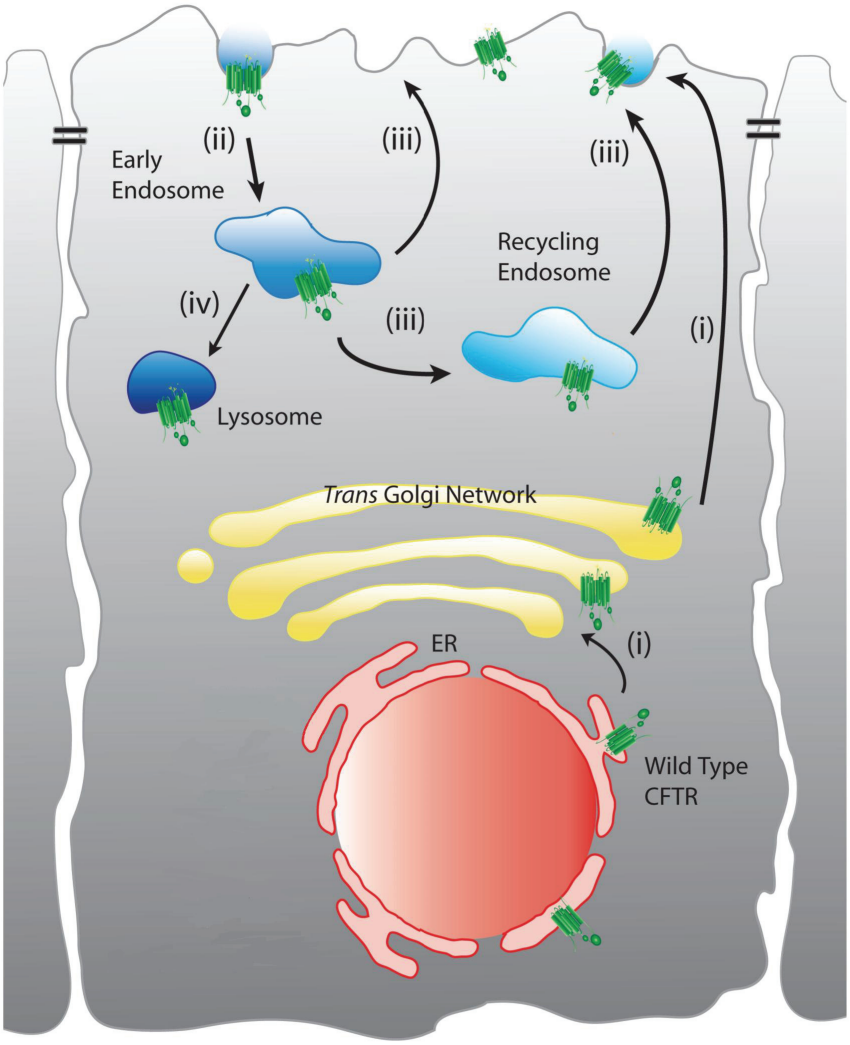

Figure I.9 - Main Trafficking Pathways of wild-type CFTR. See text for details (Ameen *et al*., 2007).

2.2.3 CFTR Functions

2.2.3.1 CFTR as a Chloride channel

Shortly after the *CFTR* gene was identified, it became clear that the protein encoded by the *CFTR* gene is dependent on ATP and cAMP for its channel activity (Anderson *et al*., 1991b). There were, however, doubts about whether the CFTR protein itself was a Cl^- channel or whether it was a regulator of Cl^- channels found in epithelial cells. This was due to the fact that up to then, no other ion channel had been identified to have any structural similarity to CFTR and the loss of Cl^- permeability in CF was compatible with both hypotheses.

However, subsequent studies showed that indeed CFTR is itself a Cl^- channel. This was demonstrated by several lines of evidence: Firstly, expressing CFTR protein into cells that do not normally exhibit cAMP-stimulated Cl^- secretion led to the appearance of cAMP-activated Cl^- conductance (Anderson *et al*., 1991b; Bear *et al*., 1991). Secondly, mutation of positively charged aa residues in the membrane-spanning domains caused a change in CFTR anion selectivity (Anderson *et al*., 1991a). Thirdly, reconstitution of purified, recombinant CFTR protein in planar lipid bilayers led to the generation of Cl^- channels regulated by cAMP-dependent phosphorylation which possessed properties ascribed to cAMP-stimulated Cl^- channels from epithelial cells (Bear *et al*., 1992).

It was then shown that CFTR is membrane-anchored via its two MSDs, which together form the channel pore (Chang *et al*., 1994). By performing experiments with anions of different sizes, it was estimated that this pore is ~5.3 Å in diameter, with an asymmetric configuration: funnel-like with the wider aperture facing the cytosol, with a

conductance of 6-10 pS (Linsdell *et al*., 1997). Figure I.10 shows the tracing from a single CFTR molecule, the small amplitude and the rapid succession of open and closed states of this channel.

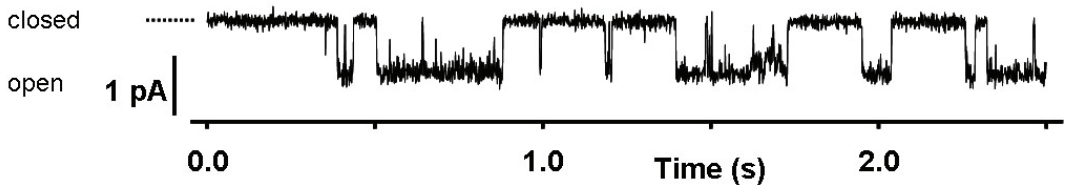

Figure I.10 - Single-channel tracing of CFTR. 2.5-s tracing from an excised, inside out membrane patch containing a single CFTR Cl^- channel. Dashed line shows closed channel, downward deflections represent channel openings. Own work, obtained during visit to DN Sheppard's lab.

Phosphorylation of the Regulatory domain (RD) and presence of ATP are required for transition between open and closed states of CFTR Cl^- channel. After cAMP-dependent activation of mainly PKA (but also PKC), these kinases are believed to phosphorylate a number of consensus sites within the RD inducing a conformational change, leading to reduced affinity of the RD to NBD1 (see Figure I.11 (Baker *et al*., 2007)).

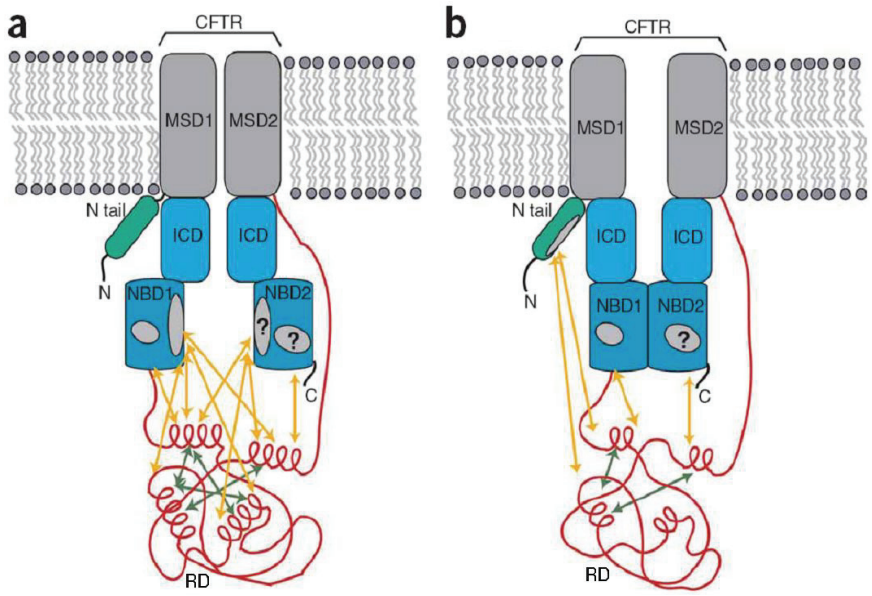

Figure I.11 - Scheme illustrating how phosphorylation-induced structural changes in the regulatory domain (RD) may lead to a redistribution of binding equilibria with various regulatory interaction partners. RD is shown in red, with

multiple helices reflecting the fractional helical structure in portions of this region. Green arrows, indicate potential tertiary interactions within the RD and gold arrows, interactions with binding partners (multiple arrows represent dynamic exchange of both multiple RD binding sites and multiple interaction interfaces, without implying involvement of specific RD segments). Other components of CFTR are also shown, including membrane spanning domains (MSDs), intracellular domains (ICDs), nucleotide-binding domains (NBDs) and cytoplasmic, helical N terminus (N tail). Gray ellipses in NBDs represent putative binding surfaces of interaction partners Interactions favoured by nonphosphorylated (a) and phosphorylated (b) RD. Upon phosphorylation, RD has a less helical structure and reduced interactions with NBD1 and possibly NBD2, as well as within the R domain. This loss of affinity for RD of NBD1 allows NBD1-NBD2 dimerisation, leading to channel opening (Baker *et al*., 2007).

The phosphorylation-induced conformational change of the RD, leading to lower affinity of RD to NBD1, allows NBD1 to bind ATP and to form the NBD1-NBD2 dimer. CFTR gating is then modulated by a cycle of hydrolysis of ATP. Once the RD is dephosphorylated, the RD-NBD1 interaction is re-established, and the channel returns to a quiescent state.

A number of different models of how gating is driven by ATP hydrolysis have been proposed. Initially, Carson *et al*. suggested that ATP binding occurs simultaneously at both NBDs in the closed conformation, hydrolysis at NBD1 then opens the channel, while hydrolysis at NBD2 closes the channel (Carson *et al*., 1995). In figure I.12 four states in the gating cycle transition between open and closed states are shown: In state 1, no ATP is bound to the NBDs and the channel is closed. In state 2, ATP binds to both NBDs, but does not open the channel. In state 3, hydrolysis has occurred at NBD1, causing the channel to open. In state 4, hydrolysis has occurred at NBD2, closing the channel.

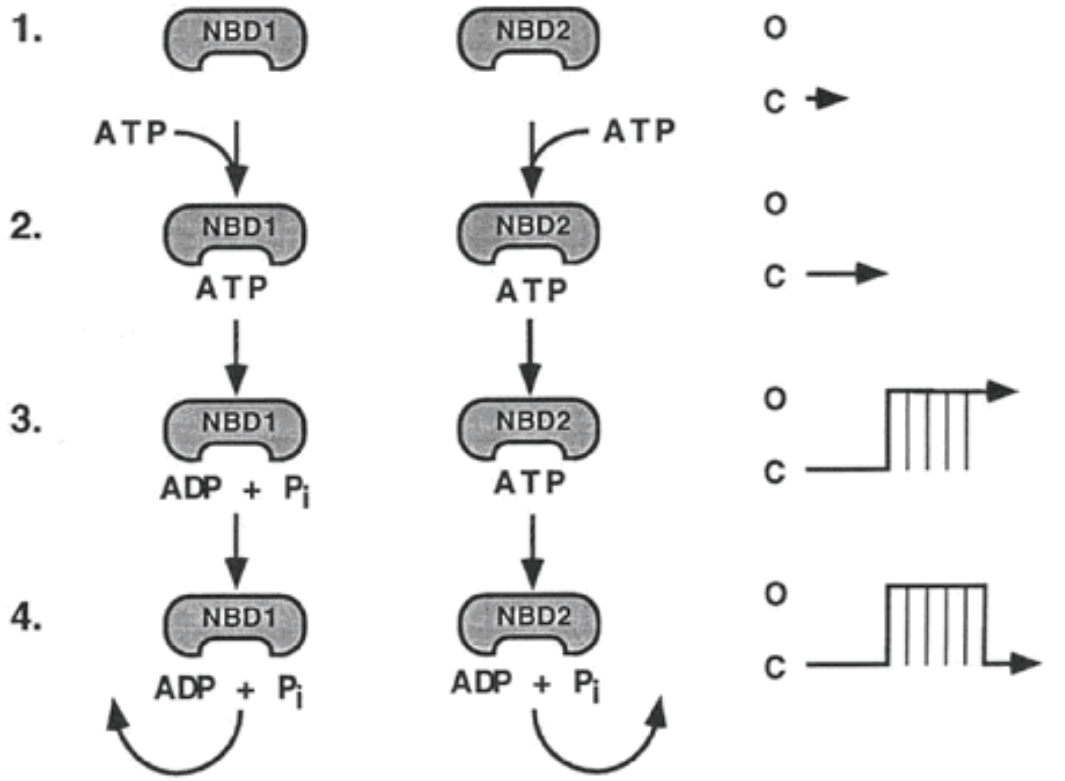

Figure I.12 - Early model of ATP-dependent gating. Vertical columns from left to right represent events at NBD1, NBD2, and the effect on channel opening and closing. “O” and “C” represent channel open and closed states, respectively. See text for details. Adapted from (Carson *et al*., 1995).

Later it became clear that the binding sites of the NBDs are located at their interface, and ATP binding at both sites is required for NBD dimerisation, while it became apparent that only ATP binding (without ATPase activity) at one site is required to open the channel, while ATPase activity appears to be required at the other site to close the channel (Vergani *et al*., 2003). Consistent with this mechanism, photoaffinity labelling studies showed that NBD1 stably binds nucleotides while NBD2 rapidly hydrolyses them (Aleksandrov *et al*., 2002). Furthermore, it was shown that dimerisation of NBDs is required for optimum hydrolysis (Kidd *et al*., 2004) and channel gating (Vergani *et al*., 2005).

Although many facets remain to be clarified, the present structural interpretation of ATP-dependent gating cycle includes two crucial steps, firstly phosphorylation of RD, and secondly binding of ATP allowing dimerisation of the NBDs. This model also accounts for the dynamics observed in CFTR channel gating (See Figure I.13) (Gadsby *et al*., 2006) and is consistent with the most recent structural studies (Baker *et al*., 2007), described above.

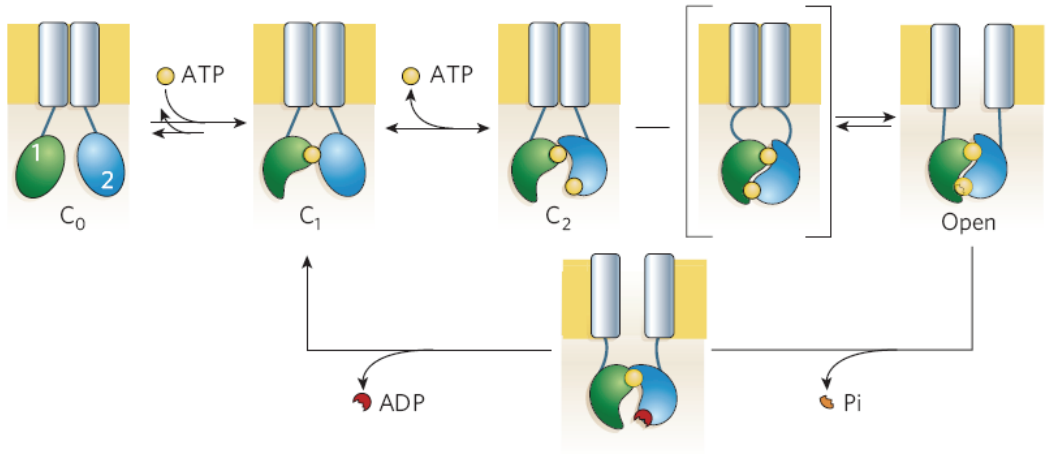

Figure I.13 - ATP-dependent gating cycle of phosphorylated CFTR channels. The RD is omitted for clarity. ATP (yellow circles) remains tightly bound to NBD1 (green) Walker motifs for several minutes, during which time any closed-open-closed gating cycles occur. ATP binding to NBD2 (blue) is followed by a slow channel opening step (C2-to-Open) that proceeds through a transition state (square brackets) in which the intramolecular NBD1-NBD2 tight heterodimer is formed but the transmembrane pore (grey rectangles) has not yet opened. The relatively stable open state becomes destabilised by hydrolysis of the ATP bound at the NBD2 composite catalytic site and loss of the hydrolysis product, inorganic phosphate (Pi). The subsequent disruption of the tight dimer interface leads to channel closure (Gadsby *et al*., 2006).

More recently, it has been suggested that the dimerisation and disruption of the dimer interface are best described as a progressive conformational changes, by which small sequential structural changes modulate gating behaviour (Csanady *et al*., 2006). Also, studies with human-murine CFTR chimeras support this model, demonstrating that NBD dimerisation is led by a conformational change in NBD1, before conformational change in NBD2, and not by simultaneous

conformational changes of the two NBDs as a dimer (Scott-Ward *et al*., 2007).

2.2.3.2 Bicarbonate and Glutathione Transporter

Besides transporting Cl^-, the CFTR channel has also been shown to be required for bicarbonate (HCO_3^-) transport in the lungs, gastrointestinal tract and in the pancreas (Hug *et al*., 2003). While CFTR has been shown to have 4-fold higher affinity for Cl^- over HCO_3^- (Poulsen *et al*., 1994), this permeation has important implications for patho-physiology, as HCO_3^- is involved in several cellular functions, including acting as a pH buffer thus enhancing the solubility of many proteins (see 2.2.3.5 Other functions). Moreover, the importance of HCO_3^- transport by CFTR was further evidenced by the correlation of HCO_3^- transport and the pancreatic function (Lee *et al*., 1999). Indeed, CFTR mutants that do not support HCO_3^- transport, but show normal (Briel *et al*., 1998) or only slightly reduced Cl^- permeability, are associated with pancreatic insufficiency (Choi *et al*., 2001). However, CFTR may exert this effect on bicarbonate transport by an indirect mechanism (see 2.2.3.3 Regulator of Epithelial Ion Transport).

Another ion described to be transported by CFTR is reduced glutathione (GSH) or γ-glutamyl-cystenyl-glycine (Kogan *et al*., 2003), the main antioxidant secreted in response to inflammation in the lung. In CF patients, it has been known for long, that GSH levels are substantially decreased in the ASL (Roum *et al*., 1993). It was later shown that CFTR itself is capable of mediating transport of GSH, possibly giving an explanation of the heightened state of inflammation found in CF lungs (Linsdell and Hanrahan, 1998; Kogan *et al*., 2003).

2.2.3.3 Regulator of Epithelial Ion Transport

In addition to its function as a selective channel, CFTR also has been implicated in the regulation of a number of channels and transporters (Kunzelmann, 2001). It is thought that the hyperabsorption of Na^+ ions observed in lung epithelia from CF patients, mediated by the epithelial Na^+ channel (ENaC), is down-regulated in normal tissue by CFTR (Briel *et al*., 1998). Several mechanisms have been proposed to explain how CFTR downregulates ENaC activity. Some data suggests that Cl^- transport by CFTR regulates ENaC activity (Konig *et al*., 2001), while others show that the absence of CFTR from the plasma membrane leads to hyperactivity of ENaC by a direct (Suaud *et al*., 2002; Suaud *et al*., 2007a) or indirect (Guggino and Stanton, 2006) interaction between the two channels and not by differences in the ion and water balance across the plasma membrane.

Furthermore, CFTR has been implicated in the regulation of other Cl^- channels: Firstly, the outwardly rectifying Cl^- channels (ORCCs), activated by cAMP, shown to be defective in CF tissues. Secondly, the Ca^{2+}-dependent Cl^- channels (CaCCs). These are transiently activated in response to changes in intracellular Ca^{2+} levels. Thirdly, the volume activated Cl^- channels, induced by osmotic stress. The mechanisms by which CFTR regulates these channels is still poorly understood, some studies suggesting a direct interaction (e.g. for ORCC, it appears that NBD1 and RD of CFTR are required (Schwiebert *et al*., 1998)), while other studies suggest an indirect interaction depending on altered ion movement (e.g. absence of CFTR is suggested to upregulate the activity of CaCCs (Tarran *et al*., 2002), while other studies suggest that CaCCs activity is dependent on basolateral K^+ channels (Mall *et al*., 2002))

CFTR also was suggested to regulate the renal outer medullary potassium (K^+) channel (ROMK) (McNicholas *et al*., 1997), expressed in the distal nephro segments of the mammalian kidney, involved in K^+ homeostasis. The mechanism by which CFTR inhibits ROMK appears to be modulated by phosphorylation and to involve the NBD1 and RD of CFTR (Cahill *et al*., 2000). Another example of a CFTR regulated transporter is the Solute carrier 26 family (SLC26), shown to be important in HCO_3^- permeation (Ko *et al*., 2002). Other ion transporters suggested to be modulated by CFTR include the Na^+/H^+ exchange regulatory factor (NHERF), putatively via ezrin binding protein of 50 kDa, (Hall *et al*., 1998), see 2.2.2 Biogenesis, Processing & Degradation), the HCO_3^-/Cl^- exchanger, (putatively regulated by CFTR via CFTR associated protein of 70kDa, CAP70, (Rossmann *et al*., 2005)), and some members of the aquaporin (AQP) family (Levin and Verkman, 2006).

2.2.3.4 Mechanism of Epithelial Ion Transport

In respiratory or intestinal tissues, CFTR can function either as an absorptive or a secretory pathway for Cl^- ions. Secretion of electrolyte by the submucosal glands covers the airways with a liquid film at their surface (airway surface liquid, ASL), which is essential for proper mucociliary clearance. Liquid secretion is also essential for mucus to exit the submucosal glands. Both secretory and absorptive processes are defective in CF, since net absorption of electrolytes is enhanced in the CF surface epithelium and secretion by the submucosal glands is inhibited (See figure I.14 (Kunzelmann, 2001)).

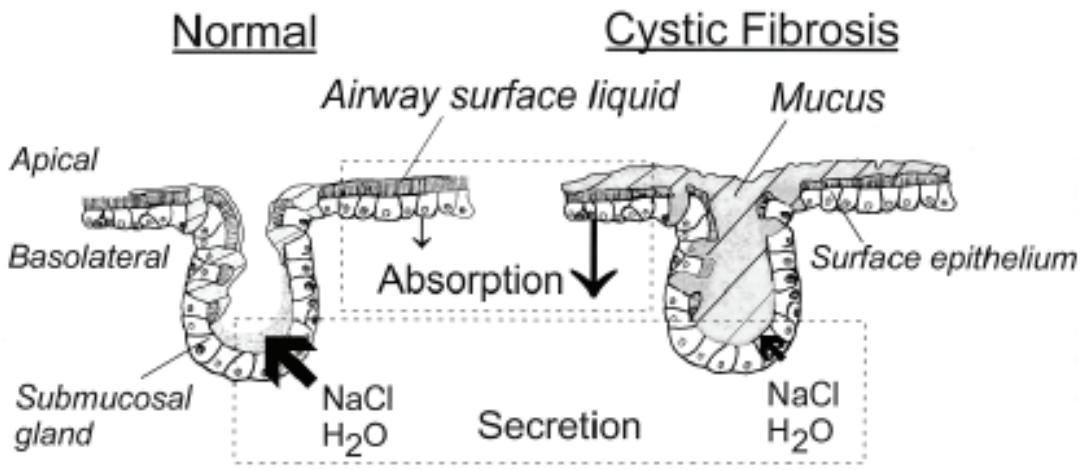

Figure I.14 - Model of airway epithelium, consisting of an absorptive surface epithelium and secretory in the submucosal glands. Normal (Left panel) and CF (Right panel) epithelium models are shown (Kunzelmann, 2001).

The apical secretion of Cl^- creates a lumen negative transepithelial voltage, leading to the secretion of cations, mainly Na^+. This in turn creates an osmotic gradient leading to secretion of fluid into the lumen. In respiratory CF tissues the absence of Cl^- secretion leads to a diminished liquid secretion with consequent thickening of mucus, termed the “low-volume” model. An alternative model has been proposed, termed the “high-salt” model, which explains the differences observed between normal and CF airway tissues by the inability of the epithelium to re-absorb Cl^- ions from the lumen, and thus removing the driving force for counter-ion absorption, leading to increased salt concentrations in the lumen. (See Figure I.15 (Rowe *et al*., 2005)).

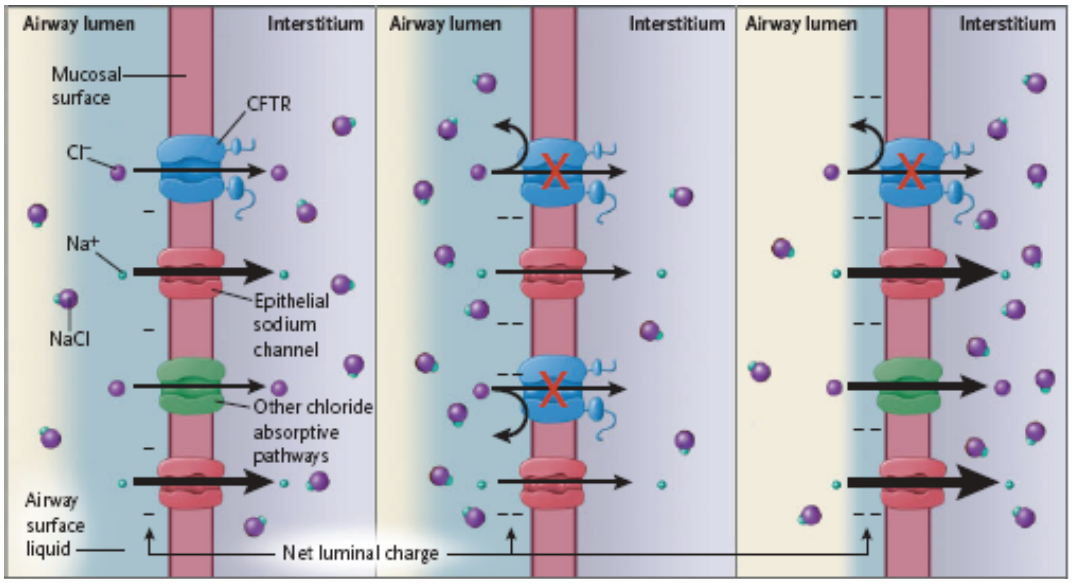

Figure I.15 - Left panel, Cl^- transport in a normal airway epithelial cell. Middle panel, "High Salt" model, dysfunction of CFTR leads to diminished absorption of counter-ions, in turn favouring accumulation of salt in the ASL. Right panel, "low volume" model, dysfunction of CFTR leads to hyperabsorption of mainly Na^+, decreasing osmotic pressure and consequent dehydration of ASL (Rowe *et al.*, 2005).

In a secretory epithelium, Cl^- is absorbed on the basolateral side by the Na^+-K^+-$2Cl^-$ (NKCC1) co-transporter, accumulating Cl^- inside the epithelial cell in preparation for secretion when cell receives appropriate stimulus at the apical side by CFTR (see Figure I.16a).

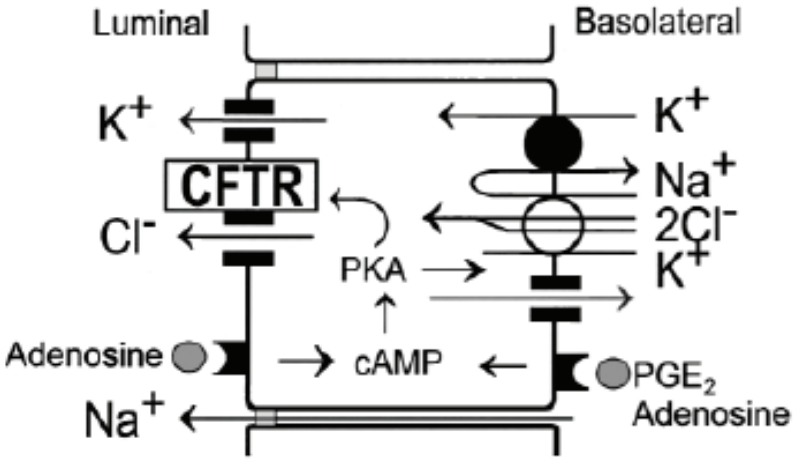

Figure I.16a - In secretory cells, Cl^- is taken up from the basolateral (blood) side by the Na^+-K^+-$2Cl^-$ cotransporter. K^+ recycles via basolateral K^+ channels, and Na^+ is pumped out of the cell by the Na^+-K^+-ATPase. Cl^- leaves the cell via luminal (apical) CFTR Cl^- channels, and Na^+ is secreted via the paracellular shunt. K^+ is also secreted to the luminal side via luminal K^+

channels. The luminal ion secretion causes H_2O to also permeate into the lumen. Depending on the tissue, intracellular cAMP is increased and secretion is activated by adenosine or prostaglandin E_2 (PGE_2) (Kunzelmann, 2001).

In sweat glands, and absorptive epithelium, the transport direction is inversed, i.e. both Cl^- and Na^+ are absorbed by the apical surface of epithelia (see Figure I.16b), accumulating inside the epithelial cell to be secreted basolaterally upon appropriate stimulus.

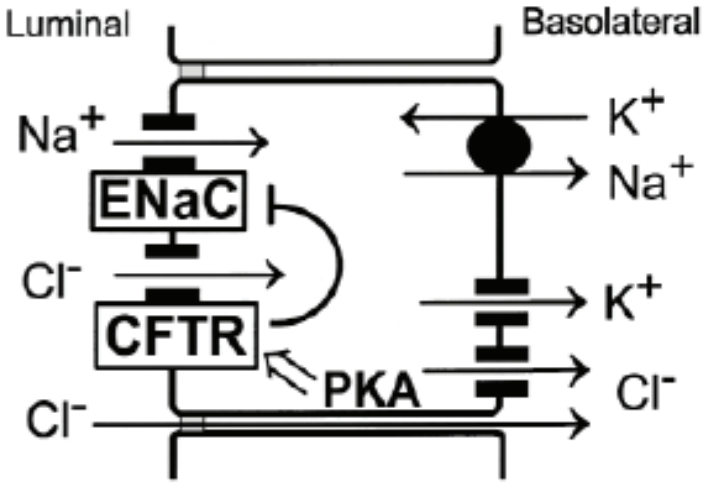

Figure I.16b - In absorptive epithelial cells, Na^+ is taken up by luminal epithelial Na^+ channels (ENaC). Cl^- is transported via the basolateral shunt and probably via CFTR Cl^- channels. Na^+ is pumped out of the cell by the basolateral Na^+-K^+-ATPase, whereas Cl^- and K^+ leave the cell via Cl^- and K^+ channels, respectively. In these absorptive epithelial cells that co-express CFTR and ENaC, stimulation of CFTR leads to inhibition of ENaC (Kunzelmann, 2001).

2.2.3.5 Other Functions

The high number of tasks assigned to CFTR, both in transporting and in regulating other channels, confirms the highly complex nature and regulation of this protein, where separate domains perform specific tasks: The membrane spanning domains are responsible (mainly) for Cl^- conductance, whereas the NBDs, besides regulating this pore,

have also been suggested to modulate the activity of other channels (Guggino and Stanton, 2006).

CFTR itself seems to be the target for a growing number of kinases, shown to interact and possibly phosphorylate CFTR. These include AMP-activated protein kinase (AMPK) and nucleoside diphosphate kinase (NDPK), which both localise to the apical membrane in a CFTR-dependent manner (Crawford *et al*., 2006). Serum- and glucocorticoid-inducible kinase (SGK1) has been shown to modulate the number of CFTR channels at the cell surface when expressed in *Xenopus* oocytes, putatively by an direct interaction with CFTR (Sato *et al*., 2007). With-no-lysine (WNK) kinases co-localise with CFTR and have also been suggested to modulate CFTR activity (Yang *et al*., 2007).

Some studies have proposed a putative role for CFTR in regulating β-adrenergic dependent stimulation of mucin secretion (Mills *et al*., 1992; McPherson and Dormer, 1994). While it remains unclear what the exact mechanism underlying this response is, it was shown in cultured pancreatic cells, that while both normal and CF cells respond to extracellular ATP stimulus with a transient intracellular Ca^{2+} mobilisation, in CF cells, contrary to normal cells, mucins fail to be secreted (Montserrat *et al*., 1996). Other studies have suggested an over-expression of certain mucins in cells defective for CFTR, increasing the propensity of CF airways for infection (Puchelle *et al*., 2002). While both Cl^- and Na^+ transport are important for hydrating the airway surface liquid (ASL), this might also be insufficient to explain the dramatic increase in mucus thickening and poor mucociliary transport that characterises this disease, leading to the designation as “mucoviscidosis”.

It has been suggested that CFTR might be of importance for mucin secretion: mucins are tightly packed in sub-apical granules which are exocytosed in response to stimulous. It has been reported that absence of CFTR increased exocytosis of mucous granules (Kuver *et al*., 2006). Furthermore, it has been suggested that mucins show a propensity for remaining compacted due to lowered pH in the extracellular fluid matrix in CF tissues (Kreda *et al*., 2007), thus augmenting the viscosity of the airway surface liquid.

As these characteristics are important in response to inflammation and bacterial challenge, this provides further support to a strategy involving the rescue of mutant CFTR to the cell membrane by small molecule CFTR correctors and potentiators, so as to restore defects associated with the absence of functional CFTR at the apical membrane (see 3.1 CFTR Rescuing Strategies).

More recently, a role for CFTR as an “anchoring platform” at the cell membrane forming specialised “microdomains” grouping together a number of proteins into the same regulatory unit was suggested (Guggino and Stanton, 2006). These include signalling molecules, kinases, transport proteins, PDZ-domain-containing proteins, myosin motors, Rab GTPases and SNAREs. Furthermore, a very large number of proteins have also been referred as being involved in the exocytic pathway of trafficking CFTR to the plasma membrane, putatively part of general cell mechanisms that enable distinction between folded and misfolded CFTR protein (Wang *et al*., 2006a).

3. CF Causing Mutations, Functional Classes and Rescuing Strategies

To date, 1546 different mutations have been identified in the *CFTR* gene (Cystic Fibrosis Genetic Analysis Consortium Report, 2007). Except for F508del, which accounts for ~70 % of CF chromosomes worldwide, all other mutations are very rare. Moreover, most have only been described at the genetic level and still lack functional description so as to explain the impact they have on expression, processing and function of CFTR protein.

While it has been shown that CFTR is capable of a number of different functions in the cell, its main function remains as a Cl^- channel. Therefore, *CFTR* gene mutations are assigned to classes depending on the type of defect they cause to CFTR Cl^- channel function. This classification was put forward by Welsh and Smith in 1993 (Welsh and Smith, 1993), grouping mutations in the *CFTR* gene into four classes: Class I, Absence of protein synthesis; class II, deficient intracellular trafficking; class III, Abnormal channel regulation; and class IV, reduced conductance. A fifth class was proposed by Wilschanski *et al.* (1995) to encompass mutations in the promoter region, which in turn leads to reduced protein production (Wilschanski *et al*., 1995), named class V. See Figure I.17 and below, with a few examples of mutations given.

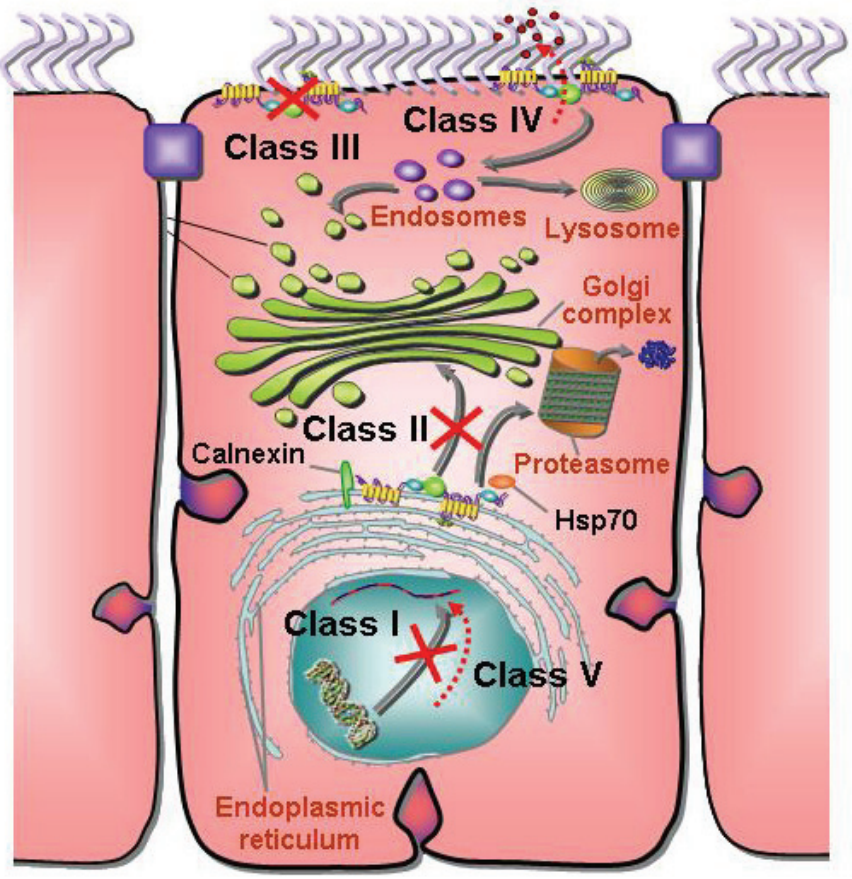

Figure I.17 - Scheme showing the different CFTR mutation classes, as described below. Figure adapted from (Amaral and Kunzelmann, 2007)

Class I - Absence of protein synthesis.

This group includes *CFTR* gene mutations that lead to the absence of protein synthesis. These comprise *nonsense* (R553X- (Hamosh *et al*., 1991) and G542X-CFTR (Schloesser *et al*., 1991)) and *frameshift* (394delTT- (Schwartz *et al*., 1994) and 3905insT-CFTR (Liechti-Gallati *et al*., 1992)) mutations which would cause truncated forms of the protein to being formed, but their synthesis is avoided by the degradation of the mRNAs with premature termination codons (PTCs) by the nonsense mediated decay (NMD) mechanism.

Class II - Deficient intracellular trafficking.

While full-length protein is produced from *CFTR* genes carrying mutations comprised in this class, the resulting CFTR protein does not

achieve a folded conformation being thus recognised by the endoplasmatic reticulum (ER) quality control (ERQC) which targets the misfolded protein for degradation via the ubiquitin (Ub) proteasome pathway (UPP). Consequently the mutant protein does not traffic to the cellular membrane. This class includes F508del (Cheng *et al*., 1990), but also many missense mutations such as A561E (Mendes *et al*., 2003) and N1303K (Osborne *et al*., 1992).

Class III - Abnormal channel regulation
CFTR Cl^- channels in this class do not respond to stimulation by cAMP, or show diminished (G1244E-CFTR (Savov *et al*., 1994)) or absent (G551D-CFTR (Parad, 1996)) function as a Cl^- channel.

Class IV - Reduced conductance
This class includes mutations with alterations in the CFTR protein channel pore leading to diminished Cl^- secretion (R117H- (Bienvenu *et al*., 1993), R334W- (Antinolo *et al*., 1997) and R347P-CFTR (Varon *et al*., 1995)).

Class V
This class contains mutations that affect the promoter region of the *CFTR* gene, and also mutations that cause exon skipping, such as G85E (Friedman *et al*., 1995), which causes exon 9 skipping. Patients carrying these mutations show reduced synthesis or partially defective processing of normal CFTR (Wilschanski *et al*., 1995).

Although about 1500 different mutations have been described, the most common CF causing mutation is a deletion of a phenylalanine residue at position 508, termed F508del-CFTR (see above). Consequently, most research efforts are focused on this class II mutation, where protein synthesis does occur, but the protein is poorly

trafficked to the cellular membrane (Cheng *et al*., 1990; Lukacs *et al*., 1994). It was found that F508del-CFTR has essentially the same biosynthesis and degradation kinetics as wild type (wt) CFTR, demonstrated to occur in a 30 minute timeframe (Lukacs *et al*., 1993; Ward and Kopito, 1994). However, and unlike its wt-CFTR counterpart, F508del-CFTR does not reach a conformation that enables it to leave the ER efficiently so as to proceed to the Golgi in significant amounts (Lukacs *et al*., 1994). It was demonstrated that this ER retention is not due to immobilisation or aggregation of F508del-CFTR (Haggie *et al*., 2002), but rather due to a prolonged interaction of the mutant protein with the Hsp70/Hdj-2 chaperone pair which consequently recruits CHIP, causing it to undergo UPP degradation (Meacham *et al*., 1999; Meacham *et al*., 2001).

Furthermore, it was proposed that the differential recruitment of co-chaperones within the Hsc70/Hsp70 machinery (Amaral, 2004) is the first checkpoint in the ER quality control which determines the fate of F508del-CFTR degradation (Farinha and Amaral, 2005) (see also Figure I.6). A second ERQC checkpoint involves calnexin, a chaperone that is also a lectin binding the glycan moiety of secretory proteins, facilitating their folding and ER export, but also their glycan-associated ER degradation (GERAD). This second checkpoint assesses proteins that are able to escape the first checkpoint (Pind *et al*., 1994). Indeed, it was shown that calnexin overexpression further enhances wt-CFTR, but not F508del-CFTR protein export from the ER, which is mostly disposed of at the first ERQC checkpoint (Farinha and Amaral, 2005). A third checkpoint was proposed to involve the selection of conformationally competent cargo into COPII vesicles (Roxo-Rosa *et al*., 2006).

3.1 CFTR Rescuing Strategies

As was described above (2.2.3 CFTR Functions) CFTR protein plays a number of important roles in the cell. Rescuing strategies therefore can be grouped by different approaches to re-establish all or some of CFTR protein functions, depending on the defect that a given mutation introduces.

The so-called "bypass" approach consists of stimulating alternative pathways for Cl^- secretion across the apical membrane or modulating channels that are normally regulated by CFTR. Supporting the feasibility of this approach is the interesting fact that CF-like symptoms (such as ASL dehydration, reduced height of the periciliary liquid layer and goblet cell metaplasia) appear in mice as a consequence of hyper-absorption of Na^+ by over-expression of β-ENaC, a subunit of ENaC (see above 2.2.3.3 Regulator of Epithelial Ion Transport), a channel normally downregulated by CFTR (Mall *et al*., 2004). Downregulating the hyper-absorption of Na^+ would likely reduce these effects in CF patients, as this hyper-absorption occurs in CF airways. However these alternative strategies, if successful, only restore one aspect of CFTR protein function. It is expected that only by having the functional CFTR protein at the cellular membrane will normal salt balance across the apical membrane be rescued and the regulatory role of CFTR of other transporters be restored.

3.1.1 The "Bypass" Approach

Indeed, as CFTR functions not only as a Cl^- channel, but also as a regulator of other membrane proteins, it is necessary to devise strategies to modulate these other membrane transporters. It seems that the presence of CFTR at the cellular membrane (even the mutant

protein) is enough for modulation of other proteins (Suaud *et al*., 2007a). While controversy remains about these findings (Suaud *et al*., 2007b), it could be that promoting mutant CFTR to the cellular membrane would restore dysfunctional interactions observed in the absence of CFGTR at the apical membrane. Pharmacological approaches aiming to correct several dysfunctions caused by the absence of CFTR have been described (Amaral and Kunzelmann, 2007), examples include dysfunctions such as:

Firstly, absence of CFTR at the apical cellular membrane leads to enhanced Na^+ conductance in surface airway epithelial cells leading to excessive absorption of electrolytes (Donaldson and Boucher, 2003). The responsible Na^+ channel ENaC can be blocked by specific inhibitors such as amiloride, benzamil or phenamil and probably by activation of protein kinase C. Also, activation of purinergic receptors by ATP or UTP inhibits ENaC. (See Figure I.18, A).

Secondly, stimulation of an alternative Cl^- channel, the Ca^+-activated Cl^- channels (CaCCs) in CF airway epithelial cells by stimulation of luminal $P2Y_2$ purinergic receptors with ATP or UTP, has been demonstrated to restore Cl^- secretion (Knowles *et al*., 1991) (See Figure I.18, B).

Thirdly, increasing of the electrical driving force of luminal Cl^- secretion by stimulation of the basolateral Ca^{2+}-activated K^+ channel SK4 by the benzimidazol compound 1-EBIO or activation of cAMP-regulated K^+ channels (KvLQT1) by agonists of the cAMP pathway, such as ß-adrenergic compounds or blockers of phosphodiesterase (PDE) like amrinone or milrinone (Kunzelmann and Mall, 2001)(See figure I.18, C).

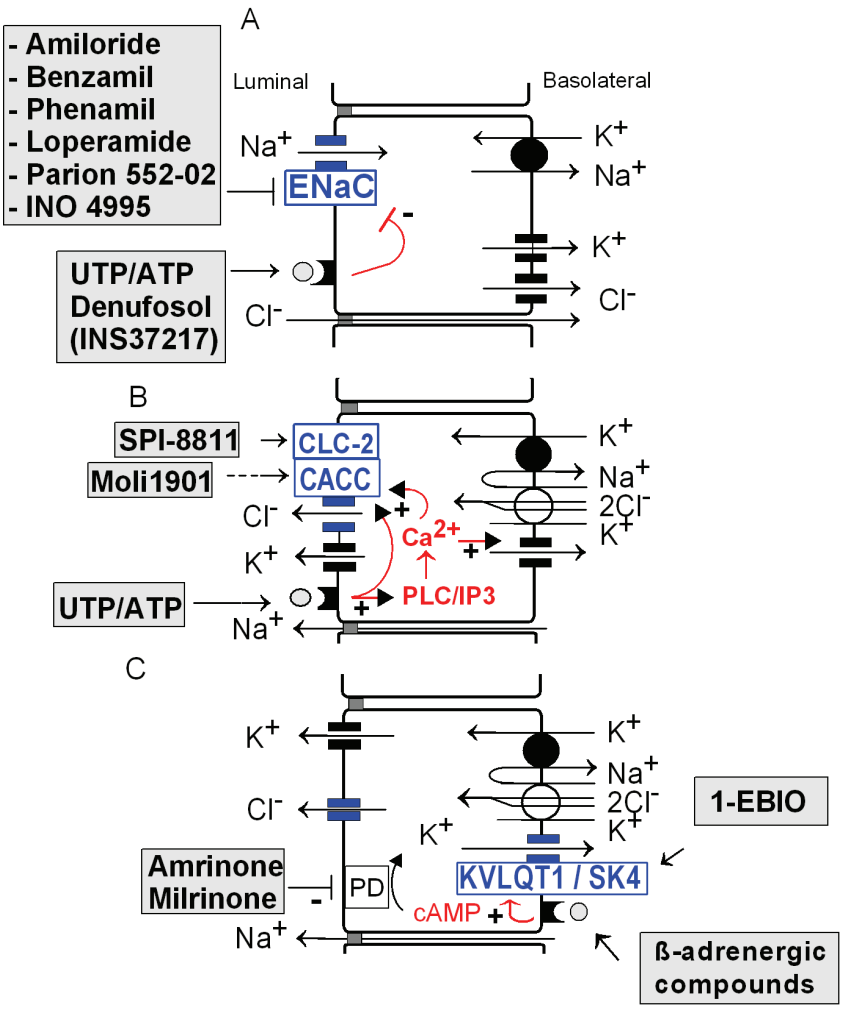

Figure I.18 - Pharmacological compounds used for the therapeutic strategies aimed at circumventing the ion channel defect in CF. (Amaral and Kunzelmann, 2007).

3.1.2 Mutation-specific Approaches

The chemical and pharmacological approach to rescuing mutant CFTR is based on restoring function according to the mutation class (See above 3. CF causing mutations) and a number of reviews validate this approach (Rubenstein, 2006; Kerem, 2006). Promising compounds targeted to the different mutation classes have recently been reviewed (Amaral and Kunzelmann, 2007) and some examples are included below:

For Class I mutations, aminoglycoside antibiotics have been described to suppress (PTCs) by disrupting translational fidelity and allowing the incorporation of an amino acid, thus permitting translation to continue (read-through) until the normal termination of the transcript. An example is the clinical trial carried out in patients with CF and CFTR stop mutations through gentamicin-induced correction of CFTR function (Wilschanski *et al*., 2003).

Compounds found to rescue Class II mutations, (where F508del is included) are more varied, but their mechanism of action is essentially to potentially stabilise protein structure, promote folding, thus allowing it to escape ER degradation and reach the cell surface (see subsequent sections). This group includes chemical, molecular or pharmacological chaperones, broadly termed 'correctors' (Pedemonte *et al*., 2005a).

CFTR potentiators such as the flavonoid genistein can overcome mutations grouped in Class III. These mutant CFTR protein potentiators, do not activate CFTR by themselves, but rather increase stimuli that activate CFTR Cl^- channel function (Moran and Zegarra-Moran, 2005). It has to be noted, that F508del-CFTR is also a class III mutant, since when it reaches the cell surface, also exhibits impaired gating (Dalemans *et al*., 1991; Haws *et al*., 1996).

Compensation for reduced conductance associated with mutations comprised in Class IV can be achieved by either increasing the overall cell surface content of these mutants (i.e. by promoting their traffic) or preferably through increased stimulation of the existing channels with potentiators.

Splicing factors that promote normal exon inclusion or factors that promote abnormal exon skipping, a feature of class V mutants can increase levels of correctly spliced transcripts. Potentiators are also useful for these mutants, since they will further enhance the activity of normal channels already at the cell surface.

3.1.3 Rescuing F508del-CFTR

3.1.3.1 Physical

An early observation soon after identification of the *CFTR* gene, was that F508del-CFTR is a temperature sensitive mutation, i.e. partial rescue of the mutant protein can be achieved by lowering the incubation temperature of cell cultures from 37 ºC to 26 ºC. While this observation has no therapeutic relevance, it does show that the trafficking block of F508del-CFTR at the ER can be surmounted, and that fully-glycosylated protein can be trafficked to the cell membrane. Furthermore, it was also shown that this temperature rescued CFTR mutant functions as a Cl^- channel at the apical membrane, albeit at reduced levels (Denning *et al*., 1992b).

Recent studies have shown that temperature rescued F508del-CFTR is less stable once it reaches the plasma membrane, indicating that low temperature does not promote correct folding of F508del-CFTR, but rather that it enables the mutant protein to bypass cellular quality control mechanisms (CM Farinha, unpublished)

3.1.3.2 Genetic

Studies have shown that rescue of the F508del-CFTR trafficking defect can be achieved by genetic manipulation. These consist of introducing

so-called second site or revertant mutations into the F508del-CFTR primary sequence. Indeed, several such second-site mutations have been identified. By replacing either glycine at position 550 by a glutamic acid residue (G550E) or isoleucine 539 by threonine (I539T), in cis with F508del, it is possible to rescue both the membrane localisation and channel activity (DeCarvalho *et al*., 2002a).

Another study showed that removal from F508del-CFTR of so-called arginine-framed tripeptide (AFT)-sequences (responsible for the ER retention and/or retrieval of other ion channels) (Zerangue *et al*., 1999) permits nascent F508del-CFTR to mature and generate functional Cl^- channels at the cell surface (Chang *et al*., 1999). There are four of these AFT-sequences in CFTR: one in the N-terminal cytoplasmic domain (R29), two in NBD1 (R516 and R555), and one in the R domain (R766). (See Figure I.19) Although the simultaneous substitution of all four arginines by lysine (K) residues (4RK: R29K, R516K, R555K and R766K) causes F508del-CFTR to traffic to the plasma membrane and function about one-third as efficiently as wt-CFTR, individual R/K substitutions at some of these positions, i.e., R29K and R555K were also described as restoring F508del-CFTR function (Teem *et al*., 1996). Recent studies assessing different combinations of these four AFTs have shown that R29K and R555K together are the most efficacious in restoring F508del-CFTR trafficking (Hegedus *et al*., 2006). Whether these AFT motifs are just ER-retention signals or, like G550E and I539T, also act as intrinsic folding determinants remains to be fully clarified (Roxo-Rosa *et al*., 2006).

Work to elucidate the mechanism involved, found that both G550E and 4RK also rescue another trafficking mutant A561E (Roxo-Rosa *et al*., 2006). These data indicate that the two second site mutations have different rescuing mechanisms: G550E likely enables the mutant

protein to fold more efficiently to a native-like conformation that allows it to be exported from the ER, while 4RK seems to enable the protein to escape (or bypass) the ER retention mechanism and thus be trafficked to the plasma membrane.

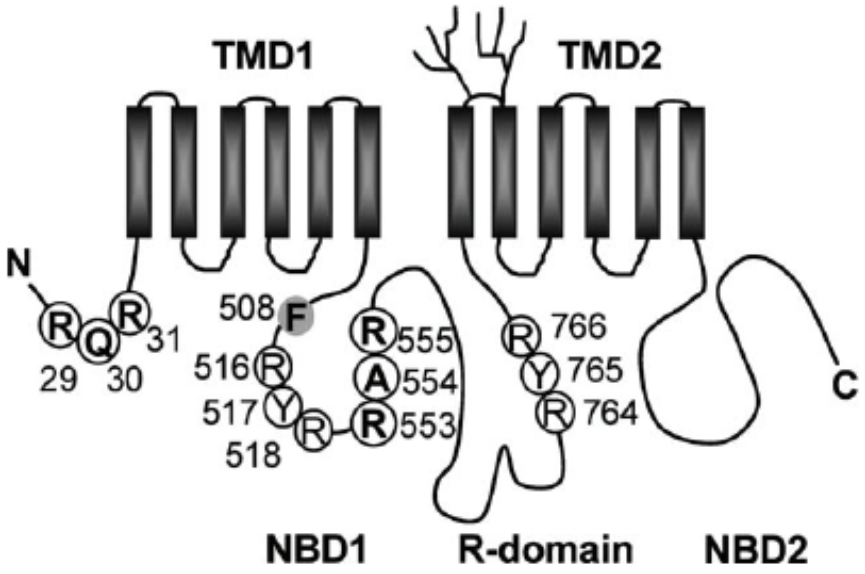

Figure I.19 - Model showing localisation of four arginine-framed tripeptides motifs, responsible for ER retention (Hegedus *et al*., 2006).

More recently, another set of second-site mutations were identified as enabling F508del-CFTR to be rescued to the plasma membrane. These were called “solubilizing mutations” as they were originally introduced to solubilise F508del-NBD1 for crystallisation purposes (Lewis *et al*., 2005). Recent work has shown that when these solubilizing mutations are introduced into full-length F508del-CFTR protein, they are able to partially rescue the mutant protein to the plasma membrane, enabling F508del-CFTR to function better as a Cl^- channel (Pissarra *et al*., 2008).

3.1.3.3 Chemical and Pharmacological Chaperones

Chemical and pharmacological approaches to rescue CFTR dysfunction can be divided into two groups. Firstly, molecules that help

F508del-CFTR to fold by generally favouring the cellular milieu, possibly through modulation of interacting proteins, such as chaperones; and secondly, specific molecules that rescue folding of CFTR mutants, putatively allowing them to function correctly at the plasma membrane. Due to their non-specificity, the former are called chemical chaperones, while the latter are termed pharmacological chaperones (or pharmaco-chaperones) (see also 3.1.2 Mutation-specific Approaches). Ideally such a pharmacological chaperone would be able to act as a CFTR corrector (i.e. promoting mislocalised protein to the plasma membrane), and also act as a CFTR Potentiator (i.e. increasing CFTR Cl^- channel function upon stimulation).

4. Objectives of this Work

The main objective of this thesis was to restore CFTR function by enhancing protein processing and channel activity. To achieve this goal, the focus primarily has been on attempting to understand the effect long term pre-treatments with small molecules has on processing and function of CFTR.

Known modulators, putatively able to enhance channel function, such as *Phyllanthus acidus* extract and genistein were tested for their ability to modulate CFTR processing and function.

Also, high throughput screening studies (Pedemonte *et al*., 2005b; Van Goor *et al*., 2006; Wang *et al*., 2006b), have identified small molecule CFTR correctors, able to partially rescue cell surface expression of F508del-CFTR. While the development of these for use in the clinic is still at an early stage, they provide valuable tools to understand how to rescue the basic defect in CF. Here, it was attempted to gain insight into their mechanism of action by use of F508del-CFTR genetic revertants, and assessing their effect on the processing of CFTR protein.

II. Materials and Methods

II. Materials and Methods

1. Cells and Culture Conditions

For the assessment of steady-state protein levels, we used untransfected BHK cells and BHK cells stably expressing either human wt- or F508del-CFTR (Farinha *et al*., 2002), and the corresponding genetic revertant mutations, G550E- and 4RK-CFTR (Roxo-Rosa *et al*., 2006). BHK cells were cultured in a 1:1 mixture of Dulbecco's Modified Eagle Medium (DMEM) and Ham's F-12 nutrient medium supplemented with 5 % v/v foetal calf serum, 100 (U/ml) penicillin and 100 (mg/ml) streptomycin (all from Invitrogen, Carlsbad, CA, USA) and (only transfected cells) with 200 (μg/ml) methotrexate (AAH Pharmaceuticals Ltd., Coventry, UK) at 37 °C in a humidified atmosphere of 5% CO_2. In some experiments BHK cells expressing F508del-CFTR were cultured at 28 ºC to overcome the processing defect of this mutation and promote its delivery to the cell membrane (Denning *et al*., 1992a).

For iodide efflux and pulse-chase/immunoprecipitation experiments, cells were seeded onto 60 mm plastic culture dishes and used when dishes were confluent (functional studies, 3-4 days (37 °C incubations) or 4-5 days (28 °C incubations); biochemical studies, 2 days). For immunocytochemistry, cells were seeded onto 8-well chamber slides (Nalge Nunc, Roskilde, Denmark) and grown for 1 day at 37 ºC prior to use. For experiments using excised inside-out membrane patches, cells were seeded onto glass coverslips and used within 60 h.

To investigate the long-term effects of genistein, we pre-incubated cells for either 2 or 24 h at 37 °C in media containing either a specific

concentration of genistein (30 or 100 μM; Sigma-Aldrich, St. Louis, USA) or an equivalent volume of the vehicle dimethyl sulphoxide (DMSO). As a control, we used the tyrosine kinase inhibitor tyrphostin 47 (100 μM; Sigma-Aldrich), testing its action under identical conditions to those used for genistein (100 μM).

To investigate the long-term effects of *Phyllanthus acidus* extract, the extract was used as described in (Sousa *et al*., 2007), and provided by Prof. K. Kunzelmann, University Regensburg (Germany). For pulse-chase experiments, cells were pre-incubated for 48 h at 37 °C in medium with 50 (μg/ml) final concentration.

To investigate the long term effects of small molecule CFTR correctors VRT-325, VRT-532, VRT-640 and C4a (Received from Vertex Pharmaceuticals (San Diego, CA,USA) and Prof. A.S. Verkman (UCSF, CA, USA) through the US Cystic Fibrosis Foundation (CFF) and Prof R. Bridges (Rosalind Franklin University, Chicago, USA)), cells were incubated for indicated times at the appropriate concentrations or equivalent vehicle (DMSO) concentrations in the culture medium, supplemented with 0.5 % (v/v) foetal calf serum. This low serum concentration was used to avoid the serum's described binding of these compounds.

2. Biochemical Analysis

2.1 Total Protein Extracts

For Western blot (WB), protein extracts were prepared by cell lysis with Laemmli sample buffer (1.5 % (w/v) SDS; 10 % (v/v) glycerol; 0.001 % (w/v) bromophenol blue; 0.5 mM dithiotreitol; 31.25 mM Tris pH 6.8) and DNA was sheared by passing the sample first through a 22G and then a 27G needle until viscosity decreased.

Total protein concentration in different samples was determined by a modified micro Lowry protein assay, being total cellular extracts diluted with water. Proteins were solubilised with 0.15% (w/v) sodium deoxycholate followed by incubation for 10 min at RT. Samples were then precipitated with 72% (w/v) trichloroacetic acid and centrifuged at 14000 g for 5 min. The supernatant was removed by suction with a needle and syringe and the pellet was resuspended in water and Reagent A (0.1 g/l $CuSO_4.5H_20$; 0.2 g/l potassium tartarate; 10 g/l Na_2CO_3; 2.5% (w/v) SDS; 0.2 M NaOH) followed by 10 min incubation at RT. Finally, Reagent B (Folin-Ciocalteau Reagent diluted 5-fold in water) was added followed by incubation for 30 min. Protein concentration was determined by measurement of A_{750} and comparison with regression line obtained for protein standards.

2.2 Western Blot

Total protein was quantified by micro Lowry method (see above) and protein extracts were separated by SDS-polyacrylamide gel electrophoresis (PAGE) on 7 % (w/v) or 6 % (w/v) mini-gels, followed by transfer onto nitrocellulose filters (Schleicher & Schuell, Dassel, Germany). After blocking with 5 % (w/v) skimmed milk in PBS

containing 0.1% (v/v) Tween (PBST) for 2 h, the filters were probed with M3A7 anti-CFTR monoclonal antibody (Cat No. MAB 3480; Chemicon, Temecula, Ca, USA), diluted 1:3000 for 2 h at RT in 5% (w/v) milk-PBST, and a secondary anti-mouse IgG at 1:4000, peroxidase-labeled monoclonal Ab (Amersham Bioscience, Piscataway, CA, USA) for 1 h at RT in 5% (w/v) milk-PBST. Blots were developed using the SuperSignal® West Pico Chemiluminescent Substrate detection system (Pierce, Rockford, IL, USA).

2.3 Pulse-chase and Immunoprecipitation

For pulse-chase experiments, BHK cells were starved for 30 min in methionine-free α-modified Eagle's medium (Gibco, Invitrogen, Carlsbad, CA, USA) and in case of drug pre-treatment, containing appropriate compound or vehicle concentration. Cells were then pulse-labelled (Farinha *et al*., 2002) in the same medium supplemented with 150 μCi/ml [^{35}S]-methionine (PerkinElmer, Boston, Ma, USA) and in case of drug pre-treatment, containing appropriate compound or vehicle concentration. After chasing for times indicated in the figures in α-modified Eagle's medium, supplemented with 0.5 % (v/v) foetal bovine serum (Gibco) and 1 mM non-radioactive methionine (Sigma-Aldrich, St. Louis, Mo, USA), and in case of drug pre-treatment, containing appropriate compound or vehicle concentration, cells were lysed in 1 ml RIPA buffer, containing: 1% w/v deoxycholic acid (Sigma-Aldrich), 1% v/v Triton X-100 (Pharmacia Biotech, GE Healthcare, Chalfont St. Giles, UK); 0.1% (w/v) SDS (Gibco); 50 mM Tris, pH 7.4 (Sigma-Aldrich) and 150 mM NaCl (Sigma-Aldrich). Immunoprecipitation (Jensen *et al*., 1995) was performed on samples after centrifugation at 14000 g for 30 min: The supernatant was incubated overnight (O/N) with 1.5 μg of anti-CFTR M3A7 antibody at

4 ºC and then Protein-G agarose beads (25 μg) (Roche, Basel, Switzerland) were added for a further 4 h at 4 ºC. Beads were washed 4 times using 1 ml RIPA buffer and protein was eluted by adding 60 μl Laemmli sample buffer (see above) for 1 h at RT. Samples were electrophoretically separated on 7% (w/v) polyacrylamide gels. Gels were pre-fixed (30% methanol, 10% acetic acid) for 30 minutes, washed thoroughly in water and then soaked in 1 M salicylic acid for 1 h for fluorographs. After drying at 80ºC under vacuum for 2 h, gels were exposed to X-ray films (Fuji, Tokyo, Japan).

Densitometry was performed on fluorographs of gels by digitising (Sharp JX-330) and integrated peak areas were then determined using ImageMaster® software (Amersham Bioscience).

Quantitative results are shown as means ± SEM of n observations. Comparisons between slopes of lines representing degradation rates (regression lines) were made using a Students *t* test (Farinha *et al*., 2002). On a regression modelling procedure, the slope is known to follow a *t* distribution (Kleinbaum D.G. *et al*., 1988; Helliwell P.S. and Jackson S., 1994; Glantz S., 1997). Therefore, slopes of two straight lines can be compared using a *t* distribution with $n_1 + n_2 - 4$ degrees of freedom where n_1 and n_2 are the number of points used on the regression procedures in groups 1 and 2, respectively.
To test for differences between groups of data at matching times of chase, an analysis of variance (ANOVA) was used. To compare only two sets of data, we used Student's *t* test.
Differences were considered as significant for p values < 0.05.

3. Functional Characterisation

3.1 Iodide Efflux

We measured CFTR-mediated iodide efflux as described before (Lansdell *et al*., 1998) by using the cAMP agonist forskolin (10 μM) and the CFTR potentiator genistein (50 μM; Sigma-Aldrich). For two reasons, we used genistein (50 μM) to potentiate CFTR activity in iodide efflux experiments: (i) the phosphorylation-dependence of genistein's action on CFTR (see (Lansdell *et al*., 2000)) and (ii) the restricted access of extracellular genistein to its binding site on the NBDs (see also the Discussion of genistein experiments). Prior to commencing experiments BHK cells were incubated for 1 h in loading buffer containing (in mM): 136 NaI, 3 KNO_3, 2 $Ca(NO_3)_2$, 20 Hepes and 11 glucose, pH 7.4 with 1 M NaOH; osmolarity, 299 ± 0.48 mOsm (n = 36) and then washed thoroughly with efflux buffer (136 mM $NaNO_3$ replacing 136 mM NaI in the loading buffer; osmolarity, 291 ± 0.24 mOsm (n = 60)). The amount of iodide in each sample of efflux buffer was determined using an iodide-selective electrode (Russell pH Ltd., Auchtermuchty, UK), at 1 min intervals, while stimulation with forskolin and genistein was only present from 0 min to 4 min (indicated by horizontal bar in figures). Experiments were performed at room temperature (RT; ~23 ºC) and results were analysed as described in 3.4 Statistics.

3.2 Patch Clamp Technique

CFTR Cl^- currents were recorded in excised inside-out membrane patches using an Axopatch 200A patch-clamp amplifier (Molecular Devices Corp., Union City, CA, USA) and pCLAMP data acquisition

and analysis software (versions 6.0.4 and 8.1.0, Molecular Devices Corp.) as described previously (Sheppard and Robinson, 1997; Lansdell *et al*., 2000). The established sign convention was used throughout; currents produced by positive charge moving from intra- to extracellular solutions (anions moving in the opposite direction) are shown as positive currents.

The pipette (extracellular) solution contained (mM): 140 N-methyl-D-glucamine (NMDG), 140 aspartic acid, 5 $CaCl_2$, 2 $MgSO_4$ and 10 N-tris[Hydroxymethyl]methyl-2-aminoethanesulphonic acid (TES), pH 7.3 with Tris ([Cl^-], 10 mM; osmolarity, 281 $\pm$ 0.5 mOsm (n = 4). The bath (intracellular) solution contained (mM): 140 NMDG, 3 $MgCl_2$, 1 CsEGTA and 10 TES, pH 7.3 with HCl, ([Cl^-], 147 mM; $[Ca^{2+}]_{free}$, $<10^{-8}$ M; osmolarity, 279 $\pm$ 0.5 mOsm (n = 7) and was maintained at 37 ºC.

After their excision, membrane patches were voltage-clamped at -50 mV and CFTR Cl^- channels activated by the addition of ATP (1 mM; Sigma-Aldrich) and the catalytic subunit of protein kinase A (PKA; 75 nM; Promega UK, Southampton, UK) to the intracellular solution within 5 min of patch excision. In this study, we used membrane patches containing large numbers of active channels from cells pre-treated with genistein. We examined the effects of genistein pre-treatment on the single-channel behaviour of CFTR by studying individual CFTR Cl^- channels active in the presence of ATP (1 mM) prior to phosphorylation by PKA.

CFTR Cl^- currents were initially recorded on digital audiotape using a digital tape recorder (Biologic Scientific Instruments, model DTR-1204; Intracel Ltd., Royston, UK) at a bandwidth of 10 kHz. On playback, records were filtered with an eight-pole Bessel filter (model 902LPF2; Frequency Devices, Inc., Haverhill, MA, USA) at a corner frequency of

500 Hz and acquired using a Digidata 1200 interface (Molecular Devices Corp.) and pCLAMP software at a sampling rate of 5 kHz. For the purpose of illustration, single-channel records were filtered at 500 Hz and digitised at 1 kHz.

We measured single-channel current amplitude (i) either by fitting Gaussian distributions to current amplitude histograms or using cursor measurements. We calculated mean burst duration (MBD) and frequency of channel opening using membrane patches that contained only bursts of single level openings with no superimposed openings that were separated from one another by a minimum of 20 ms in the presence of ATP (1 mM) as described previously (Carson *et al*., 1995; Cai *et al*., 2006).

3.3 Noise analysis

To study genistein-induced changes in gating kinetics, we applied the spectral analysis technique to macroscopic CFTR Cl^- currents using pCLAMP software (version 8.1.0; (Lindemann and Van Driessche, 1977; Venglarik *et al*., 1996; Scott-Ward *et al*., 2004). We filtered records with an eight-pole Bessel filter (Frequency Devices, Inc., model 902LPF2) at a corner frequency of 5 kHz and acquired data using a Digidata 1200 interface (Molecular Devices Corp.) and pCLAMP software at a sampling rate of 20 kHz. Digitised records were partitioned into short segments of 8,192 points (409.6 ms) and multiplied point-by-point using a Von Hanning window function before Fourier transformation. Power density spectra were calculated and averaged using a minimum of 100 segments of data. We determined the power density spectrum of background noise when CFTR Cl^- currents were quiescent and subtracted this from those of CFTR Cl^- currents. We fitted power density spectra with the sum of either two or

three Lorentzian functions using the Levenberg-Marquardt least-squares method. The Lorentzian functions used had the form:

$$S(f) = \sum_{i=1}^{n} S_{(0)i} / [1 + (f/f_c)^2], \qquad (1)$$

Where i indicates the Lorentzian component, S(f) is the power density spectrum at the frequency of f, $S_{(0)i}$ is the zero-frequency asymptote of Lorentzian component i, and f_c is the corner frequency of the component i. We chose a frequency range of 10 Hz to 3 kHz for the fitting of Lorentzian functions because tolbutamide block of CFTR introduced a high frequency Lorentzian component into power density spectra (f_c = 650 Hz;(Venglarik *et al.*, 1996)).

3.4 Statistics

Results are expressed as means ± SEM of n observations. To test for differences between groups of data, an analysis of variance (ANOVA) was used. To compare only two sets of data, we used Student's t-test. Differences were considered statistically significant for p values < 0.05. Tests were performed using either Microsoft Excel or SigmaStat™ (version 2.03, Jandel Scientific GmbH, Erkrath, Germany).

4. Preparation of Reagents

Forskolin was dissolved in methanol, while genistein, tyrphostin 47 and the small molecule CFTR correctors were dissolved in dimethyl sulfoxide (DMSO). Stock solutions of forskolin, genistein and tyrphostin 47 were stored at –20 ºC, *Phyllanthus acidus* extract was stored at +4 ºC and stock solutions of small molecule CFTR correctors were stored at RT. ATP was prepared immediately before each experiment and stored at +4 ºC. Stock solutions of all reagents were diluted in either BHK media or intracellular solution to achieve final concentrations immediately before use. Precautions against light-sensitive reactions were observed when using genistein, tyrphostin 47 or the small molecule CFTR correctors. Acute treatment with DMSO did not affect the Cl^- channel activity of CFTR (Sheppard and Robinson, 1997; Li *et al*., 2004).

III. Results and Discussion

III. Results and Discussion

Chapter 1

Prolonged treatment of cells with genistein modulates the expression and function of the cystic fibrosis transmembrane conductance regulator.

Schmidt , A. *et al*. (2008) *Br.J. Pharmacol.* **153**, 1311- 1323

1. Abstract

Cystic fibrosis (CF) is caused by dysfunction of the cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channel. In the search for new CF therapies, small molecules have been identified that rescue the defective channel gating of CF mutants (termed CFTR potentiators). Here, we investigate the long-term effects of genistein, the best-studied CFTR potentiator, on the expression and function of CFTR.

To achieve these objectives, we pre-treated baby hamster kidney (BHK) cells expressing wild-type or F508del-CFTR (the most common CF mutant) with concentrations of genistein that potentiate (30 μM) or inhibit (100 μM) CFTR function for 2 or 24 h at 37 ºC before examining CFTR maturation, expression and single-channel activity.

Using the iodide efflux technique, we found that genistein pre-treatment failed to restore function to F508del-CFTR, but altered that of wild-type CFTR. Pre-treatment of cells with genistein for 2 h had

little effect on CFTR processing, whereas pre-treatment for 24 h either augmented (30 μM genistein) or impaired (100 μM genistein) CFTR maturation. Using immunocytochemistry, we found that all genistein pre-treatments increased the localisation of CFTR protein to the cell surface. However, following the pre-treatment of cells with genistein (100 μM) for 2 h, individual CFTR Cl^- channels exhibited characteristics of channel block upon channel activation.

In conclusion, genistein pre-treatment alters the maturation, cell surface expression and single-channel function of CFTR in ways distinct from its acute effects. Thus, CFTR potentiators have the potential to influence CFTR by mechanisms distinct from their effects on channel gating.

2. Introduction

Knowledge of how F508del-CFTR disrupts CFTR-mediated Cl^- transport in CF is leading to rational new approaches to therapy (Rubenstein, 2006; Cai *et al*., 2007). Restoration of channel function to F508del-CFTR requires the use of both a drug to deliver the mutant protein to its correct cellular location (termed a CFTR corrector) and a drug to rescue defective channel gating (termed a CFTR potentiator). To date, most studies of CFTR potentiators have examined the acute effects of these agents on CFTR Cl^- channel function. However, when used as drugs, CFTR expressing cells will be exposed to CFTR potentiators for prolonged periods.

To explore the long-term effects of CFTR potentiators on the CFTR Cl^- channel, the aim of the present study was to investigate the effects of prolonged exposure to genistein on the expression, localisation and function of CFTR. We selected for study this flavonoid because, as the

most-studied CFTR potentiator, its mechanism of action is best understood (Hwang and Sheppard, 1999; Lansdell *et al*., 2000; Ai *et al*., 2004b). For practical reasons, we employed baby hamster kidney (BHK) cells engineered to express high levels of CFTR protein rather than polarised epithelial cells expressing endogenous CFTR. Using the iodide efflux technique, we investigated the effects of prolonged genistein exposure on the activity of large populations of wild-type and F508del-CFTR in intact BHK cells. Then using biochemical and cell biological techniques, we explored the long-term effects of genistein on the maturation and expression of CFTR protein and with electrophysiological methods, we examined the drug's long-term effects on the single-channel function of CFTR. We discovered that genistein pre-treatment altered the processing, cell surface localisation and single-channel function of wild-type CFTR in ways distinct from its acute effects on the channel. Thus, CFTR potentiators have the potential to influence CFTR by mechanisms distinct from their effects on channel gating.

3. Results

3.1 Pre-treating cells with genistein fails to rescue the function of F508del-CFTR, but alters that of wild-type CFTR.

To investigate the long-term effects of CFTR potentiators on the CFTR Cl^- channel, we selected for study the flavonoid genistein that potentiates robustly CFTR channel gating (Hwang *et al*., 1997) and treated cells with the drug for 2 or 24 h prior to evaluating CFTR expression and function. Because elevated concentrations of genistein inhibit CFTR (Lansdell *et al*., 2000), we tested concentrations

of genistein that both potentiate (30 μM) and inhibit (100 μM) the CFTR Cl^- channel. To explore whether genistein pre-treatment alters the cell surface expression of CFTR, we used BHK cells engineered to express high levels of wild-type and F508del-CFTR hereafter termed BHK-wt-CFTR and BHK-F508del-CFTR cells, respectively. As a control, we studied untransfected BHK cells.

We began by assessing the acute effects of genistein on the function of a large population of CFTR Cl^- channels in intact cells using the iodide efflux technique. At 37 ºC, treatment of cells with the cAMP agonist forskolin (10 μM) and genistein (50 μM) stimulated a large transient efflux of iodide from BHK-wt-CFTR cells, but were without effect on either BHK-F508del-CFTR or untransfected BHK cells (Figure III.1.1a). Genistein (50 μM) enhanced the magnitude of CFTR-mediated iodide efflux stimulated by forskolin (10 μM) 6-fold and accelerated the time to peak 2.5-fold (Figure III.1.1b inset). At 28 ºC, BHK-F508del-CFTR cells generated a small sustained CFTR-mediated iodide efflux consistent with the delivery of some F508del-CFTR protein to the cell membrane at this temperature (Denning *et al*., 1992a), while the magnitude of CFTR-mediated iodide efflux from BHK-wt-CFTR cells was attenuated 0.6-fold, albeit not significantly (P = 0.07; Figure III.1.1a). As a further control, we pre-treated untransfected BHK cells, BHK-wt-CFTR and BHK-F508del-CFTR cells with DMSO (0.1 % v/v, the vehicle for genistein (100 μM)) for 24 h at 37 ºC and demonstrated that it was without effect on the magnitude and time-course of CFTR-mediated iodide efflux (P > 0.05; Figure III.1.1b).

Next, we analysed the effects of genistein pre-treatment on CFTR function using the same approach. Figure III.1.1c and d summarises the effects of incubating BHK-wt-CFTR and BHK-F508del-CFTR cells

with genistein (30 μM) at 37 ºC for either 2 or 24 h, respectively. These pre-treatments failed to rescue CFTR function in BHK-F508del-CFTR cells, but altered CFTR-mediated iodide efflux in BHK-wt-CFTR cells (Figure III.1.1c and d). Figure III.1.1c shows that pre-treatment with genistein (30 μM) for 2 h attenuated the magnitude of iodide efflux, albeit not significantly (P = 0.39) and accelerated the response (P = 0.03). By contrast, pre-treatment with genistein (30 μM) for 24 h accentuated the magnitude of iodide efflux, albeit not significantly (P = 0.10) and accelerated the response (P < 0.01; Figure III.1.1d). These data suggest that short pre-treatments with genistein (30 μM) have little or no effect on CFTR, whereas longer pre-treatments might enhance its Cl^- channel function.

Figure III.1.1e and f summarises the effects of incubating BHK-wt-CFTR and BHK-F508del-CFTR cells with genistein (100 μM) at 37 ºC for either 2 or 24 h, respectively. These pre-treatments were without effect on iodide efflux from either BHK-F508del-CFTR cells or untransfected BHK cells (Figure III.1.1e and f). However, they markedly attenuated CFTR-mediated iodide efflux from BHK-wt-CFTR cells (P < 0.05), while accelerating the response (P < 0.05; Figure III.1.1e and f). Thus, pre-treating cells with concentrations of genistein that either potentiate or inhibit the CFTR Cl^- channel fails to rescue the activity of F508del-CFTR, but alters that of wild-type CFTR.

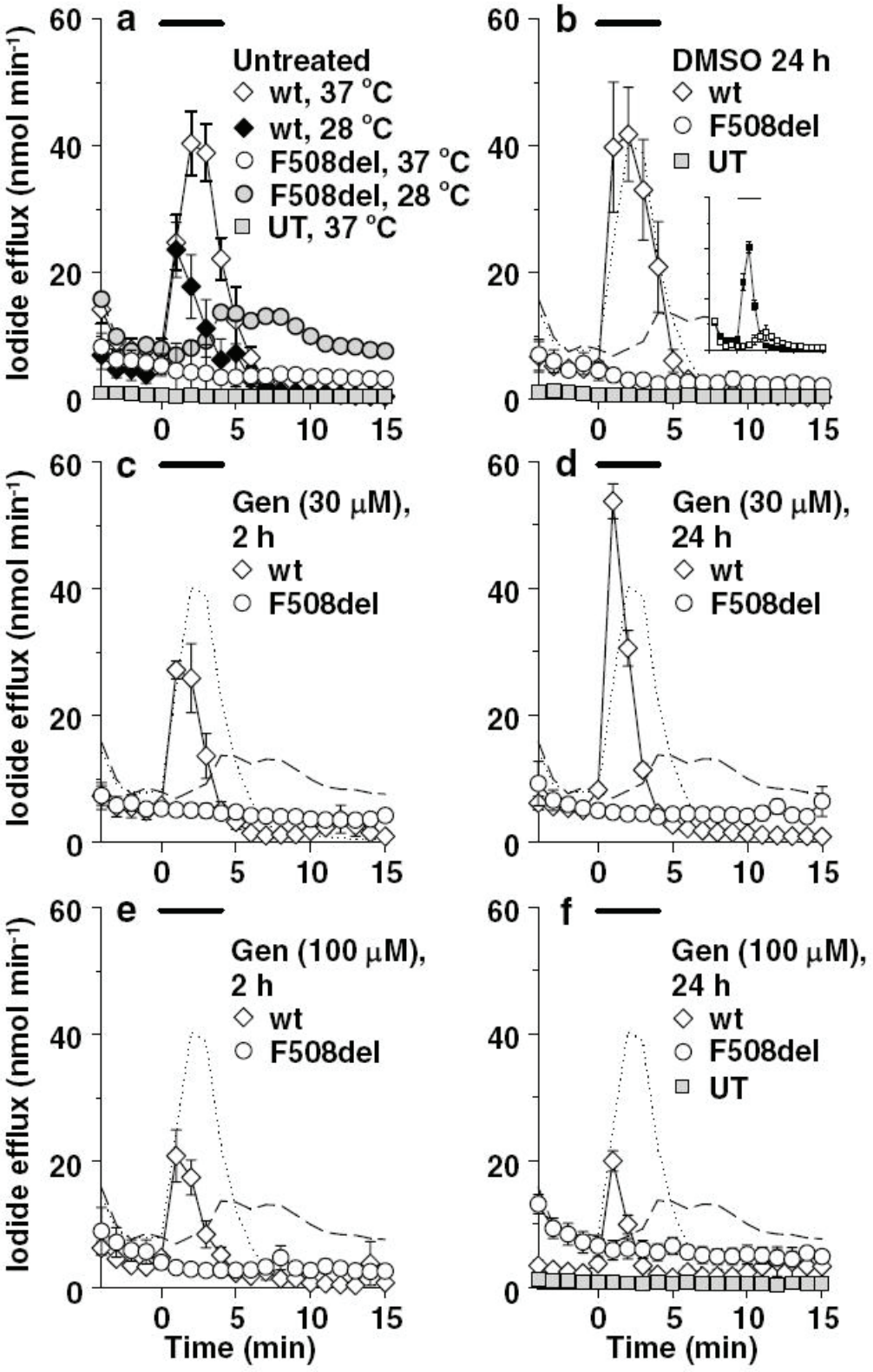

Figure III.1.1 - The effects of genistein pre-treatment on iodide efflux from BHK cells expressing wild-type and F508del-CFTR. Data show the time course of iodide efflux from BHK-wt-CFTR and BHK-F508del-CFTR cells pre-treated with genistein (30 or 100 μM) for 2 or 24 h. Diamonds and circles represent data from BHK-wt-CFTR and BHK-F508del-CFTR cells,

respectively, while grey squares denote data from untransfected BHK cells (UT). (a, b) controls; (a) cells cultured at 37 and 27 °C. (b) cells pre-treated for 24 h with DMSO (0.1% v/v) the vehicle for genistein (100 μM) at 37 ºC. The inset compares the time course of iodide efflux from untreated BHK-wt-CFTR cells stimulated by forskolin (10 μM) alone (open squares) and forskolin (10 μM) plus genistein (50 μM) (filled squares). Abscissa: time, -5 - 15 min; ordinate: iodide efflux; 0 – 60 nmol min^{-1}. Values are means ± SEM (n = 6). (c, d) cells pre-treated with genistein (30 μM) for either 2 h (c) or 24 h (d) at 37 ºC. (e, f) cells pre-treated with genistein (100 μM) for either 2 h (e) or 24 h (f) at 37 ºC. In (b-f), dotted and dashed lines indicate iodide efflux from untreated BHK-wt-CFTR cells grown at 37 ºC and untreated BHK-F508del-CFTR cells grown at 27 ºC, respectively, as shown in (a). During the periods indicated by bars forskolin (10 μM) and genistein (50 μM) were added to the efflux buffer. Symbols and error bars indicated means ± SEM (n = 6) for each condition. Where not shown, error bars are smaller than symbol size.

3.2 Genistein pre-treatment modulates the processing efficiency of CFTR protein.

In principle, genistein pre-treatment might modulate CFTR activity in one or a combination of three ways: first, by altering the number of active channels at the cell surface (trafficking); second by varying current flow through open channels; third, by regulating channel gating and hence, open probability (P_o). To investigate how genistein pre-treatment affects the number of CFTR Cl^- channels at the cell surface and their behaviour, we employed biochemical, cell biological and electrophysiological techniques.

When immunoprecipitation is used to investigate the biosynthesis of CFTR, two different forms of wild-type CFTR protein are visualised: an immature core-glycosylated form that is found in the ER (150 kDa; band B) and a mature fully-glycosylated form that has been processed through the Golgi apparatus and delivered to the cell membrane (170-180 kDa; band C; (Cheng *et al*., 1990). To evaluate the effects of

genistein pre-treatment on the turnover rate of band B and the efficiency of its processing into band C, we used pulse-chase analyses followed by CFTR immunoprecipitation.

Figure III.1.2a demonstrates that both the immature and mature forms of CFTR were detected in untreated BHK-wt-CFTR cells as well as in those pre-treated with either genistein (30 μM) or its vehicle DMSO (0.03 % v/v) for 2 or 24 h at 37 ºC. In contrast, only the immature form of CFTR was detected in untreated BHK-F508del-CFTR cells as well as those pre-treated with either genistein (30 μM) or DMSO (0.03 % v/v) for 24 h at 37 ºC (Figure III.1.2g). Pre-treatment of BHK-wt-CFTR cells with either genistein (30 μM) or DMSO for 2 and 24 h were without effect on the turnover rate of band B (Figure III.1.2c and d). Similarly, the turnover rate of band B was unaffected when BHK-F508del-CFTR cells were pre-treated with these agents for 24 h (Figure III.1.2h). However, these pre-treatments altered the efficiency of processing into band C. Figure III.1.2e shows that at early chase times (30 and 60 min) CFTR processing efficiency was accelerated by 2 h pre-treatments, albeit not significantly ($P > 0.05$). Pre-treatment for 24 h with DMSO was without effect, whereas genistein (30 μM) pre-treatment enhanced CFTR processing efficiency, but not significantly ($P > 0.05$; Figure III.1.2f). We interpret these data to suggest that prolonged pre-treatments with genistein (30 μM) appear to increase the maturation efficiency of wild-type CFTR.

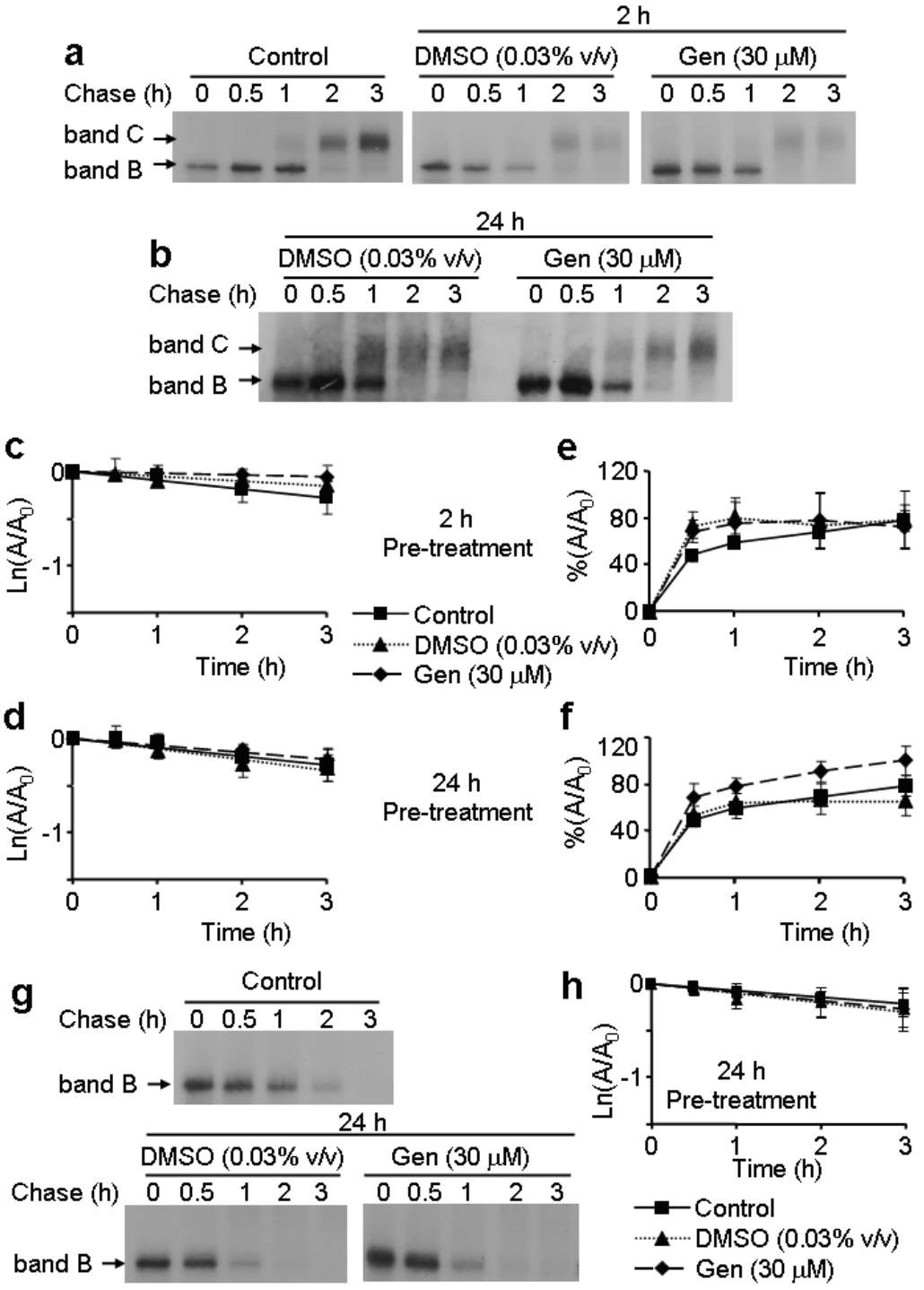

Figure III.1.2 - Turnover and processing of CFTR protein following the pre-treatment of BHK cells expressing wild-type and F508del-CFTR with genistein (30 μM). (a,b) autoradiographs of immunoprecipitated CFTR protein from BHK-wt-CFTR cells pre-treated with either DMSO (0.03% v/v) or genistein (30 μM) for 2 and 24 h at 37 ºC. (g) autoradiographs of immunoprecipitated CFTR protein from BHK-F508del-CFTR cells pre-treated with either DMSO (0.03% v/v) or genistein (30 μM) for 24 h at 37 ºC.

Following drug pre-treatments, BHK-wt-CFTR and BHK-F508del-CFTR cells were radiolabelled and chased for the indicated periods before CFTR was immunoprecipitated and separated by SDS-PAGE followed by fluorography. The positions of bands B and C are indicated by arrows. (c, d and h) effect of genistein pre-treatment on the turnover of band B. Data are presented as the natural logarithm of the amount of band B at a given time of the chase (A) relative to the amount at the beginning of the experiment (A_0). (e, f) effect of genistein pre-treatment on the efficiency of processing into band C. Data are presented as the percentage of band C detected at a given time of the chase (A) relative to the amount at the beginning of the experiment (A_0). Squares, triangles and diamonds represent data from untreated control cells, cells pre-treated with DMSO (0.03% v/v) and cells pre-treated with genistein (30 μM), respectively; (c and e) 2 h pre-treatment; (d, f and h) 24 h pre-treatment. Symbols and error bars are means ± SEM (c and e, n = 3; d, f and h, n = 5). In (c, d and h), the lines are the fit of first-order regressions to the data.

Figure III.1.3a and b demonstrates the presence of the immature and mature forms of CFTR in BHK-wt-CFTR cells pre-treated with genistein (100 μM) for 2 and 24 h at 37 ºC and the equivalent volume of DMSO for 2 or 24 h at 37 ºC. In contrast, only the immature form of CFTR was detected in untreated BHK-F508del-CFTR cells as well as those pre-treated with either genistein (100 μM) or DMSO (0.1 % v/v) for 24 h at 37 ºC (Figure III.1.3g). Interestingly, after pre-treatment with genistein (100 μM) for 24 h at 37 ºC CFTR appeared as a diffuse band of higher mobility than band C, but lower than that of band B (Figure III.1.3b). Like the effects of low concentrations of the drug (Figure III.1.2c and d), incubating BHK-wt-CFTR cells with either genistein (100 μM) or DMSO for 2 and 24 h was without effect on the turnover rate of band B (Figure III.1.3c and d). The turnover rate of band B was also unaffected when BHK-F508del-CFTR cells were pre-treated with these agents for 24 h (Figure III.1.3h). However, these pre-treatments altered the efficiency of processing to band C. Figure III.1.3e shows that pre-treatment for 2 h with genistein (100 μM) was without effect, whereas pre-treatment with DMSO decreased processing efficiency, but not significantly ($P > 0.05$). The data also

suggest that processing efficiency was similar when BHK-wt-CFTR cells were pre-treated for 2 h at 37 ºC with genistein (30 or 100 μM; Figures III.1.2e and III.1.3e). Conversely, pre-treatment for 24 h with genistein (100 μM) alters the maturation of CFTR protein to band C, whereas pre-treatment with DMSO was without effect (Figure III.1.3f). The band of intermediate mobility between bands B and C (Figure III.1.3b) indicates that some CFTR protein underwent post-ER processing and was delivered to the cell membrane where it mediated iodide efflux (Figure III.1.1f). However, the mobility of this band suggests that genistein (100 μM) impairs, albeit not completely, the glycosylation of CFTR in the Golgi apparatus.

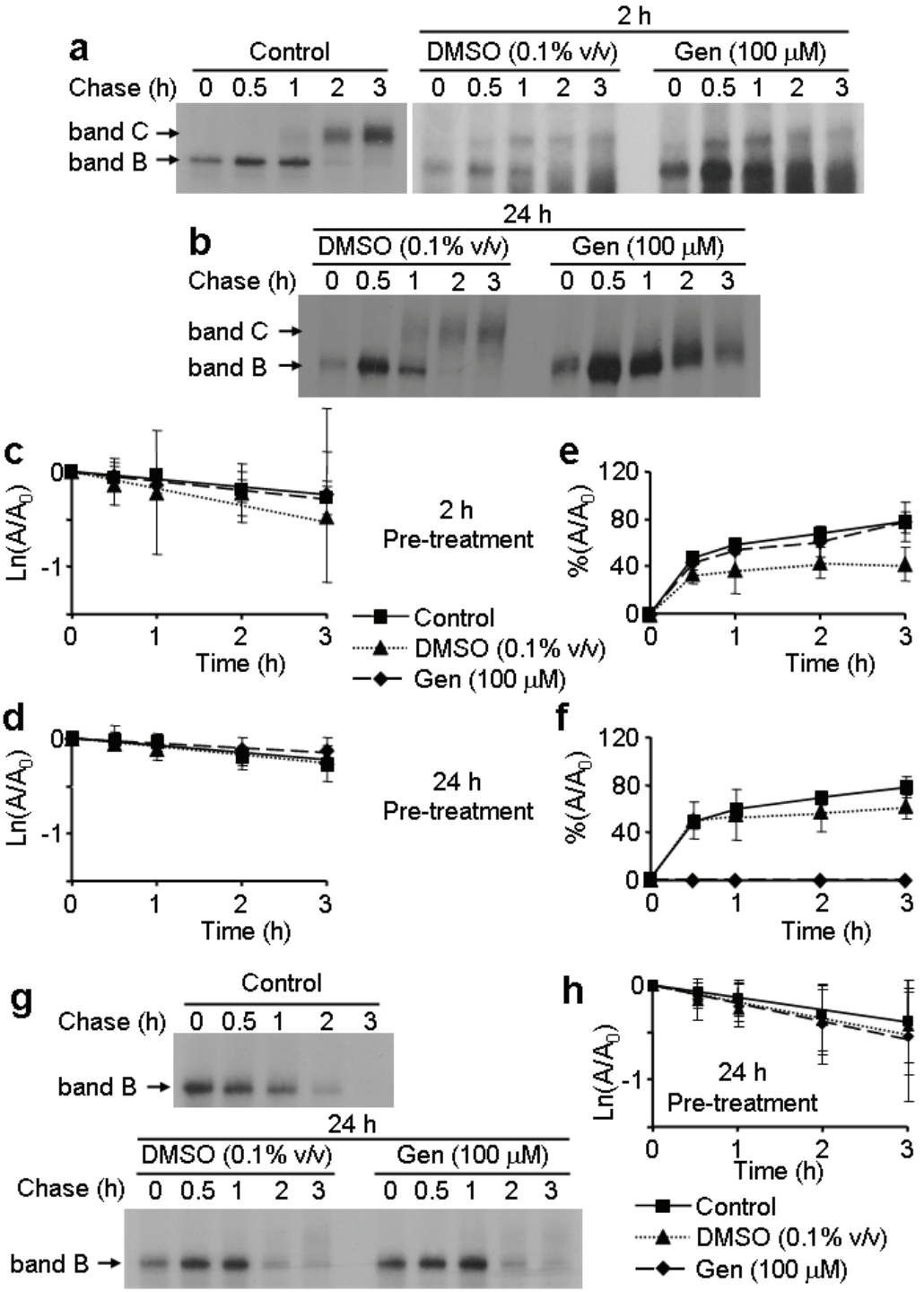

Figure III.1.3 - Pre-treatment of BHK cells expressing wild-type and F508del-CFTR with genistein (100 μM) impairs the maturation of wild-type CFTR, but is without effect on F508del-CFTR. (a,b) autoradiographs of immunoprecipitated CFTR protein from BHK-wt-CFTR cells pre-treated with either DMSO (0.1% v/v) or genistein (100 μM) for 2 and 24 h at 37 ºC. For comparison, the control autoradiograph is the same as Figure III.1.2a. (g) autoradiographs of immunoprecipitated CFTR protein from BHK-F508del-

CFTR cells non-treated control and pre-treated with either DMSO (0.1% v/v) or genistein (100 μM) for 24 h at 37 ºC. Following drug pre-treatments, BHK-wt-CFTR and BHK-F508del-CFTR cells were radiolabelled and chased for the indicated periods before CFTR was immunoprecipitated and separated by SDS-PAGE followed by fluorography. The positions of bands B and C are indicated by arrows. (c, d and h) effect of genistein pre-treatment on the turnover of band B. Data are presented as the natural logarithm of the amount of band B at a given time of the chase (A) relative to the amount at the beginning of the experiment (A_0). (e, f) effect of genistein pre-treatment on the efficiency of processing into band C. Data are presented as the percentage of band C detected at a given time of the chase (A) relative to the amount at the beginning of the experiment (A_0). Squares, triangles and diamonds represent data from untreated control cells, cells pre-treated with DMSO (0.1% v/v) and cells pre-treated with genistein (100 μM), respectively; (b and d) 2 h pre-treatment; (d, f and h) 24 h pre-treatment. Symbols and error bars are means ± SEM (c and e, n = 5; d, f and h, n = 3). In (c, d and h), the lines are the fit of first-order regressions to the data.

Besides acting as a CFTR potentiator, genistein is a potent tyrosine kinase inhibitor (Akiyama and Ogawara, 1991). This raises the possibility that the effects on CFTR processing observed when BHK-wt-CFTR cells are pre-treated with genistein (100 μM) for 24 h at 37 ºC result from genistein's action as a tyrosine kinase inhibitor rather than its role as a CFTR potentiator. To explore this possibility, we selected for study tyrphostin 47, a tyrosine kinase inhibitor that enhances CFTR-mediated transepithelial Cl^- transport, albeit less efficaciously than genistein (Sears *et al*., 1995). Using identical conditions to those employed with genistein (100 μM), we tested the effects of tyrphostin 47 on CFTR processing.

Figure III.1.4a demonstrates that pre-treatment of BHK-wt-CFTR cells with tyrphostin 47 (100 μM) for 24 h at 37 ºC led to the appearance of a diffuse band of higher mobility than band C, but lower than that of band B. This band of intermediate mobility between bands B and C was absent in untreated BHK-wt-CFTR cells and those pre-treated with tyrphostin 47's vehicle DMSO (Figure III.1.4a). Moreover,

only the immature form of CFTR was detected in BHK-F508del-CFTR cells pre-treated with either tyrphostin 47 (100 μM) or DMSO (0.1 % v/v) for 24 h at 37 ºC (Figure III.1.4d). Like the effect of genistein (100 μM) (Figure III.1.3d and h), pre-treatment of BHK-wt-CFTR and BHK-F508del-CFTR cells with tyrphostin 47 (100 μM) for 24 h at 37 ºC failed to alter the turnover rate of band B (Figure III.1.4b and e). Similarly, pre-treatment of BHK-wt-CFTR cells with either genistein (100 μM) or tyrphostin 47 (100 μM) for 24 h at 37 ºC had identical effects on processing efficiency (Figures III.1.3f and III.1.4c). Because both these tyrosine kinase inhibitors generated bands of intermediate mobility between bands B and C (Figures III.1.3b and III.1.4a), but tyrphostin 47 is a weaker CFTR potentiator than genistein (Sears *et al*., 1995), we suggest that post-ER processing of CFTR might be regulated by tyrosine phosphorylation.

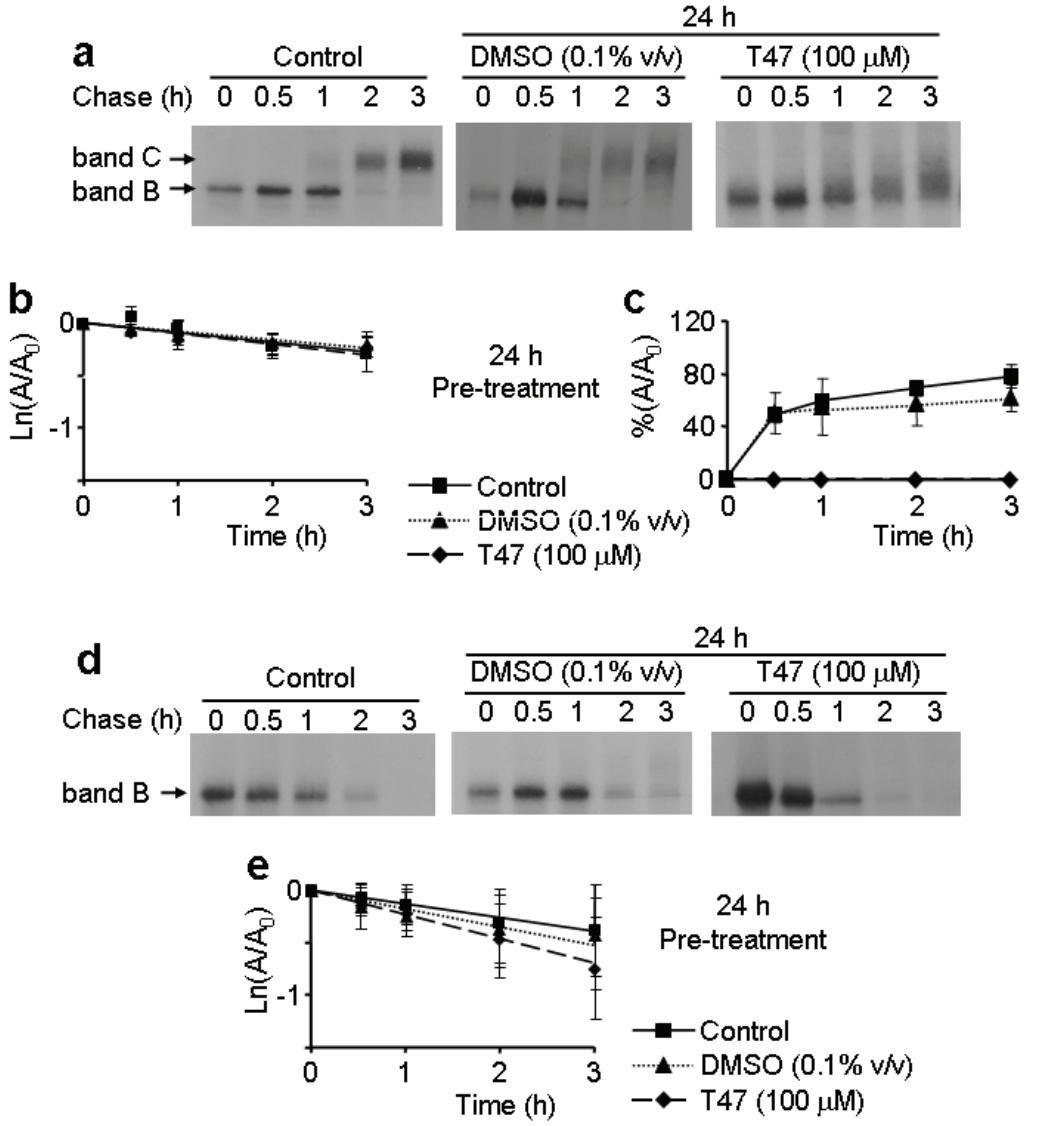

Figure III.1.4 - The tyrosine kinase inhibitor tyrphostin 47 impairs the maturation of wild-type CFTR. (a, d) autoradiographs of immunoprecipitated CFTR protein from BHK-wt-CFTR and BHK-F508del-CFTR cells, respectively, pre-treated with either DMSO (0.1% v/v) or tyrphostin 47 (T47; 100 μM) for 24 h at 37 ºC. Following drug pre-treatments, BHK-wt-CFTR and BHK-F508del-CFTR cells were radiolabelled and chased for the indicated periods before CFTR was immunoprecipitated and separated by SDS-PAGE followed by fluorography. The positions of bands B and C are indicated by arrows. (b, e) effect of tyrphostin 47 pre-treatment on the turnover of band B. Data are presented as the natural logarithm of the amount of band B at a given time of the chase (A) relative to the amount at the beginning of the experiment (A_0). (c) effect of tyrphostin 47 pre-treatment on the efficiency of processing into band C. Data are presented as the percentage of band C detected at a given time of the chase (A) relative to the amount at the beginning of the experiment (A_0). Squares, triangles and diamonds represent data from untreated control

cells, cells pre-treated with DMSO (0.1% v/v) and cells pre-treated with tyrphostin 47 (100 μM), respectively. Symbols and error bars are means ± SEM (control and DMSO pre-treatment, n = 5; tyrphostin 47 pre-treatment, n = 3). In (b, e), the lines are the fit of first-order regressions to the data.

3.3 The effects of genistein pre-treatment on the localisation of CFTR protein

Our biochemical studies demonstrate that genistein pre-treatment (2 or 24 h) alters the processing of CFTR protein. This suggests that incubating cells with the drug might alter the distribution of CFTR protein within cells. To test this idea, we performed immunocytochemical studies to localise specifically CFTR protein. Consistent with previous work (Denning *et al*., 1992a), in untreated control cells, wild-type CFTR protein was located both intracellularly and at or near the cell membrane, whereas F508del-CFTR was located only intracellularly (Figure III.1.5a). Visual inspection of the images shown in Figure III.1.5b and c suggests that pre-treatment of BHK-wt-CFTR and BHK-F508del-CFTR cells with either genistein (30 and 100 μM) or equivalent volumes of DMSO for 2 and 24 h at 37 ºC had similar effects on the localisation of CFTR protein. For BHK-wt-CFTR cells, pre-treatment resulted in the accumulation of more CFTR protein at or near the cell membrane (Figure III.1.5b and c). Moreover, for BHK-F508del-CFTR cells, pre-treatment led to a more diffuse distribution of CFTR protein within cells (Figure III.1.5b and c). Thus, these data suggest that the cell surface expression of wild-type CFTR is enhanced following the pre-treatment of cells with either genistein or its vehicle DMSO.

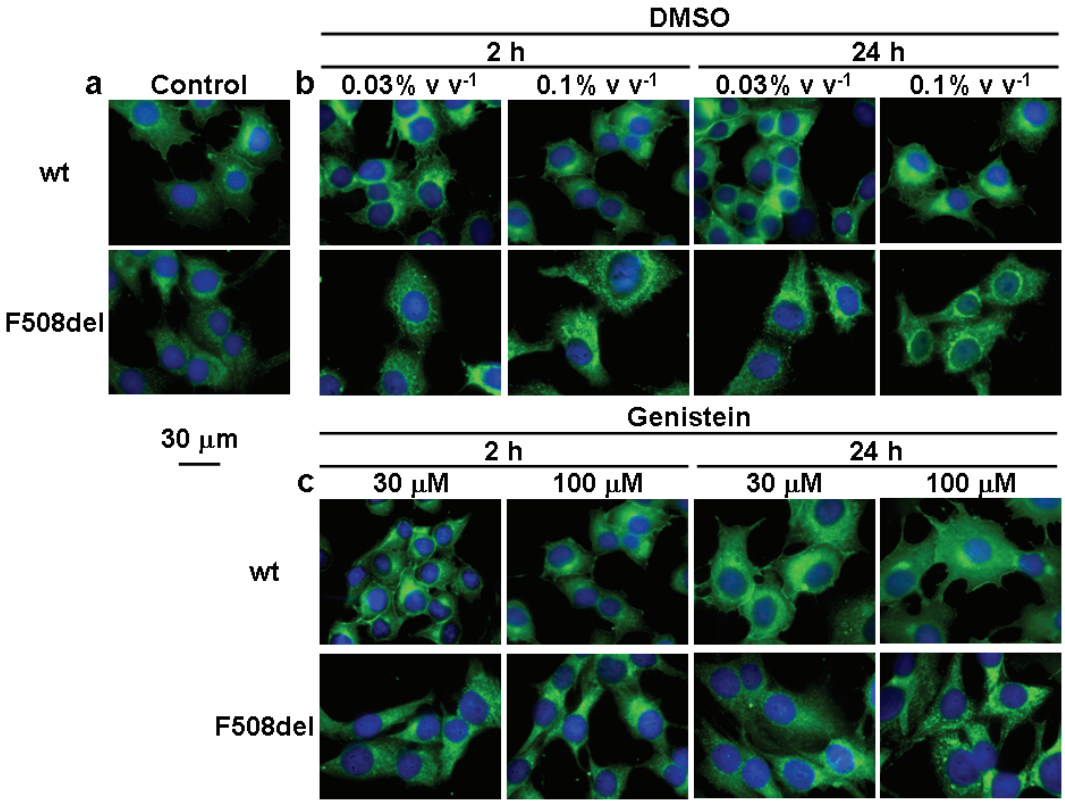

Figure III.1.5 - The effects of genistein pre-treatment on the expression of CFTR protein. Fluorescent immunocytochemical images of BHK-wt-CFTR and BHK-F508del-CFTR cells pre-treated with either DMSO or genistein for 2 and 24 h. (a) untreated controls. (b) cells pre-treated with DMSO (0.03 and 0.1% v/v) for 2 and 24 h. (c) cells pre-treated with genistein (30 and 100 μM) for 2 and 24 h. The FITC signal is represented in green and the DAPI nuclear stain in blue. Bar = 30 μm. The results are representative of two independent experiments. No background staining or autofluorescence were observed with untransfected BHK cells (data not shown). Data obtained by F. Mendes.

3.4 The single-channel properties of CFTR are altered by genistein pre-treatment

Previous work has demonstrated that genistein interacts with CFTR at multiple sites to modulate channel activity (Wang *et al*., 1998; Lansdell *et al*., 2000). Low micromolar concentrations of genistein potentiate CFTR channel gating by interacting directly with the NBDs (Wang *et al*., 1998; Randak *et al*., 1999). By contrast, elevated concentrations of

the drug inhibit the channel both by interfering with NBD-driven channel gating and occluding weakly the channel pore (Lansdell *et al*., 2000). To learn how genistein pre-treatment alters the function of wild-type CFTR, we used excised inside-out membrane patches. Disappointingly, despite repeated efforts (n > 125), we were unable to record CFTR Cl^- currents from BHK-wt-CFTR cells pre-treated at 37 ºC with (i) genistein (30 and 100 μM) for 24 h, (ii) genistein (30 μM) for 2 h and (iii) equivalent volumes of DMSO under similar experimental conditions because it was not possible to form high-resistance seals on pre-treated cells.

When we excised inside-out membrane patches from BHK-wt-CFTR cells pre-treated with genistein (100 μM) at 37 ºC for 2 h, we observed small numbers of active channels in the presence of ATP (1 mM) only and large macroscopic CFTR Cl^- currents after the phosphorylation of CFTR with PKA (75 nM; Figure III.1.6). Visual inspection of channel openings prior to PKA-dependent phosphorylation suggests that genistein pre-treatment altered the single-channel properties of CFTR decreasing current amplitude without altering the duration of channel openings (Figure III.1.6a and b). To quantify the effects of genistein pre-treatment, we measured i, MBD and frequency of channel gating in the presence of ATP (1 mM) alone. (We did not calculate interburst interval (IBI) because the number of active channels in the membrane patch was unknown in the absence of PKA). Figure III.1.6c - f demonstrates that a 2 h pre-treatment with genistein (100 μM): (i) depressed i ($P < 0.05$), (ii) decreased, albeit not significantly MBD ($P > 0.05$) and (iii) did not alter the amount of time spent in the open state nor the frequency of channel gating ($P > 0.05$). We interpret these data to suggest that 2 h pre-treatments with genistein (100 μM) inhibit CFTR activity by reducing current flow through open channels.

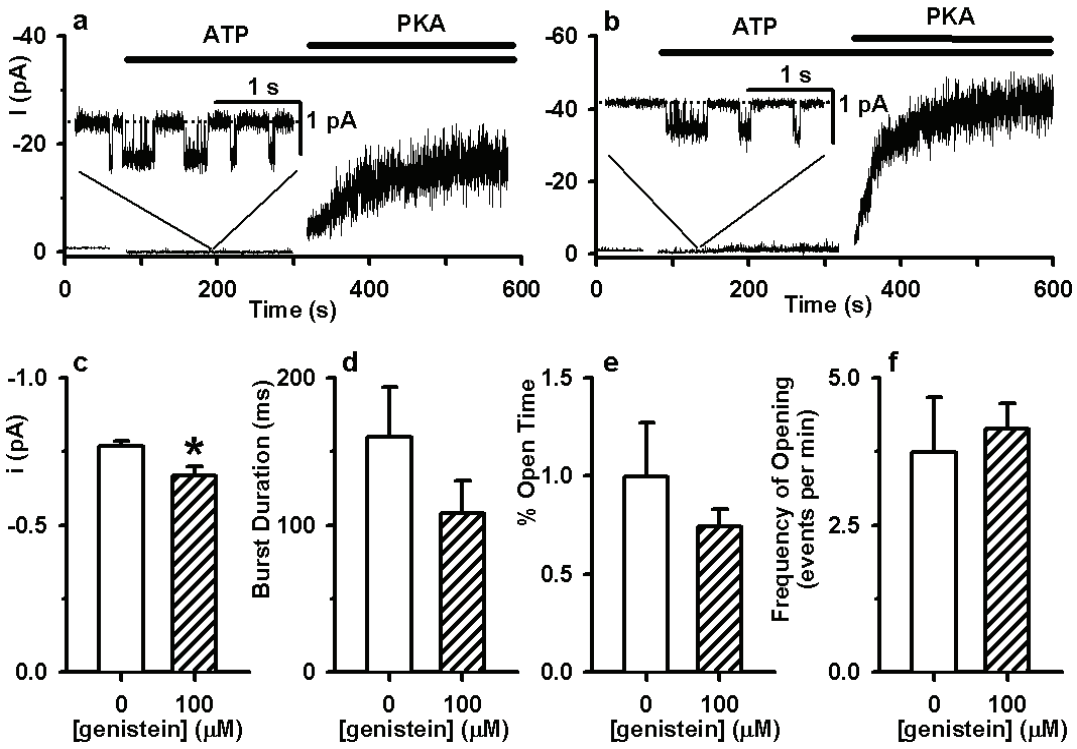

Figure III.1.6 - Pre-treating BHK-wt-CFTR cells with genistein alters the single-channel properties of CFTR (a, b) time courses of CFTR Cl^- currents in excised inside-out membrane patches from BHK-wt-CFTR cells. (a) membrane patch from an untreated control cell. (b) membrane patch from a cell pre-treated with genistein (100 μM) for 2 h prior to patch excision. ATP (1 mM) and PKA (75 nM) were present in the intracellular solution during the periods indicated by the bars. Voltage was -50 mV and there was a large Cl^- concentration gradient across the membrane ($[Cl^-]_{int}$ = 147 mM; $[Cl^-]_{ext}$ = 10 mM). For the purpose of illustration, time courses have been inverted so that upward deflections represent inward currents. The inserts show sections of the records prior to CFTR phosphorylation by PKA on an expanded time scale. The dotted lines indicate where channels are closed and downward deflections of the traces correspond to channel openings. (c, d, e and f) effect of genistein (100 μM) pre-treatment on i, MBD, percent time open and events per minute. Measurements were made in the presence of ATP (1 mM) prior to CFTR phosphorylation with PKA. Abbreviations: 0, untreated; 100, pre-treated with genistein (100 μM) for 2 h prior to patch excision. Columns and error bars indicate means + SEM (number of burst analysed: control, n = 11 and genistein, n = 17 - 27). The asterisk indicates a value that is significantly different from the control value ($P < 0.05$).

3.5 Noise analysis of the effects of genistein pre-treatment on CFTR function

The decrease in i caused by pre-treating BHK-wt-CFTR cells with genistein (100 μM) is reminiscent of several open-channel blockers of the CFTR Cl^- channel (e.g. tolbutamide (Venglarik *et al*., 1996) and niflumic acid (Scott-Ward *et al*., 2004)). To investigate the effects of tolbutamide on the kinetics of channel gating, Venglarik *et al*. (1996) used the spectral analysis technique to derive information about gating kinetics from membrane patches containing large numbers of active channels. We therefore adopted a similar strategy to investigate the effects of genistein pre-treatment on the kinetics of channel gating following CFTR phosphorylation with PKA. Consistent with previous data (Venglarik *et al*., 1996; Fischer and Machen, 1994), in untreated BHK-wt-CFTR cells power density spectra of CFTR Cl^- currents were best fitted with two Lorentzian components described by corner frequencies f_{c1} and f_{c2} (Figure III.1.7 and Table III.1.1). The dominant low frequency Lorentzian component (f_{c1}) corresponds to the bursting pattern of channel gating controlled by the interaction of ATP with the NBDs, whereas the intermediate frequency Lorentzian component (f_{c2}) with minimal power (S_{02} = 3.7% of S_{01}; n = 3) represents the brief closures that interrupt bursts of channel openings (Fischer and Machen, 1994; Venglarik *et al*., 1994).

Both when CFTR Cl^- currents were acutely treated with genistein (100 μM) and when BHK-wt-CFTR cells were pre-treated with genistein (100 μM) prior to channel activation, power density spectra of CFTR Cl^- currents were best fitted with three Lorentzian components described by corner frequencies f_{c1}, f_{c2} and f_{c3} (Figure III.1.7 and Table III.1.1). Like tolbutamide inhibition of CFTR (Venglarik *et al*., 1996),

the corner frequency of the genistein-induced component (f_{c3}) was distinguished by its high frequency (Figure III.1.7 and Table III.1.1). Moreover, the corner frequency was the same irrespective of whether CFTR Cl^- currents were acutely treated with genistein or BHK-wt-CFTR cells were pre-treated with the drug prior to channel activation (Table III.1.1). We speculate that this high frequency Lorentzian component corresponds to the very rapid binding of genistein to and its dissociation from individual CFTR Cl^- channels.

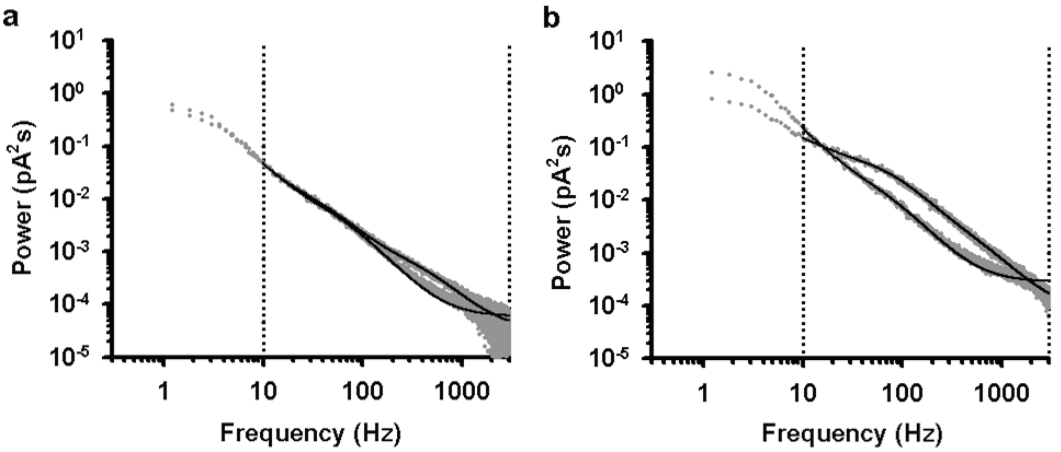

Figure III.1.7 - **Spectral analysis of genistein inhibition of the CFTR Cl^- channel.** (a) power density spectra calculated from data recorded under control conditions (ATP (1 mM) and PKA 75 (nM)) and after the addition of genistein (100 μM) to the intracellular solution of an excised membrane patch from a BHK-wt-CFTR cell. Voltage was –50 mV and there was a large Cl^- concentration gradient across the membrane ($[Cl^-]_{int}$ = 147 mM; $[Cl^-]_{ext}$ = 10 mM). The solid lines represent the fit of two and three Lorentzian components to the control and genistein (100 μM) data, respectively. Control spectra displayed corner frequencies (f_{c1} and f_{c2}) of 1.23 Hz and 57.08 Hz with maximum variances per unit frequency (S_{01} and S_{02}) of 2.75 pA^2s and 6.2 x 10^{-3} pA^2s. In the presence of genistein (100 μM), corner frequencies (f_{c1}, f_{c2} and f_{c3}) were 2.93 Hz, 43.80 Hz and 457.04 Hz with maximum variances (S_{01}, S_{02} and S_{03}) of 0.48 pA^2s, 8.3 x 10^{-3} pA^2s and 5.7 x 10^{-4} pA^2s. (b) power density spectra calculated using data from a membrane patch excised from a BHK-wt-CFTR cell pre-treated with genistein (100 μM) for 2 h prior to patch excision. The solid lines represent the fit of three Lorentzian components to the genistein (100 μM) data. For comparison, control spectra from (a) and the fit of two Lorentzian components to these data are shown. The spectra from the genistein (100 μM) pre-treated cell had corner frequencies (f_{c1}, f_{c2} and f_{c3}) of 7.76 Hz, 75.98 Hz and 633.04 Hz with maximum variances (S_{01}, S_{02} and

S_{03}) of 0.26 pA^2s, 5.3 x 10^{-2} and 1.3 x 10^{-3}. Power density spectra were constructed as described in II. Methods (Lindemann and Van Driessche, 1977; Venglarik *et al*., 1996; Scott-Ward *et al*., 2004).

Table III.1.1 - Effect of genistein on corner frequencies derived from spectral analysis of CFTR Cl^- channels

Condition	f_{c1} (Hz)	f_{c2} (Hz)	f_{c3} (Hz)	n
Control	4.05 ± 2.10	74.60 ± 6.23	-	5
Genistein (100 μM) acute treatment	3.64 ± 1.01	58.14 ± 4.74	570.54 ± 45.80	5
Genistein (100 μM) 2 h pre-pre-treatment	4.25 ± 2.19	67.56 ± 6.28	539.40 ± 104.66	3

Corner frequencies were measured using the indicated conditions. Under control conditions, the behaviour of CFTR Cl^- channels is described by the sum of two Lorentzian components (f_{c1} and f_{c2}). Either when excised membrane patches are treated directly with genistein (100 μM) or when BHK-wt-CFTR cells are pre-treated with genistein (100 μM) prior to membrane patch excision, an additional high frequency Lorentzian component (f_{c3}) is required to fit the data. Values are means ± SEM of n observations. Using a one-way ANOVA, values of f_{c1} and f_{c2} did not change significantly between the conditions tested (P > 0.1), neither did those of f_{c3} between the two conditions tested (P > 0.75). Measurements were made in the presence of the catalytic subunit of PKA (75 nM) and ATP (1 mM) in the intracellular solution. Voltage was –50 mV and there was a large Cl^- concentration gradient across the membrane ($[Cl^-]_{int}$ = 147 mM; $[Cl^-]_{ext}$ = 10 mM).

4. Discussion and Conclusions

The goal of this study was to investigate the long-term effects of the CFTR potentiator genistein on the expression and function of the CFTR Cl^- channel. We demonstrate that pre-treatment of BHK-wt-CFTR cells with concentrations of genistein that either potentiate or inhibit the CFTR Cl^- channel alter the processing, cell surface localisation and single-channel function of wild-type CFTR.

Genistein is the best-studied CFTR Potentiator (Hwang and Sheppard, 1999; Cai *et al*., 2007). The drug does not open quiescent channels. Instead, it augments the gating of phosphorylated CFTR Cl^- channels by increasing the frequency and duration of channel openings (Wang *et al*., 1998). To explain these effects of genistein, Ai *et al*. (2004a)employed the ATP-driven NBD dimerisation model of CFTR channel gating (Vergani *et al*., 2003). Ai and colleagues speculated that genistein accelerates channel opening by promoting NBD dimerisation and slows channel closure by stabilising the NBD1:NBD2 dimer conformation (Ai *et al*., 2004a). The authors also proposed that the genistein-binding site might be located at the NBD1:NBD2 dimer interface, a prediction supported by studies of genistein docking to a molecular model of the NBD1:NBD2 dimer (Moran *et al*., 2005). Consistent with functional data (e.g. (Wang *et al*., 1998)), the predicted genistein-binding site in this molecular model is distinct from the two ATP-binding sites of CFTR (Moran *et al*., 2005).

Interestingly, Cui *et al*. (2007) demonstrated that CFTR constructs lacking NBD2 exit the ER, while F508del arrests their maturation. These data argue that NBD dimerisation is not a prerequisite for CFTR processing. This suggests that CFTR potentiators, such as genistein, which stabilise the NBD1:NBD2 dimer conformation are unlikely to

rescue F508del-CFTR folding. Instead, the data presented by Cui and colleagues predict that agents, which bind to NBD1 or enhance the packaging of NBD1 with the MSDs would promote F508del-CFTR folding and hence, rescue its trafficking defect (Cui *et al*., 2007).

In the present study, we demonstrate that pre-treatment of BHK-wt-CFTR cells with genistein alters CFTR-mediated iodide efflux. These effects of genistein might reflect the direct interaction of the drug with CFTR. Alternatively, they might result from the actions of genistein on proteins with which CFTR interacts during its biogenesis within the cell and function at the cell surface. As discussed below, the effects of genistein (100 μM) pre-treatment on the single-channel behaviour of CFTR argue that the drug interacts directly with the CFTR Cl^- channel. Thus, there are similarities between the acute and chronic actions of the drug on CFTR. However, our data also suggest that genistein pre-treatment might alter CFTR function by other mechanisms.

First, genistein is a potent inhibitor of tyrosine kinases (Akiyama and Ogawara, 1991). This suggest that pre-treating BHK-wt-CFTR cells with the drug might perturb the tyrosine phosphorylation status of proteins within these cells and hence, the function of CFTR. Consistent with this idea, prolonged pre-treatment of BHK-wt-CFTR cells with high concentrations of genistein and a second tyrosine kinase inhibitor tyrphostin 47 generated a band of intermediate mobility between bands B and C. The data suggest that these agents impair, albeit not completely, the glycosylation of CFTR in the Golgi apparatus. Because BHK-wt-CFTR cells pre-treated with genistein (100 μM) for 24 h at 37 ºC generated an efflux of iodide, this intermediate form of CFTR protein is probably delivered to the cell surface where it exhibits some function. Thus, the late stages of CFTR processing are directly or indirectly regulated by tyrosine phosphorylation.

Second, genistein might interact with lipids to modulate the activity of CFTR, itself, or CFTR-interacting proteins (CIPs). Consistent with this possibility, Hwang and colleagues demonstrated that genistein modulates the behaviour of gramicidin A channels by altering the mechanical properties of the phospholipid bilayer in which the channel is imbedded (Hwang *et al*., 2003).

Third, it is feasible that genistein pre-treatment might have untoward effects on cell proliferation and viability. In support of this idea, Lim *et al*. (2004) demonstrated that concentrations of genistein exceeding 5 μM caused a dose-dependent loss of the viability of IB3-1 cells ((F508del/W1282X) bronchial epithelial cells) after 72 h of treatment, while Li *et al*. (2004) reported similar results with Madin Darby canine kidney epithelial cells using genistein (100 μM). However, pre-treatment of BHK-wt-CFTR cells with genistein (30 and 100 μM) or DMSO (0.03 and 0.1 % v/v) for 24 h at 37 ºC was without effect on cell viability (data obtained by H. Li, n = 4 – 8; $P > 0.05$; data not shown), suggesting that the genistein pre-treatment-induced changes in iodide efflux are not a consequence of cytotoxic effects of the drug.

By following the fate of radiolabelled CFTR protein, we evaluated the effects of genistein pre-treatment on CFTR maturation. Our data reveal that genistein pre-treatment is without effect on the stability of immature CFTR protein in cells expressing wt- and F508del-CFTR. However, in cells expressing wt-CFTR genistein alters the efficiency with which immature CFTR protein is processed into its mature form. They also suggest that pre-treatment with genistein (100 μM) has widespread effects on the cellular pathways responsible for CFTR processing and trafficking. While the identity of the CIPs affected by genistein (100 μM) are unknown, Lim *et al*. (2004) demonstrated that pre-treatment of IB3-1 cells with genistein (5 μM) for 48 h at 37 ºC was

without effect on the chaperones Hsp70, Hsc70, calreticulin and calnexin.

Lehrich and colleagues (Lehrich and Forrest, Jr., 1995) found that acute treatment of shark rectal glands with genistein (100 μM) promoted the trafficking of CFTR protein to the apical membrane of gland-lining epithelial cells (Lehrich *et al*., 1998), while Lim and colleagues (Lim *et al*., 2004) showed that long-term treatment of IB3-1 cells with genistein (5 μM) led to a dispersed distribution of F508del-CFTR protein throughout the cytoplasm. Consistent with these data, our immunocytochemical images demonstrate that all genistein pre-treatments tested led to the accumulation of wild-type CFTR protein at or near the cell membrane and a diffuse distribution of F508del-CFTR protein within cells. However, our biochemical data reveal that different concentrations of genistein have disparate effects on protein maturation. A likely explanation for this apparent incongruence is the pool of CFTR protein studied. Pulse-chase experiments only detect radiolabelled protein generated after cells are pre-treated with genistein. By contrast, immunocytochemistry visualises all CFTR protein in the cell including protein produced before cells are treated with genistein.

Elevated concentration of genistein inhibit CFTR by two mechanisms: allosteric and open-channel block (Lansdell *et al*., 2000). Here, we show that following a 2 h pre-treatment of BHK-wt-CFTR cells with genistein (100 μM), CFTR Cl^- channels exhibit characteristics of open-channel block (Hille, 2001; Venglarik *et al*., 1996; Scott-Ward *et al*., 2004). For two reasons, this result is curious. Firstly, genistein is a low-affinity open-channel blocker of CFTR (K_d(0 mV) ~1800 μM (Lansdell *et al*., 2000). Secondly, both drug-treated cells and excised membrane patches were thoroughly washed prior to study. Under

these conditions, we would expect genistein to be absent from the solutions bathing the intra- and extracellular sides of excised membrane patches. This suggests that the most likely source of genistein is the cell membrane based on the data of (Hwang *et al*., 2003) and the hydrophobic nature of this flavonoid. This raises the interesting question of how genistein within the lipid bilayer causes open-channel block of CFTR. Genistein might alter the mechanical properties of the cell membrane or access its drug-binding site within the CFTR pore directly from the cell membrane. However, the simplest interpretation of the data ((Lansdell *et al*., 2000); present study) is that genistein within the cell membrane enters the intracellular solution where its anionic form is swept deep inside the CFTR pore by the prevailing electrochemical gradient. When genistein binds, its large size occludes the CFTR pore preventing Cl^- permeation. Alternatively, during drug pre-treatment genistein entering the cell might saturate the environment and bind to CFTR protein en route to the cell surface. This explanation is consistent with the observed decrease in mobility of CFTR protein when treated with genistein (100 μM) for 24 h at 37 ºC.

Because of difficulties forming high-resistance seals, we did not determine the effects on CFTR function of incubating BHK-wt-CFTR cells with genistein (30 μM). Based on the data of (Hwang *et al*., 2003), our failure to form high-resistance seals on genistein pre-treated cells might result from the drug's effects on the mechanical properties of the lipid bilayer. Interestingly, (Bulteau-Pignoux *et al*., 2002) demonstrated that brief (~2 min) exposure of CHO cells expressing wild-type CFTR to genistein (25 μM) prior to forskolin (2.5 μM) slowed CFTR activation and attenuated markedly current magnitude. The authors interpreted their data to suggest that the binding of genistein to the NBDs might impede R domain phosphorylation by PKA. However, the interaction

of genistein with the cell membrane (Hwang *et al*., 2003) might influence directly either CFTR channel gating or current flow through the channel.

DMSO is widely used as a vehicle for CFTR modulators. When used acutely, it is without effect on CFTR function (e.g. (Sheppard and Robinson, 1997)). By contrast, prolonged exposure of cells to DMSO influences the expression and localisation of CFTR in IB3-1 and BHK cells ((Lim *et al*., 2004); present study). However, our data argue that the effects of DMSO do not explain those of genistein on CFTR expression and function. Previous work has shown that DMSO acts as an epithelial differentiating agent, which enhances tight junction organisation (Bebok *et al*., 1998b). Bebök and colleagues exploited this property of DMSO to promote the delivery of F508del-CFTR to the apical membrane of LLC-PK_1 renal epithelia using high doses for prolonged periods. Interestingly, DMSO was without effect on F508del-CFTR expressed in mouse L cells, a fibroblast cell line, suggesting that the effects of DMSO on F508del-CFTR are cell-type specific (Bebok *et al*., 1998b). Taken together, these data are an important reminder that studies of CFTR correctors and potentiators require judicious selection of cell lines and rigorous controls.

In conclusion, we demonstrated that genistein modulates the biosynthesis, localisation and function of the CFTR Cl^- channel. All concentrations of the drug tested promoted the accumulation of CFTR protein at the cell surface. However, the effects of genistein were concentration-dependent: low micromolar concentrations augmented protein maturation. By contrast, higher concentrations impaired protein maturation and inhibited channel activity by attenuating current flow through open channels. As a result, when we evaluated CFTR function in intact cells using the iodide efflux technique, we found that low

concentrations of genistein augmented CFTR-mediated iodide efflux, whereas high concentrations had the converse effect. These data highlight the importance of a multidisciplinary approach when investigating how CFTR potentiators rescue CF mutants. They also demonstrate the potential of CFTR potentiators to influence CFTR activity by mechanisms distinct from their effects on channel gating. Significantly, some CFTR potentiators (e.g. VRT-532; (Van Goor *et al*., 2006; Wang *et al*., 2006b)) have been identified that act as pharmacological chaperones, rescuing the cell surface expression of F508del-CFTR and enhancing its Cl^- channel function.

Chapter 2

An extract from the medicinal plant *Phyllanthus acidus* and its isolated compounds induce airway chloride secretion: A potential treatment for cystic fibrosis.

Sousa, M., *et al*. (2007) *Mol. Pharmacol.* **71**, 366-376

1. Abstract

According to previous reports, flavonoids and nutraceuticals correct defective electrolyte transport in cystic fibrosis (CF) airways. Traditional medicinal plants from China and Thailand contain phyto-flavonoids and other bioactive compounds. We examined the herbal extract of the common Thai medicinal Euphorbiaceous plant *Phyllanthus acidus (P. acidus)* for its potential effects on epithelial ion transport. Functional assays by Ussing chamber, patch-clamp, two-electrode voltage-clamp and Ca^{2+} imaging demonstrated activation of Cl^- secretion and inhibition of Na^+ absorption by *P. acidus*. No cytotoxic effects of *P. acidus* were detected. Mucosal application of *P. acidus* to native mouse trachea suggested transient and steady-state activation of Cl^- secretion by increasing both intracellular Ca^{2+} and cAMP. These effects were mimicked by a mix of the isolated components adenosine, kaempferol, and hypogallic acid.

Additional experiments in human airway cells and CFTR expressing BHK cells and *Xenopus* oocytes confirm the results obtained in native tissues. Cl^- secretion was also induced in tracheas of CF mice homozygous for F508del-CFTR and in F508del-CFTR homozygous human airway epithelial cells. Taken together, *P. acidus* corrects

defective electrolyte transport in CF airways by parallel mechanisms including i) increasing the intracellular levels of second messengers cAMP and Ca^{2+}, thereby activating Ca^{2+}-dependent Cl^- channels and residual CFTR Cl^- channel function; ii) stimulating basolateral K^+ channels; iii) redistributing cellular localisation of CFTR; iii) directly activating CFTR; and v) inhibiting ENaC through activation of CFTR.

These combinatorial effects on epithelial ion transport may provide a novel complementary nutraceutical treatment for CF lung disease.

2. Introduction

Pharmacological interventions attempt to correct defective ion transport among other pulmonary phenotypes. Recent strategies make use of natural food components because of their ready accessibility and low toxicity (DeCarvalho *et al*., 2002b; Egan *et al*., 2004). These compounds act in different ways, such as correcting the trafficking defect of mutant CFTR or potentiating residual CFTR activity (Moran and Zegarra-Moran, 2005; Kunzelmann and Mall, 2003; Van Goor *et al*., 2006). Medicinal plants have been the basis for traditional herbal remedies for many centuries.

Around 500 different herb-based medicines have been identified in Thailand (Mueller-Oerlinghausen *et al*., 1971). They are used for the treatment of a variety of diseases such as cardiovascular failure, diabetes and cancer. In addition their diuretic, anti-inflammatory, anti-asthmatic and anti-hypertensive properties are exploited (Panthong *et al*., 1986). An increasing number of Thai medicinal plants are taken to laboratories for purification and analysis. Through this approach, a number of novel compounds have been identified. In this work, we

assessed the effects of the *P. acidus* extract on the turnover and processing in BHK overexpressing of F508del- and wt-CFTR.

While metabolic pulse-chase experiments revealed no effect of *P. acidus* extract on turnover and processing, data obtained during these studies (in Prof. Kunzelmann's laboratory) assessing function of the CFTR protein at cell membrane, in model systems such as epithelial cells and *Xenopus* oocytes, indicate that an extract from *P. acidus* activates electrolyte secretion in epithelial tissues by means of intracellular second messengers and also by directly increasing the membrane expression and activity of Ca^{2+}-activated Cl^- channels. Thus, the medicinal plant extract from *P. acidus* may represent a novel and effective tool to correct defective electrolyte transport in CF.

3. Results

When immunoprecipitation is used to investigate the biosynthesis of CFTR, two different forms of wild-type CFTR protein are visualised: an immature core-glycosylated form that is found in the ER (150 kDa; band B) and a mature fully-glycosylated form that has been processed through the Golgi apparatus and delivered to the cell membrane (170-180 kDa; band C) (Cheng *et al*., 1990). To evaluate the effects pre-treatment with *P. acidus* extract on the turnover rate of band B and on the efficiency of its processing into band C, we used pulse-chase analysis followed by CFTR immunoprecipitation.

In these metabolic pulse-chase experiments, we found that the turnover rate of the core-glycosylated form (band B) of either wt-CFTR or F508del-CFTR was not affected by 48 h pre-treatment with 50 µg/ml *P. acidus* (Figures III.2.1C and III.2.1D). Moreover, *P. acidus* did not

alter the efficiency of wt-CFTR maturation, i.e. the conversion of band B into band C when assessed by densitometry (Figure III.2.1E).

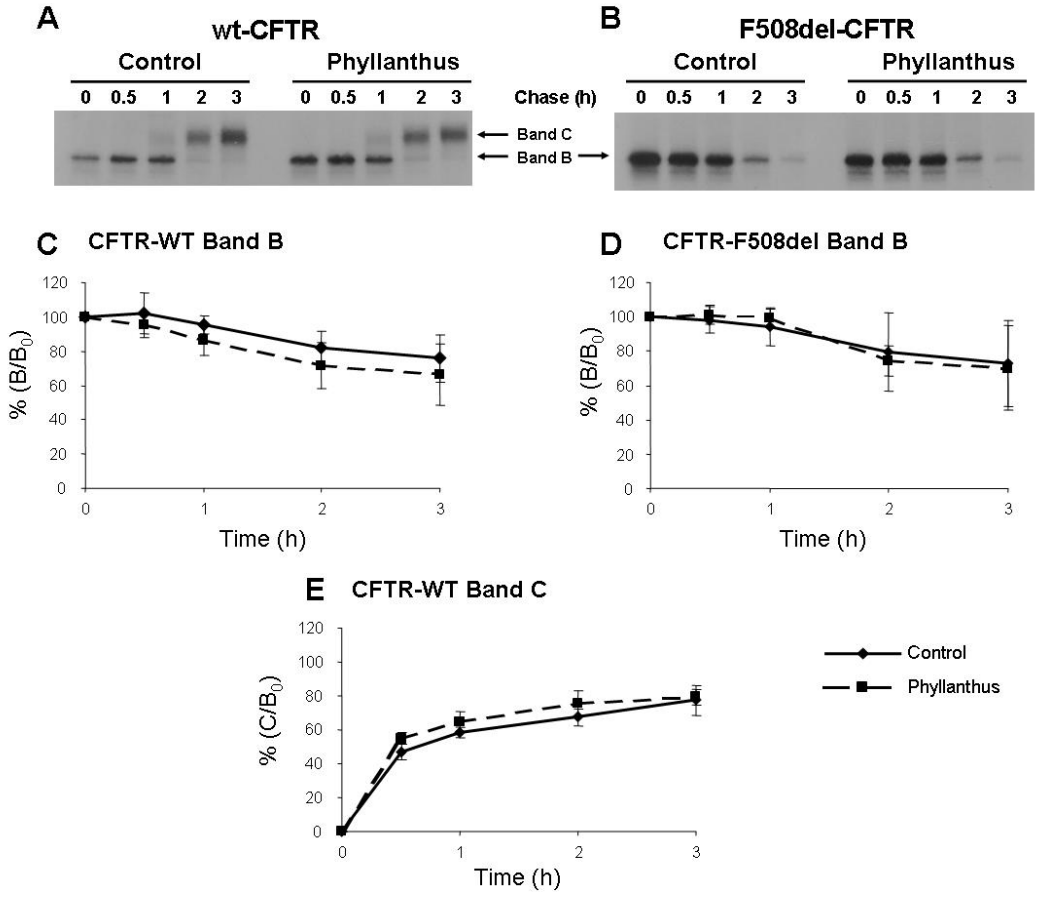

Figure III.2.1 - Turnover and processing of wt- and F508del-CFTR following pre-treatment with 50 μg/ml Phyllanthus for 48 h. BHK cells stably expressing (A) wt- or (B) F508del-CFTR were pre-treated with 50 μg/ml Phyllanthus or not treated. After 48 h, cells were pulse-labelled and chased for the times indicated in the figure. Cells were then lysed and immunoprecipitated with M3A7 anti-CFTR antibody. After electrophoresis and fluorography, images were analysed by densitometry. Disappearance of band B of wt-CFTR (C) and F508del-CFTR (D) are shown as a percentage of B, the amount of band B at time *t*, over B_o, the amount of band B at *t*=0. Similarly, appearance of band C for wt-CFTR (E) is shown as a percentage of C, the amount of band C at time *t*, over B_o, the amount of band B at the start of the experiment (n = 3 for each set of conditions).

4. Discussion and Conclusions

Experiments performed in the laboratory of Prof. Kunzelmann on cultured human epithelial cells endogenously expressing CFTR, pre-treated with *P. acidus* extract (or the purified components), show a significantly increased CFTR dependent Cl^- current compared to untreated controls. This observation was made for both wt- and low temperature cell surface rescued F508del-CFTR. Consistently, Ussing chamber experiments on tracheal biopsies from *cftr*$^{F508del/F508del}$ mice, showed *P. acidus* treatment also stimulated a Cl^- current. A transient Cl^- current was attributed to Ca^{2+} dependent Cl^- channels, while a sustained Cl^- current was shown to be CFTR dependent, as referred current could be blocked by a known CFTR inhibitor. Furthermore, whole cell patch-clamp experiments on tracheal biopsies from *cftr*$^{F508del/F508del}$ mice and on human epithelial cells from CF bronchial tissue, showed acute treatment with *P. acidus* extract (or a combination the purified components) also potentiate Cl^- currents. In *Xenopus* oocytes, expressing high levels of CFTR, results indicate that there is a direct potentiating effect of *P. acidus* extract on CFTR channel activity. Specifically, *P. acidus* extract, contains adenosine, an agonist that leads to receptor-mediated increase of intracellular cAMP and Ca^{2+} levels, leading to CFTR dependent Cl^- secretion, i.e. in normal, but not in CF tissue (Clancy *et al*., 1999). Increased Cl^- currents measured in both wt- and F508del-CFTR cDNA injected oocytes could be explained by an earlier observation by Lin *et al*. (2004), which showed that *P. acidus* extract components can increase CFTR Cl^- channel numbers at the plasma membrane.

However, our data on the processing of F508del-CFTR after prolonged pre-treatment with *P. acidus* extract did not evidence any effect on maturation of the mutant protein. A possible explanation for this

observation could be the type of cells used: BHK cells are fibroblasts, which display a notably different F508del-CFTR localisation when compared to epithelial cells (Penque *et al*., 2000).

Apart from adenosine, *P. acidus* extract also contains other components, which are likely to affect electrolyte transport in the airways, such as the flavonoid kaempferol and 2,3-dihydroxybenzoic acid (DHBA). (Li and Wang, 2004; Illek and Fischer, 1998). When comparing the effects of *P. acidus* with the effect of commercially purchased adenosine, kaempferol and DHBA and dissected out the underlying signalling pathways and the conductances affected, the data obtained by functional assays in this study, indicates that extracts from *P. acidus* activate electrolyte secretion in epithelial tissues by means of intracellular second messengers. Again, this could also be due to the previously described effects of the *P. acidus* extract components in promoting an intracellular pool of CFTR to the plasma membrane (Lim *et al*., 2004). This mechanism could also explain how our biochemical assays failed to detect a change in the levels of F508del-CFTR, as this observation would depend on the existing sub-apical CFTR, which would be promoted by the extract components to the cell surface. Thus pulse-chase experiments assessing the effect of the *P. acidus* extract on newly synthesised protein would not be able to detect differences in efficiency of processing of either F508del- or wt-CFTR.

All functional data for P. acidus treatment effects discussed in this chapter obtained by M. Sousa and J. Ousingsawat, in Prof. K. Kunzelmann's laboratory, Universität Regensburg, Deutschland.

Chapter 3

Investigation of the mechanism of action of several small molecule CFTR correctors in rescuing the trafficking defect of F508del-CFTR.

Schmidt A. *et al.* (manuscript in preparation)

1. Abstract

The focus of this work is to investigate mechanisms of action of new therapies aimed at treating the basic defect underlying the genetic disease Cystic Fibrosis (CF). CF is caused by loss of function of the cystic fibrosis transmembrane conductance regulator (CFTR) protein, a chloride (Cl^-) channel that regulates salt and water transport in epithelia. Most CF mutants cause disease either by preventing the cell surface expression of CFTR and/or by disrupting the function of CFTR as a Cl^- channel. Thus, the specific objective of the present work is to identify the mechanism of action of new compounds identified by high-throughput screening that restore the cell surface expression of CF mutants, namely of the most frequent one (F508del) which is mostly retained intracellularly at the endoplasmatic reticulum (ER). Here, we determine the effects of new compounds on CFTR biosynthesis, maturation and channel activity. The new compounds that we test are novel chemical/pharmacological chaperones: small molecule CFTR correctors that facilitate folding of the mutant protein, allowing it to proceed through the various checkpoints that constitute the ER quality control (ERQC) and cause retention of the mutants. Such compounds hence rescue the cell surface expression of CFTR mutants (F508del-

CFTR in particular), with possible effects on the activity of CFTR as Cl^- channels.

To investigate the effects of such compounds, namely VRT-325, VRT-532, VRT-640 and C4a, on the stability and processing efficiency of wild-type (wt) and mutant CFTR heterologously expressed in mammalian cells, a biochemical approach (metabolic pulse-chase followed by immunoprecipitation) was used. This approach helps to identify the mechanism of action of these rescuing agents and evaluate their therapeutic potential. The results of this research provide important insights into the molecular pharmacology of the CFTR Cl^- channel, and might also lead to the development and establishment of rationale new therapies for CF. Moreover, if different small molecule CFTR correctors are shown to act by distinct mechanisms of rescuing F508del-CFTR, they can putatively be used jointly as basis for therapeutic strategies, so as to maximise efficacy of rescuing F508del-CFTR.

2. Introduction

The major cause of the genetic disease Cystic Fibrosis (CF) is one single mutation, named F508del (a 3 bp deletion that causes the loss of the phenylalanine residue at position 508 of CFTR), which accounts for about 70% of CF chromosomes worldwide and is associated with a severe clinical phenotype (Collins, 1992). F508del disrupts the function of CFTR in three ways: firstly, F508del causes defects in protein folding that prevent the traffic of CFTR to its correct cellular location, the apical membrane of epithelial cells (Cheng *et al*., 1990). Secondly, F508del causes defects in channel regulation that impair greatly channel opening (Wang *et al*., 2000). Thirdly, once rescued to the cell surface the mutant channel exhibits reduced stability, characterised by

augmented internalisation and reduced recycling back to the cell membrane (Sharma *et al*., 2004). Knowledge of how F508del and other CF mutations cause a loss of Cl^- channel function is leading to new, more rational, approaches to therapy for CF patients aiming to correct such basic defects (Amaral and Kunzelmann, 2007). Gene transfer protocols aimed at overcoming defects in CFTR biosynthesis, came to a standstill due to major technical hurdles and difficulties. Thus, the currently most promising therapeutic strategies under investigation use pharmacological agents to augment the expression and function of the CFTR Cl^- channel.

Original studies by Denning and colleagues demonstrated that the F508del mutation causes a temperature-sensitive folding defect in the resulting CFTR protein (Denning *et al*., 1992a). This observation stimulated a search for drugs that correct the folding defect of F508del-CFTR, termed chemical chaperones when promoting protein folding unspecifically, or pharmacological chaperones, if acting in a substrate-specific manner. Indeed, original studies by Sato and colleagues showed that 10 % v/v glycerol rescued the cell surface expression of F508del in recombinant HEK cells (Sato *et al*., 1996). However, at these concentrations glycerol is highly toxic precluding its use in CF patients.

Recently, less toxic and more specific agents have been identified from high-throughput screening (HTS) efforts so as to achieve this goal. These approaches focus on the identification of small molecule CFTR correctors, which can be grouped into two classes, according to their effect on mutant CFTR. Firstly, the so-called ‘corrector’ compounds (Van Goor *et al*., 2006), which aim to overcome the trafficking defect of mutant CFTR. These include: i) VRT-325, a quinazolinium derivative (Van Goor *et al*., 2006), ii) VRT-640, chemically engineered from VRT-

325 (Presentation by Vertex at Williamsburg conference 2004); and iii) C4a, a benzamide derivative (Pedemonte *et al*., 2005a). The first two corrector compounds mentioned here were developed by Vertex Pharmaceuticals (San Diego, USA) and the third was developed by A. Verkman (UCSF, San Francisco, USA). Secondly, the so-called 'potentiator' compounds (Wang *et al*., 2006b), aiming to enable to enhance Cl^- channel function of protein which is already at the cell surface. One of these compounds, VRT-532, despite being identified as a potentiator of apically located F508del-CFTR Cl^- channel function, was later found to also rescue the trafficking defect of F508del-CFTR, albeit at higher concentration than for its potentiator effect (Wang *et al*., 2006b).

The biosynthesis and maturation of CFTR is a complex and inefficient process, even for its wild-type (wt) form. Correctly folded CFTR traffics through the Golgi, where it undergoes full glycosylation, until it reaches the plasma membrane. In contrast, misfolded, core-glycosylated CFTR (namely F508del-CFTR) is retained in the endoplasmic reticulum (ER) and targeted for ER-associated degradation (ERAD) at the proteasome (Chang *et al*., 1999; DeCarvalho *et al*., 2002a; Farinha *et al*., 2002). In heterologous systems over-expressing F508del-CFTR, most of the protein is recognised as misfolded at various checkpoints of the ERQC which cause its retention in the ER and degradation through the ERAD pathway. Moreover, in these systems, a substantial portion (up to ~75% in some cell types) of wt-CFTR is also retained by the ERQC and subject to ERAD, suggesting that expression of CFTR in non-epithelial cells is an inefficient process.

Indeed, we have recently investigated (Roxo-Rosa *et al*., 2006) the mechanism of action of two different types of genetic revertants (second site mutations) previously demonstrated to rescue the

trafficking defect of F508del-CFTR, namely: G550E (DeCarvalho *et al*., 2002a) and 4RK, the simultaneous mutation of four arginine-framed tripeptides (AFTs), namely R29K, R516K, R555K and R766K (Chang *et al*., 1999). These so-called revertants enable the mutant CFTR protein to be trafficked to the cell membrane. Our data indicated that distinct mechanisms take place when each of these revertants rescues trafficking of F508del-CFTR (Roxo-Rosa *et al*., 2006): whereas G550E appears to exert its effect on the folding of F508del-CFTR, 4RK seems to enable the mutant to escape retention/retrieval ("bypassing") at the endoplasmatic reticulum (ER) quality control (ERQC), suggesting thus a third (AFT-mediated) checkpoint for assessment of secretory protein folding in addition to the two previously described (Farinha and Amaral, 2005).

In the present studies, we investigated whether the above-mentioned small molecule CFTR correctors have an additive effect with that of the revertant mutations G550E and 4RK on the rescue of cell surface expression of F508del-CFTR. Therefore, the studies performed here were aimed at gaining insight into their mechanism of rescue by assessment of their effects on genetic revertants of F508del-CFTR. Importantly, these compounds have not passed clinical safety tests, they are only used as "tool-compounds" to investigate mechanisms of CFTR biosynthesis.

3. Results

In the search for the mechanism of action of these novel compounds, in rescuing the cell surface expression of F508del-CFTR, their effects were assessed at the level of turnover and processing of F508del-CFTR alone or of F508del-CFTR having in *cis* each of the two genetic

revertants, namely F508del-4RK-CFTR and F508del-G550E-CFTR by biochemical experiments.

To study the effects of VRT-325, VRT-532, VRT-640 and C4a on steady-state levels of CFTR, biochemical approaches, namely, Western blotting was used. To assess turnover rate and processing efficiency *in vivo* radioactive labelling (commonly termed as "pulse-chase" experiments), followed by CFTR immunoprecipitation (IP) and SDS-PAGE were used (see also II.Methods).

These biochemical analyses evidence the occurrence of two different forms for wt-CFTR protein: an immature core-glycosylated form that is localised to the ER (150 kDa; band B) and a fully glycosylated form that has been processed (or matured) through the Golgi apparatus and assumed to have been delivered to the cell membrane (170-180 kDa; band C).

3.1 Steady-state analysis to determine concentrations and pre-treatment times to be used in processing studies.

The analysis of the effects of the compounds on steady-state levels of the different CFTR variants was intended to determine the concentration of compound and/or pre-treatment time to be used for the pulse-chase experiments. The main finding of the steady-state analysis however was that all compounds showed a degree of toxicity in the cellular system used here (BHK cells), in the following order VRT-325 < VRT-532 < VRT-640 < C4a.

Western blot analysis (Figure III.3.1a) of cells pre-treated with 2 μM VRT-325 for long periods of time (8h, 16h, 24h or 96h), does not evidence any significant change in the steady-state levels of wt-CFTR. However, a significant stabilisation effect is observed on the steady-state levels of F508del-CFTR after 8h of pre-treatment with VRT-325. Under this low sensitivity analysis, no maturation of F508del-CFTR could be detected. We also determined the effect of this compound on F508del-CFTR when in *cis* with the revertant mutations G550E and 4RK. Steady-state data for these F508del-CFTR variants are suggestive that the effect of VRT-325 is additive to that of either 4RK or G550E, again after 8h of pre-treatment. Interestingly, this increased stabilisation appears to be more striking for 4RK-F508del-CFTR than for F508del-G550E-CFTR. While Van Goor and colleagues determined an EC_{50} value of ~0.8 μM, for this compound in the efflux assays, they used a higher concentration (6.7 μM) to maximise its effect in pulse-chase experiments (Van Goor *et al*., 2006), which was thus the concentration we chose for further experiments.

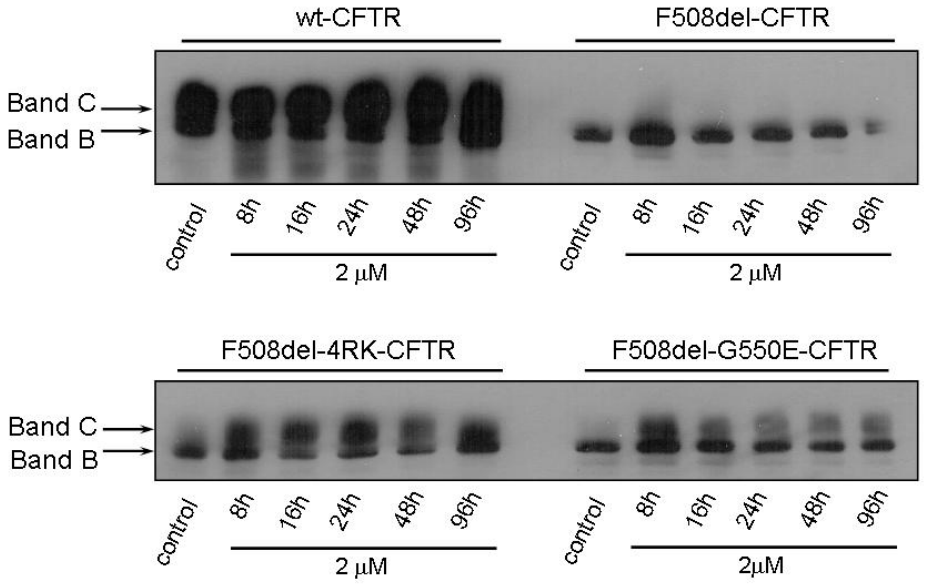

Figure III.3.1a - Steady-state levels of wt-, F508del-, F508del-4RK and F508del-G550E-CFTR after pre-treatment with VRT-325. BHK cells stably expressing wt- F508del-, F508del-4RK or F508del-G550E-CFTR were pre-treated with 2 μM VRT-325 (for 8h, 16h, 24h or 96h) or treated with vehicle

(DMSO) (as indicated). Cells were then lysed and CFTR-protein detected by Western Blot (as described in II.Methods).

Western blot analysis (Figure III.3.1b) of cells treated for brief periods of time (1 h or 2 h) with VRT-532 (4 μM) shows no significant effect on steady-state levels of CFTR variants. VRT-532 was initially described as a potentiator of temperature rescued F508del-CFTR (Van Goor *et al*., 2006), but was later shown to rescue F508del-CFTR after long pre-treatment (48 h) at higher concentrations (20 μM) (Wang *et al*., 2006b), which was thus the concentration chosen for the pulse-chase experiments.

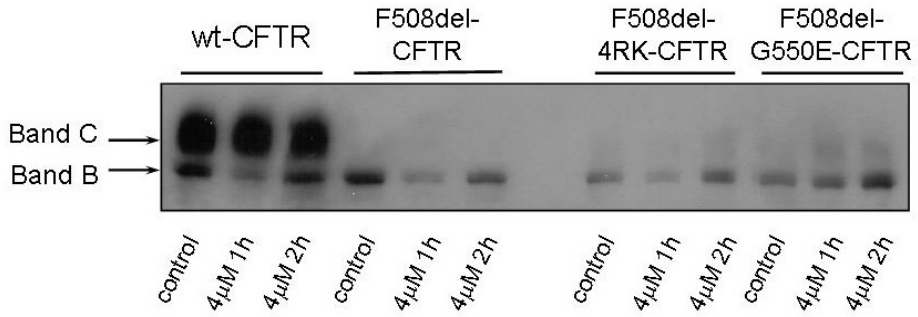

Figure III.3.1b - **Steady-state levels of wt-, F508del-, F508del-4RK and F508del-G550E-CFTR after pre-treatment with VRT-532.** BHK cells stably expressing wt-, F508del-, F508del-4RK or F508del-G550E-CFTR were pre-treated with 4 μM VRT-532 (for 1h or 2h) or treated with vehicle (DMSO) (as indicated). Cells were then lysed and CFTR-protein detected by Western Blot (as described in II.Methods).

Western blot analysis (Figure III.3.1c) of wt- and F508del-CFTR after 24h pre-treatment with VRT-640 at different concentrations shows that this compound has a concentration dependent cytotoxic effect on the cells. While steady-state levels of fully-glycosylated band C wt-CFTR remain unaffected, immature band B levels decrease in a concentration dependent manner. Several concentrations (ranging from 2 to 10 μM) and pre-treatment times (ranging from 6 to 24 h) of VRT-640 were assessed for cell viability (data not shown). This

compound showed notable cytotoxic effects, slowing down cell proliferation and causing some cell death, as assessed by microscopy observations, in a dose dependent manner (data not shown). Despite this toxicity, treatment of cells with VRT-640 (6 μM to 10 μM) for 24 h rescued F508del-CFTR processing (See figure III.3.1c, right panel). Therefore the lowest possible concentration of VRT-640 where rescue of F508del-CFTR was observed was used for the pulse chase experiments.

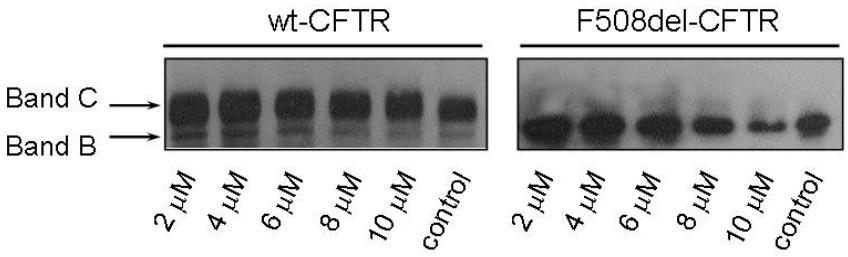

Figure III.3.1c - **Steady-state levels of wt- and F508del-CFTR after pre-treatment with VRT-640.** BHK cells stably expressing wt- or F508del-CFTR were pre-treated with VRT-640 at indicated concentrations (with 2, 4, 6, 8, 10 μM) or treated with vehicle (DMSO) (as indicated) for 24 h. Cells were then lysed and CFTR-protein detected by Western Blot (as described in II.Methods).

The C4a molecule showed very high toxicity in BHK cells, and it was not possible to pre-treat the BHK cells without significant (up to ~50%) cell death (data not shown). Given the toxicity of C4a compound in these studies, we only added the compound to the media during the pulse-chase experiments, as described by Pedemonte and colleagues (Pedemonte *et al*., 2005a).

3.2 Pre-treatment with VRT-325 rescues F508del-, increases processing efficiency of F508del-4RK- but not of F508del-G550E-CFTR.

To evaluate the effects of VRT-325 pre-treatment on the rate of turnover of band B and on the efficiency of its processing into band C, we used pulse-chase analysis prior to immunoprecipitating CFTR protein using the anti-CFTR mouse monoclonal antibody M3A7 (Chemicon), which recognises NBD2 and the C-terminus of CFTR (see II. Methods). Figure III.3.2A, C and D shows treatment of the three CFTR variants with compound vehicle DMSO (0.067 % v/v), while III.3.2B - D shows effect of VRT-325 (6.7 μM) added to the medium 24 h prior to pulse- chase experiments. Data in Figure III.3.2B, lanes 1-5, clearly evidences the appearance of the processed form of F508del-CFTR (band C), in contrast to the vehicle-treated cells (Figure III.3.2A, lanes 1-5), for which only the immature form (band B) is detected (see legend of Figure III.3.2 for details) as previously reported in the literature for F508del-CFTR expressed in BHK cells. These data indicate that in VRT-325 pre-treated cells, F508del-CFTR protein has exited the endoplasmic reticulum (ER), and undergone full processing through the Golgi, plausibly reaching the cell surface, thus confirming previous data (Loo *et al*., 2005; Van Goor *et al*., 2006). Our data also show that F508del-G550E-CFTR processing remains unaffected by VRT-325 (in Figure III.3.2, compare panel A, lanes 11-15 and panel B, lanes 11-15). However, F508del-4RK-CFTR is significantly more efficiently processed ($p < 0.05$) after pre-treatment with VRT-325 (in Figure III.3.2, compare panel A, lanes 6-10 and panel B, lanes 6-10). The 4RK genetic revertant and VRT-325 thus seem to have an additive effect in rescuing F508del-CFTR. This possibly indicates that VRT-325 is able to further enhance exit of F508del-4RK-CFTR from

the ER, allowing the protein to be more efficiently trafficked to the plasma membrane. Notably, the VRT-325 does not affect the turnover rate of neither F508del-CFTR, nor F508del-CFTR genetic revertants.

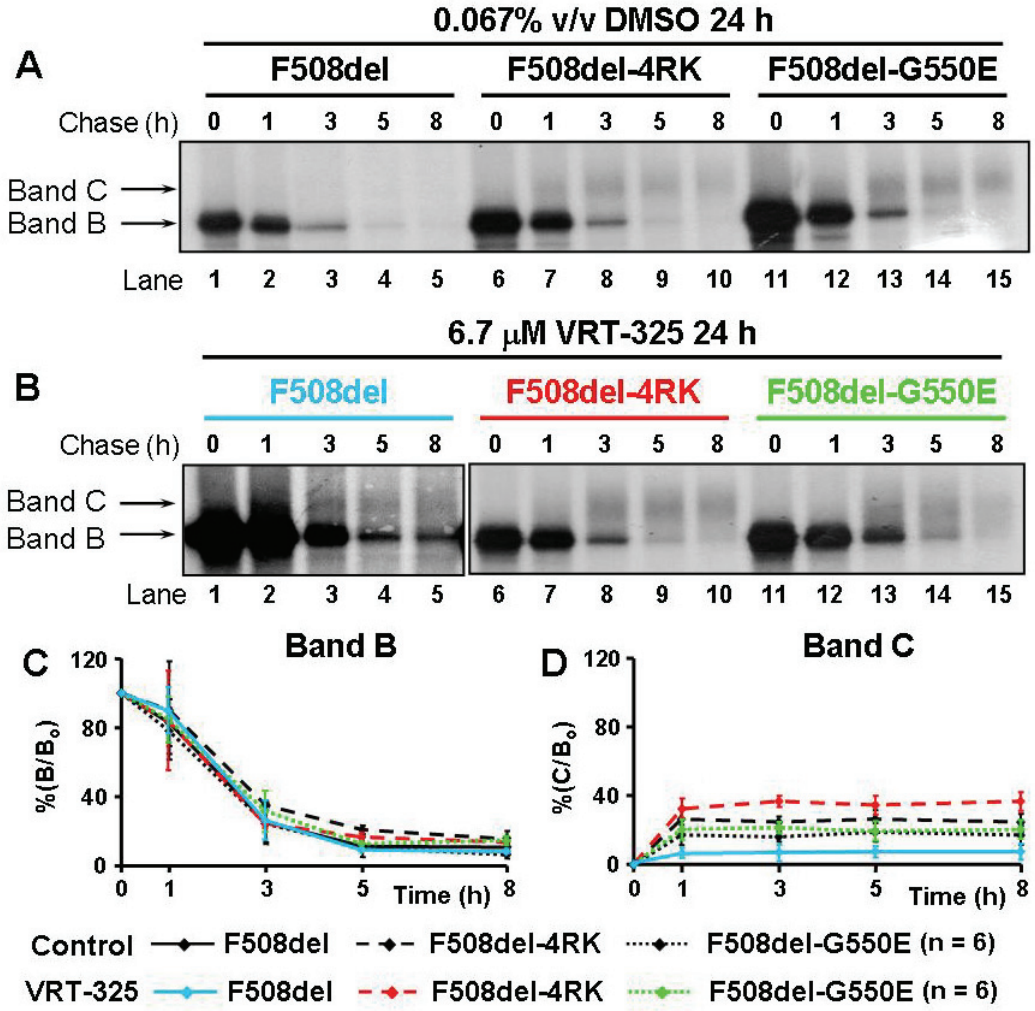

Figure III.3.2 - **Processing efficiency and turnover rate of F508del-, F508del-4RK- and F508del-G550E-CFTR, after pre-treatment for 24 h with (A, C and D) DMSO (vehicle) or (B-D) VRT-325 (6.7 μM)**, was assessed by pulse-chase followed by CFTR immunoprecipitation. Cells were radiolabelled with [^{35}S]methionine for 20 min, then chased at indicated times (0, 1, 3, 5, 8 h) before lysis with RIPA-buffer. Immunoprecipitation (IP) was performed with the anti-CFTR antibody M3A7 (Chemicon, see II.Methods). After electrophoresis and fluorography, images were analysed by densitometry. No significant difference is observed in the turnover rate among the three CFTR variants after VRT-325 treatment (in panel C, compare black and coloured lines). F508del-CFTR (lanes 1-5, in panels A and B) is processed to mature band C albeit at low levels (~6.0%) and F508del-G550E (lanes 11-15, in panels A and B) maturation seems to be unaffected after treatment with VRT-325. However, F508del-4RK-CFTR

(lanes 6-10, in panels A and B) is significantly more efficiently processed after compound pre-treatment than after pre-treatment with the vehicle, DMSO (see panel D black (control) and red (compound) dashed lines, $p < 0.05$). In panel C, data presented as the percentage of band B at a given time of the chase (B) relative to the amount at the beginning of the experiment (B_o). Panel D shows appearance of band C presented as the percentage of band C detected at a given time of the chase (C) relative to the amount at the beginning of the experiment (B_o). Continuous, dashed and dotted lines represent control, black; and VRT-325, blue, red and green lines, from F508del-, F508del-4RK and F508del-G550E-CFTR expressing cells, respectively.

3.3 Pre-treatment with VRT-532 rescues F508del-CFTR, but has no effect on the processing of the genetic revertants.

Although VRT-532 compound was developed as a potentiator (i.e., a compound rescuing the gating defects of mutant channels), it was later described to also act as a corrector of F508del-CFTR (Wang *et al*., 2006b). Here, our analysis by pulse-chase confirms rescue of F508del-CFTR protein to ~6.5% of wt-CFTR, whereas processing of revertant variants of F508del-CFTR remains unaltered. Figure III.3.3A, C and D shows treatment of the three CFTR variants with compound vehicle DMSO (0.2 % v/v), while III.3.3B - D shows effect of VRT-532 (20 μM) when added to the medium 48 h prior to pulse-chase experiments. Data in Figure III.3.3B, lanes 1-5, clearly evidences the appearance of the processed form of F508del-CFTR (band C), in contrast to the vehicle-treated cells (Figure III.3.3A, lanes 1-5), for which only the immature form (band B) is detected (see legend of Figure III.3.3 for details). The present data indicate that in VRT-532 pre-treated cells, F508del-CFTR protein has exited the endoplasmic reticulum (ER), and undergone full processing through the Golgi, plausibly reaching the cell surface, also confirming previous data

(Wang *et al*., 2006b). However, the efficiency of processing of both F508del-4RK- (in Figure III.3.3, compare panel A, lanes 6-10 and panel B, lanes 6-10) and F508del-G550E-CFTR (in Figure III.3.3, compare panel A, lanes 11-15 and panel B, lanes 11-15) remains unaffected by VRT-532. Indeed, this compound does not seem to affect the turnover and processing of either F508del-CFTR genetic revertant (figure III.3.2D).

Moreover, VRT-532 compound had a notable toxic effect in the three BHK cell lines studied here. VRT-532 compound essentially arrests cell proliferation, affecting cell morphology and adhesion, with many cells displaying a dome-like shape (data not shown).

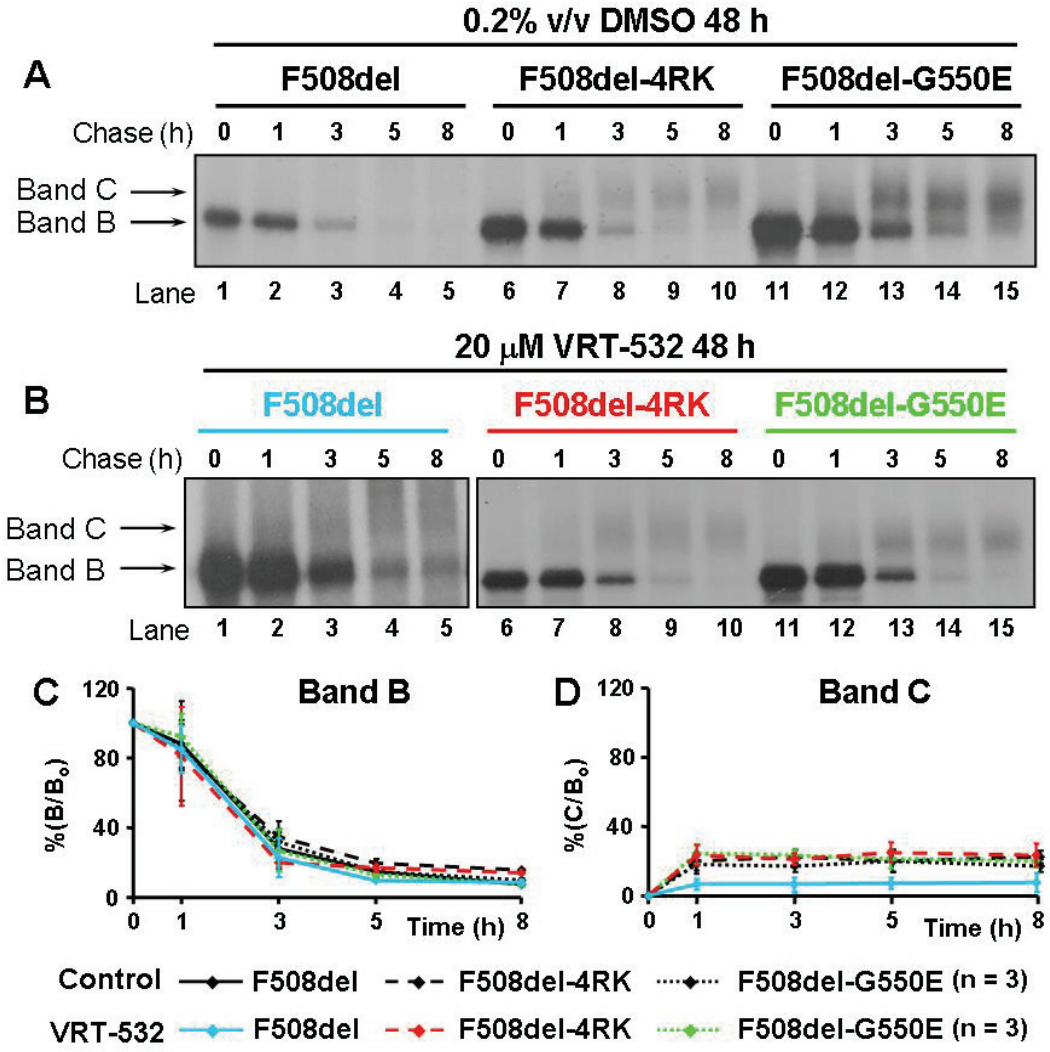

Figure III.3.3 - Processing efficiency and turnover rate of F508del-, F508del-4RK- and F508del-G550E-CFTR after 48 h pre-treatment with (A, C and D) DMSO (vehicle) or (B-D) VRT-532 (20 μM) was determined by pulse-chase followed by IP. F508del-CFTR is processed to its fully mature band C form (panel B, lanes 1-5) after 48h pre-treatment with VRT-532 (20 μM), when compared to vehicle (DMSO 0.2% v/v) control (panel A, lanes 1-5), albeit at low levels (~6.5%). No increase in turnover rate of band B was observed for neither F508del-4RK- (panel B, lanes 6-10 and panel C) nor F508del-G550E-CFTR (panel B, lanes 11-15 and panel C) after pre-treatment with VRT-532, compared to the vehicle control experiments (panel A, lanes 6-10 and lanes 11-15, respectively, and panel C) (Other details as in Figure III.3.2).

3.4 VRT-640 stabilises immature F508del-CFTR and rescues F508del-CFTR, but has no effect on the genetic revertants.

Pulse-chase data in Figure 3.4B, lanes 1-5, show that VRT-640 (6 μM) was less effective (~4.5 %) at rescuing F508del-CFTR compared to ~ 6.0 % of VRT-325, both relative to wt-CFTR levels. However, VRT-640 was able to markedly stabilise immature band B after pre-treatment (Figure III.3.4B, lanes 1-5), when compared to vehicle (DMSO 0.06 %v/v) control experiments (Figure III.3.4A, lanes 1-5): the amount of immature CFTR protein (band B) is increased nearly two-fold ($p < 0.05$) after 5 h of chase when F508del-CFTR expressing cells pre-treated with vehicle (DMSO 0.06 % v/v) (Figure III.3.4A, lane 4, and panel C) are compared with cells pre-treated with VRT-640 (6 μM) (Figure III.3.4B, lane 4, and panel C). Turnover of band B and maturation to fully-glycosylated mature form (band C) remained unaltered after pre-treatment with VRT-640 for F508del-4RK- (Figure III.3.4B, lanes 6-10 compared to A, lanes 6-10, and panel D) or F508del-G550E-CFTR (Figure III.3.4B, lanes 11-15 compared to A, lanes 11-15, and panel D) expressing cells.

Furthermore, VRT-640 showed notable cytotoxic effects on BHK cells, slowing down cell proliferation and causing some cell death (data not shown).

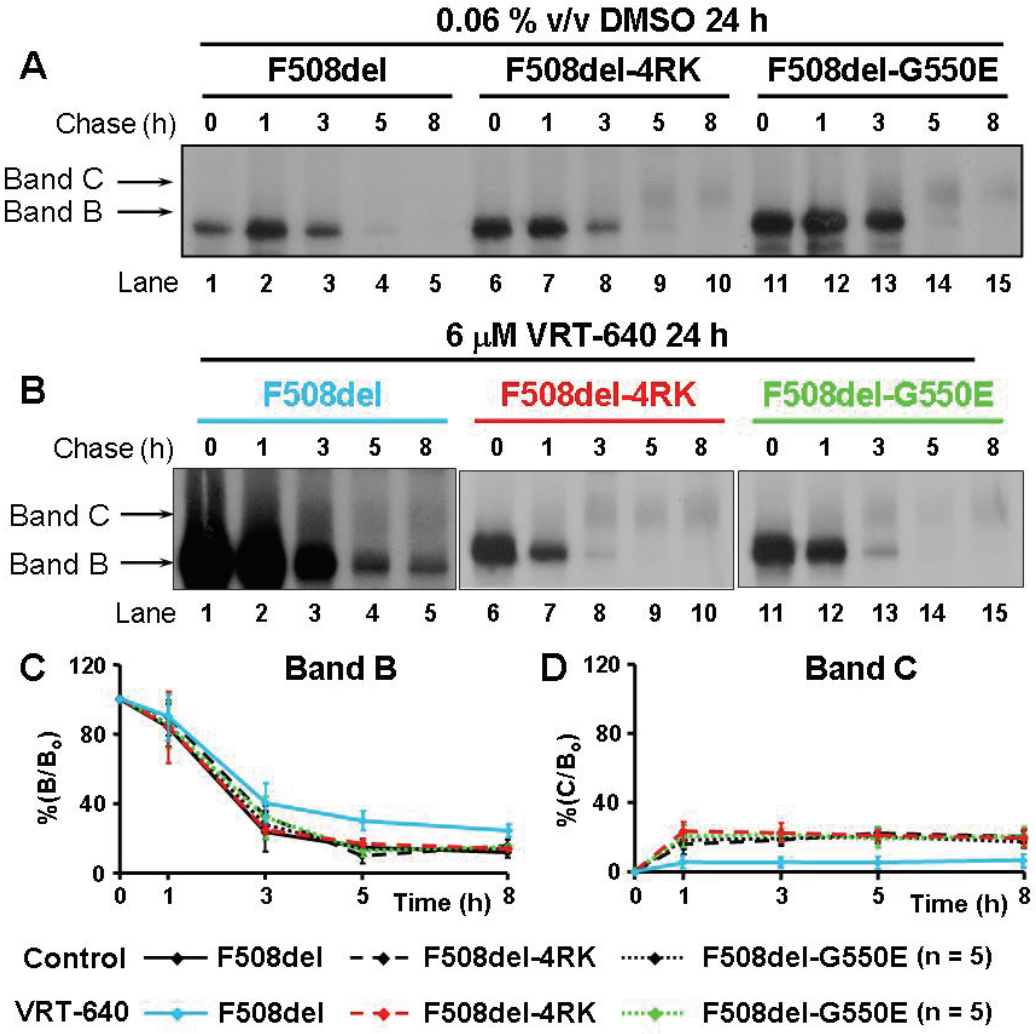

Figure III.3.4 - Processing efficiency and turnover rate of F508del-, F508del-G550E- and F508del-4RK-CFTR, after pre-treatment for 24 h with (A, C and D) DMSO (vehicle, 0.06 % v/v) or (D-F) VRT-640 (6 μM) assessed by pulse-chase followed by CFTR immunoprecipitation. The immature protein (band B) of F508del-CFTR is significantly stabilised after pre-treatment with VRT-640 (D, lanes 1-5) when compared to the turnover rate of the other CFTR variants (4RK (D, lanes 6-10), p = 0.03; and G550E (D, lanes 11-15) p = 0.04) or to control F508del-CFTR (A, lanes 1-5) (p = 0.05). There is also a low level correction of F508del-CFTR (D, lanes 1-5) (~4.5% compared to wt-CFTR) to its fully-glycosylated form, while the processing efficiency of F508del-4RK- (panel B, lanes 6-10) and F508del-G550E-CFTR (panel B, lanes 11-15) when compared to control (panel A, lanes 6-10 and lanes 11-15, respectively) remains unaffected (Other details as in Figure III.3.2).

3.5 C4a rescues F508del-CFTR, but has no effect on the genetic revertants.

C4a was identified by high throughput screening in Alan Verkman's laboratory (University of California, San Francisco, USA), and was described to rescue F508del-CFTR in human bronchial epithelial cells (Pedemonte *et al*., 2005a). This small molecule CFTR corrector showed very high toxicity in BHK cells, and it was not possible to pre-treat the BHK cells without significant (up to ~50%) cell death (data not shown). We therefore only added the compound to the media during the pulse-chase experiments, as described by Pedemonte and colleagues (Pedemonte *et al*., 2005a). Furthermore, this high toxicity made rigorous comparative experiments difficult.

Figure III.3.5 shows pulse-chase analysis in BHK cells stably expressing F508del-, F508del-4RK- and F508del-G550E-CFTR. Acute treatment (i.e. no pre-treatment, but instead addition of the compound during starvation, pulse and chase) with C4a compound (10 μM) has no effect on the turnover rate of band B, but rescues F508del-CFTR protein (Figure III.3.5B, lanes 1-5), albeit at very low levels (2% of wt-CFTR) when compared to vehicle (DMSO 0.1 % v/v) control (Figure III.3.5A, lanes 1-5). Furthermore, when acutely treated with C4a, the turnover of band B and efficiency of processing into band C of F508del-4RK- (Figure III.3.5B, lanes 6-10) and F508del-G550E-CFTR. (Figure III.3.5.B, lanes 11-15) were unaffected compared to vehicle controls of each cell line (Figure III.3.5A, lanes 6-10 and lanes 11-15, respectively).

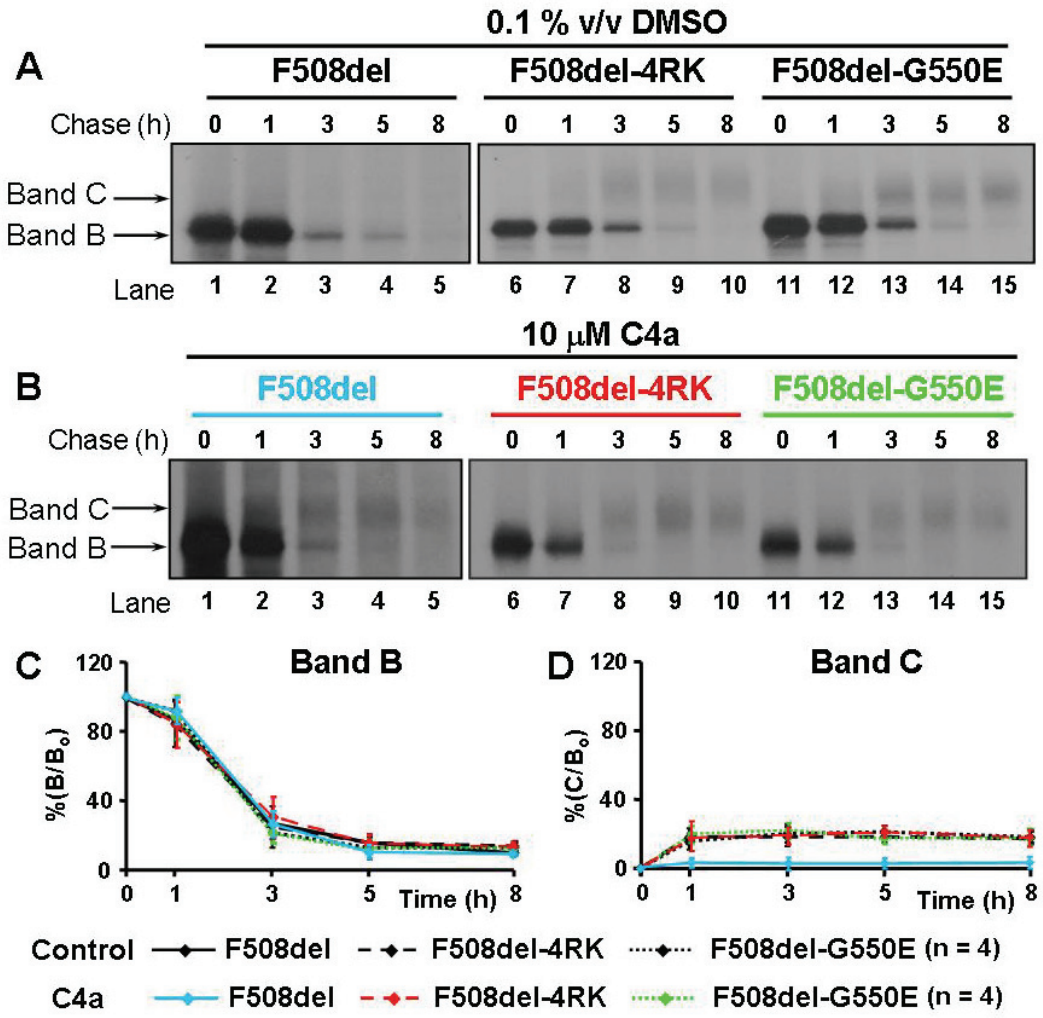

Figure III.3.5 - Processing efficiency and turnover rate of F508del-, F508del-4RK- and F508del-G550E-CFTR, under (A, C and D) DMSO (vehicle, 0.1 % v/v) or (B-D) C4a (10 μM) acute treatment. There is a low level correction of F508del-CFTR (~2%) to its fully-glycosylated form (B, lanes 1-5, and panel D), when compared to vehicle treatment (panel A, lanes 1-5), while the turnover rate of band B of F508del-CFTR remains unaffected (panel C). The turnover rate of band B and maturation efficiency to band C under C4a acute treatment in F508del-4RK- (panel B, lanes 6-10, panels C and D), or F508del-G550E-CFTR (panel B, lanes 11-15, panels C and D) expressing cells is unaffected, when compared to controls (panel A, lanes 6-10, panels C and D; and panel A, lanes 11-15, panels C and D, respectively) (Other details as in Figure III.3.2).

3.6 Quantitave analysis for the effects of compounds on rescuing F508del-CFTR and on F508del-CFTR genetic revertants.

The small molecule CFTR correctors under study all show a low level of rescue of F508del-CFTR to the fully-glycosylated form. The rescuing effects for band C were respectively VRT-325, ~6 %; VRT-532, ~6.5%; VRT-640, ~4.5 %; and C4a, ~2 % (percentage compared to wt-CFTR maturation, see Figure III.3.6a). These data indicate that in small molecule CFTR corrector treated cells, a small amount of F508del-CFTR protein has exited the endoplasmic reticulum (ER), and undergone full processing through the Golgi, plausibly reaching the cell surface. The results confirm previous data demonstrating that the small molecule CFTR correctors can rescue the F508del-CFTR mutant from its intracellular (ER) localisation to post-Golgi compartments (Loo *et al*., 2005; Van Goor *et al*., 2006; Pedemonte *et al*., 2005a).

Of note, VRT-640 markedly stabilised immature band B after pre-treatment, when compared to vehicle (DMSO 0.06 %v/v) control experiments (Figure III.3.4A and B). The amount of immature protein (band B) after 5 h of chase elevated nearly two-fold ($p < 0.05$) with VRT-640 (6 μM) pre-treatment when in compared to pre-treatment with vehicle (DMSO 0.06 % v/v).

In the case of F508del-G550E-CFTR, all the small molecule CFTR correctors neither effected the turnover of band B, nor the efficiency of maturation to fully-glycosylated form band C (see Figure III.3.6b).

Results of pulse chase experiments with F508del-4RK-CFTR expressing cells show a differential effect of VRT-325. This compound is able to increase amounts of fully-glycosylated form band C of F508del-4RK-CFTR after 3 h of chase by ~1.4-fold (see Figure III.3.6c), when compared to vehicle control experiments ($p < 0.05$).

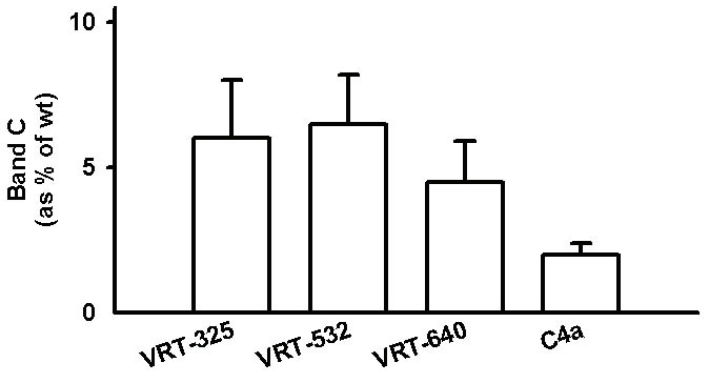

Figure III.3.6a - Amount of rescued F508del-CFTR (Band C) determined by pulse chase experiments (after 3 h chase, shown as % of wt) after treatment with **VRT-325** (~6 %), **VRT-532** (~6.5 %), **VRT-640** (~4.5 %) and **C4a** (~2 %). Data are means ± SEM after 3 h chase of n experiments (VRT-325, n = 6; VRT-532, n = 3; VRT-640, n = 5; C4a, n =4).

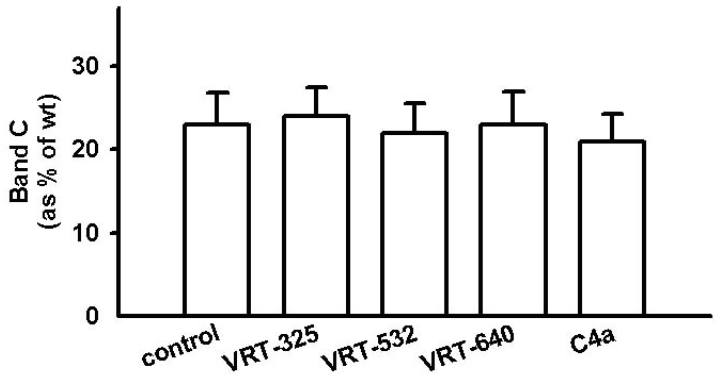

Figure III.3.6b - Amount of F508del-G550E-CFTR (Band C) determined by pulse chase experiments (after 3 h chase, shown as % of wt) of **vehicle control (DMSO 0.2 % v/v)** (~23 %), **VRT-325** (~24 %), **VRT-532** (~22 %), **VRT-640** (~23 %) and **C4a** (~21 %). Data are means ± SEM after 3 h chase of n experiments (control, n = 3, VRT-325, n = 6; VRT-532, n = 3; VRT-640, n = 5; C4a, n =4).

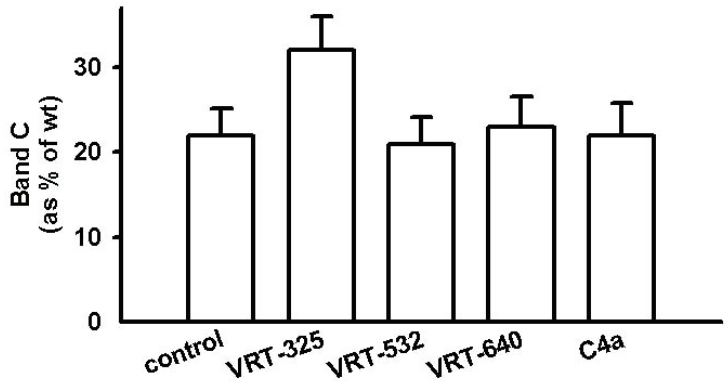

Figure III.3.6c - Amount of F508del-4RK-CFTR (Band C) determined by pulse chase experiments (after 3 h chase, shown as % of wt) of **vehicle control (DMSO 0.2 % v/v)** (~22 %), **VRT-325** (~32 %), **VRT-532** (~21 %), **VRT-640** (~23 %) and **C4a** (~22 %). Data are means ± SEM after 3 h chase of n experiments (control, n = 3, VRT-325, n = 6; VRT-532, n = 3; VRT-640, n = 5; C4a, n =4).

4. Discussion and Conclusions

The results show that F508del-CFTR is processed to its fully-glycosylated form, albeit at low levels, after pre-treatment with: VRT-325 (~6.0%), VRT-532 (~6.5%), VRT-640 (~4.5%) and C4a (~2.0%). VRT-325 was able to rescue F508del-CFTR. Moreover, the results indicate that VRT-325 and the 4RK revertant mutation act synergistically to rescue F508del-CFTR, whereas the effect caused by VRT-325 and G550E revertant are not additive, so they plausibly act at the same checkpoint of the ERQC. Consistent with this putative effect on channel gating (Roxo-Rosa *et al*., 2006), VRT-325 has no p differential effect on the genetic revertants assessed here for trafficking and maturation efficiency. VRT-640 was chemically engineered based on the structure of another corrector compound, VRT-325. In our laboratory however, VRT-640 showed less correction of F508del-CFTR than VRT-325. Interestingly, VRT-640 was able to markedly stabilise the immature form (band B) of the protein. C4a was also able to rescue F508del-CFTR, but showed no additive rescuing with the genetic revertants.

No differential effects were observed for any of the compounds with F508del-G550E-CFTR. This is perhaps not surprising, as data obtained in our laboratory (Roxo-Rosa *et al*., 2006), suggest that this revertant probably corrects the folding of F508del-CFTR. Since these small molecule CFTR correctors were identified as hits by screening for a similar effect on F508del-CFTR, they would thus be expected not to further affect this F508del-CFTR genetic revertant. However, this interpretation remains speculative, as the possible effect of these small molecule CFTR correctors might be masked by their varying degree of toxicity.

The additive effects of VRT-325 and the genetic revertant mutation 4RK suggest that they promote the maturation of F508del-CFTR by different mechanisms. Data in our lab (Roxo-Rosa *et al*., 2006) indicates that the 4RK revertant mutation putatively enables mutant protein to escape ER retention, it seems plausible that VRT-325 acts at a different cellular quality control checkpoint.

Finally, it has to be noted again that all compounds tested showed a degree of cytotoxicity in BHK cells, making rigorous comparisons of quantitative results difficult. In any case, these compounds might be good “lead-compounds“ for further chemical engineering in the search for suitable small molecule CFTR correctors that rescue F508del-CFTR without any untoward effects. Moreover, they can still be useful to identify cellular mechanism in other cells, such as human bronchial epithelial cells (Pedemonte *et al*., 2005a; Van Goor *et al*., 2006) where they have been described to be less toxic.

The major goal of this study however, was to gain insight on how small molecule CFTR correctors, known to rescue the trafficking defect of F508del-CFTR (Loo *et al*., 2005; Van Goor *et al*., 2006; Pedemonte *et al*., 2005a), affect genetic revertants of F508del-CFTR by assessing their impact on the turnover rate of the immature protein (band B) and processing efficiency into its full glycosylated mature band C form. The two genetic revertants under study here, putatively rescue F508del-CFTR by different mechanisms (Roxo-Rosa *et al*., 2006): G550E seems to promote correct folding, and allow the protein to pass through the cellular quality control mechanisms, while 4RK, on the other hand, does not appear to correct folding, but rather to allow misfolded protein to escape the ERQC by a “bypass” mechanism. Therefore, if small molecule CFTR correctors evidence different impacts on the processing of the two genetic revertants, this could be

interpreted as a rescuing mechanism and thus it would be possible to determine whether these compounds rescue plasma membrane expression of F508del-CFTR at the level of folding correction or by interfering with the cellular quality control machinery. At least one of the compounds studied here showed such an effect. Indeed, VRT-325 seemed to have an additive effect with F508del-4RK-CFTR, while the putatively correctly folded F508del-G550E-CFTR remained unaffected by pre-treatment with this compound. It thus seems reasonable to conclude that the VRT-325 compound acts at the same level as G550E, i.e. folding, and not by enabling the protein to escape the cellular quality control mechanisms. It was previously shown that VRT-325 is able to restore CFTR-dependent Cl^- secretion (Van Goor *et al*., 2006) by promoting maturation of immature form band B of F508del-CFTR to fully-glycosylated mature form band C in human bronchial epithelial cells. However it was also shown that the VRT-325 does not specifically rescue F508del-CFTR, because this compound also rescues misprocessed P-glycoprotein (P-gp) (Loo *et al*., 2005). Since P-gp is also an ABC transporter, it remains to be demonstrated whether similar rescue can also be exerted by VRT-325 on misfolded non-ABC-transporters. The same group also concluded that VRT-325 interferes with domain assembly of the respective proteins, namely the association between MSD1 and NBD1 (Loo *et al*., 2005). This would possibly give an explanation for the findings here, namely why F508del-G550E-CFTR maturation remains unaffected, and maturation of F508del-4RK-CFTR is enhanced.

Maturation of F508del-G550E-CFTR is not further enhanced by VRT-532, VRT-640 or C4a, indicating that these small molecule CFTR correctors compounds putatively act at the same level as the G550E revertant mutation, *i.e.* folding. However, since F508del-4RK-CFTR processing is equally unaffected by pre-treatment with VRT-532 or

VRT-640, and also by acute treatment with C4a, so far no further conclusion can be drawn regarding the rescuing mechanism.

In the case of VRT-532, a likely explanation seems to be that the compound was first identified as a potentiator of temperature rescued F508del-CFTR (Van Goor *et al*., 2006). The interaction of the VRT-532 with rescued protein is able to potentiate channel function upon forskolin stimulation. Much higher concentrations of VRT-532 are required to rescue F508del-CFTR trafficking (Wang *et al*., 2006b) than those concentrations described to be required for potentiation (Van Goor *et al*., 2006). Moreover, in contrast to VRT-325, VRT-532 did not to rescue misfolded P-gp (Wang *et al*., 2006b), suggesting that VRT-532 acts specifically on F508del-CFTR. Likely, it does not promote domain-domain interactions of the kind observed for the VRT-325 compound. We therefore speculate that VRT-532 might stabilise rescued F508del-CFTR at the cell membrane.

VRT-640, a chemical derivative of VRT-325, markedly stabilised the immature form band B of F508del-CFTR. A possible explanation for this effect is that VRT-640 essentially arrested cell proliferation, providing an explanation for the accumulation of immature form band B of F508del-CFTR, while masking any differential effects on the F508del-CFTR genetic revertants. Another speculative explanation is an effect at the level of ERAD inhibition.

Pre-treatment of BHK cells with C4a induced high levels of cell death (~50% after 24 h), making steady state analysis impossible. However, when C4a was added at the beginning of pulse chase experiments, as described previously (Pedemonte *et al*., 2005a), and despite the notable cytotoxic effects observed at the end of experiments, such as altered cell morphology and cell proliferation arrest, it was possible to

confirm rescue of F508del-CFTR by this compound (Pedemonte *et al*., 2005a). But these cytotoxic effects probably disguise any possible differential effects of C4a on F508del-CFTR genetic revertants.

Altogether, these results suggest that VRT-325 promotes (unspecific) folding and that the four compounds under study seem to have different mechanisms of rescue of F508del-CFTR.

Other approaches are required to elucidate of the mechanism of action of these small molecules. Moreover, their effects on other CFTR variants remain to be determined. Trafficking and endocytosis assays would likely allow a better understanding of the specificity of the compound and allow elucidation of the possible sites of action. Another approach is to evaluate the effect of these compounds combined with low-temperature. Indeed, data by Varga *et al*. (2008), which demonstrate that VRT-325 and C4a slow down the rate of internalisation of temperature rescued F508del CFTR at the cell membrane in epithelial cells, provides insight into how these small molecule CFTR correctors rescue F508del-CFTR.

IV. General Considerations

IV. General Considerations

Since the *CFTR* gene, which is responsible for CF when mutated, was first identified, it was hoped that a cure for this lethal ailment would be rapidly discovered. However, and despite the great advances made in understanding the underlying pathophysiological mechanisms, it still remains a debilitating life-threatening disease. While many organs are affected, the main cause of mortality is progressive lung disease (Welsh and Smith, 1995), caused by the accumulation of viscous mucus that is inefficiently cleared from the airways. Such *mucoviscidosis* causes increasing blockage of the small airways with mucus plugs and air trappings (bronchiectasis), accompanied by a propensity for bacterial infections, mainly by *Pseudomonas aeruginosa* (Knowles and Durie, 2002). These infections in turn lead to an chronic exacerbated airway neutrophil-mediated inflammation, causing major epithelial tissue injury and remodelling which leads to progressive lung deterioration, and ultimately patient death by respiratory failure.

To date, all progress made in improving the quality of life and median expected survival of patients has been achieved by tackling symptoms of the disease, rather than aiming to restore the basic defect causing CF. Globally, almost 90 % of patients carry the F508del mutation in one allele, which although not preventing CFTR protein synthesis, causes major intracellular retention at the level of the ER. Since the initial finding that F508del-CFTR could be rescued to the cell surface upon the incubation of cells at low temperature (i.e. 26 - 30 °C), a number of strategies have been devised to promote cell-surface expression of the retained protein. While some of these strategies have been well succeeded in cellular and in *in vitro* models, most will never be applicable in the clinical setting, but have nevertheless furthered our understanding of the mechanisms of CF disease.

Accordingly, besides low temperature incubation, strategies such as the use of compounds, which unspecifically promote folding of F508del-CFTR protein (i.e. chemical chaperones) will also never be utilised in the clinic. Therefore, the two most promising strategies to cure CF by restoring CFTR mediated Cl^- transport in target tissues, are gene therapy and a pharmacological approach, using small molecules that can specifically and efficaciously rescue F508del-CFTR from the ER to the cell surface (i.e. pharmacological chaperones).

Gene therapy, despite the still ongoing efforts to develop safe vector systems that can efficiently deliver a copy of the normal CF gene to the appropriate cells, is yet to prove its efficacy in CF. As an example, the UK CF Gene Therapy Consortium aims to evaluate this goal in a current clinical trial by delivering the vector by a bronchial endoscopic procedure into the airways of CF patients.

The most pursued pharmacological approach takes advantage of the fact that most CF patients have a least one allele with the F508del-mutation. Therefore, it is assumed that the CFTR protein is already in the target cells. The strategy is hence to promote at least part of the existing pool of ER retained F508del-CFTR to the cell surface. As this cell surface rescued mutant protein still displays deficient Cl^- channel function, a potentiator should be used in combination so as to restore normal CFTR dependent Cl^- secretion.

The first candidates for such pharmacological rescue were compounds that were suggested to bind CFTR directly, through their ability to modulate CFTR. According to the model put forward for misfolded vasopressin receptors (Morello *et al*., 2000), these could either be potentiators of CFTR channel activity (e.g. by interacting with the

NBDs) or compounds that inhibit Cl^- secretion (e.g. by occluding the channel pore).

Testing of drugs already available in the pharmacy for effects on mutant CFTR yielded some interesting results. Examples include compounds such as vitamin C, which was found to regulate CFTR mediated Cl^- secretion (Fischer *et al*., 2004). Citrus limonoids were also found to increase Cl^- conductance in epithelial cells to an extent comparable to genistein (DeCarvalho *et al*., 2002b). Another example is curcumin, a major constituent of tumeric spice, which has been proposed by some groups to rescue the cell surface expression of F508del-CFTR (Egan *et al*., 2002; Egan *et al*., 2004), but not by others (Loo *et al*., 2004). Nevertheless, curcumin was admitted to clinical trial, but the results were inconclusive (Mall and Kunzelmann, 2005). Another compound reported to rescue the F508del-CFTR trafficking defect is sildenafil (Dormer *et al*., 2005), the active compound in Viagra (Pfizer Ltd.). Recently, KM11060 (7-chloro-4-{4-[(4-chlorophenyl) sulfonyl] piperazino}quinoline), a structural analogue of sildenafil was also found to rescue F508del-CFTR cell surface expression at nanomolar concentrations (Robert *et al*., 2008), allowing the mutant protein to function as a Cl^- channel. The same was described for N-butyldeoxynojirimycin, the active compound of miglustat (Zavesca), used to treat Gaucher disease, as an inhibitor of glucosyltransferases (Norez *et al*., 2006). Recent results to assay miglustat in human CF epithelial cells and nasal biopsies from *cftr*$^{F508del/F508del}$ mice, suggest that besides its putative effect on rescuing F508del-CFTR, this drug also normalises the ENaC-dependent Na^+-hyperabsorption observed in CF cells (Noel *et al*., 2008). A phase II clinical trial is under way to test efficacy in CF patients.

When the present work began, the premise was to test known CFTR modulators for their ability to rescue F508del-CFTR processing and function. The initial compounds selected for study were chosen for their effects in modulating CFTR activity and included (i) genistein (see Chapter 1) and (ii) the *P. acidus* extract (see Chapter 2). Later, as small molecule CFTR correctors became available (through Cystic Fibrosis Foundation Therapeutics (CFFT), USA, distributed by Prof. R. Bridges Rosalind Franklin University, Chicago, USA), the CFTR correctors VRT-325, VRT-532, VRT-640 and C4a were also tested.

To explore the long-term effects of CFTR potentiators on wt- and F508del-CFTR, we employed the well characterised CFTR modulator genistein to investigate the effects of prolonged pre-treatments with this compound on the biogenesis, degradation, localisation and function of CFTR. We selected this flavonoid because, as the most-studied CFTR potentiator, its mechanism of action is well understood (Hwang and Sheppard, 1999; Lansdell *et al*., 2000; Ai *et al*., 2004b). Our studies demonstrated that in addition to modulating function of the CFTR Cl^- channel, genistein modulates the biosynthesis and localisation of wt-CFTR. The effects of genistein were concentration dependent: low micromolar concentrations augmented protein maturation. By contrast, higher concentrations impaired the full maturation of CFTR protein and inhibited channel activity by attenuating current flow through open channels. When we evaluated CFTR function in intact cells using the iodide efflux technique, we found that low concentrations of genistein augmented CFTR-mediated iodide efflux, whereas high concentrations had the converse effect. These data demonstrate the potential of CFTR potentiators to influence CFTR activity by mechanisms distinct from their effects on channel gating, and thus highlight the importance of a multidisciplinary approach when investigating how CFTR potentiators rescue CF

mutants. Significantly, some CFTR potentiators (for example, VRT-532; Van Goor et al., 2006; Wang et al., 2006) have been identified that act as pharmacological chaperones, rescuing the cell surface expression of F508del-CFTR and enhancing its Cl^- channel function.

The extract from the medicinal plant *P. acidus* contains various bioactive compounds, such as adenosine, kaempferol and hypogallic acid. The effects of these compounds include: i) increasing the intracellular second messengers cAMP and Ca^{2+} and thereby activating CFTR and Ca^{2+}-dependent Cl- channels; ii) activating CFTR directly, as demonstrated for flavonoids; iii) increasing membrane expression of CFTR; iv) enhancing the driving force for luminal Cl^- exit by activating basolateral K^+ channels; v) reducing ENaC activity through activation of CFTR, thereby reducing NaCl absorption and preventing dehydration of the airway surface liquid (ASL). Furthermore, functional data obtained during this study (by M. Sousa and J. Ousingsawat), demonstrate that the extract activates F508del-CFTR Cl^- channel function in biopsies from trachea of *cftr*$^{F508del/F508del}$ mice and in F508del/F508del human bronchial epithelial cells.

The components of *P. acidus* were thus shown to affect membrane ion transport in this collaborative study. Our studies aimed at assessing the effects of *P. acidus* on the processing of wt- and F508del-CFTR expressed in BHK cells, however, could not detect any significant effects. Our experience with this heterologous expression system indicates that its quality control for misfolded proteins is very stringent. By contrast, using epithelial tissue, both from human and murine origin, many studies have been able to detect residual levels of F508del-CFTR at the apical membrane or at a sub-apical localisation (Penque *et al*., 2000).

Apart from activating CFTR directly, components of the *P. acidus* extract have also been shown to inhibit endoplasmic reticulum Ca^{2+}-ATPase and to stimulate mitochondrial Ca^{2+} uptake (Montero *et al*., 2004), which may affect ER chaperones and thus CFTR membrane traffic. They might also lead to a favourable redistribution of F508del-CFTR within cellular compartments, without directly affecting processing of the protein (Lim *et al*., 2004). This may explain why *P. acidus* had only modest effects on biogenesis of CFTR, but activated F508del-CFTR currents after incubation of *Xenopus* oocytes or acute application to F508del-CFTR expressing BHK cells.

The effect of these potentiators on wt- and F508del-CFTR in long term treatments has allowed an insight into how these compounds may affect processing and function of the normal and mutant CFTR Cl^- channel.

Over the last half decade, great efforts were devoted to the development of systems that would allow screening of compound libraries, the so-called high-throughput screening (HTS). The Verkman laboratory developed an assay for CFTR-mediated halide efflux using a halide-sensitive fluorescent protein (Galietta *et al*., 2001). This enabled the discovery of inhibitors (Ma *et al*., 2002a) and potentiators (Ma *et al*., 2002b) of wild type CFTR. The inhibitors identified can potentially block the chronically active CFTR in secretory diarrhoeas (Ma *et al*., 2002a), whereas the potentiators enhance the activity of cell surface rescued mutant CFTR activated by cAMP-dependent phosphorylation (Yang *et al*., 2003). Then, additional libraries were screened for compounds with the ability to rescue cell surface expression of F508del-CFTR in prolonged treatments. These efforts led to the identification of a number of compounds that indeed are able to promote the misfolded protein to the plasma membrane (Pedemonte

et al., 2005a). Simultaneously, in an industrial effort to achieve the same goal by independent library screens, other small molecule CFTR correctors able to rescue F508del-CFTR were identified by Vertex Pharmaceuticals (Van Goor *et al*., 2006).

Subsequent research in the CF field has focused on understanding how some of these small molecule CFTR correctors rescue F508del-CFTR surface expression and restore Cl^- channel function. A compound originally identified as a potentiator of temperature rescued F508del-CFTR, VRT-532 (Van Goor *et al*., 2006), was later also shown to act as a specific rescuing agent for F508del-CFTR (Wang *et al*., 2006b). Other studies on temperature rescued F508del-CFTR to assess the effect of these small molecule CFTR correctors have shown that F508del-CFTR cell-surface stability is greatly increased by the presence of both VRT-325 and C4a (Varga *et al*., 2008). Also, studies assessing the effects of compound C4a, suggest that this compound binds directly to the CFTR protein (Wang *et al*., 2007b). Furthermore, data obtained using a crosslinking assay on an ER-retained variant of CFTR, suggests that this small molecule CFTR corrector promotes correct folding of the mutant protein which thus allows it to traffic to the cellular membrane (Loo *et al*., 2008).

In the present work, we assessed whether and how the small molecule CFTR correctors differentially affect the G550E and 4RK genetic revertants of F508del-CFTR. These were previously described to rescue cell-surface expression by different mechanisms (Roxo-Rosa *et al*., 2006): G550E by putatively allowing correct folding of F508del-CFTR, and thus normal maturation of F508del-CFTR on a path to the cell membrane and 4RK by enabling the mutant protein not to be recognised by the cellular quality control mechanisms, and thus “bypass” intracellular retention to be localised at the cell surface. Our

results show that one of the small molecule CFTR correctors studied here, VRT-325, markedly increased maturation efficiency of F508del-4RK-, but not of F508del-G550E-CFTR. This finding suggests that the compound acts at the same level as G550E, i.e. folding, to promote cell-surface expression of F508del-CFTR. The additive effect of VRT-325 and the genetic revertant 4RK on F508del-CFTR cell surface rescue suggests that VRT-325 and 4RK plausibly act at distinct checkpoints of the ERQC (See Figure I.6 in I. Introduction)

It seems reasonable to assume that results from studies on small molecule CFTR correctors will elucidate the mechanism of action by which they rescue CFTR, and hence enable a better understanding of the underlying dysfunction that causes CF disease. Ultimately, since most CF patients do actually produce CFTR protein, albeit a misfolded form that is retained in the ER, the knowledge accumulated by the study of small molecule CFTR correctors will lead to therapeutics that will be applicable to the vast majority of CF patients. Potentially, if different compounds have different mechanisms of rescue of F508del-CFTR, these could be combined in a multi-target approach to maximise the level of CFTR rescue. Furthermore, rescued F508del-CFTR will most likely require a potentiator once it is localised at the cell surface, to restore normal Cl^- channel function.

Future Directions

While the small molecule CFTR correctors are good “tools” to investigate the mechanisms of mutant CFTR dysfunction in cell lines (Chapter 3), they are usually quite toxic. In any case, they are good lead compounds for chemical engineering so as to increase their potency and specificity and reduce their toxicity. For example C4a has meanwhile been utilised as the starting point for chemical engineering, yielding a substantially more potent and less toxic compound (Yoo *et al*., 2008). One compound, VX-809, based on those initially identified by Vertex pharmaceuticals (Van Goor *et al*., 2006) has recently successfully passed efficacy and safety trials and been admitted for further clinical trials (phase 2B).

It has been suggested (Skach, 2007), that the different small molecule CFTR correctors could act at different steps along the folding pathway and thus a multi-drug approach to CF disease might be most appropriate to target this and other misfolding disorders. Indeed, studies assessing simultaneous treatment with several compounds, indeed showed additive effects (Wang *et al*., 2007a). If the structural determinants of the compounds for different modes of F508del-CFTR rescue are elucidated, perhaps it is possible to conceive a compound that combines the properties of two or more such small molecule CFTR correctors. Most appealing would be the combination of such small molecule CFTR correctors with potentiator activity, an idea validated by the fact that the original premise of the screens undertaken by the Verkman group was to identify potentiators, which would act as correctors in prolonged treatments.

Additional approaches are required to elucidate the mechanism of action of these small molecule CFTR correctors. The effect of these

compounds remains to be determined on other CFTR variants: trafficking and endocytosis mutants would likely allow a better understanding of the specificity of the compound and reveal the possible sites of action on CFTR protein. Another approach is to evaluate the effect of these compounds combined with low-temperature. Indeed, data by Varga *et al*. (2008), suggesting that VRT-325 and C4a slow down the rate of internalisation of temperature rescued F508del CFTR at the cell membrane in epithelial cells, helping to understand the mechanism of F508del-CFTR rescue.

The prospects of identifying a compound that will rescue mutant CFTR to its correct localisation, and is safe for clinical use, are good. The question as to how much CFTR is enough, has been the subject of discussion, but it seems that only 5% of wild type levels (Ramalho *et al*., 2002) is sufficient to yield a significantly improved clinical phenotype. Therefore, it seems reasonable to assume, that if a compound which rescues at least part of the intracellularly retained mutant CFTR, is clinically safe and has a high specificity for CFTR, CF patients' quality of life and life expectancy would be greatly improved.

V. References

V. References

Accurso, F. J. (1997): Early pulmonary disease in cystic fibrosis. *Curr. Opin. Pulm. Med.* **3**, 400-403.

Ai, T., Bompadre, S. G., Sohma, Y., Wang, X., Li, M. and Hwang, T. C. (2004a): Direct effects of 9-anthracene compounds on cystic fibrosis transmembrane conductance regulator gating. *Pflugers Arch.* **449**, 88-95.

Ai, T., Bompadre, S. G., Wang, X., Hu, S., Li, M. and Hwang, T. C. (2004b): Capsaicin potentiates wild-type and mutant cystic fibrosis transmembrane conductance regulator chloride-channel currents. *Mol. Pharmacol.* **65**, 1415-1426.

Akiyama, T. and Ogawara, H. (1991): Use and specificity of genistein as inhibitor of protein-tyrosine kinases. *Methods in Enzymology* **201**, 362-370.

Aleksandrov, L., Aleksandrov, A. A., Chang, X. B. and Riordan, J. R. (2002): The First Nucleotide Binding Domain of Cystic Fibrosis Transmembrane Conductance Regulator Is a Site of Stable Nucleotide Interaction, whereas the Second Is a Site of Rapid Turnover. *J. Biol. Chem.* **277**, 15419-15425.

Amaral, M. D. (2004): CFTR and chaperones: processing and degradation. *J. Mol. Neurosci.* **23**, 41-48.

Amaral, M. D. and Kunzelmann, K. (2007): Molecular targeting of CFTR as a therapeutic approach to cystic fibrosis. *Trends Pharmacol. Sci.* **28**, 334-341.

Ameen, N. and Apodaca, G. (2007): Defective CFTR apical endocytosis and enterocyte brush border in myosin VI-deficient mice. *Traffic.* **8**, 998-1006.

Ameen, N., Silvis, M. and Bradbury, N. A. (2007): Endocytic trafficking of CFTR in health and disease. *J. Cyst. Fibros.* **6**, 1-14.

Anderson, D. H. (1938): Cystic fibrosis of the pancreas and its relations to celiac disease: A clinical and pathologic study. *Am.J.Dis.Child.* **56**, 344-399. (cited by Welsh *et al*., 1995)

Anderson, D. H. and Hodges, R. G. (1946): Celiac syndrome: genetics of Cystic fibrosis of the pancreas with a consideration of etiology. *Am J Dis Child* **72**, 62-70. (cited by Welsh *et al*., 1995)

Anderson, M. P., Gregory, R. J., Thompson, S., Souza, D. W., Paul, S., Mulligan, R. C., Smith, A. E. and Welsh, M. J. (1991a): Demonstration that CFTR is a chloride channel by alteration of its anion selectivity. *Science.* **253**, 202-205.

Anderson, M. P., Rich, D. P., Gregory, R. J., Smith, A. E. and Welsh, M. J. (1991b): Generation of cAMP-activated chloride currents by expression of CFTR. *Science.* **251**, 679-682.

Antinolo, G., Borrego, S., Gili, M., Dapena, J., Alfageme, I. and Reina, F. (1997): Genotype-phenotype relationship in 12 patients carrying cystic fibrosis mutation R334W. *J. Med. Genet.* **34**, 89-91.

Baker, J. M., Hudson, R. P., Kanelis, V., Choy, W. Y., Thibodeau, P. H., Thomas, P. J. and Forman-Kay, J. D. (2007): CFTR regulatory region interacts with NBD1 predominantly via multiple transient helices. *Nat. Struct. Mol. Biol.* **14**, 738-745.

Bals, R., Weiner, D. J., Meegalla, R. L. and Wilson, J. M. (1999): Transfer of a cathelicidin peptide antibiotic gene restores bacterial killing in a cystic fibrosis xenograft model. *J. Clin. Invest.* **103**, 1113-1117.

Bannykh, S. I., Bannykh, G. I., Fish, K. N., Moyer, B. D., Riordan, J. R. and Balch, W. E. (2000): Traffic pattern of cystic fibrosis transmembrane regulator through the early exocytic pathway. *Traffic.* **1**, 852-870.

Barasch, J. and Al-Awqati, Q. (1993): Defective acidification of the biosynthetic pathway in cystic fibrosis. *J. Cell Sci. Suppl.* **17**, 229-233.

Bear, C. E., Duguay, F., Naismith, A. L., Kartner, N., Hanrahan, J. W. and Riordan, J. R. (1991): Cl- channel activity in Xenopus oocytes expressing the cystic fibrosis gene. *J. Biol. Chem.* **266**, 19142-19145.

Bear, C. E., Li, C. H., Kartner, N., Bridges, R. J., Jensen, T. J., Ramjeesingh, M. and Riordan, J. R. (1992): Purification and functional reconstitution of the cystic fibrosis transmembrane conductance regulator (CFTR). *Cell.* **68**, 809-818.

Bebok, Z., Mazzochi, C., King, S. A., Hong, J. S. and Sorscher, E. J. (1998a): The mechanism underlying cystic fibrosis transmembrane conductance regulator transport from the endoplasmic reticulum to the proteasome includes Sec61beta and a cytosolic, deglycosylated intermediary. *J. Biol. Chem.* **273**, 29873-29878.

Bebok, Z., Venglarik, C. J., Panczel, Z., Jilling, T., Kirk, K. L. and Sorscher, E. J. (1998b): Activation of DeltaF508 CFTR in an epithelial monolayer. *Am. J. Physiol.* **275**, C599-C607.

Bienvenu, T., Beldjord, C., Adjiman, M. and Kaplan, J. C. (1993): Male infertility as the only presenting sign of cystic fibrosis when homozygous for the mild mutation R117H. *J. Med. Genet.* **30**, 797.

Boucher, R. C., Stutts, M. J., Knowles, M. R., Cantley, L. and Gatzy, J. T. (1986): Na+ transport in cystic fibrosis respiratory epithelia. Abnormal basal rate and response to adenylate cyclase activation. *J Clin Invest.* **78**, 1245-1252.

Bradbury, N. A., Clark, J. A., Watkins, S. C., Widnell, C. C., Smith, H. S. and Bridges, R. J. (1999): Characterization of the internalization pathways for the cystic fibrosis transmembrane conductance regulator. *Am. J. Physiol.* **276**, L659-L668.

Briel, M., Greger, R. and Kunzelmann, K. (1998): Cl- transport by cystic fibrosis transmembrane conductance regulator (CFTR) contributes to the inhibition of epithelial Na+ channels (ENaCs) in Xenopus oocytes co-expressing CFTR and ENaC. *J. Physiol.* **508**, 825-836.

Bulteau-Pignoux, L., Derand, R., Metaye, T., Joffre, M. and Becq, F. (2002): Genistein modifies the activation kinetics and magnitude of phosphorylated wild-type and G551D-CFTR chloride currents. *J. Membr. Biol.* **188**, 175-182.

Cahill, P., Nason, M. W., Jr., Ambrose, C., Yao, T. Y., Thomas, P. and Egan, M. E. (2000): Identification of the cystic fibrosis transmembrane conductance regulator domains that are important for interactions with ROMK2. *J. Biol. Chem.* **275**, 16697-16701.

Cai, Z., Chen, J. H., Hughes, L. K., Li, H. and Sheppard, D. N. (2007): The Physiology and Pharmacology of the CFTR Cl^- Channel. In *Advances in Molecular and Cellular Biology, Vol. 38*, ed. M.Pusch. Elsevier Ltd.), 109-143.

Cai, Z., Taddei, A. and Sheppard, D. N. (2006): Differential sensitivity of the cystic fibrosis (CF)-associated mutants G551D and G1349D to potentiators of the cystic fibrosis transmembrane conductance regulator (CFTR) Cl- channel. *J. Biol. Chem.* **281**, 1970-1977.

Carson, M. R., Travis, S. M. and Welsh, M. J. (1995): The two nucleotide-binding domains of cystic fibrosis transmembrane conductance regulator (CFTR) have distinct functions in controlling channel activity. *J. Biol. Chem.* **270**, 1711-1717.

Chang, X. B., Cui, L., Hou, Y. X., Jensen, T. J., Aleksandrov, A. A., Mengos, A. and Riordan, J. R. (1999): Removal of multiple arginine-framed trafficking signals overcomes misprocessing of delta F508 CFTR present in most patients with cystic fibrosis. *Mol. Cell.* **4**, 137-142.

Chang, X. B., Hou, Y. X., Jensen, T. J. and Riordan, J. R. (1994): Mapping of cystic fibrosis transmembrane conductance regulator membrane topology by glycosylation site insertion. *J. Biol. Chem.* **269**, 18572-18575.

Cheng, S. H., Gregory, R. J., Marshall, J., Paul, S., Souza, D. W., White, G. A., O'Riordan, C. R. and Smith, A. E. (1990): Defective intracellular transport and processing of CFTR is the molecular basis of most cystic fibrosis. *Cell.* **63**, 827-834.

Choi, J. Y., Muallem, D., Kiselyov, K., Lee, M. G., Thomas, P. J. and Muallem, S. (2001): Aberrant CFTR-dependent HCO3- transport in mutations associated with cystic fibrosis. *Nature.* **410**, 94-97.

Clancy, J. P., Ruiz, F. E. and Sorscher, E. J. (1999): Adenosine and its nucleotides activate wild-type and R117H CFTR through an A2B receptor-coupled pathway. *Am. J. Physiol.* **276**, C361-C369.

Collins, F. S. (1992): Cystic fibrosis: molecular biology and therapeutic implications. *Science.* **256**, 774-779.

Crawford, R. M., Treharne, K. J., Best, O. G., Riemen, C. E., Muimo, R., Gruenert, D. C., rnaud-Dabernat, S., Daniel, J. Y. and Mehta, A. (2006): NDPK-A (but not NDPK-B) and AMPK alpha1 (but not AMPK alpha2) bind the cystic fibrosis transmembrane conductance regulator in epithelial cell membranes. *Cell Signal.* **18**, 1595-1603.

Csanady, L., Nairn, A. C. and Gadsby, D. C. (2006): Thermodynamics of CFTR channel gating: a spreading conformational change initiates an irreversible gating cycle. *J. Gen. Physiol.* **128**, 523-533.

Cui, L., Aleksandrov, L., Chang, X. B., Hou, Y. X., He, L., Hegedus, T., Gentzsch, M., Aleksandrov, A., Balch, W. E. and Riordan, J. R. (2007): Domain interdependence in the biosynthetic assembly of CFTR. *J. Mol. Biol.* **365**, 981-994.

Cutting, G. R., Kasch, L. M., Rosenstein, B. J., Zielenski, J., Tsui, L. C., Antonarakis, S. E. and Kazazian, H. H., Jr. (1990): A cluster of cystic fibrosis mutations in the first nucleotide-binding fold of the cystic fibrosis conductance regulator protein. *Nature.* **346**, 366-369.

Dalemans, W., Barbry, P., Champigny, G., Jallat, S., Dott, K., Dreyer, D., Crystal, R. G., Pavirani, A., Lecocq, J. P. and Lazdunski, M. (1991): Altered chloride ion channel kinetics associated with the delta F508 cystic fibrosis mutation. *Nature.* **354**, 526-528.

Davidson, A. L. and Chen, J. (2004): ATP-binding cassette transporters in bacteria. *Annu. Rev. Biochem.* **73**, 241-268.

Davies, J. C., Alton, E. W. and Bush, A. (2007): Cystic fibrosis. *BMJ.* **335**, 1255-1259.

DeCarvalho, A. C., Gansheroff, L. J. and Teem, J. L. (2002a): Mutations in the nucleotide binding domain 1 signature motif region rescue processing and functional defects of cystic fibrosis transmembrane conductance regulator delta f508. *J. Biol. Chem.* **277**, 35896-35905.

DeCarvalho, A. C., Ndi, C. P., Tsopmo, A., Tane, P., Ayafor, J., Connolly, J. D. and Teem, J. L. (2002b): A novel natural product compound enhances cAMP-regulated chloride conductance of cells expressing CFTR[delta]F508. *Mol. Med.* **8**, 75-87.

Denning, G. M., Anderson, M. P., Amara, J. F., Marshall, J., Smith, A. E. and Welsh, M. J. (1992a): Processing of mutant cystic fibrosis transmembrane conductance regulator is temperature-sensitive. *Nature.* **358**, 761-764.

Denning, G. M., Ostedgaard, L. S., Cheng, S. H., Smith, A. E. and Welsh, M. J. (1992b): Localization of cystic fibrosis transmembrane conductance regulator in chloride secretory epithelia. *J. Clin. Invest.* **89**, 339-349.

Donaldson, S. H. and Boucher, R. C. (2003): Update on pathogenesis of cystic fibrosis lung disease. *Curr. Opin. Pulm. Med.* **9**, 486-491.

Dormer, R. L., Harris, C. M., Clark, Z., Pereira, M. M., Doull, I. J., Norez, C., Becq, F. and McPherson, M. A. (2005): Sildenafil (Viagra) corrects DeltaF508-CFTR location in nasal epithelial cells from patients with cystic fibrosis. *Thorax.* **60**, 55-59.

Dosanjh, A., Lencer, W., Brown, D., Ausiello, D. A. and Stow, J. L. (1994): Heterologous expression of delta F508 CFTR results in decreased sialylation of membrane glycoconjugates. *Am. J. Physiol.* **266**, C360-C366.

Drumm, M. L., Pope, H. A., Cliff, W. H., Rommens, J. M., Marvin, S. A., Tsui, L. C., Collins, F. S., Frizzell, R. A. and Wilson, J. M. (1990): Correction of the cystic fibrosis defect in vitro by retrovirus-mediated gene transfer. *Cell.* **62**, 1227-1233.

Egan, M. E., Glockner-Pagel, J., Ambrose, C., Cahill, P. A., Pappoe, L., Balamuth, N., Cho, E., Canny, S., Wagner, C. A., Geibel, J. and Caplan, M. J. (2002): Calcium-pump inhibitors induce functional surface expression of Delta F508-CFTR protein in cystic fibrosis epithelial cells. *Nat. Med.* **8**, 485-492.

Egan, M. E., Pearson, M., Weiner, S. A., Rajendran, V., Rubin, D., Glockner-Pagel, J., Canny, S., Du, K., Lukacs, G. L. and Caplan, M. J. (2004): Curcumin, a major constituent of turmeric, corrects cystic fibrosis defects. *Science.* **304**, 600-602.

Ellsworth, R. E., Jamison, D. C., Touchman, J. W., Chissoe, S. L., Braden, M., V, Bouffard, G. G., Dietrich, N. L., Beckstrom-Sternberg, S. M., Iyer, L. M., Weintraub, L. A., Cotton, M., Courtney, L., Edwards, J., Maupin, R., Ozersky, P., Rohlfing, T., Wohldmann, P., Miner, T., Kemp, K., Kramer, J., Korf, I., Pepin, K., ntonacci-Fulton, L., Fulton, R. S., Minx, P., Hillier, L. W., Wilson, R. K., Waterston, R. H., Miller, W. and Green, E. D. (2000): Comparative genomic sequence analysis of the human and mouse cystic fibrosis transmembrane conductance regulator genes. *Proc. Natl. Acad. Sci. U. S. A.* **97**, 1172-1177.

Farber, S (1945): Some organic digestive disturbances in early life. *J Mich Med Soc* **44**, 587. (cited by Welsh *et al*., 1995)

Farinha, C. M. and Amaral, M. D. (2005): Most F508del-CFTR is targeted to degradation at an early folding checkpoint and independently of calnexin. *Mol. Cell Biol.* **25**, 5242-5252.

Farinha, C. M., Nogueira, P., Mendes, F., Penque, D. and Amaral, M. D. (2002): The human DnaJ homologue (Hdj)-1/heat-shock protein (Hsp) 40 co-

chaperone is required for the in vivo stabilization of the cystic fibrosis transmembrane conductance regulator by Hsp70. *Biochem. J.* **366**, 797-806.

Fischer, H. and Machen, T. E. (1994): CFTR displays voltage dependence and two gating modes during stimulation. *J. Gen. Physiol.* **104**, 541-566.

Fischer, H., Schwarzer, C. and Illek, B. (2004): Vitamin C controls the cystic fibrosis transmembrane conductance regulator chloride channel. *Proc. Natl. Acad. Sci. U. S. A.* **101**, 3691-3696.

Freedman, S. D., Blanco, P. G., Zaman, M. M., Shea, J. C., Ollero, M., Hopper, I. K., Weed, D. A., Gelrud, A., Regan, M. M., Laposata, M., Alvarez, J. G. and O'Sullivan, B. P. (2004): Association of cystic fibrosis with abnormalities in fatty acid metabolism. *N. Engl. J. Med.* **350**, 560-569.

Friedman, K. J., Blalock, M. L., Heim, R. A. and Silverman, L. M. (1995): Relatively high prevalence of the CFTR mutations, G85E and 1154insTC. *Hum. Mutat.* **6**, 95-96.

Frizzell, R. A., Rechkemmer, G. and Shoemaker, R. L. (1986): Altered regulation of airway epithelial cell chloride channels in cystic fibrosis. *Science* **233**, 558-560.

Gadsby, D. C., Vergani, P. and Csanady, L. (2006): The ABC protein turned chloride channel whose failure causes cystic fibrosis. *Nature.* **440**, 477-483.

Galietta, L. V., Jayaraman, S. and Verkman, A. S. (2001): Cell-based assay for high-throughput quantitative screening of CFTR chloride transport agonists. *Am. J. Physiol Cell Physiol.* **281**, C1734-C1742.

Gentzsch, M., Chang, X. B., Cui, L., Wu, Y., Ozols, V. V., Choudhury, A., Pagano, R. E. and Riordan, J. R. (2004): Endocytic trafficking routes of wild type and DeltaF508 cystic fibrosis transmembrane conductance regulator. *Mol. Biol. Cell.* **15**, 2684-2696.

Glantz S. Primer of Biostatistics. (1997): **4th**. New York, McGraw Hill.

Gregory, R. J., Rich, D. P., Cheng, S. H., Souza, D. W., Paul, S., Manavalan, P., Anderson, M. P., Welsh, M. J. and Smith, A. E. (1991): Maturation and function of cystic fibrosis transmembrane conductance regulator variants bearing mutations in putative nucleotide-binding domains 1 and 2. *Mol. Cell Biol.* **11**, 3886-3893.

Guggino, W. B. and Stanton, B. A. (2006): New insights into cystic fibrosis: molecular switches that regulate CFTR. *Nat. Rev. Mol. Cell Biol.* **7**, 426-436.

Haggie, P. M., Stanton, B. A. and Verkman, A. S. (2002): Diffusional mobility of the cystic fibrosis transmembrane conductance regulator mutant, delta F508-CFTR, in the endoplasmic reticulum measured by photobleaching of GFP-CFTR chimeras. *J. Biol. Chem.* **277**, 16419-16425.

Hall, R. A., Ostedgaard, L. S., Premont, R. T., Blitzer, J. T., Rahman, N., Welsh, M. J. and Lefkowitz, R. J. (1998): A C-terminal motif found in the beta2-adrenergic receptor, P2Y1 receptor and cystic fibrosis transmembrane conductance regulator determines binding to the Na+/H+ exchanger regulatory factor family of PDZ proteins. *Proc. Natl. Acad. Sci. U. S. A.* **95**, 8496-8501.

Hamosh, A., Trapnell, B. C., Zeitlin, P. L., Montrose-Rafizadeh, C., Rosenstein, B. J., Crystal, R. G. and Cutting, G. R. (1991): Severe deficiency of cystic fibrosis transmembrane conductance regulator messenger RNA carrying nonsense mutations R553X and W1316X in respiratory epithelial cells of patients with cystic fibrosis. *J. Clin. Invest.* **88**, 1880-1885.

Haws, C. M., Nepomuceno, I. B., Krouse, M. E., Wakelee, H., Law, T., Xia, Y., Nguyen, H. and Wine, J. J. (1996): Delta F508-CFTR channels: kinetics, activation by forskolin, and potentiation by xanthines. *Am. J. Physiol.* **270**, C1544-C1555.

Heeckeren, A., Walenga, R., Konstan, M. W., Bonfield, T., Davis, P. B. and Ferkol, T. (1997): Excessive inflammatory response of cystic fibrosis mice to bronchopulmonary infection with Pseudomonas aeruginosa. *J. Clin. Invest.* **100**, 2810-2815.

Hegedus, T., Aleksandrov, A., Cui, L., Gentzsch, M., Chang, X. B. and Riordan, J. R. (2006): F508del CFTR with two altered RXR motifs escapes from ER quality control but its channel activity is thermally sensitive. *Biochim. Biophys. Acta.* **1758**, 565-572.

Helliwell P.S. and Jackson S. (1994): Relationship between weakness and muscle wasting in rheumatoid arthritis. *Ann. Rheum. Dis.* **53**, 726-728.

Higgins, C. F. (1992): ABC transporters: from microorganisms to man. *Annu. Rev. Cell Biol.* **8**, 67-113.

Hille, B. (2001): *Ionic Channels of Excitable Membranes*. (Sunderland,MA: Sinauer Associates Inc.).

Hug, M. J., Tamada, T. and Bridges, R. J. (2003): CFTR and bicarbonate secretion by [correction of to] epithelial cells. *News Physiol Sci.* **18**, 38-42.

Hughes, A. L. (1994): Evolution of the ATP-binding-cassette transmembrane transporters of vertebrates. *Mol. Biol. Evol.* **11**, 899-910.

Hwang, T. C., Koeppe, R. E. and Andersen, O. S. (2003): Genistein can modulate channel function by a phosphorylation-independent mechanism: importance of hydrophobic mismatch and bilayer mechanics. *Biochemistry.* **42**, 13646-13658.

Hwang, T. C. and Sheppard, D. N. (1999): Molecular pharmacology of the CFTR Cl- channel. *Trends Pharmacol. Sci.* **20**, 448-453.

Hwang, T. C., Wang, F., Yang, I. C. and Reenstra, W. W. (1997): Genistein potentiates wild-type and delta F508-CFTR channel activity. *Am. J. Physiol.* **273**, C988-C998.

Illek, B. and Fischer, H. (1998): Flavonoids stimulate Cl conductance of human airway epithelium in vitro and in vivo. *Am. J. Physiol.* **275**, L902-L910.

Jensen, T. J., Loo, M. A., Pind, S., Williams, D. B., Goldberg, A. L. and Riordan, J. R. (1995): Multiple proteolytic systems, including the proteasome, contribute to CFTR processing. *Cell.* **83**, 129-135.

Joo, N. S., Lee, D. J., Winges, K. M., Rustagi, A. and Wine, J. J. (2004): Regulation of antiprotease and antimicrobial protein secretion by airway submucosal gland serous cells. *J. Biol. Chem.* **279**, 38854-38860.

Kerem, B., Rommens, J. M., Buchanan, J. A., Markiewicz, D., Cox, T. K., Chakravarti, A., Buchwald, M. and Tsui, L. C. (1989): Identification of the cystic fibrosis gene: genetic analysis. *Science* **245**, 1073-1080.

Kerem, E. (2006): Mutation specific therapy in CF. *Paediatr. Respir. Rev.* **7**, S166-S169.

Kidd, J. F., Kogan, I. and Bear, C. E. (2004): Molecular basis for the chloride channel activity of cystic fibrosis transmembrane conductance regulator and the consequences of disease-causing mutations. *Curr. Top. Dev. Biol.* **60**, 215-249.

Kleinbaum D.G., Kupper L.L. and Muller K.E. Applied Regression Analysis and Other Multivariated Methods. (1988): **2nd**. Pacific Grove, CA, Duxburry Press.

Knowles, M. R., Carson, J. L., Collier, A. M., Gatzy, J. T. and Boucher, R. C. (1981): Measurements of nasal transepithelial electric potential differences in normal human subjects in vivo. *Am Rev. Respir Dis.* **124**, 484-490.

Knowles, M. R., Clarke, L. L. and Boucher, R. C. (1991): Activation by extracellular nucleotides of chloride secretion in the airway epithelia of patients with cystic fibrosis [see comments]. *N. Engl. J Med* **325**, 533-538.

Knowles, M. R. and Durie, P. R. (2002): What is cystic fibrosis? *N. Engl. J. Med.* **347**, 439-442.

Knowles, M. R., Stutts, M. J., Yankaskas, J. R., Gatzy, J. T. and Boucher, R. C. J. (1986): Abnormal respiratory epithelial ion transport in cystic fibrosis. *Clin Chest Med* **7**, 285-297.

Knowlton, R. G., Cohen-Haguenauer, O., Van Cong, N., Frezal, J., Brown, V. A., Barker, D., Braman, J. C., Schumm, J. W., Tsui, L. C. and Buchwald, M. (1985): A polymorphic DNA marker linked to cystic fibrosis is located on chromosome 7. *Nature* **318**, 380-382.

Ko, S. B., Shcheynikov, N., Choi, J. Y., Luo, X., Ishibashi, K., Thomas, P. J., Kim, J. Y., Kim, K. H., Lee, M. G., Naruse, S. and Muallem, S. (2002): A molecular mechanism for aberrant CFTR-dependent HCO(3)(-) transport in cystic fibrosis. *EMBO J.* **21**, 5662-5672.

Koehler, D. R., Downey, G. P., Sweezey, N. B., Tanswell, A. K. and Hu, J. (2004): Lung inflammation as a therapeutic target in cystic fibrosis. *Am. J. Respir. Cell Mol. Biol.* **31**, 377-381.

Kogan, I., Ramjeesingh, M., Li, C., Kidd, J. F., Wang, Y., Leslie, E. M., Cole, S. P. and Bear, C. E. (2003): CFTR directly mediates nucleotide-regulated glutathione flux. *EMBO J.* **22**, 1981-1989.

Konig, J., Schreiber, R., Voelcker, T., Mall, M. and Kunzelmann, K. (2001): The cystic fibrosis transmembrane conductance regulator (CFTR) inhibits ENaC through an increase in the intracellular Cl- concentration. *EMBO Rep.* **2**, 1047-1051.

Kreda, S. M., Okada, S. F., van Heusden, C. A., O'neal, W., Gabriel, S., Abdullah, L., Davis, C. W., Boucher, R. C. and Lazarowski, E. R. (2007): COORDINATED RELEASE OF NUCLEOTIDES AND MUCIN FROM HUMAN AIRWAY EPITHELIAL CALU-3 CELLS. *J. Physiol.* **.**.

Kunzelmann, K. (2001): CFTR: interacting with everything? *News Physiol Sci.* **16**, 167-170.

Kunzelmann, K. and Mall, M. (2003): Pharmacotherapy of the ion transport defect in cystic fibrosis: role of purinergic receptor agonists and other potential therapeutics. *Am. J. Respir. Med.* **2**, 299-309.

Kunzelmann, K. and Mall, M. (2001): Pharmacotherapy of the ion transport defect in cystic fibrosis. *Clin. Exp. Pharmacol. Physiol.* **28**, 857-867.

Kuver, R., Wong, T., Klinkspoor, J. H. and Lee, S. P. (2006): Absence of CFTR is associated with pleiotropic effects on mucins in mouse gallbladder epithelial cells. *Am. J. Physiol Gastrointest. Liver Physiol.* **291**, G1148-G1154.

Kwon, S. H., Pollard, H. and Guggino, W. B. (2007): Knockdown of NHERF1 enhances degradation of temperature rescued DeltaF508 CFTR from the cell surface of human airway cells. *Cell Physiol Biochem.* **20**, 763-772.

Lansdell, K. A., Cai, Z., Kidd, J. F. and Sheppard, D. N. (2000): Two mechanisms of genistein inhibition of cystic fibrosis transmembrane conductance regulator Cl- channels expressed in murine cell line. *J. Physiol.* **524**, 317-330.

Lansdell, K. A., Kidd, J. F., Delaney, S. J., Wainwright, B. J. and Sheppard, D. N. (1998): Regulation of murine cystic fibrosis transmembrane conductance regulator Cl- channels expressed in Chinese hamster ovary cells. *J. Physiol.* **512**, 751-764.

Lee, M. G., Choi, J. Y., Luo, X., Strickland, E., Thomas, P. J. and Muallem, S. (1999): Cystic fibrosis transmembrane conductance regulator regulates luminal Cl-/HCO3- exchange in mouse submandibular and pancreatic ducts. *J. Biol. Chem.* **274**, 14670-14677.

Lehrich, R. W., Aller, S. G., Webster, P., Marino, C. R. and Forrest, J. N., Jr. (1998): Vasoactive intestinal peptide, forskolin, and genistein increase apical CFTR trafficking in the rectal gland of the spiny dogfish, Squalus acanthias. Acute regulation of CFTR trafficking in an intact epithelium. *J. Clin. Invest.* **101**, 737-745.

Lehrich, R. W. and Forrest, J. N., Jr. (1995): Tyrosine phosphorylation is a novel pathway for regulation of chloride secretion in shark rectal gland. *Am. J. Physiol.* **269**, F594-F600.

Levin, M. H. and Verkman, A. S. (2006): Aquaporins and CFTR in ocular epithelial fluid transport. *J. Membr. Biol.* **210**, 105-115.

Lewis, H. A., Zhao, X., Wang, C., Sauder, J. M., Rooney, I., Noland, B. W., Lorimer, D., Kearins, M. C., Conners, K., Condon, B., Maloney, P. C., Guggino, W. B., Hunt, J. F. and Emtage, S. (2005): Impact of the deltaF508 mutation in first nucleotide-binding domain of human cystic fibrosis transmembrane conductance regulator on domain folding and structure. *J. Biol. Chem.* **280**, 1346-1353.

Li, H., Cai, Z., Chen, J. H., Ju, M., Xu, Z. and Sheppard, D. N. (2007): The cystic fibrosis transmembrane conductance regulator Cl(-) channel: a versatile engine for transepithelial ion transport. *Sheng Li Xue. Bao.* **59**, 416-430.

Li, H., Findlay, I. A. and Sheppard, D. N. (2004): The relationship between cell proliferation, Cl- secretion, and renal cyst growth: a study using CFTR inhibitors. *Kidney Int.* **66**, 1926-1938.

Li, H. and Wang, Q. (2004): Evaluation of free hydroxyl radical scavenging activities of some Chinese herbs by capillary zone electrophoresis with amperometric detection. *Anal. Bioanal. Chem.* **378**, 1801-1805.

Liechti-Gallati, S., Bonsall, I., Malik, N., Schneider, V., Kraemer, L. G., Ruedeberg, A., Moser, H. and Kraemer, R. (1992): Genotype/phenotype association in cystic fibrosis: analyses of the delta F508, R553X, and 3905insT mutations. *Pediatr. Res.* **32**, 175-178.

Lim, M., McKenzie, K., Floyd, A. D., Kwon, E. and Zeitlin, P. L. (2004): Modulation of deltaF508 cystic fibrosis transmembrane regulator trafficking and function with 4-phenylbutyrate and flavonoids. *Am. J. Respir. Cell Mol. Biol.* **31**, 351-357.

Lindemann, B. and Van Driessche, W. (1977): Sodium-specific membrane channels of frog skin are pores: current fluctuations reveal high turnover. *Science* **195**, 292-294.

Linsdell, P. and Hanrahan, J. W. (1998): Glutathione permeability of CFTR. *Am. J. Physiol.* **275**, C323-C326.

Linsdell, P., Tabcharani, J. A., Rommens, J. M., Hou, Y. X., Chang, X. B., Tsui, L. C., Riordan, J. R. and Hanrahan, J. W. (1997): Permeability of wild-type and mutant cystic fibrosis transmembrane conductance regulator chloride channels to polyatomic anions. *J. Gen. Physiol.* **110**, 355-364.

Loo, T. W., Bartlett, M. C. and Clarke, D. M. (2008): Correctors promote folding of CFTR in the endoplasmic reticulum. *Biochem. J.* **ePub 25/03/2008**.

Loo, T. W., Bartlett, M. C. and Clarke, D. M. (2004): Thapsigargin or curcumin does not promote maturation of processing mutants of the ABC transporters, CFTR, and P-glycoprotein. *Biochem. Biophys. Res. Commun.* **325**, 580-585.

Loo, T. W., Bartlett, M. C. and Clarke, D. M. (2005): Rescue of DeltaF508 and other misprocessed CFTR mutants by a novel quinazoline compound. *Mol. Pharm.* **2**, 407-413.

Lu, N. T. and Pedersen, P. L. (2000): Cystic fibrosis transmembrane conductance regulator: the purified NBF1+R protein interacts with the purified NBF2 domain to form a stable NBF1+R/NBF2 complex while inducing a conformational change transmitted to the C-terminal region. *Arch. Biochem. Biophys.* **375**, 7-20.

Lu, Y., Xiong, X., Helm, A., Kimani, K., Bragin, A. and Skach, W. R. (1998): Co- and posttranslational translocation mechanisms direct cystic fibrosis transmembrane conductance regulator N terminus transmembrane assembly. *J. Biol. Chem.* **273**, 568-576.

Lukacs, G. L., Chang, X. B., Bear, C., Kartner, N., Mohamed, A., Riordan, J. R. and Grinstein, S. (1993): The delta F508 mutation decreases the stability of cystic fibrosis transmembrane conductance regulator in the plasma membrane. Determination of functional half-lives on transfected cells. *J. Biol. Chem.* **268**, 21592-21598.

Lukacs, G. L., Mohamed, A., Kartner, N., Chang, X. B., Riordan, J. R. and Grinstein, S. (1994): Conformational maturation of CFTR but not its mutant counterpart (delta F508) occurs in the endoplasmic reticulum and requires ATP. *EMBO J.* **13**, 6076-6086.

Ma, T., Thiagarajah, J. R., Yang, H., Sonawane, N. D., Folli, C., Galietta, L. J. and Verkman, A. S. (2002a): Thiazolidinone CFTR inhibitor identified by high-throughput screening blocks cholera toxin-induced intestinal fluid secretion. *J. Clin. Invest.* **110**, 1651-1658.

Ma, T., Vetrivel, L., Yang, H., Pedemonte, N., Zegarra-Moran, O., Galietta, L. J. and Verkman, A. S. (2002b): High-affinity activators of cystic fibrosis transmembrane conductance regulator (CFTR) chloride conductance identified by high-throughput screening. *J. Biol. Chem.* **277**, 37235-37241.

Mall, M., Gonska, T., Thomas, J., Hirtz, S., Schreiber, R. and Kunzelmann, K. (2002): Activation of ion secretion via proteinase-activated receptor-2 in human colon. *Am. J. Physiol Gastrointest. Liver Physiol.* **282**, G200-G210.

Mall, M., Grubb, B. R., Harkema, J. R., O'neal, W. K. and Boucher, R. C. (2004): Increased airway epithelial Na+ absorption produces cystic fibrosis-like lung disease in mice. *Nat. Med.* **10**, 487-493.

Mall, M. and Kunzelmann, K. (2005): Correction of the CF defect by curcumin: hypes and disappointments. *Bioessays.* **27**, 9-13.

McNicholas, C. M., Nason, M. W., Jr., Guggino, W. B., Schwiebert, E. M., Hebert, S. C., Giebisch, G. and Egan, M. E. (1997): A functional CFTR-NBF1 is required for ROMK2-CFTR interaction. *Am. J. Physiol.* **273**, F843-F848.

McPherson, M. A. and Dormer, R. L. (1994): Cystic fibrosis gene and mucin secretion. *Lancet.* **343**, 7.

Meacham, G. C., Lu, Z., King, S., Sorscher, E., Tousson, A. and Cyr, D. M. (1999): The Hdj-2/Hsc70 chaperone pair facilitates early steps in CFTR biogenesis. *EMBO J.* **18**, 1492-1505.

Meacham, G. C., Patterson, C., Zhang, W., Younger, J. M. and Cyr, D. M. (2001): The Hsc70 co-chaperone CHIP targets immature CFTR for proteasomal degradation. *Nat. Cell Biol.* **3**, 100-105.

Mendes, F., Roxo, R. M., Dragomir, A., Farinha, C. M., Roomans, G. M., Amaral, M. D. and Penque, D. (2003): Unusually common cystic fibrosis mutation in Portugal encodes a misprocessed protein. *Biochem. Biophys. Res. Commun.* **311**, 665-671.

Meusser, B., Hirsch, C., Jarosch, E. and Sommer, T. (2005): ERAD: the long road to destruction. *Nat. Cell Biol.* **7**, 766-772.

Mills, C. L., Pereira, M. M., Dormer, R. L. and McPherson, M. A. (1992): An antibody against a CFTR-derived synthetic peptide, incorporated into living submandibular cells, inhibits beta-adrenergic stimulation of mucin secretion. *Biochem. Biophys. Res. Commun.* **188**, 1146-1152.

Montserrat, C., Merten, M. and Figarella, C. (1996): Defective ATP-dependent mucin secretion by cystic fibrosis pancreatic epithelial cells. *FEBS Lett.* **393**, 264-268.

Moran, O., Galietta, L. J. and Zegarra-Moran, O. (2005): Binding site of activators of the cystic fibrosis transmembrane conductance regulator in the nucleotide binding domains. *Cell Mol. Life Sci.* **62**, 446-460.

Moran, O. and Zegarra-Moran, O. (2005): A quantitative description of the activation and inhibition of CFTR by potentiators: Genistein. *FEBS Lett.* **579**, 3979-3983.

Morello, J. P., Salahpour, A., Laperriere, A., Bernier, V., Arthus, M. F., Lonergan, M., Petaja-Repo, U., Angers, S., Morin, D., Bichet, D. G. and Bouvier, M. (2000): Pharmacological chaperones rescue cell-surface expression and function of misfolded V2 vasopressin receptor mutants. *J. Clin. Invest.* **105**, 887-895.

Morris, A. P., Cunningham, S. A., Benos, D. J. and Frizzell, R. A. (1993): Glycosylation status of endogenous CFTR does not affect cAMP-stimulated Cl- secretion in epithelial cells. *Am. J. Physiol.* **265**, C688-C694.

Mueller-Oerlinghausen, B., Ngamwathana, W. and Kanchanapee, P. (1971): Investigation into Thai medicinal plants said to cure diabetes. *J. Med. Assoc. Thai.* **54**, 105-112.

Muir, A., Soong, G., Sokol, S., Reddy, B., Gomez, M. I., van, H. A. and Prince, A. (2004): Toll-like receptors in normal and cystic fibrosis airway epithelial cells. *Am. J. Respir. Cell Mol. Biol.* **30**, 777-783.

Noel, S., Wilke, M., Bot, A. G., de Jonge, H. R. and Becq, F. (2008): Parallel improvement of sodium and chloride transport defects by miglustat (n-butyldeoxynojyrimicin) in cystic fibrosis epithelial cells. *J. Pharmacol. Exp. Ther.* **325**, 1016-1023.

Norez, C., Noel, S., Wilke, M., Bijvelds, M., Jorna, H., Melin, P., Dejonge, H. and Becq, F. (2006): Rescue of functional delF508-CFTR channels in cystic fibrosis epithelial cells by the alpha-glucosidase inhibitor miglustat. *FEBS Lett.* **580**, 2081-2086.

Nufer, O., Guldbrandsen, S., Degen, M., Kappeler, F., Paccaud, J. P., Tani, K. and Hauri, H. P. (2002): Role of cytoplasmic C-terminal amino acids of membrane proteins in ER export. *J. Cell Sci.* **115**, 619-628.

O'Riordan, C. R., Lachapelle, A. L., Marshall, J., Higgins, E. A. and Cheng, S. H. (2000): Characterization of the oligosaccharide structures associated with the cystic fibrosis transmembrane conductance regulator. *Glycobiology.* **10**, 1225-1233.

Osborne, L., Santis, G., Schwarz, M., Klinger, K., Dork, T., McIntosh, I., Schwartz, M., Nunes, V., Macek, M., Jr., Reiss, J. and . (1992): Incidence and expression of the N1303K mutation of the cystic fibrosis (CFTR) gene. *Hum. Genet.* **89**, 653-658.

Panthong, A., Kanjanapothi, D. and Taylor, W. C. (1986): Ethnobotanical review of medicinal plants from Thai traditional books, Part I: Plants with anti-inflammatory, anti-asthmatic and antihypertensive properties. *J. Ethnopharmacol.* **18**, 213-228.

Parad, R. B. (1996): Heterogeneity of phenotype in two cystic fibrosis patients homozygous for the CFTR exon 11 mutation G551D. *J. Med. Genet.* **33**, 711-713.

Pedemonte, N., Lukacs, G. L., Du, K., Caci, E., Zegarra-Moran, O., Galietta, L. J. and Verkman, A. S. (2005a): Small-molecule correctors of defective DeltaF508-CFTR cellular processing identified by high-throughput screening. *J. Clin. Invest.* **115**, 2564-2571.

Pedemonte, N., Sonawane, N. D., Taddei, A., Hu, J., Zegarra-Moran, O., Suen, Y. F., Robins, L. I., Dicus, C. W., Willenbring, D., Nantz, M. H., Kurth, M. J., Galietta, L. J. and Verkman, A. S. (2005b): Phenylglycine and sulfonamide correctors of defective delta F508 and G551D cystic fibrosis transmembrane conductance regulator chloride-channel gating. *Mol. Pharmacol.* **67**, 1797-1807.

Penque, D., Mendes, F., Beck, S., Farinha, C., Pacheco, P., Nogueira, P., Lavinha, J., Malho, R. and Amaral, M. D. (2000): Cystic fibrosis F508del patients have apically localized CFTR in a reduced number of airway cells. *Lab Invest.* **80**, 857-868.

Picciano, J. A., Ameen, N., Grant, B. D. and Bradbury, N. A. (2003): Rme-1 regulates the recycling of the cystic fibrosis transmembrane conductance regulator. *Am. J. Physiol Cell Physiol.* **285**, C1009-C1018.

Pier, G. B., Grout, M. and Zaidi, T. S. (1997): Cystic fibrosis transmembrane conductance regulator is an epithelial cell receptor for clearance of Pseudomonas aeruginosa from the lung. *Proc. Natl. Acad. Sci. U. S. A.* **94**, 12088-12093.

Pind, S., Riordan, J. R. and Williams, D. B. (1994): Participation of the endoplasmic reticulum chaperone calnexin (p88, IP90) in the biogenesis of the cystic fibrosis transmembrane conductance regulator. *J. Biol. Chem.* **269**, 12784-12788.

Pissarra, L. S., Farinha, C. M., Xu, Z., Schmidt, A., Thibodeau, P. H., Cai, Z., Thomas, P. J., Sheppard, D. N. and Amaral, M. D. (2008): Solubilizing Mutations Used to Crystallize One CFTR Domain Attenuate the Trafficking and Channel Defects Caused by the Major Cystic Fibrosis Mutation. *Chem. Biol.* **15**, 62-69.

Poulsen, J. H., Fischer, H., Illek, B. and Machen, T. E. (1994): Bicarbonate conductance and pH regulatory capability of cystic fibrosis transmembrane conductance regulator. *Proc. Natl. Acad. Sci. U. S. A.* **91**, 5340-5344.

Prince, L. S., Peter, K., Hatton, S. R., Zaliauskiene, L., Cotlin, L. F., Clancy, J. P., Marchase, R. B. and Collawn, J. F. (1999): Efficient endocytosis of the cystic fibrosis transmembrane conductance regulator requires a tyrosine-based signal. *J. Biol. Chem.* **274**, 3602-3609.

Puchelle, E., Bajolet, O. and Abely, M. (2002): Airway mucus in cystic fibrosis. *Paediatr. Respir. Rev.* **3**, 115-119.

Quinton, P. M. and Bijman, J. (1983): Higher bioelectric potentials due to decreased chloride absorption in the sweat glands of patients with cystic fibrosis. *N. Engl. J Med.* **19**, 1185-1189.

Ramalho, A. S., Beck, S., Meyer, M., Penque, D., Cutting, G. R. and Amaral, M. D. (2002): Five percent of normal cystic fibrosis transmembrane conductance regulator mRNA ameliorates the severity of pulmonary disease in cystic fibrosis. *Am. J. Respir. Cell Mol. Biol.* **27**, 619-627.

Randak, C., Auerswald, E. A., ssfalg-Machleidt, I., Reenstra, W. W. and Machleidt, W. (1999): Inhibition of ATPase, GTPase and adenylate kinase activities of the second nucleotide-binding fold of the cystic fibrosis transmembrane conductance regulator by genistein. *Biochem. J.* **340**, 227-235.

Rich, D. P., Anderson, M. P., Gregory, R. J., Cheng, S. H., Paul, S., Jefferson, D. M., McCann, J. D., Klinger, K. W., Smith, A. E. and Welsh, M. J. (1990): Expression of cystic fibrosis transmembrane conductance regulator corrects defective chloride channel regulation in cystic fibrosis airway epithelial cells. *Nature.* **347**, 358-363.

Riordan, J. R., Rommens, J. M., Kerem, B., Alon, N., Rozmahel, R., Grzelczak, Z., Zielenski, J., Lok, S., Plavsic, N., Chou, J. L. and . (1989): Identification of the cystic fibrosis gene: cloning and characterization of complementary DNA. *Science.* **245**, 1066-1073.

Robert, R., Carlile, G. W., Pavel, C., Liu, N., Anjos, S. M., Liao, J., Luo, Y., Zhang, D., Thomas, D. Y. and Hanrahan, J. W. (2008): Structural analog of sildenafil identified as a novel corrector of the F508del-CFTR trafficking defect. *Mol. Pharmacol.* **73**, 478-489.

Rommens, J. M., Iannuzzi, M. C., Kerem, B., Drumm, M. L., Melmer, G., Dean, M., Rozmahel, R., Cole, J. L., Kennedy, D., Hidaka, N., Zsiga, M., Buchwald, M., Riordan, J. R., Tsui, L.-C. and Collins, F. S. (1989): Identification of the cystic fibrosis gene: chromosome walking and jumping. *Science* **245**, 1059-1065.

Rossmann, H., Jacob, P., Baisch, S., Hassoun, R., Meier, J., Natour, D., Yahya, K., Yun, C., Biber, J., Lackner, K. J., Fiehn, W., Gregor, M., Seidler, U. and Lamprecht, G. (2005): The CFTR associated protein CAP70 interacts with the apical Cl-/HCO3- exchanger DRA in rabbit small intestinal mucosa. *Biochemistry.* **44**, 4477-4487.

Roum, J. H., Buhl, R., McElvaney, N. G., Borok, Z. and Crystal, R. G. (1993): Systemic deficiency of glutathione in cystic fibrosis. *J Appl. Physiol* **75**, 2419-2424.

Rowe, S. M., Miller, S. and Sorscher, E. J. (2005): Cystic fibrosis. *N. Engl. J. Med.* **352**, 1992-2001.

Roxo-Rosa, M., Xu, Z., Schmidt, A., Neto, M., Cai, Z., Soares, C. M., Sheppard, D. N. and Amaral, M. D. (2006): Revertant mutants G550E and 4RK rescue cystic fibrosis mutants in the first nucleotide-binding domain of CFTR by different mechanisms. *Proc. Natl. Acad. Sci. U. S. A.* **103**, 17891-17896.

Rubenstein, R. C. (2006): Targeted therapy for cystic fibrosis: cystic fibrosis transmembrane conductance regulator mutation-specific pharmacologic strategies. *Mol. Diagn. Ther.* **10**, 293-301.

Sadlish, H. and Skach, W. R. (2004): Biogenesis of CFTR and other polytopic membrane proteins: new roles for the ribosome-translocon complex. *J. Membr. Biol.* **202**, 115-126.

Sagel, S. D. and Accurso, F. J. (2002): Monitoring inflammation in CF. Cytokines. *Clin. Rev. Allergy Immunol.* **23**, 41-57.

Saiman, L. and Prince, A. (1993): Pseudomonas aeruginosa pili bind to asialoGM1 which is increased on the surface of cystic fibrosis epithelial cells. *J Clin. Invest* **92**, 1875-1880.

Sato, J. D., Chapline, M. C., Thibodeau, R., Frizzell, R. A. and Stanton, B. A. (2007): Regulation of human cystic fibrosis transmembrane conductance regulator (CFTR) by serum- and glucocorticoid-inducible kinase (SGK1). *Cell Physiol Biochem.* **20**, 91-98.

Sato, S., Ward, C. L., Krouse, M. E., Wine, J. J. and Kopito, R. R. (1996): Glycerol reverses the misfolding phenotype of the most common cystic fibrosis mutation. *J. Biol. Chem.* **271**, 635-638.

Savov, A., Jordanova, A., Gavrilov, D., Angelicheva, D. and Kalaydjieva, L. (1994): G1244V: a novel missense mutation in exon 20 of the CFTR gene in a Bulgarian cystic fibrosis patient. *Hum. Mol. Genet.* **3**, 513-514.

Saxena, S. K., Kaur, S. and George, C. (2006): Rab4GTPase modulates CFTR function by impairing channel expression at plasma membrane. *Biochem. Biophys. Res. Commun.* **341**, 184-191.

Schloesser, M., Arleth, S., Lenz, U., Bertele, R. M. and Reiss, J. (1991): A cystic fibrosis patient with the nonsense mutation G542X and the splice site mutation 1717-1. *J. Med. Genet.* **28**, 878-880.

Schneider, E. and Hunke, S. (1998): ATP-binding-cassette (ABC) transport systems: functional and structural aspects of the ATP-hydrolyzing subunits/domains. *FEMS Microbiol. Rev.* **22**, 1-20.

Schwartz, M., Anvret, M., Claustres, M., Eiken, H. G., Eiklid, K., Schaedel, C., Stolpe, L. and Tranebjaerg, L. (1994): 394delTT: a Nordic cystic fibrosis mutation. *Hum. Genet.* **93**, 157-161.

Schwiebert, E. M., Morales, M. M., Devidas, S., Egan, M. E. and Guggino, W. B. (1998): Chloride channel and chloride conductance regulator domains of CFTR, the cystic fibrosis transmembrane conductance regulator. *Proc. Natl. Acad. Sci. U. S. A.* **95**, 2674-2679.

Scott-Ward, T. S., Cai, Z., Dawson, E. S., Doherty, A., Da Paula, A. C., Davidson, H., Porteous, D. J., Wainwright, B. J., Amaral, M. D., Sheppard, D. N. and Boyd, A. C. (2007): Chimeric constructs endow the human CFTR Cl- channel with the gating behavior of murine CFTR. *Proc. Natl. Acad. Sci. U. S. A.* **104**, 16365-16370.

Scott-Ward, T. S., Li, H., Schmidt, A., Cai, Z. and Sheppard, D. N. (2004): Direct block of the cystic fibrosis transmembrane conductance regulator Cl(-) channel by niflumic acid. *Mol. Membr. Biol.* **21**, 27-38.

Sears, C. L., Firoozmand, F., Mellander, A., Chambers, F. G., Eromar, I. G., Bot, A. G., Scholte, B., de Jonge, H. R. and Donowitz, M. (1995): Genistein and tyrphostin 47 stimulate CFTR-mediated Cl- secretion in T84 cell monolayers. *Am. J. Physiol.* **269**, G874-G882.

Sharma, M., Pampinella, F., Nemes, C., Benharouga, M., So, J., Du, K., Bache, K. G., Papsin, B., Zerangue, N., Stenmark, H. and Lukacs, G. L. (2004): Misfolding diverts CFTR from recycling to degradation: quality control at early endosomes. *J. Cell Biol.* **164**, 923-933.

Sheppard, D. N. and Robinson, K. A. (1997): Mechanism of glibenclamide inhibition of cystic fibrosis transmembrane conductance regulator Cl- channels expressed in a murine cell line. *J. Physiol.* **503**, 333-346.

Short, D. B., Trotter, K. W., Reczek, D., Kreda, S. M., Bretscher, A., Boucher, R. C., Stutts, M. J. and Milgram, S. L. (1998): An apical PDZ protein anchors the cystic fibrosis transmembrane conductance regulator to the cytoskeleton. *J. Biol. Chem.* **273**, 19797-19801.

Skach, W. R. (2000): Defects in processing and trafficking of the cystic fibrosis transmembrane conductance regulator. *Kidney Int.* **57**, 825-831.

Skach, W. R. (2007): Pharmacological chaperoning: two 'hits' are better than one. *Biochem. J.* **406**, e1-e2.

Smith, J. J., Travis, S. M., Greenberg, E. P. and Welsh, M. J. (1996): Cystic fibrosis airway epithelia fail to kill bacteria because of abnormal airway surface fluid. *Cell.* **85**, 229-236.

Sousa, M., Ousingsawat, J., Seitz, R., Puntheeranurak, S., Regalado, A., Schmidt, A., Grego, T., Jansakul, C., Amaral, M. D., Schreiber, R. and Kunzelmann, K. (2007): An extract from the medicinal plant Phyllanthus acidus and its isolated compounds induce airway chloride secretion: A potential treatment for cystic fibrosis. *Mol. Pharmacol.* **71**, 366-376.

Suaud, L., Li, J., Jiang, Q., Rubenstein, R. C. and Kleyman, T. R. (2002): Genistein restores functional interactions between Delta F508-CFTR and ENaC in Xenopus oocytes. *J. Biol. Chem.* **277**, 8928-8933.

Suaud, L., Yan, W., Carattino, M. D., Robay, A., Kleyman, T. R. and Rubenstein, R. C. (2007a): Regulatory interactions of N1303K-CFTR and ENaC in Xenopus oocytes: evidence that chloride transport is not necessary for inhibition of ENaC. *Am. J. Physiol Cell Physiol.* **292**, C1553-C1561.

Suaud, L., Yan, W. and Rubenstein, R. C. (2007b): Abnormal regulatory interactions of I148T-CFTR and the epithelial Na+ channel in Xenopus oocytes. *Am. J. Physiol Cell Physiol.* **292**, C603-C611.

Swiatecka-Urban, A., Boyd, C., Coutermarsh, B., Karlson, K. H., Barnaby, R., Aschenbrenner, L., Langford, G. M., Hasson, T. and Stanton, B. A. (2004): Myosin VI regulates endocytosis of the cystic fibrosis transmembrane conductance regulator. *J. Biol. Chem.* **279**, 38025-38031.

Swiatecka-Urban, A., Talebian, L., Kanno, E., Moreau-Marquis, S., Coutermarsh, B., Hansen, K., Karlson, K. H., Barnaby, R., Cheney, R. E., Langford, G. M., Fukuda, M. and Stanton, B. A. (2007): Myosin Vb is required for trafficking of the cystic fibrosis transmembrane conductance regulator in Rab11a-specific apical recycling endosomes in polarized human airway epithelial cells. *J. Biol. Chem.* **282**, 23725-23736.

Tarran, R., Loewen, M. E., Paradiso, A. M., Olsen, J. C., Gray, M. A., Argent, B. E., Boucher, R. C. and Gabriel, S. E. (2002): Regulation of murine airway surface liquid volume by CFTR and Ca2+-activated Cl- conductances. *J. Gen. Physiol.* **120**, 407-418.

Teem, J. L., Carson, M. R. and Welsh, M. J. (1996): Mutation of R555 in CFTR-delta F508 enhances function and partially corrects defective processing. *Receptors. Channels.* **4**, 63-72.

Tsui, L. C., Buchwald, M., Barker, D., Braman, J. C., Knowlton, R., Schumm, J. W., Eiberg, H., Mohr, J., Kennedy, D. and Plavsic, N. (1985): Cystic fibrosis locus defined by a genetically linked polymorphic DNA marker. *Science* **230**, 1054-1057.

Van Goor, F., Straley, K. S., Cao, D., Gonzalez, J., Hadida, S., Hazlewood, A., Joubran, J., Knapp, T., Makings, L. R., Miller, M., Neuberger, T., Olson, E., Panchenko, V., Rader, J., Singh, A., Stack, J. H., Tung, R., Grootenhuis, P. D. and Negulescu, P. (2006): Rescue of DeltaF508-CFTR trafficking and gating in human cystic fibrosis airway primary cultures by small molecules. *Am. J. Physiol Lung Cell Mol. Physiol.* **290**, L1117-L1130.

Varga, K., Goldstein, R. F., Jurkuvenaite, A., Chen, L., Matalon, S., Sorscher, E. J., Bebok, Z. and Collawn, J. F. (2008): Enhanced cell-surface stability of rescued DeltaF508 cystic fibrosis transmembrane conductance regulator (CFTR) by pharmacological chaperones. *Biochem. J.* **410**, 555-564.

Varon, R., Stuhrmann, M., Macek, M., Jr., Kufardjieva, A., Angelicheva, D., Magdorf, K., Jordanova, A., Savov, A., Wahn, U., Macek, M. and . (1995): Pancreatic insufficiency and pulmonary disease in German and Slavic cystic fibrosis patients with the R347P mutation. *Hum. Mutat.* **6**, 219-225.

Venglarik, C. J., Schultz, B. D., DeRoos, A. D., Singh, A. K. and Bridges, R. J. (1996): Tolbutamide causes open channel blockade of cystic fibrosis transmembrane conductance regulator Cl- channels. *Biophys. J.* **70**, 2696-2703.

Venglarik, C. J., Schultz, B. D., Frizzell, R. A. and Bridges, R. J. (1994): ATP alters current fluctuations of cystic fibrosis transmembrane conductance regulator: evidence for a three-state activation mechanism. *J. Gen. Physiol.* **104**, 123-146.

Vergani, P., Lockless, S. W., Nairn, A. C. and Gadsby, D. C. (2005): CFTR channel opening by ATP-driven tight dimerization of its nucleotide-binding domains. *Nature.* **433**, 876-880.

Vergani, P., Nairn, A. C. and Gadsby, D. C. (2003): On the mechanism of MgATP-dependent gating of CFTR Cl- channels. *J. Gen. Physiol.* **121**, 17-36.

Wainwright, B. J., Scambler, P. J., Schmidtke, J., Watson, E. A., Law, H. Y., Farrall, M., Cooke, H. J., Eiberg, H. and Williamson, R. (1985): Localization of cystic fibrosis locus to human chromosome 7cen- q22. *Nature* **318**, 384-385.

Wang, F., Zeltwanger, S., Hu, S. and Hwang, T. C. (2000): Deletion of phenylalanine 508 causes attenuated phosphorylation-dependent activation of CFTR chloride channels. *J. Physiol.* **524**, 637-648.

Wang, F., Zeltwanger, S., Yang, I. C., Nairn, A. C. and Hwang, T. C. (1998): Actions of genistein on cystic fibrosis transmembrane conductance regulator channel gating. Evidence for two binding sites with opposite effects. *J. Gen. Physiol.* **111**, 477-490.

Wang, X., Matteson, J., An, Y., Moyer, B., Yoo, J. S., Bannykh, S., Wilson, I. A., Riordan, J. R. and Balch, W. E. (2004): COPII-dependent export of cystic fibrosis transmembrane conductance regulator from the ER uses a di-acidic exit code. *J. Cell Biol.* **167**, 65-74.

Wang, X., Venable, J., LaPointe, P., Hutt, D. M., Koulov, A. V., Coppinger, J., Gurkan, C., Kellner, W., Matteson, J., Plutner, H., Riordan, J. R., Kelly, J. W., Yates, J. R., III and Balch, W. E. (2006a): Hsp90 cochaperone Aha1 downregulation rescues misfolding of CFTR in cystic fibrosis. *Cell.* **127**, 803-815.

Wang, Y., Bartlett, M. C., Loo, T. W. and Clarke, D. M. (2006b): Specific rescue of cystic fibrosis transmembrane conductance regulator processing mutants using pharmacological chaperones. *Mol. Pharmacol.* **70**, 297-302.

Wang, Y., Loo, T. W., Bartlett, M. C. and Clarke, D. M. (2007a): Additive effect of multiple pharmacological chaperones on maturation of CFTR processing mutants. *Biochem. J.* **406**, 257-263.

Wang, Y., Loo, T. W., Bartlett, M. C. and Clarke, D. M. (2007b): Correctors promote maturation of cystic fibrosis transmembrane conductance regulator (CFTR)-processing mutants by binding to the protein. *J. Biol. Chem.* **282**, 33247-33251.

Ward, C. L. and Kopito, R. R. (1994): Intracellular turnover of cystic fibrosis transmembrane conductance regulator. Inefficient processing and rapid degradation of wild-type and mutant proteins. *J. Biol. Chem.* **269**, 25710-25718.

Welsh, M. J. and Smith, A. E. (1993): Molecular mechanisms of CFTR chloride channel dysfunction in cystic fibrosis. *Cell.* **73**, 1251-1254.

Welsh, M. J. and Smith, A. E. (1995): Cystic fibrosis. *Sci. Am.* **273**, 52-59.

Welsh, M. J., Tsui, L.-C., Boat, T. F. and Beaudet, A. L. (1995): Cystic Fibrosis. In *The Metabolic and Molecular Basis of Inherited Disease*, eds. C.R.Scriver, A.L.Beaudet, W.S.Sly, and D.Valle. (New York: McGraw-Hill, Inc.), 3799-3876.

Wendeler, M. W., Nufer, O. and Hauri, H. P. (2007): Improved maturation of CFTR by an ER export signal. *FASEB J.* **21**, 2352-2358.

Wilschanski, M., Yahav, Y., Yaacov, Y., Blau, H., Bentur, L., Rivlin, J., Aviram, M., Bdolah-Abram, T., Bebok, Z., Shushi, L., Kerem, B. and Kerem, E. (2003): Gentamicin-induced correction of CFTR function in patients with cystic fibrosis and CFTR stop mutations. *N. Engl. J. Med.* **349**, 1433-1441.

Wilschanski, M., Zielenski, J., Markiewicz, D., Tsui, L. C., Corey, M., Levison, H. and Durie, P. R. (1995): Correlation of sweat chloride concentration with classes of the cystic fibrosis transmembrane conductance regulator gene mutations. *J. Pediatr.* **127**, 705-710.

Yang, C. L., Liu, X., Paliege, A., Zhu, X., Bachmann, S., Dawson, D. C. and Ellison, D. H. (2007): WNK1 and WNK4 modulate CFTR activity. *Biochem. Biophys. Res. Commun.* **353**, 535-540.

Yang, H., Shelat, A. A., Guy, R. K., Gopinath, V. S., Ma, T., Du, K., Lukacs, G. L., Taddei, A., Folli, C., Pedemonte, N., Galietta, L. J. and Verkman, A. S. (2003): Nanomolar affinity small molecule correctors of defective Delta F508-CFTR chloride channel gating. *J. Biol. Chem.* **278**, 35079-35085.

Yoo, C. L., Yu, G. J., Yang, B., Robins, L. I., Verkman, A. S. and Kurth, M. J. (2008): 4'-Methyl-4,5'-bithiazole-based correctors of defective delta F508-CFTR cellular processing. *Bioorg. Med. Chem. Lett.* **18**, 2610-2614.

Yoo, J. S., Moyer, B. D., Bannykh, S., Yoo, H. M., Riordan, J. R. and Balch, W. E. (2002): Non-conventional trafficking of the cystic fibrosis transmembrane conductance regulator through the early secretory pathway. *J. Biol. Chem.* **277**, 11401-11409.

Zerangue, N., Schwappach, B., Jan, Y. N. and Jan, L. Y. (1999): A new ER trafficking signal regulates the subunit stoichiometry of plasma membrane K(ATP) channels. *Neuron* **22**, 537-548.