Approaches to Pharmacological Treatment and Gene Therapy of Cystic Fibrosis

BY

ANCA DRAGOMIR

ACTA UNIVERSITATIS UPSALIENSIS
UPPSALA 2004
Dissertation presented at Uppsala University to be publicly examined in B21, BMC, Uppsala, Friday, January 16, 2004 at 13:15 for the degree of Doctor of Philosophy (Faculty of Medicine). The examination will be conducted in English.

Abstract

Cystic fibrosis (CF) is the most common lethal genetic disease in the white population. It is due to mutations in the gene coding for the cystic fibrosis transmembrane conductance regulator (CFTR), a protein that functions mainly as a cAMP-activated chloride channel. The disease impairs ion and water transport in epithelia-lined organs such as airways, digestive tract, reproductive epithelium and sweat glands. At present the only therapy is symptomatic and development of curative treatment depends on uncovering the links between the defective CFTR and the disease, as well as on improving end-point measurements.

A method has been established for studying ion transport in an easily accessible cell type (nasal epithelial cells) from normal and cystic fibrosis patients by X-ray microanalysis. This method represents a rather simple and direct way of measuring simultaneously several chemical elements of biological interest.

Studies of chloride transport by means of a fluorescent indicator (MQAE) in nasal epithelial cells from CF patients showed that the phenotype cannot exclusively be explained by the CFTR activity in patients with severe genotype.

A common Portuguese CFTR mutation (Δ561E) causes protein mislocalization in the endoplasmic reticulum similar to the most common CF mutation (ΔF508) and thus it should be possible to treat it with the same pharmacological strategies.

Chronic treatment of CF airway epithelial cells with nanomolar concentrations of colchicine increased the chloride eflux via chloride channels other than CFTR, strengthening the notion that colchicine could be beneficial to CF patients.

Successful in vitro transfection of CF airway epithelial cells with cationic vectors was possible with short incubation times. Heparin added at the end of the transfection incubation time could help to maintain the viability of the cells, without interfering with the transfection efficiency. It seems possible that heparin could be an adjuvant for non-viral mediated gene therapy.

Keywords: airway epithelium, colchicine, cystic fibrosis, chloride transport, genotype, heparin, phenotype, transfection, X-ray microanalysis

Anca Dragonir, Department of Medical Cell Biology, Box 571, Uppsala University, SE-75123 Uppsala, Sweden

© Anca Dragonir 2004

ISSN 0282-7476
ISBN 91-554-5822-X
urn:nbn:se:uu:diva-3845 (http://urn.kb.se/resolve?urn=urn:nbn:se:uu:diva-3845)
To my beloved ones

“It is only with the heart that one can see rightly; what is essential is invisible to the eye.”

Antoine de Saint-Exupéry, “The Little Prince”
List of Papers

This thesis is based on the following papers, reproduced with permission of the publishers:


Preface

“WOE TO THAT CHILD which when kissed on the forehead tastes salty. He is bewitched and soon must die”. This adage is an early reference from Northern European folklore to the disease today known as cystic fibrosis.

Cystic fibrosis is the most common lethal inherited disorder in the Caucasian population. It is due to abnormalities in salt and water transport in the epithelia of many organs. This causes the body to produce thick, sticky mucus that clogs the lungs, leading to infection, and blocks the pancreas, stopping digestive enzymes from reaching the intestines. Abnormally high salt content in sweat is characteristic for cystic fibrosis and also used to diagnose the disease.

At present the only treatment is symptomatic: daily physiotherapy and mucolytic agents to remove the viscous mucus from the airways, frequent hospitalisation to treat the repeated bacterial infections, dietary regulations and pancreatic enzyme supplementation. Most male patients are infertile due to the early blockage of the spermatic duct. Many of them still become fathers with the help of modern fertilization techniques. Patients with end-stage lung disease are candidates for lung or heart-lung transplantation. The survival of CF patients has increased constantly during the past decades due to improvements in the healthcare and the average life span is now over 30 years.

For years “Sixty-Five Roses” has been used by children to name their disease because the words are much easier for them to pronounce. But making it easier to say doesn’t make it any easier to live with... It started in 1965, when a mother learned her three little boys had the disease. She became a volunteer for the Cystic Fibrosis Foundation and her duty was to call and raise money in supporting research for a cure. Her four-year-old son overheard his mother making many phone calls and told his mom, “I know what you are working for.” She was surprised because she had not told her children what she was
doing, nor that they had cystic fibrosis. “What am I working for, Ricky?” she asked. “You are working for Sixty-Five Roses”, he answered so innocently. She hugged her son tightly so he could not see the tears streaming down her cheeks, “Yes Ricky, I’m working for Sixty-Five Roses” [http://www.65roses.com].

The Sixty-Five Roses story has captured the hearts and emotions of all who have heard it. Many cystic fibrosis organizations have adopted one of the “sixty five roses” as their symbol, an appropriate choice since roses are beautiful for only a short time before they wilt. The rose has become a symbol of hope for those living with cystic fibrosis. Our hope is that basic research will expand our understanding and lead to novel therapies that will improve and extend lives.
Contents

Introduction ................................................................................................................... 1
The gene and its product ........................................................................................ 2
The CFTR protein ................................................................................................... 4
The disease ................................................................................................................ 7
The treatment ......................................................................................................... 11

Aims ............................................................................................................................... 16

Methods ........................................................................................................................17
  Cells .......................................................................................................................... 17
  Protein expression .................................................................................................. 18
  CFTR function ....................................................................................................... 20
  Morphology ............................................................................................................. 27
  Transfections .......................................................................................................... 27
  Lipids ........................................................................................................................ 28
  Statistical analysis ................................................................................................... 28

Results and Discussion ............................................................................................... 29
  Investigations of CFTR function ........................................................................ 29
  Investigation of patients ........................................................................................ 31
  Therapeutic strategies ............................................................................................ 32

Conclusions and outlook ........................................................................................... 37

Acknowledgements ..................................................................................................... 39

References .....................................................................................................................41
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AF508</td>
<td>Deletion of phenylalanine at position 508 in the CFTR structure</td>
</tr>
<tr>
<td>16HBE</td>
<td>Human bronchial epithelial cell line (normal)</td>
</tr>
<tr>
<td>ASL</td>
<td>Airway surface liquid</td>
</tr>
<tr>
<td>BHK</td>
<td>Baby hamster kidney fibroblast cell line</td>
</tr>
<tr>
<td>Calu-3</td>
<td>Human airway submucosal cell line (normal)</td>
</tr>
<tr>
<td>CF</td>
<td>Cystic fibrosis</td>
</tr>
<tr>
<td>CFBE</td>
<td>Cystic fibrosis (ΔF508/ΔF508 homozygous) human bronchial cell line</td>
</tr>
<tr>
<td>CFSME</td>
<td>Cystic fibrosis (ΔF508/unknown) human airway submucosal cell line</td>
</tr>
<tr>
<td>CFTR</td>
<td>Cystic fibrosis transmembrane conductance regulator</td>
</tr>
<tr>
<td>DMEM</td>
<td>Doblecco’s minimal essential medium with Glutamax</td>
</tr>
<tr>
<td>EMEM</td>
<td>Eagle’s minimal essential medium with Glutamax</td>
</tr>
<tr>
<td>ENaC</td>
<td>Epithelial sodium channel</td>
</tr>
<tr>
<td>HEPES</td>
<td>N-(2-hydroxyethyl)piperazine-N-(2-etanesulphonic acid)</td>
</tr>
<tr>
<td>IBMX</td>
<td>3-isobutyl-1-methylxanthine</td>
</tr>
<tr>
<td>MDR</td>
<td>Multidrug resistance protein</td>
</tr>
<tr>
<td>MQAE</td>
<td>N-(ethoxycarbonylmethyl)-6-methoxyquinolinium bromide</td>
</tr>
<tr>
<td>NBD</td>
<td>Nucleotide binding domain</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PI</td>
<td>Pancreatic insufficiency</td>
</tr>
<tr>
<td>SR</td>
<td>Standard Ringer’s solution</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris buffered saline</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
</tr>
<tr>
<td>wt-CFTR</td>
<td>Wild-type CFTR</td>
</tr>
<tr>
<td>XRMA</td>
<td>X-ray microanalysis</td>
</tr>
</tbody>
</table>
Introduction

Cystic fibrosis is the most common lethal genetic disorder in the Caucasian population, characterized by impaired ion and water transport across epithelia-lined organs such as airways, digestive tract, reproductive epithelium and sweat glands. Cystic fibrosis (CF) is caused usually by a homozygous genetic defect that leads to a variety of abnormalities in the protein named “cystic fibrosis transmembrane conductance regulator” (CFTR), a chloride channel present in the epithelia of many organs.

The CFTR gene is located on the long arm of the chromosome 7 (7q31) and over 1000 mutations causing the disease have been described [www.genet.sickkids.on.ca/cftr]. The most common mutation, ΔF508, is present in 70% of CF chromosomes and 90% of the CF patients have this mutation in at least one allele [Kerem et al. 1989]. This mutation consists of the deletion of 3 base-pairs, which results in the loss of a phenylalanine at position 508 of the putative protein.

The incidence of mutations in the Caucasian population is in the heterozygous state as high as 1/25 and in the homozygous state it ranges between 1/2200 to 1/7700 live births [Dodge et al. 1997; Kollberg 1982]. In the Finnish population the incidence of CF is much lower (1/25 000), as it is in non-Caucasian populations (1/17000 in African-Americans to 1/320 000 in Japanese) [Lucotte et al. 1995].

The high frequency of CF and ΔF508 mutation in the Caucasian population suggests that mutant CF alleles confer a selective advantage. There is a hypothesis that ΔF508 heterozygotes may be protected against CFTR-mediated secretory diarrhoea induced by the cholera toxin, typhoid fever or other enterotoxins and this hypothesis is supported by experimental results [Gabriel et al. 1994; Pier et al. 1998].
The gene and its product

The identification of CFTR gene in 1989 [Riordan et al. 1989] has sharply accelerated the research on cystic fibrosis. The gene spans approximately 250 kilobases (kb) of nucleotide sequences together with its promoters and regulatory regions (figure 1). The CFTR gene has 27 exons, which form a 6.5 kb long coding sequence [Rommens et al. 1989].

The introns allow alternative splicing of CFTR messenger RNA (mRNA), which has clinical significance because it may decrease the amount of mature CFTR protein expressed, and thus be responsible for the variable severity of cystic fibrosis. [Nissim–Rafinia et al. 2000; Pagani et al. 2003].

The studies of CFTR promoters and regulatory regions indicate that the CFTR gene has some resemblance to the “housekeeping genes” (expressed in every tissue and at any time) but in addition it has tissue-specific regulation [Yoshimura et al. 1991]. As a housekeeping gene, the functional activity of CFTR is modulated in embryonic lung (rat) by steroid hormones suggesting translational or posttranscriptional regulation [Sweezey et al. 1997]. However, the in vivo expression of the mRNA and protein shows a highly regulated pattern, both spatially and temporally. In adults, CFTR is expressed in the epithelial cells (usually only by a subpopulation) of the respiratory tract, intestine, pancreas, gallbladder, kidney, genital tract, salivary and sweat glands.

In the airway surface epithelium, CFTR is expressed at high levels in the first gestation trimester in all distal epithelia and small airways. In the second trimester the expression in the future alveolar space is reduced, while in the third trimester and neonatal period there is no alveolar or tracheal expression. Therefore, in the surface airway epithelium, the CFTR presents a bronchial centrifugal expression gradient, diminishing from bronchi to bronchioles and to prealveolar tubes, whereas the mesenchyme does not express the CFTR mRNA [Horster 2000].

The highest level of expression was detected in the serous tubules of the submucosal glands in the trachea and large bronchi. The relatively high levels of CFTR expression in the fetal lung are in marked contrast with the low levels in the adult lung, where only one-two copies of CFTR mRNA per cell are present [Yoshimura et al. 1991].

In the gastrointestinal tract, the CFTR mRNA expression levels are higher than in the lung at all embryonic stages and CFTR is found specifically in the progenitor cells of the intestinal crypts, with a decreasing gradient of expression along the crypt-villous axis. As observed for the cell lines in vitro, the polar membrane distribution of the protein is acquired with cell differentiation, after the first trimester. In the kidney and pancreas, the CFTR expression pattern is characterised by early appearance (first trimester) in the apical membrane of the epithelial cells. CFTR levels decline progressively during late-gestation morphogenesis until birth and remain unchanged thereafter [Horster 2000]. In the absorptive duct of the sweat gland CFTR is localised not only to the apical
but also to the basolateral membrane of the epithelial cells [Reddy and Quinton 1989].

CFTR was identified in a variety of other tissues such as brain or myocardium, without known relevance for the disease. CFTR was also detected in the membrane of secretory vesicles in tracheal submucosal glands, evidence that CFTR may have an intracellular function [Jacquot et al. 1993].

Figure 1. From the CF gene to the CFTR protein. TMD: transmembrane domain; NBD: nucleotide-binding domain; R: regulatory domain.
The CFTR Protein

The coding sequence of the CFTR predicts a 1480 amino acid long protein with a molecular mass of ~168 kDa. In the endoplasmic reticulum the CFTR is glycosylated and undergoes an ATP-dependent conformational change in order to become stable. This process is inefficient and only 20-25% of the immature CFTR is transported in the Golgi for further glycosylation and then to the plasma membrane. In contrast, the ΔF508-CFTR is processed abnormally and 95% is retained in the endoplasmic reticulum, from where it is degraded by the ubiquitin-proteasome system [Ward and Kopito 1994].

Once in the cell membrane, the turnover is relatively fast and the wild-type (wt) CFTR has a half-life time of ~72 hours, while the ΔF508 has ~4 hours [Heda et al. 2001]. The CFTR is recycled by endocytosis or degraded by the lysosomal proteases.

The protein consists of two repeated motifs, each containing a membrane-spanning domain and a hydrophilic, nucleotide-binding region (NBD) (figure 1). The membrane-spanning domains consist of six α-helices with charged amino acids, a feature characteristic for the ion-channel proteins. A small region between transmembrane domain 7 and 8 contains two potential glycosylation sites and is predicted to be exposed to the exterior surface. With these features, the CFTR molecule shares sequence homology with a group of membrane-bound proteins, which are involved in the active transport of molecules across membranes (ATP-binding cassette superfamily). Unique to the CFTR is the presence of a highly charged cytoplasmic regulatory (R) domain, which unites the two symmetrical motifs. This contains several phosphorylation sites for the protein kinases A and C [Riordan et al. 1989].

Cross-species analysis shows significant conservation in structure between the human CFTR cDNA and its bovine, mouse, rat and even shark homologues, especially for some of the transmembrane domains, glycosylation sites, nucleotide-binding folds and the R domain [Diamond et al. 1991; Marshall et al. 1991]. This supports the hypothesis that the CFTR may be equally important in evolutionary diverse organism and is an argument in favour of using animal models for the study of cystic fibrosis.

The main function of CFTR is to act as a cAMP-mediated chloride channel. The two membrane spanning domains represent the pore of the channel. The CFTR chloride channel is regulated by phosphorylation of the R domain. Protein kinase A is the primary activator of the chloride transport by CFTR in humans, although protein kinase C also stimulates it, but to a lower extent [Dulhanty and Riordan 1994].

Once phosphorylated, the CFTR channel requires hydrolysable nucleotides to be active. Experimental results [Ikuma and Welsh 2000] suggest a gating cycle for CFTR in which ATP binding by any NBD opens the channel and either hydrolysis or dissociation of the nucleotide leads to channel closure.
The two NBDs are not totally equal in function, NBD1 having a greater and more stable nucleotide trapping effect than NBD2 [Aleksandrov et al. 2001]. Mutations affecting the first NBD (among them the frequent ΔF508 mutation) are more common and severe than mutations affecting the second NBD, another indication that their functional importance may be different [Tsui 1992]. Once cAMP is removed, membrane-associated phosphatases probably dephosphorylate CFTR resulting in closure of the channel. [Hanrahan et al. 1996].

From an electrophysiological point of view, CFTR has a relatively small halide conductance of 9 pS for Cl\(^-\) and less for I\(^-\) and Br\(^-\). It shows a linear intensity-voltage relationship and no voltage-dependent activation or inactivation. Channel open probability is not altered by stilbenes like DIDS (4,4'-diisothiocyanostilbene-2,2'-disulphonic acid) and very little reduced by other Cl\(^-\) channel blockers such as NPPB (5-nitro-2-(3-phenylpropylamino) benzoic acid) and DPC (diphenylamine-2-carboxylate) [Bear et al. 1992]. A commonly used CFTR blocker is the anti-diabetic drug glibenclamide, although it is not very specific. Recently, high-throughput screening has identified more specific blockers of CFTR. Some of them (for example CFTRinh-172) are active at nanomolar concentration and have immediate use in the exploration of other functions of the CFTR protein, development of CF animal models or characterisation of new drugs [Ma et al. 2002; Yang et al. 2003].

CFTR can also conduct the bicarbonate anion, allowing its movement out of the cells, but blocking its re-entry by virtue of cytoplasmic anionic charge [Reddy and Quinton 2001]. CFTR activated by cAMP and ATP appears to conduct both HCO\(_3\)\(^-\) and Cl\(^-\) with an estimated selectivity ratio of 0.2 to 0.5.

CFTR enhances ATP release by a separate channel, stimulated by hypotonic challenge in order to strengthen autocrine control of cell volume regulation [Braunstein et al. 2001]. Recent studies have shown that CFTR directly mediates the transport of the glutathione molecule, playing thus a central role in the control of the oxidative stress in the airways [Kogan et al. 2003].

In addition to serving as a Cl\(^-\) channel, there is compelling evidence that CFTR inhibits the amiloride-sensitive, epithelial sodium channel (ENaC) (figure 2). The mechanism of coupling is not known but most likely involves physical interactions between the channels, perhaps mediated by an intermediate protein that impinges on other transport proteins [Kunzelmann et al. 2001]. In CF patients sodium absorption is increased in airways and colon, but paradoxically absent in the sweat glands [Kunzelman 1999]. Reddy et al. [1999] suggest that in freshly isolated, normal sweat ducts, ENaC activity is dependent on and increases with CFTR activity.

ENaC is not the only channel regulated by CFTR. Several data show that CFTR may be a regulator for potassium and other chloride channels, as well as for aquaporins. CFTR downregulates \textit{in vitro} the activity of the calcium-regulated chloride channel, especially in the pancreas [Wei et al. 1999], and stimulates the activity of the outwardly rectifying chloride channel [Schwiebert
et al. 1999]. Expression of CFTR alters the K⁺ currents through the rectifying outer medullary potassium channels in several cell lines; activation of these channels provides, in part, the driving force for apical Cl⁻ channel activation. Therefore, they could play a critical role in maintaining normal Na⁺ and Cl⁻ balance in the airway. Stimulation of wild-type CFTR by cAMP activates aquaporin-3 protein and increases the osmotic water permeability of the plasma membrane [Schreiber et al. 2000].

Several studies have identified the interaction between the PDZ (PSD-95, discs-large, ZO-1)-binding domain at the extreme COOH terminus of CFTR and anchoring proteins of the cytoskeleton, required in the polarisation of the

**Figure 2.** The role of CFTR in the ion transport across epithelial cells. TJ: tight-junctions, G/AC: G-coupled adenylyl cyclase, Kᵦ: basal K⁺ conductance channel, ENaC: epithelial Na⁺ channel, ORCC: outward-regulated Cl⁻ channel, CaCC: Ca²⁺ regulated Cl⁻ channel, PKA: protein kinase A.
CFTR to the apical membrane, or with the Na+/H+ exchanger regulatory factor and other transport proteins [Kunzelmann, 2001]. This supports the hypotheses concerning accessory proteins, linker proteins, or regulatory cofactors that may confer CFTR regulation on separate yet closely associated ion channel proteins.

Besides its important functions in regulating ion transport, CFTR is also involved in the secretion of specific proteins from the cells in response to physiological stimulants, both in exocrine epithelia such as the pancreatic acini and in the lymphocytes [McPherson et al. 2001; Bubien 2001].

At the subcellular level, there is evidence that CFTR is involved in exocytosis and endocytosis, membrane recycling and in acidification of trans-Golgi network, prelysosomes and endosomes [Ameen et al. 2000; Barasch et al. 1991]. This is important for the processes of sulfation, sialylation and glycosylation of proteins and mucins [Scharfman et al. 1996].

CFTR may be involved in the metabolism of essential fatty acids and in their incorporation into phospholipids, which could explain the lipid imbalance found in CF patients, despite their controlled diet [Freedman et al. 1999; Buhra-Bandali et al. 2000].

CFTR is necessary for closing of gap junctional communication during an inflammatory response. Defects in this mechanism may contribute to the excessive inflammatory response of CF airway epithelium [Chanson et al. 2001].

The disease

Cystic fibrosis is defined as the presence in an individual of two defective genes previously associated with the CF phenotype. At the cellular level, the CF phenotype most commonly identified is the Cl− secretion in response to cAMP stimulation: the cells from CF patients do not have the ability to transport chloride to the same extent as normal cells. In view of the emerging functions of CFTR, testing for the CF phenotype should also include testing of these new features.

Based on their effect on protein synthesis and function, the numerous CFTR mutations are classified as follows [Vankeerberghen et al. 2002]:

- **Class I**: mutations that produce no protein due to a stop mutation or fatal errors in the CFTR mRNA synthesis,
- **Class II**: mutations in which the native CFTR fails to reach the apical membrane because of defective processing (e.g., ΔF508 CFTR is not properly folded in the ER and therefore is destroyed almost completely),
- **Class III**: mutations that produce a protein that reaches the plasma membrane but fails to respond to cAMP,
- **Class IV**: mutations that produce a cAMP-responsive channel with reduced conductance,
- **Class V**: mutations that cause reduced synthesis or partially defective processing of normal CFTR due to incorrect splicing,
- **Class VI**: mutations that produce defective regulation of other channels.

Despite the fact that CF is a monogenic disease, the genotype-phenotype correlation is very complex. Homozygosity for the common mutation ΔF508, or compound heterozygosity for ΔF508 and another mutation causes the classic phenotype: progressive obstructive lung disease, pancreatic insufficiency, male infertility and elevated sweat chloride concentrations. Each organ affected in CF requires a different level of CFTR function. Decreasing levels of CFTR function are associated with progressive involvement of more organ or systems and with more severe phenotype, in the following sequence of sensitivity: vas deferens > lungs > sweat duct > pancreas.

There are several hypotheses about how the defective CFTR leads to the airway disease (figure 3). These hypotheses try to link the abnormal composition and/or volume of the airway surface liquid (ASL) to the chronic colonization of the lung with only few characteristic pathogens (*Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Haemophylus influenzae*).

The composition, volume and physical properties of ASL depend mainly on secretions of the airway submucosal glands and the absorptive properties of the surface cells. The “high salt hypothesis” proposes that the salt re-absorption form the ASL is defective in CF, leading to inactivation of the natural antimicrobials, the defensins [Goldman et al. 1997]. The “low pH hypothesis” focuses on the role of CFTR as a HCO₃⁻ transporter and proposes an acid ASL, which inhibits cilia motility and thus promotes infection [Coakley and Boucher 2001]. Defective CFTR fails to inhibit Na⁺ and water absorption from the ASL (the “low ASL volume hypothesis”), resulting in a viscous and dehydrated mucus that impairs the mucociliary clearance and promotes bacterial colonization [Boucher 1999].

Thickening of the mucus layer of the ASL can lead to impaired diffusion of oxygen, which promotes the transformation and selection of mucoid strains of bacteria. These strains are very resistant to antibiotic treatment and constitute the foundation of the chronic colonization with *P. aeruginosa* [Worlitzsch et al. 2002]. Some evidence has suggested that wild-type CFTR functions as a receptor for *P. aeruginosa*, helping to its internalisation and bacterial clearance, while defective CFTR fails to do so [Pier et al. 1996].

Another theory on the pathophysiology of cystic fibrosis is the cellular defect of specific protein secretion in response to physiological stimuli: mucins and serous proteins from the exocrine epithelia [McPherson et al. 2001]; antibodies and cytokines from lymphocytes [Bubien 2001]. A circumstantial piece of evidence is that lung-transplanted CF patients remain chronically ill. While immunosuppressive therapy may contribute to the chronic illness, the phenomenon is more acute in CF lung-transplant patients than in non-CF lung-transplant recipients receiving the same immunosuppressive therapy. A defect
in regulated secretion of antibodies and cytokines in response to antigens may be the source of a long suspected, but as yet unproved CFTR-mediated immunological defect underlying the pulmonary morbidity and mortality in cystic fibrosis.

Once initiated, bacterial infection elicits an inflammatory response that restricts the infection to the airway but does not eradicate the pathogen. CFTR may contribute to dysregulation of the inflammatory response via opsonophagocytic mismatch, defective apoptosis, excessive oxidant formation and impaired antioxidant secretion. Bacterial phenotype transformation, damage to the epithelium and exposure of matrix proteins that increase bacterial adherence, contribute also to the prolonged inflammatory response. The vicious cycle of infection, inflammation and impaired mucociliary clearance ultimately leads to bronchiectasis and irreversibly evolves to respiratory insufficiency.

![Defective CFTR Diagram](image)

**Figure 3.** The current pathophysiological hypothesis in the cystic fibrosis airway disease [after Verkman et al. 2003].
The second important affected system in CF patients is the gastrointestinal tract. The exocrine pancreas is affected in virtually all symptomatic CF patients. Approximately 85% of CF patients have a deficiency of digestive enzymes (pancreatic insufficiency, PI) due to the obstruction of the ducts. This process begins \textit{in utero} and continues until the complete destruction of the organ. The volume of fluid secretion is reduced and salt and HCO$_3^-$ levels are lower than normal due to CFTR dysfunction. It is likely that the lack of HCO$_3^-$ leads to failure to maintain a high pH in order to prevent pro-enzyme activation and subsequently causes autolysis of the pancreatic tissue. The loss of normal volumes of pancreatic juice to rapidly flush enzymes from the pancreatic duct is probably the primary cause of pancreatic failure in CF patients [Quinton 2001].

Looking at the PI, a stronger genotype-phenotype correlation has been found, unlike in the lung, where the type of mutation does not fully predict the severity of the disease.

In addition to PI, defective CFTR in the crypts of the small intestine, in the colon, and in the biliary ducts leads to physically and biologically altered secretion of fluids and mal-digestion, which in association with defective absorptive function leads to malnutrition. Most CF patients present an altered plasma lipid profile, due mainly to pancreatic and hepatic dysfunction. In the salivary glands, $\beta$-adrenergic agonist induced mucin and amylase secretion is reduced. One characteristic form of presentation of CF is meconium ileus, the obstruction of the neonatal intestine with inspissated meconium, and its adult equivalent, distal occlusion syndrome.

Another constant feature of CF is male infertility, present in 95-97% of the patients. The majority has abnormalities in the structures derived from the Wolffian duct (vas deferens, epididymis), an argument that CFTR is involved in normal ontogenesis of these structures. It seems that the male reproductive structures require a higher level of CFTR for proper development and function, compared with the other organs and systems affected.

In addition to these CF phenotypic marks, the patients have a high frequency of sinus disease and nasal polyposis (more than 25% of the patients), liver disease (5%), and increased metabolic rate. CFTR is highly expressed in the kidney, but no pathology has been demonstrated in this organ except for a lower salt recovery capacity, presumably because of the compensatory effect of the other ion channels and the corrective effect of hyperosmolarity on the defective $\Delta F508$-CFTR.

Diagnostic criteria for CF are [Cutting 2000]:

- One or more clinical markers for CF:
  - Chronic sinopulmonary disease
  - Gastrointestinal and nutritional abnormalities
  - Salt loss syndrome
  - Male urogenital abnormalities, obstructive azoospermia

OR

- A history of CF in siblings
OR
  - A positive newborn screening test
AND
  - Evidence of CFTR dysfunction:
    o Elevated sweat chloride concentration (>60 mM)
    o Presence of CF-producing mutations in each CFTR gene
    o Characteristic abnormalities of nasal potential difference measurement.

The treatment

At present, the only treatment is symptomatic: antibiotic therapy to combat bacterial infections, mucolytic agents and constant physiotherapy to remove the viscous mucus from the airway, pancreatic enzyme supplementation to compensate for the PI, and dietary regulations to optimise energy intake. Patients with end-stage lung disease are candidates for lung lobe or heart-lung transplantation. The survival of CF patients has increased constantly over the past decades due to improvement in the healthcare and at present the average life span is 30 years. Understanding the cellular defects that follow a mutated CFTR gene helps developing etiologic treatments for the disease. There are two alternative pathways for research: pharmacological treatment and gene therapy.

Pharmacological treatment

The pharmacological treatment can be directed specifically to a certain class of mutation or can approach all types of mutations. By taking into consideration only the function of CFTR as a chloride channel, one general solution would be to bypass the defective chloride transport by using alternate chloride channels.

The Ca²⁺-activated Cl⁻ channel (CaCC) has been reported to compensate for the absence of CFTR in mediating Cl⁻ and HCO₃⁻ secretion in CF pancreatic tissue and murine gallbladder. CaCC has been identified in the human airway epithelium, although its role in ASL regulation is uncertain. Its prototypical agonists are short-lived nucleotides (ATP and UTP), and its responses are rapidly down-regulated [Zsembery et al. 2000; Clarke et al. 2000]. Development of more stable purinergic agonists is foreseeable and could be of help in improving the chloride conductance of affected epithelia.

Sodium hyperabsorption, another hallmark of CF, can be reduced by topical application of amiloride, an inhibitor of the epithelial sodium channel (ENaC), regardless of the class of mutation. However, this compound has a short half-life and a more stable analogue, benzamil, was proposed for further clinical investigation [Hofmann et al. 1998].

A small number of mutations belonging to the class I defect (e.g., G542X, R553X), causing premature stop of the translation of CFTR, can benefit from
treatment with the aminoglycoside antibiotic gentamicin. This treatment can suppress the premature stop and allow for the production of some functional protein [Wilschanski et al. 2000].

Class II mutations, produced by defective maturation of CFTR, can benefit from strategies directed to prevent CFTR retention in the endoplasmic reticulum and its eventual destruction. Low temperature and a variety of chemical chaperones can rescue the defective protein. Sodium 4-phenylbutyrate (4PBA), a drug used commonly as an ammonia scavenger in renal disorders, can reduce the formation of complexes between ΔF508-CFTR and the chaperone protein Hsc70, thereby allowing for a larger proportion of the mutant CFTR to escape to the plasma membrane. 4PBA showed positive effects both in vitro [Rubenstein et al. 1997] and in vivo [Rubenstein and Zeitlin 1998]. It should be borne in mind though that the drug can have cytotoxic effects on several cell types, and at high dosage it can inhibit chloride efflux [Loffing et al. 1999; Pelidis et al. 1998].

Substituted benzo[c]quinolizinium compounds such as MBP-07 and MPB-91 have been reported to increase the maturation of ΔF508-CFTR and the chloride conductance, both in epithelial cells and native airway epithelial cells form CF patients [Dormer et al. 2001].

S-nitrosothiols are a class of compounds naturally present in the airways of the CF patients, although at lower concentrations than in healthy subjects. S-nitroso glutathione (GSNO) at physiological concentrations has recently been shown to increase maturation of CFTR and restore the function of the cAMP-dependent chloride transport in cultured human airway epithelial cells [Andersson et al. 2002].

Once in the cell membrane, defective CFTR proteins belonging to classes of mutation II, III or IV have very little cAMP-activated chloride transport compared with the normal protein. For example, ΔF508-CFTR, when present at the cell plasma membrane has a 7-fold reduced rate of activation compared with the wild-type CFTR, even in the presence of maximal cAMP stimulation [Haws et al. 1996; Hwang et al. 1997; Wang et al. 2000]. However, their response could be increased by phosphodiesterase inhibitors such as 3-isobutyl-1-methylxanthine (IBMX) and 8-cyclopentyl-1,3-dipropyl-xanthine (CPX). These xanthines are active at very low concentrations that would not affect the intracellular cAMP concentration or the normal CFTR. It has been suggested that they interact directly with CFTR by binding at the NBD1, prolonging the burst duration and/or keeping the channel open [Cohen et al. 1997]. In addition, CPX could correct the trafficking defect of ΔF508-CFTR and induce changes in the gene expression [Srivastava et al. 1999].

Flavonoids, which act as inhibitors of tyrosine kinase, represent another interesting class of compounds for activation of normal or defective CFTR. Genistein is the prototype of this class and is naturally present in soy beans. Its clinical importance resides in the fact that it can interfere with the processes of signal transduction and genistein has been shown to suppress the growth of a
variety of cancer cells [Messina et al. 1994]. It is suggested that genistein binds to at least two sites on CFTR and prolongs the opening time of the channel [Wang et al. 1998]. However, at higher concentrations than those normally used for stimulation, genistein appears to inhibit CFTR, possibly by an interaction with NBD1 and by blocking a basolateral $K^+$ conductance [Illek et al. 1996]. Apigenin, another flavonoid, is the strongest activator of CFTR from this category.

The high-throughput screening process has recently identified a number of new compounds, some of them more powerful wt-CFTR activators than apigenin [Ma et al. 2002]. They were active at nanomolar concentration, non-toxic and CFTR-selective, but it must be noted that they were unable to activate $\Delta F_{508}$-CFTR. Recently six new classes of compounds unrelated to the wt-CFTR activators were identified, that were able to specifically activate $\Delta F_{508}$-CFTR at a nanomolar concentration, probably by a direct interaction mechanism [Yang et al. 2003]. Their clinical use in conjunction with compounds or manoeuvres that correct the defective $\Delta F_{508}$-CFTR cellular trafficking seems feasible.

Phosphatase inhibitors, benzimidazolones, chlorzoxazone, psoralens, cytochalasin-D and colchicine are just a few other pharmacological treatments with the potential of activating the mutated CFTR, once the protein has reached the cell membrane [Roomans 2001, 2003].

Genetic Treatment
Gene therapy involves the introduction of normal, healthy genes into cells in order to correct for the underlying cause of a wide variety of inherited and acquired diseases. Genetic therapy of cystic fibrosis is a favoured approach because it could replace all functions of the CFTR protein, including any that have not yet been recognised.

Effective gene therapy depends on several conditions. The vector must be able to enter the target cells efficiently and deliver the corrective gene without damaging the target cell. The corrective gene should be stably expressed in the cells to allow continuous production of functional CFTR protein. Neither the vector nor the proteins produced from it should cause an immune reaction in the patient. Also, the foreign gene should not be present in the germinal cells of the patient. The degree to which intra- and extra-cellular barriers interfere with gene delivery is dependent on the vector system and the target tissue.

In vitro studies have indicated that correcting as little as 5-6% of the cells produces a monolayer with essentially normal physiologic function at least with regard to chloride secretion and bacterial adherence [Johnson et al. 1987]. In contrast, sodium hyperabsorption requires higher levels of CFTR gene transfer (up to 90%) in order to correct the dysregulation of ENaC [Goldman et al. 1995; Johnson et al. 1995] or protein sulfation [Zhang et al. 1998]. In vivo, however, the presence of at least 5% of normal levels of mRNA CFTR
correlates with mild pulmonary disease, an argument in favour of the hypothesis that the chloride defect is the major culprit in cystic fibrosis [Ramalho et al. 2002].

The target of gene transfer is still under debate, since CFTR is expressed at widely different levels in cellular populations with different patophysiological relevance. The choice is between the surface airway epithelium, the submucosal glands, or their currently unknown precursor stem cells [Jiang and Engelhardt, 1998].

Initial clinical trials provided proof for gene transfer to the airways, the most accessible for transfection, but efficiency was low and limited in time [Griesenbach and Alton 2001]. One explanation could be that the viral promoters used for controlling the expression of CFTR gene are rapidly downregulated and the target airway cells have a limited life-span of a few weeks. Improvement of plasmid constructs and targeting of the airway stem cells could be an option.

Several vectors are under continuing investigation [Flotte and Laube 2001; West and Rodman 2001]. Liposomes made of polycationic lipids and cationic polymers that attach the plasmid DNA are favoured because of their low adverse effects and lack of immune response, but their efficiency and the duration of CFTR production in the target cells are low. Increased toxicity at doses that become therapeutic and the inability to transfect non-dividing cells are also major obstacles. Adenoviruses can efficiently infect lung cells; in humans they naturally cause airway infections, such as the common cold. The first generation of adenovirus-based vectors lacked parts of the viral genome to prevent virus reproduction in the patient cells and carried instead the CFTR gene. The modified adenovirus vector still produced some viral proteins that stimulated the patient’s immune responses and limited its applicability. Adeno-associated viruses are small viruses that infect human cells without causing an inflammatory or immune response. Their safety was proven by phase II clinical trials [Wagner et al. 2002], and a further study showed small indication of improved lung function [“Targeted Genetic” press release, 2003]. Retroviral and lentiviral vectors offer several potential advantages for attaining persistent expression of a therapeutic gene in airway epithelia. However, several safety problems have limited their application [Blomer et al. 1997; Wang et al. 2000].

Because CF is produced mainly by point mutations, one alternative to cDNA-based gene therapy strategies is to correct endogenous mutant sequences by targeted replacement with the wild-type homologue. Small fragments of genomic wt-CFTR DNA (400-800 base-pairs) were transfected into CF epithelial cells with positive results in vitro [Goncz et al. 2001].

Another option is to use artificial chromosomes (minichromosomes), which are claimed to be the ideal vectors for gene therapy: they are stably retained in the host cells, not immunogenic, protected from mutagenesis and large enough to incorporate a large gene as that of CFTR with its promoter and regulator regions [Auriche et al. 2002].
End-point measurements

The clinical end-point of any rationale treatment of cystic fibrosis should be the improvement of life quality and longevity of the CF patients. Patients with CF develop already from birth severe and irreversible structural changes in several organs and systems, including lung, pancreas and intestine. Thus the treatment will only be able to slow down further deterioration of their condition.

The platform for developing curative treatments spans a large array of cystic fibrosis models, from cell lines naturally or heterogeneously expressing the human CFTR, to CF animal models (mouse, and soon sheep), tissue from CF patients (nasal, bronchial or colonic epithelium) and patients enrolled in clinical trials. Each of these models has advantages and disadvantages, which encourages their concurrent use.

The lung disease is considered the main target, because it is the cause of death in 90% of the patients. Dynamic indicators such as pulmonary parameters (the forced expiratory volume in the first second - FEV1, the frequency of infections, the levels of inflammatory cytokines), body mass index, frequency of hospitalization, etc., are the most important for deciding whether a treatment is beneficial on long-term. Considering that the population of patients available for trials is limited, and the need to use non-invasive outcome measurements, most of the drug/therapy development process has to be done with end-point measurements at the cellular level, where changes in CFTR activity should be easy to determine after only a short-term treatment.

At the cellular level, end-point measurements are CFTR expression and function. Expression can be tested at several levels: gene expression, mRNA production, protein synthesis, maturation and membrane insertion. However, CFTR function is the most relevant indicator of the treatment and the most commonly used end-point measurement is the ability to transport chloride in response to physiological stimuli. This can be measured by a variety of methods: patch-clamp, trans-epithelial currents, radioactive or fluorescent indicators of chloride efflux, X-ray microanalysis. In addition, end-point measurements of the other function of CFTR relevant for CF patophysiology need to be tested, such as the ability to secrete bicarbonate, the regulation of other ion channels (most importantly of ENaC), protein glycosylation, or bacterial adherence/clearance properties.
The overall aim of the studies included in this thesis was to gain further knowledge of cystic fibrosis genotype-phenotype correlations, develop end-points measurements and rationale treatment strategies.

The specific aims of the individual papers were to:

- Establish a method for studying ion transport in normal and cystic fibrosis nasal epithelial cells by X-ray microanalysis,
- Study chloride transport in nasal epithelial cells from CF patients and investigate a possible correlation between chloride transport in these cells and the phenotype of the CF patients,
- Study the properties of a common Portuguese CFTR mutation,
- Study the effect of chronic treatment with colchicine on chloride efflux in airway epithelial cells,
- Test a non-viral transfection system, the effect of heparin on viability and the correlation between transfection and phospholipid composition of the cell membranes.
Methods

Several methods were used to determine the expression of CFTR at the molecular and cellular level (Western blot, pulse-chase, immunocytochemistry), its functionality as a chloride channel (X-ray microanalysis and MQAE fluorescence assay), cell morphology (fluorescence and electron microscopy), expression of transfected genes (flow cytometry) or multidrug resistance proteins (Western blot), lipid composition of cell membranes (gas chromatography). A summary of these methods will follow, while for detailed information the reader is referred to the individual papers.

Cells

Nasal epithelial cells

Epithelial cells were harvested with 0.6 mm sterile cytology brushes from the inferior nasal turbinate of healthy volunteers or CF patients that had given informed consent. The projects were approved by the Ethical Committee of Huddinge University Hospital, Sweden.

For paper I, we used cells from 16 patients (mean age 29 years), 8 patients with the genotype ΔF508/ΔF508 and the rest with compound heterozygote genotype. For paper II, a total of 19 CF patients with severe genotype participated in the study. All patients were in good clinical condition, without signs of low-grade infection. Their clinical score and average age were representative for the Swedish adult cystic fibrosis population.

The cells were transported and preserved alive in Ham’s F-12 culture medium (Gibco BRL, Grand Island, NY, USA) supplemented with 100 µg/ml streptomycin and 100 UI/ml penicillin. The cells were cultured overnight under regular conditions (37°C and 5% CO₂/air) prior to experiments.
Cultured cell lines

Cells were grown under regular conditions on impermeable plastic supports in medium supplemented according to the requirements of each cell line.

Normal human bronchial epithelial cells 16HBE14o- (16HBE), their cystic fibrosis counterparts CFBE41o- (CFBE, homozygous for the ΔF508 mutation) and the cystic fibrosis submucosal epithelial cell line CFSMEo- (CFSME, genotype ΔF508/unknown) were cultured in adherent flasks (Sarstedt, Landskrona, Sweden) in EMEM (SVA, Uppsala, Sweden) supplemented with 10% foetal calf serum, 100 UI/ml penicillin and 100 µg/ml streptomycin sulphate. The normal human serous cell line Calu-3 (ATCC, Manassas, VA, USA) was grown in a similar medium, containing 1 mM sodium pyruvate and 1% non-essential aminoacids (both from Sigma, St. Louis, MO, USA). The human colonic adenocarcinoma cell line T84 was grown in DMEM:Ham's F-12 medium (SVA) supplemented with 6% foetal calf serum, 15 mM HEPES, and antibiotics.

Baby hamster kidney (BHK) cell lines stably expressing wild type (wt)-CFTR, ΔF508-CFTR or A561E-CFTR were generated by transfection and CFTR-expressing cells were selected and further cultured in DMEM medium (SVA) supplemented with 10% foetal calf serum containing 500 µM methotrexate (Apoteket, Stockholm, Sweden). For determining the effects of low temperature on the processing of CFTR, in paper III cells were grown at 26°C for 24 and 48 hours.

For paper IV, colchicine-resistant cells were obtained by culturing the original cells in medium containing 0.5 nM colchicine (Sigma) until confluence and incrementally increasing the concentration with 0.5 nM after each passage, up to 4-6 nM colchicine.

Protein expression

Western blotting

For the evaluation of CFTR expression in paper III, the cells were lysed with Laemmli sample buffer and total protein extracts were analysed after separation by SDS-PAGE on 7% polyacrylamide mini-gels followed by transfer onto nitrocellulose filters. The filters were probed with the mouse monoclonal M3A7 anti-CFTR antibody (Chemicon International, Temecula, CA, USA) and developed using the enhanced chemiluminiscent reagent (ECL) detection system (Amersham Pharmacia Biotech, Buckinghamshire, UK).

For immunodetection of the multidrug resistance (MDR) protein in paper IV, the cells were grown to confluence on adherent plastic flasks and collected.
by trypsinization. The cell line T84 was used as positive control. Proteins were extracted with Laemmli lysis buffer and the total protein concentration was determined by a modified Pierce method (Bio-Rad Laboratories, Hercules, CA, USA). Protein samples were run on a 7.5% SDS-polyacrylamide gel then transferred to a nitrocellulose membrane. The membranes were probed with a rabbit anti-hMDR polyclonal antibody (H-241 from Santa Cruz Biotechnology, Inc., USA) and detected using the ECL system. Semi-quantitative assessment of MDR expression was done by densitometry of immunoblots radiographs using the ImagePro 4.5 software (Media Cybernetics, Silver Spring, MD, USA).

Pulse-chase and CFTR immunoprecipitation

In paper III, metabolic labelling and immunoprecipitation were carried out essentially as described [Farinha et al. 2002]. After incubation in methionine-free α-Minimal Essential Medium (MEM) for 30 min, cells were pulse-labelled in the same medium containing 100 μCi/ml ^{35}S)methionine (>1000 Ci/mmol; ICN Biomedicals, Irvine, CA, USA) for 30 minutes at 37°C. For chasing, the labelling medium was replaced with 5% serum and 1 mM methionine for indicated times. Immunoprecipitates obtained with M3A7 anti-CFTR antibody were analysed by SDS-PAGE and fluorography. Fluorograms were analysed by ImageMaster software (Amersham Bioscience).

CFTR immunocytochemistry

For paper III cells were grown on glass slides (Nalge Nunc, Roskilde, Denmark) at 37°C or 26°C for 48 hours, rinsed twice with cold phosphate buffered saline (PBS) and fixed in 4% formaldehyde, 3.7% sucrose in PBS. The cells were permeabilized with 0.2% Triton X-100 in PBS for 20 minutes, and blocked with 1% bovine serum albumin (BSA)/PBS for 45 minutes prior to incubation overnight at 4°C with M3A7 anti-CFTR antibody. The cells were then washed 3 times with PBS, for 10 minutes each, and incubated with the fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG (Jackson ImmunoResearch Laboratories, Baltimore, MD, USA) diluted 1:100 for 45 minutes. The slides were mounted with Vectashield (Vector Laboratories, Burlingame, CA, USA) containing DAPI (4,6-diamino-2-phenylindole, from Sigma) for nuclei staining and covered with a glass coverslip. Immunofluorescence staining was observed and recorded on an Axioskop fluorescence microscope (Zeiss, Jena, Germany) with the Power Gene 810/Probe & CGH software system (PSI, Chester, UK).

For paper IV, cells grown on glass coverslips were fixed in methanol at –20 °C for 5 minutes, rinsed with Tris-buffer (TBS) (150 mM NaCl, 10 mM Tris-HCl pH 8.0), permeabilized with 0.2% saponin, then incubated with the mouse monoclonal MATG-1061 anti-CFTR antibody (Transgene, Strasbourg, France)
diluted 1:500 in TBS, for 1 hour at room temperature. After rinsing, the cells were incubated with an HRP/Fab polymer conjugate followed by 3-aminobis(ethylcarbazole) (AEC) chromogen detection (Zymed Laboratories Inc., San Francisco, CA, USA). The nuclei were counterstained with haematoxylin for 1-2 minutes and the cover slips were mounted in aqueous medium (Aquatex, from Merck, Darmstadt, Germany). Pictures were taken with an optic microscope equipped with a digital camera (Leica Microsystems Ltd., Heerbrugg, Switzerland).

**CFTR function**

**X-ray microanalysis**

The nasal epithelial cells resuspended in a small volume of medium were seeded onto titanium grids pretreated with Formvar (Merck) and Cell-Tack (Becton Dickinson, Bedford, MA, USA). The cells were incubated 30 minutes at 37°C to let them adhere to the grids.

As a control, the grids with cells were incubated for 5 minutes at room temperature in standard Ringer’s solution (SR): 140 mM NaCl, 5 mM KCl, 5 mM glucose, 5 mM N-(2-Hydroxyethyl)piperazine-N’-(2-ethanesulfonic acid) (HEPES), 1.5 mM CaCl₂ and 1 mM MgCl₂. In order to stimulate the chloride efflux through the CFTR channel the experimental grids were incubated in SR containing 20 µM forskolin and 100 µM IBMX, (both from Sigma) for 5 minutes at room temperature. The chloride efflux through the calcium-regulated channel was stimulated by incubating the grids in SR containing 100 µM ATP (Sigma) for 5 minutes at room temperature.

At the end of the incubation the cells were briefly washed (5-10 seconds) in order to remove the experimental solutions with one of the following isotonic solutions: ice-cold distilled water, 0.3 M mannitol, 0.3 M glucose or 0.15 M ammonium acetate. The grids were rapidly frozen in liquid propane cooled by liquid nitrogen, freeze-dried overnight and slowly warmed to room temperature. The grids were then covered with a thin conductive carbon layer to prevent charging in the electron microscope.

X-ray microanalysis (XRMA) of the intracellular elemental content was performed in a Hitachi H7100 electron microscope in the scanning-transmission mode at 100 kV accelerating voltage with a Link ISIS energy-dispersive spectrometer system (Oxford Instruments, Oxford, UK). Due to the spreading of the electron beam, the measurements reflect the concentration of the elements at the cellular level. For each experiment, 20–60 measurements were made on separate cells or clusters.
Quantitative analysis was carried out based on the ratio of characteristic peaks to background intensity ratio in the same energy region and compared with those obtained on standards [Roomans, 1988]. Elemental concentrations are expressed as millimols per kilogram dry weight (mmol/kg). Phosphorus was used as an internal standard to correct for the effects causing specimen-unrelated variation in the concentration data.

**MQAE fluorescence assay**

The fluorescence assay for measuring the intracellular chloride concentration and efflux is based on the quenching properties of halides on a quinoline compound fluorescence (N-(ethoxycarbonylmethyl)-6-methoxyquinolinium bromide, MQAE). The relationship between fluorescence intensity and halide concentration under physiological conditions is described by the Stern-Volmer equation [Stern and Volmer 1919]:

$$\frac{F_0}{F} - 1 = K_{SV} \cdot [Q]$$

(1)

where

- \( F_0 \) is the fluorescence intensity in the absence of halides or other quenching ions (expressed as arbitrary units, a.u.),
- \( F \) is the fluorescence intensity in the presence of quencher (a.u.),
- \( K_{SV} \) is the Stern-Volmer constant (l/mol),
- \([Q]\) is the concentration of the quencher (i.e., the halides) in mol/l. For physiological experiments the only relevant halide is chloride, so \([Q]\) actually represents the intracellular chloride concentration.

All these values are defined in the absence of background fluorescence.

In paper II the nasal cells were centrifuged and resuspended in 50 µl of SR; and loaded with 10 mM MQAE (Molecular Probes, Eugene, OR) for 45 minutes. The cells were attached to glass coverslips coated with Cell-Tack and were placed at the bottom of a perfusion chamber on the stage of an inverted microscope (Diaphot; Nikon, Tokyo, Japan). The temperature was maintained at 37°C by heating the chamber holder and the objective separately. A monochromator, part of a Quanticell 700 image-processing system (VisiTech International, Sunderland, U.K.), provided excitation light at 353 nm (10 nm bandwidth). The emission was measured at 460 nm using an analogue CCD camera. The cells were bathed in SR, and clusters of cells with beating cilia were chosen for analysis.

The MQAE signal was calibrated against [Cl\(^-\)]; by exposing the cells to a K\(^+\)-rich HEPES buffer (pH 7.0) containing various Cl\(^-\) concentrations, with NO\(_3\) as the substituting anion. The ionophores tributyltin acetate (20 µM) and nigericin (20 µM) were used to equilibrate [Cl\(^-\)] and the extracellular chloride concentration. Nigericin abolishes transcellular H\(^+\) and OH\(^-\) gradients.
intra- and extracellular OH\textsuperscript{−} activities are the same, cell chloride activity approximately equals extracellular chloride activity by the action of tributyltin \cite{Krapf1988}. At the end of the experiments, the background fluorescence was obtained by quenching the MQAE signal with a HEPES-buffered KCSN (150 mM, pH 7.2) solution. The Stern-Volmer quenching constant $K_{SV}$ for the nasal epithelial cells was calculated to be 12.5 M\textsuperscript{−1} in a separate set of experiments.

For the chloride efflux experiments, chloride efflux was induced by changing from a 150 mM chloride buffer to a chloride-free buffer with NO\textsubscript{3} as the substituting anion. Each experiment measured first the basal efflux, and then the efflux after stimulation with forskolin (20 µM) plus IBMX (50 µM) or with ATP (100 µM). Forskolin plus IBMX were added 4 min and ATP 1 min before anion substitution. At the end, $F_{20}$ (fluorescence at [Cl\textsuperscript{−}] = 20 mM) and autofluorescence were determined in the presence of ionophores. The rate of efflux, $dCl / dt$, was calculated from \cite{Chao1990}:

$$
\frac{dCl}{dt} = \frac{F_0}{K_{SV} \cdot (F_{Cl})^2} \cdot \frac{dF_{Cl}}{dt}.
$$

$dF_{Cl} / dt$ was determined from the initial changes in $F_{Cl}$ after changing to the NO\textsubscript{3} buffer. The difference between the unstimulated and stimulated efflux for each experiment was calculated, and the data represented as the average difference based on all clusters analysed from each patient. A response of more than 0.05 mM/s was considered positive. At least two or three experiments were done for each patient, except for three non-responding patients on whom only one measurement was carried out.

For papers III, and IV, cells were cultured on glass slides, loaded with MQAE and efflux experiments performed as described above. The agonist cocktail used for activating CFTR consisted of 20 µM forskolin and 100 µM IBMX and for the intracellular calibration the buffer contained the ionophores tributyltin (10 µM) and nigericin (10 µM). \textit{In situ} double point calibration was used for determination of $K_{SV}$ for each experiment.

In order to increase the objectivity of chloride efflux rate measurements, a series of computer-aided steps was implemented, bypassing most of the manual conversion. For example, the inevitable process of MQAE dye bleaching and leaking can be fitted by a simple exponential function, (during the initial period of exposure to SR, \textit{i.e.}, under chloride equilibrium conditions) and the constant of decay determined can be used for correction \cite{Kaneko2001}.

The next step, transformation of the MQAE fluorescence into chloride concentration, requires the use of the Stern-Volmer equation (equation 1), where $K_{SV}$ and $F_0$ need to be known. Until recently, the general approach was to determine the $K_{SV}$ value in a separate set of experiments and to assume that the rest of the cells measured for the actual experiments have the same
quenching constant [Eberhardson et al. 2000]. For different experimental settings, this assumption could lead to erroneous results, since the quenching value in a cell depends on a series of variables: viscosity, osmotic pressure, protein content, cytoskeleton, buffering capacity and temperature, and is thus unique for each cell [Krapf et al. 1988; Oliver et al. 2000; Kaneko et al. 2001]. This is highly relevant for the experiments where the treated cells were grown and examined at lower temperature (paper III) or were expressing high levels of MDR protein compared to control cells (paper IV).

The $K_{SV}$ problem can be easily solved if a double-point calibration is performed at the end of each experiment. If intracellular calibration of MQAE fluorescence is performed for example for the values of 20 and 80 mM Cl$^-$, we can record $F_{20}$ and $F_{80}$ respectively. It is important to mention that $F_0$ is difficult to obtain and prolonged exposure of cells to chloride free solution can lead to cell damage.

By solving equation 1 for these values, we obtain:

$$K_{SV} = \frac{F_{80} - F_{20}}{0.02 \cdot F_{20} - 0.08 \cdot F_{80}},$$

(3)

and

$$F_0 = F_{20} \cdot (1 + 0.02 \cdot K_{SV}).$$

(4)

The intracellular chloride concentration [Cl$^-$] for each time-point is immediately solved as

$$[\text{Cl}^-](t) = \left( \frac{F_0}{F_t} - 1 \right) \cdot \frac{1}{K_{SV}}.$$  

(5)

In order to determine the chloride efflux rate, one simple procedure would be to use $d[\text{Cl}^-]/dt$, or the slope of the plot $\Delta[\text{Cl}^-]/\Delta t$, in the discontinuity points ($t_0$) as an expression of the chloride efflux. Due to the delay of the cell response and because the graph is not smooth, these discontinuity points cannot be determined exactly. The first derivative of [Cl$^-$], usually produces a plot where it is obvious that the estimation of the discontinuity point and of the chloride efflux is difficult (figure 4).

Under standard conditions, the chloride efflux in non-excitable epithelial cell systems follows the laws of passive diffusion, down its electrochemical gradient and can be described by a transcendental equation following a generalization of the Nernst-Planck equation. Because the extracellular volume is much larger than the intracellular volume, and rapidly renewed, the efflux experiment is performed under “sink conditions” and after a finite time, the electrochemical gradient and the flux will equal 0. As long as the cell is exposed to isotonic solutions, the changes in the cell volume inherent to chloride efflux should be minimal. Indeed, the measurement of the relative cell volume made with the
fluorochrome fura-2 at its isobestic wavelength showed changes of less than 10% during exposure of the Calu-3 cells to the experimental solutions (data not shown). Hartman and Verkman [1990] have mathematically modelled the transport regulation of the airway epithelial cells and found that changes of volume less than 10% have very little influence on the model prediction.

Under these conditions the flux equation $J$ for a single ion is:

$$ J = -D \cdot \left[ \frac{dC}{dx} + \frac{Z \cdot F}{R \cdot T} \cdot C \cdot \frac{d\Psi_M}{dx} \right] $$

(6)

where

- $D$ = the diffusion coefficient (cm$^2$/s),
- $C$ = the molar concentration of the ion (mols/l),
- $x$ = the distance from the reference point on the diffusion path (cm),
- $Z$ = the valence of the ion,
- $F$ = Faraday constant (9.6 $10^4$ C/mol),
- $R$ = the ideal gas constant (8.31 J/mol·K),
- $T$ = the absolute temperature (K),
- $\Psi_M$ = the membrane electric potential.

The minus sign indicates the direction of the diffusion.

This equation does not give information on the time dependency for the global rate of diffusion. For a cell system, the diffusion coefficient $D$ at a certain moment depends on the number of specific channels for the ion in the membrane that are open. In case of a sudden increase in Cl$^-$ conductance, the result is an immediate depolarisation leading to changes in the basolateral K$^+$

Figure 4. The plot of intracellular chloride concentration (a) and efflux (b). The rectangle above the time axis indicates the chloride concentration (mM) in the extracellular buffer.
conductance as well (an increase in the activity of the Na⁺/K⁺/2Cl⁻ symport), and within a short time to a new value for the equilibrium membrane potential [Hartman and Verkman 1990]. In the absence of agonists, or in the presence of a constant and prolonged exposure to pharmacological agonists, the number of channels open in the membrane (thus \( D \)) is constant in time. Because epithelial cells have probably no voltage-activated channels in their membrane, the concentration gradient will not affect the number of open channels in time. Thus, the parameters that can affect the rate of diffusion are \( dC/d\xi \), the concentration gradient and \( d\Psi_M/d\xi \), the electrical gradient.

In epithelial secretory cells chloride transport is electroneutral, meaning that \( \Psi_M \) is also constant in time and uniformly distributed across the membrane, giving a constant contribution of the second term to the flux. Under these assumptions the ion transport follows the global flux equation [Macey, 1980]

\[
J = P \cdot Q(Z) \cdot \left( C_0 - C_i \cdot e^{\frac{Z F \Psi_M}{RT}} \right),
\]

where
- \( P \) = the permeability coefficient (cm/s),
- \( Q(Z) \) describes the global membrane potential distribution across a membrane,
- \( C_0 \) = the extracellular ion concentration,
- \( C_i \) = the intracellular ion concentration.

The term \( C_i \cdot e^{\frac{Z F \Psi_M}{RT}} \) represents the value of intracellular chloride concentration in the presence of a membrane potential, and the only time dependence is given by \( C_i \). Since all the other parameters in equation 7 are constant in time, the solution in the case of chloride efflux in the epithelial cell system can be described by an exponential function:

\[
J_{[CT]} = P \cdot Q_{[CT]} \cdot \Delta C(0) \cdot e^{-t/\tau},
\]

\( \Delta C(0) \) = the initial gradient of concentration,

\( \tau \) = the exchange time constant.

With the following notation \( J(0) = P \cdot Q_{[CL]} \cdot \Delta C(0) \), the chloride efflux rate can be written as

\[
J_{[CT]} = J(0) \cdot e^{-t/\tau}.
\]

The extremis of this function is \( J(0) \), the initial rate of efflux, which is dependent on the initial chloride gradient and the membrane permeability to chloride. The exchange time \( \tau \) and the initial chloride efflux \( J(0) \) are thus the
parameters that best characterize the activity of the chloride channels involved in the efflux.

Most software analytical programs have built-in equations or can be programmed to fit an exponential function to the experimental data using the template:

\[
\begin{align*}
\text{If } t &< t_0, \text{ then } f(t) = \text{Plateau} \\
\text{If } t &\geq t_0, \text{ then } f(t) = \text{Bottom} + (\text{Plateau} - \text{Bottom}) \cdot e^{\text{-}K \cdot (t - t_0)} \\
\end{align*}
\]

The term \((\text{Plateau} - \text{Bottom})\) actually represents the initial gradient of concentration \(\Delta C(0)\) from equation 8 and \(K\) is \(1/\tau\).

The software uses a least-sum-of-squares method and a convergence criterion to fit the data to the theoretical curve. The regression line on the data plot is drawn and the parameters of the theoretical curve are returned: \(\text{Bottom}, \text{Plateau}, K, t_0\), the goodness of fit \(R^2\), etc. These parameters are now sufficient to determine the initial chloride efflux rate \(f(0)\) and describe the membrane permeability for chloride transport.

The chloride efflux rate is defined for the fitting equation as in the next template:

\[
\begin{align*}
\text{If } t &< t_0, \text{ then } df(t)/dt = 0 \\
\text{If } t &\geq t_0, \text{ then } df(t)/dt = -K \cdot (\text{Plateau} - \text{Bottom}) \cdot e^{\text{-}K \cdot (t - t_0)} \\
\end{align*}
\]

Using this setup, an objective and reproducible method for determining the intracellular chloride concentration and efflux was established and used for the current experiments.

The successive exposure of the same cells to different conditions allows a powerful and meaningful comparison of the effects of agonists in a paired-way fashion. This is helpful especially when cells behave heterogeneously and the variability of responses impairs statistical analyses. At the same time it offers an advantage when scarce and sensitive material such as cells collected from human donors is to be examined. Although there are reports that cells behave differently after successive exposure to a chloride gradient [Davidson and McLachlan, 2002], the cells used for this study were tested and found to respond identically and thus the difference noticed in the agonist exposed response can be attributed completely to the agonist used.
Morphology

Cytoskeleton
For immunostaining of the cytoskeleton, cells were grown on glass coverslips until confluent and rinsed with 37°C warm phosphate buffer saline (PBS, from SVA). Cells were fixed in 37°C warm cytoskeleton stabilising buffer (10 mM HEPES pH 6.9, 138 mM KCl, 3 mM MgCl₂, 2 mM EGTA, 0.2% TritonX-100) with 4% formaldehyde for 20 minutes. Then, unspecific epitopes were blocked with 1% BSA in TBS containing 0.05% Tween-20 (TBST-BSA) for 30 minutes. The glass slides were incubated for 1 hour with the monoclonal mouse antibody anti-α-tubulin (Sigma) diluted 1:200 in TBST-BSA.

After rinsing, the cells were incubated for 1 hour in the goat anti-mouse rhodamine-conjugated secondary antibody (Southern Biotechnology Associates, Birmingham, AL, USA) diluted 1:50 in TBST-BSA and 5 µg/ml Phalloidin-FITC (Sigma). The coverslips were rinsed and mounted in 90% glycerol, 2% N-propyl-gallate, pH 9 containing 1 µg/ml DAPI.

Pictures were taken with a fluorescence microscope equipped with a digital camera (Leica).

Electron Microscopy
For transmission electron microscopy (TEM), the cells were cultured and transfected as described. Two hour post-transfection the cells were washed once with PBS (SVA) and then fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer overnight. Then the cells were post-fixed with osmium tetroxide, dehydrated in a graded ethanol series, embedded in Agar 100 epoxy resin (Agar Scientific, Standsted, UK), and sectioned for TEM. Sections were contrasted with uranyl acetate/lead citrate and micrographs were taken with a Hitachi 7100 TEM.

Transfections
For transfections, the cells were grown on 24-well plates, until 50-70% confluent, and then transfected with a green fluorescent protein plasmid, pEGFP.Luc (Clontech, Palo Alto, USA) at 1 µg/well complexed with the cationic commercial liposome Geneporter2 (GP) (Gene Therapy Systems, San Diego, USA) or with the 25 kDa Polyethylenimine (PEI) (Sigma-Aldrich), in serum- and antibiotic-free medium.

At the end of the incubation (2 hours) the cells were thoroughly rinsed with medium containing serum and further cultured under normal conditions. At
specified intervals after transfection, low molecular weight heparin (average molecular weight 3000 Da, from Sigma) was added to the cells to a final concentration of 10 µM, for 24 hours.

24 hours post-transfection, and the successive days, the cells were collected by trypsinization and preserved in suspension on ice until their analysis in a BD-FACSCalibur™ flow cytometer (Becton Dickinson Biosciences, San Jose, CA, USA). Using the software BD-CellQuest, (Becton Dickinson) 10000 cells were analysed for each sample. Staining of dead cells with 50 µg/ml propidium iodide (Sigma) was used in order to identify dead cells specific position in a FSC/SSC plot, and gates of living cells were drawn accordingly [Ross et al. 1989]. Transfection efficiency was measured by determining the percentage of green fluorescent cells in the population of living cells. In order to study the cytotoxicity, the percent relative viability was used, expressed as (percentage of treated alive cells)/(percentage control alive cells) ×100.

Lipids

For lipid analysis, cells were grown under regular conditions in T75 flasks, and when confluent, collected by trypsinization, rinsed twice in physiological saline and preserved at −70°C until further processing.

The lipids from the cellular membranes were first extracted in a methanol:chloroform mixture, then separated on chromatographic columns, followed by trans-methylation of the phospholipid fraction. The fatty acid composition was characterised by capillary gas-chromatography, (Hewlett-Packard GC5880A), equipped with a 30 m HP225 column (inner diameter 0.32 mm) with helium as the carrier gas. Identification of fatty acid peaks was made by comparison with authenticated standards (Sigma, Sweden). Results are expressed as molar percentage of total fatty acid [Ellin et al. 1991].

Statistical analysis

Where appropriate, Student's t test for paired data or one-way analysis of variance (ANOVA) followed by Dunnet's or Bonferroni's post-hoc test for multiple comparison of selected pairs of means was used. The level of significance was set to a p value less than 0.05. Spearman's non-parametric correlation analysis was used to study the relationship between the chloride efflux response and phenotypic parameters.
Results and Discussion

The results of this thesis can be divided into three categories according to the main focus: investigation of the functions of CFTR, characterization of patient genotype-phenotype correlation and testing therapeutic strategies.

Investigations of CFTR function

X-ray microanalysis of nasal epithelial cells

X-ray microanalysis of whole cells in the scanning-transmission electron microscope represents a rather simple and direct way of measuring simultaneously several chemical elements of biological interest. Nasal epithelial cells have a series of advantages over other types of cell systems for studying cystic fibrosis. The cell harvesting procedure is simple and minimally invasive, and can be repeated at short intervals of time, the properties of the freshly isolated cells are presumably closest to their in vivo counterparts. In general the genotype is known and experiments can be more versatile. Measuring the chloride efflux in these cells has been considered representative for the rest of the ciliated airway epithelium [Stern et al. 1995].

When the X-ray microanalysis technique is used to determine the concentration of diffusible ions in whole cells, special handling of the specimen is needed. In order to correctly determine the intracellular composition of the cells, a washing step is necessary to remove the experimental solution, rich in NaCl, from the surface of the cells. Our results suggest that freshly isolated nasal cells from non-CF subjects are very sensitive to the type of rinsing, because rinsing with cold water or ammonium acetate yielded a K/Na ratio much lower than that normally obtained for living cells. Furthermore, attempts
to stimulate chloride efflux through the CFTR channel under these experimental conditions did not result in any significant changes in ion composition, which suggests that this rinsing method introduces artefacts. In contrast, rinsing with isotonic mannitol preserved the ionic composition. Stimulation of cells from healthy controls with forskolin and IBMX in a chloride-containing medium caused a significant (28 ± 6%) decrease in chloride concentration, which is indicative of net chloride efflux. Under similar conditions, stimulation with ATP induced a 29 ± 5% decrease in the chloride concentration. Stimulation of cells from CF patients with forskolin and IBMX in a chloride-containing medium caused no significant change in the intracellular chloride concentration, while ATP stimulation induced a similar response to that obtained in cells from healthy controls. Thus, X-ray microanalysis of nasal epithelial cells may be used to determine chloride secretion in an easily accessible cell type from CF patients.

A new class II mutation

The novel CF missense mutation A561E, in which alanine is replaced by glutamic acid at position 561 of the CFTR polypeptide accounts for 3% of Portuguese CF genes. A561E, located at exon 12, is part of a cluster of missense mutations that affect the highly conserved amino acid residues within the putative nucleotide-binding fold, NBD1 of CFTR. Functional assessment of native colonic epithelia of patients showed that CFTR-mediated Cl- secretion was absent in these patients [Hirtz et al. 2003, submitted for publication].

In order to explore the molecular mechanism that leads to defective Cl- transport in A561E patients we have studied the processing, localization and function of A561E-CFTR overexpressed in BHK cell lines. The results demonstrate that A561E belongs to the trafficking defect or class II mutations. Like ΔF508, A561E-CFTR is not processed correctly and, as a consequence, is not delivered to the plasma membrane. Instead, the core-glycosylated immature form of A561E-CFTR is retained in the ER where it is degraded at the same rate as ΔF508-CFTR. The properties of A561E-CFTR revert towards those of wt-CFTR as the incubation temperature is reduced. When the processing defect is partially corrected, cAMP-regulated Cl- channels appear in the plasma membrane indicating that A561E mutation does not completely abolish CFTR function.

As suggested for ΔF508, the retention of A561E-CFTR in the ER is probably caused by abnormal folding that is recognized by the cellular quality control machinery. Theoretically, A561E could be restored to the protein trafficking pathway by manipulation of chaperone protein/CFTR interactions with chemical chaperones or drugs that affect gene regulation such as genistein and xanthines. Thus, the pharmacological therapies that have been developed
for patients bearing ΔF508 could be also useful for those with the A561E mutation.

Investigation of patients

CF is a complex disease, with a diverse spectrum of mutations and clinical phenotypes. Genotype–phenotype studies show that the correlation between genotype and phenotype differs between organs, and is highest for the pancreatic status and lowest for the pulmonary disease [Mickle and Cutting 2000; Zielenski, 2000]. Parameters other than the CFTR mutation, i.e., genetic [Rozmahel et al. 1996] and/or environmental, may play a role in the disease. In the course of a study, in which we were collecting baseline data on cAMP-dependent chloride transport in nasal epithelial cells from CF patients with severe mutations in order to determine the effects of pharmacological treatment, we found unexpectedly that three of the 19 patients had functional cAMP-dependent chloride transport, comparable with the non-CF subjects. We then wanted to test the hypothesis that residual functional cAMP-activated chloride transport in CF patients with severe mutations is related to a milder phenotype.

No significant relationships were seen between the response to cAMP and phenotypic parameters like the Shwachman score, the Bhalla score, the number of antibiotic courses, bacterial colonization of the lower respiratory tract, or the levels of polyunsaturated fatty acids in serum phospholipids.

The CF patients in the study were homozygous for the ΔF508 mutation, or heterozygous with ΔF508 combined with 394delTT, 3659delC or 2183AA→G, where no or little CFTR is believed to reach the plasma membrane. However, recent studies have shown that the ΔF508-CFTR is located in the apical region of ciliated cells from the airway [Kälin et al. 1999; Penque et al. 2000]. The presence of the ΔF508-CFTR in the plasma membrane could explain the chloride secretion seen in cells from some of the CF patients in the present study, although so far no studies have established a connection between the location of the ΔF508-CFTR at the subcellular level and the rate of chloride efflux by an individual cell.

In a study on ΔF508 homozygous children [Sermet-Gaudelus et al. 2000], patients who still showed activity of the cAMP-stimulated anion-conducting pathway also had less severe disease with regard to respiratory function and nutritional status. In adult patients many additional factors, such as clinical routines and compliance with treatment, are likely to influence clinical status. Selection of adult patients may exclude some extremely severe cases, but the population selected for the present study was representative of the adult Swedish CF patient population described recently [Schaedel et al. 2002].
A factor that could possibly influence the variation in phenotype for the same genotype may be altered fatty acid metabolism of the patients [Carlstedt-Duke et al. 1986; Strandvik et al. 1996; Roulet et al. 1997]. A membrane lipid imbalance has been shown to play a role in phenotypic expression in CF mice [Freedman et al. 1999]. The fatty acid pattern of serum phospholipids was analysed in the present CF patients to see if this could be a possible explanation for the differences in phenotype and response to cAMP between patients, but no such correlation was found. Our results could be due to the supplements of essential fatty acids (both oral and intravenous) that the patients receive regularly. Also, analysis of the fatty acid pattern in the epithelial cell membrane may have given a result different from that of analysis of the serum phospholipids.

Expression of the multi-drug resistance protein (MDR) is another possible factor that could influence the phenotype. MDR has been shown to regulate volume-activated chloride channel activity [Miwa et al. 1997; Vanoye et al. 1999; Idriss et al. 2000]. Hence differential expression of MDR between patients might influence chloride efflux characterization.

In conclusion, a mild phenotype may sometimes be explained by the fact that the respiratory cells still have the ability to secrete chloride in response to cAMP, but factors other than CFTR-mediated chloride secretion also seem to play a role in the development of the phenotype.

Therapeutic strategies

Colchicine

CFTR and MDR proteins belong to the same family of ATP-binding transporters and are expressed in native tissue in the epithelium and glands of the human airway [Wioland et al. 2000]. Polymorphic expression of the MDR1 protein in the lung parenchyma may explain part of the differences in lung symptomatology observed in the CF patients carrying the same mutation [Johannesson et al. 1997]. Colchicine is used for the treatment of acute gouty arthritis, biliary cirrhosis, familial Mediterranean fever, psoriasis, sarcoidosis, amyloidosis and other rheumatic diseases [Ben-Chetrit and Levy 1998]. Its anti-inflammatory effects are in part explained by a potent inhibition of leukocyte chemotaxis. The concentrations used in this study were in the low nanomolar range, in good agreement with the clinically tolerated daily dosage.

Colchicine at low concentrations inhibits microtubule self-assembly, and at high concentrations it can induce complete disassembly of preformed microtubules. [Taylor, 1965; Deery and Weisenberg 1981]. At the concentration used in our studies, the microtubuli were not affected, as shown by the
cytoskeleton immunochemistry, indicating true resistance of the cells to colchicine.

It has been well established that continuous exposure of cells to chemotherapeutical drugs induces the appearance of a "multidrug resistance phenotype" characterised by concomitant resistance to high concentrations of unrelated drugs and at the molecular level, by the up-regulation and expression of the ABC protein family, including MDR1 [Roepe 1995]. At nanomolar concentrations of colchicine, we found in our colchicine-resistant cells an increase in the relative amount of MDR protein, but without significant changes in the expression and localisation of the CFTR protein. Cao et al. [2003] observed that at high concentrations of the drug doxorubicin (128 µM), MDR1 overexpression was associated with a reduction in CFTR chloride channel activity, due to a promoter mediated decrease of CFTR mRNA and simultaneous increase of MDR1 mRNA. Importantly, co-expression of MDR1 and CFTR had no effect on CFTR function.

In the three cell lines studied we have noticed with increasing colchicine concentrations, a steady increase in the intracellular chloride concentration in resting conditions and a similar increase in the basal chloride efflux, which became significant for all cell lines at colchicine concentrations higher than 2-4 nM. The stimulated chloride efflux in the presence of the cAMP agonists forskolin and IBMX increased significantly in the cells resistant to colchicine at concentrations higher than 2-4 nM. For the CF cell lines, there was no difference in the basal and stimulated chloride efflux rate, neither for the control cells nor for the colchicine-resistant cells.

Our observations of increased intracellular chloride concentration at higher colchicine concentration could be the result of changes in intracellular pH and membrane electric potential, in agreement with the “altered partitioning model” [Roepe 1995; Roepe et al. 1996]. The long–term consequences of elevated intracellular Cl– might be up-regulation of other outward Cl– transport pathways and an increase in membrane permeability to Cl–. Activation of alternative chloride channels is an accepted strategy for pharmacological treatment of cystic fibrosis [Roomans 2001, 2003]. Thus, in addition to its anti-inflammatory properties, colchicine would be beneficial to CF patients by increasing the chloride efflux from airway epithelial cells, thereby potentially alleviating the fluid transport defect. Whether the effect of colchicine treatment would have clinical significance is still an open question, which would need to be answered by clinical trials.

**Cationic vector-mediated transfections**

The aim of gene therapy of cystic fibrosis lung disease is the expression of the normal CFTR protein in the well-differentiated cells that line airway lumen and submucosal glands. *In vitro*, cationic vector-mediated CFTR cDNA resulted in
the recovery of bacterial clearance activity already when at least 5% transfected cells were present in a cell mixture [Biffi et al. 1999]

We have employed normal and cystic fibrosis cell lines with well-differentiated characteristics, cultured under similar conditions. After 2 hours of transfection, both the liposome Geneporter (GP) and the cationic polymer polyethylenimine (PEI) were able to satisfactorily transfect the cell lines in this study, although with variable efficiency among the cell lines.

Cationic molecules that are strongly charged are known to destabilise the cell membrane and induce cytotoxicity and this effect was also noted in our results. The mechanism by which PEI harms the cells is probably by permeabilizing the cellular membrane [Godbey et al. 1999] and one study showed that PEI could disrupt phosphatidyl serine liposomes. By lateral diffusion, the initial “flip-flop” can lead to abnormal distribution of the lipid in the cell membrane, which can alter the fluidity and function of the membrane and lead to cell death [Oku et al. 1986].

Heparin is a natural anionic polymer that has been shown to be well tolerated for in vivo use (in nebulisation) with potential beneficial effect in the inhalation injury syndrome and bronchospasm [Bendstrup et al. 2002]. In our study, heparin was able to significantly reduce the toxicity of the cationic complexes, when added 2 hours post-transfection, as shown by both morphological study (electron microscopy) and flow cytometry. The relative viability of the transfected CF cell lines after heparin treatment increased on average with 15-20%, approaching the control values, without significant decrease of the transfection efficiency. This agrees well with other studies showing cyto-protective effects of polyanionic molecules, such as proteoglycans, in combination with cationic DNA transfection [Belting and Petersson, 1999; Ruponen et al. 2001]. Experiments were not conducted to investigate the mechanism of the protective effect of heparin treatment, but we speculate that heparin can increase the rate of lipoplex destabilization, and also speed up the recovery of the cell membrane lipid distribution and electric potential.

An interesting observation was the constant higher transfection rate and lower viability of the cystic fibrosis airway epithelial cells compared with their normal counterparts, both for the liposome and for the cationic polymer. A possible explanation might be the different intracellular trafficking in the cystic fibrosis cell line, due to defective acidification, regulation of endosome fusion and membrane recycling [Fajac et al. 1999].

Another factor might be the direct interaction between the plasma membrane of the cells and the transfection complexes. Bailey and Cullis [1997] showed that the success of transfection with cationic liposomes depends not only on charge attraction of the complexes, but also on the lipid composition of the target membranes, which determines the propensity of the target membrane to undergo fusion. This may also affect the sensitivity of cells to the
cytotoxic effect of polycations. We have therefore investigated the lipid composition of the cell membranes (phospholipids) in order to see whether there was any correlation between this composition and the transfection.

Despite near identical growth conditions, the results of the lipid analysis showed consistent changes in the concentrations of poly-unsaturated fatty acids (PUFA). This category includes the essential fatty acids linoleic acid (18:2ω6, LA) and linolenic acid (18:3ω3), as well as the LA derivative arachidonic acid (20:4ω6, AA) and the linolenic acid derivatives docosapentaenoic acid (20:5ω3, EPA) and docosohexaenoic acid (22:6ω3, DHA). As a general observation, the concentration of PUFA was elevated in the human CF cell lines when compared to the human wt-CFTR carrying cell lines, but no significant differences were noticed for the two BHK cell lines. A correlation between the fatty acid pattern in membrane phospholipids and the relative viability of the transfected cells could be noticed, with the most “sensitive” cells having more LA and 18:3ω3 compared to the other cells.

The first question that arose was whether the observed differences in fatty acid could explain the difference in transfection efficiency and relative viability. A possible explanation is that the cells with elevated PUFA have increased fluidity of the cell membrane and presumably this makes them more susceptible to the cytotoxic effect of the polycations. A more clear answer to this hypothesis might be provided by comparative investigations of the lipid composition in the plasma membrane before and after lipofection or polyfection.

The second question was whether any of the observed differences in essential fatty acid composition is directly related to the mutation in CFTR. It has been shown repeatedly that the plasma of CF patients has reduced linoleic acid and DHA levels and normal/increased levels of arachidonic acid, despite adequate dietary and pancreatic supplementation [Lloyd-Still et al. 1981; Farrell et al. 1985; Clandinin et al. 1995; Strandvik et al. 2001; Christophe and Robberecht, 1996]. Cystic fibrosis may result in a defect in the utilisation of LA [Bhura-Bandali et al. 2000]. Blockage of the Cl-/HCO3- exchanger in airway epithelial cells resulted in increased linoleic acid incorporation into triacylglycerol and decreased incorporation into phospholipids [Kang et al. 1992]. The cells from this study carrying the ΔF508-CFTR are known to have impaired Cl- transport in response to cAMP agonists. This may explain the difference in LA level observed in the human CF cell lines. Another factor that could influence the membrane lipid content is the different individual background of the human cell lines.

We have shown that successful in vitro transfection is possible with short incubation times. Low molecular mass heparin added at the end of the incubation time could help to maintain the viability of the cells, without interfering with the transfection efficiency. It seems possible that heparin could be an adjuvant for non-viral mediated gene therapy.
Conclusions and outlook

The work undertaken in this thesis contributes to advancing the knowledge of cystic fibrosis pathophysiology and development of treatment strategies, both pharmacological and genetic. It also continues the development of end-point measurements of CFTR function.

- A method has been established for studying ion transport in an easily accessible cell type (nasal epithelial cells) from normal and cystic fibrosis patients by X-ray microanalysis.
- We have studied the chloride transport in nasal epithelial cells from CF patients and found that the phenotype cannot exclusively be explained by the CFTR activity in patients with severe genotype.
- A common Portuguese CFTR mutation (A561E) causes protein mislocalization in the endoplasmic reticulum similar to the most common CF mutation (ΔF508) and thus it should be possible to treat it with the same pharmacological strategies.
- Chronic treatment of airway epithelial cells with nanomolar concentrations of colchicine increased the chloride efflux via chloride channels other than CFTR, strengthening the notion that colchicine could be beneficial to CF patients.
- Heparin added at the end of the cationic-mediated transfection incubation time could help to maintain the viability of the cells, without interfering with the transfection efficiency. The differences in the lipid composition of the cell membranes for the cell lines included in our study correlated with the viability of the cell lines after transfection, but not with the transfection efficiency. It seems possible that heparin could be an adjuvant for non-viral mediated gene therapy.

Uncovering the connections between the CFTR defect and the disease is a prerequisite for development of rational and curative treatment. Despite
considerable research, the exact functions of CFTR are still not completely known or available for testing. Further studies should not only investigate chloride transport but also the importance of CFTR interaction with other proteins and guide the new therapeutic strategies accordingly. The complex manifestations of the disease, where phenotype is not absolutely linked to CF genotype suggest that the treatment should have multiple targets, from the regulation and expression of the CFTR protein itself to additional CF modifier genes.

It is reasonable to expect that pharmacological treatment of the most common CF mutation (ΔF508) is an achievable goal, both by means of high-throughput screening and analytical development of new drugs. However, the compounds that have so far been described to remedy the folding or stability defect are either unsuitable for pharmacological use because of inherent toxicity (e.g., chemical chaperones) or too poorly characterized at the cellular or molecular level [Gelman and Kopito 2002]. The optimal strategy will be to simultaneously target the defect in folding, activation and stability of ΔF508-CFTR.

Gene therapy in combination with a pharmacogenomic approach seems also promising for the cure of the disease. Increased toxicity at doses that become therapeutic and the inability to transfect the non-dividing cells are the major obstacles in case of the non-viral agents. Physical and immunological defence mechanisms limit the successful use of the viral gene delivery to the lung. Development of better gene transfer agents, identification of more relevant targets and delivery routes, and overcoming the extra- and intracellular barriers for transfection are likely to continue in the near future and will hopefully lead to a definitive cure.
Acknowledgements

This work was carried out at the Department of Medical Cell Biology, Uppsala University. Financial support was provided by the Swedish Medical Research Council, the Swedish Heart Lung Association, the Claes Groschinski Foundation, the Swedish Cystic Fibrosis Association and grants from the Faculty of Medicine at Uppsala University.

I would like to express my sincere gratitude to my mentor, Professor Godfried Roomans for his continuous encouragements, support and guidance. My warmest thanks for introducing me to the fascinating world of research and for offering me the opportunity to be part of it.

Many thanks to Dr. Lena Hjelte for a fruitful collaboration and for the possibility to keep in contact with the clinical side of research. Sincere thanks to Dr. Deborah Penque for involving me in new fascinating projects across borders. I appreciate the contribution of my collaborators and especially that of Charlotte Andersson to our common projects.

I am grateful to Marianne Ljungkvist, Anders Ahlander, and Leif Ljung for their expert technical assistance.

Many thanks to the present and former members of the X-group for the chance of learning so much about each other, and for the joyful atmosphere. A special heartfelt thank to Jacqueline for her friendship and help. VP, Inna, Swatee and Janna, thanks for making the days pass easier! My thanks go to all at the Department of Medical Cell Biology for the administrative support, great lunch and coffee breaks, lively discussions and tasteful wine evenings.

I greatly appreciate the friendship of Birgitta and Gunnar Tibell who welcomed me and Nic into their family. Always close to my heart are my parents and my brother with his family and I thank them for being a constant source of joy, support and great pride.

Last but not least, I want to thank Nic for being my soul mate and for his endless love.
References


Bubien JK. CFTR may play a role in regulated secretion by lymphocytes: a new hypothesis for the pathophysiology of cystic fibrosis. *Pflugers Arch.* 2001; 443: S36-9.


A doctoral dissertation from the Faculty of Medicine, Uppsala University, is usually a summary of a number of papers. A few copies of the complete dissertation are kept at major Swedish research libraries, while the summary alone is distributed internationally through the series Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine. (Prior to October, 1985, the series was published under the title “Abstracts of Uppsala Dissertations from the Faculty of Medicine”.)