CFTR
FUNCTION, REGULATION AND CHARACTERIZATION OF A MOUSE MODEL FOR THE ΔF508 MUTATION

CFTR
FUNCTIE, REGULATIE
EN DE KARAKTERIZERING VAN EEN MUIS MODEL
VOOR DE ΔF508 MUTATIE

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aan Nancy
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3.2 **Genistein Activates CFTR via a Tyrosine Kinase and Protein Phosphatase Independent Pathway** (1996) P.J. French, J. Bijman, A.G. Bot, W. Boomaars, B.J. Scholte, and H.R. de Jonge. *Am. J. Physiol.* submitted

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3.3 **Fluid Transport in the Trachea of Normal and CF mouse** (1997) P.J. French, H.P.C. Peters, J.H. van Doorninck, W.H. Colledge, R. Ratcliff, M.J. Evans, B.J. Scholte and J. Bijman. *manuscript in preparation*

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3.4 **CFTR expression and mucin secretion in cultured mouse gallbladder epithelial cells** (1996) R.H.P.C. Peters, P.J. French, J.H. van Doorninck, G. Lamblin, R. Ratcliff, M.J. Evans, W.H. Colledge, J. Bijman, and B.J. Scholte. *Am. J. Physiol.* 271:G1074-G1083

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3.5 **A mouse model for the cystic fibrosis ΔF508 mutation** (1995) J.H. van Doorninck, P.J. French, E. Verbeek, R.H.P.C. Peters, H. Morreau, J. Bijman, and B.J. Scholte. *EMBO J.* 14:4403-4411

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3.6 A ΔF508 Mutation in Mouse Cystic Fibrosis Transmembrane Conductance Regulator Results in a Temperature-sensitive Processing Defect In Vivo (1996) P.J. French, J.H. van Doorninck, R.H.P.C. Peters, E. Verbeek, N.A. Ameen, C.R. Marino, H.R. de Jonge, J. Bijman, and B.J. Scholte. *J. Clin. Invest.* 98:1304-1312

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1.1 General introduction

Cystic Fibrosis (CF) is the most common lethal autosomal recessive disease in the caucasian population, affecting ≈1:2500 newborns (1,2). There are approximately 1000 CF patients in the Netherlands (3). Patients are characterized by recurrent pulmonary infections, pancreatic insufficiency and elevated sweat salt concentrations. Although improved therapy has resulted in an average lifespan of ≈29 years, CF remains a serious destructive disease with a grim prognosis. Cystic Fibrosis is caused by mutations in the gene encoding the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) (4-6), a cAMP regulated Cl^- channel expressed in the apical (luminal) membrane of many exocrine epithelia (5). The most common mutation in CFTR is a single amino acid deletion at position 508, ΔF508, found in ≈70% of all CF alleles (6). The ΔF508 mutation causes a defect in CFTR maturation resulting in an incorrect targeting to the apical membrane; the mutant protein is retained and degraded in the endoplasmic reticulum (ER) by the quality control mechanism (7).

Although the gene defective in CF patients has been identified, CF disease pathogenesis is still a not well understood process. In order to explain the CF pathogenesis, a detailed understanding of the CFTR protein is essential. For this, we have studied the regulation of CFTR, its physiological functions, and the effects of the ΔF508 CFTR mutation. Based on the functions of CFTR and the specific properties of CFTR mutants, strategies towards a therapy for CF can be developed.

1.2 History

Long before the first comprehensive description of CF in 1938 (8), a folkloric anecdote existed of midwives licking the forehead of newborns. If the sweat tasted abnormally salty, the infant was predicted to die of pulmonary congestion and its side effects. This folklore may relate to the observation of elevated sweat salt levels in CF by Di Sant-Agnese in 1953 (9). To date, the elevated sweat NaCl is still used in many clinics to help establish the diagnosis of CF. In 1981 it was observed that CF patients have an increased potential difference (PD) across pulmonary epithelia (10). This increased PD was also present across CF sweat duct epithelia. This was ascribed to a chloride impermeability of the tissue, resulting in a reduced NaCl uptake (11,12). Finally, in 1989, the CF gene was discovered, as one of the first genes to be isolated by positional cloning (4-6). The gene product was named the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR), and encodes a cAMP regulated Cl^- channel (13-17), expressed on the apical membrane of many epithelial cells (18-22).

1.3 Clinical phenotype

The major life threatening clinical manifestation of CF is recurrent pulmonary infection. However, CF is a multi organ disease affecting, besides the respiratory tract, the gastrointestinal tract (liver, gallbladder, pancreas and intestine), the genito-urinary tract and several other tissues, e.g. sweatgland. In addition, CF related organ insufficiency may cause several secondary disease symptoms like diabetes and vitamin deficiencies. The more prominent organ related disease symptoms are listed below (for comprehensive reviews see (1,2)).

1.3.1 Respiratory tract: At birth, no obvious pulmonary abnormalities are observed, except for dilation of submucosal gland duct and acinus. One of the first pulmonary lesions is mucus obstruction and infection of conducting airways (bronchioles). As the disease progresses, observed pathology include broncheolar and brocheal inflammation, submucosal gland hypertrophy and, hyperplasia of mucus producing goblet cells. Some small airways are completely obstructed. In advanced stages of the disease, CF is manifested by prominent bronchiectic cysts, brochiolar stenosis, areas of destructive emphysema and, peribroncheolar and peribronchial fibrosis. The progressive airway destruction in CF is initiated by mucus obstruction of conductive airways and the subsequent colonization by *Pseudomonas aeruginosa* (especially of the mucoid type), *Staphylococcus aureus* and, *Hemophilus influenza*. Several other bacterial strains have also been found. In the nose, polyp formation is frequently observed (15-20%) associated with mucus accumulation and chronic rhinitis.

1.3.2 Gastrointestinal tract: Meconium ileus, affecting 5-10% of CF neonatals, is one of the first major signs of gastrointestinal tract disease in CF. Less severe forms of meconium ileus like meconium plug syndrome (large intestinal obstruction), may also be observed. Later in life, small intestinal obstruction (meconium ileus equivalent) occurs frequently (20%) in the CF population. Virtually all CF patients develop pancreatic abnormalities. Pancreatic insufficiency is caused by mucus obstruction of the pancreatic duct, and the subsequent loss of acinar cells. These are replaced by fibrous tissue. Hepatobiliary disease in CF includes focal biliary cirrhosis and mucus plugging of intrahepatic ducts. An abnormal gallbladder is also frequently observed. Pancreatic insufficiency and hepatobiliary disease impairs both protein and fat digestion and so contributes to intestinal malabsorption in CF. Additionally, independent of pancreatic and liver function, the CF intestine may exhibit defects in nutrient absorption. Reduced nutrient uptake due to gastrointestinal malfunctioning in CF results in growth retardation. A side effect of fat malabsorption, are deficiencies in fat soluble vitamins

(A, D, E, and K), and their related diseases.

1.3.3 Other manifestations: Most male and part of the female CF patients are infertile. In males the main cause of infertility is the absence of the vas deferens. In females infertility may be secondary to malnutrition and/or lung infections, but it is also possible that thick cervical mucus may prevent sperm passage. The fibrosis of pancreatic acini isolates and obstructs blood flow to the pancreatic islets of Langerhans, potentially priming CF patients for diabetes. Finally, CF patients exhibit elevated sweat NaCl, with sweat Cl^- concentrations exceeding 60 meq/l (23).

1.4 CFTR

The cystic fibrosis gene was cloned and characterized in 1989 (4-6). The cystic fibrosis gene product was named the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR). A model of CFTR is depicted in figure 1.1. Its encodes a protein of 1480 amino acids consisting of two transmembrane domains (TM's), each spanning the membrane six times, two nucleotide binding domains (NBD's) and a highly charged cytoplasmic domain (the R domain) containing consensus sequences for phosphorylation. The most common mutation in CFTR, found in ≈70% of all CF alleles is a single amino acid deletion of a phenylalanine at position 508 (ΔF508) (6).

CFTR forms a cAMP regulated chloride channel with linear current-voltage relationship in transfected Hela cells (13), Sf9 insect cells (15), CHO cells (14), and IEC cells (17). Reconstitution of purified CFTR into planar lipid bilayers (16) and altered ion selectivity upon mutation of residues lining the putative pore (24) indicated that CFTR itself is a chloride channel, rather than being a channel regulator. The channel is expressed in the apical membrane of epithelia involved in the transport of fluid and electrolytes (18-22) and has a linear conductance of 8-10 pS (14,16,25).

CFTR bears homology to the protein superfamily of traffick ATPases or ATP Binding Cassette (ABC) transporters (5,26). In general, these proteins are involved in the active transport of substances across the membrane, for review see (26-29). Members of the ABC transporter superfamilly include the mammalian multidrug resistant P-glycoprotein (reviewed in (30), the yeast STE6 gene product (31,32), the epithelial basolateral chloride conductance regulator EBCR (33), the sulfonylurea receptor SUR, and several bacterial periplasmic permeases (reviewed in (27)).

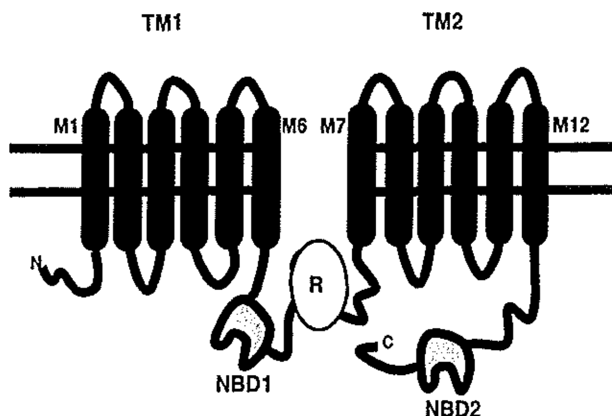

Figure 1.1: Topological model of CFTR, showing the two transmembrane domains (TM1 and TM2), each consisting of 6 membrane spanning segments (M1-M12), the two nucleotide binding domains (NBD1 and NBD2) and the regulatory domain (R).

1.4.1 CFTR expression: The expression of *CFTR* mRNA and protein is primarily restricted to epithelial tissues affected in CF (5). In the gastrointestinal tract, high *CFTR* mRNA expression was found in the small intestine and colon, with declining message from crypt to villus (34,35). *CFTR* expression was also found in epithelial cells of the pancreatic duct (34,35) and gallbladder (35,36), whereas low *CFTR* expression was observed in the stomach (34,35). In the respiratory tract, expression of CFTR protein was found predominantly in submucosal glands (22) and was not detectable in surface epithelium (18,22). However, pulmonary surface epithelia do express detectable amounts of *CFTR* mRNA albeit at low levels.

In addition to the gastrointestinal and respiratory tracts, CFTR expression was also found in other secretory and/or absorptive tissues, some of which apparently are not involved in CF pathogenesis. CFTR expression was found in salivary gland-, sweat duct- and thyroid epithelia (for reviews see (37-39)), and renal epithelia (for review see (40)). In the brain, the CFTR protein was detected in the choroid plexus and ventricular epithelium (41) and in various other areas (42). Non secretory cells expressing CFTR include human lymphocytes (43) and cardiac myocytes (44,45).

1.4.2 Regulation of CFTR expression: The CFTR promotor contains several potential transcriptional regulatory elements. Among these are 2 potential AP1 binding sites, which,

for some genes, are involved in the transcription response to phorbol esters (46). Indeed, long term incubation with phorbol esters markedly decreased *CFTR* mRNA (46-49) and protein (49), with only 10-20% of normal *CFTR* mRNA levels detectable after a 12 hr incubation. An increase in CFTR mRNA was observed after a long term incubation with the PKC inhibitors H7 and staurosporin (48). In addition to inhibition of protein synthesis by phorbol esters, these agents may also promote CFTR protein degradation (50). The *CFTR* promotor also contains a potential cAMP response element (46). Prolonged exposure to agents that elevate the intracellular cAMP concentration induce CFTR protein expression ≈3 fold (51). The inflammatory agents IFNγ (52) and TNFα (53) both reduce *CFTR* mRNA and protein expression. This downregulation of CFTR expression in response to cytokines may occurs as a tissue's response to a bacterial infection.

1.4.3 CFTR isoforms: CFTR is expressed in cardiac myocytes, but is present in a slightly different isoform. In these cells, CFTR lacks a stretch of 30 amino acids, $CFTR_{\Delta 164\text{-}193}$, corresponding to a sequence in the first cytoplasmic loop (45,54). In the kidney, a *CFTR* mRNA was isolated which encodes for a protein lacking the second TM domain and NBD2, TNR-CFTR. *TNR-CFTR* mRNA was caused by an alternative spicing between exon 13 and 14, which resulted in a premature stop codon. When expressed in *Xenopus* oocytes, TNR-CFTR could form a cAMP regulated Cl^- channel with similar properties compared to wildtype (wt) CFTR (55). Truncated CFTR proteins can dimerize to form functional Cl^- channels, as was shown for the D836X mutant (56).

1.4.4 Domains of CFTR:

1.4.4.1 The transmembrane domains: On the basis of hydrophobicity analysis, CFTR is predicted to consist of a tandem repeat of six membrane spanning segments (M1-12) with three extracellular and two intracellular loops (5). This membrane topography has been confirmed by insertion of N-glycosylation consensus sequences (57). As the transmembrane (TM) domains are in the lipid bilayer, they are likely to contribute to the pore and its (an-) ion selectivity. In search for the pore, CFTR deletion mutants have been generated.

The first transmembrane domain of CFTR appears important for the formation of the channel pore. This was based on the ability of CFTR which lacked the second TM domain and NBD2, D836X, to form cAMP regulated chloride channels when expressed in *Xenopus* oocytes (56). In contrast, CFTR lacking the first TM domain and NBD1 failed to form a Cl^- channel (56). Synthetic peptides of M6 and M2 by themselves can form anion selective channels in lipid bilayers (58). Another way to identify the pore forming motif is to identify

the amino acids lining the pore. Akabas and coworkers have done this within M1 by mutating a single amino acid of each of the residues 91-99 to a cysteine (59). They hypothesized that the cysteine can interact with sulfhydryl specific methanethiosulfate only when the cysteine lined the channel pore. G91, K95 and E98 were found to react. The periodicity is consistent with an α-helical structure of M1 (59). With a similar approach, I331, L333, R334, K335, F337, S341, I344, R347, T351, R352 and Q353 were found to interact with the 6th membrane spanning domain (60).

Charged residues within the pore are likely to contribute to the ion-selectivity of the channel (for review see cf (61)). There are a number of basic (positively charged) residues within the membrane spanning domains, several of which have been implicated in anion selectivity of the pore. For example, mutation of lysine 95 or 335 to aspartic acid and glutamate respectively, resulted in altered anion selectivity (24). Mutation of arginine 334 or 347 in M6 decreased channel conductance (62). Thus, positively charged residues lie within the pore and contribute to ion selectivity and conductance of CFTR. The importance of positively charged residues within M6 (R334, K355 and R347) could not be confirmed in a recent study (63). Although this study showed that the relative anion permeability of CFTR is not influenced when these residues are mutated to either glutamate or histidine, the single channel (chloride) conductance may still have been altered, which could not be analyzed in this study as it used whole cell patch clamp analysis.

1.4.4.2 The R domain: The R domain is a unique sequence, not shared by other ABC transporters. It contains multiple cAK and PKC phosphorylation consensus sequences (5) (see also chapter 1.5.3). Phosphorylation of CFTR on the R domain is required for channel opening (13-16). It has been suggested that the R domain, when not phosphorylated, blocks the pore via a "ball and chain" mechanism. In agreement with this, the deletion of the R domain resulted in a constituitively active CFTR Cl^- channel (64,65) and, in lipid bilayers, addition of exogenous R domain protein to CFTR resulted in a phosphorylation dependent block of the channel (66). Phosphorylation results in an increased negative charge on the R domain which might be sufficient for release of the pore-block as mutation of 8 (but not 4 (67,68)) serines for aspartic acid led to constituitively active CFTR Cl^- channels (68). Upon cAK but not PKC phosphorylation the R domain changes its conformation (69).

1.4.4.3 The nucleotide binding domains: One of the puzzling characteristics of the CFTR protein is the presence of the two nucleotide binding domains. Other ABC transporters use ATP hydrolysis for the transport of solutes against a gradient, whereas chloride transport is passive, i.e. Cl^- distributes according to the electrochemical gradient. Therefore, the first

question to be answered was whether CFTR channel opening required ATP hydrolysis or just ATP binding. ATP and several analogues were found to directly bind to CFTR (70). Also, mutations in the NBD's caused an altered relation between channel activation and ATP concentration, indicative of a direct interaction between ATP and CFTR (71). Whether CFTR actually required ATP hydrolysis was investigated by using triphosphate analogues. Hydrolyzable triphosphates (ATP, GTP, ITP, UTP and CTP) were able to induce channel opening, non- or poorly hydrolyzable triphosphates (AMP-PNP, ATPγS, AMP-PCP, AMP-CPP) could not (72). Direct ATP hydrolysis was measured at NBD1 in fusion with the maltose binding protein (73). It thus appears that ATP binding and hydrolysis is required to open the channel. ADP and AMP could not support channel activity (72,74). Both NBD's contain Walker A (GXXXXGKT/S) and B (ZZZZD, Z= hydrophobic) consensus motifs for ATP binding (75), lying, in ABC transporters, at a distance of 133-166 aa from each other (76). Mutations in the NBD's were generated to investigate individual roles of the NBD's in CFTR function, and are summarized below.

1.4.4.4 The role of NBD1: Mutations interfering with nucleotide binding, like G551D (77), or hydrolysis in NBD1 show decreased CFTR channel Po (71,78,79), reduced forskolin and IBMX activation (80), or reduced the access to the activated state (81). From these observations it can be concluded that ATP hydrolysis at NBD1 is required for CFTR channel opening.

1.4.4.5 The role of NBD2: At NBD2, the situation is somewhat more complex. Disrupting or impairing nucleotide binding, or interfering with the hydrolysis of ATP result in a low channel Po (78,79) and decrease access to the activation state in *Xenopus* oocytes (81). Thus, ATP hydrolysis at NBD2 is required for CFTR channel activation. In addition, ATP hydrolysis at NBD2 is required for channel closure as mutations that interfere with ATP hydrolysis, besides decreasing Po, increased the channel burst duration (78,79). Similarly, these mutations stabilized the active state of CFTR (81).

The kinetics of CFTR opening and closure was studied in detail by Gunderson and colleagues (79). A second open state (O_2) of CFTR was detected with slightly increased channel conductance. Mutations at NBD2 (K1250A, K1250G, K1250M, K1250T and D1370N) interfere with the transition from O_1 to O_2 and caused a large increase in burst duration. From these data, the authors hypothesized that the O_1 state of CFTR is entered upon ATP hydrolysis at NBD1. The O_2 state of the channel is entered upon ATP hydrolysis at NBD2. From the O_2 state the channel can close spontaneously. Thus mutations that interfere with ATP hydrolysis at NBD2 interfere with channel closure (79). Non hydrolyzable nucleotides e.g. AMP-PNP (78,82), or agents that interfere with ATP hydrolysis like PPi (83)

or VO_4 and BeF_3 (84) also induce a prolonged burst duration of CFTR when administered in the presence of ATP. This can be explained by a model in which these agents interfere with ATP hydrolysis at NBD2. The detailed understanding of the complex model of CFTR channel gating provides a novel pathway for the development of pharmacotherapy for CF, as it appears possible to increase CFTR channel activity by interfering with ATP hydrolysis at NBD2. Increasing CFTR channel activity may be of benefit for patients with residual CFTR activity or, in future, support CFTR expression elicited via gene transfer.

1.4.5 Regulation of CFTR activity: The regulation of the CFTR Cl^- channel is complex; both multisite phosphorylation on the R domain by cAMP dependent protein kinase (cAK) and the binding and hydrolysis of ATP on the nucleotide binding domains (NBD's) are required for channel opening (figure 1.2). Once phosphorylated by cAK, ATP alone is sufficient for channel opening (72). It has been suggested by Gadsby and coworkers that differences in the phosphorylation state of CFTR correspond to differences in channel activity (85), perhaps due to the presence of a labile phosphorylation site (78). For an excellent review on CFTR channel gating see (86). Below, an overview is given of the complex activation of CFTR by various protein kinases and nucleotide triphosphates.
1.4.5.1 cAMP regulation: CFTR can be activated in intact cells by the second messenger cAMP or, in excised membrane patches, by cAK (an effector enzyme of cAMP) and ATP, as was shown in transfected Hela cells (13), Sf9 insect cells (15), CHO cells (14), IEC cells (17) and upon reconstitution of purified CFTR in planar lipid bilayers (16). CFTR might be a direct target for cAK as the initial analysis of the primary protein sequence of CFTR revealed 10 putative cAK consensus sequences, 9 of these are located within the R domain. (5). Indeed, CFTR is phosphorylated by cAK both *in vitro* and *in vivo* (65,67,68). Multiple sites are phosphorylated as the stoichiometry of phosphate incorporation in the R domain of CFTR was 5-6 mol/mol (69,87).

A search was conducted to identify the sites phosphorylated by cAK. 13 sites phosphorylated by cAK were identified *in vitro*, 4 of which (S660, S737, S795 and S813) were also phosphorylated *in vivo* (65). Analysis of phosphorylation sites in CF2, a peptide corresponding to most of the R domain ($CFTR_{645-835}$), or wt CFTR revealed a similar pattern: S660, S700, S737, S813 and either S768 or S795, or both, are phosphorylated *in vitro*, and at least S660 and S700 *in vivo* (87). Mutation of the 4 primarily phosphorylated sites *in vivo*, S660A, S737A, S795A and S813A (the quad mutant) abolished cAMP induced chloride secretion (65), indicating that these serines were important for cAK regulation. However, the quad mutant expressed at high levels did reveal cAK regulated CFTR activity, albeit with lower Po (67,68). Residual cAK regulated CFTR activation of the quad mutant indicated other potential regulatory phosphorylation sites. Substitution of all 8 consensus serine

phosphorylation sites in the R domain (8SA) (68) or all 10 putative phosphorylation sites in CFTR (10SA) (67) still produced cAK regulated chloride channels. Only when the complete R domain was deleted in combination with S660A mutation (ΔR S660A) cAK regulation was absent (68).

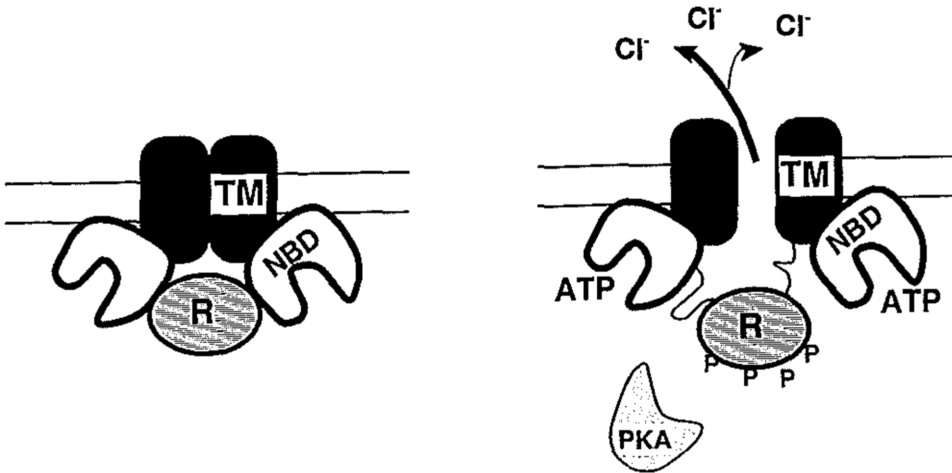

Figure 1.2: Opening of the CFTR Cl⁻ channel requires both ATP binding and hydrolysis at the nucleotide binding domains (NBD) and multisite phosphorylation on the regulatory domain (R). TM: transmembrane domains.

It is interesting to note that the putative cAK phosphorylation sites were identified with the R-R/K-x-S*/T* consensus sequence, asterix indicates the putative phosphorylated residue. Although this is the preferred cAK phosphorylation consensus sequence, both R-x-x-S*/T* and R-x-S*/T* sequences are also recognized by cAK (reviewed in (88)). Mutation of all the R-R/K-x-S sequences in CFTR (the 10SA mutant) did not completely abolish cAK regulation (67) and could be phosphorylated, likely on S753 (89). Interestingly, the S753 has the consensus sequence R-x-S. The S753A mutation in combination with the 10SA mutant further reduces CFTR activity (89) showing involvement of this phosphoserine, and the importance of other cAK consensus sequences in the regulation of CFTR.

No single phosphorylation site is highly important for CFTR activation as mutagenesis of single phosphorylation sites (S660A, S737A, S795A or S813A) did not alter channel activity (65). Rather, a general increase in negative charge accumulation elicited by phosphorylation appears important for CFTR activation as substitution of serine for the negatively charged aspartic acid on 6-8 putative phosphorylation sites generates constituitively active channels (68). When only 4 or 5 mutations were introduced S660D/E, S737D/E, S795D/E and S813D/E (and S700D) no constituitively active channels were observed (67,68),

indicating that a large accumulation of negative charge is required.

1.4.5.2 PKC regulation: 7 putative phosphorylation sites for PKC, the Ca^{2+} and phospholipid dependent protein kinase, were identified (5). PKC directly phosphorylates CFTR *in vitro* (90) and *in vivo* on serines S686 and S790 (87). It was found that PKC could directly activate CFTR at 10-15% of cAK activity (14,90). Furthermore, Cl^- secretion was induced by phorbol myristate acetate (PMA, an activator of PKC) in CFTR transfected C127i cells (91) but not in mouse L-cells (92). Cardiac CFTR Cl^- channels could be submaximally activated in intact cells by 4β-phorbol 12,13-dibutyrate (PDBu, an activator of PKC) stimulation (93). In addition to a being a direct activator of CFTR, PKC was reported to potentiate cAK activation CFTR (14,94), even with CFTR mutated for all consensus cAK phosphorylation sites (10SA) (67). The potentiating effect was not found by Berger and coworkers (90) and thus might be cell type dependent. Prolonged exposure to PDBu, 0.5-4 hr, did not induce a chloride current by itself, but could potentiate the cAMP response by 31% in pancreatic duct cells (95). In addition, inhibition of PKC slowed the rate of CFTR rundown in the whole cell patch (95). In summary, PKC has a twofold effect on CFTR activity. Firstly, PKC can directly activate CFTR, but the effect is relatively small when compared to cAK activation of CFTR. Secondly, PKC appears to have a potentiating effect on cAK activated CFTR. Both PKC effects however, appear to be cell type dependent.

1.4.5.3 cGMP regulation: A major class of microbial enterotoxins, i.e. the family of heat stable enterotoxins secreted by E-coli (STa), as well as the endogenous STa like peptide hormone guanylin, result in the accumulation of the second messenger cyclic GMP (cGMP) via the activation of a guanylyl cyclase (GC-C). The cGMP response in the enterocyte is involved salt and water secretion across the intestinal epithelium. As the cGMP induced chloride secretion is defective in CF epithelia, the involvement of CFTR in this pathway was suggested (for review see (96)). Several mechanisms have been proposed to link cGMP to the CFTR Cl^- channels, one of which is a cross-activation by cGMP of cAMP dependent protein kinase. This was shown in T84 intestinal epithelial cells (97,98) and Caco2 cells (99). cAK crossactivation by cGMP was based on the following observations: The cGMP response could be inhibited by PKI, an inhibitor of cAK (98,99) (but see (100)) but not with KT5823, an inhibitor of cGKI (99,101). The cGMP response could not be evoked by specific activators of cGKIα and β (97). cGMP did not modulate the cAMP concentration (98). Also, the concentrations of cGMP analogues used or the cGMP evoked (by STa) in these studies exceeds the concentration at which cGMP cross-activates cAK. The Ka of cAK for cAMP is 0.02 μM, for cGMP it is 4.1 μM (102).

cGMP may also activate CFTR via activation of cGMP dependent protein kinases (cGK's). To date, two isoenzymes of cGK's are known, cGKI and cGKII, for review see (103,104). Both cGKI (25,87,90) and cGKII (25) are capable of phosphorylating CFTR (and/or CF2) *in vitro* on similar sites as phosphorylated by cAK (25,87). cGK's can phosphorylate the cAK consensus sequence R-R/K-x-S/T as the cGK consensus sequences are R(K)-R(K)-R(K)-x-S/T and R(K)-R(K)-x-S/T (88). Although both cGK's can phosphorylate CFTR *in vitro*, cGKI proved virtually incapable of activating CFTR in excised patches of 3T3 fibroblasts (25,90), but could activate CFTR like Cl^- channels in T84 human colonic epithelial cells (105). cGKII could activate CFTR (25), albeit with slower kinetics than cAK. Lin and coworkers (105) found CFTR-like Cl^- channels in excised patches from T84 cells when stimulated only by ATP and cGMP. They concluded that a membrane bound cGK was responsible for CFTR activation. cGKII is attached to the membrane via N-terminal myristrylation, suggesting that cGKII is a good candidate for CFTR activation in these experiments.

Recently, a third mechanism of cGMP regulation of CFTR was proposed in which cGMP directly activates CFTR in an allosteric fashion (106). cGMP activation of CFTR chloride channels was demonstrated by the two electrode voltage clamp technique following injection of CFTR mRNA into *Xenopus* oocytes. This response was insensitive to both cAK and cGKI inhibitors. A putative cyclic nucleotide binding site in CFTR was found. Upon mutation of residues lining the cGMP binding site, altered cGMP responses were found in 2/7 mutations (106). However, studies in mammalian expression systems, including our own data did not show evidence for a direct activation of CFTR by cGMP and ATP in intact cells or excised patches (25,107).

1.4.5.4 Tyrosine kinases: In addition to regulation of CFTR by the serine/threonine protein kinases cAK, cGKII and PKC, several groups have studied the involvement of tyrosine kinases in the regulation of CFTR. Although no tyrosine residues on CFTR appeared to be phosphorylated (65), genistein, a tyrosine kinase inhibitor (108,109), was able to activate CFTR in the CFTR transfected cell lines IEC-CF7 and NIH-3T3 (110,111) and in CFTR expressing HT29/B6 adenocarcinoma and T84 colonic epithelial cells (112). Furthermore, genistein induced a chloride secretion in the shark rectal gland (113) and enhanced the cAMP induced chloride secretion across rat distal colon (114). In both tissues, the genistein induced chloride secretion was dependent on the activation of CFTR-Cl^- channels. Genistein did not activate CFTR by increasing the $[cAMP]_i$ (110,111), but its action required basal cAMP levels (112) suggesting a possible action as a protein phosphatase inhibitor (112,115). In chapter 3.2 we provide evidence that genistein neither acts via tyrosine kinase inhibition nor

via inhibition of a protein phosphatase. Instead, we propose that genistein directly acts on CFTR (French et al, submitted). Among other tyrosine kinase inhibitors tested, only tyrphostin 47 (110) and B42 (111) but not tyrphostin 51 and 25, herbimycin, AG126 and, cantharidin were able to activate CFTR in NIH-3T3 fibroblasts (H.R. de Jonge, unpublished observation). Similar observations were made using other cell types (112).

1.4.5.5 Other protein kinases: The calcium-calmodulin dependent protein kinase-II (CaMKII) could not activate CFTR in the excised patch (90), nor did CamKII inhibitors affect the STa induced CFTR Cl^- secretion (98). No *in vitro* phosphorylation of CFTR (90) and CF2 (87) was observed with CaMKII. CaMKI but not CamKIII or Casein kinase II was able to phosphorylate CF2 *in vitro* (87). Whether CamKI has a role in CFTR activation is presently unclear.

1.4.5.6 Dephosphorylation: As phosphorylation activates the channel, its counterpart, dephosphorylation, should inactivate the channel. The investigations on CFTR dephosphorylation do not give a consistent picture as to which protein phosphatases are involved. The protein phosphatases 1 and 2B neither affected CFTR channel currents in the excised patch nor did they dephosphorylate CFTR *in vitro* (90). Phosphatase 2A decreased the cAK induced CFTR current by 63% in transfected NIH-3T3 fibroblasts, and this phosphatase could also directly dephosphorylate CFTR *in vitro* (90). Thus, phosphatase 2A and not 1 or 2B is involved in CFTR dephosphorylation. In contrast, phosphatase 1 and 2A inhibitor okadaic acid did not prevent channel rundown (deactivation) or increase Po in transfected CHO cells (14,116) (but see below).

Alkaline phosphatase decreased CFTR channel Po (14) and directly counteracted cAK induced CFTR phosphorylation (116). Alkaline phosphatase inhibitors (VO_4, IBMX or Br-t), inhibited CFTR channel rundown upon excision of a membrane patch (116). However, these experiments were performed in the absence of extracellular ATP, thus delayed channel rundown could have been caused by a reduced ATP dissociation upon excision rather than CFTR dephosphorylation. In a different study, alkaline phosphatase was found to decrease CFTR activity, but upon removal of the phosphatase CFTR activity was restored. The authors suggested that the ATPase activity of alkaline phosphatase caused channel deactivation (90). In summary, it is likely that protein phosphatase 2A and perhaps alkaline phosphatase, and not 1 or 2B, mediate the dephosphorylation of CFTR. However, further analysis is required to accurately determine which protein phosphatase is involved in CFTR dephosphorylation.

1.4.6 CFTR modulation of other ion channels: CFTR has been reported to modulate other ion conductances, which is not an uncommon principle for ABC transporters (for review see

(117)). There have been several reports on a regulatory role for CFTR on the outward rectifying chloride channel, ORCC, an ubiquitously expressed chloride channel. Initially it was thought that defective cAK regulation of this channel was the underlying cause of cystic fibrosis (for review see (118)). However, the cloning and characterization of the CF gene revealed that CFTR and the ORCC are distinct proteins, see e.g. (119), encoded by separate genes (see also (17)).

Under specific whole cell patch clamp conditions (5 mM ATP in the pipette), cAMP induces Cl^- currents that are derived from both CFTR and ORCC (120). Both currents are absent in CF cells, indicating a potential cross talk between ORCC and CFTR. Similar experiments revealed that CFTR mutant A455E but not G551D was able to regulate the ORCC (121). A regulatory role for CFTR on the ORCC was further suggested by single channel patch clamp experiments, showing ORCC activation by cAK and ATP only in CFTR expressing cells, but not in CF cell lines (119,122). Reconstitution of CFTR and ORCC in lipid bilayers showed similar results; the presence of CFTR was required for cAK activation of the ORCC (123), and could not be replaced by the inactive CFTR mutant G551D (124).

An interesting model was proposed by Schwiebert and colleagues linking CFTR to the ORCC (125). Conductance of ATP through CFTR activates the P_{2U} receptor subsequently activating the ORCC. The model was modified later when it was shown that extracellular ATP by itself was insufficient to activate the ORCC but that the actual presence of (G551D) CFTR was required (124). G-proteins were hypothesized to modulate the additional interaction between CFTR and the ORCC (126). However, the model does not unify the above mentioned data and there remain considerable controversies. Firstly, the model requires that CFTR modulates ATP secretion. However, there is little evidence that CFTR conducts ATP itself (127-129). A previous study which did show ATP conductivity of CFTR (130) failed to provide convincing data that CFTR was in fact the channel studied. Secondly, in bilayer experiments (123), the P_{2U} receptor is likely to be absent (due to purification of the reconstituted proteins). Finally, CFTR was not detected in patches from mouse nasal epithelia, but still conferred cAK sensitivity to the ORCC (119). In summary, the activation of the ORCC by CFTR via ATP is a highly controversial issue. This will remain so until the sometimes conflicting data is explained, experiments are reproduced by independent observers, and gaps in the theory (e.g. how does ATP exit the cell, which G-protein is involved, and what messengers are required to activate the ORCC) are filled.

An increased amiloride-sensitive potential difference was found across pulmonary epithelia of CF patients (10). Amiloride blocks epithelial apical Na^+ channels (ENaC's) and it was concluded that the increased amiloride response in CF tissues was the result of increased

Na^+ absorption (10), reviewed in (131). Transfection of CFTR to a CF tissue, reduces the amiloride sensitive short circuit current (Isc) to values seen in controls (132). It was concluded that CFTR restored the Na^+ hyperabsorption to normal. Subsequently, several groups have investigated a relation between CFTR and ENaC's in more detail. Chinet and colleagues found that a specific type of Na^+ channels had a higher Po in CF patients (133). Also, coexpression of CFTR and ENaC's led to a lower Na^+ current compared to the Na^+ current in absence of CFTR (134). These results suggest that the presence of CFTR has a negative regulatory effect on the ENaC's. The most convincing evidence of an interaction between CFTR and the ENaC came from reconstitution experiments in lipid bilayers. In these experiments, the simple presence of CFTR reduced the channel Po of the ENaC (135).

Although CFTR might negatively regulate ENaC's, we doubt the relevance of Na^+ hyperabsorption in CF. Because, in order to have net flux of Na^+, anions (Cl^-) have to follow, otherwise just a maximal Na^+ gradient is created. In the Ussing chamber, the current required for short circuiting the tissue (the Isc) is only a measure for actively transported ions like Na^+, and not passively diffusing ions like Cl^- (136). The short circuit conditions do not represent the *in vivo* situation where actively transported Na^+ flux has to be followed by passive anion flux through a shunt pathway that may utilize CFTR chloride channels. Therefore, increased Isc is not necessary correlated with increased flux *in vivo* (Bijman, unpublished observations). Thus, the increased amiloride sensitive Isc seen in CF, does not imply Na^+ hyperabsorption *in vivo*; in CF no Cl^- ions are capable of following the Na^+ flux. Thus, although CFTR might reduce the ENaC channel activity, its relevance for Na^+ absorption is debatable.

1.5 The ΔF508 mutation

Mutations which result in a loss of channel activity in CFTR can be classified into four major categories, class I-IV (fig 1.3), reviewed in (137). Class I mutations have a defective protein production. Examples of CFTR mutants belonging to this category are all mutations leading to a premature stop, thus stopcodon-, frameshift-, splice- mutations as well as promotor mutations. Class II mutations have defective processing, meaning that CFTR protein is made, but it is not correctly targeted to the apical plasma membrane. ΔF508, ΔI507, N1303K and several others belong to this category. NBD1 appears very sensitive for processing mutations, as all (except for N1303K) have been found in this area. Class III mutations have a defective regulation; CFTR is expressed in the apical membrane, but can not be activated sufficiently. A typical example is the G551D mutation. Class IV mutations have defective conductance, meaning that CFTR is present in the apical membrane but its

conductance is severely reduced. CFTR mutations R117H, R334W and R347P are examples of this category. Logically, these mutants are predominantly positively charged residues lining the pore as these comprise anion-selectivity and conductance of CFTR.

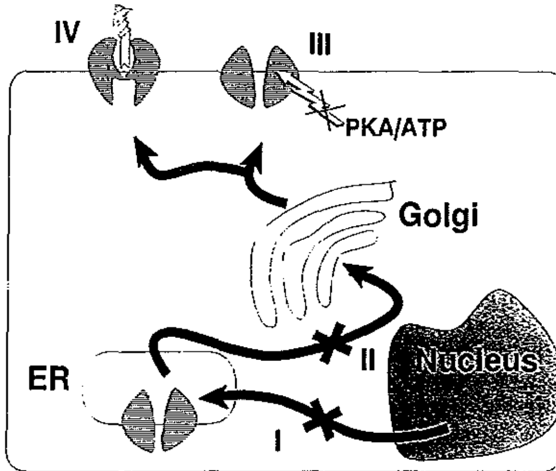

Figure 1.3: Schematic drawing of the different classes of mutation in CFTR. I) defective protein production. II) defective processing. III) defective regulation. IV) defective conductance. ER: endoplasmic reticulum.

1.5.1 The ΔF508 mutation is a class II mutation: The most common mutation in CFTR is a single amino acid deletion at position 508 (ΔF508) (6). Topologically, phenylalanine 508 lies within the first NBD, between the Walker A and B ATP binding sequences. Initially the phenylalanine was thought to lie within a loop of NBD1 not participating in ATP hydrolysis (26). However, a more detailed examination revealed that it is situated just a few amino acids downstream of a site potentially involved in ATP hydrolysis (76). ΔF508-CFTR expressing cells do not exhibit a cAMP stimulated Cl^- conductance (13). For review on ΔF508 CFTR see (7).

CFTR migrates as a triplet, band A, B, and C (in the order of reduced mobility) on a western blot (138). Band A represents the unglycosylated form of CFTR, band B is a core glycosylated form, and band C is a complex glycosylated form. The addition of complex glycosylation patterns to core glycosylated proteins occurs in the Golgi. In ΔF508 expressing cells, band C was absent, whereas band B was normally present (139,140). This was the first indication that the ΔF508 mutation was a processing defect; the presence of band B indicated that the protein is produced, whereas the absence of band C indicated that the protein is not

properly processed to the Golgi. Subsequently, it was shown by immunocytochemistry that ΔF508 CFTR is not localized at the plasma membrane but has a cytoplasmic (perinuclear) localization (19,21,22,139,141).

It was shown that the processing defect is temperature sensitive; reducing cell culture temperatures for ≥2 days at ≤30°C resulted in the appearance of C-band CFTR on western blot, and the appearance of cAMP stimulated Cl^- permeability by whole cell patch clamping (141,142). This finding was of some interest because it implied that ΔF508 CFTR expressing cells contain potential Cl^- channel activity, and that CF therapy could be aimed at the relocation of ΔF508 CFTR. Several other groups have shown ΔF508 CFTR expression on the cell membrane; Sf9 insect cells (143) and *Xenopus* oocytes (80), systems that are traditionally cultured at low temperatures, express ΔF508 CFTR on their cell surface. ΔF508 CFTR was also observed on the surface of vaccinia infected (vertebrate) Vero cells (144).

Expression of active ΔF508 CFTR on the cell membrane allowed biophysical characterization of the channel. ΔF508 CFTR appeared to have normal anion conductance and selectivity (143,144) (PJF, unpublished observations). The channel can be activated by cAK when reconstituted in lipid bilayers (143) and, in the cell-attached patch clamp configuration by cAMP agonists (144) (PJF, unpublished observations) albeit with reduced cAMP sensitivity i.e. requiring higher cAMP concentrations (80) (but see (145)). A 3-4 fold reduction in channel Po was found in vertebrate cells (142,144,145) but not in Sf9 insect cells (143). A marked reduction in Po was also found in excised patches from NIH-3T3 fibroblasts expressing ΔF508 CFTR (PJF, unpublished observation). Except for the reduced Po, all other parameters were similar to wt CFTR (145). The reduced Po of ΔF508 CFTR indicates that the ΔF508 mutation is not only a processing mutant, but also a class III mutant with defective regulation. As was discussed before, the ΔF508 mutation lies with in a position putatively involved in ATP hydrolysis (76), making it tempting to speculate that the reduced Po is caused by a reduced ATP hydrolysis at NBD1. ΔF508 CFTR is not a class I mutant as mRNA levels are normal (22,146).

1.5.2 Processing of wt and ΔF508 CFTR: When it became clear that the most common mutation in CFTR, ΔF508, is a processing mutant, several groups started studying the process of CFTR maturation. Translation of *CFTR* mRNA into protein occurs in the endoplasmic reticulum (ER), where the protein attains its proper configuration. For complex proteins like CFTR the folding process is aided by chaperones (for review see (147)). HSP70 (148) and calnexin (149) are chaperones with affinity for CFTR. Once the protein has attained its proper folding configuration, the protein is transported to the Golgi. Surprisingly, only a

small percentage (10-50%) of wt CFTR is transported to the Golgi (149-152), the remainder is retained in the ER (as is all ΔF508 CFTR). The halflife for both wt and ΔF508 CFTR in the ER is ≈0.5 hr (149,150,152). Improperly folded CFTR is degraded in non-lysosomal (152) proteasomes (153,154) via the ubiquitin pathway (153). A small percentage of wt CFTR attains a stable, protease resistant form in the ER, which is assumed to be properly folded CFTR primed for transport to the Golgi (150,152). In the Golgi CFTR attains complex glycosylation and is subsequently transported to the apical plasma membrane. In the plasma membrane, the halflife for wt CFTR was reported to be ≥24 hr (155) and 7-8 hr (150) and ≤4 hr for ΔF508 CFTR (155). Mature CFTR is probably degraded in lysosomes (148).

One of the reasons that proteins are processed incorrectly is an aberrant folding pattern. Incorrect folding due to the ΔF508 mutation was shown in experiments using a synthetic peptide spanning the ΔF508 mutation, $CFTR_{450-516}$. In these studies, the ΔF508 mutation de-stabilized the secondary structure of the peptide, resulting in a loss of β-sheet structure (156-158). Therefore, incorrect folding of ΔF508 CFTR may underlie the processing defect.

1.5.3 Correcting the processing defect: Since it became clear that most CF patients have potentially functional CFTR in their cells, strategies were aimed at correcting the mislocalization of CFTR. The process of CFTR protein folding and routing is a poorly understood mechanism which requires further investigation (see the discussion chapter 2.3). It was hypothesized that inhibition of CFTR degradation might result in a larger fraction of mature wt CFTR and perhaps also ΔF508 CFTR. However, this approach did not result in increased mature (band C) CFTR (153,154). Ubiquitinylation serves as a degradation signal for proteins (159) and directs CFTR degradation (153) but the inhibition of CFTR ubiquitinylation does not result in increased mature CFTR expression (153), suggesting that the signal to retain CFTR in the ER lies either upstream or is different from the ubiquitinylation pathway.

Few agents have been proposed to correct the processing defect. Firstly, phenylbutyrate was shown to increase Cl^- secretion as well as the fraction of band C in several CF cell lines (160). The role of phenylbutyratre on the ΔF508 CFTR protein was based on the assumption that a very low level of ΔF508 CFTR does reach the apical plasma membrane. A general transcription activator like phenylbutyrate, might increase the total amount of protein processed to the apical plasma membrane. Experiments in our laboratories failed to reproduce these results (PJF, unpublished observations and HR de Jonge, unpublished observations). Secondly, glycerol (10%) caused the appearance of 3-8% mature ΔF508 CFTR (wt≈25%) and

the appearance of a cAMP activated Cl^- conductance in cell culture (161). Compounds which, like glycerol, stabilize proteins against thermally induced aggregation are referred to as chemical chaperones. Several of these compounds were reported to increase the amount of mature ΔF508 CFTR e.g. deuteriated water, trimethylamino N-oxide (162) and DMSO (163). Mutations in ΔF508 CFTR may revert the processing defect. These revertant mutations were found in studies using chimeric proteins of CFTR NBD1 and yeast STE6, a member of the ABC transporters involved in a-factor (a yeast mating factor) secretion (164). In yeast, several NBD1-STE6 chimeric proteins appeared functional in a-factor secretion, whereas chimeric proteins in which the ΔF508 mutation was introduced were not. Interestingly, the additional mutants R553Q and R553M both were able to revert the phenotype caused by the ΔF508 mutation. Introduction of these mutations in human ΔF508 CFTR increased the fraction of mature CFTR (R553M) and/or restored channel Po to normal. Interestingly, a single patient carrying an R553Q mutation in combination with a ΔF508 mutation on the same chromosome (R553X on the other) was described. The patient exhibited a complex phenotype of a borderline sweattest in combination with typical CF symptoms of gastrointestinal and pulmonary disease (165). However, the R553Q mutation is in itself a CF causing mutation (166).

1.6 Functions of CFTR

In the mid-fourties, Cystic fibrosis was named "mucoviscidosis" i.e. thick mucus. The function of a mucoid layer is to protect and lubricate epithelial cells (for an excellent review on mucus and mucins see (167)), and there are several indications that the mucoid layer is altered in CF. Alterations in mucin secretion or properties may reduce mucus clearance and so cause obstruction of small ductular systems. These mucus plugs create a suitable environment for bacterial growth in pulmonary epithelia. The thick mucus in CF implies a physiological function for CFTR, be it either directly or due to secondary effects, in the alteration of mucus properties. In this chapter possible physiological functions of CFTR are discussed.

1.6.1 The role of CFTR in mucus synthesis: One of the first speculations on the basic defect in CF was a defect in mucus metabolism. Indeed, mucus isolated from CF pulmonary epithelial tissue showed increased sulfation (168-172), not observed by Gupta and coworkers (173), and decreased (glycoprotein) sialylation (174,175). Several reports have shown altered intestinal mucin composition in CF, i) increased fucosylation and decreased sialylation (in mucins isolated from meconium, the first infants stool) (176), ii) decreased sialylation and an increased fucose/hexosamine ratio in mucins isolated from CF duodenal fluid (177), and iii)

increased fucosylation without decreased sialylation (178). No increased fucosylation was observed in CF bronchial mucus (179). Fucosylation, sialylation and sulfation appear to be among the last steps in the biosynthesis of glycoproteins. Thus, in CF there may be a defect in the terminal glycosylation of glycoproteins.

Changes in terminal glycosylation pattern of mucus glycoproteins as observed in CF may alter the physical properties of mucus. Firstly, the cleavage of sialic acid from mucus increases the elasticity of mucus (180), and reduces its viscosity (181). Secondly, decreased sialylation may result in increased bacterial adhesion. The desialylated glycoproteins, asialo-GM1 and 2 and fucoasialo-GM1 are specific binding sites for many bacterial strains, whereas the sialylated forms of GM1-3 are not (174,182,183). Decreased sialylation might therefore result in increased bacterial adhesion to CF glycoproteins (184).

An interesting hypothesis has been postulated linking CFTR to alterations in mucin glycosylation. The fucosyl-, sialyl- and sulfo- transferases are localized in the distal Golgi or trans Golgi network and have distinct pH optima (for review see (185)). If CFTR is involved in the acidification of the distal Golgi or trans Golgi network, one can imagine a different pattern of terminal glycosylation in CF. For instance, a more basic environment would result in increased fucosylation (pH optimum 7-8.5) and decreased sialylation (pH optimum 5.8). Furthermore, fucosylation and sialylation are mutually exclusive processes (186).

The rationale of decreased acidification of endosomal and Golgi compartments in the absence of CFTR is excellently reviewed by Al Awqati (185). The theory is based on the requirement of a counterion to generate a significant ΔpH. In the absence of a counterion, ΔpH is limited by the potential difference (PD) created by active proton pumping (fig. 1.4). Chloride may serve as the counterion and, via CFTR, may passively follow the electrical gradient generated by the proton flux. The CFTR dependent acidification hypothesis requires both the presence of CFTR in the Golgi and its involvement in the acidification process. Both points are discussed below.

The presence of CFTR in the Golgi or *trans* Golgi network has not been demonstrated directly. However *de novo* CFTR synthesis, derived from the ER, and the fusion of the *trans* Golgi with (CFTR containing) vesicles derived from the endosomal pathway are two potential sources of CFTR in the Golgi. The presence of CFTR has been demonstrated by immunohistochemical analysis in clathrin coated vesicles (187) and early endosomes (188). The functional presence of CFTR in endosomes can be monitored by the rate of deacidification upon addition of a protonophore which is accelerated by CFTR dependent co-efflux of Cl^- counterions. With this assay, the functional expression of CFTR in endosomes has been demonstrated (189,190)

Although several groups have examined the role of CFTR in the process of acidification, most studies have been confined to the study of vesicles of the endosomal pathway, not measuring Golgi compartments. However, a direct relation between endosomal acidification and reduced glycoprotein sialylation was shown in CHO mutants with temperature-sensitive defective receptor-mediated endocytosis (191). A role for CFTR in endosomal and early lysosomal acidification was suggested in studies using CF pulmonary epithelial cells (175)[1] and in Swiss 3T3 fibroblasts (190)[2]. Furthermore, it was found that C127 cells, expressing CFTR, acidify when treated with cycloheximide, whereas ΔF508 expressors did not (192). As cellular acidification is a prerequisite for apoptotic DNA cleavage, the investigators found that ΔF508 expressing cells are less primed to apoptotic stimuli (192). In contrast to the studies listed above, other studies have failed to confirm a role for CFTR in the acidification of both endosomal (189,193,194) and Golgi (193,194) compartments. Thus, there is as yet no conclusive evidence for a role of CFTR in organelle acidification.

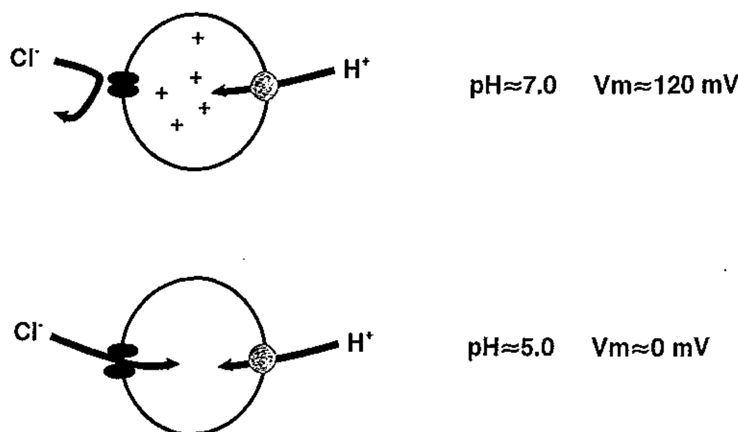

Figure 1.4: The role of a counterion in endosomal acidification. In absence of a counterion, proton pumping is limited by the generated membrane potential.

[1] The experimenters compared endosomal acidification in CF nasal polyp cells with control tracheal cells, hardly the ideal control.

[2] A decreased endosomal acidification was found in ΔF508 CFTR compared to wt CFTR expressing Swiss 3T3 fibroblasts. Mock transfected cells showed an acidification not different from wt CFTR transfectants, suggestive of a specific role of ΔF508 CFTR.

Although there are some indications that CF mucins are altered, whether or not due to defective trans-Golgi acidification, a summary of the published literature does not provide a conclusive answer. A single key experiment still has to be performed in which CFTR is transfected in CF epithelial cells, and normalization of the terminal mucus glycosylation pattern is observed. Presently the differences in terminal glycosylation of CF mucins can be attributed equally well to genetic variability of patient material (most investigators use epithelial cells isolated from very few patients, while variability between individuals is high (195)). Also, an altered mucus expression pattern may be the result of the CF condition rather than a cause of the disease.

1.6.2 Mucus secretion is increased in CF: Several investigators have found an increased mucus secretion in CF pulmonary tissues (170,196), which could well explain the characteristic CF feature of mucus plugging of airway and small ducts. Increased mucus secretion may simply be explained as a cellular response to inflammation, but may also be the result of an active (inhibitory) role of CFTR in the process of mucus secretion (197). This contrasts the suggested stimulatory role for CFTR in this process in experiments which showed an impaired β-adrenergic (cAMP) regulation of mucus secretion in CF submandibular glands (198) and bronchi (179). Moreover, antibodies directed against CFTR inhibited β-adrenergic mucus secretion in normal rat submandibular glands (199). In conclusion, several experiments suggest a stimulatory role for CFTR in the secretion of mucus but they do not explain the observed increase mucus secretion in CF tissues. Basal mucus secretion was normal in human bronchial secretions (179) and in cell cultures of nasal polyps and turbinate (171). Therefore basal mucus secretion does not depend on CFTR expression.

It remains puzzling how CFTR can be involved in mucus secretion in all tissues as there is little or no CFTR expression in mucus producing cells of pulmonary tissue and in the digestive tract. I.e. in lung, CFTR is mainly detected in the serous, not mucous, cells of submucosal glands (22,39). However, in the small intestine a candidate-CFTR protein was detected in mucus goblet cells (200). Perhaps the amount of CFTR required to regulate mucus secretion is low and below immunohistochemical detection limits. Alternatively, the role of CFTR may be, by inducing Cl^- (and water) secretion, to create a suitable environment for mucus secretion, as was shown in *Xenopus* subepidermal glands (201). In this case, CFTR expression and mucus secretion need not be colocalized within the same cell type.

1.6.3 The role of CFTR in fluid and electrolyte transport:

1.6.3.1 Altered fluid and electrolyte resorption. A model for active epithelial NaCl uptake is

shown in fig. 1.5A. In this model, Na^+ is transported against its electrochemical gradient from the mucosal to the serosal side via an apical amiloride sensitive Na^+ channel and basolateral Na^+/K^+ pumping. CFTR dependent Cl^- transport accompanies the Na^+ flux passively. In CF, a reduced Cl^- permeability will impair Na^+ uptake as the sum of the net transported charge should be zero (Kirchhoff's law) as depicted in figure 1.5B. This was shown more than a decade ago in sweat duct epithelium where a Cl^- impermeability actually reduces NaCl uptake. As a consequence, sweat NaCl concentrations are elevated in CF (202).

In CF upper airway surface fluid the luminal NaCl concentrations are elevated (203,204), like in the sweat of CF patients. This suggests that, according to our model (fig 1.5A) normal airway epithelia absorb salt from the surface fluid whereas the Cl^- impermeability in CF impairs the NaCl uptake (fig 1.5B). As a result, CF airway surface fluid NaCl concentrations are increased. The increased osmolality in airway surface fluid not only influences the viscoelasticity and charge of mucins which changes dramatically when the salt concentration in- or decreases (180,181,205), but also reduces the effectivity of epithelia to kill bacteria (206).

In fluid absorptive epithelia, isotonic fluid transport is dictated by the active transport of ions. Both salt absorptive and fluid absorptive epithelia may utilize similar transporters, but they differ in their ability to generate an osmotic gradient. The H_2O arrow in fig. 1.5A can be applied to fluid absorptive epithelia. The model predicts that CF epithelia, because of reduced NaCl uptake, show reduced fluid absorption (fig. 1.5B).

1.6.3.2 Altered fluid and electrolyte secretion. There have been several studies showing impaired fluid secretion in CF epithelia. For example, pancreatic (207-209) and pulmonary (210,211) (see also chapter 3.3) epithelia show reduced cAMP induced fluid secretion in CF. CFTR dependent cAMP induced fluid secretion was also reduced in ADPKD (autosomal dominant polycystic kidney disease) cyst epithelial cells (212). In addition, CF mouse gallbladder (213) and jejunum (PJF, unpublished observations) do not secrete fluid in response to forskolin, a $[cAMP]_i$ elevating agent. Finally, CF-/- mice intestine do not secrete fluid in response to cholera toxin (214). There is evidence that CFTR itself may conduct H_2O (215). In the absence of epithelial fluid secretion, the protective mucus layer is not sufficiently hydrated. Dehydrated mucus is viscid and difficult to clear, and the change in rheogenic properties may explain the mucus plugging and subsequent inflammation of small ducts in CF.

In fluid transporting epithelia like gallbladder and renal proximal tubule, isotonic fluid transport is directed by the active transport of ions. A model for active epithelial electrolyte, and thus fluid, secretion is presented in figure 1.5C. Apical CFTR dependent Cl^- secretion is followed by cations either trans (K^+) or paracellular (Na^+). Basolateral Cl^- pumping is

mediated via the bumethanide sensitive $Na^+/K^+/Cl^-$ transporter and cellular ion-gradients are maintained by the basolateral Na^+/K^+ ATPase. In the absence of apical CFTR Cl^- channels, the model predicts reduced fluid and electrolyte secretion, shown in figure 1.5D.

1.6.3.3 the pH. CF pancreas exhibits reduced Cl^- and HCO_3^- secretion (209). CFTR itself may conduct HCO_3^-, and thus can influence luminal pH (216). The pH can influence the viscoelastic properties of mucus (205). However, there are no reports on altered luminal pH in CF pulmonary epithelium.

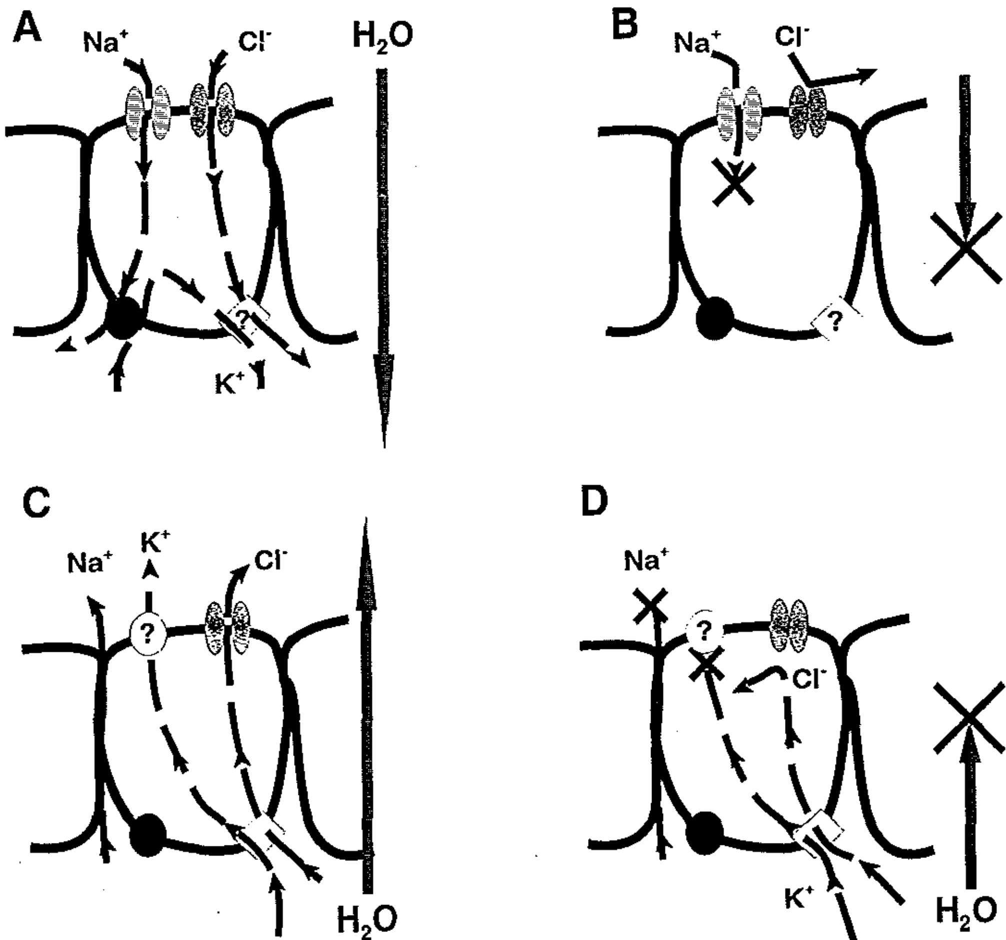

Figure 1.5: Models for fluid and electrolyte transport across epithelia. A) absorptive model: Na^+ is actively transported to the serosa by apical amiloride sensitive Na^+ channels and basolateral Na^+/K^+ pumping. Cl^- passively follows the cation flow. B) In absence of apical chloride conductance (CFTR), Na^+ (and thus fluid) uptake is limited by the created electrical potential. C) secretory model: opening of apical CFTR Cl^- channels induces Cl^- efflux followed by either transcellular K^+ or paracellular Na^+ or both. In absence of CFTR (D) both cation and fluid fluxes are reduced in CF.

1.6.4 The role of CFTR in pathogen affinity, internalization and killing: Recurrent pulmonary infections, a characteristic clinical manifestation of CF, may be caused by a reduced clearance of bacteria. A reduced bacterial clearance in CF epithelia can be caused by an increased bacterial adhesion due to the altered terminal glycosylation pattern of CF mucus (see chapter 1.6.1). Indeed, an increased *Pseudomonas aeruginosa* adhesion to CF cells was found (217). Increased *P. aeruginosa* binding was also found in CFPAC (CF pancreatic duct cells, homozygous for the ΔF508 mutation) and IB3 (CF bronchial cells, heterozygous ΔF508/W1282X) cells compared to the same cell lines transfected with wt CFTR (184). *P. aeruginosa* may not be the only bacteria displaying increased adhesion to CF epithelia, *Staphylococcus aureus* and *P. aeruginosa* compete for the same binding sites (184). In addition to increased *P. aeruginosa* adhesion, there may also be decreased *P. aeruginosa* internalization in CF (218), with the first predicted extracellular domain of CFTR acting as a *P. aeruginosa* receptor (218). Bacterial internalization may play a role in the host defence mechanism. Finally CF epithelia may have a reduced effectivity to kill bacteria (206) (discussed in chapter 1.6.3).

1.6.5 The role of CFTR in endo- and exocytosis: Bradbury and coworkers have found a CFTR regulated inhibition of endocytosis and a stimulated exocytosis in CFPAC cells, upon complementation with wt CFTR (219). In contrast, cAMP stimulation in T84 cells did not induce increased apical CFTR expression (20,220). However, human nasal polyp cells did show increased CFTR expression upon forskolin stimulation (221). Absence of CFTR regulated endo- and exocytosis can contribute to CF disease pathogenesis as cAMP regulated processes, i.e. fluid and electrolyte secretion, may be amplified by increased apical expression of proteins involved in this process.

1.6.6 Summary: In this part of the thesis several hypotheses have been discussed which associate a defect in an epithelial Cl^- channel to the devastating disease pathology of CF. Apparently multiple causes may underlie the clinical manifestations of CF, as CF epithelia may have i) an increased pathogen affinity (chapter 1.6.4) ii) increased mucus secretion (chapter 1.6.2) iii) a reduced mucus clearance (due to altered viscoelastic properties, chapter 1.6.1 and 1.6.3) and, iv) a reduced pathogen killing (chapter 1.6.3). The combination of these result in an environment favorable for bacterial growth and mucus plugging of small ducts.

1.7 Mouse models for CF:

Mouse models for CF can be relevant for understanding the pathogenesis of CF and the basic physiological function of CFTR. They may also be used to aid research aimed at the

development for new strategies leading to a cure for CF. Upon the cloning of the gene encoding human CFTR (4-6) and the subsequent isolation of the mouse homologue (222), it was possible to generate mouse models for CF. To date, most CF mouse models have been generated for CFTR class I mutations (for CFTR mutation classification see figure 1.3) which contain a premature stopcodon in exon 2 (223), exon 3 (224) or exon 10 (225-227). In addition, several mouse models have been generated for the most common CFTR mutations like (class II) ΔF508 (228-230) and (class III) G551D (231).

Knockout CF mouse models (223-227,232) display a phenotype very similar to human CF patients. The only knockout mouse model for CF not displaying obvious disease symptoms was created by an insertion vector, resulting in a duplication of exon 10. The inserted exon 10 contains a premature stop (226). Due to alternative splicing, the mutant exon can be skipped and, as a result, some ≈10% wt *Cftr* mRNA is generated (233). Apparently this is sufficient to prevent most of the obvious disease symptoms.

Cftr knockout animals display a general failure to thrive, amounting to 10-50% reduced weight compared to wt animals (223-225,232). The mice show increased lethality, especially in the first weeks of life, with only 5-10% of the animals surviving into adulthood (223-225,232). Increased mortality is most likely caused by intestinal obstruction of the ileum and subsequent peritonitis. Intestinal crypts are dilated and filled with eosinophilic material (224,225,232). A wormlike caecum was observed (225). Other organs of the gastrointestinal tract are not as severely affected as the intestine, but do show some signs of pathology. Gallbladders may be filled with black bile in some animals, several others may show a distended or even ruptured gallbladder (225). Variable disease symptoms have been observed in the pancreas, varying from a dilation and block or accumulation of eosinophilic material in several small ducts of ≈50% of all CF animals (225,232), to mild infection of the main pancreatic duct (224). There have been no obvious signs of liver disease (223,224)

In contrast to male human CF patients, male CF mice are fertile (223-225). However, females are reported to be sterile (223). Several glandular structures have aberrant morphology in CF mice, i) acinar dilation of the lacrimal gland (232) ii) atrophy of serous glands and nose dorsolateral sinuses (225), iii) disruption of serous acini in salivary glands (225) (also reported to be normal (224)), iv) dilation and accumulation of eosinophilic secretions in the Brunner's glands (the submucosal mucoid secreting glands in the proximal part of the duodenum) (225), reported to be normal by others (224), v) dilation of acini and accumulation of eosinophilic material of the minor sublingual glands (224).

Perhaps the most striking difference between human CF patients and CF mouse models is the absence of lung disease in the CF mouse (223,224,232). There may be several

explanations for this. Firstly, the mouse respiratory tract almost completely lacks submucosal glands (234). Mucus plugging of the submucosal glands are among the first signs of pulmonary disease in CF in human newborns (1). If the submucosal glands are the site of respiratory pathogenesis, their virtual absence in mice may provide an explanation for the absence of lung disease. Secondly, absence of lung disease in CF mice may also be explained by the presence of a large Ca^{2+} activated Cl^- current in mouse pulmonary epithelium compensating for Cftr (235), as was also recently suggested for the mouse intestine (236). Thirdly, it may be that CF mice simply do not have long enough life span for lung disease to develop. Finally, keeping mice under pathogen free conditions (SPF) limits pathogen exposure and infection. There is evidence that when CF mice with residual Cftr activity are challenged with pathogens (*S. aureus* and *P. cepacia*) they do develop lung disease (237).

Electrophysiological measurements were performed to confirm the knockout of the *Cftr* gene. Ussing chamber experiments all consistently showed in several parts of the intestine, an absence (or reduction) of a cAMP activated Cl^- conductance in homozygous -/- knockout mice (223,224,226,232,238,239). Cultured *Cftr* -/- mouse nasal and tracheal epithelium also did not exhibit a cAMP activated Cl^- conductance (238). In nasal epithelium *in vivo*, an increased basal PD was observed (226,239), or no response to forskolin in cultured nasal (and tracheal) epithelium (238). These observations are consistent with an absence of a CFTR dependent Cl^- conductance in these tissues.

Mouse models generated with a specific point mutation (228-231) (see also chapters 3.5 and 3.6) generally show a similar disease pathology and electrophysiology, although the phenotype is less severe than in knockout mice. Mice have a failure to thrive (228-231) and most groups have reported an increased mortality due to intestinal obstruction (229-231). It is important to note that most mouse models for CFTR mutations have been generated by a procedure leaving an intronic selection marker to reside within the *Cftr* gene. Through the generation of antisense mRNA by the selection marker, the expression of the desired gene may be reduced. Alternatively, the selection marker may interfere with transcription of the *Cftr* gene or the efficiency of Cftr protein processing. Reduced *Cftr* mRNA levels have been found in all mutant mouse models thus created (229-231), but not in a mouse model for the ΔF508 mutation with an undisrupted intronic sequence (228,240). Thus, except for the mouse model generated in our lab (228), most CF mouse models reflect a combination of the mutation (ΔF508 class II, or G551D class III) with reduced mRNA (and protein) expression (class I).

Importantly, the ΔF508 mutation in mouse Cftr results in a temperature sensitive processing defect (230,240), quite like human ΔF508 CFTR (cf (142)). These mice therefore are excellent models for human ΔF508 CFTR and may facilitate studies on the processing of

CFTR (see also the summary and discussion chapter 2). It has not yet been shown that mouse G551D Cftr, like human G551D CFTR, is a class III mutation. Human G551D CFTR exhibits severe defective channel regulation with a reduced Po.

1.8 Scope of the thesis

Although it is widely accepted that CFTR encodes a cAMP regulated chloride channel, it is not clear how the absence of CFTR on the apical membrane relates to the disease symptoms of CF. One of the main characteristics in CF pathogenesis is mucus obstruction of small airways and several ducts and several hypotheses have been postulated to explain the mucus obstruction in relation to CFTR (discussed in chapter 1.7). A detailed examination of CFTR regulation and the physiological functions of CFTR may help to further understand the process of CF disease pathogenesis, and so aid the development of a therapy for CF. Therefore, mechanisms of CFTR activation (chapter 3.1 and 3.2) and physiological functions of CFTR (chapter 3.3 and 3.4) have been investigated and are described in this thesis, discussed below in more detail. An alternative approach towards a therapy for CF is aimed at the relocation of ΔF508 CFTR to the apical plasma membrane. An important step to facilitate research in this direction is the development of a mouse model for the human ΔF508 CFTR mutation. The generation and characterization of a ΔF508-CFTR mouse model is described in chapters 3.5 and 3.6.

Regulation of CFTR activity: The 'classical' way to open CFTR is via activation of cAMP dependent protein kinase (cAK) in the presence of ATP. The role of other protein kinases have not yet been fully elucidated, but other regulatory mechanisms are likely to be present. For instance, CFTR appears to be constituitively open in human upper airways (nasal epithelia). A role for cGMP in the regulation of CFTR became apparent from the absence of a cGMP induced chloride secretion across CF intestinal epithelia. One of the pathways which may link cGMP to CFTR activation is via the activation cGMP dependent protein kinases (cGK's). Therefore, in chapter 3.1, we studied the role of cGK's in the activation of CFTR. This mode of regulation is of particular importance in intestinal physiology and in the pathogenesis of diarrheal disease.

Apart from regulation of CFTR activity by the serine/threonine protein kinases cAK and cGKII, several groups have studied the involvement of tyrosine kinases in the regulation of CFTR. Indeed, genistein, a tyrosine kinase inhibitor, could activate CFTR in a variety of cell systems, and induce or enhance a CFTR dependent chloride secretion across intact

epithelia (discussed in chapter 1.5.3). Although genistein is a tyrosine kinase inhibitor, genistein has a variety of other effects which can not be attributed to tyrosine kinase inhibition. Therefore, the mechanism of the genistein induced CFTR activation remained to be elucidated, and was investigated in chapter 3.2 of this thesis. Our finding that genistein interacts directly with the channel, and acts as an opener of both wt and ΔF508 CFTR chloride channels offers perspectives for pharmacotherapy for CF.

Functions of CFTR: In chapters 3.3 and 3.4, we aimed to further understand the physiological role of CFTR in epithelia. First, we tested the hypothesis that in CF thick and sticky mucus (mucoviscidose) is caused by defective mucus hydration, implying a role for CFTR in the transepithelial transport of fluid (chapter 3.3). Second, we examined whether CF tissues show increased mucus secretion, or whether mucus secretion is dependent on CFTR activation (chapter 3.4).

2 Summary and Discussion

Cystic Fibrosis (CF) is the most common lethal genetic disease in the caucasian population affecting ≈ 1 in 2500 newborns (1). The disease is caused by mutations in the gene encoding the Cystic Fibrosis Transmembrane Conductance Regulator, CFTR (5,6), a small (8-10 pS) ATP and cAMP regulated chloride channel which is expressed in the apical membrane of many epithelia (13-16). The most common mutation in CFTR is a deletion of a phenylalanine at position 508, ΔF508 (6). This deletion prevents processing of ΔF508 CFTR to its mature form. Instead, it is retained and degraded in the endoplasmic reticulum (152-154).

Although it has been established that CFTR is a cAMP regulated Cl^- channel, it is not clear how a defective epithelial Cl^- channel relates to the pathology of CF. In this thesis we have tried to gain more insight in the role of CFTR in epithelia by studying the regulation (chapters 3.1 and 3.2) and physiological functions (chapter 3.3 and 3.4) of CFTR. In addition, we describe a mouse model carrying the ΔF508 CFTR mutation which we created and subsequently characterized in our laboratory (chapters 3.5 and 3.6). This ΔF508 mouse model may help to develop new pharmaceutical strategies based on relieving the processing defect.

2.1 Regulation of CFTR: The 'classical' way to open CFTR chloride channels operates via the activation of cAMP dependent protein kinase (cAK) and multisite phosphorylation of the CFTR protein in the presence of ATP (72), but other regulatory mechanisms are also likely to be present. In chapter 3.1 and 3.2 of this thesis, we have investigated two alternative regulatory pathways in the activation of CFTR.

In intestinal epithelia, accumulation of the second messenger cyclic GMP (cGMP) causes fluid and electrolyte secretion through the activation of apical chloride channels. This response is absent in CF tissues, indicating a role for CFTR in this pathway. Several mechanisms have been proposed to link cGMP to CFTR activation, one of which is a direct activation of CFTR by cGMP dependent kinases (cGK's). To date, two isoforms of cGMP dependent kinases have been characterized, cGKI and cGKII. cGKI is expressed in many tissues and is mainly localized in the cytosol whereas cGKII has a more restricted expression pattern (brain, intestine) and is membrane bound. In the excised patch we have shown that cGKII but not cGKI, was able to activate CFTR in cell lines stably transfected with CFTR (Chapter 3.1). A direct interaction between cGMP dependent kinases and CFTR was shown by *in vitro* phosphorylation studies. Although both kinases appear to phosphorylate CFTR *in vitro*, in intact cells, the differential subcellular distribution of the kinases are likely to be the

cause of the difference in the ability to activate CFTR (241).

A model proposed to explain the cGMP induced fluid and electrolyte secretion across intestinal epithelia is shown in fig. 2.1 (96). Membrane bound guanylyl cyclase-C (GC-C) is activated by guanylin or heat stable enterotoxin of E. coli (STa). The increase in $[cGMP]_i$ generated by GC-C, activates cGKII resulting in phosphorylation and activation of CFTR. We have confirmed this model by directly measuring *in vivo* fluid secretion in the mouse jejunum. STa was able to induce fluid secretion in normal mice, but failed to act as a secretagogue in CFTR -/- knockout mice (241a).

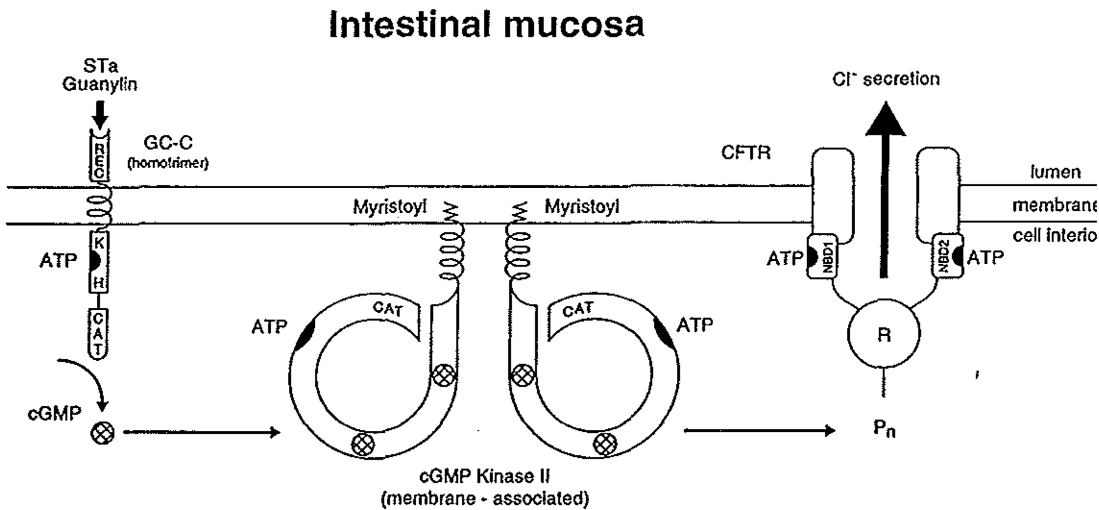

Figure 2.1 Model depicting the molecular mechanism by which heat-stable enterotoxin (STa) or guanylin is able to provoke intestinal chloride secretion. cGMP accumulation, upon stimulation of guanylyl cyclase-C (GC-C), activates cGMP dependent protein kinase II, and subsequently CFTR. The resultant is an efflux of Cl^- followed isoosmotically by H_2O.

Recently, several groups have shown cAMP independent activation of CFTR by the tyrosine kinase inhibitor genistein (110-112). In chapter 3.2 we have investigated the mechanism of this activation. We show that, in the excised patch, genistein, in the absence of protein kinases, is unable to activate CFTR, but genistein can further increase (hyperactivate) preactivated CFTR via an increase in the channel open probability. Hyperactivation appeared independent of protein phosphatases, and was not due to the inhibition of a tyrosine kinase. Instead, we propose that genistein hyperactivates CFTR by a direct interaction with CFTR,

possibly at NBD2. This hypothesis is supported by the following evidence i) genistein binds to the ATP domain of tyrosine kinases (108), but also has affinity for other nucleotide binding domains (242), ii) mutations or pharmacological agents that interfere with the hydrolysis of ATP at the second nucleotide binding domain of CFTR, like genistein, increase channel burst duration. Interestingly, genistein not only hyperactivates wt CFTR but also CFTR mutants (ΔF508) when expressed on the plasma membrane (P.J. French and HR de Jonge, unpublished observations). Hyperactivation of mutant CFTR may be of importance for the treatment of CF patients with residual CFTR activity. Therefore, our next step will be to screen rectal biopsies of CF patients that exhibit a residual CFTR Cl^- secretion in the Ussing chamber (23) for potentiating effects of genistein. If genistein enhances the CFTR dependent Cl^- secretion in these patients, it may be considered to be used as a therapeutic agent. As genistein is present in soy food, and as blood concentration may approach the level required for CFTR activation in intact epithelial cells (110,112,113,243), simply a dietary supplement could prove beneficial to patients with residual CFTR activity. The efficacy of a soy based diet supplementation can be studied in our ΔF508 CFTR mouse model which exhibits residual CFTR activity (228,240). The measurement of the potential difference across nasal epithelium can be used to monitor increases in chloride permeability.

2.2 Functions of CFTR: It is still an unresolved and intriguing problem how to relate the absence of a cAMP regulated chloride channel to the disease symptoms of CF. In chapter 3.4 we have attempted to address this question by studying the relation between CFTR and mucus secretion. An increased mucus secretion in CF pulmonary tissue has been found by several groups (170,244), indicating a possible function for CFTR in this process. Such an increase may lead to the mucus plugging of small conductive airways (if the production rate exceeds the clearance rate), creating a suitable environment for bacterial growth. Alternatively, a stimulatory effect of CFTR on mucus secretion was suggested from experiments showing an impaired cAMP dependent mucus secretion in CF (179,198). In this case, the impaired mucus secretion may result in the accumulation of mucus in goblet cells and, when these lyse, mucus is released into the lumen. This aberrantly secreted mucus may be insufficiently hydrated or buffered, and cause obstruction due to changes in the rheological properties. A provocative hypothesis relates defective mucus secretion in CF to the presumed intracellular role of CFTR in endo and exocytosis (219,221), endosome fusion (245) or endosomal acidification (175). We tested the role of CFTR in mucin secretion by investigating whether CFTR modulates mucin secretion in primary cultures of murine gallbladder epithelium. Apparently, CF tissues do not exhibit increased mucin secretion, nor is mucin secretion stimulated by agonists which activate CFTR in wt cells. We conclude that CFTR does not modulate mucin secretion in

mouse gallbladder epithelium.

Several groups have reported an abnormal structure of mucus in CF (168-172,174-178). An abnormal mucus structure alters the physical properties of mucus (180,181) which may impair mucus clearance and so cause mucus plugging of small conductive airways and ducts. In addition, the altered mucus structure in CF may enhance bacterial adhesion (182). In the CF mouse gallbladder, our preliminary data show an abnormal mucus composition, evidenced by a reduced periodate activated staining of mucus (B.J. Scholte, unpublished observations). Since periodate reacts with free hydroxyl groups, it follows that CF mucus exhibit reduced amounts of free hydroxyl groups, possibly due to an increased mucus sulfation. This remains to be investigated.

Mucus obstruction in CF may also be caused by a defective mucus hydration. Improper hydration of the mucoid gel layer alters its viscoelastic properties, resulting in reduced pulmonary clearance of mucus and attached pathogens. As fluid transport is secondary to active ion transport, and as CFTR is a chloride channel, defective chloride permeability could result in defective fluid transport. Furthermore, CFTR is mainly expressed in tissues involved in salt and water transport. We tested this hypothesis by directly measuring fluid transport across intact murine trachea (chapter 3.3), gallbladder (213) and jejunum (PJF, unpublished observations). In the trachea, we did not observe differences in the amounts of fluid transported between normal and CF -/- mice. This can be attributed to the low expression of CFTR in this tissue, and the high expression of other (perhaps compensatory) chloride channels. In fact, we could not detect CFTR electophysiologically in the murine trachea. In contrast, tissues with a high expression of CFTR, do exhibit CFTR dependent fluid secretion. Both murine wt gallbladder (213) and small intestine (214) (PJF, unpublished observations) secrete fluid in response to cAMP stimulation. This response is absent in CF -/- mice, implying a role for CFTR in fluid secretion. Under fluid secretory conditions, like in mouse gallbladder and jejunum, apical Cl^- secretion is likely to be directed by the basolateral $Na^+/K^+/Cl^-$ cotransporter (fig. 1.5C). The cotransporter, using the Na^+ gradient, increases the cellular Cl^- concentration and so creates an outward driving force for Cl^-. Cations, either transcellular K^+ or paracellular Na^+, passively follow the apical anion secretion (see also fig. 1.5C).

Fluid secretion was also studied in a ΔF508 CFTR mouse model with residual CFTR channel activity (228). The residual CFTR activity completely failed to induce CFTR dependent fluid secretion in both gallbladder (R.H.P.C. Peters, unpublished observations) and jejunum (PJF, unpublished observations), within the limits of our detection assay. As the ΔF508 CFTR mouse model has a mild phenotype compared to most knockout strains, there apparently is no simple correlation between defective fluid secretion and CF pathology. Thus,

absence of significant amounts of fluid secretion is not the only determinant of disease pathogenesis. A residual physiological function of CFTR other than fluid secretion could explain the mild phenotype of our mouse model. What functions of CFTR are important for the onset of the disease remains as yet unresolved. The mild phenotype may also be explained by the genetic background of the mouse strain (81) or breeding conditions, or by the assumption that a very low level of fluid secretion (below the threshold of our assay) is sufficient to prevent pathology.

In summary, several hypothesis have been proposed which link CFTR to the accumulation and obstruction of mucus. The various presumed physiological functions of CFTR have been combined in a model recently proposed by J. Bijman. This model is based on the requirement of a counterion for cation dependent transport systems. In the absence of a counterion, cation transport down its chemical gradient is limited by the electrical gradient that it creates. As different cell types may require Cl^- counterions for the functioning of a multitude of transporters, this may explain the wide variety of CF related disease symptoms. For example, Cl^- used as a counterion for H^+, is required for endosome acidification (185), see also fig 1.4. CFTR dependent Cl^- permeability is required for NaCl and fluid uptake in both sweat duct and the respiratory tract (Cl^- is used as a counterion for Na^+) see also figure 1.5A and B. The theory predicts that Na^+ and fluid absorption is decreased in CF airways, contradictory to what has been suggested before based on short circuit measurements in the Ussing chamber (246). Cl^- used as a counterion for Na^+ coupled transport systems may be required for intestinal nutrient uptake. For instance, the Na^+/glucose cotransporter was reported to be increased in short circuit current measurements of CF intestine mounted in Ussing chamber (247), quite like Na^+ uptake in airways. Non epithelial cell types, like cardiomyocytes and lymphocytes, may require CFTR expression to provide counterions for their specific transport systems. In summary, the hypothesis states that CF is not simply caused by a defect in one specific transport function of CFTR, but by a multitude of cellular transport functions; CF is caused by a general Cl^- impermeability associated with the active transport of cations.

If the theory also applies to intestinal epithelium, it predicts a reduction in the uptake of glucose and/or amino acids across CF intestine. This can be studied in CF mouse models either *in vivo*, or *in vitro* using radiolabelled ligands. In pulmonary epithelia, the model can be verified by studying fluid absorption across cultured pulmonary epithelium as described (210,211). Much of our present knowledge of CFTR functioning stems from short circuit current measurements (Isc) of tissue mounted in the Ussing chamber, however, the Isc measurements are not suitable to verify a possible defect in Na^+ coupled nutrient uptake in CF because they only reflect the active transport of Na^+ ions and not of passively diffusing ions like the Cl^- counterion (136).

2.3 Characterization of a mouse model for the ΔF508 CFTR mutation: The most common mutation in CFTR found in ≈70% of all CF alleles, is a deletion of a phenylalanine at position 508, ΔF508 (6). This mutation affects correct processing to the apical plasma membrane causing accumulation and degradation of the mutant protein in the endoplasmic reticulum (19,21,22,139-141,149,150,152). Interestingly, ΔF508 CFTR can be functional as a CFTR-like chloride channel when expressed on the apical membrane (80,141-143). Correction of the processing defect therefore is an interesting area for new therapeutic strategies.

In chapter 3.5 we describe the generation and characterization of a mouse model homozygous for the ΔF508 mutation. We show that this mouse model has a CF phenotype, but not as severe as the previously published KO mouse models (223-225,227,232). The mild phenotype of our mouse model can be explained by a residual chloride secretion observed in pulmonary (nasal), intestinal (jejunum, ileum, and caecum), and gallbladder epithelium. In a more detailed characterization (chapter 3.6), we show that mouse ΔF508 Cftr, like human ΔF508 CFTR, is misprocessed. Moreover, this processing defect is temperature sensitive. A small amount of ΔF508 Cftr appears to escape the processing defect which explains the residual cAMP induced chloride secretion observed in Ussing chamber experiments. The channel characteristics of wt and ΔF508 Cftr are identical. We conclude that the ΔF508 Cftr mouse model is an excellent model for the human ΔF508 CFTR mutation.

Two other groups have also generated mouse models for the ΔF508 mutation (229,230). In contrast to the ΔF508 mouse model described in this thesis, these mice were generated by a procedure leaving an intronic selection marker to reside within the mouse *Cftr* gene. Interestingly, these ΔF508 mouse models have a much more severe CF phenotype in comparison to our ΔF508 mouse model (229,230). The severe phenotype appears to be due to a reduced expression of ΔF508 *Cftr* mRNA, which is not found in our mouse model (chapter 3.6). The presence of an intronic selection marker is a likely cause of reduced ΔF508-*Cftr* mRNA expression. Therefore, the mouse models which have an intronic selection marker do not reflect true processing mutants because they combine reduced CFTR mRNA expression (class I mutant) with a processing defect (class II mutant).

The ΔF508 Cftr mouse model may find application, as *in vivo* model to test new pharmacological approaches aimed at circumventing or correcting the processing defect. For example, phenylbutyrate was reported to increase the amount of ΔF508 CFTR band C in cultured cells measured by western blot analysis (160). However, in our experiments, the use of phenylbutyrate did not increase the amount of ΔF508-Cftr expression on the plasmamembrane of cultured mouse gallbladder epithelia (PJF, unpublished observations). Clearly, further *in vivo* studies in the ΔF508 Cftr mouse model are required to verify its

potential effect.

Future studies should also focus on the basic mechanisms of protein processing and folding. It may prove worthwhile to follow processing of ΔF508 CFTR in cells overexpressing specific chaperones. A ΔF508 mutation in mouse Cftr results in a partial processing defect (Chapter 3.5 and 3.6), whereas this mutation generally causes a complete processing defect in human cells. Minor differences in mouse Cftr or processing machinery may explain the "leaky" processing of ΔF508 Cftr in the mouse. Cross-species transfection experiments could discriminate between the two, and can pinpoint the subtle but pathophysiologically important differences. Also, mouse models exhibiting residual CFTR activity may to help establish the amount of CFTR required for disease prevention. This is of crucial importance for gene transfer studies.

It is interesting to note that all processing mutations (except N1303K) can be found within a relatively small area of CFTR, comprising the amino acids 455-574 (A455E, K464M, ΔI507, ΔF508, F508R, S549I/R, A559T, D572N, P574H and N1303K (139,140,248)), although a recent report suggests that mutations in other parts of CFTR may also cause processing defects (249). This stretch almost completely covers the first NBD of CFTR. It would be interesting to study the importance of this stretch in the processing of CFTR. Several basic questions remain to be answered, for example, do chaperonins bind to the stretch and is its topology within CFTR important. Our basic understanding of the folding and processing of proteins may be greatly enhanced by studying proteins, like CFTR in which a single point mutation causes a complete block in protein processing.

Abbreviations:

Br-t	(-) -P- bromotetramisole
cAK	cAMP dependent protein kinase
C_{ak}	cAMP dependent protein kinase, catalytic subunit
CF	Cystic Fibrosis
CFTR	Cystic Fibrosis Transmembrane Conductance Regulator
CF2	$CFTR_{645-835}$
cGK	cGMP dependent protein kinase
DMSO	dimethyl sulfoxide
ER	endoplasmic reticulum
GM	ganglioside
IBMX	3-isobutyl 1-methyl xanthine
NBD	nucleotide binding domain
PDBu	4β-phorbol 12,13-dibutyrate
PKC	Ca^{2+} and phospholipid dependent protein kinase
PMA	phorbol myristate acetate
Po	open probability
TM	transmembrane domain
wt	wild type

Single letter amino acid code:

A:ala; C:cys; D:asp; E:glu; F:phe; G:gly; H:his; I:ile; K:lys; L:leu; M:met; N:asn; P:pro; Q:gln; R:arg; S:ser; T:thr; V:val; W:trp; Y:tyr

(1) Boat, T.F., Welsh, M.J., and Beaudet, A.L. *Cystic Fibrosis*; Boat, T.F., Welsh, M.J., and Beaudet, A.L., Ed., 1989; Vol. 6, pp 2649-2680.

(2) Welsh, M.J., Tsui, L.-C., Boat, T.F., and Beaudet, A.L. *Cystic Fibrosis*; Welsh, M.J., Tsui, L.-C., Boat, T.F., and Beaudet, A.L., Ed., 1995; Vol. 7, pp 3799-3876.

(3) Wildhagen, M.F., Verheij, J.B., Hilderink, H.B., Kooij, L., Tijmstra, T., ten Kate, L.P., Gerritsen, J., Bakker, W., Dik, J., and Habbema, F. (1996) *Thorax* **51**, 298-301

(4) Rommens, J.M., Iannuzzi, M.C., Kerem, B., Drumm, M.L., Melmer, G., Dean, M., Rozmahel, R., Cole, J.L., Kennedy, D., Hidaka, N., et al. (1989) *Science* **245**, 1059-65

(5) Riordan, J.R., Rommens, J.M., Kerem, B., Alon, N., Rozmahel, R., Grzelczak, Z., Zielenski, J., Lok, S., Plavsic, N., Chou, J.L., et al. (1989) *Science* **245**, 1066-73

(6) Kerem, B., Rommens, J.M., Buchanan, J.A., Markiewicz, D., Cox, T.K., Chakravarti, A., Buchwald, M., and Tsui, L.C. (1989) *Science* **245**, 1073-80

(7) Welsh, M.J., Denning, G.M., Ostedgaard, L.S., and Anderson, M.P. (1993) *J Cell Sci Suppl* **17**, 235-9

(8) Andersen, D.H. (1938) *Am J Dis Child* **56**, 344-399

(9) Di Sant-Agnese, P.A., Darling, R.C., Perera, G.A., and Shea, E. (1953) *Pediatrics* **12**, 549-

(10) Knowles, M., Gatzy, J., and Boucher, R. (1981) *N Engl J Med* **305**, 1489-95

(11) Quinton, P.M., and Bijman, J. (1983) *N Engl J Med* **308**, 1185-9

(12) Quinton, P.M. (1983) *Nature* **301**, 421-2

(13) Anderson, M.P., Rich, D.P., Gregory, R.J., Smith, A.E., and Welsh, M.J. (1991) *Science* **251**, 679-82

(14) Tabcharani, J.A., Chang, X.B., Riordan, J.R., and Hanrahan, J.W. (1991) *Nature* **352**, 628-31

(15) Kartner, N., Hanrahan, J.W., Jensen, T.J., Naismith, A.L., Sun, S.Z., Ackerley, C.A., Reyes, E.F., Tsui, L.C., Rommens, J.M., Bear, C.E., et al. (1991) *Cell* **64**, 681-91

(16) Bear, C.E., Li, C.H., Kartner, N., Bridges, R.J., Jensen, T.J., Ramjeesingh, M., and Riordan, J.R. (1992) *Cell* **68**, 809-18

(17) Bijman, J., Dalemans, W., Kansen, M., Keulemans, J., Verbeek, E., Hoogeveen, A., De Jonge, H., Wilke, M., Dreyer, D., Lecocq, J.P., et al. (1993) *Am J Physiol* **264**, L229-35

(18) Crawford, I., Maloney, P.C., Zeitlin, P.L., Guggino, W.B., Hyde, S.C., Turley, H., Gatter, K.C., Harris, A., and Higgins, C.F. (1991) *Proc Natl Acad Sci U S A* **88**, 9262-6

(19) Puchelle, E., Gaillard, D., Ploton, D., Hinnrasky, J., Fuchey, C., Boutterin, M.C., Jacquot, J., Dreyer, D., Pavirani, A., and Dalemans, W. (1992) *Am J Respir Cell Mol Biol* **7**, 485-91

(20) Denning, G.M., Ostedgaard, L.S., Cheng, S.H., Smith, A.E., and Welsh, M.J. (1992) *J Clin Invest* **89**, 339-49

(21) Denning, G.M., Ostedgaard, L.S., and Welsh, M.J. (1992) *J Cell Biol* **118**, 551-9

(22) Engelhardt, J.F., Yankaskas, J.R., Ernst, S.A., Yang, Y., Marino, C.R., Boucher, R.C., Cohn, J.A., and Wilson, J.M. (1992) *Nature Genet* **2**, 240-8

(23) Hermans, C.J., Veeze, H.J., Drexhage, V.R., Halley, D.J., and van den Ouweland, A.M. (1994) *Hum Mol Genet* **3**, 1199-200

(24) Anderson, M.P., Gregory, R.J., Thompson, S., Souza, D.W., Paul, S., Mulligan, R.C., Smith, A.E., and Welsh, M.J. (1991) *Science* **253**, 202-5

(25) French, P.J., Bijman, J., Edixhoven, M., Vaandrager, A.B., Scholte, B.J., Lohmann, S.M., Nairn, A.C., and de Jonge, H.R. (1995) *J Biol Chem* **270**, 26626-31

(26) Hyde, S.C., Emsley, P., Hartshorn, M.J., Mimmack, M.M., Gileadi, U., Pearce, S.R., Gallagher, M.P., Gill, D.R., Hubbard, R.E., and Higgins, C.F. (1990) *Nature* **346**, 362-5

(27) Ames, G.F., Mimura, C.S., and Shyamala, V. (1990) *Fems Microbiol Rev* **6**, 429-46

(28) Ames, G.F., and Lecar, H. (1992) *Faseb J* **6**, 2660-6

(29) Higgins, C.F. (1992) *Annu Rev Cell Biol* **8**, 67-113

(30) Endicott, J.A., and Ling, V. (1989) *Annu Rev Biochem* **58**, 137-71

(31) Kuchler, K., Sterne, R.E., and Thorner, J. (1989) *Embo J* **8**, 3973-84

(32) McGrath, J.P., and Varshavsky, A. (1989) *Nature* **340**, 400-4

(33) Van Kuijk, M.A., Van Aubel, R.A.M.H., Busch, A.E., Lang, F., Russel, F.G.M., Bindels, R.J.M., Os, C.H., and Deen, P.M.T. (1996) *Proc Natl Acad Sci USA* **93**, 5401-06

(34) Trezise, A.E., and Buchwald, M. (1991) *Nature* **353**, 434-7

(35) Strong, T.V., Boehm, K., and Collins, F.S. (1994) *J Clin Invest* **93**, 347-54

(36) Dray-Charier, N., Paul, A., Veissiere, D., Mergey, M., Scoazec, J.Y., Capeau, J., Brahimi-Horn, C., and Housset, C. (1995) *Lab Invest* **73**, 828-36

(37) Riordan, J.R. (1993) *Annu Rev Physiol* **55**, 609-30

(38) Frizzell, R.A., and Morris, A.P. (1994) *curr topics membr* **42**, 173-214

(39) Tizzano, E.F., and Buchwald, M. (1995) *Ann Intern Med* **123**, 305-8

(40) Schwiebert, E.M., Lopes, A.G., and Guggino, W.B. (1994) *curr topics membr* **42**, 265-315

(41) Hincke, M.T., Nairn, A.C., and Staines, W.A. (1995) *J Neurochem* **64**, 1662-8

(42) Mulberg, A.E., Resta, L.P., Wiedner, E.B., Altschuler, S.M., Jefferson, D.M., and Broussard, D.L. (1995) *J Clin Invest* **96**, 646-52

(43) McDonald, T.V., Nghiem, P.T., Gardner, P., and Martens, C.L. (1992) *J Biol Chem* **267**, 3242-8

(44) Levesque, P.C., Hart, P.J., Hume, J.R., Kenyon, J.L., and Horowitz, B. (1992) *Circ Res* **71**, 1002-7

(45) Hart, P., Warth, J.D., Levesque, P.C., Collier, M.L., Geary, Y., Horowitz, B., and Hume, J.R. (1996) *Proc Natl Acad Sci U S A* **93**, 6343-8

(46) Yoshimura, K., Nakamura, H., Trapnell, B.C., Dalemans, W., Pavirani, A., Lecocq, J.P., and Crystal, R.G. (1991) *J Biol Chem* **266**, 9140-4

(47) Trapnell, B.C., Zeitlin, P.L., Chu, C.S., Yoshimura, K., Nakamura, H., Guggino, W.B., Bargon, J., Banks, T.C., Dalemans, W., Pavirani, A., et al. (1991) *J Biol Chem* **266**, 10319-23

(48) Bargon, J., Trapnell, B.C., Yoshimura, K., Dalemans, W., Pavirani, A., Lecocq, J.P., and Crystal, R.G. (1992) *J Biol Chem* **267**, 16056-60

(49) Shen, B.Q., Barthelson, R.A., Skach, W., Gruenert, D.C., Sigal, E., Mrsny, R.J., and Widdicombe, J.H. (1993) *J Biol Chem* **268**, 19070-5

(50) Breuer, W., Glickstein, H., Kartner, N., Riordan, J.R., Ausiello, D.A., and Cabantchik, I.Z. (1993) *J Biol Chem* **268**, 13935-9

(51) Breuer, W., Kartner, N., Riordan, J.R., and Cabantchik, Z.I. (1992) *J Biol Chem* **267**, 10465-9

(52) Besancon, F., Przewlocki, G., Baro, I., Hongre, A.S., Escande, D., and Edelman, A. (1994) *Am J Physiol* **267**, C1398-404

(53) Nakamura, H., Yoshimura, K., Bajocchi, G., Trapnell, B.C., Pavirani, A., and Crystal, R.G. (1992) *Febs Lett* **314**, 366-70

(54) Horowitz, B., Tsung, S.S., Hart, P., Levesque, P.C., and Hume, J.R. (1993) *Am J Physiol* **264**, H2214-20

(55) Morales, M.M., Carroll, T.P., Morita, T., Schwiebert, E.M., Devuyst, O., Wilson, P.D., Lopes, A.G., Stanton, B.A., Dietz, H.C., Cutting, G.R., and Guggino, W.B. (1996) *Amer J Physiol Renal Fl Elect* 39, F1038-F1048

(56) Sheppard, D.N., Ostedgaard, L.S., Rich, D.P., and Welsh, M.J. (1994) *Cell* **76**, 1091-8
(57) Chang, X.B., Hou, Y.X., Jensen, T.J., and Riordan, J.R. (1994) *J Biol Chem* **269**, 18572-5
(58) Oblatt-Montal, M., Reddy, G.L., Iwamoto, T., Tomich, J.M., and Montal, M. (1994) *Proc Natl Acad Sci U S A* **91**, 1495-9
(59) Akabas, M.H., Kaufmann, C., Cook, T.A., and Archdeacon, P. (1994) *J Biol Chem* **269**, 14865-8
(60) Cheung, M., and Akabas, M.H. (1996) *Biophys J* **70**, 2688-2695
(61) Imoto, K. (1993) *Febs Lett* **325**, 100-3
(62) Sheppard, D.N., Rich, D.P., Ostedgaard, L.S., Gregory, R.J., Smith, A.E., and Welsh, M.J. (1993) *Nature* **362**, 160-4
(63) Hipper, A., Mall, M., Greger, R., and Kunzelmann, K. (1995) *Febs Lett* **374**, 312-6
(64) Rich, D.P., Gregory, R.J., Anderson, M.P., Manavalan, P., Smith, A.E., and Welsh, M.J. (1991) *Science* **253**, 205-7
(65) Cheng, S.H., Rich, D.P., Marshall, J., Gregory, R.J., Welsh, M.J., and Smith, A.E. (1991) *Cell* **66**, 1027-36
(66) Ma, J.J., Tasch, J.E., Tao, T., Zhao, J.Y., Xie, J.X., Drumm, M.L., and Davis, P.B. (1996) *J Biol Chem* **271**, 7351-7356
(67) Chang, X.B., Tabcharani, J.A., Hou, Y.X., Jensen, T.J., Kartner, N., Alon, N., Hanrahan, J.W., and Riordan, J.R. (1993) *J Biol Chem* **268**, 11304-11
(68) Rich, D.P., Berger, H.A., Cheng, S.H., Travis, S.M., Saxena, M., Smith, A.E., and Welsh, M.J. (1993) *J Biol Chem* **268**, 20259-67
(69) Dulhanty, A.M., and Riordan, J.R. (1994) *Biochemistry* **33**, 4072-9
(70) Travis, S.M., Carson, M.R., Ries, D.R., and Welsh, M.J. (1993) *J Biol Chem* **268**, 15336-9
(71) Anderson, M.P., and Welsh, M.J. (1992) *Science* **257**, 1701-4
(72) Anderson, M.P., Berger, H.A., Rich, D.P., Gregory, R.J., Smith, A.E., and Welsh, M.J. (1991) *Cell* **67**, 775-84
(73) Ko, Y.H., and Pedersen, P.L. (1995) *J Biol Chem* **270**, 22093-6
(74) Schultz, B.D., Venglarik, C.J., Bridges, R.J., and Frizzell, R.A. (1995) *J Gen Physiol* **105**, 329-61
(75) Walker, J.E., Saraste, M., Runswick, M.J., and Gay, N.J. (1982) *Embo J* **1**, 945-51
(76) Yoshida, M., and Amano, T. (1995) *Febs Lett* **359**, 1-5
(77) Logan, J., Hiestand, D., Daram, P., Huang, Z., Muccio, D.D., Hartman, J., Haley, B., Cook, W.J., and Sorscher, E.J. (1994) *J Clin Invest* **94**, 228-36
(78) Carson, M.R., Travis, S.M., and Welsh, M.J. (1995) *J Biol Chem* **270**, 1711-7
(79) Gunderson, K.L., and Kopito, R.R. (1995) *Cell* **82**, 231-9
(80) Drumm, M.L., Wilkinson, D.J., Smit, L.S., Worrell, R.T., Strong, T.V., Frizzell, R.A., Dawson, D.C., and Collins, F.S. (1991) *Science* **254**, 1797-9
(81) Wilkinson, D.J., Mansoura, M.K., Watson, P.Y., Smit, L.S., Collins, F.S., and Dawson, D.C. (1996) *J Gen Physiol* **107**, 103-19
(82) Hwang, T.C., Nagel, G., Nairn, A.C., and Gadsby, D.C. (1994) *Proc Natl Acad Sci U S A* **91**, 4698-702
(83) Carson, M.R., Winter, M.C., Travis, S.M., and Welsh, M.J. (1995) *J Biol Chem* **270**, 20466-72
(84) Baukrowitz, T., Hwang, T.C., Nairn, A.C., and Gadsby, D.C. (1994) *Neuron* **12**, 473-82
(85) Hwang, T.C., Horie, M., and Gadsby, D.C. (1993) *J Gen Physiol* **101**, 629-50
(86) Gadsby, D.C., Hwang, T.C., Baukrowitz, T., Nagel, G., Horie, M., and Nairn, A.C. (1994) *Jpn J Physiol* **44**, S183-92

(87) Picciotto, M.R., Cohn, J.A., Bertuzzi, G., Greengard, P., and Nairn, A.C. (1992) *J Biol Chem* **267**, 12742-52

(88) Kennelly, P.J., and Krebs, E.G. (1991) *J Biol Chem* **266**, 15555-8

(89) Seibert, F.S., Tabcharani, J.A., Chang, X.B., Dulhanty, A.M., Mathews, C., Hanrahan, J.W., and Riordan, J.R. (1995) *J Biol Chem* **270**, 2158-62

(90) Berger, H.A., Travis, S.M., and Welsh, M.J. (1993) *J Biol Chem* **268**, 2037-47

(91) Dechecchi, M.C., Tamanini, A., Berton, G., and Cabrini, G. (1993) *J Biol Chem* **268**, 11321-5

(92) Dho, S., and Foskett, J.K. (1993) *Biochim Biophys Acta* **1152**, 83-90

(93) Collier, M.L., and Hume, J.R. (1995) *Circ Res* **76**, 317-24

(94) Bajnath, R.B., Groot, J.A., De Jonge, H.R., Kansen, M., and Bijman, J. (1993) *Pflugers Arch* **425**, 100-8

(95) Winpenny, J.P., McAlroy, H.L., Gray, M.A., and Argent, B.E. (1995) *Am J Physiol* **268**, C823-8

(96) Vaandrager, A.B., and De Jonge, H.R. (1994) *Adv Pharmacol* **26**, 253-83

(97) Forte, L.R., Thorne, P.K., Eber, S.L., Krause, W.J., Freeman, R.H., Francis, S.H., and Corbin, J.D. (1992) *Am J Physiol* **263**, C607-15

(98) Chao, A.C., de Sauvage, F.J., Dong, Y.J., Wagner, J.A., Goeddel, D.V., and Gardner, P. (1994) *Embo J* **13**, 1065-72

(99) Tien, X.Y., Brasitus, T.A., Kaetzel, M.A., Dedman, J.R., and Nelson, D.J. (1994) *J Biol Chem* **269**, 51-4

(100) Zielenski, J., Markiewicz, D., Lin, S.P., Huang, F.Y., Yang-Feng, T.L., and Tsui, L.C. (1995) *Clin Genet* **47**, 125-32

(101) Hidaka, H., and Kobayashi, R. (1992) *Annu Rev Pharmacol Toxicol* **32**, 377-97

(102) Shabb, J.B., Ng, L., and Corbin, J.D. (1990) *J Biol Chem* **265**, 16031-4

(103) De Jonge, H.R., and Rao, M.C. *cyclic nucleotide dependent kinases*; De Jonge, H.R., and Rao, M.C., Ed., 1990, pp 191-207.

(104) Vaandrager, A.B., and de Jonge, H.R. (1996) *Mol Cell Biochem* **157**, 23-30

(105) Lin, M., Nairn, A.C., and Guggino, S.E. (1992) *Am J Physiol* **262**, C1304-12

(106) Sullivan, S.K., Agellon, L.B., and Schick, R. (1995) *Curr Biol* **5**, 1159-67

(107) Markert, T., Vaandrager, A.B., Gambaryan, S., Pohler, D., Hausler, C., Walter, U., De Jonge, H.R., Jarchau, T., and Lohmann, S.M. (1995) *J Clin Invest* **96**, 822-30

(108) Akiyama, T., and Ogawara, H. (1991) *Methods Enzymol* **201**, 362-70

(109) Akiyama, T., Ishida, J., Nakagawa, S., Ogawara, H., Watanabe, S., Itoh, N., Shibuya, M., and Fukami, Y. (1987) *J Biol Chem* **262**, 5592-5

(110) Sears, C.L., Firoozmand, F., Mellander, A., Chambers, F.G., Eromar, I.G., Bot, A.G., Scholte, B., De Jonge, H.R., and Donowitz, M. (1995) *Am J Physiol* **269**, G874-82

(111) Illek, B., Fischer, H., Santos, G.F., Widdicombe, J.H., Machen, T.E., and Reenstra, W.W. (1995) *Am J Physiol* **268**, C886-93

(112) Illek, B., Fischer, H., and Machen, T.E. (1996) *Am J Physiol* **39**, C265-C275

(113) Lehrich, R.W., and Forrest, J., Jr. (1995) *Am J Physiol* **269**, F594-600

(114) Diener, M., and Hug, F. (1996) *Eur J Pharmacol* **299**, 161-170

(115) Reenstra, W.W., Dam, A., and Raman, S. (1995) *Ped Pulmonol Suppl* **12**, 188

(116) Becq, F., Jensen, T.J., Chang, X.B., Savoia, A., Rommens, J.M., Tsui, L.C., Buchwald, M., Riordan, J.R., and Hanrahan, J.W. (1994) *Proc Natl Acad Sci U S A* 91, 9160-4

(117) Higgins, C.F. (1995) *Cell* **82**, 693-6

(118) Welsh, M.J. (1990) *Faseb J* **4**, 2718-25

(119) Gabriel, S.E., Clarke, L.L., Boucher, R.C., and Stutts, M.J. (1993) *Nature* **363**, 263-8

(120) Schwiebert, E.M., Flotte, T., Cutting, G.R., and Guggino, W.B. (1994) *Am J Physiol* **266**, C1464-77

(121) Fulmer, S.B., Schwiebert, E.M., Morales, M.M., Guggino, W.B., and Cutting, G.R. (1995) *Proc Natl Acad Sci U S A* **92**, 6832-6

(122) Egan, M., Flotte, T., Afione, S., Solow, R., Zeitlin, P.L., Carter, B.J., and Guggino, W.B. (1992) *Nature* **358**, 581-4

(123) Jovov, B., Ismailov, I.I., and Benos, D.J. (1995) *J Biol Chem* **270**, 1521-8

(124) Jovov, B., Ismailov, I.I., Berdiev, B.K., Fuller, C.M., Sorscher, E.J., Dedman, J.R., Kaetzel, M.A., and Benos, D.J. (1995) *J Biol Chem* **270**, 29194-200

(125) Schwiebert, E.M., Egan, M.E., Hwang, T.H., Fulmer, S.B., Allen, S.S., Cutting, G.R., and Guggino, W.B. (1995) *Cell* **81**, 1063-73

(126) Ismailov, I.I., Jovov, B., Fuller, C.M., Berdiev, B.K., Keeton, D.A., and Benos, D.J. (1996) *J Biol Chem* **271**, 4776-80

(127) Reddy, M.M., Quinton, P.M., Haws, C., Wine, J.J., Grygorczyk, R., Tabcharani, J.A., Hanrahan, J.W., Gunderson, K.L., and Kopito, R.R. (1996) *Science* **271**, 1876-9

(128) Li, C., Ramjeesingh, M., and Bear, C.E. (1996) *J Biol Chem* **271**, 11623-6

(129) Grygorczyk, R., Tabcharani, J.A., and Hanrahan, J.W. (1996) *J Membr Biol* **151**, 139-48

(130) Reisin, I.L., Prat, A.G., Abraham, E.H., Amara, J.F., Gregory, R.J., Ausiello, D.A., and Cantiello, H.F. (1994) *J Biol Chem* **269**, 20584-91

(131) Quinton, P.M. (1990) *Faseb J* **4**, 2709-17

(132) Johnson, L.G., Boyles, S.E., Wilson, J., and Boucher, R.C. (1995) *J Clin Invest* **95**, 1377-82

(133) Chinet, T.C., Fullton, J.M., Yankaskas, J.R., Boucher, R.C., and Stutts, M.J. (1994) *Am J Physiol* **266**, C1061-8

(134) Stutts, M.J., Canessa, C.M., Olsen, J.C., Hamrick, M., Cohn, J.A., Rossier, B.C., and Boucher, R.C. (1995) *Science* **269**, 847-50

(135) Ismailov, I.I., Awayda, M.S., Jovov, B., Berdiev, B.K., Fuller, C.M., Dedman, J.R., Kaetzel, M., and Benos, D.J. (1996) *J Biol Chem* **271**, 4725-32

(136) Steward, M.C., and Case, R.M. *Principles of ion transport across epithelia*; Steward, M.C., and Case, R.M., Ed., 1989.

(137) Welsh, M.J., and Smith, A.E. (1993) *Cell* **73**, 1251-4

(138) Gregory, R.J., Cheng, S.H., Rich, D.P., Marshall, J., Paul, S., Hehir, K., Ostedgaard, L., Klinger, K.W., Welsh, M.J., and Smith, A.E. (1990) *Nature* **347**, 382-6

(139) Cheng, S.H., Gregory, R.J., Marshall, J., Paul, S., Souza, D.W., White, G.A., O'Riordan, C.R., and Smith, A.E. (1990) *Cell* **63**, 827-34

(140) Gregory, R.J., Rich, D.P., Cheng, S.H., Souza, D.W., Paul, S., Manavalan, P., Anderson, M.P., Welsh, M.J., and Smith, A.E. (1991) *Mol Cell Biol* **11**, 3886-93

(141) Yang, Y., Devor, D.C., Engelhardt, J.F., Ernst, S.A., Strong, T.V., Collins, F.S., Cohn, J.A., Frizzell, R.A., and Wilson, J.M. (1993) *Hum Mol Genet* **2**, 1253-61

(142) Denning, G.M., Anderson, M.P., Amara, J.F., Marshall, J., Smith, A.E., and Welsh, M.J. (1992) *Nature* **358**, 761-4

(143) Li, C., Ramjeesingh, M., Reyes, E., Jensen, T., Chang, X., Rommens, J.M., and Bear, C.E. (1993)

Nature Genet **3**, 311-6

(144) Dalemans, W., Barbry, P., Champigny, G., Jallat, S., Dott, K., Dreyer, D., Crystal, R.G., Pavirani, A., Lecocq, J.P., and Lazdunski, M. (1991) *Nature* **354**, 526-8

(145) Haws, C.M., Nepomuceno, I.B., Krouse, M.E., Wakelee, H., Law, T., Xia, Y., Nguyen, H., and Wine, J.J. (1996) *Amer J Physiol Cell Physiol* **39**, C1544-C1555

(146) Trapnell, B.C., Chu, C.S., Paakko, P.K., Banks, T.C., Yoshimura, K., Ferrans, V.J., Chernick, M.S., and Crystal, R.G. (1991) *Proc Natl Acad Sci U S A* **88**, 6565-9

(147) Thomas, P.J., Qu, B.H., and Pedersen, P.L. (1995) *Trends Biochem Sci* **20**, 456-9

(148) Yang, Y., Janich, S., Cohn, J.A., and Wilson, J.M. (1993) *Proc Natl Acad Sci U S A* **90**, 9480-4

(149) Pind, S., Riordan, J.R., and Williams, D.B. (1994) *J Biol Chem* **269**, 12784-8

(150) Ward, C.L., and Kopito, R.R. (1994) *J Biol Chem* **269**, 25710-8

(151) Marshall, J., Fang, S., Ostedgaard, L.S., O'Riordan, C.R., Ferrara, D., Amara, J.F., Hoppe, H.4., Scheule, R.K., Welsh, M.J., Smith, A.E., et al. (1994) *J Biol Chem* **269**, 2987-95

(152) Lukacs, G.L., Mohamed, A., Kartner, N., Chang, X.B., Riordan, J.R., and Grinstein, S. (1994) *Embo J* **13**, 6076-86

(153) Ward, C.L., Omura, S., and Kopito, R.R. (1995) *Cell* **83**, 121-7

(154) Jensen, T.J., Loo, M.A., Pind, S., Williams, D.B., Goldberg, A.L., and Riordan, J.R. (1995) *Cell* **83**, 129-35

(155) Lukacs, G.L., Chang, X.B., Bear, C., Kartner, N., Mohamed, A., Riordan, J.R., and Grinstein, S. (1993) *J Biol Chem* **268**, 21592-8

(156) Thomas, P.J., Shenbagamurthi, P., Ysern, X., and Pedersen, P.L. (1991) *Science* **251**, 555-7

(157) Thomas, P.J., Ko, Y.H., and Pedersen, P.L. (1992) *Febs Lett* **312**, 7-9

(158) Thomas, P.J., Shenbagamurthi, P., Sondek, J., Hullihen, J.M., and Pedersen, P.L. (1992) *J Biol Chem* **267**, 5727-30

(159) Ciechanover, A. (1994) *Cell* **79**, 13-21

(160) Rubenstein, R., and Zeitlin, P. (1995) *Ped Pulmonol* **12**, 234

(161) Sato, S., Ward, C.L., Krouse, M.E., Wine, J.J., and Kopito, R.R. (1996) *J Biol Chem* **271**, 635-8

(162) Welch, W.J., Brown, C.R., Brown, L.Q., Biwersi, J., Verkman, A.S., Tatzelt, J., and Prusiner, S.B. (1996) *Ped Pulmon Suppl* **13**, 258

(163) Bebok, Z., Venglarik, C.J., and Sorscher, E.J. (1996) *Ped Pulmon Suppl* **13**, 259

(164) Teem, J.L., Berger, H.A., Ostedgaard, L.S., Rich, D.P., Tsui, L.C., and Welsh, M.J. (1993) *Cell* **73**, 335-46

(165) Dork, T., Wulbrand, U., Richter, T., Neumann, T., Wolfes, H., Wulf, B., Maass, G., and Tummler, B. (1991) *Hum Genet* **87**, 441-6

(166) Greil, I., Wagner, K., Eber, E., Zach, M., and Rosenkranz, W. (1995) *Wien Klin Wochenschr* **107**, 464-9

(167) Rose, M.C. (1992) *Am J Physiol* **263**, L413-29

(168) Boat, T.F., Kleinerman, J.I., Carlson, D.M., Maloney, W.H., and Matthews, L.W. (1974) *Am Rev Respir Dis* **110**, 428-41

(169) Boat, T.F., Cheng, P.W., Iyer, R.N., Carlson, D.M., and Polony, I. (1976) *Arch Biochem Biophys* **177**, 95-104

(170) Frates, R., Jr., Kaizu, T.T., and Last, J.A. (1983) *Pediatr Res* **17**, 30-4

(171) Cheng, P.W., Boat, T.F., Cranfill, K., Yankaskas, J.R., and Boucher, R.C. (1989) *J Clin Invest* **84**,

68-72

(172) Zhang, Y., Doranz, B., Yankaskas, J.R., and Engelhardt, J.F. (1995) *J Clin Invest* **96**, 2997-3004

(173) Gupta, R., and Jentoft, N. (1992) *J Biol Chem* **267**, 3160-7

(174) Saiman, L., and Prince, A. (1993) *J Clin Invest* **92**, 1875-80

(175) Barasch, J., Kiss, B., Prince, A., Saiman, L., Gruenert, D., and al-Awqati, Q. (1991) *Nature* **352**, 70-3

(176) Clamp, J.R., and Gough, M. (1979) *Clin Sci* **57**, 445-51

(177) Dische, Z., di Sant Agnese, P., Pallavicini, C., and Youlos, J. (1959) *Pediatrics* , 74-91

(178) Wesley, A., Forstner, J., Qureshi, R., Mantle, M., and Forstner, G. (1983) *Pediatr Res* **17**, 65-9

(179) Rogers, D.F., Alton, E.W., Dewar, A., Lethem, M.I., and Barnes, P.J. (1993) *Exp Lung Res* **19**, 37-53

(180) Litt, M., Khan, M.A., Shih, C.K., and Wolf, D.P. (1977) *Biorheology* **14**, 127-32

(181) Mian, N., Pope, A.J., Anderson, C.E., and Kent, P.W. (1982) *Biochim Biophys Acta* **717**, 41-8

(182) Krivan, H.C., Roberts, D.D., and Ginsburg, V. (1988) *Proc Natl Acad Sci U S A* **85**, 6157-61

(183) Krivan, H.C., Ginsburg, V., and Roberts, D.D. (1988) *Arch Biochem Biophys* **260**, 493-6

(184) Imundo, L., Barasch, J., Prince, A., and Al-Awqati, Q. (1995) *Proc Natl Acad Sci U S A* **92**, 3019-23

(185) al-Awqati, Q., Barasch, J., and Landry, D. (1992) *J Exp Biol* **172**, 245-66

(186) Paulson, J.C., Prieels, J.P., Glasgow, L.R., and Hill, R.L. (1978) *J Biol Chem* **253**, 5617-24

(187) Bradbury, N.A., Cohn, J.A., Venglarik, C.J., and Bridges, R.J. (1994) *J Biol Chem* **269**, 8296-302

(188) Webster, P., Vanacore, L., Nairn, A.C., and Marino, C.R. (1994) *Am J Physiol* **267**, C340-8

(189) Lukacs, G.L., Chang, X.B., Kartner, N., Rotstein, O.D., Riordan, J.R., and Grinstein, S. (1992) *J Biol Chem* **267**, 14568-72

(190) Biwersi, J., and Verkman, A.S. (1994) *Am J Physiol* **266**, C149-56

(191) Roff, C.F., Fuchs, R., Mellman, I., and Robbins, A.R. (1986) *J Cell Biol* **103**, 2283-97

(192) Gottlieb, R.A., and Dosanjh, A. (1996) *Proc Natl Acad Sci U S A* **93**, 3587-91

(193) Dunn, K.W., Park, J., Semrad, C.E., Gelman, D.L., Shevell, T., and McGraw, T.E. (1994) *J Biol Chem* **269**, 5336-45

(194) Seksek, O., Biwersi, J., and Verkman, A.S. (1996) *J Biol Chem* **271**, 15542-15548

(195) Lamblin, G., Lhermitte, M., Degand, P., Roussel, P., and Slayter, H.S. (1979) *Biochimie* **61**, 23-43

(196) Chace, K.V., Leahy, D.S., Martin, R., Carubelli, R., Flux, M., and Sachdev, G.P. (1983) *Clin Chim Acta* **132**, 143-55

(197) McPherson, M.A., and Dormer, R.L. (1994) *The Lancet* **343**, 7

(198) McPherson, M.A., Dormer, R.L., Bradbury, N.A., Dodge, J.A., and Goodchild, M.C. (1986) *The Lancet* **2**, 1007-8

(199) Mills, C.L., Pereira, M.M., Dormer, R.L., and McPherson, M.A. (1992) *Biochem Biophys Res Commun* **188**, 1146-52

(200) Hoogeveen, A.T., Keulemans, J., Willemsen, R., Scholte, B.J., Bijman, J., Edixhoven, M.J., De Jonge, H.R., and Galjaard, H. (1991) *Exp Cell Res* **193**, 435-7

(201) Engelhardt, J.F., Smith, S.S., Allen, E., Yankaskas, J.R., Dawson, D.C., and Wilson, J.M. (1994) *Am J Physiol* **267**, C491-500

(202) Bijman, J., and Quinton, P.M. (1984) *Am J Physiol* **247**, C3-9

(203) Joris, L., Dab, I., and Quinton, P.M. (1993) *Am Rev Respir Dis* **148**, 1633-7

(204) Gilljam, H., Ellin, A., and Strandvik, B. (1989) *Scand J Clin Lab Invest* **49**, 121-4

(205) Bansil, R., Stanley, E., and LaMont, J.T. (1995) *Annu Rev Physiol* **57**, 635-57

(206) Smith, J.J., Travis, S.M., Greenberg, E.P., and Welsh, M.J. (1996) *Cell* **85**, 229-36

(207) Kopelman, H., Durie, P., Gaskin, K., Weizman, Z., and Forstner, G. (1985) *N Engl J Med* **312**, 329-34
(208) Davidson, G.P., Kirubakaran, C.P., Ratcliffe, G., Cooper, D.M., and Robb, T.A. (1986) *Acta Paediatr Scand* **75**, 145-50
(209) Kopelman, H., Corey, M., Gaskin, K., Durie, P., Weizman, Z., and Forstner, G. (1988) *Gastroenterology* **95**, 349-55
(210) Jiang, C., Finkbeiner, W.E., Widdicombe, J.H., McCray, P., Jr., and Miller, S.S. (1993) *Science* **262**, 424-7
(211) Smith, J.J., Karp, P.H., and Welsh, M.J. (1994) *J Clin Invest* **93**, 1307-11
(212) Davidow, C.J., Maser, R.L., Rome, L.A., Calvet, J.P., and Grantham, J.J. (1996) *Kidney Int* **50**, 208-218
(213) Peters, R.H.P.C., van Doorninck, J.H., French, P.J., Ratcliff, R., Evans, M.J., Colledge, W.H., Bijman, J., and Scholte, B.J. (1996) *Hepatology (in press)*
(214) Gabriel, S.E., Brigman, K.N., Koller, B.H., Boucher, R.C., and Stutts, M.J. (1994) *Science* **266**, 107-9
(215) Hasegawa, H., Skach, W., Baker, O., Calayag, M.C., Lingappa, V., and Verkman, A.S. (1992) *Science* **258**, 1477-9
(216) Smith, J.J., and Welsh, M.J. (1992) *J Clin Invest* **89**, 1148-53
(217) Zar, H., Saiman, L., Quittell, L., and Prince, A. (1995) *J Pediatr* **126**, 230-3
(218) Pier, G.B., Grout, M., Zaidi, T.S., Olsen, J.C., Johnson, L.G., Yankaskas, J.R., and Goldberg, J.B. (1996) *Science* **271**, 64-7
(219) Bradbury, N.A., Jilling, T., Berta, G., Sorscher, E.J., Bridges, R.J., and Kirk, K.L. (1992) *Science* **256**, 530-2
(220) Prince, L.S., Tousson, A., and Marchase, R.B. (1993) *Am J Physiol* **264**, C491-8
(221) Spilmont, C., Hinnrasky, J., Zahm, J.M., Jacquot, J., Puchelle, E., and Kuhry, J.G. (1996) *Biochem Biophys Res Commun* **227**, 182-188
(222) Tata, F., Stanier, P., Wicking, C., Halford, S., Kruyer, H., Lench, N.J., Scambler, P.J., Hansen, C., Braman, J.C., Williamson, R., et al. (1991) *Genomics* **10**, 301-7
(223) Hasty, P., O'Neal, W.K., Liu, K.Q., Morris, A.P., Bebok, Z., Shumyatsky, G.B., Jilling, T., Sorscher, E.J., Bradley, A., and Beaudet, A.L. (1995) *Somat Cell Mol Genet* **21**, 177-87
(224) O'Neal, W.K., Hasty, P., McCray, P., Jr., Casey, B., Rivera-Perez, J., Welsh, M.J., Beaudet, A.L., and Bradley, A. (1993) *Hum Mol Genet* **2**, 1561-9
(225) Snouwaert, J.N., Brigman, K.K., Latour, A.M., Malouf, N.N., Boucher, R.C., Smithies, O., and Koller, B.H. (1992) *Science* **257**, 1083-8
(226) Dorin, J.R., Dickinson, P., Alton, E.W., Smith, S.N., Geddes, D.M., Stevenson, B.J., Kimber, W.L., Fleming, S., Clarke, A.R., Hooper, M.L., et al. (1992) *Nature* **359**, 211-5
(227) Colledge, W.H., Ratcliff, R., Foster, D., Williamson, R., and Evans, M.J. (1992) *The Lancet* **340**, 680
(228) van Doorninck, J.H., French, P.J., Verbeek, E., Peters, R.H., Morreau, H., Bijman, J., and Scholte, B.J. (1995) *Embo J* **14**, 4403-11
(229) Zeiher, B.G., Eichwald, E., Zabner, J., Smith, J.J., Puga, A.P., McCray, P., Jr., Capecchi, M.R., Welsh, M.J., and Thomas, K.R. (1995) *J Clin Invest* **96**, 2051-64
(230) Colledge, W.H., Abella, B.S., Southern, K.W., Ratcliff, R., Jiang, C., Cheng, S.H., MacVinish, L.J., Anderson, J.R., Cuthbert, A.W., and Evans, M.J. (1995) *Nature Genet* **10**, 445-52
(231) Delaney, S.J., Alton, E.W., Smith, S.N., Lunn, D.P., Farley, R., Lovelock, P.K., Thomson, S.A., Hume, D.A., Lamb, D., Porteous, D.J., Dorin, J.R., and Wainwright, B.J. (1996) *Embo J*

15, 955-63

(232) Ratcliff, R., Evans, M.J., Cuthbert, A.W., MacVinish, L.J., Foster, D., Anderson, J.R., and Colledge, W.H. (1993) *Nature Genet* 4, 35-41

(233) Dorin, J.R., Stevenson, B.J., Fleming, S., Alton, E.W., Dickinson, P., and Porteous, D.J. (1994) *Mamm Genome* 5, 465-72

(234) Pack, R.J., Al-Ugaily, L.H., Morris, G., and Widdicombe, J.G. (1980) *cell tissue res* 208, 65-84

(235) Clarke, L.L., Grubb, B.R., Yankaskas, J.R., Cotton, C.U., McKenzie, A., and Boucher, R.C. (1994) *Proc Natl Acad Sci U S A* 91, 479-83

(236) Rozmahel, R., Wilschanski, M., Matin, A., Plyte, S., Oliver, M., Auerbach, W., Moore, A., Forstner, J., Durie, P., Nadeau, J., Bear, C., and Tsui, L.C. (1996) *Nature Genet* 12, 280-7

(237) Davidson, D.J., Dorin, J.R., McLachlan, G., Ranaldi, V., Lamb, D., Doherty, C., Govan, J., and Porteous, D.J. (1995) *Nature Genet* 9, 351-7

(238) Clarke, L.L., Grubb, B.R., Gabriel, S.E., Smithies, O., Koller, B.H., and Boucher, R.C. (1992) *Science* 257, 1125-8

(239) Smith, S.N., Steel, D.M., Middleton, P.G., Munkonge, F.M., Geddes, D.M., Caplen, N.J., Porteous, D.J., Dorin, J.R., and Alton, A.W.F.W. (1995) *Am J Physiol* 268, C297-C307

(240) French, P.J., Vandoorninck, J.H., Peters, R., Verbeek, E., Ameen, N.A., Marino, C.R., Dejonge, H.R., Bijman, J., and Scholte, B.J. (1996) *J Clin Invest* 98, 1304-1312

(241) Vaandrager, A., Tilly, B., Smolenski, A., Schneider-Rasp, S., Bot, A.G.M., Edixhoven, M., Scholte, B.J., Jarchau, T., Walter, U., Lohmann, S.M., Poller, W.C., and de Jonge, H.R. (1997) *J Biol Chem* 272, 4195-200

(241a) de Jonge, H.R., Ameen, N.A., Boomaars, W.E.M., French, P.J., Bijman, J., Scholte, B.J., Marino, C.R. (1996) *Ped Pulmonol suppl* 13, 218

(242) Markovits, J., Linassier, C., Fosse, P., Couprie, J., Pierre, J., Jacquemin-Sablon, A., Saucier, J.M., Le Pecq, J.B., and Larsen, A.K. (1989) *Cancer Res* 49, 5111-7

(243) Barnes, S. (1995) *J Nutr* 125, 777S-783S

(244) Chace, K.V., Leahy, D.S., Martin, R., Carubelli, R., Flux, M., and Sachdev, G.P. (1983) *Clin Chim Acta* 132, 143-55

(245) Biwersi, J., Emans, N., and Verkman, A.S. (1996) *Proc Natl Acad Sci Usa* 93, 12484-12489

(246) Boucher, R.C., Jackson Stutts, M., Knowles, M.R., Cantley, L., and Gatzy, J.T. (1986) *J Clin Invest* 78, 1245-52

(247) Baxter, P., Goldhill, J., Hardcastle, J., Hardcastle, P.T., and Taylor, C.J. (1990) *Gut* 31, 817-20

(248) Sheppard, D.N., Ostedgaard, L.S., Winter, M.C., and Welsh, M.J. (1995) *Embo J* 14, 876-83

(249) Seibert (1996) *Ped Pulmonol suppl* 13, 226

Samenvatting

Cystische Fibrose (CF) is een veel voorkomende erfelijke ziekte in de westerse samenleving. De ziekte wordt voornamelijk gekenmerkt door recidiverende pulmonaire infecties welke de belangrijkste oorzaak vormen voor het vroegtijdig overlijden van CF patienten. De gehele europese gemeenschap telt ongeveer 25000 CF patienten waarvan zo'n 1000 in Nederland. Hoewel de gemiddelde leeftijd van CF patienten de laatste decenia flink is toegenomen tot circa 29 jaar blijft CF een zeer ernstige aandoening met een slechte prognose waarvoor tot op heden een effectieve therapie ontbreekt. Aangezien CF een erfelijke ziekte is, zal er bij deze patienten een verandering zijn opgetreden in het DNA, de drager van ons erfelijk materiaal. Het gen[1] wat gemuteerd is in CF patienten is, na intensief onderzoek, ontdekt in 1989. Het gen bleek te coderen voor CFTR (Cystic Fibrosis Transmembrane Conductance Regulator), een membraan-geassocieerd eiwit dat functioneert als een chloride kanaal[2]. Dit kanaal komt bij CF patienten niet, of niet actief, tot expressie op de celmembraan. Momenteel is het nog niet duidelijk waarom de afwezigheid van een chloride kanaal kan leiden tot CF. Om dit te verklaren is een gedetailleerd onderzoek nodig naar de regulatie en fysiologische functie van het CFTR eiwit. Een beter inzicht in het CFTR eiwit kan nieuwe perspectieven bieden in de ontwikkeling van een therapie voor CF.

Eén van de manieren om het CFTR-chloride kanaal te openen (te activeren) is door op speciefieke plaatsen in het CFTR eiwit een fosfaatgroep te incorporeren. De incorporatie van deze fosfaatgroepen (fosforylering) wordt gemedieerd door zogenaamde proteine kinasen, waarvan er vele soorten in de cel aanwezig zijn. Eén van deze proteine kinasen is cGKII (cyclisch GMP afhankelijke proteine kinase type II). In hoofdstuk 3.1 wordt beschreven dat cGKII het CFTR eiwit zowel kan fosforyleren als activeren. Activering van CFTR door cGKII resulteert in een efflux van chloride uit de cel welke gevolgd wordt door een efflux van kationen (natrium of kalium) en water. De rol van CFTR bij zout en water secretie in de darm betekent dat CFTR ook een rol speel bij secretoire diarree (cholera)

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[1] DNA is opgebouwd uit de bouwstenen C, G, A en T. Het menselijke DNA bestaat uit zo'n 2 miljard van deze basen. De volgorde van de basen vormt de blauwdruk voor in totaal ongeveer 80000 eiwitten. Elk stuk DNA wat codeert voor 1 eiwit wordt een gen genoemd.

[2] De ion balans in de cel verschilt van de extracellulaire ion concentraties. Om de de intracellulaire ion balans te handhaven, beschikt elke cel over verscheidene ion kanalen en ion transporteurs.

In hoofdstuk 3.2 is gekeken naar een andere vorm van CFTR aktivering. Recentelijk werd beschreven dat genistein, een remmer van tyrosine kinase (een andere proteine kinase), CFTR kon activeren. Onze data laten echter zien dat genistein niet werkt via de inhibitie van tyrosine kinase, maar waarschijnlijk direkt aangrijpt op het CFTR eiwit zelf. De observatie dat genistein CFTR kan activeren kan van groot belang zijn voor de ontwikkeling van een therapie voor CF. Het blijkt namelijk dat een subpopulatie van CF patienten wel CFTR chloride kanalen tot expressie brengt op de celmembraan (hoewel sterk verminderd in aantal of met een sterk verlaagde activiteit). Voor deze groep patienten met residuele activiteit kan een extra activering van het CFTR chloride kanaal door genistein wellicht het CF ziekte proces vertragen. Aangezien genistein aanwezig is in bepaalde voedingsmiddelen (mn in soya producten) en in redelijke hoeveelheden opgenomen kan worden door ons lichaam, zou men deze patientengroep kunnen behandelen met een genistein-rijk dieet. Aanvullend onderzoek zal aantonen of dit dieet leidt tot curatieve genistein concentraties in het bloed. Voor dit onderzoek kan bv het door ons laboratorium ontwikkelde muis model voor CF gebruikt worden (zie later).

Zoals eerder vermeld is het nog onduidelijk waarom de afwezigheid van het CFTR cloride kanaal leidt tot CF. Om de pathogenese van CF te begrijpen is onderzoek verricht naar de fysiologische functie(s) van CFTR. De resultaten van dit onderzoek staan beschreven in hoofdstuk 3.3 en 3.4 van dit proefschrift. Eén van de eerste en meest kenmerkende ziekteverschijnselen in CF is mucus[3] obstructie van ducten en lagere luchtwegen. Deze mucus obstructie vormt waarschijnlijk een goede voedingsbodem voor bacteriën welke uiteindelijk leidt tot pulmonaire infecties. De mucus obstructie kan op verschillende manieren worden veroorzaakt. Twee hypothesen werden getest en staan beschreven in dit proefschrift. Als eerste werd gekeken naar de rol van CFTR in vloeistoftransport. Bij een onvoldoende hydratering van mucus wordt mucus taai en moeilijk te verwijderen. Aangezien CFTR een chloride kanaal is en vloeistoftransport wordt gereguleerd door ionstromen, zou een defect in dit chloride kanaal kunnen leiden tot een defect in vloeistoftransport. De data beschreven in hoofdstuk 3.3 tonen aan dat CFTR niet betrokken is bij het vloeistoftransport in de muizetrachea. Dit in tegenstelling tot andere weefsels waaronder de galblaas en dunne darm van de muis waar CFTR wel degelijk betrokken is bij het vloeistoftransport (niet beschreven in dit proefschrift). De observatie dat CFTR een rol speelt in het actieve transport van vloeistof over een epithelium (muv de muizetrachea) zou een goede onderliggende verklaring kunnen zijn voor het ontstaan van de CF ziekteverschijnselen.

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[3] Mucus bestaat uit hoogmoleculaire glycoproteinen en wordt uitgescheiden door epitheliale cellen. Een mucus laag dient ter bescherming van de cel.

Mucus obstructie in CF kan ook worden veroorzaakt door een verandering in mucus secretie. Deze hypothese veronderstelt een actieve rol van CFTR in de secretie van mucus. Indien bijvoorbeeld de afwezigheid van CFTR leidt tot een toename in mucussecretie kan dit obstructie ten gevolgen hebben (als de aanvoer groter is dan de afvoer). Aan de andere kant kan een afname in de secretie van mucus in CF leiden tot een ophoping van mucus in cellen. Wanner deze ophoping leidt tot celdood komt mucus vrij welke onvoldoende gebufferd of gehydrateerd is. De op deze manier in het milieu vrijgekomen mucus is taai en kleverig en kan niet adequaat worden afgevoerd. In hoofdstuk 3.4 is het onderzoek naar de rol van CFTR bij de secretie van mucus door gekweekte galblaascellen van de muis beschreven. Onze resultaten laten zien dat CFTR niet betrokken is bij de secretie van mucus door deze cellen. Deze data kunnen de CF pathogenese dan ook niet verklaren.

De meest voorkomende mutatie in het *cftr* gen leidt tot de deletie van een enkel aminozuur (het CFTR eiwit is in totaal opgebouwd uit 1480 aminozuren), een fenylalanine op positie 508 (ΔF508) van het CFTR eiwit. Deze mutatie is interessant omdat het ΔF508 CFTR nog wel codeert voor een relatief normaal gereguleerd chloride kanaal. Dit kanaal komt echter niet tot expressie op de celmembraan. Het ΔF508 CFTR wordt na *de novo* synthese verhinderd van transport uit het endoplasmatisch reticulum en wordt uiteindelijk afgebroken. De observatie dat deze maturatie mutant nog wel kan functioneren als een relatief normaal CFTR chloride kanaal betekent dat de meeste patienten (±90%) potentieel funtionele chloride kanalen bezitten mits het ΔF508 CFTR maar getranslokeerd kan worden naar de celmembraan. Een muis model voor de ΔF508 CFTR mutatie zou een belangrijke stap zijn in het onderzoek naar de maturatie van ΔF508 CFTR en kan de basis vormen van alternatieve therapiën voor CF die zich richten op de relocatie van ΔF508 CFTR naar de celmembraan. In dit proefschrift wordt de generatie en de karakterisering van een muis model voor de ΔF508 CFTR mutatie beschreven (hoofdstuk 3.5 en 3.6).

Isotype-specific Activation of Cystic Fibrosis Transmembrane Conductance Regulator-Chloride Channels by cGMP-dependent Protein Kinase II*

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Type II cGMP-dependent protein kinase (cGKII) isolated from pig intestinal brush borders and type Iα cGK (cGKI) purified from bovine lung were compared for their ability to activate the cystic fibrosis transmembrane conductance regulator (CFTR)-Cl$^-$ channel in excised, inside-out membrane patches from NIH-3T3 fibroblasts and from a rat intestinal cell line (IEC-CF7) stably expressing recombinant CFTR. In both cell models, in the presence of cGMP and ATP, cGKII was found to mimic the effect of the catalytic subunit of cAMP-dependent protein kinase (cAK) on opening CFTR-Cl$^-$ channels, albeit with different kinetics (2–3-min lag time, reduced rate of activation). By contrast, cGKI or a monomeric cGKI catalytic fragment was incapable of opening CFTR-Cl$^-$ channels and also failed to potentiate cGKII activation of the channels. The cAK activation but not the cGKII activation was blocked by a cAK inhibitor peptide. The slow activation by cGKII could not be ascribed to counteracting protein phosphatases, since neither calyculin A, a potent inhibitor of phosphatase 1 and 2A, nor ATPγS (adenosine 5′-*O*-(thiotriphosphate)), producing stable thiophosphorylation, was able to enhance the activation kinetics. Channels preactivated by cGKII closed instantaneously upon removal of ATP and kinase but reopened in the presence of ATP alone. Paradoxically, immunoprecipitated CFTR or CF-2, a cloned R domain fragment of CFTR (amino acids 645–835) could be phosphorylated to a similar extent with only minor kinetic differences by both isotypes of cGK. Phosphopeptide maps of CF-2 and CFTR, however, revealed very subtle differences in site-specificity between the cGK isoforms. These results indicate that cGKII, in contrast to cGKIα, is a potential activator of chloride transport in CFTR-expressing cell types.

Guanosine 3′,5′-cyclic monophosphate (cGMP) has been identified as an important intracellular mediator of salt and water secretion in intestinal epithelium (1–3). Secretagogues acting through the cGMP-signaling pathway include the family of heat-stable enterotoxins (STs),[1] low molecular weight peptides secreted by enteropathogenic bacteria, and guanylin, a recently discovered endogenous ST-like peptide hormone (3, 4). Binding of ST or guanylin to the receptor domain of an intestine-specific isoform of guanylyl cyclase (GC-C) triggers cyclase activation, cGMP accumulation, and stimulation of net fluid secretion through the activation of apical Cl$^-$ channels in parallel with inhibition of coupled NaCl transporters (3, 5, 6). The cystic fibrosis transmembrane conductance regulator (CFTR), an epithelial Cl$^-$ channel mutated in CF patients (7, 8), appears to be involved in the Cl$^-$ secretory response to ST and cGMP analogues, as evidenced by the absence of this response in CF intestine (9, 10).

Several mechanisms have been proposed to link cGMP to the CFTR-Cl$^-$ channels, including (i) cGMP cross-activation of cAMP-dependent protein kinase (11–13) followed by multisite-phosphorylation of CFTR (14), (ii) direct interaction of cGMP with the CFTR protein (15), and (iii) cGMP activation of an intestine-specific isoform of cGMP-dependent protein kinase (type II cGK; Refs. 16–19). cGKII was discovered as a cGMP-sensitive 86-kDa phosphoprotein localized in intestinal brush border membranes (16), which comigrated with a cGMP receptor protein on one- and two-dimensional gels (17, 18). The intestinal isoform is clearly distinct from the homodimeric type Iα and Iβ cGK (153–156 kDa) identified in other mammalian tissues, as illustrated by differences in subcellular localization, subunit composition, isoelectric point, phosphopeptide maps, immunoreactivity, and affinity for cyclic nucleotide analogues (17–19). Recently, molecular cloning of cGKII from mouse brain (20) and rat intestine (21) demonstrated that cGKII is a different gene product than cGKIα and Iβ (22, 23).

In the present study, evidence for a functional difference between cGK isoenzymes was obtained from studies of the activation of CFTR-Cl$^-$ channels in excised membrane patches of an intestinal cell line (IEC-CF7; Ref. 24) or NIH-3T3 fibroblasts stably expressing recombinant CFTR (25). In both models, exposure of patches to a combination of cGMP and ATP failed to elicit Cl$^-$ channel activity. However, the further addition of purified cGKII, but not cGKIα, resulted in almost full activation of CFTR-Cl$^-$ currents.

Differential activation of the CFTR-Cl$^-$ channel by cGKII is the first example of isotype specificity in cGK regulation of

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[1] The abbreviations used are: ST, heat-stable enterotoxin; CFTR, cystic fibrosis transmembrane conductance regulator; CF, cystic fibrosis; cGK, cGMP-dependent protein kinase; cAK, cAMP-dependent protein kinase; PKI, Walsh inhibitor peptide (PKI(5–24)-amide); ATPγS, adenosine 5′-*O*-(thiotriphosphate); PAGE, polyacrylamide gel electrophoresis.

cellular functions and provides a plausible explanation for the prominent role of cGMP as a regulator of Cl^- transport in intestinal epithelium in comparison to other CFTR-expressing cell types in which cGKII expression is marginal or absent (19–21).

EXPERIMENTAL PROCEDURES

Materials—Calyculin A was obtained from Calbiochem, San Diego, CA. PKI, the Walsh inhibitor peptide (PKI(5–24)-amide) was obtained from Dr. U. Walter, Würzburg, Germany. CF-2, a cloned R domain peptide of CFTR (AA645–835), was produced in bacteria and purified as described (26). Adenosine 5′-triphosphate, sodium salt (ATP), and cGMP were obtained from Boehringer Mannheim. [γ-^{32}P]ATP was obtained from Amersham, UK. All other chemicals were from Sigma.

Cells and CFTR Expression Systems—Two cell types that stably express CFTR were prepared and maintained as described previously (24, 27). IEC-CF7 cells were obtained by stable transfection of the rat fetal intestine-derived IEC-6 cell line with a plasmid encoding CFTR (24); NIH-3T3 cells expressed CFTR after infection with a retroviral vector encoding CFTR (27).

Isolation of Protein Kinases—cGKII was purified from the small intestine of adult pigs (donated by the Department of Experimental Cardiology, Erasmus University). The small intestine was dissected from anesthetized pigs, rinsed with ice-cold 0.9% NaCl, and frozen in liquid nitrogen. Brush border membrane vesicles were prepared from the intestinal pieces by a freeze-thaw procedure and subsequent differential Mg^{2+} precipitation and centrifugation as described previously (28). cGKII extraction from the vesicles and purification by affinity chromatography on 8-(2-aminoethyl)-amino-cAMP-Sepharose was performed essentially as described (17, 18) with a slight modification. To obtain detergent-free enzyme for use in the patch clamp experiments, elution of cGKII from the affinity gel with 1 mM cGMP was performed in the presence of 4 mM octyl glucoside rather than Triton X-100. Subsequently, cGMP and octyl glucoside were removed by dialysis in detergent-free patch clamp medium (see below).

The catalytic subunit of type II cAK and cGKI (characterized by antibody analysis to be primarily the Iα isoform; Ref. 29) were purified from bovine heart and bovine lung, respectively, as described (30, 31). The specific activities (units/mg protein) of the purified protein kinases as determined by the Kemptide phosphorylation assay (32) were 4.2 (cAK), 2.0 (cGKI), and 1.6 (cGKII), respectively. A monomeric constitutively active cGKI fragment was obtained by limited trypsinization as described (33).

Patch Clamp Technique—Patch clamp experiments were performed as described by Hamill *et al.* (34). Glass (borosilicate) pipettes were pulled to a resistance of 308 megohms and heat polished. Pipette potential refers to the voltage applied to the pipette interior with reference to the bath potential. Upward deflections denote negative charge flowing out of the pipette. A List EPC-7 amplifier was used for current amplification and voltage clamping. Membrane voltage was continuously clamped at −40 mV, except when performing current-voltage relationships. Data were monitored on an oscilloscope and stored on a VCR. The recorded data were filtered at 50 or 100 Hz, digitized at 200 Hz, and analyzed on a personal computer. Data analysis was performed as described by Kansen *et al.* (35). In view of the fact that the high density of CFTR-Cl^- channels in membrane patches of the 3T3-CFTR fibroblasts hinders the accurate determination of open state probability or number of channels, channel activity in these patches was expressed in pA, rather than as the number of open channels. The composition of the bath and pipette solutions was (in mM): 140 *N*-methyl-D-glucamine, 1 EGTA, 3 $MgCl_2$, and 10 Hepes-HCl (pH 7.3, final Cl^- concentration 147 mM). In some experiments a low Cl^- pipette buffer was used containing (in mM): 140 *N*-methyl-D-glucamine, 100 L-aspartic acid, 2 $MgCl_2$, 5 $CaCl_2$, and 10 Hepes-HCl (pH 7.3; final Cl^- concentration 49 mM). Excised patches were studied in a solution exchange compartment (volume 1 ml), as described previously by Kansen *et al.* (36). Experiments were performed at room temperature.

In Vitro Phosphorylation of CFTR and CF-2 and Phosphopeptide Mapping—CFTR was immunoprecipitated from T84 cells using specific CFTR antibodies and protein A-Sepharose beads as described (26). CFTR attached to protein A-Sepharose beads (10 μl of suspension) or CFTR antibody plus beads alone was incubated at 30 °C for 40 min in 100 μl of buffer containing 10 mM $MgCl_2$, 1 mM EGTA, 10 mM Hepes, pH 7.3, 50 μM MgATP, 20 μM cGMP, 10 Ci/mmol [γ-^{32}P]ATP, and purified protein kinases (catalytic subunit of cAK, 2 milliunits/ml; cGKI, 7.5 milliunits/ml; cGKII, 9.4 milliunits/ml). The phosphorylated samples were washed, resuspended in SDS-stop solution, and analyzed on 6% SDS-PAGE as described previously (26). Phosphorylation conditions fo CF-2 (5 μM) were similar to those for CFTR. For kinetic experiment linear incorporation of ^{32}P with time was ensured by (i) restricting th incubation time to 5 min, (ii) varying the CF-2 concentrations betwee 0.1 and 0.5 μM, (iii) increasing the [γ-^{32}P]ATP concentration to 500 μ (1 Ci/mmol), and (iv) using equal concentrations (25 nM) of each protei kinase. Reactions were terminated by the addition of 20 μl of 70 trichloroacetic acid, and protein pellets were washed three times wit 0.2 ml of ice-cold H_2O, suspended in 50 μl of SDS-stop buffer, boiled fo 2 min, and subjected to 12% SDS-PAGE as described (26). ^{32}P-Labele CF-2 or CFTR were excised from the dried gels, washed with tw changes of 10% acetic acid/30% methanol and three changes of 50 methanol, and lyophilized. In the kinetic experiments, the incorpora tion of ^{32}P into CF-2 was quantified by liquid scintillation spectrometry For two-dimensional phosphopeptide mapping of CF-2 and CFTR, 1 m of 50 mM NH_4CO_3, pH 8.0, containing L-1-tosylamido-2-phenylethy chloromethyl ketone-treated trypsin (50 μg/ml) was added to the drie gel pieces and the mixture was incubated at 37 °C for 20 h (26). The ge pieces were washed with 0.5 ml of 50 mM NH_4HCO_3 at 37 °C for 4 h, an the collected supernatants were lyophilized. Phosphopeptides were sep arated on thin layer cellulose sheets (20 × 20 cm, Eastman Kodak Co. by electrophoresis in the first dimension, followed by chromatograph in the second dimension. Dried sheets were subjected t autoradiography.

RESULTS

Activation of CFTR-Cl^- Channels by Protein Kinases—In agreement with earlier studies of Cl^- channel activation in excised, inside-out membrane patches from 3T3-CFTR fibroblasts (25, 37, 38), the addition of catalytic subunit of cAK (2 milliunits/ml) to the bath, in the presence of 2 mM MgATP, resulted in rapid activation (lag time < 1 min) of multiple anion-selective channels (average current increase per patch 40 ± 38 pA at −40 mV holding potential; n = 15) showing characteristic properties of the CFTR-Cl^- channel (*i.e.* Cl^- selectivity, linear current-voltage relationship in symmetrical Cl^- concentrations, 8 pS single channel conductance; results not shown). A similar low conductance channel, occurring at a much lower density (2–6 channels/patch) was activated by cAK in excised patches from the rat intestinal IEC-CF7 cell line stably expressing CFTR (24). In addition to cAK, the effect of cGK on CFTR-Cl^- channel opening was examined in excised, cell-free patches from both cell lines. Addition of MgATP (2 mM) and cGMP (50 μM) together did not activate current in 3T3-CFTR patches during 5–15 min observation (Fig. 1*A*). Inclusion of the cGKI isoform purified from bovine lung (10 milliunits/ml) likewise failed to open the CFTR-Cl^- channel (Fig. 1*A*), confirming earlier observations by Berger *et al.* (38). However, the subsequent addition of saturating concentrations of the cGKII (10 milliunits/ml) elicited a large Cl^- current, reaching a value after 15 min that was 78 ± 24% (n = 5) of the maximal current attained upon addition of saturating amounts of cAK (2 milliunits/ml) to the same patch (Fig. 1*A*). Half-maximal cGKII-activation (48 ± 9% of the maximal cGKII response at 15 min; n = 5) was observed in the presence of 2 milliunits/ml cGKII, whereas the threshold for current activation (10 ± 6%; n = 5) was found at 0.5 milliunits/ml cGKII (data not shown). In comparison to cAK, CFTR-Cl^- current activation by cGKII was a relatively slow process (time required to reach half-maximal activation following addition of 10 milliunits/ml cGKII: 8 ± 1 min; for 2 milliunits/ml cAK: 0.7 ± 0.2 min, n = 6; Fig. 1, *A* and *B*). This slow activation was unlikely to result from the activity of counteracting protein phosphatases, since neither calyculin A (10^{-7} M), a potent inhibitor of phosphatase 1 and 2A (recently implied in CFTR regulation; Refs. 38 and 39), nor the additional presence of ATPγS (1 mM) to produce stable thiophosphorylation, was able to enhance the CFTR activation kinetics (results not shown).

The cGKII isoform, but not cGKI, was capable of activating CFTR-Cl^- channels also in excised patches from IEC-CF7 cells

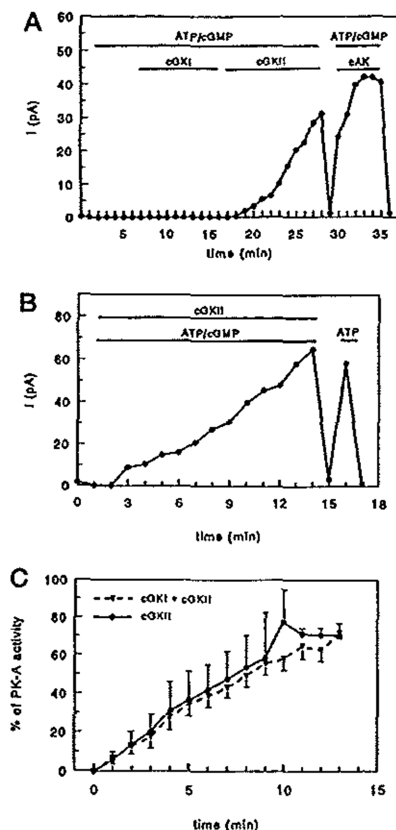

FIG. 1. **cGKII but not cGKI activates CFTR-Cl⁻ channel current in excised, inside-out membrane patches from 3T3-CFTR fibroblasts.** ATP (2 mM), cGMP (50 μM), cGKII (10 milliunits/ml), and catalytic subunit of cAK (2 milliunits/ml) were present in the cytosolic (bath) solution during the times indicated by the *bars*. *Panel A*, comparison of the effects of cGKI, cGKII, and cAK. The amount of current activated by cGKII was 78 ± 24% ($n = 5$) of the current measured after subsequent addition of cAK. *Panel B*, a representative example of an experiment showing that the cGK-activated currents rapidly returned to near base-line values upon removal of the kinase and ATP from the bath (also shown for the cAK-activated currents in *panel A*), but could be restored almost instantaneously by the readdition of ATP alone. *Panel C*, time course of activation of CFTR-Cl⁻ current by cGKII alone (●; 10 milliunits/ml) or a combination of cGKI (10 milliunits/ml) and cGKII ( ); $n = 6$. Current levels are expressed as a percentage of the maximal CFTR-Cl⁻ current observed upon subsequent addition of 2 milliunits/ml cAK.

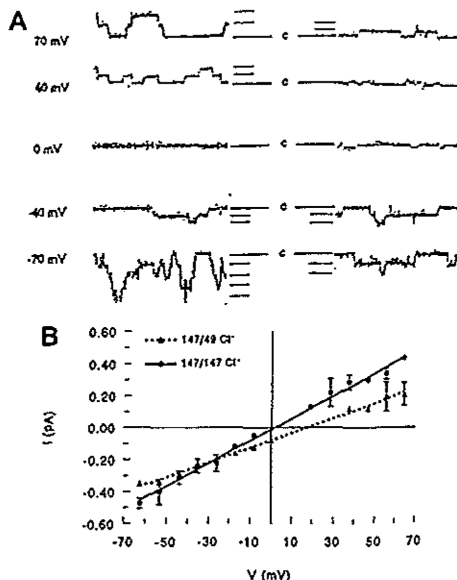

FIG. 2. **Biophysical characteristics of the cGKII-activated channel in excised, inside-out membrane patches from IEC-CF7 cells.** *Panel A*, current tracings of cGKII-activated channels. *Left*, symmetrical 147/147 mM chloride solutions. *Right*, pipette buffer was replaced by a low (49 mM) chloride buffer. Tracings were obtained at the indicated voltages. *C*, all channels closed; *dotted line*, single-channel current levels. *Panel B*, I-V characteristics of the channel. The channel conductance was 8.0 ± 0.6 pS ($n = 5$). ●, symmetrical Cl⁻ solution (147/147 mM); , reduction of Cl⁻ in the pipette to 49 mM by replacement with aspartic acid.

(Fig. 2). In this low expression model, single-channel events could be monitored (Fig. 2*A*), which had a linear I-V relation in symmetrical Cl⁻ concentrations, a channel conductance of 8 pS, and a rightward shift in current reversal potential upon lowering of the Cl⁻ concentration in the pipette (Fig. 2*B*). The mean open probability (P_o) of CFTR-Cl⁻ channels measured at the plateau phase of activation by saturating concentrations of cGKII (0.22 ± 0.09; $n = 9$) did not differ significantly from the P_o of cAK-activated channels (0.23 ± 0.12; $n = 8$).

Additional proof of the identity of the cGKII-activated channel as CFTR came from the observation that cGKII could not further enhance channel activity in excised patches from 3T3-CFTR cells following their pre-phosphorylation by cAK (2 milliunits/ml) and ATP (2 mM) (results not shown). Another similarity between CFTR-Cl⁻ channel regulation by cAK and cGKII was the observation that the currents rapidly returned to near base-line values upon the removal of either kinase and ATP from the bath, but could be restored almost instantaneously by the readdition of 2 mM ATP alone, confirming the crucial role of ATP in CFTR-Cl⁻ channel functioning (Fig. 1*B*; *cf.* Refs. 25, 38, and 40).

To eliminate the possibility that cGKII activation of CFTR resulted from a contamination of the cGKII preparation with cAK, a specific peptide inhibitor of cAK, PKI (0.1 μM) was added to the bath. Under this condition CFTR-Cl⁻ channel activation in 3T3 membrane patches by cAK (2 milliunits/ml) was completely abolished, whereas channel activation by cGKII (2 milliunits/ml) was not significantly affected (42 ± 3% of the maximal channel activity evoked by cAK in the same patch following removal of PKI from the bath, as compared to 37 ± 5% in the absence of PKI; $n = 5$). Moreover, PKI was unable to inhibit phosphorylation of Kemptide or CF-2 by cGKII (not shown).

Finally, the possibility was considered that cGKI, in spite of its failure to open CFTR-Cl⁻ channels by itself, could interfere with the activation of CFTR-Cl⁻ channels by the other cGK isoform. However, neither preincubation of 3T3 patches with cGKI (10 milliunits/ml, 15 min; results not shown) nor the simultaneous addition of cGKI (10 milliunits/ml) and cGKII (10 milliunits/ml) had any effect on the rate or extent of CFTR-Cl⁻ channel activation as compared to cGKII alone (Fig. 1*C*).

Phosphorylation of CFTR and CF-2 by cGK Isotypes—Phosphorylation studies carried out with CFTR immunoprecipitates (Fig. 3*A*) and CF-2, the recombinant R domain of CFTR (Fig. 3*B*) confirmed previous reports that both proteins are excellent *in vitro* substrates for cAK and cGKI (26, 38). This discrepancy

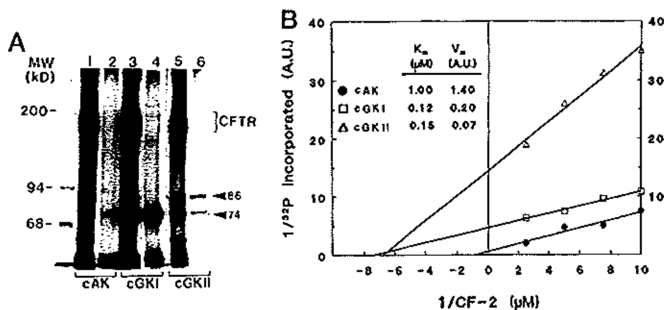

FIG. 3. **Phosphorylation of CFTR (*panel A*) and CF-2 (*panel B*) by purified protein kinases.** *Panel A*, CFTR was immunoprecipitated from T84 cells and phosphorylated as described under "Experimental Procedures." The reactions also contained: catalytic subunit of cAK (2 milliunits/ml; *lanes 1* and *2*), cGKI (7.5 milliunits/ml; *lanes 3* and *4*), and cGKII (9.4 milliunits/ml; *lanes 5* and *6*). *Lanes 2, 4*, and *6*, control experiments in which CFTR was omitted. The ^{32}P-labeled proteins were separated by 6% SDS-PAGE. The gel was dried and exposed to x-ray film. CFTR migrates as a broad band of 180 kDa ("band C"; see Ref. 14). The 86- and 74-kDa bands represent residual amounts of autophosphorylated cGKII (*lanes 5* and *6*, intact 86-kDa form + 74-kDa proteolytic fragment; *cf.* Ref. 17) and cGKI (*lanes 3* and *4*, intact 74-kDa form), respectively, remaining following the washing steps. *Panel B*, Lineweaver-Burk plots of CF-2 phosphorylation by equal concentrations (25 nM) of cAK, cGKI, and cGKII. The experimental conditions needed to ensure linear rates of ^{32}P incorporation are specified under "Experimental Procedures." The *inset* shows the kinetic constants (K_m, V_{max}) calculated from the Lineweaver-Burk plots. *A.U.*, arbitrary units. Data represent the mean of three experiments.

between phosphorylation and functional studies was even more apparent from the kinetics of *in vitro* phosphorylation of CF-2 by cGKI and cGKII isoenzymes (Fig. 3*B*). CF-2 was a better substrate for cGKI than for cGKII (similar K_m; 3-fold higher V_{max}), and the plateau level of phosphate incorporation into CFTR (Fig. 3*A*) was also higher for cGKI (140 ± 11% of the cGKII level; $n = 8$).

Two-dimensional Phosphopeptide Mapping of CF-2 and CFTR Phosphorylated by cAK, cGKI, and cGKII—Up to 10 serine residues within the R domain have been suggested to be involved in the activation of CFTR (for review, see Ref. 39). In order to investigate whether the isotype specificity of cGK activation of the CFTR-Cl$^-$ channel might be related to the pattern of specific serine residues that are phosphorylated, two-dimensional phosphopeptide mapping was performed of CF-2 and CFTR phosphorylated by cAK, cGKI, and cGKII (Fig. 4). All three kinases phosphorylated the same peptides in CF-2 with very slight differences being observed in the intensities of phosphorylation of individual peptides (Fig. 4, *A–C*). All three kinases also phosphorylated the CFTR on a subset of the peptides phosphorylated in CF-2 (Fig. 4, *D–F*), arguing against the location of novel cGKII sites outside the R domain. Several sites, in particular those located on peptides 1, 2, and 4 (the latter containing serine 700; see Ref. 26), were phosphorylated to a similar level. In contrast, one prominent peptide, labeled 9 (see Ref. 26 for numbering) was preferentially phosphorylated by cAK and cGKII, but was phosphorylated to a much lower level by cGKI (Fig. 4, *D–F*, and data not shown).

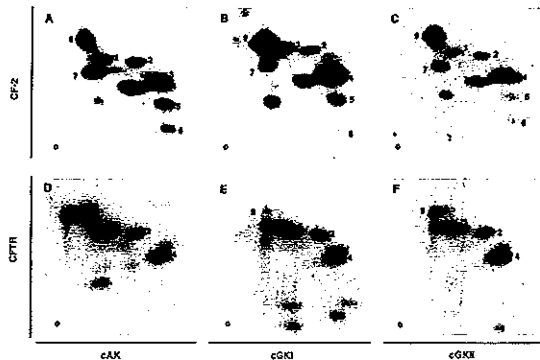

FIG. 4. **Two-dimensional tryptic phosphopeptide maps of CF-2 and CFTR.** *Upper* and *lower panels*, phosphopeptide maps of CF-2 and CFTR, respectively, showing phosphorylation by cAK (*A* and *D*), cGKI (*B* and *E*), and cGKII (*C* and *F*), respectively. Purified CF-2 and immunoprecipitated CFTR were phosphorylated for 40 min at 30 °C in the presence of cAK (2 milliunits/ml), cGKI (7.5 milliunits/ml), or cGKII (9.4 milliunits/ml) as described under "Experimental Procedures." The ^{32}P-labeled proteins were separated by 6% (CFTR) or 12% (CF-2) SDS-PAGE and visualized by autoradiography. Radioactive bands of CF-2 (pooled middle and upper bands running at 30 and 32 kDa, respectively; *cf.* Ref. 26) and CFTR (see Fig. 3*A*) were excised from gels, washed, and digested for 20 h with 50 μg/ml L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin. Tryptic digests were separated by electrophoresis in the first dimension at 400 V in 10% acetic acid, 1% pyridine, pH 3.5, and by chromatography in the second dimension using pyridine: 1-butanol:water:acetic acid (10:15:12:3%). *O*, origin. *Left*, +; *right*, −. Phosphopeptides are numbered from 1 to 9, and correspond to those in Ref. 26. The results shown are representative of three independent experiments. In two other sets of CF-2 maps, an additional phosphopeptide migrating closely to peptide 9 could be distinguished, indicating that the peptide 9 spot may contain more than one peptide or more than one phosphoacceptor site in one peptide, occasionally giving rise to alternative tryptic digestion.

DISCUSSION

In this study a recently cloned cGK isoform, cGKII (20, 21), expressed at high levels in the luminal membrane of intestinal epithelial cells (16–19, 21, 41) and to a lower extent in kidney and brain (21), is identified as a novel potential regulator of the CFTR-Cl$^-$ channel. Using a reconstitution assay consisting of a detergent-free preparation of solubilized and purified cGKII, inside-out membrane patches from CFTR-transfected intestinal cells (IEC-CF7), or NIH-3T3 fibroblasts, Mg-ATP and cGMP, the enzyme could almost fully mimic the effect of cAK on CFTR-Cl$^-$ channel opening, albeit with a slower time course. It is possible, however, that the rate-limiting step in the *in vitro* assay is the anchoring of the cGKII to the membrane, and that this delayed opening does not necessarily imply a similar kinetic disadvantage for cGKII *in vivo*, considering its colocalization with CFTR in the luminal membrane (19, 41). The amount of Cl$^-$ channel current reached (78 ± 24% of that measured after the subsequent addition of cAK) was considerably greater than the current level reported after addition of another activating kinase, PKC, using similar assay conditions (15 ± 8% of cAK; Ref. 38). In accordance with these functional data, the phosphopeptide maps made from CF-2 and CFTR phosphorylated *in vitro* by cGKII and cAK were virtually iden-

tical and clearly different from the pattern generated by PKC (26). The plateau level of phosphate incorporation into CFTR reached with cAK and cGKII was also similar (Fig. 3*A*), although the rate of CF-2 phosphorylation by cGKII was slower (Fig. 3*B*). However, such a close correlation between *in vitro* phosphorylation and functional data should be interpreted with caution: cGKIα, another mammalian cGK isoform expressed in many non-intestinal cell types (30, 31) failed to activate CFTR-Cl^- channels in this and an earlier study (38) or to potentiate cGKII activation of the channel, in spite of its ability to phosphorylate *in vitro* three of the four major cAK/cGKII sites in CFTR (Fig. 4).

The molecular basis for the differential activation of the CFTR-Cl^- channel by cGK isotypes is presently unclear. One possibility is that cGKIα may not recognize one or more phosphoacceptor sites in CFTR that are crucial for its activation. However, the results from the two-dimensional peptide mapping studies indicate that the pattern of phosphorylation of CF-2 is similar for cGKI and cGKII and essentially the same as that of cAK. Furthermore, the pattern of phosphorylation of CFTR by the three kinases was also similar with the exception that one peptide (peptide 9) was phosphorylated to only a very low level by cGKI (Fig. 4). Although initial mutagenesis studies suggested that the multisite phosphorylation of the R domain is degenerate and that no single phosphorylation site is critical for CFTR-Cl^- channel function (14), more recent studies provide evidence for a distinct role of subsets of phosphorylation sites in controlling the function of the two nucleotide binding domains in CFTR (39), and for the existence of stimulatory and inhibitory phosphorylation sites in the R domain (42). However, the possible presence of a predominant inhibitory site phosphorylated by cGKIα but not by cGKII or cAK seems unlikely since CFTR-Cl^- channel activation by cGKII or cAK was not inhibited by cGKI (Fig. 1*C*). A second possibility is that the CFTR protein in its natural membrane environment is differentially accessible to the cGK isoenzymes and that this difference is lost following immunoprecipitation or in CF-2 peptide studies. Conceivably, differences in size or quaternary structure between the monomeric cGKII protein (17–19) and the cGKI dimer do not play a major role because limited trypsinization of cGKIα generating a monomeric C-terminal fragment failed to improve CFTR-Cl^- channel activation. On the basis of secondary structure analysis, it has been argued that the structural determinants accounting for the tight association of the type II cGK with membranes may reside in the N-terminal region (21). Earlier topological studies in intestinal brush borders have also pointed to a role of a 15-kDa N-terminal fragment in anchoring cGKII to the microvillar cytoskeleton (17, 18). In particular the presence of a consensus sequence for N-terminal myristoylation in cGKII (21) may have functional significance. This sequence is absent in cGKIα (43), which may explain in part its inability to activate CFTR. As a third possibility, cGK activation of the CFTR-Cl^- channel may depend on the additional phosphorylation of a CFTR-associated regulatory protein ubiquitously expressed in cells of epithelial origin (IEC-CF7) and non-epithelial cells (3T3 fibroblasts). Small phosphorylatable proteins have been recently implicated in the regulation of a different class of Cl^- channels (44, 45). However, the opening of CFTR-Cl^- channels in lipid bilayers by cAK does not require an auxiliary protein (46). Similar studies performed with the cGK isotypes are clearly needed to discriminate between a direct and indirect model of CFTR regulation.

The demonstration of cGKII regulation of CFTR-Cl^- channels in membrane patches together with the high expression level of cGKII and virtual absence of cGKI in intestinal epithelium (17–19, 41) strongly support a model depicting cGKII as the major effector of the action of cGMP and cGMP-linked secretagogues (ST, guanylin) in this tissue. Recent immunological and ion transport experiments (41) also show a tight correlation between cGKII expression and 8-Br-cGMP-provoked, but not 8-Br-cAMP-provoked Cl^- secretion in rat intestinal segments and human colonic cell lines (T84, CaCo-2). With one possible exception (47), the latter cell lines do not express detectable levels of cGKII (41) but are still able to activate CFTR in response to ST, apparently as a consequence of excessive cGMP accumulation followed by cross-activation of cAK (11–13). However, in a previous study no low affinity binding of cGMP to cAMP receptors in the brush border membrane could be detected following luminal exposure of rat small intestine to ST *in vivo* (48). Moreover, in contrast to 8-Br-cAMP, ST did not further enhance Cl^- secretion in rat small intestinal mucosa and proximal colon *in vitro* beyond the level reached in the presence of exogenous cGMP analogues (41). The apparent need for coexpression of cGKII and CFTR may also explain why cGMP activation of Cl^- secretion is not universally observed in other CFTR-expressing cell types, including human airway epithelium (38).

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REFERENCES

1. Field, M., Graf, L. H., Laird, W. J., and Smith, P. L. (1978) *Proc. Natl. Acad. Sci. U. S. A.* 75, 2800–2804
2. Hughes, J. M., Murad, F., Chang, B., and Guerrant, R. L. (1978) *Nature* 271, 755–756
3. Field, M., Rao, M. C., and Chang, E. B. (1989) *N. Engl. J. Med.* 321, 800–806 and 879–883
4. Currie, M. G., Fok, K. F., Kato, J., Moore, R. J., Hamra, F. K., Duffin, K. L., and Smith, C. E. (1992) *Proc. Natl. Acad. Sci. U. S. A.* 89, 947–951
5. Schultz, S., Green, C. K., Yuen, P. S. T., and Garbers, D. L. (1990) *Cell* 63, 941–948
6. Vaandrager, A. B., Schulz, S., De Jonge, H. R., and Garbers, D. L. (1993) *J. Biol. Chem.* 268, 2171–2179
7. Collins, F. S. (1992) *Science* 256, 774–779
8. Welsh, M. J., and Smith, A. E. (1993) *Cell* 73, 1251–1254
9. De Jonge, H. R., Bijman, J., and Sinaasappel, M. (1987) *Pediatr. Pulmonol. Suppl.* 1, 54–57
10. Baxter, P. S., Goldhill, J., Hardcastle, P. T., and Taylor, C. J. (1988) *Nature* 335, 211
11. Forte, L. R., Thorne, P. K., Eber, S. L., Krause, W. J., Freeman, R. H., Francis, S., and Corbin, J. D. (1992) *Am. J. Physiol.* 263, C607–C615
12. Tien, X.-Y., Brasitus, T. A., Kaetzel, M. A., Dedman, J. R., and Nelson, D. J. (1994) *J. Biol. Chem.* 269, 51–54
13. Chao, A. C., de Sauvage, F. J., Dong, Y.-J., Wagner, J. A., Goeddel, D. V., and Gardner, P. (1994) *EMBO J.* 13, 1065–1072
14. Cheng, S. H., Rich, D. P., Marshall, J., Gregory, R. J., Welsh, M. J., and Smith, A. E. (1991) *Cell* 66, 1027–1036
15. Sullivan, S. K., Agellon, L. B., Schick, R., Gregory, R. J., and Paul, S. (1994) *Pediatr. Pulmonol. Suppl.* 10, 187–188
16. De Jonge, H. R. (1976) *Nature* 262, 590–593
17. De Jonge, H. R. (1981) *Adv. Cyclic Nucleotide Res.* 14, 315–333
18. De Jonge, H. R., and Rao, M. C. (1990) *Textbook of Secretory Diarrhea* (Lebenthal, E., and Duffey, M., eds.) pp. 191–207, Raven Press, New York
19. Vaandrager, A. B., and De Jonge, H. R. (1994) *Adv. Pharmacol.* 26, 253–283
20. Uhler, M. D. (1993) *J. Biol. Chem.* 268, 13586–13591
21. Jarchau, T., Häusler, C., Markert, T., Pöhler, D., Vandekerckhove, J., De Jonge, H. R., Lohmann, S. M., and Walter, U. (1994) *Proc. Natl. Acad. Sci. U. S. A.* 91, 9426–9430
22. Wernet, W., Flockerzi, V., and Hofmann, F. (1989) *FEBS Lett.* 251, 191–196
23. Sandberg, M., Natarajan, V., Ronander, I., Kalderon, D., Walter, U., Lohmann, S. M., and Jahnsen, T. (1989) *FEBS Lett.* 255, 321–329
24. Bijman, J., Dalemans, W., Kansen, M., Keulemans, J., Verbeek, E., Hoogeveen, A., De Jonge, H. R., Wilke, M., Dreyer, D., Lecocq, J.-P., Pavirani, A., and Scholte, B. (1993) *Am. J. Physiol.* 264, L229–L235
25. Anderson, M. P., Berger, H. A., Rick, D. P., Gregory, R. J., Smith, A. E., and Welsh, M. J. (1993) *Cell* 67, 775–784
26. Picciotto, M. R., Cohn, J. A., Bertuzzi, G., Greengard, P., and Nairn, A. C. (1992) *J. Biol. Chem.* 267, 12742–12752
27. Anderson, M. P., Gregory, R. J., Thompson, S., Souza, D. W., Paul, S., Mulligan, R. C., Smith, A. E., and Welsh, M. J. (1991) *Science* 253, 202–205
28. Van Dommelen, F. S., Hamer, C. M., and De Jonge, H. R. (1986) *Biochem. J.* 236, 771–778
29. Keilbach, A., Ruth, P., and Hofmann, F. (1992) *Eur. J. Biochem.* 208, 467–473
30. Kaczmarek, L. K., Jenning, K. R., Strumwasser, F., Nairn, A. C., Walter, U., Wilson, F. D., and Greengard, P. (1980) *Proc. Natl. Acad. Sci. U. S. A.* 77,

7487–7491
31. Walter, U., Miller, P., Wilson, F., Menkes, D., and Greengard, P. (1980) *J. Biol. Chem.* **255**, 3757–3763
32. Kemp, B. E., Graves, D. J., Benjamini, E., and Krebs, E. G. (1977) *J. Biol. Chem.* **252**, 4888–4894
33. Monken, C. E., and Gill, G. N. (1980) *J. Biol. Chem.* **255**, 7067–7070
34. Hamill, O. P., Marty, A., Neher, E., Sackmann, B., and Sigworth, F. J. (1981) *Pfluegers Arch.* **395**, 85–100
35. Kansen, M., Bajnath, R., Groot J., De Jonge, H. R., Scholte, B. J., Hoogeveen, A. T., and Bijman, J. (1993) *Pfluegers Arch.* **422**, 539–545
36. Kansen, M., Keulemans, J., Hoogeveen, A. T., Scholte, B. J., Vaandrager, A. B., Van der Kamp, A. W. M., Sinaasappel, M., Bot, A. G. M., De Jonge, H. R., and Bijman, J. (1992) *Biochim. Biophys. Acta* **1139**, 49–56
37. Gregory, R. J., Cheng, S. H., Rick, D. P., Marshall, J., Hekir, K., Ostedgaard, L., Klinger, K. W., Welsh, M. J., and Smith, A. E. (1990) *Nature* **347**, 382–386
38. Berger, H. A., Travis, S. M., and Welsh, M. J. (1993) *J. Biol. Chem.* **268**, 2037–2047
39. Gadsby, D. C., and Nairn, A. C. (1994) *Trends Biochem. Sci.* **19**, 513–518
40. Berger, H. A., Anderson, M. P., Gregory, R. J. Thompson, S., Howard, P. W., Maurer, R. A., Mulligan, R., Smith, A. E., and Welsh, M. J. (1991) *J. Cli Invest.* **88**, 1422–1431
41. Markert, T., Vaandrager, A. B., Gambaryan, S., Pöhler, D., Häusler, C Walter, U., De Jonge, H. R., Jarchau, T., and Lohmann, S. M. (1995) *J. Cli Invest.* **96**, 822–830
42. Wilkinson, D. J., Strong, T. V., Collins, F. S., and Dawson, P. C. (1994) *Pediat Pulmonol. Suppl.* **10**, 179–180
43. Takio, K., Wade, R. D., Smith, S. B., Krebs, E. G., Walsh, K. A., and Titani, I (1984) *Biochemistry* **23**, 4207–4218
44. Moorman, J. R., Palmer, C. J., John, J. W., III, Durieux, M. F., and Jones, I R. (1992) *J. Biol. Chem.* **267**, 14551–14554
45. Attali, B., Guillemare, E., Lesage, F., Honoré, E., Romey, G., Lazdunski, M and Barhanin, J. (1993) *Nature* **365**, 850–852
46. Bear, C. E., Li, C. H., Bartner, N., Bridges, R. J., Jensen, T. J., Ramjeesing, M and Riordan, J. R. (1992) *Cell* **68**, 809–818
47. Lin, M., Nairn, A. C., and Guggino, S. E. (1992) *Am. J. Physiol.* **262** C1304–C1312
48. Van Dommelen, F. S., and De Jonge, H. R. (1986) *Biochim. Biophys. Acta* **886** 135–142

Genistein Activates CFTR via a Tyrosine Kinase and Protein Phosphatase Independent Mechanism[1]

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Previous studies have revealed a cAMP independent activation of CFTR-chloride channels by the tyrosine kinase inhibitor genistein. To further investigate its mechanism of action, we attempted to reconstitute genistein activation of CFTR in excised inside-out membrane patches from CFTR transfected NIH-3T3 fibroblasts. In the presence or absence of ATP, genistein was unable to open silent CFTR-chloride channels. However, upon CFTR prephosphorylation by the catalytic subunit of cAMP dependent protein kinase (cAK), genistein enhanced (hyperactivated) CFTR activity 2 fold in comparison with ATP alone. This hyperactivation of CFTR resulted from a prolonged burst duration, causing an increased channel open probability. Genistein could also hyperactivate partially phosphorylated CFTR, in the absence of cAK, and therefore is different from 5'-adenylylimido diphosphate (AMP-PNP) which required fully phosphorylated CFTR. Therefore, subtle differences in the basal phosphorylation state of CFTR in intact cells may contribute to the variable efficacy of genistein as a CFTR activator. Phosphatase resistant thiophosphorylation also primed the CFTR-chloride channel for hyperactivation by genistein. Replacement of ATP by GTP as a hydrolyzable nucleotide triphosphate for CFTR did not impair the ability of genistein to hyperactivate thiophosphorylated CFTR, despite the fact that GTP is a poor substrate for tyrosine kinases. These findings argue against a possible role of protein phosphatases or tyrosine kinases, but suggest a direct interaction of genistein with CFTR, possibly at the level of NBD2.

Cystic Fibrosis is a common lethal autosomal recessive disease, caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR[2]) (1,2). CFTR encodes an epithelial chloride channel which is activated upon phosphorylation by cAMP dependent protein

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[2] The abbreviations used are: CF, cystic fibrosis; CFTR cystic fibrosis transmembrane conductance regulator; cAK, cAMP dependent protein kinase; cGK, cGMP dependent protein kinase; PKC, Ca^{2+} and phospholipid dependent protein kinase; ATPγS, adenosine 5'-O-(3-thiotriphosphate); AMP-PNP, 5'-adenylylimido diphosphate; NBD, nucleotide binding domain; Po, open probability; PKI, PKI(5-24)-amide

kinase (cAK) in the presence of ATP (3-5). CFTR can also be activated by a specific isoform of cGMP dependent protein kinase, cGKII (6) and to a minor extent by the Ca^{2+} and phospholipid dependent protein kinase, PKC (5,7).

Apart from regulation of CFTR by the serine/threonine protein kinases cAK, cGKII and PKC, several groups have studied the involvement of tyrosine kinases in the regulation of CFTR. Indeed, genistein, a tyrosine kinase inhibitor (8,9), was able to activate CFTR in CFTR transfected cell lines (10-12) and in CFTR expressing HT29/B6 adenocarcinoma and T84 colonic epithelial cells (10,13). Furthermore, genistein provoked chloride secretion in the shark rectal gland (14), and enhanced the cAMP induced chloride secretion across rat distal colon (15). In both tissues, the genistein induced chloride secretion was dependent on the activation of CFTR-chloride channels. Genistein did not activate CFTR by increasing the $[cAMP]_i$ (10,11) or by activation of cAK (12), but its action was dependent on basal cAMP levels (13) and cAK activity (12), suggesting a possible action as a protein phosphatase inhibitor (12,13). Among other tyrosine kinase inhibitors tested, only tyrphostin 47 (10) and B42 (11), but not tyrphostin 51 and 25, herbimycin, AG126 and cantharidin (H.R. de Jonge, unpublished data) were able to activate CFTR in NIH-3T3 fibroblasts. Similar observations were made using other cell types (13).

Although genistein is a tyrosine kinase inhibitor, the involvement of a tyrosine kinase in CFTR regulation has not been demonstrated directly. Genistein has a variety of other effects, which can not be attributed to tyrosine kinase inhibition. For example, genistein analogues that are inactive in tyrosine kinase inhibition, like daidzein (9), were as effective as genistein in the inhibition of L-type calcium currents (16) and the inhibition of platelet activation (17). Also, the concentrations used for genistein inhibition of prostaglandin synthesis failed to correlate with a reduction in tyrosine phosphorylation (18). Furthermore, the voltage gated potassium channel in vascular smooth muscle cells was blocked by genistein through a tyrosine kinase-independent pathway (19). Finally, genistein inhibited DNA topoisomerase II (20), probably through a direct interaction at the ATP site.

In this study we further examined the mechanism by which genistein activates CFTR in NIH-3T3 fibroblasts. CFTR activation by genistein was previously demonstrated in this cell type by ^{125}I-efflux assays and cell-attached or whole-cell patch clamp analysis (10-12). Here we present conditions in which genistein remains capable of enhancing CFTR-chloride channel activity in excised inside-out membrane patches in the absence of cytosolic factors. These conditions allowed us to demonstrate that genistein neither acts via the inhibition of a tyrosine kinase, as was postulated initially (10), nor by the inhibition of a protein phosphatase as was suggested later (12,13). Instead, we propose that genistein directly acts on CFTR. The possibility is discussed that genistein competes with ATP binding at one of the two nucleotide binding domains of CFTR.

EXPERIMENTAL PROCEDURES

Materials: Genistein, genistin, AMP-PNP, ATP, GTP, and ATPγS were all obtained from Sigma. PKI, the Walsh inhibitor peptide (PKI(5-24)-amide) was obtained from Dr. U. Walter, Würzburg, Germany. The catalytic subunit of type II cAMP dependent protein kinase A (cAK) was purified from bovine heart as

described (21).

Cells and CFTR Expression Systems: IEC-CF7 cells were obtained by stable transfection of the rat fetal intestine-derived IEC-6 cell line with a plasmid encoding CFTR (22). NIH-3T3 fibroblasts expressed CFTR after infection with a retroviral vector encoding CFTR (7). Cells were maintained as described (7,22).

Patch Clamp Technique: Patch clamp experiments were performed as described by Hamill et al (23), Patch pipettes, borosilicate glass (Clark CG150-TF) were pulled to a resistance of 3-5 MOhm and heat polished. Excised inside-out patches were transferred to a solution exchange compartment (24). Pipette potential refers to the voltage applied to the pipette interior with reference to the bath potential. Upward defections denote negative charge flowing out of the pipette. A List EPC-7 amplifier was used for current amplification and voltage clamping. Membrane voltage was continuously clamped at +40 or -40 mV, except when performing current-voltage relationships. Data were filtered using an eight-pole Bessel filter, digitized with a Digidata 1200 (Axon instruments) and stored on a personal computer. Data was analyzed by means of pClamp, version 6.0.2 (Axon Instruments). CFTR-channel activity was observed as anion channels (Cl^- is the only conductive ion in the Ringer's solution) with a linear slope conductance of 7.2±0.2 pS (n=6), activated by cAK and ATP. In the presence or absence of genistein CFTR exhibited similar slope conductance and linearity. CFTR expression varied per batch and passage number, as is apprent from a comparison of the maximal currents shown in Figs. 1 and 3. The high density of CFTR-Cl^- channels in membrane patches of NIH-3T3-CFTR fibroblasts hinders the accurate determination of the number of channels, therefore, channel activity in these patches was generally expressed as the average current (after leak current substraction), rather than as the number of open channels. The composition of bath and pipette solutions was (mM) 140 N-Methyl-D-Glucamine, 1 EGTA, 3 $MgCl_2$ and 10 Hepes-HCl (pH 7.3, final Cl^- concentration 147 mM). Experiments were performed at 37°C. Data given represent average ± SE.

RESULTS

Genistein action requires ATP and prephosphorylated CFTR: NIH-3T3-CFTR fibroblasts stimulated with 100 μM genistein displayed a CFTR like Cl^- channel activity in cell attached membrane patches (not shown), as previously reported (11) and in agreement with Cl^- efflux data (10-12). We next examined whether CFTR could be activated by genistein in excised membrane patches using patch clamp analysis. Membrane patches were incubated for 7 minutes with 2 milliunits/ml catalytic subunit of cAMP dependent protein kinase (cAK) and 2 mM ATP to obtain maximal CFTR activation (cf. Ref. 3). Subsequent addition of 100 μM genistein caused a further increase in CFTR activity by 115±18% (n=10; see Fig. 1A). The increased activity, i.e. hyperactivation, occurred within 30 seconds and was fully reversible. Since phosphorylated CFTR can be opened upon the addition of ATP alone (3), we tested whether subsequent addition of genistein could still cause hyperactivation of CFTR. As shown in Fig. 1C, CFTR activity was reduced by ≈30% upon removal of cAK, most likely as a result of partial dephosphorylation (cf. Refs. 3,6). However, even in the absence of cAK, genistein remained

able to further enhance CFTR activity (64.5±11.4% increase compared with activation by ATP alone; Fig. 1C). Reversal of the order of administration, i.e. ATP *plus* genistein first, followed by ATP alone provided similar results (51.4±12.0% increase in activity as compared with ATP alone; Fig. 1C). These findings not only exclude the possibility that genistein hyperactivates CFTR by increasing cAK activity, but also argue against the concept that genistein inhibits CFTR dephosphorylation. Under these conditions, genistein action was critically dependent on the presence of ATP, as no CFTR activity was observed in the absence of ATP (Fig. 1B). In addition, genistein action required a (partially) prephosphorylated state of CFTR, as no CFTR activity was observed in freshly excised membrane patches exposed to genistein in either the presence or absence of 2 mM ATP (not shown). This finding also rules out the possibility that genistein acts through activation of an endogenous, CFTR-activating, protein kinase or the inhibition of a counteracting protein phosphatase associated with the membrane patch. In summary, we find that the minimal requirement for genistein activation of CFTR in excised membrane patches is i) a cAK (pre-) phosphorylated state of CFTR and ii) the continuous presence of ATP.

A lower concentration of genistein (20μM) was also effective in the hyperactivation of phosphorylated CFTR (Fig 2); the average increase in CFTR activity was 70±26% (n=3), compared with maximal activation by cAK and ATP alone. Genistin, the 7-glucose isoflavone form of genistein is often used as an inactive analogue of genistein, like daidzein, in tyrosine phosphorylation assays. Upon addition of 100μM genistin, the increase in CFTR activity, as compared with maximal cAK *plus* ATP activity, was only minor (34±14%; n=5; see Fig. 2). DMSO, the solvent of genistein and genistin, had no effect on CFTR activity in this assay: 0.1% DMSO resulted in a 3.5±8.0% increase in CFTR activity (n=3).

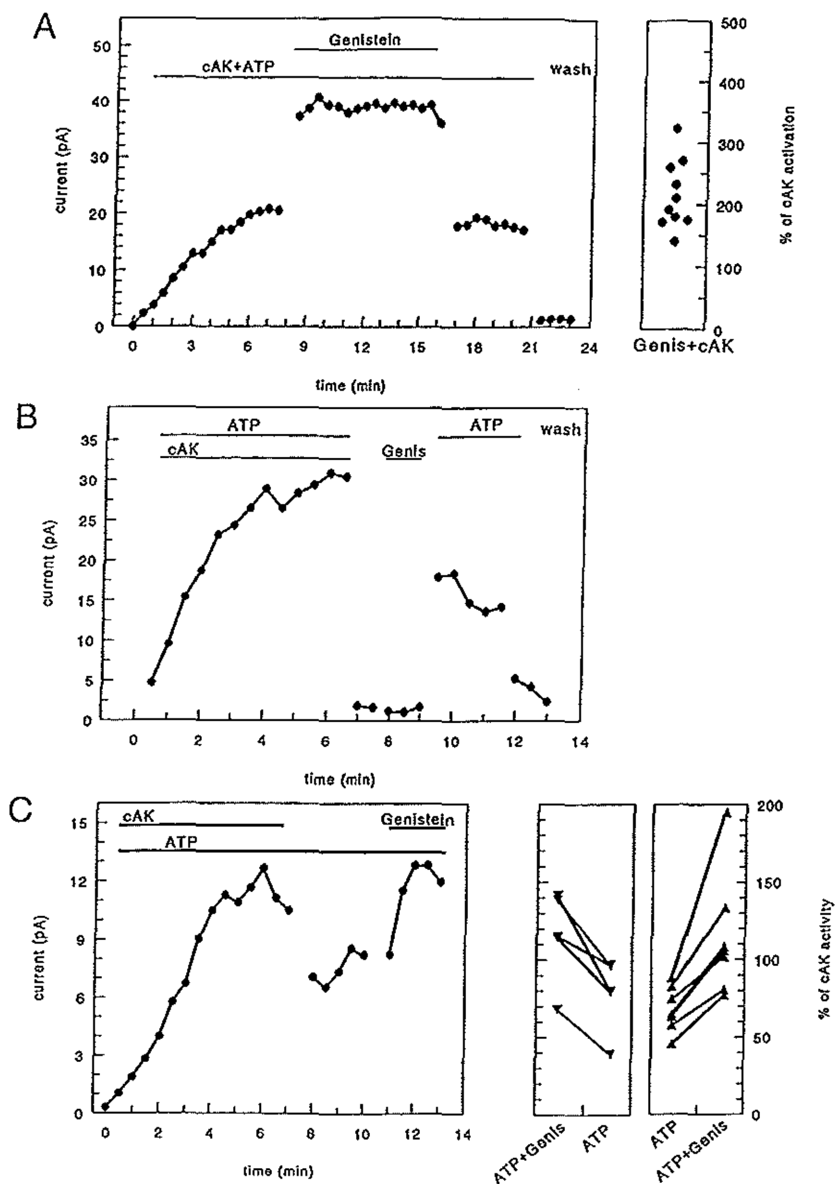

Fig. 1. Activation of CFTR by genistein in the excised inside-out membrane patch from NIH-3T3 CFTR fibroblasts. Data points represent average current levels during 30 second intervals. Panel A) Genistein (100μM) increases CFTR activity in excised patches. CFTR maximally activated by cAK and ATP can be further activated by genistein, and this hyperactivation is reversible. Left panel, typical example of an individual experiment, right panel: summary of all experiments. Panel B) In the absence of ATP, genistein failed to activate CFTR. Panel C) Hyperactivation of CFTR does not require the continuous presence of cAK. Left panel: typical example of an individual experiment. Middle and right panels, summary of all experiments. Middle panel: Pre-phosphorylated CFTR was incubated with genistein and ATP, followed by incubation with ATP alone. Right panel: similar experiments, but with a reversed order of addition.

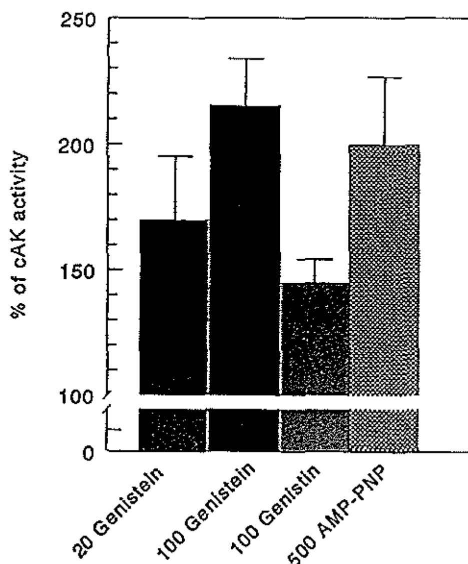

Fig. 2. Activation of CFTR by genistein, genistin and AMP-PNP. A protocol similar to Fig. 1A was followed. The plateau level of CFTR activity reached in the presence of cAK and ATP could be elevated further by addition of 100μM (see also Fig. 1A) or 20μM genistein. In comparison with genistein, 100μM genistin had only a minor effect on CFTR activity under these conditions. AMP-PNP (0.5 mM), like genistein, also increased CFTR activity in this assay. Addition of 0.1% DMSO had no effect (not shown).

Genistein does not hyperactivate CFTR via the inhibition of a protein phosphatase: Thiophosphorylation is relatively resistant to protein phosphatases (25), and thus can be used to study the involvement of protein phosphatases in the hyperactivation of CFTR by genistein. First, we established standard conditions to obtain CFTR thiophosphorylation. To achieve this, we incubated a membrane patch with 2 milliunits/ml cAK and 3 mM ATPγS for 7-9 minutes. During this incubation, CFTR remains virtually inactive (Figs. 3 and 4). Replacement of cAK and ATPγS by 2 mM ATP and 0.2 μM PKI, the Walsh inhibitor peptide of cAK, immediately resulted in a low level of CFTR channel activity (Figs. 3 and 4), indicating that CFTR thiophosphorylation had occurred. Under these conditions however, critical phosphorylation sites in CFTR became only partially thiophosphorylated as the subsequent incubation with 2 milliunits/ml cAK and 2 mM ATP further increased CFTR channel activity by 1290±380% (n=4; see Fig. 3A). Nevertheless, these subsaturating thiophosphorylation conditions appeared ideally suited to obtain large stimulatory effects of genistein in the presence of 2 mM ATP. 100μM genistein increased CFTR channel activity by 483±139% (n=4; see Figs. 3B and 4C). This effect of genistein on protein phosphatase resistant, thiophosphorylated CFTR, in the absence of cAK, eliminates the possibility that genistein exerts its action via the inhibition of a protein phosphatase. In contrast, a 1.0±28.2% decrease in CFTR channel activity (n=4; see Fig. 6) was observed in presence of 100μM genistin. This argues against a non-specific interaction of genistein with CFTR under our assay conditions.

Genistein does not hyperactivate CFTR via the inhibition of a tyrosine kinase: Tyrosine kinases

do not efficiently use hydrolyzable nucleotide triphosphates other than ATP as a phosphate donor for phosphorylation (26). In contrast, several of these compounds were able to interact with the nucleotide binding domains (NBD's) and to substitute for ATP in CFTR activity assays (3). The difference in nucleotide triphosphate specificity between CFTR and tyrosine kinases was exploited to investigate the involvement of tyrosine kinases in the hyperactivation of CFTR by genistein. Similar to previous experiments, we first thiophosphorylated CFTR in the excised patch by the addition of cAK and ATPγS. After a 7-9 minute incubation we removed the kinase and ATPγS and replaced it by 2 mM GTP and 0.2 μM PKI. We found that the addition of GTP, in absence of ATP, is sufficient to open CFTR chloride channels (Figs. 5A and C). This confirms the initial observation that GTP can open prephosphorylated CFTR at an efficiency of ≈60% of maximal activation by ATP (3). Subsequent addition of 100μM genistein caused a rapid and large increase in channel activity, amounting to 653±222% of the activity in the presence of GTP alone (n=4; see Figs. 5B and 6). This stimulation is slightly higher than the hyperactivation of thiophosphorylated CFTR by genistein in the presence of ATP. Under these conditions, the involvement of a tyrosine kinase was virtually excluded by the use of GTP. In conclusion, genistein does not seem to hyperactivate CFTR by means of tyrosine kinase inhibition.

Genistein increases the mean open burst time of CFTR: In a few patches, the channel frequency was sufficiently low to allow the analysis of the effects of genistein on the single channel characteristics of CFTR. In Fig. 7, a tracing is shown of an excised patch containing a single CFTR chloride channel. Following its phosphorylation by cAK and ATP the mean open burst time was 334±43 ms, which increased upon the addition of 100μM genistein to 1002±180 ms. The increase in open burst time resulted in a marked increase in channel open probability (Po) from 0.10 to 0.31. A qualitatively similar effect was observed in IEC-CF7 intestinal epithelial cells which showed an increase in Po from 0.34±0.05 to 0.46±0.06 (n=3; not shown).

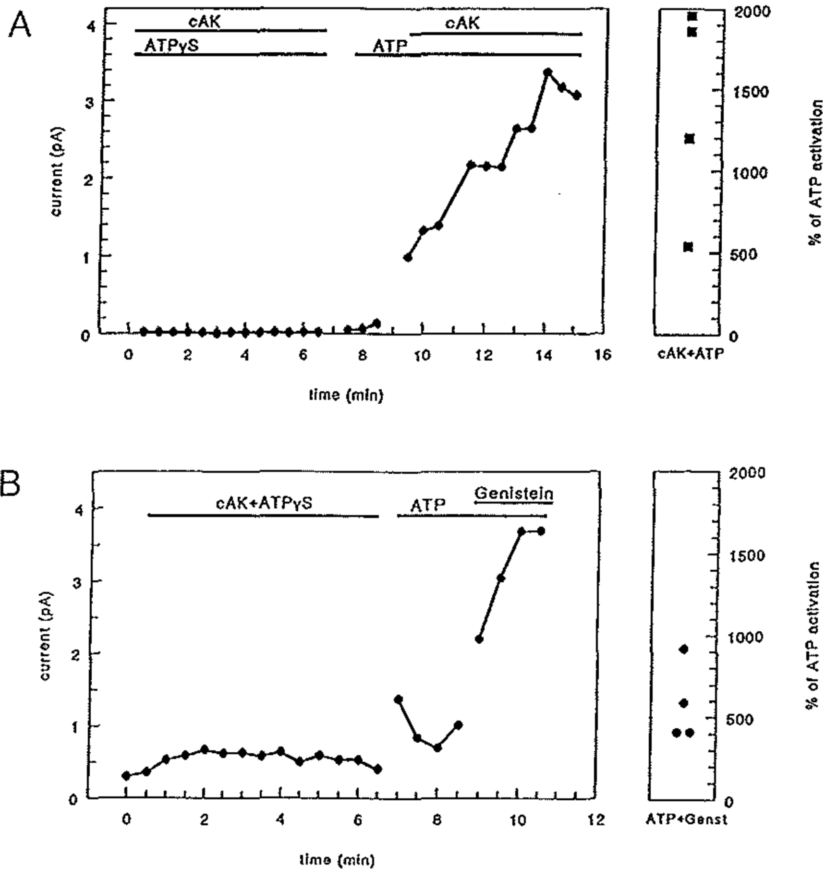

Fig. 3. **Thiophosphorylation assay of CFTR.** Panel A) An assay was developed to obtain stable, phosphatase resistant thiophosphorylation of CFTR. Incubation of CFTR with 2 milliunits/ml cAK and 3 mM ATPγS did not result in channel activity. Upon removal of the kinase and ATPγS, the addition of 2 mM ATP (in the presence of 0.2μM PKI) resulted in some CFTR channel activity. Subsequent incubation of CFTR with 2 milliunits/ml cAK (in the absence of PKI) resulted in a further increase in channel activity, demonstrating that the thiophosphorylation of CFTR was only partial. Panel B) thiophosphorylated CFTR could still be hyperactivated by genistein in the absence of cAK, demonstrating that genistein does not act via phosphatase inhibition. Left panels: example of a typical experiment, right panels: Increase in CFTR activity as compared to ATP alone, summary of all experiments.

Genistein hyperactivates CFTR via a unique mechanism: Our results show that genistein hyperactivates CFTR by an increase in the Po and open burst time. Interestingly, 5'-adenylyl imido diphosphate (AMP-PNP), a nonhydrolysable triphosphate, was also reported to increase the Po of CFTR by increasing the open time (27,28). Therefore, we compared the effect of 0.5 mM AMP-PNP with genistein in the excised patch. We found that AMP-PNP indeed hyperactivated CFTR following its phosphorylation by cAK and ATP (Fig. 2). The increase in activation was 99.8±26.7% (n=4), similar to the enhancement observed by 100μM genistein. In contrast, AMP-PNP failed to potentiate thiophosphorylated CFTR in the presence of 2 mM ATP (Fig. 6). Rather, a decrease by 38±12.3% (n=4) as compared with the activity in the

presence of ATP was observed. This contrasts with the large stimulatory effect of genistein under the same assay conditions (Fig. 6). These results confirm the concept that AMP-PNP can only potentiate fully phosphorylated CFTR, and has no effect on partially phosphorylated CFTR (27,28). Because genistein action does not require fully phosphorylated CFTR, it is also clear that the interaction between CFTR and genistein is different from its interaction with AMP-PNP.

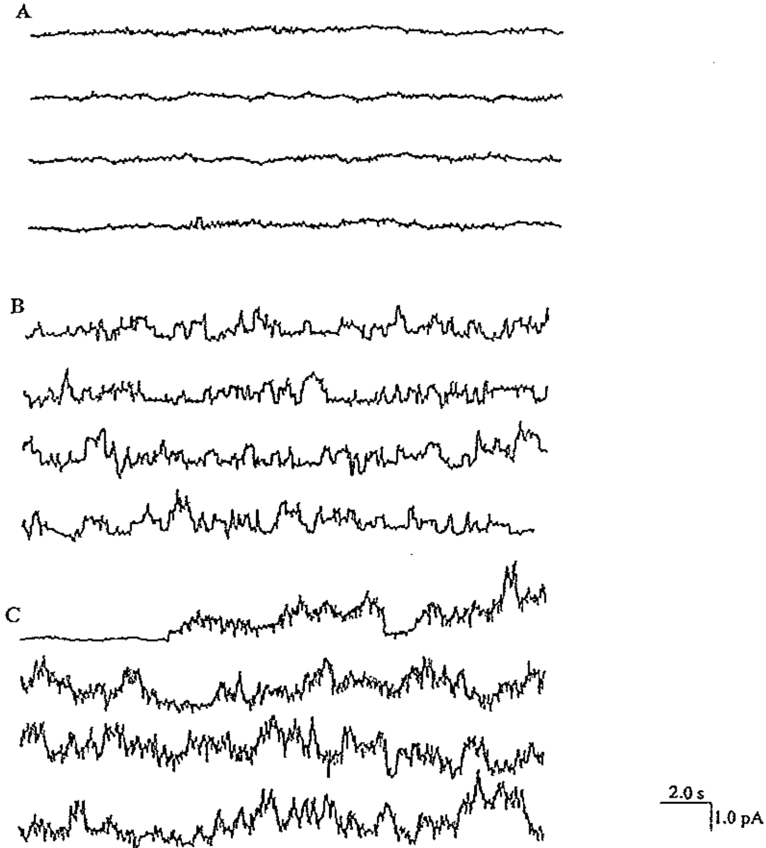

Fig. 4. **Genistein does not hyperactivate CFTR by phosphatase inhibition.** A typical example of an experiment, performed under conditions of Fig. 3B, with a low amount of channels present. Panel A) Incubation of CFTR with 2 milliunits/ml cAK and 3 mM ATPγS showed virtually no CFTR activation. Panel B) Removal of cAK and ATPγS followed by addition of 2 mM ATP resulted in a low level of channel activity. Panel C) Subsequent addition of 100μM genistein caused a large increase in channel activity. Note the absence of a significant lag-time for the effect of genistein on CFTR; the membrane patch was manipulated in a way that it instantaneously faced the genistein *plus* ATP containing solution.

DISCUSSION

In this study we successfully reconstituted CFTR activation by genistein in excised inside-out membrane patches. We used this approach to further delineate its mechanism of action. Previous studies on the effect of the tyrosine kinase inhibitor genistein on CFTR chloride

channels have been confined to intact or permeabilized cells in which chloride channel activity was measured by $^{125}I^-$ efflux, Ussing chamber, or whole- and cell-attached patch clamp analysis (10-15). First we found that genistein alone or in the presence of ATP was unable to activate non-phosphorylated CFTR in membrane patches from NIH-3T3 fibroblasts, and therefore does not act as a channel opener like NS004 (29). Instead, genistein activation of CFTR required both ATP and prior phosphorylation of CFTR by cAK. The cAK dependence was suggested previously on the basis of inhibitory effects of the cAK inhibitor H-89 on genistein-provoked ^{125}I efflux in NIH-3T3-CFTR fibroblasts (12). In conclusion, genistein is only able to further activate (hyperactivate) already active CFTR. Since hyperactivation of pre-phosphorylated CFTR was also observed in the absence of cAK, genistein did not enhance CFTR activity by increasing cAK activity, or by facilitating the net phosphorylation of CFTR.

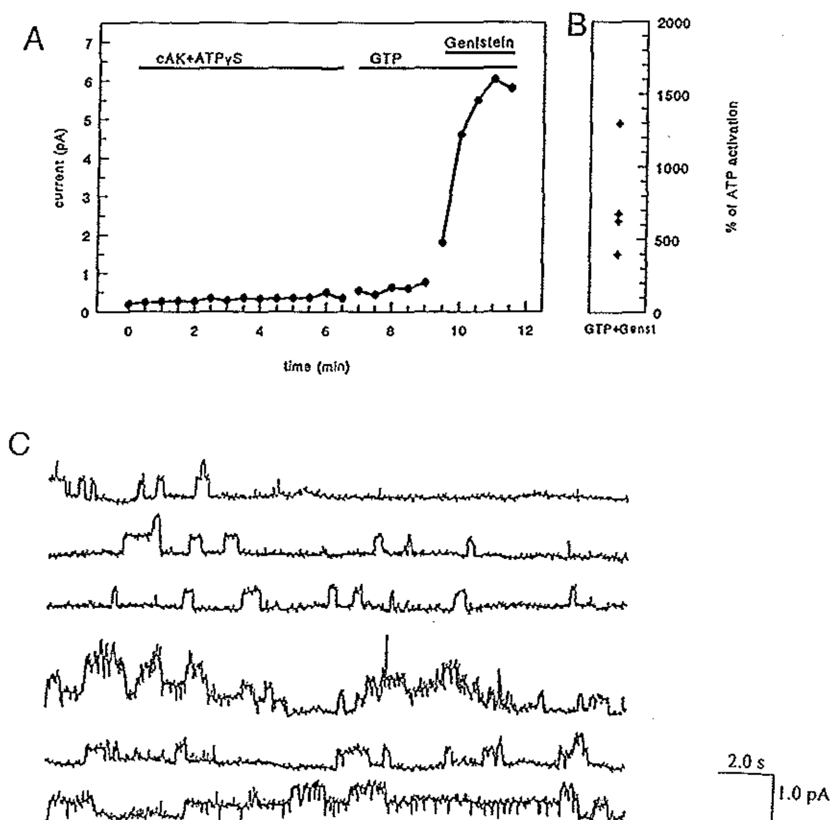

Fig. 5. **Genistein does not hyperactivate CFTR by tyrosine kinase inhibition.** Panel A) Thiophosphorylated CFTR can be activated by the addition of GTP. Subsequent addition of 100µM genistein drastically increased channel activity. Panel B) Summary of all experiments performed. Panel C) Typical example of an experiment shown in panel A with low numbers of CFTR chloride channels. Top three tracings: Thiophosphorylated CFTR in the presence of 2 mM GTP and PKI. Lower three tracings: 100µM genistein *plus* 2 mM GTP.

Partial phosphorylation is sufficient for CFTR opening by genistein: Using thiophosphorylation conditions described in this paper, we could subsequently activate CFTR upon the sole addition of ATP. However, CFTR was not fully (thio)phosphorylated, as i) subsequent incubation with cAK and ATP increased channel activity ≥12 fold, and ii) AMP-PNP did not further increase the activity of thiophosphorylated CFTR. This hydrolysis-resistant ATP analogue requires fully phosphorylated CFTR to increase channel activity (27,28). Our observation that partially phosphorylated CFTR corresponded to an intermediate state of CFTR activity is in agreement with the model presented by Gadsby and coworkers (30). Interestingly, a low level of pre-phosphorylation is apparently sufficient to allow hyperactivation of CFTR by genistein. The relative enhancement of CFTR activity is even more pronounced in partially (thio)phosphorylated CFTR (483%) than in fully phosphorylated CFTR (118%; see results).

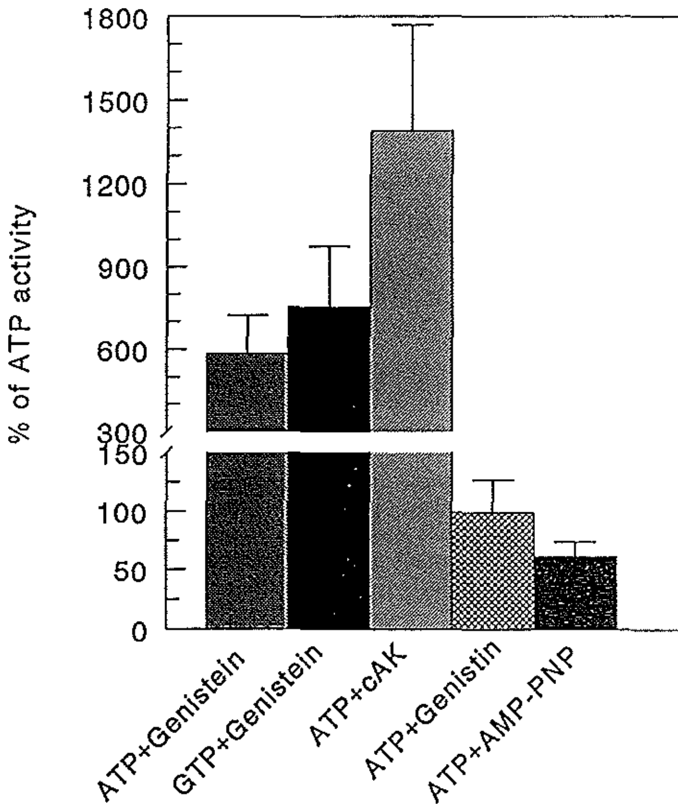

Fig. 6. **Activation of thiophosphorylated CFTR.** A protocol was followed similar to the one used in Fig. 3. Thiophosphorylated CFTR in the presence of ATP, or GTP where indicated can be further activated by 2 milliunits/ml cAK or 100μM genistein, but not by 100μM genistin or by 0.5 mM AMP-PNP.

These results, when extrapolated to intact cells, suggest that a very low level of CFTR phosphorylation is a prerequisite for genistein activation of CFTR. As a consequence, subtle differences in the basal phosphorylation state of CFTR between different cell types (or different assay conditions) would explain the variable efficacy of genistein as a CFTR activator. For example, the rather small effect genistein has on chloride secretion in the rat distal colon (15) and in the HT-29.cl.19A colonic cell line (31) may result from a very low basal state of CFTR phosphorylation. However, we cannot rule out the possibility that the action of genistein on CFTR in intact cells is more complex and involves additional interactions, lost by membrane excision. Genistein was recently reported to promote net phosphorylation of CFTR in intact NIH-3T3 fibroblasts, possibly through inhibition of CFTR dephosphorylation (12). However, the data from this study do not allow to discriminate between possible (inhibitory) effects of genistein on protein phosphatase activities, or on the susceptibility of the substrate (i.e. a putative CFTR-genistein complex) to undergo dephosphorylation.

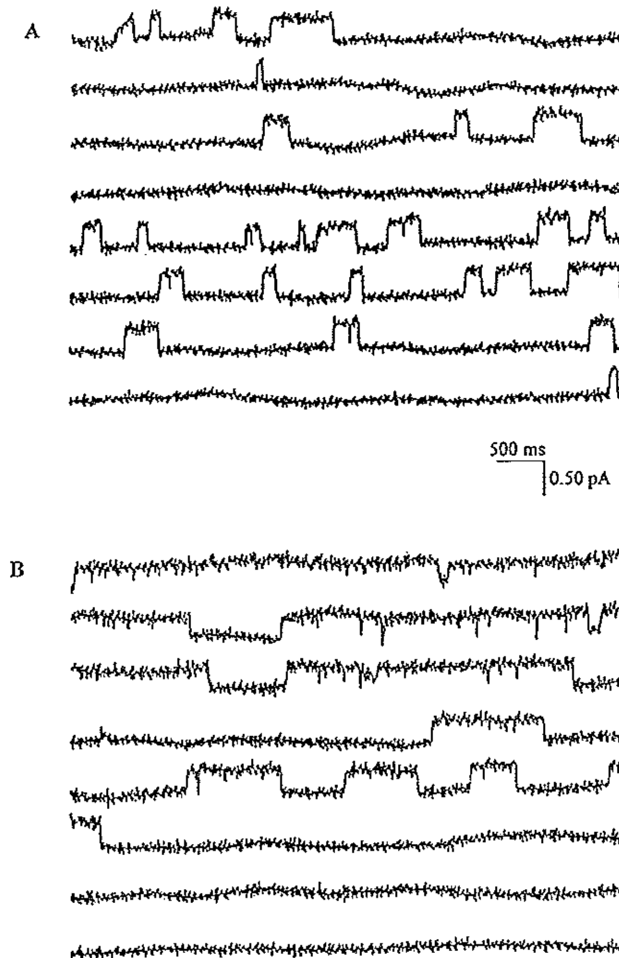

Fig. 7. **Genistein prolongs burst duration of CFTR.** Panel A) CFTR was maximally activated by cAK and ATP. Panel B) Subsequent addition of 100μM genistein resulted in an approximate 3- fold increase in burst duration.

Genistein hyperactivates CFTR by direct interaction with NBD2: Our results clearly indicate that genistein is able to hyperactivate thiophosphorylated CFTR, eliminating the possibility that genistein modulates CFTR activity via the inhibition of a protein phosphatase (Figs. 3B and 4). Furthermore, we found that replacement of ATP for GTP as a hydrolyzable triphosphate to open CFTR, did not impair the ability of genistein to enhance CFTR activity (Fig. 5). As GTP is a poor phosphate donor in tyrosine kinase assays, this finding strongly argues against the hypothesis that genistein hyperactivates CFTR via tyrosine kinase inhibition. We also found that genistein induces a prolonged burst duration of CFTR. This action was not observed before in excised inside out patches from 3T3 fibroblasts (32). A prolonged burst duration is a typical characteristic of CFTR mutant proteins that fail to perform ATP hydrolysis at NBD2 but not NBD1 (28,33). From these data it was hypothesized that ATP hydrolysis at NBD1 causes channel opening whilst hydrolysis at NBD2 causes channel closure. Hence, agents that prevent or interfere with ATP hydrolysis, i.e. AMP-PNP (27,28), PPi (34), or VO_4 and BeF_3 (35), can induce a prolonged burst opening. We therefore suggest that genistein likewise induces prolonged burst duration by intervening with ATP hydrolysis at one of the NBD's.

Genistein inhibits tyrosine kinase activity by binding to nucleotide binding domains (9). Genistein may also interact with the nucleotide binding domains of other proteins, as was suggested before for DNA-topoisomerase II (20). A common sequence characteristic of the ATP site shared by protein tyrosine kinases, is G X G X X G....K. This sequence is also found in PKA and PKC, and is common to Walker A type ATP binding motifs. Alignment of sequences between tyrosine kinases and DNA-topoisomerase II yielded a striking homology (20). Interestingly, when a search was conducted for the sequence GXGXXG...K within the CFTR protein, we identified sequence homologies at both NBD's. The highest homology match was found at aa 1236-1253 in NBD2 with homology stretching N-terminal to the actual ATP-binding motif.

```
H topo II       K T L A V S G L G V V G   R D K Y G V
CFTR 1236-1253  P G Q R V G L L G R T G   S G K S T L
c-erb-B2        R K V K V L G S G A F G T V Y K G I
CFTR 450-467    R G Q L L A V A G S T G   A G K T S L
```

The location of a potential genistein binding site within NBD2 suggests that genistein may hinder ATP hydrolysis at this site. This putative genistein binding site lies within the conserved P-loop, which contacts the polyphosphoryl moiety of bound nucleotide triphosphates. Mutations of the conserved lysine within this loop (K1250) result in prolonged burst duration (28,33), quite like observed in this study. We therefore postulate that genistein binds to NBD2, which causes an impairment of ATP hydrolysis at this site, resulting in a prolonged channel open time. Interaction of genistein with this site, in contrast to the binding of ATP or AMP-PNP (27), apparently does not require a fully phosphorylated form of CFTR, but also occurs efficiently in a partially phosphorylated state.

CFTR is not the first ion transporter to be modulated by genistein. Effects of genistein on ion channels and transporters include i) inhibition of the $Na^+K^+Cl^-$ cotransporter in brain capillary endothelial cells (36), ii) inhibition of a basal and calcium activated potassium channel in rat distal colon (15), iii) inhibition of a basolateral potassium current in HT-29/B6 (13), iv) inhibition of a voltage gated potasium current in vascular smooth muscle cells (19), v) inhibition of L-type calcium currents in rat ventricular cells (16), vi) inhibition of neutral NaCl absorption and Na^+-H^+ exchange in rabbit brush border membranes (37), and vii) inhibition of MRP-1, another member of the ATP-binding cassette (ABC) transporter superfamily (38). Although the mechanism underlying ion transport inhibition by genistein has not yet been established, it is tempting to speculate that genistein, analogous to its putative action on CFTR, may interact directly with the transporters at an ATP-modulatory site.

Therapeutic potential of genistein: At present, genistein is under investigation as a possible anticancer therapeutic (39-41). Genistein is present in soy-food, and diet supplementation might be sufficient for beneficial effects (42). The results of the present and previous studies suggest that genistein might also be of benefit to some CF patients in which a residual CFTR activity is present. Our preliminary attempts to activate ΔF508 CFTR-chloride channels in NIH-3T3 fibroblasts revealed a strong synergistic activation of mutant CFTR by a combination of genistein and cAMP agonists (43). This property of genistein would render it an interesting therapeutic for CF, as genistein would only hyperactivate mutant CFTR channels in the additional presence of an endogenous secretagogue. The IC50 for CFTR activation is about 12.5-40μM (10,13,14), just above the presumed upper limit for the serum genistein concentration under high soy diet (13.2μM;39). Currently, we are investigating the *in vitro* effects of genistein on chloride secretion in intestinal mucosa from CF patients. Also, we are exploring the possibility of diet supplementation as a CF therapeutic in a ΔF508 CFTR mouse model with residual CFTR activity (44,45). Finally, the distinction between the action of genistein as a CFTR-Cl^- channel opener and as a tyrosine kinase inhibitor revealed by the present study may open perspectives for the development of genistein-based CF therapeutics showing increased affinity for CFTR but reduced affinity for tyrosine kinases or other ion transporters.

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REFERENCES

(1) Kerem, B., Rommens, J. M., Buchanan, J. A., Markiewicz, D., Cox, T. K., Chakravarti, A., Buchwald, M., and Tsui, L. C. (1989) *Science* 245, 1073-1080

(2) Riordan, J. R. (1993) *Ann. Rev. Physiol.* 55, 609-630

(3) Anderson, M. P., Berger, H. A., Rich, D. P., Gregory, R. J., Smith, A. E., and Welsh, M. J. (1991) *Cell* 67, 775-784

(4) Bear, C. E., Li, C. H., Kartner, N., Bridges, R. J., Jensen, T. J., Ramjeesingh, M., and Riordan, J. R.

(1992) *Cell* **68**, 809-818

(5) Tabcharani, J. A., Chang, X. B., Riordan, J. R., and Hanrahan, J. W. (1991) *Nature* **352**, 628-631

(6) French, P. J., Bijman, J., Edixhoven, M., Vaandrager, A. B., Scholte, B. J., Lohmann, S. M., Nairn, A. C., and de Jonge, H. R. (1995) *J. Biol. Chem.* **270**, 26626-26631

(7) Anderson, M. P., Rich, D. P., Gregory, R. J., Smith, A. E., and Welsh, M. J. (1991) *Science* **251**, 679-682

(8) Akiyama, T., and Ogawara, H. (1991) *Methods Enzymol.* **201**, 362-370

(9) Akiyama, T., Ishida, J., Nakagawa, S., Ogawara, H., Watanabe, S., Itoh, N., Shibuya, M., and Fukami, Y. (1987) *J. Biol. Chem.* **262**, 5592-5595

(10) Sears, C. L., Firoozmand, F., Mellander, A., Chambers, F. G., Eromar, I. G., Bot, A. G., Scholte, B., De Jonge, H. R., and Donowitz, M. (1995) *Am. J. Physiol.* **269**, G874-G882

(11) Illek, B., Fischer, H., Santos, G. F., Widdicombe, J. H., Machen, T. E., and Reenstra, W. W. (1995) *Am. J. Physiol.* **268**, C886-C893

(12) Reenstra, W.W., Yurko-Mauro, K., Dam, A., Raman, S., and Shorten, S. (1996) *Am. J. Physiol.* **271**, C650-C657

(13) Illek, B., Fischer, H., and Machen, T. E. (1996) *Am. J. Physiol.* **39**, C265-C275

(14) Lehrich, R. W., and Forrest, J., Jr. (1995) *Am. J. Physiol.* **269**, F594-F600

(15) Diener, M., and Hug, F. (1996) *Eur. J. Pharmacol.* **299**, 161-170

(16) Yokoshiki, H., Sumii, K., and Sperelakis, N. (1996) *J. Mol. Cell. Cardiol.* **28**, 807-814

(17) Nakashima, S., Koike, T., and Nozawa, Y. (1991) *Mol. Pharmacol.* **39**, 475-480

(18) Kniss, D. A., Zimmerman, P. D., Su, H. C., and Fertel, R. H. (1996) *Prostaglandins* **51**, 87-105

(19) Smirnov, S. V., and Aaronson, P. I. (1995) *Circ. Res.* **76**, 310-316

(20) Markovits, J., Linassier, C., Fosse, P., Couprie, J., Pierre, J., Jacquemin-Sablon, A., Saucier, J. M., Le Pecq, J. B., and Larsen, A. K. (1989) *Cancer Res.* **49**, 5111-5117

(21) Kaczmarek, L. K., Jennings, K. R., Strumwasser, F., Nairn, A. C., Walter, U., Wilson, F. D., and Greengard, P. (1980) *Proc. Natl. Acad. Sci. U S A* **77**, 7487-7491

(22) Bijman, J., Dalemans, W., Kansen, M., Keulemans, J., Verbeek, E., Hoogeveen, A., De Jonge, H. R., Wilke, M., Dreyer, D., Lecocq, J. P., J.-P Pavirani, A., and Scholte, B. J. (1993) *Am. J. Physiol.* **264**, L229-L235

(23) Hamill, O. P., Marty, A., Neher, E., Sakmann, B., and Sigworth, F. J. (1981) *Pflugers Arch.* **391**, 85-100

(24) Kansen, M., Bajnath, R. B., Groot, J. A., de Jonge, H. R., Scholte, B., Hoogeveen, A. T., and Bijman, J. (1993) *Pflügers Arch.* **422**, 539-545

(25) Eckstein, F. (1985) *Ann. Rev. Biochem.* **54**, 367-402

(26) Hunter, T., and Cooper, J.A. (1985) *Ann. Rev. Biochem.* **54**, 897-930

(27) Hwang, T. C., Nagel, G., Nairn, A. C., and Gadsby, D. C. (1994) *Proc. Natl. Acad. Sci. U S A* **91**, 4698-4702

(28) Carson, M. R., Travis, S. M., and Welsh, M. J. (1995) *J. Biol. Chem.* **270**, 1711-1717

(29) Gribkoff, V. K., Champigny, G., Barbry, P., Dworetzky, S. I., Meanwell, N. A., and Lazdunski,

M. (1994) *J. Biol. Chem.* **269**, 10983-10986

(30) Hwang, T. C., Horie, M. , and Gadsby, D. C. (1993) *J. Gen. Physiol.* **101**, 629-650

(31) de Jonge, H. R., Bot, A. G. M., Scholte, B., Sears, C. L., and Donowitz, M. (1994) *Pediatric Pulmonol.* **Suppl. 10**, 188

(32) Fisher, H., Illek, B., and Machen, T. E. (1994) *Mol. Biol. Cell.* **5**, 319a

(33) Gunderson, K. L., and Kopito, R. R. (1995) *Cell* **82**, 231-239

(34) Carson, M. R., Winter, M. C., Travis, S. M., and Welsh, M. J. (1995) *J. Biol. Chem.* **270**, 20466-20472

(35) Baukrowitz, T., Hwang, T. C., Nairn, A. C., and Gadsby, D. C. (1994) *Neuron* **12**, 473-482

(36) Vigne, P., Lopez Farre, A., and Frelin, C. (1994) *J. Biol. Chem.* **269**, 19925-19930

(37) Donowitz, M., Montgomery, J. L., Walker, M. S., and Cohen, M. E. (1994) *Am. J. Physiol.* **266**, G647-G656

(38) Versantvoort, C. H., Broxterman, H. J., Lankelma, J., Feller, N., and Pinedo, H. M. (1994) *Biochem. Pharmacol.* **48**, 1129-1136

(39) Barnes, S. (1995) *J. Nutr.* **125**, 777S-783S

(40) Peterson, G. (1995) *J. Nutr.* **125**, 784S-789S

(41) Fotsis, T., Pepper, M., Adlercreutz, H., Hase, T., Montesano, R., and Schweigerer, L. (1995) *J. Nutr.* **125**, 790S-797S

(42) Hendrich, S., Lee, K. W., Xu, X., Wang, H. J., and Murphy, P. A. (1994) *J. Nutr.* **124**, 1789S-1792S

(43) de Jonge, H. R., Bot, A. G. M., French, P. J., Bijman, J., Sinaasappel, M., and Veeze, H. J. (1995) *Pediatric Pulmonol.* **Suppl 12**, 186

(44) van Doorninck, J. H., French, P. J., Verbeek, E., Peters, R. H., Morreau, H., Bijman, J., and Scholte, B. J. (1995) *EMBO J.* **14**, 4403-4411

(45) French, P. J., Doorninck, J. H., Peters, R. H. P. C., Verbeek, E., Ameen, N. A., Marino, C. R., de Jonge, H. R., Bijman, J., and Scholte, B. J. (1996) *J. Clin. Invest* **98**, 1304-1312

Fluid Transport In The Trachea Of Normal And CF Mouse

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We set out to examine the role of CFTR in active fluid transport in the murine trachea. For this aim, normal- and CF murine trachea's were mounted in a modified Ussing-chamber to measure net flux of water ($J_{H_2O\ net}$), transmural Potential Difference- (PD_{tr}) and Resistance (R_{tr}). Compounds known to interfere with NaCl-transport (amiloride, carbachol, IBMX, forskolin) across the tissue were evaluated for their effect on $J_{H_2O\ net}$ and electrical parameters. A basal state of net fluid resorption from the lumen was observed (0.41±0.23 µl/trachea/2hrs) in the unstimulated mouse trachea. With amiloride (0.1 mmol/l) in the lumen I_{eq} decreased and net water movement was abolished (0.080±06 µl/trachea/2hrs). The addition of forskolin (10 µmol/l), or forskolin and IBMX (0.2 mmol/l), resulted in a hyperpolarization of PD_{tr} indicating activation of a luminal chloride conductance. Although forskolin/IBMX tended to decrease fluid resorption, it failed to reach statistical significance. A combination of forskolin/IBMX and amiloride did not result in a net secretion of water, suggesting that the murine trachea is not capable of secreting significant amounts of fluid. Electrophysiological parameters, $J_{H_2O\ net}$, and the net changes in $J_{H_2O\ net}$ to amiloride and forskolin/IBMX, were similar in both normal (CFTR +/+ and +/-) and CF (CFTR -/-)- mouse trachea, indicating that CFTR is not involved in the fluid transport across mouse tracheal epithelium. Our results suggest that both in normal- and CFTR(-/-)- mouse trachea amiloride sensitive net fluid uptake is present which is attenuated similarly in the two tissues by forskolin/IBMX, suggesting that CFTR is not involved in this process.

Cystic Fibrosis (CF[1]) is a common lethal genetic disease in the Caucasian population, affecting 1:2500 new-borns (for review see (35)). The disease is caused by mutations in the

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[1] The abbreviations used are: CF, cystic fibrosis; CFTR cystic fibrosis transmembrane conductance regulator; DIDS, 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid; DPC, diphenylamine 2-carboxylate; IBMX, 3-isobutyl-

gene encoding the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) (22, 29, 30) which forms a cAMP and ATP regulated chloride channel (2a, 5, 7, 21, 34) and is expressed in the apical membrane of various epithelial cells (11, 14). Although the gene defective in CF has been identified, CF disease pathogenesis is still a poorly understood process. It has been proposed that mucus plugging of small airways and ducts, a characteristic CF disease symptom, is caused by a defective transepithelial fluid transport, resulting in an insufficient hydration of mucus. The dehydrated mucus is viscid and difficult to clear, and these changes in the rheological properties may explain the mucus plugging and subsequent inflammation in CF.

In fluid transporting epithelia like gallbladder and renal proximal tubule, isotonic fluid transport is dictated by the active transport of ions. In the absence of apical CFTR chloride channels the transepithelial fluid transport therefore may be altered. Indeed, a reduced cAMP induced fluid secretion was found in CF pancreatic (13, 23, 24) and pulmonary (19, 32) epithelia. In addition, both CF mouse intestine (15 and PJF, unpublished observations) and gallbladder (26) fail to secrete fluid when stimulated with cAMP agonists. A CFTR dependent fluid secretion was also found in ADPKD (autosomal dominant polycystic kidney disease) cells (12).

Although these observations clearly indicate an active role for CFTR in the process of fluid secretion, measurements of fluid transport across pulmonary epithelia have been restricted to cultured cell systems. The presence of multiple cell types (e.g. ciliated and goblet cells) in intact pulmonary tissue, suggests a more complex model system than cell culture, and electrophysiological differences between cell culture and the intact system have been reported (17). As a consequence, cultured pulmonary epithelia may not truly reflect the *in vivo* situation. We therefore studied the role of CFTR in fluid transport across the intact murine trachea in a CF mouse model (28). The measurement of CFTR dependent fluid transport across intact pulmonary epithelium may help understand CF airway pathogenesis.

MATERIAL AND METHODS

Tissue preparation: Mice were weighed and anaesthetised with hypnodil, 0.2 mg/g. Murine tracheas were carefully excised from either adult C57BL/6 mice, or from age matched CF mice (28) (homozygous -/- CFTR knockout mice), and normal mice (hetero-(+/-) and homozygous(+/+) litter mates). CF mice had a significant lower weight compared to their normal littermates, 24.5 ± 5.5gr (n=22) vs. 19.4 ± 3.5gr (n=16), respectively ($P<0.01$). After excision, trachea's were mounted at 4°C in a modified Ussing chamber (Fig. 1). The average length of excised trachea's was 4.7 mm, average diameter 0.85 mm. Fluid measurements were corrected for the number of cartilage rings. On average, an excised trachea will account for approx. 0.11 cm^2 of epithelium.
PD_{tr} and Resistance (R_{tr}) measurement: In the perfused trachea (40 µl/min) PD_{tr} was measured between agar bridges inside the lumen of the trachea (± 1 mm distal from the pipette tip opening) and the bath. A small (0.2

1-methyl-xantine; I_{eq}, equivalent short circuit current; Isc, short circuit current; $J_{H2O}net$, net fluid transport; PD_{tr}, transmural potential difference; R_{tr}, transmural resistance.

mm diameter) platinum wire was inserted into the lumen and one in the bath (ground) for homogenous current sending across the trachea. Current pulses (0.5-1 μA, 1 sec duration, 5 sec interval) were sent to calculate R_{tr}.

Fluid transport measurement: Net water movement ($J_{H_2O\ net}$) across the tissue was determined in the non perfused trachea. PD_{tr} was measured before and after measuring $J_{H_2O\ net}$, also in a non perfused set-up. For measuring $J_{H_2O\ net}$ the trachea and holding pipettes were filled with water-saturated mineral oil. Subsequently, a small defined droplet of Ringer's solution was positioned within the lumen of trachea. The volume of the fluid was calculated by using a thin calibrated 0.3 mm internal diameter poly-ethylene capillary. The resolution of this method for net volume flow determination is ~40 nl (the volume in 0.5 mm length of capillary). After a period of two hours the droplet was recovered in the same tubing and its volume was redetermined. By rapid installation and recovery of the droplet in the trachea (< 1 min. incubation), we determined the dead space of the trachea, 0.09 ± 0.04 μl (n=5), and all data described in this paper are corrected for this volume.

Solutions and chemicals: experiments were performed in a modified, air saturated, Ringer's solution containing (10^{-3} M); 133 NaCl, 5 KCl, 1.5 $CaCl_2$, 1 $MgCl_2$, 0.4 NaH_2PO_4, 0.3 Na_2HPO_4, 5 Hepes, 5.5 Glucose, pH 7.4. 10^{-5} M indomethacin was added to the Ringer's solution in trachea experiments to block prostaglandin synthesis. Measurements were begun after a slow rise of the bath temperature (~20 min) to 37°C. Amiloride (0.1 mM) was added to the mucosal side of the tissue. Indomethacin (10^{-5} M), Carbachol (10^{-5}) and forskolin (10^{-5} M) were added to the serosal side of the tissue. DMSO (1‰ V/V) and ethanol (1‰ V/V) as solvents for amiloride and forskolin respectively, did not affect R_{tr} and PD_{tr} (n=5; not shown). All data are expressed as mean±SD.

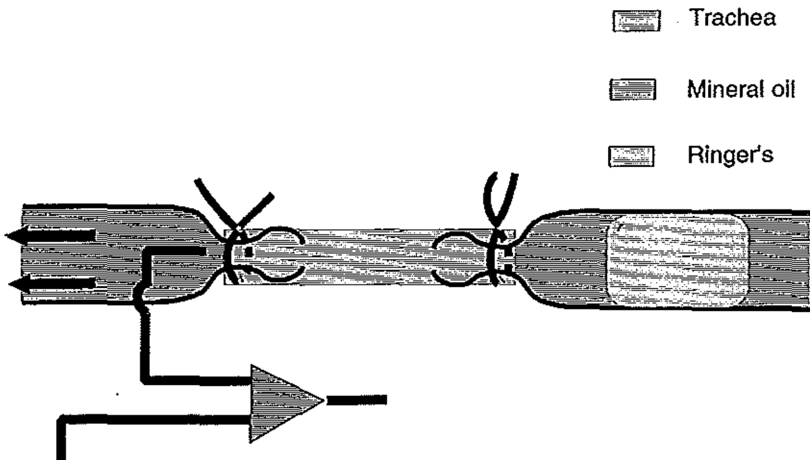

Fig 1. Schematic drawing of the set-up. A trachea is mounted onto two pipettes. First a Ringer's solution is perfused trough the trachea, during which the PD was measured. In some experiments, a 0.2 mm platinum wire was inserted into the lumen and one placed in the bath for homogeneous current sending across the trachea (not shown). Then, water-saturated mineral oil is perfused through the trachea. Within the oil a precisely defined droplet of Ringer's solution was placed. The droplet was recovered after two hours, and differences in volume of the droplet was determined.

RESULTS

Bioelectrical properties of perfused normal and CF mouse trachea: In order to determine the contribution of the ion-channels and transporters present in murine tracheal epithelium, we first measured the effects of various agonists (amiloride, forskolin/IBMX, carbachol, bumetanide, phloridzin) on the PD_{tr} and R_{tr} of the trachea of CFTR -/- mice (CF mice) and their normal

homozygous +/+ and heterozygous +/- litter mates (normal mice). Typical examples of experiments are shown in figure 2. Depicted in figure 3 are the equivalent short circuit currents (I_{eq}, calculated from PD_{tr} and R_{tr} using Ohm's law) of the perfused trachea.

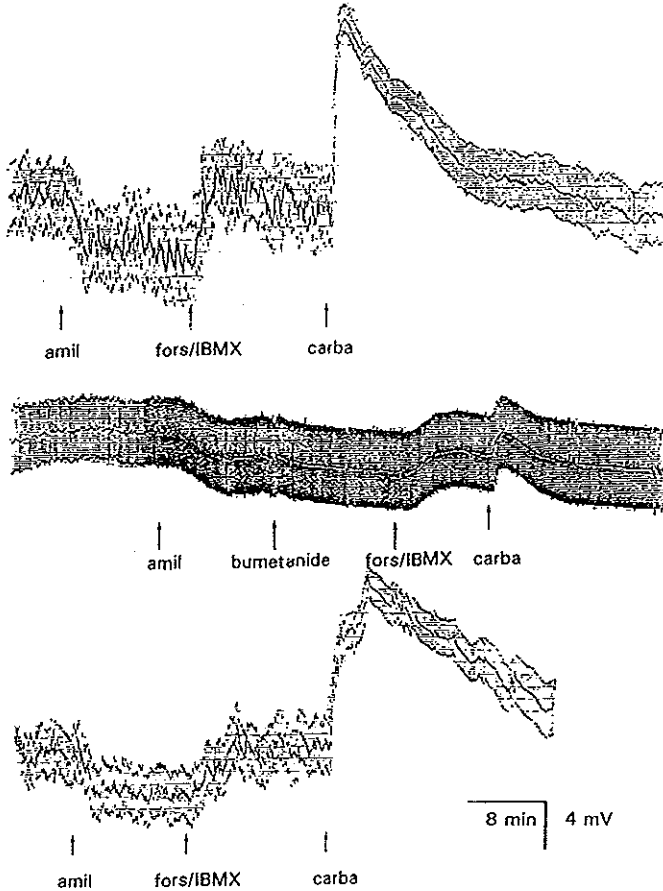

Fig 2. Typical examples of PD responses of normal mouse trachea's to amiloride, forskolin/IBMX and carbachol. Voltage deflections are responses to ±1 μA current pulses. Upper and middle tracing: normal mouse trachea, lower tracing: CF mouse trachea. Amiloride depolarises the lumen, whereas forskolin/IBMX induce hyperpolarization. Carbachol induces a large hyperpolarization in combination with a marked decrease in resistance. In the presence of bumetanide, the baseline PD_{tr} decreases, and responses to forskolin/IBMX and carbachol are reduced.

Blocking the apical sodium conductance by the addition of amiloride resulted in a significant depolarisation with small effect on R_{tr} in both normal (fig 2, upper tracing) and CF mice (fig 2, lower tracing). The subsequent addition of forskolin/IBMX induced a luminal hyperpolarization in both normal and CF mice (fig. 2, upper and lower tracing), with only minor effect on R_{tr}. This luminal hyperpolarization was due to an increased chloride permeability as reduction of the luminal chloride concentration resulted in a further hyperpolarization of the luminal membrane (not shown). In both normal and CF mice, the

tissues response to forskolin/IBMX could not be inhibited by the addition of DIDS, a blocker of Ca^{2+} activated chloride channels, but the forskolin/IBMX response could be prevented completely by the addition of DPC, a blocker of the CFTR chloride channel (not shown). Carbachol, by increasing the intracellular Ca^{2+} activity, induced a marked hyper-polarisation combined with a large reduction of tissue resistance in both normal and CF mice. Blocking the basolateral $Na^+/K^+/Cl^-$ cotransporter by addition of bumetanide resulted in a slow decrease in PD_{tr} and increase in R_{tr}, (average I_{eq} reduction: 29±17%, n=4, figure 2, middle tracing). Furthermore, addition of bumetanide markedly reduced the forskolin/IBMX and carbachol induced chloride secretory response, average reduction in I_{eq}: 83±0.2% (n=2) resp 77±18% (n=4). Phloridzin, an inhibitor of the Na^+/glucose cotransporter present in pulmonary epithelia (3, 20), had no electrophysiological effect on the mouse trachea (not shown).

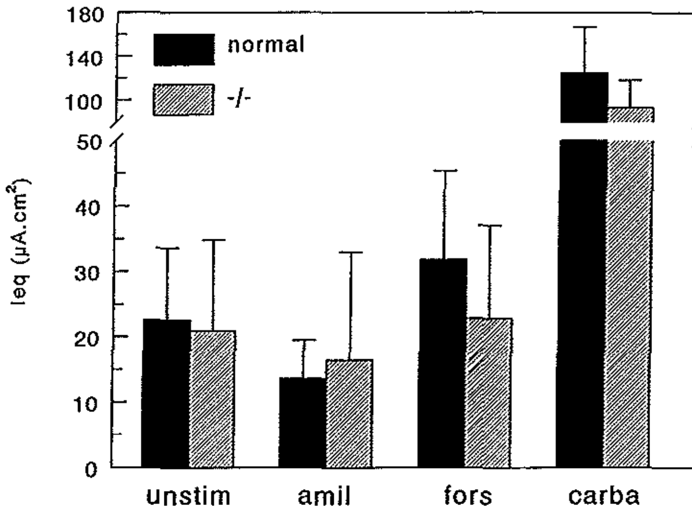

Fig 3. A summary of data calculated from tracings shown in fig 2, perfused set-up. In the basal state, normal mice tracheas absorb sodium via amiloride sensitive chloride channels. No significant differences in I_{eq}'s between normal and CF mice were detected.

The bio-electrical properties of the mouse trachea as measured with our set-up were qualitatively similar to those previously reported for the mouse pulmonary epithelia *in vivo* (33), freshly excised (31, 17), cell culture (9, 17) and pulmonary tissues from other animals (1, 2). We did not detect differences in the electrophysiological responses between normal and CF mouse trachea. This was also reported by other groups for intact mouse trachea (4, 17) but not for cultured mouse trachea (17). As the bioelectrical properties of the CF mouse trachea is similar to previously published, we concluded that our set-up provides an excellent model to study intact mouse pulmonary epithelium.

Fluid transport in mouse trachea: Prior to the measurement of fluid transport, we determined tissue viability of the mouse trachea by measuring the PD in a non perfused set-up (a similar condition as used to measure fluid transport). Under these conditions, baseline PD's were -9.3±3.7 mV for normal mouse trachea. Interestingly, a significant hyperpolarisation was observed in the CF mouse trachea (-12.0±4.6 mV, $P<0.05$). Responses to agonists (amiloride, forskolin/IBMX or both) were qualitatively similar to the perfused set-up in both normal and CF mice (data not shown). Fig. 4 shows the net transport of fluid in the trachea of B57Bl/6 mice. A net absorption was observed in the unstimulated trachea. When a combination of 10 μM forskolin and 0.1 mmol/l amiloride was applied to the medium (bath and lumen), the net fluid absorption was almost completely inhibited. The same method was used to investigate fluid transport in the trachea of the CF and normal mouse. The results are shown in fig. 5 and 6. In normal mice we observed a net fluid absorption of 0.37±0.25μl/trachea/2hrs in the basal, unstimulated state. Net fluid absorption in normal and CF mice tracheas were comparable (see fig 6) Application of 0.1 mM amiloride to both sides of the trachea almost completely inhibited net fluid absorption in the normal mouse trachea to 0.05±0.05 μl/trachea/2hrs. A combination of 10 μM forskolin and 0.2 mM IBMX, did not significantly inhibit net fluid absorption, neither in normal mice nor in CF mice (fig 6). A combination of both amiloride and forskolin/IBMX completely inhibited net fluid absorption in the normal mouse trachea, but no significant amounts of fluid secretion were observed.

Tissue viability after experimentation was checked by redetermining the PD in (unstimulated) C57Bl/6, normal, and CF mouse tracheas, and were -4.9±0.5 mV (n=3),-2.5±1.4 mV (n=8), -2.5±1.4 mV (n=5) respectively. The response to carbachol after experimentation was -13.6±3.3 mV (n=3) in normal mice and -15.7±6.7 mV (n=3) in CF mice.

DISCUSSION

Water transport in normal mouse trachea: To our knowledge this report is the first in which measurements of fluid transport in intact, excised (mouse) tracheas have been performed. Our results indicate a basal state of fluid absorption of approximately 0.4 μl/trachea/2hrs, corresponding to ~1 μl/cm^2/h. The addition of amiloride almost completely inhibited the fluid absorption, indicating that amiloride sensitive Na^+ transport is responsible for the fluid absorption in the basal state of the mouse trachea. The addition of forskolin and IBMX did not have a significant effect on fluid absorption, and no significant net fluid secretion was observed in the combined presence of amiloride and forskolin/IBMX.

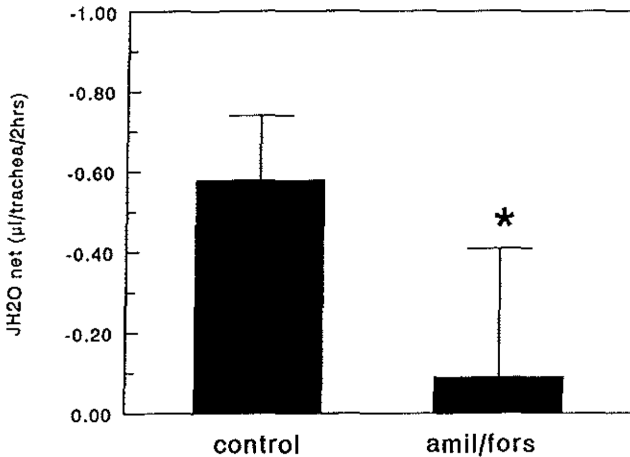

Fig 4. Net fluid transport in C57b/6 mice trachea. Unstimulated trachea's absorb net fluid. Addition of forskolin and amiloride markedly reduces the net fluid absorption.

Previous reports of studies on fluid transport across cultured pulmonary epithelia show similar results: i) a basal state of absorption (range 0.13 - 4.4 µl/cm^2/h (6, 19, 31)), ii), forskolin/IBMX do not significantly inhibit fluid absorption (31), and, iii) amiloride almost completely inhibits absorption (6, 19, 31). Thus, in spite of differences in tissue origin (human nasal epithelium), cell culture, and experimental set-up, our data are qualitatively similar and quantitatively within the same order of magnitude as previously published data by other groups. A calculation of the maximal amounts of fluid transported (under basal conditions) by electrogenic ion transport (I_{eq} of 20 µA/cm^2) predicts a J_{H_2O} net of 1.1 (µl/trachea.2hr or 4.1 µl/cm^2.h), thus also within the same order of magnitude as our results.

A major difference between our data and those reported by others (19) in cultured human pulmonary tissue, is that we did not observe significant amounts of net fluid secretion in the combined presence of amiloride and forskolin/IBMX. This discrepancy may reflect a species differences between mouse and human pulmonary epithelium, but alternatively may be due to differences between cell culture and intact tissues. It follows that, contrary to human pulmonary epithelium (19), mouse tracheal epithelium can only modify the airway surface fluid via active fluid absorption and not by active fluid secretion. The inability of the mouse trachea to actively secrete significant amounts of fluid indicates that the airway surface fluid lining the pulmonary epithelium is derived from a different, more distal, source. In humans, the submucosal glands may provide a source for proximal airway fluid secretion, these are however virtually absent in the murine trachea (25). There may not be a need for the mouse trachea to actively secrete large amounts of fluid, as there is a constant fluid supply from distal

airways, and the large decrease in surface area in proximal airway fluid transport primarily will require an absorptive state of the epithelium. Furthermore, humidification of inspired air occurs mainly by the nasal epithelium (18). Therefore, a reduction in fluid absorption could be sufficient to modify the surface liquid and secure airway clearance.

water transport in the CF mouse trachea: Our results indicate that the basal state of the CF mouse trachea is fluid absorption amounting to 0.40±0.2 µl/trachea/2hrs (~1 µl/hr/cm^2). We did observe, in the nonperfused set-up only, an increased PD_{tr} in the CF mouse trachea; a typical characteristic for CF epithelia (see e.g. (8, 27)). However, as the basal fluid transport across murine tracheal epithelium is similar between CF and normal mice, the increased PD_{tr} of CF mouse trachea (non perfused set-up) does not correlate with an increased fluid absorption. Therefore, in contrast to the hyper absorption observed in human CF pulmonary epithelia by Jiang et al (19), intact murine CF tracheal epithelium do not hyper absorb fluid. A normal fluid absorption was also found by others in cultured CF human pulmonary epithelium (31). The differential observations may be due to differences in experimental set-up, as Jiang and co-workers (19) have used a perfused set-up, whereas our data, as well as those obtained by Smith et al (31) have been obtained in a static, non perfused set-up. In the more physiological situation of a static set-up, the generation of supraepithelial salt gradients may determine the actual amount of salt and fluid transported, whereas in a perfused set-up fluid and electrolyte absorption is determined by the bath composition.

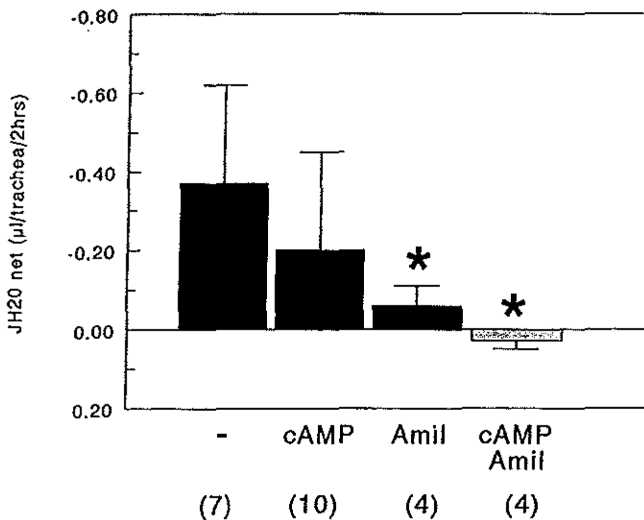

Fig 5. Net fluid transport in normal mice trachea. Unstimulated normal mice trachea's absorb fluid. forskolin and IBMX inhibit net absorption, amiloride almost completely inhibits net fluid absorption whereas a combination of amiloride, forskolin and IBMX completely abolishes net absorption.

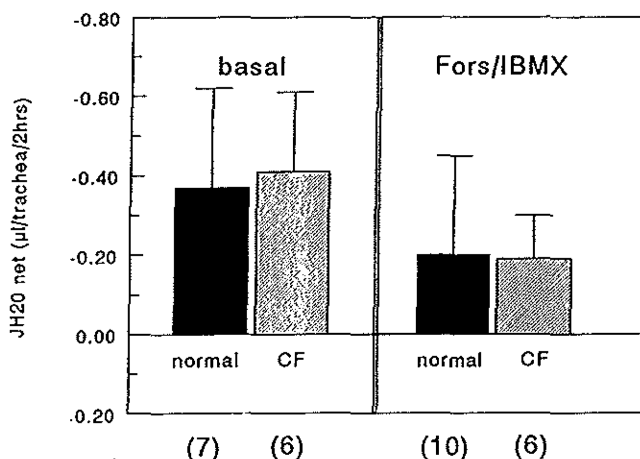

Fig 6. Net fluid transport in normal and CF mice trachea. CF mice trachea absorb fluid at a rate no greater than normal mice trachea. Forskolin and IBMX inhibit net fluid absorption in the CF mice trachea; the magnitude of the inhibition is similar to the inhibition seen in normal mice trachea.

In CF mice, like in normal mice, we could observe a trend towards a decrease in absorption when trachea's were stimulated by forskolin/IBMX. However, both normal and CF mice respond to forskolin/IBMX to a similar extent, and therefore we conclude that CFTR does not play a role in the active fluid transport across mouse tracheal epithelium. As isotonic fluid transport is dictated by the active transport of ions, a similar conclusion can be deduced from the almost identical bioelectrical properties between normal and CF mouse trachea. Our results show a several times larger tissues response to the Ca^{2+} agonist carbachol than to cAMP agonists, therefore the Ca^{2+} activated currents may be of more importance to the physiology of the mouse trachea. It has been suggested that a Ca^{2+}activated chloride channel can compensate for CFTR in CF mouse epithelia (10, 16, 36).

Summarising, the mouse trachea has a basal state of amiloride sensitive Na^+ absorption. The role of CFTR in fluid transport is minor, and it is questioned whether CFTR can be detected electrophysiologically in the mouse trachea. We conclude that CFTR does not play an important role in the mouse trachea, thus providing an explanation for the absence of pulmonary pathology in the CF mouse. As it is difficult to detect CFTR in the mouse trachea, the suitability of this tissue for gene therapy protocols seems rather questionable.

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REFERENCES

1) Al-Bazzaz, F.J., Tarka, C., Farah, M. (1991) *Am. J. Physiol.* **260**: L594-L602
2a) Anderson, M.P, Rich, D.P., Gregory, R.J., Smith, A.E., Welsh, M.J. (1991) *Science* **251**: 679-82
2) Ballard, S.T., Schepens, S.M., Falcone, J.C., Meininger, G.A., Taylor, A.E. (1992) *J. Appl. Physiol.* **73**: 2021-2027
3) Barker, P.M., Boyd, C.A.R., Ramsden, C.A., Strang, L.B., Walters, D.V. (1989) *J. Physiol.* **409**: 15-27
4) Barker, P.M., Brigman, K.K., Paradiso, A.M., Boucher, R.C. Gatzy, J.T. (1995) *Am. J. Respir. Cell. Mol. Biol.* **13**: 307-313
5) Bear, C.E., Li., C.H., Kartner, N., Bridges, R.J., Jensen, T.J., Ramjeesingh, M., Riordan, J.R. (1992) *Cell* **68**: 809-18
6) Benali, R., Pierrot, D., Zahm, J.M., Debentzmann, S., Puchelle, E. (1994) *Am. J. Resp. Cell. Mol. Biol.* **10**:363-368
7) Bijman, J., Dalemans, W., Kansen, M., Keulemans, J., Verbeek, E., de Jonge, H.R., Wilke, M., Dreyer, D., Lecocq, J.P. et al (1993) *Am. J. Physiol* **264**: L229-35,
8) Boucher, R.C., Jackson Stutts, M., Knowles, M.R., Cantley, L. Gatzy, J.T (1986). *J. Clin. Invest.* **78**: 1245-1252
9) Clarke, L.L., Burns, K.A., Bayle, J.-Y., Boucher, R.C., Van Scott, M.R. (1992) *Am. J. Physiol.* **263**: L519-L525
10) Clarke, L.L., Grubb, B.R., Yankaskas, J.R., Cotton, C.U., McKenzie, A., Boucher, R.C. (1994) *Proc. Natl. Acad. Sci. USA* **91**: 479-483
11) Crawford, I, Maloney, P.C., Zeitlin, P.L., Guggino, W.B., Hyde, S.C., Turley, H., Gatter, K.C., Harris, A., Higgins, C.F. (1991) *Proc. Natl. Acad. Sci. USA* **88**: 9262-9266
12) Davidow, C.J., Maser, R.L., Rome, L.A., Calvet, J.P., Grantham, J.J. (1996) *Kidney International* **50**: 208-218
13) Davidson,G.P., Kirubakaran, C.P., Ratcliffe, G., Cooper, D.M., Robb, T.A. (1986) *Acta Pead. Scand* **75**:145-50
14) Engelhardt, J.F., Yankaskas, J.R., Ernst, S.A., Yang, Y., Marino, C.R., Boucher, R.C., Cohn, J.A., Wilson, J.M. (1992) *Nature Genet.* **2**: 240-248
15) Gabriel, S.E., Brigman, K.N., Koller, B.H., Boucher, R.C., Stutts, M.J. (1994) *Science* **266**:107-109
16) Grubb, B.R., Vick, R.N., Boucher, R.C. (1994) *Am. J. Physiol.* **266**:C1478-C1483
17) Grubb, B.R., Paradiso, A.M., Boucher, R.C. (1994) *Am. J. Physiol.* **267**: C293-C300
18) Ingelstedt, S. (1956) *Acta Otolaryngol. Suppl.* **131**:1-80
19) Jiang, C.W., Finkbeiner, W.E., Widdicombe, J.H., McCray, P.B., Miller, S.S. (1993) *Science* **262**:424-427
20) Joris, L and Quinton, P.M. (1989) *Pflügers Arch.* **415**:118-120
21) Kartner, N., Hanrahan, J.W., Jensen, T.J., Naismith, A.L., Sun, S.Z., Ackerly, C.A., Reyes, E.F., Tsui, L.-C., Rommens, J.M., Bear, C.E., et al, (1991) *Cell* **64**: 681-91
22) Kerem, B., Rommens, J.M., Buchanan, J.A., Markiewicz, D., Cox, T.K., Chakravarti, A., Buchwald, M., Tsui, L.C. (1989) *Science* **245**: 1073-1080
23) Kopelman, H., Durie, P., Gaskin, K., Weizman, Z., Forstner, G. (1985) *N. Engl. J. Med.*, **312**:329-334
24) Kopelman, H., Corey, M., Gaskin, K., Durie. P., Weizman, Z., Forstner, G. (1988) *Gastroenterol.*, **95**: 349-355
25) Pack, R.J., Al-Ugaily, L.H., Morris, G., and Widdicombe, J.G (1980). *Cell Tissue Res.* **208**: 65-84
26) Peters, R.H.P.C., van Doorninck, J.H., French, P.J., Ratcliff, R., Evans, M.J., Colledge, W.H., Bijman, J. and Scholte, B.J. *Hepatology*, in press
27) Quinton, P.M and Bijman, (1983) J.*New Engl. J. Med.* **308**: 1185-1189
28) Ratcliff, R., Evans, M.J., Cuthbert, A.W., Macvinish, J.L., Foster, D., Anderson, J.R., Colledge, W.H. (1993) *Nature. Genet.* **4**: 35-41
29) Riordan, J.R., Rommens, J.M., Kerem, B., Alon, N., Rozmahel, R., Grzelczak, Z., Zielenski, J., Lok, S., Plavsic, N., Chou, J.L. et al. (1989) *Science* **245**: 1066-73
30) Rommens, J.M., Iannuzzi, M.C., Kerem, B., Drumm, M.L., Melmer, G., Dean, M., Rozmahel, R., Cole, J.L., Kennedy, D., Hidaka, N., et al. (1989)*Science* **245**: 1059-65
31) Smith, J.J., Welsh, M.J. (1993) *J. Clin.Invest.*, **91**: 1590-1597
32) Smith, J.J, Karp, P.H., Welsh, (1994) M.J.*J. Clin Invest.* **93**: 1307-1311
33) Takahashi, M., Freed, A.N., Croxton, T.L. (1995) *Resp. Physiol.* **99**: 19-27, .
34) Tabcharani, J.A., Chang, X.B., Riordan, J.R., Hanrahan, J.W. (1991) *Nature* **352**: 628-31
35) Welsh, M.J., Tsui, L.-C, Boat, T.F., Beaudet, A.L. Cystic Fibrosis.In Sriver, C.R., Beaudet, A.L., Sly, W.S. and Valle, D (eds), *the metabolic bases of inherited disease* (1995) 3799-3876
36) Wilschanski, M.A., Rozmahel, R., Beharry, S., Kent, G., Li, C., Tsui, L.-C., Durie,. P., Bear, C.E. (1996) *Biochem. Biophys. res. comm* 219: 753-759

CFTR expression and mucin secretion in cultured mouse gallbladder epithelial cells

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Peters, Richard H. P. C., Pim J. French, J. Hikke van Doorninck, Genevieve Lamblin, Rosemary Ratcliff, Martin J. Evans, William H. Colledge, Jan Bijman, and Bob J. Scholte. CFTR expression and mucin secretion in cultured mouse gallbladder epithelial cells. *Am. J. Physiol.* 271 (*Gastrointest. Liver Physiol.* 34): G1074–G1083, 1996.—Dysfunction of the cystic fibrosis transmembrane conductance regulator (CFTR) in humans is frequently associated with progressive liver disease, which appears to result from obstruction of biliary ducts with mucous material. CFTR in the liver is expressed in the biliary epithelium. With the use of a mouse model for cystic fibrosis (CF) we have studied the relationship between CFTR expression and glycoprotein secretion in primary culture of mouse gallbladder epithelial cells (MGBC). MGBC in culture maintain a well-differentiated phenotype as shown by microscopy. The cells produce CFTR mRNA to levels comparable to the intact tissue. With patch-clamp analysis we could frequently observe a linear protein kinase A-regulated Cl^- channel that shows all the major characteristics of human CFTR, although its conductance is lower (5 pS compared with 8 pS). MGBC in culture produce and secrete high molecular weight glycoproteins (HMG) in a time-dependent and temperature-sensitive manner. Secretion of HMG was not stimulated significantly by either adenosine 3',5'-cyclic monophosphate (cAMP), Ca^{2+}, or protein kinase C agonists in this system. High concentrations (3 mM) of extracellular ATP stimulated secretion threefold, but low concentrations (0.3 mM) had no effect. Approximately one-third of the HMG produced and secreted consisted of mucin. Cultured MGBC from CFTR-deficient mice produced and secreted mucin to a similar extent as normal cells. We conclude that cultured mouse gallbladder cells are a convenient model to study both CFTR function and mucin secretion. In this system, we found no evidence for a direct link between mucin secretion and CFTR activity, as has been suggested for other cell types.

cystic fibrosis transmembrane conductance regulator; glycoproteins

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THE INTRAHEPATIC BILE DUCTS, as well as the cystic duct and gallbladder, are lined with epithelial cells with distinct morphology. This epithelial cell layer plays an important role in the regulation of bile volume and bile pH, using a combination of ion-transport systems situated in both apical and basolateral membranes. In addition to performing regulated ion transport, bile duct cells have a secretory function. Many subapical vesicles can be observed in electron micrographs of biliary epithelium. Mucins are prominent among the products secreted from this intracellular pool (36). The physiological function of mucin secretion is supposedly the formation of an extracellular gel protecting the tissue against the aggressive biliary environment. The regulation of mucin secretion in biliary epithelium has not been studied in detail.

Cystic fibrosis (CF) is a recessive inherited disease with a high incidence in the Caucasian population (6, 60). It is caused by mutations in an adenosine 3',5'-cyclic monophosphate (cAMP)-regulated Cl^- channel [cystic fibrosis transmembrane conductance regulator (CFTR)] situated in the apical membranes of a variety of epithelial cells (49). CFTR is involved in the regulation of isosmotic fluid transport. CFTR dysfunction results in a complex phenotype characterized by recurrent lung infections, pancreatic insufficiency, and malabsorption. Morbidity and mortality in CF is mainly associated with lung disease. However, hepatobiliary abnormalities are quite common in CF patients. Obstruction of biliary ducts, focal, or multilobular biliary cirrhosis was reported in over 50% of adult CF patients at autopsy (5, 33, 59). Microgallbladder and high incidence of gallstones is also observed (41, 42, 52). CFTR in the liver is mainly expressed in bile duct cells, as shown for rats (21) and humans (13, 55). The pathogenesis of liver disease in CF remains to be elucidated. Defective isosmotic fluid transport may result in decreased solubility and subsequent precipitation of bile components. In support of this, studies with a mouse model for CF in our laboratory showed that CFTR is required for cAMP-induced fluid secretion across the biliary epithelium (44a). On the other hand, there may be additional reasons for the abnormal properties of mucus secretions in CF epithelia. First, CFTR dysfunction affects membrane recycling (7, 45) and possibly secretion (20, 38, 50) in several cell types. Second, CFTR mutations can result in abnormal glycosylation and sulfation of glycoproteins (11, 16, 34, 64). Biliary mucins are implicated as promoting factors in the formation of bile stones (1, 9). Therefore, both increased secretion of mucins and abnormal mucin structure could contribute to hepatic disease in CF.

We have studied the relationship between CFTR and mucin secretion in cultured mouse biliary epithelial cells using a mouse model of CF. We show here that primary cultures of mouse gallbladder epithelium maintain many characteristics of the original tissue, including CFTR expression and mucin synthesis.

METHODS

Materials. Sepharose CL-4B was from Pharmacia (Uppsala, Sweden), and guanidinium Cl^- was from Merck (Darmstadt, Germany). Heparitinase I (Heparinase III) from *Flavobacterium heparinum*, keratanase (*Pseudomonas* species), and hyaluronidase from *Streptomyces hyalurolyticus* were purchased from Sigma (Saint Canteen Fallavier, France), and chondroitinase ABC from *Proteus vulgaris* was from Seikagaku (Tokyo, Japan). The enhanced chemoluminescence (ECL) glycoprotein system, the ECL Western blotting detection system, and the nitrocellulose membrane Hybond C extract were from Amersham (Little Chalfont, UK). Scintillation cocktail was Aqualite from Baker (Deventer, Holland).

Animals. All animal experiments were performed in compliance with the guidelines issued by the Dutch government concerning animal care. All experiments not involving CF(−/−) mice shown in this study were performed with C57BL6 animals. Pilot studies showed that good explant cultures could also be obtained with BCBA or BALB/c mice. A colony of mice with a lesion in the *cftr* gene resulting in complete loss of function in C57BL6 genetic background (47) was bred under pathogen-free conditions in our transgenic unit. The genotype of each individual animal was tested by Southern blotting of tail DNA.

Mouse gallbladder epithelial cell culture. Gallbladders were removed from the animal and cut open lengthwise. After the bile was washed out with RPMI 1640 medium supplemented with 100 U/ml penicillin, 0.1 g/l streptomycin, and 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), pH 7.2, the gallbladders were cut into small pieces and embedded in 1-mm collagen gels (Sigma, Calf skin type I). The explant cultures were incubated at 37°C in a humidified 10% CO_2-air mixture in Dulbecco's modified Eagle's medium (DMEM) supplemented with 100 U/ml penicillin, 0.1 g/l streptomycin, 2 mM glutamine, and 10% fetal calf serum. Mouse gallbladder epithelial cells could be cultured for up to 3 wk, with medium changes every 2–3 days.

Light and electron microscopy. Cells cultured on collagen were either fixed in phosphate-buffered saline containing 4% paraformaldehyde for light microscopy, or in 0.15 M cacodylate buffer, pH 7.4, containing 2.5% glutaraldehyde for electron microscopy. The glutaraldehyde-fixed cells were postfixed with 1% osmium tetroxide and embedded in Epon 812. Ultrathin sections stained with uranyl citrate were examined and photographed in a Philips CM100 electron microscope at 80 kV. The paraformaldehyde-fixed cells were dehydrated and embedded in paraffin. Sections were made at 5–10 μm and stained with hematoxylin and eosin.

Quantitative polymerase chain reaction analysis. Two mouse-specific oligonucleotides, MCF1 (5' GCAGAAACAAGAGTATAAAG 3') and MCF4 (5' CTGCTGTAGTTGGCAAG 3'), were synthesized. The sequences are localized in exons 8 and 10, respectively and generate a mouse CFTR mRNA-specific 459-bp fragment in reverse transcriptase (RT)-polymerase chain reaction (PCR) analysis. Total RNA was isolated by extraction of various mouse tissues or cultured cells in guanidine isothiocyanate and centrifugation through a 5.2 M CsCl step gradient. The RNA was reverse transcribed to cDNA by adding 10 U Avian myoblastoma virus (AMV)-RT in 20 μl cDNA buffer [7 μg heat-denatured RNA, 0.8 μM antisense oligonucleotide primer MCF4, 10^{-3} M dNTPs, 0.05 M KCl, 0.02 M tris(hydroxymethyl)aminomethane (Tris)-HCl, pH 8.4, 0.0025 M $MgCl_2$, 0.1 g/l bovine serum albumin, and 20 U RNasin] for 1 h at 37°C. The 50-μl PCR assay contained 0.3 μM sense and antisense oligonucleotide primer (MCF1 and MCF4), 0.2 mM Tris-HCl, pH 8.4, 2.5 mM $MgCl_2$, and 0.1 g/l bovine serum albumin. After denaturing at 94°C, 5 U *Taq* polymerase were added at 72°C ("hot start"). The standard cycling program was 30 cycles of denaturation for 2 min at 93°C, primer annealing for 2 min at 47°C, and elongation for 4 min at 72°C. Aldolase A oligonucleotide primers, which amplify a 442-bp cDNA fragment, were as in Bremer et al. (8). The amplification of aldolase A sequences was done in parallel, under the same conditions as described above for CFTR. PCR products were separated on a 1.5% agarose gel. For quantitative analysis of the PCR reaction, equal amounts of total RNA isolated from different tissues were subjected to an RT-PCR protocol in parallel incubations. Aliquots were withdrawn at regular intervals from the PCR and subjected to Southern blotting. Radioactivity was measured with a PhosphorImager (Molecular Dynamics).

Patch-clamp analysis. Patch-clamp experiments, data sampling, and analysis were performed as previously described (27). Patch pipettes of borosilicate glass (Clark GC150-TF) were pulled to a resistance of 3–8 MΩ. Excised patches were transferred to a solution exchange compartment (27); this allowed us to add various substrates [ATP, protein kinase A (PKA), and glibenclamide] as well as to change the buffer composition to a I^-, F^-, NO_3^-, Br^-, or gluconate buffer. Pipette (external) and bath (internal) solutions contained (in mM) 140 NaCl, 5 KCl, 1.2 $MgCl_2$, 0.15 $CaCl_2$, 1 ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid, and 5 HEPES. The final Ca^{2+} concentration was 10^{-8} M, pH 7.4. Low (0.05 M) Cl^- buffer was identical except for NaCl (0.043 M); 0.155 M mannitol was added to adjust the osmolarity of the buffer. High (0.427 M) Cl^- buffers contained 0.42 M NaCl. Other buffers replaced 0.14 M NaCl for either NaI, NaBr, $NaNO_3$, NaF, or sodium gluconate, all 140 mM. All experiments were performed at 37°C.

Mucin secretion. Normal mouse gallbladder epithelial cells were incubated with 6 μCi [6-^{3}H]glucosamine hydrochloride (25 × 10^3 Ci/mol) in 2 ml culture medium for 20 h. After the radiolabeling, the cells were rinsed three times in serum-free medium (RPMI 1640 supplemented with 100,000 U/l penicillin, 0.1 g/l streptomycin, and 0.02 M HEPES). The secretion experiment was started by adding 0.75 ml medium to the cells. The incubation was performed at 37°C or 4°C in 95% air plus 5% CO_2 in a humidified atmosphere. After 1 h, a potential secretagogue was added to the cells. At different time points, the incubation medium was replaced, and the cells were rinsed to remove the adherent mucin. At the end of the incubation period, the cells were lysed with 0.1% Triton X-100. The labeled glycoproteins in the medium samples and the cell lysates were washed with deionized water by centrifugation through a 10,000 molecular weight Millipore filter, until no unincorporated [^{3}H]glucosamine was detectable in the eluate and lyophilized. Part of the samples was used for scintillation counting, and part was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 5.5% gels. The gels were fixed in 10% methanol-10% acetic acid, incubated in Amplify (Amersham), and dried. The radiolabeled glycoproteins in the gel were made visible by exposing the gels at −70°C to X-ray films (Kodak).

Analysis of mucins from CF(−/−) and normal cells. Gallbladder explant cultures (8 days) from 8 CF(−/−) and 7 control litter mates (+/− and +/+) were incubated for 16 h with 5 μCi/ml [^{3}H]glucosamine (20–30 Ci/mmol) in culture medium. The medium was collected, the cells were washed and incubated for 20 h at 37°C in serum free medium. After the incubation, the cells were lysed with 0.1% Triton X-100. The medium samples (overnight labeling medium and chase) and cell lysates were lyophilized in three aliquots, after extensive washing to remove the unincorporated label, and used for further investigation.

Enzymatic digestion. An aliquot of each sample was solubilized in 0.3 ml of 0.2 M Tris/acetate buffer, pH 7.4, containing 2×10^{-3} M $CaCl_2$ and 0.02% sodium azide and digested with a glycosaminoglycan-degrading enzyme cocktail (GAG cocktail) composed of 1.5 U hyaluronidase, 0.75 U chondroitinase ABC, and 1.5 U heparitinase for 18 h at 37°C. Each fraction was then dialyzed, freeze dried, and subjected to gel filtration on Sepharose CL-4B (46).

Nonreductive alkaline degradation. Samples obtained from cell lysates were subjected to β-elimination in 1 ml of 0.05 M NaOH at 45°C for 16 h. After neutralization with acetic acid, the treated material was analyzed on Sepharose CL-4B columns.

Fractionation on Sepharose CL-4B. An aliquot of each sample was fractionated on a Sepharose CL-4B (32 × 1 cm diam) in 6 M guanidinium chloride. The flow rate was 24 ml/h, and 1-ml fractions were collected. Radioactivity was assayed in a Beckman S 3801 liquid scintillation counter after mixing 0.1 ml of each eluted fraction with 2 ml of Aqualite.

RESULTS

In this study, we used a knockout mouse model for CF (47). These mice suffer from a complete loss of CFTR function due to a disruption of exon 10. The CF(−/−) animals in our facility display less severe mortality due to intestinal obstruction as reported for the Cambridge colony [20% in the first 2 wk in Rotterdam, compared with 80% in the Cambridge colony (47)]. This may be due to differences in breeding conditions. However, all physiological parameters measured, nasal potential difference (PD), and intestinal and gallbladder PD measurements (Ref. 57 and French, unpublished observations) confirmed the CF phenotype of our animals. In particular, no cAMP-induced Cl^- transport (57) or fluid secretion (Peters, unpublished observations) was observed in intact gallbladders of CF(−/−) animals, in contrast to controls. The animals used for the experiments reported here were 5–6 wk old, without obvious signs of disease or discomfort, average weight 23.5 ± 2.7 g for CF(−/−) and 23.0 ± 4.4 for their normal [CF(+/+) and CF(+/−)] litter mates.

Cultured mouse gallbladder cells. Mouse gallbladder epithelial cells grow from small (1- to 4-mm^2) pieces of embedded mouse gallbladder tissue as a monolayer on a collagen gel (Fig. 1*A*, see METHODS). The outgrowth finally results in holes in the collagen gel (Fig. 1*A*, *top right*) as cells cover both the upper and lower surface of the gel. Mouse gallbladder epithelial cells proliferate for up to 20 days under these conditions to approximately 10 times the original surface area of the epithe-

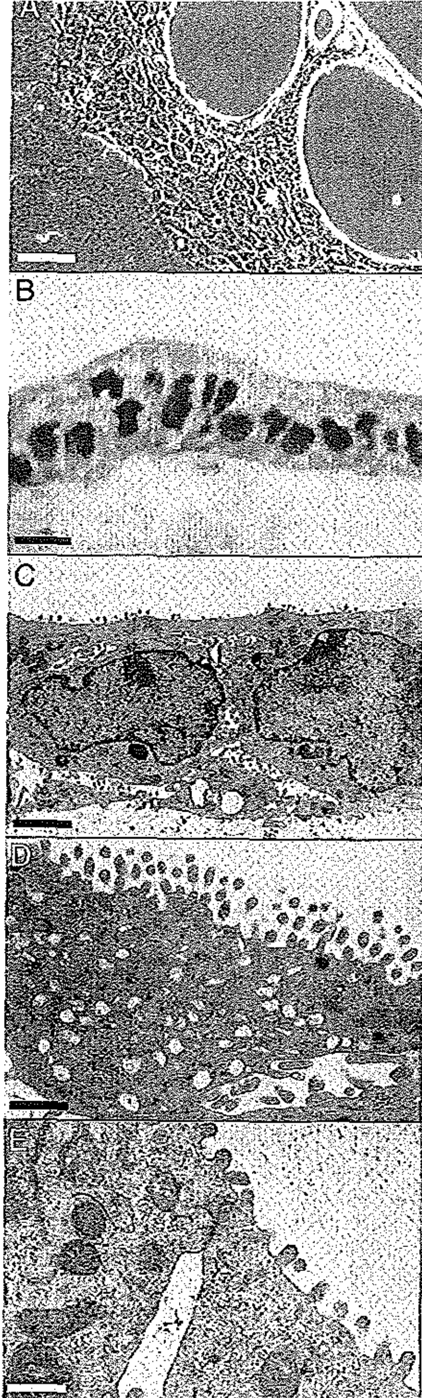

Fig. 1. Light and electron micrographs of cultured mouse gallbladder epithelial cells (MGBC). *A*: primary culture of MGBC (12 days); cells are unstained and were viewed under light microscopy. Epithelial cells grown from fragments of mouse gallbladder are cultured on collagen gel, as described in METHODS (bar = 40 μm). *B*: paraffin section of MGBC in culture (eosin and hematoxylin), showing high cuboid polarized epithelium on collagen matrix (*bottom*; bar = 7 μm). *C*: electron micrograph and cultured MGBC with apical and lateral microvilli (bar = 1.6 μm). *D*: electron micrograph showing apical microvilli and vesicles (bar = 0.5 μm). *E*: electron micrograph showing tight junctions, apical villi, and mitochondria (bar = 0.5 μm).

lium. Microscopic analysis of the cells growing on collagen for 7–14 days showed a morphology highly reminiscent of the intact tissue (58, 61): a polarized high cuboid epithelium (Fig. 1, *B* and *C*) with tight junctions (Fig. 1*E*), apical microvilli (Fig. 1, *C-E*), extensive lateral villi (Fig. 1, *C* and *D*), and numerous mitochondria (Fig. 1, *C* and *E*). Clusters of vesicles similar to those found in native tissue were routinely observed as seen with electron microscopic analysis (Fig. 1*D*). The morphology of cultured gallbladder epithelial cells from CF(−/−) mice is normal (light microscopy, not shown). On morphological criteria (light- and electron-microscopic analysis) no significant outgrowth of fibroblasts was observed under these conditions. After prolonged incubation (>20 days), clusters of fibroblasts were sometimes observed in the open spaces in the collagen gel. All experiments described below were performed with explant cultures <3 wk old with typical epithelial morphology (Fig. 1).

CFTR expression in cultured mouse gallbladder cells. The expression of the cAMP-dependent Cl^- channel CFTR in cultured mouse gallbladder cells was examined with two different methods. RT-PCR analysis demonstrated that cultured mouse gallbladder cells produce CFTR mRNA at levels comparable to the intact tissue (Fig. 2, *A-C*). Intestinal tissues of normal mice (ileum and cecum) give a >10-fold higher level of expression than gallbladder in this assay (Peters, unpublished observations). However, these tissues were negative in CF(−/−) mice, which confirms the absence of full-length CFTR mRNA in these animals (data not shown). Patch-clamp analysis allows us to detect single-channel activity in membrane patches sealed to a microelectrode. During on-cell recording, the cAMP agonist forskolin induced the activity of small linear channels. These channels were inactive after the membrane patches were excised but could be activated with the cAMP-dependent PKA plus ATP (Fig. 3*A*). The voltage-current characteristic of this channel is linear with a conductance of 5 pS in 150 mM NaCl (Fig. 3*B*). The ion selectivity of the channels was determined by exchanging the bath solution with low-Cl^- buffer. This resulted in a shift of the reversal potential (current = 0) expected of an anion-selective channel (Fig. 3*B*). In this way the relative ion selectivity was determined as $NO_3^- > Br^- = Cl^- > Fl^- \gg I^- =$ gluconate. This channel is very similar to the human CFTR, although we and others (4, 17, 48) found a higher conductance for human CFTR (8 pS). On average, 6 ± 5 (n = 10) CFTR channels per patch were observed in this way. In five successful patch-clamp protocols with CF(−/−) cells, not one Cl^- channel of this type was observed. These observations are consistent with the conclusion that cultured normal mouse gallbladder cells express the mouse homologue of human CFTR. A different Cl^- channel frequently observed was a 25-pS outward rectifying channel that often appeared on sustained hyperpolarization of the membrane. This channel was not activated by PKA plus ATP. Glibenclamide (10^{-4} M) reduced the conductivity and induced channel flickering. The ion selectivity of this channel was determined as $NO_3^- > I^- = Br^- > Cl^- \gg F^- >$ gluconate (n = 3) and was also observed in cells from the CFTR-deficient [CF(−/−)] mice (n = 2). This channel is very similar to the outward rectifying Cl^- channel (ORCC) described previously in human cells (23, 51).

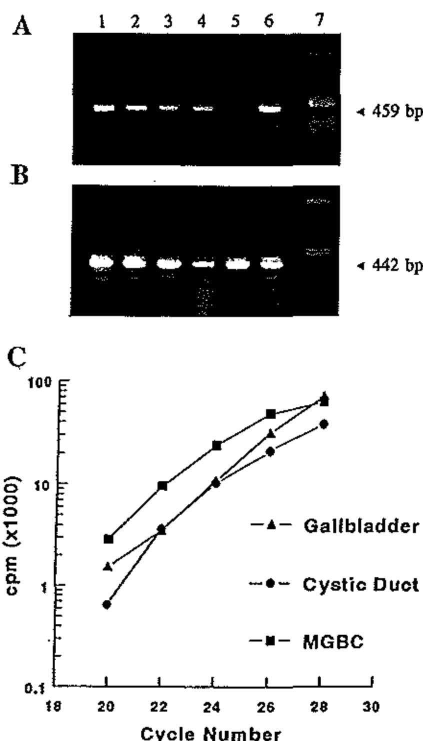

Fig. 2. Expression of cystic fibrosis transmembrane conductance regulator (CFTR) mRNA in cultured mouse gallbladder epithelial cells (MGBC). Agarose gel electrophoresis (ethidium bromide stain) of reverse transcription (RT)-polymerase chain reaction (PCR) products obtained with CFTR primers (*A*) and aldolase primers as a control (*B*). Total RNA was isolated from murine cystic duct (*lane 1*), gallbladder (*lane 2*), lungs (*lane 3*), kidney (*lane 4*), total liver (*lane 5*), and cultured MGBC (*lane 6*). *Lane 7*, size marker (1,018, 517/506, 396, 344, and 289 bp). *C*: amount of CFTR RT-PCR product as a function of the number of PCR cycles was determined as described in METHODS. Data indicate that cultured cells produce CFTR mRNA at levels comparable to epithelial cells of cystic duct or intact gallbladder. cpm, Counts per minute; bp; base pairs.

Glycoprotein secretion from cultured mouse gallbladders. Cultured mouse gallbladder cells produced high molecular weight glycoproteins (HMG) as shown by SDS-PAGE analysis of [^{3}H]glucosamine-labeled proteins (Fig. 4*B*). Labeled HMG are also observed in the culture medium (Fig. 4*A*). Transport of HMG to the medium is time and temperature dependent, as expected for active secretion (Fig. 5*A*). Cells passaged once on collagen gels had similar morphology (light microscopy) but secreted HMG at a lower rate than primary cells (compare Fig. 5, *A* and *B*). On testing the effect of possible activators of secretion (Fig. 5*B*), we found a twofold stimulation by 3 mM ATP. However,

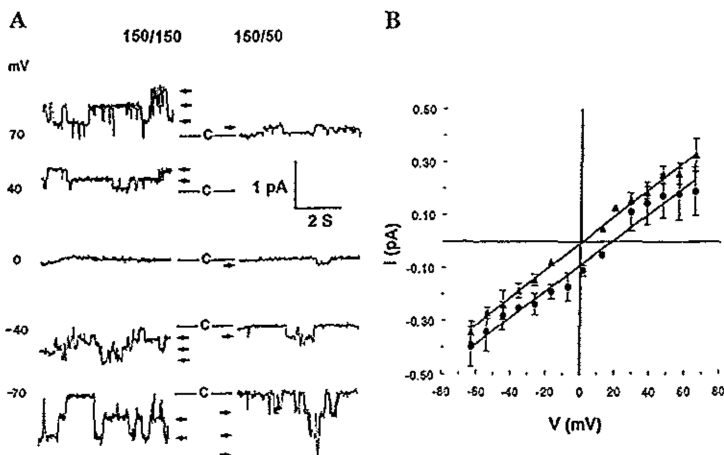

Fig. 3. Patch-clamp analysis. Detection of mouse CFTR channel activity in cultured mouse gallbladder cells with patch-clamp analysis was performed as described in METHODS. *A*: typical pipette current recording (pA) of mouse CFTR activity at different clamp voltages (mV). C, closed state of the channel; arrows, single-channel open-state levels. *Left*: 150 mM Cl^- in pipette and bath. *Right*: 0.05 M Cl^- in bath. *B*: current (*I*)-voltage (*V*) relationship of 5-pS channel after activation with protein kinase A and ATP at 0.15 M Cl^- in pipette and bath (▲) and at 0.05 M Cl^- in bath and 0.15 M in pipette (●). Shift of reversal potential (at which current is zero) indicates ion selectivity of the channel.

lower concentrations of ATP (0.3 mM) had little effect on basal secretion rates (not shown). Forskolin, which increases the intracellular cAMP concentration, had no significant effect. This suggests that in these cells there is no direct link between CFTR activation and glycoprotein secretion and also that the glycoprotein secretion mechanism is not directly regulated by intracellular cAMP. Calcium ionophore or phorbol ester (phorbol 12-myristate 13-acetate) alone or in combination (not shown) did not induce mucin secretion. In conclusion, the cells show active secretion of HMG, probably from the pool of subapical vesicles. The secretion apparently occurs mainly via a constitutive (nonregulated) pathway. The HMG were further analyzed by column chromatography. Part of the labeled HMG found in the cell lysates was excluded from a Sepharose CL4B column (Fig. 6*A*). This material is insensitive to enzymes known to break down glycosaminoglycans but is sensitive to alkaline degradation, suggesting an *O*-glycosyl attachment of carbohydrate chains. These data show that the labeled HMG excluded from the column consist mainly of mucins. The partially included peak possibly corresponds to the 300-kDa band on the PAGE gel (Fig. 4) and has similar properties with respect to enzyme and alkali sensitivity (Fig. 6*A*). This material may consist of precursors or breakdown products of mucins. The labeled HMG secreted into the medium consists in part of mucins, as shown by column chromatography, sensitivity to enzymes (Fig. 6*B*), and carbohydrate staining on Western blots. The 300-kDa mucin component is less prominent than in cell lysates (compare Figs. 4 and 6), suggesting that this material is not actively secreted. The peak in the column volume (23 ml) presents a smear of labeled material on a 5–15% PAGE gel in which no specific products could be identified (data not shown). Since this component was not prominent in short secretion experiments (Fig. 4), it may represent a mixture of low molecular weight glycoproteins and breakdown products of high molecular weight components.

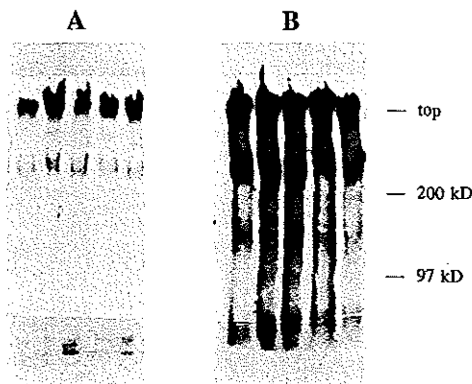

Fig. 4. SDS-PAGE of ^{3}H-labeled glycoproteins secreted from cultured mouse gallbladder epithelial cells. Cultured mouse gallbladder epithelial cells preincubated for 16 h with [^{3}H]glucosamine were washed and incubated in fresh medium for 30 min at 37°C. After incubation, medium was collected and cells were lysed with Triton X-100. Incubation media from 5 separate dishes (*A*) and associated cell lysates (*B*) were subjected to SDS-PAGE on a 5.5% acrylamide gel (90% of total samples). ^{3}H-labeled glycoproteins were visualized by autoradiography. Both the medium samples and cell lysates contain a prominent high molecular weight component on the top of the gel, indicative of mucins.

Mucin secretion by CF(−/−) cells. Cells of CF(−/−) mice also produce and secrete mucins, as shown by column chromatography and by enzyme and alkali sensitivity (Fig. 6, *C* and *D*). Quantitative analysis of total tritium-labeled HMG and the material excluded from the Sepharose column (Table 1) shows that there is no significant difference between normal and CF(−/−) cells with respect to the rate of basal secretion of [^{3}H]glucosamine incorporated in HMG and in mucins, either expressed as percentage of total amount produced or by comparing the amount secreted per dish of culture cells. We conclude that CFTR is not required for either accumulation or basal secretion of glycoprotein, including mucins in cultured mouse gallbladder cells.

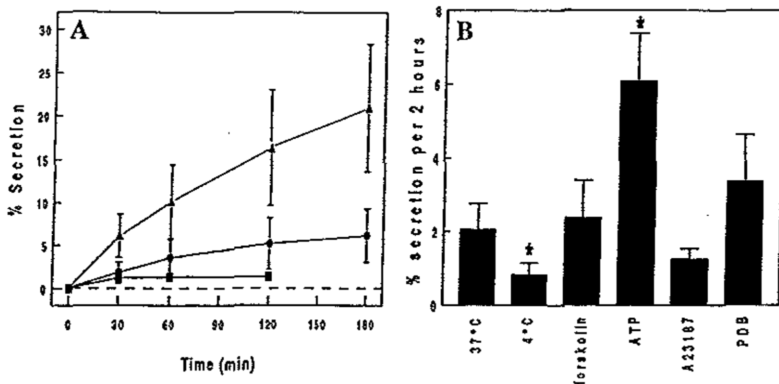

Fig. 5. Glycoprotein secretion from cultured mouse gallbladder epithelial cells (MGBC). *A*: time and temperature dependence of glycoprotein secretion. Cultured MGBC and NIH/3T3 cells as a negative control (~30% confluent/3.5-cm dish) were preincubated for 16 h with [^{3}H]glucosamine to label cellular glycoproteins. Dishes were washed and incubated for 3 h at 4°C or 37°C as described in METHODS. Samples were collected and analyzed as described in METHODS at intervals indicated. Finally, cells were lysed and secretion from cells, as a percentage of total labeled glycoprotein, was calculated [%secretion = 100 × (medium/medium + lysate); values are averages of 7 dishes ± SD]. Data show a steady secretion of high molecular weight glycoproteins from mouse gallbladder cells at 37°C (▲), which is significantly reduced at 4°C (●). Less than 2% of total labeled glycoproteins were secreted by NIH/3T3 cells at 37°C (■). *B*: effect of different agonists on glycoprotein secretion. In a parallel experiment, MGBC passaged once on collagen gels were labeled with [^{3}H]glucosamine (*n*, no. of dishes), washed, and incubated for 60 min in label-free medium as indicated. Medium was then replaced by medium without additions at 37°C ($n = 5$) or at 4°C ($n = 5$), with 10^{-5} M forskolin ($n = 4$), 3×10^{-3} M ATP ($n = 3$), 5×10^{-7} M Ca^{2+} ionophore (A-23187) ($n = 4$), or 5×10^{-7} M phorbol dibutyrate (PDB) ($n = 5$) followed by a 2-h incubation. Medium was collected, cells were subsequently lysed, and the release of high molecular weight glycoprotein to the medium was expressed as percentage of secretion. * Significant difference with control at 37°C (Student's *t*-test).

DISCUSSION

We have established and characterized a primary culture system for mouse gallbladder epithelial cells. The cells we obtained have many of the morphological characteristics of fully differentiated biliary epithelium and can therefore be considered a valid model for this tissue (Fig. 1). Similar results have been obtained wit gallbladder cells from other species, including human (62) and dogs (28, 43). In contrast to these system however, we found that the mouse cells could not b passaged regularly without senescence of the cells an fibroblast overgrowth. Therefore, the experiments de

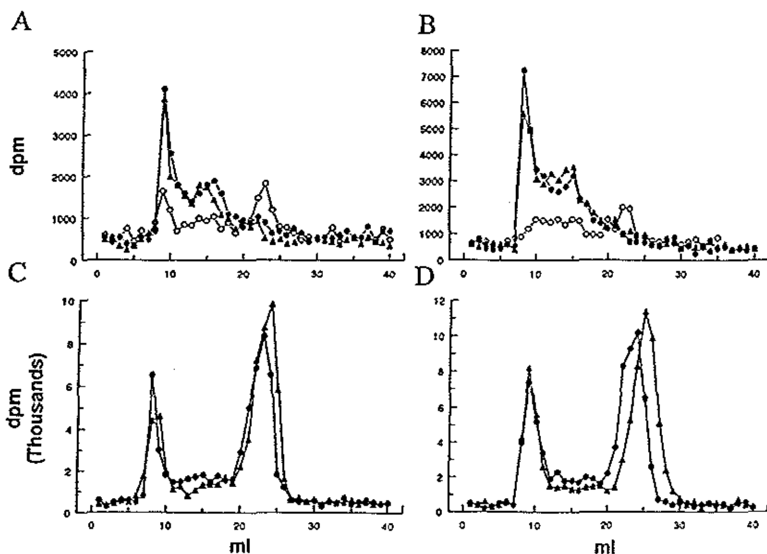

Fig. 6. Column chromatography of la beled glycoproteins of normal and cys tic fibrosis [CF(−/−)] mouse cells. La beled glycoproteins were collected fron cell lysates (*A*, *C*) and culture media (*B* *D*) of normal (*A*, *B*) and CF(−/−) (*C*, *D* mouse gallbladder epithelial cells i culture, as described in METHODS Samples were analyzed on Sepharos CL4B columns either without furthe treatment (●), after incubation with a mixture of heparitinase, hyaluroni dase, and chondroitinase (▲), or wit alkaline degradation (O). dpm, Disinte grations per minute.

Table 1. *Secretion of HMG by cultures of normal and CF(−/−) mouse gallbladder epithelial cells*

	Control	CF(−/−)
[^{3}H]HMG medium (chase), dpm	92,000	96,000
[^{3}H]HMG medium (overnight), dpm	100,000	96,000
[^{3}H]HMG cell lysate, dpm	92,000	175,000
Total [^{3}H]HMG, dpm	282,000	367,000
[^{3}H]HMG secreted, %	68	52
[^{3}H]mucin medium (total), dpm	42,000	60,000
[^{3}H]mucin cell lysate, dpm	36,000	69,000
Total [^{3}H]mucin, dpm	78,000	129,000
[^{3}H]mucin secreted, %	53	48

Primary explant cultures of normal ($n = 7$) and cystic fibrosis [CF(−/−); $n = 8$] gallbladders were labelled with [^{3}H]glucosamine overnight. The medium was collected and replaced by unlabeled medium (chase). High molecular weight glycoproteins (HMG) of medium samples and cell lysates [expressed as disintegrations per minute (dpm) of ^{3}H] were determined by ultrafiltration as described. Combined medium samples and cell lysates were analyzed by gel filtration (Fig. 6) to determine the mucin component of HMG. Percent of mucin and HMG secreted is the percentage of material in the combined media of the total.

scribed in this study were performed with cells grown from primary explants or with cells passaged once to collagen gels.

Our data show that CFTR mRNA is produced in cultured mouse gallbladder cells with levels comparable to intact tissue (Fig. 2). Patch-clamp analysis revealed the presence of a small Cl^- channel with characteristics very similar to human CFTR (Fig. 3). We conclude that these cells express the mouse counterpart of human CFTR, providing the first description of this Cl^- channel. The number of Cl^- channels per patch in these cells compares favorably to other primary epithelial cells in culture (24) or even colon carcinoma cell lines (HT-29, T84) that are often used as a model for CFTR expression (2, 56). Cultured MGBC, therefore, are a convenient model for the study of CFTR activity in a well-differentiated epithelial cell in primary culture. This is particularly important in view of the availability of several mouse models of CF. These include loss of function mutants (44, 47, 54) and more recently the prevalent ΔF508 processing mutant (14, 57, 63) and a G551D mutant (15). Other CFTR mutations are likely to follow.

An additional interesting feature of the cultured MGBC is that they produce and secrete glycoproteins (Figs. 4 and 5), including mucins (Fig. 6). Mucin secretion has been shown for cultured human (62) and dog (29, 43) gallbladder epithelial cells. Mucin secretion in cultured dog epithelial cells was reported to be stimulated by prostaglandin E_2, possibly by increasing the intracellular cAMP concentration (29). In contrast, our results suggest that in cultured MGBC glycoprotein secretion occurs via a constitutive pathway. Forskolin, Ca^{2+} ionophore, and phorbol ester had no significant effect on glycoprotein release in these cells. This suggests that an increase in intracellular cAMP, Ca^{2+}, or protein kinase C activity is not sufficient to induce secretion (Fig. 5). The effect of extracellular ATP is difficult to interpret. Since 0.3 mM ATP had no effect (not shown), the activity of a P_2 purinergic receptor seems excluded. Possibly, aspecific effects of ATP on membrane permeability (18) are involved.

As pointed out in the introduction, several observations in the literature suggest a relationship between CFTR activity and mucin secretion. This could contribute to the development of liver disease in CF. In bronchial segments, the β-adrenergic secretion stimulus observed in normal tissue is reduced in CF patients (50). In this case, the coupling could be indirect. Since CFTR is mainly expressed in serous cells of submucosal glands (19), reduced output from serous acini could impair mucin transport. Mucus secretion in airway goblet cells is under purinergic rather than cAMP control (32). In the intestinal crypt, mucin-secreting Goblet cells are stimulated by Ca^{2+} agonists but not by cAMP (25). In isolated submandibular salivary glands, a significant reduction of cAMP-induced secretion was observed in human CF patients (38) and in a CF mouse model (39). In these cells, mucin release is triggered by an increase of intracellular Ca^{2+} (40). Therefore, the exact relationship between CFTR and secretion is not yet clearly established. Our current data show that MGBC from mice with a loss of function mutation of the *cftr* gene produce and secrete labeled glycoproteins, including mucin, to a similar extent as normal cells, as determined by [^{3}H]glucosamine labeling (Fig. 6, Table 1). Therefore, neither activation of CFTR by forskolin nor absence of CFTR has a marked effect on glycoprotein secretion. This does not support the notion that, in gallbladder cells, CFTR function and secretion are closely coupled, as suggested by experiments with Neomycin-selected dog gallbladder cell clones that express CFTR from an expression vector (28). However, we cannot exclude that the mucins from CF-deficient cells differ from normal with respect to carbohydrate structure and protein/carbohydrate ratio. CFTR deficiency in humans results in increased sialation and sulfation of glycoproteins, and abnormal carbohydrate structures were observed (10, 11, 34, 35). The exact cause of this phenomenon is not known, but it has been suggested that it relates to an effect of CFTR on acidification of intracellular vesicles (3). Therefore, it is possible that the mucins produced by CFTR-deficient gallbladder and biliary cells are abnormal due to increased sulfation or aberrant branching, as in human salivary and respiratory mucins (10, 34). Abnormal biliary mucin structure could contribute to the development of liver disease in CF patients. The amount of material that can be recovered from the present mouse gallbladder cell cultures does not allow a careful analysis of mucin carbohydrate molecular structure. We are presently attempting to increase the yield of cultured cells by crossing the ΔF508 CFTR mouse model that we recently produced (57) with a mouse producing a temperature-sensitive version of Large T-antigen. In this model, we can study the effect of CFTR dysfunction on mucin structure and membrane recycling.

Cells expressing CFTR show a marked cAMP-dependent inhibition of endocytosis and CFTR internal-

ization that is not observed in CFTR-deficient cells (7, 45). CFTR apparently can affect regulated membrane trafficking. An interesting observation in this context is that the cAMP agonist secretin induced release of a fluid-phase marker from an endocytotic compartment in isolated rat biliary duct cells. Similarly, the columnar intestinal crypt cells that express CFTR release vacuolar content in response to cAMP agonists (25). It would be interesting to observe the effect of cAMP on uptake and release of fluid-phase markers in normal and CF MGBC. It is possible that the cells contain vesicular compartments with different regulation of secretion.

CF mice do not develop histological liver abnormalities, at least not at the age we usually study them (<3 mo). The same is probably true for a majority of young human CF patients. The development of liver disease in CF patients does not necessarily correlate with a severe clinical phenotype (lung disease, pancreas dysfunction) (59). CFTR dysfunction apparently is not the only determinant. One other contributing factor may be the presence of other Cl^- channels in the biliary cells (37), which may partially compensate for CFTR dysfunction in some tissues (12). Where expression of this compensating activity becomes the limiting factor, i.e., in CFTR mutants, species and individual differences in pathology can be expected.

It is possible that cultured gallbladder cells behave differently from cells in intact biliary tissue. There is evidence that prostaglandins (30) and cholecystokinin (CCK) (58) stimulate mucin release from intrahepatic duct cells. CCK activates the phosphoinositol pathway rather than intracellular cAMP. There is no compelling evidence that cAMP directly affects mucin release in the intact biliary tract. cAMP agonists do activate biliary fluid and bicarbonate secretion in vivo (31). Fluid secretion is dependent on CFTR activity, as was shown for the mouse gallbladder (44a), intestine (22), and cultured airway epithelium (26, 53). Future in vivo studies in the CF mouse model may allow us to determine the effect of agonists on mucin and fluid output from the intrahepatic biliary system. Available data point at reduced fluid output rather than increased mucin secretion as the major factor in the development of CF liver disease.

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REFERENCES

1. **Afdhal, N. H., N. Niu, D. Gantz, D. M. Small, and B. F. Smith.** Bovine gallbladder mucin accelerates cholesterol monohydrate crystal growth in model bile. *Gastroenterology* 104: 1515–1523, 1993.
2. **Bajnath, R. B., J. A. Groot, H. R. De Jonge, M. Kansen, and J. Bijman.** Synergistic activation of non-rectifying small-conductance chloride channels by forskolin and phorbol esters in cell-attached patches of the human colon carcinoma cell line HT-29cl.19A. *Pfluegers Arch.* 425: 100–108, 1993.
3. **Barasch, J., B. Kiss, A. Prince, L. Saiman, D. Gruenert, a[…] Q. Alawqati.** Defective acidification of intracellular organell[…] in cystic fibrosis. *Nature Lond.* 352: 70–73, 1991.
4. **Bijman, J., W. Dalemans, M. Kansen, J. Keulema[…] E. Verbeek, A. Hoogeveen, H. Dejonge, M. Wilke, D. Drey[…] J. P. Lecocq, A. Pavirani, and B. Scholte.** Low-conductan[…] chloride channels in IEC-6 and CF nasal cells expressing CFT[…] *Am. J. Physiol.* 264 (*Lung Cell. Mol. Physiol.* 8): L229–L23[…] 1993.
5. **Bilton, D., R. Fox, A. K. Webb, W. Lawler, R. F. T. McMaho[…] and J. M. T. Howat.** Pathology of common bile duct stenosis […] cystic fibrosis. *Gut* 31: 236–238, 1990.
6. **Boat, T., M. J. Welsh, and A. Beaudet.** Cystic fibrosis. I[…] *Metabolic Basis of Inherited Disease,* edited by C. Scrive[…] A. Beaudet, W. Sly, and D. Valle. New York: McGraw Hill, 1989, […] 2649–2860.
7. **Bradbury, N. A., T. Jilling, G. Berta, E. J. Sorscher, R. […] Bridges, and K. L. Kirk.** Regulation of plasma membran[…] recycling by CFTR. *Science Wash. DC* 256: 530–532, 1992.
8. **Bremer, S., T. Hoof, M. Wilke, R. Busche, B. Scholte, J. F[…] Riordan, G. Maass, and B. Tummler.** Quantitative expressio[…] patterns of multidrug-resistance P-glycoprotein (MDR1) an[…] differentially spliced cystic fibrosis transmembrane-conductanc[…] regulator messenger RNA transcripts in human epithelia. *Eu[…] J. Biochem.* 206: 137–149, 1992.
9. **Carey, M. C.** Pathogenesis of gallstones. *Am. J. Surg.* 165 410–419, 1993.
10. **Carnoy, C., R. Ramphal, A. Scharfman, J. Lo-Guidice N. Houdret, A. Klein, C. Galabert, G. Lamblin, and P[…] Roussel.** Altered carbohydrate composition of salivary mucin[…] from patients with cystic fibrosis and the adhesion of *Pseudomonas aeruginosa. Am. J. Respir. Cell Mol. Biol.* 9: 323–334, 1993.
11. **Cheng, P. W., T. F. Boat, K. Cranfill, J. R. Yankaskas, and R. C. Boucher.** Increased sulfation of glycoconjugates by cultured nasal epithelial cells from patients with cystic fibrosis. *J. Clin. Invest.* 84: 68–72, 1989.
12. **Clarke, L. L., B. R. Grubb, J. R. Yankaskas, C. U. Cotton, A. McKenzie, and R. C. Boucher.** Relationship of a non-cystic fibrosis transmembrane conductance regulator-mediated chloride conductance to organ-level disease in CFTR(−/−) mice. *Proc. Natl. Acad. Sci. USA* 91: 479–483, 1994.
13. **Cohn, J. A., T. V. Strong, M. R. Picciotto, A. C. Nairn, F. S. Collins, and J. G. Fitz.** Localization of the cystic fibrosis transmembrane conductance regulator in human bile duct epithelial cells. *Gastroenterology* 105: 1857–1864, 1993.
14. **Colledge, W. H., B. S. Abella, K. W. Southern, R. Ratcliff, C. Jiang, S. H. Cheng, L. J. MacVinish, J. R. Anderson, A. W. Cuthbert, and M. J. Evans.** Generation and characterisation of a ΔF508 cystic fibrosis mouse model. *Nature Genet.* 10: 445–452, 1995.
15. **Delaney, S. J., S. Thomson, D. Hume, D. Lamb, D. Porteous, J. D. Dorin, and B. J. Wainwright.** Cystic fibrosis mice carrying the murine mutation G551D replicate human genotype-phenotype correlations. *EMBO J.* 15: 955–963, 1996.
16. **Dosanjh, A., W. Lencer, D. Brown, D. A. Ausiello, and J. L. Stow.** Heterologous expression of ΔF508 CFTR results in decreased sialylation of membrane glycoconjugates. *Am. J. Physiol.* 266 (*Cell Physiol.* 35): C360–C366, 1994.
17. **Drumm, M. L., H. A. Pope, W. H. Cliff, J. M. Rommens, S. A. Marvin, L. C. Tsui, F. S. Collins, R. A. Frizzell, and J. M. Wilson.** Correction of the cystic fibrosis defect in vitro by retrovirus-mediated gene transfer. *Cell* 62: 1227–1233, 1990.
18. **El-Moatassim, C., J. Dornand, and J. C. Mani.** Extracellular ATP and cell signalling. *Biochim. Biophys. Acta* 1134: 31–45, 1992.
19. **Engelhardt, J. F., J. R. Yankaskas, S. A. Ernst, Y. Yang, C. R. Marino, R. C. Boucher, J. A. Cohn, and J. M. Wilson.** Submucosal glands are the predominant site of CFTR expression in the human bronchus. *Nature Genet.* 2: 240–248, 1992.
20. **Engelhardt, J. F., M. Zepeda, J. A. Cohn, J. R. Yankaskas, and J. M. Wilson.** Expression of the cystic fibrosis gene in adult human lung. *J. Clin. Invest.* 93: 737–749, 1994.
21. **Fitz, J. G., S. Basavappa, J. McGill, O. Melhus, and J. A. Cohn.** Regulation of membrane chloride currents in rat bile duct epithelial cells. *J. Clin. Invest.* 91: 319–328, 1993.

22. **Gabriel, S. E., K. N. Brigman, B. H. Koller, R. C. Boucher, and M. J. Stutts.** Cystic fibrosis resistance to cholera toxin in the cystic fibrosis mouse model. *Science Wash. DC* 266: 107–109, 1994.
23. **Gabriel, S. E., L. L. Clarke, R. C. Boucher, and M. J. Stutts.** CFTR and outward rectifying chloride channels are distinct proteins with a regulatory relationship. *Nature Lond.* 363: 263–266, 1993.
24. **Gray, M. A., C. E. Pollard, H. L. Coleman, J. R. Greenwell, and B. E. Argent.** Anion selectivity and block of the small-conductance chloride channel on pancreatic duct cells. *Am. J. Physiol.* 259 (*Cell Physiol.* 28): C752–C761, 1990.
25. **Halm, D. R., S. T. Halm, D. R. Dibona, R. A. Frizzell, and R. D. Johnson.** Selective stimulation of epithelial cells in colonic crypts: relation to active chloride secretion. *Am. J. Physiol.* 269 (*Cell Physiol.* 38): C929–C942, 1995.
26. **Jiang, C., W. E. Finkbeiner, J. H. Widdicombe, P. B. McCray, and S. S. Miller.** Altered fluid transport across airway epithelium in cystic fibrosis. *Science Wash. DC* 262: 424–426, 1993.
27. **Kansen, M., R. B. Bajnath, J. A. Groot, H. R. Dejonge, B. Scholte, A. T. Hoogeveen, and J. Bijman.** Regulation of chloride channels in the human colon carcinoma cell line HT29.cl19A. *Pfluegers Arch.* 422: 539–545, 1993.
28. **Kuver, R., N. Ramesh, S. Lau, C. Savard, S. P. Lee, and W. R. A. Osborne.** Constitutive mucin secretion linked to CFTR expression. *Biochem. Biophys. Res. Commun.* 203: 1457–1462, 1994.
29. **Kuver, R., C. Savard, D. Oda, and S. P. Lee.** PGE generates intracellular cAMP and accelerates mucin secretion by cultured dog gallbladder epithelial cells. *Am. J. Physiol.* 267 (*Gastrointest. Liver Physiol.* 30): G998–G1003, 1994.
30. **Lamont, J. T., B. S. Turner, D. DiBenedetto, R. Handin, and A. L Schafer.** Arachidonic acid stimulates mucin secretion in prairie dog gallbladder. *Am. J. Physiol.* 245 (*Gastrointest. Liver Physiol.* 8): G92–G98, 1983.
31. **Lenzen, R., G. Alpini, and N. Tavoloni.** Secretin stimulates bile ductular secretory activity through the cAMP system. *Am. J. Physiol.* 263 (*Gastrointest. Liver Physiol.* 26): G527–G532, 1992.
32. **Lethem, M. I., M. L. Dowell, M. Van Scott, J. R. Yankaskas, T. Egan, R. C. Boucher, and C. W. Davis.** Nucleotide regulation of goblet cells in human airway epithelial explants: normal exocytosis in cystic fibrosis. *Am. J. Respir. Cell. Mol. Biol.* 9: 315–322, 1993.
33. **Lindblad, A., R. Hultcrantz, and B. Strandvik.** Bile-duct destruction and collagen deposition—a prominent ultrastructural feature of the liver in cystic fibrosis. *Hepatology* 16: 372–381, 1992.
34. **Lo-Guidice, J., J. Wieruszeski, J. Lemoine, A. Verbert, P. Roussel, and G. Lamblin.** Sialylation and sulfation of the carbohydrate chains in respiratory mucins from a patient with cystic fibrosis. *J. Biol. Chem.* 269: 18794–18813, 1994.
35. **Lukacs, G. L., X. B. Chang, N. Kartner, O. D. Rotstein, J. R. Riordan, and S. Grinstein.** The cystic fibrosis transmembrane regulator is present and functional in endosomes—role as a determinant of endosomal pH. *J. Biol. Chem.* 267: 14568–14572, 1992.
36. **Madrid, J. F., M. T. Castells, J. A. Martinez-Menarguez, M. Aviles, and F. Hernandez.** Subcellular characterization of glycoproteins in the principal cells of human gallbladder. *Histochemistry* 101: 195–204, 1994.
37. **McGill, J. M., M. S. Yen, S. Basavappa, A. W. Mangel, and A. P. Kwiatkowski.** ATP-activated chloride permeability in biliary epithelial cells is regulated by calmodulin-dependent protein kinase II. *Biochem. Biophys. Res. Commun.* 208: 457–462, 1995.
38. **McPherson, M. A., and R. L. Dormer.** Cystic fibrosis gene and mucin secretion (Abstract). *Lancet* 343: 7, 1994.
39. **Mills, C. L., J. R. Dorin, D. J. Davidson, E. W. Porteus, F. W. Alton, R. L. Dormer, and M. A. McPherson.** Decreased β-adrenergic stimulation of glycoprotein secretion in CF mice submandibular glands: reversal by the methylxanthine, IBMX. *Biochem. Biophys. Res. Commun.* 215: 674–681, 1995.
40. **Mills, C. L., M. B. Hallett, M. A. McPherson, and R. L. Dormer.** Beta-adrenergic mobilization of Ca^{2+} from an intracellular store in rat submandibular acini. *Biochem. J.* 293: 691–695, 1993.
41. **Nagel, R. A., A. Javaid, H. B. Meire, A. Wise, D. Westaby, J. Kavani, M. G. Lombard, R. Williams, and M. E. Hodson.** Liver disease and bile duct abnormalities in adults with cystic fibrosis. *Lancet* 2: 1422–1425, 1989.
42. **O'Neal, W. K., P. Hasty, P. McCray, B. Casey, J. Rivera-Perez, M. J. Welsh, A. L. Beaudet, and A. Bradley.** A severe phenotype in mice with a duplication of exon 3 in the cystic fibrosis locus. *Human Mol. Genet.* 2: 1561–1569, 1993.
43. **O'Brien, S., M. Keogan, M. Casey, G. Duffy, D. McErlean, M. X. Fitzgerald, and J. E. Hegarty.** Biliary complications of cystic fibrosis. *Gut* 33: 387–391, 1992.
44. **Oda, D., S. P. Lee, and A. Hayashi.** Long term culture and partial characterization of dog gall bladder epithelial cells. *Lab. Invest.* 64: 682–692, 1991.
44a. **Peters R. H. P. C., J. H. van Doorninck, P. J. French, R. Ratcliff, M. J. Evans, W. H. Colledge, J. Bijman, and B. J. Scholte.** Cystic fibrosis transmembrane conductance regulator mediates the cAMP induced fluid secretion but not the inhibition of resorption in mouse gallbladder epithelium. *Hepatology*. In press.
45. **Prince, L. S., R. B. Workman, and R. B. Marchase.** Rapid endocytosis of the cystic fibrosis transmembrane conductance regulator chloride channel. *Proc. Natl. Acad. Sci. USA* 91: 5192–5196, 1994.
46. **Rahmoune, H., H. P. Rounding, W. J. McDonald-Gibson, G. Lamblin, R. L. Hall, and P. Roussel.** Sulfated O-glycoproteins secreted by guinea pig trachea in organ culture. *Am. J. Respir. Cell Mol. Biol.* 4: 156–165, 1991.
47. **Ratcliff, R., M. J. Evans, A. W. Cuthbert, L. J. MacVinish, D. Foster, J. R. Anderson, and W. H. Colledge.** Production of a severe cystic fibrosis mutation in mice by gene targeting. *Nature Genet.* 4: 35–41, 1993.
48. **Rich, D. P., M. P. Anderson, R. J. Gregory, S. H. Cheng, S. Paul, D. M. Jefferson, J. D. McCann, K. W. Klinger, A. E. Smith, and M. J. Welsh.** Expression of cystic fibrosis transmembrane conductance regulator corrects defective chloride channel regulation in cystic fibrosis airway epithelial cells. *Nature Lond.* 347: 358–363, 1990.
49. **Riordan, J. R.** The cystic fibrosis transmembrane conductance regulator. *Annu. Rev. Physiol.* 55: 609–630, 1993.
50. **Rogers, D. F., E. W. Alton, A. Dewar, M. I. Lethem, and P. J. Barnes.** Impaired stimulus-evoked mucus secretion in cystic fibrosis bronchi. *Exp. Lung Res.* 19: 37–53, 1993.
51. **Schwiebert, E. M., M. E. Egan, T. Hwang, S. B. Fulmer, S. S. Allen, G. R. Cutting, and W. B. Guggino.** CFTR regulates outwardly rectifying chloride channels through an autocrine mechanism involving ATP. *Cell* 81: 1063–1073, 1995.
52. **Scottjupp, R., M. Lama, and M. S. Tanner.** Prevalence of liver disease in cystic fibrosis. *Arch. Dis. Child.* 66: 698–701, 1991.
53. **Smith, J. J., P. H. Karp, and M. J. Welsh.** Defective fluid transport by cystic fibrosis airway epithelia. *J. Clin. Invest.* 93: 1307–1311, 1994.
54. **Snouwaert, J. N., K. K. Brigman, A. M. Latour, N. N. Malouf, R. C. Boucher, O. Smithies, and B. H. Koller.** An animal model for cystic fibrosis made by gene targeting. *Science Wash. DC* 257: 1083–1088, 1992.
55. **Strong, T. V., K. Boehm, and F. S. Collins.** Localisation of cystic fibrosis transmembrane conductance regulator mRNA in the human gastrointestinal tract by in situ hybridisation. *J. Clin. Invest.* 93: 347–354, 1994.
56. **Tabcharani, J. A., W. Low, D. Elie, and J. W. Hanrahan.** Low-conductance chloride channel activated by cAMP in the epithelial cell line T84. *FEBS Lett.* 270: 157–164, 1990.
57. **Van Doorninck, J. H., P. J. French, E. Verbeek, R. P. C. Peters, H. Morreau, J. Bijman, and B. J. Scholte.** A mouse model for the cystic fibrosis ΔF508 mutation. *EMBO J.* 14: 4403–4411, 1995.

58. **Wahlin, T., G. D. Bloom, and A. Danielsson.** Effect of cholecystokinin (CCK-PZ) on glycoprotein secretion from mouse gallbladder epithelium. *Cell Tissue Res.* 171: 425–435, 1976.
59. **Waters, D. L., S. F. Dorney, M. A. Gruca, H. C. O. Martin, R. Howman-Giles, A. E. Kan, M. De Silva, and K. J. Gaskin.** Hepatobiliary disease in cystic fibrosis patients with pancreatic sufficiency. *Hepatology* 21: 963–969, 1995.
60. **Welsh, M. J.** The path of discovery in understanding the biology of cystic fibrosis and approaches to therapy. *Am. J. Gastroenterol.* 89: S97–S105, 1994.
61. **Yamada, E.** The fine structure of the gall bladder epithelium of the mouse. *J. Biophys. Biochem. Cytol.* 1: 445–458, 1955.
62. **Yoshitomi, S., K. Miyazaki, and F. Nakayama.** Demonstration and maintenance of mucus secretion in cultured human gallbladder epithelial cells. *In Vitro Cell. Dev. Biol.* 23: 559–566, 1987.
63. **Zeiher, B. G., E. Eichwald, J. Zabner, J. J. Smith, A. P. Puga, P. B. McCray, M. R. Capecchi, M. J. Welsh, and K. R. Thomas.** A mouse model for the ΔF508 allele of cystic fibrosis. *J. Clin. Invest.* 96: 2051–2064, 1995.
64. **Zhang, Y., B. Doranz, J. R. Yankaskas, and J. F. Engelhardt.** Genotypic analysis of respiratory mucous sulfation defects in cystic fibrosis. *J. Clin. Invest.* 96: 2997–3004, 1995.

A mouse model for the cystic fibrosis ΔF508 mutation

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Most cystic fibrosis (CF) patients produce a mutant form (ΔF508) of the cystic fibrosis transmembrane conductance regulator (CFTR), which is not properly processed in normal cells but is active as a chloride channel in several experimental systems. We used a double homologous recombination ('Hit and Run') procedure to generate a mouse model for the ΔF508 mutation. Targeted embryonic stem (ES) cells (Hit clones) were found; of these either 80 or 20% of the clones had lost the ΔF508 mutation, depending on the distance between the linearization site in the targeting construct and the ΔF508 mutation. Correctly targeted clones underwent a second selection step resulting in ES cell clones (Run clones) heterozygous for the ΔF508 mutation with an efficiency of 2–7%. Chimeric mice were generated and offspring homozygous for the ΔF508 mutation showed electrophysiological abnormalities in nasal epithelium, gallbladder and in the intestine, and histological abnormalities in the intestine, typical of CF. Our data suggest that the ΔF508 mice have residual ΔF508 CFTR activity which would explain the mild pathology of the ΔF508 mice. The ΔF508 mouse may provide a useful model for the study of the processing defect of ΔF508 CFTR and for the development of novel therapeutic approaches based on circumvention of the processing block.

Keywords: animal model/embryonic stem cells/gene targeting/Hit and Run procedure/protein processing

Introduction

Cystic fibrosis (CF) is a lethal autosomal recessive disease affecting ~1 in 2500 Caucasians (Boat *et al.*, 1989). The mutated gene is the cystic fibrosis transmembrane conductance regulator (CFTR) gene, which is expressed in several epithelial tissues (Riordan *et al.*, 1989; Crawford *et al.*, 1991; Denning *et al.*, 1992a). CFTR protein functions as an apical cAMP-regulated Cl^- channel (reviewed in Riordan, 1993; Sferra and Collins, 1993) and is presumably involved in osmotic water transport (Quinton, 1990). Malfunction of this chloride channel in CF patients is associated with intestinal malabsorption, obstruction and inflammation of airways, pancreatic ducts, intestine and bile ducts and absence of the vas deferens. Mortality in CF patients is at present mainly due to recurrent pulmonary infections (Boat *et al.*, 1989).

Over 450 different mutations have been described in CF patients (Cystic Fibrosis Genetic Analysis Consortium). The most common mutation is a deletion of a phenylalanine residue at amino acid position 508 of the protein (ΔF508). It is found in 70% of the CF chromosomes (Kerem *et al.*, 1989; Riordan *et al.*, 1989), and therefore 90% of the CF patients have at least one ΔF508 allele. The mutant protein cannot be processed to its mature glycosylated form (Cheng *et al.*, 1990; Gregory *et al.*, 1991). It behaves like a temperature-sensitive processing mutant (Denning *et al.*, 1992b) which is active as a cAMP-regulated chloride channel under permissive conditions (Dalemans *et al.*, 1991; Drumm *et al.*, 1991; Bear *et al.*, 1992; Li *et al.*, 1993). Consequently, it has been suggested that circumvention of the processing block could lead to a therapeutic strategy for CF (Denning *et al.*, 1992b; Yang *et al.*, 1993; Pind *et al.*, 1994). A mouse model expressing the ΔF508 form of CFTR would be very helpful to develop novel therapeutic approaches. Animal models for CF with a complete disruption of the mouse *cftr* gene are available and these show several pathological and electrophysiological abnormalities characteristic of CF patients (Snouwaert *et al.*, 1992; O'Neal *et al.*, 1993; Ratcliff *et al.*, 1993). The mouse exon 10 amino acid sequence is highly homologous to the human sequence and there is no mismatch around the ΔF508 site (Tata *et al.*, 1991). Therefore, we would expect that mouse CFTR with a deletion of the same phenylalanine at position 508 has properties similar to the human counterpart.

To introduce a mutation into the mouse genome, one can choose several strategies. Since we wanted to obtain a mutant *cftr* gene without possible effects of transcriptional interference, the use of a replacement construct with a neomycin gene in the intron next to the mutated ΔF508 exon (Deng *et al.*, 1993) did not seem ideal. One alternative is a two-step selection procedure, 'Hit and Run' (Hasty *et al.*, 1991), which would result in a mutated exon without selection marker genes or plasmid sequences in the intron structure of the gene. In this report, we describe the successful use of this procedure to generate mouse embryonic stem cells (ES cells) carrying the ΔF508 mutation in the endogenous *cftr* gene. Injection of the ES cells into blastocysts resulted in germline transmission. Our data show that mice homozygous for the ΔF508 mutation have abnormalities characteristic of CF.

Results

Targeted integration of a mutant exon in the ES cell genome

We used the 'Hit and Run' procedure (Hasty *et al.*, 1991) to introduce the ΔF508 mutation into the endogenous

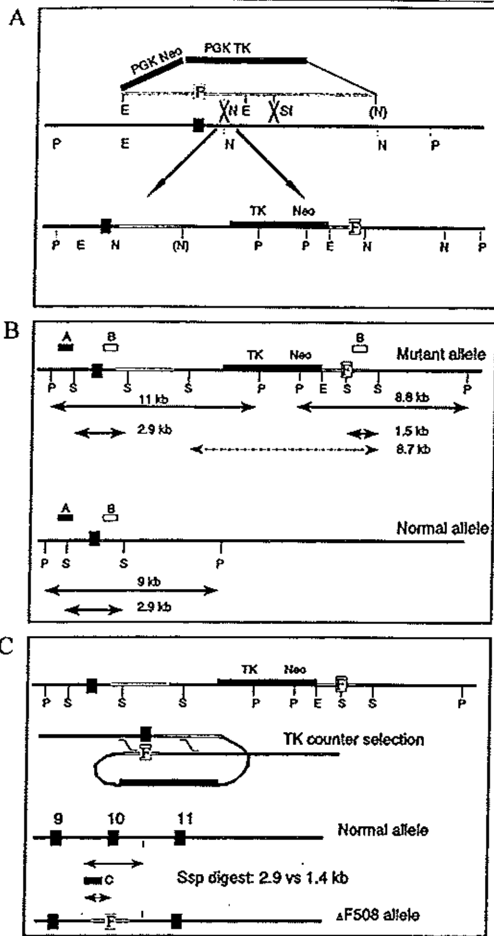

Fig. 1. Hit and Run procedure. (A) The 5.7 kb targeting construct containing exon 10 with the ΔF508 mutation was linearized at the *Nsi*I or the *Sfi*I site and transfected into ES cells which were then selected for the expression of the neomycin gene. The DNA of the construct aligns with the endogenous *cftr* allele and is integrated. The result is a duplication of 5.7 kb spaced by the plasmid and the selectable markers [P = *Pst*I, E = *Eco*RI, N = *Nsi*I, (N) = disrupted *Nsi*I site, Sf = *Sfi*I, S = *Ssp*I; only relevant *Eco*RI and *Ssp*I sites are shown]. (B) A correct integration is detected with a *Pst*I digest and probe A outside the integration construct. The 5′ duplicate generates a new 11 kb *Pst*I fragment, whereas the normal allele has a 9 kb *Pst*I fragment. A randomly integrated construct does not hybridize with this probe. A *Ssp*I digest shows the presence of the ΔF508 mutation with probe B. A correctly integrated construct has a 1.5 kb *Ssp*I digest, while the normal allele and the 5′ duplicate have a 2.9 kb fragment. A repaired integration shows a 8.7 kb fragment. In a correct Hit clone there is a 2:1 ratio of the 2.9 and 1.5 kb fragment. (C) Cells which lost the TK gene will be resistant to selection with FIAU. A homologous cross-over 3′ of the exons generates a normal allele, while a cross-over event 5′ of the exons will result in the desired *cftr* allele with a normal genomic organization but with a mutant exon 10. Clones are digested with *Ssp*I and hybridized with probe C. Correct Run clones have a 1:1 ratio of the 2.9 versus a new 1.4 kb fragment and no neomycin, TK or plasmid sequences.

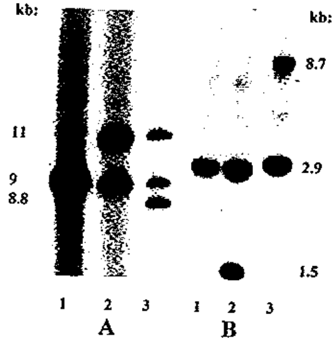

Fig. 2. Southern blot analysis of Hit clones. (A) *Pst*I digest of E14 control DNA (lane 1) and Hit DNA (lane 2) hybridized with probe A lying outside of the transfected construct. Lane 3 is as lane 2 but hybridized with the internal probe B. (B) *Ssp*I digests hybridized with probe B to check the introduced ΔF508/*Ssp*I site (see Figure 1B). Lane 1 is a E14 control, lane 2 is a correct Hit clone and lane 3 is a Hit clone which lost the ΔF508/*Ssp*I site.

mouse *cftr* gene. The first step involves targeted integration of a mutant construct containing 5.7 kb of isogenic genomic mouse DNA, into the mouse *cftr* exon 10 region (Figure 1A). The ΔF508 mutation in the construct was created six nucleotides from a new *Ssp*I restriction site, which did not change the amino acid sequence and was used in the screening (see Materials and methods). After transfection of mouse ES cells, the linearized construct aligns with its homologous endogenous counterpart and integrates at the site of linearization. This results in a duplication of 5.7 kb of genomic DNA, one part with the normal exon 10 and the other part with the ΔF508 exon, separated by plasmid sequences and a neomycin (PGKNeo) plus thymidine kinase (PGKTK) selection marker cassette (Figure 1). ES cell colonies obtained by selection for G418 resistance were analyzed by Southern hybridization. Probe A, which lies outside of the targeting construct, shows a 9 kb *Pst*I fragment for a normal allele and an 11 kb fragment in the case of successful integration (Figures 1B and 2A). We found that 8% (68/800) of the G418-resistant colonies, obtained with the construct linearized at an *Nsi*I site 600 bp downstream of the ΔF508 mutation, showed the 11 kb *Pst*I band with probe A, indicating a correct targeting event (Figure 2A, lane 2). The internal probe B showed the additional 8.8 kb band from the 3′ duplication (Figure 2A, lane 3). Any random integrations would give an extra band with this probe, but none were found in any of the correctly targeted clones. The *Nsi*-Hit clones were checked for the presence of the *Ssp*I site diagnostic for the ΔF508 mutation. This new *Ssp*I site results in a 1.5 kb *Ssp*I fragment from the 3′ duplicate while the 5′ duplicate and the normal allele both give a 2.9 kb fragment (Figures 1B and 2B, lane 2). Out of 25 *Nsi*-Hit clones, 21 clones (84%) had lost the *Ssp*I site diagnostic of the ΔF508 mutation, which resulted in a new 8.7 kb fragment (Figure 2B, lane 3). Since the *Nsi*I site was rather close to the mutation (600 bp), we chose another linearization site further away from the mutation to test whether this would affect the process. With linearization at the *Sfi*I site, 1600 bp downstream from the ΔF508 mutation, only 20% of the *Sfi*-Hit clones had

Table I. Genotype and phenotype of FIAU-resistant clones

Hit clone	Hit clone cultured on 2 μM FIAU for 2 days				Hit clone cultured for 3 p. on G418; 2 p. on G418⁻; 0.4 μM FIAU for 10 days			
	No. of TK⁻ clones analyzed	Genotype			No. of TK⁻ clones analyzed	G418-sensitive	Genotype	
		Wild	Hit	Scrambled			Wild	ΔF508
155	3	0	0	3	206	61	60	1
B30	157	157	0	0	ND			
B47	39	39	0	0	ND			
B73	33	2	28	3	198	113	99	14
B84	69	11	51	7	170	115	108	7

p. indicates passages; ND, not determined.

lost the *Ssp*I site (2/11 colonies), while the targeting efficiency was in the same range (5%, 13/279 colonies) as for *Nsi*I linearization. These data suggest that a repair mechanism is active around the integration site with a preference for the endogenous chromosomal sequence.

ES cell clones heterozygous for the ΔF508 mutation

The next step of the procedure (Run) involves the removal of the duplication plus the intervening plasmid sequences via an intra-chromosomal homologous recombination (Figure 1C). This leads to the loss of either the mutant or the wild-type exon (Figure 1C). The latter case gives us the desired clone with a mutant ΔF508 exon 10 in an otherwise normal genomic organization of the *cftr* gene. In both cases, recombinants will be resistant to the toxic thymidine kinase substrate 1-(2-deoxy, 2-fluoro-β-D-arabinofuranosyl)-5 (FIAU) and sensitive to G418. The correct Run clone is identified by a new 1.4 kb *Ssp*I fragment, generated by an *Ssp*I site outside of the integrated construct and the *Ssp*I site in the mutated exon 10 (Figure 1C). Different Hit clones with a correct karyotype were subjected to selection with 2 μM FIAU for 2 days. Resistant clones were picked after 7–10 days and screened by Southern analysis. In initial experiments, we found either wild-type cells with two copies of the 2.9 kb *Ssp*I fragment, or cells which had no genomic rearrangement compared with Hit clones (Table I). The latter presumably represented TK mutants. A few clones showed a rearranged and scrambled genotype. To reduce the possibility of contaminating wild-type cells in the population, we cultured the Hit cells for three passages in the presence of G418 before the FIAU selection. This decreased the number of FIAU-resistant clones 10- to 100-fold. In addition, we screened the resultant clones for G418 sensitivity to avoid Southern analysis of clones with a mutation in the TK gene. When we started the FIAU selection the day after ending the G418 selection, we did not find any G418-sensitive cells (0/42 colonies). However, if we cultured the cells for two passages in non-selective medium prior to FIAU selection, 30–70% of the clones were G418 sensitive (Table I). Of these, 2–7% had the 1.4 kb *Ssp*I fragment of the desired ΔF508 Run clone. (Table I, Figure 3A). No rearrangements of the *cftr* gene were observed, as other digests show a wild-type restriction pattern (Figure 3B). Further, hybridization with the neomycin probe does not show a signal with any of the Run clones (Figure 3A, lanes 6–8). These results confirm the loss of the duplication and the normal genomic organization of the ΔF508 allele in the Run clones.

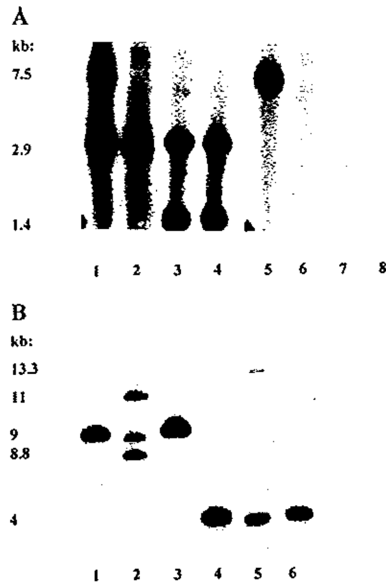

Fig. 3. Southern blot analysis of Run clones hybridized with probe C, 5′ of exon 10. (A) *Ssp*I digests of Hit clone DNA (lane 1), E14 control DNA (lane 2) and two Run clones (lanes 3 and 4). Lanes 5–8: the same blot hybridized with a neomycin probe demonstrating the absence of PGKNeo in the Run clones. (B) *Pst*I digests (lanes 1–3) and *Nsi*I digests (lanes 4–6) of respectively E14 DNA, Hit clone DNA and Run clone DNA hybridized with probe C were used to assay the correct chromosomal structure of the Run clones.

Generation of a ΔF508 CFTR mouse strain

Cells of 11 Run clones derived from three different Hit clones, with a correct karyotype and heterozygous for the ΔF508 mutation, were injected into blastocysts and gave rise to sex conversion and coat color chimerism ranging from 20 to 100%. Two male chimeras showed full germline transmission in a cross with FVB mice, as indicated by coat color and Southern analysis (using the same procedure as for the Run clone analysis, results not shown). To check the correct transcription of the ΔF508 allele, we isolated intestinal RNA from normal and mutant mice and performed a nested RT-PCR analysis with primers in

Fig. 4. (A) A RT-PCR was performed with intestinal RNA of a ΔF/ΔF mouse (lanes 1 and 2), a N/ΔF mouse (lanes 3 and 4) and a N/N mouse (lanes 5 and 6) with primers located in exon 8 and 10. The 403 bp PCR products (odd lanes) were digested with *Ssp*I (even lanes) which cuts the products of a ΔF508 allele resulting in a 359 bp fragment. (B) The sequence of the PCR products shows the deletion of the phenylalanine and the silent base pair substitutions which generated a *Ssp*I restriction site (AATATT) in the ΔF/ΔF mouse. Since the second (Run) recombination took place in intron 9, the sequence was verified from exon 9–10 (not shown).

exons 8 and 10. Since the ΔF508 allele contains the *Ssp*I site in exon 10, we can distinguish the normal from the mutant PCR product. Mice heterozygous (ΔF/N) or homozygous (ΔF/ΔF) for the ΔF508 mutation produced the predicted 359 bp fragment after *Ssp*I digestion of the PCR product (Figure 4A). Sequence analysis of the PCR products showed that the mutant form of CFTR mRNA had the predicted sequence (Figure 4B).

ΔF/ΔF mice have a phenotype typical of CF

Mice homozygous for the ΔF508 mutation (ΔF/ΔF) are viable and do not show severe disease symptoms, such as runting or meconium ileus. However, the ΔF/ΔF mice are growth retarded, as is apparent from the significant difference in weight ($P<0.05$) between the ΔF/ΔF mice (average age 42 days: 17.9 g ± 4.4, $n = 6$) and their normal, N/ΔF and N/N, littermates (average age 39 days: 23.4 g ± 2.6, $n = 7$). Mice were sacrificed at 5–7 weeks and used for histological and electrophysiological studies. In the small intestine of ΔF/ΔF mice, focal hypertrophy of goblet cells was observed in the crypts of Lieberkuhn (Figure 5b). The hypertrophy of goblet cells was predominantly seen in deep crypts, though a few hypertrophic cells were found at the crypt–villus border. The crypts containing hypertrophic goblet cells appeared to be located in clusters. The majority of the intestine appeared normal. Crypts in the colon showed some mucus retention and slight dilatation (Figure 5e). A layer of thick mucus on top of the crypts could also be noticed. We did not observe dramatic distention of the crypts in the small intestine or complete intestinal obstruction either in the ileum or colon, as reported in *cftr* knock-out mice (Figure 5c and f, data from a cftr^{m1Cam} mouse, Ratcliff *et al.*, 1993). So far, we have not seen abnormalities or stasis of inspissated mucus in lungs, pancreas, liver bile ducts, vas deferens and salivary glands.

Electrophysiological abnormalities in ΔF/ΔF mice

The reduced chloride permeability of the epithelium due to CFTR dysfunction causes typical abnormalities in the ion transport of different epithelia (Quinton, 1990). An increased nasal potential difference (PD) is characteristic of CF patients (Knowles *et al.*, 1983; Alton *et al.*, 1990) and is also observed in *cftr* knock-out mice (Grubb *et al.*, 1994a; P.J.French, unpublished data). The basal *in vivo* nasal PD of ΔF/ΔF mice was significantly higher than in normal littermates (Figures 6 and 7c). Consequently, the net effect of the sodium channel inhibitor amiloride, which would block net ion movement in a CF mouse, was higher than normal (Figure 6). The nasal PD in ΔF/ΔF mice increased in response to a large chloride gradient created by substitution of chloride by gluconate in the superfusion solution (Figures 6 and 7c). This gluconate response was quantitatively similar to the response of control littermates (Figure 6). Since cftr^{m1Cam} knock-out mice do not show a significant response to gluconate under our conditions (Table II), we conclude that the response we observe in the ΔF/ΔF mouse is indicative of a residual chloride conductance in nasal epithelium.

Human CF epithelial tissues do not respond to forskolin, which activates CFTR through an increase of $[cAMP]_i$ (Riordan, 1993). The adult excised ΔF/ΔF mouse trachea shows a forskolin response comparable with normal mice (P.J.French, unpublished data). This is also observed in the *cftr* knock-out mouse trachea (Grubb *et al.*, 1994b). In this tissue, forskolin can apparently activate calcium-dependent chloride channels (Clarke *et al.*, 1994) through a cross-talk mechanism which is currently not understood. In the gallbladder, the ΔF/ΔF mice showed a markedly reduced PD response to forskolin compared with normal littermates in Ussing chamber experiments (Figures 6 and 7a). This reduced forskolin response was not due to aspecific tissue damage, as both the electrical resistance and the peak response to the Ca^{2+} agonist carbachol were normal (Figures 6 and 7a). In the ileum, the initial PDs and equivalent short circuit currents (Ieq) were significantly lower in ΔF/ΔF mice compared with normal littermates (Figure 6). Luminal hyperpolarization induced by forskolin was significantly reduced in ΔF/ΔF mice, whereas the response to glucose addition, which activates the Na^+/glucose co-transporter (Wright, 1993), was normal (Figures 6 and 7b). In Table II, the responses to forskolin of ΔF/ΔF mice are compared with those responses in cftr^{m1Cam} knock-out mice. Knock-out mice lack forskolin responses in ileum and gallbladder, whereas the homozygous ΔF/ΔF mice exhibit residual activity. This is in agreement with the observed residual chloride permeability in ΔF/ΔF nasal epithelia (Table II).

Discussion

We have generated mice with a mutation analogous to the frequently occurring temperature-sensitive processing mutation in CF, ΔF508. We intend to use this mouse to study the interaction between ΔF508 CFTR and processing proteins such as calnexin (Pind *et al.*, 1994) and hsp70 (Yang *et al.*, 1993) in intact tissues. This might lead to

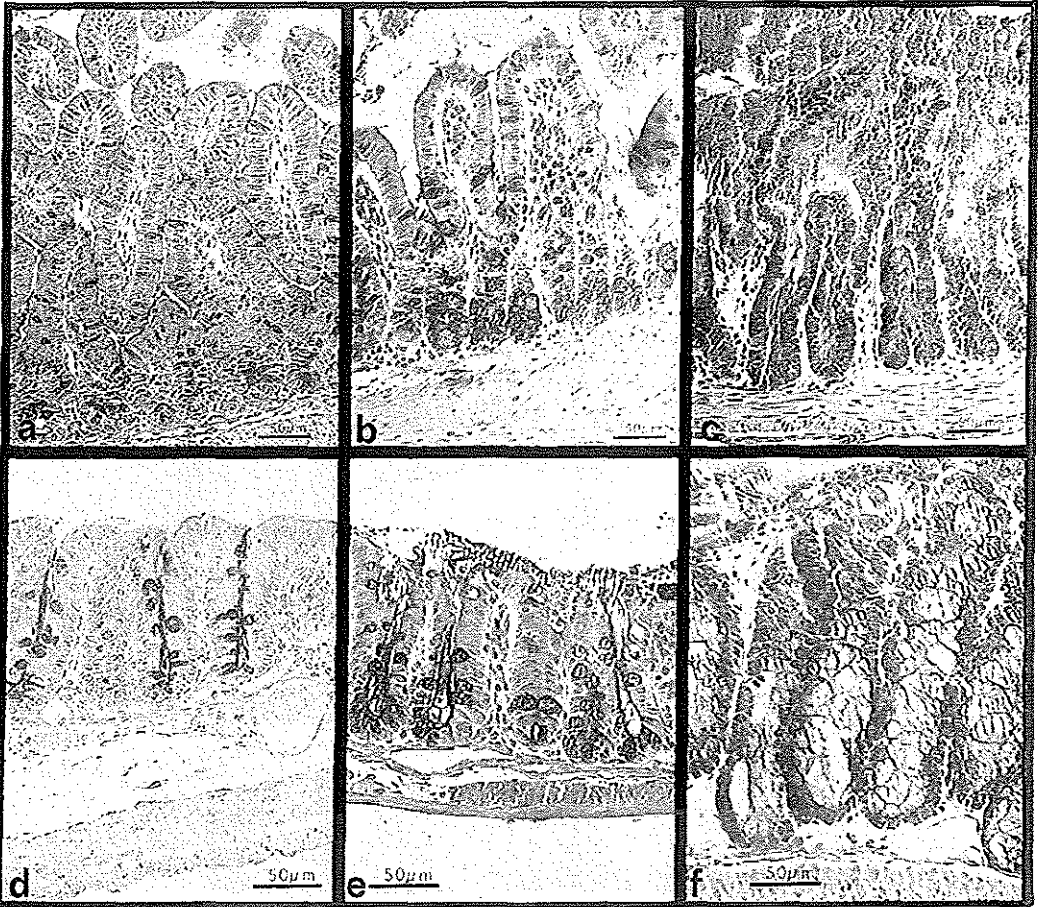

Fig. 5. Histopathology of ΔF508 mice. Sections of the small intestine (a–c) and colon (d–f) of normal (littermates of ΔF/ΔF) (a and d), ΔF/ΔF (b and e) and *cftr*^{Cam} knock-out mouse (c and f). In the ΔF/ΔF mice, focal hypertrophy of goblet cells was found in crypts of the small intestine (b), though not as severe as the ileum of a *cftr*^{Cam} knock-out mouse showing extensive goblet cell hyperproliferation, increased mucus accumulation and luminal obstruction (c). Colon of a ΔF/ΔF mouse with moderate mucus accumulation and distension of the crypts (e) compared with the colon of a *cftr*^{Cam} knock-out with massive goblet cell hyperplasia and hypertrophy (f).

novel therapeutic approaches for the 90% of CF patients carrying a ΔF508 mutation.

Hit and Run procedure

We found integration of the targeting construct into the mouse *cftr* locus in 5–8% of the G418-resistant ES cell colonies. This is comparable with the frequencies found by Dickinson *et al.* (1993) for integration of a targeting construct near exon 10. When we used a linearization site relatively close (600 bp) to the ΔF508/*Ssp*I site, 84% of the Hit clones had lost the diagnostic *Ssp*I restriction site. Since we observed no rearrangement in the exon 10 area, we concluded that the plasmid DNA is preferentially repaired using the endogenous DNA as a template. The mechanism of this repair might be through gap formation and repair (Valancius and Smithies, 1991). We never found the appearance of the *Ssp*I site in both exons nor did we find it only in the 5′ duplication. This implies that, during our Hit procedure, mismatch heteroduplex repair and migration of the Holliday junctions across the mutations are rare (Hasty and Bradley, 1993). The usage of a restriction site 1600 bp away from the ΔF508/*Ssp*I site resulted in only 20% loss of the mutation, confirming previous observations that the frequency of loss depends on the distance between the break and the mutation (Valancius and Smithies, 1991; Deng *et al.*, 1993)

The overall frequency of a correct Run clone (ΔF/N) is ~$1/10^6$–10^7 cells in the original population used for selection, and 2–7% of the clones screened by Southern analysis. In order to find these Run clones, we had to adapt our screening protocol by selecting against wild-type contaminants and by allowing time for the cells to perform an intra-chromosomal recombination. Screening for the loss of G418 resistance further reduced the number of clones analyzed by Southern analysis (Table I). On the basis of the length of the homologous regions involved

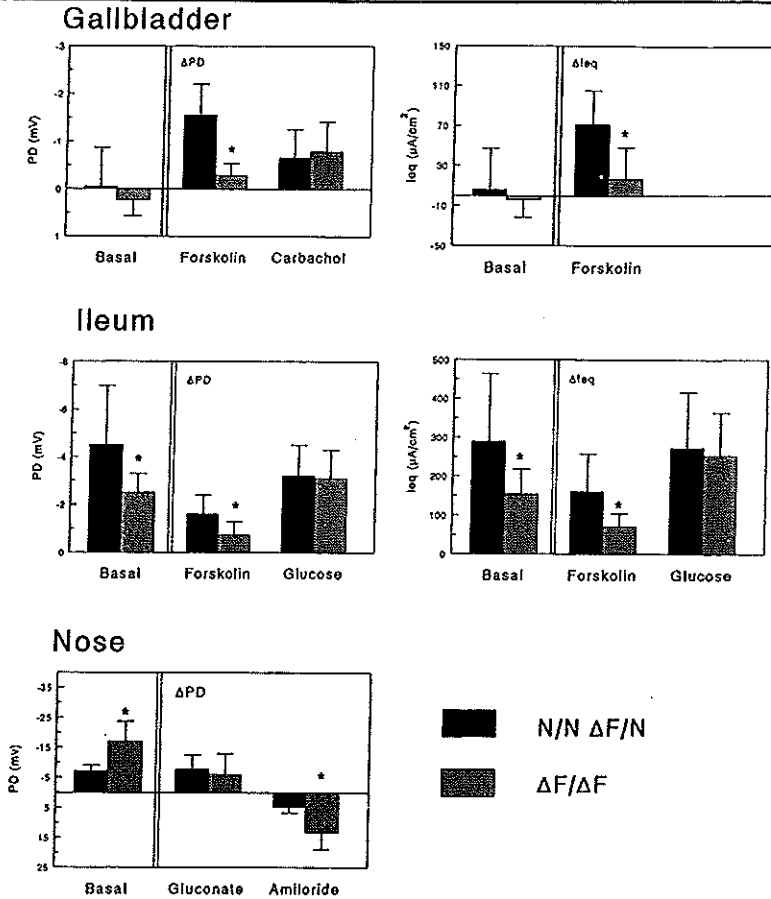

Fig. 6. Summary of the electrophysiological data obtained from six ΔF/ΔF, five ΔF/N and three N/N mice. Left panels: PD, right panels: Ieq. Top: gallbladder. The basal PD and Ieq as well as the peak response to carbachol of ΔF/ΔF mice and normal (N/N or ΔF/N) littermates are comparable. Forskolin (10^{-5} M) responses, however, were markedly reduced in the ΔF/ΔF gallbladder ($P<0.001$, nine tissue samples from six mice) compared with normal (eight tissue samples from eight mice). Middle: ileum. ΔF/ΔF ilea (12 tissue samples from six mice) had a significantly reduced basal PD and Ieq ($P<0.02$) compared with their normal littermates (14 tissue samples from eight mice). Responses to forskolin were reduced in the ΔF/ΔF ileum ($P<0.01$). Lower: nasal PD. In ΔF/ΔF mice the *in vivo* basal PDs were significantly higher than their control littermates ($P<0.01$). Both normal ($n = 6$) and ΔF/ΔF ($n = 6$) nasal epithelia responded to a chloride gradient [153 mM chloride replaced gluconate (133 mM), final Cl^- concentration 10 mM]. Responses to the sodium channel inhibitor amiloride were significantly higher in ΔF/ΔF nasal epithelia ($P<0.02$), resulting in a similar residual PD. Values are averages ± SD. Statistical analyses were performed with the student's *t*-test.

in the two possible Run recombinations, one would expect to find ~25% ΔF508 Run clones (Figure 1C). However, the actual ratio of ΔF508 to wild-type recombinants (± 1/20) does not seem to follow this simple rule. This may be due to cryptic sequence preferences in the recombination process.

The Hit and Run procedure has previously only been used to produce the mutant Hoxb-4 mouse (Ramírez-Solis *et al.*, 1993). The possible drawback of the 'Hit and Run' procedure compared with a one-step replacement protocol is the number of passages under selective pressure that is involved. The starting passage of our E14 cells was 24; selections with both G418 and FIAU plus several expansion steps lead to ΔF508 clones of around passage 57 (assuming about three divisions per passage). The blastocyst injections showed that germline chimeras can still be made with these cells. However, in Run clones derived from the B84 Hit clone, loss of totipotency was observed (20% chimerism, sex-reversal rate 1.4 and no germline transmission).

ΔF/ΔF mice have a CF phenotype

The Hit and Run procedure resulted in mice which produce ΔF508 CFTR mRNA with the predicted sequence (Figure 4B). The heterozygous ΔF/N mice do not show significant abnormalities in any of our assays. We therefore consider them phenotypically normal. ΔF/ΔF mice show a phenotype typical of CFTR dysfunction, including failure to grow to normal weight, histological abnormalities in the intestine (Figure 5) and electrophysiological abnormalities in nasal epithelium, gallbladder and intestine (Figures 6 and 7). We did not observe lethal obstruction of the

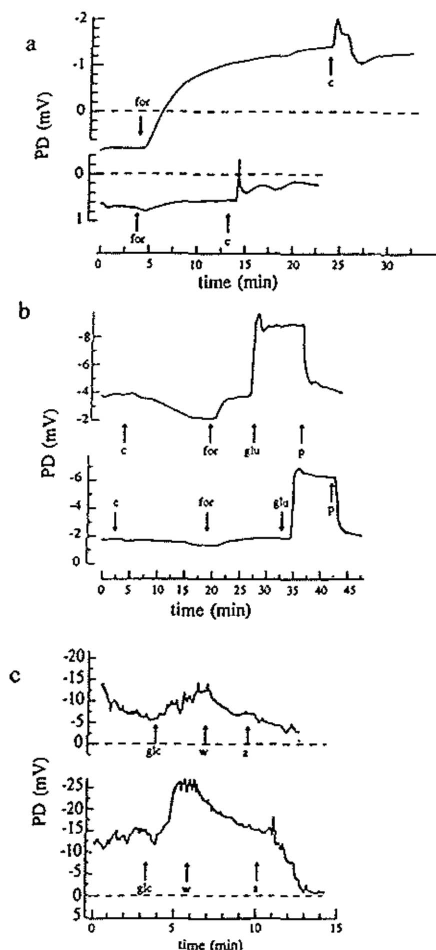

Fig. 7. Representative Ussing chamber tracings from normal mice (upper tracings) and ΔF/ΔF mice (lower tracings) tissue.
(a) Gallbladders of ΔF/ΔF mice had a reduced response to forskolin (for) compared with normal littermates, whereas peak responses to carbachol (c) were similar. (b) Ilea of ΔF/ΔF mice had a reduced response to forskolin (for) compared with normal littermates. Responses to 30 mM glucose (glu) and subsequent 0.2 mM phloridzin (p), activating and inhibiting the Na^+/glucose transporter (Wright, 1993) respectively, were similar. Responses to carbachol (c) on average caused luminal depolarization and an increase in tissue resistance. (c) Nasal epithelia of ΔF/ΔF mice had a higher basal PD and an increased response to amiloride (a) but did respond to a chloride gradient [from 153 to 10 mM Cl^-, chloride replaced gluconate (glc, 133 mM)]. w = wash.

intestine in ΔF/ΔF mice. Meconium ileus, i.e. obstruction of the intestine, is diagnosed in only 10% of neonatal CF patients, and those patients show mucus obstruction in the crypts. Other CF patients do not show severe mucus accumulation but have distended glands and goblet cell hypertrophy similar to our ΔF/ΔF mice (Thomaidis and Arey, 1963). Another typical feature of CF, progressive lung inflammation accompanied by accumulation of viscous mucus (Boat *et al.*, 1989), is not observed in either ΔF/ΔF or knock-out mice under normal conditions. However, it has been shown recently that CF mice, when challenged with lung pathogens, do show increased pathology compared with normal littermates (Davidson *et al.*, 1995). It would therefore be interesting to subject the ΔF/ΔF mice to a similar protocol. As many abnormalities in CF patients develop with age, further histological analysis will be done with mice older than those of 5–7 weeks which we used for this study.

The virtual absence of lethal intestinal obstruction in ΔF/ΔF mice differs from the *cftr* knock-out models described earlier. The latter were reported to have a 50–90% mortality rate in the first 4 weeks, mainly due to intestinal obstructions (Snouwaert *et al.*, 1992; O'Neal *et al.*, 1993; Ratcliff *et al.*, 1993). The $cftr^{m1HGU}$ insertion mutant (Dorin *et al.*, 1992) expresses low levels of normal CFTR (Dorin *et al.*, 1994) and has a low mortality rate. This suggests that the mild phenotype of the ΔF/ΔF mice is related to the residual chloride permeability observed. However, mortality rates may prove a rather misleading parameter. The colony of $cftr^{m1Cam}$ mice in the Rotterdam animal facility has a considerably lower mortality rate than reported (<40% in the first 4 weeks versus 80% in the first 5 days, Ratcliff *et al.*, 1993). Apparently, the breeding conditions have a major impact on mortality rates. The phenotypic effect of residual chloride permeability may be revealed by challenging knock-out and ΔF/ΔF mice with pathogens in parallel experiments.

Residual CFTR activity in ΔF/ΔF mice

The small but significant forskolin response observed in ileum and gallbladder, and the response to a chloride gradient in the nasal epithelium (Figures 5 and 6) of the ΔF/ΔF mouse might be due to residual ΔF508 CFTR activity. An alternative chloride channel (Clarke *et al.*, 1994) seems a less plausible explanation since $cftr^{m1Cam}$ knock-out mice completely lack a forskolin response in the gallbladder (Table II) and intestine (Table II, Clarke *et al.*, 1992; Cuthbert *et al.*, 1994) and show no significant gluconate response in the nasal PD measurements (Table II). Ca^{2+}-activated chloride channels appear absent in the ileum, as the ileum does not respond to carbachol with a luminal hyperpolarization (Figure 7). Thus, the cAMP-induced activation of Ca^{2+}-dependent chloride channels as described in mouse trachea (Grubb *et al.*, 1994b) seems not to occur in the ileum or the gallbladder. It is conceivable that the mouse ΔF508 CFTR is partially processed into mature ΔF508 CFTR protein reaching the plasma membrane. At present, it is not known whether the same phenomenon occurs in CF patients. Residual intestinal chloride transport activity, observed in the minority of ΔF/ΔF patients, is correlated with mild disease (Veeze *et al.*, 1994). Since this has not yet been analyzed at the level of single chloride channels, this activity cannot be attributed unequivocally to CFTR. Further experiments should determine the channel characteristics of the mouse ΔF508 CFTR and the processing kinetics of ΔF508 CFTR in differentiated mouse epithelial cells. The mouse and human processing systems may differ with respect to the kinetics of their interactions with the ΔF508 CFTR protein, and the mouse ΔF508 CFTR may have different properties from the human ΔF508 form. The ΔF508 mouse model gives us an opportunity to study the CFTR processing

Table II. Responses to forskolin or gluconate of ΔF/ΔF and cftr^{m1Cam} –/– mice (ΔPD in mV)

	ΔF/ΔF	Controls	*P**	–/–	Controls	*P**	*P#*
Gluconate:							
Nose	–5.3 $n = 6$	–8.3 $n = 6$	ns	–2.5 $n = 11$	–7.0 $n = 11$	<0.001	ns
Forskolin:							
Gallbladder	–0.2 $n = 9$	–1.5 $n = 6$	<0.001	0.0 $n = 6$	–1.2 $n = 10$	<0.001	<0.01
Intestine	–0.5 $n = 13$	–1.5 $n = 13$	<0.001	0.05 $n = 6$			<0.001

Control mice were heterozygous and homozygous wild-type littermates. Median values are given, with *n* indicating the number of experiments. Mann–Whitney test: $P^* = P$ value comparing CF mice with littermates, and $P\# = P$ value comparing ΔF/ΔF mice with –/– cftr$^{m1Cam\ mice}$. ns, not significant.

defect observed in a majority of CF patients in more detail and allows us to test novel approaches for therapy of CF *in vivo*.

Materials and methods

Embryonic stem cells

E14 ES cells (a gift of M.Hooper, Edinburgh) were cultured on 0.1% gelatine-coated dishes in DMEM/60% Buffalo rat liver-conditioned medium/10% fetal calf serum (FCS) (Smith and Hooper, 1987) supplemented with 1% non-essential amino acids (Gibco BRL, Life Technologies), 0.1 mM 2-mercaptoethanol, antibiotics and 1000 U/ml LIF (ESGRO, Gibco BRL, Life Technologies) and passaged every 2–3 days.

Targeting constructs

CFTR genomic DNA was cloned from a 129/Ola λ library (provided by G.Grosveld). A 2 kb *Eco*RI fragment containing exon 10 of the *cftr* gene was mutated using standard site-directed mutagenesis technology to introduce a ΔF508 deletion and a *Ssp*I restriction site (AAT ATC ATC TTT = amino acids NIIF to *AAT ATT* AT. ..T = amino acids NII.). This fragment of the mouse *cftr* gene was enlarged to 5.7 kb by insertion of a 4 kb *Nsi*I fragment between the *Nsi*I site of the *Eco*RI fragment and the *Pst*I site of the pBluescript vector (pESEN7). A cassette with a thymidine kinase (TK) gene driven by a phosphoglycerate kinase promoter (PGKTK, a gift of N.van der Lugt, Amsterdam) and a neomycin gene driven by a PGK promoter (PGKNeobpA, Soriano *et al.*, 1991) was introduced as a PGKTK–PGKNeo *Sal*I fragment into the *Xho*I site of pESEN7, generating pESTNΔF+ (Figure 1A). This construct contains a unique *Nsi*I linearization site at 600 bp from the ΔF508 and a unique *Sfi*I site at 1600 bp from the ΔF508. The mutant exon and surrounding sequences were checked by sequence analysis.

Selection of integration mutants (Hit clones)

E14 ES cells (10^7) were electroporated in PBS (Ca^{2+}- and Mg^{2+}-free) with 5–10 μg linearized plasmid in a Progenetor II, PG200, Hoefer Gene pulser at 350 V/cm, 1200 μF, 10 ms. Selection was started at 300 μg/ml G418 (Gibco BRL, Life Technologies) the next day. Genomic DNA of G418-resistant clones was digested as indicated, Southern blotted and hybridized to probe A, a *Hae*III fragment upstream of the construct, to probe B, a *Hin*dIII–*Nsi*I fragment starting from the second *Hin*dIII site in exon 10 or to probe C, a *Bgl*II–*Hin*dIII fragment ending on the first *Hin*dIII site in exon 10 (Figure 1B). Targeted clones (Hit clones) with a normal karyotype were used for the next step.

Selection of TK revertants (Run clones)

To obtain independent ΔF508 clones, a Hit clone was grown on G418 for three passages, followed by two passages on medium without G418, on 10 different plates which were kept separate during the whole procedure. To select for TK-negative clones, the cells were plated at 3×10^6 cells per 10 cm dish and FIAU (Bristol Myers, Squibb) was added at a concentration of 0.4 μM for 10 days. Colonies were picked at days 7–10 and transferred to 24-well plates. FIAU-resistant clones derived from the same plate were not considered to be independent. When confluent, a clone was divided into three parts, for testing loss of G418 resistance, for freezing and for DNA analysis. G418-resistant clones were discarded.

Generation and screening of ΔF508 mice

Blastocyst injection and re-implantation were performed as described by Bradley (1987). Male chimeras were crossed with FVB mice. DNA was isolated from tail segments (Laird *et al.*, 1991) and analyzed on Southern blots as described for the Run clones.

Analysis of the ΔF508 allele

To check the correct transcription of the ΔF508 allele, intestinal RNA was isolated from ΔF/ΔF mice and control mice with the LiCl/urea method (Auffray and Rougeon, 1980). A PCR (30 cycles: 2 min 94°C, 2 min 47°C, 4 min 72°C) was carried out with primers MCF1 and MCF4 located in exon 8 and at the 3′ end of exon 10. A nested PCR was done with the same cycle parameters and with primers MCF2 and MCF4 located inside and adjacent to the first primer pair. (5′→3′ sequence MCF1:gcagaaacaagagtataaag, MCF2: aatgaccacaggcataatc, MCF3: caacactcttatatctgt, MCF4: ctgctgtagttggcaag). Part of the product was digested with *Ssp*I, which cuts the introduced *Ssp*I site adjacent to the ΔF508 mutation. The PCR products derived from ΔF/ΔF mice and N/N mice were cloned and sequenced.

Histological analysis of the ΔF/ΔF mice

The ΔF508 mouse strain has a 129/Ola×FVB/N background and was kept in a pathogen-free environment. Tissues were fixed in 40% ethanol, 5% HAC, 3.7% formaldehyde, 45% saline pH 7.3, and paraffin sections were stained with Alcium blue, haematoxylin and eosin.

Electrophysiological analysis

Gallbladder and ileum were mounted in an Ussing chamber, under open circuit conditions. Experiments were performed at 37°C. The electrical resistance of the epithelium was measured at intervals by the voltage deflection following a calibrated current pulse. The basic perfusion solutions for gallbladder and ileum consisted of (mM): 105 NaCl, 4.7 KCl, 1.3 $CaCl_2$, 1.0 $MgCl_2$, 20.2 $NaHCO_3$, 0.4 NaH_2PO_4, 0.3 Na_2HPO_4, 10 HEPES, saturated with 95% O_2 and 5% CO_2, pH 7.4. To the serosal side of the ileum, 5 mM glucose was added. Nasal PD measurements were performed *in vivo* essentially as described by Grubb *et al.* (1994b). Data were corrected for liquid junction potentials. The basic perfusion solution consisted of (mM): 133 NaCl, 5 KCl, 1.5 $CaCl_2$, 1 $MgCl_2$, 0.4 NaH_2PO_4, 0.3 Na_2HPO_4, 5.5 glucose and 5 mM HEPES pH 7.4, 37°C.

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References

Alton,E.W.F.W., Currie,D., Logan-Sinclair,R., Warner,J.O., Hodson,M.E. and Geddes,D.M. (1990) Nasal potential difference: a clinical diagnostic test for cystic fibrosis. *Eur. Respir. J.*, 3, 922–926.

Auffray,C. and Rougeon,F. (1980) Purification of mouse immunoglobulin heavy-chain messenger RNAs from total myeloma tumor. *Eur. J. Biochem.*, 107, 303–314.

Bear,C.E., Jensen,T.J. and Riordan,J.R. (1992) Functional capacity of the major mutant form of the cystic fibrosis transmembrane conductance regulator. *Biophys. J.*, 61, A127.

Boat,T., Welsh,M.J. and Beaudet,A. (1989) Cystic Fibrosis. In Scriver,C., Beaudet,A., Sly,W. and Valle,D. (eds), *Metabolic Basis of Inherited Disease*. McGraw Hill, New York, pp. 2649–2860.

Bradley,A. (1987) Production and analysis of chimaeric mice. In Robertson,E.J. (ed.), *Teratocarcinomas and Embryonic Stem cells—A Practical Approach*. IRL Press, Oxford, pp. 113–153.

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

Clarke,L.L., Grubb,B.R., Gabriel,S.E., Smithies,O., Koller,B.H. and Boucher,R.C. (1992) Defective epithelial chloride transport in a gene-targeted mouse model of cystic fibrosis. *Science*, 257, 1125–1128.

Clarke,L.L., Grubb,B.R., Yankaskas,J.R., Cotton,C.U., McKenzie,A. and Boucher,R.C. (1994) Relationship of a non-cystic fibrosis transmembrane conductance regulator-mediated chloride conductance to organ-level disease in *Cftr* (-/-) mice. *Proc. Natl Acad. Sci. USA*, 91, 479–483.

Crawford,I., Maloney,P.C., Zeitlin,P.L., Guggino,W.B., Hyde,S.C., Turley,H., Gatter,K.C., Harris. and Higgins,C.F. (1991) Immunocytochemical localization of the cystic fibrosis gene product CFTR. *Proc. Natl Acad. Sci. USA*, 88, 9262–9266.

Cuthbert,A.W., MavVinish,L.J., Hickman,M.E., Ratcliff,R., Colledge, W.H. and Evans,M.J. (1994) Ion-transporting activity in the murine colonic epithelium of normal animals and animals with cystic fibrosis. *Pflügers Arch. Eur. J. Phys.*, 428, 508–515.

Dalemans,W. *et al.* (1991) Altered chloride ion channel kinetics associated with the ΔF508 cysic fibrosis mutation. *Nature*, 354, 526–528.

Davidson,D.J., Dorin,J.R., McLachlan,G., Ranaldi,V., Lamb,D., Doherty,C., Govan,J. and Porteous,D.J. (1995) Lung disease in the cystic fibrosis mouse exposed to bacterial pathogens. *Nature Genet.*, 9, 351–357.

Deng,C., Thomas,K.R. and Capecchi,M.R. (1993) Location of crossovers during gene targeting with insertion and replacement vectors. *Mol. Cell. Biol.*, 13, 2134–2140.

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

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

Dickinson,P., Kimber,W.L., Kilanowski,F.M., Stevenson,B.J., Porteous, D.J. and Dorin,J.R. (1993) High frequency gene targeting using insertional vectors. *Hum. Mol. Genet.*, 2, 1299–1302

Dorin,J.R. *et al.* (1992) Cystic fibrosis in the mouse by targeted insertional mutagenesis. *Nature*, 359, 211–215.

Dorin,J.R., Stevenson,B.J., Fleming,S., Alton,E.W., Dickinson,P. and Porteous,D.J. (1994) Long-term survival of the exon 10 insertional cystic fibrosis mutant mouse is a consequence of low level residual wild-type *Cftr* gene expression. *Mamm. Genome*, 5, 465–472.

Drumm,M.L., Wilkinson,D.S., Smit,L.S., Worrell,R.T., Strong,T.V., Frizzel,R.A., Dawson,D.C. and Collins,F.S. (1991) Chloride conductance expressed by ΔF508 and other mutant CFTRs in *Xenopus* oocytes. *Science*, 254, 1797–1799.

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

Grubb,B.R., Vick,R.N. and Boucher,R.C. (1994a) Hyperabsorption of Na^+ and raised Ca^{2+}-mediated Cl^- secretion in nasal epithelia of CF mice. *Am. J. Phys. Cell Physiol.*, 35, C1478–C1483.

Grubb,B.R., Paradiso,A.M. and Boucher,R.C. (1994b) Anomalies in ion-transport in CF mouse tracheal epithelium. *Am. J. Phys. Cell Physiol.*, 36, C293–C300.

Hasty,P. and Bradley,A. (1993) Gene targeting vectors for mammalian cells. In Joyner,A.L. (ed.), *Gene Targeting—A Practical Approach*. IRL Press, Oxford, pp. 1–31.

Hasty,P., Ramírez-Solis,R., Krumlauf,R. and Bradley,A. (1991) Introduction of a subtle mutation into the Hox-2.6 locus in embryonic stem cells. *Nature*, 350, 243–246.

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

Knowles,M., Gardzy,J. and Boucher,R.C. (1983) Increased bioelectric potential difference across respiratory epithelia in cystic fibrosis. *N. Engl. J. Med.*, 305, 1489–1495.

Laird,P.W., Zijderveld,A., Linders,K., Rudnicki,M.A., Jaenisch,R. and Berns,A. (1991) Simplified mammalian DNA isolation procedure. *Nucleic Acids Res.*, 19, 4293.

Li,C., Ramjeesingh,M., Reyes,E., Jensen,T., Chang,X., Rommens,J.M. and Bear,C.E. (1993) The cystic fibrosis mutation (ΔF508) does not influence the chloride channel activity of CFTR. *Nature Genet.*, 3, 311–316.

O'Neal,W.K., Hasty,P., McCray,P.Jr, Casey,B., Rivera-Perez,J., Welsh, M.J., Beaudet,A.L. and Bradley,A. (1993) A severe phenotype in mice with a duplication of exon 3 in the cystic fibrosis locus. *Hum. Mol. Genet.*, 2, 1561–1569.

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

Quinton,P. (1990) Cysic fibrosis: a disease of electrolyte transport. *FASEB J.*, 4, 2709–2717.

Ramírez-Solis,R., Zheng,H., Whiting,J., Krumlauf,R. and Bradley,A. (1993) Hoxb-4 (Hox-2.6) mutant mice show homeotic transformation of a cervical vertebra and defects in the closure of the sternal rudiments. *Cell*, 73, 279–294.

Ratcliff,R., Evans,M.J., Cuthbert,A.W., MacVinish,L.J., Foster,D., Anderson,J.R. and Colledge,H. (1993) Production of a severe cystic fibrosis mutation in mice by gene targeting. *Nature Genet.*, 4, 35–41.

Riordan,J.R. (1993) The cystic fibrosis transmembrane conductance regulator. *Annu. Rev. Physiol.*, 55, 609–630.

Riordan,J.R. *et al.* (1989) Identification of the cystic fibrosis gene: cloning and characterization of the complementary DNA. *Science*, 245, 1066–1073.

Sferra,T.J. and Collins,F.S. (1993) The molecular biology of cystic fibrosis. *Annu. Rev. Med.*, 44, 133–144.

Smith,A.G. and Hooper,M. (1987) Buffalo rat liver cells produce a diffusible activity which inhibits the differentiation of murine embryonal carcinoma and embryonic stem cells. *Devel. Biol.*, 121, 1–9.

Snouwaert,J.N., Brigman,K.K., Latour,A.M., Malouf,N.N., Boucher, R.C., Smithies,O. and Koller,B.H. (1992) An animal model for cystic fibrosis made by gene targeting. *Science*, 257, 1083–1088.

Soriano,P., Montgomery,C., Geske,R. and Bradley,A. (1991) Targeted disruption of the c-*src* proto-oncogene leads to osteopetrosis in mice. *Cell*, 64, 693–702.

Tata,F. *et al.* (1991) Cloning the mouse homolog of the human cystic fibrosis transmembrane conductance regulator gene. *Genomics*, 10, 301–307.

Thomaidis,T.S. and Arey,J.B. (1963) The intestinal lesions in cystic fibrosis of the pancreas. *J. Pediatr.*, 63, 444–453.

Valancius,V. and Smithies,O. (1991) Double-strand gap repair in a mammalian gene targeting reaction. *Mol. Cell. Biol.*, 11, 4389–4397.

Veeze,H.J., Halley,D.J., Bijman,J., de Jongste,J.C., de Jonge,H.R. and Sinaasappel,M. (1994) Detection of mild clinical symptoms in cystic fibrosis patients. Residual chloride secretion measured in rectal biopsies in relation to the genotype. *J. Clin. Invest.*, 93, 461–466.

Wright,E.M. (1993) The intestinal Na^+/glucose cotransporter. *Annu. Rev. Physiol.*, 55, 575–589.

Yang,Y., Janich,S., Cohn,J.A. and Wilson,J.M. (1993) The common variant of cystic fibrosis transmembrane conductance regulator is recognized by hsp70 and degraded in a pre-Golgi nonlysosomal compartment. *Proc. Natl Acad. Sci. USA*, 90, 9480–9484.

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A ΔF508 Mutation in Mouse Cystic Fibrosis Transmembrane Conductance Regulator Results in a Temperature-sensitive Processing Defect In Vivo

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Abstract

The most prevalent mutation (ΔF508) in cystic fibrosis patients inhibits maturation and transfer to the plasma membrane of the mutant cystic fibrosis transmembrane conductance regulator (CFTR). We have analyzed the properties of a ΔF508 CFTR mouse model, which we described recently. We show that the mRNA levels of mutant CFTR are normal in all tissues examined. Therefore the reduced mRNA levels reported in two similar models may be related to their intronic transcription units. Maturation of mutant CFTR was greatly reduced in freshly excised oviduct, compared with normal. Accumulation of mutant CFTR antigen in the apical region of jejunum crypt enterocytes was not observed, in contrast to normal mice. In cultured gallbladder epithelial cells from ΔF508 mice, CFTR chloride channel activity could be detected at only two percent of the normal frequency. However, in mutant cells that were grown at reduced temperature the channel frequency increased to over sixteen percent of the normal level at that temperature. The biophysical characteristics of the mutant channel were not significantly different from normal. In homozygous ΔF508 mice, we did not observe a significant effect of genetic background on the level of residual chloride channel activity, as determined by the size of the forskolin response in Ussing chamber experiments. Our data show that like its human homologue, mouse ΔF508-CFTR is a temperature sensitive processing mutant. The ΔF508 mouse is therefore a valid in vivo model of human ΔF508-CFTR. It may help us to elucidate the processing pathways of complex membrane proteins. Moreover, it may facilitate the discovery of new approaches towards therapy of cystic fibrosis. (*J. Clin. Invest.* 1996. 98:1304–1312.) Key words: cystic fibrosis • chloride channel • antibody • animal models • patch clamp

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Introduction

Cystic fibrosis is the most common lethal genetic disease in Caucasian population (1, 2). It is caused by mutations in gene encoding the cystic fibrosis transmembrane conducta regulator (CFTR)[1] (3). CFTR is a cAMP-regulated chlor channel, which is expressed in the apical membrane of m epithelia. The most common mutation in cystic fibrosis ((patients is a deletion of a phenylalanine at position : (ΔF508) (4). This mutation affects correct processing of protein to its mature, fully glycosylated form (5, 6). The ΔF5 form of CFTR is retained in the endoplasmic reticulum a degraded, and does not reach the apical plasma membrane 8). However, ΔF508-CFTR can function as a cAMP regulat chloride channel, both in the endoplasmic reticulum (9) a when expressed on the apical plasma membrane. The latter curs when ΔF508-CFTR expressing cells are cultured at l temperatures (10) and when Vaccinia expression vectors a used (11). Cells cultured at lower temperatures, Sf9 insect ce (12) and *Xenopus* oocytes (13), also express functional ΔF50 CFTR on the plasma membrane when transduced with ΔF508-CFTR expression vector. The clinical significance this observation is that it may lead to a new approach towar the therapy of CF based on relieving the processing defect. an attempt to facilitate research in this field we have recent reported the generation of a mouse model with the ΔF50 CFTR mutation using the "hit and run" mutagenesis proc dure (14). In this model the intron structure is not disturbed contrast to two similar models presented (15, 16). In our pre ous studies we demonstrated severely reduced chloride perm ability in several epithelial tissues, confirming a CF phenotyp However, in Ussing chamber experiments with intact gall bla der and intestinal tissue from mutant mice we observed a sma residual cAMP regulated chloride secretion, possibly due t the presence of some functional ΔF508-CFTR (14). O present study shows that the mouse ΔF508-CFTR is not pr cessed efficiently to the fully glycosylated form in vivo. Hov ever, the mutant protein is expressed as functional chlorid channels in the plasma membrane of cells cultured at reduce temperature. Furthermore, we could show that the electr physiological characteristics of the mouse ΔF508-CFTR chan nels were indistinguishable from normal.

1. *Abbreviations used in this paper:* CF, cystic fibrosis; CFTR, cystic fi brosis transmembrane conductance regulator.

Methods

Animals. All animal experiments were performed according to the guidelines issued by the Dutch government concerning animal care. Mice with the ΔF508 mutation were described by Van Doorninck et al. (14). Mice with a targeted disruption in the CFTR gene ($cftr^{m1cam}$ knockout mice, CF−/−) resulting in complete loss of function were obtained from Ratcliff et al. (17). All animals were bred under pathogen-free conditions in our transgenic unit. The genotype of each individual animal was tested by Southern blotting of tail DNA. The CF−/− animals in our facility display less severe runting and lower mortality due to intestinal obstruction as reported for the Cambridge colony, which may be due to breeding conditions. All physiological parameters measured, nasal potential differences (PD), intestinal and gall bladder PD measurements (14, 17*a*), confirmed the CF−/− phenotype of these animals. The animals used for the experiments reported here were 5–6 wk old, without obvious signs of disease or discomfort, with an average weight of 23±3 grams. All experiments involving ΔF508 mice were performed with the strain in 129/FVB genetic background (14) using littermates as control in parallel experiments. The experiments presented in Fig. 7 were performed with three different ΔF508 CFTR mouse colonies as described in text.

Quantitative PCR analysis. Two mouse specific oligonucleotides MCF2 (5'-AATGACCACAGGCATAATC-3'), and MCF3 (5'-CAACACTCTTATATCTGTAC-3') were synthesized. The sequences are localized in exons 8 and 10, respectively, and generate a mouse CFTR mRNA-specific 403-bp fragment in RT-PCR analysis. Total RNA was isolated by extraction of various mouse tissues or cultured cells in guanidine isothiocyanate and centrifugation through a 5.2 mol/liter CsCl step-gradient. The RNA was reversed transcribed to cDNA by adding 10 U Avian myoblastoma virus reverse transcriptase (AMV-RT) in 20 μl cDNA buffer (7 μg heat denatured RNA, 0.8 μmol/liter antisense oligonucleotide primer MCF3), 10^{-3} mol/liter dNTP's, 0.05 mol/liter KCl, 0.02 mol/liter Tris-HCl pH 8.4, 0.0025 mol/liter $MgCl_2$, 0.1 grams/liter bovine serum albumin and 20 U of RNasin for 1 h at 37°C. The 50 μl PCR assay contained $0.3.10^{-6}$ mol/liter sense and antisense oligonucleotide primer (MCF2 and MCF3, $0.2.10^{-3}$ mol/liter dNTP's, 4 μl cDNA mix, 0.05 mol/liter KCl, 0.02 mol/liter Tris-HCl ph 8.4, $2.5.10^{-3}$ mol/liter $MgCl_2$, and 0.1 grams/liter bovine serum albumin. After denaturing at 94°C, 5 U Taq polymerase was added at 72°C. Standard cycling program: 30 cycles of denaturation for 2 min at 93°C, primer annealing for 2 min at 47°C, and elongation for 4 min at 72°C. Aldolase A oligonucleotide primers, which amplify a 442-bp cDNA fragment, were as in Bremer et al. (18). To check the quality of the RNA and the PCR procedure, the amplification of aldolase A sequences was done in parallel, under the same conditions as described above for CFTR. PCR products were separated on a 1.5% agarose gel. For quantitative analysis of the PCR reaction, equal amounts of total RNA isolated from different tissues were subjected to an RT-PCR protocol in parallel incubations. Aliquots were withdrawn at regular intervals from the PCR and subjected to Southern blotting. Radioactivity was measured with a Phosphorimager (Molecular Dynamics, Inc., Sunnyvale, CA).

Western blot analysis of oviduct. Female mice of different phenotypes were anesthetized with ether. The lower abdomen was opened and the oviducts were dissected. After rapid excision the oviducts from one mouse were pooled and immediately solubilized by vortexing followed by brief sonication in 30 μl of modified Laemmli sample buffer (Tris-HCl 0.06 mol/liter, 2% (wt/vol) SDS, 15% (wt/vol) Glycerol, 2% (vol/vol) β-mercaptoethanol, 0.05 grams/liter leupeptin, 0.05 grams/liter soybean trypsin inhibitor, 0.03 grams/liter phosphoramidon, 10^{-4} mol/liter Pefabloc (Boehringer Mannheim, Mannheim, Germany), 10^{-3} mol/liter benzamidine, 0.1% (wt/vol) bromophenolblue, pH 6.8). The samples were incubated for ten minutes at 37°C and centrifuged (2 min, 8,000 g). Samples of the supernatants (10 μl, containing about 20 μg protein) were separated on 6% poly-acrylamide slabgels using a Bio-Rad Miniprotean apparatus (Bio-Rad Laboratories, Herfordshire, UK). Proteins were subsequently electroblotted onto nitrocellulose paper (0.1 μm pore size, Schleicher and Schuell, Inc., Keene, NH) in 0.025 mol/liter Tris, 0.192 mol/liter glycine, 20% (vol/vol) methanol. The blots were then incubated overnight at 4°C with 0.02 mol/liter Tris-HCl, 0.5 mol/liter NaCl, 0.05% (wt/vol) Tween20 (TTBS), followed by an overnight incubation at 4°C with a 1:3000 dilution of affinity purified antibody R3195 in TTBS. The blots were washed three times in TTBS, incubated with peroxidase conjugated anti-rabbit IgG (Tago Inc., Burlingame, CA; 1:3000 in TTBS for 2 h), and washed four times with TTBS. Peroxidase activity was detected with bioluminescence reagents (Amersham, Braunschweig, Germany) on x-ray film. The rabbit polyclonal antibody R3195 was developed against the 13-amino acid COOH-terminal peptide sequence of rodent CFTR, conjugated to bovine thyroglobulin. The antibody was affinity-purified on a peptide-epoxide activated Sepharose column, eluted with 4.9 mol/liter $MgCl_2$, dialyzed and concentrated.

Immunocytochemistry. Non-fasted mice were killed after inhalation anaesthesia. Intestinal tissues were quickly removed and frozen in OCT embedding medium (Miles Lab., Elkhardt, In.) using liquid nitrogen cooled 2-methylbutane. Cryosections (5 μm) were fixed with 3% (wt/vol) paraformaldehyde (10 min) and methanol (20 min), washed with phosphate-buffered saline supplemented with 1% bovine serum albumin and protease inhibitors before incubation with the anti CFTR antibody R3195 (1/500). Antibody labelling was detected with FITC conjugated anti-rabbit F(ab)2 fragments (Boehringer, Mannheim, Germany).

Gall bladder epithelial cell culture. Gall bladders were removed from the animal. After the bile was washed out with RPMI-1640 medium supplemented with 100 U/ml penicillin, 0.1 grams/liter streptomycin, and 0.02 mol/liter Hepes pH 7.2, the gallbladders were cut into small pieces and embedded in 1 mm collagen gels (Sigma Chemical Co., St. Louis. MO; Calf skin type I). The explant cultures were incubated at 37 °C in a humidified 10% CO_2/air mixture in Dulbecco's Modified Eagle's Medium (DME) supplemented with 100 U/ml penicillin, 0.1 grams/liter streptomycin, 0.002 mol/liter glutamine, and 10% fetal calf serum. Mouse gall bladder epithelial cells could be cultured for up to 3 wk, with medium changes every 2–3 d.

Patch clamp analysis. Patch clamp experiments, data sampling and analysis were performed as previously described (19). Patch pipettes, borosilicate glass (Clark GC150-TF) were pulled to a resistance of 3-8 MOhm. Excised patches were transferred to a solution exchange compartment (19). This allowed us to add various substrates (ATP, protein kinase A) and to change the buffer composition to a I^-, F^-, $NO3^-$, Br^-, or gluconate buffer. Pipette (external) and bath (internal) solutions contained (mol/liter) 0.14 NaCl, 5.10^{-3} KCl, $1.2.10^{-3}$ $MgCl_2$, 0.15. 10^{-3} $CaCl_2$, 10^{-3} EGTA, and 5.10^{-3} Hepes. Final Ca^{2+} concentration was 10^{-4} M, pH 7.4. Low chloride buffer was identical except that it contained 0.14 mol/liter Na-gluconate instead of NaCl, except for the low chloride pipet buffer used to analyzes normal mouse CFTR. This buffer contained (mol/liter) 0.140 N-methyl-d-glucamine (NMDG), $5.10^{-3}CaCl_2$, $2.10^{-3}MgCl_2$, 10^{-2} Hepes and 0.1 D-Aspartic acid adjusted to pH 7.2, final chloride concentration 49.10^{-3}. High (0.427 mol/liter) chloride buffers contained 0.420 mol/liter NaCl. In other buffers 0.140 mol/liter NaCl was replaced with either NaI, NaBr, $NaNO_3$, NaF, or Na-Gluconate, all 0.140 mol/liter. All experiments were performed at 37°C. A List LM-EPC 7 (Darmstadt, Germany) amplifier was used for current amplification and voltage clamping. Data were digitalized (Sony PCM-F1) and stored on videotape. For analysis, data were filtered at 50 Hz, sample frequency 100 Hz, and transferred to a personal computer (Tulip 386sx). Pipette potential refers to the voltage applied to the pipette interior with respect to the (grounded) bath. Positive (upward) currents represent negative charge flowing out of the pipette. $V_{I=0}$ was corrected for the liquid junction potential. Experiments presented in Fig. 6 were performed blinded with respect to the genotype of the cells under study.

Ussing chamber experiments. Mouse gallbladder and caecum were mounted in an Ussing chamber, basal electric potential and re-

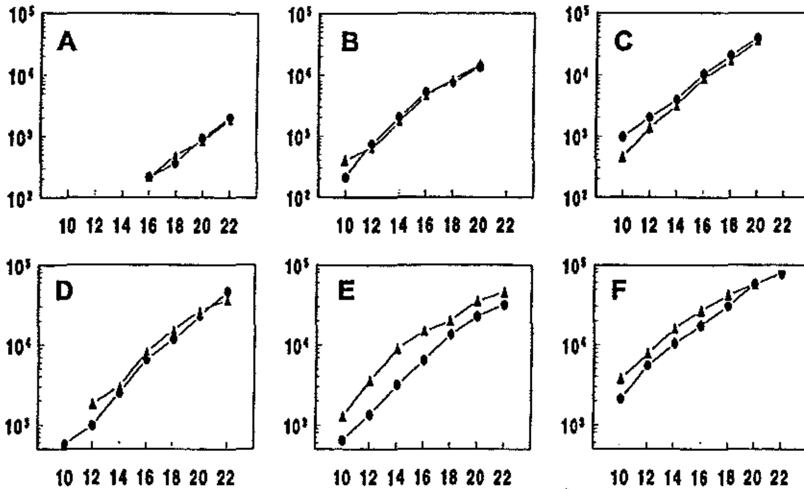

Figure 1. Quantitative analysis of CFTR mRNA expression. Total RNA from normal and mutant mice was isolated from different tissues and subjected to RT-PCR analysis in parallel experiments as described. Specific primers in mouse *Cftr* exons 8 and 10 yielded a 403-bp fragment, which contained an SspI site only when the product was initiated on a ΔF508-CFTR mRNA (14). Samples collected at different cycle intervals were subjected to Southern blotting using the labeled 403-bp fragment as a probe. Data shown here represent the amount of radioactivity per band in arbitrary units. Data from different panels are not corrected for differences in blotting efficiency. PCR products from heterozygous mice (ΔF/+) were digested with SspI before Southern analysis (*A–C*). Data indicate the amount of PCR product from the normal allele (403 bp, ●-●) and from the ΔF508 allele (359 bp, ▲-▲) when RNA was isolated from salivary gland (*A*), caecum (*B*), and jejunum (*C*) of heterozygous mice. PCR products from homozygous normal (●-●) and homozygous ΔF508 mice (▲-▲) were compared in parallel, using RNA isolated from salivary gland (*D*), caecum (*E*), and jejunum (*F*). The data show that there is no significant difference in the levels of mutant and normal CFTR mRNA. RNA from CFTR knockout mice (17) did not produce a PCR product in a parallel experiment whereas the aldolase primers produced comparable amounts of PCR product in all samples tested (not shown).

sponses to forskolin (10^{-5} mol/liter) were determined as described by van Doorninck et al. (14).

Statistical analysis. Significance analysis was tested with linear regression analysis for the current-voltage data presented in Fig. 5, and with a Student's *t* test (unequal variance) for the channel frequency determination (Fig. 6)

Results

ΔF508 mice have normal CFTR mRNA levels. The ΔF508 mouse model from our laboratory was generated by the "hit and run" procedure. With this method the mutation is introduced in an otherwise normal mouse *cftr* gene. Therefore, CFTR mRNA transcription and processing rates are expected to be normal. This is particularly important as low ΔF508-CFTR mRNA levels were reported in tissues of two other ΔF508-CFTR mouse models which were made while introducing a selectable marker in the intron structure (15,16). To investigate this we have performed quantitative RT-PCR analysis with RNA isolated from heterozygous and homozygous ΔF508 mice. As controls we have used normal littermates and homozygous *cftr* knockout mice (17). The PCR product from the mutant allele contains an SspI restriction site which is not present in the wild type allele (14). This allowed us to accurately determine the ratio of normal and mutant CFTR mRNA in a single PCR experiment with RNA from a heterozygous animal. With this approach we can rule out variations introduced by differences in reaction conditions and quality of RNA preparations. Our data show that the steady state levels of normal and ΔF508-CFTR mRNA are virtually identical in different regions of the intestine and in salivary gland (Fig. 1, *A–C*). Therefore, the mutation that we have introduced does not significantly affect mRNA synthesis processing or stability in epithelial tissues. In experiments with RNA isolated from homozygous animals ΔF508-CFTR, mRNA levels did not differ significantly from normal values (Fig. 1, *D–F*). This confirms that also in homozygous deficient animals expression of the gene in these tissues is not reduced.

Abnormal processing of ΔF508-CFTR in mouse oviduct. Western blot analysis (N = 3) of mouse CFTR from normal oviduct (Fig. 2, +/+) showed the characteristic pattern as observed for human CFTR, a core-glycosylated form (band *B*)

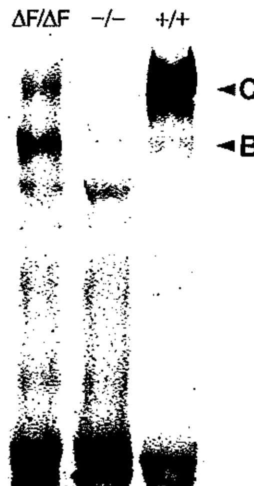

Figure 2. CFTR in mouse oviduct. Oviduct from normal (+/+), homozygous ΔF508 (ΔF/ΔF), and CFTR deficient (−/−) mice were subjected to Western blot analysis as described in the methods section. *C* indicates the fully glycosylated form of mouse CFTR antigen, *B* indicates the core-glycosylated precursor. The data show that ΔF508-CFTR is not glycosylated to the same extent as the normal form.

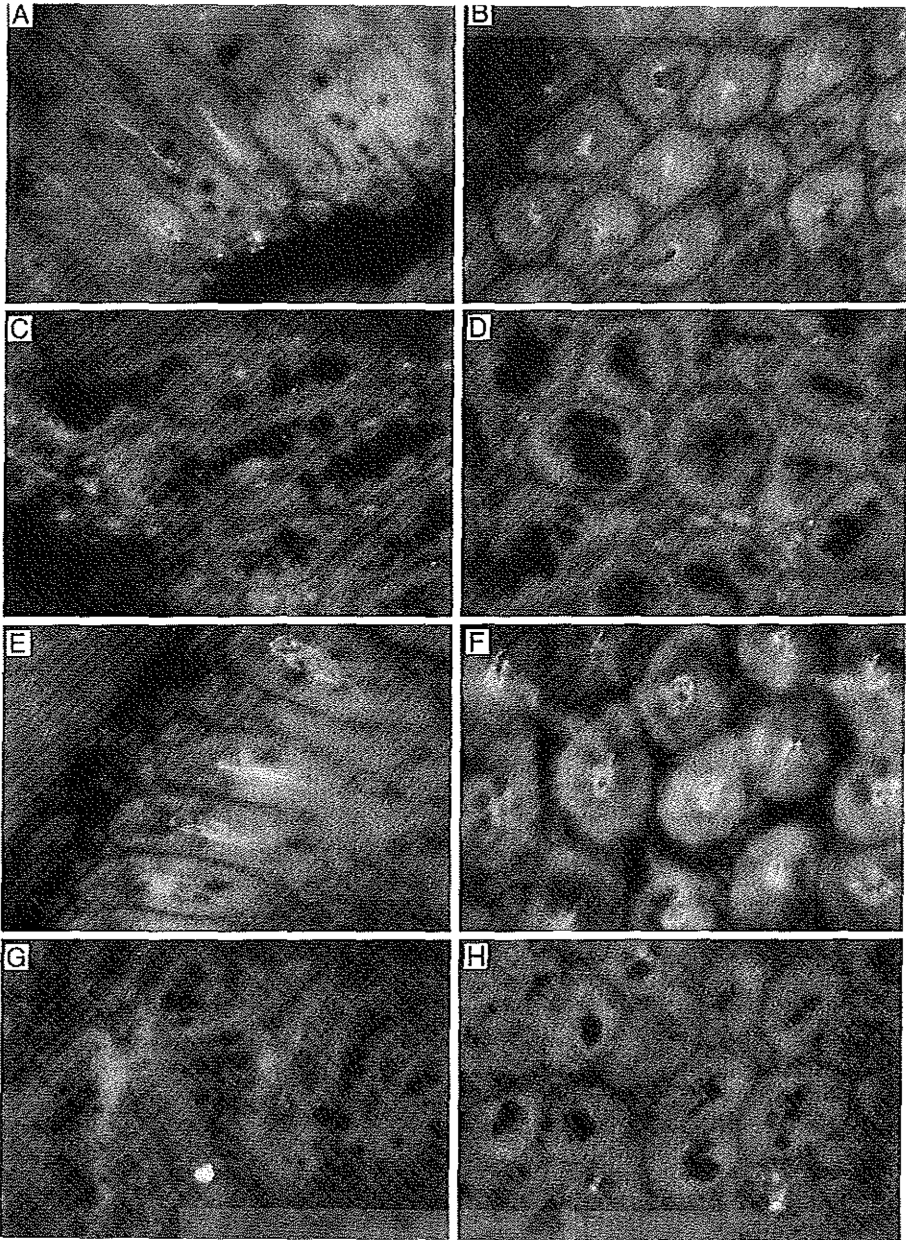

Figure 3. CFTR in mouse jejunum. Immunocytochemical detection of CFTR antigen in jejunal crypts from normal (*A* and *B*, +/+ $cftr^{m1cam}$ colony; *E* and *F*, +/+ ΔF508cftr colony), CFTR deficient (−/− $cftr^{m1cam}$) mice (*C* and *D*), and homozygous ΔF508 mice (*G* and *H*), and was performed as described in the methods section, using a polyclonal antibody against the murine CFTR carboxy terminal. Normal crypts show intense staining of the apical region of the epithelial cells. This is not observed with crypts from CFTR −/− mice or in ΔF/ΔF mice.

and forms containing complex N-linked oligosaccharides (band C) (3). In oviduct from a mouse with a complete loss of function mutation (Fig. 2, −/−) (17) these bands were not observed. Oviduct from a homozygous ΔF508 mouse (Fig. 2, ΔF/ΔF) produced a prominent band B and some band C antigen. The ratio of B over C is increased over 50-fold compared with the normal ratio as estimated by scanning the original radiograph. This strongly suggests that processing and subsequent glycosylation of mouse ΔF508 CFTR is severely affected in this tissue, as described previously for the human form of ΔF508 CFTR (5, 6). Gallbladder, pancreas and intestine did not give reproducible data with this technique.

Mouse ΔF508-CFTR is not efficiently transported to the apical membrane. Immunocytochemical detection of CFTR antigen in normal jejunal crypt shows intense staining of the apical region of all crypts (N = 7, Fig. 3 *A*, *B* and *E*, *F*). This is in accordance with immunocytochemical localization studies published previously for human intestine (20). In jejunal crypts from mice with a loss of function mutation (17), no CFTR-specific apical staining is observed (N = 6, Fig. 3, *C* and *D*). This confirms the specificity of the staining. Jejunum crypts of homozygous ΔF508 mice generally showed no clear apical CFTR antigen (N = 6, Fig. 3, *G* and *H*). In some preparations (three out of six) a diffuse cytoplasmic stain was observed in the epithelial crypt cells. The immunocytochemical method used here is characterized by an inherently nonlinear relationship between signal intensity and local antigen density. Moreover the absolute level of staining intensity varies per experiment. Therefore we can neither quantitate the level of correct processing nor the level of antigen expression in homozygous ΔF508 mice with any accuracy. The data presented show that despite the normal CFTR mRNA levels in ΔF/ΔF mice (Fig. 1) the amount of apical CFTR antigen in the jejunum crypt is greatly reduced. This is in agreement with our functional studies in intestinal tissue of mouse (14) and human (21) ΔF508-CFTR mutants. Moreover, it is in agreement with immunocytochemical studies in airway cells (22) and in cell culture (23). Other tissues that we have tested for CFTR specific staining with this method (gallbladder, cystic duct, pancreas) did not show consistent specific staining above background signals observed in tissues from knockout mice.

Patch clamp analysis of mouse ΔF508-CFTR. CFTR mRNA is expressed in mouse gallbladder epithelial cells, both in situ and in primary culture (17*a*). We have determined the number and properties of normal and ΔF508-CFTR chloride channels by patch clamp analysis of mouse gallbladder epithelial cells in primary culture. In excised membrane patches from unstimulated normal (+/+ and +/ΔF) cells, a linear 5.1±0.1 pS (SE) could be activated by the addition of protein kinase A in the presence of ATP (Fig. 4 *A*). In cell attached membrane

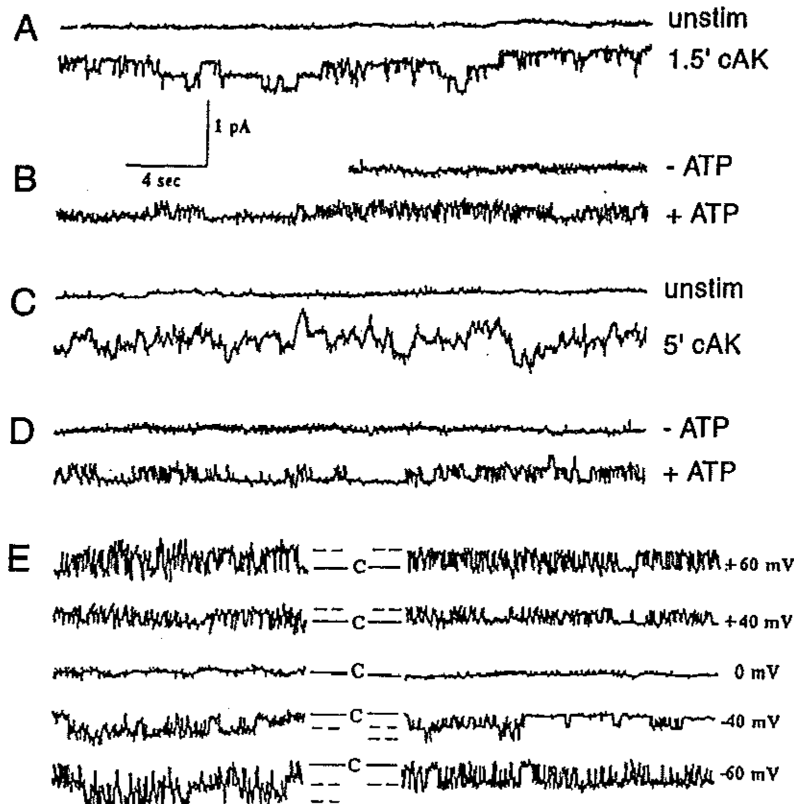

Figure 4. Characterization of normal and ΔF508 CFTR chloride channel activity. Current tracings of CFTR activity in excised patches from normal (*A* and *B*) and ΔF508 (*C* and *D*) mouse gallbladder epithelial cells cultured at 27°C. Unstimulated excised, inside-out patches from cultured gallbladder epithelial cells generally showed no channel activity. Upon a short incubation with the catalytic subunit of protein kinase A (cAK, 2 mU/ml) in the presence of 2.10^{-3} mol/liter ATP, a low conductance channel was observed in both normal and ΔF508 cells (*A* resp. *C*). Active patches of forskolin stimulated cells became silent upon excision (*B* and *D*, −ATP), but could be reactivated by the addition of 2.10^{-3} mol/liter ATP alone both in normal (*B*) and ΔF508 CFTR (*D*, +ATP). (*E*) Current tracings at different holding potentials from normal (*left tracings*) and ΔF508 CFTR (*right tracings*). Holding potential was +40 mV scale bar as indicated, except for *C* where the holding potential was + 60 mV, scale bar 4 s, 1.5 pA.

patches of forskolin-stimulated normal cells channels were observed at an average of 27.6±16.5 (SEM) per patch (Fig. 6 *left*). Excision of the membrane inactivated the channel but it could be reopened by addition of ATP (Fig. 4 *B*). Considering the resemblance with human CFTR (3) we conclude that these linear chloride channels represent normal mouse CFTR. The only difference observed between mouse and human CFTR is the lower conductance of the channel (human CFTR 8.0±0.6 pS) (3). In homozygous mutant cells cultured at 37°C the number of functional CFTR channels per patch is about 1% of normal (+/+ and +/ΔF, $P < 0.02$) (Fig. 6), but not zero. These data confirm our conclusion that the expression of CFTR activity on the plasma membrane of homozygous mutant cells is greatly reduced but not completely absent (14). In cells expressing human ΔF508-CFTR an increase of apical channel activity has been observed when cells were grown at reduced temperature, which is associated with a conversion of core-to-complex chain glycosylation and increased apical localization of ΔF508-CFTR (10). In normal (+/+) and heterozygote (ΔF/+) mouse gall bladder cells grown at 27°C for 3–7 d we observed a twofold decrease in CFTR channel frequency compared to 37°C (Fig. 6). In contrast, ΔF/ΔF cells cultured at 27°C show a considerable increase in ΔF508-CFTR channel frequency compared to cells cultured at 37°C ($P < 0.05$). The average number of channels per patch was 16% of the average observed in normal cells cultured at 27°C (Fig. 6). The high frequency at which the ΔF508-CFTR channel was observed in cells cultured at lower temperatures enabled us to study its properties in detail. ΔF508-CFTR could be observed in cell attached patches after addition of forskolin. The channel became silent upon excision but could be reactivated by the addition of ATP (Fig. 4 *D*). Excised membrane patches from unstimulated ΔF/ΔF cells were silent, but CFTR-like channel activity was observed by the addition of protein kinase A plus ATP (Fig. 4 *C*). These data show that mouse ΔF508-CFTR has a normal protein kinase and ATP regulation. Mouse ΔF508-CFTR is a linear 4.9±0.1 pS chloride channel, similar to normal mouse CFTR, 5.1±0.1 (SE) (Fig. 5, *A–B*). Lowering or increasing the chloride concentration in the bath results in a shift in the reversal potential ($V_{I=0}$), indicative for anion selectivity (Fig. 5, *A–B*). The relative cation to anion permeability, P_{cat}/P_{Cl} = 0.14 and 0.16 for normal and ΔF508-CFTR respectively (calculated from the least permeant anion, i.e., F^-). When chloride was substituted for other monovalent anions the relative permeability was determined as $NO_3^- > Br^- > Cl^- >> \text{gluconate} > F^-$ for both normal (Fig. 5 *C*) and ΔF508-CFTR (Fig. 5 *D*). Iodine had a high affinity but low permeability for both normal and ΔF508-CFTR (Fig. 5, *C* and *D*) which is a characteristic for CFTR (3). The open probability (Po) of ΔF508-CFTR (0.33±0.04) is comparable with normal mouse CFTR (0.35±0.04) (n = 5, values±SE, excised patches from forskolin activated cells in the presence of ATP). The Po of both normal and mutant CFTR was voltage independent. Channel "flickering," i.e., the number of closing events during a period of activation, also did not differ significantly between ΔF/ΔF (3.99±0.70 s^{-1}, n = 12) and normal (+/+) cells (3.23±0.55 s^{-1}, n = 7 patches, ±SE). We conclude that, similar to human ΔF508-CFTR, trafficking of the mouse ΔF508-CFTR to the plasma membrane is greatly increased at reduced temperature. Moreover, the biophysical properties of the mouse ΔF508-CFTR channel do not differ significantly from the wild type form.

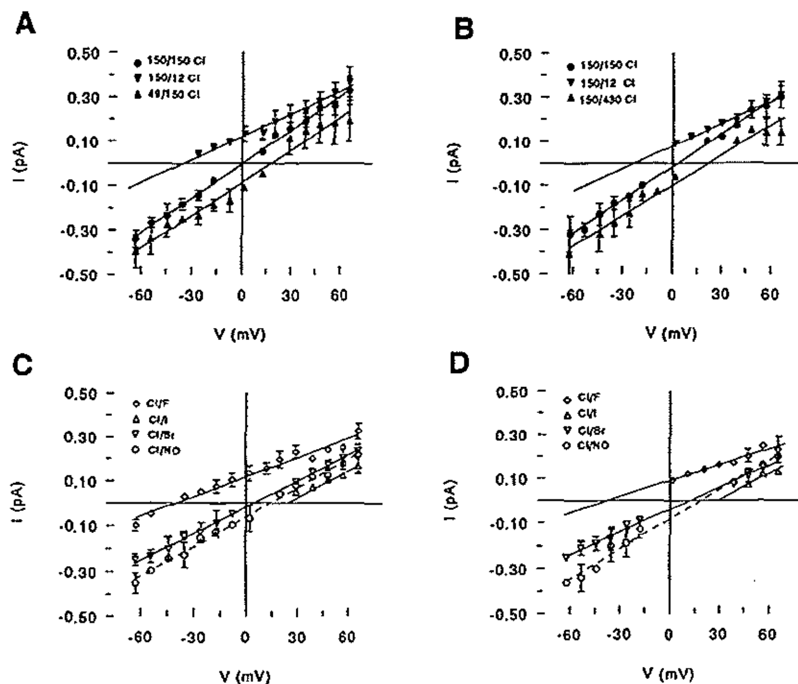

Figure 5. Current-voltage characteristics and relative anion permeability of normal and ΔF508 mouse CFTR. Current-voltage relationships of normal (*A* and *C*) and ΔF508-CFTR (*B* and *D*). At equal pipet and bath chloride concentrations (●–●, 0.150 mol/liter) both normal and ΔF508-CFTR show a linear conductance of 5.1±0.1 resp. 4.9±0.1 pS. A shift in the reversal potential ($V_{I=0}$) expected for a chloride channel was observed when bath chloride concentration was higher (▲–▲) or lower (▼–▼) than the pipet concentration (*A* and *B*). By replacing chloride in the bath with other anions as indicated (*C* and *D*), the relative ion selectivity was determined as $NO_3^- > Br^- > Cl^- >> \text{gluconate} > F^-$ for both normal (*C*) and ΔF508-CFTR (*D*). Iodine has a high affinity but low permeability in both normal and ΔF508-CFTR, as in human CFTR.

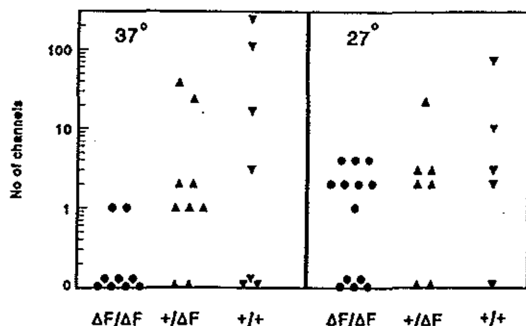

Figure 6. CFTR chloride channels in gall bladder plasma membrane. CFTR channel activity on plasma membrane of cultured gallbladder epithelial cells was recorded with patch clamp analysis as described. Cells were cultured either at 37°C (*left*) or at 27°C (*right*) for 3–7 d before analysis. Forskolin stimulated cells were recorded for two minutes in the on cell patch clamp configuration, followed by a 1–10-min recording in the excised patch configuration in the presence of 2 mM ATP. CFTR channel frequency was calculated from the equation $n = I_a/(I_1 * Po)$ where n is the calculated number of levels, I_a is the average current in the patch, I_1 is the current of one channel at holding potential, and Po is the open probability. When 4 or less levels of activity were observed in the patch the equation was not used, in these cases the number of channels correspond directly to the number of current levels observed. Patches which showed no CFTR activity were scored as zero. Cells were obtained from parallel cultures of littermates. (●) Homozygous mutant, ΔF/ΔF (cells from 3 mice at 37°C and 8 mice at 27°C); (▲) heterozygous mutant +/ΔF (3 and 4 mice); (▼) homozygous normal +/+ mice (2 and 1 mice).

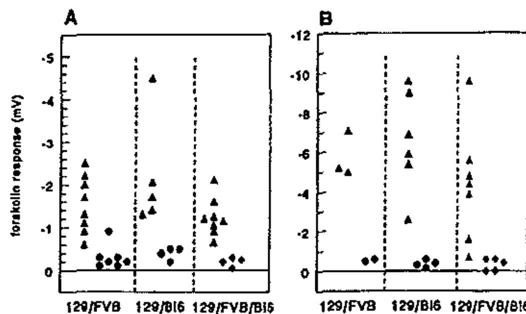

Figure 7. Forskolin responses in homozygous ΔF/ΔF mice gallbladder and caecum in mice strains with different genetic backgrounds. Gallbladder (*A*) and caecum (*B*) of mice from ΔF508 CFTR strains with different genetic backgrounds were mounted in the Ussing chamber and monitored for their electrical response to forskolin (ΔPD, mV). 129/FVB and 129/Bl6 were obtained by blastocyst injection and crossing into FVB and C57BL/6 type animals. 129/FVB/Bl6 was obtained by crossing ΔF/+ (129/FVB) with +/− cftrm^{1cam} (129/C57BL6). (▲) +/+, +/Δ and +/− mice; (●) ΔF/ΔF mice; (◆) ΔF/− mice.

Inefficient processing of mouse ΔF508 CFTR is observed in different genetic backgrounds. The most sensitive available measure of apical CFTR activity is probably the steady state response to forskolin in Ussing chamber experiments. Using this method, we have previously shown low but significant residual ΔF508-CFTR activity in different tissues of homozygous ΔF508 mice when compared to cftr^{m1cam} knockout mice that showed no activity (14). Next, we studied whether genetic background influences the level of ΔF508-CFTR processing. The 129*FVB and 129*C57Bl/6 ΔF strains were obtained by introduction of the original mutant clone (129/Ola) in FVB and C57BL/6 blastocysts respectively, ΔF/+ (129*FVB) mice were crossed with cftr^{m1cam} knockout mice (KO) that are 129*C57BL/6. The forskolin response of gallbladder and caecum of these different ΔF mouse strains was monitored in Ussing chamber experiments. For all strains the forskolin response was equally reduced in homozygous ΔF/ΔF and in ΔF/− mice (Fig. 7). All individual measurements from ΔF mutant mice are in the same range with only one exception observed (Fig. 7). The data show no evidence for dominant alleles in either the 129/Ola, FVB or C57BL/6 inbred genetic backgrounds that significantly increase or reduce the level of ΔF508-CFTR processing. However, one homozygous ΔF/ΔF mouse in a 129*FVB background did show a response to forskolin in the normal range using gallbladder (Fig. 7 *A*) and ileum (not shown). Therefore, more complex relationships involving several genetic loci can not yet be excluded and require further study.

Discussion

CFTR mRNA expression is normal in mutant mice. Using the "hit and run" procedure, we have generated a ΔF508-CFTR mouse model without modifying the intron structure of the CFTR gene (14). Our quantitative PCR data show that in heterozygotes the expression levels of normal and mutant alleles are comparable in intestine and salivary gland. Also in homozygote normal and mutant mice the expression levels are not significantly different. In two other ΔF508-CFTR mouse models created by homologous recombination, low CFTR mRNA levels were reported in salivary glands and intestine (15, 16). In these models an expression cassette encoding a selectable marker was introduced into the flanking intron, in addition to the ΔF508 mutation in exon 10. Comparison with our data suggests that the presence of this transcription unit affects the CFTR mRNA levels by interfering with RNA polymerase activity or RNA processing.

Maturation of mouse ΔF508-CFTR is abnormal. Immunochemical analysis of CFTR expression in intact mouse tissues is difficult with the available antisera. This can be attributed in part to relatively low expression levels as in gallbladder tissue (17*a*) and to proteolytic activity, especially in intestinal tissue (H.R. de Jonge, unpublished data). This limits the scope of our investigations to tissues that give consistent and reproducible data. We were able to make reproducible western blots of total oviducts from normal and mutant mice with a purified antibody raised against the carboxy terminus of rodent CFTR (Fig. 2). The data show that CFTR antigen is produced in both normal (+/+) and mutant (ΔF/ΔF) mice, but not in CFTR deficient (−/−) mice. However, the proportion of mature, fully glycosylated CFTR is severely reduced in homozygous mutant. We conclude that the ΔF508 mutation in the mouse CFTR protein results in a processing defect similar to that observed with human ΔF508-CFTR. The available evidence suggests that transfer to the Golgi system and subsequent full glycosylation of the human mutant protein does not occur, because proper chaper-

onin-dependent folding is arrested in the endoplasmic reticulum (7, 8, 10). With the same antibody as used for western blot analysis, we could show specific staining of the apical region of normal mouse jejunum crypt epithelial cells (Fig. 3, *A, B, E,* and *F*). Specificity of the staining was verified with serum preabsorbed with the antigenic peptide (not shown) and jejunum of CFTR-deficient mice (Fig. 3, *C* and *D*). This is in agreement with localization studies in human intestine (20) and rat (24). In jejunum crypts of mice homozygous for the ΔF508 mutation we did not observe a preferential staining of the apical region. Most crypts seemed devoid of specific staining. However, with this method we cannot rule out that cytoplasmic CFTR is present (note that also in control cells no specific cytoplasmic staining above background can be seen Fig. 3). In several preparations some crypts did stain more intensely than background but the signal was not concentrated to the apical region as in normal crypts. These observations are in agreement with a processing defect of mouse ΔF508-CFTR that prevents translocation of the protein to the apical region.

Temperature dependent apical targeting of ΔF508-CFTR. Airway cells of ΔF508 mutant mice in primary culture have increased cAMP-induced chloride conductance when cultured at reduced temperature (16). We were unable to detect CFTR chloride channel activity in primary airway cells using patch clamp single channel analysis (P.J. French, unpublished data). In contrast, cultured mouse gall bladder cells provide a convenient system for the study of CFTR with this method. This allowed us to show that the actual frequency of active mutant CFTR molecules at the plasma membrane is increased by incubation of the gallbladder cells at low temperature. In homozygous mutant cells cultured at 37°C few CFTR channels were observed, i.e., 1% of the normal level at this temperature (Fig. 6). The expression of ΔF508-CFTR channels increased to 16% of normal levels when cells from homozygous mutants were grown at 27°C (Fig. 6). A similar behavior was observed with human ΔF508-CFTR (10).

Channel activity of ΔF508-CFTR. The channel conductance and anion selectivity of mouse ΔF508-CFTR is in our analysis indistinguishable from normal mouse CFTR (Fig. 4). In addition, the open probability (Po) and number of closing events during a burst of activity of ΔF508-CFTR are normal. Studies of human ΔF508-CFTR are inconsistent at this point as two groups reported normal conductance but reduced Po for human ΔF508-CFTR (10, 11) whereas others claimed a normal conductance and Po (25). Since the activity of the CFTR chloride channel depends on a number of parameters in particular the state of phosphorylation of the protein, the reported difference in relative Po may result from differences in experimental conditions.

Residual activity of ΔF508-CFTR. Both Ussing chamber experiments (14) (Fig. 7), and patch clamp analysis (Fig. 6) in our model indicated a low but significant level of apical activity of mouse ΔF508-CFTR at physiological temperature. This is confirmed by the western blot analysis (Fig. 2), which shows a low level of fully glycosylated CFTR. Interestingly, a minority of human ΔF508 CFTR homozygotes possess a small residual intestinal chloride permeability, which correlates with a relatively mild clinical status (21). Unpublished data showed that in some, but not all, cases this activity is insensitive to the channel blocker DIDS (H. Veeze, personal communications). This would suggest that in these cases the residual activity is due to ΔF508-CFTR. It is possible therefore, that a significant level of correct processing is also present in a subgroup of human ΔF508 CFTR homozygotes. Immunocytochemical analysis of airway cells from ΔF508 CFTR patients seem to confirm this (26). It will be of great interest to analyze the genetic and environmental factors which contribute to this phenomenon. Our current data indicate that the level of mutant CFTR activity, as determined by Ussing chamber experiments, is quite constant in the different genetic backgrounds tested (Fig. 7). The one exception to this rule we found sofar is intriguing, but extensive further studies are required to establish a genetic basis for this effect. The level of ΔF508-CFTR processing could differ in various tissues and small variations in apical activity levels could have profound effects on pathology. Our electrophysiological data, supported by immunochemical data, show that in all epithelial tissues studied sofar the ΔF508 mice have severely reduced apical activity. However, a careful analysis of ΔF508-CFTR processing kinetics may reveal subtle differences between cell types.

Applications of the ΔF508 mouse model. Our data show that the tissues of the ΔF508 mouse model provide a valid model for the processing defect of the human ΔF508-CFTR mutation. It gives us the opportunity to study several aspects of CFTR function in more detail in intact tissues. CFTR is active in intracellular compartments (9), possibly affecting the pH of vesicular compartments. This could explain why CFTR deficient cells produce mucins and surface glycoproteins with abnormal carbohydrate structures (27–31). These abnormalities may contribute to the formation of abnormal secretions and to the reduced clearance of airway pathogens characteristic of CF. CFTR is involved in regulated endocytosis and exocytosis in some cell types (32, 33). In addition, recent evidence suggests that CFTR either directly or indirectly interacts with other ion transport systems (34, 35). ΔF508-CFTR expressed in epithelial cells may be partially active in these processes. Therefore, we may expect to observe differences between ΔF508 mice and mice with complete loss of function mutations (knockout). It will be interesting to compare ΔF508 mice with knockout mice in an experiment in which the mice are challenged with lung pathogens (36). Furthermore, the ΔF508 mouse model will allow us to study conditions that may enhance the activity of ΔF508-CFTR at the apical epithelial membrane in vitro and in vivo. Processing of normal CFTR involves chaperonin mediated folding and core glycosylation at the endoplasmic reticulum. This is followed by full glycosylation in the Golgi system and transport to the apical membrane. It is a rather inefficient process since approximately 75% of normal CFTR is degraded in immature form by cellular proteases (7, 8). Processing of ΔF508-CFTR to a fully glycosylated apical form is severely reduced. The striking stimulating effect of reduced temperature on ΔF508-CFTR expression, both with human and mouse mutants, suggests that the processing block can be relieved within the range of physiological conditions. We can consider several approaches towards finding therapeutic substances. Compounds which interfere with ΔF508-CFTR-chaperonin interaction may improve processing by relaxing the quality control mechanism. An example of this may be glycerol, which has an effect comparable to low temperature (37). Inhibition of CFTR degradation may improve processing of mutant CFTR, although this approach has not been successful to date (7). CFTR at the plasma membrane is activated by ATP binding and multi-site phosphorylation of the large cytoplasmic regulatory domain (3, 38). Therefore, substances that stabilize the

open state of CFTR (39, 40) or inhibit protein phosphatases (41–43) are expected to improve the activity of both normal and ΔF508-CFTR. These studies may lead to new developments, not only in our understanding of membrane protein processing, but also towards effective therapies for CF.

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References

1. Boat, T., M.J. Welsh, and A. Beaudet. 1989. Cystic Fibrosis. *In* Metabolic Basis of Inherited Disease. C. Scriver, A. Beaudet, W. Sly, and D. Valle, editors. McGraw Hill, New York. 2649–2860.

2. Welsh, M.J. 1994. The path of discovery in understanding the biology of cystic fibrosis and approaches to therapy. *Am. J. Gastroenterol.* 89 (8):S97–S105.

3. Riordan, J.R. 1993. The cystic fibrosis transmembrane conductance regulator. *Ann. Rev. Physiol.* 55:609–630.

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

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

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

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

8. Ward, C.L., S. Omura, and R.R. Kopito. 1995. Degradation of CFTR by the ubiquitin-proteasome pathway. *Cell.* 83:121–127.

9. Pasyk, A.E., and J.K. Foskett. 1995. Mutant (ΔF508) CFTR Cl channel is functional when retained in endoplasmic reticulum of mammalian cells. *J. Biol. Chem.* 270:12347–12350.

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

11. Dalemans, W., P. Barbry, G. Champigny, S. Jallat, K. Dott, D. Dreyer, R.G. Crystal, A. Pavirani, J.P. Lecocq, and M. Lazdunski. 1991. Altered chloride ion channel kinetics associated with the ΔF508 Cystic Fibrosis mutation. *Nature (Lond.).* 354:526–528.

12. Li, C.H., M. Ramjeesingh, E. Reyes, T. Jensen, X.B. Chang, J.M. Rommens, and C.E. Bear. 1993. The cystic fibrosis mutation (ΔF508) does not influence the chloride channel activity of CFTR. *Nat. Genet.* 3:311–316.

13. Drumm, M.L., D.J. Wilkinson, L.S. Smit, R.T. Worrell, T.V. Strong, R.A. Frizzell, D.C. Dawson, and F.S. Collins. 1991. Chloride conductance expressed by ΔF508 and other mutant CFTRs in xenopus oocytes. *Science (Wash. DC).* 254 (5039):1797–1799.

14. Van Doorninck, J.H., P.J. French, E. Verbeek, R.P.C. Peters, H. Morreau, J. Bijman, and B.J. Scholte. 1995. A mouse model for the cystic fibrosis ΔF508 mutation. *EMBO (Eur. Mol. Biol. Organ.) J.* 14:4403–4411.

15. Zeiher, B.G., E. Eichwald, J. Zabner, J.J. Smith, A.P. Puga, P.B. McCray, M.R. Capecchi, M.J. Welsh, and K.R. Thomas. 1995. A mouse model for the ΔF508 allele of cystic fibrosis. *J. Clin. Invest.* 96:2051–2064.

16. Colledge, W.H., B.S. Abella, K.W. Southern, R. Ratcliff, C. Jiang, S.H. Cheng, L.J. MacVinish, J.R. Anderson, A.W. Cuthbert, and M.J. Evans. 1995. Generation and characterisation of a ΔF508 cystic fibrosis mouse model. *Nat. Genet.* 10:445–452.

17. Ratcliff, R., M.J. Evans, A.W. Cuthbert, L.J. Macvinish, D. Foster, J.R. Anderson, and W.H. Colledge. 1993. Production of a severe cystic fibrosis mutation in mice by gene targeting. *Nat. Genet.* 4 :35–41.

17*a*. Peters, R.P.C., J.H. van Doorninck, P.J. French, R. Ratcliff, M.J. Evans, W.H. Collins, J. Bijman, and B.J. Scholte. 1996. Cystic fibrosis transmembrane conductance regulator mediates the cAMP induced fluid secretion but not the inhibition of resorption in mouse gallbladder epithelium. *Hepatology.* In Press.

18. Bremer, S., T. Hoof, M. Wilke, R. Busche, B. Scholte, J.R. Riordan, G. Maass, and B. Tummler. 1992. Quantitative expression patterns of multidrug-resistance P-glycoprotein (MDR1) and differentially spliced cystic-fibrosis transmembrane-conductance regulator messenger RNA transcripts in human epithelia. *Eur. J. Biochem.* 206:137–149.

19. Kansen, M., R.B. Bajnath, J.A. Groot, H.R. De Jonge, B. Scholte, A.T Hoogeveen, and J. Bijman. 1993. Regulation of chloride channels in the human cc lon carcinoma cell line HT29.cl19A. *Pflugers Arch. Eur. J. Physiol.* 422:539–545

20. Crawford, I., P.C. Maloney, P.L. Zeitlin, W.B. Guggino, S.C. Hyde, H Turley, K.C. Gatter, A. Harris, and C.F. Higgins. 1991. Immunocytochemical lo calization of the Cystic Fibrosis gene product CFTR. *Proc. Natl. Acad. Sci. USA* 88:9262–9266.

21. Veeze, H.J., D.J. Halley, J. Bijman, J.C. De Jongste, H.R. De Jonge, an M. Sinaasappel. 1994. Detection of mild clinical symptoms in cystic fibrosis pa tients. Residual chloride secretion measured in rectal biopsies in relationship t the genotype. *J. Clin. Invest.* 93:461–466.

22. Puchelle, E., D. Gaillard, D. Ploton, J. Hinnrasky, C. Fuchey, M.C. Bout terin, J. Jacquot, D. Dreyer, A. Pavirani, and W. Dalemans. 1992. Differentia localization of the cystic fibrosis transmembrane conductance regulator in norma and cystic fibrosis airway epithelium. *Am. J. Resp. Cell Molec. Biol.* 7:485–491.

23. Dalemans, W., J. Hinnrasky, P. Slos, D. Dreyer, C. Fuchey, A. Pavirani and E. Puchelle. 1992. Immunocytochemical analysis reveals differences between the subcellular localization of normal and delta-Phe508 recombinant cystic fibrosis transmembrane conductance regulator. *Exp.Cell Res.* 201 :235–240.

24. Ameen, N.A., T. Ardito, M. Kashgarian, and C.R. Marino. 1995. A unique subset of rat and human intestinal villus cells express the cystic fibrosis transmembrane conductance regulator. *Gastroenterol.* 108:1016–23.

25. Li, C., M. Ramjeesigh, E. Reyes, T. Jensen, X. Chang, J.M. Rommens, and C.E. Bear. 1993. The cystic fibrosis mutation (ΔF508) does not influence the chloride channel activity of CFTR. *Nat. Genet.* 3:311–316.

26. Dupuit, F., N. Kalin, S. Brezillon, J. Hinnrasky, B. Tummler, and E. Puchelle. 1995. CFTR and differentiation markers expression in non-CF and ΔF508 homozygous CF nasal epithelium. *J. Clin. Invest.* 96:1601–1611.

27. Carnoy, C., R. Ramphal, A. Scharfman, J. Lo-Guidice, N. Houdret, A. Klein, C. Galabert, G. Lamblin, and P. Roussel. 1993. Altered carbohydrate composition of salivary mucins from patients with cystic fibrosis and the adhesion of Pseudomonas aeruginosa. *Am. J. Respir. Cell Mol. Biol.* 9:323–334.

28. Lo-Guidice, J., J. Wieruszeski, J. Lemoine, A. Verbert, P. Roussel, and G. Lamblin. 1994. Sialylation and sulfation of the carbohydrate chains in respiratory mucins from a patient with cystic fibrosis. *J. Biol. Chem.* 269:18794–18813.

29. Dosanjh, A., W. Lencer, D. Brown, D.A. Ausiello, and J.L. Stow. 1994. Heterologous expression of ΔF508 CFTR results in decreased sialylation of membrane glycoconjugates. *Am. J. Physiol.* 266:C360–366.

30. Cheng, P.W., T.F. Boat, K. Cranfill, J.R. Yankaskas, and R.C. Boucher. 1989. Increased sulfation of glycoconjugates by cultured nasal epithelial cells from patients with Cystic Fibrosis. *J. Clin. Invest.* 84:68–72.

31. Zhang, Y., B. Doranz, J.R. Yankaskas, and J.F. Engelhardt. 1995. Genotypic analysis of respiratory mucous sulfation defects in cystic fibrosis. *J. Clin. Invest.* 96:2997–3004.

32. Bradbury, N.A., T. Jilling, G. Berta, E.J. Sorscher, R.J. Bridges, and K.L. Kirk. 1992. Regulation of plasma membrane recycling by CFTR. *Science (Wash. DC).* 256:530–532.

33. Prince, L.S., R.B. Workman, and R.B. Marchase. 1994. Rapid endocytosis of the cystic fibrosis transmembrane conductance regulator chloride channel. *Proc. Natl. Acad. Sci. USA.* 91:5192–5196.

34. Schwiebert, E.M., M.E. Egan, T. Hwang, S.B. Fulmer, S.S. Allen, G.R. Cutting, and W.B. Guggino. 1995. CFTR regulates outwardly rectifying chloride channels through an autocrine mechanism involving ATP. *Cell.* 81:1063–1073.

35. Stutts, M.J., C. M. Canessa, J.C. Olsen, M. Hamrick, J.A. Cohn, B.C. Rossier, and R.C. Boucher. 1995. CFTR as a cAMP-dependent regulator of sodium channels. *Science (Wash. DC).* 269:847–850.

36. Mills, C.L., J.R. Dorin, D.J. Davidson, D.J. Porteus, E.W.F. W. Alton, R.L. Dormer, and M.A. Mcpherson. 1995. Decreased beta-adrenergic stimulation of glycoprotein secretion in CF mice submandibular glands: reversal by the methylxanthine, IBMX. *Biochem. Biophys. Res. Comm.* 215:674–681.

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

38. Gadsby, D.C., and A.C. Nairn. 1994. Regulation of CFTR channel gating. *Trends Biochem. Sci.* 19:513–518.

39. Hwang, T.C., G. Nagel, A.C. Nairn, and D.C. Gadsby. 1994. Regulation of the gating of CFTR Cl channels by phosphorylation and ATP analysis. *Proc. Natl. Acad. Sci. USA.* 91:4698–4702.

40. Gribkoff, V.K., G. Champigny, P. Barbry, S.I. Dworetzky, N.A. Meanwell, and M. Lazdunski. 1994. The substituted benzimidazole NS004 is an opener of the cystic fibrosis channel. *J. Biol. Chem.* 269:10983–10986.

41. Hanrahan, J.W., F. Becq, J.H. Tabcharani, T.J. Jensen, X.B. Cheng, and J.R. Riordan. 1995. Use of phosphatase inhibitors as therapeutic agents to activate mutant CFTR. *Ped. Pulmonol.* suppl 12:152–153.

42. De Jonge, H.R., A.G.M. Bot, P.J. French, J. Bijman, M. Sinaasappel, and H.J. Veeze. 1995. Synergistic activation of ΔF508-CFTR Cl-channels by cyclic AMP agonists and the tyrosine kinase inhibitor genistein. *Ped. Pulmonol.* suppl 12:186.

43. Becq, F., T.J. Jensen, X.B. Chang, A. Savoia, J.M. Rommens, L.C. Tsui, M. Buchwald, J.R. Riordan, and J.W. Hanrahan. 1994. Phosphatase inhibitors activate normal and defective CFTR chloride channels. *Proc. Natl. Acad. Sci. USA.* 91:9160–9164.

Curriculum Vitae

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Isotype-specific Activation of Cystic Fibrosis Transmembrane Conductance Regulator-Chloride Channels by cGMP-dependant protein kinase II. (1995) P.J. French, J. Bijman, M. Edixhoven, A.B. Vaandrager, B.J. Scholte, S.M. Lohmann, A.C. Nairn and H.R. de Jonge. *J. Biol. Chem.* **270**:26626-26631

A Mouse Model for the cystic fibrosis ΔF508 mutation. (1995) J.H. van Doorninck, P.J. French, E. Verbeek, H.P.C. Peters, H. Morreau, J. Bijman and B.J. Scholte. *EMBO J.* **14**:4403-4411

CFTR expression and mucin secretion in cultured gallbladder epithelium. (1996) H.P.C. Peters, P.J. French, J.H. van Doorninck, R. Ratcliff, M.J. Evans, W.H. Colledge, J. Bijman and B.J. Scholte. *Am. J. Physiol.* **271**:G1074-G1083

CFTR is required for active fluid secretion in biliary epithelium. (1996) H.P.C. Peters, J.H. van Doornink, P.J. French, R. Ratcliff, M.J. Evans, W.H. Collins, J. Bijman and B.J. Scholte. *Hepatology*

Fluid transport in the trachea of normal and CF mouse. (1997) P.J. French, H.P.C. Peters, J.H. van Doorninck, W.H. Colledge, R. Ratcliff, M.J. Evans, B.J. Scholte and J. Bijman. *manuscript in preparation*

A ΔF508 mutation in Mouse CFTR Results in a Temperature Sensitive Processing Defect *in vivo.* (1996) P.J. French, J.H. van Doorninck, H.P.C. Peters, E. Verbeek, N.A. Ameen, C.R. Marino, H.R. de Jonge, J. Bijman and B.J. Scholte *J. Clin. Invest.* **98**:1304-1312, 1996.

Genistein activates CFTR via a tyrosine kinase and phosphatase independent pathway. (1997) P.J. French, J. Bijman, A.G. Bot, W. Boomaars, B.J. Scholte and H.R. de Jonge. *submitted*

Antibodies directed to the Calmodulin-binding domain of B-50 (GAP-43) inhibit Ca^{2+}-induced dopamine release from permeated synaptosomes. (1996) J.J.H. Hens, G. Hoogland, P.J. French, F. Boomsma and P.N.E. de Graan. *Neurosc. Res. Comm.* **19**:9-17

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