

European Working Group on CFTR Expression

September 2001

CF Cystic Fibrosis
European
Network

Volume 2, Issue 3

Editorial

Dear members of the CFTR Working Group,

In this Newsletter you will find important information about the next Meeting of the Working Group (p. 11). In Focus 1 includes two main articles one (p. 2) by M. Mall and K. Kunzelmann defend their hypothesis on ENaC regulation by CFTR, and another G. Nagel explains the seeming increase of ENaC by enhanced driving force for Na⁺ (p.4). This controversial discussion is introduced by D. Sheppard and M. Hug (p.1).

In Focus 2 (p. 6) includes an article by K. Kirk summarizing

topics about detection and validation of CFTR interacting Proteins.

The web page of our Working Group has a new outfit and a new URL at the Gulbenkian Institute of Science, Oeiras, Portugal. Please read the short description on p. 8.

As always, we publish the reports of visits scientists exchange, sponsored by the Working Group (p. 12).

Looking forward to receiving your contributions,
Sebastian Beck, Lisboa (Portugal)

In Focus I

NaCl Transport across epithelia

The role of CFTR in vectorial sodium chloride (NaCl) transport across epithelia has been a puzzling issue for many years. Indeed, in recent years there has been a heated debate about the role of CFTR in airway epithelia and how loss of CFTR function leads to cystic fibrosis (CF).

The airways, the colon and the sweat ducts all express CFTR and the epithelial sodium (Na⁺) channel, (ENaC), in their apical membranes.

However, these epithelia utilise strikingly different mechanisms to transport NaCl (see Fig. 1). In the airways, Na⁺ is absorbed by passing through the cell (transcellularly), whereas chloride (Cl⁻) is transported between cells (paracellularly; Fig. 1A). When CFTR is stimulated by cAMP-dependent phosphorylation, the cell switches from absorption to secretion by activating CFTR channels and inhibiting ENaC channels. In contrast, in the

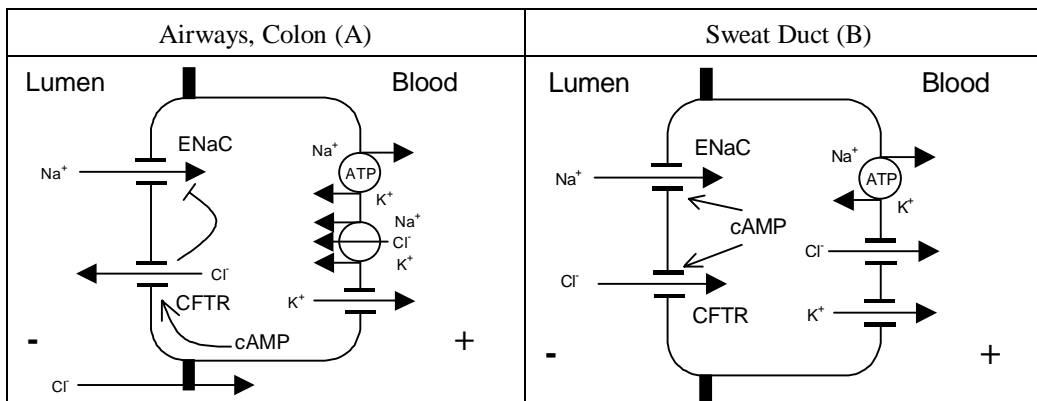


Fig. 1 Schematic models of transport mechanisms proposed for the airways, the colon, (A) and the sweat duct (B). Ion channels are denoted by short parallel lines with arrows indicating the direction of ion movement. The Na⁺ /K⁺ ATPase is denoted by a circle labelled ATP and the Na⁺ / K⁺ / 2Cl⁻ co-transporter is denoted by an open circle. The signs at the bottom refer to the polarity of the transepithelial voltage. For further explanation, see the text.

In this issue...

Editorial	1
In Focus I	1
Introduction	1
CFTR: A Regulator of ENaC	2
What is the Problem?	4
In Focus II	6
Detecting and Validating Protein Interactions with CFTR	
Proceedings	8
The Virtual Repository	9
Exchange of Scientists	9
Forum	
The Next Meeting	11
Contacts	11

sweat duct, both Na^+ and Cl^- are absorbed transcellularly following the activation of CFTR and ENaC channels in parallel by the cAMP signalling pathway (Fig. 1B).

In this issue of the Newsletter, two expert groups in the field of CFTR research outline their ideas about the role of CFTR in transepithelial NaCl transport. Marcus Mall and Karl Kunzelmann discuss their data obtained from *Xenopus* oocytes co-expressing recombinant CFTR and ENaC and human nasal and colonic biopsies that endogenously express CFTR and ENaC. They conclude that there is strong evidence for the inhibition of ENaC by stimulated CFTR and suggest that this inhibition might be mediated by an as yet unidentified factor.

Georg Nagel has reinvestigated the regulation of ENaC by CFTR in the *Xenopus* oocyte system. He finds no evidence of a functional interaction between CFTR and ENaC. Instead, he highlights how the activation of CFTR in the presence of a NaCl gradient enhances the driving force for Na^+ movement. This provides a convincing explanation of the co-activation of

Na^+ and Cl^- transport in the sweat duct by Quinton and co-workers (Reddy *et al.* (1999) *Nature* **402**, 301-304).

It is obvious that understanding of the role of CFTR in different epithelia will have an enormous impact on future therapeutic strategies to treat CF. Readers might interpret the juxtaposition of two articles with contradictory conclusions as an attempt by us to further muddy the water. However, we would argue that these articles serve two important purposes. First, they highlight the care that must be taken with the interpretation of experimental data, as well as with the details of the methods used to produce them. Second, they challenge our concepts about the mechanisms of NaCl transport by epithelia. We eagerly await new data about the roles of CFTR and ENaC in transepithelial ion transport, favourably performed on native tissues.

Martin J. Hug, Münster, Germany

David N. Sheppard, Bristol, UK

CFTR: A Regulator of the Amiloride-sensitive Epithelial Na^+ channel, ENaC

Cystic Fibrosis (CF) airway epithelia are characterized by a defect in cAMP dependent chloride (Cl^-) conductance and by an increased sodium (Na^+) conductance (1). Following the identification and cloning of the cystic fibrosis transmembrane conductance regulator (CFTR) and the amiloride-sensitive epithelial Na^+ channel (ENaC), it became possible to investigate the molecular basis of transepithelial ion transport in normal and CF airways. In various heterologous cells co-expressing CFTR and ENaC (including MDCK cells, 3T3 fibroblasts and *Xenopus* oocytes), it was demonstrated that cAMP-dependent activation of wild type (wt) but not mutant CFTR inhibits ENaC activity (3;5;6;9;13). These results suggested that CFTR acts as a cAMP dependent regulator of ENaC and that enhanced Na^+ absorption in CF airways may be explained by lack of down-regulation by mutant CFTR. Further studies on the interaction of CFTR and ENaC revealed that CFTR activation reduces the open probability (P_o) of ENaC (14) and that CFTR dependent down-regulation of ENaC depends on the presence of anions and requires that NBF1 of CFTR is structurally intact (2;12).

In contrast to these previous findings, a recent study by Nagel *et al.* reported lack of evidence for regulation of ENaC by CFTR in *Xenopus* oocytes and concluded that co-expression of CFTR and ENaC results in non-specific activation of ENaC, due to electrochemical coupling, rather than specific inhibition (10). As a possible explanation for their results, Nagel *et al.* demonstrated that functional assessment of ion channels in *Xenopus* oocytes by double electrode voltage clamp experiments can be compromised by expression of large CFTR and ENaC currents, when using a single high resistance bath electrode (10).

While the results may certainly apply to the experimental conditions chosen by Nagel *et al.*, they do not explain previous reports of specific CFTR-ENaC interactions. In our previous double electrode voltage clamp experiments using *Xenopus* oocytes co-expressing CFTR and ENaC, the resistance of the single bath electrode was much smaller ($\sim 700 \Omega$) and the expressed ENaC and CFTR conductances were much lower than

reported by Nagel *et al.* (10). Furthermore, as shown in Fig. 1, we observed that cAMP-dependent activation of CFTR in co-expressing *Xenopus* oocytes did not necessarily result in changes of the whole cell conductance, yet a CFTR Cl^- conductance was activated as demonstrated by the inhibition of whole cell conductance due to removal of extracellular Cl^- (5Cl^-). At the same time, the amiloride sensitive ENaC conductance was inhibited, as demonstrated by the attenuated amiloride response after CFTR activation. In these experiments, the increase in Cl^- conductance (G_{Cl^-}) and decrease of ENaC ($G_{\text{Amiloride}}$) were of similar magnitude and as a result a net increase in whole cell conductance (G_m) was not detected. Accordingly, CFTR-mediated inhibition of ENaC in *Xenopus* oocytes does not require large changes in the whole cell conductance. The series resistance of the bath electrode in our experiments did not exceed $\sim 4\%$ of the measured whole cell resistance and was therefore negligible (2;9;12). Furthermore, inhibition of ENaC by CFTR was also observed when two bath electrodes and a virtual-ground headstage were used (M.M. & K.K., unpublished data). Using this electrode configuration, the voltage drop across the series resistance is effectively zero. Under these experimental conditions inhibition of ENaC currents following CFTR activation cannot be explained by potential technical artifacts.

While the molecular mechanism of the interaction between CFTR and ENaC is still not well understood, the basic observation of CFTR-mediated down-regulation of ENaC has been confirmed in mammalian cells that express both proteins endogenously (7). In freshly excised human and murine nasal and colonic tissues mounted on small aperture Ussing chambers, CFTR activation inhibited amiloride-sensitive Na^+ conductance in normal tissues, but increased Na^+ conductance in CF tissues (4;8). According to these studies, the increased Na^+ absorption observed in CF airways and distal colon is likely to be caused by defective regulation of ENaC by mutant CFTR. Reciprocal regulation of CFTR and ENaC allows relatively leaky epithelia, e.g. airway and colonic epithelia, to switch from active transepithelial Na^+ absorption to Cl^- secre-

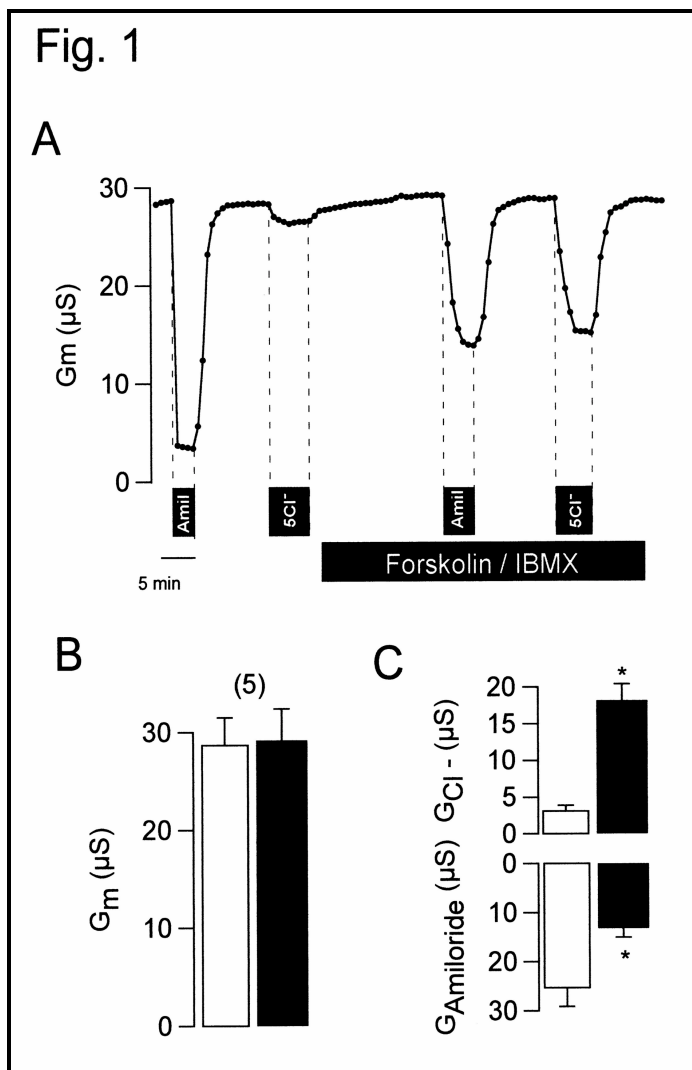


Fig. 1: Continuous recording (A) and summary data (B, C) of whole cell conductances from *Xenopus* oocytes co-expressing CFTR and ENaC obtained using the double electrode voltage-clamp technique. In the experiments shown, activation of CFTR did not result in changes of the net whole cell conductance. However, when the effects of amiloride (10 $\mu\text{mol/l}$) and low bath Cl⁻ concentration (5 mmol/l) were compared before (open bars) and after (filled bars) cAMP-dependent stimulation with IBMX (1 mmol/l) and forskolin (10 $\mu\text{mol/l}$) it is apparent that CFTR activation increases the Cl⁻ conductance (G_{Cl^-}), but decreases the ENaC conductance ($G_{\text{Amiloride}}$). Asterisks indicate statistical significance (paired t-test, $p < 0.05$). The number of experiments is indicated in parentheses.

tion in order to regulate luminal surface liquid. In CF tissues, increased Na⁺ conductance leads to volume depletion of the luminal surface liquid, resulting in markedly increased mucus viscosity that plays an important role in the pathogenesis of CF airway disease, meconium ileus of the newborn and intestinal obstruction observed in older CF patients.

In contrast, it has been demonstrated that CFTR activates ENaC mediated absorption in the sweat duct (11). The sweat duct epithelium is a tight epithelium that does not allow significant paracellular movement of Na⁺, Cl⁻ and H₂O. The main function of the sweat duct is to perform coordinated transcellular absorption of Na⁺ and Cl⁻, explaining why a defect of the CFTR Cl⁻ channel leads to an increase in both Na⁺ and Cl⁻ in the sweat of CF patients. Therefore, positive regulation of ENaC by CFTR is in accordance with the physiology

of the sweat duct and points to tissue specific interaction between ENaC and CFTR. These findings indicate that the interaction between the two proteins may not be direct, but rather mediated by a third, yet unidentified factor.

In conclusion, further studies are required to identify accessory elements that are responsible for tissue specific regulation of ENaC by CFTR. This work will contribute to our understanding of how CFTR regulates the activity of ENaC in different organ systems.

References

- Boucher, R. C., M. J. Stutts, M. R. Knowles, L. Cantley, and J. T. Gatz. Na⁺ transport in cystic fibrosis respiratory epithelia. Abnormal basal rate and response to adenylate cyclase activation. *J Clin Invest.* 78: 1245-1252, 1986.
- Briel, M., R. Greger, and K. Kunzelmann. Cl⁻ transport by CFTR contributes to the inhibition of epithelial Na⁺ channels in *Xenopus* oocytes coexpressing CFTR and ENaC. *J. Physiol. (Lond)* 508.3: 825-836, 1998.
- Chabot, H., M. F. Vives, A. Dagenais, C. Grygorczyk, Y. Berthiaume, and R. Grygorczyk. Downregulation of epithelial sodium channel (ENaC) by CFTR co-expressed in *Xenopus* oocytes is independent of Cl⁻ conductance. *J Membr. Biol* 169: 175-188, 1999.
- Grubb, B. R., R. N. Vick, and R. C. Boucher. Hyperabsorption of Na⁺ and raised Ca²⁺-mediated Cl⁻ secretion in nasal epithelia of CF mice. *Am J Physiol* 266: C1478-C1483, 1994.
- Ji, H. L., M. L. Chalfant, B. Jovov, J. P. Lockhart, S. B. Parker, C. M. Fuller, B. A. Stanton, and D. J. Benos. The cytosolic termini of the beta- and gamma-ENaC subunits are involved in the functional interactions between cystic fibrosis transmembrane conductance regulator and epithelial sodium channel. *J Biol Chem.* 275: 27947-27956, 2000.
- Jiang, Q., J. Li, R. Dubroff, Y. J. Ahn, J. K. Foskett, J. Engelhardt, and T. R. Kleyman. Epithelial sodium channels regulate cystic fibrosis transmembrane conductance regulator chloride channels in *Xenopus* oocytes. *J Biol Chem.* 275: 13266-13274, 2000.
- Letz, B. and C. Korbmayer. cAMP stimulates CFTR-like Cl⁻ channels and inhibits amiloride-sensitive Na⁺ channels in mouse CCD cells. *Am. J. Physiol.* 272: C657-C666, 1997.
- Mall, M., M. Bleich, R. Greger, R. Schreiber, and K. Kunzelmann. The amiloride-inhibitable Na⁺ conductance is reduced by CFTR in normal but not in CF airways. *J Clin Invest* 102: 15-21, 1998.
- Mall, M., A. Hipper, R. Greger, and K. Kunzelmann. Wild type but not F508 CFTR inhibits Na⁺ conductance when coexpressed in *Xenopus* oocytes. *FEBS Lett.* 381: 47-52, 1996.
- Nagel, G., T. Szellas, J. R. Riordan, T. Friedrich, and K. Hartung. Non-specific activation of the epithelial sodium channel by the CFTR chloride channel. *EMBO Rep.* 2: 249-254, 2001.
- Reddy, M. M., M. J. Light, and P. M. Quinton. Activation of the epithelial Na⁺ channel (ENaC) requires CFTR Cl⁻ channel function. *Nature* 402: 301-304, 1999.
- Schreiber, R., A. Hopf, M. Mall, R. Greger, and K. Kunzelmann. The first nucleotide binding domain of the cystic fibrosis transmembrane conductance regulator is important for inhibition of the epithelial Na⁺ channel. *Proc. Natl. Acad. Sci. USA* 96: 5310-5315, 1999.
- Stutts, M. J., C. M. Canessa, J. C. Olsen, M. Hamrick, J. A. Cohn, B. C. Rossier, and R. C. Boucher. CFTR as a cAMP-dependent regulator of sodium channels. *Science* Vol. 269: 847-850, 1995.
- Stutts, M. J., B. C. Rossier, and R. C. Boucher. Cystic fibrosis transmembrane conductance regulator inverts protein kinase A-mediated regulation of epithelial sodium channel kinetics. *J. Biol. Chem.* 272: 14037-14040, 1997.

Marcus Mall, Freiburg, Germany
Karl Kunzelmann, Brisbane, Australia

CFTR as a Regulator of the Epithelial sodium Channel - What's the Problem?

The search for the molecular defect causing cystic fibrosis (CF) has taken many routes. In 1968, Schulz and Frömter suggested that in **sweat gland ducts** from CF patients "chloride permeability is decreased and sodium leak permeability is increased". The observation that the activity of the amiloride-sensitive, epithelial sodium (Na^+) channel (ENaC) is increased in the **airways** of CF patients is also old: Knowles et al. (1981) made the important discovery that the electrical potential of CF nasal mucosa was twice as negative as that of controls. Knowles et al. (1981) postulated that the increased electrical potential of CF airways was due to increased Na^+ absorption from the airway surface liquid (ASL), resulting in increased fluid absorption and decreased volume of ASL. Quinton (1983) was the first to demonstrate convincingly that the main defect in **sweat ducts** from CF patients is a strongly reduced Cl^- conductance. The indirect conclusions of Quinton (1983) from simple potential measurements were subsequently confirmed by Bijman and Frömter (1986). Using transepithelial potential and resistance measurements, Bijman and Frömter (1986) demonstrated that the transepithelial resistance is high in CF sweat ducts and not changed by chloride (Cl^-) replacement, whereas in control ducts the transepithelial resistance is low and increases dramatically by Cl^- replacement.

Recently Quinton's group reported that the ENaC conductance in the **sweat duct** is enhanced by activation of cystic fibrosis transmembrane conductance regulator (CFTR), whereas in CF sweat ducts ENaC conductance is low and unresponsive to stimulation (Reddy et al., 1999). It is important to realize the "technicalities" of these demanding experiments: they were current-clamp experiments, i.e. current was clamped and potential measured. The conductance was inferred from current pulses. Because a large gradient of NaCl concentration was imposed across the epithelium, CFTR stimulation induced a voltage shift from $\sim +10$ mV to $\sim +90$ mV in the presence of amiloride and from ~ -10 mV to $\sim +45$ mV in its absence (see fig. 1 in Reddy et al., 1999). However, using a large NaCl concentration gradient, the conductance of the voltage-independent channels CFTR and ENaC is expected to be "voltage dependent" (from the Goldman-Hodgkin-Katz equation, see e.g. Hille 1992). The theoretical current-voltage relationship for ENaC, derived for a Na^+ gradient of 150 mM to 10 mM, is shown in fig. 1. From this relationship it follows that the observed increase of ENaC conductance by CFTR activation can qualitatively be explained by the CFTR-induced voltage shift. However, the **sweat duct** experiments suggest that inhibition of ENaC by stimulated CFTR is unlikely. Thus, these experiments leave open the question of how ENaC reacts to stimulation of CFTR at an unchanged ("clamped") membrane potential.

The cloning of ENaC from rat colon initiated a flurry of studies of CFTR-ENaC interactions at the molecular level. Stutts et al. (1995) first described the inhibition of ENaC conductance by activation of CFTR conductance in MDCK cells coexpressing ENaC and CFTR. Subsequently, similar findings were made by Mall et al. (1996) and then by more

authors (e.g. Briel et al., 1998, Chabot et al., 1999) in oocytes coexpressing ENaC and CFTR.

Using an experimental system designed to study CFTR regulation at high time resolution (Nagel et al., 1992; Weinreich et al., 1999), we set out to investigate the nature of CFTR-ENaC interactions (Nagel et al., 2001). We used giant excised inside-out membrane patches from *Xenopus laevis* oocytes, incorporating thousands of ENaC and CFTR channels. To our disappointment, we observed neither stimulation nor inhibition of ENaC by ATP-induced activation of CFTR (see fig. 3 in Nagel et al., 2001). Because using excised membrane patches soluble cytoplasmic factors are lost, we examined ENaC-CFTR interactions in whole oocytes by measuring the ENaC-specific uptake of the radioactive Na^+ isotope $^{22}\text{Na}^+$. We observed increased Na^+ uptake when CFTR was activated by raising intracellular [cAMP] in an external solution with 120 mM Cl^- (see fig. 1 in Nagel et al., 2001). Like in the sweat duct study of Reddy et al (1999), in this experiment membrane voltage was not clamped with the result that the activation of CFTR induced a voltage shift. We therefore concluded that tracer uptake measurements in **oocytes** confirm the observation by Reddy et al. (in **sweat duct**) that stimulation of CFTR activates Na^+ uptake, but only under non-voltage-clamp conditions.

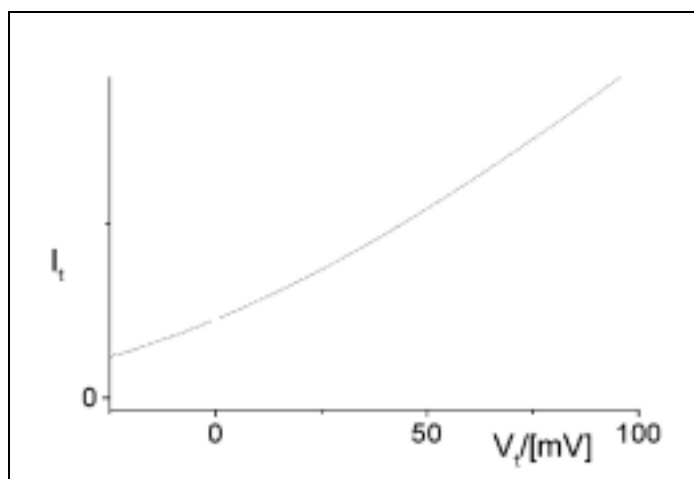


Fig. 1 Current-voltage dependence of a voltage-independent Na^+ channel under conditions of 150 mM NaCl on the luminal side and 10 mM NaCl on the permeabilized basolateral side. Current is calculated according to the Goldman-Hodgkin-Katz equation and plotted against transepithelial voltage. A shift to more positive potential clearly increases sodium conductance: an apparent "activation".

Finally we studied the ENaC conductance before, during, and after activation of the CFTR Cl^- conductance in voltage-clamped oocytes with the two-electrode voltage-clamp technique. In contrast to previous work (e.g. Mall et al., 1996, Briel et al., 1998, Chabot et al., 1999), we observed no influence of the CFTR Cl^- conductance on amiloride-sensitive ENaC conductance (see fig. 2A,B in Nagel et al., 2001). When examining the experimental conditions of published

work, we noticed that in some studies a potentially non-negligible series resistance was introduced by a single bath reference Ag/AgCl-electrode and the "bridge" electrically connecting the bath solution and the bath electrode. When we simulated such an arrangement in our two-bath-electrodes voltage-clamp experiment, we immediately observed inhibition of ENaC by activation of CFTR (see fig. 2C, D in Nagel et al., 2001). Based on these data, we propose that a "series resistance artifact" might cause inhibition of ENaC by stimulated CFTR. This series resistance hypothesis is supported by findings from other electrophysiologists (for further information, contact the author).

In summary, I propose that CFTR is not a specific regulator of ENaC (in either oocytes expressing recombinant ENaC and CFTR or native epithelia) because:

1.) ENaC conductance is not influenced by activation of CFTR conductance in oocytes, if voltage-clamp conditions are carefully controlled.

2.) Apparent "activation" of ENaC by CFTR-stimulation can be observed in non-voltage clamped **oocytes** (Nagel et al., 2001) or **sweat gland** tissue (Reddy et al., 1999) under conditions of a Na⁺ concentration gradient. Under such conditions, any conductance becomes "voltage dependent" (see fig. 1).

I argue that the Cl⁻ channel function of CFTR can explain most, if not all, of the various results, reported in studies of different tissues. If not taboo, we should re-examine rigorously by appropriate experiments the function of CFTR as a regulator of other ion channels.

Acknowledgements

I thank Klaus Hartung (MPI Biophysik) and Eberhard Frömter (Univ. Frankfurt) for their advice on careful voltage-clamping, Tanjef Szellas (MPI Biophysik) for giant patch experiments and help in preparing fig. 1, Klaus Hartung for critically reading this contribution and Ernst Bamberg for his continuing support. Supported by the German Research Foundation (Deutsche Forschungsgesellschaft, DFG).

References

- Bijman, J., Frömter, E. (1986) Direct demonstration of high transepithelial chloride-conductance in normal human sweat duct which is absent in cystic fibrosis. *Pflügers Arch. (Eur. J. Physiol.)*, **407**, S123-S127
- Briel, M., Greger, R. and Kunzelmann, K. (1998) Cl⁻ transport by cystic fibrosis transmembrane conductance regulator (CFTR) contributes to the inhibition of epithelial Na⁺ channels (ENaCs) in *Xenopus* oocytes co-expressing CFTR and ENaC. *J Physiol (Lond)*, **508**, 825-36.
- Chabot, H., Vives, M.F., Dagenais, A., Grygorczyk, C., Berthiaume, Y. and Grygorczyk, R. (1999) Downregulation of epithelial sodium channel (ENaC) by CFTR co-expressed in *Xenopus* oocytes is independent of Cl⁻ conductance. *J Membr Biol*, **169**, 175-88.
- Hille, B. (1992) *Ionic channels of excitable membranes*. Sinauer Associates Inc., Sunderland, Massachusetts.
- Knowles M, Gatzky J, Boucher R. (1981) Increased bioelectric potential difference across respiratory epithelia in cystic fibrosis. *N Engl J Med*. **305**:1489-95.
- Mall, M., Hipper, A., Greger, R. and Kunzelmann, K. (1996) Wild type but not deltaF508 CFTR inhibits Na⁺ conductance when coexpressed in *Xenopus* oocytes. *FEBS Lett*, **381**, 47-52.
- Nagel, G., Hwang, T.C., Nastiuk, K.L., Nairn, A.C. and Gadsby, D.C. (1992) The protein kinase A-regulated cardiac Cl⁻ channel resembles the cystic fibrosis transmembrane conductance regulator. *Nature*, **360**, 81-4.
- Nagel G, Szellas T, Riordan JR, Friedrich T, Hartung K. (2001) Non-specific activation of the epithelial sodium channel by the CFTR chloride channel. *EMBO Rep* **2**:249-54
- Quinton, P.M. (1983) Chloride impermeability in cystic fibrosis. *Nature*, **301**, 421-2.
- Reddy, M.M., Light, M.J. and Quinton, P.M. (1999) Activation of the epithelial Na⁺ channel (ENaC) requires CFTR Cl⁻ channel function. *Nature*, **402**, 301-4.
- Schulz I.J., Frömter E. (1968) Mikropunktionsuntersuchungen an Schweißdrüsen von Mucoviscidose-Patienten und gesunden Versuchspersonen. In: Windorfer H., Stephan U. (eds.) Mucoviscidose. Georg Thieme Verlag, Stuttgart, pp. 12-21.
- Stutts, M.J., Canessa, C.M., Olsen, J.C., Hamrick, M., Cohn, J.A., Rossier, B.C. and Boucher, R.C. (1995) CFTR as a cAMP-dependent regulator of sodium channels. *Science*, **269**, 847-50.
- Weinreich, F., Riordan, J.R. and Nagel, G. (1999) Dual effects of ADP and adenylylimidodiphosphate on CFTR channel kinetics show binding to two different nucleotide binding sites [see comments]. *J Gen Physiol*, **114**, 55-70.

Georg Nagel, Frankfurt/M, Germany

New Homepage of the Working Group on CFTR Expression

<http://central.igc.gulbenkian.pt/cftr/>

Detecting and Validating Protein Interactions with CFTR

A growing area in CFTR research is the identification of proteins that can bind to and regulate this epithelial chloride channel. These efforts have been driven in part by the development and refinement of new technologies that enable the rapid screening for potential protein-protein interactions (*e.g.*, yeast two hybrid assays, affinity purification techniques and mass spectrometric detection methods). More importantly, there is a good scientific reason to search for CFTR binding partners. CFTR appears to be functionally coupled to several other cellular processes including parallel ion transport pathways (1), cell signaling pathways (2) and possibly membrane traffic (3). Conceivably, protein-protein interactions could help integrate CFTR channel activity with these and other epithelial cell functions.

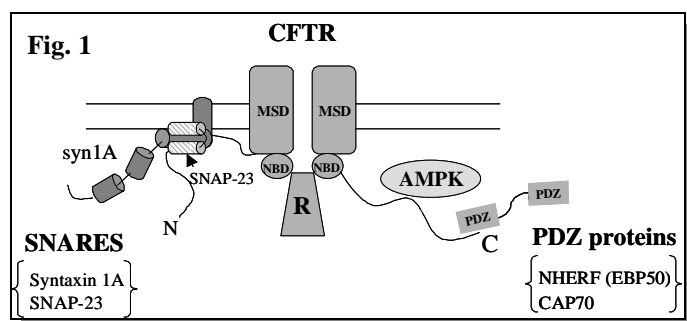


Fig. 1 shows a schematic of several CFTR binding proteins that have been identified to date. The opposing cytoplasmic tails appear to engage in distinct sets of protein-protein interactions. The amino terminal tail physically associates with components of the SNARE machinery that mediates membrane fusion in eukaryotic cells (4-6 and unpublished data). The carboxy terminal tail binds to two kinds of proteins: (i) PDZ domain-containing proteins that bind to the last 3 amino acids of CFTR (7-9) and (ii) a metabolically regulated kinase (AMP kinase[®]) that binds upstream of the PDZ binding motif (10). Each of these interactions has been shown to modulate the functional properties of CFTR chloride channels in expression systems and cultured epithelial cells and, therefore, appears to be functionally relevant. In addition, SNAREs and PDZ-domain containing proteins have been shown to physically and functionally interact with a number of other ion channels and transporters. Thus, these types of interactions constitute more general paradigms in ion channel biology that include but are not limited to CFTR. SNARE interactions may help couple the activities of certain ion channels to membrane traffic in tissues for which coordinating these two processes is advantageous. PDZ interactions may serve a scaffolding func-

* Abbreviations

AMPK – adenosine monophosphate kinase; EBP50 – ERM (ezrin-radixin-moesin)-binding phosphoprotein 50 (also called NHERF, Na⁽⁺⁾/H⁽⁺⁾ exchanger regulatory factor); GST – glutathione-S- transferase; MSD – membrane spanning domain; NBD – nucleotide binding domain; PDZ domain – the term ‘PDZ’ is derived from the first letters in the names of the three proteins in which this domain was originally characterized: PSD-95, Disc-large and ZO-1; R – regulatory domain; SNAP soluble NSF (N-ethylmaleimide sensitive factor) attachment protein; SNARE – SNAP (see above) receptor.

tion to promote CFTR channel clustering (homotypic interactions) and/or interactions with other membrane proteins that may serve a regulatory function (heterotypic interactions).

The protein-protein interactions involving the opposing tails of CFTR were initially discovered using different strategies. The carboxy terminal interacting proteins were identified using yeast two hybrid assays to screen for proteins that are capable of binding to this region of the CFTR polypeptide. SNARE-CFTR interactions were initially discovered in our laboratory using biochemical assays such as pull down assays in which CFTR protein is ‘pulled down’ from cell extracts using purified SNARE protein fragments with affinity tags (typically, glutathione-S- transferase (GST); reviewed in 11). The latter assays were specifically directed toward testing for interactions between CFTR channels and certain SNAREs (notably, syntaxin 1A) because of initial patch clamp results which indicated that this SNARE could modulate CFTR-mediated currents in epithelial cells (4). In fact, we first performed these biochemical experiments as control experiments with the expectation that there would be no direct physical interaction between CFTR channels and SNAREs. To our initial surprise, however, we observed a direct and specific interaction between the CFTR amino tail and syntaxin 1A (but not other syntaxins that were tested), and have evidence that the physical association of these proteins is required for the regulation of CFTR activity by SNAREs (4-6).

Cell based genetic screens (*e.g.*, yeast two hybrid screens) and biochemical strategies for detecting protein-protein interactions have distinct advantages and disadvantages. Both strategies can cast a ‘wide net’ in the search for interacting proteins, especially when the biochemical strategies (*e.g.*, pull downs from cell extracts using purified protein as ‘bait’) are coupled with a generic detection method such as protein microsequencing or, more recently, mass spectroscopy. The genetic screens have the advantage that the detected protein-protein interactions occur in intact cells, which enhances the possibility (but does not ensure) that the interaction occurs between proteins or polypeptide fragments in their native conformations. The genetic screens can also be highly sensitive since the typical ‘read out’ is the expression of a reporter gene. Until recently this was a significant advantage over biochemical strategies, however, the advent of sensitive and generally available mass spectroscopic methods has made the biochemical strategies the methods of choice in some cases. Biochemical screens using purified proteins as bait are not limited by one of the major shortcomings of the genetic screens, which is the requirement that the interacting proteins (or, more typically, fragments of these proteins) can be expressed

and appropriately localized (typically to the nucleus) in eukaryotic cells. On the other hand, the biochemical assays rely on the availability of purified and appropriately folded 'bait' polypeptides. Unless one has a method for purifying sufficient quantities (mg quantities) of the native protein of interest from a tissue source, this involves expressing and purifying recombinant bait protein usually in bacteria. Potential pitfalls with this approach include polypeptide insolubility or degradation and the lack of certain posttranslational modifications of bacterially expressed proteins (*e.g.*, lipid modifications). There are ways around most of these problems (*e.g.*, using the baculovirus/insect cell system to promote posttranslational modifications), although some trial and error is usually required.

Of course, the biggest concern regarding any method for detecting protein-protein interactions is the identification of false-positives. How does one validate the putative interac-

ing assay has its own limitations but, if they all give the same result, then there is greater confidence that the detected interaction is relevant. Each of the interactions schematized in Fig. 1 has been detected using several biochemical assays and, therefore, has satisfied this first criterion.

The second criterion is that the identified binding protein should colocalize with native CFTR in epithelial cells. Verifying this point is complicated by the fact that CFTR itself is a low abundance protein that is often difficult to detect by conventional immunolocalization methods. However, the available functional and immunolocalization data clearly indicate that a portion of CFTR resides at the apical surfaces of epithelial cells in airways, gut and exocrine glands. Thus, given that each of the CFTR binding proteins shown in Fig. 1 also has been shown to localize in part to the apical poles of epithelial cells (6-10), this criterion appears to have been largely satisfied for these CFTR binding proteins.

Table 1 – Minimal Criteria for Validating Protein Interactions with CFTR

1. Interaction can be detected using multiple biochemical assays
 - yeast two hybrid
 - pull down assays
 - direct, pairwise binding assays
 - co-immunoprecipitation (ideally, from epithelial cell extracts)
2. Binding protein colocalizes with native CFTR in epithelial cells
 - Immunocytochemistry
3. Detected interaction has functional consequences
 - heterologous expression systems (*e.g.*, *Xenopus oocytes*)
 - epithelial cells (*e.g.*, patch clamp studies)

tions that are detected using genetic screens or biochemical strategies? Table 1 lists the minimal criteria for a physiologically relevant protein-protein interaction involving CFTR. First, the interaction must be detectable using multiple biochemical assays. For example, a positive result from a yeast two hybrid screen should be followed up by performing pull down assays and/or direct binding assays using purified binding proteins. Negative controls (*e.g.*, GST alone or irrelevant fusion proteins) must be performed to verify specificity. Protein concentrations should be varied to generate binding curves from which the saturability and apparent affinity of the interaction can be assessed. Ideally, biochemical interactions between native proteins in epithelial cells could also be detected. Co-immunoprecipitation (with appropriate controls) would be the first method of choice in this regard, although this requires that the interaction is stable under the selected lysis conditions and/or that the complex is abundant. Many physiologically relevant interactions are too dynamic to efficiently capture using co-immunoprecipitation techniques. Methods to stabilize interactions between native proteins in intact cells or cell extracts can be tried if necessary (*e.g.*, chemical cross-linking). The main point is that the initial discovery of a protein interaction with CFTR should be validated using multiple and complementary binding assays. Each bind-

Finally, functional tests must be performed to determine to what extent the detected interaction influences the function or fate of CFTR. Typically, the first approach is to co-express CFTR with the protein of interest in a heterologous system such as *Xenopus oocytes* or fibroblasts. Such experiments can provide clues as to whether the protein in question has the capacity to influence CFTR function (and in what direction), and the conditions under which such regulation can occur. Of course, such heterologous expression experiments have the potential problem that the levels of recombinant proteins may far exceed those normally exhibited by the corresponding native proteins in epithelial cells. Consequently, any detected functional interactions must be followed up in studies of epithelial cells that normally express CFTR and the protein of interest. Antisense experiments to knock out or knock down the expression of the gene of interest can be tried, although in our experience the currently available methods are often ineffective. If one can identify reagents (*e.g.*, peptides or antibodies) that specifically prevent or disrupt the biochemical interaction between CFTR and the binding protein of interest, then these reagents can be tested for their effects of CFTR traffic and channel activity in epithelial cells. In this regard, we were fortunate during our initial studies of the CFTR-syntaxin 1A interaction in oocytes to identify several soluble peptides and

a syntaxin binding protein (Munc-18) that could rescue CFTR currents from syntaxin 1A inhibition by blocking the physical interaction between these proteins. In whole cell patch clamp studies we observed that these same reagents stimulated native CFTR currents when introduced into cultured epithelial cells (4,6). Thus, by building on the results of our initial oocyte experiments and extending those experiments into epithelial cell models, we were able to provide evidence for a functional interaction between native CFTR and syntaxin 1A in epithelial cells.

What are the outstanding issues regarding protein-protein interactions involving CFTR? First, it seems likely that there are other yet to be discovered CFTR binding proteins that could play important roles in CFTR biology. The ongoing development and refinement of strategies for detecting protein-protein interactions including novel 'function-based assays' (12) should help in this regard. Second, the existence of different protein interactions with the opposing cytoplasmic tails of CFTR (Fig 1) begs the question of whether CFTR channels can exist in distinct macromolecular complexes. Resolving this issue will require biochemical methods to stabilize and purify such complexes (*e.g.*, chemical cross-linking in combination with co-immunoprecipitations) and to identify the protein components of these complexes (*e.g.*, using mass spectroscopic methods). Lastly, it will be essential to verify that the detected interactions take place *in vivo*. In this regard, none of the protein-protein interactions involving CFTR that have been identified to date has been shown yet to occur in animal models. Transgenic approaches or cell permeant reagents will be required to establish that CFTR function is indeed modulated by such interactions *in vivo*. Until then, the

physiologic importance of any detected interaction with CFTR will be uncertain.

References:

1. Stutts, M.J., Rossier, B.C. and Boucher, R.C. (1997) *J. Biol. Chem.* 272(22): 14037-14040.
2. Schwiebert, L.M., Estell, K. and Propst, S.M. (1999) *Am. J. Physiol* 276 (2 Pt 1): C700-C710.
3. Biwersi, J., Emans N. and Verkman, A.S. (1996) *Proc. Natl. Acad. Sci. USA* 93:12484-12489.
4. Naren, A.P., Nelson, D.J., Xie, W., Jovov, B., Pevsner, J., Bennett, M.K., Benos, D.J. and Kirk, K.L. (1997) *Nature* 390: 302-305.
5. Naren, A.P., Quick, M.W., Collawn, J.F., Nelson, D.J. and Kirk, K.L. (1998) *Proc. Natl. Acad. Sci. USA* 95:10972-10977.
6. Naren, A.P., Di, A., Cormet-Boyaka, E., Boyaka, P.N., McGhee, J.R., Zhou, W., Akagawa, K., Fujiwara, T., Thome, U., Engelhardt, J.F., Nelson, D.J. and Kirk, K.L. (2000) *J. Clin. Invest.* 105:377-386.
7. Moyer, B.D., Denton, J., Karlson, K.H., Reynolds, D., Wang, S. S., Mickle, J.E., Milewski, H., Cutting, G.R., Guggino, W.B., Li, M. and Stanton, B.A. (1999) *J. Clin. Invest.* 104:1353-1361.
8. Wang, S., Yue, H., Derin, R.B., Guggino, W.B. and Li, M. (2000) *Cell* 103(1):167-179.
9. Raghuram, V., Mak, D. and Foskett, J.K. (2001) *Proc. Natl. Acad. Sci. USA* 98(3):1300-1305.
10. Hallows, K.R., Raghuram, V., Kemp, B.E., Witters, L.A. and Foskett, J.K. (2000) *J. Clin. Invest.* 105(12): 1711-1721.
11. Naren, A.P. *Methods in Molecular Medicine*; ed. W.R. Skatch (in press).
12. Remy, I. And Michnick, S.W. (2001) *Proc. Natl. Acad. Sci. USA* 98(14):7678-7683.

Kevin Kirk, Birmingham, USA

Proceedings

The new Webpage of the Working Group on CFTR Expression

The web page of our Working Group was re-designed and has a new host at the Gulbenkian Institute of Science, Oeiras, Portugal: <http://central.igc.gulbenkian.pt/cftr>. You can find the following information:

CFTR Expression – An overview about the project: the objectives and aims, coordinating centre and scientific board. Furthermore, you can see the list of participants and information on how to join the Working Group on CFTR Expression.

Newsletters – Download the published newsletters.

Virtual Repository – A collection of protocols/models (either published or reviewed by referees) that were sent to the Working Group on CFTR Expression by contributing members. This part is subdivided into 6 Chapters:

- A. Transcript Analysis; qualitative and quantitative methods.
- B. Cell Biology and Histology.
- C. Protein Biochemistry and Biophysics.
- D. Cell Physiology.
- E. *In vivo* and *ex vivo* Functional Assessment
- F. Cellular and Animal Models

Email Forum – We created an Email forum for all scientists who would like to benefit from the communication possibilities the web offers. After subscribing to this forum you can write mails simultaneously to all other subscribers, read the message archive and receive instantly the messages from the other subscribers.

Links – You will find useful links to other web resources about Cystic Fibrosis research.

Downloads – At the moment you will find here easy-to-handle sequences of the *CFTR* gene and the Abstract book of our 2001 Meeting. Submissions are welcome.

Annual Meetings – Visit this page to get the last information about the next Meeting of the Working Group on CFTR Expression.

We hope this new page will – with the help from all of you – become a growing and useful tool for researchers in the field of Cystic Fibrosis.

For any comments, suggestions or contributions, please do not hesitate in contacting me.

Sebastian Beck, Lisboa, Portugal

The Virtual Repository

Immortalization of Lymphocytes

The following protocol on EBV (Epstein-Barr-Virus)-transformation of Lymphocytes was sent by Els Dequeker, Leuven, Belgium in response to the request for protocols to establish an European Task Force to create new cell lines as tool for Cystic Fibrosis Research.

Material needed:

- fresh heparinized blood
- accuspin 1077 tubes
- sterile physiological water
- sterile plastic tubes of 15 ml
- EBV medium RPMI 1640 or DME-F12 w/o TES & HEPES 10% FCS
- EBV sup containing the virus
- sterile culture flasks F25 and F75
- Cyclosporin A = Sandimmun 50 mg/ ml Sandoz

Procedure

Centrifuge accuspin 1077 tubes for 30 sec at 2500 rpm at room temperature.

Bring 3 ml to 6 ml fresh heparinized blood on top of filter of accuspin tube.

Centrifuge for 15 min at 2500 rpm at room temperature; if the separation is not clear or clean repeat centrifugation (possible cause = old or cold blood; too old = no separation !)

After centrifugation remove plasma carefully until 0.5 cm of

opaque band which contains the cells

Transfer cells in 15 ml tube and wash with physiological water, centrifuge 15 min at 4°C at 1200 rpm

Wash the pellet with physiological water and centrifuge 10 min at 4°C at 800 rpm

Resuspend cells in 8 ml EBV medium with 2µg / ml cyclosporine

Start the cell culture in 2 F25 flasks standing up, 4 ml/flask and add 1 ml EBV sup containing the virus to each flask

After one week add 2 ml EBV medium / flask and again 2 ml after another week

Check the culture two times a week for cell growth (clump forming) and add fresh medium as needed

If enough growth (mostly after 4 weeks) split

Transfer ½ medium to another small falcon and add fresh medium

2 F25 flasks to 4 F25 flasks:

- 1 F25 for caryotyping
- 3 F25 to 2 F75 for freezing

Grow cells until enough to freeze 10^8 cells in 2 ml DME-F12 w/o Tes & Hepes + 15% FCS + 5%

DMSO/amp. (5 amp./patient)

Els Dequeker, Leuven, Belgium

Training / Exchange of Scientists

Report on the visit of Alexandra Efthymiadou, Athens, Greece to the Centre of Human Genetics, Instituto Nacional de Saúde (Lisboa, Portugal) from 11-29 June 2001

Alexandra Efthymiadou, PhD student, Department of Medical Genetics, University of Athens, Aghia Sophia Children's Hospital, Athens, Greece, under supervision of Associate Professor Emmanuel Kanavakis and Dr. Maria Tzetis, visited the Cystic Fibrosis Research Laboratory, headed by Dr. Deborah Penque and Prof. Margarida Amaral, Centro de Genética Humana, Instituto Nacional de Saúde, Lisboa, Portugal, between 11th June and 29th June 2001.

The purpose of the visit was training in immunofluorescence technique in order to study the intracellular localization of CFTR protein. This technique was performed in both nasal epithelial cells collected by brushing from non-CF and CF individuals homozygous for F508del and cell lines stably expressing wt or F508del CFTR. Eight antibodies against CFTR

were tested and compared under the same conditions. The results showed that most of the antibodies were able to detect CFTR with similar specificity in those cells.

This methodology will be now applied to the study of Greek CF patients, especially those bearing non-F508del CFTR mutations. This will contribute to the better characterization of CFTR expression in native epithelial tissues as well to the elucidation of the disease pathophysiology for unusual CFTR mutations.

Alexandra Efthymiadou, Maria Tzetis,
Emmanuel Kanavakis, Athina, Greece, and
Margarida Amaral, Deborah Penque, Lisboa, Portugal

It has been shown that, regardless of the CF mutation, CFTR localization can be affected by the epithelial maturation. We addressed the question whether a tridimensional (3-D) culture of human airway epithelial cells – known to reproduce *in vitro* the airway epithelium properties – could mimic the *in vivo* phenomenon of differentiation and polarization enabling a correct trafficking and apical localization of CFTR.

We used in a first step non-CF nasal polyps or mature human fetal trachea (ranging from 17 to 20 SA). After dissociation with 1% pronase (overnight at 4°C), isolated cells were completely dissociated with a 0.5 mm diameter needle. Then, the airway epithelial cells were placed in Ham's F12 medium supplemented with 2% Ultrosor G and 1% antibiotics (penicillin/streptomycin) and submitted to a permanent rotation (100 rpm) in a 6 wells plate at 37°C with 5% CO₂. The medium was changed after 24H (after gentle centrifugation – 100g, 20 seconds) and at 48 H, the spheroids structures were placed in a 25 mL tissue culture flask with 5mL of Ham's F12. The medium was changed twice a week.

Then we analyze the kinetics of epithelial differentiation (5, 15, 25 and 35 days) in the 3-D spheroids structures using scanning and transmission electron microscopy (SEM and TEM) and immunocytochemical analysis of markers of polarity (Ezrin, CD59), junctionality (ZO-1) and airway epithelial differentiation (mucins).

For **SEM**, at 5, 15, 25 and 35 days, we centrifuged the spheroids (100g – 2 minutes). The pellet was placed on collagen IV coated slides for 1 hour at 37°C. Once attached, the spheroids were fixed with 2% glutaraldehyde (in 0.1M PBS) for 1h at 4°C. Then they were dehydrated in increasing concentrations of ethanol ranging from 30% to 100%. Critical point drying was carried out according to the technique described by Anderson. The slides were then stuck on stubs and coated with a conductive film of gold-palladium in a vacuum evaporator. The different samples were observed by a Philips SEM (XL30).

For **TEM** observation, at similar periods, the pellet was fixed in 2% glutaraldehyde (in 0.1M PBS) for 1 h at room temperature and then postfixated with 1% osmium tetroxide at 4°C. The pellet was also dehydrated with the same procedure as described for SEM and embedded in Epon for polymerization during 78h at 60°C. Semithin sections (2 µm) were first cut with an ultramicrotome to localize the spheroids. Then ultrathin sections (80 nm) were mounted on copper grids, stained with uranyl acetate and lead citrate before the observation on a JEOL 200X transmission electron microscope.

For **immunolocalization**, the pellet was rinsed in 0.1M PBS and embedded in optimum cutting temperature compound (O.C.T.) and frozen in liquid nitrogen. Frozen sections (5µm thick) were placed on tanned slides and fixed in precooled methanol (-20°C) for 15 minutes. To saturate non-specific sites, the sections were incubated with 1% bovine serum albumin (BSA) in 0.1M PBS. They were then sequentially treated as follows: exposition to the primary antibody (diluted in PBS-BSA 1%) for 1 hour at room temperature ; two washes (15 minutes in PBS) ; incubation with 2% PBS-BSA (15 minutes) ; exposition to the biotinylated antibody (1:50 in 1% PBS-BSA) for 1 hour at room temperature ; two washes (15 minutes in PBS) and finally, revelation with streptavidin-coupled fluorescein isothiocyanate. The observations were made under an Axiophot microscope with epifluorescence and Nomarski differential interference illumination. Two controls were carried out according to the same protocol : one without the primary antibody and with the corresponding immunoglobulin.

For **CFTR functionality**, spheroids were incubated with the SPQ probe (1:2 in chloride buffer – 130mM) for 10 minutes. Then, one of them was fixed by a microcapillary and filmed during 14 minutes (7 images every other 2 minutes) in nitrate buffer (100mM) on the stage of an inverted microscope at 37°C. We successively add amiloride (10 µM), forskolin (25 µM) and ATP (10 µM) . Chloride secretion of the spheroids was analyzed by measuring SPQ fluorescence variations obtained from excitation light at 365 nm and emission light at 395 nm. The recorded images were digitized as a 512 ´ 512 pixel. The fluorescence variations were analyzed in regions of interest on each image of the temporal series.

Now, our present project is to analyze, by *in situ* hybridization, the expression of CFTR mRNA. In collaboration with Ann Harris's lab and her team, I learned this technique using a CFTR sheep probe on fetal sheep tissue. We will now soon apply this technique at different periods of maturation of the spheroids and adapt the protocol that we learned in Ann Harris's lab to our specific tissue using the human mRNA CFTR probe.

Nicolas Castillon, Reims, France
Edith Puchelle, Reims, France
Ann Harris, Oxford, UK

Forum

2002 Meeting of the CFTR Expression Working Group

The third meeting of the Working Group on CFTR Expression will be held between 12-14 April 2002 in a place (near Lisboa) not yet defined. Sintra, a small historic village 30 Km off Lisboa is a strong candidate.

There will be a limited number of invited speakers, which will be mostly chosen among active contributors to the Virtual Repository/Newsletter, since the meeting is aimed at discussing methods/models used in CFTR expression and functional studies. Therefore, submission of such contributions to the Virtual Repository becomes essential in two aspects for the planning of the meeting. To become eligible for a travel grant for the 2002 meeting, contributions to the Virtual Repository are accepted until 31 December 2001. If the number of contributions exceeds the number of grants, these will be allocated among those who have contributed most.

For additional information on the procedure to submit contributions., please visit the page:

<http://pen2.igc.gulbenkian.pt/cftr/meetings.html>

Sebastian Beck, Margarida Amaral, Lisboa, Portugal

Who is eligible to receive a travel grant for the 2002 meeting?

Any author submitting one or more new contribution(s) to the Virtual Repository arriving until 31 December 2001!

The Fifteenth Annual North American Cystic Fibrosis Conference

Orlando World Center Marriott, Orlando, Florida
25-28 October 2001

The 25th European Cystic Fibrosis Conference

Genoa, Italy 20-23 June 2002
Deadline for Abstracts: 15 January 2002

Co-ordinating Centre - Contacts

cfnet@insa.min-saude.pt

Margarida D. Amaral: mdamaral@igc.gulbenkian.pt

Sebastian Beck: sebastian_beck@hotmail.com

Deborah Penque: deborah.penque@insa.min-saude.pt

Scientific Board

Ann Harris, Oxford, UK

Edith Puchelle, Reims, France

Aleksander Edelmann, Paris, France

David Sheppard, Bristol, UK

Burkhard Tümmler, Hannover, Germany

Bob Scholte, Rotterdam, the Netherlands

Thank you, Filipa!

Filipa Cristina, who has been a scientific co-worker of the CFTR Expression Working Group, is leaving this project. She is going to the USA for a research project on haematopoietic differentiation. We are grateful for her valuable work during the past 2 years and wish her good luck.