2005 – European Cystic Fibrosis Conference
New Frontiers in Basic Science of Cystic Fibrosis

Évora, Portugal

Chairpersons
Margarida D. Amaral and Karl Kunzelmann

Hotel da Cartuxa

14 – 17 April 2005

Dear Colleagues and Friends,

It is a great pleasure to invite you to the second European Cystic Fibrosis Conference entirely dedicated to Basic Science, which will take place in Portugal, in the historic city of Évora, a World Heritage site since 1986.

The ECFS believes the activities of the basic scientists are speeding up our understanding of CF with the development of new therapies being adopted by clinicians. Thus, the ECFS is proud to provide you with a platform to discuss your ideas with your colleagues during this important conference. The ECFS prides itself on providing conferences based on a tradition of excellence. They bring together the best of European and International expertise in Cystic Fibrosis Research and your active participation will contribute to a productive exchange of information.

The success story of the basic science conferences can be mainly attributed to the splendid organization of Prof Margarida Amaral and her team. So, on behalf of the ECFS, I extend a very warm welcome to all basic scientists for an exciting conference of high scientific quality.

Gerd Döring
ECFS President

It is a great pleasure to invite you to attend the 2005 -ECFS Basic Science Conference: "New Frontiers in Basic Science of Cystic Fibrosis", which will take place in the Portuguese historic city of Évora, in the heart of the province of Alentejo. This is a region that has been inhabited by mankind since time immemorial, and all around Évora there is a remarkable heritage of megalithic monuments. Once a roman city, today a world heritage site, still evoking memories of its Visigothic period and of the Moorish presence, Évora, protected by a vast ring of fortified walls, has more than two millennia of history.

The “New Frontiers in Basic Science of Cystic Fibrosis” Conferences have grown on the tradition of the CF Network meetings and are characterised by active discussion of data and ideas at the forefront of research on CF and CFTR, in an informal, co-operative environment. They bring together the best of European and International experts, but also post-graduate students, engaged in cystic fibrosis research who have thus an opportunity to interact and discuss science in a great atmosphere.

This year the conference will gather scientists actively engaged in CF and CFTR research from gene expression and genomics, protein structure and biochemistry, cell biology, physiology and novel therapies, and also key experts in these fields, to discuss the latest ideas and the most recent unpublished data. There will be symposia with invited presentations and posters. Some authors of submitted abstracts will be invited to give short talks. Importantly, plenty of time will be allowed for discussions.

To foster close interaction amongst participants, the conference will be open to a maximum of 80 scientists (invited speakers and registrants). There will be no parallel session and all participants will be encouraged strongly to attend the full duration of the conference.

We welcome all scientists studying CFTR and CF to an exciting conference of high scientific quality in the spirit of the traditional Portuguese hospitality. Besides participating in an exciting conference you will have the opportunity to visit and get acquainted with the remarkable and peaceful city of Évora, a cross-road of cultures and civilizations and a delight for those with a passion for life and beauty.

Welcome to Évora!

Margarida D Amaral and Karl Kunzelmann
Conference Chairpersons

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2005 ECFS Conference
New Frontiers in Basic Science of Cystic Fibrosis

(Évora – Portugal, 14 – 17 April 2005)
Programme

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13:00-16:00	*Registration / Light meal / Set up of Posters*	
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SYMPOSIUM 1 – CFTR in the Nucleus: Localization, Activation, Transcription and Splicing
Chair: Ann Harris, Oxford (UK)

S1.1 Transcription-Dependent Spatial Arrangements of CFTR and Adjacent Genes in Human and Mouse Cell Nuclei

Daniele Zink[1], Margarida D Amaral[2], Andreas Englmann[1], Susanne Lang[1, 3], Luka Clarke[2], Carsten Rudolph[4], Felix Alt[1], Kathrin Luther[1, 5], Carla Braz[2], Nicolas Sadoni[1], Joseph Rosenecker[4] and Dirk Schindelhauer[6]

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[6]University of Munich (TU), Institute of Human Genetics, 81675 Munich and Life Science Center Weihenstephan, Hochfeldweg 1, 85354 Freising, Germany

Recent results suggested that the spatial organization of genomes in the cell nucleus contributes to their functional regulation. In order to investigate the relationships between transcriptional regulation and spatial organization at the level of individual genes, we investigated in different human cell types nuclear positioning and transcriptional regulation of the functionally unrelated genes GASZ, CFTR, and CORTBP2, mapping to adjacent loci on human chromosome 7q31. When inactive, GASZ, CFTR, and CORTBP2 preferentially associated with the nuclear periphery and with perinuclear heterochromatin, whereas in their actively transcribed states the gene loci preferentially associated with euchromatin in the nuclear interior. Adjacent genes associated simultaneously with these distinct chromatin fractions localizing at different nuclear regions, in accordance with their individual transcriptional regulation. Together, the results show that small chromosomal sub-regions can display highly flexible nuclear organizations, which are regulated at the level of individual genes in a transcription dependent manner. Furthermore, the results suggest that the nuclear localization of gene therapy vectors might impact the expression levels of CFTR on these constructs. Interestingly, although CFTR displays a similar transcription-dependent nuclear localization in 8 different human primary and tumor cell types investigated so far, the nuclear localization of CFTR appears to be different in mouse nuclei. This phenomenon is currently investigated and recent results will be discussed.

S1.2 Regulation of Expression of the CFTR Gene

Ann Harris

Weatherall Institute of Molecular Medicine, Oxford University, John Radcliffe Hospital, Oxford, UK

S1.3 Regulation of Expression of the CFTR Gene

F Pagani[1] and FE Baralle[2]

[1]Human Molecular Genetics, Trieste, Italy
[2]Molecular Pathology Departments International Centre for Genetic Engineering and Biotechnology, Trieste, Italy

It is well established that exonic sequences contain regulatory elements of splicing that overlap with coding capacity. However, studies on genomic variability in fields as diverse as clinical genetics and molecular evolution mainly focus on the effect of mutations on protein function. In the human CFTR exons 9 and 12, we have previously defined the Composite Exonic Regulatory Elements of Splicing (CERES). They are short context-dependent 6-8 nucleotide sequences in which natural and site-directed mutants may increase or decrease the exon splicing efficiency. Skipping of these CFTR exons removes a highly conserved region encoding part of the first nucleotide binding fold of CFTR rendering the protein non functional. Depending on the extent of exon skipping and thus on the residual levels of normal transcript, splicing mutations in CFTR may result in severe or non-classical forms of Cystic Fibrosis. Synonymous variations in particular, are assumed to be functionally neutral both in clinical diagnosis and when measuring evolutionary distances between species. However when analyzed for splicing efficiency in the CFTR exon 12 minigene, about one quarter of synonymous variations resulted in exon skipping and hence in an inactive CFTR protein. Furthermore, comparative splicing evaluation of mammalian sequence divergences showed that artificial combinations of CFTR exon 12 synonymous and non-synonymous substitutions are incompatible with normal RNA processing. In particular, the combination of the mouse synonymous with the human missense variations causes exon skipping. These results indicate that synonymous changes cannot evolve freely as they are significantly constrained by splicing requirement. In addition, the composition of exons during evolution has to be selected to ensure accurate and efficient splicing, which is an essential requirement to maintain optimal protein-coding capacity. The fact that any new genomic variation has to be compatible with the pre mRNA splicing process has to be kept in mind when analyzing apparently neutral polymorphisms in diagnostic genomic scans, which may be erroneously classified as innocuous.

P1

S1.4 Very Long PCR

Dirk Schindelhauer

Life Sciences Center Weihenstephan, Technical University of Munich, Germany

Genomic engineering is often hampered by the lack of rare cutter restriction sites. Gene BACs/PACs can contain more than one gene, or lack regions of interest and usually need further engineering to come up with a construct functioning as intended. PCR bears the risk of mutations, as do repeated growth steps of gene constructs in E. coli. Methods have been developed and will be presented, which reduce the number of growth steps in E. coli and allow robust PCR of 30-50 kb of human sequences with a very low error rate (previous limits were up to 30 kb , but often below 15 kb). The flexible introduction of restriction sites via primers, and the sureness of having lowest error rates enforce the engineering of functional gene loci. Thus, a very important technical prerequisite for the introduction of a functional CFTR gene on human artificial chromosomes has been achieved.

SYMPOSIUM 2 – ER Quality Control and Traffic
Chair: Margarida D Amaral, Lisboa (Portugal)

S2.1 Simultaneous Recruitment of the p97 ATPase and Ubiquitin Ligases to the Site of Retro-Translocation at the ER Membrane

Yihong Ye[1], Yoko Shibata1, Marjolein Kikkert[2], Sjaak van Voorden[2], Emmanuel Wiertz[2] and Tom Rapoport[1]

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Misfolded proteins are eliminated from the endoplasmic reticulum (ER) by a pathway termed retro-translocation, in which misfolded polypeptides are exported into the cytosol can degraded by the proteasome. This pathway can be co-opted by certain viruses to destroy folded cellular proteins such as MHC class I heavy chains. How substrate proteins cross the ER membrane is unclear, but it requires poly-ubiquitination and subsequent movement of the modified substrates into the cytosol by the p97 ATPase (also called VCP or, in yeast, Cdc48). In higher eukaryotes, p97 is associated with the ER membrane by a membrane protein complex, containing Derlin-1 and VIMP. Here we report that the p97 ATPase interacts directly with the ubiquitin ligases gp78 and Hrd1 and mediates their binding to Derlin-1. During retro-translocation, substrate first interacts with Derlin-1 before p97 and other factors join the complex. These data, together with the fact that Derlin-1 is a multi-spanning membrane protein forming homo-oligomers, support the idea that Derlin-1 is part of a retro-translocation channel, which is associated with both the poly-ubiquitination and p97 -ATPase machineries.

S2.2 Gaining Insights into the Chaperone-Assisted Degradation of CFTR

Simon Alberti, Verena Arndt, Christina Daniels and Jörg Höhfeld

Institute of Cell Biology, University of Bonn

Molecular chaperones of the Hsp70 and Hsp90 families are of central importance during CFTR maturation. They assist the folding of large cytoplasmic domains of CFTR. However, they also participate in CFTR degradation. This is evident from the characterization of the chaperone-associated ubiquitin ligase CHIP. CHIP associates with Hsp70 and Hsp90, and mediates the attachment of ubiquitin chains to chaperone-bound CFTR. In this way CHIP triggers the proteasomal destruction of the ion channel. Here we describe novel factors that control the activity of CHIP and modulate protein quality control processes underlying CFTR biogenesis.

S2.3 Functions for the Hsc70 System in Partitioning Nascent CFTR Between Folding and Degradation

JM Younger, Hong-Yu Ren, L Chen, C-Y Ran and Douglas M Cyr

Department of Cell Biology, UNC Chapel Hill

F508del-CFTR exhibits a correctable protein-folding defect that leads to its misfolding and premature degradation that is the cause of cystic fibrosis (CF). Herein we report on the characterization of the F508del-CFTR biogenic intermediate that is selected for proteasomal degradation and identification of cellular components that polyubiquitinate F508del-CFTR. Non-ubiquitinated F508del-CFTR accumulates in a kinetically trapped, but folding competent conformation, that is maintained in a soluble state by cytosolic Hsc70. Ubiquitination of Hsc70 bound F508del-CFTR requires CHIP, a U-box containing cytosolic co-chaperone. CHIP is demonstrated to function as a scaffold that nucleates the formation of a multisubunit E3 ubiquitin ligase whose reconstituted activity toward CFTR is dependent upon Hdj2, Hsc70, and the E2 UbcH5a. Inactivation of the Hsc70/CHIP E3 leads F508del-CFTR to accumulate in a soluble conformation, which upon lowering of cell growth temperatures can fold and reach the cell surface. Inhibition of F508del-CFTR ubiquitination can increase its cell surface expression and may provide an approach to treat CF.

S2.4 Most F508del-CFTR is Targeted to Degradation at an Early Folding Checkpoint and Independently of Calnexin

Carlos M Farinha and Margarida D Amaral

Department of Chemistry and Biochemistry, Faculty of Sciences, University of Lisboa, Portugal
Center for Human Genetics, National Institute of Health, Lisboa, Portugal

Biosynthesis and folding of multidomain transmembranar proteins is a complex process. Structural fidelity is monitored by the endoplasmic reticulum (ER) quality control (ERQC) that targets misfolded proteins to ER associated degradation (ERAD) through the ubiquitin (Ub)-proteasome pathway. Calnexin, one of the components of ERQC, was previously shown to form complexes with both wild-type (wt) and F508del-CFTR [1]. These data suggest that calnexin has some function in the ERQC of CFTR. However a direct role of calnexin in folding and/or degradation of CFTR has not been demonstrated.

We have looked at the *in vivo* involvement of calnexin and of the N-glycan moiety of CFTR on its degradation. For that purpose, we modulated the *in vivo* levels of calnexin and found that overexpression of this chaperone increases degradation of immature F508del-CFTR having no effect on the wt protein, whereas its downregulation by RNAi impairs processing of immature wt-CFTR. Cells expressing wt- or F508del-CFTR cells were also treated with different inhibitors of enzymes responsible for processing of the glycan moiety, namely castanospermine (CAS) and 1-deoxymannojirimycin (DMM). Results show that wt-CFTR is strongly stabilized by both these inhibitors of glycan processing enzymes whereas neither of them stabilizes F508del-CFTR, contrary to most substrate glycoproteins.

The existence of a degradation pathway that is independent of CFTR glycan moiety was confirmed by genetically abrogating the two consensus N-glycosylation residues both in wt- and F508del-CFTR, for which stability was found to be drastically impaired in comparison to their glycosylated counterparts.

We also investigated the possible involvement of EDEM, known to accelerate the degradation of misfolded client proteins in the ER [2] and, although its presence was detected both in wt- and F508del-CFTR complexes, it was found to occur in reduced amounts in the latter.

Altogether, our data support a novel model by which wt- and F508del-CFTR undergo ERAD from two distinct checkpoints, the mutant being disposed of independently of N-glycosidic residues and calnexin, probably mediated by the Hsc70/Hsp70 machinery, and some (unfolded) wt-CFTR undergoing glycan-mediated ERAD, from the calnexin checkpoint.

References:
[1] Pind *et al*. (1994) *J Biol Chem* **269**, 12784-12788.
[2] Oda *et al*. (2003) *Science* **299**, 1394-1397.

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S2.5 Endosomal SNARE Proteins and CFTR Trafficking in Polarized Cells

M Nacfer[1], F Bilan[1], C Norez[2], A Cantereau[3], F Becq[2], A Kitzis[1] and V Thoreau[1]

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CFTR targeting in polarized cells is still poorly understood. Identification of proteins involved in CFTR trafficking is essential to determine intracellular pathways followed by CFTR. We have recently shown that endosomal SNARE proteins syntaxin 8, VAMP8 and vti1b directly bind CFTR *in vitro* (Bilan *et al.* 2004). In CFTR-transfected CHO cells, the overexpression of each endosomal SNARE proteins (syn7, syn8, VAMP8 or vti1b) resulted in the inhibition of the CFTR chloride channel activity. This could be explained by the fact that SNARE protein overexpression disrupts cell surface CFTR trafficking. Indeed, in COS-7 cells, GFP-CFTR no longer targeted to the plasma membrane but co-localized with overexpressed SNAREs in a perinuclear region. In the case of syntaxin 8, we identified this co-localization compartment as recycling endosomes.
In order to study CFTR trafficking in epithelial polarized cells, we generated LLCPK1 cells co-expressing VSV-G tagged CFTR (Costa de Beauregard *et al.* 2000) and one SNARE protein, respectively syntaxin 1A, syntaxin 3, syntaxin 7, syntaxin 8, VAMP8 or vti1b. By radioactive iodide efflux assays, we demonstrated that each endosomal SNARE protein or syntaxin 1A overexpression inhibited CFTR activity. By contrast, overexpression of syntaxin 3 (not interacting with CFTR) had no effect upon CFTR activity. Moreover, SNARE protein overexpression did not modify other chloride currents as ICl_{swell} and ICl_{Ca}, indicating that SNARE inhibitory effects appeared specific to CFTR. We obtained similar results in polarized Caco-2 cells endogenously expressing CFTR and overexpressing each SNARE protein respectively.
We then focused on physical interactions between CFTR and SNAREs. In our LLCPK1 cell lines, we co-immunoprecipitated CFTR along with each endosomal SNARE protein, signifying that these proteins belonged to a same complex. Moreover, we showed by GST pull-down experiments that VAMP8 and vti1b preferably interacted with CFTR N-terminus domain and, in a lesser extent, with the R domain.
By immunofluorescence experiments, we studied CFTR and SNARE protein localization in our LLCPK1 cell lines. Overexpression of each endosomal SNARE disturbed CFTR apical targeting. CFTR and SNARE were co-localized in a region that we are currently identifying. Pulse-chase experiments demonstrated that SNARE overexpression had no effect upon CFTR glycosylation status, indicating that CFTR/endosomal SNARE interactions should occur in a post-golgi compartment.

This work was supported by "Vaincre La Mucoviscidose".

P3

Role of NHERF1 in Rescuing of F508del-CFTR Activity

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There is evidence that CFTR interacting proteins play critical roles in the proper expression and function of CFTR. Na^+/H^+ exchanger regulatory factor (NHERF) was the first protein to be identified that binds CFTR. Here we further clarify the role of NHERF in the regulation of CFTR expression and activity in two human bronchial epithelial cell lines: the normal, 16HBE14o-, and the F508del-CFTR, CFBE41o-. Immunofluorescence studies showed that NHERF1 was localized in the plasma membrane in 16HBE14o- cells while its distribution was cytoplasmic in CFBE41o- cells. To determine the dependence of CFTR activity on NHERF1, we transfected 16HBE14o- monolayers with vectors encoding for Wild type (Wt) NHERF1 or NHERF1 mutated in the binding groove of the PDZ domains or truncated for the ERM domain. Fluorimetric measurements using a Cl^- sensitive dye, MQAE, showed that both of the NHERF1 PDZ domains and the ERM domain play fundamental roles in regulating CFTR activity.
Importantly, CFTR-dependent chloride efflux was potentiated by the overexpression of Wt NHERF1 leading us to hypothesize a role for NHERF1 in regulating CFTR localization on the apical membrane in CF cells. To explore this possibility, we transfected CFBE41o- cells with the cDNA encoding for Wt NHERF1. Immunofluorescence showed that Wt NHERF1 overexpression in CFBE41o- cells shifted the expression of both NHERF1 and CFTR to the plasma membrane and this was paralleled by a large increase in CFTR-dependent chloride efflux demonstrating that the CFTR protein expressed on the membrane was functional. All together these data suggest that the overexpression of NHERF1 may favour the rescue of F508del-CFTR activity in CF affected cells even if the precise molecular and cellular mechanisms remain to be clarified.

SYMPOSIUM 3 – ABC Transporters: Structure and Function
Chair: David Sheppard, Bristol (UK)

Overview – DN Sheppard

Department of Physiology, University of Bristol, Bristol, UK

S3.2 Purification, Characterisation and 3D Structure of Recombinant Human CFTR with a deca-His Tag

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Human recombinant CFTR with a deca-His tag at the C-terminus was solubilised from membranes isolated from a BHK cell line using dodecyl maltoside as a detergent. The protein was then purified in a two-stage affinity chromatography procedure utilising (i) the deca-His tag (with a Ni-NTA column) and (ii) the N-linked glycosylation of extracellular loop 4 (with a WGA column). The protein was assayed by SDS-PAGE and for ATPase and chloride channel activity and shown to be highly purified and very active. Crystallisation of the purified protein into 2-dimensional ordered arrays was carried out using a hanging droplet/air-water interface method, and structural data for the two forms were collected by electron microscopy after negative staining of the crystals. Two different crystal forms of CFTR were identified; both in the *p1* two-dimensional plane group, but with slightly differing unit cell parameters. Projection maps calculated for these two crystal forms suggested that monomeric CFTR existed in a different conformation in each crystal form. Similar conformational states had also been found in structural studies of another ABC transporter, P-glycoprotein. Three-dimensional structures for each crystal form (conformation) of CFTR were calculated to a resolution of about 20Å, and these confirmed the strong similarity to P-glycoprotein structures. In contrast to the latter, however, both conformational states can be observed for CFTR in the presence or absence of nucleotide, which may be related to the role of CFTR as an ion channel rather than as a transporter. It is possible that the conformations could represent open and closed states of the channel. Attempts have been made to identify the location of the C-terminus in CFTR by exploiting the deca-His tag which can be labelled with 1.8nm-diameter Nanogold spheres coupled to Ni-NTA. Single particle alignment and averaging of detergent-solubilised CFTR molecules visualised in the electron microscope led to a 3D reconstruction of Nanogold-labelled CFTR. The location of the 1.8nm diameter Nanogold sphere was clearly delineated in the 3D structure. The location implies that the C-terminus in CFTR is not disordered but rather has a discrete location, and modelling of the 3D structure suggests that the C-terminus could be intimately associated with the interface between the nucleotide binding domains. Location of the R-domain in the 3D structure of CFTR is still uncertain, but recent cryo-electron microscopy data will be presented that may throw some light on this issue. CFTR appears to have a very similar low resolution 3D structure to other ABC transporters studied so far, suggesting that its specialisation as a channel is likely to be revealed only with higher resolution data, collection of which is underway.

S3.3 Side Chain and Backbone Contributions of F508del to CFTR Folding

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Mutations in the cystic fibrosis transmembrane conductance regulator (CFTR), an integral membrane protein, cause cystic fibrosis (CF). The most common CF-causing mutant, deletion of F508del, fails to properly fold. To elucidate the role F508del plays in the folding of CFTR, missense mutations at this position were generated. Only one missense mutation had a pronounced effect on the stability and folding of the isolated domain in vitro. In contrast, many substitutions, including those of charged and bulky residues, disrupted folding of full length CFTR in cells. Structures of two mutant nucleotide-binding domains (NBDs) reveal only local alterations of the surface near position 508. These results suggest that the peptide backbone plays a role in the proper folding of the domain, whereas the side chain plays a role in defining a surface of NBD1 that potentially interacts with other domains during the maturation of intact CFTR.

P4

S3.4 Protein Kinase CK2: the Key to F508del's Importance in wild type CFTR

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Aim: To determine whether the F508del region of CFTR has the potential to bind associated protein(s), the identity of the protein(s), whether the interaction is F508del-dependent and the functional consequences.

Introduction: The F508del mutation [1,2] disrupts CFTR's function in multiple ways perturbing CFTR biosynthesis [3], cell surface expression [4], channel gating [5] and regulation of epithelial ion channels [6]. Structural studies indicate that F508del is located on the surface of NBD1 in a position where it might participate in protein-protein interactions [7]. However, to date, the molecular role of F508del in wild type CFTR remains uncertain with studies confined to the phenotypical consequences of absent F508del.

Our Approach: We turned the problem on its head and investigated why F508del might be needed in wild type CFTR. We speculated that the F508del region could act as a binding site for a regulatory associated protein, such as a protein kinase, and searched for phosphorylation consensus sequences within the CFTR protein local to F508del. We noticed that II**F**$_{508}$GV**S**$_{511}$Y**DE**YR forms a consensus sequence for the pleiotropic protein kinase CK2 [8,9] which is involved in gene transcription, protein trafficking, cell proliferation and development [11,12].

Methods: We constructed peptides and generated recombinant NBD1 protein corresponding to wild type and F508del CFTR to test whether CK2 could bind and phosphorylate these constructs. We used co-immunoprecipitation and confocal immunofluorescence to determine whether CK2 is associated with CFTR *ex vivo* and 4,5,6,7-tetrabromobenzotriazole (TBB), a specific inhibitor of CK2 [10], to determine whether CK2 activity is required for CFTR Cl^- transport activity.

Results: Protein kinase CK2 binds and phosphorylates only wild-type NBD1-CFTR at S511. In contrast PKA phosphorylates both wild type and F508del-CFTR. CK2 co-immunoprecipitates with CFTR from wild type airway cell membranes and the two proteins co-localise at the apical membrane. Importantly, the interaction of CK2 with NBD1 of CFTR is abrogated by the F508del mutation and CK2 is absent from the membranes of an airway cell line homozygous for F508del. Thus, CK2-dependent phosphorylation of S511 does not occur in F508del-CFTR but PKA phosphorylation (S660) is unaffected. We confirmed that TBB inhibits the PKA-stimulated CFTR Cl^- channel activity using Ussing chambers and in single channel excised patches.

Conclusion: CK2 is a novel, F508del-dependent CFTR-associated protein with a phosphorylation target at S511. CK2 phosphorylation appears to be required for normal channel gating by ATP and PKA. We propose that this F508del-dependent disruption of the CK2-CFTR interaction provides a molecular mechanism to explain the function of F508del in wild type CFTR.

References :
[1] Riordan *et al*. (1989) *Science* **245**, 1066-73.
[2] Welsh *et al*. (2001) *The Metabolic and Molecular Basis of Inherited Disease*. Eds. Scriver, C R, Beaudet, AL, Sly, W S & Valle, D. McGraw-Hill Inc., New York. 5121-5188.
[3] Cheng, *et al*. (1990) *Cell* **63**, 827-34.
[4] Lukacs *et al*. (1993) *J Biol Chem* **268**, 21592-8.
[5] Dalemans *et al*. (1991) *Nature* **354**, 526-8.
[6] Stutts *et al*. (1995) *Science* **269**, 847-50.
[7] Lewis *et al*. (2004) *J Biol Chem.*
[8] Litchfield *et al*. (2003) *Biochem J* **369**, 1-15.
[9] Meggio, F and Pinna, LA (2003) *Faseb J* **17**, 349-68.
[10] Sarno *et al*. (2001) *FEBS Lett* **496**, 44-8.
[11] Guerra, B and Issinger, OG (1999) *Electrophoresis* **20**, 391-408.
[12] Wang *et al*. (2001) *Mol Cell Biochem* **227**, 167-74.

P5

S3.5 Pyrophosphate Potentiates CFTR Cl^- Currents by Interacting with the Second Nucleotide-Binding Domain

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The identification of drug-binding sites on CFTR is crucial for the development of rational new therapies for diseases associated with CFTR malfunction. One agent that robustly potentiates human CFTR Cl^- currents is the inorganic phosphate analogue pyrophosphate PP_i [1]. Interestingly, when investigating the properties and regulation of murine CFTR, we demonstrated that PP_i fails to potentiate the murine CFTR Cl^- channel [2]. Based on this observation, we have employed human-murine CFTR chimeras to investigate the protein regions that form the PP_i-binding site. Using homologous recombination, we constructed human-murine CFTR chimeras by replacing nucleotide-binding domain 1 (NBD1), NBD2 or the regulatory domain (RD) of human CFTR with the equivalent regions of murine CFTR to form the chimeras hmNBD1, hmNBD2 and hmRD, respectively. When expressed transiently in HEK293 cells, wild-type and chimeric CFTRs expressed both the immature (band B) and mature (band C) forms of the CFTR protein, demonstrating that chimeric CFTRs are correctly processed. To investigate the properties and regulation of CFTR chimeras, we employed excised inside-out membrane patches from CHO cells transiently expressing CFTR. The external solution contained 10 mM Cl^- and the internal solution contained 147 mM Cl^-, ATP (0.3 mM) and PKA (75 nM) at 37 ?C; voltage was –50 mV. Under these conditions, the single-channel current amplitude (i) and open probability (P_o) of CFTR chimeras resembled that of human CFTR. To test the effects of PP_i on CFTR chimeras, we added increasing concentrations of PP_i (0.03 – 20 mM) to the intracellular solution. For wild-type human CFTR, the relationship between PP_i concentration and CFTR Cl^- current was bell-shaped with a peak value of 161 ? 6% (mean ± SEM; n = 5) at PP_i (5 mM) and weak current inhibition observed at PP_i (20 mM; n = 5). In contrast, for murine CFTR, little or no potentiation of CFTR Cl^- current was observed over the range PP_i (0.03 – 5 mM), but strong current inhibition occurred at PP_i (20 mM; n = 4). Of note, the PP_i-sensitivity of hmNBD1 mirrored that of human CFTR (n = 4), whereas the PP_i-sensitivity of hmNBD2 paralleled that of murine CFTR (n = 4). Interestingly, the relationship between PP_i concentration and CFTR Cl^- current was bell-shaped for hmRD, with a peak value of 179 ? 6% (n = 2) at PP_i (1 mM) and no current inhibition observed at PP_i (20 mM). We interpret our data to suggest that NBD2 is a key determinant of the PP_i-binding site, but that the R domain might influence the efficacy of PPi as a CFTR potentiator.

References:
[1] Carson *et al*. (1995) *J Biol Chem* **270**: 20466-72.
[2] Lansdell *et al*. (1998) *J Physiol* **508**: 379-92.

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P6

S3.6 Processing and Function Properties of CFTR Mutants Located at the NBDs

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Many of the CF-causing mutations located in the nucleotide-binding domains (NBDs) of CFTR have not been biochemically nor functionally characterized. Here, the following CFTR mutations were introduced into CFTR cDNA cloned into the pCDNA3 vector: S549N, Y563N (both in NBD1 and described as disease-causing [1]). These mutants were also produced in *cis* with previously described revertant mutations, namely, 1) for S549N: G330S and T1134P (the latter identified as revertant of Ste6 [2]); and 2) for Y563N: G550E [3] and 4RK [4] (R29K/R516K/R555K/R766K), both identified as revertants of the F508del trafficking defect.

Following transient transfection of HEK293 cells with each of these recombinant plasmids, the processing of the resulting mutant proteins was assessed by Western blotting. S549N-CFTR and its related double mutants (S549N/G330S and S549N/T1134P-CFTR) are fully processed into mature CFTR (band C) as well as the isolated secondary mutants (G330S and T1134P-CFTR). In contrast, Y563N-CFTR and both related double mutants (Y563N/G550E and Y563N/4RK-CFTR) only appear as the endoplasmic reticulum (ER)-specific core-glycosylated form (band B) failing to be detected as band C by this technique.

Chloride (Cl^-) channel activity of these mutants was assessed by the iodide efflux assay [5]. Relatively to wt-CFTR, S549N- and T1134P-CFTR seem to have a diminished forskolin-stimulated response, which appears to be delayed for S549N. The addition of T1134P to S549N seems to abolish this delay. G330S/S549N-CFTR appears to lack Cl^- channel function. G330S-CFTR is currently under study. Consistent with the lack of band C, no activity has been detected for Y563N-CFTR neither for its derived revertant mutants.

The presence or absence of a trafficking restoring effect by these revertant mutations is expected to provide structural insights into the architecture of CFTR NBD1 or NBD2 and help to elucidate the structural features responsible for processing defects.

References:
[1] CFTR Mutation database (http://www.genet.sickkids.on.ca/cftr).
[2] Proff C and Kolling R (2001) *MolGenGenet* **264**: 883-893.
[3] DeCarvalho *et al*. (2002) *JBiolChem* **277**(39) : 35896-905.
[4] Chang *et al*. (1999) *MolCell* **4**(1): 137-142.
[5] Hughes *et al*. provided through the European Working group on CFTR Expression.

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P7

Impact of the N-Terminus on CFTR Processing

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Misfolding of the CFTR protein with consequent defective intracellular transport is the principal molecular defect caused by CFTR mutations, of which F508del mutation is the most frequent example.

We have detected a novel mutation in exon 1, 120del23 (corresponding to the deletion of 23 nucleotides, 120 to 142, which span the translation initiation codon) in a Portuguese CF patient, in compound heterozygosity with F508del. Previously, CFTR function was assessed in the colon of this patient and no detectable cAMP-mediated chloride (Cl^-) secretion was found [1]. The impact of this mutation on the transcripts levels was assessed in native tissues, as described before [2] and the results shown no significant difference between the level of F508del and 120del23 transcripts.

Our aim here was to test the influence of this mutation at the protein level. The recombinant pNUT-CFTR cDNA vector was used to produce the 120del23 mutant CFTR cDNA, by direct mutagenesis. This recombinant vector was used to transfect BHK cells and thus generates a novel stable line expressing the mutant protein in significant amounts. Protein expression was assessed by immunoblot using the anti-CFTR M3A7 antibody and a band similar to the immature form of CFTR (band B) but of lower molecular mass was detected. We propose that this band corresponds to the protein resulting from the use of the M150 alternative initiation in-frame codon, as previously suggested [3]. This smaller protein expressed by the CFTR 120del23 mutant (missing the first 150 aa) does not appear to be fully-glycosylated, since no higher molecular mass band could be detected. However, in preliminary functional analysis using the iodide efflux assay, residual channel activity was detected for this protein. Altogether, results for this mutant suggest that the N-terminus of CFTR has a major impact on its folding and processing, although not completely abolishing function. The fact that Cl^- secretion could not be detected in the colon of a patient carrying this mutation [1] is suggestive that other factors influence CFTR regulation in native tissues.

References:
[1] Hirtz *et al* (2004) *Gastroenterology* **127**: 1085-1095.
[2] Ramalho *et al* (2002) *Am J Respir Cell Mol Biol* **27**: 619-627.
[3] Carrol *et al* (1995) *JBC* **270**: 11941-11946.

Work supported by POCTI/MGI/47382/2002 (FCT, Portugal) research grant. AS Ramalho was a recipient of SFRH/BD/3085/2000 PhD fellowship (FCT, Portugal).

SYMPOSIUM 4 – Membrane Traffic and Lipids
Chair: Gerd Schmitz, Regensburg (Germany)

S4.1 Lipid Rafts and the Regulation of Exocytosis

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Exocytosis, the fusion of intracellular vesicles with the plasma membrane, is essential for the targeting of newly synthesised proteins and lipids to the plasma membrane and also for the secretion of various molecules from the cell. Exocytosis occurs constitutively in all cell types, but can also be tightly regulated. Examples of regulated exocytosis pathways include the release of neurotransmitters from nerve endings, adrenaline secretion from adrenal chromaffin cells and insulin release from pancreatic beta cells. A multitude of proteins have been identified that function in exocytosis. Central to this process are 'SNARE' proteins; SNAREs present at the plasma membrane interact with a SNARE protein localised to the vesicle membrane to form a 'SNARE complex'. The formation of this highly stable complex bridging the two membranes is generally believed to promote exocytic membrane fusion. SNAP25 and SNAP23 are plasma membrane SNARE proteins essential for regulated exocytosis in diverse cell types. Several recent studies have shown that these proteins are partly localised in lipid rafts, domains of the plasma membrane enriched in sphingolipids and cholesterol. Targeting of SNAP25/23 to lipid raft domains is dependent upon the multiple palmitoylation of a central cysteine-rich domain. Interestingly, modifying the number of cysteine residues within the cysteine-rich domains of these proteins has a marked effect on raft association. Raft-targeting mutants of SNAP25/23 were employed to examine whether raft association of these proteins is important for the regulation of exocytosis. PC12 cells were engineered which express the light chain of botulinum neurotoxin; in these cells all of SNAP25 was cleaved to a lower molecular weight form, and regulated exocytosis was essentially absent. Exocytosis was rescued by expressing toxin-resistant SNAP25 or wild-type SNAP23, which is naturally toxin-resistant. Remarkably, a mutant SNAP25 protein with an increased affinity for rafts displayed a reduced ability to support exocytosis, whereas SNAP23 mutants with a decreased affinity for rafts displayed an enhancement of exocytosis when compared to wild-type SNAP23. The effects of the mutant proteins on exocytosis were dependent upon the integrity of the plasma membrane and lipid rafts. These results provide the first direct evidence that rafts regulate SNARE function and exocytosis, and identify the central cysteine-rich region of SNAP-25/-23 as an important regulatory domain.

S4.2 Role of ABCA1 in Membrane Phospholipid and Cholesterol Trafficking: New Insights from Interactive Proteins and Transcriptional Regulators

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Mutations in the gene for the ABC transporter ABCA1 cause high-density lipoprotein (HDL) deficiency syndromes such as Tangier disease. ABCA1 functions as critical regulator of cellular cholesterol and phospholipid export which exerts its activity that within the cellular lipid export machinery rather as a facilitator than a genuine pump. A number of transcription factors that control the expression of ABCA1 have thus far been characterized. In particular, we provided evidence for the involvement of SP1/3, the E-box binding factor HIF2alpha/ARNT and oncostatin M in the control of the ABCA1 promoter activity. Moreover, recent work showed that the zinc finger protein ZNF202 acts as a major repressor of ABCA1 transcription through binding of the universal corepressor KAP1. Recent data from ZNF202 knockout mice demonstrating an increase in pre-beta-HDL and a shift from fast to slowly migrating alpha-HDL underscore the physiological significance of ZNF202 in HDL metabolism in vivo. Of specific importance for the understanding of the workings of ABCA1 is the identification and characterization of its molecular interaction partners. We found that the PDZ-protein beta2-syntrophin physically associates with the ABCA1 C-terminal amino acid sequence, which forms a perfect docking site for PDZ domains. PDZ-proteins have been implicated in apical and basolateral sorting. Immunoprecipitation experiments confirmed the interaction between ABCA1 and beta2-syntrophin. Utrophin, a protein known to couple beta2-syntrophin was identified as an additional component of the ABCA1 complex. Moreover, we identified additional PDZ-proteins that potentially interact with ABCA1 including GAIP C-terminus interacting protein, membrane associated guanylate kinase MAG13, PAPIN, scribble and TIP1. The physical association of ABCA1 and PDZ-proteins suggests a role of ABCA1 in apical and basolateral targeting of transport vesicles. Because SNARE proteins are potential players in fusion processes involving ABCA1 containing vesicles, we tested the hypothesis that syntaxins, members of the SNARE family, interact directly with ABCA1. M-CSF induced differentiation of human monocytes and cholesterol loading resulted in co-induction of syntaxins 3, 6 and 13 and ABCA1. Immunoprecipitation experiments revealed a direct association of syntaxin 13 and full length ABCA1. Also, we found that ABCA1 and syntaxin 13 co-localize in macrophages. Silencing of syntaxin 13 by small interfering RNA (siRNA) led to reduced ABCA1 protein levels associated with a significant decrease in apoA-I dependent choline-phospholipid efflux. Moreover, the uptake of latex phagobeads was enhanced in ABCA1 deficient fibroblasts and ABCA1, syntaxin 13 as well as flotillin-1 were identified as components of the phagosome complex. Finally, we identified a novel G-protein receptor, which is involved in potassium trafficking, that regulates the ABCA1/AP-3 dependent lipid secretory pathway suggesting the intimate functional association of ABCA1 and an ion flux, a constellation analogous to CFTR and SUR1. In conclusion, we have identified novel members of the ABCA1 dependent lipid export complex and critical regulators of ABCA1 expression which highlight the complex involvement of this transporter in cellular lipid trafficking.

S4.3 Cystic Fibrosis and Lipids: Fatty Acid Imbalances and Membrane Microdomains

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An association has been reported between alterations in fatty acid metabolism and cystic fibrosis (CF), consisting of a decrease in linoleate and a deficiency of docosahexaenoate. We hypothesized that these alterations are specific for a particular lipid component(s), and the result of a specific metabolic defect. The different lipid classes were examined for fatty acid changes using pancreatic homogenates and primary cultures of pancreatic acini from cftr-/- (CF) and wild-type (WT) mice. Lipid classes and phospholipids were separated by aminopropyl column chromatography and HPLC, and fatty acid methyl esters analyzed. The results indicate that in CF mice: (1) 18:2n-6 was decreased in phospholipids but not in neutral lipids; (2) there was an increase in 20:3n-6 and in 22:5n-6, the terminal fatty acid of the n-6 pathway, in all lipid classes; and (3) the 22:5n-6/22:6n-3 ratio was significantly elevated in all phospholipids. DHA supplementation of mice (40mg/day for 7 days) resulted in (1) a marked decrease in the conversion of 18:2 n-6 to more desaturated and elongated fatty acids, such as 22:5 n-6; (2) a decreased metabolism of 20:5 n-3 to 22:5 n-3; (3) increased DHA content in all phospholipids tested, without any specific localization in a particular phospholipid, as well as (4) increased localization of 18:2 n-6 and 18:3 n-6 into phospholipids in CF animals, but not in wt. Taken together, these results indicate that the fatty acid defect(s) characteristic of CF are localized to membrane neutral phospholipids. In our search for a potential explanation to this fatty acid imbalance, and given that the cell membrane contains microdomains of specific lipid composition, we hypothesized that the membrane localization of CFTR may be relevant to the dynamics of phospholipids and fatty acids. We studied the distribution of CFTR in the membrane microdomains of Calu-3 cells. Cells were lysed in the presence of 1% Triton X-100, and subjected to ultracentrifugation in a discontinuous sucrose gradient (40, 30, 5%). The fractions corresponding to the 5-30% interphase (Triton non-soluble, or rafts) and the 40% density region (Triton soluble) were collected separately and analyzed by western blot for CFTR localization. The results showed a major distribution of CFTR in the soluble fraction, but a significant (9% of total) presence in the raft fraction. The analysis of biotinylated surface proteins showed up to 29% of total CFTR in the raft fraction. In addition, raft and non-raft fractions were isolated from primary cultures of pancreatic acinar and duct cells of $cftr^{-/-}$ (CF) and wild-type (WT) mice. A similar distribution pattern was obtained in wild-type pancreas. To test for differences in lateral distribution of membrane proteins between CF and wt pancreas cells, the localization of the chloride channel CLC-2 was also studied. CLC-2 was found both in rafts and non-rafts in the wt. However, it was totally absent from the raft fraction of CF cells. These results suggest (1) that CFTR is unevenly distributed within membrane microdomains, and (2) that microdomain distribution of membrane proteins may be altered in CF.

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Lipopolysaccharide Binding Protein, Cytokine Production in Whole Blood and Lipoproteins in Cystic Fibrosis

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According to the endotoxin lipoprotein hypothesis lipoproteins may down regulate cytokine production by neutralizing lipopolysaccharide (LPS) binding protein (LBP) complexes. We investigated the correlation between lipoproteins, LBP, cytokine production and clinical status in F508del (homozygous) individuals.

CF patients with mild disease (Shwachman score 71-100 pts, group A, n=22, median FEV_1 80%) were compared with those with more severe disease (Shwachman score 41-55 pts, group B, n=22, median FEV_1 59%) and age matched controls (group C, n=22). LBP (median A/B/C 4.3/7.2/4.0, $p<0.016$), interleukin-8 (IL-8 median A/B/C 15/25.1/8.0 pg/ml, $p<0.005$), tumor necrosis factor alpha (TNF alpha median A/B/C 43/56/30 pg/ml, $p<0.0001$) using a chemiluminescent immunometric assay (DPC Bad Nauheim, Germany) and fat intake (A/B/C 1.88/2.38/1.28 g/kg, $p<0.007$) as well as serum triglycerides (TG median A/B/C 106/90/76 mg/dl, n.s.), cholesterol (CHOL median 116/124/168 mg/dl, $p<0.0001$), very low density lipoprotein (VLDL median 21/19/15 mg/dl, n.s.), low density lipoprotein (LDL median A/B/C 51.5/56/91 mg/dl, $p<0.0001$), high density lipoprotein (HDL median A/B/C 43.5/45.5/55 mg/dl, n.s.) were measured. In more severe disease there was a correlation between MEF_{25} and HDL ($r=0.555$, $p<0.021$). To adjust for the influence of colonization with *P. aeruginosa*, those who were colonized with *P. aeruginosa* (n=25) were analysed separately: There was a significant correlation between LBP and FEV_1 ($r=-0.588$, $p<0.004$).

Lipoproteins may have a modulating effect in more advanced disease and are not influenced by fat intake. LBP correlates (in Psa +) with clinical status as well as lung function and may be a critical molecule regulating LPS induced inflammation.

SYMPOSIUM 5 – Epithelial Ion Transport
Chair: Karl Kunzelmann, Regensburg (Germany)

S5.1 **TRPV4 Channel in the Physiology and Pathophysiology of Ciliated Epithelia**

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The vertebrate transient receptor potential cationic channel TRPV4 has been proposed as an osmo- and mechanosensor channel. Studies using knock-out animal models have further emphasized the relevance of the TRPV4 channel in the maintenance of the internal osmotic equilibrium and mechanosensation. However, at the cellular level there is still one important question to answer: Does the TRPV4 channel generate the Ca^{2+} signal in those cells undergoing a Ca^{2+}-dependent regulatory volume decrease (RVD) response? RVD in human airway epithelia requires the generation of a Ca^{2+} signal to activate Ca^{2+}-dependent K^+ channels. The RVD response is lost in airway epithelia affected with cystic fibrosis (CF). Defective RVD in CF epithelia is linked to the lack of swelling-dependent activation of Ca^{2+}-dependent K^+ channels, a process related to the absence of a TRPV4-mediated Ca^{2+} signal and the subsequent activation of Ca^{2+}-dependent K^+ channels.
The role of TRPV4 channel in epithelia might not be restricted to the regulation of cell volume. Epithelial ciliated cells are responsible for the mechanical clearance of mucus and trapped substances from the airways and the transport of gametes and embryos through the oviduct. A primary determinant of mucociliary transport is the ciliary beat frequency (CBF), which is regulated by a variety of chemical and mechanical stimuli. Our most recent data suggest the participation of the TRPV4 in the maintenance of the ciliary beat frequency under conditions of varying mucus viscosity. Therefore, TRPV4 emerges as a candidate to participate in the coupling of different stimuli to the generation of the Ca^{2+} signal required for the correct functioning of ciliated epithelia.

S5.2 CLIC Proteins: Novel Intracellular and Plasma Membrane Cl^- Channels?

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Chloride Intracellular Channel (CLIC) genes, and their homologues encoding p64 and parchorin, are very widely expressed in multicellular animals, and most mammalian cells probably contain at least one CLIC protein. Although CLIC1 and CLIC4 in particular have been associated with ion channel activity, they remain controversial candidates for pore-forming proteins in cells [1]. Firstly, the proteins show a highly unusual dual cytosolic and membrane location, because despite sharing the same fold as soluble, omega-class GSTs, soluble CLICs can "autoinsert" into membranes, forming integral membrane proteins that appear to contain a single transmembrane domain. Their novel insertional activity is reminiscent of some soluble apoptosis-associated proteins, and indeed CLIC4 itself is pro-apoptotic [2]. It certainly contrasts strongly with the normal cellular processing of other membrane proteins, including all the known pore-forming subunits of human and other mammalian ion channels. Secondly, although recombinant CLICs form channels in artificial membranes, their properties have yet to be compared in detail to CLIC-related channels in cells, with the further caveat that currents recorded from cellular CLICs have almost always followed protein over-expression, and do not represent recordings from native CLICs in unmodified cells. Thirdly, and perhaps most significantly, mutations in the likely pore-forming region (the putative transmembrane domain) of CLIC1 and CLIC4 have not been shown to give rise to identical functional changes (e.g. in conductance or ionic selectivity) when the recombinant channels are reconstituted in vitro and expressed in vivo. Nevertheless, despite all these untested hypotheses, CLIC proteins remain very strong candidates for novel anion channels, and they are particularly interesting because over-expression can "drive" the proteins to the plasma membrane (the basis for patch-clamp recording of CLIC-associated channels), suggesting they could in principle form anion channels that might compensate for mislocalised or defective CFTR channels in CF. In recent work with monolayers (Langmuir-Blodgett films) and planar lipid bilayers, we found that the insertional activity of CLIC1 and CLIC4, and their ability to form anion channels, reflected different aspects of a multi-step process, and pore formation is largely controlled by the lipid composition of the membrane. In addition, CLIC1 channels are inhibited by a highly specific anti-CLIC1 antiserum that also inhibits brain microsomal anion channels previously reconstituted in our laboratory, suggesting that CLIC1 or a CLIC1-like protein is a normal component of native intracellular anion channels, not an artefact of protein over expression. Finally, CLIC1 and CLIC4 contain several cysteine residues, similar to soluble GSTs. One cysteine, predicted to be external or intraluminal in the membrane form of the proteins, is very highly-conserved across species, and CLIC1 channels are functionally modulated by redox activity, suggesting a further way in which novel channel activity might be controlled to improve chloride ion transport in CF.

References:
[1] Ashley (2003) *Mol Membr Biol* **20**: 1-11.
[2] Fernandez-Salas *et al*. (2002) *Mol Cell Biol* **22**: 3610-20.

S5.3 Purinergic $P2Y_6$ Receptors Induce Ca^{2+} and CFTR Dependent Cl^- Secretion in Mouse Trachea

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In airways Cl^- secretion is activated and Na^+ absorption is inhibited when $P2Y_2$ receptors are stimulated by ATP or UTP. Both nucleotides are subject to degradation to ADP and UDP by ecto-nucleotidases. Here we show that these metabolites change electrolyte transport by stimulation of $P2Y_6$ receptors in mouse trachea. Immunohistochemistry confirmed luminal and basolateral expression of $P2Y_6$ receptors. In Ussing chamber experiments luminal ADP or UDP induced both transient and persistent increase in short circuit currents (I_{SC}). Activation of I_{SC} was inhibited by the $P2Y_6$ receptor blocker PPADS. The transient response was inhibited by DIDS, whereas the persistent I_{SC} was inhibited by glibenclamide. Moreover, sustained activation of I_{SC} by luminal UDP was inhibited by blocking basolateral K^+ channels with 293B. Possible effects of diphosphates on $P2Y_1$ or adenosine receptors were excluded by the inhibitors MRS2179 and 8-SPT, respectively. Inhibition of amiloride sensitive Na^+ absorption was only seen after blocking basolateral K^+ channels with 293B. In contrast, Cl^- secretion activated by basolateral ADP or UDP was only transient and was blocked by the sk4 K^+ channel blocker clotrimazole. In summary, activation of luminal $P2Y_6$ receptors in the airways shifts electrolyte transport towards secretion by increasing both intracellular Ca^{2+} and cAMP.

S5.4 Inhibitory Control of Pancreatic HCO_3^- Secretion: Role of SLC26 Transporters

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The pancreatic ductal epithelium secretes an alkaline fluid in response to both cAMP and Ca^{2+}-mobilizing agonists, that may contain up to 140 mM $NaHCO_3$. The physiological function of this alkaline secretion is to wash digestive enzymes down the ductal tree and into the duodenum, and to neutralize the acid chyme entering the duodenum from the stomach. Failure of ductal HCO_3^- secretion, as occurs in cystic fibrosis, has serious consequences for the integrity of the pancreas and often leads to pancreatic failure. While the regulatory pathways that switch on pancreatic ductal HCO_3^- secretion are well described, much less is known about the inhibitory control of ductal secretion. Such inhibitory pathways may be physiologically important in terms of limiting the hydrostatic pressure within the lumen of the duct (thus preventing leakage of enzymes into the parenchyma of the gland), and in terms of switching off pancreatic secretion after a meal.

We originally showed in 1990 that substance P (SP) inhibited cAMP-stimulated fluid and HCO_3^- secretion from isolated rat pancreatic ducts, indicating that this peptide exerts its effects directly on the ductal epithelium. SP interacts with tachykinin receptors, which are seven transmembrane span receptors coupled to the Gq/G11 family of G proteins. Our more recent experiments have confirmed that a similar inhibition is seen in isolated guinea pig ducts, and have further elucidated the mechanism of action of this neurotransmitter. SP had no significant effect on basolateral HCO_3^- transporters or on CFTR Cl^- channels, and inhibited cAMP-stimulated secretion by modulating a Cl^--dependent HCO_3^- efflux step on the apical membrane of the duct cell. This inhibitory effect of SP was mimicked by phorbol 12, 13-dibutyrate (PDBu), an activator of protein kinase C (PKC). Moreover, bisindolylmaleimide I, a blocker of PKC, relieved the inhibitory effect of both SP and PDBu on HCO_3^- secretion. In microperfused ducts, luminal H_2DIDS caused intracellular pH to alkalinize (consistent with inhibition of HCO_3^- efflux) and, like SP, inhibited cAMP-stimulated HCO_3^- secretion. However, SP did not reduce HCO_3^- secretion further when H_2DIDS was applied to the lumen, suggesting that SP and H_2DIDS inhibit the same transporter on the apical membrane. Our data therefore indicate that SP inhibits an H_2DIDS-sensitive HCO_3^- transport step at the luminal membrane of the duct cell. As CFTR is not affected by DIDS, then the most likely candidate is a Cl/HCO_3^- exchanger. Both SLC26A3 (DRA) and SLC26A6 (PAT-1) have recently been localized to the apical membrane of human pancreatic duct cells using immunocytochemistry. However, as SLC26A3 is generally considered to be DIDS-insensitive the most likely target for SP and PKC is SLC26A6.

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S5.5 Bile Acid Dysfunction in the Cystic Fibrosis Intestine

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The intestinal epithelial transport defect affects both absorption and secretion. Bile acids are released into the duodenum where they facilitate processing of dietary fat with subsequent reabsorption in the terminal ileum where 95% of bile salts are actively reabsorbed by the sodium dependent ileal bile acid transporter (IBAT). Possibly interactions between IBAT and CFTR were investigated using the CF mouse model.

Mucosal application of bile acids caused biphasic increase in the short circuit current generated by ileal sheets from both normal and wild type mice. This initial phase was sodium dependent but unaffected by the chloride channel blocker DPC, the NKCC inhibitor furosemide and a chloride free medium suggesting that it reflected the activity of IBAT. A second phase was observed representing bile acid-induced secretion which was sodium independent, but inhibited by DPC, furosemide and the absence of chloride. In more proximal regions of intestine, when applied serosally, bile acids induced a monophasic secretory response. This response involved the activation of mast cells that released secretory stimulants including histamine and 5-HT. In the case of taurocholic acid, but not ursodeoxycholic acid, a neural component was also present.

In tissues from CF mice (F508del) the secretory response to bile acids was absent in all regions of the intestine and the absorptive component in the ileum was reduced.

Taurocholate absorption measured directly in everted intestinal sacs demonstrated sodium dependent bile acid uptake in both normal and wild type mice. In CF ileum taurocholic acid uptake was significantly lower, however, this was not confirmed using brush-border membrane vesicles, suggesting an intracellular component was required for this effect. In jejunum and mid-intestine taurocholic acid uptake was passive and there was no difference between wild-type and CF tissues.

These studies confirm that bile acid function is disturbed in CF intestine. The absence of bile acid-induced secretion in CF may contribute obstructive symptomatology such as meconium ileus and distal ileal obstruction.

SYMPOSIUM 6 – Models of Airway Disease
Chair: Marcus Mall, Heidelberg (Germany)

S6.1 Gene Expression Changes in CF Mouse Tissues

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Although the primary mutation responsible for CF is known, the way in which loss of CFTR channel activity results in the disease phenotype is still largely unresolved. It is increasingly recognised, however, that other factors contribute to the disease severity. Variation in disease severity between individuals with identical CFTR mutations suggests that genetic factors may ameliorate or exacerbate the disease. Several loci have been identified that improve the survival of CF mice. A characterization of the gene expression changes that occur in CF and a critical assessment of the role that these changes play in disease progression will improve our understanding of CF pathogenesis and may allow the development of new therapeutic strategies.
We have used filter microarrays from Clontech to examine gene expression alterations in the airways and intestinal tract of null and F508del mouse models of CF. Thirty genes that showed a potential change in their level of expression were selected for further analysis by real-time qRT-PCR. Several of these have been confirmed to show consistent alterations in gene expression. In mouse CF colon, intelectin, carbonic anhydrase IV and guanylate cyclase activator 2A are all reduced compared to normal tissue. In the mouse CF lung, calbindin 3 and trefoil factor 2 are down regulated, while lipocalin 2 is up regulated. A common theme for some of these genes is that they may be involved as modulates of innate immunity. Of particular interest is the intelectin gene (also known as the lactoferrin receptor), which is expressed by Paneth cells in the crypts of the intestine and has been suggested to play a role in defence against bacterial pathogens. In 129 strain mice, there are two highly related intelectin homologues and both these are expressed at much higher levels in normal colon than in CF mouse colon raising the possibility that reduced expression might increase the susceptibility to pathogens in CF.

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S6.2 Embryonic Lung Growth in the *cftr*-knockout Murine Model

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Background

The assertion that CFTR does not play a role in antenatal lung growth and development has been challenged by recent studies that demonstrate marked expression of *CFTR* in early foetal life [1] with ~100 fold reduction in postnatal airways. In the CF knockout mouse, *in utero* gene transfer at 15-16 days gestation has been reported to correct the CF phenotype in *cftr* -/- pups [2]. The same group report that the frequency of surviving "rescued" pups is less than expected suggesting that CFTR may have a critical role before day 15 gestation [3]. We used an established model to examine the impact of *cftr* knockout on early murine survival and embryonic lung growth. In addition we also assessed airway peristalsis, which has recently been recognised as a critical factor for normal embryonic lung growth [4].

Methods

Timed mated pregnancies of *cftr*+/- (*cftr*^{tm1Cam}) mice were generated to harvest embryos at day 11.5 (equivalent to 37 days post conceptional age in the human). Lungs were microdissected and cultured under standard conditions on membrane inserts for 78 hours. Morphometric measurements were made of total lung area, perimeter and branching (bud count) at 6, 30, 54 and 78 hours. Inter-contraction intervals (ICI) of each lung specimen were analysed to provide a quantitative measure of airway peristalsis. All measurements were undertaken without knowledge of genotype.

Results

45 embryonic lungs were studied from 9 litters (10 = *cftr* -/- (CF), 23 = *cftr* +/-(Heterozygote) and 12 = *cftr* +/+ (Wild Type)). All CF embryos had evidence of normal lung growth and branching (CF vs. Wild Type vs. Heterozygote; NS, Mann Whitney test). Peristalsis was observed in lung specimens at 54 and 78 hours. There was no significant difference in ICI between groups.

Conclusions

In the embryo, the lung is a liquid filled secretory organ. Liquid secretion is driven by active transepithelial chloride transport and is an important component of normal lung growth. CFTR is expressed in significant amounts in the embryonic and foetal airway but apparently is not critical for normal lung growth. These data provide evidence that murine embryonic lung growth and budding is normal in the *cftr*-knockout mouse model. In addition CFTR does not appear to have a critical role with respect to embryonic lung peristalsis. Finally, the yield of viable embryonic lungs does not support increased *in utero* death in *cftr*-knockout embryos.

References:

[1] (2002) *Hum Mol Genet* **11**: 125-31.

[2] (1997) *Lancet* **349**: 619-20.

[3] (2000) *Am J Physiol Lung Cell Mol Physiol* **279**: 333-41.

[4] (2005) *Am J Respir Cell Mol Biol* **32**: 118-27.

S6.3 The ENaC Overexpressing Mouse as an *in vivo* Model for Cystic Fibrosis Lung Disease l

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Chronic progressive airway disease with mucus obstruction, inflammation and bacterial infection remains the major factor contributing to morbidity and mortality in cystic fibrosis (CF) patients. Progress in the understanding of the pathophysiology and development of effective treatments for CF lung disease has been hampered by the lack of an animal model. While gene-targeted *Cftr* 'knock-out' (*Cftr* $^{-/-}$) mice presented with severe intestinal disease, *Cftr* $^{-/-}$ mice did not develop CF-like lung disease. The observation that human CF airways typically exhibit increased Na^+ absorption along with defective CFTR-mediated Cl^- secretion led to the hypothesis that increased activity of epithelial Na^+ channels (ENaC, also known as SCNN1) may play an important role in the pathogenesis of CF lung disease. To test this hypothesis *in vivo*, we generated a transgenic mouse model with airway-specific overexpression ENaC. ENaC is a heteromultimeric protein composed of three subunits (?, ?, ?). Interestingly, we found that overexpression of ?-ENaC (also known as *Scnn1b*) alone was sufficient to cause a significant increase in Na^+ transport across freshly excised airway tissues, suggesting that the expression level of the ? subunit is rate limiting for airway Na^+ absorption. Increased airway Na^+ absorption significantly altered the homeostasis of the airway surface liquid (ASL): the height of the periciliary liquid layer (PCL) was reduced, and the mucus concentration (% solids) was increased in ?-ENaC overexpressing mice compared to wild-type littermate controls. This ASL volume depletion resulted in slowed mucociliary clearance (MCC) and mucus adhesion to airway surfaces, and caused a severe spontaneous lung disease sharing key features with CF in humans. ?-ENaC overexpressing mice showed significant postnatal pulmonary mortality (~40% survival at 4 weeks). Histopathologic evaluation of lungs from deceased ?-ENaC transgenic mice revealed severe mucus obstruction and goblet cell metaplasia throughout the airways, suggesting that death was caused by mucus plugging. Studies on surviving ?-ENaC transgenic mice demonstrated that airway Na^+ hyperabsorption and mucus obstruction were associated with sterile neutrophilic inflammation and slowed clearance of bacterial pathogens such as *Pseudomonas aeruginosa* and *Haemophilus influenzae*. Taken together, the ?-ENaC overexpressing mouse demonstrates that increased airway Na^+ absorption *in vivo* is sufficient to initiate CF-like lung disease. We expect that this novel animal model will allow us to evaluate various factors contributing to the complex pathophysiology of CF lung disease, and of novel therapeutic interventions to prevent or ameliorate CF lung disease *in vivo.*

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S6.4 Mouse Models for Mucus Hypersecretion

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The accumulation of mucus is a key factor in the pathogenesis of cystic fibrosis lung disease. Mucin is composed of water, electrolytes, and mucins. Mucins are glycoproteins with a protein backbone to which abundant carbohydrate side chains are attached and are encoded by MUC genes. The regulation of the expression of MUC genes or of the secretion in incompletely understood. Many factors contribute to this complex process. It is the goal of this presentation to summarize mechanisms of mucus biology highlighting the methods used to investigate mucus secretion in vitro and in vivo.

In vitro different cell types are currently used to study the regulation and secretion of secretory mucins. Cell lines might significantly differ from primary airway epithelial cells. Most studies use quantitative RT-PCR to measure the amounts of transcripts and apply antibody or lectin based methods to detect the mucin amount.

Different animal models are used to study mucus secretion. The mouse represents the most important models based on the availability of genetically modified strains. Nevertheless, the airway anatomy and physiology differ from the human. Morphological, molecular biological and immunological methods are used to study mucin expression. Different mouse models are presented and discussed.

S6.5 Animal Models of the Chronic *P. aeruginosa* Lung Infection in Cystic Fibrosis

Niels Høiby

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Characterization of Novel Airway Submucosal Gland Cell Models for Cystic Fibrosis Studies

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CFTR is expressed in most native epithelial tissues at very low levels, with the exception of the intestine and the airway submucosal glands which appear to have higher endogenous CFTR expression. The submucosal glands have also been proposed as the primary site for initiating and sustaining CF airway disease [1].

Due to the very low levels of endogenous expression, as well as to the limited availability and size of native epithelial tissues, primary cultures and immortalized cell lines constitutively synthesizing the protein have been developed to characterize the biochemical and genetic mechanisms underlying CF. However, because most of these are non-epithelial and/or are non-polarized epithelial cells or do not normally express CFTR they have a limited applicability for the assessment of vectorial ion transport, secretion, trafficking and other differentiated functions. Recently, a number of *in vitro* studies have used an airway adenocarcinoma cell line, Calu-3 producing wild-type CFTR mRNA and protein in significant amounts [2]. As a complement to this cell line we have more fully characterized two previously isolated CF tracheobronchial gland epithelial cell lines, CFSMEo- and 6CFSMEo- [3].

Following PCR-amplification and DNA sequencing of genomic DNA, these cell lines were found to be compound heterozygotes for the F508del and Q2X mutations. We further characterized these cells regarding their CFTR RNA and protein expression, as well as their electrophysiological properties. We determined that both these cell lines only have residual CFTR mRNA expression and do not produce CFTR protein in detectable amounts. Electrophysiologically, both cell lines are characteristically CF as they lack cAMP-induced Cl^- currents.

Together with Calu-3 cells, these airway epithelial cell systems provide defined culture systems to study the biology and pathology of CF. They may also constitute a useful null-CFTR control cell line required by numerous studies, namely those involving gene therapy through CFTR complementation or gene targeting.

References:
[1] McNicholas *et al*. (1996) *Proc Natl Acad Sci USA* **93**: 8083-8.
[2] Farinha *et al*. (2004) *Mol Cell Probes* **18**: 235-42.
[3] Cozens *et al*. (1992) *Proc Natl Acad Sci USA* **89**: 5171-5.

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SYMPOSIUM 7 – Host Bacterial Interactions
Chair: Gerd Döring, Tubingen (Germany)

S7.1 New Insights into the Interaction of *Pseudomonas aeruginosa* with the CF Lung Epithelium

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Whereas the pathogenesis of the chronic bacterial infection and the neutrophil-dominated inflammatory response is well described in the endobronchial space of patients with the hereditary disease cystic fibrosis (CF), little is known in this context about the alveolar region. We quantified macrophage and neutrophil numbers and elastin and collagen fibers in peripheral lung tissue from CF patients who underwent lung transplantation and in healthy controls. Furthermore, we stained alveolar tissues for the inflammatory markers nF?B, ICAM-1, IGF-1, IL-8 and for *P. aeruginosa*. Lastly, we measured the elastin degradation products desmosine and isodesmosine in urine samples from both groups. Significantly increased numbers of highly NF?B and IGF-1-positive macrophages and elastase-positive neutrophils were present in alveola of CF patients. In addition, also CF alveolar epithelial cells revealed the presence of inflammatory markers NF?B, ICAM-1, IGF-1, and IL-8, in contrast to controls. Taken together, the data reveal increased inflammation and suggest tissue remodeling. Indeed, alveolar remodeling was demonstrated by a significantly increased collagen and decreased elastin fiber content of alveolar septa. Lung elastin degradation decreasing with age was also evident by high desmosine and isodesmosine urine concentrations in CF patients. We hypothesise that single bacteria entering the alveoli via aerosols, derived from the endobronchial space, trigger alveolar inflammation permanently and thus causing more or less rapid lung function decline in CF. Indeed, exopolysaccharide producing *P. aeruginosa* bacteria were detected in some but not all inflamed CF alveoli.

S7.2 The CGD Mouse Model for Assessing *Burkholderia cepacia* Complex Virulence

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In patients with cystic fibrosis (CF), bacteria of the *Burkholderia cepacia* complex (BCC) can cause long-term asymptomatic airway colonization or severe lung infection, leading to rapid pulmonary decline [1]. Little is known about the virulence factors of BCC strains associated with poor clinical outcome in CF patients. Appropriate host models to study BCC infection may be helpful in this context, but existing models do not mimic the clinical course of BCC infection in CF patients. Here we present evidence that CF-related virulence of BCC strains can be monitored in mice with a null allele of the gene involved in X-linked chronic granulomatous disease (CGD) [2]. Groups of $gp91^{phox-/-}$ mice, challenged intratracheally with 10^3 cells of 5 *B. cepacia* complex strains, differing largely in CF-related virulence, also differed largely in granuloma formation and time to death. *B. cenocepacia* J2315, a member of the highly transmissible and virulent lineage ET12, caused extensive necrotising pneumonia, abscess formation and death within 3 days after challenge, whereas a clinical CF isolate of *B. vietnamensis* was completely avirulent. Furthermore, several mutants from two wild type strains, defective in either exopolysaccharide biosynthesis [3] or quorum sensing mechanism [4] revealed largely diminished or absent granuloma formation and mortality. Taken together, we show that BCC strains differing in virulence in CF patients can be distinguished in CGD mice. This animal model may therefore be helpful in assessing virulence in BCC strains as well as providing rapid prognostic information for CF patients infected with BCC strains.

References:
[1] Govan, JRW and Deretic, V (1996) *Microbiol. Rev.* **60**: 539-574.
[2] Pollock, JD *et al.* (1995) *Nat. Gen.* **9**: 202-209.
[3] Moreira, LM *et al.* (2003) *Biochem. Biophys. Res. Commun.* **312**: 323-333.
[4] Huber, B *et al.* (2001) *Microbiology* **147**: 2517-2528.

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S7.3 Gallium Prevents Biofilm Formation by *P. aeruginosa*.

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In the chronic airway infections that afflict cystic fibrosis patients, *P. aeruginosa* live in matrix-encased groups known as biofilms. Biofilms are often thought of as communities of bacteria because the cells occupy a defined territory, they can exhibit coordinated behaviour facilitated by communication, and because key biofilm characteristics (like their marked resistance to killing) cannot be generated by the bacteria living as individuals. Work from several laboratories suggests that the concentration of iron in the environment is a key signal that promotes biofilm formation. For example, the Fe-binding protein lactoferrin, found in high concentrations in pulmonary secretions, prevents biofilm formation by promoting surface motility. Because free iron concentrations are already very low in mucosal secretions, we have explored alternative methods to produce iron limitation in bacteria. Gallium (fw=69.7) has many features similar to Fe^{3+}, including a nearly identical ionic radius. Ga^{3+}, efficiently enters cells via the same mechanism that cells utilize to acquire Fe, however substitution of Ga for Fe in Fe-containing enzymes renders them inactive since Ga^{3+}, in contrast to Fe^{3+}, is not able to undergo redox cycling. In this presentation we will present evidence showing that Ga can disturb *P. aeruginosa* biofilm formation, most likely by interfering with Fe acquisition/metabolism. Administering gallium may be a promising therapeutic approach as $Ga(NO_3)_3$ is already approved for use in humans and has a relatively low side effect profile.

P14

S7.4 Modulation by Dietary n-3 and n-6 Fatty Acids of Lung Mucin Expression After Infection by P. *aeruginosa*

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Overproduction of mucus with altered rheologic properties in cystic fibrosis contributes to mucus obstruction of the airways. Mucus obstruction is the culmination of several complex processes including mucin gene regulation. Mucins are complex O-glycoproteins with a high-M_r. The two main proteins secreted in the lung are the two mucins MUC5B and MUC5AC while MUC2 is present at low level. It has been suggested that MUC5AC may have the mechanical function of facilitating ciliary clearance of mucus while MUC5B may form the basis of the gel, which helps with the clearance of specific pathogens like *P. aeruginosa*. Among membrane-bound mucins, MUC4 is well characterized and expressed at the luminal surface of the epithelium. To date, most studies in the lung have focused on MUC2 and MUC5AC upregulation while MUC5B regulation has been largely ignored. It has been reported using airway culture cells and explants that LPS and *P. aeruginosa* both upregulate these two genes. An alternative promising therapeutic strategy to immune suppressive pharmaceutics could be to decrease the immune response using an adapted diet in order to slow down or inhibit the pathogenesis of cystic fibrosis. It has been demonstrated that supplemented diets with ? 3 PUFAs inhibit several parameters of inflammatory reactions.
In this study, we evaluated blindly the impact of PUFAs on mucin gene expression using mice infected or not by *P. aeruginosa* (PA01 strain). Male C57BL/6 mice were fed for 5 weeks with either a regular blend or a blend supplemented with either ? 3 or ? 6 PUFAs. One-day, 4-days or 7-days post-infection by *P. aeruginosa*, lungs RNAs were retro-transcribed. We have developed an absolute real-time PCR strategy to study the differential expression of the three mucins Muc5b, Muc5ac and Muc4. Duplex PCR amplifications were carried out using the 18S RNA as an internal positive control. Real-time PCR were performed on the ABI Prism 7700 SDS. Statistical analysis was conducted using the statistical package StatXact® 6.0 for exact nonparametric inference and a p-value = 0.05 was considered statistically significant.
cDNA plasmid standards were obtained. Standard curves for Muc genes and the endogenous 18S control gene were generated with a very efficient and sensitive amplification as indicated by slopes and y-intercepts. In the absence of infection by *P. aeruginosa*, Muc5b and Muc5ac were barely detectable in contrast to Muc4 and there were no difference between the three diet groups. Infection by *P. aeruginosa* induced an up-regulation of Muc5b and a drastic down-regulation of Muc4 contrasting with a slight decrease of Muc5ac RNA expression. Up-regulation of Muc5b is significantly modulated by the diet after infection with the lower expression of the mucin for mice fed with a blend supplemented with n-3 PUFAs and the higher expression for mice fed with a blend supplemented with n-6 PUFAs. Finally, we found strong correlations between Muc5b, Muc5ac and Muc4 expressions suggesting that (i) infection by *P. aeruginosa* may induce a transdifferentiation of ciliated cells to a secretory phenotype or differentiation of progenitor cells and (ii) the differentiation could be modulated by fatty acids.

P15

CF Blood Neutrophils Demonstrate Delayed Apoptosis and are Resistant to the Early Killing Effect of TNF-a

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Aims The inflammatory response in CF is neutrophil dominated. Neutrophil apoptosis is a key anti-inflammatory mechanism. Previous studies identified that neutrophil apoptosis is delayed in the airways of CF subjects. We have examined CF neutrophil apoptosis in the vascular compartment in the presence or absence of GM-CSF and TNF-a.
Method Blood was taken from twelve CF subjects with age matched healthy controls. Neutrophils were purified by discontinuous Percoll gradients. Freshly harvested neutrophils were suspended at 5 x 10^6/ml in Iscoves MDM supplemented with 10 % autologous serum and cultured at 37º C in flat bottom 96-well Falcon flexiwell plates in presence or absence of test reagents (TNF-a 200 U/ml, GM-CSF 10 ng/ml). Apoptosis was assessed morphologically using oil immersion light microscopy. Apoptotic neutrophils were defined as cells containing darkly stained condensed pyknotic nuclei.
Results At six hours there was no difference in percentage survival between CF and control neutrophils (CF 3.6±0.8 %, control 6.5±1.3 %, n=12, p=0.07). At twenty hours CF neutrophils demonstrated significantly increased survival (CF 43±4.7 %, control 56±3.1 %, n=12, p=0.0046). Both CF and control neutrophils responded to the pro-survival effect of GM-CSF at twenty hours equally (CF 30±5 %, control 26±3.4 %, n=11, p=0.6). However, at six hours there was a significant difference in the pro-apoptotic effect of TNF-a (CF 4.2±1.6 %, control 10.8±2.1 %, p=0.04).
Conclusion The identification of a pro-survival phenotype in the CF neutrophil and it's resistance to TNF-a early killing may contribute to the over exuberant inflammatory response seen in CF.

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P16

Defective CFTR Enhances PAO1-Stimulated Inflammatory Response in Epithelial Cells

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Rationale: the pathogenesis of CF lung disease is not fully elucidated. The hallmark of CF patients is chronic colonisation by *Pseudomonas aeruginosa (PA)* in the lung leading to neutrophil-mediated inflammation. Airway epithelial cells express chemokines in response to a variety of stimuli and therefore contribute to the overall inflammation within the lung. Infection plays a major role in stimulating inflammation in the airways; however there is a consensus that in CF the response to infection is dysregulated and excessive. **Aim:** to study the inflammatory response to *PA* infection in epithelial cell lines expressing mutated CFTR protein. **Methods:** Cell lines: C127, mouse epithelial with expression of CFTR w/t or F508del; IB3, human bronchial epithelial CFTR F508del/W1282X; S9, IB3 corrected with CFTR w/t. Infection: ranging doses (0.05-5 cfu/cell) of a laboratory strain of *PA* (PAO1). Inflammatory response: ICAM-1 and IL-8 mRNA levels quantitated by real time PCR; IL-8 protein secretion measured by ELISA. Bacterial binding: metabolically labelled [^{35}S] PAO1. **Results:** very high levels of ICAM-1 mRNA (up to 400 fold) are measured in C127 cells after 4 hours of infection. ICAM-1 mRNA induction is significantly higher in C127 CFTR F508del than in CFTR w/t cells in respect to steady-state unstimulated level (314 ? 106 vs 149 ? 63 fold induction, n=4, $p<0.04$ by Student's t test). A sharply increased response of C127 F508del cells appears within 2 hours after infection and this does not correlate with augmented bacterial binding to the cells. Infection of IB3 cells with PAO1 up-regulates both ICAM-1 and IL-8 mRNA up to 25 and 1000 fold respectively. Induction of ICAM-1 mRNA, IL-8 mRNA and IL-8 protein secretion are significantly higher in IB3 than in S9 cells ($p<0.03$, $p<0.007$, $p<0.008$ by ANOVA). **Conclusions:** these results indicate that defective CFTR protein enhances the inflammatory responses elicited by PA infection of epithelial cells.

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P17

Anti-Pseudomonas IgY – A New Drug for the Treatment and Prophylaxis Against *Pseudomonas aeruginosa* in Patients with Cystic Fibrosis

Hans Kollberg and Anders Larsson

Hypothesis: Gargling with a solution of anti-pseudomonas IgY is a new therapy that will prevent infections with *P. aeruginosa* due to inhibition of adhesion of bacteria to epithelium.

Immunoglobulin Y (IgY) in eggs is a maternally derived passive immunity that protects chicken from infections. Hens vaccinated with microbes produce specific anti-IgY. We have vaccinated hens with *P. aeruginosa to* produce anti-pseudomonas IgY. Our anti-pseudomonas IgY inhibits adhesion of *P. aeruginosa* to epithelial cells. Gargling with IgY in the evening gives IgY activity in saliva overnight. IgY is generally recognized as safe (U S Code Federal Regulations): Oral IgY does not activate any cell activators or mediators of inflammation. Pathogens will not develop resistance against IgY. Humans do not produce anti-IgY antibodies against orally administered IgY.

Infections with *P. aeruginosa* occur in virtually all CF-patients and are impossible to eradicate from a patient with chronic colonization. All *P. aeruginosa* have to pass oropharynx to infect the lungs. The passage happens mainly during night.

Results: Our study on anti-pseudomonas IgY for CF has gone on for 10 years. Thirteen CF patients have gargled daily with anti-pseudomonas IgY, altogether for about 80 patient-years. The results to April 2001 were reported in Pediatric Pulmonology 2003. Nov. 2003 the Swedish Medical Products Agency granted license for anti-pseudomonas IgY (produced according to GMP-standards) to CF-patients, provided that observations for effect and adverse events continued. The frequency of positive PA cultures over all the years is still about 1/40 months of treatment and the need of antibiotics is substantially decreased. Only one patient has become chronically colonized with a non-mucoid P. aeruginosa *strain.* All patients continue to do well with maintained pulmonary functions and very few days of illness. No cultures have shown *B. cepacia*, and very few *Aspergillus fumigatus* – probably due to reduced use of antibiotics. There have been no advert events.

Conclusion: Anti-pseudomonas IgY is a valuable drug for prevention and early treatment of *Pseudomonas aeruginosa.*

SYMPOSIUM 8 – Novel Therapies
Chair: Olga Zegarra-Moran, Genoa (Italy)

S8.1 Progress towards Novel Therapeutic Modulators of CFTR

A Singh

S8.2 Pharmacologic Activation of Chloride Transport in CF Mutants

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Most mutations cause CF either by reducing the CFTR channel function (gating defect) or by causing a folding defect, an arrest of protein processing, and thus the absence of CFTR on the plasma membrane (trafficking defect). The F508del, which is present in about 70% of all mutated alleles, displays both defects. In the last years, we have used a high throughput screening approach to identify small organic molecules that, acting al low concentrations, are able to restore normal chloride permeation in CF cells. Indeed, it is believed that restoring CFTR chloride channel activity would improve the clinical conditions of CF patients. The primary screening of chemical libraries has been done using a fluorescent assay on Fisher rat thyroid cells cotransfected with the halide-sensitive yellow fluorescent protein, YFP, and mutant CFTR. Up to date 150,000 compounds have been screened for CFTR correctors (drugs able to correct the trafficking defect), and 200,000 for CFTR potentiators (drugs able to correct the gating defect). Once hits were identified, sublibraries were created and screened. To define the potency and specificity of active drugs we used electrophysiological methods on transfected cell lines and on primary cultures of human bronchial epithelial cells. While screening for CFTR correctors have yielded only a few compounds that worked at relatively high concentrations on primary bronchial epithelial cells, many families of potentiators have been identified. These compounds neither increase cAMP concentration nor inhibit cell phosphatases, suggesting a direct action on CFTR and are active on transfected cell lines as well as on primary epithelial cells. Some of the potentiators, such as benzothiophenes, phenylglycines, and sulfonamides displayed very high potencies. The most potent phenylglycine reversibly activated F508del-CFTR in the presence of forskolin with Kd ~70 nM, and was also active on other gating mutants such as G1349D, D1152H and G551D. The affinity for G1349D was similar to that displayed for the F508del protein, while the affinity for G551D was shifted toward much higher values (~1000 nM). The Kd for D1152H was in-between these values. In contrast with phenylglycine, benzothiophenes and sulfonamides were very potent on F508del (Kd ~20 nM), but inactive on G551D and G1349D. We investigated the activating mechanism of these compounds in cell-attached patches of F508del cells. We found that all of them increased the channel open probability from about 0.04 in the presence of forskolin alone, to 0.3-0.4. Kinetics analysis indicated that the increase in open probability was mainly due to a reduction of the interburst closed time rather than an increase of the mean open time. Our results suggested that these compounds might be useful for monotherapy of CF disease caused by gating mutants and possibly for a subset of F508del subjects with significant CFTR protein in the plasma membrane.

This work was supported by the Cystic Fibrosis Foundation Therapeutics, Italian Cystic Fibrosis Foundation and Telethon-Italy.

S8.3 Anti-Sense Therapy by Targeting EnaC-Expression

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The epithelium sodium channel (ENaC) is assumed to play a major role in the pathogenesis of chronic lung disease in cystic fibrosis patients. Its natural regulation by the cystic fibrosis transmembrane conductance regulator (CFTR) appears to be compromised based on the impaired function of CFTR. The missing downregulation of the channel results in increased absorption of sodium ions and fluid across airway epithelia leading to the depletion of the perciliary liquid layer and to the depression of mucus clearance.
Several observations suggest that a downregulation of ENaC restores the perciliary liquid layer, thereby rehydrating the mucus and improving ciliary clearance in the lung. Therefore, specifically downregulating EnaC expression by RNA interference is a promising approach.
In this talk new means of nucleic acid precision targeting both on the molecular and macroscopic level will be presented to target ENaC-Expression in the airways.

S8.4 Change of Ion Transport by Thai Medicinal Plant Extracts from *Phyllanthus acidus* and their Potential Application for the Treatment of Cystic Fibrosis

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Previous reports suggested that naturally occurring flavonoids and nutraceuticals may be used to target the defective electrolyte transport in cystic fibrosis (CF) airways and thus may serve as low cost clinical treatments for CF. Since many of the traditional medicinal plants from China and Thailand contain phyto-flavonoids, we examined the effects of a herbal plant extract from the Euphorbiaceae *Phyllanthus acidus* (*PA*) on epithelial transport and tried to identify the underlying mechanisms. In Ussing chamber recordings of mouse trachea *PA* induced a DIDS- sensitive Cl^- secretion in a concentration dependent manner, when applied to the luminal side of the epithelium. The short circuit current activated by *PA* had a transient and a steady state component, which was due to increase of both intracellular Ca^{2+} and cAMP. Stimulation by UDP as well as the purinergic blockers suramin and PPADS interfered with the effect of *PA* on Cl^- secretion, while ATP or carbachol induced a Cl^- secretion in addition to that induced by *PA*. Parallel K^+ and Cl^- conductances were activated by *PA* in patch clamp experiments with airway epithelial cells. Effects of *PA* on *Xenopus* oocytes expressing cystic fibrosis transmembrane conductance regulator and epithelial Na^+ channels demonstrate activation of CFTR by *PA* and parallel inhibition of ENaC. Since a main component of *PA* is adenosine, the steady state response was inhibited by the A2B blocker 8-SPT. Taken together, these experiments suggest that traditional Thai medicinal plants may be useful for the treatment of cystic fibrosis.

S8.5 Bacterial Transfer of Functional DNA into Mammalian Cells

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Efficient transfer of chromosome-based vectors into mammalian cells is difficult, mostly due to their large size and most gene delivery systems do not allow efficient transfer of large (> 100 kb) DNA fragments, thus limiting their functional analysis. The currently used viral derived vectors do not provide sufficient packaging capacity; non-viral delivery systems based on lipofection, combined or not with polycations, have no size limits and have been used with some success to transfer intact BAC DNA. However, this technique requires production and purification of the DNA construct prior to transfection, a step which can impair its integrity. Intracellular attenuated bacteria such as *Shigella*, invasive *Escherichia coli*, *Salmonella*, and *Listeria* can transfer functional genes to a very broad range of mammalian cells. We have shown that invasive *E. coli*, that undergo lysis upon entry into mammalian cells because of impaired cell wall synthesis due to diaminopimelate auxotrophy, can deliver plasmid DNA to host cells. Plasmid pGB2? *inv-hly* containing the *inv* gene from *Yersinia pseudotuberculosis* and the *hly* locus from *Listeria monocytogenes* has been introduced into the dap auxotroph *E. coli* BM2710. The *inv* gene confers to *E. coli* the ability to invade non-phagocytic cells provided they express ?1-integrins. The *hly* gene product, listeriolysin O, is a pore forming cytolysin that allows escape of the bacteria, or of its cytoplasmic content, from the vacuole of entry. Transfer of functional DNA into a variety of mammalian cell lines occurs after simple co-incubation with the bacterial vector.
Using this genetically engineered invasive *Escherichia coli* vector, alpha satellite DNA cloned in P1 based artificial chromosome (PAC) was stably delivered into HT1080 cell line and efficiently generated human artificial chromosomes (HACs) de novo. Similarly, a large genomic CFTR construct of 160 kb was stably propagated in the bacterial vector and transferred into HT1080 where it was transcribed, after correct splicing, indicating transfer of an intact and functional locus of at least 80 kb. These results demonstrate that bacteria represent a valuable tool for the transfer of large, intact and functional fragments of genomic DNA. Engineered bacteria allow the cloning and propagation of large DNA fragments and their subsequent direct delivery in cells for functional analysis.

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S8.6 Development of a High Throughput Assay for the Identification of Novel ENaC Blockers

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The pyrazinoylguanidines (e.g. amiloride, benzamil) currently represent the only known class of ENaC blockers. Amiloride is widely used as a K+-sparing diuretic and has also been demonstrated to enhance mucociliary clearance in Cystic Fibrosis (CF) when delivered into the airways as an aerosol. The limited clinical efficacy of amiloride inhalation in CF was believed to be due to low target potency (IC50 on ENaC ~ 500nM) combined with rapid clearance of drug substance out of the airway lumen. A novel ENaC blocking molecule with improved potency and a pharmacokinetic profile well suited to inhaled delivery would represent a novel potential therapy for CF lung disease. To this end we have developed a high throughput, cell-based assay to screen compound libraries for novel scaffolds showing activity at ENaC.

Murine kidney epithelial cells (M1) were cultured in 96 or 384 well plates for 5-7 days in PC-1 media supplemented with glutamine (5mM). This period in culture was sufficient for the cells to reach confluence and to observe the formation of "domes" indicative of a transport phenotype. On the day of assay, media was aspirated and the cells washed 3X in assay buffer (HBSS containing 20mM HEPES; pH 7.4). Cells were then incubated with membrane potential dye (Molecular Devices) in assay buffer for 30-60 minutes (37°C). Plates were then placed into a Fluorometric Imaging Plate Reader (FLIPR, Molecular Devices) and baseline fluorescence monitored for 60s at which time test compound was added on-line. Following a further 240s a maximal concentration of amiloride (30µM) was added to each well to enable the calculation of the maximal response. From the changes in fluorescence it was possible to construct concentration-response data to confirm the appropriate pharmacology for ENaC with a blocker potency order of benzamil (50nM) > amiloride (2µM) >> dimethylamiloride (8µM). Screening of a commercially available ion channel-focussed library (BIOMOL, USA) resulted in the identification of only known ENaC blockers (phenamil, dichlorobenzamil).

This assay can therefore be utilized to screen large compound collections to identify potentially novel scaffolds with ENaC-blocking activity. Such compounds will enable the initiation of drug discovery efforts to design and optimise ENaC blockers suitable for inhalation therapy in CF.

P19

S8.7 N1, A Medicine Prescribes for an Orphan Disease, is Able to Restore the Defective Trafficking of F508del-CFTR

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The cystic fibrosis transmembrane conductance regulator (CFTR) is a plasma membrane-associated glycoprotein. The protein can exist in three different molecular weight forms, representing either nonglycosylated, core glycosylated, or fully mature complex glycosylated CFTR. It has been reported that only the fully mature form reaches to the surface membrane, where it functions as a chloride channel. Newly synthesized glycoproteins interact during folding and quality control in the ER with calnexin, a lectin specific for monoglucosylated oligosaccharides. The most common mutation in cystic fibrosis (CF) results in the synthesis of F508del-CFTR that is incompletely glycosylated and defective in its trafficking to the cell surface.

In this study, we bring to light the effect of N1 on the abnormal trafficking F508del-CFTR from the human airway epithelial CF cell line JME/CF15 (F508del/F508del). Immunoprecipitation, in conjunction with endoglycosidase-H digestion and western-blot analysis show appearance of mature glycosylated band C form of F508del-CFTR protein, which indicate that CFTR exit the ER and undergoes post-synthetic processing in Golgi complex. Confocal immunofluorescence studies show restoration of the mature F508del-CFTR at the cell surface after a treatment of 2 hours at 37°C. Moreover, the recovery of the F508del-CFTR chloride channel activity is demonstrated by iodide efflux and patch-clamp recordings. Additional experiments show a correction of defective trafficking of F508del-CFTR from others CF types cells after a 2 hours of N1 treatment: i) electrophysiological and iodide efflux analysis reveal the functional expression of F508del-CFTR at the cell surface of GFP-F508del-CFTR cos-7. ii) N1 restores an iodide efflux on the human CF tracheal submucosal gland serous cell line CF-KM4 (F508del/F508del). iii) Exposure to N1 of human nasal epithelial cells, obtained by brushing from individuals with CF, results in recruitment of F508del-CFTR to the apical membrane. iv) Lastly, Ussing chamber studies show that N1 corrects the abnormal localization and chloride secretion of F508del in intestine from F508del/F508del mice.

We conclude from the present study that N1 restores the abnormal trafficking of F508del-CFTR via the inhibition of calnexin/F508del-CFTR interaction. Consequently, N1 treatment interferes with the ability of the ER quality control machinery to interact and retain the F508del protein into the ER. N1 interest reside in the fact that it is already a medicament for the treatment of another orphan disease. A phase IIA clinical trial is under preparation with this agent for cystic fibrosis.

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Adenovirus Vector Receptor Interactions and Early Pro-Inflammatory Signalling

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Since the cloning of the CFTR gene, different virus-derived vectors such as adenoviruses (Ad), adeno-associated parvoviruses (AVV) and lentiviruses (LV) have been developed for the transfer of CF gene into the epithelial cells of the respiratory tract. This study is addressed to add information on the signalling pathways involved in the early induction of the inflammatory response associated with gene transfer vector. We previously reported that one mechanism of inflammation is the induction of the pro-inflammatory Intercellular Adhesion Molecule (ICAM)-1 by Ad vectors, which is mediated by MAPKs and Nuclear Factor-kB (NF-kB), even in the absence of viral gene transcription. Signal transduction through the heparan-sulfate glycosoaminoglycans (HS-GAGs), which are receptors of Ad, AAV and LV has been studied. We used the Ad vector model since it is known that Ad binds to cell through three receptors, namely HS-GAGs, the coxsackie adenovirus receptor (CAR) and ?$_v$-integrin. Ad domains, ?$_v$-integrins and extracellular receptor components have been tested in A549 respiratory cells. Results indicate that the activation of MAPKs and NF-kB in the time lapse between 10 min to 4 hrs upon exposure of the cells to Ad domains is due to CAR, but not to HS-GAGs or ?$_v$-integrins. Therefore, HS-GAGs does not play a role in the early signals relevant to the inflammatory response. This study contributes to a better understanding of Ad domains interactions with the cellular receptors in the early induction of pro-inflammatory signals and may help in developing novel and safe vectors for gene therapy applications.

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New Screening Method Based on Calcium Measurement to Identify Drugs Able to Correct F508del-CFTR

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The most common mutation in cystic fibrosis, F508del, results in cystic fibrosis transmembrane conductance regulator (CFTR) protein that is retained in the endoplasmic reticulum (ER). Retention is dependent upon chaperone proteins, many of which require Ca^{++} for optimal activity. Interfering with chaperone activity by depleting ER Ca^{++} stores might allow functional F508del-CFTR to reach the cell surface. Treatment with the ER Ca^{++} pump inhibitors (thapsigargin, curcumin), releases ER-retained F508del-CFTR to the plasma membrane, where it functions effectively as a Cl^- channel. In order to discover new agents able to relocalize a functional F508del-CFTR to the cell surface, we are routinely screening pharmacological agents using iodide efflux technique.

Recently, we develop a complementary method which consist for each drug to determine the capacity to mobilize the intracellular Ca^{++} store by confocal microscopy Ca^{++} measurement with a fluorescent Ca^{++} indicator (Fluo-4). This new aspect of the screening allow us to find new correctors of F508del-CFTR which mechanism of action involves a calcium-dependent pathway. Two protocols are used to show the intracellular calcium mobilization. The first one consists in the measurement of the calcium mobilisation during 2 hours which correspond to the treatment time using the iodide efflux technique. The second one is realised during 5 minutes and uses ATP as control to empty the calcium stores on F508del-CFTR human nasal epithelial cells. The response obtained with new drugs is compared to the ATP-response. Finally, we confirmed that the mobilisation of calcium comes from the empty of endoplasmic reticulum in extracellular free calcium medium.

Using this new screening method, we found new drugs. For example CFTR-T1 which permit the relocalization of F508del-CFTR to the plasma membrane while producing an empty of the intracellular calcium store. We show that CFTR-T1 produces an important phasic elevation of calcium. This new agent CFTR-T1 induces the same calcium response in presence or in absence of extracellular calcium, this suggest that the mobilization is due to the empty of intracellular calcium store. To confirm this point, we used 2-APB (an inhibitor of IP3 receptor) and observed that 2-APB induces a total inhibition of the calcium mobilization. Moreover, using the iodide efflux technique, we show that 2-APB leads to a partial inhibition of the F508del-CFTR activity produced by CFTR-T1 treatment. These results suggest two mechanisms of action for CFTR-T1: one dependent and one independent of the intracellular calcium.

This new screening method which compiles iodide efflux technique and confocal calcium measurement should help to discover new calcium-dependent molecules able to correct F508del-CFTR.

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P22

Compatible Solutes as Potential Therapeutic Agents for Cystic Fibrosis

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The assembly, trafficking and turnover of CFTR is a very complex process that is not fully understood. Experimental evidence shows that the most frequent mutation -F508del- causes the resulting CFTR protein to misfold, being thus mostly retained by the endoplasmic reticulum quality control (ERQC) and subsequently targeted for degradation, resulting in a trafficking defect. Because F508del-CFTR retains some function and owing to its temperature sensitivity in folding, compounds known to stabilize proteins against thermal treatments could prove useful in the management of CF. Compatible solutes are stabilizers of biological structures produced by extremophilic microorganisms to provide protection from extreme environmental conditions like *e.g.*, heat, UV radiation or dryness [1]. In particular, compatible solutes from thermophilic organisms are very effective in protecting proteins against heat inactivation [2]. In order to test whether these compounds correct the protein folding defect of F508del-CFTR, here we assessed the impact of compatible solutes effect in wild type (wt) and F508del-CFTR processing and function. The following compounds (kindly provided by Bitop AG, Witten, Germany) were tested: DIP (di-myo-inositolphosphate), DGP (diglycerolphosphate), Firoin-A (mannosylglyceramide), Firoin (mannosylglycerate), Ectoine and Hydroxyectoine. Preliminary toxicity tests were carried out without and with osmolarity correction (from 350 Osm to 500 Osm by addition of 100 mM NaCl) in order to enhance compound uptake. Cellular viability was evaluated using the trypan blue assay in BHK cells stably transfected with wt- or F508del-CFTR, following 24h-treatment with 0.02-2 mM of each solute. Cells were found to be tolerant to the various compatible solutes in this concentration range (in triplicate experiments). The effect of each compatible solute on the processing of wt- and F508del-CFTR was evaluated by Western-blot (anti-CFTR Lis-1 antibody [4]). The same cells were incubated for 24h with 0.5 mM, 1 mM and 2 mM of each solute. Results did not evidence any detectable effect of the studied compatible solutes in F508del-CFTR processing. However, treatment with Firoin for 24h (with osmolarity correction) seems to enhance the amount of processed wt-CFTR (band C). Functional assays (iodide efflux) are currently underway to confirm these results. Moreover, evaluation of higher doses of compatible solutes (up to 200mM) on processing of wt- and F508del-CFTR are currently being investigated, as these high concentrations are required to cause significant protein stabilization in vitro [3].

References:
[1] Santos H and da Costa MS (2001) *Methods Enzymol* **334**: 302-15.
[2] Lamosa *et al*. (2000) *Appl Environ Microbiol* **66** : 1974-1979.
[3] http://www.bitop.de/
[4] Farinha *et al*. (2004) *Mol Cell Probes* **18**: 235-42.

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SYMPOSIUM 9 – Modifier Genes / Transciptomics & Proteomics
Chair: Alexander Edelman, Paris (France)

S9.1 Characterization of Cystic Fibrosis Related Gene Expression by Microarray Analysis

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Our aim is to identify genes that show robust differential expression both in human Cystic Fibrosis (CF) cell lines and native tissues, for potential use as diagnostic markers of CF on a concurrently developed novel magnetoresistive sensor-based array platform. A comparison of mRNA abundance between selected pairs of "CF" and "non-CF" tissues and cells was thereforemade by using genome-wide "40K" (40,000 probes) oligonucleotide microarrays (MWG-Biotech, Germany). Firstly, we screened two pairs of human CF-related cell lines: (a) CFBE-41o- (*CFTR: F508del-homozygous*) paired with 16HBE-14o- (*CFTR: wt*; both human bronchial epithelial); (b) 6CFSMEo- (*CFTR: F508del/2QX*) paired with Calu-3 (*CFTR: wt*; both human bronchial submucosal epithelial). Secondly, we screened two pools of total RNAs extracted from nasal epithelial cells from CF patients (CFTR = *F508del-homozygous*) and healthy control subjects matched for sex (equal numbers of males and females in each pool) and age (subjects aged between 7 and 12). Total RNAs were extracted from all paired cell lines and tissues and used as templates for transcription of double stranded DNA followed by amplification of Cy-3 and Cy-5-labelled cRNAs, which were subsequently hybridized to the arrays. Gene expression ratios were tested for statistical significance and ranked using a Bayesian method based on the Normal distribution. Scanning of the 40K microarrays generated lists of significantly regulated genes for each pair of CF cell lines (74/41 upregulated and 57/86, downregulated, respectively), including genes involved in oxidative stress, apoptosis, inflammation, ion transport, intracellular signalling, extracellular matrix etc. These results are currently being validated using the *in vivo* data from the nasal epithelial cells. Furthermore, complementary data is also being generated using reduced pools of nasal cell RNAs hybridized to custom lung arrays (Affymetrix HsAirwaya520108F). It is expected that through the comparison of all data sets that is currently being undertaken, a very short list (5-10) of strongly regulated CF marker genes will be generated which can be used to design oligonucleotide probes for a novel magnetic sensor-based microchip array for high-throughput CF diagnosis.

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S9.2 Differential Gene Expression in CF vs. Normal Nasal Cells

Alexander Edelman

Cystic fibrosis (CF) is a human genetic disease due to mutations in *cftr* gene encoding for a cAMP-activated Cl^- channel (CFTR). However, the pathophysiology of this disease that is characterized by abnormal fluid transport across secretory epithelia and chronic inflammation in lungs, pancreas and intestine cannot be explained solely by mutations in *CFTR* and dysfunction of CFTR/chloride channel. Among different experimental strategies used to identify factors that are involved in the development of a variety of clinical phenotypes observed in CF patients, the proteomic approach (i.e. comparison of differential expression of proteins in a given tissue) offers the advantage to compare the large number of proteins between normal and "sick" samples. We are using this approach to compare the protein expression patterns for different experimental models (cell lines expressing CFTR or F508del-CFTR, mice normal vs. KO CFTR mice, nasal cells). The analysis of 2D gels unmasks different high abundance proteins that are differentially expressed in normal vs. CF tissues (keratin 8/18 i.e. cystoskeletal proteins, annexin 1 i.e. an anti-inflammatory protein). These proteins represent potential factors that play a role in the development of CF pathogenesis. The subsequent experiments using classical cell biology approaches and/or the exploration of patient's cells suggest that the identified proteins might be either targets for pharmacotherapy for CF (keratin 18) or an index of inflammation for stop codon mutations. Finally, the investigation of human nasal cells by bi-dimensional electrophoresis points to the necessity to develop new methodologies, which will allow a better sensitivity for the detection of proteins on 2D gels, and increased sensitivity for the subsequent identification of protein spots by mass spectrometry.

S9.3 Genomics Approaches to Host Defense Gene Discovery in Human Airway Epithelia: PLUNC and LPLUNC1

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We used cDNA library sequencing and expression profiling to identify novel transcripts in primary air-liquid interface cultures of human airway epithelia (HAE). Two transcripts, termed PLUNC ("Palate, Lung, Nasal Epithelium Clone") and LPLUNC1 ("long PLUNC1"), were among the most abundant messages in HAE derived from both cystic fibrosis (CF) and non-CF epithelia. The genes encoding PLUNC and LPLUNC1 belong to a larger cluster on chromosome 20q11.2. Other genes in this cluster include the lipid transfer/lipopolysaccharide binding (LT/LBP) family of proteins. PLUNC and LPLUNC1 share homology with bactericidal/permeability increasing protein (BPI), an innate immune molecule with both antibacterial and endotoxin-binding properties. We hypothesized that PLUNC and LPLUNC1 are secreted proteins involved in the innate immune responses of airway epithelia. To test this hypothesis, we used RT-PCR and microarray analysis to screen HAE for expression of PLUNC family members, and regulation of PLUNC and LPLUNC1 gene expression was investigated using quantitative RT-PCR. These approaches confirmed that PLUNC and LPLUNC1 are the most highly expressed LT/LBP family members in airways. Both gene products are expressed in the surface epithelium and submucosal glands of the conducting airways. Western analysis demonstrates that both PLUNC and LPLUNC1 are secreted products of airway epithelia, and both proteins were identified in HAE apical secretions and bronchoalveolar lavage (BAL) fluid. Preliminary functional studies suggest that LPLUNC1 associates with endotoxin, supporting a possible role in host defense responses. In conclusion, PLUNC and LPLUNC1 are highly expressed in conducting airway epithelia, regions that encounter microbial and other environmental insults on a daily basis. Ongoing studies are investigating possible host defense functions of these proteins. These studies should lead to a better understanding of the role that PLUNC and LPLUNC1 play in airway host defense.

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S9.4 The Protein Phosphatase 2A Binds to and Dephosphorylates the R Domain of CFTR

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In order to identify new proteins that interact with the R domain of CFTR, we performed yeast two hybrid screenings on a total human lung library with the R domain (amino acid 671-855) as bait. PR65, a structural subunit of the protein phosphatase 2A (PP2A), was found to interact directly with the R domain. This interaction was confirmed in two different independent biochemical assays. Pull down experiments showed that GST-PR65, expressed and purified from bacteria, bound to both the R domain and wt CFTR synthesized by *in vitro* transcription/translation in a rabbit reticulocyte lysate system. *In vivo* experiments showed that PR65 and CFTR could be co-immunoprecipitated from the cell lysate of COS cells transfected with CFTR cDNA. After *in vitro* phosphorylation by PKA, the R domain could be dephosphorylated by PP2A. This dephosphorylation was inhibited after addition of ocadaic acid, an inhibitor of PP2A. Further yeast two hybrid experiments could delineate the interaction domain in PR65 to the first ten HEAT repeats of PR65, were also the third variable PP2A B subunits bind. The catalytic subunit of PP2A (PP2AC) is known to interact with HEAT repeats 11-15. Therefore a ternary CFTR-PR65-$PP2A_C$ is possible and PR65 might recruit the catalytic subunit to the R domain. Overexpression of the first ten HEAT repeats in CaCO2 cells, showed a significant delay in deactivation of the chloride channel as shown in whole cell patch-clamp experiments. Probably these first ten heat repeats can compete with the binding of endogenous PR65 to the R domain, thereby also preventing $PP2A_C$ binding and interfering with the dephosphorylation and deactivation of the CFTR channel. Involvement of PP2A in the deactivation process is corroborated by the fact that treatment with ocadaic acid ion CaCO2 cells resulted in a prolonged deactivation of the CFTR channel. Taken together our results show a direct and functional interaction between CFTR and PP2A. The interaction of PR65 with the R domain probably also brings the catalytic subunit to this domain, which leads to dephosphorylation and inactivation of the CFTR channel. We are currently developing a strategy to determine the site of interaction within PR65 and CFTR. We hope that this knowledge can bring a more comprehensive insight into the network of CFTR interacting proteins and that this will help to elucidate CF pathology.

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S9.5 8.1 Ancestral Haplotype (AH) Protects Against Colonization in Cystic Fibrosis (CF): Could this Caucasian Haplotype Affect the Maintenance of this Lethal Disease?

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The 8.1.AH, common in Caucasians, consists of among others HLA-B8, DQ2 and DR3, TNFAB?a2b3, TNF-? -308A (TNF2), C4A?Q0 alleles. In a previous study we demonstrated that RAGE -429C and HSP70-2G alleles are new candidate members of the 8.1 AH [1]. Mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene cause CF, modifier genes are believed to affect clinical features of the disease. Colonization –particularly with Pseudomonas aeruginosa- and its consequences determine the progression of CF. Our aim was to test whether the 8.1 AH known to be associated with alterations in the immune response could affect colonization in CF. 98 Hungarian CF patients (47 homozygous, 51 heterozygous for F508del) were genotyped for TNF-? -308 polymorphism by SSP-PCR, HSP70-2 and RAGE -429 polymorphisms by PCR-RFLP. Simultaneous carriage of TNF2, RAGE -429C and HSP70-2G alleles was considered carriage of the 8.1 AH. To date 36 CF patients (18 homozygous, 18 heterozygous for F508del) had clinical data available in terms of colonization (*Pseudomonas, S. aureus*).

Results:	8.1 AH-		8.1 AH+		
	colonized	non-colonized	colonized	non-colonized	p
F508del homozygous	12	2	1	3	0.0441
F508del heterozygous	9	1	1	7	0.0029
?	21	3	2	10	0.0004

72.4% of TNF2 carriers of all CF patients are positive for 8.1 AH while it is 48% for healthy adults; i.e. CF patients carry the *TNF2 allele as part of the 8.1 AH* in much higher proportion than observed in healthy controls. Our novel observations indicate that the 8.1 AH may protect from colonization in CF. 8.1 AH carriers are characterized among others by an increase in activated T cells probably partially as a result of increase in circulating immune complexes (due to the presence of C4A?Q0 allele), by increased TNF-? production due to the presence of TNFAB?a2b3 and TNF2 alleles; all advantageous for coping with infections. On the other hand in carriers of the 8.1 AH a decrease is observed in lymphocyte count, in macrophage function, in NK activity and in neutrophil chemotaxis (concerning CF these could avoid prolonged, no more "useful" inflammation). During evolution the 8.1 AH could exert advantage against infectious diseases due to this genetically determined alteration of the immune response, the exact mechanism not known though. That could be the reason why this haplotype is present in more than 10 million Europeans [2]. The same effect could modify colonization in CF. Progression of CF is very much determined by condition of the lungs which is dependent on colonization. The 8.1 AH may protect from colonization. F508del carriers who carried the 8.1 AH as well could have a possible selection advantage during evolution. Taking into consideration that the frequency of the 8.1 AH is among the highest where the frequency of F508del is the highest in Europe, we may hypothesize that the interaction of the 8.1 AH and F508del allele could have an important impact on the maintenance of cystic fibrosis.

References:
[1] ESHG Conference 2005, Prague.
[2] Caruso (2000) *Hum Immunol* **61**: 942-949.

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S9.6 Search for Potential Biological Markers for CF Lung Disease – A Proteomic Approach

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In order to identify biological markers for Cystic Fibrosis (CF) lung disease, we analyzed total protein extracts of nasal cells from F508del-homozygous CF-patients *vs* non-CF individuals using a proteomics approach.

Nasal cells (n = 8 and n = 7 individuals in each group, respectively) were lysed and the total protein extracts were analysed by two-dimensional electrophoresis (2DE) as previously described [1]. Following silver and/or Coomassie-staining of proteins, digitalized images of the 2DE gels were analysed using the ImageMaster™ 2D Platinum software (Geneva Bioinformatics SA/ Amersham Biosciences) in order to generate reference maps. From these, 63 spots were selected and identified by mass spectrometry (MS), being the majority of these ubiquitously expressed proteins (*e.g.*, Hsc70, ?-actin, tubulins ? 1, ? 6 and ? ?etc). MS results further confirmed the epithelial nature of the tissue, since a number of the identified proteins have epithelial-specific expression. These include: cathepsin D; keratin, type I cytoskeletal 19; keratin, type II cytoskeletal 5 and 8; Plunc; and SCCA1.

Comparison of the protein profiles obtained for the two groups of individuals analysed also evidenced a set of proteins that are differentially expressed ($p < 0.05$). These were clustered into groups that included proteins related to: 1) chronic inflammation; 2) oxidative stress injury; and 3) changes in the cytoskeleton organization of CF airways cells. Moreover, differences in expression levels were also found for some proteins that have not been related to CF lung disease so far. Further work will help to understand the involvement of such proteins in CF pathophysiology and whether they are potential targets for CF therapy.

References:
[1] Roxo Rosa *et al*. (2004) *Journal of Cystic Fibrosis* **3 (S1).**

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