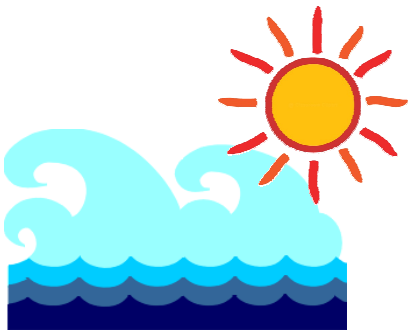
2007

European Cystic Fibrosis Society

New Frontiers in Basic Science of Cystic Fibrosis

Tavira, Algarve, Portugal



Chairpersons

Margarida D. Amaral and Ray Frizzell

25 – 29 April 2007

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Welcome to Tavira

Dear Friends and Colleagues,

It is a great pleasure to invite you to the European Cystic Fibrosis Conference entirely dedicated to Basic Science which in 2007 will take place in Tavira, Algarve, Portugal. I would like to acknowledge the success of this series of conferences to Professor Margarida Amaral and her team, and welcome Professor Ray Frizzell as this year's co-Chairperson.

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. We are thus proud to provide you with a platform to discuss your ideas with your colleagues during this important conference, which will comprise a series of symposia with invited European and International expert guest speakers. Your active participation will contribute to a productive exchange of information.

The Basic Science conferences are characterised by active discussion of data and ideas at the forefront of research on CF and CFTR, in an informal, co-operative environment. Post-graduate students engaged in cystic fibrosis research are encouraged to attend and the conference provides them ample opportunity to interact and discuss science.

I extend a very warm welcome to an exciting conference of high quality.

Marie Johannesson
ECFS President

Welcome from the Conference Chairpersons

It is a great pleasure to welcome you to the 2007 - ECFS Basic Science Conference: "New Frontiers in the Basic Science of Cystic Fibrosis". Our aim in organizing this venture is to help foster new research interactions towards a better understanding of the pathogenesis of CF and of other related disorders. We have encouraged our invited speakers to present their most recent unpublished results and wish to ensure lively discussion of data and ideas at the forefront of research, all in an informal, co-operative atmosphere.

Welcome to Tavira.

Margarida D. Amaral
University of Lisboa, Portugal

Ray Frizzell
University of Pittsburgh, USA

2007 ECFS Conference
New Frontiers in Basic Science of Cystic Fibrosis

Portugal, 25 – 29 April 2007
Programme

Wednesday, 25 April 2007

13:00-17:00	Registration, Light Meal & Set-up of Posters
17:00-17:30	**Official opening of the meeting** President of the European Cystic Fibrosis Society Conference Chairpersons: Margarida Amaral (Lisboa, Portugal) / Ray Frizzell (Pittsburgh, USA)
17:30-18:30	**Opening lecture:** William Balch (La Jolla, California, USA) **–** The Systems Biology of Cystic Fibrosis Chairs: Margarida Amaral (Lisboa, Portugal) / Ray Frizzell (Pittsburgh, PA, USA)
18:30-20:30	**Reception** - 'Port of Honour' partially sponsored by ROCHE
20:30-21:30	*Dinner*

Thursday, 26 April 2007

07:30-09:00	*Breakfast*
09:00-10:30	**Symposium 1 – CFTR Structure** Chairs: Ray Frizzell (Pittsburgh, PA, USA) / Martina Gentzsch (Chapel Hill, NC, USA)
09:00-09:20	Invited Speaker 1 –Bill Skach (Portland, OR, USA) – CFTR Targetting and Folding in the ER Membrane
09:20-09:40	Invited Speaker 2 – Phil Thomas (Dallas, TX, USA) – F508del Affects NBD1 First and Foremost
09:40-10:00	Invited Speaker 3 – Piotr Zielenkiewicz (Warsaw, Poland) - Molecular Dynamics study of the native and F508del NBD1 domains
10:00-10:15	John Richardson (Dallas, TX, USA) - Identification of a non-native state of NBD1 that is affected by F508del
10:15-10:30	Nuri Awayn (Manchester, UK) - Production and analysis of 2D crystals of CFTR
10:30-11:00	*Coffee break* & **Poster viewing**
11:00-12:30	**Symposium 2 – What have we learned about CF from Omics?** Chairs: Rainer Pepperkok (Heidelberg, Germany) / William Balch (La Jolla, CA, USA)
11:00-11:20	Invited Speaker 1 – Paul McCray (Iowa City, IA, USA) – Searching the Genome for Clues to Pulmonary Innate Immunity
11:20-11:40	Invited Speaker 2 – Aleksander Edelman (Paris, France) – Building the functional CFTR interactome: experimental and in sillico analysis
11:40-12:00	Invited Speaker 3 – Rainer Pepperkok (Heidelberg, Germany) – Large Scale Functional Screens to Study Secretory Protein Traffic
12:00-12:15	Anabela S. Ramalho (Lisbon, Portugal) - Cystic Fibrosis due to Abnormal Processing of CFTR Transcripts
12:15-12:30	Heather Love (Belfast, UK) - Pulmonary Surfactant is an Important Lubricant for Cystic Fibrosis Sputum
12:30-14:30	*Lunch*
14:30-16:00	**Symposium 3 – Membrane Traffic of CFTR and lessons from Other Proteins** Chairs: Martina Gentzsch (Chapel Hill, NC, USA)
14:30-14:50	Invited Speaker 1 – Agnes Urban (Pittsburgh, PA, USA) – Myosin Motors Regulate CFTR Trafficking

14:50-15:10	Invited Speaker 2 – Robert Ford (Manchester, UK) – Low resolution structures of other ABC proteins: What can they tell us about CFTR?
15:10-15:30	Invited Speaker 3 – Robert Tampé (Frankfurt, Germany) – ABC Transporters in Adaptive Immunity and Virus Escape Mechanisms
15:30-15:45	Anurag Singh (Hannover, Germany) - The role of Slc26a6 and CFTR in acid-induced and prostaglandin E_2-stimulated duodenal bicarbonate secretion *in vivo*
15:45-16:00	Ana Carina Da Paula (Lisbon, Portugal) – Folding, Processing and Function of Human-Murine CFTR Chimeras: Structural Implications
16:00-16:30	*Coffee Break* & **Poster Viewing**
16:30-18:00	**Symposium 4 – Epithelial Physiology** Chairs: Mike Gray (Newcastle, UK) / Bob Dormer (Cardiff, UK)
16:30-16:50	Invited Speaker 1 – Mike Gray (Newcastle, UK) – CFTR and Pancreatic Bicarbonate Secretion.
16:50-17:10	Invited Speaker 2 – Mauri Krouse (Stanford, CA, USA) – Submucosal glands and surface epithelium: the dynamic duo of the airway
17:10-17:30	Invited Speaker 3 – Ursula Seidler (Hannover, Germany) – Intestinal acid/base transport 2007: New Transporters, Regulatory Pathways, and New Therapeutic Strategies
17:30-17:40	Nabila Hamdaoui (Paris, France) - Estrogens increases CFTR expression in MDCK and MCF7 epithelial cell lines.
17:40-17:50	Francine de Courcey (Belfast, UK) - Characterization of volume-regulated anion channels (VRAC) in normal and cystic fibrosis epithelial cells: regulation of VRAC by CFTR and receptor agonists
17:50-18:00	Nico Derichs (Hannover, Germany) - Ex vivo chloride secretion measurements as outcome parameter of the CF basic defect
18:30-20:00	**Special Group Discussion IA – What is wrong with F508del-CFTR structure and how can we rescue it?** Chairs: Bob Ford (Manchester, UK) / Phil Thomas (Dallas, TX, USA) Bob Ford (Manchester, UK) – Structure of CFTR in the phosphorylated state, as determined by cryo-EM **Special Group Discussion IB – pH and CF Disease**. Chairs: Mauri Krouse (Stanford, CA, USA) & Mike Gray (Newcastle, UK)
20:30-21:30	*Dinner*
21:30-23:30	**Poster Session** – All Presenters Free drinks kindly sponsored by GRUPO TAPER

Friday, 27 April 2007

07:30-09:00	*Breakfast*
09:00-10:30	**Symposium 5 – ENaC: Traffic and Activity** Chairs: Karl Kunzelmann (Regensburg, Germany) / Deborah Baines (London, UK)
09:00-09:20	Invited Speaker 1 – Joe Pilewski (Pittsburgh, PA, USA) – Protease Regulation of ENaC in Cystic Fibrosis
09:20-09:40	Invited Speaker 2 – Karl Kunzelmann (Regensburg, Germany) – Casein Kinase 2 May Regulate Epithelial Na^+ Channels by Rescuing from Nedd4-2 Mediated Degradation
09:40-10:00	Invited Speaker 3 – Deborah Baines (London, UK) – Forskolin increases transepithelial Na^+ transport across H441 cell monolayers via induction of cell shrinkage and translocation of EGFP-human □ENaC to the apical membrane
10:00-10:10	Joana Almaça (Lisbon, Portugal) - Regulation of ENaC Membrane Traffic by Casein Kinase 2
10:10-10:20	Anurag Singh (Hannover, Germany) – Role of the NHERF adapter proteins NHERF1, NHERF2/E3KARP and NHERF3/PDZK1 in the regulation of murine duodenal bicarbonate secretion in vivo
10:20-10:30	Bettina Schock (Belfast, UK) - Toll-like receptor expression in CFBE and HBE cells
10:30-11:00	*Coffee break* & **Poster viewing**

11:00-12:30	**Symposium 6 – Biogenesis and Quality Control of Membrane Proteins** Chairs: William Skach (Portland, OR, USA) / Margarida Amaral (Lisboa, Portugal)
11:00-11:20	Invited Speaker 1 – Ray Frizzell (Pittsburgh, PA, USA) – F508DEL CFTR is targeted for ERAD by sHsp-mediated sumoylation
11:20-11:40	Invited Speaker 2 – Zsuzsa Bebok (Birmingham, AL, USA) – CFTR Expression Regulation by the Unfolded Protein Response
11:40-12:00	Invited Speaker 3 – Martina Gentzsch (Chapel Hill, NC, USA) – Processing and turnover of CFTR in highly differentiated cultures of human airway epithelia
12:00-12:10	Karl Enquist (Stockholm, Sweden) - Membrane Integration Efficiency of Individual Transmembrane Segments of the Multispanning Membrane Protein CFTR
12:10-12:20	Alexandre Mironov (Chieti, Italy) - Pathways for intracellular transport of CFTR
12:20-12:30	Carlos M Farinha (Lisbon, Portugal) - Role of Casein Kinase 2 in the Early Steps of CFTR Biogenesis
12:30-14:30	*Lunch*
	Free Afternoon - Tavira Guided Tour
18:30-20:00	**Special Group Discussion IIA – Is regulation of ENaC tissue - specific?** Chairs: Karl Kunzelmann (Regensburg, Germany) / Ray Frizzell (Pittsburgh, PA, USA) **Special Group Discussion IIB – Endo/Exocytic Regulation of CFTR** Chairs: Agnes Urban (Pittsburgh, PA, USA) + Martina Gentzsch (Chapel Hill, NC, USA)
20:00-21:30	*Dinner*
21:30-23:30	**Poster Session** – All Presenters Free drinks kindly sponsored by BONSAI

Saturday, 28 April 2007

07:30-09:00	Breakfast
09:00-10:30	**Symposium 7 – Models for airway signalling** Chairs: M. Chanson (Geneva, Switzerland) / Carla Ribeiro (Chapel Hill, NC, USA)
09:00-09:25	Invited Speaker 1 – Carla Ribeiro (Chapel Hill, NC, USA) – Airway Epithelial Infection, ER Stress, Ca^{2+} Signals and Inflammation: What is the Connection?
09:25-09:45	Invited Speaker 2 – Hugo De Jonge (Rotterdam, The Netherlands) – Airway Signalling in CF Mice
09:45-10:05	Invited Speaker 3 – Marc Chanson (Geneva, Switzerland) – Influence of lung fibroblasts on proliferation and differentiation of human airway epithelial cells: specific expression profile of gap junction forming connexins
10:05-10:15	Christine Tertilt (Mainz, Germany) - Role of CFTR in dendritic cell function upon infection with Pseudomonas aeruginosa
10:15-10:25	René Barro Soria (Regensburg, Germany) - Bestrophin 1 and 2, important components of the calcium activated chloride channel in mouse trachea epithelium
10:25-10:30	Discussion
10:30-11:00	*Coffee break* & **Poster viewing**
11:00-12:30	**Symposium 8 – Epithelial Cell Biology, Inflammation and Pathogens** Chairs: Anil Mehta (Dundee, UK) / Joe Pilewski (Pittsburgh, PA, USA)
11:00-11:20	Invited Speaker 1 – Deborah Nelson (Chicago, IL, USA) – CFTR: Regulation of Intracellular Acidification and Bacterial Killing in Alveolar Macrophages
11:20-11:40	Invited Speaker 2 – Aura Perez (Cleveland, OH, USA) – CFTR Inhibition Mimics the Cystic Fibrosis Inflammatory Profile
11:40-12:00	Invited Speaker 3 – Madeleine Ennis (Belfast, UK) - Inflammation and the Cystic Fibrosis Epithelium
12:00-12:15	Giulio Cabrini (Verona, Italy) - Inhibitory effect of medicinal plant extracts from Bangladesh on *P.aeruginosa*-dependent induction of pro-inflammatory genes in CF bronchial epithelial cells
12:15-12:30	Maria Cristina Dechecchi (Verona, Italy) - Correctors of F508del CFTR reduce the inflammatory response to *Pseudomonas aeruginosa* in CF respiratory epithelial cells

12:30-14:00	*Lunch*
14:30-16:00	**EuroCareCF Meeting Session 1 – WP7 (Resources) Overview & Discussion** Chair: Margarida Amaral (Lisboa, Portugal) / Bob Dormer (Cardiff, UK) / Scott Randell (Chapel Hill, NC, USA)
14:30-14:40	Margarida Amaral (Lisboa, Portugal) - Overview
14:40-15:00	Scott Randell (Chapel Hill, NC, USA) – Primary Epithelia and Novel Cell Lines for CF Research
15:00-15:20	Bob Dormer (Cardiff, UK) – Endpoints for the Assessment of Therapy Aimed at the CFTR Defect
15:20-16:00	Future Perspectives and General Discussion (possible coordination with the *US CFTR Folding Consortium)*
15:30-16:00	*Coffee Break*
16:00-18:00	**EuroCareCF Meeting Session 2 – WP6 (Animal Models) Overview and Discussion** Chair: Bob Scholte (Rotterdam, The Netherlands)
16:00-16:10	Bob Scholte (Rotterdam, The Netherlands) – Introduction. CF mouse models, a quarrelling family.
16:10–16:30	Scott Randell (Chapel Hill, NC, USA) – Update on the ENaC-β mouse
16:30-16:50	Carole Planès (Paris, France) - The β-Liddle mutation of the epithelial sodium channel increases alveolar fluid clearance and reduced the severity of hydrostatic pulmonary oedema in mice.
16:50-17:10	Joern Karhausen - The relevance of hypoxia to mucosal inflammatory processes
17:10-17:30	Teresinha Leal (Leuven, Belgium) - Azithromycin modulates IL-1β overexpression in alveolar macrophages from CF mice
18:00-19.15	**Special Group Discussion IIIA – Protein interactions: CFTR and its accomplices** Chairs: Anil Mehta (Dundee, UK) / William Balch (La Jolla, CA, USA) **Special Group Discussion IIIB – Immune Mechanisms in ASL** Chairs: Aura Perez (Cleveland, USA) / Scott Randell (Chapel Hill, NC, USA) Scott Randell (Chapel Hill, NC, USA) - Novel Cell Lines for CF Research
20:00	*Social Function*

Sunday, 29 April 2007

08:00-09:00	Breakfast
09:00-10:30	**Symposium 9 – Strategies Aimed at Correcting CFTR Mutants** **Chairs:** Zsuzsa Bebok (Birmingham, AL, USA) / David Sheppard (Bristol, UK)
09:00-09:20	Invited Speaker 1 – David Sheppard (Bristol, UK) – Overview: How much CFTR is enough?
09:20-09:40	Invited Speaker 2 – Fred Van Goor (San Diego, CA, USA) – Pharmacological Rescue of F508del-CFTR Function for the Treatment of Cystic Fibrosis
09:40-10:00	Invited Speaker 3 – Luis Galietta (Genoa, Italy) – Potentiators and Correctors for the Mutant CFTR Channel
10:00-10:20	Invited Speaker 4 – Melissa Ashlock (Bethesda, MD, USA) – Novel approaches in therapy aimed at correcting the basic defect in CF
10:20-10:30	André Schmidt (Lisbon, Portugal) - Investigation of the Mechanism of Action of Several Small Corrector Molecules by Means of F508DEL-CFTR Genetic Revertants
10:30-10:40	Short break
10:40-11:15	Closing Lecture – Mike Welsh (Iowa City, IA, USA) – The CF Pig Model
11:15-11:30	**Closing remarks**
12:00	End of the Meeting - Departure

POSTERS

P.1 **Membrane Integration Efficiency of Individual Transmembrane Segments of the Multispanning Membrane Protein CFTR**
Enquist, K. and Nilsson, I.

P.2 **Regulation of ENaC Membrane Traffic by Casein Kinase 2**
Joana Almaça, Tanja Bachhuber, Toby Scott-Ward, Rainer Schreiber, Margarida Amaral and Karl Kunzelmann

P.3 ***In vivo* effect of BAG-1 on the turnover and processing of CFTR**
Filipa Mendes, Isabel Vieira, Paulo C Alves, Carlos M Farinha, Margarida D. Amaral

P.4 **Pathways for intracellular transport of CFTR**
A. Mironov, S. Shityakov, M. Micaroni, G. Beznoussenko, R. Parashuraman, A. Luini

P.5 **Role of Casein Kinase 2 in the Early Steps of CFTR Biogenesis**
Carlos M Farinha, Luísa Pissarra and Margarida D. Amaral

P.6 **Solubilizing mutations in the NBD1 of CFTR rescue the processing and channel gating defects of the cystic fibrosis mutation F508del**
Luísa S. Pissarra, Zhe Xu, André Schmidt, Carlos M. Farinha, Zhiwei Cai, Patrick H. Thibodeau, Philip J. Thomas, David N. Sheppard, Margarida D. Amaral

P.7 **Exploiting Isolated Domains of CFTR to Capture Novel CFTR-interacting Proteins and Quantify the Strength of CFTR-chaperone Interactions**
Toby S Scott-Ward, Diana Faria, Luisa Alessio, Margarida D. Amaral

P.8 **Preclinical evidence that sildenafil and vardenafil correct cystic fibrosis defects**
Bob Lubamba, Hugues Lecourt, Pierre Wallemacq, Alice Bot, Jean Lebacq, Patrick Lebecque, Hugo De Jonge, Teresinha Leal

P.9 **Medicinal plant extracts from *Phyllanthus acidus* may represent a potential treatment for Cystic Fibrosis**
Marisa Sousa, Jiraporn Ousingsawat, Roswitha Seitz, Supaporn Puntheeranurak, Ana Regalado, André Schmidt, Tiago Grego, Chaweewan Jansakul, Margarida D. Amaral, Rainer Schreiber, Karl Kunzelmann

P.10 **Investigation of the Mechanism of Action of Several Small Corrector Molecules by Means of F508del-CFTR Genetic Revertants**
André Schmidt and Margarida D. Amaral

P.11 **Rescue of F508del-CFTR channel gating by the ATP analogue 2'-deoxy-ATP**
Min Ju, Zhiwei Cai and David N. Sheppard

P.12 **Functional properties and protein interactions of Bestrophins**
Joana Raquel Martins, Melanie Spitzner, Jiraporn Ousingsawat, René Barro Soria, Kerstin Scheidt, Toby Scott-Ward, Rainer Schreiber, Margarida Amaral and Karl Kunzelmann

P.13 **The role of Slc26a6 and CFTR in acid-induced and prostaglandin E_2-stimulated duodenal bicarbonate secretion *in vivo.***
Anurag Singh, Markus Sjöblom, Anja Krabbenhöft, Brigitte Riederer, William Colledge, Manoocher Soleimani, and Ursula Seidler

P.14 **Folding, Processing and Function of Human-Murine CFTR Chimeras: Structural Implications**
Ana Carina Da Paula, Marisa Sousa, Elizabeth S. Dawson, Ann Doherty, David N. Sheppard, A. Christopher Boyd and Margarida D. Amaral

P.15 **$CFTR_{inh\text{-}172}$ and glibenclamide inhibit the murine CFTR Cl^- channel**
Zhiwei Cai, Min Ju and David Sheppard

P.16 **Estrogens increases CFTR expression in MDCK and MCF7 epithelial cell lines.**
Nabila Hamdaoui, Noura Bensalem, Delphine Roussel, Meriem Garfa-Traore, Fatna Makaci, Naziha Bakouh, Isabelle Sermet-Gaudelus, Aleksander Edelman, Maryvonne Legros, Janine Fritsch & Gabrielle Planelles

P.17 **Role of the NHERF adapter proteins NHERF1, NHERF2/E3KARP and NHERF3/PDZK1 in the regulation of murine duodenal bicarbonate secretion *in vivo.***
Anurag Singh, Anja Krabbenhöft, Brigitte Riederer, Boris Hogema, Hugo de Jonge, Mark Donowitz, Edward J. Weinman, Oliver Kocher, and Ursula Seidler

P.18 **Characterization of volume-regulated anion channels (VRAC) in normal and cystic fibrosis epithelial cells: regulation of VRAC by CFTR and receptor agonists**
Francine de Courcey, Alexander Zholos, Prof Madeleine Ennis, Prof J Stuart Elborn

P.19 **Bestrophin 1 and 2, important components of the calcium activated chloride channel in mouse trachea epithelium**
R. Barro-Soria, Marisa Sousa, R. Schreiber, K. Kunzelmann

P.20 **Toll-like receptor expression in CFBE and HBE cells**
Bettina C. Schock, A. Bingham and J.S. Elborn

P.21 **Inhibitory effect of medicinal plant extracts from Bangladesh on *P.aeruginosa*-dependent induction of pro-inflammatory genes in CF bronchial epithelial cells**
Ilaria Lampronti, Maria Cristina Dechecchi, Monica Borgatti, Elena Nicolis, Valentino Bezzerri, Irene Mancini, Anna Tamanini, Federica Quiri, Paolo Rizzotti, Giulio Cabrini, Roberto Gambari,

P.22 **Correctors of F508del CFTR reduce the inflammatory response to *Pseudomonas aeruginosa* in CF respiratory epithelial cells**
Maria Cristina Dechecchi,; Elena Nicolis, Caroline Norez, Valentino Bezzerri, Federica Quiri, Monica Borgatti, Irene Mancini, Paolo Rizzotti, Roberto Gambari, Frederic Becq, and Giulio Cabrini

P.23 **A transcription factor "decoy" strategy to control leukocyte chemotaxis activated by *P.aeruginosa* in bronchial epithelial cells**
Valentino Bezzerri, Monica Borgatti, Irene Mancini, Elena Nicolis, Maria Cristina Dechecchi, Anna Tamanini, Federica Quiri, Ilaria Lampronti, Paolo Rizzotti, Roberto Gambari, Giulio Cabrini,

P.24 **An Investigation of the role of CK2 in the CF Inflammatory Response**
Alexandra K Winter, Megan L Crichton, Kate J Treharne, Anil Mehta, Stephen C Land

P.25 **Evidence for an IgG binding site in NBD1 of CFTR.**
Kate J. Treharne and Anil Mehta

P.26 **Factors influencing sputum cytokine levels in cystic fibrosis patients**
S.Schmitt-Grohé, L.v.d.Boom, D.N´Gampolo, O.Eickmeier, R.Schubert, S.Zielen, B.Zur, M.J.Lentze

P.27 **Effect of Moisture Tent on the Ion Content of Nasal Fluid in Patients with Cystic Fibrosis**
Inna Kozlova, Georgia Varelogianni, Lena Hjelte, and Godfried M. Roomans

P.28 **Azithromycin modulates IL-1β overexpression in alveolar macrophages from CF mice**
Magali Meyer, François Huaux, Sybille van den Brûle, Patrick Lebecque, Dominique Lison, Pierre Wallemacq, Teresinha Leal

P.29 **Reduction of the immunogenicity of the lentiviral vector-technology for pulmonary gene therapy**.
Jaan Toelen, Rik Gijsbers, Kris De Boeck, Jan Deprest and Zeger Debyser

P.30 **Non-invasive long term follow-up of pulmonary gene transfer with a lentiviral vector in a fetal rat model.**
Jaan Toelen, Christophe M Deroose, Rik Gijsbers, Kris De Boeck, Jan Deprest and Zeger Debyser

P.31 **Nasal epithelial cell culture from cystic fibrosis patients.**
Francine de Courcey, Grzegorz Skibinski, J Stuart Elborn, Madeleine Ennis

P.32 **A Model of the CFTR Folding Defect through Usage of MDR/CFTR Chimeras in *C. elegans* defective for Heavy Metal Resistance**
Mário F. Neto[1,2], Susana M. Garcia[2], Maria Catarina Silva[1,2], Richard I. Morimoto[2], Margarida D. Amaral[1,3]

P.33 **Role of CFTR in dendritic cell function upon infection with *Pseudomonas aeruginosa***
Christine Tertilt, Yaqin Xu, Lena Reichenbach, Hansjörg Schild, Stefan Worgall

P.34 **Three-Dimensional Structure of CFTR investigated by Electron Microscopy and Single Particle Analysis**
Liang Zhang, Nuri H. Awayn, Luba Aleksandrov, John R. Riordan, Robert C. Ford

P.35 **Production and analysis of 2D crystals of CFTR**
Awayn Nuri H., Rosenberg Mark F., Aleksandrov Luba A., Riordan John R. and Ford Robert C.

P.36 **Identification of a non-native state of NBD1 that is affected by F508del**
John M. Richardson, Patrick H. Thibodeau, Jarod Watson, and Philip J. Thomas

P.37 **Distinct NBD1 binding sites for ATP and Cyclic AMP revealed by ligand binding to wild type and mutant CFTR proteins.**
M.M.C. Pereira, M. Pomeroy, R.L. Dormer

P.38 **A novel array for CFTR mutation detection: preliminary binding studies**
Luka A. Clarke & Margarida D. Amaral

P.39 **Genetic predisposition to osteoporosis in Polish children with cystic fibrosis.**
A. Norek, D. Sands, D. Chmielewski, K. Szamotulska, A. Sobczynska-Tomaszewska, J. Bal

P.40 **The peculiarities of immunological reactions in children with cystic fibrosis**
S.Sciuca, E.Chioroglo, O.Turcu, V.Salaru

P.41 **Cystic Fibrosis due to Abnormal Processing of CFTR Transcripts**
Anabela S. Ramalho, Margarida D Amaral

P.42 **Pulmonary Surfactant is an Important Lubricant for Cystic Fibrosis Sputum**
H. Love, J.S. Elborn and B.C. Schock

P.43 **Ex vivo chloride secretion measurements as outcome parameter of the CF basic defect**
N. Derichs, C. Stolpe, B. Tümmler, M. Ballmann.

Wednesday 25 April -17:30-18:30

Opening Lecture

The Systems Biology of Cystic Fibrosis

William E. Balch[1,2,5], Darren Hutt[1], Atanas Koulov[1], Bogdan Tanasa[1], Wendy Kellner[1], Helen Plutner[1], Paul Lapointe[1], Scott Stagg[1], Clint S. Potter[1,4], Bridget Carragher[1,4], Jeffery W. Kelly[3,4] and John R. Yates III[1]

The Scripps Research Institute, Departments of Cell[1] and Molecular Biology[2],and Chemistry[3], The Skaggs Institute for Chemical Biology[4], The National Resource for Automated Molecular Microscopy[5], and The Institute for Childhood and Neglected Diseases[6], 10550 North Torrey Pines Road, La Jolla, CA 92037, USA

Physiological correction of cystic fibrosis transmembrane conductance regulator (CFTR) trafficking and restoration of normal channel function at the cell surface remains a fundamental challenge to the CF field. We are now beginning to appreciate that CFTR operates in the context of an extensive network of interactions (the *CFTR interactome*) that dictates its flow through exocytic and endocytic pathways, and regulates channel function at the cell surface. These networks require the assistance of molecular chaperones that define the chemical and biological folding buffer of the cell (the *chaperome*). Cells exploit the variable composition of the chaperome environment to maintain protein folding homeostasis through the kinetic and thermodynamic properties of the protein fold. Perturbation of this relationship, as occurs in misfolding diseases such as CF, results in an imbalance leading to disruption of normal physiology. By understanding the relationship between folding energetics and the modular biological networks controlling CFTR flow and function at cell surface, we should be able to generate an integrated systems biology view of CF disease that accurately reflects the contributions of the local tissue environment, host inflammatory responses, and genetic modifiers. Knowledge of these relationships will allow us to minimize the pathophysiology of disease through chemical biology.

Thursday 26 April – 09:00-10:30 Room: Master

SYMPOSIUM 1 – CFTR Structure

Chair: Ray Frizzell (Pittsburgh, PA, USA) / Martina Gentzsch (Chapel Hill, NC, USA)

S1.1 - CFTR Targetting and Folding in the ER Membrane

Steven Grund and William R. Skach

Department of Biochemistry, Oregon Health & Science University, 97239 Portland, USA

A major challenge in CFTR biogenesis is to understand how its transmembrane segments (TMs) are properly oriented, inserted and folded within the hydrophobic environment of the lipid bilayer. This process occurs coincident with protein synthesis at specialized sites in the endoplasmic reticulum (ER). During the initial stages of CFTR folding, the ribosome nascent chain (RNC) complex is targeted to the ER membrane by signal recognition particle and then transferred to the Sec61 translocon(1). The resulting ribosome translocon complex (RTC) establishes protein topology by providing a dynamic pathway that controls axial movement of nascent elongating polypeptide into the ER lumen and cytosol as well as lateral movement of TM segments into the membrane(2, 3).

In the simplest model, protein movement through the RTC is alternately directed into lumenal and cytosolic compartments as sequential signal and stop transfer activities encoded within TM segments open and close the translocation pathway. CFTR uses a variation on this model that reflects different topogenic behavior and timing of TM segment insertion (4). Although ER targeting and protein translocation are normally tightly coupled events, the first signal sequence of CFTR, encoded by TM1, can effectively target the RNC to the ER membrane but is unable to efficiently initiate translocation due to the presence of two polar residues (Glu92 and Lys95) within its hydrophobic core(5). Using modified suppressor aminoacyl-tRNAs to probe the molecular environment of the nascent CFTR polypeptide within the RTC, we show that TM1 also contacts Sec61α in the absence of TM2 but does not efficiently open the translocon channel. The net result of this behavior is that ER targeting and translocation are temporally uncoupled during the initial stages of CFTR synthesis. The first extracellular loop (ECL1) therefore transiently resides between the base of the docked ribosome and the cytosolic face of the translocon until translation has proceeded sufficiently for TM2 to open the translocon and enable ECL1 to move into the ER lumen. Photocrosslinking experiments also reveal that TM1 and TM2 simultaneously contact Sec61α during ECL1 translocation, suggesting that they insert together into the translocon. Our data provide a mechanistic basis for CFTR topogenesis and explain how the first two TM segments establish CFTR N-terminus topology by uncoupling SRP-mediated targeting from signal sequence-mediated Sec61 gating. They also provide a general means to examine more precisely, how charged and polar residues in CFTR TM segments impact the timing of polypeptide exposure and folding in different cellular compartments.

Supported by NIH (DK53457, W.S. and DK069031, S.G.) and by CFFTI.

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S1.2 - F508del Affects NBD1 First and Foremost.

Patrick H. Thibodeau, Wei Wang, John Richardson, Kai Du, Juan Mendoza, Linda Millen, Jarod Watson, Gergely Lukacs, Kevin Kirk, and Philip J. Thomas

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The deletion of phenylalanine 508 (F508del) in the first nucleotide binding domain (NBD1) of the cystic fibrosis transmembrane conductance regulator (CFTR) is directly associated with >90% of all cases of cystic fibrosis. This mutant protein fails to traffic out of the ER and is subsequently degraded by the proteasome. However, the effects of this mutation may be reversed by manipulation of conditions--expression at low temperature or the introduction of second-site suppressor mutations restore at least partial trafficking and function to F508del CFTR. A large number of studies have described differential impacts of the F508del mutation on the structure of full-length CFTR and recent high-resolution crystal structures have provided insight into the local structural effects of the mutation on the isolated NBD1. However, the exact mechanism by which this mutation impacts the folding and assembly of full-length CFTR is unclear. To elucidate the mechanisms and effects of the F508del mutation on CFTR biosynthesis, we have probed CFTR structure utilizing a series of mutations and deletions to evaluate their effects on the folding and maturation of CFTR enabling a better understanding of the critical steps in the process and their recognition by the cellular quality control machinery. These results indicate that the primary manifestation of the F508del defect lies within the NBD1 itself and that, while other domains of CFTR likely respond to alterations in NBD1 structure, the defect first presents itself and is recognized within the context of NBD1. These findings provide insight into how single domains of multi-domain proteins impact the maturation of these complex systems. Finally, the data suggest that proper NBD folding represents a step in the biosynthetic pathway that should be targeted while screening for small molecule correctors of the F508del defect.

S1.3 - Molecular Dynamics study of the native and F508del NBD1 domains

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We present a Molecular Dynamics study on the native and F508del CFTR NBD1 domains. Crystalographically resolved structures of NBD1 domains from human F508del CFTR mutant and CFTR without this deletion — Protein Data Bank entries 1XMJ and 2BBO, respectively — both complexed with ATP, were the starting points for the study. The averaged from the 20ns MD trajectories solvent accessible surface of the domains was 11983Å^2 for F508del and 11434Å^2 for wild-type protein, while the average solvent accessible surface of hydrophobic residues were 2651Å^2 and 2611Å^2, and the values for frames of the highest degree of accessibility of hydrophobic residues are 3087Å^2 and 3001Å^2, respectively. The most of the surface changes on F508del protein takes its origings in the increase of conformational freedom gained by a linker between subdomains ABCα and ABCβ of NBD1 domain — aminoacids from 492 to 499. The surface of both subdomains becomes more variable then in wild type case. Lack of stabilizing influence, which the Phe508 residue imposes on the linker, mainly on Met498 and Trp496 seems to play important role. As the following effect, both the subdomains are allowed to bend more significantly then in the wild-type protein, exposing their hydrophobic interior substantially. The results show that F508del CFTR has much more conformational freedom compared to the wild type, and exposes its hydrophobic interior to the solution. This might be the reason for the recognition of mutated CFTR by the housekeeping proteins and its premature degradation. This, in turn results in reduction of population of functional channels at the epithelial cell surface and disease phenotype.

S1.4 - Identification of a non-native state of NBD1 that is affected by F508del

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Protein misfolding is the basis for a multitude of human diseases; however, the mechanisms underlying misfolding are not well understood [1]. Most cases of cystic fibrosis are associated with mutations--including the most common, deletion of phenylalanine 508 (F508del) in NBD1--that interfere with the folding of the cystic fibrosis transmembrane conductance regulator (CFTR) [2]. The resulting loss of functional CFTR causes the disease. Thus, elucidating how F508del, affects the folding of CFTR is critical to understanding the pathology of the disease. CFTR is composed of five domains: two integral membrane transmembrane domains (TMDs), a regulatory domain (R), and two nucleotide binding domains (NBDs) [3]. F508del occurs in the N-terminal NBD1. Both the murine wildtype and F508del NBD1 can be expressed in bacteria and purified to near homogeneity. While the soluble expression yield of F508del is lower than the wild type under identical conditions, F508del achieves a native conformation similar to wild type as monitored by a variety of hydrodynamic and spectroscopic characteristics such as analytical size exclusion chromatography, circular dichroism, and fluorescence. Recently, a non-native, but folded, species has been detected under mildly denaturing conditions. Far-UV CD reveals a time and temperature dependent conversion from a mixed alpha/beta native conformation to a less helical non-native conformation, while fluorescence measurements reveal a parallel blue shift in the peak emission intensity from 343 to 330 nm. This non-native species is in a more open conformation as determined by both limited proteolysis and the change in retention time on analytical size exclusion chromatography. The conversion to this state is inhibited by the native state ligand (ATP) and by the presence of the second site suppressors (G550E, R553Q/M, and R555K). Notably, the F508del NBD1 protein populates this state under milder conditions than the wild type NBD1. These studies reveal the properties of the native state and its conversion to a partially folded state that is affected by the F508del mutation. This data highlight the proximal affect of the disease causing mutation and provide a means of assessing strategies designed to correct the defect.

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S1.5 - Production and analysis of 2D crystals of CFTR

Nuri H. Awayn [1], Mark F. Rosenberg [1], Luba A. Aleksandrov [2], John R. Riordan [2] and Robert C. Ford [1]

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The Cystic Fibrosis Transmembrane Conductance Regulator protein (CFTR) is a member of the ATP-binding cassette (ABC) superfamily of membrane proteins which are involved in many essential physiological processes. Mutations in CFTR cause cystic fibrosis. The CFTR protein is unusual as an ABC protein because it is an ion channel and has an extra regulatory domain (R) located between NBD1 and TMD2. Elucidation of the structure and transportation mechanism is very important for the design of suitable drugs for cystic fibrosis treatment. Here, we have studied the CFTR structure by using crystallographic methods.

First, by using a hanging drop method we have successfully produced 2D crystals of CFTR under about ten different conditions depending on the activator or inhibitor that was added (Ni-NTA nanogold, ATP, PKA, Genistein). From the analysis of these data we can divide them to two different groups of conditions: (1) Conditions that produce only one crystal form, (2) Conditions that produce two different types of crystal forms. Low resolution 3D structures from a few interesting conditions were produced and will be presented.

Secondly, 2D crystals were produced by reconstitution of the nucleotide-free CFTR protein into lipid vesicles using Biobeads and dialysis methods and are now under analysis. Ongoing efforts to generate higher resolution data from 2D crystals observed under cryo-conditions will be described.

Thursday 26 April – 11:00-12:30 Room: Master

SYMPOSIUM 2 – What have we learned about CF from Omics?

Rainer Pepperkok (Heidelberg, Germany) / William Balch (La Jolla, CA, USA)

S2.1 - Searching the Genome for Clues to Pulmonary Innate Immunity

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The airway epithelium plays an important role in pulmonary innate immunity. We are using genomics-based approaches to study epithelial biology and identify genes involved in host defense. We prepared and sequenced a cDNA library from well-differentiated human airway epithelia and used this focused gene set to make a custom Affymetrix genechip. This ~23K gene set includes ESTs not represented on commercial arrays. Primary air-liquid interface epithelial cultures were treated for 24 hr with a cocktail of IL-1, TNF-alpha, and IFN gamma and mRNA harvested and prepared for microarray hybridization. Many genes with known or putative innate immune function were identified with constitutive or inducible expression. Two lipid transfer/LPS binding protein family members, PLUNC and LPLUNC1, showed constitutive, high level expression. Ongoing studies indicate that PLUNC is expressed by both surface epithelia and neutrophils. This very hydrophobic protein exhibits surface tension lowering properties, and therefore may modify the composition of respiratory secretions and the function of surface epithelial cells. Inducible genes included STAT1, HBD-2, IL-8, CCL8, CCL20, CXCL9, CXCL10, SERPINA3, SERPINB7, and DUOX2. Of note, the DUOX1 and DUOX2 gene products are key participants in an epithelial host defense system based on the generation of the inorganic microbicide hypothiocyanate ($OSCN^-$). Other participants in this host defense system include lactoperoxidase from submucosal glands and transcellular thiocyanate (SCN^-) transport via CFTR and other pathways. Interestingly, we present data indicating that defective SCN transport via CFTR in CF epithelia leads to a defect in mucosal host defense. Large-scale expression profiling in airway epithelia provides novel insights into the repertoire of responses of this mucosal surface, reflecting its dynamic role in innate immunity.

S2.2 - Building the functional CFTR interactome: experimental and in silico analysis

F. Brouillard[1], S. Trudel[1], F. Borot[1], P. Pawlowski[2], J. Fritsch[1], N. Davezac[1], N. Bensalem[1], M. Dadlez[2], C. Guerrera[3], P. Zielenkiewicz[2], M. Ollero[1], Aleksander Edelman[1]

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Plateau Proteome Necker, IFR94, Paris, France[3]

A growing body of evidence indicates that CFTR protein is part of large protein complexes involved in several cell functions. The formation of such complexes in a time-dependent manner is necessary for the correct localization, degradation, and regulation of the function of other proteins, inflammatory processes, etc. Thus, identification of new partners interacting with CFTR represents an important challenge for the CF research community. This knowledge will not only lead to a better understanding of CFTR functions but will ultimately result in the identification of new targets for CF pharmacotherapy.. In recent years, the challenge has become a little easier because of the development of new proteomic approaches. Progress in separation of protein samples and identification of proteins of interest has not only been made in the analysis of hydrophilic but also of hydrophobic membrane proteins (1,2). The growing number of proteins that might interact with CFTR/Fdel508CFTR (3) points to the question of whether CFTR is a hub protein, i.e. a protein that interacts with several partners in a time- and space-dependent manner (4).
The goal of our research is to identify new functional partners of CFTR, and considering the already known interactions, to analyze if CFTR is a hub protein.
We have used a classic proteomic approach (1D and 2D gels and mass spectrometry) to identify proteins with a CFTR-linked expression level, and we have subsequently tested them as possible CFTR partners. Among the >20 proteins that we have identified as differentially expressed in CF protein samples derived form cell lines, KO mice, and human nasal cells (classical proteomic approach:) we have identified at least 2 proteins that might be involved in CF physiopathology and form a complex with CFTR. They are cytokeratin 18 (CK18)-a cytoskeletal protein, and annexin 1(A1)-an anti-inflammatory protein. By co-immunoprecipitation of CFTR under mild conditions using different CFTR-expressing cell lines and M3A7 CFTR antibodies we have identified CK18 and A1 as potential members/components of a CFTR complex. Other cystoskeletal proteins have also been identified in co-immunoprecipitation experiments, suggesting that these may be important players in CFTR function. CK18 has been found to be involved in the delivery of F508 CFTR to the plasma membrane (5), whereas A1 seems to be important for CFTR localization to lipid microdomains under proinflammatory conditions.
In silico analysis of interactions (indexed in databases as physical interactions) indicates that CFTR interacts with >13 proteins, 6 of them interacting themselves with >8 proteins. Statistical analysis of the discussed interactome fragment indicates that CFTR and its interaction partners follow the double exponential model of a node degree distribution, similar in characteristics to interactomes of entire organisms. With its 13 partners CFTR falls in the middle of the range of observed values of node degrees. It is positioned within the border area of sizes characteristic for small hubs. About 46% of its interactions occur with other hubs in the network. This suggests that CFTR may be a hub protein: depending on its localization and interacting partners, the functions it regulates might be different.

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S2.3 - Large Scale Functional Screens to Study Secretory Protein Traffic

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One of the greatest challenges facing biology today is the conversion of huge amounts of genomic data into functional information about the proteins encoded. We have developed and applied functional assays to assess on a large scale the effect of knock-ins by cDNA overexpression and knock-downs by RNAi, on processes such as constitutive protein transport, Golgi integrity and function of vesicular coat complexes.
In order to achieve the throughput such analyses require, we have developed a fully automated high content screening microscopy platform including sample preparation, image acquisition and analysis. We have applied this technology to a genome-wide siRNA screen to identify comprehensively the genes involved in constitutive protein secretion.

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S2.4 - Cystic Fibrosis due to Abnormal Processing of CFTR Transcripts

Anabela S. Ramalho[1,2], Margarida D Amaral[1,2]

Centre of Human Genetics, National Institute of Health Dr. Ricardo Jorge, Lisboa, Portugal[1]
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Any mutation that disrupts or diminishes the efficiency of the splicing process will have an impact on disease manifestation. In the Cystic Fibrosis (CF) transmembrane conductance regulator (CFTR) gene more than 1,500 mutations were identified [1] most of them being disease-causing. Among these ~13% are classified as splicing mutations because they result in the disruption of canonical splice consensus sequences [1]. However, the concept of what is a splicing mutation is evolving, including nowadays mutations other than those just altering the nucleotides within or close to consensus splice sites, being critical for the clinical setting to determine the actual effect of gene mutations at the mRNA level and directly in native tissues [2].
In this study, we analysed CFTR transcripts from native tissues of CF patients bearing *CFTR* splicing or missense mutations (in one allele and F508del in the other), so as to determine their impact on splicing and thus gain insight on how they influence the disease outcome. Total RNA was extracted and used for RT-PCR amplification with one fluorescently labelled primer in the region spanning the exon with the mutation. Aliquots were collected at different PCR cycles within the reaction log phase. Products were separated by polyacrylamide gel electrophoresis on the ABI Prism 1100™ automatic sequencer allowing distinction between F508del and non-F508del transcripts (3 nt difference).
The 1898+1G>T (intron 12) mutation analysed here results in the skipping of exon 12, as expected. However, the largest percentage of transcripts resulting from this allele lacks both exon 9 and 12. The G576A (exon 12) missense mutation was confirmed here as causing alternative splicing though the alteration of a regulatory splicing element (CERES - composite exonic regulatory element of splicing [3]) that was identified in this exon.
Another missense mutation I1234V (exon 19) was also found to interfere with the normal CFTR splicing, by creating a novel donor splice site and resulting in alternative spliced CFTR transcripts lacking the last 18 nucleotides of exon 19. Data obtained clearly demonstrate that the deleterious effect of this mutation is not due to the amino acid change but rather due to abnormal splicing. Indeed, only alternatively spliced transcripts result from this allele. The processing and function characterization of the truncated protein is in progress.
In conclusion, the analysis of a given mutation at the transcript level directly in native affected tissues is critical not just for disease diagnosis and prognosis, but also to gain insight into the basic mechanisms underlying RNA processing.

Work supported by pluriannual funding from CIGMH (FCT, Portugal). ASR is a recipient of postdoctoral fellowship SFRH/BPD/20622/2004 (FCT, Portugal),

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S2.5 - Pulmonary Surfactant is an Important Lubricant for Cystic Fibrosis Sputum

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Background: Efficient mucociliary clearance is vital to maintaining healthy airways. Ciliated epithelial cells aid the propulsion of mucus out of the respiratory tract. The pericellular fluid layer (PCL) bathes epithelial cilia while separating epithelial cell surfaces from airway mucus. A layer of surfactant phospholipids separates the PCL from mucus functioning as a lubricant. However, in Cystic Fibrosis (CF) the efficiency of mucociliary clearance is greatly reduced. CF sputum tends to be more viscous and altered in composition compared to 'healthy' mucus. In the lower airways, surfactant is responsible for lowering surface tension preventing the collapse of the alveoli. During expiration some surfactant also reaches the upper airways. Here, surfactant may prove beneficial for mucociliary clearance.
Hypothesis: Addition of a natural source pulmonary surfactant (BLES^{TM}) will reduce the surface tension and increase the fluidity of CF sputum.
Methods: We used an in-house fluidity-meter for measurement of fluidity of sputum treated with BLES^{TM}, (bovine lipid extract surfactant), a commercially available pulmonary surfactant (BLES Biochemicals Inc., Canada.)
Fresh expectorated CF sputum samples (n=9) were obtained from patients during routine physiotherapy at the Regional CF Clinic at the Belfast City Hospital. Sputum plugs were collected, 0.5 g samples weighed into 6-well plates, then treated with 0.9% NaCl, treated with pulmonary surfactant BLES^{TM} at 0-400 µg/g and incubated at 37^{o}C (without humidity) for 2 hours. Fluidity was measured with the fluidity-meter where samples were drawn through a narrowing tube to a specified graduation. The time for each sample (100 µl) to reach the graduation was recorded.
Phospholipid concentrations were measured using a commercially available Phospholipids B Enzymatic kit (Wako, Germany). Total protein was analysed using the Lowry method.
Results: The ratio of phospholipid to protein significantly decreased in sputum from CF patients (median: 12.37 (4.14-33.49) µg /mg vs. 1.74 (0.21-1.14) µg /mg, n=6; Mann Whitney U p=0.002), suggesting a relative deficiency of pulmonary surfactant in CF airways. Addition of BLES^{TM} appeared to decrease surface tension in the sputum samples. Visually, after incubation, samples treated with higher concentrations of BLES^{TM} were distributed more evenly in the wells and appeared more fluid. Measurements showed that fluidity [time] decreased in a dose response curve with addition of BLES^{TM}.
However, subsequent fluidity measurements of the same samples showed a very low fluidity, similar to the untreated sputum.
Conclusions: Pulmonary surfactant increases sputum fluidity in a dose dependently, repeated measurements indicate that this may be a pure surface effect with surfactant coating the sputum plugs. The beneficial effects of pulmonary surfactant in airway clearance may be a result of its lubricating effects rather than any mucolytic effect on mucus. However, a sufficient concentration of pulmonary surfactant may be essential for the effective mucociliary transport in the upper airways.

We wish to thank the Staff and Patients of the Regional Cystic Fibrosis Clinic at the Belfast City Hospital. BLES™ was a kind gift of BLES Biochemicals Inc., London, Ontario, Canada.

Thursday 26 April – 14:30-16:00 Room: Master

SYMPOSIUM 3 – Membrane Traffic of CFTR and lessons from Other Proteins

Chairs: / Martina Gentzsch (Chapel Hill, NC, USA)

S3.1 - Myosin Motors Regulate CFTR Trafficking

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University of Pittsburgh School of Medicine, Department of Cell Biology and Physiology, Pittsburgh, PA, USA[2]

The Institute of Physical and Chemical Research (RIKEN), Fukuda Initiative Research Unit, 2-1 Hirosawa, Wako, Saitama, Japan[3]

Graduate School of Life Sciences, Tohoku University, Department of Developmental Biology and Neurosciences, Laboratory of Membrane Trafficking Mechanisms, Aobayama, Aoba-ku, Japan[4]

University of California at San Diego, Section of Cell and Developmental Biology, La Jolla, CA, USA[5]

University of North Carolina at Chapel Hill, Department of Cell and Molecular Physiology, Chapel Hill, NC, USA[6]

University of Massachusetts at Amherst, Department of Biology, Amherst, MA, USA[7]

Dartmouth Medical School, Department of Physiology, Hanover, NH, USA[8]

CFTR-mediated Cl^- secretion across fluid-transporting epithelia is regulated, in part, by modulating the number of CFTR Cl^- channels in the plasma membrane by adjusting CFTR endocytosis and recycling. However, the mechanisms that regulate CFTR endocytosis and recycling in human airway epithelial cells are incompletely understood, at least in part, because the endocytic trafficking itineraries of CFTR in these cells are incompletely understood. Trafficking of CFTR through the endocytic and recycling pathway requires dynamic, spacially and temporally regulated CFTR interactions with macromolecular adaptor protein complexes. As an example, the protein complexes involved in CFTR endocytosis include clathrin, the clathrin adaptor complex AP-2, the large GTPase dynamin, and the Rab5 protein complex. Recycling of CFTR is regulated by the PDZ domain interactions and the epsin homology domain containing protein, Rme1, and the Rab11 protein complex. Our recent data demonstrate that two non-conventional, actin-based, processive mechanoenzymes, namely myosin VI and myosin Vb facilitate the endocytic trafficking of CFTR in human airway epithelial cells. CFTR endocytosis is facilitated by a protein complex that includes myosin VI and its adaptor Dab2 in human airway epithelial cells. Dab2 binds directly to the globular tail of myosin VI and recruits myosin VI to the sites of endocytosis at the plasma membrane. Myosin VI is unique among the processive motors because it is the only known myosin that moves on actin filaments exclusively from the plus to the minus end. Such direction of movement supports the role of myosin VI in CFTR endocytosis. Unlike myosin VI, myosin Vb is a plus-end directed, actin-based mechanoenzyme. The direction of movement of myosin Vb, from the minus to the plus-end of actin filaments supports its role in anterograde trafficking including protein recycling. Myosin Vb is recruited to Ra11a-specific apical recycling vesicles in polarized cells and to recycling vesicles in non-polarized cells. Myosin Vb binds directly to Rab11a and facilitates polarized movement of recycling vesicles to the plasma membrane. CFTR undergoes trafficking in Rab11a-specific apical recycling endosomes in human airway epithelial cells. Myosin Vb is required for CFTR recycling in Rab11a-specific apical recycling endosomes in polarized human airway epithelial cells. This study was supported by a Shwachman Award SWIATE03QO from the Cystic Fibrosis Foundation (ASU), NIH grant P20-RR018787 from the National Center for Research Resources (ASU), NIH grant RO1-DK45881 (BAS), NIH grant RO1-DK34533 (BAS), NIH grant P20-RR018787 from the National Center for Research Resources (BAS), a Research Development Program grant from the Cystic Fibrosis Foundation (BAS), a March of Dimes Birth Defects Foundation grant 6-FY02-150 (TH) and a NIH grant RO1-EY12695 (TH) NIH grant RO1-DC03299 (REC).

S3.2 - Low resolution structures of other ABC proteins: What can they tell us about CFTR?"

Robert Ford,

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S3.3 - ABC Transporters in Adaptive Immunity and Virus Escape Mechanisms

Robert Tampé

Johann Wolfgang Goethe-University, Frankfurt – Institute of Biochemistry, Germany

A challenging task for the adaptive immune system of vertebrates is to identify and eliminate intracellular antigens. Therefore, a highly specialized antigen presentation machinery has evolved to display fragments of newly synthesized proteins to effector cells of the immune system at the cell surface. After proteasomal degradation of unwanted proteins or defective ribosome products, resulting peptides are translocated into the endoplasmic reticulum by the transporter associated with antigen processing and loaded onto MHC class I molecules. Peptide-MHC complexes traffic via the secretory pathway to the cell surface, where they are then inspected by cytotoxic T lymphocytes, which can trigger an immune response. The current view of the translocation machinery and of viral immune escape mechanisms to circumvent destruction by the host will be discussed.

S3.4 – The role of Slc26a6 and CFTR in acid-induced and prostaglandin E_2-stimulated duodenal bicarbonate secretion *in vivo*

Anurag Singh[1], Markus Sjöblom[1], Anja Krabbenhöft[1], Brigitte Riederer[1], William Colledge[2], Manoocher Soleimani[3], and Ursula Seidler[1]

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University of Cincinnati, Cincinnati, USA[3]

Background & Aims: Duodenal mucosal HCO_3^- secretion is a key factor in epithelial protection against gastric acid. We elucidated the role of the apical chloride/base exchanger Slc26a6, and the CFTR Cl^- channel in basal and stimulated state in murine duodenal HCO_3^- secretion *in vivo*. **Methods:** Basal, forskolin-, PGE_2- and luminal acid-stimulated HCO_3^- secretion was examined in Slc26a6- and CFTR-deficient and wt mice. The isolated proximal duodenum was perfused *in situ* with isotonic saline, and HCO_3^- secretion was determined by back-titration. **Results:** The basal secretory rates of bicarbonate were similar in Slc26a6-deficient and WT mice, but significantly reduced in mice deficient in CFTR. Luminal acidification induced the same increase in HCO_3^- secretion in Slc26a6-deficient and WT mice. Slc26a6-deficient mice displayed a significant reduction in HCO_3^- secretion in response to 10 µM luminal PGE_2 compared to WT mice, while forskolin-stimulated HCO_3^- secretion was not different. CFTR-deficient mice displayed a substantial reduction in both PGE_2-stimulated and acid-induced HCO_3^- secretion. **Conclusion:** Slc26a6 plays an important role in PGE_2-stimulated murine duodenal HCO_3^- secretion *in vivo*. The HCO_3^- secretory response to acid, however, is not influenced by the lack of Slc26a6, whereas it is markedly depressed in the absence of CFTR.

S3.5 - Folding, Processing and Function of Human-Murine CFTR Chimeras: Structural Implications

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A powerful approach to investigate the structure and function of the CFTR protein is to examine interspecies differences and identify regions of conservation and divergence. Although little is known about its global structure, it has been shown that the first nucleotide-binding domain (NBD1) of both murine (m) and human (h) CFTR possess essentially the same fold [1,2]. However, functional studies demonstrate important differences in single-channel behaviour between hCFTR and mCFTR. When compared with hCFTR, mCFTR is characterised by 1) reduced single-channel conductance; 2) decreased open-probability; 3) an altered pattern of channel gating and 4) insensitivity to channel potentiation by pyrophosphate (PP_i) [3]. To understand the structural basis for these differences in function, we generated hmCFTR chimeras containing mCFTR domains on an hCFTR backbone and investigated their biochemical properties. We evaluated the *in vivo* folding of hmCFTR chimeras by transfecting cells with chimeric constructs of hmNBD1, hmNBD2 and hmR domain (RD), in which all or part of NBD1, NBD2 and the RD of hCFTR were replaced by the equivalent regions of mCFTR. Like wt-hCFTR, most chimeric proteins were processed within the cell. However, two chimeras failed to be processed: clone 12b (mNBD1 residues 518-585) and clone 114c (mNBD2 residues 1260-1412). When we compared the murine amino acid (aa) sequences of these chimeras with the corresponding human regions, we found 12 (clone 12b) and 27 (clone 114c) aa substitutions. We selected the most divergent aa changes in clone 12b and 114c in terms of physico-chemical distance (PCD) to perform *in vitro* mutagenesis in the context of both hCFTR and mCFTR backbones. For clone 12b the mutations are: E527Q, E528Q (PCD = 29); S531T (PCD = 58); K536Q (PCD = 53), I539T (PCD = 89) and K584E (PCD = 56). In addition, for clone 114c the mutations are: P1290T (PCD = 38), K1302Q (PCD = 53), Y1307N (PCD = 143), S1311K (PCD = 121), C1344Y (PCD = 194), D1394G (PCD = 94) and E1409D (PCD = 45). Biochemical analyses of these mutants expressed in BHK cells revealed that the following mutants are processed: E527Q, S531T, K536Q and I539T, whereas K584E is not. Additional biochemical analysis of these mutants is underway while functional characterization of these constructs is planned to gain insight into the individual role of these residues in CFTR structure and function. It is expected that identification of differences caused by small variation in the polypeptidic chain will identify critical residues responsible for conformational changes, thus explaining functional differences between human and murine CFTR.

Work supported by the BBSRC and grant POCTI/MGI/47382/2002 (FCT, Portugal) and pluriannual funding of CIGMH (FCT, Portugal). AC DaPaula is a recipient of PhD fellowship SFRH/BD/17475/2004 (FCT, Portugal).

1. Lewis H et al (2004) *EMBO J* 23. 282-93.
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Thursday 26 April – 16:30-18:00 Room: Master

SYMPOSIUM 4 – Epithelial Physiology

Chair: Mike Gray (Newcastle, UK) & Robert Dormer (Cardiff, UK)

S4.1 – Novel effect of bile acids on pancreatic bicarbonate secretion

Mike Gray[1], Viktória Venglovecz,[2] Zoltán Rakonczay Jr.,[2] Béla Ózsvári,[2] Barry E. Argent,[1] Péter Hegyi [2,3].

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S4.2 - Submucosal glands and surface epithelium: the dynamic duo of the airway

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Innate mucosal defences, which include secretion of mucus from submucosal glands and secretion/absorption of salt water from the airway surface liquid (ASL) by the surface epithelium, keep the airways sterile by mucociliary clearance and the bacteriostatic properties of the mucus. Gland density is about 1/mm^2 in the upper airway and gland volume per mm^2 is ~10 fold larger than the surface epithelium. Acutely, the glands can secrete fluid 10 fold faster than the surface can absorb (Widdicombe et al *Eur Resp J.* 1997). Chronically, the glands add mucins and antimicrobials to a mucus blanket that grows deeper and moves faster as it travels up the steadily decreasing surface area of the airways. The increased abundance of upper-airway mucus and its enrichment with antimicrobials is important because most of the interaction between inhaled air and airway mucus occurs in the nose and first few airway generations. In cystic fibrosis (CF) the airway innate defences are clearly dysfunctional, and the airway mucus becomes chronically infected by opportunistic, gram negative bacteria, particularly *Pseudomonas aeruginosa.* These bacteria also infect immune compromised individuals and people who have indwelling devices, such as catheters. This may provide a clue about why CF airways are vulnerable: their innate immune system is compromised, such that static mucus patches may provide the stable surface the bacteria require colony formation, and the lack of bio-available antimicrobials permits their growth. Exactly how the loss of CFTR function produces these changes is not yet definitively established. Our work emphasizes the role of CFTR as an anion channel that mediates Cl^- and HCO_3^- dependent fluid secretion. Loss of CFTR in surface epithelia means that dehydrated airways don't secrete fluid under passive conditions, but complete mucus stasis and cilia collapse is avoided by the operation of a CFTR-independent secretion pathway that depends upon mechanically-stimulated ATP release (Boucher and colleagues). Like the surface epithelia, the glands also have both CFTR-dependent and CFTR independent mechanisms for fluid secretion. As shown by Ballard and colleagues, if all fluid secretion is blocked with transport inhibitors, macromolecular secretion continues in stimulated glands and viscous mucus clogs the glands. Fortunately, this extreme condition does not occur in cystic fibrosis glands, which can continue to secrete mucus in response to strong cholinergic input. However, in unprovoked airways, glands are predicted to produce a low level of tonic secretion, and this kind of secretion appears to depend importantly on the CFTR pathway. What happens to gland mucus in the absence of the CFTR pathway? Using time-lapse, differential interference contrast (DIC) microscopy, we have filmed mucus as it transits isolated airway submucosal glands. In control glands, heterogeneous, viscoelastic mucus flows at different velocities within a duct. CF gland mucus moves more slowly and retracts when agonists are removed suggesting that the mucus strand is tethered and can stretch. Defective gland mucus may involve the premature entanglement of the mucin strands as they explode from the packed granules into an environment deficient in bicarbonate (Quinton hypothesis), water (Wine hypothesis) or both, since these conditions will limit the rate of expansion.

S4.3 –Intestinal Acid/Base Transport 2007: New Transporters, Regulatory Pathways, and New Therapeutic Strategies

Ursula Seidler

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Intestinal HCO_3^- transport is of major importance for the barrier function of all intestinal segments, and a crucial element of electrolyte and fluid movement in all digestive organs. The major transport pathways for HCO_3^- and many aspects of its biological significance have been defined in the pre-molecular period. The advances in molecular biology in the last decade brought forth a larger number of candidates for the long-sought transport proteins involved in intestinal HCO_3^- transport than expected, and we are currently facing the difficult task to attribute the already known transport processes and regulatory events to the potential molecular players. In this overview, the current concepts of the ion transport proteins involved in HCO_3^- transport in the intestine are discussed.

The last decade has also led to the identification of a multitude of proteins involved in the regulation of intestinal acid/base transport. Elegant studies in heterologous expression systems have defined the crucial role for the formation of multiprotein signalling complexes in the regulation of CFTR activity as well as its interaction with other ion transporters. In particular, the NHERF family of PDZ-domain adapter proteins have been shown to interact with CFTR and regulate its membrane abundancy and function in a variety of cellular systems. Studies on the significance of these findings in the native intestinal epithelium are now available and will be discussed. These proteins may also be of crucial importance for the interaction of CFTR with members of the SLC26 family of anion exchangers in the coordinated secretion of Cl^- and HCO_3^- ions

Will these findings lead to new therapeutic options? They may. A recent review in Nature drug development calls the targeting of PDZ interactions in disease pathways “a new magic bullet”. Unfortunately, the PDZ-interactions sites are barely drugabble shallow grooves. But the recent demonstration that the mere overexpression of normal NHERF1 leads to an increase membrane retention, as well as conductivity, of a CFTR trafficking mutant in an airway cell line is impressive indeed.

S.4.4 - Estrogens increases CFTR expression in MDCK and MCF7 epithelial cell lines.

Nabila Hamdaoui, Noura Bensalem, Delphine Roussel, Meriem Garfa-Traore, Fatna Makaci, Naziha Bakouh, Isabelle Sermet-Gaudelus, Aleksander Edelman, Maryvonne Legros, Janine Fritsch & Gabrielle Planelles

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Introduction. The regulatory factor NHERF-1 is an apical PDZ protein that interacts with the carboxy terminus DTRL motif of CFTR. It has been reported that NHERF-1 overexpression (after transfection in human bronchial epithelial cell lines) increases both expression and channel activity of CFTR and redistributes F508 CFTRCFTR from the endoplasmic reticulum to the plasma membrane (Guerra et al, 2005, J Biol Chem, 280: 40925). Interestingly, NHERF-1 is a primary response gene for estrogen: estrogen induces a tremendous up-regulation of NHERF-1 expression (Ediger et al, 2002, Mol Endocrinology 16:1828). Thus, it may be that estrogens physiologically modulate CFTR expression and function, via NHERF. At present time, the effects of estrogen and flavonoids on CFTR are debated (Illek & Fisher, 2002, Phytochem 60: 205; Singh et al, 2000, J Pharm Exp Ther, 295: 195).
Aim of the study We investigate the effect of estrogens and estrogen derivatives on cultured cell lines. MDCK cells derive from renal distal tubule, and are a model for polycystic disease ; it is considered that CFTR plays a major role in the cysts formation in this genetic pathology. MCF7 cells are endowed with estrogen receptors and derived from human epithelial breast cancer; in the human mammary epithelium, CFTR and ENaC are considered to play major role in the ductal NaCl absorption.
Methods In MDCK and in MCF7 cells, the effects at different time exposure of various concentrations of 17 alpha- and 17 beta-estradiol, and of several flavonoids are studied. Western blot analyses are performed on total proteins and on total membrane proteins, using the CFTR 24-1 antibody or NHERF-1 antibody. CFTR 24-1 antibody was used to localize CFTR in cyto-immunochemistry experiments. Short circuit current measurements are performed in control MDCK cells versus estrogen-treated MDCK cells (liquid-liquid or in air-liquid cultures) : in the presence of amiloride (10 µM) and of barium chloride (5 mM), current changes induced by a CFTR-activating cocktail (forskolin, 20 µM+ IBMX, 100 µM + cAMP, 100 µM) are measured. The glycine hydrazide GlyH101 (50 µM), recently reported to block the CFTR pore from the extracellular side (Muanprasat et al, 2004, J Gen Physiol 124 : 125), is then added to confirm that current change induced by the activating cocktail is related to the activation of CFTR.
Results and conclusion In MDCK cells and in MCF7 cells treated by 17 beta-estradiol and by resveratrol, Western-blots analysis show a time- and concentration-dependent increase of CFTR endogenous expression (total proteins and total membrane proteins). In MCF7 cells, estrogens also increase NHERF-1 expression (total proteins). Immunocytolocalization experiments confim that estrogens increase the CFTR expression. This increase in CFTR expression during treatment is first detected in the cytoplam, then at the plasma membrane. Confocal x-z sections show that CFTR is apically located. Short-circuit current measurements are under investigation to determine if the increase in membrane CFTR expression is associated with a functional change in CFTR-mediated current.

This study is supported by the French Association "Vaincre la Mucoviscidose"

S4.5 - Characterization of volume-regulated anion channels (VRAC) in normal and cystic fibrosis epithelial cells: regulation of VRAC by CFTR and receptor agonists

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Cystic fibrosis (CF) is a life-threatening disease caused by a defect in the cystic fibrosis trans-membrane conductance regulator (CFTR). CFTR primarily moves chloride ions but can also regulate other channels that move ions. Volume-regulated anion channels (VRACs) are found on many cell types and are critical for cell volume regulation and other functions (e.g., cell proliferation and apoptosis). Little attention has been applied to the volume related channel (VRAC) which is an anion channel highly permeable to chloride (and even larger molecules) critical for cell volume regulation. VRAC is likely to undertake some of the functions of CFTR by transporting chloride, other solutes and water. It is possible that by modulating VRAC function in CF cells that some of the problems with airways surface liquid could be restored. We believe that the amount of VRAC and/or its function may be altered in CF and might contribute to CF disease severity.
Initial studies on the amount and function of VRAC were carried out on two bronchial epithelial cell lines, a F508del homozygote CFBE41o-, and a normal cell line 16HBE14o-, and also on primary nasal epithelial cells (NECs) grown from nasal brushings from CF patients and normal controls using patch-clamp technology.
Cultured cells were separated by a brief trypsin treatment and plated on glass cover slips forming the bottom of 0.5 ml chambers which were placed on the stage of an inverted Nikon Eclipse TE2000-S microscope. Small volume of the recording chambers allowed rapid (<1 s) complete replacement of the external solution. The culture medium was replaced with standard external solution (SES) before establishing whole-cell configuration. Following formation of the whole-cell configuration, cell input resistance was evaluated. If input resistance exceeded 1 GΩ further electrical recordings were performed. For precise measurements of the steady-state currents at different test potentials and evaluation of the voltage-dependent kinetics of the currents, we employed a voltage-step protocol which consisted of 1 s pulses to test potentials ranging from -100 to 120 mV (applied every 5 to 20 s depending on the type of the experiment) with an increment of 10 mV.
Our results have shown that forskolin-evoked Cl^- currents are present in 16HBE14o-, but not in CFBE41o- cells. Moreover, normal cells compared to CF cells have substantially higher resting conductance, which is explained by the presence of several types of constitutively active channels as observed in on-cell patches.
Both primary cells cultures (normal and CF) were characterised. The CF NECs were found to be highly responsive to hypotonic conditions, such that they sensed even as little as 15% reduction of osmolarity and generated VRAC similar to normal HNECs. Interestingly, CF-HNEC also generated VRAC-like current in response to infusion of GTPγS.
These preliminary data indicate that in CF cells, VRAC expression and function is not grossly impaired and therefore vectorial transport of osmolytes and water required for normal epithelial function can be achieved in CF cells via this alternative pathway.

Supported by Queen's University Belfast and the Cystic Fibrosis Trust

S4.6 - Ex vivo chloride secretion measurements as outcome parameter of the CF basic defect

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Ex vivo chloride (Cl^-) secretion measurements (Intestinal current measurement, ICM) in cystic fibrosis (CF) patients have been established over the past 15 years to study the CF transmembrane conductance regulator (CFTR) basic defect in more functional detail. Modified circulating Micro-Ussingchambers are used to registrate the transepithelial short-circuit current (I_{sc}) in freshly obtained human rectal suction biopsies as a measure of ion transport after stimulation with secretagogues of the relevant intracellular pathways (Veeze 1991, Veeze 1994, Bronsveld 2000). Hereby, the CFTR Cl^- channel, its amount of residual function in CF and alternative Cl^- channels can be investigated by a standardised protocol (de Jonge 2004), which can be also applied for diagnostic purposes in individuals with equivocal results in the standard diagnostic tests.
In the course of the development of CFTR pharmacotherapeutics as well as agents activating alternative Cl^- channels, ICM may function as a useful outcome parameter in CF clinical trials. It is easy to perform repeatedly at all patient´s ages and comprises the safety advantages of an ex vivo method which is relevant especially for early study phases. Previous ICM experiences exhibited reproducible intraindividual interpretation of secretory responses. Aim of this study was to describe reference values and quantify the intraindividual variability of different ICM parameters.
Patients and methods: A total of n=574 rectal biopsies from n=212 individuals with pancreatic insufficient (PI)-CF (n=22; mean age 14.5 years), pancreatic sufficient (PS)-CF (n=9; 10.8 years), excluded CF by ICM diagnostics (n=169; 12.8 years) and healthy control (n=12; 26.3 years) was included into analysis. For calculation of intraindividual variability, 2-4 biopsies per patient were compared with respect to basal tissue resistance ($R_{t\ basal}$), basal open circuit potential difference ($PD_{\ basal}$), basal short circuit current ($I_{sc\ basal}$) and the I_{sc} responses to stimulation with carbachol (10^{-4} mol/l, serosal), 8-bromo-cyclic monophosphate (cAMP) (10^{-3} mol/l, mucosal+serosal) + forskoline (10^{-5} mol/l, serosal) and histamine ($5x10^{-4}$ mol/l, serosal).
Results: We determined ICM reference values for the groups of PI-CF ($I_{sc\ basal}$ 23.4 ± 18.1 µA/cm², $\Delta I_{sc\ carbachol}$ 0.4 ± 3.6 µA/cm², $\Delta I_{sc\ cAMP/forskoline}$ 2.0 ± 2.9 µA/cm², $\Delta I_{sc\ histamine}$ -3.1 ± 4.6 µA/cm²), PS-CF ($I_{sc\ basal}$ 45.6 ± 31.5 µA/cm², $\Delta I_{sc\ carbachol}$ 3.4 ± 6.8 µA/cm², $\Delta I_{sc\ cAMP/forskoline}$ 8.0 ± 10.4 µA/cm², $\Delta I_{sc\ histamine}$ 6.3 ± 10.8 µA/cm²), and healthy control ($I_{sc\ basal}$ 39.0 ± 26.1 µA/cm², $\Delta I_{sc\ carbachol}$ 27.3 ± 7.1 µA/cm², $\Delta I_{sc\ cAMP/forskoline}$ 15.2 ± 10.6 µA/cm², $\Delta I_{sc\ histamine}$ 27.4 ± 15.2 µA/cm²). For the total cohort (n=212), mean coefficients of variation were: $R_{t\ basal}$ 29%, $PD_{\ basal}$ 48%, $I_{sc\ basal}$ 49%, $\Delta I_{sc\ carbachol}$ 59%, $\Delta I_{sc\ cAMP/forskoline}$ 64%, $\Delta I_{sc\ histamine}$ 80%.
Conclusion: This first comprehensive analysis of the intraindividual variability of ICM basal tissue and Cl^- secretion parameters provides the basis for the method as a useful outcome measure for future clinical trials aiming to rescue the CFTR basic defect. Possible effects of pharmacological therapeutics in CF relevant human epithelia have to be adequately interpreted with respect to subject variability and laboratories´ reference data. Ex vivo Cl^- secretion measurements have the potential of being an essential step in the evaluation process of CFTR-correcting/potentiating agents on their way from laboratory screening to the application in human CF tissue without any risk of toxicity.

Thursday 26 April – 18:30-20:00 Room: Gilão

Special Group Discussion – IA

What is wrong with F508del-CFTR structure and how can we rescue it?

Chairpersons: Bob Ford (Manchester, UK) & Phil Thomas (Texas, USA)

The most common cystic fibrosis-causing mutation leads to a loss of a single phenylalanine residue at position 508 (F508del) in the first Nucleotide Binding Domain (NBD1) of the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR). This mutation interferes with the maturation of CFTR through the secretory apparatus and, thus, causes a dramatic reduction in active CFTR at the plasma membrane. Aberrant folding of the NBD1 and CFTR and subsequent recognition by cellular quality control systems underlies the poor maturation efficiency of the mutant protein. Notably, correction of the folding/maturation defects by a variety of manipulations restores at least some CFTR function at the membrane suggesting a therapeutic opportunity. Effective exploitation of this information will require detailed structural knowledge of CFTR and F508del-CFTR. Recent changes in our understanding of the structures of intact ABC transporter homologues of CFTR and isolated CFTR domains provide significant insight into the possible mechanisms by which the F508del mutation exerts its damaging effects.

The objectives of the SGD are to:

--summarize the relevance of published ABC transporter and CFTR domain structures to understanding the dysfunction and correction of F508del CFTR.

--present the latest unpublished progress toward structural information of CFTR itself, identify areas where progress is currently stymied and ask whether there are any new techniques that might offer hope for advancement of our knowledge.

--interpret available data on folding, function, misfolding and quality control recognition within the context of these structures.

--discuss opportunities for effective interventions to circumvent the effects of the mutation and obstacles to this goal.

--other burning issues arising from earlier sessions at the Tavira meeting.

Thursday 26 April – 18:30-20:00 Room: Arade

Special Interest Discussion Group - IB

Chair: Mauri Krouse (Stanford, CA, USA) & Mike Gray (Newcastle, UK)

pH and CF Disease

Cystic fibrosis is caused by a defect in CFTR, an anion channel that conducts both chloride and bicarbonate ions. Since bicarbonate (and CO_2) is the main determiner of pH in the body, defects in transport of this anion will have important consequences for epithelial tissues. In CF, defective CFTR leads to a loss of bicarbonate in the secreted fluid making it more acidic. This loss of bicarbonate may lead to an environment in the airway that is conducive to bacterial growth, may lead to activation of enzymes within the pancreas which lead to its destruction, and may reduce mucosal protection in the intestine. In addition to bicarbonate movement CFTR may also provide counterions for H^+ movement in intracellular organelles in both epithelial and non-epithelial cells.

This Special Group discussion will explore whether defects in bicarbonate and pH are primary defects, exacerbating factors or unimportant in CF disease.

Friday 27 April – 09:00-10:30

SYMPOSIUM 5 – ENaC: Traffic and Activity

Chair: Karl Kunzelmann (Regensburg, Germany) & Deborah Baines (London, UK)

S5.1 - Altered serine protease-protease inhibitor balance in the proteolytic regulation of ENaC: A mechanism for sodium hyperabsorption in Cystic Fibrosis

Michael M. Myerburg, Erin E. McKenna, Joseph D. Latoche, Raymond A. Frizzell, Thomas Kleyman, Joseph M. Pilewski

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Efficient pulmonary clearance of mucus and pathogens requires an optimal airway surface liquid (ASL) volume, which is maintained by the regulated transport of Na+ and Cl-. When the balance between Na+ absorption, through the epithelial sodium channel (ENaC), and Cl- secretion, through CFTR, is disrupted, as in CF, ASL volume is reduced and mucus clearance is impaired. Despite the evidence that Na+ hyperabsorption is critical to the pathogenesis of CF airways disease, the mechanism by which ENaC becomes dysregulated is incompletely understood. Recent studies have demonstrated that proteolytic cleavage of ENaC activates the channel, and that serine protease inhibitors (PIs) block ENaC activity. We therefore investigated the role that PIs and channel activating proteases (CAPs) play in the regulation of ENaC in normal and CF primary human airway epithelial cells (HAEC).

We reasoned that under physiological conditions endogenous PIs might prevent proteolytic activation of near-silent ENaC by CAPs. To assess this, we measured the amiloride sensitive current (I_{Na}) response to serine protease inhibition with aprotinin and stimulation with trypsin in HAEC cultured at air-liquid conditions and following ASL volume expansion. Following ASL volume expansion, I_{Na} increased more than two fold. This increase in I_{Na} with ASL volume expansion is due to dilution of endogenous PIs as (i) aprotinin did not affect I_{Na} at air-liquid conditions, but decreased I_{Na} in ASL volume expansion conditions, (ii) trypsin increased I_{Na} at air-liquid conditions, but not under ASL volume expansion conditions, and (iii) reconstitution of the expanded ASL volume with acetone-precipitated apical secretions from HAEC prevented the increase in I_{Na} and the decrease in trypsin response associated with ASL volume expansion. We also investigated whether normal and CF HAEC differ in susceptibility to trypsin and PIs. In contrast to normal HAEC, I_{Na} in CF HAEC was inhibited by 39% following exposure to aprotinin at air-liquid conditions (versus 18.2 % in normal HAEC, p=0.043), while the activating effect of trypsin on I_{Na} in CF HAEC was half of that seen in normal HAEC.

Collectively, these data indicate that (i) protease inhibitors in the ASL prevent the activation of near-silent ENaC, (ii) when the ASL depth is increased, endogenous protease inhibitors become diluted, allowing for proteolytic activation of near silent channels, and (iii) ENaC channels are constitutively active in CF, suggesting that protease-antiprotease imbalance is involved in the development of sodium hyperabsorption and CF airways disease.

More recent studies have begun to determine whether the serpin, protease nexin-1 (PN-1), regulates ENaC in HAEC and whether deficient PN-1 expression causes excessive proteolytic activation of ENaC in CF. PN-1 formed an inactive 82 kDa complex with prostasin in HAEC. Addition of recombinant PN-1 (0-1 μM) increased the intensity of this 82 kDa band and decreased Na^+ conductance. While PN-1 expression was modestly reduced in CF compared to normal HAEC lysate, the biological significance of this finding is uncertain. These results suggest that PN-1/prostasin interactions are involved in the proteolytic regulation of ENaC activity in HAEC, however, the mechanism for protease-protease inhibitor imbalance in CF remains to be defined.

S5.2 - Casein kinase 2 may regulate epithelial Na^+ channels by rescuing from Nedd4-2 mediated degradation

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Department of Maternal and Child Health Sciences, University of Dundee, Ninewells Hospital, Dundee, DD1 9SY, UK. [3]
[#]TB and JA contributed equally

Cystic fibrosis transmembrane conductance regulator (CFTR) is a protein kinase A and ATP-regulated Cl^- channel that also controls the activity of other transport proteins such as the epithelial Na^+ channel (ENaC). We recently demonstrated that CFTR binds the pleiotropic casein kinase (CK) 2 and translocates it to the cell membrane. CK2 activates CFTR and also phosphorylates ENaC, as demonstrated by the Garty group. CFTR could affect ENaC expression or activity by either competing for CK2 binding or by clustering CK2 in particular cell compartments. We therefore examined the potential regulation of ENaC by CK2. We found that the CK2 inhibitor 4,5,6,7-tetrabromobenzotriazole (TBB; 1 – 100 µM) acutely and dose dependently inhibits ENaC activity in epithelial tissues from trachea, distal colon and cultured mouse collecting duct cells, as measured in Ussing chamber experiments. ENaC was also inhibited by TBB in double electrode voltage clamp experiments of ENaC expressing oocytes. Overexpression of a TBB insensitive double mutant of CK2 abrogated the inhibitory effect of TBB on ENaC. Also, mutating the CK2 phosphorylation sites in beta-ENaC (S631A) or gamma-ENaC (T599A) reduced ENaC activity by more than 90 % and rendered the remaining ENaC activity insensitive to TBB. Moreover, membrane expression of ENaC was largely reduced for the mutated ENaC - channel. In contrast to wtENaC, which translocates CK2 to the cell membrane, mutant ENaC is unable to enrich CK2 in the plasma membrane of *Xenopus* oocytes. As recently demonstrated by the group of D. Cook, the G protein-coupled receptor kinase (Grk2) phosphorylates the C terminus of the channel beta subunit and renders the ENaC channels insensitive to inhibition by Nedd4-2. Amiloride sensitive Na^+ currents in mouse salivary duct cells, however, were insensitive to TBB. Since the Grk2 phosphorylation site (S633) is in close proximity to the CK2 phosphorylation site (S631), we speculate that CK2 phosphorylation similarly reduces Nedd4-2 mediated inhibition of ENaC. Depending on the type of tissue, the metabolic state of the cell or the cellular compartment either Grk2 or CK2 may take the lead in ENaC regulation.

S5.3 Forskolin increases transepithelial Na^+ transport across H441 cell monolayers via induction of cell shrinkage and translocation of EGFP-human αENaC to the apical membrane

Woollhead, A and Baines, D L

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Activation of β2-adrenoceptors and elevation of intracellular cAMP increases fluid re-absorption in the lung by raising amiloride-sensitive Na^+ transport through apically localised epithelial Na^+ channels (Olver *et al.*, 1986; Folkesson *et al.*, 1998) . Elevation of cAMP in both fetal and adult rat lung epithelial cells increases Na^+ transport (Niisato *et al.*, 1999; Ramminger *et al.*, 2000). Evidence indicates that elevation of cAMP inhibits retrieval of epithelial Na^+ channel (ENaC) proteins from the apical membrane and also increases translocation of subunits to the apical membrane from an intracellular pool. However, the signaling pathways mediating translocation of ENaC proteins to the lung cell membrane are not well understood. To address this, we have used resistive monolayers of human H441 airway epithelial cells to investigate changes in function and the cellular localisation of green fluorescent protein (EGFP)-labelled human αENaC in response to elevation of cAMP.

We show that inhibition of protein tyrosine kinase (PTK) with genistein and protein kinase A (PKA) with KT5720, decreased forskolin-stimulated amiloride-sensitive short circuit current (I_{sc}) across H441 adult human lung epithelial cell monolayers. KT5720 also decreased basal I_{sc}. RT-PCR and immunoblot analysis of stable clones expressing green fluorescent protein (EGFP)-labelled human αENaC in H441 cells revealed two clones expressing a truncated (αC3-5) and full length (αC3-3) EGFP-hαENaC protein. Only the αC3-3 clone exhibited a 50% increase in basal and forskolin-stimulated amiloride-sensitive I_{sc} indicating that the full length protein was required for functional activity. Apical surface biotinylation and real-time confocal microscopy demonstrated that EGFPhαENaC (αC3-3) translocated to the apical membrane in response to forskolin in a Brefeldin A-sensitive manner. This effect was completely inhibited by Genistein but only partially inhibited by KT5720. Forskolin also induced a reduction in the height of cells within αC3-3 monolayers, indicative of cell shrinkage. This effect was inhibited by KT5720 but not by Genistein or Brefeldin A. These data show that forskolin activates PKA-sensitive cell shrinkage in adult human H441 lung epithelial cell monolayers, which induces a PTK-sensitive translocation of EGFP-hαENaC subunits to the apical membrane and increases amiloride-sensitive Na^+ transport.

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S5.4 - Regulation of ENaC Membrane Traffic by Casein Kinase 2

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Cystic fibrosis (CF) is caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene that encodes a cAMP-dependent Cl^- channel. Among many other cellular defects observed in CF, epithelial Na^+ absorption via the epithelial Na^+ channel ENaC is enhanced, due to defective regulation of ENaC by CFTR [1,2]. The mechanisms involved in CFTR dependent downregulation of ENaC are not yet completely understood. Wild-type CFTR, but not F508del-CFTR has been shown to bind and translocate casein kinase 2 (CK2) to the cell membrane, suggesting a role of CK2 for regulation of ENaC [3]. In the present study, we examined the role of the pleiotropic CK2 in the regulation of ENaC and CFTR. For this purpose, we performed two-electrode voltage clamp analyses in *Xenopus* oocytes, heterologously expressing the three (alpha beta gamma) subunits of rat ENaC and also human CFTR. Data demonstrated that CK2 phosphorylation of wt-CFTR is required for normal channel gating by PKA. In addition, data show that CK2 phosphorylation sites in beta ENaC (S631) and gamma ENaC (T599) are necessary for functional expression of ENaC and that mutation of these residues to alanine largely reduces amiloride-sensitive conductances to values below 1 microS. Inhibition of CK2 by its specific inhibitor 4,5,6,7-tetrabromobenzotriazole (TBB) induced a significant decrease in the amiloride-sensitive conductance measured either in *Xenopus* oocytes or in the mouse renal epithelial cell line M1. Moreover, chemiluminescence experiments in *Xenopus* oocytes show that the membrane expression of the alpha ENaC subunit requires phosphorylation by CK2, suggesting a requirement of this enzyme for proper assembly of ENaC subunits and trafficking of a functional channel to the cell membrane.
For a clarification of the molecular mechanism of CK2-dependent trafficking of ENaC to the cell surface, which is suggestive of a direct/indirect interaction between these two proteins, a search for interacting protein partners of ENaC is underway. To this end, the C terminus of each human ENaC subunit was cloned into the pET-SUMO-1 vector (Invitrogen) fused to the SUMO protein for enhanced solubilisation and to a polyhistidine (pHis) tag. Constructs produced were: alphaENaC (aa 584-669, alpha ENaC-Cterm), betaENaC (aa 554-640, beta ENaC-Cterm); and gammaENaC (aa 563-649, gamma ENaC-Cterm). After isopropylthio-beta-galactoside (IPTG)-induced expression of the construct in BL21 bacteria and purification, each of the three ENaC-Cterm polypepdides was immobilised onto metal-affinity resin and used to capture interacting protein partners from human pulmonary cell line H441 total and sub-cellular lysates. Protein-containing fractions recovered ENaC-Cterm-coated and blank resins are subjected to 2D-gel and protein identification.
The functional characterization of the interactions among the captured proteins, ENaC and CK2 is expected to help elucidating the membrane trafficking of ENaC and the regulation of sodium transport in CF.
Work supported by DFG SFB 699 A6 (Germany) and pluriannual funding of CIGMH. J Almaça is a recipient of doctoral fellowship SFRH/BD/28663/2006 (from FCT, Portugal).

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S5.5 - Role of the NHERF adapter proteins NHERF1, NHERF2/E3KARP and NHERF3/PDZK1 in the regulation of murine duodenal bicarbonate secretion in vivo

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Background & Aims: Heterologous expression studies have demonstrated that a family of adapter proteins called the NHERF family bind to the cystic fibrosis membrane regulator (CFTR) and modulate its membrane expression, conductivity and interaction with other transport proteins. Their role in the regulation of CFTR-dependent intestinal anion secretion *in vivo* has not been studied. The aim of this investigation was to elucidate the role of these adaptor proteins in regulation of murine duodenal bicarbonate secretion *in vivo.* **Methods:** Basal and stimulated duodenal HCO_3^- secretion was examined in the NHERF1, E3KARP (NHERF2), PDZK1 (NHERF3), double (NHERF1 and PDZK1) and triple (NHERF1/E3KARP/PDZK1) +/+ and -/- mice. The triple ko mice displayed a delayed growth rate but no gross pathology. After anaesthesia, the proximal duodenum was cannulated and perfused with isotonic saline and HCO_3^- secretory rates in the basal state and after stimulation with luminal forskolin was determined by back-titration. **Results:** The basal secretory rates of bicarbonate were similar in E3KARP-deficient and wt mice, but significantly reduced in mice deficient for NHERF1 (40% of the respective wt littermate), for PDZK1 (45%), for NHERF1/PDZK1 (38%) and for NHERF1/E3KARP/PDZK1 (53%). The secretory response to luminal perfusion with 100 µM forskolin was significantly reduced only in the NHERF1-deficient (47% of the respective wt mice), the NHERF1/PDZK1-deficient (36%) and the NHERF1/E3KARP/PDZK1-deficient mice (53%). **Conclusions:** 1) By studying basal and cAMP stimulated duodenal HCO3$^-$secretion we demonstrated that different NHERF proteins are necessary for basal vs stimulated secretion. 2) The absence of the adapter proteins NHERF1 as well as NHERF3/PDZK1 resulted in significantly reduced basal HCO_3^- secretion in a non-additive manner. This indicates both NHERF1 and NHERF3/PDZK1 are necessary for some aspects of the basal pathway. 3) In contrast, the duodenal HCO_3^- secretory response to elevated cAMP only dependent on NHERF1. This suggests not only that cAMP turns on additional regulatory steps involved in HCO3$^-$ secretion but that it turns off the NHERF3/PDZK1 steps involved in basal HCO3$^-$ secretion. 4) The major NHERF adaptor protein involved in regulation of duodenal bicarbonate secretion is NHERF1.

S5.6 - Toll-like receptor expression in CFBE and HBE cells

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Background: *Pseudomonas aeruginosa* (**PA**) infection is an important cause of pulmonary inflammation in Cystic Fibrosis (**CF**) lung disease, associated with increased decline in lung function, morbidity and mortality. Toll-like receptors (**TLR**) are part of the innate immune system responsible for recognition of pathogen-associated molecular patterns. The immune response to PA involves binding and signalling via TLR2, where TLR2 recognises conserved microbial products (lipopetides, LPS, bacteria). Compared to non-CF cells, the activation of in CF bronchial epithelium induces prolonged and excessive pro-inflammatory signalling with secretion of e.g. interleukin-8 (IL-8). Upon binding of PA or PA-LPS, aggregation of TLR2 on the cell surface initiates the intracellular signal transduction. The receptor complex is then internalized and TLR2 is degraded/recycled.
Hypothesis: Increased pro-inflammatory signalling in CF epithelium may be due to increased TLR2 expression.
Methods: We used the well utilised cell lines HBE (human bronchial epithelial cells) and CFBE (CF bronchial epithelia cells, F508del). As both cell lines have different cell size (HBE<CFBE), we first established the optimal seeding concentrations necessary to achieve similar confluency prior to LPS challenge using microscopic evaluation of confluency and mitochondrial activity by MTT test. Cells were seeded at $1x10^4$-$15x10^4$/ml (12 well plates). Furthermore, to determine the best confluency for a maximal LPS response, cells were stimulated with PA-LPS (50 □g/ml) for 24 h and IL-8 determined by ELISA (R&D Systems, UK). Finally, using the optimal seeding concentration and confluency, cells were stimulated with PA-LPS for up to 72 h. TLR2 expression was determined using a monoclonal antibody to TLR2 (IMGENEX, USA) and FACS analyses. IL-8 was determined by ELISA.
Results: Our initial experiments showed that HBE cells reach their maximum IL-8 secretion at ~ 80-85% confluency, while CFBE cells secrete even higher amounts of IL-8 at 100% confluency. Moreover, at similar confluency (80-90%) unstimulated HBE cells often secrete more IL-8 than unstimulated CFBE cells. The optimal seeding concentration to reach a confluency of ~75% for LPS challenge and subsequent IL-8 analyses after 24 h was found to be 1.5x105/ml for HBE and $1x10^5$/ml for CFBE cells.
TLR2 analyses revealed that CFBE cells have a higher expression of TLR2 protein on the cell surface than HBE cells (4-12 h PA-LPS exposure). Surprisingly we found TLR2 to be expressed at higher levels intracellularly, with CFBE cells showing higher intracellular TLR2 expression at 1h, 8h and 12 h of PA_LPS exposure ($p<0.05$ for 8 and 12h). This high expression of TLR2 coincided with increased secretion of IL-8 (24 h and 72h) in CFBE compared to HBE cells.
Conclusion: The optimal confluency (and seeding concentrations) is essential for experiments comparing physiological responses between HBE and CFBE cells and may have to be determined when culture conditions change. Activation of surface expressed TLR2 is important to initiate signalling. Prolonged intracellular signalling has been shown for Epidermal Growth Factor Receptor when the receptor does not enter the degradation pathway, but remained in a perinuclear compartment and our data suggest further signalling by intracellular TLR2 in CFBE compared to HBE cells.

Friday 27 April – 11:00-12:30

SYMPOSIUM 6 – Biogenesis and Quality Control of Membrane Proteins

Chairs: William Skach (Portland, USA) & Margarida Amaral (Lisboa, Portugal)

S6.1 -F508del CFTR is targeted for ERAD by sHsp-mediated sumoylation

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The endoplasmic reticulum (ER) provides mechanisms that facilitate the appropriate folding of newly synthesized proteins, and processes that eliminate misfolded proteins via ER associated degradation (ERAD). Steps in ERAD involve the recognition and removal of misfolded proteins from the ER, followed by their cytoplasmic ubiquitylation and proteasome-dependent digestion. CFTR was the first integral membrane mammalian protein to be implicated as an ERAD substrate (1,2). Due to its complex folding scheme, the majority of WT CFTR and nearly 100% of the common folding mutant, F508del CFTR, are degraded by the ubiquitin-proteasome pathway (3).

A system that leads to selective degradation of F508del CFTR was first identified using a genomic screen in yeast, which degrade WT CFTR as though it were the F508del variant (4). Yeast expressing CFTR, showed elevated transcript levels of a small heat shock protein (sHsp), Hsp26, and CFTR degradation was blocked in strains lacking the two known yeast sHsps, *HSP26* and *HSP42*. Mammalian cells express 10 sHsp homologues; two of these, αA-crystallin and Hsp27, were detected in Calu-3 and primary HBE airway cells. Their co-expression with WT or F508del CFTR elicited proteasome-dependent reductions in F508del CFTR levels at steady-state and enhanced mutant protein degradation in pulse-chase experiments, while WT CFTR degradation was not affected. αA-crystallin and Hsp27 selectively associated with F508del CFTR in co-immunoprecipitation (IP) experiments. These data indicate that sHsps selectively increase the exposure of F508del CFTR to ubiquitin-proteasome degradation pathways.

Similar to ubiquitin, the small ubiquitin-like modifier, SUMO, is added to its substrates by serial thiol transfer reactions, and Hsp27 was reported, by Y2H, to interact with the SUMO E2 conjugating enzyme, Ubc9 (5). We verified this interaction by co-IP and found also that Ubc9 and the SUMO protease, SENP1, were expressed in airway epithelial cells. Expression of Ubc9 decreased, while expression of SENP1 increased, the levels of F508del CFTR, and pulse-chase studies implicated changes in ERAD in their actions on F508del levels. Similar to Hsp27, these actions were selective for F508del CFTR. Bacterially expressed and purified NBD1 (Phil Thomas, UTSW) was sumoylated by E1 plus E2 enzymes in vitro. SUMO was added to F508del or WT NBD1, and this process was facilitated by Hsp27. In vivo, F508del CFTR sumoylation was enhanced by Ubc9 or Hsp27 co-expression. These findings indicate that small heat shock proteins selectively interact with F508del CFTR, leading to its degradation by the proteasome. This process appears to be mediated by addition of SUMO to NBD1, and perhaps other sites. The relation of SUMO addition to the ubiquitylation and disposal of mutant CFTR remains to be explored.

Supported by grants from the NIH and the Cystic Fibrosis Foundation

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S6.2 - CFTR Expression Regulation by the Unfolded Protein Response

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We recently reported that the unfolded protein response (UPR) decreases endogenous wild-type (WT) CFTR expression. As a follow up, we investigated the role of the folding deficient, F508del CFTR on ER stress and UPR induction. We also identified the main mechanism by which the UPR decreases endogenous CFTR expression. For these studies, we developed Calu-3ΔF clones expressing different ratios of recombinant F508del and endogenous WT CFTR. For endogenous F508del CFTR expression, CFPAC-1 cells were tested. Our results indicate that the UPR is constitutively activated in Calu-3 ΔF Clone 5, expressing high levels of recombinant mutant CFTR. No UPR activity was detected in CFPAC-1 or in Calu-3 ΔF Clone 3. However, induction of recombinant F508del CFTR expression with sodium butyrate in Calu-3 ΔF Clone 3 causes ER stress and activates the UPR. Furthermore, pharmacological induction of the UPR in CFPAC-1 and Calu-3 ΔF cells diminishes endogenous CFTR mRNA to virtually undetectable levels. The decrease in endogenous CFTR mRNA levels under ER stress is not the result of decreased mRNA half-life. In contrast, using a human CFTR promoter driven, firefly luciferase reporter vector (Hu*CFTR*Lu) we show a transcriptional suppression of the human CFTR promoter under ER stress. Considering the significant efforts directed towards the rescue of F508del CFTR as therapeutic approach for CF, it is important that F508del CFTR expressed at *in vivo* levels does not cause ER stress and activate the UPR. Our results also reveal that F508del CFTR rescue agents need to be carefully tested for UPR activation since suppression of endogenous F508del CFTR transcription may contribute to inefficient rescue in native cells and tissues.

S6.3 - Processing and Turnover of CFTR in Highly Differentiated Cultures of Human Airway Epithlia

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CFTR is a glycoprotein synthesized on ER-associated ribosomes and subsequently transported to the Golgi and plasma membrane. In various heterologous expression systems less than half of native immature CFTR is converted to mature fully glycosylated protein. Although it has been suggested that the total pool of native immature CFTR matures in epithelial cell cultures [1], we find that maturation of endogenous CFTR in epithelial cell lines derived from lung, colon or pancreas is not efficient (~30-60%) and occurs to a similar extent in highly differentiated primary cultures of human airway epithelia (HAE).

To visualize turnover of cell surface CFTR in HAE cultures we employed adenoviral vectors with a CFTR construct containing an extracellular epitope tag (Extope-CFTR). After labelling the apical pool of CFTR with an antibody recognizing this tag, we followed internalization of CFTR over time, as done previously in non-polarized cells [2]. Wild-type CFTR internalization occurred more slowly in HAE cultures than in non-polarized BHK-21 cells. In BHK-21 cells the internalized CFTR is first detected in association with EEA1-containing early endosomes and transferrin-positive recycling compartments and later with lysosomal markers [2]. Co-localization of endocytic CFTR in HAE cultures revealed overlap with EEA1-positive compartments and partial co-localization with lysosomal markers. Therefore although CFTR endocytosis occurs more slowly in HAE cells, the overall itinerary appears to be similar to that in BHK-21 cells.

The most common mutation in cystic fibrosis, F508del, causes misfolding and retention of the immature protein at the ER. Low temperature, chemical chaperones and more recently some newly identified small molecule correctors were shown to overcome this processing block [3]. We expressed Extope-F508del CFTR in fully differentiated HAE cultures and observed temperature-rescued mature F508del at the apical membrane.

In heterologous non-polarized systems it has been demonstrated that the cell surface life-time of rescued F508del is significantly shorter than of wild-type CFTR due to reduced recycling and rapid degradation of the internalized protein [2,4]. A recent study in a polarized airway cell line confirmed the shorter half-life of rescued apical F508del and attributed it to an increased in endocytosis [5]. When we labelled the cell surface pool of temperature-rescued Extope-F508del CFTR in HAE cultures and followed the turnover, we found that the mutant protein transited from the apical membrane to endocytic compartments much more rapidly than the wild-type. Virtually all of the apical pool of the mutant protein had been internalized by the time the apically-derived wild-type protein first appeared intracellularly.

In conclusion wild-type CFTR matures as inefficiently in HAE cultures as in non-polarized heterologous systems, but is more stable at the epithelial apical membrane than at the plasma membrane of non-polarized cells. However, once internalized, wild-type CFTR appears to traffic through similar compartments in polarized and non-polarized cells. Temperature-rescued F508del CFTR also has a generally similar fate in differentiated airway cells as in non-polarized cells. Thus it has a shorter half-life at the epithelial apical membrane than wild-type CFTR and moves more rapidly to intracellular endocytic compartments, from which it is rapidly eliminated.

Supported by CFF and NIH.

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S6.4 - Membrane Integration Efficiency of Individual Transmembrane Segments of the Multispanning Membrane Protein CFTR

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The topogenesis of multi-spanning membrane proteins is dictated by determinants contained within the primary sequence of the polypeptide chain. The classical notion is that hydrophobic stretches of primary sequence integrate into the membrane, while the hydrophilic ones are either translocated across or remain on the inside of the membrane. However, in polytopic membrane proteins, transmembrane (TM) segments can be found that normally would not qualify as membrane spanning, judging by the relatively low hydrophobicity of these segments. It is thought that these marginally hydrophobic stretches are dependent upon determinants outside of the membrane-spanning region, such as flanking residues and neighboring helices, for proper membrane integration. In our lab, the efficiencies of integration into the endoplasmic reticulum (ER) membrane of putative TM segments of the cystic fibrosis transmembrane conductance regulator (CFTR) were measured individually. Using a recently characterized system where the studied TM segment is engineered into the bacterial transmembrane protein leader peptidase, followed by expression in the presence of ER microsomes and subsequent mapping of glycosylations, it was found that TM1-4 of CFTR individually integrate efficiently both with and without their natural flanks. These TMs all have negative predicted free energy changes of integration and are therefore expected to efficiently integrate into the membrane. TM6 and TM8, however, both have positive predicted free energy changes of integration and are expected to be poor integrators. In the experiments, TM6 exhibited efficient integration only in the presence of the natural flanks. Further studies narrowed down the flank important for TM6 integration to the adjacent valine residue on the C-terminal side of the TM segment. TM8 exhibited poor integration efficiency both with and without the natural flanks and preliminary data indicate that the efficiency is enhanced when TM8 is preceded by TM7 in the cotranslational membrane integration. The results suggest that proper membrane integration of marginally hydrophobic TM segments in polytopic membrane proteins is ensured by sequence elements outside of the TM segment itself.

S6.5 - Pathways for intracellular transport of CFTR

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Cystic fibrosis (CF) is one of the most widespread of the monogenic diseases, and it arises from mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) protein. The mechanisms involved in the transport of the CFTR protein remain almost unknown. Here, we aim to elucidate the mechanisms of intracellular transport of CFTR through the application of a combination of morphological, *in situ* and biochemical analyses that could provide a precise synchronization of transport of CFTR and other protein cargoes. To this end, IB3-1 (bronchial cells from patients with F508del CFTR) and S9 (IB3-1 cells stably transfected with wt CFTR) cells have been examined. We showed that wtCFTR did not co-localized with giantin, although some patches of wtCFTR were localized near the Golgi region (but never co-localizing with giantin or ManII), some were integrated into the PM. In contrast, F508del CFTR remained mostly in the ER (Fig. 1). In IB3-1 and S9 cells, F508del CFTR was mostly localized in the ER, close to the nucleus (perinuclear region), and wtCFTR was scattered all over the cell, forming vacuoles. To visualize CFTR at the apical PM, we incubated confluent monolayers of IB3-1 and S9 cells on ice with the antibody. After washout and labelling with a secondary antibody, the signal from IB3-1 cells was significantly different from that of S9 cells, suggesting that, indeed, in IB3-1 cells F508del CFTR is not seen at the PM. Careful electron microscopic examination of IB3-1 and S9 cells revealed striking differences between the structures of the Golgi in these cells. In IB3-1 cells, the Golgi was normal, with the usual compartments; in S9 cells stably transfected with wtCFTR, there was hypertrophy of the intermediate compartment and appearance of the characteristic reticular subdomain within it. In some cases, this subdomain was within the forming autophagosomes. These data suggest that CFTR uses an unusual transport pathway.

S6.6 - Role of Casein Kinase 2 in the Early Steps of CFTR Biogenesis

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The most frequent mutation in the cystic fibrosis (CF) gene, F508del, causes retention of its protein product, F508del-CF transmembrane conductance regulator (CFTR) in the endoplasmic reticulum (ER) as a core-glycosylated intermediate that is rapidly degraded. F508del-CFTR therefore fails to traffic to the plasma membrane, where wild-type (wt) CFTR normally functions as a chloride (Cl^-) channel. Retention, however, is not due to lack of function, since the mutant still retains some function if it reaches the membrane. Instead, it results from misfolding which is recognized by the ER quality control (ERQC) in which many intervenients, including molecular chaperones, participate.

It was recently shown that casein kinase II (CK2) binds wt CFTR near the F508 residue, phosphorylating its first nucleotide binding domain (NBD1) at S511 [1]. Interestingly, deletion of F508 abrogates this interaction, which is the first described F508del-dependent protein-protein interaction. Our aim here was to identify whether CK2 interaction affects the early steps of CFTR biogenesis, turnover and processing.

Novel BHK cells were produced which stably express wt- or F508del-CFTR in which the consensus residue S511 was substituted by either a neutral (alanine - S511A) or an acidic residue (aspartate - S511D). Metabolic labelling and pulse-chase experiments followed by CFTR immunoprecipitation were performed in these lines. After quantification of bands B (immature form) and C (mature form) of CFTR, these preliminary results show that substitution of S511 does not affect the turnover or processing of either wt- or F508del-CFTR.

The effect of CK2 inhibition on the turnover and processing of CFTR was also studied. Cells were incubated with 20 μM of the CK2 inhibitor tetrabromobenzotriazole (TBB) for 90 min and pulse-chase experiments performed as above. Results under TBB treatment show that: 1) the steady-state levels of both wt- and F508del-CFTR are reduced; 2) the turnover of wt-CFTR (but not of F508del-CFTR) is increased; and 3) processing of wt-CFTR is decreased.

Our data suggest a putative stabilizing role for CK2 upon wt-CFTR in these cells. However, in our preliminary results this effect does not appear to be dependent on residue 511 (nor on the putative charge added by the kinase on this residue), thus suggesting that this effect may be indirect.

Work supported by pluriannual funding of CIGMH and POCTI/SAU/MMO/58425/2004 grant (FCT, Portugal). LS Pissarra is recipient of doctoral fellowship BD/9095/2002 (from FCT, Portugal).

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Friday 27 April – 18:30-20:00 Room: Arade

Special Interest Discussion Group – IIA

Is regulation of ENaC tissue-specific?

Chair: Karl Kunzelmann (Regensburg, Germany) & Ray Frizzell (Pittsburgh, PA, USA)

In human airway, the epithelial sodium channel (ENaC) plays an important role in fluid absorption and the maintenance of an optimal airway surface liquid (ASL) layer. Together with the impaired activity of CFTR, increased ENaC-mediated Na^+ absorption leads to insufficient airway surface hydration, impaired mucociliary clearance, persistent bacterial infection, inflammation and bronchiectasis. This progressive airway disease accounts for most of the morbidity and mortality in CF. This Special Discussion Group will examine the regulation of airway ENaC activity, contrasting these processes with the control of its better understood counterpart in distal nephron and salivary duct. Topics for discussion will focus on the regulation of ENaC gating and trafficking by agonists (e.g. cAMP, nucleotides), protease-mediated subunit cleavage, bacterial products. To what degree is airway ENaC regulation is due to changes in membrane resident channel gating as opposed to the regulation of its density in the apical membrane via altered trafficking? Differences in these processes that have the potential to explain the enhanced Na absorption characteristic of the CF airway, and treatment strategies will also be discussed. Attendees of the SDG are welcomed to contact the discussion leaders regarding brief presentations of their thoughts/data during this session.

Friday 27 April – 18:30-20:00 Room: Gilão

Special Interest Discussion Group - IIB

Endo/Exocytic Regulation of CFTR

Chair: Agnes Urban (Pittsburgh, PA, USA) & Martina Gentzsch (Chapel Hill, NC, USA)

CFTR is a highly regulated chloride channel expressed in the apical plasma membrane of epithelial cells. CFTR mediated chloride transport is regulated by modulating channel activity and by controlling the total number of CFTR channels at the cell surface. The latter is achieved by the removal (i.e. endocytosis) and the insertion (i.e. recycling) of CFTR at the plasma membrane. This discussion group will outline the processes that control the plasma membrane trafficking of CFTR. An increasing number of CFTR-interacting adaptor proteins have been identified that control the expression of CFTR in the plasma membrane. These adaptors include ER chaperons, PDZ proteins, SNARE proteins, Rho and Rab GTPases, myosin motors, protein kinases, and components of the ubiquitin-dependent endosomal sorting machinery. In addition several sequence motifs in the polypeptide sequence of CFTR have been identified that regulate the ER retention, apical targeting, or internalization of CFTR. We will review current knowledge on the intracellular trafficking of CFTR and specifically address differences between cultured non-polarized cell lines and highly differentiated airway epithelial cultures.
F508del CFTR, the most common mutant protein in cystic fibrosis, has a folding defect and is retained by quality control in the ER. Low temperature and chemical chaperones were shown to promote the transit of F508del to the plasma membrane and more recently several new components have been identified that allow a small proportion of F508del CFTR to mature. However, temperature rescued F508del CFTR is rapidly targeted for lysosomal degradation while the majority of internalized wild-type CFTR is recycled back to the plasma membrane. We will explore approaches that not only aim at the ER rescue of F508del CFTR but also intend to restore its plasma membrane stability.

In this Special Interest Discussion Group we will gather together experts from various disciplines with different research focus to discuss their current understanding of the endo/exocytic regulation of CFTR.

Saturday 28 April – 09:00-10:30

SYMPOSIUM 7 – Models for Airway Signalling

Chairs: Marc Chanson (Geneva, Switzerland) & Carla Ribeiro (Chapel Hill, NC, USA)

S7.1 Airway Epithelial Infection, ER Stress, Ca2+ Signals and Inflammation: What is the Connection?

Mary E. Braun and Carla M. Pedrosa Ribeiro.

Department of Medicine and Cystic Fibrosis/Pulmonary Research and Treatment Center, The University of North Carolina at Chapel Hill, Chapel Hill, North Carolina, USA.

We have reported expansion of the endoplasmic reticulum (ER) and its Ca^{2+} stores in infected and inflamed native human airway epithelia from diseases such as cystic fibrosis (CF) and primary ciliary dyskinesia. The ER/Ca^{2+} store expansion was reproduced *in vitro* by exposing mucosal surfaces of primary cultures of human bronchial epithelia (HBE) to supernatant of mucopurulent material (SMM) from bacteria-infected and inflamed CF airways, suggesting a link of airway epithelial ER/Ca^{2+} store expansion to luminal infection and inflammation. Indeed, increased ER/Ca^{2+} stores can contribute to airway inflammation by amplifying Ca^{2+}-dependent inflammatory pathways and by increasing the ER capacity for the synthesis of inflammatory mediators. The molecular mechanisms leading to infection and inflammation-induced epithelial ER/Ca^{2+} store expansion in the airways are not known. Neither is it clear whether the ER/Ca^{2+} store expansion is a beneficial or a detrimental response that leads to hyperinflammation in chronically infected and inflamed airways.

Our data suggest that infection of HBE *in vivo* or *in vitro* induces ER stress, recognized as the unfolded protein response (UPR), coupled to stimulation of inositol requiring 1 (IRE1)-dependent mRNA splicing (activation) of X-box binding protein 1 (XBP-1). Spliced XBP-1 (XBP-1s) is a transcription factor shown in other cell types to expand the ER and the secretory pathway, increase lipid biosynthesis, and augment the ER protein folding capacity. Our *in vitro* findings are consistent with UPR activation via XBP-1s in HBE during luminal infection and inflammation, since 1) SMM up-regulates lipid biosynthesis and secretory pathway genes; 2) studies in cells stably expressing XBP-1s or a dominant negative XBP-1 (DN-XBP-1) suggest a key role for this pathway on infection-induced inflammation and ER expansion; and 3) *Pseudomonas aeruginosa* (*P. aeruginosa*) induces inflammation and XBP-1s coupled to ER expansion. The relevance of the UPR in airway inflammation *in vivo* is suggested by initial studies linking *P. aeruginosa* infection to XBP-1s and ER expansion in inflamed murine airway epithelia, and increased levels of XBP-1s and XBP-1s-induced target genes in native CF HBE.

Our findings suggest that activation of the UPR in airway epithelia by infection and inflammation is an important epithelial response to airways infection. We are currently utilizing a variety of *in vitro* and *in vivo* studies manipulating UPR pathways to address the role and significance of UPR-mediated events during airway inflammation. Unraveling the functions of UPR pathways such as IRE1/XBP-1s should help determine if therapies targeted to manipulate pathway activity would be likely to improve lung function in patients with CF and other chronic inflammatory airway diseases.

Funded by The CFF.

S7.2 - Airway Signalling in CF Mice

Hugo de Jonge, Martina Wilke, Huub Jorna, Alice Bot, Marcel Bijvelds, Boris Hogema

Department of Biochemistry, Erasmus University Medical Center, Rotterdam, The Netherlands

Studies of CFTR and ENaC regulation in CF mice have sofar been limited mainly to tracheal and nasal epithelium. However, CF mouse tracheae seem less qualified as a model in view of major anatomical and physiological differences with human trachea[1]. In contrast, the hyperactivity of ENaC, a prime diagnostic criterium in CF patients, is faithfully reproduced in nasal epithelium from both CFTR-/- and F508del CFTR mice, irrespective of the genetic background[2]. Immunostaining of β-ENaC in this tissue was rather uniform in all epithelial cell types and strongly enhanced in CF mice. CFTR immunoreactivity was highest in the microvilli of brush cells and nearly absent in ciliated cells of the respiratory epithelium. The relative amount of residual mature CFTR (band C on Western blots) in nasal epithelium from F508del CFTR mice (~30% of WT) greatly exceeded the amount detected in other mouse tissues (<10%), corresponding with virtually normal forskolin/cAMP induced transepithelial chloride currents (Isc) in the Ussing chamber. However the *in vivo* change in nasal potential difference (NPD) in response to Cl^--free+forskolin superfusion was typically only 40-50% of WT and may be used as a biomarker for pharmacological correction of F508del CFTR in mouse airways.

Recent reports that the NO/guanylyl cyclase/cyclic GMP pathway and inhibitors of the cGMP-hydrolyzing phosphodiesterase PDE5 (e.g. sildenafil) can normalize the endosomal pH in CF airway cells possibly through inhibition of ENaC[3], and that sidenafil corrects CFTR mistrafficking and dysfunctioning in nasal epithelial cells from F508del CF patients *ex vivo*[4] has prompted us to investigate NO/cGMP regulation of ENaC and CFTR in mouse nasal epithelium in more detail. Unexpectedly, the NO donor nitroprusside, sildenafil and 8-Br-cGMP all failed to inhibit the amiloride-sensitive Isc (reflecting ENaC activity in the luminal membrane) in both CF and WT mice. However, all cGMP agonists, in the presence of amiloride, caused a strong stimulation of Isc in WT and F508del CF mice (equalling 40% of forskolin activation) but not in CFTR-/- mice (congenic FVB), indicative for cGMP-activated, CFTR-dependent anion secretion.

8-Br-cGMP activation of Isc could neither be mimicked by inhibitors of PDE3, a cGMP-inhibitable phosphodiesterase, nor by a Ca^{2+} ionophore or Ca^{2+}-linked agonists (carbachol; mucosal UTP), arguing against cGMP acting through cAMP or through cGMP-provoked Ca^{2+} release/uptake and Ca^{2+} activation of basolateral K^+ channels. Surprisingly, cGMP stimulation of Isc persisted in nasal epithelium from transgenic mice lacking the cGMP-dependent protein kinases type I and type II, excluding a conventional protein kinase-mediated signalling pathway. Alternative mechanisms, including cGMP gating of K^+-selective CNG channels or of CFTR itself, possibly involving direct binding of cGMP[5], are under present investigation. In addition, KO mouse models for the PDZ domain proteins NHERF1-3 are exploited to investigate the role of these CFTR binding partners in cAMP-and cGMP-regulation of anion secretion and in CFTR-ENaC interaction in native airway epithelium.

1. Grubb BR & Boucher RC 1999. *Physiol Rev Suppl* 79: S193-S214
2. Scholte BJ 2004 *J Cystic Fibrosis* 3: 183-190
3. Poschet JF et al. 2006 *EMBO Rep* 7: 553-559
4. Dormer RL et al. 2005 *Thorax* 60: 55-59
5. Pereira MMC et al. 2007 *Biochem J manuscript* 20061879

S7.3 - Influence of lung fibroblasts on proliferation and differentiation of human airway epithelial cells: specific expression profile of gap junction forming connexins

Marc Chanson

Laboratory of Clinical Investigation III, Geneva University Hospitals, 1211 Geneva, Switzerland

We have developed an airway cell model to allow studying the differentiation of human airway epithelial cells *in vitro*. Airway epithelial cells (AEC) were seeded onto permeable supports that carried on their undersurface a preformed feeder layer of primary human airway fibroblasts. As compared to previously described methods, the use of fibroblast feeder layers strongly stimulated the proliferation of epithelial cells, allowing the expansion of the cell pool with successive passages. AEC at increasing passage were seeded onto supports undercoated with airway fibroblasts and exposed to air. Either freshly isolated or amplified AEC could differentiate into a pseudostratified mucociliated epithelium for at least 9 months. These airway epithelia showed apical expression of CFTR and cAMP-dependent Cl^- currents in Ussing chambers. In comparison, cultures of CF airway epithelium showed elevated Na^+ transport, drastic hyper-absorption of surface liquid and absence of cAMP-induced Cl^- secretion. They were also characterized by thick apical secretion that hampered the movement of cell surface debris by cilia but did not exhibit increased production of mucins or IL-8. To evaluate the differentiation state of these airway epithelia, we documented the expression of a family of epithelial gap junction connexins (Cx26, Cx30, Cx30.3, Cx31, Cx31.1, Cx32 and Cx43). Gap junctions are important regulator of cellular functions by providing direct pathways for intercellular communication. These functions are ensured by the expression of tissue-specific connexins whereas alteration in connexin expression is associated with abnormal tissue functions. We observed that maturation of AEC was associated with the down regulation of Cx26 and Cx43 whereas Cx30 was mostly present between ciliated cells and Cx31 between basal cells. Dye coupling assays using the fluorescent tracer Lucifer Yellow revealed limited gap junctional communication between differentiated AEC. In contrast, cultures of airway epithelium generated in the absence of feeder layers showed extensive dye coupling and large expression of Cx43. Of note, the expression of Cx30 and Cx31 but not Cx43 was confirmed in the mouse airway epithelium. These results are supportive of the establishment of signal-selective interaction between the feeder layers and AEC, likely contributing to support airway epithelium maturation. In addition, they lay the ground for studying the role of connexin-mediated cell-cell communication during repair following AEC injury and exploring connexin-targeted interventions to modulate the healing process in the normal and CF airway epithelium.

S7.4 - Role of CFTR in dendritic cell function upon infection with Pseudomonas aeruginosa

Christine Tertilt[1,2], Yaqin Xu[2], Lena Reichenbach[1], Hansjörg Schild[1], Stefan Worgall[2]

Department of Immunology, Mainz Gutenberg University, Germany[1]; Department of Pediatrics, Weill Medical School of Cornell University, New York, USA.[2]

Cystic fibrosis patients are highly susceptible to pulmonary infection with *Pseudomonas* (P.) *aeruginosa*. While this is in part attributed to the defect in mucociliary clearance caused by the typical changes in mucus consistency, it is now recognized that CFTR defects also lead to more specific immune abnormalities. It is further hypothesized that CFTR deficiency leads to a hyperinflammatory state in the lung with excess recruitment of granulocytes even in the absence of infection, and that this may contribute to lung pathology. Dendritic cells (DC) are the most potent antigen presenting cells and play a crucial role in the initiation and regulation of immune responses. Changes in DC function can contribute to abnormal immune responses on multiple levels, such as antigen processing and presentation, expression of costimulatory molecules, and production of cytokines. We therefore addressed the response of mouse bone marrow derived DC isolated from wildtype and CFTR deficient mice to *in vitro* stimulation with LPS and *P. aeruginosa*. We were able to show by flow cytometric analysis that CFTR is expressed at low levels by immature DC and strongly upregulated upon stimulation. In order to search for potential differences in the response of DC to *P. aeruginosa*, we isolated RNA from DC generated from wildtype and CFTR deficient mice and performed microarray analysis. Stimulation with LPS or *P. aeruginosa* both induced profound changes of expression levels of genes involved in inflammation and chemotaxis, signalling, cell cycling and apoptosis. While the gene expression pattern of resting wildtype and CFTR deficient cells was similar, we were able to identify 35 genes with more than 5fold induction upon exposure to *P. aeruginosa* in wildtype but not in CFTR deficient DC, 15 genes with more than 5fold induction in CFTR deficient but not wildtype DC, 9 genes with more than 5fold downregulation in wildtype but not CFTR deficient cells, and 4 genes which showed more than 5fold downregulation in CFTR deficient but not wildtype cells. Differential expression of select genes of interest has been confirmed by real-time PCR. We are now in the process of investigating in which way these changes affect dendritic cell function. Our results provide insight into the changes in the immune system in the absence of a functional CFTR. These findings are important not only to further elucidate the mechanism of *P. aeruginosa* susceptibility in cystic fibrosis, but may also need to be taken into account for the development of *P. aeruginosa* vaccines which will confer protection in cystic fibrosis patients.

S7.5 - Bestrophin 1 and 2, important components of the calcium activated chloride channel in mouse trachea epithelium

R. Barro-Soria[1], Marisa Sousa[12], R. Schreiber, K[1]. Kunzelmann[1]

Institut für Physiologie, Universität Regensburg, D-93053 Regensburg, Germany[1]
Instituto Nacional de Saúde Dr. Ricardo Jorge, Lisboa, Portugal[2]

Ca^{2+} activated Cl^- channels (CaCCs) play an important role in many physiological processes and diseases such as cystic fibrosis and Polycystic kidney disease. It has been proposed that the activation of CaCCs could be a therapy for cystic fibrosis but this has been impeded in part due to the unknown molecular identity of these channels. Bestrophin1 has been proposed to form a new family of Cl^- channels; however there are still many controversial issues to be solved. For instance, ATP, CCH, ADP and Ionomycin induced short circuit current (I_{sc}) in mouse trachea and colon from mbest 1 knockout mice (VMD2-/-) showed no differences when compare to wild type (VMD2+/+). We studied the contribution of the different mouse bestrophins for the Ca^{2+} activated Cl^- secretion in primary trachea epithelial cells (TEC). ATP-induced whole cell currents in the VMD2-/- TEC, expressing only bestrophin 2 and 3, were two times smaller than that obtained in the VMD2+/+ TEC. Interestingly, heterologous expression of mbest1 in VMD2-/- TEC exhibited ATP-induced whole cell currents similar to that of the wild type. Moreover, short interfering RNA (siRNA) for mbest2 suppressed ATP induced whole cell currents in VMD2-/- TEC by 70% but only by 38% in the VMD2+/+ TEC. ATP-activated currents were also significantly inhibited in VMD2+/+ TEC, treated with either siRNA targeting mbest1 or a combination of siRNA for mbest 1+2. Immunostaining analysis confirmed reduced expression of bestrophin in siRNA transfected cells. These results suggest a role of mbest1 and mbest2 for the CaCCs in the trachea. It is also likely that these proteins form part of a large complex together with other membrane proteins.

Supported by DFG SFB699A7 and Else-Kröner-Fresenius Stiftung

Saturday 28 April – 11:00-12:30

SYMPOSIUM 8 – Epithelial Cell Biology, Inflammation and Pathogens

Chairs: Anil Mehta (Dundee, UK) & Joe Pilewski (Pittsburgh, PA, USA)

S8.1 – CFTR: Regulation of Intracellular Acidification and Bacterial Killing in Alveolar Macrophages

A.Di[1], L. Deriy[1], M.E. Brown[1], A.P. Naren[2] V. Bindokas[1], H.C. Palfrey[1], and D.J. Nelson[1]

The University of Chicago, Chicago, IL 60637, USA[1]
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The family of phagocytic immune cells plays a major role in host defense against microbial infection. The site of the most clinically important pathology in CF is the lung and mutations in CFTR lead to an increased propensity for pulmonary infection in susceptible subjects. The mechanism by which the genetic defect in CF leads to bacterial respiratory infections and associated chronic inflammation remains one of the major obstacles in the design of long-term effective therapeutic strategies. It is well-established that neutrophil-dependent airway inflammation exists in CF, but alveolar macrophages (AMs) have received relatively little attention. We chose to study AM function as modulated by CFTR expression in CFTR-null and mutant mice, as well as human CF tissue, and examine the potential role these cells might play in the pathogenesis of the disease. AMs were found to express functional CFTR Cl^- channels at the cell surface as well as on various internal membranes by electrophysiological and immunocytochemical criteria. While phagocytosis per se was not affected by CFTR function, we found that the elimination of internalized bacteria as well as lysosomal and phagosomal acidification was greatly reduced in the absence of normal CFTR. By contrast, neutrophils did not show bactericidal defects in CF and these cells may use an alternate chloride conductance for both internal acidification and bacterial killing. Defective lysosomal acidification was also observed in AMs isolated from F508del CFTR mutant mice. We have also recently explored the possibility that the enhanced state of inflammation seen in the CF lung may be exacerbated due to an induction of apoptotic signaling in response to the delayed clearance of microbial pathogens. Comparative examination of AMs isolated from WT and F508del CFTR mice by TUNEL labeling revealed a significant increase in the population of apoptotic AMs in bronchoalveolar lavages from mutant mice over that observed in WT controls. We are currently assessing whether the difference in the apoptotic population is related to the lack of acidification in the lysosomal compartment. The defects in AM lysosomal acidification in the CF lung might be pleiotropic, leading to a decrease in bacterial clearance, disruption in cytokine secretion, and activation of pro-apoptotic signaling cascades. While compromised epithelial cell function and resultant changes in the mucus layer are important factors in promoting bacterial persistence our results suggest that a defective innate immune response may contribute significantly to the pathogenesis of CF.

S8.2 - CFTR Inhibition Mimics the Cystic Fibrosis Inflammatory Profile

Aura Perez [1], Amanda C. Issler [1], Calvin U. Cotton [1,2], Thomas J. Kelley [1,3], Alan S. Verkman [5], and Pamela B. Davis [1,2,4]

Departments of Pediatrics [1], Physiology & Biophysics [2], Pharmacology [3], and Molecular Biology & Microbiology [4], School of Medicine, Case Western Reserve University, Cleveland, OH; and Departments of Medicine and Physiology [5], University of California, San Francisco, California, USA

Primary airway epithelial cells grown on filters in air-liquid interface (ALI) differentiate into cultures that resemble native epithelium morphologically, express ion transport similar to those *in vivo*, and secrete cytokines in response to appropriate stimuli. Comparisons of cultures derived from normal and CF individuals are difficult to interpret due to genetic differences apart from CFTR. The recently discovered thiazolidinone CFTR inhibitor, $CFTR_{inh}$-172, was used to create a CF model with its own control to test if loss of CFTR-Cl^- conductance alone was sufficient to initiate the CF inflammatory response. Continuous inhibition of CFTR-Cl^- conductance for 3-5 days resulted in significant increase in IL-8 secretion at basal ($p = 0.006$) and in response to 10^9 *Pseudomonas* ($p = 0.0001$), a four-fold decrease in Smad3 expression ($p = 0.02$), a 3-fold increase in RhoA expression, and increased NF-κB nuclear translocation upon TNFα/IL-1β stimulation ($p < 0.000001$). CFTR inhibition by $CFTR_{inh}$-172 over this period does not increase ENaC activity, so lack of Cl^- conductance alone can mimic the inflammatory CF phenotype. $CFTR_{inh}$-172 does not affect IL-8, IL-6 or GM-CSF secretion in two CF phenotype immortalized cell lines: 9/HTEo^- pCEP-R and 16HBE14o^- Antisense, or IL-8 secretion in primary CF cells, and inhibitor withdrawal abolishes the increased response, so $CFTR_{inh}$-172 effects on cytokines are not direct. Five-day treatment with $CFTR_{inh}$-172 does not affect cells deleteriously as evidenced by LDH assays, trypan blue exclusion, ciliary activity, EM histology, and inhibition reversibility (59% and 70% of CFTR-Cl^- conductance is recovered at 1 and 4h, respectively, after removal of $CFTR_{inh}$-172). Our results support the hypothesis that lack of CFTR activity is responsible for the onset of the inflammatory cascade in the CF lung.

S8.3 - Inflammation and the Cystic Fibrosis Epithelium

Madeleine Ennis[1], F de Courcey[1], F Dunlevy[1], JS Elborn[1], H Love[1], BC Schock[1], G Skibinski[1], A Zholos[2],M Williams[1]

Respiratory Medicine Research Cluster [1]and Cell and Metabolic Signalling Research Group[2], The Queen's University of Belfast, Grosvenor Road, Belfast BT12 6BN, UK

Despite being exposed to a variety of insults e.g. pollution, bacteria, viruses etc., lungs of the healthy population are not inflamed. In contrast, in the cystic fibrosis population there is a vicious cycle of chronic inflammation and infection. Neutrophil numbers and IL-8 are elevated in sputum and bronchoalveolar lavage fluid from patients with cystic fibrosis compared to healthy control subjects. We now realise that the epithelium is not a simple barrier but plays a crucial role in host defence and produces a wide variety of mediators. In order to study the epithelium in cystic fibrosis, a number of approaches have been adopted. A significant amount of work has been published using epithelial cells isolated from excised recipient lungs from patients with cystic fibrosis obtained at transplantation. However, these organs are from patients with end-stage disease and epithelial cell function may not necessarily reflect that seen in patients with milder disease. Epithelial cells can also be obtained by growing samples of nasal polyps in explant cultures. This approach enables relatively easy comparison of healthy controls and patients with cystic fibrosis. However, although nasal polyps are relatively common in patients with cystic fibrosis, our own clinic sees very few patients with polyps. Other researchers have opted to investigate immortalised cell lines. This approach results in a uniform and ready cell supply but contradictory results have been obtained with cell lines. We have successfully grown epithelial cells from bronchial brushings of control subjects as well as patients with asthma or COPD. We therefore attempted to repeat this procedure in cystic fibrosis. However, the results were extremely disappointing. Furthermore, our cystic fibrosis patients did not enjoy the bronchoscopic procedure. We therefore turned to nasal brushings, an area where we had had success in patients with asthma. This work is still at a preliminary stage but we believe that we now have access to a ready supply of primary nasal epithelial cells from patients with cystic fibrosis. The procedure is well tolerated by patients and control subjects. In this presentation, the responses of the different types of epithelial cell cultures will be compared and our method will be described.

We thank the Cystic Fibrosis Trust for their generous support of our work.

S8.4 - Inhibitory effect of medicinal plant extracts from Bangladesh on *P.aeruginosa*-dependent induction of pro-inflammatory genes in CF bronchial epithelial cells

Ilaria Lampronti[2]; Maria Cristina Dechecchi[1]; Monica Borgatti[2]; Elena Nicolis[1]; Valentino Bezzerri[1]; Irene Mancini[2]; Anna Tamanini[1]; Federica Quiri[1]; PaoloRizzotti[1]; Giulio Cabrini[1]; Roberto Gambari[2];

Laboratory of Molecular Pathology, Laboratory of Clinical Chemistry and Haematology, University Hospital of Verona, Verona, Italy[1].. Department of Biochemistry and Molecular Biology, University of Ferrara, Ferrara, Italy. [2]

Innovative pharmacological approaches to control the massive neutrophil infiltrates into the bronchial lumen of CF patients are thought to be beneficial to reduce the extensive tissue damage and the progressive respiratory insufficiency. The activation of expression of pro-inflammatory genes upon interaction of *P.aeruginosa* with bronchial epithelial cells is a central mechanism to be targeted with novel therapies. Medicinal plants from the so-called traditional Asian medicine are attracting a growing interest because of their potential safety, already tested in large scale applications in human diseases. However, due to the presence of different active principles in each plant extract, whose multifunctional effects may even result contradictory, understanding the molecular mechanisms of action of each component is mandatory to pursue selective and reproducible applications. A panel of Bangladeshi medicinal plant extracts have been firstly screened for their capacity of interfering in the binding of nuclear transcription factor proteins (TF) with DNA consensus sequences identified in the promoters of the pro-inflammatory genes, thus for their potential inhibitory action on gene expression. Extracts from *Emblica officinalis, Aegle marmelos, Moringa oleifera, Terminalia arjuna, Vernonia anthelmintica, Oroxylum indicum, Saraca asoka, Rumex maritimus, Lagerstroemia speciosa, Red sandal, Cuscuta reflexa, Argemone mexicana, Hemidesmus indicus, Polyalthia longifolia*, *Cassia sophera, Paederia foetida, Hygrophilla auriculata, Ocimum sanctum, Aphanamixis polystachya* have been screened for their ability to interfere with the TF NF-kB, whose consensus sequence has been identified in several pro-inflammatory genes, and extended to other TFs such as AP-1, GATA-1, STAT-3 and CREB, with Electrophoretic Mobility Shift Assay (EMSA). We found a wide variety of effectiveness and specificity in inhibiting TF/DNA interactions, which anyway opens the possibility of potential applications to down-regulate expression of pro-inflammatory genes. After observing the ability of *Emblica officinalis* (EO) in inhibiting the interaction with DNA of different TFs, this was tested in IB3-1 CF bronchial cells exposed to the *P.aeruginosa* laboratory strain PAO1. EO (500 µg/ml) inhibited the PAO1-dependent transcription of IL-8 and GRO-α by 80%, of the InterCellular Adhesion Molecule (ICAM)-1, GRO-γ and IL-6 by 50%. Then, one of the active principles of EO, namely pyrogallol, was tested in IB3-1 cells. Pyrogallol (20 µM) inhibited the transcription of the neutrophil chemokines IL-8, GRO-α and GRO-γby 75%, of the InterCellular Adhesion Molecule (ICAM)-1 and the pro-inflammatory cytokine Interleukin 6 by 50% as quantified by real-time RT PCR. In conclusion, extracts from plants of the traditional Asian medicine can inhibit expression of pro-inflammatory genes possibly by interfering with the TF proteins/DNA interactions. Screening active principles purified from medicinal plants and dissecting their molecular mechanism(s) of action could result useful to identify safe and innovative pharmaceutical molecules to control lung inflammation in the lung of CF patients.

Supported by Italian Cystic Fibrosis Research Foundation (to RG)
and by Fondazione CariVerona – Bando 2005 – Malattie rare e della povertà (to GC) Azienda Ospedaliera di Verona and Legge 548/93 - Finanziamento Ricerca Fibrosi Cistica 2004.

S8.5 - Correctors of F508del CFTR reduce the inflammatory response to *Pseudomonas aeruginosa* in CF respiratory epithelial cells

Maria Cristina Dechecchi,; Elena Nicolis, Caroline Norez, Valentino Bezzerri, Federica Quiri, Monica Borgatti, Irene Mancini, Paolo Rizzotti, Roberto Gambari, Frederic Becq, and Giulio Cabrini

University Hospital of Verona, Laboratory of Molecular Pathology, Verona, Italy

Rationale: Chronic lung inflammation in cystic fibrosis (CF) is specifically characterized by predominant endobronchial neutrophil infiltrates, colonization by *Pseudomonas aeruginosa* (*P.aeruginosa*) and elevated levels of cytokines and chemokines, first of all interleukin (IL)-8. This neutrophil-dominated inflammation seems to be related to an excessive pro-inflammatory signalling, originating from the same surface epithelial cells, in which the underlying mechanisms is intensively investigated. An increasing number of evidence report that diseases due to mutations causing protein misfolding with accumulation in the ER are accompanied by inflammation. We have previously demonstrated that the corrector MPB-07 down modulates the *P.aeruginosa* dependent expression of the pro-inflammatory mediators IL-8 and ICAM-1 in CF bronchial cells [Dechecchi et al, Am. J. Resp. Cell. Mol Biology, 2007], supporting the feasibility to reduce the excessive lung inflammatory response in CF through the correction of the misfolding defect. Knowledge of the pharmacological modulation of CFTR is on exponential increase and candidate small molecule correctors of defective processing are available. **Aim**: to evaluate the possibility to reduce the pro-inflammatory status through the pharmacological modulation of the CFTR defect, by using other F508del CFTR correctors, different from MPB-07. To this aim, the effect of the compound Miglustat, already described as a F508del CFTR corrector [Norez et al, FEBS letters, 2006] is studied. **Methods:** Human bronchial cell lines: IB3-1 (F508del CFTR/W1282X), CuFi-1 (F508del CFTR/F508del). Inflammatory response: ICAM-1 and IL-8 mRNA levels quantitated by real time PCR after infection with a laboratory strain of *P.aeruginosa* (PAO1). CFTR function assay: by single-cell fluorescence imaging, using the potential-sensitive probe DiSBAC2 [Renier et al, Hum. Gene Ther. 6, 1275-1283, 1994]. Electrophoretic Mobility Shift Assay (EMSA): by analysis of binding of NF-kB and AP-1 transcription factors to labelled DNA target [Borgatti et al, J. Biol. Chem. 2003]. **Results:** Miglustat significantly reduces the expression of IL-8 by 90% in IB3-1 and by about 70% in CuFi-1 cells and the expression of ICAM-1 by 80% in IB3-1 and by about 50% in CuFi-1 cells. In parallel correction of F508del CFTR function is observed after Miglustat treatment in both IB3-1 and CuFi-1 cells. Miglustat has no major effects on overall binding activity of transcription factors NF-*k*B and AP-1, which play a central role in the immune response to *P. aeruginosa* infection in respiratory epithelial cells. **Conclusion**: we report that both Miglustat and MPB-07 reduce the inflammatory response to *P.aeruginosa* in CF cells, besides the correcting effect on F508del CFTR.

***Supported by** : Italian Cystic Fibrosis Research Foundation (FFC #16/2006 to MCD, sponsored by Associazione Veneta per la lotta contro la fibrosi cistica; Fondazione CariVerona – Bando 2005 – Malattie rare e della povertà, Azienda Ospedaliera di Verona and Legge 548/93, Finanziamento Ricerca Fibrosi Cistica 2004. Studies from CN et FB are supported by VLM.*

Saturday 28 April 14:30-16:00

EuroCareCF Meeting – Workpackage 7 (Resources)

Chair: Margarida Amaral (Lisboa, Portugal)

1. Overview

Margarida Amaral

Department Chemistry and Biochemistry, Faculty of Sciences, University of Lisboa, Portugal

2. Primary Epithelia and Novel Cell Lines for CF Research

SH Randell

Cystic Fibrosis/Pulmonary Research and Treatment Center, The University of North Carolina, Chapel Hill, NC, USA

Primary human tracheobronchial epithelial (hTBE) cells cultured at an air-liquid interface (ALI) regulate ion transport, mucin secretion, and cilia beating to recapitulate mucociliary clearance, a key physiologically-regulated protective mechanism that fails in cystic fibrosis (CF). Thus, ALI hTBE cell cultures are crucial for studying CF pathogenesis and for developing/validating novel therapies. Different research centers use alternative protocols that enable reproducible creation of ALI hTBE cell cultures. In 2005, ~390 lung transplant procedures were performed worldwide for CF; however, the majority of explanted lungs were most likely not used for research. The lack of networks and protocols to procure and process CF and non-CF lungs is a major obstacle to CF research that should be overcome. Limited hTBE cell availability has driven the creation of cognate cell lines, accomplished mainly by the introduction of viral oncogenes. Viral oncogenes disrupt normal cell physiology causing cellular hyperplasia, abundant apoptosis, genomic instability and, sometimes, poor epithelial polarization / differentiation, rendering the cells less useful than primary cells for assessing CFTR function. Furthermore, genetically unstable cell lines will continuously acquire changes independent of *CFTR* genotype. Bmi-1 is a polycomb group protein that controls cell cycle and cell identity via epigenetic regulation of chromatin, maintaining stem cells by inhibiting expression of the cyclin dependent kinase inhibitor p16. Bmi-1 expression has been used to create cell lines that recapitulate normal cell structure and function better than viral oncogene-immortalized cell lines. Using HIV lentiviral vectors, we introduced Bmi-1 and the catalytic subunit of telomerase to enhance the growth of 3 different F508del homozygous CF and 3 non-CF primary human airway epithelial cell preparations. All 6 new cell lines grew for at least 40 population doublings, while their normal counterparts senesced prior to 20 doublings. At passage 14-15, the new cell lines had a diploid karyotype compared to grossly abnormal chromosomes in cells immortalized by viral oncogenes. Ussing chamber analysis of ALI cultures at passage 14-15 revealed variable transepithelial resistances among the cell lines ranging from 125 - >1000 Ω*cm^2, but short circuit current (I_{sc}) responses stimulated by forskolin and inhibited by compound CFTR172 were true to the cell's genotype and usually similar to early passage primary cells. Amiloride sensitive and UTP-stimulated I_{sc}'s were present but were more variable in magnitude in comparison to the currents observed in primary cells. ALI cultures exhibited a pseudostratified morphology with prominent apical membrane polarization, few apoptotic bodies, numerous mucous secretory cells and occasional ciliated cells. All CF and non-CF cell lines in culture produced similar levels of IL-8 at baseline and CF and non-CF cells equally increased IL-8 secretion in response to IL-1β, TNFα and the Toll-like 2 receptor agonist, Pam3Cys. While it is important to improve the utilization of CF and non-CF lung tissue resources worldwide to increase the availability of primary hTBE cells, novel cell lines will also help fill gaps currently hindering CF research and therapeutic development.

3. Endpoints for the Assessment Therapy Aimed at the CFTR Defect

R.L. Dormer

Dept. Medical Biochemistry & Immunology, School of Medicine, Cardiff University, Heath Park, Cardiff, UK

Subjects for discussion will be presented as follows:

What function of CFTR needs to be measured? There are a number of *in vivo* (nasal and lower airways P.D., sweat test) and *ex vivo* (rectal tissue P.D., nasal brushing cells) assays of the ion transport function of CFTR in surface epithelial cells in CF patients. Such assays give information about the effect of potential therapies (gene replacement or drug) on sodium and chloride absorption, chloride secretion under basal and stimulated conditions, CFTR localisation. The question remains as to whether a positive result in one or more of these assays is sufficient to merit progress to clinical trials. This is especially relevant in the lungs where airways surface liquid is primarily derived from submucosal gland secretion where there is growing evidence to support earlier work on salivary glands and airways gland cell cultures that the basic defect in these cells is hyposecretion. The question is also important in terms of cost, particularly for compounds identified at random by high throughput screening as opposed to drugs already in clinical use for other diseases that have been identified from hypothesis-driven or screening studies.

Are alternative pathways available that can be targeted? The possibility of therapy by stimulation of chloride channels other than CFTR in airways surface epithelial cells has been proposed. For gland cells an important question remains as to whether the hyposecretion defect is explained by lack of fluid secretion alone or by a defect in exocytotic protein secretion as well. Evidence suggests that there is an alternative pathway for fluid secretion but not for exocytosis in gland cells.

What can be usefully measured by the use of mouse models? CF mice that carry specific mutations such as F508del or G551D provide an important link for development of therapies, in that administration can be *in vivo*. Although the mouse is limited as a model of CF disease, particularly as it does not develop characteristic lung disease, it can be used to test a wider range of *ex vivo* functions on tissues (airways, G-I tract, gland cells) that are not available from CF patients.

Can surrogate marker proteins be identified? A large number of proteins contribute to the biosynthesis, trafficking and multiple functions of CFTR although few have been shown to interact differently with mutant forms of CFTR. This type of study is progressing with the application of proteomic techniques that will allow detection of changes in expression or interaction with CFTR, of specific proteins in tissues or fluids that are available from CF patients in an acceptably non-invasive and repeatable manner. This may allow development of new endpoints for functions that must be corrected to provide clinical benefit.

4. Future Perspectives and General Discussion

Saturday 28 April 16:30-18:00

EuroCareCF Meeting - Workpackage 6 (Animal Models)

Chair: Bob Scholte (Rotterdam, The Netherlands)

1. CF mouse models, a quarrelling family

BJ Scholte

Erasmus University Medical Center, Cell Biology dept, Rotterdam, the Netherlands

Several mouse strains with Cftr mutations have been made by homologous recombination, comprising complete loss of function mutants ('knockout') and several specific mutations that are frequently found in the human population. Together, these strains form a valuable tool in the study of CF pathology and novel therapeutics, ranging from gene therapy vectors to compounds that correct CFTR processing defects. EuroCareCF supports the distribution of the $Cftr^{tm1Cam}$ Knockout mouse and the $Cftr^{tm1eur}$ F508del (F508del) Cftr 'hit and run' mutant mouse.

CF is a disease with extremely complex and variable pathophysiology humans. It is not surprising that also CF mouse pathology is far from straightforward. In general, CF mutant mice are considered a good model of intestinal disease with typical absence of Cftr driven chloride and fluid secretion, mucus plugging and goblet cell hyperplasia, associated with reduced lipid resorption. In this workshop we will study the effect of hypoxia on barrier function of CF epithelia. However, pancreas function is normal in all models investigated, in contrast to most CF patients. CF mutant mice do have an airway and lung phenotype, as shown by abnormal nasal electrophysiology, increased inflammation and tissue remodelling, and reduced clearance of pathogens. Data from our laboratory show that CF mutant mice respond to lung injury with a more sustained production of extracellular matrix components (Scholte et al submitted). Recently, evidence was presented that also alveolar fluid secretion is defective in CF mutant mice (Lindert et al 2007). While these studies are certainly valid and rewarding, it has been called into question whether this phenotype is really relevant to CF lung disease in humans. CF mutant mice do not display spontaneous chronic lung infections with opportunistic pathogens, associated with mucus plugging and neutrophilaemia. This can easily be explained by the complexities of host-pathogen interactions, and the different cellular architecture of the mouse lung compared to the human. Nevertheless, there is a need for a model that more closely resembles the phenomena observed in human CF lung disease. An example of this is the mouse strain that overexpresses the beta subunit of the sodium channel EnaC in airway cells. This is based on the idea that CF lung pathology is caused by a shift in the balance between EnaC dependent fluid resorption, and Cftr pependent fluid secretion. In this workshop we present this model and a novel one with a mutation that causes hyper activity of ENaC (as in Liddle's disease), and both present an intriguing lung phenotype. The question is whether these are the 'real' CF models, approaching conditions in human CF lung better than Cftr mutant strains, or whether these models represent a interesting new disease in mouse that does not exist as such in humans.

The group of Deborah Nelson has reported that alveolar macrophages of CF mutant mice are not capable of killing phagocytosed bacteria. Several members of the leukocyte family, including macrophages, mast cells and dendritic cells, express functional CFTR, and are potentially dysfunctional in CF. In this workshop we present novel data on the phenotype of alveolar macrophages from CF mutant mice.

2. Update on the βENaC Mouse

SH Randell[1], BR Grubb[1], WK O'Neal[1], A Livraghi[1], B Button[1], J Schwabe[1], EJ Hudson[1], JR Harkema[2], RJ Pickles[1], M Mall[3] and RC Boucher[1]

Cystic Fibrosis/Pulmonary Research and Treatment Center, The University of North Carolina, Chapel Hill, NC, USA[1]; Michigan State University, Lansing Michigan, USA[2]; University of Heidelberg, Germany[3]

Relative dehydration of airway surface liquid (ASL) that ultimately inhibits mucus clearance is a major defect leading to lung infection in cystic fibrosis (CF). *Cftr* knockout or mutant mice display lesions in several of the organs affected in human CF but, due to a minor role for CFTR in mouse lower airway epithelial ion transport, CF mice do not exhibit lung pathology similar to the human disease. Because ASL hydration is determined, in part, by Na^+ absorption through the epithelial Na^+ channel (ENaC), increased ENaC activity is predicted to deplete ASL and inhibit mucus clearance. Mall *et al.* individually over-expressed the three constituent ENaC subunits (α,β, γ encoded by the *Scnn1a, b* and *g* genes, respectively) in transgenic mice using the lung epithelial-specific secretoglobin 1A1 promoter (Nat Med. 10:487-93, 2004). Scnn1b mice exhibited greater amiloride-sensitive short circuit currents (I_{sc}), ASL depletion, and defective mucus clearance compared to control mice. Scnn1b mice developed pathology including mucus adhesion, airway plugging, mucous secretory cell hyperplasia, neutrophilic inflammation and neonatal mortality. Surviving Scnn1b mice did not clear inhaled bacteria as efficiently as control mice. Our group has undertaken several lines of investigation to better understand disease pathogenesis due to airway Na^+ hyper-absorption, ASL dehydration and impaired mucus clearance in Scnn1b mice, and make the following observations:

- Na^+ transport and the severity of lung pathology appear to be correlated
- Genetic background modifies phenotypic outcome in Scnn1b mice
- Signaling through the IL-4 receptor alpha is involved in the normal neonatal abundance of mucous secretory cells in mice, but does not contribute to chronic inflammation and goblet cell hyperplasia in Scnn1b mice
- Mucus stasis and the pre-existing inflammatory milieu modifies the response to viral infection

Scnn1b mice confirm the importance of Na^+ transport in ASL homeostasis and are a useful model to understand the pathogenesis of diseases such as CF characterized by impaired mucus clearance.

3. Effect of Mutations of the beta-ENaC gene (SCNN1b) on Mouse Lung Alveolar Fluid Balance

Nadia Randrianarison [1,2], Brigitte Escoubet [2,3,4], Chrystophe Ferreira [4], Alexandre Fontayne [1,2], Nicole Fowler-Jaeger [5], Christine Clerici [1,2], Edith Hummler [5], Bernard C. Rossier [5], Carole Planès [1,6]

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Transepithelial sodium (Na^+) transport via alveolar epithelial Na^+ channels and Na,K-ATPase constitutes the driving force for removal of alveolar oedema fluid. Decreased activity of the amiloride-sensitive epithelial Na^+ channel (ENaC) in the apical membrane of alveolar epithelial cells impairs sodium-driven alveolar fluid clearance (AFC) and predisposes to pulmonary oedema. We hypothesized that hyperactivity of ENaC in the distal lung could improve AFC and facilitate the resolution of pulmonary oedema. AFC and lung fluid balance were studied at baseline and under condition of hydrostatic pulmonary oedema in the β-Liddle (L) mouse strain harbouring a gain-of-function mutation (R566-stop) within the *Scnn1b* gene.
Histological examination of the lungs revealed no morphological difference between wild-type (+/+) and □-ENaC (L/L) mice. Expression levels of α- and γ-ENaC mRNA transcripts as assessed by real-time RT-PCR and of α- and γ-ENaC subunit proteins quantified by Western-blotting in bronchioloalveolar epithelial cells isolated from (+/+) and (L/L) mice were comparable. As compared with wild type (+/+), baseline AFC was increased by 2- and 3-folds in heterozygous (+/L) and homozygous mutated (L/L) mice respectively, mainly as a result of increased amiloride-sensitive AFC. The beta2-agonist terbutaline stimulated AFC in (+/+) and (+/L) mice, but not in (L/L) mice. The CFTR inhibitor glycine hydrazide GlyH-101 (5.10^{-5} M) failed to inhibit baseline AFC in both (+/+) and (L/L) mice. Acute volume-overload induced by saline infusion (40% of body weight over 2 hours) significantly increased extravascular (i.e. interstitial and alveolar) lung water as assessed by the bloodless wet-to-dry lung weight ratio in (+/+) and (L/L) mice, as compared with baseline. However, the increase was significantly larger in (+/+) than in (L/L) group ($p=0.01$). Volume-overload also increased the volume of the alveolar epithelial lining fluid in (+/+) mice, indicating the presence of alveolar oedema, but not in (L/L) mice. Cardiac function as evaluated by echocardiography was comparable in both groups.
In conclusion, this study highlights the crucial role of ENaC in transepithelial alveolar Na^+ transport and alveolar fluid balance in the mouse. Indeed, our finding that ENaC hyperactivity in the distal lung results *in vivo* in increased AFC and reduced severity of hydrostatic pulmonary oedema suggests that new therapies targeted at enhancing ENaC activity and AFC could hasten the resolution of hydrostatic pulmonary oedema in patients.

Supported by the French CF Association, Vaincre la Mucoviscidose

4. The relevance of hypoxia to mucosal inflammatory processes

J Karhausen.[1]; H.E. Etzschig[1]; S.P Colgan.[2]

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In cystic fibrosis (CF), the efficacy of antiinflammatory therapies underscores the importance of inflammation to cause loss of lung function while enhanced NFkB signaling and increased interleukin (IL)-8 and neutrophils numbers in CF bronchial lavage fluid suggest the significance of abnormal inflammation for the CF disease process. Controversy continues as to whether defective CF transmembrane conductance regulator (CFTR) might directly dysregulate inflammation leading to a pro-inflammatory vs. anti-inflammatory imbalance. However, since inflammation is a key contributor to the pathogenesis of CF disease, the understanding of the involved processes must assume a pivotal role when trying to identify potentially therapeutic mechanisms.

Our own work has focused on the role of hypoxia as a pathogenetic factor in inflammation. In this context, active inflammation is characterized by dramatic shifts in tissue metabolism. These changes include diminished availability of oxygen (hypoxia) with subsequent lactate accumulation and resultant metabolic acidosis. Pathophysiologic changes related to hypoxia include changes in resident cell phenotype, abnormalities of epithelial and endothelial permeability, mucosal secretory changes, recruitment and accumulation of inflammatory cells, and transcriptional induction of proinflammatory cytokines.

Recent studies have also identified hypoxia-elicited factors that counterregulate such proinflammatory circuit, thereby functioning as endogenous antiinflammatories. Central to this latter pathway is extracellular adenosine (Ado). In addition, through activation of surface-expressed receptors on a variety of cells, Ado dampens ongoing inflammation and promotes woundhealing. Our recent work demonstrated that hypoxia coordinates both transcriptional and metabolic control of the surface ectonucleotidases CD39 and CD73, and thereby amplifies extracellular accumulation of Ado via increased phosphohydrolysis from precursor adenine nucleotides (ATP, ADP, and AMP). Additional mechanisms also exist to amplify Ado signaling during hypoxia, including coordinate changes at the adenosine-receptor level.

Here, we present our ongoing work in the context of pulmonary and intestinal inflammation and will demonstrate relevance of the hypoxia model to CF pathogenesis. Both as a model for inflammation and through its characteristic enhancement of adenosine signaling, data have direct implications for pathways relevant to CF and may provide valuable tools for the better understanding of the involved inflammatory mechanisms and potential adaptive pathways.

5. Azithromycin modulates IL-1β overexpression in alveolar macrophages from CF mice

Magali Meyer[1], François Huaux[2], Sybille van den Brûle[2], Patrick Lebecque[3], Dominique Lison[2], Pierre Wallemacq[1], Teresinha Leal[1]

Clinical Chemistry[1], Industrial Toxicology and Occupational Medicine[2], Pediatric Pulmonology[3]; Université Catholique de Louvain, Brussels, Belgium

We have previously demonstrated in cystic fibrosis (CF) mice homozygous for the F508del mutation that *in vivo* treatment with azithromycin attenuates cellular infiltration in both baseline and induced inflammatory condition, and inhibits pro-inflammatory cytokine release in lipopolysaccharide (LPS)-induced inflammation (Legssyer et al, 2006).

This study aimed at investigating the effect of azithromycin on primary-cultured macrophages isolated from wild type (WT) or F508del-CF mice (van Doorninck et al, 1995). Macrophages were harvested by peritoneal or bronchoalveolar lavage. Cells were then plated, purified by adhesion and stimulated with LPS (*Pseudomonas aeruginosa*, 0.1µg/ml) either in the presence or in the absence of azithromycin (1µg/ml). Macrophage inflammatory status was investigated by assessing pro-inflammatory (TNFα and IL-1β) and anti-inflammatory (IL-10) cytokine expression at mRNA or protein level.

Our results indicated that following LPS stimulation, peritoneal macrophages isolated from F508del-CF mice produced higher TNFα and lower IL-10 levels than macrophages harvested from WT mice. Likewise, IL-1β mRNA levels, assessed by RT-qPCR, were significantly higher in peritoneal macrophages from CF mice as compared with those cells obtained from WT animals. Azithromycin had no significant effect on the expression of any of the three monitored markers in macrophages obtained from peritoneal lavage. Interestingly, an upregulation of IL-1β, that was significantly downregulated following azithromycin treatment, was detected in CF alveolar macrophages. Contrarily to that observed in peritoneal macrophages, TNFα did not appear to be overexpressed in CF and IL-10 expression did not differ between CF and WT mice in macrophages obtained from alveolar lavage.

In conclusion, we found that primary-cultured murine peritoneal and alveolar macrophages show different patterns of cytokine expression in response to LPS. An inflammatory imbalance, characterized by pro-inflammatory cytokine upregulation and anti-inflammatory cytokine downregulation, was observed in CF peritoneal cells. Azithromycin modulates IL-1β overexpression in CF alveolar macrophages. This finding supports the anti-inflammatory activity of this macrolide and identifies, at least partly, alveolar macrophages as possible target cells for its effects.

Supported by the French CF Association, Vaincre la Mucoviscidose

Saturday 28 April – 18:30-20:00 Room: Gilão

Special Interest Discussion Group - IIIA

Protein Interactions: CFTR and its accomplices

Chair: William Balch, (La Jolla, USA) and Anil Mehta (Dundee, UK)

CFTR is multi-membrane spanning chloride channel whose assembly during co-translational insertion in the endoplasmic reticulum (ER) and trafficking through the exocytic and endocytic pathways requires multiple protein-protein interactions. Evidence for the role in of CFTR in regulating (or being regulated by) a variety of components of components at the cell surface that control cellular and extracellular ion and fluid balance, and components required for controlled recycling and degradation of CFTR in the endocytic pathway are now readily apparent from both functional and, more recently, proteomic studies characterizing CFTR interactions using mass spectrometry. In CF disease, one (or more likely) many interactions are disrupted reflecting energetic destabilization of the protein fold in response to different mutations identified in patients. The most prominent mutation, the Phe 508 deletion, results in loss of export of CFTR from the ER and efficient targeting to ER-associated degradation pathways. Analysis of the folding pathways involved in wild-type and mutant CFTR using mass spectrometry has revealed a surprising wealth of chaperone and co-chaperone components that likely contribute to folding for export and/or targeting to degradation. Moreover, whole cell proteomics and genomic analyses of human tissues reveals a number of pathophysiological responses leading to changes in the cell and tissue message and protein composition- both cell autonomous and cell non-autonomous- likely reflecting the cellular response to the adverse environment created by the dysfunctional CFTR channel and its linked regulated activities. The goal of the group discussion is to try began to integrate our understanding of these diverse cellular pathways- both initiated and responsive- to give a more global systems biology view of CFTR physiology and pathophysiology in health and disease.

Saturday 28 April – 18:30-20:00 Room: Arade

Special Interest Discussion Group – IIIB

Immune Mechanisms in ASL

Chair: Aura Perez and Scott H. Randell

There is no doubt that the cystic fibrosis (CF) airway is predisposed to infection by characteristic pathogens, that it typically becomes inflamed out of proportion to pathogen load and that inflammation is slow to, or even fails to resolve. However, the precise sequence of events and relative importance of different contributing factors is still under considerable debate. There is general consensus that abnormal ion transport due to absence of functional CFTR results in relative dehydration of the airway surface layer (ASL), causing mucus to be hyper-viscous, static and poorly cleared by cough. The goal of this small group discussion is to critically analyze the likely events leading from mucus dehydration and stasis to inflammation and chronic infection. We will explore the series of pathogenic events, including whether or not thickened mucus per se is intrinsically inflammatory, if environmental agents and paracrine mediators accumulate in static ASL, how thickened mucus promotes the unique environment exploited by CF pathogens, the potential role of defective anti-microbial defense, mechanisms by which inflammatory cell function is impaired, why CF airway inflammation does not resolve and finally, how innate and acquired immunity interact in the CF airway environment. Our goal is to highlight the type of experiments needed in order to test alternative hypotheses to ultimately reach a consensus regarding key steps in the pathogenesis of CF airway disease.

Sunday 29 April – 09:30-11:00

SYMPOSIUM 9 – Strategies Aimed at Correcting CFTR Mutants

Chairs: Zsuzsa Bebok (Birmingham, AL, USA) & David Sheppard (Bristol, UK)

S9.1 Overview – How much CFTR is Enough?

David Sheppard

Department of Physiology, University of Bristol,School of Medical Sciences, University Walk, Bristol, UK

Knowledge of how cystic fibrosis (CF) mutations disrupt cystic fibrosis transmembrane conductance regulator (CFTR)-mediated transepithelial ion transport is leading to rational new approaches for therapy (Cai *et al*. 2007). Many mutations, including the most common F508del, disrupt the processing of CFTR and its delivery to the apical membrane. Restoration of channel function to these mutants requires the use of a drug (termed a CFTR corrector), which delivers the mutant protein to its correct cellular location. By contrast, other mutant proteins reach the cell surface, where they form Cl^- channels with defective function. Restoration of channel function to these mutants requires the use of a drug (termed a CFTR potentiator) that enhances channel gating.

A plethora of agents that rescue the function of CF mutants in recombinant cells have been identified in recent years, most notably using high-throughput screening. The challenge now is to carefully decide which of the candidate drugs to pursue further in the quest to develop rational therapies for CF. One fundamental question for the development of drug therapies for CF is how much CFTR function is required to rescue CF mutants?

The amount of function that must be restored to individual CF mutants to rescue defective transepithelial ion transport is currently unknown. One approach to estimate the amount of function required is to analyse published data on the relationship between genotype, phenotype and CFTR Cl^- channel function. Using such an approach, Van Goor *et al.* (2006) speculated that restoration of between 5 – 30% of wild-type function to CF patients bearing the F508del mutation would be of therapeutic benefit.

The Cl^- channel function of CFTR in the apical membrane of an epithelium (I^{CFTR}(apical)) is determined by the product of the number of CFTR Cl^- channels in the apical membrane (N), the current amplitude (i) of an individual CFTR Cl^- channel and the probability (P_o) that a single CFTR Cl^- channel is open: $I^{CFTR}(apical) = N \times i \times P_o$. Using biochemical (N) and functional (i and P_o) data, the apical CFTR Cl^- current generated by CF mutants can be predicted. By measuring CFTR-mediated apical membrane Cl^- currents with the Ussing chamber technique, the predicted I^{CFTR}(apical) for CF mutants can be compared with the actual I^{CFTR}(apical) measured. Such analyses reveal good agreement between predicted and measured currents. Based on these data, we have investigated how much function different CFTR potentiators restore to CF mutants. Our data suggest that the hydrolysable ATP analogue 2'-deoxyATP (1 mM) increases the predicted current of the CF mutant G551D from 2% to 20% that of wild-type CFTR (Cai *et al*. 2007). Encouraging as these data are, the challenge now is to determine what level of CFTR function is required to rescue defective epithelial ion transport in the respiratory airways of CF patients.

1. Cai Z et al. (2007). In Pusch M, (ed). Chloride Movements across Cellular Membranes. *Elsevier Limited*: San Diego. pp 109-143.
2. Van Goor F et al. (2006). *Am. J. Physiol*. 290, L1117-1130.

S9.2 - Pharmacological Rescue of F508DEL-CFTR Function for the Treatment of Cystic Fibrosis

Fredrick Van Goor

Vertex Pharmaceuticals, Department of Ion Channels, San Diego, CA, USA

Cystic fibrosis (CF) is a fatal genetic disease caused by mutations in *cftr*, a gene encoding a PKA-regulated Cl^- channel (CFTR). The most common mutation results in a deletion of phenylalanine at position 508 (F508del-CFTR) that leads to a reduction in the cell surface density and gating activity of the anion channel. In the airway, these defects alter salt and fluid transport, leading to chronic infection, inflammation, and bronchiectasis. Here we describe two classes of potent small molecules identified by high-throughput screening and medicinal chemistry optimization that restore the cell surface density and gating activity of F508del-CFTR in airway epithelia isolated from multiple CF patients. The changes in F508del-CFTR activity are sufficient to improve airway epithelial function and support the rationale of a drug discovery strategy based on rescue of the basic genetic defect responsible for CF.

S9.3 – Potentiators and Correctors for the Mutant CFTR Channel

Nicoletta Pedemonte, Emanuela Caci, Elvira Sondo, Antonella Caputo, Olga Zegarra-Moran, Roberto Ravazzolo, Luis J.V. Galietta

Laboratorio di Genetica Molecolare, Istituto Giannina Gaslini, Genova, Italy

Many cystic fibrosis (CF) mutations decrease CFTR protein function by impairing the process of channel opening. Class III mutants like G551D and G1349D are characterized by a severe impairment of channel activity. After correction of the trafficking defect by low temperature incubation, F508del also shows a reduced channel activity although this defect is less severe than that of class III mutants. The defective activity of G551D, G1349D, and F508del can be corrected by small molecules called potentiators. Interestingly, some potentiators, like phenylglycines, show a general ability to stimulate various CFTR mutants. Conversely, other potentiators, like sulfonamides and benzothiophenes, seem to be active only on F508del (Pedemonte et al., Mol. Pharmacol. 67: 1797-1807, 2005; Yang et al., J. Biol. Chem. 278: 35079-35085, 2003).

Potentiators have been identified by various investigators in hypothesis-driven studies or in high-throughput screenings of large chemical libraries. We have performed a screening of a library containing many FDA/EMEA approved drugs. This screening has detected anti-hypertensive 1,4-dihydropyridines (DHPs) as an interesting family of CFTR potentiators (Pedemonte et al., Mol. Pharmacol. 68: 1736-1746, 2005). Among common DHPs, felodipine was the most potent, having activity on G551D, G1349D and F508del mutants. More recent results suggest that felodipine, as well as other potentiators, may be active also on CF mutations lying outside the nucleotide binding domains.

Many evidences indicate that DHPs stimulate CFTR with a mechanism independent of the block of voltage-dependent calcium channels (VDCCs, the primary target of DHPs as anti-hypertensive agents). To improve the selectivity of DHPs for CFTR, we have recently performed a study on 333 felodipine analogs having various chemical substitutions in the basic scaffold. To understand the structural features required for CFTR channel activation and VDCC block, we have used specific cell-based assays. For CFTR, we have used FRT cells expressing the halide-sensitive yellow fluorescent protein. For VDCC, we have designed an assay based on the calcium-sensitive fluorescent probe Fluo-4 and H9C2 muscle cells, which have an endogenous expression of VDCCs. Analysis of data obtained from the two different assays has identified critical modifications of the DHP structure that allow improved activity on CFTR mutants and, at the same time, 500-fold decrease of potency for calcium channels. These results indicate the possibility to generate selective potentiators of the CFTR channel.High-throughput screenings have also identified putative correctors of the F508del trafficking defect (Pedemonte et al., J. Clin. Invest. 115: 2564-2571, 2005; Van Goor et al., Am. J. Physiol. 290: L1117-L1130, 2006). Such molecules include bithiazoles (corr-4a) and quinazolines (VRT-345 and VRT-640). We are comparing potency and maximal effect of such compounds in different cell systems expressing the F508del protein to assess the possibility of cell type specific effects. In addition, by combining different treatments together, we are looking for possible additive/synergic effects which would be suggestive of different mechanisms of action.

S9.4 – Novel approaches in Therapy aimed at Correcting the Basic Defect in CF

Melissa Ashlock

Cystic Fibrosis Foundation Therapeutics, Department of Drug Discovery, Bethesda, MD, USA

Cystic Fibrosis Foundation Therapeutics (CFFT) is the non-profit drug discovery and development affiliate of the US national Cystic Fibrosis Foundation (CFF). One of the primary goals of CFFT is to fund research for the discovery and development of therapies directed at restoring function to mutant CFTR. Over the past several years, CFFT has made several significant investments in industry and academia to accomplish this goal. These investments include those made in 1) high-throughput screening of small molecular libraries to identify positive modulators of CFTR gating and trafficking (potentiators and correctors, respectively); 2) CFTR protein structure determination to facilitate structure-based drug design; 3) *in silico* modelling of CFTR structure and potential drug binding sites and 4) novel target identification strategies using approaches such as siRNA to probe the CFTR trafficking pathway for proteins that could be used as targets for drug discovery projects. The presentation will cover promising aspects of these investments and will also include a description of other CFFT efforts to provide resources to the CF community. Additional topics include a synopsis of unresolved issues encountered in CF drug development and how CFFT attempts to overcome these hurdles.

S9.5 - Investigation of the Mechanism of Action of Several Small Corrector Molecules by Means of F508DEL-CFTR Genetic Revertants

[1,2] André Schmidt and [1,2] Margarida D Amaral

Center for Human Genetics, National Institute of Health, Lisboa, Portugal. [1]
Department of Chemistry and Biochemistry, Faculty of Sciences of University of Lisboa, Lisboa, Portugal[2].

We have recently investigated the mechanism of action of two different types of revertant mutations previously demonstrated to rescue the trafficking defect of F508del, namely: G550E [1] and 4RK, the simultaneous mutation of four arginine-framed tripeptides (AFTs): R29K, R516K, R555K, R766K [2]. Our data indicated distinct mechanisms of rescuing by these revertants [3]: whereas G550E appears to exert its effect on the folding of F508del-CFTR, 4RK seems to enable the mutant to escape retention/retrieval at the endoplasmic reticulum (ER) quality control (ERQC), suggesting a third (AFT-mediated) checkpoint for assessment of secretory protein folding besides the two we have previously described [4].
Recent studies describe novel potent small molecules, identified from high-throughput screening of compound libraries, which restore the function of F508del-CFTR in both recombinant cells and cultures of human bronchial epithelia from CF patients [5]. These molecules were described to partially rescue the trafficking defect of F508del-CFTR by facilitating exit from the ER.
Our aim here is to determine the mechanism of action of these small corrector molecules on the processing of F508del-CFTR by testing its effect on the above-mentioned genetic revertants.
Pulse-chase analyses, i.e., radio-labelling experiments with [^{35}S]methionine, followed by CFTR immunoprecipitation, were performed to determine the efficiency of conversion of the core-glycosylated (band B) into the fully-glycosylated form (band C) of F508del-CFTR, F508del-G550E-CFTR, and F508del-4RK-CFTR in the presence of 6.7 μM VRT-325 (a quinazoline derivative) or 20 μM VRT-532 (a pyrazole derivative shown to act as a potentiator of the channel activity).
These experiments confirmed the rescuing effect of these small molecules on F508del-CFTR. Moreover, VRT-325 was able to increase the conversion of band B from F508del-4RK-CFTR into the mature form, but not for F508del-G550E-CFTR suggesting that this small molecule exerts its effect directly on the folding of F508del-CFTR and not at the cellular ERQC.
Additional data shows that 6 μM VRT-640 (another quinazoline derivative) also rescues F508del and the effects of this and other small molecules F508del-G550E-CFTR, and F508del-4RK-CFTR are currently under assessment.

Work supported by grant POCTI/MGI/47382/2002 and pluriannual funding of CIGMH (FCT, Portugal). A. Schmidt is a recipient of SFRH/BD/19415/2004 doctoral fellowship (FCT, Portugal).The authors are grateful to Vertex and Cystic Fibrosis Therapeutics (CFT) for making the small molecules available for this study.

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Room: Museum

Poster Session – Biogenesis, ER Quality Control & Traffic

P.3

In vivo effect of BAG-1 on the turnover and processing of CFTR

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CFTR protein, undergoes a fairly complex biosynthetic pathway due to its multi-domain structure. Several molecular chaperones and co-chaperones have been shown to participate in this process, namely: Hsp70/Hdj-2, calnexin, CHIP, Hsp90, Hdj-1/Hsp40 [1]. Biogenesis of CFTR initiates with synthesis and folding in ER where the protein is core-glycosylated, originating an immature precursor form, know as band B. Wt-CFTR, but not F508del-CFTR, matures into the Golgi, originating a ~160-kDa form with complex glycosylation (band C). Depending of the cell type, only about 25% to 70% of precursor wt-CFTR matures, the remaining undergoing, like most F508del-CFTR, ubiquitination and degradation by the proteasome.
We have previously shown that overexpressing Hsp70 together with its co-chaperone Hdj-1 (Hsp40), stabilizes wt- but not F508del-CFTR [1]. Moreover, we have shown that wt- and F508del-CFTR interact differently with calnexin (an ER-specific chaperone) and EDEM (a novel ER degradative factor) [2]. These results, and those from other groups, led us to propose a model for the ER quality control (ERQC) in which wt- and F508del-CFTR are distinctly recognized by molecular chaperones which cause them to be discarded at two different ERQC checkpoints, involving the Hsp70/CHIP machinery and the calnexin/EDEM, respectively [3]
Bcl-2-associated athanogenes (BAGs) constitute a protein family all sharing a BAG domain for binding to Hsc70 (co-chaperones) and acting as nucleotide exchange factors in the ATP-dependent chaperone cycle of Hsc70 [4]. BAG-1, initially described by its anti-apoptotic activity, possesses a Ub-like domain that associates the co-chaperone with the proteasome [5]. BAG-1 modulates the Hsc70/Hsp70 *in vitro* chaperone by promoting substrate release and ATP hydrolysis through accelerating the ADP/ATP exchange rate [6].
We studied the *in vivo* effect of BAG-1 on the turnover and processing of wt- and F508del-CFTR. Results show that under BAG-1 overexpression: 1) for wt-CFTR, neither the degradation rate of the immature form (Band B) nor the efficiency of its conversion into the mature form (band C) are significantly altered; 2) the immature form of F508del-CFTR (band B) is significantly stabilized in CHO cells, but not in BHK cells. These results suggest that BAG-1 plays a stabilizing role on a conformational-intermediate of CFTR, which seems to be more abundant for F508del-CFTR.
In order to test whether CFTR interacts *in vivo* with BAG-1, we performed pull-down assays and observed co-immunoprecipitation of CFTR and BAG-1, thus suggesting the occurrence of such interaction. However, as Hsc70 is also present in the precipitates, it is not clear whether this is a direct or indirect (Hsc70-mediated) interaction. To clarify this issue, we carried out the same assays with BAG-1 deletion mutants lacking the Hsc70 binding domain and/or the Ubiquitin-like region. Data indicate that Bag-1 interacts *in vivo* with CFTR, through its ubiquitin-like region and most probably also independently of Hsc70.

Work supported by Fundação Calouste Gulbenkian, CIGMH pluriannnual funding (FCT, Portugal) and post-doctoral fellowships Praxis XXI BPD/18811/98 (IV) and SFRH/BPD/1905/2004 (FM). We thank Prof R Morimoto and J Song for BAG-1 constructs.

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Solubilizing mutations in the NBD1 of CFTR rescue the processing and channel gating defects of the cystic fibrosis mutation F508del

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Recently, the crystal structure of the first nucleotide-binding domain (NBD1) of CFTR bearing the most frequent mutation, F508del was elucidated [1]. While this structure shows only minor conformational changes relative to that of wt-NBD1, several additional mutations (F494N/Q637R or F429S/F494N/Q637R), chosen on the basis of their natural occurrence in mouse or fish orthologs and their lack of association with CF, were required for domain solubility and crystal formation [2]. Inspection of the published data reveals that these mutations produce an increase in the thermodynamic stability of the "solubilized" domain, as measured by the ΔG^0 of unfolding in denaturation experiments. We therefore hypothesised that these solubilizing mutations might have an impact on the overall structure of NBD1. To test this idea, we investigated the effects of the solubilizing mutations F494N/Q637R (termed double) and F429S/F494N/Q637R (termed triple) on wt- and F508del-CFTR by studying (i) the solubility of NBD1, (ii) the processing and trafficking of CFTR protein and (iii) the Cl^- channel function of CFTR. Both sets of mutations overcame the loss of solubility of F508del-NBD1, rendering F508del-NBD1 more soluble than wt-NBD1. The double and triple sets of solubilizing mutations also led to the partial maturation of F508del-CFTR and to glycosylation patterns that were intermediate between those of immature and mature CFTR protein. The solubilizing mutations rescued the defective channel gating of F508del-CFTR with the triple mutation being most efficacious because it strongly attenuated the prolonged interburst interval of F508del-CFTR. However, in contrast to the revertants G550E and 4RK [3], neither solubilizing mutations enhanced wt-CFTR channel gating. In conclusion, our data demonstrate that in addition to improving the solubilization of F508del-NBD1, the double and triple mutations when in *cis* with F508del promote the maturation of the mutant CFTR protein and enhance its function as a Cl^- channel.

Work supported by POCTI/MGI/47382/2002, PTDC/SAU-GMG/64471/2006 grants and pluriannual funding of CIGMH (Portugal/European Union), the BBSRC and CF Trust (UK). LP and AS are recipients of BD/9095/2002 and SFRH/BD/19415/2004, respectively doctoral fellowships (FCT, Portugal) and ZX is supported by the University of Bristol and an ORSAS award.

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Exploiting Isolated Domains of CFTR to Capture Novel CFTR-interacting Proteins and Quantify the Strength of CFTR-chaperone Interactions

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The deletion of a phenylalanine residue at position 508 (F508del) in the first nucleotide-binding domain (NBD1) of the cystic fibrosis transmembrane conductance regulator (CFTR) is the principal cause of cystic fibrosis (CF). The altered interaction of F508del CFTR with endoplasmic reticulum (ER) quality control proteins, primarily chaperones, promotes its proteasomal degradation. However, it is believed that crucial CFTR-interacting proteins (CIPs) remain unknown [1]. Moreover, there is little information currently available on the strength of CIP-CFTR interactions. We are employing a novel strategy to isolate unidentified CIPs: purified NBD1 is immobilised onto metal-affinity resin and used to capture CIPs from epithelial cell lysates. Using this approach, CIPs were captured from human respiratory cell (Calu-3) lysates and analysed by 2D-electrophoresis. Relevant protein spots so far identified by mass spectrometry include: 1) Raichu404X (Thr/Ser kinase); 2) Profilin 2 isoform b; 3) Annexin A5; 4) Ifapsoriasin (intermediate filament-associated Ca^{2+} regulatory protein); 5) MGC35308 (member of the ER reticulon family). Current analyses aim to determine their functional roles.

In additional experiments, we used surface plasmon resonance (SPR; Biacore™) to quantify real-time binding of CIPs to bacterially expressed wt- and F508del-NBD1. Hsc70/Hsp70 was covalently immobilised onto the surface of carboxymethyl dextran (CM5) sensor chips (600 ± 200 µM) and the real-time binding of purified NBD1 and control proteins quantified. In control experiments, anti-Hsc70 antibody 1B5 (20 nM; against residues 373-650) bound specifically to immobilised Hsc70 with high affinity (K_D, 0.46 ± 0.07 nM; n = 3) whereas bovine serum albumin (15 µM) did not interact (n = 10). Wt human (h) NBD1 displayed dose-dependent binding to immobilised Hsc70 (K_D^{app}, 1.46 ± 0.28 µM; B_{max}^{app}, 405 ± 39 pmol NBD1 nmol $Hsc70^{-1}$; n = 3). Consistent with recent studies [2], the difficulties associated with expression of hNBD1 carrying the F508del mutation precluded its use in SPR analyses. We thus used purified murine (m) NBD1 to study the effect of F508del on the strength of CFTR-chaperone interactions. The affinity of mNBD1 binding to immobilised Hsc70 was dramatically lower when F508 was present (wt, K_D^{app}, 0.50 ± 0.06 µM; F508del, K_D^{app}, 0.13 ± 0.04 µM; n = 3; $P < 0.05$). In summary, we used SPR to demonstrate that: (i) NBD1 of CFTR bind specifically to Hsc70/Hsp70 with micromolar affinity, and (ii) the F508del mutation enhances the association of NBD1 with Hsc70. Presently we are investigating the effect of co-chaperones and small molecules on the interaction of Hsc/Hsp70 with wt- and F508del-NBD1 and extending these studies to the other cytoplasmic domains of CFTR to obtain quantitative data about inter-domain interactions.

The authors are grateful to Prof. John Findlay for training in SPR methodology and to Prof. Philip Thomas, Dr. Patrick Thibodeau and the CF Folding Consortium (www.cftrfolding.org) for supplying the purified NBD1 proteins used in SPR studies. Work supported by grant POCTI/SAU/MMO/58425/2004 and pluriannual funding of CIGMH (FCT, Portugal). TSS-W is a recipient of post-doctoral fellowship SFRH/BPD/18989/2004 (FCT, Portugal).

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Room: Museum

Poster Session – Rescuing the Basic Defect in CF

P.8

Preclinical evidence that sildenafil and vardenafil correct cystic fibrosis defects

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Rationale: Sildenafil has been implicated in the activation of cystic fibrosis transmembrane conductance regulator (CFTR) protein. The effect was observed *in vitro* and in the presence of doses roughly 300 larger than those commonly used for treating erectile dysfunction.
Objectives: To evaluate *in vivo* therapeutic efficacy of clinical doses of sildenafil and vardenafil, two clinically approved type V phosphodiesterase inhibitors, for correcting cystic fibrosis defects.
Methods: We measured transepithelial potential difference *in vivo* across the nasal mucosa as a readout for sodium and chloride conductance. The effect of a single intraperitoneal injection of sildenafil (0.7 mg/kg) or vardenafil (0.14 mg/kg) was investigated in F508del/F508del, *cftr* knockout and normal homozygous mice. In addition, acute effects of sildenafil on transepithelial ion currents in excised nasal tissue were monitored in Ussing chambers.
Measurements: and Main Results: In F508del/F508del, but not in *cftr* knockout mice, chloride conductance, evaluated by perfusing the nasal mucosa with a chloride-free solution in the presence of amiloride and with forskolin, was corrected 1 h after sildenafil administration. A more prolonged effect, persisting at least for 24 h, was observed with vardenafil. The forskolin response was increased after sildenafil and vardenafil in both normal and F508del mutant animals. No effect on sodium conductance was detected in any group of animals. Sildenafil enhanced CFTR chloride currents and failed to affect sodium currents in excised nasal mucosa of F508del/F508del mice.
Conclusions: Our results provide preclinical evidence that both drugs correct chloride transport defects of F508del-CFTR protein.

Supported by grants from the French CF Association (VLM) and by an educational grant from Pfizer Belgium.

Medicinal plant extracts from *Phyllanthus acidus* may represent a potential treatment for Cystic Fibrosis

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According to previous reports flavonoids and nutraceuticals correct defective electrolyte transport in CF airways. Traditional medicinal plants from China and Thailand contain phyto-flavonoids and other bioactive compounds. We examined herbal extracts of the common Thai medicinal Euphorbiaceous plant *Phyllanthus acidus (P. acidus)* for their potential effects on epithelial transport. Functional assays by Ussing chamber, patch-clamping, double electrode voltage-clamp and Ca^{2+} imaging demonstrate activation of Cl^- secretion and inhibition of Na^+ absorption by *P. acidus* [1]. No cytotoxic effects of *P. acidus* could be detected. Mucosal application of *P. acidus* to native mouse trachea suggested transient and steady-state activation of Cl^- secretion by increasing both intracellular Ca^{2+} and cAMP [1]. These effects were mimicked by a mix of the isolated components adenosine, kaempferol, and hypogallic acid [1].
Additional experiments in human airway cells and CFTR expressing BHK cells and *Xenopus* oocytes confirm the results obtained in native tissues. Cl^- secretion was also induced in tracheas of CF mice homozygous for F508del-CFTR and in F508del-CFTR homozygous human airway epithelial cells. Taken together, *P. acidus* corrects defective electrolyte transport in CF airways by parallel mechanisms including i) increasing the intracellular levels of second messengers cAMP and Ca^{2+}, thereby activating Ca^{2+} - dependent Cl^- channels and residual CFTR-Cl^- conductance; ii) stimulating basolateral K^+ channels; iii) redistributing cellular localization of CFTR; iii) directly activating CFTR; and v) inhibiting ENaC through activation of CFTR [1]. These combinatorial effects on epithelial transport may provide a novel complementary nutraceutical treatment for the CF lung disease. The effect of this and other compounds are currently being tested in human native tissues, namely in rectal biopsies.

Work supported by the Bayerische Forschungsstiftung, Mukoviszidose e.V., the Else Kröner-Fresenius Stiftung and by the research grant POCTI/MGI/47382/2002 and CIGMH (FCT, Portugal and FEDER, EU). AR and AS are recipients of post-doctoral and PhD fellowships SFRH/BD/19415/2004 and SFRH/BPD/14653/2004, respectively (FCT, Portugal).

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Rescue of F508del-CFTR channel gating by the ATP analogue 2'-deoxy-ATP

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In the search for new therapies for cystic fibrosis (CF), small molecules have been identified that interact directly with CFTR to enhance the function of the cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channel and CF mutants. One such agent is the hydrolysable ATP analogue 2'-deoxy-ATP (2'-dATP), which gates phosphorylated CFTR Cl^- channels more effectively than ATP and efficaciously rescues the gating behaviour of the CF mutants G551D and G1349D (Aleksandrov *et al*. 2002; Cai *et al*. 2006). Here we investigate whether 2'-dATP rescues the defective channel gating of F508del-CFTR, the most common CF mutant.

For this study, we used inside-out membrane patches excised from C127 cells stably expressing F508del-CFTR grown at 27 ºC to promote the delivery of F508del-CFTR to the cell surface. The pipette (extracellular) solution contained 10 mM Cl^- and the bath (intracellular) solution contained 147 mM Cl^-, 1 mM ATP or 1 mM 2'-dATP and 75 nM PKA at 37 ºC; voltage was –50 mV. Data are expressed as means ± SEM of n observations. To compare sets of data, we used Student's t test.

The gating behaviour of F508del-CFTR is characterised by short bursts of channel openings interrupted by prolonged channel closures. As a result the open probability (P_o) of F508del-CFTR is attenuated markedly compared with that of wild-type CFTR (wild-type, $P_o = 0.52 \pm 0.04$ (n = 18); F508del-CFTR, $P_o = 0.11 \pm 0.03$ (n = 5); $p < 0.05$). Substitution of ATP (1 mM) in the intracellular solution by 2'-dATP (1 mM) did not change single-channel current amplitude, but altered channel gating. 2'-dATP prolonged considerably the duration of channel openings and enhanced markedly their frequency with the result that the P_o of F508del-CFTR increased by more than three-fold (ATP, $P_o = 0.11 \pm 0.03$; 2'-dATP, $P_o = 0.36 \pm 0.02$; n = 5; $p < 0.05$).

To evaluate the efficacy with which 2'-dATP restored function to F508del-CFTR, we calculated the predicted macroscopic CFTR Cl^- current (I^{CFTR}) in the presence of ATP and 2'-dATP. I^{CFTR} is determined by the product of the number of CFTR Cl^- channels in the cell membrane (N), the current amplitude (i) of an individual CFTR Cl^- channel and the probability (P_o) that a single CFTR Cl^- channel is open: $I^{CFTR} = N \times i \times P_o$. In the presence of ATP, the I^{CFTR} of F508del-CFTR is just 1.2% that of wild-type CFTR (Cai *et al*. 2007). By contrast in the presence of 2'-dATP, the I^{CFTR} of F508del-CFTR rose to 32% that of wild-type CFTR. For comparison, phloxine B (1 μM) potentiated the I^{CFTR} of F508del-CFTR to 22% that of wild-type CFTR (Cai *et al*. 2007). Thus, 2'-dATP is an efficacious potentiator of F508del-CFTR. It or related analogues might be of value in the development of new therapies for CF.

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Room: Museum

Poster Session – CFTR, Other Channels and Transporters

P.12

Functional properties and protein interactions of Bestrophins

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Cystic fibrosis (CF) is an inherited disorder dominated by airway disease and resulting from absence of functional cystic fibrosis transmembrane conductance regulator (CFTR) in the apical membrane of epithelia. CFTR and Ca^{2+}-activated Cl^- channels (CaCCs), stimulated by cAMP and Ca^{2+}, respectively, are main Cl^- channels present in respiratory epithelia [1]. Data demonstrating an upregulation of CaCC when CFTR is absent or defective [2,3] have triggered further studies of these channels.

Although searched for long, the molecular identity of CaCC(s) is still under debate. The role of bestrophin family proteins as putative candidates for CaCC(s) is discussed controversially. Bestrophin 1 (Best-1) has been proposed to form Ca^{2+} activated Cl^- channels in epithelial cells. CaCCs have been shown to support cell proliferation, namely in the development of cancer. Here we investigated the correlation between expression levels of Best-1 and proliferation rates of original T84 colonic carcinoma (T84-slow) and in spontaneously transformed T84 cells (T84-fast). We observed that T84-slow have a small proliferation rate and express low amounts of Best1, while T84-fast express high levels of this protein. Best-1 is spontaneously active in T84-fast. Best-1-RNAi inhibited Ca^{2+} activated Cl^- conductance and cell proliferation, therefore establishing a novel role of bestrophins in cell proliferation, which maybe be of relevance for the regeneration of the epithelia in CF and also alternative therapies CF.

In order to better understand the role and function of bestrophin(s) in epithelial cells, a search for interacting protein partners of bestrophin is currently being pursued. Moreover, the above coordinated regulation between CaCC and CFTR, prompts further elucidation of the functional (and possibly also physical) interactions between these two channels. For this purpose, two putative cytoplasmic domains of Best1: polyhistidine -tagged (pHis) N-term (aa 1-30, Best1-N) and C-term (aa 291-584, Best1-C) have been cloned into the Pet-SUMO bacterial expression vector. These domains are then immobilised onto metal-affinity resin to capture interacting proteins from human T84 whole cell and sub-cellular lysates. Protein-containing fractions recovered from Best1-N/C-coated and blank resins are subjected to 2D-gel and protein identification. It is expected that functional characterization of the interaction between the captured proteins and Best1 will give new insights into the biological role, plausibly of relevance to CF.

Work supported by DFG SFB699 A7 and pluriannual funding of CIGMH. JR Martins is a recipient of doctoral fellowship SFRH/BD/28663/2006 (from FCT, Portugal).

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$CFTR_{inh-172}$ and glibenclamide inhibit the murine CFTR Cl^- channel

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$CFTR_{inh-172}$ (3-[(3-trifluoromethyl)phenyl]-5-[(4-carboxyphenyl)methylene]-2-thioxo-4-thiazolidinone) and glibenclamide are widely used inhibitors of the cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channel (Ma *et al*. 2002; Sheppard & Welsh 1992). Glibenclamide is an open-channel blocker of CFTR (Sheppard & Robinson 1997), while $CFTR_{inh-172}$ acts by an allosteric mechanism (Taddei *et al*. 2004). Because the single-channel behaviour of murine CFTR differs from that of human CFTR, the aim of this study was to investigate the effects of $CFTR_{inh-172}$ and glibenclamide on the murine CFTR Cl^- channel. For this study, we used inside-out membrane patches excised from CHO cells stably expressing wild-type murine CFTR. The external solution contained 10 mM Cl^- and the internal solution contained 147 mM Cl^-, 1 mM ATP and 75 nM PKA at 37 ºC; voltage was -50 mV. Data are expressed as means ± SEM of n observations. To compare sets of data, we used Student's t test. In the absence of drug, the gating behaviour of murine CFTR is characterised by prolonged openings to a sub-conductance state (O_1) and only brief openings to the full open-state (O_2) (Lansdell *et al*. 1998). When added to the internal solution, visual inspection of channel records suggested that both $CFTR_{inh-172}$ (2 μM) and glibenclamide (50 □M) blocked murine CFTR by decreasing both open probability (P_o) and single-channel current amplitude (i) (n = 4 – 6). Values of the O_2 state in the absence and presence of $CFTR_{inh-172}$ (2 μM) were: control, P_o = 0.08 ± 0.01, i = -0.44 ± 0.02 pA; $CFTR_{inh-172}$, P_o = 0.05 ± 0.02, i = -0.35 ± 0.02 pA, n = 4, $p < 0.05$). Consistent with the drug's effects on human CFTR (Taddei *et al*. 2004), $CFTR_{inh-172}$ (2 □M) decreased the P_o of murine CFTR mainly by prolonging long closures between bursts of channel openings (n = 4). We interpret these data to suggest that (i) compared to their effects on human CFTR, the concentrations of $CFTR_{inh-172}$ and glibenclamide tested in this study inhibit murine CFTR with lower potency; (ii) inhibition of murine CFTR by $CFTR_{inh-172}$ shows similarities (reduced P_o) and differences (decreased i) to the drug's effects on human CFTR; (iii) $CFTR_{inh-172}$ and glibenclamide are valuable tools to probe the pore structure of murine CFTR.

Supported by the BBSRC and CF Trust.

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Room: Museum

Poster Session – Epithelial Inflammation / Pathogens

P.23

A transcription factor "decoy" strategy to control leukocyte chemotaxis activated by *P.aeruginosa* in bronchial epithelial cells

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The massive intraluminal infiltrates of neutrophils in the conductive airways of CF patients, while ineffective in eradicating the bacterial colonization, are one of the main causes of progressive tissue damage and lung function decline. Interaction of pathogens (*S.aureus* and *P.aeruginosa*) with Toll-like and AsialoGM1 receptors expressed on bronchial epithelial cells induces a series of kinases and adapters leading to the activation of transcription factors (e.g. NF-κB, AP-1, Sp1, NF-IL6, NF-AT, Elk-1, C/EBP, CREB), which ultimately results in transcription of chemo/cytokine genes driving the recruitment of leukocytes inside the bronchial lumen. To find new treatment options focused on the reduction of neutrophil chemotaxis, without abolishing the immune response against pathogens, we are exploring the transcription factor (TF) "decoy" strategy, in which oligodeoxynucleotides (ODN) mimicking the consensus sequences for the TFs proteins identified in the promoters of different chemo/cytokines are delivered inside the cell in order to interfere with gene transcription. CF bronchial epithelial cells IB3-1 have been exposed to the *P.aeruginosa* strain PAO1. A clear PAO1-dependent activation of TFs such as NF-κB, Sp1, AP-1, NF-AT, NF-IL6 was confirmed by Electrophoretic Mobility Shift Assay (EMSA). In parallel, transcription of genes involved in innate immune response, like the adhesion molecule ICAM-1, the chemokines IL-8, GRO-γ and the pro-inflammatory cytokines IL-1β and IL-6, has been observed and quantified by real-time RT PCR. Consensus sequences for the TFs NF-κB, AP-1, Sp-1, NF-AT and NF-IL6 have been identified in the promoters of the genes induced by PAO1. Importantly, the homology of the consensus sequences recognized by the same TFs differs considerably from one gene to another. "Decoy" ODNs directed against the TF NF-κB, AP-1, Sp1, NF-IL6, NF-AT have been designed on the consensus sequences identified in different gene promoters and tested for their ability to interfere in TF protein/DNA binding with EMSA assays. Transfection of IB3-1 cells with HIV-1 LTR and Igκ chain NF-κB ODN "decoys" complexed with Lipofectamine, performed 30 hrs before challenge with PAO1, strongly inhibited PAO1-dependent IL-8 mRNA induction by 80 to 85 % but not PAO1-induced transcription of GRO-γ, IL-1β, IL-6 and ICAM-1. Other TF "decoy" ODNs have been also tested: a) ODN for NF-kB from IL-8 promoter inhibited both IL-8, Gro-γ and IL-6 by 50%, b) ODN for Sp1 from HIV-1 genome inhibited IL-6 by 50%, c) ODN for AP-1 from IL-8 promoter inhibited both IL-8 and GRO-γ by 50%. A TF "decoy" molecule designed as peptide-DNA chimera mimicking the consensus sequence of HIV LTR NF-kB strongly and selectively inhibited IL-8 transcription. In conclusion, transcription of chemo/cytokines induced by *P.aeruginosa* in CF bronchial epithelial cells *in vitro* can be inhibited with different efficiency and selectivity by TF "decoy" molecules. These results provide useful hints for a gene-targeted anti-inflammatory approach and add further information on the regulation of expression of pro-inflammatory genes induced by *P.aeruginosa* in bronchial epithelial cells.

Supported by Italian Cystic Fibrosis Research Foundation (to RG) and by Fondazione CariVerona – Bando 2005 – Malattie rare e della povertà (to GC), Azienda Ospedaliera di Verona and Legge 548/93 - Finanziamento Ricerca Fibrosi Cistica 2004.

An Investigation of the role of CK2 in the CF Inflammatory Response

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Previous work in this laboratory has demonstrated that the pleiotropic protein kinase CK2, which controls protein trafficking, cell proliferation and development, binds wild-type CFTR near F508 and phosphorylates it at S511. Both the interaction and phosphorylation are abrogated by the F508del mutation. Point mutation of CFTR at S511 disrupts channel gating, confirming that this is a regulatory target.
Preliminary work presented at this meeting last year suggested that CK2 phosphorylation at S511 plays a role in the CF inflammatory response.
Here, we present further evidence that CK2 has such a role using a HeLa cell expressing either wild-type or F508del CFTR.
These data suggest that inflammatory agents (live *P.aeruginosa* and purified *P.aeruginosa* lipopolysaccharide (LPS)) interact with CFTR to induce NFκB activity by a pathway that is modulated by CK2.
LPS stimulation of wild-type CFTR-expressing HeLa cells increased NFκB activity 2.5 fold, and CK2 inhibition (using 1μM or 5μM 4,5,6,7-tetrabromobenzotriazole) abolished this rise, suggesting that CK2 activity in wild-type CFTR-expressing HeLa cells is pro-inflammatory. Notably, 5μM BAY11-7082 (a specific inhibitor of IκK) did not alter the LPS effect.
By contrast, in F508del CFTR expressing HeLa cells, we found that CK2 activity was anti-inflammatory. NFκB activity in both LPS-stimulated and unstimulated F508del CFTR expressing HeLa cells rose 6 and 4 fold respectively when CK2 was inhibited using TBB. This dramatic rise in activity was abolished by treating cells with 5μM BAY11-7082, and could not be restored by inhibiting both CK2 and IκK.
Thus, our studies have shown that the inflammatory pathways relating to wild-type and F508del CFTR are complex and distinct from each other. CK2 and IκK have antagonistic roles but are differentially activated in the presence of wild-type or F508del CFTR.

This work was supported by the Wellcome Trust, CF Trust, Anonymous Trust and Tenovus, Scotland.

Evidence for an IgG binding site in NBD1 of CFTR

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The results presented here comprise data from several experimental methods that suggest CFTR has a binding site for IgG in NBD1. Two peptides corresponding to amino acids 503-516 in wild-type CFTR and the equivalent with the F508del sequence were synthesised (referred to as KENIIF and KENII respectively). Whilst screening for interacting proteins using dot-blots of these peptides, we observed that 'negative' controls, omitting the primary antibody and probing only with the secondary antibody, produced a positive result that was specific for the KENIIF peptide. We investigated this unexpected finding further and found that three different anti-IgG antibodies all bound specifically to KENIIF but not either KENII or unrelated peptides derived from remote sequences in CFTR or parts of a CFTR-associated kinase.
This serendipitous result suggested that IgG could interact with CFTR in the region of F508.
Similarity searches revealed significant homology between all of the four antibody-binding domains of protein L, a bacterial virulence-related protein from *Peptostreptococcus magnus*, and KENIIF.

fswimpg **tik**e**n**i**if**gv sydeyryrsv	Human CFTR 494-
||| |+||+	
pepeeev **tik**a**n**l**if**ad gstqnaefkg	Protein L 251-
eepkeev **tik**v**n**l**if**ad gktqtaefkg	Protein L 323-
eepkeev **tik**v**n**l**if**ad gkiqtaefkg	Protein L 397-
eepkeev **tik**v**n**l**if**ad gktqtaefkg	Protein L 471-

In contrast with proteins A and G, which bind to the heavy chain subunit of IgG, protein L interacts specifically with κ but not λ, light chains. This suggested that CFTR could bind to the κ light chain of IgG, however, binding assay experiments suggested that KENIIF can bind to either κor λlight chains.
We immobilised KENIIF, KENII and a control peptide on Biacore chips and used human IgG at various concentrations as analyte. Positive interactions were seen with both peptides and K_D was determined to be 69 nM for the KENIIF peptide and 86 nM for the KENII peptide. The interaction was thus similar for both peptides, suggesting that there may be another mechanism regulating the IgG-CFTR interaction.
Immunofluorescence analysis revealed an IgG-reactive protein that colocalised with CFTR at the apical membrane in primary human ciliated cells. Preincubation of the cells with 5nM myristoylated KENIIF peptide abolished the apical localisation of IgG.
Thus, we present several lines of evidence that suggest CFTR has the capacity to bind IgG in the same region as we have previously reported to interact with the protein kinase CK2. Further work is underway to determine whether CK2 phosphorylation can modulate the CFTR-IgG interaction.
This work was supported by the Wellcome, CF and Anonymous Trusts.

Factors influencing sputum cytokine levels in cystic fibrosis patients

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Following LPS inhalation the inflammatory response in the lung depends on hematopoetic cells (Hollingsworth et al 2005). ICAM-1 is in involved in neutrophil recruitment to the alveolar space following LPS exposure (Mooreland et al 2002). TGFß increases alveolar epithelial permeability (Sheppard 2004).

Previously we reported a significant negative correlation between Interleukin-8 in whole blood and clinical status in cystic fibrosis and a significantly higher difference between whole blood (WB) and induced sputum (IS) values for Interleukin-8 (IL-8) than for TNF alpha.

The aim of this study was to assess the factors influencing sputum cytokine levels in patients with the Delta F 508 (homozygous) mutation.

Twenty six patients (median age 20.5 y, m/f 16/10, BMI 19.55 kg/m^2, shwachman score 75, FEV_1(%) 80.1) and 15 contols (median age 30 y, m/f 5/10, BMI 21.4 kg/m^2) were examined. The following tests were performed: Interleukin-8 (patients: median WB 10.4/IS 5791 pg/ml; controls: median WB 5.0/IS 62.5 pg/ml) and tumor necrosis factor alpha (patients: median WB 11.55/IS 29.6 pg/ml; controls: median WB 17.8/IS 15.3 pg/ml) using a chemiluminescent immunometric assay (DPC Bad Nauheim, Germany). ICAM-1 (289.25 µg/ml) and TGFß (35.8 µg/ml) in serum were measured using an Elisa kit (R&D Systems, Wiesbaden, Germany) in patients. Sodium (148 mmol/l), chloride (143 mmol/l) and leucocytes (2.7 K/Mikrol) in sputum were measured as well. There was only a correlation ($r=0.477$, $p<0.039$) between sodium and leucocytes in sputum as well as a correlation ($r=0.473$, $p<0.041$) between leucocytes and IL-8 in sputum in patients. There is a trend for a negative correlation for TGFß in serum and lung function (FEV1) ($r=-0.521$, $p<0.056$)

Conclusion:.ICAM1 and TGFß in serum have no impact on sputum cytokines. But sodium levels in sputum might indirectly influence IL-8 levels in sputum.

Effect of Moisture Tent on the Ion Content of Nasal Fluid in Patients with Cystic Fibrosis

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Sleeping in a moisture tent has been used as a treatment for cystic fibrosis (CF) patients, in order to hydrate the viscous mucus and make it easier to remove by coughing (Matthews et al., 1968). However, the efficiency of the method has been questioned, and its use was largely discontinued. With a new method to measure the ion content of human nasal fluid the effect of sleeping in a moisture tent on the ion content of nasal airway surface liquid (ASL) in CF-patients and healthy controls was determined. The CF-patients and healthy controls spent a night (8h) in a moisture tent, and samples of the nasal ASL were taken before the experiment, after the period in the tent, and then at each hour during 4h after the persons had left the tent. Samples of nasal fluid were collected on Sephadex G-25 beads that had been mounted on strips of filter paper, which were then put into the nasal cavity of the CF patients or controls for 10-15 min. The strips were removed, and the beads were isolated, dried, and analyzed by X-ray microanalysis (Vanthanouvong et al., 2006).

The concentration of Na, Cl, and K in the nasal fluid of CF patients was 132, 147, and 50 mM, respectively, before the patient entered the tent, significantly higher than the levels in the nasal fluid of the controls (Na 118 mM, Cl 107 mM, and K 19 mM). During the period in the tent, the ion content decreased, to levels of Na 44 mM, Cl 88 mM, and K 19 mM (CF-patients) and Na 32 mM, Cl 45 mM, and K 9 mM (controls) immediately after leaving the tent. After leaving the moisture tent the ion levels in the nasal fluid increased, reaching after 4 h values of Na 131 mM, Cl 176 mM, and K 59 mM (CF-patients) and Na 133 mM, Cl 157 mM and K 37 mM (controls), which was for both groups higher than before entering the tent. No major changes in the ion content of the ASL occurred after 4h.

The salt content of the ASL may be relevant in CF, since the ASL is known to contain anti-bacterial proteins, defensins, that are sensitive to the salt concentration (Bals et al., 1998). Hence, the higher salt concentrations in the ASL of CF-patients may have negative consequences for the anti-bacterial defense system in the lungs, and conversely, the decrease in ion concentrations, caused by spending time in a moisture tent, may have positive effects. However, with currently used procedures, the effect of sleeping in a moisture tent on the ion content of the nasal ASL is short-lived.

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Room: Museum

Poster Session – Cellular and Animal Models

P.29

Reduction of the immunogenicity of the lentiviral vector-technology for pulmonary gene therapy.

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Gene delivery has the potential to offer effective treatment to patients with life-threatening lung diseases such as cystic fibrosis. Phase I/II clinical trials have shown that, in principle, gene transfer to the lung is feasible and safe. However the host immune system represents a major barrier to successful gene transfer to the lung. To overcome these immunological obstacles we have designed an efficient lentiviral vector for optimal transduction of airway epithelium with minimal immunogenicity. We hypothesised that by using selective mi-RNA target sequences the vector will still efficiently express the transgene in pulmonary cells without expression in immunological cells.
Due to the ethical and financial constraints of extensive animal experiments, an in vitro assay using immunological and pulmonary cell lines has been developed to test the vector in vitro.
Materials and Methods: Several lentiviral constructs were cloned starting from a construct where enhanced Green Fluorescent Protein is driven by the ubiquitous CMV promoter (pCHMWS-eGFP). We subsequently cloned micro-RNA target sequences into the transfer plasmids immediately behind the transgene. The target sequences were selected from miRNA-databases for expression in immunological cells and absent expression in pulmonary cells. Three miRNA target sequences were selected: mi142.3p, mi 23.a, mi126. These constructs were named pCHMWS-eGFP-mi142.3p, pCHMWS-eGFP-mi23.a, pCHMWS-eGFP-mi126. Vectors were produced by transient transfection on 293T producer cell lines. They were pseudotyped with the VSV-G envelope (Vesicular Stomatitis Virus protein). A549 (human pulmonary carcinoma cell line, alveolar type), Calu 3 (human pulmonary adenocarcinoma cell line, epithelial type), U937 cells (human histiocyte cell line), MT4 (human T-lymphocyte derived cell line) and 293T cells (control cell line) were grown in their respective mediums for 24h. The cells were transduced for 6 h with different amounts of lentiviral vector. After 72 h the cells were analyzed for eGFP fluorescence using FACS-analysis. Total DNA extraction from the transduced cell lines was used for RT-PCR to evaluate integration of the transgene DNA into the cellular genome.
Results: The vector produced from pCHMWS-eGFP resulted in strong expression of eGFP in all cell lines. Incorporation of the mi142.3p target sequence in the vector resulted in complete inhibition of transgene expression in the immunological cell lines but with normal expression in the pulmonary cell lines. The RT-PCR did however show integration of the transgene DNA into the cellular genome in all cell lines. Target sequences for mi23.a and mi126 failed to suppress eGFP expression in the immunological cell lines.
Conclusion: The complementary micro-RNA sequence (mi142.3p) results in complete inhibition of transgene expression in immunological cell lines, despite clear evidence of integration of the transgene by the lentiviral vector. Expression in pulmonary cell lines remains unchanged. Previous experiments have shown that inhibition of transgene expression in immune cells (eg. antigen presenting cells) effectively prevents an adaptive immune response and results in long term expression of the transgene (Naldini et al, 2006).
Bioengineering and in vitro testing of the lentiviral vector to overcome the barriers encountered in the current gene therapy trials results in promising vectors for future animal experiments.

Non-invasive long term follow-up of pulmonary gene transfer with a lentiviral vector in a fetal rat model.

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Objective: Fetal pulmonary gene therapy is currently being evaluated for genetic conditions such as cystic fibrosis. Fetal therapy has several advantages including small target volume, presence of progenitor and stem cells, long contact time between vector and target cells and reduced immune response. In the current study we examined non-invasively the long-term expression of a transgene and subsequent immune response after injection of a lentiviral vector in a fetal rat model.
Study Design: Time mated pregnant Wistar rat (term=21 days) underwent at E19 general anesthesia, laparotomy and exposition of both uterine horns. Fetuses were transthoracically injected under the right axilla with a bicistronic lentiviral vector expressing enhanced green fluorescent protein (eGFP) and firefly-luciferase (n=10). A second cohort was injected with the same vector in the liver (n=9). Control animals were injected using normal saline (n=10). After birth surviving pups were followed up using a bioluminescence (BLI) camera. Scans were performed at week 1, 3, 10 and 30. Histological examination was performed using immunohistochemistry against eGFP.
As proof of principle one animal was injected with a lentiviral vector coding for HSV-TK and scanned at the age of 1 month using a micro-PET.
Seroconversion for the transgene was assessed by Western blot analysis, serum from intravenous injected adult animals was used a positive control.
Results: In the first (lung) and the second (liver) group one fetus did not survive the procedure, in the group injected wirth normal saline 2/10 fetuses died. Of the remaining fetuses 8/9 and 8/8 in the lung and liver group respecively were found positive on BLI scan, whereas none of the fetuses injected with normal saline were positive. The BLI signal stayed detectable upto 30 weeks.
In the animal scanned with the micro-PET a clear site of tracer accumulation was visualized in the right upper lung region.
No seroconversion against the transgene could be detected using Western blot analysis in the fetuses, adult animals did show seroconversion.
Conclusion: In the rat model fetal gene therapy into pulmonary and hepatic tissue using a lentiviral vector results in long term gene expression with no detectable humoral immune response.

Nasal epithelial cell culture from cystic fibrosis patients

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In order to study inflammatory pathways in cystic fibrosis (CF) epithelial cells a variety of model systems have been employed such as cell lines, primary cells derived from lungs being removed at transplant and primary cells derived from nasal polyps. Primary cells obtained at transplant are from patients with end stage disease and although nasal polyps are relatively common, in our centre only ca. 1 patient per year has surgery for polyps. We have attempted to culture bronchial epithelial cells from patients with CF but in contrast to our results with brushings from patients with asthma or COPD were unsuccessful. We therefore turned our attention to nasal brushings. These are relatively easy to obtain with only mild discomfort for the patient or healthy control volunteer. We can now successfully culture primary nasal epithelial cells from patients with CF (success rate of ~80% from CF and 100% from normal controls). This enables us to investigate the effect of different genotypes or phenotypes on epithelial cell function.

Nasal brushings are obtained using a bronchial cytology brush (3mm, Telemed systems). Two brushings are obtained from the external turbinate of each nostril, providing the patient tolerates the procedure. After each brushing, the cytology brush is washed by placing it in a 15ml tube containing 5ml PBS. On the last brushing the brush is cut off with wire cutters and placed in the 4th the tube containing PBS. Transport media containing antibiotics is added to the tubes for transport back to the lab. Cells are agitated from the brush, tubes centrifuged and the resulting pellets combined and resuspended in airway epithelial growth medium and seeded into a collagen coated T25 flask. Stocks are made from the initial T25 for 4 days, making 4 T25's. Once confluency is reached in each flask, they are further expanded into T75s. Cells tolerate being frozen in liquid nitrogen and no problems have been encountered in resuspension.

In addition, these cells have been successfully grown on Snapwells at an air-liquid interface for 4 to 6 weeks. Using an Evom Voltohmmeter with STX electrodes, good transepithelial resistances of in excess of 1000 Ω were recorded. This suggests that the cells grown in this manner form tight junctions as *in vivo.*

Although there have been many reports using cultured epithelial cells from patients with cystic fibrosis, we have successfully cultured cells derived from nasal brushings. Previous work has used cells derived from lungs of patients with end stage disease at transplantation surgery or from nasal polyps. Our work will extend the results from these previously published reports and offers the advantage that it provides a relatively simple method to obtain source of epithelial cells from cystic fibrosis patients.

A Model of the CFTR Folding Defect through Usage of MDR/CFTR Chimeras in *C. elegans* defective for heavy metal resistance

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The highly variable clinical phenotypes of CF airway disease suggest that a number of genetic factors, other than *CFTR*, play a role in its pathophysiology. Some of these modifier genes are expected to play a role in the endoplasmic reticulum quality control (ERQC), since the major defect caused by F508del is misfolding, retention and degradation of the mutant protein by this cellular surveillance system.

The nematode *Caenorhabditis elegans* is an excellent multicellular genetic model, which has been successfully used in studies of human diseases. Its ~20,000-genes genome has been fully sequenced, it has short life-span (2-3 weeks) and life cycle (~ 3.5 days) and it is easily cultured and amenable for gene disruption, both by knockout or RNAi.

We are generating a *C. elegans* model for the CFTR folding defect, as to take advantage of this model for the identification of genes involved in CFTR folding and/or degradation. Since there is no CFTR orthologue described in *C. elegans*, we constructed a previously described human P-gp-CFTR chimeric protein [1,2] to be used as an *in vivo* folding substrate in this organism. We used human P-gp backbone where a 9 aminoacid motif (^{483}TIAENIRYG^{491}) of NBD1 was replaced by the equivalent CFTR region, i.e., either ^{501}TIKENIIFG^{509} (P-gp/wt-CFTR), or ^{501}TIKENIIG^{509} (P-gp/F508del-CFTR) [1,2]. These two chimeric cDNAs and the intact human P-gp cDNA were cloned into the *C. elegans* pS235 vector of ubiquitous expression and injected into previously described nematode mutant strains for multidrug resistance genes [3]. The effect on the worms phenotype was assessed by an assay of heavy metal sensitivity (2.0 mM arsenite), as described [3].

Our quantitative results show that the P-gp/wt-CFTR chimera increases the resistance to arsenite when injected into the *pgp-1*/*pgp-3 C. elegans* double mutant, whereas P-gp/F508del-CFTR causes no effect. These preliminary results indicate that it is possible to generate two distinct nematode phenotypes caused by each of the transgenic chimeras (P-gp/wt-CFTR and P-gp/F508del-CFTR). These data also suggest that the same folding defect that impairs F508del-CFTR function in CF may also be responsible for the loss of heavy metal resistance function by the Pgp/F508del-CFTR chimera.

Analysis of these strains by RT-PCR showed a reduced expression of the two P-gp/CFTR chimeras at the mRNA level. This led us to additional strategies, in terms or more robust constructs so as to obtain an increased difference in P-gp/wt-CFTR and P-gp/F508del-CFTR phenotypes, such as testing their expression under the *C. elegans* endogenous *pgp-1* and other intestinal-specific promoters, or developing P-gp chimeras with a full CFTR-NBD1.

Eventually, these *C. elegans* models will be used in genome-wide RNAi screens to identify genes involved in ERQC of these P-gp/CFTR chimeras, thus suggesting a putative role in CF for their human orthologues.

Work supported by grant POCTI/BIA-BCM/56609/2004 and pluriannual funding of CIGMH; Mário F. Neto is the recipient of doctoral fellowship SFRH/BD/28741/2006 (FCT, Portugal). The authors are grateful to Rita Aroeira for valuable technical assistance.

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Room: Museum

Poster Session – Protein Structure

P.34

Three-Dimensional Structure of CFTR investigated by Electron Microscopy and Single Particle Analysis

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CFTR (cystic fibrosis transmembrane conductance regulator) is an ATP binding cassette (ABC protein) as well as an ion channel. It utilizes ATP hydrolysis as the energy source to open or close the channel which allows the passage of Cl^- ions. Mutations of CFTR result in cystic fibrosis. Topologically, the channel contains two transmembrane domains (TMD) and two nucleotide binding domains (NBD). The two halves of the molecule are linked by the regulatory domain (R domain): TMD_1-NBD_1-R-TMD_2-NBD_2. To better understand the CFTR function, three dimensional structures of this protein in various states become essential. Using Ni-NTA-nanogold to label the C-terminal poly-histidine tag of CFTR, the position of NBD_2 was located. Two CFTR models could be fitted into the structure suggesting a dimeric architecture of CFTR in the detergent micelle. A density close to the nanogold was not present in comparable studies of P-glycoprotein and hence was suspected to be a candidate for the R domain. Furthermore, higher resolution structures of CFTR in the absence and presence of ATP and PKA were solved. As well as the confirmation of the dimeric association of the protein, the transmembrane regions show a clear triangular top view and a hole can be seen in the plus ATP and PKA structure whilst not shown in the structure with no ATP and PKA present. The difference could be attributed to the phosporylated channel. In contrast, no obvious changes were observed at the nucleotide binding domains for the two structures at this resolution. Further structural studies at other states should reveal more information on the channel formed by the CFTR protein.

Distinct NBD1 binding sites for ATP and Cyclic AMP revealed by ligand binding to wild type and mutant CFTR proteins.

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Activation of CFTR Cl^- channel function is thought to result from phosphorylation by PKA followed by ATP binding to NBD1 and NBD2, then dimerization of the two domains [1]. Direct activation by cyclic AMP, not involving PKA, has also been reported [2], a mechanism supported by our demonstration that cyclic AMP binds directly to the first cytoplasmic domain of CFTR [3]. To investigate the relationship between cyclic AMP and ATP binding, we synthesised soluble his-tagged NBD1-R proteins comprising residues 357-856 of CFTR and developed equilibrium radioligand-binding assays. Wild type NBD1-R protein bound [^{3}H]-ATP with half-maximal displacement by non-radioactive ATP at 3.5μM and 3.1mM. By comparison TNP-ATP binding showed half-maximal binding at 2.7μM. Reported values for half maximal concentration of ATP required to open CFTR chloride channels differ widely (1-100μM) and such discrepancies may be explained by the two affinities for ATP binding demonstrated by our methods. [^{3}H]-cyclic AMP also bound to NBD1-R protein with half-maximal displacement by cyclic AMP at 2.6μM and 167μM. Introduction of a mutation (T421A) in a motif predicted to be important for cyclic nucleotide binding decreased the higher affinity binding of cyclic AMP to 9.2μM, without effect on ATP binding. NBD1-R proteins incorporating the common disease-causing mutations, G551D and F508del, have also been synthesised. G551D protein showed a 42% reduction in ATP binding whereas cyclic AMP bound at the same level as for wild type. F508del protein showed an apparent increase in the low affinity binding of cyclic AMP to 1.9mM, compared to wild type (0.2mM). The data demonstrate that the NBD1-R protein is a good model of the effects of mutations on nucleotide binding to NBD1 and further suggest that the 392-428 region of CFTR is the site of cyclic AMP binding. The effects of mutations suggest that cyclic AMP and ATP binding sites are distinct. It is possible that the relative action of cyclic AMP and ATP binding may determine which function of CFTR predominates. Small molecules such as benzo(c)quinolizinium (MPB) compounds activate wild type and mutant CFTR without increasing cyclic AMP levels and are selective stimulators of F508del-CFTR trafficking. MPB compounds also displaced nucleotides from NBD1-R protein. In conclusion, we suggest that the cyclic nucleotide-binding site is an important regulatory site in CFTR function and should be investigated as a mechanism for correction by drugs of mutant CFTR.

This work was supported by the Cystic Fibrosis Trust and the Wellcome Trust.

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Room: Museum

Poster Session – Omics

P.38

A novel array for CFTR mutation detection: preliminary binding studies

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Rapid identification of mutations is a key challenge in CF, a disease with more than 1,500 mutations in the CFTR gene identified to date, and a complex spectrum of symptoms often defined by the genotype. Our work supports the development of an integrated mutation detection system using CF as a model disease, whose modules (for: 1. DNA extraction and purification from blood samples, 2. DNA amplification, and 3. mutation or SNP detection) will be integrated within a single microelectronic system, with automated movement of samples within and between modules, in a 'lab-on-a-chip' concept.
Our current work is focused on development of an integrated mutation detection array utilizing a base stacking approach for washing temperature-based discrimination between perfectly matched and mismatched oligonucleotide duplexes. As a preliminary approach, we have created a glass slide custom array with multiple, randomly distributed spots containing "fixed" wild-type (wt) and mutant oligonucleotide probes for some of the most common CF mutations, from exon 10 (F508del, F508C, I507del and I506V), exon 11(G551D and G542X) and exon 21 (N1303K) of the CFTR gene. These fixed probes were 3'-thiolated for surface immobilization, and terminated at the 5' end with the variant sequence to be detected. In our detection assay we added exon-specific single-stranded DNA amplified by asymmetrical PCR from CF patient genomic DNA samples, and complementary to the fixed probes. We also added mutation specific "free" probes 5'-labelled with the Cy-3 fluorochrome. The free probes were designed to hybridize to the target DNA immediately upstream of and adjacent to the mutation site-containing part of the fixed probe. Both mutant (mut) and wt target sequences hybridized to the complementary fixed probes and the fluorescently-labelled free probes. However, the thermodynamic stability for duplexes containing perfectly matched complementary sequences (mut:mut or wt:wt) is significantly higher than that for duplexes containing a mismatch (mut:wt), due both to the extra hydrogen bonds between complementary bases at the variant sites, and to the extra stability imparted by the vertical "base stacking" energy between the adjacent fixed probe 5'-ends and the free probe 3'-ends. A perfectly matched duplex will therefore withstand a higher washing temperature, thus providing a simple temperature-dependent method for discrimination between perfectly matched and mismatched duplexes by detection of bound labelled free probe in mutant and wild type spots, using a standard microarray scanner following a series of temperature specific washes.
We present data from our prototype CF mutation arrays on detection of single and multiple mutations from patient samples, and we discuss the implications of these data on the development of our integrated mutation detection system.

Work supported by FP6-2004-IST-NMP-2-016833 (SNiP2CHIP). We thank Dr. Ants Kurg (Asper Biotech, Tartu, Estonia) for printing of arrays, and Prof. Salomé Pais (ICAT, Lisboa) for use of the array scanner.

Room: Museum

Poster Session – Others

P.39

Genetic predisposition to osteoporosis in Polish children with cystic fibrosis

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Introduction: Multidisciplinary management of CF patients lead to an increase of interest in musculoskeletal system pathology in these patients.
Aims: The aims of this study were to determine bone mineral density (BMD) and bone turnover in the group of Polish CF children and to identify determinants of reduced BMD.
Methods: The group of 72 unrelated Polish (all Caucasian) CF children (40 girls, 32 boys), aged 5-20 years (mean age 12.5 years), with known genotype were enrolled in the study between January and May 2006. Fifty DNA samples from healthy volunteers without clinical symptoms of CF and alterations in musculoskeletal system were selected. Clinical data were recorded and analyzed. The blood samples of CF patients were analyzed for serum level of calcium, phosphates, alkaline phosphatase, parathormon, osteocalcin and C-telopeptide. BMD was measured, at L_1-L_4 lumbar spine in the group of CF patients and healthy volunteers, by dual absorptiometry, using LUNAR DPX IQ (#2898) densitometer. DNA was isolated from peripheral blood leucocytes. Polymorphisms in *COLIA1* (*Bal*I, *Rsa*I), *VDR* (*Apa*I, *Bsm*I, *Fok*I, *Taq*I) and *CALCR* (*Alu*I) genes and F508del allele in *CFTR* gene were identified, using DNA-RFLP technique. Differences in polymorphisms distribution between CF patients and control group were analyzed. Association between clinical and molecular parameters and *Z-score* was evaluated. Correlation between FEV_1, biochemical parameters and *Z-score* was estimated. Data analysis was undertaken using SPSS version 12.0 (SSCP Inc., Chicago, Illinois, USA).
Results: In 52% of cases (37 patients) decreased BMD at L_1-L_4 lumbar spine was identified. Sixteen patients (22.5%) had *Z-score* in the range of <-1 to >-2 and in 21 subjects (29.6%), *Z-score*<-2 was observed. Boys had lower *Z-score* (but not significantly, $P = 0.88$) in comparison to girls (-1.25 vs -1.10). In 84.7% of patients (80% girls and 90.6% boys) high bone turnover was observed. More severe lung disease, pancreatic insufficiency, corticoid therapy and male sex were independently associated with reduced BMD. Possible correlations between s (*Bal*I, *COLIA1*), B (*Bsm*I, *VDR*), f (*Fok*I, *VDR*), t (*Taq*I, *VDR*) C (*Alu*I, *CALCR*) and F508del (*CFTR*) alleles and decreased BMD were found. The *COLIA1, VDR* and *CALCR* genes and F508del allele in *CFTR* gene were shown to be one of the factors determining BMD in 72 Polish CF children.
Conclusions: Reduced BMD is common in Polish children with cystic fibrosis. Results of this study indicate several genes participation in alteration of musculoskeletal system development in Polish CF children. Further studies, involving higher number of CF patients and control individuals are needed to confirm these findings.

P.40

The peculiarities of immunological reactions in children with cystic fibrosis

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Aim: To study immunopathologic mechanisms of reaginic and immunocomlex types and the humoral immunity in children with cystic fibrosis (CF).
Methods and materials: In study we have evaluated 17 children with CF ages from 4 till 17 years old, most of them older then 12 years old. The determination of summary IgE was performed by immunoenzymatic method with standard test utilization. CIC concentration – by precipitation in poliethilenglicol method. The IgA, IgM, IgG levels in serum were determinate by immunodiffusion by Mancini. The genetic research in CF children was effectuated by DNA examination by PCR reaction for 5 CFTR gene mutation: F508DEL, R334W, R347R, G551D, R553X. Clinical signs were dominated by severe lung affecting with pulmonary fibrosis in 8 children, bronchiectasises – 5 cases, fibroatelectasises – 2 cases, chronic bronchitis – 11 cases. The severity of bronchopulmonary manifestations was confirmed by respiratory insufficiency with restrictive and obstructive disorders of II – III grade (10 children). In children with cystic fibrosis pulmonary affecting is severe and is characterized by expressed restrictive and obstructive impairments. Growth and development disorders were estimated in 2/3 of cases.
Results: Immunologic researches in children with CF determined hyperimmunoglobulinemia E in 61,5% with the variety 112,5-1366,0 Ul/ml. The concentration of IgE in all children with CF was significantly increased - 313,13±93,95 Ul/ml ($p<0,05$) in comparison to age norms (56,8±4,83 Ul/ml). Immunopathological mechanisms in this children was supplemented by CIC system disorders, which presented significantly ($p<0,01$) increased levels in the exacerbation period - 109,1±9,01 Ul/ml (age norm 65,17±5,04 Ul/ml). The humoral immunologic reactions in children with CF demonstrated considerable increased ($p<0,001$) level of the IgA -1,47±0,22 g/l (in healthy children – 1,18±0,05 g/l) and IgM - 1,8±0,19 g/l (in healthy children – 1,26±0,24 g/l), but concentration of the IgG was not significantly reduced ($p>0,05$) – 9,6±0,68 g/l (in healthy children 11,04±0,74 g/l).
Conclusion: The immunological reactivity in children with cystic fibrosis is determined by immunohumoral reactions effective on the background of reaginic and immunocomplex hyperergic immunopathological reactions, which further to the development of severe disorders of the bronchopulmonary system.

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