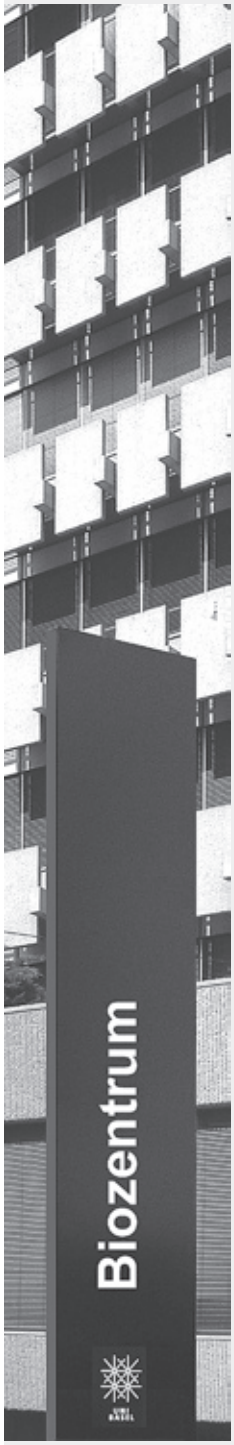

BIENNIAL REPORT 2002 - 2003

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UNIVERSITY OF BASEL

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A printed copy of this report can be obtained via the Chairman‘s secretary at the above address or e-mail.
An online copy is available on our homepage.

Cover

The hexagon stands for a recurrent theme in the life-sciences as can be seen by the benzol ring as part of many biologically relevant macromolecules, the hexagonal layout of certain crystal structures, and honeycomb.

We have therefore chosen this synonym to graphically represent the individual research focii of the different groups at the Biozentrum. Although they are highly individual with respect to their specific research topics, complementary and strengthening interaction is finally the power achieved at the Biozentrum.

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Werner Indlekofer, Margrit Jaeggi

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PREFACE

During the last four years, a renewal of the Biozentrum faculty has taken place. 13 new professors have been hired which accounts for about one third of the total faculty. The tenure track system has been implemented and the first evaluations and promotions from assistant professor to associate professor have been made. We are particularly delighted that we could attract a group of four young assistant professors to create the Bioinformatics division, supported, in addition, by two adjoint professors working at Novartis and F. Hoffmann-La Roche. In 2002, a new curriculum "Nano Sciences" has been launched at the University of Basel, a joint effort of the Departments of Physics, Chemistry, and the Biozentrum.

The international visibility of the Biozentrum remains high. In 2003, the Biozentrum faculty gave some 200 lectures at international conferences, workshops, and related events. This level is also attested by prestigious prizes awarded to its members. Walter Gehring received the Balzan Prize 2002 and became honorary member of the St. Petersburg Society of Naturalists; Stephan Grzesiek received the 2002 Award of the European Society of Applied Physical Chemistry; Ueli Aebi was awarded with the Gregor Mendel Medal, the highest award of the Czech Academic Society; Mike Hall received the Dr. Max Cloëtta Prize 2003; Silvia Arber was awarded the Swiss National Latsis Prize 2003 and the Eppendorf Young Investigator Award 2003; Urs A. Meyer will receive the Robert Pfleger Research Prize and Yves-Alain Barde the Perl-UNC Neuroscience Prize. Joachim Seelig was elected member of the Royal Netherlands Academy of Sciences. Several other prizes and Doctores honoris causae were awarded to Biozentrum members.

The Maurice E. Müller foundation has been the most important single sponsor of the Biozentrum with donations of about 30 Mio CHF since 1986. We are most grateful to Professor Maurice Müller for his generous support, in particular, since the Foundation has expressed its intention to extend its financial commitment for another 8 years.

The present Biennial Report is the last one which is organized according to the classical "Divisions" of the Biozentrum. In a number of discussions and retreats, the members of the Biozentrum have decided to define "Focal Areas" and "Core Programmes" in order to better define the research activities and the methodological interests. At present the areas are classified as follows:

Focal Areas
- Neurobiology
- Infection Biology
- Cell Growth & Development

Core Programmes
- Structural Biology & Biophysics
- Genome Scale Biology & Bioinformatics

In the reorganization of the Biozentrum, our international Advisory Board with its president Beat Gähwiler was extremely helpful, and we express our sincere gratitude.

Recently, it has been decided that the ETH Zürich will set up a Center for Systems Biology in Basel with about 20 research groups. This will considerably strengthen basic research in Life Sciences and we are very much looking forward to collaborating with our new colleagues.

Joachim Seelig
Chairman Biozentrum
April 2004

TEACHING

TEACHING ACTIVITIES

The Biozentrum educates scientists at the bachelor, master, doctoral and postdoctoral level. The particular advantage of the education at the Biozentrum is its direct integration into research. All lecturers are active, grant-holding scientists. The ratio of lecturers to students is also ideal and ensures optimal support for every student.

Bachelor in Biology

In 2003 the current biology curriculum introduced in 2000 was adjusted to the Bachelor/Master system according to the Bologna convention. The Bachelor curriculum takes three years to complete and is taught by lecturers of the Biozentrum and the Department of Integrative Biology.

During the first two years of basic studies, the students receive the essential grounding in mathematics/ statistics, physics, and chemistry, as well as introductory courses in biology. In addition, they are trained in ethics and attend optional courses in biology and non-biological subjects.

After having completed the basic studies, the students must choose either molecular biology or organismic biology to be the main focus of their further studies, because the content of the third year, which consists of block courses lasting several weeks each, determines the Major of the Biology Bachelor. The block courses provide theoretical information which the students then turn into practice in the laboratory or in the field.

Master in Molecular Biology

Students with a Bachelor in Biology, Major Molecular Biology, are admitted to the fast track Master program that takes 1 year. The students work on their Master thesis and take additional courses in the field of molecular biology. The Master thesis will replace the current Diploma thesis as of 2004 and is the students' real introduction to research. At the Biozentrum, the students can specialize in Biochemistry, Bioinformatics, Biophysics, Cell Biology, Developmental Biology, Genetics, Immunology, Infection Biology, Microbiology, Neurobiology, Pharmacology, and Structural Biology. A complete list of the diploma theses completed during the report period can be found on pp 7-9.

The interaction between students and the lecturers is additionally supported by the so called career mentoring: at the beginning of their studies, every student is assigned to a certain lecturer, which implies an early contact between researchers and students. This lecturer, the career tutor, is the student's contact person and personal advisor during the entire duration of his/her studies.

The PhD program

The Biozentrum also offers a Graduate Teaching Program. Besides the work on his/her dissertation, the student must attend lectures that take place in regular cycles within a time frame of 2 to 3 years. The cycles of the new Graduate Teaching Program cover infection biology, neuroscience, cell biology, integrated biological systems, structure and function of macromolecules, biomolecular interactions and structure, genomics and bioinformatics, plant sciences, molecular biology, and molecular medicine. After approval of the dissertation by the Natural Science Faculty of the University of Basel, the studies are terminated with a detailed oral examination. A complete list of the dissertations completed during the report period can be found on pp 10-12.

Seminars at the Biozentrum

The Biozentrum has a formal seminar series, the "Biozentrumsseminare", planned several months in advance and generally given by a senior scientist. Many other seminars are also given that cannot be fitted into the formal series either because they are arranged on short notice or because of scheduling conflicts. We call these "Informelle Seminare", informal seminars. They are, however, just as widely announced as the formal ones and many speakers are surprised to find themselves giving their informal seminar to a packed auditorium. These seminars play an important role in the life of the Biozentrum. They are perhaps the most important post-graduate educational activity that we offer, and many a graduate student has secured his first post-doctoral position while sharing a post-seminar drink with the speaker.

DIPLOMA THESIS

*work performed outside the Biozentrum

Allan Martin G., *Structural Studies of Bacterial Antibiotic Resistance Regulator Proteins by Nuclear Magnetic Resonance Spectroscopy* (S. Grzesiek)

*Andrejszki Laura, *Mutants of the leucine zipper transcription factors c-Jun and c-Fos as HIV-1 enhancer binding polypeptides inhibiting proviral gene expression* (B. Gutte, M.Spiess)

Bacher Christian, *Expression profiling of drug-regulated genes in human liver using oligonucleotide microarrays* (M. Oscarson, U.A. Meyer)

Baeriswyl Simon, *Biochemical approaches to identify mediators of CtrA proteolysis in* C. *crescentus* (U. Jenal)

Bamert Roky, *Localization and biochemical characterization of the Lem3 protein family members* (A. Kralli)

Berthold Guenda, *The promoter structure of UME6* (M. Primig)

Binggeli Simone, *Genome-wide gene expression pattern of* Bartonella henselae *during macrophage infection analyzed by microarray technology* (C. Dehio)

Brogli Kathrin, *The coactivator PGC-1 regulates transcriptional activity of the orphan nuclear receptor ERR*α (A. Kralli)

Broz Petr, *Characterization of the external part of the* Yersinia enterocolitica *injectisome* (G.R. Cornelis)

*Buser Andres, *Purification and characterization of new anti-MAL antibodies and immunoprecipitation of MAL from human tissue* (N. Schären-Wiemers, M.A. Rüegg)

Cartron Marie, *An online microarray data uploading module for GermOnline* (M. Primig)

Casagrande Fabio, *Recombinant MIP: expression, purification and two-dimensional crystallization* (A. Engel)

Christen Beat, *The cause of morphological divergence in experimental evolution of Caulobacter crescentus* (U. Jenal, M. Ackermann)

Cueni Luzius, *Identification and characterisation of genes upregulated by the formation of postsynaptic structures in vivo* (M.A. Rüegg, H. Reichert*)*

Dürr Stefan, *Characterisation of whmC, a multiple drug resistance gene in* Mycobacteria (M. Folcher, Ch.J. Thompson)

Duijts Andreas, *Towards establishing the functional size of the nuclear pore complex by using recombinant Hepatitis virus core capsids* (U. Aebi)

Eglin Daniel, *Confirmation of the dependence of the flagellar protein FliF on ClpAP for degradation using an in vitro approach in Caulobacter crescentus* (U. Jenal, S. Tawfilis)

Eng Cathrine, *Statistical analysis of microarray Expression profiling data of a wild-type versus an abf1-1 mutant strain* (M. Primig)

Engel Eveline, *Role of alternative splicing at the neuromuscular junction: site specific overlap PCR mutagenesis in vitro* (M.A. Rüegg, H. Reichert)

Fierz Beat, *Dynamics of intramolecular contact formation in model peptides* (T. Kiefhaber)

Fischer Franziska, *YsaK, a putative chaperone from the* Yersinia enterocolitica *Ysa Type III secretion system* (G.R. Cornelis)

*Forro Gaby, *Regulated intramembrane proteolysis (RIP): A signalling pathway for teneurin-2* (R. Chiquet-Ehrismann, M.A. Rüegg)

Garcia Patrick L., *Exploring molecular mechanisms of the methyl-dependent restriction enzymes Mrr and McrA* (P. Janscak, T.A. Bickle)

Gregorini Marco, *Structure determination of a bovine and an insect V-type ATPase by electron crystallography* (A. Engel)

Güntherodt Gabriela, *Imaging native nuclear pore complexes by Atomic Force Microscope - attempts to track cargoes* en route (U. Aebi)

Guye-Vuillème Patrick, *Analysis of the* virB-*operon regulation in* B. tribocorum *by promoter fusion to the gene encoding green fluorescent protein* (C. Dehio)

Helfer Hanspeter, *Effects of myosin gene deletions on growth and development of the filamentous fungus* Ashbya gossypii (P. Philippsen)

Hungerbühler Katrin, *Cell cycle regulation in* Ashbya gossypii*: Analysis of B-type cyclin homologs* (P. Philippsen)

Huschauer Viola, *Purification of the needle-part from the* Yersinia enterocolitica *injectisome* (G.R. Cornelis)

*Joset Pascal, *Rab-GDP Dissociation Inhibitor plays a role in commissural axon pathfinding in the chicken spinal cord* (E. Stöckli, M.A. Rüegg)

Kaufmann Andreas, AgBNI1 *and* AgHOF1*: Two genes involved in hyphal development in the filamentous fungus* A. gossypii (P. Philippsen)

Kobialka Szymon, *Recruitment of the AP-1B clathrin adaptor to membranes* (M. Spiess)

Keller Sandro, *Effects of DD-substitutions on self-association and lipid binding of Alzheimer's β-amyloids* (J. Seelig)

*Keller Seraina, *A tight regulation of vesicle fusion is crucial for axon guidance at choice points* (E. Stöckli, M.A. Rüegg)

Köhli Michael, *Functional analysis of the Rho GTPases Cdc42, Rho1a, Rho1b and Rho3 in the filamentous fungus* Ashbya gossypii *by directed mutagenesis* (P. Philippsen)

Kukulski Wanda, *Purification, 2D-crystallization and Electron Microscopy of the plant aquaporin PM28A* (A. Engel)

Kyburz Andrea, *Molecular characterization of the yeast cleavage and polyadenylation factor subunit Ydh1/Cft2* (W. Keller)

Langheld Stefan, *Folding and stability of Hsp15* (T. Kiefhaber)

Lützelschwab Silke, *Characterization of a non-conventional nuclear localization signal in* the Y. enterocolitica *type III effector YopM* (G.R. Cornelis)

Maeder Céline, *Characterization of the Bul1p-Rsp5p protein ubiquitin ligase complex in Saccharomyces cerevisiae* (S. Helliwell)

* Mallaun Michel, *Characterization of the ARE-binding protein BRF1 (ZFP36L1)* (C. Moroni, M. Spiess)

Meinen Sarina, *Proteolytic processing of agrin in vivo (*M.A. Rüegg, H. Reichert*)*

Meng Alexandra, *CRP in* Streptomyces coelicolor (Ch.J. Thompson)

Nijas Alijoski, *Pilot experiments to establish medium-density DNA microarrays for transcriptional profiling in* Ashbya gossypii (P. Philippsen)

Nyfeler Beat, *Identification of ERGIC-53 as a target of the unfolded protein response* (H.-P. Hauri)

Paris Marcel, *Purification of McrC and production of antibodies* (S. Schmid-Nuoffer, T.A. Bickle)

Portmann Thomas, *Molecular dissection of ETS transcription factor activity* (S. Arber, M. Affolter)

*Ranft Christina, *Inhibition of thrombopoietin receptor (c-mpl) function by the splice variant mpl-truncated (mpl-tr) in cell lines* (R. Skoda, J. Coers, U.A. Meyer)

Reisch Adrian, *Electrophysiological Characterization of Sleep-Related Rhythms in the Thalamus of the Mouse* (Anita Lüthi, M.A. Rüegg)

Rhomberg T.A., *Identification of outer membrane proteins from* Bartonella henselae *ATCC 49882 H-1 by proteomic means* (C. Dehio)

Rischatsch Riccarda, *Activity of syntenic* Ashbya gossypii *promoters in Saccharomyces cerevisiae* (P. Philippsen)

*Ruiz Christian, *Tenascin-C induces specific signaling in tumor cells* (R. Chiquet-Ehrismann, G. Orend, M.A. Rüegg)

Ryf Beat, *In vitro and in vivo studies on the involvement of calcium-signalling pathways in phenobarbital induction of cytochrome P450s 2C45 and 2H1* (U.A. Meyer)

*Schaefer Quirino, *Antigen presentation by CD1* (G. De Libero, M. Spiess)

Stalder Jacqueline, *Interaction of the chaperone encoded by ORF155 with Yops from* Yersinia enterocolitica (G.R. Cornelis)

Stettler Hansruedi, *Developmental genes controlled by BldD and AdpA in* Streptomyces coelicolor (Ch.J. Thompson)

Ströbel Simon, *Expression of ETS transcription factors and cadherin in developing mouse spinal cord* (S. Arber, M. Affolter)

Uhlmann Thomas, *Identification of PGC-1 interacting protein* (A. Kralli)

*Vrieseling Eline, *An electrophysiological and anatomical study on ER81 knock out mice: Rescue of ER81$^{-/-}$ phenotypes by EWS-Pea3 fusion genes?* (S. Arber, W. Wadman)

*Wegmüller Daniel, *Analysis of ARE-dependent mRNA turnover by siRNA targeted to AU-binding proteins* (C. Moroni, M. Spiess)

Wong Christine, *mTOR at the neuromuscular junction* (M.A. Rüegg)

Wüest Esther, *Role of the N-terminal secretory domain of YopE and YopH in TTS secretion process* (M. Feldman, G.R. Cornelis)

Zaugg Sacha, *Upregulation of the neuronal genes SNAP-25 and NSE in normal appearing white matter in Multiple sclerosis is restricted to neurons* (N. Schären-Wiemers, M.A. Rüegg)

Zeis Thomas, *Purification and characterization of new anti-MAL antibodies and immunoprecipitation of MAL from human tissue* (N. Schären-Wiemers, M.A. Rüegg)

Zuest Roland, *The regulon of the Abf1 transcription factor* (M. Primig)

DOCTORAL DISSERTATIONS

* work performed outside the Biozentrum

Anderson Ayuk Agbor, *JP-45, a novel protein of the skeletal muscle sarcoplasmic reticulum junctional face membrane: molecular cloning, tissue distribution, developmental expression, and functional interaction with other sarcoplasmic reticulum proteins involved in skeletal muscle excitation contraction coupling* (A. Engel, H.-R. Brenner)

Baader Manuel, *Drug-mediated induction of cytochrome P450 genes in chicken. Identification of pheno-barbital-responsive enhancer units and characterization of their interactions with the nuclear receptor CXR.* (U.A. Meyer, U.M. Zanger)

Blindenbacher Alex, *Molecular analysis of liver regeneration* (U.A. Meyer, M. Heim, M. Hall)

Boncristiano Sonia, *Cerebral amyloidosis in a transgenic mouse model of Alzheimer's disease: impact and therapy* (M.A. Rüegg, M. Jucker, P. Kelly)

Bondolfi Luca, *Neurodegeneration and neurogenesis in mouse models of aging and Alzheimer's disease* (M.A. Rüegg, M. Jucker, K.-H. Krause)

Bonenfant Débora, *Mass spectrometric analysis of the rapamycin-sensitive phosphorylation sites of the yeast protein kinase NPR1* (M.N. Hall, P. Jenö, J. Hofsteenge)

Boudko Sergei, *Structure, stability and folding of trimeric fibrous proteins* (J. Engel, T. Kiefhaber)

Braun Thomas, *2D-crystallization and 3D-structures of membrane channels and transporters* (A. Engel, T. Kiefhaber)

*Brodmann Peter Daniel, *Development and validation of PCR-methods in food analysis with a main focus on quantitative GMO detection and species identification* (T.A. Bickle, Ph. Hübner)

Brooks Hilary, *The carnitine transporter OCTNI2 – just how important is it for carnitine transport?* (U.A. Meyer, S. Krähenbühl, B. Stieger)

Demirovic Alma Rubio, *Therapeutic targeting of tumor blood vessels with VEGF receptor 2-specific biomolecules* (H.-P. Hauri, K. Ballmer-Hofer, N. Hynes)

De Paola Vincenzo, *Pre-synaptic terminal dynamics in the Hippocampus* (M. Rüegg, P. Caroni, A. Matus)

Dettwiler Sabine, *Characterization of factors involved in cleavage and polyadenylation of mammalian messenger RNA precursors* (W. Keller, W. Filipowicz)

Dorn Gabriele, *Purinoreceptors $P2X_2$ and $P2X_3$ are involved in pain* (U.A. Meyer, W. Wishart, R. Häner)

Drabinowski Krzysztof, *Investigations on Ten-1 in* Caenorhabditis elegans *(*J. Engel, R. Chiquet-Ehrismann, J. Hagmann)

Ebner Andreas, *Tracheal development in Drosophila melanogaster as a model system for studying the development of a branched organ* (M. Affolter, W.J. Gehring)

Erb Michael, *Differential expression and MAL-dependent targeting of the L-MAG and S-MAG isoforms to myelin membranes* (M. Rüegg, N. Schaeren-Wiemers, H. Reichert)

Eusebio Alexander, *Role of agrin isoforms in neuromuscular junction gene expression and in mouse models for muscular dystrophies* (M.A. Rüegg, Th. Meier)

Foser Stefan, *Structural and functional characterization of positional isomers of monopegylated interferon alpha-2a* (T.A. Bickle, K. Weyer, U. Certa)

*Foultier Boris, *Caractérisation du locus* ysa *codant un appareil de secretion de type III chez les* Y. enterocolitica *du biotype 1B* (G.R. Cornelis)

Fuchs Thomas, *Functional analysis of CicA, a novel morphogene in caulobacter crescentus* (U. Jenal, Th. A. Bickle, C.J. Thompson)

Gemperle Angela, *Comparative investigations of haloperidol, clozapine and iloperidone on prefrontal cortex physiology in reference to cognitive dysfunctions in schizophrenia* (U.A. Meyer, H.R. Olpe, W. Schaffner)

Graumann Ursula, *Characterisation of molecular alterations in normal appearing white matter from multiple sclerosis patients* (M. Rüegg, N. Schaeren-Wiemers, H. Reichert)

Green Janelle, *Exploring amyloid structure, assembly and toxicity by electron and atomic force microscopy* (U. Aebi, J. Kistler)

Hergovich Alexander, *Studying the localization and regulation of the von Hippel-Lindau tumour suppressor protein* (M. Spiess, W. Krek, H.-P. Hauri)

Hofsäss Ulrike, *Identification of genes differentially expressed in rat brain during postnatal development* (M.A. Rüegg, J. Kapfhammer, S. Arber)

Huang Wentao, *A mechanism for the anti-adhesive properties of tenascin-C and its influence on cell proliferation* (J. Engel, R. Chiquet-Ehrismann, M.A. Rüegg)

Jauslin Matthias, *A cellular model for Friedreich Ataxia and its application in drug discovery* (M.A. Rüegg, T. Meier, M. König)

Karelson Gunnar, *Cerebral metabolism of rat studied with in vivo NMR spectroscopy and in vivo microdialysis* (J. Seelig, M. Rudin)

Kaufmann Isabelle, *New components of mammalian pre-mRNA 3'-end processing factors* (W. Keller, W. Filipowicz)

Kiefer Fabrice, *Heart angiogenesis in vitro in response to hypoxia and growth factors; an assay for investigating angiogenesis of the heart in vitro* (M.A. Rüegg, E. Battegay, K.G. Hofbauer)

Knechtle Philipp, *AgSPA2 and AgBOI control landmarks of filamentous growth in the filamentous* Ashbya gossypii (P. Philippsen, M.-P. Gulli)

Knutti Darko, *Cloning and characterization of PGC-1, a tissue-specifically expressed coactivator of nuclear receptors* (M.N. Hall, A. Kralli, T. Grange)

Kong, Xian Chu, *RNA interference: a new tool to study gene functions in adult mammalian muscle in vivo* (M.A. Rüegg, W. Filipowicz)

*Kosel Markus M., *Métabolisme hépatique et extrahépatique de citalopram, un médicament antidépresseur de la classe des inhibiteurs de la recapture de la sérotonine* (P. Baumann, A. Telenti, U.A. Meyer, C.B. Eap, B. Testa)

Landwojtowicz Ewa, *Multidrug transporter P-glycoprotein: from kinetics of drug-induced ATPase activation in intact cells to structure – activity relationship* (J. Seelig, A. Seelig, T. Kiefhaber)

Lemire-Brachat Sophie, *Exploring the potential of complete sequence information and synteny: Comparative genome annotation and analysis of Ashbya gossypii and Saccharomyces cerevisiae* (P. Philippsen, Y. Barral)

Leu Marco, *ErbB2 function in the peripheral and central nervous systems* (M.A. Rüegg, U. Müller, H.R. Brenner)

*Levano Soledad, *Expression of cytochrome P450 aromatase and the detection of aromatase variant messenger RNAs in human breast cancer* (A.N. Eberle, U. Eppenberger, U.A. Meyer, F. Schoumacher)

*Marenne Marie-Noëlle, *Rôle de LihDC, LcrV et YopN dans la mise en place et le fonctionnement de l'injectisome de type III de* Yersinia enterocolitica (G.R. Cornelis)

Mascarenhas Joseph Bosco D, *Structure of the NtA domain of agrin and characterisation of the agrin-laminin interaction* (J. Engel, M. Rüegg)

Möller Clemens, *Development and application of single-molecule atomic force microscopy methods in biology* (A. Engel, G. Büldt)

Mueggler Thomas, *Functional magnetic resonance imaging of the murine brains: applicaton to a transgenic model of Alzheimer's disease* (J. Seelig, M. Rudin)

Müller Patrick Y., *Sequence variations in the genes encoding sterol-regulatory element-binding protein-1 and –2: Impact on lipoprotein metabolism* (U.A. Meyer, A.R. Miserez, U. Certa)

Nufer Oliver, *Lectins and traffic early in the secretory pathway* (H.-P. Hauri, M. Spiess)

Pedruzzi Ivo, *The Ras/cAMP pathway in yeast: Identification and analysis of new effectors/ activators involved in cell proliferation control* (M.N. Hall, C. De Virgilio, Th. Boller)

Podvinec Michael, *Molecular mechanism of drug-dependent transcriptional regulation of ALAS1 and cytochromes P450. A coordinative link between the induction of cytochrome P450 hemoproteins and the upregulation of heme biosynthesis* (U.A. Meyer, B. Desvergne)

Prince Frédéric, *Identification of a novel Antennapedia cofactor in Drosophila melanogaster* (W.J. Gehring, S. Arber)

Radimerski Thomas, *A genetic and biochemical analysis of Drosophila melanogaster dS6K upstream and downstream signaling elements* (W.J. Gehring, G. Thomas, E. Hafen)

Ribeiro Carlos, *Cellular and molecular analysis of branching morphogenesis in Drosophila melanogaster* (M. Affolter, W.J. Gehring)

Sadowski Martin, *Molecular characterization of yeast factors involved in pre-messenger RNA 3'-end processing and of their function in transcription termination by RNA polymerase II* (W. Keller, W. Filipowicz)

Sanchez Ignacio Enrique, *Rate-equilibrium free energy relationships in protein folding* (T. Kiefhaber, J. Seelig)

*Spengler Marianne, *Isolierung von GAL4-Neuinserierungen auf 2. und 3. Chromosom-Charakterisierung GAL4-induzierte Struktur-variationen in adulten zentralen Gehirnen von Drosophila melanogaster* (M. Affolter, W.J. Gehring)

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The research projects pursued in the Division of Cell Biology include developmental genetics of *Drosophila*, with a major focus on organ development and evolution of developmental pathways, neurobiology in the mouse and the genetic and biochemical characterization of the RNA processing machinery. The division consists of four research groups, headed by Markus Affolter, Silvia Arber, Walter J. Gehring and Walter Keller. The research activities of the individual groups are described below.

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Cell signaling and cell rearrangement during organ morphogenesis

The organization of body pattern in developing multicellular organism is controlled to a large extend by cell-cell signaling. Great efforts have been devoted in the past decades to identify and characterize the molecular components of a relatively small number of diverse signaling cascades conserved throughout evolution. We have been studying two important developmental signals (Dpp/BMP and Fgf), and our efforts concentrated first on characterizing the signaling pathways in detail and decipher their molecular logic, and second on understanding how these recurring pathways control exquisite cellular behavior during development.

Cell signaling in organ formation

It has been proposed more than a century ago that the organization of body pattern might be controlled by so-called morphogen gradients. Only recently has it been possible to demonstrate that secreted proteins of the Wnt, Hedgehog and transforming growth factor β (TGFβ) families specify positional information by this mechanism. *Drosophila* Dpp is a member of the TGFβ superfamily and over the past ten years we have characterized the Dpp signaling pathway in detail, in collaboration with the group of Konrad Basler in Zürich. Surprisingly, two nuclear proteins, Schnurri (Shn) and Brinker (Brk), play major key roles in Dpp signaling and are responsible for much of Dpp's function as a morphogen. Brk is a DNA-binding transcription factor that counteracts responses to Dpp; Loss of Brk function causes overproliferation and ectopic expression of Dpp target genes, while gain of Brk function causes a general loss of Dpp signaling. We find that throughout development, Dpp causes the graded transcriptional downregulation of the *brk* gene, and it turns out that the inverse gradient of *brk* generated by the Dpp gradient is instructive and essential for organ development.

Since the conversion of an extracellular Dpp gradient into an inverse transcriptional gradient of *brk* is a (the) primary important event in the morphogen readout, we concentrated our efforts in understanding the events involved at the molecular level. The dissection of the *brk* regulatory region identified two elements with opposing properties involved in the morphogen readout, a constitutive enhancer and a Dpp morphogen-regulated

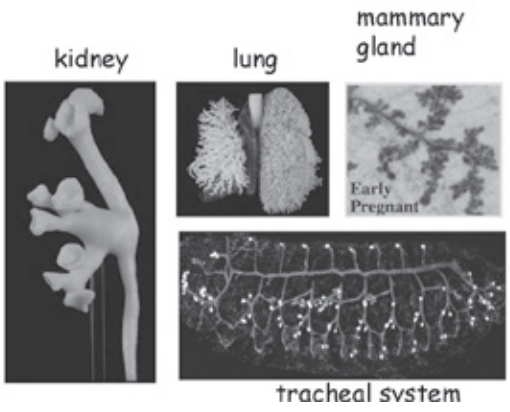

Fig. 1: Many organs undergo branching morphogenesis

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silencer. Furthermore, we find that the *brk* silencer serves as a direct target for a protein complex consisting of the Smad homologs Mad/Medea (which transduce the Dpp signal to the nucleus) and the zinc finger protein Schnurri. Together, these results provide the molecular framework for a mechanism by which the extracellular Dpp morphogen establishes a finely tuned, graded read-out of transcriptional repression.

Although ligand-induced gene repression is not a general property to most signaling pathways, we recently discovered that Dpp represses many genes via the same mechanism with which it represses *brk*, regulating (in addition to morphogen readout) the decision to remain a germ line stem cell, or decisions involved in the establishment of the dorsal-ventral axis of the fly. Therefore, the novel and simple repression mechanism we identified controls key aspects of development. We are now interested in finding out whether similar molecular scenarios exist in other developmental signaling pathways, and whether ligand-induced repression is more widespread than presently anticipated.

Cell rearrangement in organ formation

To gain insight into how signaling pathways control complex cellular decisions during organ morphogenesis, we investigate the formation of the *Drosophila* tracheal system, an epithelial branched network similar to the lung, the kidney or the vasculature. Tracheal development serves as a paradigm to understand how

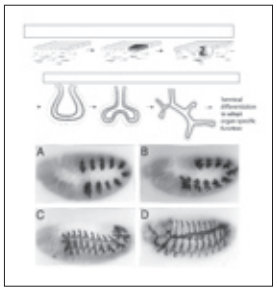

Fig. 2:
Branching morphogenesis of the Drosophila tracheal system during embryogenesis

epithelial sheets can be transformed by cell signaling and cell-cell or cell-matrix interactions into complex three-dimensional networks, a process generally referred to as branching morphogenesis. Our approach has been to identify genes involved in the process by genetic analysis, and the characterization of relevant gene products by *in vivo* and *in vitro* analysis. In addition, we have devoted major efforts to characterize branching morphogenesis at the cellular level, using avant-garde live imaging technology. Over the past decade, these studies have provided a framework for understanding complex processes involved in the architectural design of developing organs, including the control of cell migration and cell rearrangement, and the regulation of the latter by extracellular components.

Studies on the development of blood vessels in higher organisms suggest strong parallels between tracheal development in insects and tube formation in the growing vasculature. Interested by this possible developmental similarity, we have initiated studies aimed at a better understanding of blood vessel development in zebrafish, one of the most promising animal systems in the study of angiogenesis available at the moment.

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Neuronal circuit assembly in the developing spinal cord

The aim of our studies is to understand the molecular and mechanistic basis involved in the establishment of specific neuronal connections within a circuit of interconnected neurons. A deep knowledge about the logic of how neuronal circuits are being assembled during development and which molecules are involved in the establishment of neuronal circuits may contribute to our understanding of the functioning of the mature nervous system. The main focus of our studies is to determine the principles of neuronal circuit formation in the developing vertebrate spinal cord. In the spinal monosynaptic reflex circuit many details of early neuronal specification as well as mature connectivity are already well understood. This neuronal circuit thus represents an ideal system to study how different neuronal subpopulations are being interconnected during development to establish mature neuronal circuits. In our studies, we mainly focus on the development of motor neurons in the ventral horn of the spinal cord and proprioceptive afferents in dorsal root ganglia (DRG) which establish monosynaptic connections with motor neurons (Ia afferents; Figure 1).

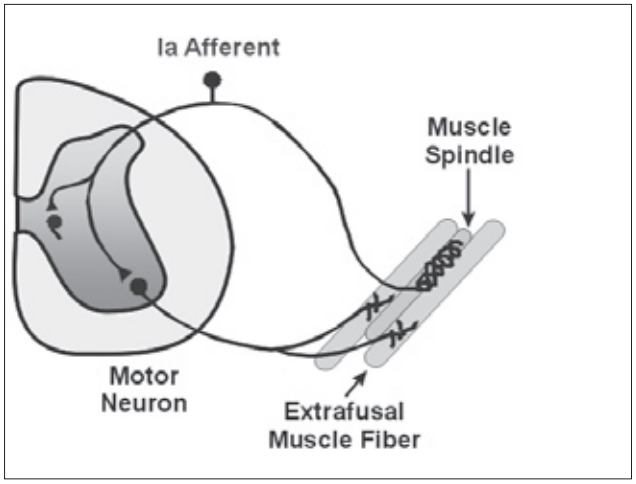

Fig. 1:
Schematic representation of the mature spinal monosynaptic reflex circuit. Motor neurons project to extrafusal muscle fibers and receive direct synaptic input from Ia Afferent sensory neurons.

Function of ETS transcription factors in the assembly of the vertebrate monosynaptic reflex circuit

Different classes of transcription factors have been shown to control sequential steps in the hierarchy of differentiation of motor and DRG sensory neurons (Chen et al., 2003). Many of the early neuronal differentiation steps are thought to be controlled by cell-intrinsic cues and thus to occur independent of an influence from the target region. In contrast, the expression of members of one transcription factor family which has been the focus of our recent studies, the ETS transcription factor family, requires limb-derived signals for induction in motor neurons and DRG sensory neurons.

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The analysis of mice mutant in the ETS transcription factor *Pea3* (Livet et al., 2002) has focused on the differentiation of two brachial motor neuron pools which express PEA3. In the absence of PEA3, the axons of these specific sets of motor neurons fail to branch appropriately within their target muscles, resulting in marked defects in neuromuscular innervation (Figure 2). We observed a strikingly similar projection defect in *GDNF* mutant mice or in mice mutant in components of the receptor complex for GDNF (Haase et al., 2002). Interestingly, Pea3 expression in motor neurons failed to be induced in these mutant mice. Moreover, the addition of GDNF to early ventral spinal cord explants was sufficient to induce Pea3 in motor neurons in the absence of limb target *in vitro*. These experiments thus demonstrate that peripherally localized GDNF is sufficient for the induction of Pea3 expression in distinct subsets of motor neurons and that Pea3 in turn is essential in these motor neurons to control target invasion and branching (Livet el al., 2002; Haase et al., 2002).

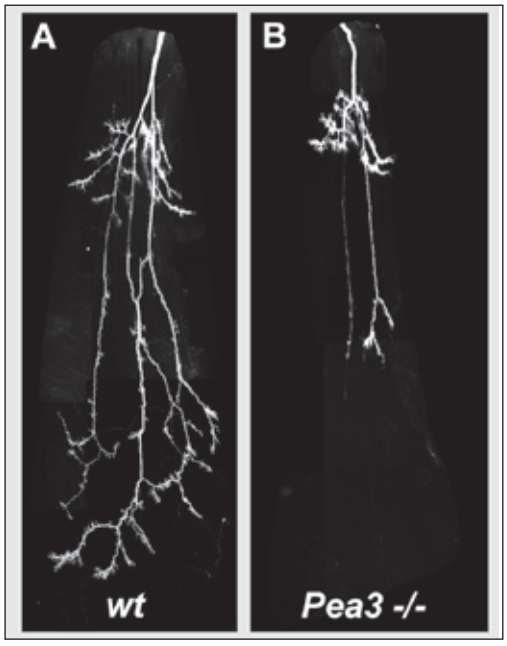

Fig. 2:
The innervation of the latissimus dorsi muscle in wild-type (A) and Pea3 mutant (B) mice is visualized by the expression of a membrane-bound eGFP fusion protein expressed from the Thy-1 promoter (from Livet et al., 2002).

In the mouse, the ETS transcription factor Er81 is prominently expressed by all proprioceptive afferent neurons in DRG. In *Er81* mutant mice, proprioceptive afferents fail to project into the ventral spinal cord and instead terminate prematurely in the intermediate spinal cord. These defects thus led to an essentially complete lack of monosynaptic connections between proprioceptive afferents and motor neurons (Arber et al., 2000). The use of an *in vitro* explant assay system with DRG isolated from a developmental stage when Er81 is not expressed yet allowed us to identify the neurotrophic factor NT3 as a candidate molecule that was capable of inducing Er81 expression in DRG neurons prematurely (Patel et al., 2003). To confirm this *in vitro* result in an *in vivo* context we analyzed *NT3* mutant mice that had been crossed to a strain of mice mutant for the proapoptotic gene *Bax* to circumvent neuronal cell death due to lack of trophic support. In *NT3/Bax* double mutant mice, Er81 failed to be expressed by proprioceptive afferents in DRG, thus confirming our *in vitro* experiments. In addition, in *NT3/Bax* double mutant mice proprioceptive afferents failed to project into the ventral horn of the spinal cord and terminated in the intermediate spinal cord, similar to the defect observed in *Er81* mutant mice. In summary, peripherally localized NT3 is sufficient for the induction of Er81 expression in proprioceptive afferents and Er81 expression in proprioceptive afferents in turn is essential to control connectivity to motor neurons in the ventral horn of the spinal cord (Arber et al., 2000; Patel et al., 2003).

These studies clearly demonstrate that neurons are not completely predetermined intrinsically with respect to the establishment of their target innervation, but instead require retrograde signals from their intermediate target region of further control their specification and differentiation. We provide molecular evidence for the existence of such pathways and show that specific peripherally derived signals are required for the induction of ETS transcription factors in defined subsets of motor neurons and dorsal root ganglia (DRG) sensory neurons respectively.

A role for *Neuregulin1* in muscle spindle differentiation

The differentiation and maturation of neuronal target cells is frequently triggered by signals supplied by ingrowing axons destined to form the presynaptic element of nascent synaptic complexes. The contribution of neural inputs to the initiation of muscle spindle differentiation has been proposed based on surgical elimination experiments and the analysis of mice mutant for genes encoding the neurotrophin *NT-3* or its receptor *TrkC* where proprioceptive afferents fail to differentiate. The identity of the postulated afferent-derived factor(s) responsible for the induction of muscle spindle differentiation has, however, remained unclear.

We have used the early expression of the transcription factors *Egr3*, *Pea3* and *Erm* by intrafusal fibers within muscle spindles to test the potential role of *Nrg1* as an inducer of muscle spindle differentiation in the mouse. We show that *Ig-Nrg1* isoforms are expressed preferentially by TrkC$^+$ DRG

sensory neurons at a developmental stage when proprioceptive afferents first invade developing muscles, whereas only a very low level of *Ig-Nrg1* is expressed in developing motor neurons. In contrast, *CRD-Nrg1* is expressed broadly by DRG neurons and motor neurons. We have compared the state of muscle spindle differentiation in two different mouse *Nrg1* mutations. Elimination of all *Nrg1* isoforms from DRG and motor neurons profoundly impairs muscle spindle differentiation, as assessed by the absence of *Egr3, Pea3* and *Erm* expression, and the failure of proprioceptive afferents to elaborate annulospiral terminals. In contrast, muscle spindle differentiation proceeds normally in mice that selectively lack the *CRD-Nrg1* isoforms. These assays reveal a critical role for Nrg1 signaling in the early induction of muscle spindle differentiation and establish the sufficiency of *Ig-Nrg1* isoforms in this inductive process.

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Master control genes in development and evolution

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Introduction

Our developmental genetic studies on Hox and Pax genes have lead us towards an understanding of developmental pathways. In accordance with Dobshansky's dictum that "nothing makes sense in biology except in the light of evolution" our genetic analysis has far reaching evolutionary implications. The finding of Pax 6 as a master control gene for eye morphogenesis which is shared among all bilaterian animals analyzed so far, has shattered the dogma that eyes have evolved some 40 to 60 times independently in the different animal phyla. I have proposed a monophyletic origin of the eye, and even Ernst Mayer in his latest book admits that his original proposal may not be correct. Meanwhile, we continue to accumulate evidence for the monophyletic origin of bilaterean eyes. The establishment of the body plan by Hox genes is still far from being understood and it also has to be considered in light of evolution.

Hox genes

We have extended our analysis of Hox genes in several directions. In collaboration with Patrick Callaerts, a former postdoctoral fellow, and Gert de Couet from the University of Hawaii we have cloned the Hox genes of the squid Euprymna and found that its homeodomain sequences are most closely related to known annelid, brachiopod and nemerteans genes. These animal phyla are evolutionarily related to molluses in the clade of Lophotrochozoa. Our data are consistent with the presence of a single Hox cluster in the squid, which corroborates the existence of a differentiated Hox gene cluster in the last common ancestor of bilaterians. These findings also indicate that a single Hox cluster is sufficient for the generation of an elaborate body plan as found in cephalopods, and that it is not necessary to have multiple clusters as found in vertebrates.

Hox genes not only contain a homeobox, but also a small YPWM motif, which is also conserved from flies to man, but of unknown function. Using the yeast two-hybrid system Fred Prince, a graduate student, has identified a protein BIP-2, which specifically interacts with the YPWM motif of Antennapedia (Antp). Sequence comparisons indicate that BIP-2 is a TATA-binding factor associated protein, homologous to TAF 155. This is the first demonstration of a direct interaction of a homeodomain protein with the basal transcriptional machinery.

Instead of dissecting gene function by serial deletions of the gene, we are now reconstructing the Antp gene function by fusing various domains into "synthetic genes". A synthetic gene consisting of the YPWM motif and the homeobox of Antp fused to GFP, if driven by distalless enhancer – gal 4, is capable of transforming the distal antenna into leg structures

(tarsus) with 100% penetrance (Daniel Felix and Raphael Fünfschilling). This serves as a starting point for an in depth analysis of synthetic genes *in vivo*.

Previously we have presented evidence that homeodomains interact with other homeodomains or with paired domains at the posttranslational level leading to epistastatic gene interactions (Plaza et al., 2001, EMBO J. 20, 802). These studies have been extended to the Hox gene proboscipedia (pb). A combination of *in vivo* and *in vitro* approaches indicates that Pb suppresses eyeless (ey) transactivation via protein-protein interactions, whereby the homeodomain of Pb binds to the paired domain of EY.

Eye development

Drosophila eye development is under the control of the two Pax 6 homologs, eyeless (ey) and twin of eyeless (toy) which directly activate the early eye specification genes sine oculis (so), eyes absent (eya) and dachshund (dac) and form a regulatory network with various feedback controls. All of these genes are shared between insects and vertebrates. Sine oculis is a direct target of ey and toy. We have identified an eye-specific enhancer in an intron of so which can restore eye development in the so1 mutant. This enhancer contains five binding sites, three of which are recognized by both EY and TOY proteins, whereas two are TOY-specific. The EY binding sites are required for compound eye development, whereas the TOY binding sites are required for the formation of ocelli. This indicates that in the course of evolution ey and toy have diverged in function.

Using DNA microarrays we are trying to decipher the gene network controlling eye morphogenesis in *Drosophila*. We have published a first paper by Michaut et al. on the earliest stage of eye development, when the eye morphogenetic field first forms in the imaginal disc. At this early stage of determination the majority of the identifyable genes that are activated, are transcription factors.

Eye evolution

In order to test our hypothesis that Pax 6 is universal among vertebrates and invertebrates we have previously shown that the mouse Pax 6 gene is capable of inducing compound eyes when expressed ectopically in *Drosophila*. We now have done the reciprocal experiment by injecting ey and toy RNA into one of the blastomeres of two-cell stage Xenopus embryos. These experiments were carried out in collaboration with Makoto Asashima's group in Japan. The two *Drosophila* homologs induce eye structures in frog embryos. In addition, to Pax 6 function, Notch signaling is also conserved in vertebrate eye development.

Previously we have shown that Pax 6 is controlling eye development in organisms ranging from planarians to humans. If Pax 6 is a universal master control gene for eye development, the eye-specific enhancers containing Pax 6 binding sites should also be universally conserved. We have tested this hypothesis by using the P3 enhancer constructed by Ernst Wimmer which is found e.g. in front of most rhodopsin genes and drives GFP expression into the compound eyes and ocelli of *Drosophila* and various other insects. In collaboration with Emili Saló's laboratory we have developed a method for electroporation leading to gene transfer in planarians. By subjecting the entire worm to electroporation we succeeded in transforming the totipotent neoblasts with a P3-GFP construct. The neoblasts are stem cells which gradually replace the somatic cells of the worm and also replenish the germ-line. The P3-GFP transformed neoblasts gradually replace the photoreceptor cells of the eyes leading to green-eyed planarians; they also replenish the germ-line and we have obtained F1 progeny with completely green eyes as well. This supports our notion that Pax 6 is a universal master control gene for eye development and allows us to study the truly totipotent stem cells in planarians.

Our attempts to find a Pax 6 homolog in jellyfish and corals have so far been unsuccessful. It seems that Coelenterates have fewer Pax genes than bilatereans and they may contain a Pax 6 precursor gene involved in eye specification. However, the relationship between Pax C and Pax B proteins of corals and the Pax 6 and Pax 2 / 5 / 8 classes in bilaterians when analyzed in *Drosophila* is not simple.

Co-evolution of blue-light photoreception and circadian rhythms

Sunlight is a primary source of energy for life. However, its UV component causes DNA damage. We suggest that the strong UV component of sunlight contributed to the selective pressure for the evolution of the specialized photoreceptor cryptochrome from photolyases involved in DNA repair and propose that early metazoans avoided irradiation by descending in the oceans during the daytime. We suggest further that it is not coincidental that blue-light photoreception evolved in an aquatic environment, since only blue light can penetrate to substantial depths in water. These photoreceptors were then also critical for sensing the decreased luminescence that signals the coming of night and the time to return to the surface. The oceans and the 24-h light dark cycle therefore provided an optimal setting for an early evolutionary relationship between blue-light photoreception and circadian rhythmicity.

Age-related Macula degeneration

Based on our findings on eye development in *Drosophila*, we have started an applied project with potential medical implications on Age-related Macula Degeneration (AMD) and Malattia Leventinese, an early onset form of AMD. While we are concentrating on *Drosophila* as a model system, ophthalmologists in Zürich and Lausanne work in parallel on mice and humans. This project is headed by Sacha Glardon, a senior postdoc.

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Processing and editing of eukaryotic messenger RNA precursors and of transfer RNAs

The processing reactions capping, splicing, 3‘ end cleavage/polyadenylation and RNA editing are important steps in the generation of nuclear messenger RNAs in eukaryotic cells. Differential splicing and RNA editing give rise to alternate protein products that can have drastically different functional properties. The cap at the 5’ end and the poly (A) tails at the 3’ end of mRNAs have multiple functions, such as increasing the efficiency of translation, mRNA export and the control of mRNA turnover. Elucidation of the functional and structural properties of the factors involved in these reactions is the main purpose of our research. Although RNA processing reactions have traditionally been considered to be “posttranscriptional” and can be studied individually *in vitro*, it is now well established that RNA processing is intimately coupled to transcription by RNA polymerase II *in vivo*.

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Mammalian pre-mRNA 3’ end processing factors

3’ end processing proceeds in two main steps. Endonucleolytic cleavage of the pre-mRNA is followed by the addition of a poly(A) tract to the 3’ end of the upstream cleavage product. The reaction takes place within a surprisingly large multi-component complex, the assembly of which precedes the first reaction step. Specificity and stability of the reaction are achieved by many weak RNA-protein and protein-protein interactions. We are studying this process with *in vitro* systems derived from mammalian cells and in the yeast *Saccharomyces cerevisiae* by genetic and biochemical methods.

The complete pre-mRNA 3’ end processing reaction in the mammalian *in vitro* system can be reconstituted with six separate components: cleavage and polyadenylation specificity factor (CPSF), cleavage stimulation factor (CstF), cleavage factors I and II (CF I_m and CF II_m; the subscript “m” stands for “mammalian” to distinguish the factors from yeast factors with the same names but different functions), poly(A) polymerase (PAP), and nuclear poly(A) binding protein I (PABN1; formerly called PABP II). The cleavage and polyadenylation reactions can be arteficially uncoupled and studied separately *in vitro*. Specific cleavage *in vitro* requires CPSF, CstF, CF I_m and CF II_m and for most pre-mRNA substrates, also PAP; specific polyadenylation of RNA substrates that end at the natural cleavage site (“pre-cleaved RNA”) requires CPSF, PAP, and PABN1.

Purified CPSF contains four core subunits with molecular masses of 160, 100, 73, and 30 kD and interacts with the canonical RNA sequence signal AAUAAA via its 160kD subunit and perhaps also its 30kD subunit. With the exception of the three to four subunits that make up CF I, all mammalian 3’ end processing factors have homologues in yeast.

Based on sequence similarity to the *Saccharomyces cerevisiae* polyadenylation factor Fip1p, we have identified human Fip1 (hFip1) and found that the protein is an integral subunit of CPSF. hFip1 interacts with PAP and, unlike its yeast homologue, has an arginine-rich RNA-binding motif that preferentially binds to U-rich sequence elements on the pre-mRNA. Recombinant hFip1 is sufficient to stimulate the *in vitro* polyadenylation activity of PAP in a U-rich element-dependent manner. Moreover, hFip1, CPSF160 and PAP form a ternary complex *in vitro*, suggesting that hFip1 and CPSF160 act together in poly(A) site recognition and in cooperative recruitment of PAP to the RNA. These results indicate that hFip1 significantly contributes to CPSF-mediated stimulation of PAP activity.

3' end processing of mRNA precursors in yeast

Biochemical fractionation of yeast whole cell extract initially defined the cleavage factors IA and IB (CF IA and CF IB) and the cleavage factor II (CF II) as sufficient to catalyze the cleavage step; polyadenylation of pre-cleaved pre-mRNA required CF IA, polyadenylation factor I (PF I) and poly(A) polymerase (Pap1p). More recently, it was found that CF II, PF I and Pap1p are associated *in vivo* in the so called cleavage and polyadenylation factor CPF. Intact CF IA and CPF can be prepared by tagging one of their subunits and subsequent affinity purification. The polypeptide composition of these factors is complex. CF IA is a hetero-tetrameric protein and CPF consists of at least fifteen different polypeptides. In contrast, CF IB is represented by a single polypeptide (Hrp1p/Nab4p). In the reporting period we have further characterized the CF IA subunit Pcf11p and the CPF subunits Yhh1p and Ydh1p, as well as Ssu72p, one of six new polypeptides identified in affinity-purified CPF preparations by a proteomics approach. One of the more surprising results that emerged from this, is the extent of the coupling between transcription and 3' end formation. Almost half of the subunits of the yeast 3' end processing factors have been found to interact with the C-terminal domain (CTD) of the largest subunit of RNA polymerase II (RNAP II) and conditional mutants in the genes coding for theses proteins in many cases caused a disruption of transcription termination. Transcription termination requires co-transcriptional recognition of a functional polyadenylation sequence but the molecular mechanisms that signal to RNAP II to terminate remain unclear. We have shown that Yhh1p, the yeast homologue of the mammalian AAUAAA interacting protein CPSF160, is a RNA-binding protein and participates in poly(A) site recognition. Interestingly, RNA-binding is mediated by a central domain composed of predicted ß-propeller-forming repeats which is also found in other proteins that function in a variety of cellular processes. We also found that Yhh1p bound specifically to the phosphorylated CTD of RNAP II *in vitro* and in a two-hybrid test *in vivo*. Furthermore, transcriptional run-on analysis demonstrated that *yhh1* mutants were defective in transcription termination suggesting that the protein functions in the coupling of transcription and 3' end formation. Moreover, certain *yhh1* mutant alleles caused a strong defect in transcription termination, but had only a mild reduction in cleavage activity. We propose that direct interactions of Yhh1p with both the RNA transcript and the CTD are required to communicate poly(A) site recognition to elongating RNAP II to initiate transcription termination. The CF IA component Pcf11p also interacts with the phosphorylated form of the CTD. Mutational analysis revealed that Pcf11p has two functional domains for CTD-binding and pre-mRNA 3' end processing that are both essential for mRNA biogenesis and cell viability. Loss of CTD-binding activity did not affect the functions of Pcf11p in cleavage and polyadenylation and *vice versa*. Transcription run-on analysis of mutant *PCF11* alleles demonstrated that the CTD-interaction domain of Pcf11p is required for correct transcription termination, whereas another domain located more C-terminal is necessary for cleavage and polyadenylation. The combined results support the view that pre-mRNA cleavage and transcription termination are independent events. 3' end processing factors bound to the CTD could travel with the elongating RNAP II until a functional 3' end processing signal emerges from the transcription complex. Transfer of the factors from the CTD to the pre-mRNA and the assembly of a 3' end processing complex might then signal to RNAP II to switch to the termination mode.

Ydh1p, the second largest subunit of CPF, is an essential component of CPF but its precise role in 3' end processing remained unclear. We have generated conditional mutations in *YDH1* and found that they were deficient in both the cleavage and the polyadenylation steps of the 3' end formation reaction *in vitro*. We also demonstrated that an important function of CPF lies in the recognition of poly(A) site sequences and RNA binding analyses suggested that like Yhh1p, Ydh1p interacts with the poly(A) site region. We showed that mutant *ydh1* strains are deficient in the recognition of the *ACT1* cleavage site *in vivo*. We also provide evidence that Ydh1p interacts with several other subunits of CPF and with the CF IA subunit Pcf11p. Although the protein also binds to the phosphorylated CTD, conditional mutants were not affected in transcription termination. We suggest that Ydh1p contributes to the formation of interaction surfaces that mediate the dynamic association of CPF with RNAP II, the recognition of poly(A) site sequences and the assembly of the polyadenylation machinery on the RNA substrate.

Ssu72p was previously found to interact with the general transcription factor TFIIB and RNAP II and recent work by several groups indicates that Ssu72p has protein phosphatase activity. However, no target proteins for dephosphorylation by Ssu72p have been identified yet. We found that Ssu72p is a component of CPF and that it bridges CPF, TFIIB and Rpb2p, the second largest subunit of RNAP II. Extracts from a temperature-sensitive mutant (*ssu72-2*) retain *in vitro* 3' end formation activity whereas *ssu72-2* cells underaccumulate polyadenylated RNAP II transcripts at non-permissive temperatures. Furthermore, *CYC1* RNAs induced in *ssu72-2* cells by the GAL1/10 promoter at 37ºC were mostly read-through products. We suggest that the protein functions to recruit CPF to gene promoters. Furthermore, genetic and biochemical analyses of *ssu72-2* mutant cells provided evidence for a negative effect of Ssu72p on transcription elongation which, when relieved during poly(A) site recognition by CPF, contributes to termination. We propose that *SSU72* has an important function in determining the commitment of RNAP II to either transcription elongation or to termination.

RNA-specific adenosine deaminases and RNA editing

RNA-specific adenosine deaminases which convert adenosine to inosine residues are divided into two families: Adenosine deaminases acting on RNA (ADARs) act on double-stranded RNA and edit several cellular pre-mRNAs whereas ADATs convert adenosine to inosine in tRNAs. Previously, we have characterized mammalian ADAR1 and ADAR2, as well as ADAT1/Tad1p from yeast, which forms inosine at position 37 (3' of the anticodon) in eukaryotic tRNA-Ala. We have also identified a yeast adenosine deaminase that generates inosine at the wobble position of tRNAs (position 34). This enzyme consists of two sequence-related subunits, ADAT2/Tad2p and ADAT3/Tad3p, which form a heterodimer. Comparison of the conserved active site domains of adenosine and cytidine deaminases revealed that ADAT2 may represent the evolutionary precursor of ADAT1 and ADARs (see below).

We have further characterized *Escherichia coli* and human tRNA:A34 deaminases. We showed that the prokaryotic enzyme tadA is encoded by an essential gene. This underscores the critical function of inosine at the wobble position in prokaryotes. Recombinant tadA protein forms homodimers and is sufficient for site-specific inosine formation at the wobble position of tRNA-Arg2, the only tRNA containing this modification in prokaryotes. With the exception of yeast tRNA-Arg2 no other eukaryotic tRNA substrates were found to be modified by tadA. However, an artificial yeast tRNA-Asp, which carries the anticodon loop of yeast tRNA-Arg, is bound and modified by tadA. Moreover, a tRNA-Arg2 minisubstrate containing the anticodon stem and loop is sufficient for specific deamination by tadA. We showed that nucleotides at positions 33 to 36 are sufficient for inosine formation in mutant Arg2 minisubstrates. The anticodon is thus a major determinant for tadA substrate specificity. TadA has sequence similarity to the yeast tRNA deaminase subunit Tad2p. We propose that tadA is the ancestor of the eukaryotic Tad2p/Tad3p proteins. We had suggested previously that Tad2p and Tad3p are paralogs that appeared by gene duplication after the divergence of prokaryotes and eukaryotes. This hypothesis is now further supported by the identification of tadA, which acts as a single polypeptide. Prokaryotic organisms encode only Tad2p-like proteins, thus the appearance of Tad3p, Tad1p and the ADARs were later events, which most probably happened after the divergence of prokaryotes and eukaryotes.

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The Division currently consists of four senior research groups headed by Ueli Aebi, Andreas Engel, Stephan Grzesiek, Tilman Schirmer, and one junior research group headed by Olga Mayans. Two further project groups are headed by Peter Burkhard and Cora-Ann Schönenberger, who successfully passed their habilitation in 2002. The Division receives generous support by the M.E. Müller Foundation via the fully integrated M.E. Müller Institute for Structural Biology. Two group leaders participate in the National Centers of Competence in Research (NCCR) programs 'Nanoscale Science' (Ueli Aebi and Andreas Engel) and 'Structural Biology' (Andreas Engel). In the year 2002, the 'G.J. Mendel Honorary Medal for Merit in the Biological Sciences' was awarded to Ueli Aebi by the Academy of Sciences of the Czech Republic, and Stephan Grzesiek received the 'Applied Physical Chemistry Award' from the European Society of Applied Physical Chemistry.

The techniques applied in the Division of Structural Biology range from light, electron and scanning probe microscopy over X-ray crystallography to NMR spectroscopy. Using rigorous combinations of these techniques, we are in a unique position to determine structure of biological matter at all levels, from entire cells to atomic detail.

The projects of the individual research groups are described below

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Structure-based functional analysis of supramolecular assemblies by hybrid methods

Our lab has a long-standing interest in a structure-based understanding of the formation, turnover and functioning of molecular machines, and more generally, supramolecular assemblies. For this to be achieved, we typically employ hybrid methods that include light, electron and scanning probe microscopies, X-ray crystallography (in collaboration with the group of P. Burkhard in the Division), biochemistry, and molecular cell biology. Being problem-driven, we focus on (1) cytoskeletal filament structure, assembly and turnover; (2) the nuclear pore complex and its involvement in nucleocytoplasmic transport; and (3) fibrillogenesis of amyloid forming peptides and how this spatially and temporally correlates with disease progression. In addition, the group is developing and implementing novel optical and mechanical nano-sensors for local diagnosis and therapy by minimally invasive interventions.

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Determining nuclear pore complex structure and function at molecular detail and beyond

The nuclear pore complex (NPC) is the sole gateway between the cytoplasm and the nucleus of interphase eukaryotic cells. Embedded in the double membrane of the nuclear envelope (NE), the NPC allows free diffusion of ions and small molecules across the NE, and it facilitates receptor-mediated nucleocytoplasmic transport of proteins, RNAs and RNPs. The NPC is composed of a set of proteins, collectively termed nucleoporins, and proteomic approaches have revealed that the NPC is composed of ~30 different nucleoporins, both in yeast and mammals. The presence of distinct FG (phenylalanine-glycine) repeat-motifs is a common feature of many nucleoporins, which mediate the main interaction between nucleoporins and soluble transport receptors.

With the goal in mind to ultimately produce a 3D envelope of the NPC suitable for atomic modelling, we have now completed tomographic reconstruction of fully native Xenopus oocyte NPCs that were embedded in a thick film of amorphous ice to optimally preserve their cytoplasmic and nuclear peripheries [Stoffler et al., 2003]. The reconstruction representing an average over more than 500 NPCs, has resolved for the first time some of the distinct peripheral components of the NPC, including the distal ring of the nuclear basket, and remnants of the cytoplasmic as well as intranuclear filaments. As a next step, we are now performing cryo-electron tomography of NPCs in the presence of cargoes and/or caught in different transport states.

In order to better understand the role of the FG-repeat domains and of other nucleoporin domains for NPC structure, function and nucleocytoplasmic transport, we are mapping the domain topography

of different nucleoporins, for example, Nup153, CAN/Nup214 and p62, within the NPC by immunogold-EM. For these studies, we are using domain-specific antibodies against the distinct domains of these nucleoporins. Moreover, we are expressing epitope-tagged versions of the different nucleoporins in Xenopus oocytes to confirm and strengthen the results we are obtaining by using the domain-specific antibodies. With this comprehensive approach, we could show that, for example, the FG-repeat domain within the C-terminus of Nup153 is tethered mobile within the NPC, i.e. the FG-repeat domain of Nup153 can localize to the nuclear and the cytoplasmic side of the NPC. In contrast, an N-terminal region and a central zinc-finger region of Nup153 are residing exclusively at the nuclear face of the NPC [Fahrenkrog et al., 2002]. Such a mobility of FG-repeat domains might have a strong impact on the mechanisms underlying nucleocytoplasmic transport [cf. Fahrenkrog & Aebi, 2003a, 2003b; Fahrenkrog et al., 2004b].

Additionally, in order to better understand the role of the distinct domains of Nup153 for NPC structure, we have started, in collaboration with K. Ullman (Huntsman Cancer Institute, University of Utah), to overexpress the distinct domains in Xenopus nuclei reconstituted from mitotic egg extracts and study the effect of the overexpressed Nup153 domains on NPC structure.

In the last few years it turned out that mediating nucleocytoplasmic transport might be the main, but not the only function of the nucleoporins and the NPC. During mitosis, for example, many nucleoporins localize to kinetochores or the spindle apparatus and mutations in nucleoporins can lead to defects in chromosome segregation and kinetochore integrity. Interestingly, our studies on the yeast nucleoporin Nic96p has lead us to the identification of a protein, termed Nma111p. Nma111p is a nuclear protein and it promotes apoptosis in yeast [Fahrenkrog et al., 2004a]. Currently we are studying this highly interesting link between the yeast NPC and yeast apoptosis more systematically.

Molecular and biophysical characterization of assembly intermediates of human vimentin

Together with microtubules and microfilaments, the ~11 nm wide intermediate filaments (IFs) constitute the integrated, dynamic filament network present in the cytoplasm of all metazoan cells. This network is critically involved in cell division, motility and plasticity. The "elementary building" block of all IFs is a highly elongated, rod-shaped α-helical coiled-coil dimer whose atomic structure is slowly but definitely emerging [cf. Strelkov et al., 2002; 2003]. By a series of distinct lateral and longitudinal interactions, this dimer eventually assembles into mature IFs [cf. Herrmann et al., 2002; Herrmann and Aebi, 2004]. In collaboration with the labs of Dr. Herrmann and Dr. Langowski at the DKFZ in Heidelberg, and the X-ray crystallography group of Dr. Burkhard in our Division, we are aiming to dissect IF structure, assembly and dynamics at atomic detail.

In the context of this initiative, we have developed an assembly protocol for the intermediate filament (IF) protein vimentin based on a phosphate buffer system which enables the dynamic formation of authentic IFs. The advantage of this physiological buffer is that the analysis of subunit interactions by chemical cross-linking of internal lysines becomes feasible. By this system, we have analyzed the potential interactions of the coiled-coil rod domains with one another by analytical ultracentrifugation, electron microscopy and chemical cross-linking [Mücke et al., 2004b]. We show that headless vimentin, which forms a dimer under low salt conditions, associates under assembly conditions into stable tetramers of the A_{22}-type configuration, indicating that one of the effects of increasing the ionic strength is the establishment of coil 2-coil 2 interactions. Furthermore, in order to obtain insight into the molecular interactions that occur during the first phase of assembly of full-length vimentin, we employed a temperature-sensitive variant of human vimentin, which is arrested at the "unit-length-filament" (ULF) state at room temperature, but starts to elongate as soon as the temperature is raised. We demonstrate by cross-linking analysis that ULF formation predominantly involves A_{11}-type interactions. The presence of A_{22} and A_{12} cross-linking products in mature IFs, however, indicates that major rearrangements occur during the longitudinal annealing and radial compaction steps of IF assembly.

In another effort, we have assessed the flexibility of vimentin IFs by atomic force microscopy (AFM) [Mücke et al., 2004a]. Depending on the solid support used, apparent persistent lengths ranging between 0.3 and 1.0 μm were measured. Making simple assumptions concerning the adsorption mechanism, we could estimate the persistence length of an IF in a dilute solution to be ~1 μm, indicating that the lower measured values reflect constraints induced by the adsorption process of the filaments on the corresponding support.

Dissecting human amylin fibril formation by substitution of single amino acid residues

Pancreatic amyloid deposits, composed of the 37 amino acid peptide amylin, represent an integral part of type 2 diabetes mellitus pathology. Human amylin (hA) forms fibrils *in vitro* and is toxic to cultured pancreatic islet β-cells. In contrast, rat amylin (rA)

which differs from hA by only six amino acid residues in the central region of the peptide, residues 18-29, does not form fibrils and is not cytotoxic.

To elucidate the role of individual residues in fibril formation, we have generated a series of full-length rA variants and examined their ability to form fibrils *in vitro* [Green et al., 2003]. Single-residue substitutions with amino acids from corresponding positions of the hA sequence, i.e. R18H, L23F, or V26I, were sufficient to render rA competent for fibril formation albeit at a small yield. Combining two or three of these substitutions generally increased the ability to produce fibrils. Variant rA fibril morphologies were examined by negative stain electron microscopy and found to be similar to those generated by hA itself. Bulk assays i.e. involving thioflavin-T (TFT) fluorescence and sedimentation, showed that the amount of fibril formation was relatively small for these rA variants when compared to hA under the same conditions. Fibril growth was demonstrated by time-lapse atomic force microscopy (AFM), and MALDI-TOF mass spectrometry was used to verify that fibrils consisted of full-length peptide. Our observations confirm previous reports that the three proline residues play a dominant negative role in fibril formation. However, their presence is not sufficient to completely abolish the ability of rA to form fibrils, as each of the other three implicated residues (i.e. R18, L23 and V26) also has a dominant modulating effect.

Poised by the increasing interest in early assembly intermediates rather than mature fibrils being the cytotoxic species of amyloid forming peptides, we have captured the smallest hA oligomers that form by time-lapse AFM [Green et al., 2004]. Accordingly, two distinct phases have been identified in hA fibrillogenesis: lateral growth of oligomers followed by longitudinal growth into mature fibrils. These observations suggest that mature hA fibrils are assembled directly via longitudinal growth of full-width oligomers, rendering assembly by lateral association of protofibirls less likely.

Assessing the biomechanical properties of normal, diseased and engineered tissue by indentation-type atomic force microscopy

The atomic force microscope (AFM) is capable of imaging, measuring and manipulating biological matter in a physiological environment from the nanometer to the millimeter scale. This bears the promise to assess soft biological tissues at all levels of their hierarchic structural organization. This prospect, in turn, prompted us, in collaboration with Dr. U.A. Daniels from the Felix Platter Hospital in Basel, and Dr. R. Raiteri from the University of Genova, to develop and validate indentation-type AFM (IT AFM) to image and measure the mechanical properties of soft biological tissues [Stolz et al., 2004].

More specifically, IT AFM which is based on piezoelectric actuators has far greater dimensional sensitivity compared to conventional clinical indentation testing devices. This technical improvement allows for higher-resolution indentation testing data and therefore suggests that IT AFM can be used to explore and better understand the effects of disease, injury and treatment on soft biological tissue, and perhaps detect early changes. To explore and validate the practical use of IT AFM, we are currently focusing on two frequent and devastating diseases, i.e. arteriosclerosis and osteoarthritis. Ultimately, we want to move IT AFM from the bench to the patient by directly bringing the AFM to the disease site via an endoscopic approach [Hunziker et al., 2002; Stolz et al., 2003].

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Membrane protein structure and function

Membrane proteins comprise more than 30% of the proteome of higher organisms. With characteristic dimensions of 5-10 nm they are membrane-embedded nanomachines that fulfill key functions such as energy conversion, solute transport, secretion and signal transduction. Their central role in a wide range of diseases and in cell-cell communication may explain the fact that 70% of all drug targets are membrane proteins. While the structure of more than 10'000 soluble proteins are solved, the number of membrane protein structures is smaller than 100. This lack of structural information is related to the instability of membrane proteins in a detergent-solubilized state, making the growth of 3D crystals difficult. To understand the function of membrane proteins knowledge of their structure is urgently required. 2D crystals of purified membrane proteins reconstituted in the presence of lipids provide a close to native environment. Electron crystallography allows the atomic structure of a membrane protein packed in a perfect 2D crystal to be elucidated at atomic resolution. In contrast, atomic force microscopy (AFM) provides the surface structure of membrane proteins from tightly packed, but disordered membranes at sub-nanometer resolution. Combining these methods we have studied different aquaglyceroporins, a bacterial photosynthetic system, and the packing arrangement of rhodopsin in murine disk membranes. In parallel, we use the Scanning Transmission Electron Microscope (STEM) for mass determination and single particle imaging to acquire information of biological relevance.

Aquaglyceroporins

Maintaining water homeostasis is essential for all living organisms. Diffusion of water through pure lipid bilayers has a high activation energy (E_a >10 kcal/mol). In contrast, the rapid flow of water through human red cell membranes requires an E_a < 5 kcal/mol, leading to the hypothesis that water pores must exist. Peter Agre, Nobel Prize laureate 2003, discovered an abundant 28 kDa red cell membrane protein, and identified its water channel function by expressing it in *Xenopus laevis* oocytes and measuring water permeation. The protein is now known as aquaporin-1 (AQP1). We have been able to grow highly ordered 2D crystals of AQP1, which eventually led to the determination of its atomic structure in a collaborative effort between the laboratories of Yoshi Fujiyoshi, Helmut Grubmüller and ourselves. Meanwhile we have crystallized two further members of this important protein family, AQP2, the regulated water channel of the renal collecting duct, and PM28A, a major aquaporin of plant plasma membranes. Both types of crystals are well ordered, allowing high-resolution analyses by electron diffraction.

Rhodopsin

G protein-coupled receptors (GPCRs) are involved in virtually all physiological processes. Crystal structures are only available for the detergent-solubilized light receptor rhodopsin. Using AFM, we have demonstrated, in collaboration with Kris Palczewski, the dimeric packing arrangement of rhodopsin and the higher order

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oligomeric state in native murine disk membranes. We have also determined the organization of opsin in native membranes obtained from *Rpe65*-/- mutant mice, which do not produce the chromophore 11-*cis* retinal. Both, rhodopsin and opsin form structural dimers that are organized in paracrystalline arrays. The intradimeric contact is likely to involve helices IV and V, whereas contacts mainly between helices I and II and the cytoplasmic loop connecting helices V and VI facilitate the formation of rhodopsin dimer rows. Contacts between rows are on the extracellular side and involve helix I. This is the first semi-empirical model of a higher order structure of a GPCR in a native membrane, and it has profound implications for the understanding of how such receptors interact with G-protein heterotrimers as well as with arrestin.

Photosynthetic system of *Rhodospirillum rubrum*

The bacterium *Rhodospirillum rubrum* contains a simple photosynthetic system, in which the reaction center (RC) receives energy from the light harvesting LH1 complex. We have used high-resolution AFM to image 2D crystals of the RC-LH1 complex of *R. rubrum*. The AFM topographs show that the RC-LH1 complex is ~94Å in height; the RC-H subunit protrudes from the cytoplasmic face of the membrane by 40Å and sits 21Å above the highest point of the surrounding LH1 ring. In contrast, the RC on the periplasmic side is at a lower level than LH1, which protrudes from the membrane by 12Å. Nanodissection of the RC by the AFM tip removed the RC-H subunit and revealed the underlying RC-L and -M subunits; LH1 complexes completely lacking the RC were also found, providing ideal conditions for imaging both rings of LH1 polypeptides for the first time by AFM. In addition we demonstrated the ellipticity of the LH1 ring at the cytoplasmic and periplasmic sides of the membrane, in both the presence and absence of the RC. In collaboration with Per Bullough and Neil Hunter, these AFM measurements have since been reconciled with previous EM and NMR data to produce a model of the RC-LH1 complex.

STEM

STEM is primarily used to determine the mass of biomacromolecules and their assemblies. Exploiting this feature leads to collaborations and projects covering a large spectrum of microbiology. We have studied the helical filaments assembled by PulG, a pseudo-pilin and derived the atomic scale model of the filament using the atomic structure of PulG determined by X-ray crystallography. The channel-forming VirE2 protein of *Agrobacterium tumefaciens* mediates the transfer ssDNA to the nucleus of infected cells. The oligomeric state of VirE2 was analyzed by cross-linking and mass-measurements in the STEM, and found to be trimeric, while single particle analysis revealed that each monomer is organized into 2 distinct regions.

Protein expression

Recombinant expression of the protein of interest is in general a necessity to produce sufficient material for structural analyses, but is difficult for mammalian membrane proteins. We have compared different systems for their expression of different membrane protein classes: Yeast, Baculovirus/Sf9 and the Semliki Forest Virus (SFV)/mammalian cells. First successes of the SFV expression system are the expression of vasopressin receptor, V2R, of human AQP3 and of the pacemaker channel HCN2. While HCN2 was functionally expressed in HEK cells as demonstrated by patch-clamp experiments by Anita Lüthi (Biozentrum), V2R expressed in BHK cells at mg/l levels was not functional. V2R and AQP3 have also been produced in Sf9 cells in the laboratory of Peters Deen (Nijmegen), using the Baculovirus/insect cell expression system. While AQP3 is in a functional state, V2R is not functional when produced in the Sf9 cells. Finally, we have also explored the yeast systems *S. cerevisiae* and *P. pastoris,* mainly for expressing PM28A and AQP6, the aquaporin of intracellular vesicles of acid-secreting alpha-intercalated cells from the renal collecting duct. High cell densities in culture and an efficient and strong induction can be obtained in the methylotrophic yeast *P. pastoris,* which achieves high expression levels with PM28A and medium levels with AQP6. The latter can still be improved by multiple integration of the AQP6 gene.

Novel processing software for electron crystallography

The MRC programs are widely used for processing images of 2D crystals, helical structures and spherical viruses. As they are an undocumented, yet powerful collection of programs some of which were written more than 30 years ago, we have initiated the creation of a new software package. It has a modular, collaborative, integrated, open-source architecture for image processing of electron micrographs, and is named 'iplt'. Designed around object oriented paradigms it is implemented using the programming languages C++ and Python. In many aspects it deviates from classical image processing approaches (see http://www.iplt.org).

Future direction

As a first step to enlarge the repertoire of 2D crystals from medically important membrane proteins, we have set up a suitable protein expression laboratory. Close to obtaining sufficient quantities of several functional human channel proteins and GPCRs, we will concentrate on producing 2D crystals thereof, using conventional reconstitution methods as well as methods related to dilution driven crystallization. To accelerate the extraction of structural information from low dose electron micrographs of 2D crystals, we will improve image processing, a project initiated within the NCCR for Structural Biology. Another project embedded in the NCCR in Nanoscale Sciences will produce sophisticated tips to simultaneously acquire multiple signals and to control the environment of biomolecules observed at work. The photosynthetic membranes and the HCN2 channels will be the first systems to which these multifunctional cantilevers will be applied. We will continue our collaborations within the house, offering STEM, AFM and cryo-electron microscopy as important tools.

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High resolution NMR of biological macromolecules

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The scientific goal of the group is the application and development of high resolution Nuclear Magnetic Resonance (NMR) methods for the elucidation of structure, function, and dynamics of biological macromolecules.

Our work is directed towards problems in structural biology where NMR can give unique information: 1. the determination of biomolecular structures and interactions which are difficult to obtain by other methods. A special emphasis is put onto biomolecular pathogens, not completely folded structures, and membrane-associated proteins. 2. the study of macromolecular complexes where NMR yields information on interaction surfaces, the strength of the interaction and the dynamics, and thereby offers insights into biomacromolecular mechanisms. 3. the study of forces that are relevant for macromolecular folding.

Structural Projects

Homoassociation of Cadherin. Cadherins are single pass transmembrane glycoproteins which mediate calcium dependent cell-cell adhesion by homophilic interactions. A detailed understanding of the calcium induced homoassociation of cadherin is still missing. In the last years, we have extensively characterized this homoassociation by NMR spectroscopy *in vitro*. We have determined interaction surfaces of the first two domains of epithelial cadherin, their relative orientation in the calcium-free, the calcium-bound monomeric, and the calcium-bound complexed state (Häussinger et al, 2002). Cadherins are made as immature precursor proteins, which are activated by the proteolytic cleavage of an N-terminal prosequence. We have been able to follow this proteolytic activation mechanism in atomic detail in a model precursor in the NMR tube. This N-terminal processing leads to substantial structural rearrangements that are observable by solution NMR spectroscopy (Figure 1). At low protein concentrations, the cleavage induces tight, intramolecular binding of the crucial tryptophan-2 residue. At higher protein concentrations, spectral changes indicate an associated form of the protein and the onset of dimerization by the exchange of the first β-strand. We have confirmed the strand exchange in mature ECAD12 by an X-ray crystallographic study. Kinetic and equilibrium constants of the strand exchange dimerization could be derived from the NMR data in calcium-bound and calcium-free forms. The data show that the process is strongly favored in the presence of calcium and that there is a severe activation barrier of ~7 kcal/mol for the strand exchange. Our findings present a dynamical view of the strand exchange mechanism (Häussinger et al. *in press*; in collaboration with J. Engel).

The multidrug resistance protein TipA. In recent years, research on multidrug resistance proteins and their regulation has gained increased interest as increasing numbers of microbial organisms develop

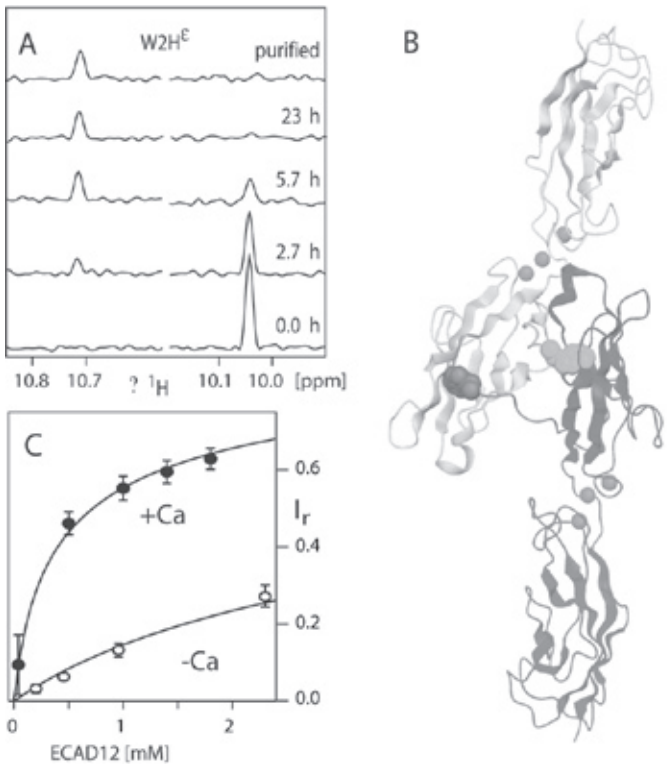

Fig. 1: Direct observation of the mechanism of proteolytic cadherin activation, which leads to the adhesion between cells. (A) NMR spectral changes in epithelial cadherin ECAD12 induced by prodomain proteolytic cleavage. (B) Our new crystal structure of ECAD12 showing the strand-exchange association of two cadherin molecules (1q1p). (C) NMR-derived binding isotherms for the strand-exchange association under Ca-bound and Ca-free conditions.

resistance to antibiotics. The TipA protein is one such multidrug resistance protein in *Streptomyces lividans*, which regulates its own expression by binding to its own promotor upon antibiotic binding. The protein consists of a multidrug binding domain and a DNA binding part. After solving the structure of the drug binding TipAS domain in the free form, we have now made significant progress in the characterization of the drug binding. The results show that upon drug binding, the protein undergoes a dramatic structural change from a half-folded form to a fully folded form. A search for fold analogues showed that the TipAS structure has weak similarity to the globin family of proteins. Thus, the classical globin fold is well adapted not only for accommodating its canonical cofactors, heme and other tetrapyrroles, but also for the recognition of a variety of antibiotics where ligand binding leads to transcriptional activation and drug resistance. At present, work is direct towards the TipA/DNA interaction and the antibiotic regulation mechanism (Kahmann et al., 2003; in collaboration with C. Thompson).

Atomic details of the folding mechanism of foldon – a natural folding nucleus and inducer of trimerization. We have carried out the NMR characterization of the folding of foldon – a trimerization domain from bacteriophage T4 fibritin. We have determined the solution structure of the trimer, followed its disintegration at low pH to a monomer, solved the monomer structure, and followed the decrease in monomer structural order during thermal denaturation in a quantitative and residue-specific manner by new residual dipolar coupling based methods. The results provide atomic details of the different forces responsible for monomer folding and trimer association (Krause et al., *in press*; in collaboration with T. Kiefhaber).

Brinker. Brinker is a recently discovered repressor of the *Drosophila* gradient morphogen Decapentaplegic (Dpp) signaling pathway, involved e.g. in *Drosophila* wing development. We have characterized the protein in its free and DNA-bound form. In the absence of DNA, Brinker is not folded. However, DNA-binding induces the transition to a well-structured, folded protein. We have determined the structure and dynamics of the Brinker protein within its complex with DNA (in collaboration with M. Affolter).

Secondary structure of the left-terminal domain of the potato spindle tuber viroid. The secondary structure of the 69-nucleotide RNA potato spindle tuber viroid left-terminal domain has been determined by NMR. This RNA domain is one of the largest, for which a secondary structure has been deduced by NMR (Dingley et al. 2003; in collaboration with A. Dingley, London and D. Riesner, Düsseldorf).

Other structural projects. Several proteins of the TOR signalling pathway are under investigation (in collaboration with M. Hall). The structure of the cold-shock domain of the duman Y-box protein YB-1 has been solved (Kloks et al., 2002; in collaboration with C. Hilbers, Nijmegen). The backbone of the 298 amino acid catalytic domain of protein tyrosine phosphatase 1B has been assigned (Meier et al., 2002; in collaboration with Novartis Pharma AG). The latter protein is an interesting drug target for the treatment of diabetes and obesity.

NMR technique development

Scalar couplings across hydrogen bonds. Hydrogen bonds (H-bonds) are essential for the stabilization of protein and nucleic acid secondary structure and often play a fundamental role in the regulation of enzymatic reactions. However, until recently, most of the evidence for hydrogen bonds in biological macromolecules was indirect either from the spatial proximity of donor and acceptor groups or from H-bond related parameters such as reduced hydrogen exchange rates. In 1998, we and others discovered that surprisingly strong NMR-observable scalar couplings exist across H-bonds. This effect can be used to “see” all three partners of a H-bond directly in an NMR experiment, i.e. the donor, the acceptor and the proton itself. Thus, the H-bond connectivity pattern of biomacromolecules (and therefore the secondary structure) can be

established directly via a COSY experiment. The effect itself is caused by the overlap of H-bond acceptor and hydrogen electronic orbitals and thus can be related to the H-bond geometry.

In the last two years, we have extended the original work to study (1) the effect of thermal denaturation on protein H-bonds, where the scalar couplings give quantitative information on the behavior of individual H-bonds during protein melting (Cordier and Grzesiek, 2002), (2) to quantitate the effect amide hydrogen/deuterium exchange on the strength of protein H-bonds, where we could show that the exchange by deuterium leads to a small decrease in donor acceptor overlap (Jaravine et al. *in press*), and (3) to C^{α}-$H^{\alpha}\cdots$O=C H-bonds in proteins. Such non-canonical C-H···O H-bonds are slowly being recognized as a rather common structural elements in chemistry and biology. Little is known about the contribution of C-H···O H-bonds to the stability of biomacromolecules. Recent ab initio calculations suggest association enthalpies D*H* of -3 kcal·mol^{-1} for C^{α}-$H^{\alpha}\cdots$O=C H-bonds in proteins, which are roughly half the size of N-H···O=C energies. We have now been able to observe for the first time H-bond scalar coupling ($^{h3}J_{CaC'}$) correlations across such C^{α}-$H^{\alpha}\cdots$O=C H-bonds in β-sheet regions of a small protein (Cordier et al., 2003). The results are in good agreement with predicted average values based on DFT/FPT calculations and should provide reliable experimental limits for theoretical models of C^{α}-$H^{\alpha}\cdots$O=C H-bonds in proteins.

Other technique developments. A new medium for weak alignment of biomacromolecules in the NMR tube and measurement of residual dipolar couplings has been developed based on mechanically strained, acrylamide/acrylate copolymers (Meier et al., 2002). We have devised new experimental schemes that measure long-range residual dipolar couplings (RDCs) between amide protons and ^{13}C nuclei as well as other amide protons in perdeuterated (but amide protonated) proteins (Meier et al., 2003). A very large number (up to 10 per residue in a small protein) of such long-range RDCs can be observed from individual amide protons. The accuracy of the coupling constants is very high and defines individual internuclear distances to within few hundredths of one Ångstroem.

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Protein X-ray crystallography

Our research relates to biological systems essential for the formation or remodelling of cell architecture and ultrastructure, where several of the proteins under study are of biomedical relevance.

Signal transduction: Multi-step activation of complex kinases

Kinase activity can be regulated by multiple mechanisms including phosphorylation, autoinhibition by pseudo-substrate segments, interaction with other domains, small molecules or intracellular effectors as well as oligomerization events. Eukaryotic multidomain kinases commonly exhibit intricate, multi-step activation mechanisms combining a number of the above. These kinases do not just confine their activity to on- and off-states, but frequently occur *in vivo* as multiple isoforms where control steps have been added, removed or modified according to tissue localization or developmental stage. To date, little is known about the interplay of regulatory events in these complex systems. To this respect, we are engaged in the study of Dystrophic Kinase, a multidomain kinase thought to be involved in modulation of plasma membrane depolarization, reorganization of the actin cytoskeleton during tissue development and, possibly, synaptic plasticity. This kinase is regulated by a combination of the mechanisms listed above. In particular, we aim at revealing the molecular basis of activation by oligomerization, which remains an uncharacterized control event. Recently, we have determined the crystal structure of the full coiled-coil domain thought to mediate the oligomerization of this kinase (*unpublished data*). Together with ultracentrifugation measurements. Structural data hint at the molecular events underlying self-assembly in this kinase family.

Molecular basis of muscle elasticity

The giant muscle protein titin constitutes the third filament system in skeletal and cardiac muscle of vertebrates. It is composed of 27'000 to 33'000 residues (according to isoforms), which fold into 300 domains approximately. Titin spans about 1 µm, connecting the Z-disc and the central M line of the sarcomere. Among other physiological roles, this colossal filament is responsible for the elastic properties of muscle as well as being involved in sarcomere assembly during development. Recently, we determined the crystal structure of the N-terminus of titin, which is formed by two Ig domains connected by a flexible linker (in collaboration with M. Wilmanns, unpublished data). This is the binding site of a small protein, named telethonin, which has been proposed to cap the titin filament and is relevant to muscle disease.

Titin is responsible for sarcomere elasticity by providing restoring force passively. This is achieved

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through the elastic properties of its central I-band region, which comprises (among other elements) tandem repeats of dozens of Ig domains. The stretch properties of linear domain arrays from titin have been extensively investigated by AFM, optical tweezers and molecular dynamics simulations, being the most widely used sample for the study of passive forces in biological materials. Due to the large size and inherent flexibility of titin, its structural characterization remains a challenge. Thus, the molecular principles underlying filament response to the directed pressures taking place in the sarcomere remain unknown. In collaboration with S.Labeit, A.Engel and D.Svergun we are investigating elastic elements of titin.

Lamina-Associated Proteins from the eukaryotic cell nucleus

Knowledge on the molecular principles governing the assembly of a functional cell nucleus is currently very limited. The nuclear lamina and its associated proteins appear to play a central role in the organization of genetic material and the nuclear dynamics during mitosis or general cellular developmental processes. However, the molecular mechanisms underlying these processes are mostly unknown. In collaboration with Roland Foisner and Yosef Gruenbaum, we are pursuing structural elucidation and biophysical characterization of several lamina-associated proteins with emphasis in revealing regions involved in nucleoskeletal assembly or in functional interactions.

Metabolic Evolution

An additional component of our research relates to evolutionary aspects of metabolic enzymes. The transfer of a ribosyl group between aromatic bases and phosphate groups is one of the most fundamental processes in the metabolism of nucleotides and amino acids. Ribosylation is performed by phospho-ribosyltransferases (PRT), while removal is catalysed by nucleoside phosphorylases (NP). Despite the similarity of the catalyses, these enzyme families are unrelated and have evolved independently and even at different times in the course of evolution. Recently, the crystal structure of anthranilate PRT (AnPRT) (Mayans et al, 2002) showed for the first time an evolutionary link between ribosylation and deribosylation processes. AnPRT is involved in ribosylating intermediates in the tryptophan biosynthetic pathway. Its structure is unrelated to that of any other PRT and, instead, share structural features with NPs from nucleotide salvage pathways. The tryptophan biosynthetic pathway is the major metabolic route under study after the glycolysis. Recent data on structure, biochemistry and evolution of its metabolic enzymes makes of this pathway a model for the elucidation of evolutionary inter-relations in metabolism.

In our latest work, we have determined the crystal structure of wild-type AnPRT in complex with its two natural substrates (anthranilate and PRPP) and metal counterions, showing the active site in four different states of occupancy (*unpublished data*). In addition, we have monitored domain motions taking place during catalysis using SAXS (in collaboration with D.Svergun), where wide-angle measurements have allowed the calculation of an atomic model of the compact conformation adopted by the enzyme during catalysis. Furthermore, the kinetic characterization of mutated AnPRT variants (performed by the collaborating group of R.Sterner), will allow deciphering possible aspects of a shared catalytic strategy between AnPTR and NPs. This work illustrates how metabolic enzymes have a high potential for interconversion of their activities and, thus, can be recruited by other cellular pathways according to organismic requirements.

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Crystallography of membrane proteins and soluble enzymes

Structural knowledge of membrane proteins at atomic resolution is still scarce. We are employing X-ray crystallography for the structure determination of mainly bacterial membrane proteins. In particular, we are interested in the structural basis of solute translocation across the outer and the inner membrane. At the same time, we are working on the structure-based inhibitor design of GABA aminotransferase and the crystal structure elucidation of various soluble proteins as in-house collaborations.

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Porins

Porins are integral membrane proteins from the outer membrane of Gram-negative bacteria. They allow the uptake of nutrients by passive diffusion through an intrinsic pore that extends along the axis of the transmembrane β-barrel structure. Over the years we have studied in detail the structural and electrophysiological determinants of ion flow through the non-specifc OmpF porin by a combination of mutagenesis, artificial lipid bilayer experiments, X-ray structure analysis, and Brownian dynamics simulations. It was found that anion/cation selectivity is crucially influenced by the charge constellation at the constriction, and that the presence of protein charges at the dipolar channel constriction increases channel conductance due to electrostatic steering.

In the last years we have focussed on the structural basis of the high efficiency of maltoporin to translocate maltodextrins across the outer membrane. We had shown earlier that the polypeptide chain is folded into an 18-stranded antiparallel β-barrel with three inwardly folded loops. Six contiguous aromatic residues line one side of the channel and form a hydrophobic path (‘greasy slide’) from the vestibule to the channel outlet. Maltoporin is the prototype of a facilitated diffusion channel. Based on sugar-maltoporin structures, we proposed that the greasy slide guides the long linear sugar substrates through the tight channel. At the start of translocation, the hydrophobic patches of the glucosyl moieties would adhere to the end of the greasy slide that is extending into the channel vestibule and which is easily accessible. This notion has recently been corroborated by results on side-directed mutants. Changing the ‘greasy slide’ residues to alanine reduced considerably the translocation efficiency *in vivo*. This is also true for the residues at the two ends of the slide that have been shown not to be involved in sugar binding at equilibrium.

After binding to the high-affinity site in the middle of the channel (as observed in the respective complex crystal structure), a maltodextrin molecule would have to perform a “register shift” for further translocation. The trajectory of this shift and the corresponding

energetics have been calculated by the “conjugated peak refinement” method (in collaboration with S. Fischer and M. Karplus, Strasbourg). It was found that, during register shift, the maltoporin channel acts as a rigid complementary matrix. Due to the smooth and uninterrupted interaction with the greasy slide and the presence of a multitude of H-bonding partners no large energy barriers have to be overcome.

Recently, several new porins displaying properties different to those of the classical and structurally characterized porin family have been reported. KdgM, a major outer membrane protein of the plant pathogen *Erwinia chrysanthemi*, is one of them. It is thought to act as an oligogalacturonate specific porin used by the bacteria during the course of plant infection. KdgM homologues have been found in other bacteria including major human pathogens, such as *Yersinia pestis* and *Salmonella typhimurium*, and may also play an important role in their virulence. In collaboration with G. Condemine (Villeurbanne, France), KdgM has been overexpressed and purified. Reproducible protein crystals have been obtained. The X-ray structure of this porin together with further functional characterization should shed light onto the structure-function relationships of this new class of porins.

Active sugar transporters of the bacterial phosphoryl-transfer-system

In the PEP-dependent carbohydrate: phosphotransferase system (PTS), the phosphoryl group is transferred indirectly from phosphoenolpyruvate to the carbohydrate via several proteins that get transiently phosphorylated. In addition to the transport function, PTS components are involved in the regulation of carbohydrate metabolism and chemotaxis. The PTS is ubiquitous in bacteria, but not found in eukaryotes. It, therefore, appears an attractive target for novel antibiotics. The PTS consists of general proteins (enzyme I and HPr) and sugar-specific components (enzymes II). Latter are comprised of several structural domains or subunits: IIA and IIB are hydrophilic cytoplasmic components and catalyze the phosphorelay, whereas IIC (IID) is integrated in the plasma membrane and catalyzes translocation.

We are working on the mannose family of the PTS and have solved the structures of the IIA and IIB domains, both exhibiting a mixed α/β fold with the phosphorylatable histidine shown to be activated by an adjacent aspartyl residue. The transition state for phosphoryl transfer between IIA and IIB has been modeled based on the individual X-ray structures. The model of the complex shows surface and charge complementarity at the interface. The penta-coordinated phosphoryl group appears to be stabilized by the partial dipole charges of two helices, one from each protein. We are intensively trying to get crystals of the IIC/IID transmembrane part of the complex following various strategies.

Aquaporin from the mammalian eye lens

Aquaporins are found in various eukaryotic membranes and in the bacterial plasma membrane. They exhibit exquisite specificity for water or glycerol. We are working on the structure determination of AQP0 (MIP26) from mammalian eye lenses. Our collaborating partners are P. Agre, Baltimore, and A. Engel, Biozentrum, who have overexpressed the protein in yeast and developed a purification scheme. We have obtained crystals that diffract to 7 Å resolution. Despite the poor resolution, we were able to determine the crystal packing by molecular replacement. Octamers that are formed by tight head-to-head association of tetramers are the building blocks of the lattice. Possibly, this interaction reflects the *in vivo* situation, where AQP0 is known to be essential for mediating the tight stacking of lens fiber cell membranes.

GABA aminotransferase

GABA aminotransferase, a vitamin B6 (PLP) dependent enzyme, is essential for homeostasis of the neurotransmitter GABA in the mammalian brain. Due to the recent improvement in the quality of the crystal diffraction data, the presence of an iron-sulfur [2Fe-2S] cluster at the center of the dimeric molecule could be, quite unexpectedly, revealed.

Fig. 1: Structure of the iron-sulfur cluster at the center of GABA aminotransferase

This raises the interesting possibility that the enzyme might be regulated by the redox state of the cell. In collaboration with R.B. Silverman (Eaveston, USA), we are studying the binding mode of novel mechanism-based inhibitors of pharmaceutical importance with the ultimate goal to improve their specificity and efficacy. We have determined the structure of liver GABA aminotransferase in complex with the commercial anti-epileptic drug vigabatrin. The inhibitor forms a covalent ternary adduct with the active site Lys329 and the PLP cofactor. Further studies are in progress.

Two-component response regulator PleD

Typically, two-component response regulators are comprised of an input and an output domain. Cognate kinases can phosphorylate the former which results in activation of a transcription factor output domain. In contrast, PleD, which is part of a signaling pathway regulating the developmental cycle of *Caulobacter crescentus*, is composed of two (CheY like) input domains and an output domain that bears no obvious structural homology to any existing fold and contains a highly conserved GG(D/E)EF sequence motif. Recently, it has been shown that the output domain, which is widely distributed amongst bacteria, acts as a diguanylate cyclase (see report U. Jenal). In collaboration with U. Jenal, Biozentrum, we have overexpressed both the output domain and the full-length protein, have identified stable proteolytic subfragments, and are making progress in crystallization. We are striving to reveal the mechanisms of activation and catalysis of this prototype of a two-component response regulator.

Plant allergens

The IgE mediated hypersensitivity reaction to birch pollen is caused by the major birch pollen allergen, Bet v 1, which is biochemically and immunologically well characterized. Yet, the biological function of Bet v 1 and the related homologous pathogenesis-related plant proteins (PR-10), which constitute a group of defense proteins, is unknown. We have determined the 3D structure of the stable, high affinity stable complex between hypoallergenic isoform Bet v 1l and two molecules of deoxycholate, a steroid molecule very similar to plant brassinosteroids. A large hydrophobic Y-shaped cavity inside the protein is partly occupied by two deoxycholate molecules, suggesting the role of Bet v 1 in the transfer of apolar ligands. We have also shown, by mass spectroscopy, that specific non-covalent interaction exist between Bet v 1l and two plant brassinosteroids. Together, our findings suggest the role of Bet v 1l as a general, rather than specific, steroid carrier.

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Nano-biology and protein *de novo* design

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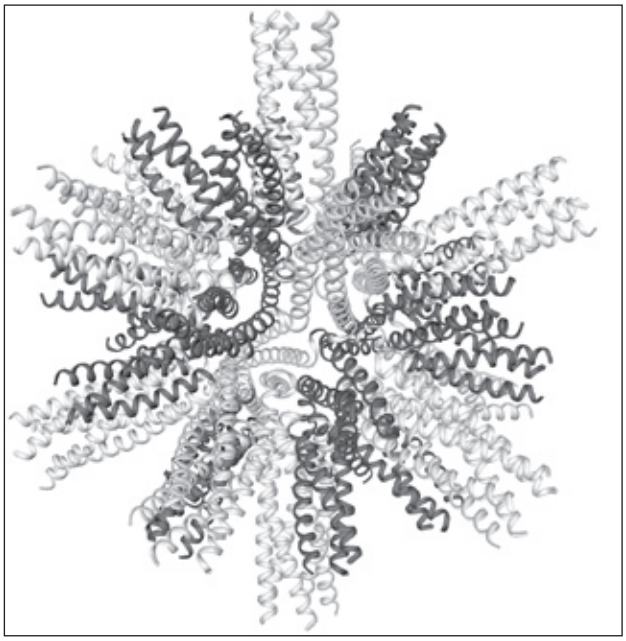
Nanoparticles

While peptides have so far been used as building blocks of nanotubes e.g. for the design of antimicrobial agents or the design of conducting nanowires, we are designing nanoparticles based on peptides as building blocks. This is achieved by rational protein de novo design. These nanoparticles are composed of protein oligomerization domains with different oligomerization states linked by a short linker peptide. The nanoparticles are characterized by regular icosahedral symmetry like small virus particles (Figure 1).

We are now in the process of developing a prototype of self-assembling functionalized peptidic nanoparticles, which can be used as a drug targeting and delivery system for the visualization and treatment of cancer. These nanoparticles will be modified to carry a drug entity (radionuclide) as well as a pathfinder molecule on each of the 60 peptide chains of the icosahedral nanoparticle.

Furthermore, such nanoparticles with regular polyhedral symmetry represent an ideal repetitive antigen display system. Surface proteins of pathogens or fragments of such proteins can easily be engineered into the peptide sequence of the nanoparticle. Notably, many surface proteins of pathogens contain coiled-coil sequences. For example, by simply extending the trimeric coiled-coil of the nanoparticle by the coiled-coil sequence of the HIV surface protein gp41, a candidate HIV vaccine can be designed. Whereas in the past, different kinds of adjuvants were tested to improve the immunogenicity of an antigen or a specific epitope, such a repetitive antigen display can strongly augment the immunogenicity of a certain epitope, thus avoiding the need for sometimes highly toxic adjuvants.

Fig 1: Computer model of a peptidic nanoparticle



De novo design of α-helical proteins

The parallel two-stranded α-helical coiled coil is the most frequently encountered subunit oligomerization motif in proteins. We have de novo designed several highly charged two-heptad repeat long peptides which are stabilized by a complex network of different possible inter- and intra-helical salt bridge arrangements in addition to the well-known hydrophobic interactions occurring along the dimer interface. We are accessing the biophysical properties of these peptides by CD-spectroscopy, analytical ultracentrifugation and X-ray structure determination. The X-ray structure of these peptides confirmed our predicted intra- and inter-helical salt bridge network. Such de novo designed peptides can be used as very short α-helical coiled coils for applications such as two-stage drug targeting and delivery systems, coiled coils as templates for combinatorial helical libraries for drug discovery, and as synthetic carrier molecules.

Structural investigations of IF proteins

Another related goal is to resolve the atomic structure of the intermediate filaments (IFs). A ~46 nm long, parallel α-helical coiled-coil dimer is the elementary building block of all IFs. We are designing a series of overlapping fragments of the 55-kDa type III IF protein vimentin for subsequent crystallization and X-ray structure determination. Knowledge of the atomic structure of an IF protein dimer is a prerequisite for a better understanding of how the many identified point mutations interfere with IF assembly, structure and dynamics.

So far we have designed, cloned, expressed and purified a series of 'miniconstructs' of the intermediate filament proteins vimentin, lamin and desmin. Biophysical and structural investigation of these constructs have revealed first details of the IF architecture. Studies on additional fragments are currently underway, and are expected to provide further information on the structure of IF proteins at atomic level. (See also U. Aebi)

Structural investigations of vitamin B6 enzymes

DOPA decarboxylase (DDC): Parkinson's disease is a chronic, progressive, neurological disorder characterized by tremor, bradykinesia, rigidity, and postural instability. The crystal structure of DDC that was recently determined in our lab will now be used to assist in the design of more potent DDC inhibitors as candidate drugs for the treatment of Parkinson's disease.

Cystathionine β-synthase (CBS): In collaboration with the group of J. Kraus we have recently determined the X-ray crystal structure of CBS. CBS is the first enzyme of the transsulfuration pathway in which the toxic homocysteine is converted to cysteine. Deficiency of CBS activity is the most common cause of homocystinuria, an inherited metabolic disease characterized by dislocated eye lenses, skeletal problems, vascular disease and mental retardation. In our future work we want to assess the structures of the catalytic intermediates and we then want to address how CBS is it regulated in its catalytic activity and whether this regulation could possibly be modulated.

O-acetylserine Sulfhydrylase (OASS): In collaboration with the group of P. Cook, we are investigating the structure-activity-relationship of OASS in more detail. Covalent binding of L-methionine as an external aldimine to OASS induces a large conformational change in the protein. When the inhibitor chloride binds to OASS this results in a new "inhibited" conformation that differs from the "open" native or "closed" external aldimine conformations.

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There is more to actin than assembling filaments in the cytoplasm

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Our laboratory is interested in actin conformations that are not present in conventional actin filaments at steady state and might be specific to distinct cellular compartments. In particular, our work is aimed at understanding the role of the 'lower dimer' (LD), the first actin dimer that is transiently formed at the onset of polymerization, in the patterning of supramolecular actin networks in cells. Observations from our laboratory indicate that the LD may indeed be a key element in the formation of branched actin structures. An uncommon conformation may well be the reason why the structure of actin in the nucleus has so far remained an enigma.

Tools for analyzing actin conformations

Because actin is a highly conserved molecule the production of anti-actin antibodies is in itself a challenge. This explains why the mushroom toxin phalloidin is predominantly used to visualize the actin cytoskeleton in cells. However, phalloidin specifically binds to filamentous actin, and non-polymerized actin, which in some cells constitutes the greater fraction of the total actin, goes undetected. The LD, in which the subunits are in a G-like conformation, is also unlikely to represent a binding site for phalloidin. Thus, carrying out structural and functional studies on the LD requires antibodies, both mono- and polyclonal that recognize distinct actin conformations. We are pursuing several strategies to coax different hosts into producing conformation-specific antibodies. One of them involves challenging the host immune system with an LD-related antigen such as cross-linked purified LD, 2D crystalline arrays of actin in which LD contacts are represented, or peptides that were designed based on an LD-like crystal structure. To increase peptide antigenicity, we will take advantage of the repetitive antigen display offered by designed nanoparticles.

From the monoclonal antibodies established so far, there is one anti-actin antibody termed 1C7, which only recognizes non-filamentous actin in cells and not actin stress fibers. This first example of a conformation-specific antibody demonstrates that we can anticipate producing additional antibodies that will recognize actin in different conformational states.

What form does actin assume in the nucleus ?

Although there are many reports of the presence of actin in the nucleus, in general they have been discounted as artifactual because of the apparent absence of phalloidin staining indicative of filaments, as well as the failure to document F-actin filaments in the nucleus by electron microscopy. However, over the past few years a role for nuclear actin in chromatin remodeling, RNA processing, and in nucleocytoplasmic transport has become evident. Still, virtually nothing is known about the form of

nuclear actin. With new functions for nuclear actin emerging, the quest for its oligomeric/polymeric structure has become a challenging task for our laboratory.

We have observed that the actin species detected by 1C7 moves to the nucleus upon treatment of the cells with Latrunculin A. Its localization in the nucleus is also clearly distinct from the actin detected by phalloidin. Conformation-specific antibodies will be valuable tools to characterize actin in the nucleus.

To increase the levels of actin in the nucleus, we have established yeast strains that express a proteinA-tagged actin marked for import into the nucleus by a conventional nuclear localization signal (NLS). We observed that NLS-ProA-actin assembled into rod-like structures in the nucleus. Electron microscopy revealed that the rodlets consisted of laterally aligned filamentous structures. Deletion of the nuclear export signals in actin had no further effect on the amount of nuclear actin. Using a temperature-sensitive actin mutant we observed that NES mediated protein export is dependent on nuclear actin.

S100A1 inhibits apoptosis via activation of the ERK1/2 signaling pathway in neonatal cardiomyocytes

S100 proteins are a family of soluble, EF-hand Ca^{2+}-binding proteins that exhibit cell- and tissue-specific expression. One member, S100A1, regulates cardiac as well as skeletal muscle Ca^{2+}-homeostasis and contractility and hence, is expressed at high levels in the myocardium. We have examined the effects of extracellularly added recombinant S100A1 on neonatal ventricular cardiomyocytes (NVCM). Immunolabeling of NVCM incubated with S100A1 showed that rhodamine-conjugated S100A1 is internalized into the endosomal compartment of NVCM via a Ca^{2+}-dependent clathrin-mediated process. S100A1-uptake protects neonatal ventricular cardiomyocytes from 2-deoxyglucose- and oxidative stress-induced apoptosis *in vitro*. S100A1-mediated anti-apoptotic effects involve the activation of the extracellular signal-regulated kinase 1/2 pro-survival pathway, including activation of phospholipase C, protein kinase C, mitogen-activated protein kinase kinase 1. Our studies provide evidence for S100A1 protein acting as a novel cardioprotective factor *in vitro*.

We are also using NVCM to examine the localization of endogenous adenoviral-mediated S100A1 overexpression and its effects on cell survival under stress. To learn more about this and other cellular functions of S100A1, we are looking for target proteins that interact with S100A1.

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DIVISION OF MOLECULAR MICROBIOLOGY

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The major research interests of the division are bacterial pathogenesis, bacterial defense mechanisms, growth and development of bacteria and fungi. The Division consists of groups headed by Tom Bickle, Guy R. Cornelis, Christoph Dehio, Urs Jenal and Charles Thompson. It also includes the Institute of Applied Microbiology, led by Peter Philippsen.

The Cornelis and Dehio groups focus on the cellular and molecular aspects of pathogenesis of bacteria endowed with specific mechanisms for intracellular toxin delivery, which are called type-III secretion and type-IV secretion, respectively. The Bickle group studies the biochemistry, genetics, and physiology of restriction enzymes. The Thompson group studies antibiotic resistance in *Mycobacterium* and differentiation in *Streptomyces*. The Jenal group studies temporal and spatial control during the bacterial cell cycle and development. The Philippsen group uses genomics to investigate growth of a filamentous fungus.

The research activities of each group are described in more detail below.

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Protein-nucleic acid interactions

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Complex restriction enzymes: NTP-driven molecular motors

Survival is assuredly the prime directive for all living organisms either as individuals or as a species. One of the main challenges encountered by bacterial populations is the danger of bacteriophage attacks, since infection of a single bacterium may rapidly propagate, decimating the entire population. In order to protect themselves against this acute threat, bacteria have developed an array of defense mechanisms, which range from preventing the infection itself *via* interference with bacteriophage adsorption to the cell surface and prevention of phage DNA injection, to degradation of the injected phage DNA. This last defense mechanism is catalysed by the bacterial restriction-modification (R-M) systems, and in particular, by nucleoside 5'-triphosphate (NTP)-dependent restriction enzymes, e.g. type I and type III R-M systems or the modification-dependent endonucleases.

Type I and type III restriction systems have dual properties. They may either act as methylases and protect the host's own DNA against restriction by methylating specific residues, or they catalyse ATP-dependent endonuclease activity so that invading foreign DNA lacking the host-specific methylation is degraded. These defense mechanism systems are further complemented by the presence of methylation-dependent, GTP-dependent endonucleases, which restricts specifically methylated DNA. Although all three types of endonucleases are structurally very different, they share a common functional mechanism. They recognise and bind to specific DNA sequences but do not cleave DNA within those target sites. They belong to the general class of DNA motor proteins, which use the free energy associated with nucleoside 5'-triphosphate hydrolysis to translocate DNA so that the subsequent DNA cleavage event occurs at a distance from the endonuclease recognition site. Moreover, DNA cleavage appears to be a random process triggered upon stalling of the DNA translocation process and requiring dimerisation of the bound endonucleases for a concerted break of both DNA strands.

Stalling normally occurs when an enzyme bumps into another enzyme translocating from another recognition site, which explains why linear DNA molecules are only cut if they contain at least two recognition sites (see Figure next page).

Characterization and Mutational Analysis of the RecQ core of the Bloom syndrome Protein

The complex restriction enzymes are all DNA helicases and the type I enzymes may have a role in recombination. This has led us to become interested in other DNA helicases with possible

roles in recombination. Bloom syndrome protein (BLM) belongs to a group of DNA helicases showing extensive homology to the *Escherichia coli* DNA helicase RecQ, a suppressor of illegitimate recombination (IR). In addition to the RecQ core, which contains a DEAH-helicase, a RecQ Ct and a HRDC domain, BLM and other eukaryotic members of the RecQ family have extensive N- and C-terminal regions that mediate interactions with other proteins. After over-production in *E. coli,* we have purified the RecQ part of BLM, encompassing the amino acid residues 642-1290. The $BLM^{642-1290}$ fragment exhibited a DNA-stimulated ATPase activity as well as a helicase activity on both partial DNA duplex and 4-way junction substrates. Gel filtration experiments indicated that $BLM^{642-1290}$ exists as a monomer both

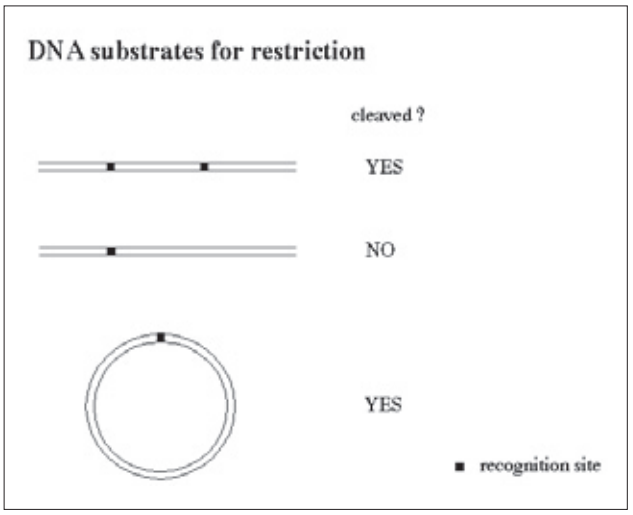

Fig. 1: All three kinds of translocating restriction enzymes normally require at least two recognition sites on a linear DNA molecule. A single site suffices on a circular molecule.

in solution and in its ssDNA-bound form, even in the presence of Mg^{2+} and ATPgS. Rates of ATP hydrolysis and DNA unwinding by $BLM^{642-1290}$ showed a hyperbolic dependence on ATP concentration, excluding a co-operative interaction between ATP binding sites. Using a Spi^- assay, we have found that expression of the $BLM^{642-1290}$ fragment in an *E. coli* strain carrying a *rec*Q mutation partially reduced the elevated illegitimate recombination observed in this strain. A deletion of 182 C-terminal amino acids of $BLM^{642-1290}$ including the HRDC domain resulted in helicase and single-stranded DNA (ssDNA) binding defects whereas kinetic parameters for ATP hydrolysis of this mutant were close to the $BLM^{642-1290}$ values. Mutations at several conserved residues within the RecQ Ct domain of BLM severely reduced ATPase and helicase activities as well as ssDNA binding of the enzyme. Together, these data define a minimal helicase domain of BLM and demonstrate its ability to act as a suppressor of IR.

Specificity of DNA cleavage by the EcoP1I type III restriction enzyme requires S-adenosylmethionine

DNA cleavage by the type III restriction endonuclease *Eco*P1I was analysed on circular and catenane DNA in a variety of buffers with different cationic and anionic salts. In the presence of the cofactor *S*-adenosyl methionine (AdoMet), and irrespective of buffer, only substrates with two indirectly repeated *Eco*P1I sites were susceptible to cleavage. Maximal activity was achieved at a Res_2Mod_2 to site ratio of ~1:1 yet resulted in cleavage at only one of the two sites. In contrast, the outcome of reactions in the absence of AdoMet was dependent upon the identity of the monovalent buffer components, in particular the identity of the cation. With Na^+ ions, cleavage was only observed on substrates with two indirectly repeated sites at elevated enzyme to site ratios (>15:1). However, with K^+ ions every substrate tested was susceptible to cleavage above an enzyme to site ratio of ~3:1, including a DNA with two directly repeated sites and even a DNA with a single site. Above an enzyme:site ratio of 2:1, substrates with two indirectly-repeated sites were cleaved at *both* cognate sites. The rates of cleavage suggested two separate events: a fast primary reaction for the first cleavage of a pair of indirectly repeated sites; and, an order-of-magnitude slower secondary reaction for the second cleavage of the pair or for the first cleavage of all other site combinations. EcoP1I enzymes mutated in either the ATPase or nuclease motifs did not support secondary cleavage reactions. Thus, AdoMet appears to play a dual role in type III endonuclease reactions: Firstly, as an allosteric activator, promoting DNA association; and secondly, as a "specificity factor", ensuring that cleavage only occurs when two endonucleases bind two recognition sites in a designated orientation. However, given the right conditions, AdoMet is not strictly required for DNA cleavage by type III enzymes.

Chemical restriction: Strand cleavage by ammonia treatment at 8-oxoguanine yields biologically active DNA

Numerous site selective DNA strand cleavage reactions at modified nucleotides are known, but none of them is leading to the formation of biologically active DNA. Most modifications are not tolerated by polymerases due to increased steric or hydrophilic interactions. We now report a significant optimization of the strand break reaction at 8-oxoguanine in ammonia by preceding oxygen saturation. This reaction was applied for the generation of ligatable single stranded overhangs at PCR amplified DNA. The elongated primers were 8-oxoguanine modified and contained the appropriate sequences to design the single stranded overhangs. As an example, the lac Z' gene was amplified, restricted by ammonia

treatment, ligated into a plasmid vector, transformed in *E. coli* cells and screened for blue colonies. With this new method efficiencies comparable to the standard cloning procedure using restriction enzymes were obtained. Additionally, the transformed recombinant plasmids were isolated and sequenced to investigate the action of the polymerases opposite the modification during PCR. The ratios of the 8-oxoguanine:adenine mispair formation by two thermostable polymerases were determined.

A new class of DNA damaging agents

The acridicinium salts are a new class of DNA intercalating agents which can cleave DNA photolytically in both the presence and the absence of oxygen. We have looked at the mechanism of cleavage under these two conditions and found that they were remarkably different. It was found that the presence of oxygen led to cleavage exclusively at guanine while cleavage was non-specific in its absence. This and other results led to the conclusion that acridicinium salts do not cut DNA directly. Rather, they generate small reactive species, singlet oxygen in the presence of oxygen and hydroxyl radicals in its absence. These are the species that react with the DNA by mechanisms that are well understood. The production of hydroxyl radicals with such low energy irradiation (360 nm) is remarkable. The acridicinium salts are thus potentially very interesting molecules for laboratory use, being capable of a number of reactions (also with proteins) under mild conditions.

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Molecular mechanisms of microbial pathogenesis: the *Yersinia* paradigm

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This report includes the work done by the group at the ICP, Brussels, during the 2002−2003 period.

Introduction

We are studying the Yop virulon, a complex weapon that allows bacteria from the genus *Yersinia* (*Y. enterocolitica*, *Y. pseudotuberculosis* and *Y. pestis*) to defeat the immune system of their host. This system allows extracellular bacteria adhering at the surface of eukaryotic cells to inject bacterial effector proteins called Yops into the cytosol of these cells, which disarms them or sabotages their communications. This system, encoded by a 70-kb plasmid, is an archetype for the so-called type III secretion (T3S) systems, encountered in more than twenty animal, plant and insect pathogens. In *Yersinia*, the effectors are YopE, YopH, YopO, YopM and YopT (Fig. 1). YopH dephosphorylates proteins from the focal adhesion, YopT modifies RhoA, YopE activates the GTPase activity of Rac and YopO (YpkA) is a kinase whose target is not known yet. YopP prevents the release of the proinflammatory cytokine TNFα by macrophages by interfering with the mitogen-activated proteins (MAP) kinases (MAPK) and NF-κB pathways. It also induces apoptosis of macrophages. Little is known about YopM, except that, unlike the other Yops, it is targeted to the nucleus of the cell. Injection of the Yop effectors requires the Ysc injectisome and the translocators. The injectisome is a secretion apparatus that spans the two bacterial membranes and ends up with a needle. The translocators are Yop proteins that form a pore in the animal target cell membrane.

During the years 2002−2003, we pursued our study of the injectisome and pore-formers as well as of the action of the effectors in eukaryotic target cells. We also pursued our study of a second T3S system present in *Y. enterocolitica.* A major outcome was the discovery that the length of the needle of the injectisome is determined by a protein acting as a molecular ruler.

The length of the needle of the injectisome is controlled by a molecular ruler

We measured the length of the injectisome needle by electron microscopy and found a length of 58 +/- 10 nm. *Y. enterocolitica* with a large deletion (codons 97 to 465 out of 515) in gene *yscP* ($yscP_{\Delta 97\text{-}465}$) produced needles with an indefinite length. When the mutation was complemented with the $yscP^{+}$ allele, control of the length was restored indicating that YscP played an essential role in length control. Protein YscP from *Y. enterocolitica* E40 ($YscP_{entero}$) carries a duplication of 60 central residues. YscP from *Y. pestis* KIM5 ($YscP_{pestisKIM5}$) is 90 % identical in sequence to $YscP_{entero}$ but it is shorter (455 residues) due to the lack of such repetition. To explore if the two proteins lead to needles of the same length, we complemented the *Y. enterocolitica* $yscP_{\Delta 97\text{-}465}$ mutation with the $yscP_{pestisKIM5}{}^{+}$ gene. The shorter *Y.*

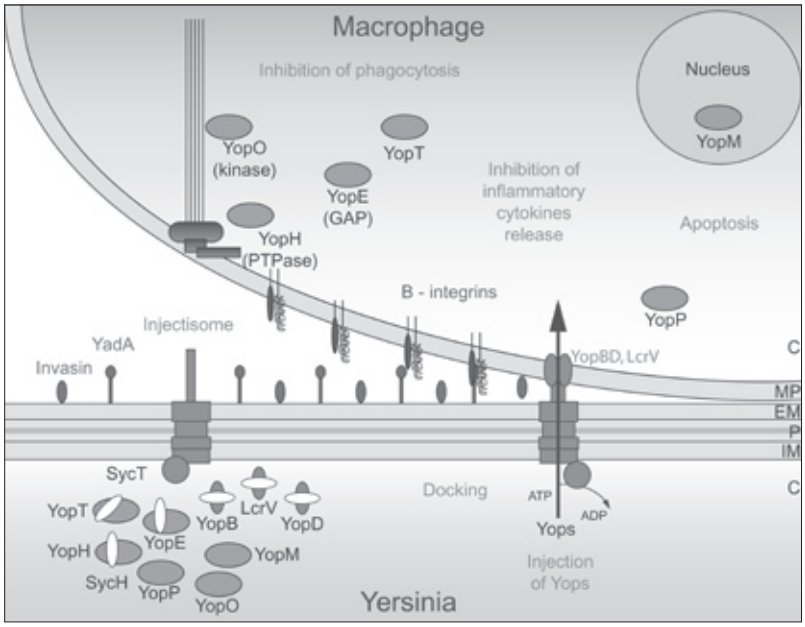

Fig. 1: The basic model.
When Yersinia *are placed at 37 °C in a rich environment, the Ysc injectisome is installed and a stock of Yop proteins is synthesized. During their intrabacterial stage, Yops are capped with their specific Syc chaperone. When the bacterium enters into contact with a target cell, it docks tightly by the interaction between its adhesins Inv and YadA and cellular β-integrins. Then, the secretion channel opens and the Yops are exported. YopB, YopD and LcrV form a pore in the target cell plasma membrane and the Yop effectors are translocated across this membrane into the cell's cytosol.*

pestis gene restored length control but programmed shorter needles (41 +/- 8 nm). To investigate if the needle length reduction was due to the shortening of YscP and not to subtle residue changes, we complemented the $yscP_{\Delta 97\text{-}465}$ mutation with the allele $yscP_{entero\Delta 222\text{-}306}{}^{+}$ encoding $YscP_{entero}$ without its repeat. The truncated $YscP_{entero\Delta 222\text{-}306}$ programmed short needles (42.5 +/- 8 nm), suggesting that the needle length indeed correlated with the size of YscP. We then engineered a set of deletions and insertions within the cloned $yscP_{entero}$ gene and used them to complement the $yscP_{\Delta 97\text{-}465}$ mutation. A strict linear relationship existed between needle length and the number of amino acid in YscP, with 1.9 Å/YscP residue, indicating that YscP acts as a molecular ruler. If this is the case, one might expect YscP to be associated with the needle, at least during the needle elongation stage. To test this, we purified needles from *Y. enterocolitica* incubated in conditions that are either non-permissive or permissive for Yop secretion. As expected, some YscP was found in the needle fraction from non-secreting bacteria but not from secreting bacteria. The model that we proposed is illustrated in Fig. 2.

LcrV and YopN play a role in the process of Yops translocation into animal cells

We tested the role of LcrV, YscF and YopN in the formation of the translocation pore in macrophages by monitoring the release of the low-molecular-weight fluorescent dye BCECF. BCECF is released through the translocation pore provided no Yop effector is trafficking through the channel. A *lcrV*, *yopQ* double mutant was deficient in pore formation while able to produce YopB and YopD. Pore formation was restored by the introduction of $lcrV^{+}$ but not $yopQ^{+}$ confirming that LcrV itself is directly required for pore formation. Bacteria secreting only YopB, YopD and LcrV could form pores, showing that YopB, YopD and LcrV are sufficient for pore formation provided they are secreted by the same bacterium. Bacteria producing normal Ysc injectisomes, including the YscF needle but no translocators did not form pores, indicating that the needle is not sufficient by itself for pore formation, as was suggested. *yopN* mutant bacteria formed needles and released BCECF even if they secreted the effectors. This observation suggests that many translocation pores are not filled in the absence of YopN and thus that YopN might form a link between the needle and the pore, guiding the effectors.

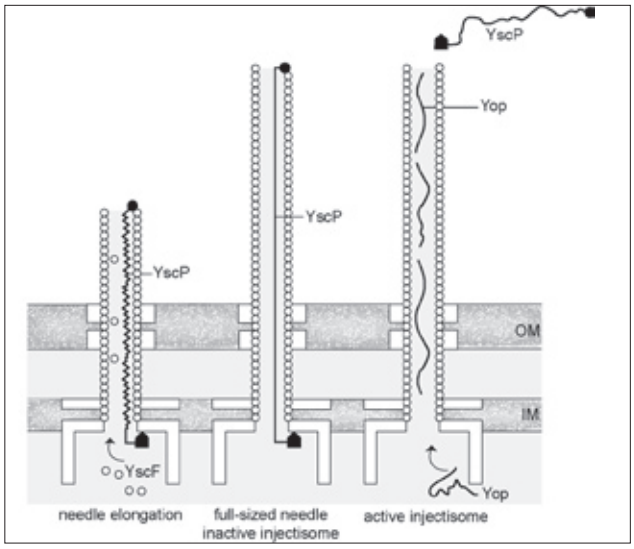

Fig. 2: Proposed model for the control of the YscF-needle length by the YscP ruler.

Yops affect the transcriptome of macrophages infected by *Y. enterocolitica*

To investigate the role of Yops, on expression of the host cell genes, we characterized the transcriptome alterations in infected mouse macrophages. PU5-1.8 macrophages were infected either with an avirulent (pYV^-), a wild type (pYV^+) or two knockout (*yopP*$^-$ and *yopM*$^-$) mutants of *Y. enterocolitica*. Among the 12000 genes represented on the array, about 4800 were expressed by the macrophages. Expression of 857 genes was altered following *Y. enterocolitica* infection. 339 of those genes were specifically regulated by the action of the Yop virulon. Further analysis of those 339 genes allowed to identify specific targets of YopP, YopM or the other pYV-encoded factors. According to these results, the main action of the Yop virulon is to counteract the host cell pro-inflammatory response to the infection. YopP is a key-effector of this inhibition, but another pYV-encoded factor appeared to be also involved in this down-regulation. Besides, YopM was found to regulate genes involved in cell cycle and cell growth.

YopH inhibits the PI 3-kinase pathway in macrophages

Phosphatidylinositol 3-kinase (PI 3-kinase) and its target protein kinase B (Akt) are involved in various cell biology processes including internalisation, chemotaxis and proliferation. We analysed the activation of Akt in J774 macrophages infected with virulent (pYV^+) or avirulent (pYV^-) *Y. enterocolitica*. During the early stage of infection with both pYV^+ and pYV^- bacteria, Akt and its targets, glycogen synthase kinase 3 (GSK-3) and forkhead transcription factor (FKHRL1) became phosphorylated. This phosphorylation induction was inhibited by wortmannin and thus dependent on PI 3-kinase. When infection was carried out with pYV^+ bacteria but not with pYV^- bacteria, Akt and its targets became dephosphorylated at later time points. Using single knockout mutants in bacterial effector genes, we have determined that the tyrosine phosphatase YopH was responsible for the inactivation of the PI 3-kinase cascade. Furthermore, in macrophages this inactivation was linked to the down-regulation of mRNA coding for monocyte chemoattractant protein 1 (MCP-1), suggesting that YopH inhibits macrophages recruitment to lymph nodes. We then analysed the effects of *Y. enterocolitica* infection on proliferation of T lymphocytes. Consistently with the observation that YopH inactivated the Akt pathway, YopH inhibited PI 3-kinase dependent secretion of interleukin 2 and proliferation.

YopE and YopT inhibit the release of interleukin-1

We observed that inhibition of Rho GTPases by the effectors YopE and YopT prevents the maturation and release of IL-1. Accordingly, caspase-1 activation was prevented by dominant negative Rac1/Cdc42, as well as by inhibition of Rho GTPases with *C. botulinum* Toxin B or the prenylation inhibitor GGTI-2147. Constitutive active Rac1/Cdc42 stimulated caspase-1 protease activity. These results not only shed light on a new function of different Yop effectors, but also implicate a novel role for Rho GTPases in the onset of the inflammatory and immune response.

The C-terminus of YopM contains a nuclear localization signal

Using a yeast approach, we observed that the three first leucine-rich repeats (LRR1-3) and the 32 C-terminal residues of YopM ($YopM_{C\text{-}ter}$) act as nuclear localization signals (NLSs) in yeast. Furthermore, by transfection of HEK293T cells, we observed that $YopM_{C\text{-}ter}$ could direct large recombinant EGFP-LexA-AD proteins into the nucleus of mammalian cells confirming that it contains a NLS. Critical residues for nuclear targeting were identified by site-directed mutagenesis in $YopM_{C\text{-}ter}$.

Identification of substrates for the second T3S of *Y. enterocolitica*

All pathogenic *Y. enterocolitica* carry the pYV plasmid encoding the Ysc-Yop T3S described here above. In addition, biovar 1B *Y. enterocolitica* strains possess a second, chromosomally encoded, T3S system called Ysa. We identified four specific Ysa substrates (YspA, YspB, YspC and YspD). Ysa also appeared to secrete the pYV-encoded YopE, indicating that YopE is a potential effector of both *Y. enterocolitica* T3S systems.

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Molecular and cellular basis of type IV secretion in bacterial pathogenesis: The *Bartonella* model

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Patent:
Schulein, R., Guye, P., Rhomberg, T., Schmid, M., Dehio, M. & Dehio, C. (2003). Polypeptides translocated into cells by the VirB/VirD4 type IV secretion system and uses thereof. *Patent application no. EP 03004826.8-1222* filed on March 6th, 2003.

The aim of our studies is to understand the molecular and cellular basis of type IV secretion (T4S) as a widespread virulence mechanism in human-pathogenic bacteria. The T4S systems of pathogenic bacteria have evolved from bacterial conjugation systems and are known to mediate the translocation of bacterial effector molecules during interaction with target cells of the infected host. The prototype of these versatile transporters is the VirB-D4 system of the plant pathogen *Agrobacterium tumefaciens*. A supramolecular assembly of 12 proteins is considered to span both Gram-negative bacterial membranes and possibly the host cell membrane, allowing translocation of a nucleo-protein complex from the bacterial cytoplasm directly into the host cell cytoplasm. Human pathogens have adapted T4S systems for transport of either multi-component protein toxins to the extracellular milieu (i.e. pertussis toxin by the agent of whooping cough, *Bordetella pertussis*) or for the delivery of individual effector proteins directly into the host cell cytoplasm (e.g. CagA by the gastric pathogen *Helicobacter pylori*). Despite this knowledge, important questions regarding the molecular mechanism of T4S and the specific role of the translocated bacterial effector proteins within host cells remain elusive. Our recent work on the pathogenesis of tumor-inducing bacteria of the genus *Bartonella* revealed the presence of two distinct T4S systems which are both essential for the infection process of these emerging pathogens (Fig. 1). The availability of appropriate genetics for *Bartonella*, as well as *in vitro* (cell culture) and *in vivo* (animal) infection systems allowed us to establish a unique model for studying both the molecular mechanistic aspects of T4S and the role of this versatile virulence mechanism in bacterial pathogenesis. Furthermore, our findings on the nature of the substrate translocation signal and its use for efficient intracellular delivery of heterologous protein substrates into human cells may lead to an exploitation of T4S systems for novel therapeutic approaches.

Bartonella infection models as a basis for studying the role of T4S in bacterial pathogenesis

The genus *Bartonella* comprises an increasing number of human and animal pathogens. Common features of bartonellae include transmission by blood-sucking arthropods and the specific interaction with endothelial cells and erythrocytes of their mammalian hosts. For each *Bartonella* species, the invasion and persistent intracellular colonization of erythrocytes is limited to a specific human or animal reservoir host. In contrast, endothelial cells are target cells in probably all mammals, including humans in case of the animal-specific species (zoonotic infections). In recent years we have established infection models for studying *Bartonella* pathogenesis in two species:

B. henselae and *B. tribocorum*. *B. henselae* causes an asymptomatic bacteremia in the feline reservoir, while it represents an important pathogen in the incidental human host. The broad range of disease manifestations includes proliferative disorders of the endothelium associated with prominent vascular tumor formation (pathological angiogenesis). We use primary human umbilical endothelial cells (HUVEC) as *in vitro* model for studying this subversion of human endothelial cell function by *B. henselae*. Laboratory rats experimentally infected with the closely related species *B. tribocorum* serve for studying the characteristic intraerythrocytic bacteremia of bartonellae in their specific reservoir host. In this model, the intraerythrocytic bacteremia was shown to be preceded by the colonization of a primary infection niche. In the primary niche, which is considered to include the vascular endothelium, bacteria get competent for the subsequent invasion of erythrocytes in the blood stream. We are using the outlined *in vitro* and *in vivo* infection models for studying the process of T4S in *Bartonella* pathogenesis.

The VirB-D4 system of *Bartonella* is essential for establishing infection *in vivo* and mediates subversion of multiple endothelial cell functions *in vitro* (Schulein *et al*., 2002, Schmid *et al*., *in press*)

In the past two years, we have extensively characterized a conserved T4S system of *B. henselae* and *B. tribocorum,* VirB-D4, which displays homology to the AvhB-TraG conjugative plasmid transfer system of *A. tumefaciens*. The conserved *virB-D4* locus of these bartonellae is encoded by an operon of 10 genes (*virB2-10*) and a downstream-located *virD4* gene.

Unlike wild-type *B. tribocorum,* mutants deleted for *virB4* or *virD4* are unable to cause intraerythrocytic bacteremia in the rat model. VirB-D4 is strictly required for infection of the primary niche, but does not seem to play a role in the subsequent phase of intraerythrocytic bacteremia.

Given that endothelial cells are considered part of the primary niche, we next tested whether VirB-D4 is required for the interaction of *B. henselae* with human endothelial cells *in vitro*. Mutants arising by deletion of either *virB4* or *virD4* were found incapable of mediating most of the known physiological changes associated with *B. henselae* infection of HUVEC. These include (i) massive rearrangements of the actin cytoskeleton, resulting in the formation of bacterial aggregates and their internalization by the invasome structure, (ii) nuclear factor κB-dependent proinflammatory activation, leading to cell adhesion molecule expression and chemokine secretion, and (iii) inhibition of apoptotic cell death, resulting in enhanced endothelial cell survival. Moreover, VirB-D4 mediated cytostatic and cytotoxic effects at high bacterial titers, which interfere with the potent VirB-independent mitogenic activity of *B. henselae*. These VirB-D4-mediated processes provoke a specific transcriptional signature as shown by genome-wide transcriptional profiling with human Affymetrix chips. Taken together, VirB-D4 was shown to be a major virulence determinant of *B. henselae*, required for targeting multiple endothelial cell functions exploited by this vasculotropic pathogen.

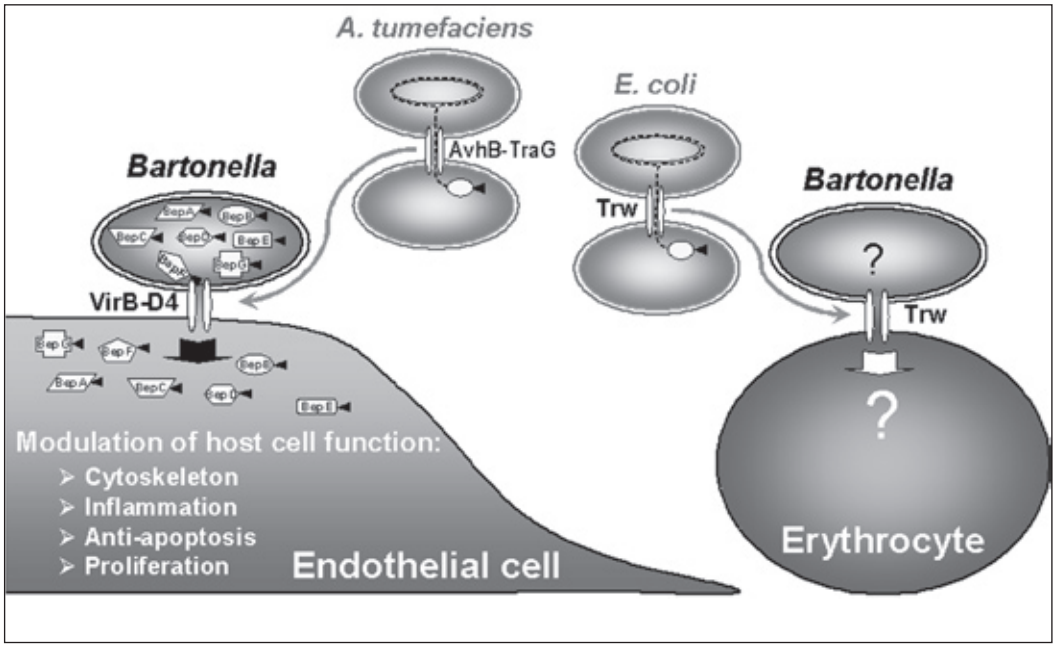

Fig. 1: Schematic representation of the evolutionary origin of the two distinct T4S system of Bartonella *and their role in mediating host cell interaction.*

Intracellular protein delivery into human target cells by the *Bartonella* VirB-D4 system (Schulein *et al*., 2003)

We have identified several genes downstream of the *virB* locus of *B. henselae*, which we have shown to encode effector proteins translocated by VirB-D4 into human endothelial cells. These Bep (*Bartonella*-translocated effector proteins) proteins share a partly conserved C-terminal translocation signal, while their heterologous N-terminus is considered to serve effector functions after translocation into endothelial cells. Three out of the seven Bep proteins carry conserved tyrosine phosphorylation motifs in this effector domain, which became phosphorylated upon translocation into endothelial cells. Deletion of the locus encoding the Bep proteins resulted in a mutant phenotype indistinguishable from *virB4* and *virD4* mutants, demonstrating that the Bep proteins mediate all VirB-D4-dependent changes of endothelial cell function (Fig. 1, previous page).

The C-terminal translocation signal of the Bep proteins is bipartite, including a novel domain of approximately 140 aa termed BID (Bep intracellular delivery) followed at the very C-terminus by an unconserved tail sequence. Hidden-Markov models allowed us to identify BID domains also in several DNA-transfer proteins of plasmid-borne conjugation systems, suggesting a recent history of adaptive evolution of the *Bartonella* VirB-D4 protein transfer system from these conjugative DNA-transfer systems.

Fusion proteins composed of a heterologous reporter protein (i.e. Cre recombinase) and the terminal translocation signal of one Bep protein, BepD, were efficiently delivered by the VirB-D4 system into endothelial cells. Recombinant *B. henselae* strains may thus be used as suitable vectors for targeting proteins of therapeutic value into infected human cells, i.e. for the treatment of vascular disorders or for vaccine delivery.

The Trw system: Recruitment of an *Escherichia coli* conjugation system for the establishment of intraerythrocytic bacteremia by *Bartonella* (Seubert *et al*., 2003)

In a genetic screen for bacterial genes specifically activated during endothelial cell infection, we have identified a second T4S system in *B. henselae*. This Trw system is present throughout the genus *Bartonella* and was characterized most thoroughly in *B. tribocorum*. Trw of *B. tribocorum* shares an extremely high level of sequence identity with the Trw conjugation machinery of the broad-host-range antibiotic-resistance plasmid R388 of *E. coli* (up to 80% amino acid identity for individual T4S components). The highly conserved T4S loci are co-linear except for the presence of numerous tandem gene duplications in *B. tribocorum*, which mostly encode variant forms of surface-exposed pilus subunits. Conservation is not only structural, but also functional: R388 mutated in either *trwD* or *trwH* encoding essential T4S components could be trans-complemented for conjugation by the homologues of the *B. tribocorum* system. Conservation even includes the transcription regulatory circuit: both T4S loci encode a highly homologous and interchangeable KorA/KorB repressor system that negatively regulates expression of all T4S components. These data suggest that the Trw T4S system of *Bartonella* and R388 have a short history of adaptive evolution from a common ancestor locus. Recent acquisition of the *trw* locus of *B. tribocorum* by horizontal gene transfer is suggested by the presence of a cryptic phage-like integrase at a distal position of the locus, as well as by the fact that flanking chromosomal regions are contiguous in the genomes of related α-proteobacteria. By these criteria, the *trw* locus is a pathogenicity island. Whether or not this T4S system translocates bacterial effector molecules into host cells similar as outlined for VirB-D4 is presently unknown. An essential role of Trw in the infection process was demonstrated by genetic means: unlike wild-type *B. tribocorum,* a mutant deleted for *trwE* was unable to cause intraerythrocytic bacteremia in the experimental rat model. In contrast, a *trwK* mutant of *B. henselae* did not show any altered phenotype in the infection of human endothelial cells. Together, these data demonstrate that the Trw T4S system is an essential pathogenicity factor in *Bartonella*, which is presumably required for the interaction with erythrocytes rather than endothelial cells (Fig. 1, previous page).

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Temporal and spatial control during the bacterial cell cycle and development

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Our main objective is to understand temporal and spatial control mechanisms of the bacterial cell cycle and development on a molecular scale. To appreciate how cells execute and coordinate proliferation and differentiation events in a four-dimensional space requires a systematic dissection of regulatory mechanisms on all possible levels including controlled synthesis, activation, localization and degradation of proteins. We study cell dynamics in *Caulobacter crescentus*, a bacterial model organism with a simple life cycle, which includes an asymmetric cell division and an obligate cell differentiation step (see Figure). Our work concentrates on the role of specific protein degradation during the *Caulobacter* cell cycle, and on regulatory principles and pathways involved in polar development.

Cell cycle-dependent proteolysis

In the last two years we have examined a range of protease substrates and have functionally analyzed several ATP-dependent proteases, including ClpXP, ClpAP and FtsH. While FtsH is required for *C. crescentus* stress response and survival, ClpXP and ClpAP have adopted specific roles in cell cycle and development, respectively. ClpXP is involved in the degradation of the master cell cycle regulator CtrA and the McpA chemoreceptor during G1-to-S transition. To identify additional ClpXP substrates we have combined genetics with global proteome analysis. Several protein spots were identified, which, upon inactivation of ClpX, show a dramatically increased stability. Future studies are geared at analyzing turnover and specific role of these proteins during the cell cycle.

ClpAP is required for the cell cycle-dependent degradation of the flagellar anchor protein FliF. FliF, an integral membrane protein, contains a degradation tag at its extreme C-terminus. The analysis of the nature of this degradation signal has revealed that both number and positioning of hydrophobic amino acids at the FliF C-terminus are critical for its degradation. These results contribute substantially to our understanding of how proteases specifically recognize and select proteins tagged for destruction. What remains to be elucidated are the regulatory mechanisms, which are responsible for the correct timing of proteolysis during the cell cycle.

Polar development: discovery of a new global secondary messenger in Bacteria

As a consequence of *C. crescentus* development, cell poles are continuously remodeled during each cell cycle. Two sensor histidine kinases, PleC and DivJ, involved in controlling pole development are dynamically positioned to the cell poles during the cell cycle (Fig. 1). We have used genetics, biochemistry and cell biology to show that the atypical response regulator PleD, which is required for normal pole development, constitutes one of the readout proteins of the PleC/DivJ regulatory system. DivJ and PleC modulate

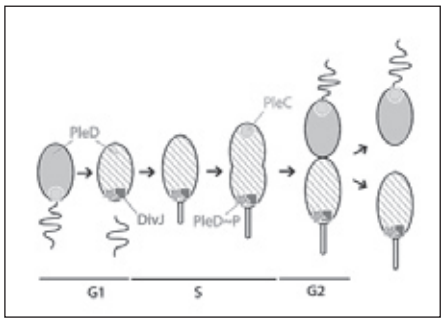

Fig. 1: Schematic of the C. crescentus cell cycle illustrating the dynamic positioning of regulatory components involved in pole development. Localization of PleC (circle), DivJ (square) and PleD~P (star) are indicated.

PleD activity during the cell cycle by acting as kinase or phosphatase of the response regulator at different stages of development. But PleC and DivJ not only control PleD activity, but also its subcellular localization. Soluble PleD dynamically localizes to the differentiating stalked pole as a function of its phosphorylation state (see Figure). Neither the added phosphoryl group per se nor the activity of the PleD output domain is responsible for polar sequestration but rather an active, dimerized conformation of the protein. Coupling PleD activity to its polar sequestration provides a mechanism that spatially restricts PleD activity to the site where it coordinates polar morphogenesis.

Activation of PleD results in the production of a novel secondary messenger, cyclic di-guanosine-monophosphate (c-diGMP). Purified PleD protein efficiently catalyzes the conversion of GTP into c-diGMP *in vitro*, but lacks detectable phosphodiesterase activity. PleD cyclase activity requires an intact C-terminal output domain, is specific for GTP, and correlates with PleD activation by phosphorylation. The observation that PleD homologs form a large, prokaryote-specific protein family strongly implies that c-diGMP represents the readout of a novel signal transduction network in bacteria. The direct link discovered between the activation of PleD and its localization to the differentiating cell pole suggests that spatially controlled diguanylate cyclase activity might constitute a general regulatory principle in bacterial growth and development.

Our future activities will concentrate on defining the c-diGMP regulatory network in *Caulobacter* by identifying target proteins and their role in development, and on elucidating the mechanisms of PleD activation and polar sequestration. As a collaborative effort with the group of Tilman Schirmer (Biozentrum) the analysis of the PleD 3-D structure is in progress and will provide the roadmap for targeted experimental approaches leading to an understanding of this new class of proteins.

Experimental evolution of bacterial senescence

We have also used *Caulobacter* to ask if bacteria are senescent and to test the hypothesis that replicative senescence could have evolved only based on cellular asymmetry. Using an experimental system that allowed us to monitor the reproductive performance and mortality of single cells, we found that *Caulobacter* stalked cells show a significant decline in growth rate and reproduction late in life. This is the first evidence for replicative senescence in bacterial cells and provides the opportunity to investigate the molecular basis of aging in a simple unicellular organism. When mutants that had been selected for increased fitness in logarithmic growth for over 7000 generations were analyzed for their senescence behavior, we found that the reproductive output of one mutant line declined much earlier than in wild-type cells, indicating that improved performance early in live has a negative effect on performance late in life. This finding offers a possible explanation for the evolution of senescence.

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Networks controlling polar growth, nuclear division and peroxisome biogenesis in fungi

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Introduction

We investigate differences and similarities in cellular networks of two eukaryotic microorganisms, the unicellular fungus *Saccharomyces cerevisiae* and the filamentous fungus *Ashbya gossypii*. Despite their differences in life style and growth habitat both organisms have 95 % of genes in common because they evolved from the same ancestor.

Two key functions, polar growth and timing of nuclear divisions, are controlled in both organisms by very similar sets of genes. The majority of these genes are present at syntenic positions confirming their evolution from common ancestral genes. During evolution, the individual role of many of these genes and the network interaction as a whole was modified to produce specific differences in growth and nuclear division observed today. Growth of *A. gossypii* is restricted to the tip thus forming long hyphae. The surface expansion rate is 20 to 40 times higher than in *S. cerevisiae* which displays alternating phases of polar and isotropic growth. We hypothesize that components controlling the actin cytoskekeleton have evolved differently in both organisms. Mitotic divisions in multinucleated hyphae of *A. gossypii* are expected to occur synchronously based on the known controls of nuclear divisions in *S. cerevisiae*. However, nuclei in *A. gossypii* divide asynchronously despite the common cytoplasm. We hypothesize that novel modes of regulation of conserved cell cycle proteins direct this nuclear autonomous divison cylce.

Also cellular components other than nuclei are challenged by the different growth patterns of our two model organisms. Therefore, we additionally focus on the dynamics and maintenance of peroxisomes; small single membrane-bound organelles involved in fatty acid metabolism. We have already identified components for slow cytoskeletal-based transport of peroxisomes in yeast and we are currently investigating how the same components could account for the rapid dynamics observed in *A. gossypii*.

Genomics as information basis

Our group initiated in 1995 a genome project with the filamentous fungus *A. gossypii*. In 1997 we gained support from Novartis Agro (now Syngenta) which led to completion of the genome sequence by April 2002. The map of 4718 ORFs shows extensive synteny to the gene order in *S. cerevisiae.* The gene by gene comparison between *A. gossypii* and *S.cerevisiae* allowed a complete annotation of the *A. gossypii* genome and an almost complete reconstruction of the ancient *S. cerevisiae* genome at the time of its duplication. The data are a rich source for novel approaches to analyze the components of

fungal growth and nuclear division including evolution of promoters. The data also allowed important re-annotations of the *S. cerevisiae* genome.

Nuclear division in multinucleated cells

Multinucleated cells are encountered in a variety of organisms and are integral to processes as diverse as the early development of the fruit fly, bone remodeling, and cancer metastasis. One molecular explanation for synchronous mitoses in multinucleated cells is that cyclin proteins and cyclin dependent kinases are shuttling between the nucleus and the cytoplasm. This enables the core cell cycle machinery to diffuse freely and influence multiple nuclei in the same cytoplasm. Thus, in multinucleated eukaryotic cells studied to date, continual communication between nuclei and the cytoplasm coordinates synchronous nuclear division.

We have begun studying a novel asynochronous nuclear divison system in the multinucleated fungus *A. gossypii*. Neighbouring nuclei in the hyphae divide at different times despite close physical proximity. Utilizing time-lapse video microscopy, we have generated nuclear pedigrees showing that most nuclei in *A. gossypii* cells have the capacity to divide but do so in nearly complete asynchrony. Analysis of spindle morphology suggests that neighboring nuclei exist in different stages of the cell cycle and that asynchrony is independent of cell shape. Additionally, asynchrony is a robust characteristic of the system and is regenerated rapidly upon release from an artificially generated synchronization. Furthermore, we showed that both G1 and mitotic cyclins appear predominantly in nuclei suggesting that one means to maintain asynchrony is to sequester cyclins in the nucleus. These data represent the first molecular, *in vivo* description of asynchronous mitoses in multinucleated cells and suggest that novel means of cell cycle regulation govern the nuclear division cycle in *A. gossypii*.

Maintenance of asynchronous mitoses in *A. gossypii*

We want to examine how *A. gossypii* establishes and maintains autonomous nuclear cycles. One possible mechanism is through nuclear sequestration of cell cycle control elements. Thus, one approach we are taking is to force key cell cycle proteins out of the nucleus to assay whether this leads to synchronous mitosis. If nuclear sequestration is the basis for asynchrony this raises the question of how newly translated proteins find the nucleus from which they originated. Using the protein-DNA interaction of the GFP-Lac repressor and Lac operator system, we are investigating whether different, nuclear-localized cell cycle proteins are always reentering their transcriptional mother nucleus. Another possible mechanism to maintain insulation between the different nuclei, is to specifically control nuclear import. Thus, we are evaluating, if the localization of different cell cycle proteins depends on various karyopherins.

In search of a link between morphogenesis and nuclear division in *A. gossypii*

How mitosis is spatially regulated in a multinucleated filamentous fungus is largely unknown. We study this problem in the ascomycete *A. gossypii*, which is closely related to *S. cerevisiae* on a genome scale. Yet, *A. gossypii* displays an entirely different pattern of growth, leading to branched filaments whose nuclei multiply by asynchronous mitosis. This further raises the question whether, unlike in budding yeast, hyphal morphogenesis is independent of cell cycle stages in *A. gossypii*.

In *S. cerevisiae*, the septins are thought to link bud formation to mitosis by recruiting Hsl1p and Hsl7p, which are required for Swe1p inactivation and entry into mitosis. The proper assembly and organization of the septin collar at the neck is essential for the function of these cell cycle regulators. In *A. gossypii*, homologues to all key players of this morphogenesis checkpoint exist. To begin to investigate possible connections between the cell cycle and morphogenesis we have focused on analyzing the structure and function of septin proteins in *A. gossypii* by using a Sep7-GFP strain. We detected some septin structures that were not described so far. Interestingly, most of the structures in *A. gossypii* seem to be composed of parallel bars, an instance which in yeast is only seen in certain mutants and is associated with higher Swe1p kinase activity. Heterologous expression of AgSep7-GFP in *S. cerevisiae* leads to the formation of normal yeast structures with continuous rings and double rings. This implies that the observed structures are not inherent to the Sep7 protein, but rather the result of different regulation or interaction in the two organisms.

Dynamics of the actin cytoskeleton in *A. gossypii*

In *A. gossypii* the actin cytoskeleton is composed of long bundles of filaments (actin cables) and small punctate structures (actin patches) localized to growth sites and required for polar growth. We analyse different components of the actin cytoskeleton which have been labelled with Green Fluorescent Protein (GFP) by means of video microscopy, studying their structural and dynamic properties. Cap1 and Cap2 are the subunits making up capping protein which binds the barbed end of actin filaments and nucleates actin polymerisation *in vitro*. Cap1-GFP and Cap2-GFP colocalize with actin patches in rhodamine-phalloidin stainings. They are concentrated at the tip of hyphae,

mostly cortical and in the subapical domain. Velocity measurements show that they are slower than actin patches in *S. cerevisiae* or *S. pombe*. Sequential recordings of the entire hypha show that the net movement of actin patches is backward. Co-stainings with the endocytosis marker FM4-64 show partial colocalization. These findings support the idea that actin patches are involved in endocytosis. Cap1-GFP and Cap2-GFP patches are immobilized by low doses of Latrunculin A, indicating that actin polymerization may be their mode of propulsion.

Actin cables were made visible by using a GFP tagged variant of ABP140, an actin binding protein. ABP140-GFP colocalizes with actin cables in rhodamine phalloidin stainings. They are mostly cortical, often helical, and can be as long as 40 µm. They are very motile and show undulating movements. This is in contrast to the short, straight actin cables in *S. cerevisiae*, which have been shown to transport exocytic vesicles to the site where a new cell wall is formed. These results suggest that the structural basis and mechanisms for polarized growth are similar in *S. cerevisiae* and *A. gossypii*, yet differ markedly in dynamic behaviour.

Role of formins and RHO proteins for hyphal growth in *A. gossypii*

Formin Homology Proteins are known nucleators of actin cables in many different organisms. We study AgBNI1 a Formin Homology Protein from *A. gossypii*. AgBNI1 is essential for hyphal formation and might be involved in tip branching, as shown by deletion mutants and activated alleles. Deletion mutants of AgBNI1 have a defect actin cytoskeleton and do not form mature hyphae. In contrast, cells carrying the activated allele show tip-branching prior to emergence of lateral branches, which is never observed in the wildtype. In addition, we identified four different members of the family of small Rho-type GTPases that are capable of binding to the AgBNI1 protein and therefore might be regulators of the latter. To further investigate the role of these members of the Rho-family we integrated activated alleles into the genome of *Ashbya gossypii*. However no single activated rho-allele was capable to mimic the phenotype of an activated AgBNI1 indicating that either several different GTPases or other so far unknown factors are necessary for activation of AgBNI1.

***A. gossypii* homologues of *S. cerevisiae BUD* genes**

In *S. cerevisiae*, a series of so-called *BUD* genes has been identified which control establishment of new axis of polarity. The function of the *Ashbya BUD1/RSR1* and *BUD2* homolog were investigated by using time-lapse video microscopy. These movies revealed that *AgBUD2* is important for a stable growth axis of the elongating hyphae. *AgRSR1/BUD1* seems to play a role in the maintenance of hyphal tip growth and in the elongation of initiated hyphal branches. *Ashbya* hyphae lacking this gene frequently paused growth at the tip and showed many unsuccessful branching events.

A. gossypii also carries homologues of *ScRAX1, ScRAX2, ScBUD7, ScBUD8, ScBUD9*. These genes encode putative transmembrane proteins. We are interested in determining the role of these homologues in the filamentous growth pattern of *A. gossypii*. In contrast to *A. gossypii* wild type, in Agrax1, Agrax2 and Agbud8 deletions up to four branches can simultaneously emerge from the main hypha, and additional new branches are formed between the already developed ones. Tip growth of the main hypha is markedly reduced when branches start growing. An altered branching pattern was not observed in the *Agbud7* deletion. AgRax2-GFP localizes at the tips of hypha and at the septum where it appears just after actomyosin ring formation, and before chitin deposition. AgBud7-GFP is found in dot-like structures which oscillate. The observed structures are able to divide keeping a similar density throughout the hyphae. We will complete the analysis by using video microscopy with different tip-located GFP marker proteins.

Activity of syntenic *A. gossypii* promoters

Due to a high degree of synteny, our two model organisms represent an ideal system to study the conservation of entire promoters and promoter elements. A selection of promising *A. gossypii* promoters were chosen and fused to a promoterless *GFP* reporter gene. Evaluation of syntenic *A. gossypii* promoter strengths in *S. cerevisiae* revealed that 44% of the promoters tested exhibited significant green fluorescence. Two of those promoters, *ENO2P* and *PFK2P*, can be used as new heterologous promoters in *S. cerevisiae.* In order to identify conserved promoter elements we compared the promoters of *A. gossypii CLN1,2*, *ENO2*, *PFK2*, *TEF2*, *H3/H4*, *H2A/H2B*. and their *S. cerevisiae* homologues. Interestingly, we observed a high frequency of order conservation between *A. gossypii* and *S. cerevisiae* transcription factor binding sites. In contrast to that, the distances between the individual sites varied considerably between the two species.

Peroxisome formation via a novel ER pathway

Impairment of peroxisomal function is the cause of a number of diseases in humans ranging from single enzyme deficiencies to severe syndromes in which peroxisomes are completely absent. Peroxisome functions in fatty acid metabolism are quite well characterized but knowledge on the biogenesis of this organelle is scarce. Focusing on peroxisome

inheritance, we could show by time-lapse microscopy that maintenance of the number of peroxisomes in *S. cerevisiae* cells involves a fission process and that peroxisomes are segregated to the daughter cell in a highly ordered process. In screens we have identified the dynamin-like protein Vps1 to be involved in a membrane fission event that is required for the regulation of peroxisome abundance. In addition, we found that efficient segregation of peroxisomes from mother to daughter cell is dependent on the actin cytoskeleton and that peroxisomes move along actin filaments driven by the class V myosin motor protein Myo2.

Furthermore, we developed a conditional genetic system that allows us to induce the de novo formation of peroxisomes. Using fluorescently tagged peroxisomal proteins we could show that in case of complete peroxisomal loss peroxisomal membrane proteins localize into the endoplasmic reticulum to recruit new membrane to form a primary peroxisomal vesicle. Using a collection of deletion mutants as well as GFP tagged alleles, we generated a map that shows that specific peroxisomal membrane proteins are required for the formation, maturation, and division of the primary peroxisome in a hierarchical and sequential manner, until the original number of small, functional peroxisomes is restored. We expect that our finding could form the basis for the recognition of additional peroxisomal diseases and facilitate new therapeutic approaches.

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Morphological development and intrinsic drug resistance in *Streptomyces*

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Scope of research

Streptomyces are filamentous soil bacteria that undergo morphological changes coordinated with the synthesis of an enormous variety of antibiotics and other pharmaceutically important metabolites. A better understanding of the regulatory mechanisms which control this developmental program is of great interest, not only from the point of view of fundamental biology, but also for the discovery and engineering of new chemotherapeutic drugs.

Unlike typical bacteria, *Streptomyces* behave as multicellular organisms that have evolved as coordinated communities of specialized, interactive cells. Whereas bacterial chromosome replication is typically followed by segregation into two cells that separate, *Streptomyces* genomes remain associated in a chain of cells forming a filament that elongates and branches. The dense interconnected network thus formed within the substrate enlarges into a colony. Developmental changes occur in localized regions of the colony generating groups of cells specialized for continued growth, programmed cell death, manufacture and circulation of nutrients, erection of aerial mycelium, spore formation, and biosynthesis of various secondary metabolites. This “tissue-specific” gene expression, coordinated by diffusible butyrolactone and peptide signaling compounds, is reminiscent of metazoans.

The erection of aerial mycelium, the most obvious visual manifestation of the developmental program, is linked to the biosynthesis of antibiotics. In general, these processes take place in substrate mycelium at about the same time; most mutants that cannot erect hair-like aerial mycelium (referred to as “bald”) are also unable to produce antibiotics. While several bald loci have been identified genetically as transcriptional and translational regulatory elements, the genetic functions they control are largely unknown.

Imbalances in physiology lead to accumulation of certain primary metabolites that may either trigger morphogenesis or be converted to novel compounds by the activation of alternative, “secondary metabolic” pathways. The secondary metabolites produced by *Streptomyces* have been the primary source of antibiotics in clinical use and also have applications as herbicides, anticancer drugs, immunoregulators, and antiparasitic compounds. In order to avoid suicide, the biosynthesis of antibiotics must be associated with the expression of antibiotic resistance genes. Antibiotic-producing *Streptomyces* share a common ancestry with *Mycobacterium* and may rely on similar mechanisms of intrinsic resistance. The remarkable antibiotic tolerance of *M. tuberculosis* is the root cause of treatment failure, relapse and acquired drug resistance in patients with tuberculosis.

Antibiotic resistance and developmental genes have been the focus of this reporting period. We have identified and analyzed systems that provide multidrug resistance in *Streptomyces* and *Mycobacterium*. Secondly, we demonstrated linkage of developmental program in *Streptomyces* to metabolic and stress response systems.

Identification of a multidrug resistance regulator in *Streptomyces* provides an important insight into intrinsic antibiotic resistance in *Mycobacterium tuberculosis.*

We isolated a spontaneous mutant of *Streptomyces lividans* that had become hypersensitive to a diverse array of chemically and functionally unrelated clinical antibiotics. The mutant allele was mapped to *mdrW*, belonging to a family of paralogous genes that are related to the *Streptomyces coelicolor* developmental regulatory gene *whiB*, and unique to the actinomycetes. Targeted null-mutants in *S. lividans*, *S. coelicolor* and in the orthologous *mdrW* genes of *Mycobacterium smegmatis* and *Mycobacterium bovis* BCG had no obvious growth or developmental defects. However, all of these mutants displayed multi-drug sensitivity. Overexpression led to a marked increase in antibiotic resistance. Heterologous expression of the streptomycete *mdrW* gene in the corresponding *M. smegmatis* mutant rescued the antibiotic-sensitive phenotype. This work strongly predicts that the *mdrW* gene of *M. tuberculosis* is a central regulator of its innate multi-drug resistance and offers an attractive target for effective therapeutic intervention.

Antibiotic-induced increases in multidrug resistance in *Streptomyces*

Studies of a family of cyclic thiopeptide antibiotics that induce resistance to diverse antibiotics led us to the discovery and subsequent investigations of the *tipA* gene in *Streptomyces lividans*. We have shown that TipAL, a transcriptional regulator of the MerR family, is activated by exposure to low concentrations of these compounds both *in vivo* and *in vitro*. Its C-terminal drug-binding domain, TipAS, defines a subfamily of broadly distributed bacterial proteins including Mta, a central regulator of multidrug resistance in *Bacillus subtilis*. Our data suggest that cyclic peptides similar to thiostrepton may be diffusible activators of multidrug resistance in gram positive bacteria. Activation of TipAL is mediated by conformational changes induced by antibiotic binding to this C-terminal domain that consequently allows promoter recognition and binding. The molecular mechanism for the broad antibiotic recognition specificity displayed by TipA and the conformational changes leading to its activation have now been partially defined by analysis of NMR spectra in collaboration with Prof. Stephan Grzesiek's laboratory in the Division of Structural Biology. TipAS is composed of a globin-like helical fold with a deep surface cleft and an unfolded N-terminal region. Antibiotics bind within this cleft, structurally reminiscent of the corresponding heme pocket in myo- and hemoglobin, and induce folding of the N-terminus. Thus, the classical globin fold is well adapted not only for accommodating its canonical cofactors, heme and other tetrapyrroles, but also for the recognition of a variety of antibiotics where ligand binding leads to transcriptional activation and drug resistance.

Differentiation in *Streptomyces coelicolor* – a specialized developmental program that relies on a coordinated network of metabolic and stress response genes

Bacteria typically undergo intermittent periods of starvation and adaptation emulated as diauxic growth in the laboratory. In association with growth arrest elicited by metabolic stress, *S. coelicolor* not only adapts its primary metabolism, but can also activate developmental programs leading to morphogenesis and antibiotic biosynthesis. We combined proteomic and metabolomic data to analyze global changes in gene expression during diauxic growth in a defined liquid medium and have created a publicly available proteomic database describing these patterns as well as identifying protein spots (http://proteom.biomed.cas.cz). These studies demonstrated that the rates of synthesis of heat shock proteins are determined not only by temperature increase but also by the relative rates of metabolic flux in the glycolytic pathway and the citric acid cycle. In addition, statistical analysis of protein synthesis during diauxic lag showed that this starvation response was accompanied by a structured regulatory program involving the sequential activation of heat-, salt-, cold-, and bacteriostatic antibiotic (pristinamycin I, PI)- induced stimulons. These studies revealed interactive regulation of metabolic and stress response systems including some proteins known to support developmental programs in *S. coelicolor*.

Biochemical and genetic studies have shown that physiological switches and stress responses underlie initiation of a key developmental stage. Metabolic studies first indicated that alterations in flux through the TCA cycle accompanied a switch to antibiotic biosynthesis and aerial hyphae formation in wild type strains but not in bald mutants (Viollier, P. H., Minas, W., Dale, G. E., Folcher, M. & Thompson, C. J. (2001) *J Bacteriol* 183, 3184-3192). We have also demonstrated a direct link between development and stress response systems by showing that a sigma factor controlling the stress response system (SigH) is subject to temporal and spatial regulation during colony development and that this tissue-specific regulation is mediated directly by the developmental transcription factor BldD (Kelemen, G. H., Viollier, P. H., Tenor, J., Marri, L., Buttner, M. & Thompson,

C. J. (2001) *Mol Microbiol* 40, 804-814). Another negative regulator of SigH, its upstream antisigma factor (*prsH*) is also needed for colonial differentiation. These results led us to speculate that a metabolic shift accompanied by an adaptive stress response is needed for development and that many of the previously isolated developmental mutants (bald) represented defects in these systems.

In support of this new concept, we showed that aerial hyphal growth is activated independently from the normal developmental program by expression of the key developmental switch gene, *ramR*. Our studies showed that aerial hyphal growth could be turned on independently from the normal developmental cascade by providing constitutive expression of functionally interactive genes within a gene cluster (*ram*). Within this cluster, *ramC*, *ramS,* and *ramR* genes were essential for normal growth of aerial hyphae and *ramR*, a response regulator gene, was a key activator of development. The *ramR* gene restored growth of aerial hyphae in all bald strains tested of which many are characterized by physiological defects. Disruption of the *ramR* gene severely delayed growth of aerial hyphae. We identified and mapped RamR targets immediately upstream of the region encoding *ramC* and *ramS*, a putative operon. Based on these results, we propose that phenotypes of bald mutations reflect an early stage in the *Streptomyces* developmental program similar to the *spo0* mutations in the unicellular bacterium *Bacillus subtilis* and that RamR is analogous to Spo0A, the *Bacillus* response regulator that integrates physiological signals before triggering endospore formation. Based on these results, we chose to focus on *adpA*, a specialized developmental gene that controls transcription of *ramR*.

In addition to transcriptional control, Keith Chater and his collaborators have shown that *Streptomyces* colonial development also depends on a translational regulatory system. *bldA*, encoding the principal tRNA for translation of the TTA (leucine) codon, is dispensable for vegetative growth but at a later stage is conditionally required for the synthesis of aerial mycelium and for biosynthesis of some antibiotics. We identified the *adpA* mRNA as a possible target for developmental control mediated by *bldA* since it is the only known *bld* locus containing a TTA codon. To test whether translation of *adpA* mRNA was dependent on *bldA*, perhaps related to accumulation of an active form of *bldA* tRNA, the unique TTA codon in *adpA* was changed to CTC. The fact that expression of this mutated gene allows aerial mycelium formation in the *bldA* mutant suggests that *adpA* is a target for translational control of differentiation.

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DIVISION OF BIOCHEMISTRY

During the past two years, the Division of Biochemistry has undergone significant change among its group leaders. Professor Howard Riezman and independent investigator Anastasia 'Natasha' Kralli departed, and Professor Jean Pieters arrived. We are grateful to our colleagues Howard Riezman and Natasha Kralli for their outstanding science and teaching. We are particularly grateful to Howard Riezman who served as head of the Division for several years. We also note that Professor Jean Pieters has integrated well as a member of the Division. Currently, the Division of Biochemistry comprises three senior groups headed by Michael Hall, Jean Pieters, and Martin Spiess, and a Protein Chemistry Service Group headed by Paul Jenö. The major research interests of the Division are protein transport and signal transduction. The Hall group studies signal transduction and growth control in yeast and mammalian cells, with particular emphasis on the evolutionarily conserved TOR signaling network. The Pieters group studies mechanisms involved in protein and membrane traffic in eukaryotic cells, using the interaction of mycobacteria with macrophages as a model system. The Spiess group studies the asialoglycoprotein receptor and intracellular sorting of membrane proteins in mammalian cells. The Protein Chemistry Service Group contributes to these studies and to the research efforts of others in the Biozentrum, and is developing microanalytical strategies for mass spectrometric determination of protein structure. The research activities of each group are described in more detail below.

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TOR signaling and control of cell growth

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Introduction

Cell growth is highly regulated. Cells respond to nutrients or other appropriate growth stimuli by upregulating macromolecular synthesis, and thereby increasing in size. Conversely, cells respond to nutrient limitation or other types of stress by downregulating macromolecular synthesis and enhancing turnover of excess mass. Thus, the control of cell growth involves balancing positive regulation of anabolic processes with negative regulation of catabolic processes. Growth is also controlled relative to cell division. In proliferating cells, growth is linked to the cell cycle such that most cells precisely double their mass before dividing. In other physiological contexts, such as load-induced muscle hypertrophy or growth factor-induced neuronal growth, cell growth is controlled independently of the cell cycle. Furthermore, in addition to the temporal control of cell growth described above, cell growth can be subject to spatial constraints. For example, budding yeast and neurons grow in a polarized manner as a result of new mass being laid down only at one end of the cell. Finally, in multicellular organisms, growth of individual cells is controlled relative to overall body growth such that the organs and tissues constituting the organism are properly proportioned.

The TOR signaling network

What are the mechanisms that mediate and integrate the many parameters of cell growth? In other words, what determines that a cell grows only at the right time and at the right place? Remarkably, the study of these mechanisms has been largely neglected, despite their clinical relevance and despite cell growth being, along with cell division and cell death, one of the most fundamental (and obvious!) aspects of cell behavior. Also remarkable is the recent finding that cell growth control, regardless of the eukaryotic organism or the physiological context, seems always to involve the same protein - the target of rapamycin TOR protein – and its namesake signaling network. TOR is a highly conserved protein kinase and the target of the immunosuppressive and anti-cancer drug rapamycin. The TOR signaling network controls cell growth by activating an array of anabolic processes including protein synthesis, transcription, and ribosome biogenesis, and by inhibiting catabolic processes such as bulk protein turnover (autophagy) and mRNA degradation, all in response to nutrients. Dysfunction of signaling pathways controlling cell growth results in cells of altered size and, in turn, causes developmental errors and a wide variety of pathological conditions. An understanding of the TOR signaling network may lead to novel drugs for the treatment of, for example, cancer, diabetes, inflammation, muscle atrophy, learning disabilities, depression, obesity, and aging.

We are studying the TOR signaling network in the yeast *Saccharomyces cerevisiae* and in mammalian cells. We have found that the two TORs in yeast, TOR1 and TOR2, mediate numerous signaling pathways that fall into one of two major signaling branches. The two signaling branches integrate the temporal and spatial control of yeast cell growth. The branch that determines when a cell grows (temporal control) utilizes TOR1 or TOR2 and is rapamycin sensitive. The branch that determines where a cell grows (spatial control) contains TOR2, but not TOR1, and is rapamycin insensitive. The rapamycin sensitive 'TOR-shared' branch controls several readouts which collectively determine the mass of the cell. These readouts include protein synthesis and degradation, mRNA synthesis and degradation, ribosome biogenesis, nutrient transport, and autophagy. Some of the effector pathways that make up this branch are known, but there are some (many?) which are not yet known. One of the known effector pathways involves inhibition of the type 2A-related phosphatase SIT4 by the essential protein TAP42. The rapamycin insensitive 'TOR2-unique' branch controls the polarized organization of the actin cytoskeleton. This branch so far contains a single effector pathway consisting of the small GTPase RHO1, Protein Kinase C (PKC1) and a PKC1 regulated MAP Kinase pathway.

The elucidation of two major TOR signaling branches that integrate temporal and spatial control of cell growth was a major step in the understanding of TOR signaling and cell growth control. However, the two signaling branches have also raised some new and interesting questions. For example, what are the molecular determinants of the specificity and diversity of TOR signaling? Why can TOR2 signal in both branches whereas TOR1 is restricted to only one branch? Furthermore, how does TOR sense and discriminate between different nutrients? To answer these questions, we recently purified TOR1 and TOR2 in the hope of identifying co-purifying accessory proteins that mediate TOR function. Two functionally and structurally distinct, membrane-bound TOR complexes were identified. TOR complex 1 (TORC1) contains LST8, the uncharacterized protein KOG1 and either TOR1 or TOR2. TOR complex 2 (TORC2) contains TOR2, LST8 and the uncharacterized proteins AVO1, AVO2 and AVO3. As suggested by the fact that TORC1 contains either TOR1 or TOR2 whereas TORC2 contains only TOR2, TORC1 mediates the TOR-shared signaling branch and TORC2 mediates the TOR2-unique signaling branch. We also showed that TORC1 is conserved in mammalian cells. mTOR forms a complex with raptor (also known as mKOG1) and mLST8, the mammalian orthologs of KOG1 and LST8, respectively. It remains to be determined whether TORC2 is conserved. The

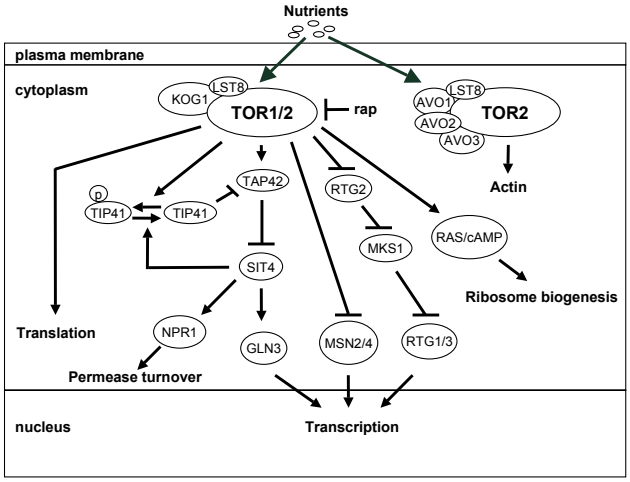

Fig. 1: TOR signaling pathways in yeast integrate temporal and spatial control of cell growth.

molecular functions of the individual TOR partner proteins are not known, but most of the partners are essential proteins (only AVO2 is not essential) and likely play an important role in determining the specificity and diversity of TOR sensing or signaling. Thus, the identification of TORC1 and TORC2 has been a significant step in the ongoing characterization of TOR signaling. Our current and future work focuses largely on further characterizing the two TOR complexes and on elucidating the roles of the TOR partner proteins. We hope thereby to determine the molecular mechanisms by which TOR senses and signals nutrient availability.

The TOR and RAS/cAMP signaling pathways are the two major pathways in yeast controlling cell growth in response to nutrients. To gain further insight into the effector pathways by which TOR controls cell growth, we examined the functional interaction between TOR and the RAS/cAMP pathway. First, activation of the RAS/cAMP signaling pathway confers pronounced resistance to rapamycin. Second, constitutive activation of the RAS/cAMP pathway prevents several rapamycin-induced responses, such as the nuclear translocation of the transcription factor MSN2 and induction of stress genes, the accumulation of glycogen, the induction of autophagy, the down-regulation of ribosome biogenesis (ribosomal protein gene transcription and Pol I/Pol III activity), and the down-regulation of the glucose transporter HXT1. Third, many of these TOR-mediated responses are independent of the previously described TOR effectors TAP42 and the type 2A-related protein phosphatase SIT4. Conversely, TOR-controlled TAP42/SIT4-dependent events are not affected by the RAS/cAMP pathway. Finally, and importantly, TOR controls the subcellular localization of both the PKA catalytic subunit TPK1 and the RAS/cAMP signaling-related kinase YAK1. Our findings suggest

that TOR signals through the RAS/cAMP pathway, independently of TAP42/SIT4. Thus, the RAS/cAMP pathway may be a novel TORC1 effector branch. We are currently investigating transcription factors that may be the targets of the RAS/cAMP pathway in the control of ribosomal genes. The control of ribosome biogenesis is a key and as yet unsolved process in the regulation of cell growth.

The research described above covers only a part of our ongoing research effort. Many other questions are being actively pursued concerning the role of TOR and the regulation of cell growth. For example, we have recently begun studying the role of TOR in the regulation of lifespan. The Leopold laboratory (Nice) has recently found that TOR controls the growth not only of the cell in which it resides, but also of distant cells. This is a major advance in the field because it represents a new, humoral mode of TOR-mediated growth control, and also because it provides a mechanism by which the growth of cells in different tissues is coordinated such that overall body growth is properly balanced. These findings also have interesting implications when considering other hormone- and nutrient-related processes, such as appetite regulation and aging. Appetite and lifespan are regulated by hormones produced in response to nutrients. However, the nutrient sensor that signals the production of such hormones is not known. The findings of the Leopold laboatory suggest that TOR might be such a sensor. Thus, TOR may play a here-to-fore unknown and key role in the control of appetite and lifespan. Indeed, a role for TOR in the control of appetite and aging is supported by the finding that TOR modulates insulin signaling which has already been implicated in the control of food intake and lifespan. In collaboration with our in house colleagues Markus Rüegg and Yves-Alain Barde we are also studying the role of TOR signaling in the postmitotic growth of muscle and neuronal cells.

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Mechanisms of membrane trafficking in the phagosomal/endocytic pathway

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Introduction

When an organism is invaded by microbes, an immune response has to be established to clear the host from the invading microorganisms. Elimination of infectious organisms is first of all ensured by this innate immune defence system. For extracellular pathogens, this innate immune defence consists largely of phagocytes that non-specifically engulf the microorganisms and destroy these within lysosomes, whereas viruses can be eliminated through cellular degradation machineries such as the proteasome. Degradation of the infectious agents also leads to the generation of fragments that can be displayed to T lymphocytes by Major Histocompatibility Complex (MHC) molecules, resulting in T cell activation and generation of specific immune responses

My laboratory is interested in the intracellular trafficking events involved in the internalization and degradation of antigens and the mechanisms that result in a proper T cell response. Pathogenic microorganisms have gained the capacity to circumvent the effectiveness of the immune response at several levels. We are interested in deciphering the mechanisms that are used to escape immune recognition.

Membrane traffic events involved in antigen presentation in dendritic cells and macrophages

An important step in generating an immune response is the activation of naïve T cells, which occurs through their stimulation by dendritic cells. Dendritic cells sample peripheral tissue for the presence of antigens, and migrate to lymphoid organs where antigenic peptides captured at the periphery can be presented to naïve T lymphocytes. For the generation of CD4+ T lymphocytes, antigens are captured within the endocytic pathway, and during migration to the secondary lymphoid organs the dendritic cells mature and increase the expression of MHC class II-peptide complexes at the cell surface in order to efficiently induce T cell activation. During migration, MHC class II molecules are redistributed from an intracellular MHC class II compartments to the cell surface. This redistribution occurs during dendritic cell development as they are converted from an antigen capturing immature dendritic cell into an MHC class II-peptide presenting mature dendritic cell, as antigen uptake and processing are down regulated and peptide-loaded class II complexes become stably expressed on the cell surface. We found that a member of the tetraspanins, CD63, is potentially involved in these cell biological alterations during dendritic cell maturation. CD63 associates with intracellularly localized MHC class II molecules in immature dendritic cells, and is posttranslationally modified by poly-N-acetyl lactosamine addition during maturation. This modification of CD63 was

accompanied by a change in morphology of MHC class II compartments from typical multivesicular organelles to structures containing densely packed lipid moieties as analyzed by immunocytochemistry. Posttranslational modification of CD63 may be involved in the functional and morphological changes of MHC class II compartments that occur during dendritic cell maturation.

How CD8+ T cells can be activated against antigens present in the periphery has been less well defined. For generation of CD8+ T lymphocytes, antigen captured in the periphery by professional antigen presenting cells has to acquire access to the MHC class I pathway. It is now becoming increasingly clear that a pathway does exist to deliver exogenously captured antigens to the MHC class I processing compartments, referred to as 'cross-presentation'. Cross presentation is crucial for the generation of immunity against virus-infected and tumor-transformed cells as well as the induction of tolerance. We are analyzing the molecular requirements for antigens to become internalized into professional antigen presenting cells to better understand the mechanisms involved in cross presentation.

Interaction of pathogenic Mycobacteria with mammalian cells

Several microorganisms have gained the capacity to invade and survive within mammalian host cells. Mycobacteria have the ability to enter and survive within eukaryotic cells, by preventing phagosome-lysosome fusion, making this an excellent model system to study both the pathogen-host interaction as well as the biochemical events involved in regulating transport events inside the host cell. We are interested in both the host as well as the mycobacterial factors contributing to mycobacterial survival.

Macrophage factors involved in the modulation of intracellular trafficking

Analysis of the constituents of mycobacterial phagosomes revealed the accumulation of a host protein, termed TACO (for tryptophane aspartate containing coat protein, also known as coronin-1) that is a crucial factor for mycobacterial survival inside macrophages. In the absence of TACO/coronin-1, mycobacteria are readily transferred to lysosomes.

Biochemical analysis revealed that TACO/coronin-1 molecules are coiled-coil-mediated homotrimeric complexes which associate with the plasma membrane and with the actin cytoskeleton via two distinct binding domains. Association with the actin cytoskeleton was mediated by the three-stranded C-terminal α-helical coiled-coil, while in the absence of the coiled-coil domain plasma membrane binding still occurred suggesting that this is mediated by the N-terminal, WD repeat-containing domain. The capacity of coronin-1 to link F-actin filaments to the leukocyte plasma membrane may serve to integrate outside-inside signalling with modulation of the actin cytoskeleton.

Mycobacterial factors involved in the modulation of intracellular trafficking

More recently, we have embarked on a search for mycobacterial proteins that are responsible for intracellular survival within macrophages. In a first approach, we have established a system to analyze the expression of mycobacterial proteins intracellularly, and to distinguish these from host protein expression. Two-dimensional IEF/SDS-PAGE combined with mass spectrometry will allow the identification of mycobacterial proteins expressed intracellularly. Together, this work will help to understand how pathogenic mycobacteria evade the normal host immune responses. In addition, this work may reveal previously unknown mechanisms involved in the normal trafficking pathways as they are employed by these intracellular pathogens.

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Membrane traffic in yeast

Endoplasmic reticulum to Golgi transport of GPI-anchored proteins

Until our recent work, it was generally thought that all secretory proteins are transported together in common ER-derived vesicles from the ER to the Golgi compartment. We showed that GPI-anchored proteins exit the ER in distinct vesicles from other secretory proteins and have proposed to identify the machinery. We have made significant progress in our studies of the mechanism of cargo protein sorting upon exit from the endoplasmic reticulum. First, we have shown that cytosolic proteins, including the Rab GTPase, Ypt1p, and other factors thought solely to be involved in vesicle tethering to the Golgi compartment, are required for cargo protein sorting upon ER exit. We also found tethering factors that were not required for this process. These findings have important implications for the mechanism of cargo protein sorting, because they suggest that the same proteins that are involved in the sorting process are also involved in targeting the vesicles to the proper location. These findings also permitted us to extend our demonstration of cargo protein sorting from our *in vitro* budding studies to the *in vivo* situation. We demonstrated that cargo protein sorting occurs at the same step *in vivo* and has the same requirements for Ypt1p, and tethering factors (Morsomme and Riezman, 2002).

The most salient finding of the past year has been our demonstration that SNARE proteins are required for sorting of GPI-anchored proteins from other secretory proteins into distinct vesicles leaving the endoplasmic reticulum. Using mutants in the ER to Golgi SNAREs, we could show that Bos1p, Bet1p, and Sec22p, but not Sed5p are required for the sorting step to occur. We showed this using an *in vitro* budding system. In addition to the experiments with the mutant extracts, we could also show that inclusion of antibodies that bind to the specific SNARE proteins into the assay inhibited the sorting step, without affecting the overall budding efficiency. Interestingly, antibodies against the specific SNARE proteins only affected entry of the individual SNARE protein into vesicles showing that the SNARE proteins are not in a cis-SNARE complex when they enter into vesicles and therefore are most likely to work as individuals rather than as a complex. Furthermore, using the mutants we could confirm that the SNARE proteins are required for protein sorting *in vivo* and we could visualize the sorting step on the ER membrane by immunoelectronmicroscopy (Morsomme et al, 2003).

We showed several years ago that ceramide or sphingolipid synthesis is required for efficient transport of GPI-anchored proteins to the Golgi compartment in yeast. This finding has frequently been cited in support of a role of “raft” structures in

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protein sorting and traffic. Therefore, we decided to obtain further information on the role of this lipid synthetic pathway. First, we determined the lipid that is required for GPI-anchored protein transport. In contrast to endocytosis, where sphingoid bases are required, ceramide synthesis is required for GPI-anchored protein transport. When we investigated the more precise location of the block in transport (budding, fusion, etc) we were surprised to find that the GPI-anchored proteins were no longer tightly membrane associated when ceramides were depleted even though the GPI-anchor was attached. We constructed a mutant GPI-anchored protein where it was rendered soluble and showed that this also causes a strong transport delay. Therefore, a likely explanation for the defect in transport of GPI-anchored protein in absence of ceramide synthesis is their lack of tight membrane association (Watanabe et al, 2002).

Endocytosis in yeast

We have published studies on the immunoelectron microscopy of the endocytic pathway in yeast and determined the order of compartments in the pathway (Prescianotto-Baschong and Riezman, 2002)

We have constructed a large series of mutants in the synthesis of ergosterol, the yeast counterpart of cholesterol and have examined the pathway of endocytosis in the various mutants as well as determining their sterol composition. The major findings from these studies were that sterols are required at multiple stages of the yeast endocytic pathway, but that the actual structural requirements in the sterol molecule were different. The first stage of the pathway where sterols are required is the internalisation step of the alpha factor receptor. This is not a general endocytic block, but a defect in the phosphorylation and ubiquitination of the receptor. There is no correlation with the ability to form rafts and the ability to phosphorylate and ubiquitinate. The next step is a post-internalisation defect that is still not very well defined. Finally, ergosterol is also required for maintenance of proper vacuole morphology (Heese-Peck et al, 2002).

We have also collaborated with Sandra Lemmon's lab on studies of endocytosis, where our role was to characterize the endocytic defects in various strains and this has resulted in another publication (Henry et al, 2002).

In another project concerning the role of lipids in the endocytic pathway we collaborated with Joost Holthuis' lab to show that two yeast P-type ATPases have an overlapping function in endocytosis that is almost certainly linked to their ability to translocate aminophospholipids across the plasma membrane. Together we could show that mutation of the transporters led to a change in the asymmetry of phosphatidylethanolamine across the plasma membrane and to a striking defect in the internalisation step of endocytosis (Pomorski et al, 2003). This work is highly significant because it provides genetic evidence for a role of an asymmetric distribution of aminophospholipids in vesicle budding.

In previous work, we have shown that the Arc35 protein of the Arp2/3 complex plays a role in endocytosis, but in addition to its endocytic role, it plays a crucial role in the cell cycle. Both roles of Arc35p require calmodulin and affect different calmodulin regulatory pathways. Exploring the role of Arc35p in the cell cycle, we could isolate high copy suppressors of the cell cycle defect and we identified subunits of casein kinase II. We showed that Arc35 interacts with calmodulin, casein kinase II, and gamma tubulin to carry out its cell cycle function (Schaerer-Brodbeck and Riezman, 2003).

Sphingolipid synthesis and transport and function

We have made progress both in the pathways of ceramide synthesis and transport. Our initial findings that the *LAG1* and *LAC1* genes are required for ceramide synthesis in yeast have been extended. We have epitope-tagged Lag1p and Lac1p and have performed purification studies. First, we could show that the solubilized and highly-enriched subunits have ceramide synthase activity *in vitro*, showing for the first time that they are indeed part of the ceramide synthase. Second, upon purification we have found another protein that co-purifies with them. We are currently analysing this protein and its function in ceramide synthesis and its regulation.

In collaboration with the Futerman group in Israel, we have obtained evidence that expression mammalian homologs of *LAG1*/*LAC1* affect ceramide synthesis in cells (Venkataram et al, 2002).

Ceramides can be synthesised from endogenous sphingoid bases or from exogenous sphingoid bases found in the medium. This latter pathway is important for free-living organisms like yeast. We have characterized this pathway and found that for efficient incorporation of sphingoid bases into ceramides, that the sphingoid bases must be phosphorylated and then dephosphosphorylated. Surprisingly, even the phosphorylation event must take place on the membrane even though a portion of the sphingoid base kinase is soluble (Funato et al, 2002).

We have provided evidence that sphingoid bases are involved in the heat stress response and have made novel insights into the mechanism of the

sensitivity of cells to heat shock. We have shown that the *lcb1-100* mutant, which is part of the serine palmitoyltransferase and is unable to make sphingoid bases at high temperatures, is exquisitely sensitive to heat stress. The reason for this sensitivity is that the mutant does not induce synthesis of the majority of the heat shock proteins. We isolated a suppressor of the heat shock sensitivity. When overexpressed, ubiquitin allowed the cells to survive a heat-shock and to grow at 37°C. We postulated that the reason for this is that ubiquitin stimulated the proteasome-dependent degradation of misfolded proteins that accumulated at 37°C in the lcb1-100 mutant. We went on to show that the mutant cells accumulated more protein aggregates with time at 37°C than wild type cells. Mutant cells overexpressing ubiquitin did not. The mutant cells showed reduced protein turnover when compared to wild type cells and protein turnover was increased when ubiquitin was overexpressed. Furthermore, the suppression phenotype of ubiquitin overexpression depended on a functional proteasome. Therefore, we could conclude that the reason why cells die during a heat shock is not the loss of activity of some crucial heat-sensitive enzyme, but rather the accumulation of toxic aggregates. Normally the aggregates are removed by a combination of their refolding using the heat shock chaperones and by degradation using the ubiqutin-proteasome system. In the absence of the chaperone system, the ubiquitin-proteasome system is sufficient if it is expressed in high enough amounts (Friant et al, 2003).

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Membrane protein topogenesis and intracellular protein sorting

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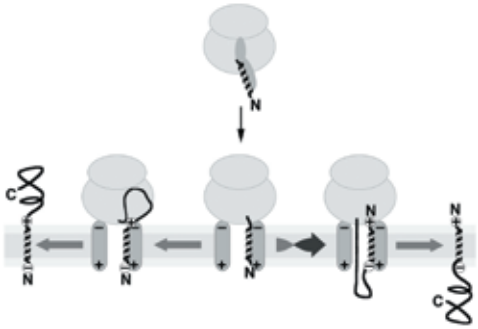
Proteins initially synthesized on cytosolic ribosomes must be sorted to the specific compartment(s) in which they perform their function. Proteins destined to the endoplasmic reticulum (ER), the Golgi apparatus, the plasma membrane, the endosomal/lysosomal system and the cell exterior are all first targeted to the ER, translocated across the membrane or inserted into the lipid bilayer, and then distributed via the secretory pathway. Our research centers on (1) how membrane proteins are sorted to the ER and acquire a defined topology in the lipid bilayer, and (2) how they are sorted at the *trans*-Golgi or in endosomes.

Membrane protein topogenesis

Secretory and membrane proteins are sorted to the ER by signal sequences which upon insertion into the membrane are either cleaved or anchor the protein in the lipid bilayer. The orientation of a signal in the membrane is determined at least in part by its flanking charges (the so-called positive-inside rule) and hydrophobicity. We are studying the insertion process *in vivo* by challenging the insertion machinery of cultured cells with diagnostic mutant proteins. Based on experiments with hydrophobic signal-anchors, the following mechanism for signal orientation emerged. N-Terminal signals initially insert head-on with a lumenal N-terminus. Depending on the flanking charges, they either remain in this orientation or invert to translocate the C-terminus. The rate of inversion increases with more positive N-terminal charge and is reduced with increasing hydrophobicity. Inversion may proceed for up to ~50 s, when it is terminated by a signal-independent process.



The driving force for signal reorientation is likely to be a local potential at the translocon. We tested whether charged amino acids of the major component of the translocation pore, Sec61a, contribute to orienting signals according to the positive-inside rule. Conserved charged residues of Sec61p of *Saccharomyces cerevisiae* were mutated and tested for the effect on the topologies of diagnostic model proteins. Mutation of R67, R74, or E382 in Sec61p reduced C-terminal translocation of a signal-anchor protein with a positive N-terminal flanking sequence and increased it for signal-anchor proteins with positive C-terminal sequences. Although these three residues do not account for the entire charge effect in signal orientation, the results showed that Sec61p contributes to the positive-inside rule.

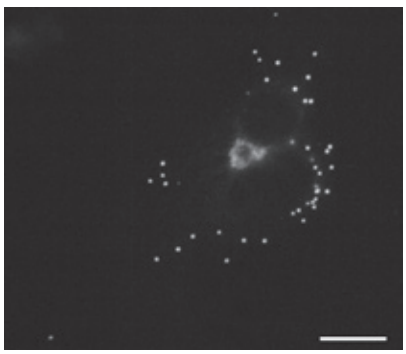
Formation of AP-1/clathrin-coated vesicles

Sorting of membrane proteins is generally mediated by cytosolic coats which serve to create a scaffold to form coated buds and vesicles, and to selectively concentrate cargo by interacting with cytosolic signals. Clathrin coats with different adaptor proteins (APs) are involved in transporting cargo proteins with characteristic tyrosine and dileucine motifs in various intracellular pathways (e.g. AP-2 in endocytosis and AP-1 in *trans*-Golgi network-to-endosome traffic). We have developed an assay in permeabilized cells to study the molecular requirements for the recycling of internalized receptors back to the plasma membrane. Depletion of AP-1 from cytosol inhibited formation of recycling vesicles, whereas readdition of purified AP-1 restored it. Vesicle formation was also stimulated by addition of clathrin and inhibited by brefeldin A. Further experiments suggest a role for rab4 and rabaptin-5 in regulating the formation of recycling vesicles at endosomes.

To analyze the molecular mechanism of AP-1/clathrin coat formation, we reconstituted the recruitment of purified AP-1 adaptors to chemically defined liposomes presenting peptides corresponding to tyrosine-based sorting motifs. AP-1 recruitment was found to be dependent on myristoylated ARF1, GTP or nonhydrolyzable GTP-analogs, tyrosine signals and small amounts of phosphoinositides, but not on any additional cytosolic or membrane-bound proteins. This suggests that cargo proteins contribute to coat formation and that the local lipid composition may specify the site of vesicle formation. Recruited AP-1 forms high-molecular weight complexes already in the absence of clathrin. The GTPase activating protein of ARF1 (ARFGAP1) triggers GTP hydrolysis in ARF1 and disassembly of the AP-1 precoat. GTP hydrolysis may therefore mediate quality control in the formation of the full coat.

Secretory granules

The peptide hormone vasopressin is synthesized as a precursor protein, sorted into secretory granules, and released from the neurohypophysis in a regulated manner to control water reabsorption in the kidney. In collaboration with Jonas Rutishauser (University Hospital, Basel), we study how mutations (some of them in the signal sequence) cause familial central *Diabetes insipidus*, a dominant degenerative disease. In the course of these experiments, we discovered by immunofluorescence that expression of pro-vasopressin and several other regulated cargo proteins in nonendocrine cells like COS and HEK293 cells generates granule-like structures which morphologically resemble secretory granules, lack markers of ER, Golgi, endosomes or lysosomes. Secretogranin II and chromogranin B show significant intracellular storage in these heterologous cells and can even be stimulated for secretion by increased intracellular calcium. This suggests that initial granule formation requires no additional machinery specific to regulated secretory cells besides the regulated cargo itself.



Immunofluorescence of pro-vasopressin expressed in COS-1 cells. Bar=20 µm.

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Friberg, M. A., Spiess, M. & Rutishauser, J. (*in press*). Degradation of wild-type vasopressin precursor and pathogenic mutants by the proteasome. *J Biol Chem*

Modulators of nuclear receptor signaling

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Lipophilic hormones play important roles in mammalian development and physiology, and have widespread applications as drugs. They exert their effects by binding and activating nuclear receptors that are ligand-regulated transcription factors. The responses elicited by the hormone-activated receptors are remarkably cell-type specific and physiological-state dependent. Our studies focus on the receptor cofactors that modulate, and thereby confer specificity or regulation to hormone signaling. To identify such modulators, we have exploited the ability of mammalian nuclear receptors to function in yeast, and identified yeast proteins that downregulate hormone responses (e.g. Pdr5p, Lem3p, and Erg6p) and mammalian modulators that enhance nuclear receptor signaling (such as the molecular chaperone p23, the chromatin assembly factor ASF1, and the transcriptional coactivators PGC-1α and PGC-1β/PERC). Understanding the mechanism of action of modulators may reveal novel intervention ways for activating, suppressing or altering the specificity of lipophilic hormone action.

The transcriptional coactivators PGC-1α and PGC-1β/PERC

PGC-1α, an inducible and tissue-specific coactivator of many nuclear receptors, has been proposed to coordinate transcriptional programs important for energy homeostasis. Increases in the levels of PGC-1α, in response to signals relaying metabolic needs, lead to the enhanced expression of programs that drive mitochondrial biogenesis, fatty acid oxidation, and gluconeogenesis. PGC-1β/PERC acts as a selective coactivator of only a few nuclear receptors, such as the estrogen and estrogen-related receptors, and carries a subset of PGC-1α functions. Decreased levels of PGC-1α and PGC-1β/PERC are associated with metabolic disease, such as insulin-resistance and diabetes. To elucidate the mechanism of action of PGC-1α and PGC-1β/PERC, we have dissected the protein domains and functions that are required for enhancement of nuclear receptor signaling, and identified interacting proteins. Current work focuses on the mechanisms that regulate the activities of the two coactivators.

The Estrogen related receptor alpha (ERRα) and mitochondrial biogenesis.

ERRα is a member of the subfamily of nuclear receptors that have no known lipophilic ligands, termed orphan receptors. Even though ERRα was the first orphan nuclear receptor to be identified in 1988, we know little about its physiological role, or the mechanisms that regulate its activity. Our recent studies have shown that ERRα is regulated by the transcriptional coactivator PGC-1α, at two levels. First, PGC-1α induces the expression of ERRα. Consistent with this, ERRα is expressed in a tissue-

specific manner, and induced in response to similar metabolic signals as PGC-1α (e.g. exposure to cold and fasting). Second, PGC-1α interacts physically with ERRα, and converts it from a factor with little or no transcriptional activity to a potent regulator of gene expression, suggesting that PGC-1α acts as a protein ligand of this orphan receptor. Our findings suggest that the two proteins act in a common pathway to regulate transcriptional responses that control cellular energy metabolism. Indeed we have shown that ERRα is an effector of the transcriptional coactivator PGC-1α, and that it regulates the expression of genes important for oxidative phosphorylation and mitochondrial biogenesis. Inhibition of ERRα, using small interfering RNAs, compromises the ability of PGC-1α to induce the expression of genes that encode mitochondrial proteins, and to increase mitochondrial DNA content. Conversely, a constitutively active form of ERRα is sufficient to elicit both responses. Functional ERRα binding sites at the transcriptional control regions of ERRα/PGC-1α-induced genes support a model where ERRα serves to recruit PGC-1α to target genes. The genes that we have identified as being regulated by ERRα are known to be expressed at reduced levels in humans that are insulin-resistant, suggesting that changes in ERRα activity could be linked to pathological changes in metabolic diseases.

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The Division of Biophysical Chemistry consists of 3 senior groups headed by Jürgen Engel, Thomas Kiefhaber and Joachim Seelig and two independent groups headed by Heiko Heerklotz and Anna Seelig. The research focuses on quantitative aspects of various biological and biochemical problems. The main topics are extracellular matrix proteins, protein folding, peptide-membrane interactions, membrane pores and *in vivo* magnetic resonance. Several methods are established in the division including optical and NMR spectroscopy, high sensitivity titration, pressure perturbation and differential scanning calorimetry, stopped-flow and laserflash kinetics, measurements of membrane permeability and peptide synthesis. In addition, Ariel Lustig provided service on analytical ultracentrifugation for the Biozentrum. J. Seelig was elected as a foreign member of the “Royal Netherlands Academy of Arts and Sciences (Koninklijke Nederlandse Akademie van Wetenschappen)” in 2003.

Extracellular matrix, cell adhesion and oligomerization

Essentially all cells of an organism are surrounded by an extracellular matrix (ECM), which mediates cell adhesion, mechanical strength of tissues, cell communication, cellular differentiation and cell migration. ECM proteins are usually very large and composed of many domains with different functions. The ECM forms a tight and multifunctional network. Oligomerization domains such as collagen triple helices and α-helical coiled-coil domains are essential for network formation. Cellular receptors and specific adhesion proteins on plasma membranes are also involved in the network and connections exist to the cytoskeleton via membrane spanning receptors such as integrins. The group explores the question how cells adhere to each other and how they communicate with their surrounding matrix. To solve the mechanisms of adhesion and interaction between matrix proteins the group applies molecular biology and biophysical techniques including structure determinations.

In particular the homoassociation of the epithelial adhesion protein E-cadherin and related classical cadherins (N-cadherin, VE-cadherin) are investigated by high-resolution electron microscopy, analytical centrifugation, spectroscopy, x-ray crystallography and nuclear magnetic resonance in collaboration with the Department of Structural Biology. For the ECM-proteins individual domains were recombinantly expressed in mammalian cells. Functional and structural information obtained for the individual domains is combined to obtain an integrated understanding of these very large and complex multidomain proteins. Examples are laminins, agrin, matrilins, thrombospondins, cartilage oligomeric matrix protein and integrin receptors.

Special attention is paid to oligomerization domains such as α-helical coiled-coil structures and collagen triple-helices, which are used in the ECM to interlink different protein subunits and to mediate formation of supramolecular structures. Very frequently a linkage between several polypeptide chains is instrumental for binding interactions and for the nucleation of folding. To explore proteins with newly introduced oligomerization domains novel proteins were designed by protein engineering. For example the trimeric phage protein foldon was fused with a collagenous domain, thus converting the very slow and concentration depending folding of three collagen chains to a triple helix into a concentration independent fast process.

The assembly of the nematocyst wall from minicollagen and a glycoprotein Nowa is studied as a relatively simple model system for a matrix structure. Nematoycysts are explosive organels and their wall withstands more than 150 atm osmotic pressure before explosion and it is of high interest how such an extremely tensile structure is constructed. They are composed of two major proteins, minicollagens and NOWA. Both proteins contain homologous Cys-rich domains, which link them by disulfide bridges. These

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intermolecular bridges are not present in precursors or recombinantly expressed proteins but are formed during maturation of the nematocyst wall.

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The mechanism of protein folding

Folding of a protein starting from the random coil is a spontaneous process *in vitro.* We use different approaches to gain insight into the mechanism of protein folding. Kinetic measurements of unfolding and refolding reactions of small model proteins are aimed at the detection and characterization of partially folded intermediates between the unfolded protein and the native state and at the characterization of the rate-limiting steps in the folding process. Studies on peptide models investigate the earliest steps in protein folding like intrachain diffusion and secondary structure formation. Different experimental techniques are applied that allow us to study conformational dynamics from the femtoseconds to the hours time range.

Elementary steps in protein folding

During folding a protein has to form interactions between specific parts of the polypeptide chain. Intrachain diffusion can thus be regarded as the elementary step in the folding process, which determines the maximum rate at which a folding polypeptide chain can explore conformational space. We developed a model system to directly measure contact formation between two defined points on a polypeptide chain using triplet-triplet energy transfer (TTET; Bieri et al., 1999).

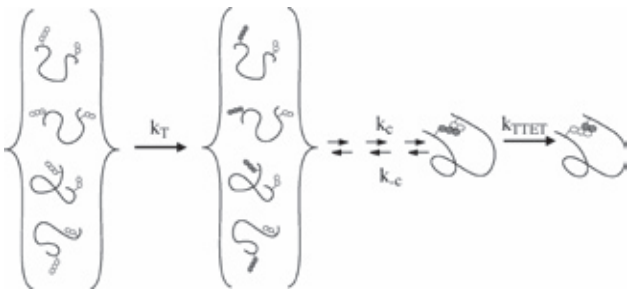

Fig 1: Schematic representation of TTET experiments from a triplet donor to a triplet acceptor group to measure intrachain diffusion in polypeptide chains.

We used various model peptides and natural loop sequences from proteins to study the effects of amino acid sequence, chain length, temperature and solvent viscosity on the process of intrachain diffusion. Single exponential kinetics of intrachain contact formation on the nanosecond time scale were observed for most peptides. The dynamics depend on the number of amino acids separating donor and acceptor and show a maximum time constant of 5-10 ns for flexible chains. These results set an upper limit for the speed of formation of the first side chain contacts during protein folding and suggest that typical protein loops can form on a timescale of 30 to 50 ns. The minimum time constant for contact formation at the ends of very tight turns is in the range of 5-10 ns.

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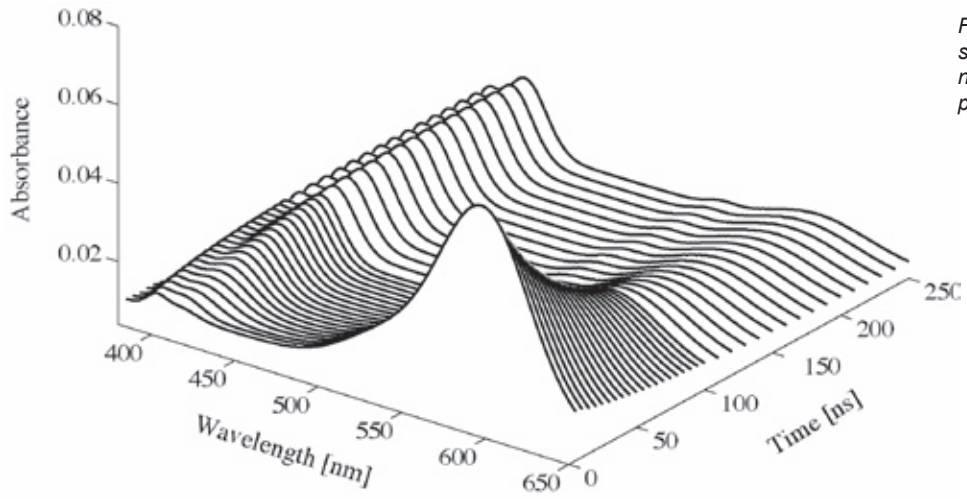

Fig. 2: Transient triplet absorbance spectra for TTET from xanthone to naphthalene attached to an unfolded polypeptide chain.

Folding mechanism of small single domain proteins

Several small proteins were shown to fold very fast (typically on the millisecond time scale) and without detectable intermediates. Our group uses several single domain proteins including the DNA-binding domain of brinker, Hsp15 and tendamistat as model systems to study folding and stability of fast folding proteins. The studies on tendamistat revealed that folding of this apparent two-state folder proceeds in at least two consecutive steps through an obligatory high energy intermediate. These results showed that defined folding pathways may also exist for apparent two-state folders (Bachmann & Kiefhaber, 2001). Comparison with the results from other apparent two-state folders revealed that obligatory high energy intermediates are general features in free energy barriers for folding of small proteins. Obviously, apparent two-state folding and folding through transiently populated intermediates share similar free energy landscapes with consecutive barriers on sequential pathways. The major difference between two-state and multi-state folding seems to be the relative stability of partially folded intermediates.

To obtain additional insight into barriers for protein folding we applied various kinetic concepts from physical organic chemistry to protein folding reactions. Analysis of folding data from our lab and of data reported in literature on other proteins allowed us to obtain a general picture of the properties of free energy landscapes for protein folding reactions. The results indicate that folding transition states are narrow regions on the free energy landscape which are robust against perturbations like mutations or changes in solvent conditions. Apparent transition state movements frequently observed in mutational studies were shown to be due to ground state effects, most commonly caused by structural changes in the unfolded state.

In summary the results on the folding mechanism of small single domain proteins showed that folding proceeds through a small number of structurally well-defined transition states. The transition states seem to be distorted native states for the major part of a protein (diffuse transition states) or for large substructures (polarized transitions states).

Evolutionary optimized protein folding reactions

Model proteins for studies on the mechanism of protein folding are usually not optimized for rapid folding but rather for function in the cell. We have investigated folding of two proteins, which have a strong evolutionary pressure for rapid and efficient folding. The first system is a protease from Semliki Forest Virus (SFVP), which has to cleave itself out of a large polyprotein chain cotranslationally during protein synthesis. Rapid folding and cleavage of SFVP is essential for the biogenesis of the more C-terminal peptide segments in the viral polyprotein, since self-cleavage of the SFVP releases a signal peptide sequence at the newly generated N-terminus of the nascent chain. This directs the remaining polyprotein to the endoplasmic reticulum. To test whether fast co-translation folding of SFVP is an intrinsic property of the polypeptide chain or whether folding is accelerated by cellular components, we investigated spontaneous folding of recombinant SFVP *in vitro*, in collaboration with the group of R. Glockshuber (ETH Zürich). The results showed that SFVP folds more than two orders of magnitude faster than any previously studied two-domain protein (τ=50 ms), and that structure formation in the N-terminal domain precedes folding of the C-terminal domain. This demonstrates that co-translational

folding of SFVP does not require additional cellular components and suggests that rapid folding is the result of molecular evolution towards efficient virus biogenesis.

The second model protein to study an evolutionary optimized folding reaction is the C-terminal domain from the trimeric phage T4 protein fibritin. The only known function of this small domain (3x27 amino acids) is the promotion of rapid folding and association of fibritin. In collaboration with the group of S. Grzesiek (Department of Structural Biology) we could show that a chemically synthesized foldon-domain folds into the same trimeric β-propeller structure as in full-length fibritin (3x486 amino acids) and undergoes a two-state unfolding transition from folded trimer to unfolded monomers.

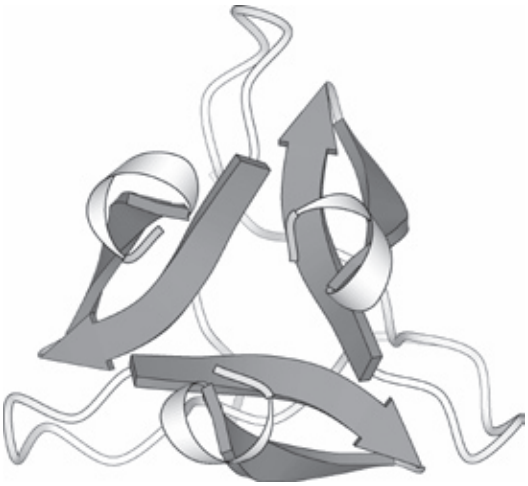

Fig. 3: Structure of the trimeric foldon domain.

The folding kinetics of the foldon domain involve several consecutive reactions. Structure formation in the region of the single β-hairpin of each monomer occurs on the submillisecond time scale. This reaction is followed by two consecutive association steps with rate constants around $6 \cdot 10^6$ $M^{-1}s^{-1}$, which is similar to the fastest reported bimolecular association reactions for folding of dimeric proteins. At low protein concentrations folding shows apparent third-order kinetics. At high protein concentrations it becomes independent of protein concentration with a half-time of about 3 ms, indicating that a first-order folding step from a partially folded trimer to the native protein becomes rate-limiting. Our results suggest that all steps on the folding/trimerization pathway of the foldon domain are evolutionary optimized for very rapid and specific initiation of trimer formation during fibritin assembly.

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Membrane interactions and *in vivo* magnetic resonance

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Thermodynamics of peptide-membrane interactions is a complex process. It depends on the chemical nature of the lipids, peptides, and carbohydrates involved and also on the mechanistic nature of the processes investigated. Different rules apply for trans-membrane insertions than for half-sided embedding. Electrostatic forces (both coulombic attraction / repulsion and dipolar interactions), hydrogen bond formation, and hydrophobic interactions play equally important roles. The enormous interest in “lipid rafts” has sensitized the scientific community to realize that the lipid part of the membrane is not simply a homogenous grease but comprises an enormous variety of lipid molecules of hitherto unknown functions. Depending on the membrane composition, groups of specific lipids may aggregate into patches with physical properties distinctly different from those of other membrane domains. The interaction of a peptide with the lipid membrane can be divided into three steps. (i) Binding is initiated by the electrostatic attraction of a usually cationic peptide to the anionic membrane. (ii) The next step is the transition of the peptide into the plane of binding which is followed by (iii) a conformational change of the peptide in the new environment (most often a transition to a partially α-helical structure).

The second topic of our research is *in vivo* magnetic resonance. *In vivo* Magnetic Resonance Imaging (MRI) provides anatomic images of living systems. Functional magnetic resonance imaging (fMRI) goes one step further and provides insight into brain activity. Magnetic Resonance Spectroscopy (MRS) detects the metabolic processes in well-defined spatial areas of the living animal without the use of invasive methods.

Hydrophobic partitioning

A good example for a purely hydrophobic partitionig is cyclosporin A, a peptide which has found wide-spread pharmaceutical applications as an immunosuppressant. It is a cyclic peptide of 11 amino acids, barely soluble in water but easily dissolved in oil. The binding (the physical adsorption) of the peptide to the lipid bilayer is associated with a considerable release of heat and the partitioning into the membrane can thus be studied with high-sensitivity isothermal titration calorimetry (ITC). The characteristic parameters determined are the partition constant $K = 2 \times 10^3\ M^{-1}$ and the partition enthalpy $\Delta H^0 = +\ 12.9$ kcal/mol at 20°C (Schote *et al.* 2002, *Pharm. Sci. 91*, 856-72).

Key-lock interactions

Specific phospholipid-peptide interactions are rare. One of the very few known exceptions is the tetracyclic peptide Ro 09-0198 (cinnamycin). Ro 09-0198 forms a tight equimolar complex with phosphatidylethanolamine. We have established the thermodynamic parameters of this equilibrium with isothermal titration calorimetry and have studied the structural consequences with nmr. Complex formation is characterized by a large binding constant of $K_0 = 10^7 - 10^8\ M^{-1}$. The same reaction was also investigated by

dissolving both compounds in hydrophobic micelles and serves as a model system for the interaction of water-insoluble membrane proteins with their substrates (Machaidze *et al*. 2002, *Biochemistry 41*, 1965-71, Machaidze & Seelig 2003, *Biochemistry 42,* 12570-6).

Thermodynamics of the membrane-induced random coil ⇄ α-helix transition

Amphipathic α-helices are the membrane binding motif in many proteins. The corresponding peptides are random coil in solution but are folded into a α-helix upon interaction with the membrane. While the thermodynamics and cooperativity of the random coil-to-α-helix transition in solution have been investigated extensively, the corresponding membrane-induced processes have been approached only recently. A systematic study was undertaken with the 23aa antibacterial frog peptide magain 2 amides. M2a is random coil in buffer but adopts an essentially α-helical conformation when bound to a negatively charged membrane. The spectral deconvolution of the CD-spectrum of the bound peptide yields an α-helix content of about 74% at room temperature. The helix content was varied by substituting two adjacent amino acids by their D-enantiomers. For d4.5 M2a the helix content is 53%, for d11,12 M2 only 29%. Using titration calorimetry it was possible to measure the corresponding binding enthalpies and binding isotherms and to plot the thermodynamic parameters as a function of helicity. To a good approximation, straight lines are obtained. From the intercept with the ordinate (0% helicity) the binding enthalpy (and the free energy) of a hypothetical random coil peptide can be deduced while the slope yields the additional contributions per helical segment (Wieprecht *et al.* 1999, *J. Mol. Biol. 294*, 785-94, Wieprecht *et al.* 2002, *Biopyhs. Chem. 96*, 191-201, Wieprecht *et al.* 2000, *Biochemistry 39*, 15297-305, Wieprecht & Seelig 2002, in *Current Topics in Membranes* Peptide-Lipid Interactions, Vol. 52, (eds. S.A. Simon and T.J. McIntosh) Elsevier Science, pp. 31-56).

Cell penetrating peptides

Cell penetrating peptides (CPPs) are highly charged molecules which traverse cell membranes of *cultured cells* very efficiently by a mechanism not yet identified. Recent theories suggest either the *formation of inverted micelles* with negatively charge lipids or the binding of CPPs to the extracellular glycosaminoglycans. We have investigated the binding of the protein transduction domain (PTD) of the human immunodeficiency virus (HIV-1) TAT peptide to negatively charged phospholipid membranes with a variety of physical chemical techniques, including high sensitivity titration calorimetry and solid state NMR. The data show that the bilayer remains intact upon TAT-PTD binding. The NMR data provide no evidence for a non-bilayer structure or for domain formation. Membrane binding is caused by essentially electrostatic attraction with little penetration into the hydrophobic core (Ziegler *et al.* 2003, *Biochemistry 42*, 9185-94). Calorimetric methods were used to elucidate the binding of HIV-1 TAT-PTD to heparin sulfate, heparan and related compounds (Ziegler & Seelig 2004, *Biophys. J. 86*, 254-63). The polysulfonated macromolecules exhibit multiple identical binding sites and the binding is essentially a charge neutralization reaction.

Membrane interactions with detergents and Cyclodextrin

The partition equilibrium of sodium dodecyl sulfate (SDS) between water and bilayer membranes was investigated with isothermal titration calorimetry. The partitioning of the dodecyl sulfate anion (DS^-) into the bilayer membrane is energetically favored by an exothermic partition enthalpy of ΔH_D^0 = -6.0 kcal/mol at 28°C. The partition enthalpy decreases linearly with increasing temperature and the molar heat capacity is ΔC_p^0 = -50 ± 3 cal mol^{-1} K^{-1}. The partition isotherm is non-linear if the bound detergent is plotted versus the free detergent concentration in bulk solution. This is caused by the electrostatic repulsion between the DS^- ions inserted into the membrane and those free in solution near the membrane surface. The surface concentration of DS^- immediately above the plane of binding was hence calculated with the Gouy-Chapman theory and a strictly linear relationship was obtained between the surface concentration and the extent of DS^- partitioning. The surface partition constant K describes the chemical equilibrium in the absence of electrostatic effects (Tan *et al.* 2002, *Biophys. J. 83*, 1547-56).

Cyclodextrins are able to bind hydrophobic molecules in their interior cavity and as such have received a great deal of attention as carriers of cholesterol, lipophilic drugs, and other sparingly soluble compounds. Despite the importance of these biochemical applications, relatively little is known about the interactions of cyclodextrins with phospholipids membranes. We have characterized the binding of randomly methylated β-cyclodextrin (mβCD) to 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC). Existing models of lipophile-membrane interactions are inadequate to describe the observed binding; we introduce a modified chemical reaction model in which the chemical activity of the phospholipids is independent of its concentration (Anderson *et al.* 2004, *Biochemistry 43*, 2251-61).

***In Vivo* magnetic resonance**

^{13}C-NMR spectroscopy at 7 Tesla provides the sensitivity and spectral resolution to detect ^{13}C-labeled metabolites in rat and mice at sub-millimolar concentration. Myocardial substrate uptake, substrate preference and metabolism are difficult to assess non-invasively. We have extended cardiac ^{13}C-NMR spectroscopy to the *in vivo* situation ('closed-chest model') and have quantitated the myocardial metabolism *in vivo*. Overnight-fasted Sprague-Dawley

rats received intravenous infusions of non-radioactive ^{13}C-labeled glucose, 3-hydroxybutyrate, and acetate as markers for glycolysis, metabolism of ketone bodies and direct incorporation into tricarboxylic acid (TCA) cycle, respectively. *In vivo* ^{13}C-NMR spectra were acquired from the myocardium with a time resolution of 6 min. 3-Hydroxybutyrate and acetate were rapidly extracted by the myocardium and supplied 42 ± 6 and 53 ± 9% of the acetyl-CoA for TCA cycle operation whereas glucose, although also well extracted, did not contribute to myocardial oxidative metabolism (Ziegler *et al.* 2002, *NMR Biomed. 15*, 1-13). We have measured the gastrointestinal transit times in mice and humans with ^{27}Al- and ^{19}F-nuclear magnetic resonance. Al^{3+} bound to ion-exchange resin and perfluorononane were administered orally as selective and specific markers for the stomach and the entire GI tract, respectively. ^{27}Al- and ^{19}F-NMR were employed to follow boli of the mixed markers. The MR-data revealed that gastric emptying in humans proceeded linearly whereas in mice an exponential decay was observed (Schwarz *et al.* 2002, *Mag. Reson. Med. 48,* 255-61). As an aside, we have characterized the unusual chemical and structural properties of our contrast agent, perfluorononane, with ^{19}F- and ^{13}C-multidimensional NMR spectroscopy (Schwarz *et al. in press*, *MCR*). In a combined *in vivo* and *in vitro* study we have investigated the metabolism of the non-steroidal anti-inflammatory drug niflumic acid in humans (Bilecen *et al.* 2003, *NMR Biomed. 16*, 144-51). Finally we have elucidated the metabolic origin of the brain osmolyte N-acetyl-aspartate by either 2 h infusion of [U-$^{13}C_6$] glucose or by long-term feeding of rat with chow containing either [U-$^{13}C_6$] glucose or [U-^{13}C] biomass (a mixture of proteins, lipids, DNA, and carbohydrates) during 3-5 days (Karelson *et al.* 2003, *NMR Biomed. 16*, 413-23).

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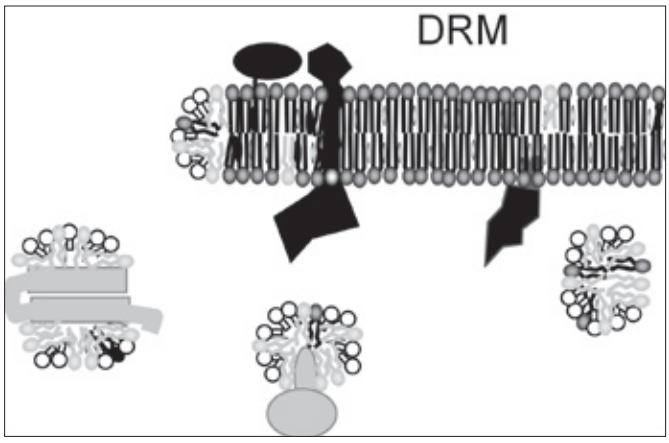
Lipid-surfactant systems

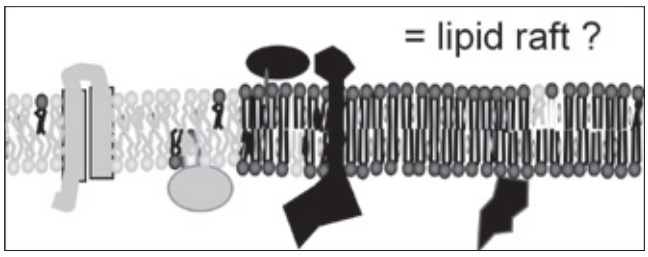
Over several years we have been studying the interaction of amphiphilic compounds such as detergents, peptides or others with lipid membranes by a variety of methods. One means to obtain new insight has been the development of new microcalorimetric assays and techniques. Rather comprehensive review articles were completed last year on both lipid-surfactant systems (Heerklotz 2004a) and microcalorimetric techniques (Heerklotz 2004b). Three major research projects have been pursued as outlined below.

Detergent-resistant membranes and lipid rafts

The raft concept is mainly based on the finding that the application of the detergent triton (white in cartoon) to cells may lead to the selective solubilization of certain lipids such as unsaturated phosphatidylcholine (PC) and proteins (grey), whereas, for example, sphingomyelin (SM), cholesterol (Cho) and GPI-anchored proteins (black) are left unsolubilized in so-called detergent-resistant membrane fragments (DRMs).



It has been argued that these DRMs may resemble functional domains, lipid rafts, that are present in the native membrane. These rafts are assumed to be formed by spontaneous de-mixing of SM/Cho from a PC-rich matrix.



Lipid rafts are believed to play a crucial role in cell signaling, trafficking, etc. However, in spite of the enormous interest and thousands of papers dealing with the topic, even the existence and basic

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properties of rafts are still debated. We have shown (Heerklotz 2002) that the detergent changes not only the properties of the PC-rich environment but also those of potentially existing SM-rich domains. In the extreme case, it can even induce domain formation in a previously homogeneous membrane. Hence, DRMs may differ from rafts in size, composition, structure, and even in existence. We have explained these effects quantitatively on the basis of lipid-lipid and lipid-detergent interactions (Heerklotz et al. 2003a).

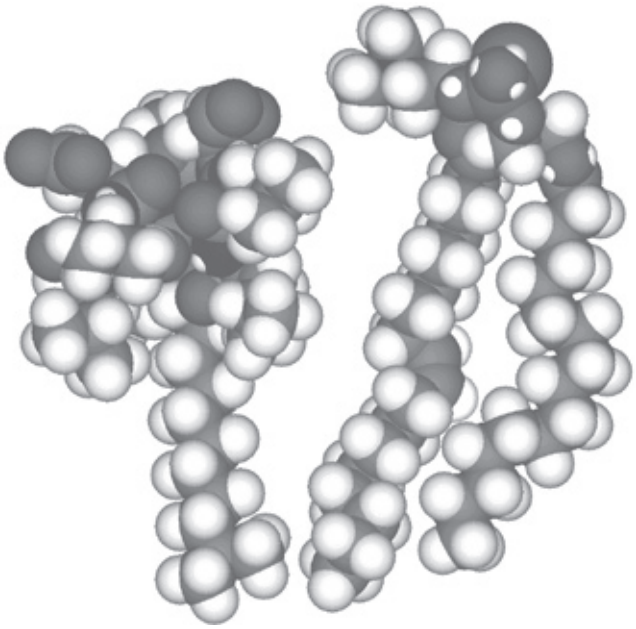
Recent reviews (Edidin 2003; Munro 2003) and the Faculty of Thousand (2003) have quoted our work as one of the most important studies challenging the recent opinion and assumptions on lipid rafts.

Pressure perturbation calorimetry (PPC)

The volume of biological macromolecules in solution and its variation with temperature are important quantities when it comes to understand the internal packing of the molecule or assembly and the effects of solvation of the accessible surface area. These properties could hardly be measured until recently, when PPC has become available. We were the first to apply the new technique to lipid vesicles (Heerklotz 2002; Heerklotz and Seelig 2002; Heerklotz et al. 2003b). Now we are also studying the volumetric effects of protein denaturation, micelle formation or micellar shape transitions in order to relate the volumetric, structural, and thermodynamic effects to each other.

Detergent-like behavior of antibiotic peptides

There is a consensus that antibiotic peptides kill bacteria by permeabilizing their membrane. They act either by forming pore-like oligomers or by a rather unspecific destabilization of the lipid bilayer ("detergent-like" mechanism).



The figure shows the antibiotic lipopeptide surfactin and a phospholipid. We have studied the effects of surfactin on lipid membranes in detail with respect to thermodynamic (Heerklotz and Seelig 2001), structural (Heerklotz and others 2004), and functional (Heerklotz and Seelig, in preparation) effects.

These studies have provided insight into the nature of membrane effects that may account for the antibiotic activity of surfactin and other host-defense peptides.

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Passive influx and active efflux by ABC transporters

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Biological membranes are selective diffusion barriers. The barrier permeability is regulated, on one hand, by the characteristics of the lipid bilayer which represents the diffusion barrier proper and, on the other hand, by the expression level of different transporters. Transporters either move hydrophilic compounds across the lipid bilayer or efflux hydrophobic compounds (e.g. drugs or toxins) from the lipid bilayer to the extracellular environment. The latter belong to the family of ATP Binding Cassette, ABC, transporters. Important exponents are P-glycoprotein, P-gp, and the multidrug resistance protein, MRP1 which play a role in multidrug resistance. To understand transport of hydrophobic drugs across a biological membrane at the molecular level, the relationship between passive influx and active efflux has to be analyzed.

Membrane binding and permeation

Most drugs permeate biological membranes by passive diffusion. The extent of permeation depends on both the properties of the lipid bilayer and the properties of the diffusing molecule. Biological membranes are highly organized, anisotropic systems that are nevertheless fluid enough to allow considerable translational, rotational, and flexing movements of the constituent lipid and protein molecules. Under physiological conditions the lipid bilayer membrane is in a liquid crystalline state (Seelig & Seelig 2002, *Encycl. Phys. Sci. Techn. Vol.9,* 355-67). In contrast, most of the membrane-mimicking systems in use today are isotropic organic solvents. A much better model system for a lipid bilayer is a lipid monolayer which has the advantage of easier handling and greater flexibility. It can be compressed to a density equivalent to that of a lipid bilayer and the penetration of drugs into the lipid phase can be analyzed in a quantitative manner (Seelig 1987, *BBA 899,* 196-204).

The parameters of the drug with the strongest impact on membrane binding and permeation are hydrophobicity (which is well reflected by the air-water partition coefficient, K_{aw}) and the cross-sectional area, A_D. Whereas partitioning into an isotropic organic solvent generally increases with the molecular volume partitioning into an anisotropic lipid bilayer decreases exponentially with increasing cross-sectional area, A_D, of the molecule (Fischer *et al.* 1998, *J. Membrane Biol. 165*, 201–11; Gerebtzoff *et al. in press*, *ChemBioChem.*).

The first step in the process of passive diffusion is binding of the compound to the lipid membrane. To understand this step we have extensively measured binding of peptides (e.g. TAT peptide (Ziegler *et al.* 2003, *Biochemistry 42,* 9185-94) or drugs to lipid bilayers. We have then shown that binding and

diffusion can be perfectly predicted on the basis of data obtained from surface activity measurements (K_{aw} and A_D) either by means of a simple calibration diagram (Fischer *et al.* 1998, *J. Membrane Biol. 165*, 201–11) or by calculation (Gerebtzoff *et al. in press*, *ChemBioChem.*).

Active efflux by ABC tansporters

P-glycoprotein, P-gp, a product of the multidrug resistance gene, *MDR1,* is an efflux transporter with a broad substrate specificity that protects cells against exogenous and endogenous toxic compounds. We have shown that the broad specificity can be well explained by assuming that P-gp and MRPI recognize not one single “pharmacophore” but two distinct acceptor patterns, called Type I and Type II units, which are formed from H-bond acceptor groups in a specific geometric arrangement and can appear in several copies, n, per substrate, where n ≈ 1 – 12 for Type I and/or n ≈ 0 – 3 for Type II units. We have also shown that the free energy of substrate binding to the transporter within the lipid membrane is the sum of the individual free energies of binding of the different patterns (Seelig 1998, *Eur. J. Biochem. 251*, 252-61; Seelig 2003, in *Drug Bioavailability/ Estimation of Solubility, Permeability, and Absorption,* 461-92; Seelig & Gatlik *in press*, *Hot Topics Issue/ Mini-Reviews in Medic. Chem.*).

Moreover, we have shown that binding of a drug from water to the transporter occurs in two steps, a partitioning step from water to the lipid membrane, characterized by a lipid-water partition coefficient, K_{lw}, and a binding step from the lipid membrane to the transporter, characterized by a binding constant, K_{lt}. The overall binding constant from water to the transporter, K_{tw} can thus be expressed as product of two individual binding constants K_{lw} and K_{tl}. The binding constants K_{lw} and K_{tw} are available experimentally, and K_{tl} can then be determied. The value of K_{tl} obtained experimentally is consistent with the model discussed above suggesting an interaction between H-bond acceptor groups in Type I and Type II units of substrates and H-bond donor groups in the transmembane sequence of the transporter (Seelig 2003, in *Drug Bioavailability/Estimation of Solubility, Permeability, and Absorption,* 461-92; Seelig & Gatlik *in press*, *Hot Topics Issue/Mini-Reviews in Medic. Chem.*).

Using a silicon-based pH sensitive potentiometric sensor (Cytosensor Microphysiometer) that monitors the extracellular acidification rate, ECAR, in real time we have measured the first P-gp activation/inhibition profile as a function of concentration in living MDR1 transfected cell lines (Landwojtowicz *et al.* 2002, *Biochemistry 41,* 8050-7).

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DIVISION OF PHARMACOLOGY/NEUROBIOLOGY

The division at present houses 5 senior research groups headed by Yves-Alain Barde, Hans-Peter Hauri, Karl Hofbauer, Urs A. Meyer and Markus Rüegg, and a junior group headed by Anita Lüthi. Karl Hofbauer holds an endowed chair financed by the Novartis Research Foundation. Yves-Alain Barde has been appointed as a new professor of the Division Pharmacolog/Neurobiology in 2003. Andreas Lüthi has been promoted to SNF professor of the Swiss National Science Foundation and was appointed as a group leader at the Friedrich Miescher Institute in Basel in 2003. Dieter Walz has retired in 2003.

In contrast to other divisions of the Biozentrum, which are part of the Natural Science Faculty of the University (Phil. II), the Division of Pharmacology/ Neurobiology is also an institute of the Medical Faculty of the University of Basel. In this context it is responsible for the teaching of pharmacology and pharmacotherapy to students of medicine and dentistry. In the Biology Curriculum, the members of the division have a major role in teaching of neurobiology, physiology, and cell biology for undergraduate students. They also teach neurobiology and molecular medicine in the graduate program of the Biozentrum.

The research activities of each group are described below.

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Molecular control of neuronal survival and axonal elongation in development and diseases

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Scope of research

We are interested in the molecular pathways controlling neuronal survival, axonal elongation and activity-dependent plasticity in higher vertebrates. To understand mechanisms relevant to development and diseases we use neurotrophins as dissecting tools, as well as cellular assays based on differentiated embryonic stem cells.

Neurotrophins

While not detected in the genome of *C. elegans* and *Drosophila melanogaster*, the members of the small neurotrophin family are involved in virtually all aspects of the biology of vertebrate neurons. These include the control of cell survival and of axonal elongation, as well as neurotransmission and activity-dependent modification of synaptic function. While this field of research has its conceptual and experimental origin in development biology, there is increasing interest in the role of neurotrophins in the adult, both intact as well as injured nervous system. For example, the neurotrophin receptor p75 plays a significant role in inhibiting axonal elongation and in causing cell death during development. But in the adult, this receptor is re-expressed by many neurons following lesions such as axotomy or in pathological conditions including brain ischemia and epilepsy. P75 is also known to bind a number of ligands others than neurotrophins, including a viral envelope glycoprotein and the plaque-forming peptide A-β, thus further suggesting a link between this receptor and pathologies of the nervous system. Remarkably, p75 is expressed at significant levels by adult basal forebrain cholinergic neurons, even in the absence of injury. These neurons are known to be involved in selective attention and memory, and we are interested to test the possibility that the growth of these neurons and of their processes is controllably held in check by the expression of p75. With regard to brain-derived neurotrophic factor (BDNF), a range of intriguing observations links the levels of expression of this neurotrophin with complex behavioural patterns in rodents, including the regulation of food intake and with experimental models of depression, and in human, with episodic memory.

To learn about the role of the neurotrophin signalling system in the adult, it is desirable to use methods allowing selective gene deletion in specific brain areas. With the help of novel *in vitro* approaches (see below) we are attempting to use chemicals to trigger selective gene excision when required.

Embryonic stem cells

Recently, our group has begun to use mouse embryonic stem cells to complement with cellular assays our *in vivo* research. While these cells have an unlimited potential to divide they can also be

selectively guided towards specific differentiation pathways. We found that stem cells can generate essentially uniform populations of neurons with the characteristics of cortical pyramidal cells. This was made possible following the accidental observation that embryonic stem cells can be directed to form an essentially pure population of a specific sub-type of neuronal precursors, namely radial glial cells. As mouse stem cells can be genetically manipulated they represent an attractive tool to test the feasibility and completeness of gene deletion in neurons using chemically-induced, targeted gene excision. Also, these cells should allow us to better understand the biochemistry of neuronal differentiation and of axonal elongation. Our stem cell-derived neurons respond to neurotrophins and we have begun to use them in biochemical experiments to study the regulation of mRNA translation by neurotrophins, including BDNF in particular. Preliminary results suggest that the pathway controlled by TOR may be involved and in collaboration with the Hall group at the Biocenter, we would like to isolate TOR and its associated proteins from our cultured, differentiated neurons. This work should shed light on the mechanisms leading to the selective localization of TOR in cellular compartments such as dendrites. We also use radial glial cells generated from embryonic stem cells in transplantation studies to examine their differentiation potential in an *in vivo* environment. To this end we use the developing chick embryo as a recipient organism and cell as well as markers to identify mouse-derived cells and their progeny in the chick developing nervous system.

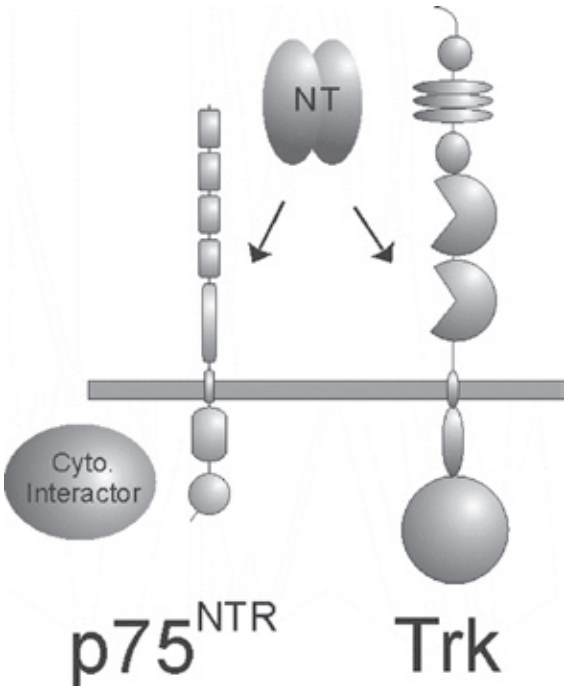

Fig.1: Neurotrophins (NT) use a dual receptor system to signal. The outcome can be as different as the prevention of cell death and the promotion of axonal elongation through the activation of tyrosine kinase receptors of the Trk family, or the prevention of axonal elongation and cell death through that of p75. The p75 receptor uses cytoplasmic interactors to transduce its message within the cell while Trk is a transmembrane receptor kinase.

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Protein traffic in the secretory pathway

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Understanding the molecular mechanisms underlying the secretory pathway is a major goal of cell biology and biomedical research. In a eukaryotic cell hundreds of different proteins are synthesized in the endoplasmic reticulum (ER) and subsequently sorted along the secretory pathway or secreted to the outside of the cell. The current challenge is to identify all key molecules that catalyze each of the numerous steps in protein sorting and to integrate their function to understand how organelles are formed and maintained with characteristic structure and function and how itinerant proteins are separated from resident proteins. We are studying major questions related to these issues in mammalian cells using morphological, biochemical, biophysical, and molecular approaches. Knowledge of these fundamental processes may ultimately lead to new strategies for the treatment of inherited and acquired diseases in which protein secretion is impaired.

Dynamics of the ER-Golgi intermediate compartment (ERGIC) in living cells

The ERGIC is an intermediary transport station between the ER and the Golgi apparatus. It consists of numerous tubulovesicular membrane clusters that can be identified by the marker protein ERGIC-53, a cycling type I membrane protein with mannose-specific lectin activity. To elucidate if the ERGIC clusters are transport vehicles moving from ER to Golgi or stable entities, we have visualized GFP-ERGIC-53 in living cells and observed that a majority of ERGIC clusters are long-lived and do not move to the Golgi apparatus. The live imaging data are inconsistent with the notion that the ERGIC clusters are simply anterograde transport vehicles. Current efforts focus on the mechanism of sorting of anterograde and retrograde protein traffic in the ERGIC in living cells.

ER-microtubule interaction

Structure and dynamics of the ER of higher eukaryotes depend on membrane-cytoskeleton interactions, primarily involving microtubules. It is assumed that this interaction is mediated by motor and non-motor proteins although little is known at the molecular level. We have discovered that the integral ER membrane protein CLIMP-63, formerly termed p63, binds microtubules *in vivo* and *in vitro*. CLIMP-63 is so far the only known integral membrane protein of the ER with microtubule-binding activity. It localizes to the reticular sub-domain of the ER but is excluded from the nuclear envelope. Since overexpression of CLIMP-63 changes ER and microtubule morphology in parallel, this protein may be an ER morphogen stabilizing ER-microtubule interaction. Using site-directed mutagenesis in conjunction with morphological and microtubule binding assays we are testing the hypothesis that the CLIMP-63-

mediated interaction of the ER with microtubules is regulated by phosphorylation.

Selective protein export from the ER

ER export is either selective or non-selective depending on the protein. We use ERGIC-53 as a model protein to uncover the mechanism of selective ER-export. ERGIC-53 is a dimeric and hexameric type I membrane protein with lectin activity that operates as a cargo receptor mediating efficient transport of some secretory glycoproteins including cathepsin C, cathepsin Z and blood coagulation factors V and VIII. We have discovered an unanticipated role of cytosolically exposed C-terminal amino acids of type I membrane proteins in ER export. Three different minimal motifs (FX or YX; LL or II; XV) were defined that facilitate ER export. The motifs are common among type I membrane proteins but also appear to be required for efficient transport of polytopic membrane proteins including presenilins and ion channels. The motifs mediate binding to coat protein II (COPII) that drives transport vesicle formation at the ER. Most strikingly a single C-terminal valine can act as a transport signal when attached to a reporter protein. We have now established a complete map of ER export determinants for ERGIC-53. ER export requires a phenylalanine motif at the C-terminus in conjunction with a critical glutamine in the cytoplasmic domain. Disulfide bond-stabilized oligomerization is also required. Efficient hexamerization depends on the presence of a polar and two aromatic residues in the transmembrane domain. ER export is also influenced by transmembrane domain length, 21 amino acids being most efficient. The results suggest an ER-export mechanism in which transmembrane and luminal determinants mediate oligomerization required for efficient recruitment of ERGIC-53 into budding vesicles via the C-terminal COPII-binding phenylalanine motif. We propose that selective export of membrane proteins requires cytosolic ER-export motifs interacting with COPII and, in many cases, oligomerization.

Cargo/receptor mechanism of ERGIC-53

ERGIC-53 is an excellent model protein to study receptor-mediated glycoprotein transport from the ER. Optimal binding of newly-synthesized glycoprotein cargo to ERGIC-53 in the ER requires glucose-trimming of the high-mannose glycans and calcium. Cargo release occurs in the ERGIC by an unknown mechanism. *In vitro* studies have now revealed efficient binding of purified ERGIC-53 to immobilized mannose at pH 7.4, the pH of the ER, but not at slightly lower pH. A conserved histidine in the center of the carbohydrate recognition domain is required for lectin activity suggesting it may serve as a molecular pH/calcium sensor. Acidification of cells inhibited ERGIC-53's association with the cargo glycoprotein cathepsinZ and dissociation was impaired by organelle neutralization. The results suggest that a pH-induced structural change of ERGIC-53 triggers glycoprotein release and establish the ERGIC as the earliest low pH compartment of the secretory pathway.

Role of animal L-type lectins in protein traffic

ERGIC-53 and the related proteins ERGL and VIP36 constitute the family of animal L-type lectins. These lectins are related to leguminous plant lectins. In order to search for new family members we established consensus profiles and screened available data bases. The search identified a new family member we termed VIPL since it is most highly related to the lectin VIP36. Unlike VIP36 and ERGIC-53 which cycle early in the secretory pathway, VIPL appears to be an ER protein that is retained in this organelle by a di-arginine motif. Overexpression of VIPL redistributes ERGIC-53 to the ER suggesting it may regulate the cycling of ERGIC-53.

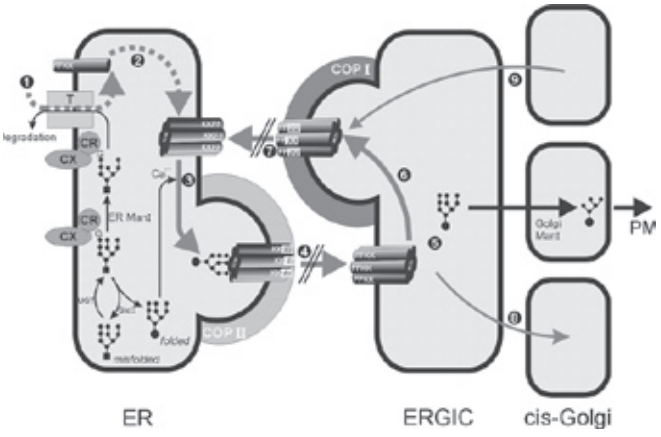

Fig. 1: The different steps in cargo receptor function and recycling of the transport receptor ERGIC-53

Function of ERGIC-53

Non-functional ERGIC-53 is the molecular basis of a human genetic disease termed combined factor V and VIII deficiency that leads to hemophilia. Surprisingly, a novel calcium-binding protein that binds to ERGIC-53, termed MCFD2, also leads to the same disease. We are currently studying the role of MCFD2 for the cargo receptor function of ERGIC-53. In search of additional functions of ERGIC-53 we have tested the possibility that ERGIC-53 is a target of the unfolded protein response (UPR). Using inducers of UPR we indeed found an upregulation of ERGIC-53 that could be fully accounted for by the ATF6 pathway

of UPR. Unlike previous reports, we postulate that in mammalian cells the UPR also affects traffic from and beyond the ER, very much like in yeast cells. It remains to be investigated, whether or not ERGIC-53 plays a role in quality control in the ERGIC.

Search for novel cycling proteins by proteomics of the ERGIC

Proteins cycling in the early secretory pathway play important roles in membrane trafficking. They tend to accumulate in the ERGIC upon treatment of cells with brefeldin A (BFA). We established a novel procedure for the isolation of ERGIC membranes with high purity from BFA-treated cells and identified several known and new cycling proteins by mass spectrometry. A novel 32kD protein localizing to the ERGIC, termed ERGIC-32, was characterized in detail. ERGIC-32 is related to Erv41p and Erv46p, two homologous proteins that play a role in ER-to-Golgi traffic in yeast. Other cycling proteins that may operate as transport receptors are being characterized. Our approach seems suitable to ultimately identify all the major proteins cycling in the early secretory pathway.

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Appenzeller-Herzog, C., Roche, A., Nufer, O. & Hauri, H. P. (*in press*). pH-induced conversion of the transport lecting ERGIC-53 triggers glycoprotein relase. *J Biol Chem*

New pharmacological concepts for the therapy of obesity and cachexia

New concepts for the pharmacotherapy of obesity continued to be the main topic of our research activities during the past two years. We were interested to find out which pharmacological approaches for the induction of weight loss would lead to a concomitant reduction of cardiovascular risk factors. Since the hypothalamic melanocortin system appears to be involved in the integration of peripheral hunger or satiety signals with the regulation of autonomic function, we focused our efforts on the melanocortin pathways in the central nervous system, in particular the hypothalamic melanocortin 4 receptor (MC4R).

Several pharmacological tools for the assessment of MC4R function have been designed and evaluated *in vitro*. Experiments with antisense oligonucleotides and siRNAs against the MC4R showed promising results in cell culture systems but still need to be characterized in animal models *in vivo*. Antibodies against the C-terminus of the MC4R should become available in the near future. A collaborative effort with the group of Prof. Andreas Engel on the expression, purification and crystallisation of the MC4R has been initiated and should help us to gain more insight into the molecular properties of this target protein.

So far the most promising *in vivo* results have been obtained with synthetic low molecular weight agonists and antagonists in chronically instrumented rats. The implantation of telemetry transmitters makes it possible to monitor blood pressure, heart rate, body temperature and locomotor activity over periods of several weeks to months. An example of such studies is a series of experiments in which we investigated the effects of the gut hormone PYY3-36 on food intake and cardiovascular function. PYY3-36 is released from the intestines after meals and has an appetite suppressing (anorexigenic) effect in experimental animals and man by an action on NPY Y 2 receptors in the hypothalamus. In our telemetry system in rats we could demonstrate that the peripheral administration of this peptide hormone increased blood pressure and heart rate by a central mechanism depending on the dietary state. While in fasted rats a small but consistent response was observed, no effects were seen in rats on a high fat diet. Rats on a standard diet showed an intermediate response. In further studies it was shown that these effects are probably mediated by the activation of MC4R.

These results show how closely the regulation of energy balance is interrelated with that of cardiovascular function. However, our data also suggest that appetite suppression via MC4R activation may always be associated with a rise in blood pressure. If this was also the case with other pharmacological approaches appetite suppressants

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in general may have an inherent risk to induce hypertension which would limit the beneficial effects of weight loss. The results of these studies have been presented at several national and international meetings and were recently published (Nordheim, U. and Hofbauer, K.G.: Stimulation of NPY Y2 receptors by PYY 3-36 reveals divergent cardiovascular effects of endogenous NPY in rats on different dietary regimens. Am J Physiol Regul Integr Comp Physiol 286: 138-142, 2004).

Our program on obesity has recently been supplemented by a program on cachexia. This syndrome, which consists of anorexia and a loss of fat and lean body mass, is frequently associated with chronic diseases such as cancer or infections. Since it represents an independent risk factor for morbidity and mortality efficient treatment would provide a significant therapeutic benefit for the patients. Our program is focused on the identification and characterization of MC4 receptor antagonists. This makes it particularly suitable for our group because we can build on our expertise in the obesity field and the MC4R as a molecular target.

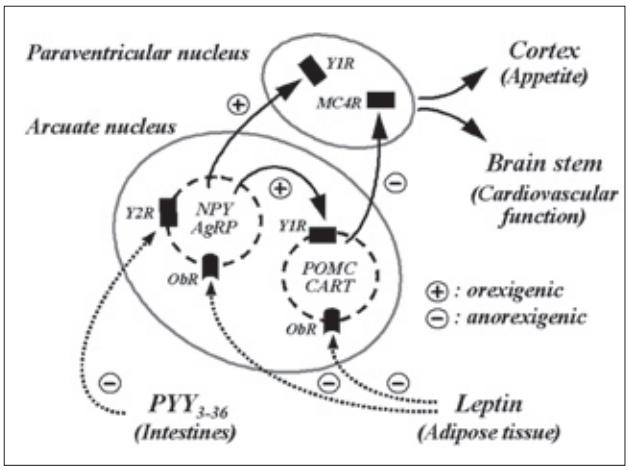

Fig. 1:
The arcuate and paraventricular nuclei are located in a brain area called hypothalamus. Circulating hormones from adipose tissue (leptin) or the intestines (PYY 3-36) can reach receptors on neurons in the arcuate nucleus because the blood-brain barrier in this region is absent or leaky. Both leptin and PYY 3-36, have an appetite suppressing (anorexigenic) effect which is mediated by the stimulation of neurons expressing anorexigenic neurotransmitters (pro-opiomelanocortin, POMC; cocaine and amphetamine related transcript, CART) and/or by the inhibition of neurons expressing orexigenic neurotransmitters (neuropeptide Y, NPY; agouti related peptide, AgRP). These pathways control not only feeding behaviour but also autonomic function and thereby influence cardiovascular parameters such as blood pressure and heart rate. Because of the importance of this brain region in the short- and long-term regulation of energy balance the various receptors located in the hypothalamic nuclei are potential targets for anti-obesity or anti-cachexia drugs (ObR: long form of leptin receptor; Y1 and Y2 R: neuropeptide Y receptor subtype 1 and 2; MC4R: melanocortin receptor subtype 4).

Our anti-cachexia program is being pursued in collaboration with a start-up company, MyoContract AG in Liestal, BL, and is funded since October 2003 by a KTI grant. Currently new animal models are being established, which include an acute model of cytokine-induced anorexia after lipopolysaccharide injection in rats and a chronic tumour model in mice. The prevention or reversal of muscle wasting in the latter model will provide the preclinical proof of concept for this therapeutic approach. Changes in lean body mass in tumour bearing mice will be determined not only by conventional biochemical analysis but also by magnetic resonance spectroscopy in collaboration with Prof. J. Seelig's group.

Because of our interest in the central control of appetite and cardiovascular function, our group has joined the newly defined focal area Neurobiology of the Biozentrum. In addition to our experimental work, we continue to contribute to several continuing education initiatives for medical doctors and pharmacists. A new series of seminars called Pharmathemen has been successfully established as a Telepoly conference between Zurich and Basel. The latest event attracted more than 100 participants. A lecture series on Obesity, Metabolism and Nutrition (OMeN) is co-organized with several colleagues at the Kantonsspital and has now been running for the third year. A book on the pharmacotherapy of obesity has been co-edited and is scheduled for publication in May 2004 (Hofbauer, K.G., Keller, U., Boss, O.: Anti-obesity Drugs. Options and Alternatives. CRC Press, Boca Raton, FL, USA). A book project on the pharmacotherapy of cachexia is in preparation.

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Cellular mechanisms of fear learning

How does the brain form memories of emotional events? My laboratory is interested in understanding the molecular and cellular mechanisms underlying the formation and retrieval of emotional memories. Using *in vitro* and *in vivo* electrophysiological methods, we are investigating activity- and experience-dependent changes in the neural circuitry of the amygdala of the mouse, a brain structure that plays a pivotal role in emotional behavior, particularly fear, in both humans and animals.

One of the most powerful models to study fear is classical fear conditioning, a form of Pavlovian conditioning. In Pavlovian fear conditioning, the subject, often a rat or a mouse, is exposed to an unconditioned stimulus (US), a noxious stimulus such as a footshock, in conjunction with a conditioned stimulus (CS), a neutral stimulus such as a tone or a light. As a result, the CS acquires aversive properties and will, on next occurrence, trigger a fear response. Behavioral and *in vivo* electrophysiological experiments suggest that activity-dependent associative synaptic plasticity in the amygdala underlies Pavlovian fear conditioning. However, the molecular and cellular mechanisms of associative synaptic plasticity in the amygdala are poorly understood.

Input specific timing-dependent plasticity mediated by voltage-gated Ca^{2+} channels

Projection neurons in the lateral amygdala (LA) receive converging thalamic and cortical sensory afferents. During fear conditioning, temporally correlated neural activity induced by CS-US pairing is thought to result in input specific changes in synaptic strength at thalamic and cortical afferent synapses. We have studied the temporal rules underlying the induction of long-term potentiation (LTP), a form of associative synaptic plasticity. Our results indicate that thalamic, but not cortical, afferent synapses are able to detect precise temporal correlations between pre- and postsynaptic activity. This so-called spike timing-dependent plasticity was mediated by the input specific activation of dendritic R-type voltage-gated Ca^{2+} channels. In contrast, pairing presynaptic activity with prolonged postsynaptic depolarization induced L-type Ca^{2+} channel-dependent plasticity at both inputs. Thus, the selective activation of dendritic voltage-gated Ca^{2+} channels enables LA projection neurons to perform input specific computations.

Heterosynaptic interactions between sensory afferents to the lateral Amygdala

In vivo electrophysiological experiments indicate that thalamic and cortical inputs are simultaneously active during fear conditioning. We found that simultaneous activation of converging cortical and thalamic afferents *in vitro*, using a stimulation paradigm consisting of random stimuli at an average frequency corresponding to the activity measured *in vivo*, specifically induced

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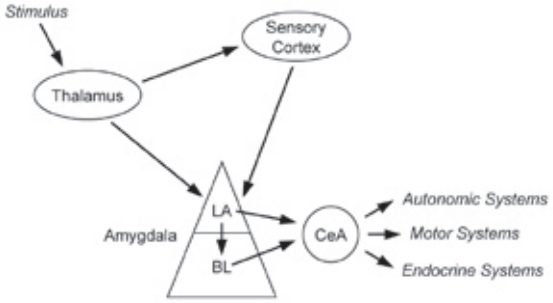

Fig. 1: Neural circuitry underlying classical fear conditioning. Acoustic and somatosensory stimuli reach the lateral amygdala (LA) directly from the thalamus or via the sensory cortex. LA projects directly, or via the basal nucleus (BL), to the central amygdala (CeA), which controls fear responses by brain stem projections. Adapted from LeDoux, 2000.

associative, NMDA receptor-dependent LTP at cortical, but not at thalamic inputs. Surprisingly, the induction of associative LTP at cortical inputs was completely independent of postsynaptic activity. Since LTP induction did not depend on network activity, this suggests that glutamate released by thalamic afferents directly activates NMDA receptors located on presynaptic terminals of cortical afferents. These findings show that input specificity of associative LTP can be entirely determined by presynaptic properties and open a new perspective for understanding integrative processes between converging afferent pathways in the mammalian central nervous system.

Modulation of synaptic plasticity by inhibitory transmission

The LA is a brain structure that is tightly controlled by GABAergic inhibition, which essentially prevents the induction of LTP. We could show that dopamine gates the induction of LTP at thalamic inputs by transiently suppressing feed-forward inhibitory control mediated by local interneurons. Our findings provide a cellular mechanism for the modulation of fear conditioning by dopamine and indicate that suppression of feed-forward inhibition represents a key mechanism for the induction of associative synaptic plasticity in the LA.

Recent experiments indicate that the induction of LTP is not only constrained by activation of ionotropic $GABA_A$ receptors, but that metabotropic $GABA_B$ receptors play an important role. Application of a weak tetanic stimulation protocol that did not result in LTP under control conditions, reliably induced LTP in the presence of a specific $GABA_B$ receptor antagonist at both cortical and thalamic afferent synapses. Interestingly, whereas facilitation of LTP at thalamic inputs required postsynaptic NMDA receptor activation and calcium influx, LTP at cortical afferents was mediated by a purely presynaptic, NMDA receptor-independent mechanism. Thus, activation of presynaptic $GABA_B$ receptors may serve to prevent the induction of non-associative LTP, thereby imparting associative properties to synaptic plasticity at cortical afferents.

Impact of early life stress on amygdala function

There is converging evidence that a number of psychiatric disorders involving anxiety and emotional dysregulation, such as posttraumatic stress disorder or borderline personality disorder, are associated with a dysfunction of the amygdala. Based on clinical observations, we are interested in the long-term consequences of early life stress on the physiology of the amygdala and the medial prefrontal cortex, a brain region that strongly modulates the amygdala. To this end, we are using mice that have been repeatedly separated from their mother during the first two weeks of postnatal development as an animal model for early life stress. We are applying behavioral, biochemical, and electrophysiological techniques to correlate changes in behavior with physiological alterations. Ultimately, we expect that a combined *in vitro* and *in vivo* approach, aimed at understanding the synaptic and cellular mechanisms of fear learning and their regulation by environmental and genetic factors in the mouse, will help us to reveal some of the mechanisms underlying anxiety disorders in humans.

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Mechanisms of rhythmogenesis in thalamocortical networks

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Why do we sleep? The full answer to this question is still ahead of us, but it is clear that the activities generated by the brain are essential for the restoration- and for the memory-promoting effects of sleep. If we are deprived of sleep, our need for sleep increases dramatically. When allowed to recover from sleep deprivation, the brain generates pronounced, rhythmic electrical activities in the slow frequency range (1-10 Hz) which are typical for deep sleep phases (so-called slow-wave sleep). This indicates that unraveling the ionic and biochemical events promoting slow-wave sleep may eventually tell us much about why we sleep. Our primary goal is to understand how slow-wave sleep rhythms are controlled in time via a) the precise regulation of ionic currents that act as pacemakers, and b) the temporal control of neuronal discharges via synaptic afferents. We also address the question of how the perturbance of such rhythms, such as it occurs during epilepsy, affect the functioning of the neurons and circuits involved in rhythm generation.

Experimentally, we apply electrophysiological techniques to *in vitro* slice preparations of the rodent thalamocortical system. The thalamocortical system is the largest neuronal network in the brain and contains specialized circuits that act as the prime generators of rhythms related to states of arousal. Moreover, we use heterologous expression systems to investigate the structure-function relationship of ionic channels and the regulation of these by neurotransmitter receptors. Computationally, we study the generic conditions for the emergence of temporally patterned population activity in artificial networks.

The regulation of native pacemaker currents by cyclic nucleotides in normal and epileptic rodents

The classically known pacemaker current is carried by membrane-spanning ion channel pores and essentially contributes to the timing of sleep-related rhythms in the thalamocortical system. The regulation of the pacemaker channels by cyclic nucleotides, predominantly by cyclic AMP (cAMP), is important for the involvement of the pacemaker current in rhythms occurring on different time scales. In spite of this recognized role of cAMP, the physiological pathways of cAMP-mediated regulation of this current are, so far, poorly understood.

We have identified a previously undescribed, powerful pathway of cAMP synthesis that targets the thalamic pacemaker current and requires the temporally coincident activation of both G_s- and $G_{i/o}$-coupled neurotransmitter receptors (Fig. 1, next page; Frère and Lüthi, 2004; Frère et al., 2004). This finding indicates that distinct pathways of cAMP synthesis target the pacemaker current, most likely

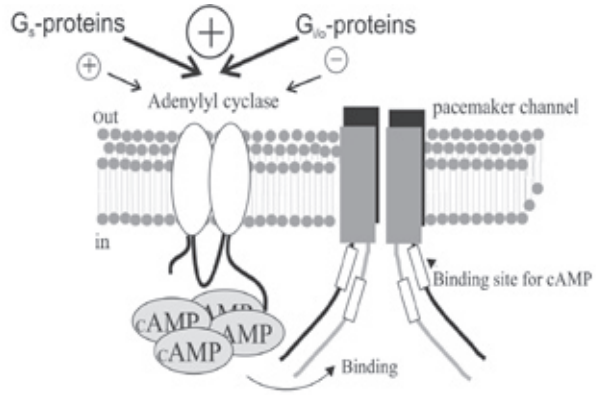

Fig. 1: Schematic diagram illustrating the regulation of pacemaker ion channels by single and synergistic activation of adenylyl cyclases by G-proteins. Plus sign indicates stimulation, Minus sign indicates inhibition of cAMP synthesis. The size of the sign represents the strength of the effect.

via molecularly distinct types of adenylyl cyclases, the primary enzymes producing cAMP. We also found that this cAMP synthesis pathway can be recruited following temporally coincident activation of β-adrenergic receptors with synaptic activation of $G_{i/o}$-coupled $GABA_B$ receptors (GABA is the principal inhibitory neurotransmitter in the brain). This is the first demonstration that synaptically located G-protein-coupled neurotransmitter receptors are involved in the control of the pacemaker currents. Thus, the recruitment of cAMP synthesis pathways could be determined by the strength of GABAergic activity occurring within thalamocortical networks during sleep. The strong cAMP-mediated enhancement of the pacemaker current may then contribute to the timing of neuronal rhythms emerging from GABAergic synaptic activity.

Recently, a number of cardiac and neurological diseases were found to be intimately associated with a dysregulation of the transcription of pacemaker channel genes, suggesting a critical contribution of pacemaker currents to the pathophysiology of excitable systems. To address whether this hypothesis also holds for epilepsies arising within the thalamocortical system, we are currently using the GAERS rat (Generalized Absence Epilepsy Rat from Strasbourg) to evaluate the expression, the properties and regulation of the pacemaker channels in thalamocortical neurons of these epileptic animals (Collaboration with D. Pinault and T. Baram).

The regulation of heterologously expressed pacemaker currents by cyclic nucleotides

We would like to characterize the biochemical mechanisms leading to a synergy between co-activated G-protein-coupled receptors in regulating the pacemaker channels. We have therefore expressed the pacemaker channel HCN2 in heterologous systems (Clones obtained from Prof. Siegelbaum, Columbia University, Collaboration with B. Bettler). We showed first hat the expressed channels are targeted by cAMP released from endogenous adenylyl cyclases, thus validating the use of expression systems to study ion channel regulation. We are currently co-expressing pacemaker channels with G_s- and $G_{i/o}$-coupled neurotransmitter receptors to characterize the regulation of the current via cAMP produced following single or combined receptor stimulation.

The structure-function relationship of pacemaker channels

In the framework of a national project in Nanosciences (NCCR in Nanosciences), we are collaborating with the Department of Structural Biology in the house (Prof. A. Engel and Dr. P. Werten) in the elucidation of the structure-function relationship of the pacemaker channels. The ultimate goal of this project is to apply atomic force microscopy (AFM) and multifunctional probing to study the gating mechanism of these channels. These channels appear suited for an evaluation of their structure-function relationship via AFM for both scientific and technical reasons. We succeeded in expressing molecularly tagged pacemaker channels in heterologous expression systems, and we verified their functionality by electrophysiological recordings. In particular, we showed that these channels, in spite of their tag, produce currents that closely resemble

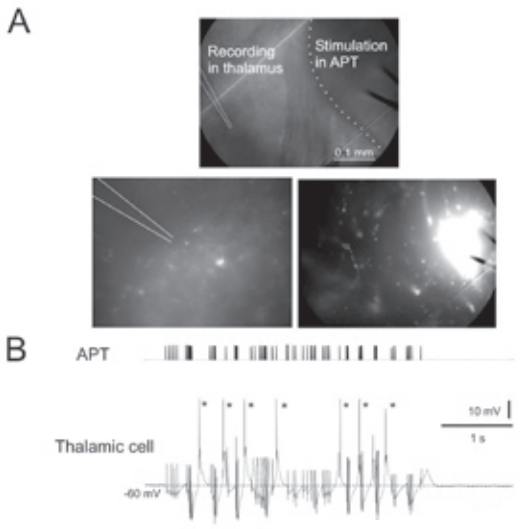

*Fig. 2: A. Visualization of the projection from the anterior pretectal nucleus into the thalamus by fluorescence microscopy. The top figure shows the area covering stimulation and recording areas in transmission light, expanded portions of these two areas in fluorescent light (red) are presented below. Note the clear fluorescence in both stimulation and recording areas, indicating successful propagation of the dye along the projection between the anterior pretectal nucleus and the thalamus. Stimulation and recording electrodes are depicted with black and grey lines, respectively. B. Application of a stimulus protocol mimicking the in vivo firing pattern of the anterior pretectal nucleus (APT) produces repetitive rhythmic burst discharges in a thalamic neuron (indicated by asterisks *).*

those generated by untagged channels in terms of their pharmacological profile and their modulation by cyclic nucleotides. This achievement represents a major prerequisite for the further purification and reconstitution of the channels in preparations that allow the application of AFM techniques. Ultimately, our approach may allow to molecularly and functionally probe the actions of drugs on pacemaker channels.

Novel afferent inhibitory pathways into the thalamus that promote rhythmic bursting

Afferents from the reticular thalamic nucleus have been traditionally regarded as the main source of inhibitory control of thalamocortical cells and the involvement of these in the rhythmic discharges underlying sleep-related oscillations. In this project, we study a second major inhibitory system that is restricted to higher order thalamic nuclei. To visualize these afferents for electrophysiological recordings, small injections of a fluorescent anterograde tracers into the anterior pretectal nucleus (APT) of the rat caudal diencephalon were performed (Collaboration with Làszlò Acsàdy). We combined extracellular stimulation of the APT in pretecto-thalamic slices from tracer-injected animals with whole-cell patch-clamp recordings in fluorescently labeled areas of higher order thalamic nuclei (Fig. 2). APT stimulation evoked a synaptic response that was a) fully blocked by antagonists of $GABA_A$ receptors b) showed a fixed latency and c) displayed all-or-none responses to incrementing stimulation intensities. These three properties strongly suggest the existence of a monosynaptic inhibitory projection from APT into higher order thalamic nuclei. The inhibitory input from APT attenuated tonic firing of thalamic cells at depolarized potentials, whereas its repetitive activation (at least 5 times at 10 Hz) robustly elicited rebound burst firing at hyperpolarized potentials. Moreover, application of stimulus protocols derived from *in vivo* recordings of spontaneously discharging APT neurons showed that repetitive high-frequency firing of APT cells promoted rhythmic bursting in thalamus. Thus, additional inhibitory synaptic activity targets thalamocortical cells and thus may exert differential control of thalamic firing during various arousal states.

The generation of periodicity in artificial neural networks

The experimental finding of spontaneous network synchronization in the thalamocortical system motivates a computational validation of cellular and synaptic processes hypothesized to contribute to this network self-organization. We numerically examined how experimentally characterized microscopic network properties, such as the type, strength and short-term plasticity of synaptic interconnections, and the intrinsic firing properties of neurons, specify the collective macroscopic organization of the network, in particular its rhythmic activity and synchrony (Wiedemann and Lüthi, 2003). Our first results include the application of correlation analysis techniques to separate mechanisms which may provide biological clocks (pacemaker effects) from other sources of synchrony generation (e.g. avalanche effects in metastable network configurations).

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Molecular mechanisms of diversity in response to drugs and chemicals in man

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Introduction

Genetic and environmental factors cause clinically important interindividual differences in the response to drugs and chemicals. Genetic polymorphisms of the genes coding for drug metabolizing enzymes, receptors and transporters are major contributors to this variability. A second major source of variability is the effect of drugs on gene expression, i.e. the transcriptional activation or repression of genes coding for drug-metabolizing enzymes, such as cytochromes P450, a phenomenon known since 40 years as “induction”. The goal of our research is to understand the molecular mechanisms of variability in human drug response and to develop simple, non-invasive methods by which individuals at risk to develop adverse drug reactions, to suffer from drug inefficacy or drug-induced disease can be recognized.

Pharmacogenetics and pharmacogenomics

Genetic polymorphisms of drug response are relatively common (>1%) monogenic traits caused by the presence of more than one allele at the same gene locus and more than one phenotype in regard to drug interaction with the organism. Pharmacogenetic polymorphisms lead to subgroups in the population with altered responses to drugs and chemicals, e.g. a higher incidence of adverse drug reactions or a higher risk to develop a certain disease, for example cancer. In the past, we have elucidated the molecular mechanisms of several common genetic polymorphisms of drug metabolizing enzymes: 1) the debrisoquine/sparteine or cytochrome P4502D6 (CYP2D6) polymorphism; 2) the mephenytoin or cytochrome P4502C19 (CYP2C19) polymorphism, and 3) the polymorphism of N-acetyltransferases (NAT). These polymorphisms are transmitted as autosomal recessive traits and cause impaired inactivation of numerous clinically important drugs or chemical carcinogens in so-called “poor metabolizer” individuals. More recently, we have collaborated with other laboratories to study the variation of cytochrome P4503A4 (CYP3A4), the enzyme responsible for the metabolism of more than 50 % of clinically used drugs. DNA tests derived from these projects are now being used in epidemiological studies throughout the world, particularly to evaluate hypotheses that implicate these polymorphisms in adverse drug reactions or in the pathogenesis of several diseases including cancer.

Mechanisms of transcriptional regulation of Cytochromes P450 (CYP) genes by drugs

Numerous drugs and chemicals induce the expression of drug- and steroid-metabolizing enzymes in the liver. Induction of these enzymes

has a major impact on drug effects including drug-drug interactions, drug-toxicity and xenobiotic carcinogenicity and contributes to interindividual variability in drug response. Induction most often occurs at the level of transcription and prototypical drug- and other xenobiotic inducers include polycyclic aromatic hydrocarbons (e.g. dioxin), phenobarbital (PB), peroxisome proliferators (e.g. clofibrate) and steroids (e.g. dexamethasone). Each of these classes of compounds induce a different but overlapping pattern of enzymes. Recent advances in our understanding of the induction response suggest the following general mechanisms of transcriptional activation: The xenobiotics interact with intracellular proteins either of the basic helix-loop-helix class (polycyclic aromatic hydrocarbons, AhR system) or with so-called orphan nuclear receptors such as PPAR (peroxisome proliferator activated receptor), CAR (constitutively activated receptor, affected by phenobarbital and phenobarbital-like-inducers) or PXR (pregnane x receptor, activated by dexamethasone, rifampicin, etc.). Each of these intracellular proteins forms heterodimers with other proteins (AhR with Arnt, CAR and PXR with the retinoid x receptor RXR) and the heterodimer binds to DNA recognition motifs, so-called drug or xenobiotic response elements. Many of the details of the induction mechanism remain to be elucidated. In particular, the endogenous ligands of PXR and CAR and the detailed mechanisms of transcriptional activation are unknown. Inducers of cytochrome P450s of the CYP3A family apparently can directly bind to the ligand binding domain and activate PXR. It remains unclear, how phenobarbital-like inducers increase CAR-RXR binding to the DNA recognition sequences characterized as phenobarbital-response units (PBRU) or motifs (PBREM). Moreover, phosphorylation/dephosphorylation events, cytokines and hormones affect the induction response by as yet unknown mechanisms. This lack of knowledge is in part due to methodological difficulties. Most of the known phenobarbital- and rifampicin-inducible enzymes are not expressed or not inducible in presently known stable mammalian cell lines.

We initially used a non-mammalian system, namely avian liver, avian hepatocytes and avian hepatoma cells (LMH), to study induction of cytochromes P450 and heme synthesis by drugs and chemicals. In chicken embryo liver in ovo, in primary cultures of chicken embryo hepatocytes, and in LMH cells cytochromes P450 remain highly inducible by phenobarbital-like inducers. We are presently developing cell lines derived from human hepatoma cells that maintain the response to phenobarbital.

In reporter gene studies in LMH cells we have discovered two phenobarbital-responsive enhancer units in CYP2H1. Within these enhancer elements, we have identified a conserved nuclear receptor consensus sequence (a DR-4 element) which mediates induction in reporter gene assays. In parallel, we have used homology concepts to clone the avian nuclear receptor CXR (chicken xenobiotic sensing receptor). This orphan nuclear receptor has properties of both PXR and CAR and may represent the evolutionary precursor of these transcription factors. CXR also affects the drug-induction in chicken liver of two additional cytochromes P450 recently cloned and characterized in our laboratory, CYP3A37 and CYP2C45. Our experiments in LMH cells provide strong evidence for evolutionary conservation of the signaling pathways triggered by PXR, CAR and CXR. Thus, the phenobarbital-responsive enhancer units (PBRUs) of mouse Cyp2b10, rat CYP2B2 and human CYP2B6 were activated by the same compounds that activate the chicken CYP2H1 PBRU. Moreover, the mammalian receptors also bind to and activate the avian enhancer sequence. We could thus demonstrate that closely related nuclear receptors, transcription factors and signaling pathways are mediating the transcriptional activation of multiple genes by xenobiotics in chicken, rodents and man. These findings have led us to develop a computer algorithm that can predict DNA sites that bind xenobiotic sensing nuclear receptors. This program is named NUBIScan and is available to the scientific community and for commercial use (www.nubiscan.unibas.ch).

Crosstalk between drug metabolism and cholesterol and bile acid homeostasis

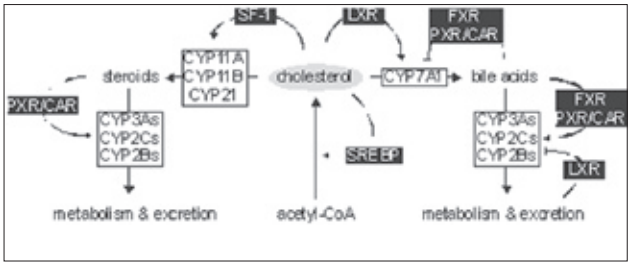

Fig. 1
Nuclear receptor cross-talk during induction of cytochromes P450

Induction of CYPs by drugs is observed in all species (a notable exception is yeast). We therefore have speculated that drug-induction of CYPs involves an endogenous regulation common to all living organisms. Firstly, CYPs catalyze not only the metabolism of xenobiotics, but also steroids, fatty acids, cholesterol and bile acids, and secondly, CAR, PXR and CXR belong to the subfamily of orphan nuclear receptors comprising also receptors involved in cholesterol homeostasis, including the oxysterol receptor LXR (liver X receptor) and the bile acid

receptor (FXR or BAR). In the absence of uptake of exogenous cholesterol, intracellular cholesterol content is controlled via de novo synthesis of cholesterol and catabolism of cholesterol to bile acids. Over the last 2 years we therefore have tested the hypothesis that the mechanism of induction of CYPs can be explained by interactions between xenobiotic-sensing nuclear receptors and nuclear receptors involved in cholesterol and bile acid homeostasis. We compared the effect of squalestatin (SQ1, a potent inhibitor of cholesterol synthesis), oxysterols and bile acids on both the basal and inducible expression of CYP2H1 and CYP3A37 in LMH cells. SQ1 potently induced both CYPs and shared signaling pathways with the drug regulation of these genes. Moreover, we discovered that bile acids also induce CYP2H1 and confirmed recent data in mammals that bile acids regulate CYPs. Interestingly, hydroxylated bile acids and hydroxycholesterols inhibit drug activation of CYP2H1 and this is due to activation of LXR. LXR apparently binds to the same response elements as CXR, CAR and PXR and inhibits the induction of drug-metabolizing CYPs. This was deduced from experiments in which cLXR was downregulated by RNAi or expressed in a constitutively active form. Moreover, experiments in mice exposed to a high (1 %) cholesterol diet or treated with pravastatin (a HMG-CoA reductase inhibitor) confirmed the interaction between cholesterol homeostasis and xenobiotic metabolism, although additional mechanisms than increased oxysterols are involved. Experiments in knockout mice (LXRα-/-, LXRβ-/- and LXRα/LXRβ-/-) revealed a very complex situation with unexpected and probably adverse effects of accumulation of cholesterol.

In an additional study we have discovered that bile acids at physiological concentrations affect the expression of CYP3A4 via a previously unknown response element for the bile acid receptor FXR/BAR. This may be relevant for the clinically important, extremely variable expression of CYP3A4.

Gene expression experiments with Affymetrix gene chips were performed with human liver samples from our own and the Stuttgart human liver bank as well as with duodenal biopsies taken in volunteers before and after treatment with the inducer-drug rifampicin. The results reveal the extensive coregulation of genes involved in drug metabolism, drug transport and in cholesterol and bile acid synthesis and breakdown, but also reveal connections to other homeostatic processes in liver and gut. A concept emerges that metabolism of lipid-soluble drugs and xenobiotics evolved in close relation to the mechanisms that keep lipids constant in the cell.

Role of heme synthesis during induction of cytochromes P450

An important aspect of CYP regulation is the role of heme synthesis in drug-mediated induction of CYP-hemeproteins. This has been a controversial issue for many years. The supply of heme for the assembly of hemeproteins in the liver is controlled by the activity of 5-aminolevulinic acid synthase (ALAS1). Drug-induction of CYPs in the liver and other tissues requires coordinated increases in ALAS1 and apocytochromes. How does the cell achieve this coordination?

The expression of the ALAS1 protein is tightly controlled by a regulatory heme pool, which affects different negative feedback mechanisms. Heme inhibits induction of ALAS1 in the liver and depresses ALAS1 activity by blocking mitochondrial import of ALAS1 preprotein and by destabilizing ALAS1 mRNA. Therefore, it was assumed that induction of apocytochromes P450 by drugs would deplete the regulatory heme pool and derepress heme-mediated inhibition of ALAS1 synthesis. Our own studies have demonstrated that induction of CYPs by drugs involves parallel increases in the transcription of ALAS1 and CYP genes and is not dependent on apocytochrome synthesis. The molecular mechanism that determines the drug-induced, tissue-specific transcription of the ALAS1 gene has not been studied. It concerns one of the main projects of our research team. We have cloned the flanking regions of chicken mouse and human ALAS1 in various cell systems and have discovered drug-responsive enhancer sequences. These sequences were found at unusually distal sites of the 5'flanking region, usually more than 15 kb upstream of the transcription start site and were named ADRES (for ALAS1-drug-responsive enhancer sequence). Because these sequences may be used to design *in vitro* systems for the prediction of xenobiotic inducers, they have been the subject of a patent application (PCT/IB02/CT258).

The mechanism by which ALAS1 is induced by drugs is of particular importance in diseases associated with deficiencies in hepatic heme synthesis, such as inducible hepatic porphyrias. In these disorders, drugs including phenobarbital, rifampicin, and other CYP inducers precipitate acute attacks of neuropsychiatric dysfunction and these attacks are associated with massively induced ALAS1. We have developed a transgenic mouse model of a partial heme deficiency which is unique as an experimental tool and has been successfully used to study the effects of heme limitation on the transcription of ALAS1 and CYP genes and to assess new treatment models to suppress the induction of

ALAS1 in patients with porphyria. We intend to use these mice to understand how xenobiotics induce ALAS1 and how this precipitates acute attacks of porphyria.

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Synapse formation and neuromuscular diseases

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Among the most remarkable features of the brain is its capability to process information, which is to a large part determined by the complexity of the neural network. The cellular units underlying information processing in the nervous system brain are synapses. To warrant efficient information transfer, synapses are highly specialized both on the pre- and on the postsynaptic site. In processes, such as learning and memory, synapses are likely to undergo structural changes in the adult. Moreover, synapses are the main site of action in several degenerative diseases in the central and the peripheral nervous system.

My laboratory follows two lines of research. In one project, we aim to identify and characterize molecules that affect the formation and the modulation of synaptic connections. In the second project, we follow up on our previous finding that specifically designed hybrids of extracellular matrix molecules can substitute for the loss of a non-homologous protein, called laminin-2, which causes a severe form of muscular dystrophy.

Development of synapses

Formation of synaptic structures is independent of electrical activity but requires the exchange of trophic factors between the presynaptic neuron and the postsynaptic target cell. It has been difficult to study synapse formation on the molecular level in the brain because of technical limitations. In contrast, the cholinergic neuromuscular junction (NMJ) allows the detailed study of its structure and function. Moreover, it offers the opportunity to investigate the function of genes *in vivo* using conventional transgenesis in mice and, most importantly, muscle-specific overexpression and knock- down technologies, such as RNA interference (e.g. Kong et al., 2004). Thus, the NMJ is amenable to a fast and reliable assessment of gene function *in vivo*. It is an old question whether the NMJ is a good model to understand synapse formation and function in the central nervous system (CNS). However, ample evidence shows that the principal mechanisms of synaptic transmission are identical at the NMJ and at neuron-neuron synapses. Thus, it is probable that the molecular principles that govern the formation and maintenance of synaptic connections are also similar.

One of the hallmarks of postsynaptic differentiation at the NMJ is the aggregation of acetylcholine receptors (AChRs) underneath the nerve terminal. Subsequently, gene transcription of “synaptic” genes is selectively ceased in non-synaptic myonuclei but is maintained in the myonuclei underneath the nerve terminal. All these events result in a highly specialized and complex structure at the site of contact between motor neuron and muscle fiber (Fig. 1). While we still know only little about the molecular mechanisms involved in presynaptic differentiation, some of the key players involved in the formation of postsynaptic structures have been identified. The heparan sulphate proteoglycan agrin, which is released from developing

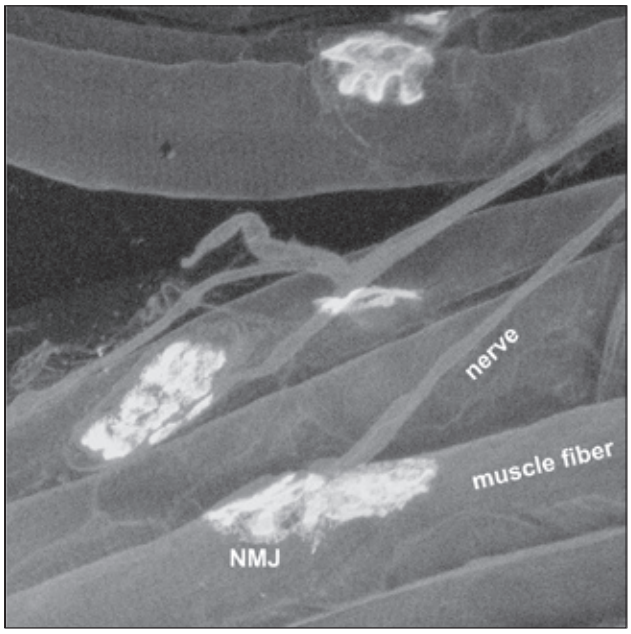

Fig. 1:
Whole mount view of muscle fibers that are innervated by single motor neurons. At the neuromuscular junction (NMJ), hallmarks of postsynaptic specializations include aggregates of acetylcholine receptors (light grey) opposite to the innervating nerve

moto neurons and tightly binds to synaptic basal lamina, is required and sufficient to induce the formation of the postsynaptic apparatus. This postsynapse-inducing activity of agrin is mediated by the activation of a receptor tyrosine kinase called MuSK. Activation of MuSK results in changes of cytoskeletal organization and induces synapse-specific gene transcription in subsynaptic myonuclei.

To specifically identify candidate genes involved in synapse formation at the NMJ, we employ a newly developed technique where postsynaptic structures are induced in non-synaptic regions of rat muscle by the application of recombinant agrin protein. This method allows impregnation of the entire muscle basal lamina by agrin which leads to the formation of many postsynapse-like structures that have all the features characteristic of the postsynaptic apparatus at the NMJ. These specializations include the activation/silencing of gene transcription in myonuclei underlying these specializations. Using this experimental paradigm, we have identified more than 20 genes whose expression is upregulated by the formation of postsynaptic structures. After confirmation of their regulation in postsynaptic differentiation in muscle, we have now also investigated their gene expression levels during synapse formation in cultured hippocampal neurons. Interestingly, several of the candidates are indeed also regulated in this experimental paradigm. Current efforts are devoted to elucidate the role of these novel genes in the formation of the NMJ *in vivo* and of central CNS synapses in cultured hippocampal neurons. The methods employed will include overexpression and knock-down of the genes of interest. These experiments are aimed at validating the hypothesis that postsynaptic differentiation in skeletal muscle and synaptogenesis at inter-neuronal connections uses similar molecular mechanisms.

Agrin-deficient mice show changes in synapses in the brain

The organizing role of agrin in the development of the NMJ and the expression of agrin during synapse formation in the brain would argue for a role as a general molecular code that orchestrates synaptogenesis. Agrin-deficient mice die perinatally because of the failure to form NMJs, thus making it impossible to analyze synapse structure and function in these mice at postnatal stages. To determine the role agrin in the CNS, we are also investigating the brain phenotype of agrin-deficient mice whose NMJ phenotype was rescued by the transgenic expression of chick neural agrin in motor neurons. Such mice survive birth but still show severe growth retardation and usually die before the age of 3 months. Morphological analysis reveals no gross abnormalities in the overall structure and the layering of the brain. However, although the number of neurons is not altered, there is an approximately 30% loss of synapses and of postsynaptic spines in pyramidal neurons. These experiments demonstrate that agrin does play a role in the development of synaptic connections in the CNS. Rather than being essential for the induction of synapses, these data indicate a role of agrin in the maintenance of synaptic connections. Current efforts are devoted to assess the molecular mechanisms underlying these changes.

Development of new therapies for neuromuscular diseases

Pathological changes of neuromuscular connections and of muscle fiber integrity result in muscle weakness, progressive muscle atrophy and muscle wasting. In the most severe cases patients die of respiratory failure. Some of the genes involved in NMJ development are also relevant for the pathology and/or the treatment of muscular diseases. For example, we have recently shown that mice suffering from a severe form of muscular dystrophy, called “merosin-deficient congenital muscular dystrophy” or MDC1A, can be helped by the transgenic overexpression of an miniaturized form of agrin (using a splice variant that cannot induce postsynaptic structures).

MDC1A is a relatively rare, autosomal recessive muscular dystrophy that often leads to early death in childhood. It is due to mutations in the α2-subunit of laminin-2, which is a major component of basement membranes in skeletal muscle (Fig. 2). The current model predicts that laminin-2 is important for the linkage of basement membrane to the F-actin cytoskeleton by its binding to integrins and dystroglycan, which in turn, are connected to the F-actin cytoskeleton (Fig. 2). This model is based on genetic evidence that mutations in components of this complex all cause

muscular dystrophies similar to MDC1A. In MDC1A patients, the mutated α2 chain of laminin-2 is replaced by the α4 homologue, which, however, does not bind to dystroglycan (Fig. 2). The carboxy-terminal end of agrin also binds to dystroglycan and its amino-terminal end confers high-affinity binding to the laminins. The fact that overexpression of a miniaturized form of agrin, which comprises only the binding sites for laminins and dystroglycan, significantly ameliorates the disease in the mouse model of MDC1A, provides strong evidence that the tethering of dystroglycan to basement membrane by specifically designed linker proteins could be used as therapy in muscular dystrophy.

Our studies are the first example of functional compensation of a diseased phenotype by an engineered molecule whose design was based on functional and not on structural similarities between the mutated α2 chain of laminin and agrin. This can be considered a major advancement in a potential treatment of MDC1A because gene therapy with this mini-agrin would offer several advantages over reinsertion of the laminin-α2 chain. First, the cDNA encoding the agrin mini-gene is small enough to allow the use of adeno-associated virus (AAV) which has been shown to be more effective than currently used vectors. Second, the notoriously low efficacy in the infection of muscle,

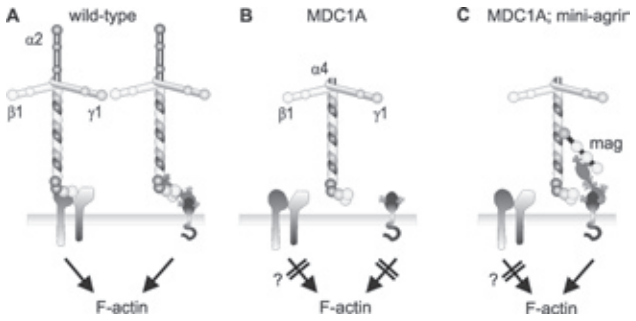

Fig. 2:
Molecular mechanisms involved in the linkage of basement membrane and cytoskeleton.

A. *In wild type mice, laminin-2 (α2;β1;γ1) is linked via integrins (left) and dystroglycan, which is posttranslationally cleaved into α-dystroglycan and β-dystroglycan (right).*

B. *In laminin α2-deficient mice, the level of α-dystroglycan at the sarcolemma is diminished although β-dystroglycan is still expressed. As a compensation for the missing α2 chain, the α4 chain is overexpressed giving rise to laminin-8. This laminin isoforms lacks domains important for the binding to α-dystroglycan and for the formation of a laminin network. There is no evidence for laminin-8 binding to integrins. Consequently, the linkage to F-actin is abrogated.*

C. *Transgenic expression of a minigene for agrin (mag) in muscle, restabilizes α-dystroglycan at the sarcolemma, and links laminin-8 to F-actin.*

which is a major drawback when gene therapy is used to bring back intramuscular genes (e.g. dystrophin), is not a problem because the protein is secreted from infected muscle fibers and could therefore also reach neighboring, non-infected muscle fibers. Third, thanks to MDC1A patients expressing agrin, immunological rejection of the mini-agrin protein would be minimal. In addition and most importantly, the mini-agrin protein could directly be applied to MDC1A patients. Thus, the potential of a mini-agrin-based therapy is high. We are now in the process of verifying this concept by using a set of other rationally designed molecules and creating transgenic mice where the production of the mini-agrin protein can be switched on only after the onset of the muscular dystrophy. These experiments are critical steps for testing the feasibility of using approaches as described above for the treatment of MDC1A patients.

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DIVISION OF BIOINFORMATICS / SWISS INSTITUTE OF BIOINFORMATICS BASEL

During the report period, the Division of Bioinformatics was joined by two new group leaders at the Assistant Professor level and now consists of four independent research groups lead by Torsten Schwede, Michael Primig, Erik van Nimwegen, and Mihaela Zavolan. In collaboration with the Swiss EMBnet node, Lorenza Bordoli provides user support and practical training courses in Bioinformatics for scientists at the University of Basel. The Biozentrum's efforts to build a Bioinformatics division were supported by the Swiss Institute of Bioinformatics (SIB), Novartis and Roche.

We have established a fruitful collaboration between the Biozentrum, the Swiss Institute of Bioinformatics (SIB), and the Friedrich Miescher Institute (FMI) in the field of Bioinformatics to create synergies concerning IT hardware, system administration, user support and training activities. This joint effort lead to the construction of the Basel Computational Biology Center $[BC]^2$ that provides a competitive infrastructure including application-, database-, and web-servers as well as large scale storage and backup facilities. This infrastructure, i.e. locally installed software and database mirrors, are available not only to the bioinformatics groups, but to all researchers in the participating institutions in Basel.

The Basel Computational Biology Conference $[BC]^2$ is an annual interdisciplinary symposium organized at the Biozentrum to provide a platform for exchange of ideas in the field of computational biology. The first event held in 2003 called "Life Sciences Meet IT" explored the advances of technology and science in the interface of IT technology and bioinformatics. The second symposium in 2004 "From Information to Simulation" has established $[BC]^2$ as the most important computational biology conference in Switzerland with more than 200 registered scientists from academic research institutes, the pharmaceutical industry and Biotech companies. In addition, a $[BC]^2$ plenary lecture series held by outstanding scientists whose ideas change the way we work and think, was initiated. The first lecture was held in 2003 by Ronald Davies from the Stanford Genome Center.

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Development of knowledgebases and microarray data management/analysis solutions. Expression profiling of gametogenesis in yeast and mammals

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Introduction

Gametogenesis is an important developmental process that leads to the formation of haploid germ cells during sexual reproduction in eukaryotes. This differentiation pathway involves reprogramming of mitotic growth such that two consecutive meiotic divisions without an intervening S-phase become possible. Genes expressed in germ cells, including many that are conserved between unicellular eukaryotes, plants and mammals, display complex meiotic transcriptional regulation. Recent expression profiling experiments using microarrays have provided insight into the coordinated transcription of several hundred genes during mitotic growth and meiotic development in budding and fission yeast as well as spermatogenesis in the worm, rodents and human. These studies identified numerous candidate loci for roles in mammalian gametogenesis and fertility.

The regulation of meiotic gene expression in yeast

UME6

The Ume6 transcription factor in yeast was shown to repress and/or activate expression of about 80 genes during growth and meiotic development. A microarray analysis was carried out to examine the effect on transcription in wild type versus ume6 deletion diploids during vegetative growth in the presence of fermentable (glucose) and non-fermentable (acetate) carbon sources. Two different strains (W303 and SK1) were examined to identify a core group of Ume6-regulated genes. 82 loci contain matches to the Ume6-binding site (URS1) in their promoters and are therefore expected to be directly regulated by Ume6. Almost half of the genes we consider to be direct Ume6 targets are induced during meiosis, with most falling into the early meiotic expression class (cluster 4), and a smaller subset in the middle and later classes (clusters 5-7). We propose that in diploid cells Ume6 couples metabolic responses to nutritional cues with the initiation and progression of meiosis. Expression patterns of genes involved in gluconeogenesis and the glucose-dependent regulation of *IME1* (inducer of meiosis) in the two strains suggest that SK1 is better adapted to respiration and W303 to fermentation. These findings, which may in part account for the more efficient and synchronous sporulation of SK1, are currently being further investigated. Moreover, the genome-wide DNA binding pattern of Ume6 will be studied during mitosis and meiosis using ChIP-chip experiments to complement the available expression data and to comprehensively identify the group of Ume6-target genes.

ABF1

ABF1 is a remarkable gene. It encodes a conserved DNA binding factor involved in processes as diverse as the transcriptional regulation of mitotic and possibly meiotic genes, gene silencing, autonomously replicating sequence (ARS) function and nucleotide excision repair. Recently, Abf1 was postulated to be involved in partitioning the genome into functional domains that

are independently regulated. A temperature-sensitive allele, abf1-1, has a point mutation in the zinc finger domain of the protein and displays reduced affinity to its target site (UAS_H). We have now finished expression profiling studies that aim at the identification of the mitotic regulon of Abf1 in rich media containing either fermentable (glucose) or non-fermentable carbon sources (acetate). In addition to that, we are in the process of analyzing the role of Abf1 in meiotic gene expression by comparing the transcription patterns of potential target genes in sporulating wild-type and abf1-1 strains. This data will be compared to a genome wide DNA binding study of Abf1 (ChIP-Chip) using samples from mitotically growing and sporulating cells. This work is currently under way in collaboration with the laboratory of Alain Nicolas (Curie Institute, Paris). This comprehensive dataset will reveal most if not all of the Abf1 target promoters and help better understand how a single protein can regulate different processes like mitotic growth and meiotic differentiation.

Identification of candidate genes for the regulation of fertility in mammals by large-scale expression profiling

We published the first comprehensive large-scale expression profiling analysis of mammalian male germ cells undergoing mitotic growth, meiosis and gametogenesis using High Density Oligonucleotide Microarrays and highly enriched cell populations. Among 11955 rat loci investigated we identified 1268 as differentially expressed in germ cells at different developmental stages as compared to samples from total testis, somatic Sertoli cells as well as brain and skeletal muscle controls. For convenience, the transcripts were organized into four expression clusters that correspond to somatic, mitotic, meiotic and post-meiotic cell types. Our work provides the community with information about expression patterns of approximately 200 genes known to be important during male germ cell development. Moreover, we identify a group of 121 transcripts expressed only in meiotic and post-meiotic germ cells but not dividing germ cells, Sertoli cells or two somatic control tissues. Finally, we demonstrate the testicular expression and transcriptional induction in mitotic, meiotic and/or post-meiotic germ cells of 293 as yet uncharacterized transcripts some of which are likely to encode factors involved in spermatogenesis and fertility. It is noteworthy that this group also contains potential germ cell specific targets for innovative approaches to reversibly inhibit male fertility. A graphical display as well as curated information on many rat genes important for meiosis and spermatogenesis is accessible through the GermOnline database at http://www.germonline.org. A web portal provides access to raw data files and the software used for data analysis at http://www.bioz.unibas.ch/personal/primig/rat_spermatogenesis. We now plan to determine the transcriptome of spermatogenesis in mouse, rat and human using the latest generation of GeneChips that contain probes to all currently annotated genes in these species. This project will be carried out in collaboration with B. Jégou (University of Rennes) and D. Wolgemuth (Columbia University, NY).

Development of a novel cross-species and subject-oriented approach to genome annotation and microarray data management

We published work on a unique knowledgebase on phenotypic and gene expression data relevant to germ line development and gametogenesis. The GermOnline project includes an interactive platform for cross-species gene annotation by research scientists. This novel approach to knowledge management partitions the genomes of important model systems into groups of genes involved in important conserved biological processes. GermOnline provides information and microarray expression data for genes involved in mitosis and meiosis, gamete formation and germ line development across species. The database has been developed, and is being curated and updated by life scientists in cooperation with bioinformaticists and professional curators. Information is contributed by scientists through an online form using free text, images, references and the controlled vocabulary developed by the GeneOntology consortium. The database is overlooked by an international board of scientists whose members ensure the highest quality of GermOnline's information content. The locus report pages include links to numerous external databases. Conversely, *Saccharomyces* Genome Database (SGD), *S. cerevisiae* GeneDB and Swiss-Prot link to the budding yeast section of *GermOnline* from their respective locus pages. GermOnline is a fully operational prototype accessible through a network of servers in the US, Europe and Japan at http://www.germonline.org (Figure 1). We are currently in the process of building up information and microarray data content.

Fig. 1: a screen shot of GermOnline's welcome page

The Ashbya Genome Database

The genome of the haploid filamentous fungus *Ashbya gossypii* was sequenced, assembled, and fully annotated by the laboratory of Peter Philippsen in collaboration with Syngenta Inc. Approximately 4720 protein-encoding loci located on seven chromosomes were identified. The vast majority of these genes are

highly homologous and syntenic (conserved gene order) to their orthologues in the budding yeast *S. cerevisiae*. Therefore, the Ashbya Genome Database (AGD) is a very useful tool for the various yeast communities as well as biologists who are interested in evolutionary aspects of genome research. AGD provides researchers with a convenient genome browser graphical user interface (GUI), and will include a three-way synteny viewer (one *Ashbya* chromosome versus two *Saccharomyces* chromosomes), and numerous external links to other important databases including SGD and Swiss-Prot. Release 1.0 of the database is savailable at http: //agd.unibas.ch/.

The Microarray Data Management and Analysis System (MIMAS)

It is our goal to develop a microarray data management and analysis system based upon a relational database solution. A sophisticated graphical user interface (GUI) was developed that will make the cumbersome but essential task of entering information about experiments most efficient and straightforward for the scientists who use array facilities in Switzerland (Figure 2). MIMAS will enable the members of the Swiss Array Consortium (SAC) to upload, analyse and share microarray data in a convenient and practically error-

Fig. 2: a screen shot of the data uploading module in MIMAS.

proof manner. Importantly, it will also let them upload the raw data prior to publication to the ArrayExpress repository maintained by the EBI virtually by a single key stroke. The database model is fully MIAME-compliant (Minimal Information about a Microarray Experiment) and partially integrated a commercially available user management solution used within the SAC (GeNet). The repository will be the data source for various analysis tools including open source (CTWC, BioConductor) and commercial software (GeneSpring). It should be emphasized that our laboratory develops this repository for joint use together with the Friedrich Miescher Institute (Edward Oakeley), the University of Geneva (Patrick Descombes), the CIG in Lausanne (Keith Harshman), the Functional Genomics Center in Zuerich (Ralph Schlapbach) and the Institute for Biological Research in Bellinzona (Francesco Bertoni).

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Protein structure bioinformatics

Three-dimensional protein structures are key to a detailed understanding of the molecular basis of protein function. Combining sequence information with 3D structure gives invaluable insights for the development of effective rational strategies for experiments such as site directed mutagenesis, studies of disease related mutations, or the structure based design of specific inhibitors. Today, the number of structurally characterized proteins is about two orders of magnitude smaller than the number of known protein sequences in the UniProt database, i.e. no direct experimental structural information is available for the vast majority of protein sequences. Theoretical methods for protein structure prediction are aiming to bridge this structure knowledge gap. As shown during the biannual CASP experiments, homology modeling is the only computational approach, that can generate reliable three-dimensional models for a protein. My group is working on the development and application of methods in molecular modeling with emphasis on large-scale automated comparative protein modeling.

Automated large-scale comparative protein structure modeling

If a protein sequence shares significant amino acid sequence similarity to at least one protein with experimentally determined three-dimensional structure, homology or comparative modeling can be applied to construct a three-dimensional model for the new protein. The huge and constantly growing number of structurally uncharacterized protein sequences together with the increasing number of available template structures motivated the development of automated modeling methods. It has been estimated that at the moment about one third of all sequences has detectable similarity to one or more known protein structures and are thereby accessible to homology modeling. With regard to ongoing structural genomics efforts, the usefulness of comparative modeling for soluble proteins is increasing steadily.

Automation of homology modeling demands high standards of the stability and reliability of the implementation and stimulates the ongoing development of better algorithms. The SWISS-MODEL server pioneered the field of web-based automated modeling and is now being further developed in my group at the Biozentrum (in collaboration with N. Guex, GSK). With more than 140’000 modeling requests in 2003, the SWISS-MODEL server is currently the most widely used web-based modeling service. In order to ensure the quality and reliability of the modeling results, SWISS-MODEL is part of the EVA project, aiming to continuously evaluate the accuracy of protein structure prediction servers. The refinement of homology modeling techniques, especially the development of expert systems for automated large-scale modeling, will be a major focus of my group during the next years.

The SWISS-MODEL repository

We have established the SWISS-MODEL Repository to provide access to an up-to-date collection of annotated models generated by automated homology modeling, bridging the gap between sequence and structure

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databases. It contains the results of automated large-scale modeling for sequences from the UniProt database using a modified SWISS-MODEL server pipeline. The SWISS-MODEL Repository has been implemented using relational database technology. During the modeling process it communicates with the SWISS-MODEL server pipeline and keeps track of the workflow for individual target sequences. The quality of each model is assessed using a partial Gromos96 force field implementation, and the empirical Anolea mean force potential in order to select reliable models to be entered into the database. Functional annotation is done by mapping InterPro descriptors to individual models.

As of January 2004, the Swiss-Model repository contained 362,904 models for 324,571 different UniProt sequence entries. The length of the models varies from 45 to 1,524 residues with an average model size of 205. The Repository is updated regularly to take into account new sequences, modifications of existing sequence entries, and new template structures released by the PDB that might allow the construction of models for previously un-modeled proteins, or might provide a better template for already existing model entries. Also, fundamental changes and improvements of the modeling pipeline initiate a new update cycle.

Integration of structural models with biochemical knowledge databases is a crucial step in any computational analysis of sequence-structure-function relationships. In the future, links established from the Swiss Model Repository to other biological knowledge bases, e.g. InterPro, will allow visualizing regions of functional relevance by highlighting them on 3-dimensional protein models.This integration will have direct applications in the validation of sequence-based functional assignments in a 3-dimensional structural context.

Structural bases of inherited human disease

Single nucleotide polymorphisms, or SNPs, are accounting for the major part of inter-individual variation in the genome. The effects of genetic variations range from neutral mutations over increased susceptibly for complex diseases, individual variation in drug response, to rare single-allele mendelian inherited diseases. Non-synonymous mutations located in protein coding regions, i.e. variations leading to an exchange of an amino acid, may possibly affect a protein's stability or function. Since protein function strongly depends on the integrity of its three-dimensional structure, structural models of proteins can be used to analyze the functional role of the mutated residue and to provide insights into possible molecular consequences of mutations. For example, using homology modeling of the membrane bound multi-domain protease TMPRSS3, we could rationalize the impact of the observed mutations on the protein, which cause a rare form of inherited deafness in Caucasians. The development of a general objective scoring function to asses the impact of mutations is still subject of ongoing research. The systematic analysis of pairs of protein structures with single point mutations provides the basis for the development of suitable comparative modeling processes.

Modeling protein ligand interactions

Structure-based and structure-guided drug design methods have made significant impact on the development of drugs in recent years. Although several compounds discovered with the help of structural information have successfully passed clinical trials and have become approved drugs, one of the bottlenecks of structure-based methods is the availability of experimental structures of the target proteins. In these cases, protein structure homology models can provide a valuable alternative. One of the open questions in this context is how errors and inaccuracies of the homology models influence the molecular modeling of the protein-ligand interaction. In collaboration with M. Meuwly (Department of Chemistry) we are using molecular dynamics approaches to quantify protein-ligand affinities based on homology models. A better model for effects of sequence variations on ligand affinity will on one side help to rationalize inter-individual variations in drug response caused by mutations in drug receptors or metabolizing enzymes. On the other side, it supports the rational design of site specific mutagenesis experiments aiming to alter the substrate specificity of enzymes, e.g. modifications in the ATP binding pocket of protein kinases.

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Principles of regulatory design

This group has been newly established in September 2003. My previous work at the Center for Studies in Physics and Biology of the Rockefeller University focused on the development of probabilistic algorithms for the analysis of large scale biological data that aim to uncover design principles in the global organization of regulatory pathways. Specific projects included:

1) Genome-wide predictions of regulons in bacterial genomes using comparative genomics. By searching for short sequence fragments that are significantly conserved between promoter regions of orthologous genes in related bacterial species one can identify putative transcription factor binding sites on a genome-wide scale. Using a probabilistic clustering algorithm thousands of such putative sites can be partitioned into putative regulons. Using this approach, my work recovered many of the known regulons in E. coli, and predicted almost one hundred new regulons.

2) Functional organization of mRNA decay rates. Here we showed, using analysis of microarray expression data, that there are common design principles in the functional organization of transcriptome-wide mRNA decay rates. For instance, from yeast to human, mRNAs that encode regulatory proteins decay significantly faster than mRNAs encoding biosynthetic proteins. We also uncovered sequence motifs, whose occurrence in 3' UTR of transcripts correlates with the mRNA's decay rate.

3) Scaling-laws in functional gene-content. By functionally annotating all currently sequenced genomes one can compare the number of genes in different functional classes across genomes ranging from the simplest bacteria to human. We performed such analyses and discovered that there are quantitative scaling laws relating the number of genes in a given functional class to the total number of genes in the genome for a large number of high-level functional classes. For example, in bacteria the number of transcription factors in a genome scales with the square of the total number of genes in the genome.

In the coming years we will further pursue comparative genomic analyses for the genome-wide annotation of transcription factor binding sites. We are particularly interested in integrating the comparative genomic analysis with analysis of microarray expression data. In addition, we will also use comparative genomic approaches for studying the evolution of gene-content in bacterial genomes, with the aim of elucidating the evolutionary origin of the scaling laws mentioned in project 3 above.

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Computational methods for studying the regulation of gene expression

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My group has been established at the Biozentrum in September 2003, when I moved from the Computational Genomics Laboratory headed by Terry Gaasterland at the Rockefeller University in New York.

We are interested in the various ways in which cells regulate the constellation of transcripts that they express at a given time:

1) A common way of regulating gene expression is by altering the rate at which genes are transcribed. This is accomplished by transcription factors that bind to specific sequences upstream of the transcription start site and either enhance or inhibit the rate of transcription. One of our on-going projects concerns the development of computational methods for genome-wide annotation of transcription factor binding sites in mammalian genomes.

2) Mammalian transcriptomes are additionally diversified by alternative splicing. It has been estimated that as much as 60% of the human and mouse multi-exon genes have multiple splice forms. While at Rockefeller, I have developed a software system to map all the known mouse transcripts to the mouse genome, and to characterize the splice variation present in this species. We are currently extending this system to provide more downstream analyses of the functionality of the alternative splice forms. We will use this software to analyze the mouse, human and rat transcriptomes.

3) Most recently it has been discovered that gene expression can be regulated at the post-transcriptional level by small regulatory RNAs. We are collaborating with the laboratory of Dr. Thomas Tuschl at the Rockefeller University and with the group of Dr. Chris Sander at the Memorial Sloan Cancer Center in New York on the annotation of small RNA sequences obtained through large-scale cloning, on the discovery of novel regulatory RNAs in these libraries, and on the characterization of the downstream targets of miRNAs (Pfeffer S., Zavolan M., Graesser F. A. et al. (2004). Identification of virus-encoded microRNAs. *Science*, 30 April 2004).

The Life Sciences Training Facility provides a microarray platform and training for researchers who carry out quantitative large-scale DNA and RNA analysis.

Introduction

High Density Oligonucleotide Microarrays (GeneChips) and PCR microarrays (spotted arrays) are available for nearly all key model systems and *H. sapiens*. GeneChips contain 25 base oligonucleotide probes directly synthesized *in situ* on glass slides using photolithography and combinatorial chemistry. 11 to 16 of these probes recognize one single transcript. PCR microarrays are standard microscopic glass slides onto which PCR fragments or oligonucleotides are spotted using a robotic device (see http://www.gene-chips.com). The arrays are hybridized with a mixture of cRNAs from two samples, each labelled with a different fluorophor (CY3 and CY5). GeneChips ultimately yield a single fluorescence intensity measurement for each gene, while PCR microarrays produce a ratio between two signals (red and green) measured simultaneously in one spot.

Today, there are three major applications for microarrays. The most common experiments are expression profiling studies that determine thousands of mRNA concentrations in parallel. It is also possible to analyze genomic DNA to identify small mutations or gene deletions. Finally, genome-wide protein DNA binding patterns of factors involved in e.g. DNA replication, recombination or transcription by combined chromatin immunoprecipitation and hybridization to microarrays (ChIP-Chip experiments) can now be studied. The latter application is limited to spotted PCR arrays for the moment since GeneChips contain probes that are usually located in the 3'-UTR of a locus. However, the next generation of "genomic" GeneChips that contain tiling oligonucleotides covering the whole genome will be available soon.

The High Density Oligonucleotide Microarray (GeneChip) platform

The LSTF provides the infrastructure for carrying out research projects based upon the Affymetrix GeneChip system. This includes training in total RNA preparation, cRNA target synthesis and quality control, microarray hybridisation and scanning. The facility is equipped with state-of-the art GeneChip Scanner 3000 and two fluidics stations 450 as well as the necessary IT-infrastructure required for array data storage, backup and processing. This is ensured by a close collaboration with the lab of Torsten Schwede, Dean Flanders (FMI) and the Swiss Institute of Bioinformatics (SIB) whose efforts lead to the construction of the Basel Computational Biology Center $[BC]^2$ (http://www.bc2.ch).

The Microarray production platform

The LSTF continues to provide indirect support for the microarray labs located at the Biozentrum

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and the Swiss Tropical Institute. This ensures that the hardware, software and know-how required for manufacturing and processing PCR microarrays are available in Basle for the benefit of the local scientific community.

Accomplished experimental work

Over the last 24 months approximately 65 projects were carried out that involved 20 laboratories and nearly 35 PhD and postdoctoral students as well as research technicians from Basle, Bern and Strasbourg. A total of 1054 GeneChips were hybridized that include arrays for *H. sapiens* (46 %), *M. musculus* (22 %), *R. norvegicus* (15 %), *D. melanogaster* (7 %) and *S. cerevisiae* (10 %). The total cost of these experiments was approximately CHF 1'100'000.-.

The Microarray data analysis platform

To support users of the LSTF we organize microarray data analysis training courses at various levels for beginners and advances users. These courses focus on commercially available software solutions that we purchased by a large number of laboratories within the Swiss Array Consortium. In addition to that we provide appropriate training and access to the local mirror of a server-based clustering tool (Coupled-Two-Way-Clustering, C2WC; Figure 1) developed by Eytan Domany. It is our intention to expand the array of tools provided for the users by including software packages from the open source community and to establish an IT-lab that is meant to serve as an analysis hub for scientists who employ GeneChips in their research.

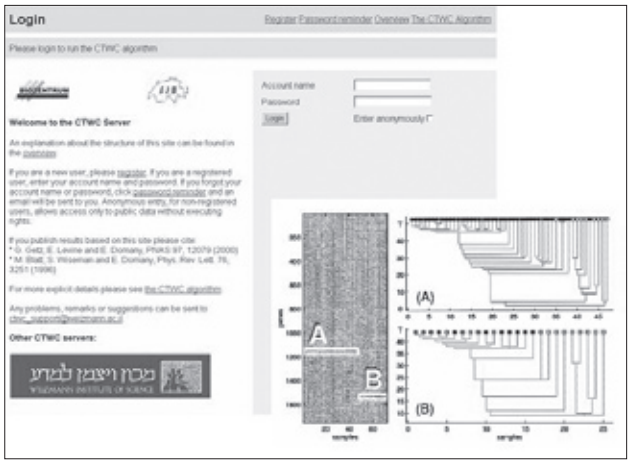

Fig. 1: the Coupled Two Way Cluster Server at the Biozentrum (http://ctwc.bioz.unibas.ch)

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PROTEIN CHEMISTRY

Functional phosphoproteomics by mass spectrometry

The yeast *Saccharomyces cerevisiae* adapts to the supply of nitrogen in the culture medium by expressing a number of permeases capable of transporting amino acids for use as sources of nitrogen and building blocks for protein synthesis. Upon nitrogen limitation or rapamycin treatment, high-affinity amino acid permeases such as the tryptophan permease TAT2 are rapidly internalised and sorted to the vacuole for degradation, while the broad specificity permease GAP1 is delivered to the plasma membrane and stabilised. The differential sorting of these two classes of permeases is mediated by the Ser/Thr kinase NPR1. Nitrogen limitation or rapamycin treatment activates NPR1 by dephosphorylation with the TOR-modulated SIT4 phosphatase. The TOR kinase promotes the association of the SIT4 phosphatase and the regulatory subunit TAP42. Upon nitrogen starvation or treatment with rapamycin, SIT4 is activated by dissociation from its inhibiting subunit TAP42. In turn, SIT4 dephosphorylates NPR1 and as a consequence, GAP1 is allowed to reach the plasma membrane (s. Figure 1).

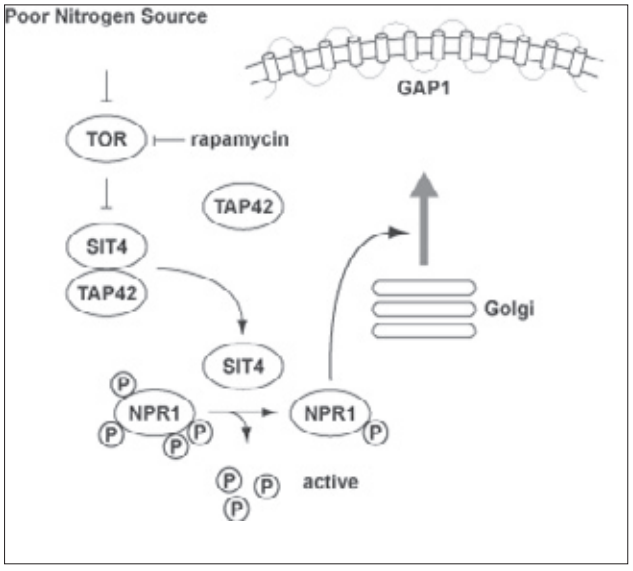

Fig. 1: Sorting of the general amino acid permease GAP1 and the tryptophan permease TAT2. Upon nitrogen starvation, or inactivation of TOR by rapamycin, the SIT4 phosphatase is activated by dissociation from its regulatory subunit TAP42. SIT4 dephosphorylates NPR1 which in turn positively influences the sorting of GAP1 from the Golgi to the plasma membrane where it is stabilised.

To characterize the rapamycin-sensitive phosphorylation sites in NPR1 (Swiss Prot database accession no. P22211), a mass spectrometric analysis was initiated. First, a method was developed to quantitate the changes of phosphorylation occuring in NPR1 when yeast cells are treated with rapamycin. The method consists of three steps: (i) enzymatic digestion in $H_2{}^{16}O$ or isotopically enriched $H_2{}^{18}O$ to label pools of NPR1 derived from untreated

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or rapamycin-treated cells, respectively; (ii) affinity selection of phosphopeptides from the combined digests by immobilized metal-affinity chromatography; and (iii) dephosphorylation with alkaline phosphatase to allow for quantitation of changes of phosphorylation by MALDI-TOF. With this method, alterations in the extent of phosphorylation of 21 phosphosites could be easily assessed and quantitated both in wild-type yeast cells treated with rapamycin and in cells lacking the SIT4 phosphatase responsible for dephosphorylating nitrogen permease reactivator protein (Figure 2). The method here is simple and allows quantitation of relative changes in the level of phosphorylation in signaling proteins.

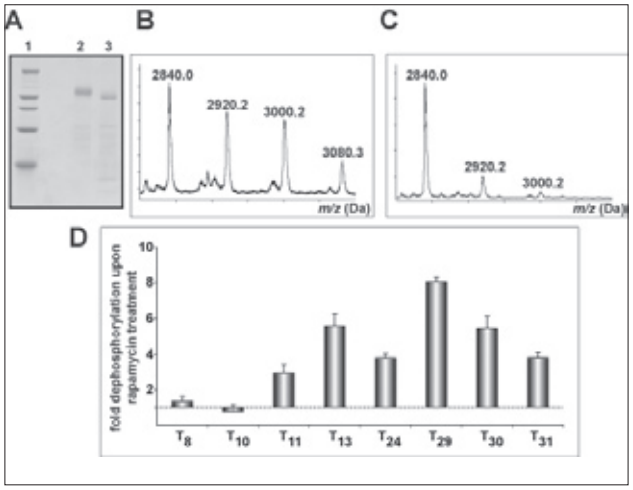

Fig. 2: Regulation of GST-NPR1 phosphorylation by rapamycin treatment. A: Coomassie-blue stained SDS gel of GST-NPR1 isolated from untreated cells (lane 2) or from cells that had been treated with 200 nM rapamaycin for 15 min (3). Lane 1 contains the molecular weight markers myosin, 200 kDa; β-galacotsidase, 116 kDa; phosphorylase b, 97 kDa; serum albumin, 66 kDa; ovalbumin, 45 kDa. B: Linear mode MALDI-TOF spectrum of the tryptic peptide T11 from untreated cells (B) and from cells which had been treated with 200 nM rapamycin for 15 min (C). The peptide with a mass/charge ratio of 2840.0 Da corresponds to unphosphorylated T11, while the peaks at 2920.2, 3000.2, and 3080.3 correspond to the singly, doubly, and triply phosphorylated peptide T11. D: Quantitation by stable isotope labelling of the extent of dephosphorylation of individual phosphopeptides upon rapamycin treatment of GST-NPR1 from untreated and from cells treated with rapamycin for 15 min.

With the precise knowledge of the sites of phosphorylation in NPR1, studies will be carried out to (a) establish the molecular function of NPR1 phosphorylation, (b) to search for possible NPR1 substrates, and (c) to search for protein kinases that activate NPR1.

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ZMB - Center of Microscopy of the University of Basel

Introduction

The ZMB is a central service unit providing microscopical imaging support and training. The service covers Transmission Electron Microscopy (TEM), Scanning Electron Microscopy (SEM) and specialized light microscopy such as Confocal Laser Scanning Microscopy (CLSM), Video- and Fluorescence Microscopy.

Administratively, the ZMB is integrated into the Biozentrum; his service commitments indeed cover the microscopical requirements of the University of Basel as a whole. Historically it has developed out of a fusion of the Interdepartmental Electron Microscopy unit of the Biozentrum and the REM-Laboratory of the University of Basel.

Services and training

The facilities of the ZMB comprise a Preparation Laboratory, two Transmission Electron Microscopes and three Scanning Electron Microscopes. In collaboration with the Biozentrum that provides certain additional instruments, the ZMB offers service training and technical support for Video-, Fluorescence- and Confocal Light Microscopy. Also a Confocal Laser Scanning Microscope (CLSM) with image processing hard- and software is at disposal on request.

Training courses

The ZMB organizes the following training courses in microscopy:

A two weeks basic workshop on microscopy for students of the biology curriculum; a one week workshop ‘applied microscopy for molecular biologists’ and a one week workshop ‘applied microscopy for biologists’.

For further details on time schedule and registration, please contact the Units’ homepage.

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* employed during part of the report period

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In collaboration with Andreas Hefti (Division of Molecular Microbiology, Biozentrum); Dean Flanders (Friedrich Miescher Institute, Basel); Reto Schaub* (Department Forschung, Kantonsspital Basel)

Introduction

BioPhIT integrates divisional and central information technology support positions in order to centrally provide support for the Biozentrum and Pharmazentrum in the area of information technology. BioPhIT also embeds its activities in the framework of the Basel Computational Biology Center ($[BC]^2$), a collaboration between the Biozentrum, the Swiss Institute of Bioinformatics (SIB), and the Friedrich Miescher Institute (FMI). The aim is to share knowledge, resources, and services to create a fertile environment for research groups in computational biology, bioinformatics, and experimental life sciences. In particular, through close collaboration with the FMI, a standardized infrastructure covering Windows and Linux application and file services, high-availability storage systems including backup and an Oracle database environment has been built.

Major outcomes of the activities

- A central Windows Active Directory Service (ADS) Infrastructure was designed and implemented for the Bio-/Pharmazentrum. The existing Windows NT 4 domains were smoothly migrated. The ADS environment includes fully automated (pushed) updates of operating system security patches and anti virus signature files. Software costs are reduced by the use of a licensing server and concurrent use type of licensing (whenever possible).
- Successful introduction of a central helpline and web--based help desk system allowed BioPhIT to further strengthen and improve the different levels of daily user support at the Bio-/Pharmazentrum
- For the new and demanding Division of Bioinformatics, a Linux based client/server working environment was setup. Beside file and application servers, an Oracle databases environment and a 24 node high performance computing cluster completes this infrastructure.
- BioPhIT provides and technically administers the platform for various static and dynamic Web-Services. This includes the home pages of the Biozentrum, the Pharmazentrum, the Basel Neuroscience Program and the Institute of Zoology, the Basel Computational Biology Conference page, the Bench-to-Bedside Symposium and the Neurofly Symposium 2004. In the scientific area, the Swissmodel Comparative Protein Modeling Server (including Swissmodel Repository), the Germonline knowledge base, the Ashbya Genome Database, and the Nubiscan nuclear receptor binding site prediction server are hosted.
- Administration, maintenance and user support for the Wisconsin Package (GCG), a software package for the analysis of DNA and protein sequence data which is widely used by researchers from institutes of the entire Basel University. Local copies of major DNA and protein sequence databases are provided and updated on a regular schedule. Recently, the users have started to migrate towards EMBOSS (the European Molecular Biology Open Software Suite) which is an open source alternative to the commercial Wisconsin Package.

Core Facilities and Administration Personnel

Core Secretariat
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Jeannette Holenstein, Ursula In-Albon, Cinar Kara, Elisabeth Loiseleur*, Marisa Müller, Doris Rossi*, Christine Saladin, Silvia Schad*, Irène Schmucki, Caroline Schneider, Vera Sinniger

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Karl-Heinz Heuser, Gioacchino Romagnoli, Hans-Rudolf Zeller

Mechanical workshop
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Electronical workshop
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Technical services
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Safety and radiation protection
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Web and Graphic Design
Werner Indlekofer
Margrit Jaeggi

Photolab
Verena Grieder, Annette Roulier, Ingrid Singh

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Anka Stark

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Renato Zedi
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