A.J.M. Driessen, M. van der Laan, S. Kol, F. de Plessis, J. de Keyzer, and N. Nouwen

**YidC – an evolutionary conserved device for the assembly of energy-transducing membrane protein complexes in bacteria**

All primary energy-transducing membranes contain a member of the cytochrome oxidase biogenesis (Oxa) membrane protein family. In mitochondrial inner membranes, Oxa1p mediates the integration of membrane proteins from the matrix side. In chloroplast thylakoid membranes, Alb3 is required for integration of light-harvesting complex proteins. Bacterial cytoplasmic membranes contain one or more family members that appear to have multiple functions in membrane protein biogenesis. The best-studied example is YidC from *Escherichia coli*, an essential membrane protein. YidC associates with the SecYEG channel and binds to inserting nascent membrane proteins. YidC also function on its own or together with unknown components. Cells that are depleted from YidC are defective in the assembly of major energy transducing complexes such as the F₀ domain of the ATPase and cytochrome o oxidase (1). By direct reconstitution, we could show that subunit c of the F₀ ATPase domain strictly requires YidC for membrane insertion, a process that precedes the formation of an oligomeric subunit c ring (2). Membrane insertion of the quinol binding subunit CyoA of cytochrome o oxidase requires both SecYEG and YidC. YidC therefore seems to fulfill a more specific role than previously anticipated. It may act as a membrane-embedded molecular chaperone that facilitates and stabilizes newly inserted membrane proteins prior to their assembly into multisubunit membrane complexes.

**Literature:**


**Contact:**

**Prof. Dr. Arnold J.M. Driessen**

University of Groningen
Department of Molecular Microbiology
Biomolecular Sciences and Biotechnology
Institute & Materials Science Center Plus
a.j.m.driessen@rug.nl

Kerklaan 30
9751 NN HAREN (The Netherlands)
P. Rehling

Transport of preproteins across the inner membrane of mitochondria

Most mitochondrial proteins are encoded in the nucleus and transported into mitochondria posttranslationally from the cytosol. The TOM complex mediates protein transport across the outer mitochondrial membrane (1). Multispanning membrane proteins with internal targeting signals destined for the inner membrane use the twin-pore translocase (Tim22 complex) for membrane insertion (1,2). In contrast, proteins that use N-terminal presequences as targeting signals are transported by the presequence translocase (TIM23 complex) across the inner membrane into the matrix or into the inner membrane. We have isolated the TIM23 complex (3) with its associated import motor (PAM) (4) and identified new proteins of both complexes that participate in protein import. Our recent analyses indicate that the presequence translocase is built as a modular multi-protein complex, which first associates physically with the TOM complex and then recruits the PAM complex for matrix protein import.

Literature:

Contact:
PD Dr. Peter Rehling
Albert-Ludwigs-University of Freiburg
Institute of Biochemistry and Molecularbiology
peter.rehling@biochemie.uni-freiburg.de
Hermann-Herder-Straße 7
D-79104 Freiburg (Germany)
The catalytic cycle of the NBD of the ABC-transporter HlyB from E. coli

In Gram-negative bacteria different pathways exist to translocate proteins across the inner and outer membranes. Haemolysin B (Hly B) is a paradigm for the so-called Type I secretion, which is a Sec-independent mechanism that shuttles various substrates in one step across both membranes of Gram-negative bacteria. All information necessary and sufficient for translocation is encoded at the C-terminus of the transported substrate (allocrite). This secretion signal normally comprises the last 50 to 60 C-terminal amino acids of the allocrite. The first identified allocrite secreted by the Type I pathway was haemolysin (Hly) A, a 107 kDa pore-forming toxin secreted by certain uropathogenic E. coli strains and member of the family of RTX-toxins. The Type I secretion apparatus is made up of three components, an ABC-transporter, a membrane-fusion (MFP) protein, and an outer-membrane protein (OMP). For the HlyA transporter complex, HlyB (ABC-transporter) and HlyD (MFP) reside in the inner-membrane of E. coli. The OMP component is TolC, which is thought to interact with the MFP to form a continuous channel across the periplasm, from the cytoplasm to the exterior. HlyB belongs to the family of ABC-transporters, which are ubiquitous, ATP-dependent transmembrane pumps or channels. Despite major advances in the understanding of ABC-proteins, the exact mechanism of ATP-hydrolysis remains an enigma. Particularly, the exact function of the conserved amino acid residues in the vicinity of the ATP-binding pocket, the reaction path of ATP-hydrolysis, and the molecular communication between NBD monomers during ATP-binding and hydrolysis is poorly understood.

Here, we report the crystal structures of each individual state of the catalytic cycle of the HlyB-NBD, which was obtained for the wild type protein or for mutants, in which amino acids essential for the function of the enzyme were selectively replaced by repressive amino acids. Based on these structure combined with biochemical and biophysical studies we will try to answer the above-mentioned questions and propose a model in which the chemical energy of ATP and the mechano-chemistry induced by the binding of ATP and subsequent dimerization of the NBD is used to coordinate allocrite transport across the two membranes of E. coli.

Contact:
Prof. Dr. Lutz Schmitt
Heinrich-Heine-University of Düsseldorf
Institute of Biochemistry
Universitätsstraße 1
lutz.schmitt@uni-duesseldorf.de
D-40225 Düsseldorf (Germany)
Mycobacterial porins - new channel proteins in unique membranes

The outer membrane of mycobacteria is unique among bacteria and provides an effective permeability barrier rendering mycobacteria intrinsically resistant to many antibiotics. Porins provide water-filled channels for diffusion of hydrophilic compounds across the mycobacterial outer membrane (1). Deletion of the *mspA* gene in *Mycobacterium smegmatis* reduced its permeability towards glucose four-fold (2) and increased its resistance to hydrophilic antibiotics 8-16-fold. These results showed that MspA is the major porin of *M. smegmatis*. An x-ray analysis of MspA revealed a homoctameric goblet-like conformation with a single central channel (3). MspA contains two consecutive membrane-spanning β barrels. This is new architecture of a channel protein and represents the first structure of a mycobacterial outer membrane protein. One of the most remarkable features of MspA is the constriction zone, which is lined by 16 aspartate residues (D90, D91). Functional analysis of mutant MspA proteins by lipid bilayer and liposome swelling experiments revealed that this constriction zone determines the channel properties of MspA. Thus, the channel properties of MspA can be easily tailored to specific needs in nanotechnological applications.

Expression of *mspA* increased the sensitivity of *Mycobacterium tuberculosis* to isoniazid, ethambutol, streptomycin and beta-lactam antibiotics. This is the first experimental proof that the porin pathway limits the efficiency of hydrophilic antibiotics and drugs against *M. tuberculosis* (4). It is concluded that increasing the permeability through porins of *M. tuberculosis* is a useful strategy to improve TB chemotherapy.

**Literature:**


**Contact:**
**Prof. Dr. Michael Niederweis**
University of Alabama at Birmingham
Department of Microbiology, BBRB Room 609
[mnieder@uab.edu](mailto:mnieder@uab.edu)
Mailbox 24
1530 3rd Ave S
Birmingham, AL 35294 (U.S.A.)
Domain interaction in the assembly and function of CFTR

CFTR is the only known ABC protein to be an ion channel. Binding of ATP at two sites and hydrolysis at one regulate the opening and closing of the permeation pathway to halide ions. However, in order that the channel not gate continuously while exposed to cytoplasmic ATP concentration far above the ATP binding constants and Km for hydrolysis, allosteric coupling between the binding sites and the channel gate is stringently controlled by phosphorylation of the central R-domain. This relatively unstructured R-domain is unique and conserved among CFTRs from different species only in the distribution patterns of multiple sites for phosphorylation by protein kinase A. While sequence homology searches yield no significant similarities to other proteins, pattern searches revealed high similarity to phosphorylated nuclear proteins of several types.

Nucleotide binding site I constituted by the Walker A motif of NBD1 and the signature sequence of NBD2 is non-hydrolytic while site II is hydrolytic. Channel gating reflects primarily ATP binding and hydrolysis at site II.

The most common CFTR mutation in patients with cystic fibrosis, ΔF508 alters a hydrophobic patch on the surface of NBD1, disrupting an interaction elsewhere in the protein. The mutated protein is recognized by ER quality control and degraded by the proteasome. Because the two NBDs closely associate to form the sites of ATP binding and hydrolysis, it was suspected ΔF508 may perturb their interaction. However, we have found that this is not the case. Indeed, CFTR can mature and pass ER quality control in the complete absence of NBD2, consistent with but not proving that NBD2 may be added post-translationally. ΔF508 renders the first transmembrane domain (TMD1) of CFTR hypersensitive to limited protease digestion suggesting that the phe508-containing surface patch on NBD1 normally interacts with TMD1, mostly likely with a cytoplasmic loop between membrane spanning helices. We speculate that this interaction may be necessary for the correct association between TMD1 and TMD2 to form the channel pore which can occur in the absence of NBD2. (Supported by the NIH.)

Literature:


Contact:
Dr. John Riordan
Mayo Clinic College of Medicine
Department of Biochemistry and Molecular Biology
riordan@mayo.edu
13400 E. Shea Boulevard
Scottsdale, AZ 85259 (U.S.A.)
V. Flockerzi

TRPC4-pore-forming subunit, transporter or accessory subunit

The TRP Superfamily of membrane proteins is comprised of six subfamilies, three of which TRPC, TRPV and TRPM, include mammalian members which share significant structural similarities to the founding member, Drosophila TRP. The mammalian proteins that display the greatest sequence similarity to Drosophila TRP belong to the TRPC subfamily and include TRPC4. TRPC4 comprises six sequence segments predicted to form transmembrane helices and is generally believed to be part of an ion conducting channel. Two variants of the TRPC4 protein have been identified in human and in mouse tissues. They include a “full length” TRPC4 protein and, in addition, a slightly shorter variant which lack a stretch of 84 amino acid residues within the cytoplasmic C-terminal part of the protein. Both TRPC4 protein variants bind calmodulin with high affinity in the presence of Ca\(^{2+}\) and we used this feature to enrich the TRPC4 containing protein complex after solubilization and affinity chromatography. In parallel we established a semi quantitative approach to determine the number of TRPC4 proteins in a single cell. Together these combined approaches will allow analysing the TRPC4 containing protein complex and correlating these data with recent functional studies.

Contact:
Prof. Dr. Veit Flockerzi
University of Saarland
Institute for experimental and clinical Pharmacology and Toxicology
Faculty of Medicine
Veit.flockerzi@uniklinik-saarland.de
Gebäude 26
D-66421 Homburg (Germany)
Regulated interaction between the Arr4p ATPase and the Gef1p CLC chloride channel in yeast

* =equal contribution

CLC chloride channels are a family of channel proteins mediating chloride transport across the plasma membrane and intracellular membranes. In contrast to cation channels, little is known about accessory proteins involved in the diversity of CLC function and subcellular localization. We have employed the yeast two-hybrid system to identify interaction partners of the single CLC protein present in *S. cerevisiae*, Gef1p.

We show that the dimeric ATPase Arr4p can be recruited to the CLC protein. The interaction was verified by co-immunoprecipitation of the two full-length proteins. The C-terminal CBS (cystathione beta-synthetase) domains are involved in the interaction of Arr4p with the Gef1p C-terminus. Arr4p is a soluble cytosolic protein that is found on different membranous compartments as a peripherally associated protein. We find that Arr4p does not represent an obligatory beta-subunit of the CLC protein and, thus, set out to determine the physiological context and relevance of the interaction.

*In-vitro* binding experiments based on proteins purified from *E. coli* revealed that a factor present in yeast but not bacterial cytosol is required to reconstitute the interaction. Subsequent fractionation of the yeast cytosolic extract showed that the factor is a small ligand rather than an additional protein required for complex formation. We show that Arr4p can act as a cytosolic sensor for this ligand in living yeast cells and propose that Arr4p adjusts transmembrane transport activities, like the one mediated by Gef1p, to optimize ion transport to the lumen of the secretory pathway with respect to the sensed parameter.

Literature:

Contact:
PD Dr. Blanche Schwappach
Ruprecht-Karls-University of Heidelberg
Centre of Molecular Biology
b.schwappach@zmbh.uni-heidelberg.de
Im Neuenheimer Feld 282
D-69120 Heidelberg (Germany)
Functional proteomics of nicotinic acetylcholine receptors in Caenorhabditis elegans

Nicotinic acetylcholine receptors (nAChRs) are ligand gated ion channels (LGICs) that mediate fast excitatory neurotransmission at post-synaptic sites on the plasma membrane of neurons and muscles. As a prototypic LGIC, understanding the function of the nAChR is of general interest, in particular because of its crucial role in mediating nicotine addiction. Little is known about proteins interacting with nAChRs, which are expected to functionally regulate them and to govern their cell surface expression.

To identify such proteins, we have purified the levamisole-sensitive nAChR from C. elegans using the tandem affinity purification, and identified the associated proteins by mass spectrometry. Five of those proteins were non-nAChR subunits that affected behavioral responses to nicotine, indicative of impaired nAChR function, when inactivated by RNAi. The nicotine resistance phenotypes were confirmed in genomic deletion mutants.

Among those proteins is a copine, which binds to the plasma membrane in a Ca^{2+}-dependent manner, and SOC-1, a substrate adaptor of the fibroblast growth factor (FGF) receptor, EGL-15. Further analysis demonstrated that loss of these proteins causes a significant reduction in (post-synaptic) cell-surface expression of the levamisole receptor, implicating those proteins in either trafficking of nAChRs to, or stabilization on, the plasma membrane. We are functionally characterizing these and additional nAChR-associated proteins, using a combination of cell biology, genetics and also direct electrophysiological analysis in nematodes.

Literature:


Contact:
Dr. Alexander Gottschalk
Johann Wolfgang Goethe-University of Frankfurt/Main
Biocenter N210
A.Gottschalk@em.uni-frankfurt.de
Marie-Curie-Straße 9
D-60439 Frankfurt/Main (Germany)
Roles of chloride transport - function unravelled by dysfunction

The phenotypes of knock-out mouse models and human inherited disease was instrumental in defining the physiological functions of many chloride transport proteins, in particular the CLC chloride channels and KICC K-Cl co-transporters studied in our laboratory. CLC chloride channels and transporters function as dimers with one pore per subunit. They form an evolutionarily old gene family that has nine members in mammals. While the first branch of this family encodes plasma membrane channels, it is now clear that channels belonging to the other two branches reside primarily in intracellular organelles. This was first recognised for CIC-5, a channel which is mutated in Dent’s disease, a disorder characterised by low molecular weight proteinuria and kidney stones. CIC-5 resides in endosomes of the proximal tubule (PT), where it co-localises with the H\(^+\)-ATPase and endocytosed proteins. This suggested a role in the acidification of the endocytotic pathway. Disrupting CIC-5 in mice affects both fluid-phase and receptor-mediated endocytosis, as well as the endocytotic retrieval of certain plasma membrane proteins in the PT. As the PT endocytoses hormones such as PTH and 25(OH)VitD\(_3\), this leads to changes in calcitropic hormone levels and to kidney stones. We have also disrupted the highly homologous CIC-3 Cl\(^-\) channel that is expressed in brain and several other organs. This led to a nearly complete degeneration of the hippocampus and photoreceptors. CIC-3 was localised to late endosomes and synaptic vesicles, to whose acidification it contributes. In contrast to CIC-3 to CIC-6, which are mainly located in endosomes, CIC-7 is prominently expressed in lysosomes. The disruption of CIC-7 led to severe osteopetrosis, which is due to a failure of osteoclasts to acidify the resorption lacuna. CIC-7 can be inserted together with the H\(^+\)-ATPase into the osteoclast ruffled border upon attachment to bone. Stimulated by this finding, we also demonstrated that human patients with severe osteopetrosis have mutations in either the CIC-7 Cl\(^-\) channel, or in a subunit of the H\(^+\)-ATPase. The lack of CIC-7 additionally led to retinal degeneration and to a severe lysosomal storage disease in the CNS. Lysosomal storage was also observed in the proximal renal tubule. Contrary to our expections, no change in steady-state lysosomal pH could be observed in neurons.

The bacterial CLC protein CIC-e1, which has been crystallized by Dutzler and colleagues, surprisingly functions as a Cl/H exchanger rather than as a Cl channel, as shown by Accardi and Miller. We are therefore investigating whether such an exchange activity may also been found in mammalian CLCs, in particular with CIC-4 and CIC-5.

Contact:
Prof. Dr. Thomas J. Jentsch
University of Hamburg,
Center for Molecular Neurobiology, ZMNH,
Jentsch@zmnh.uni-hamburg.de
Falkenried 94
D-20246 Hamburg (Germany)
A. Simon, S.H. Karbach, and E.I. Closs

**Substrate supply of nitric oxide synthase: Functional crosstalk of transporters for amino acids and peptides**

We have established that endothelial and neuronal nitric oxide synthases (eNOS and nNOS) use distinct intracellular substrate sources. While both isoforms can obtain arginine from protein breakdown, only eNOS has access to arginine derived from recycling of citrulline to arginine. This is in spite of an active recycling reaction in nNOS expressing cells. Extracellular glutamine reduces eNOS activity by reducing intracellular citrulline levels, but has no effect on nNOS activity. In contrast, histidine interferes with substrate supply to both isoforms. Inhibition of intracellular protein breakdown by the proteasome inhibitor epoxomycin and the lysosome inhibitor chloroquine in endothelial cells, leads to a 80% reduction in eNOS activity which can be reversed by arginine or arginine-containing dipeptides. Histidine attenuates the recovery of eNOS activity by the dipeptides, suggesting that a transporter accepting both, peptides and histidine, is responsible for providing extracellular peptides as a substrate source for eNOS. In fact, the peptide and histidine transporter PHT1 (but not PHT2) is highly expressed in endothelial cells. Taken together, our data demonstrate that not only arginine transporters, but also transporters for neutral amino acids and peptides are involved in substrate supply to NOS.

**Contact:**
Dr. Ellen I. Closs
Johannes Gutenberg University of Mainz
Department of Pharmacology
Closs@Mail.Uni-Mainz.de
Obere Zahlbacher Straße 67
D-55101 Mainz (Germany)
Searching for substrates: Discovery of the ergothioneine transporter

Recently, associations between chronic inflammatory diseases (rheumatoid arthritis and Crohn's disease) and polymorphisms within the SLC22A4 gene have been reported. SLC22A4 codes for OCTN1 which belongs to the amphiphilic solute facilitator (ASF) family of integral membrane transporters. OCTN1 (= novel organic cation transporter) is supposed to transport tetraethylammonium (TEA), and to operate as H⁺/organic cation antiporter. Also, OCTN1 has been designated as "multispecific" cationic drug transporter with a broad substrate specificity. However, in our experiments the transport of TEA by OCTN1 was always very low. We presumed that the substrate specificity of OCTN1 was unresolved and thus have developed a new strategy of comprehensive substrate search.

Our strategy, termed LC-MS Difference Shading, is based on color-coded comparative image analysis of LC-MS data of cell lysates. A stable cell line was created based on 293 cells in which the transporter expression can be turned on by the addition of doxycycline to the culture medium. Cells with and without transporter expression were incubated in paired assays with diluted human plasma - a complex mixture of potential substrates. Cell lysates were directly analyzed by LC-MS, which generates large data sets with dimensions of m/z, time, and intensity. In order to detect robustly differences between both data sets, we have developed an algorithm that converts intensities to scales of gray and then combines RGB pixel information from both images. In the resulting difference image, compounds only present in the active or inactive set are shaded as red or cyan, respectively, while compounds present in equal amounts in both sets remain scales of grey.

A substrate lead, stachydrine (alias proline betaine), was thus identified. Analysis of transport efficiency of stachydrine-related solutes, affinity, and Na⁺ dependence indicates that the physiological substrate is ergothioneine (ET). The related carnitine transporter OCTN2 did not transport ET at all. Since ET is transported over 100 times more efficiently than TEA and carnitine, we propose the functional name ETT instead of OCTN1. ET, all of which is absorbed from food, is an intracellular antioxidant with metal ion affinity. The real-time PCR expression profile of human ETT, with strong expression in CD71⁺ cells, is consistent with a pivotal function of ET in erythrocytes. Moreover, prominent expression of ETT in monocytes suggests a protective role of ET in chronic inflammatory disorders.

Contact:
PD Dr. Dirk Gründemann
University of Köln
Department of Pharmacology
University Hospital
dirk.gruendemann@uni-koeln.de
Gleueler Straße 24
D-50931 Köln (Germany)
Pharmacology of human organic cation transporters

Monoamine neurotransmitters and many drugs with a positive net charge permeate cell membranes via "organic cation transporters" (OCTs). The human organic cation transporters hOCT1, hOCT2 and hOCT3 (also known as EMT, extraneuronal monoamine transporter) belong to the SLC22A solute carrier gene family whose genes are localized on chromosome 6 (q26-27). OCT1 is expressed in the liver and small intestine, OCT2 in the kidney and in human placenta, whereas OCT3 mRNA is detected in various tissues such as liver, kidney, heart, small intestine and placenta. The mRNAs of the OCTs have also been found in the brain, and functional hOCTs have been identified in glial cells. Transport of organic cations by OCTs is independent of Na$^+$ and Cl$^-$ and is driven by the membrane potential. The monoamines dopamine, noradrenaline, adrenaline, serotonin and histamine are substrates of all three hOCTs but they exhibit different affinities and transport efficacies. The $K_m$ value for uptake of the substrate MPP$^+$ (1-methyl-4-phenylpyridinium) is similar at all three hOCTs, and all three hOCTs are inhibited with high potency by cyanine dyes (e.g. decynium22). A variety of drugs such as metformin, cimetidine, prazosine, verapamil and chindine are (or seem to be) transported by OCTs. By means of IC$_{50}$ values for inhibition of $^3$H-MPP$^+$ uptake in cells transfected with the cDNAs encoding hOCT1-3, affinities (as OCT substrates or inhibitors) of a variety of drugs have been examined. We showed that O-methyl-isoprenaline more selectively inhibits hOCT3 than corticosterone and that $\beta$-estradiol preferentially inhibits hOCT3 and hOCT1.

Since hOCTs are expressed in the brain (e.g. in glial cells), we examined a series of psychoactive drugs (antidepressants, antipsychotics, and psychostimulants) for their affinities to hOCTs expressed in stably transfected HEK293 cells by measuring inhibition (IC$_{50}$) of $^3$H-MPP$^+$ (15 nM) uptake. All examined drugs caused 50% inhibition of all three hOCTs in concentrations below 500 $\mu$M. With the exception of risperidon and amphetamine which preferentially inhibited hOCT2 (in low micromolar concentrations), all examined drugs showed higher potencies at hOCT1 than at the other two hOCTs. With IC$_{50}$ values of about 2 $\mu$M desipramine and levomepromazine exhibited the highest potency at hOCT1. These results indicate that some psychoactive drugs may at therapeutic plasma concentrations inhibit glial OCTs and thereby increase extracellular levels of monoamine neurotransmitters in the CNS.

Contact:
Prof. Dr. Heinz Bönisch
University of Bonn
Institute of Pharmacology and Toxicology
boenisch@uni-bonn.de
Reuterstraße 2b
D-53113 Bonn (Germany)
Pharmacogenomics of drug transporters

In addition to phase I and phase II drug metabolism, drug transport through biological membranes is now recognized as an important determinant of drug disposition and effects. Efflux transporter such as the MDR1 gene product P-glycoprotein are expressed in apical membranes of organs with excretory function (intestine, liver, kidney) thereby limiting bioavailability of orally administered drugs and promoting drug excretion from the body [1]. Moreover, P-glycoprotein expression in the blood-brain-barrier and in the placenta have a protective function for the CNS and the fetus, respectively. Recently, a number of single nucleotide polymorphisms (SNPs) have been identified in MDR1 [2]. Currently available data on the potential influence of MDR1 SNPs/haplotypes on P-glycoprotein expression, drug plasma concentrations, treatment outcome (e.g. HIV) and host susceptibility to diseases (e.g. refractory seizures) will be discussed.

In addition to efflux transporters, there is increasing knowledge on the relevance of uptake transporters for drug disposition. For example, in vitro evidence suggests that the HMG-CoA reductase inhibitor pravastatin is transported from the blood into the hepatocytes by the uptake transporter OATP-C (SLC21A6 or SLCO1B1, also known as OATP1B1, OATP2, and LST-1). Accordingly, the hepatic extraction ratio of pravastatin appears to be relatively high, 0.47. The OATP-C 521T>C and -11187G>A SNPs were associated with an increased systemic exposure to pravastatin [3], possibly explaining parts of the interindividual variability in lipid lowering effects of statins.

Literature:

Contact:
Prof. Dr. Martin F. Fromm
Friedrich-Alexander-University of Erlangen-Nürnberg
Institute of Clinical and Experimental Pharmacology and Toxicology
fromm@pharmakologie.med.uni-erlangen.de
Fahrstraße 17
D-91054 Erlangen (Germany)
New members of the SLC10 transporter family

Sodium-dependent bile acid transporters (SBATs) constitute the family of solute carriers SLC10A. Two members of this family are well known, the Na\(^+\)/taurocholate cotransporting polypeptide (NTCP) and the ileal sodium-dependent bile salt transporter (ISBT/ASBT). These carriers maintain the enterohepatic circulation of bile salts forming a pathway for bile salts across the membrane barriers in the liver (NTCP) and the intestine (ISBT) and, therefore, also participate in the homeostasis of cholesterol.

Recently, we identified a new member of the SLC10 family, the sodium-dependent organic anion transporter (SOAT). This carrier protein consists of 377 amino acids and shows 44% and 30% overall amino acid sequence identity to the ISBT and NTCP, respectively. The SOAT gene is localized at chromosome 4q21.3 and consists of six exons. The highly conserved domain “ALGMMPL” was found in all human and animal NTCP/Ntcp, ISBT/Isbt and SOAT/Soat sequences and is regarded as new SLC10 signature motif. However, despite the high sequence homology, SOAT is not a bile acid transporter. Transport studies in Xenopus laevis oocytes and stably transfected HEK293 cells revealed sodium-dependent transport of estrone-3-sulfate and dehydroepiandrosterone sulfate (DHEAS) with K\(_m\) of 31 µM and 30 µM, respectively. However, further substances including estradiol-17β-glucuronide, ouabain, digoxin, and taurocholate were not transported. SOAT mRNA expression was detected in multiple tissues and was highest in heart, lung, spleen, testis, small intestine, adrenal gland, testis, placenta and uterus. Reflecting the hormonal activity in these tissues, transport of estrone-3-sulfate and DHEAS may be linked to this carrier.

In conclusion: Soat is a new extraordinary non-bile acid transporting carrier of the SLC10A transporter family permitting selective estrone-3-sulfate and DHEAS uptake which is unusual within this group of Na\(^+\)-dependent bile acid transporters.

Literature:


Contact:
Dr. Joachim Geyer
Justus-Liebig-University of Giessen
Institute of Pharmacology and Toxicology
Joachim.M.Geyer@vetmed.uni-giessen.de
Frankfurter Straße 107
D-35392 Gießen (Germany)
B. Ugele

**Essential role of steroid sulfate transporters in para-/intracrine action of steroid sulfates**

Transformation of the adrenal steroids DHEA-S and DHEA into androgens and estrogens as well as the hydrolysis/activation of other steroid sulfates in peripheral target tissues depends upon the level of expression of the various steroidogenic enzymes in each of these tissues. This sector of endocrinology that focuses on the intracellular hormone formation and action has been called intracrinology. The sulfoconjugated steroids, DHEA-S and estrone sulfate (E1S), are present in the blood in concentrations which are orders of magnitude greater than those of the unconjugated steroids. Thus, they form a large reservoir of precursors, which is available for conversion into active hormones in numerous peripheral tissue sites. This is completed by the fact, that the first enzyme of this pathway, the microsomal steroid sulfatase, is ubiquitously expressed in many cells. However, at physiological pH steroid sulfates are negatively charged and belong to the chemically heterogenous substance group of “organic anions”, which can not enter the cells by free diffusion. Thus, the action of steroid sulfate transporters (SST) in the cell membrane are a prerequisite for the intracellular activation and intracrine activity of all steroid sulfates. In the last years, it was demonstrated that different cloned transporter polypeptides for organic anions also transport steroid sulfates. The so far known SSTs are expressed by genes of 4 different gene families. The human **SLCO**-super family codes for more than 10 organic anion transporting polypeptides (OATPs) and the **SLC22A**-familiy codes for more than 4 organic anion transporters (OATs). **OATP1A2**, -1B1, -1B3, -2B1, -3A1, -4A1, and OAT-3 and -4 are well known/putative SST. From the two well-characterised genes of the **SLC10A**-familiy only the **Na^+**/taurocholate co-transporting polypeptide (NTCP) of the liver also transports E1S. Additionally, a heterodimeric organic solute transporter (OSTα/β) was identified, which also transports E1S with low affinity. NTCP, OATP1B1 and -1B3 are highly liver specific, whereas all others SST are also expressed in many other normal and even cancer tissues and cells. OATP2B1 transports highly specific E1S whereas other SST also transport DHEA-S. In conclusion, it seems that the tissue-specific expression and the different substrate specificities of the various SSTs are indeed an additional important factor in the intracrine activity of the different steroid sulfates.

**Contact:**
**Dr. Bernhard Ugele**
Ludwig-Maximilians-University of München
University Hospital
Department of Gynaecology and Obstetrics
[bernhard.ugele@med.uni-muenchen.de](mailto:bernhard.ugele@med.uni-muenchen.de)
Maistraße 11
D-80337 München (Germany)
Solid-state NMR Concepts to study Multi-drug Efflux Pumps of the SMR and ABC Families

Multi-drug efflux pumps are found in all major transporter families. Unsolved problems include the mechanism of drug recognition, drug translocation, and characterisation of the catalytic cycle as well as resolving their 3D structure. Solid-state NMR is an emerging technique for the investigation of membrane proteins which has the potential to allow a detailed look at these transport systems. A number of experiments based on magic angle sample spinning (MAS) NMR are available to provide inter nuclear distances, drug binding and dynamic information when combined with suitable isotope labelling schemes. Here, we will present applications of these solid-state NMR concepts to members of the small multidrug resistance (SMR) family as well as to the *L. lactis* ABC transporter LmrA.

We have successfully over-expressed a number of SMR proteins (from *E. coli* EmrE, SugE, YdgE/YdgF, from *H. salinarium* Hsmr, from *M. tuberculosis* Tbsmr and from *S. typhimurium* Yfw). Expression was done both in *E. coli* and by *in vitro* translation/transcription. These expression systems together with *L. lactis* allow a number of specific 15N, 2H, 13C isotope labelling schemes. Chemical shift information and MAS-NMR lineshapes are sensitive to local structure and dynamics. In addition, long range distance constraints are required for studying conformational re-organisations during the transport cycle or for defining the oligomeric state of the transport systems under investigation. 19F-19F dipolar couplings provide an useful approach to obtain long-range information with suitable sensitivity. Single and double cysteine substitution mutants of both EmrE and LmrA have been expressed and 19F-labeled through the attachment of a trifluoroacetone group by a disulfide linkage. First 19F-MAS-NMR dipolar recoupling experiments on these transporters will be presented and discussed.

Besides novel solid-state NMR concepts, progress in functional assays is required in order to understand the coupling between drug and proton translocation in SMR proteins. Here, we will present first data based on a fluorescence assay using a controlled pH gradient obtained by co-reconstituting the efflux pump EmrE with the light-driven proton pump bacteriorhodopsin.

Contact:
Prof. Dr. Clemens Glaubitz
Johann Wolfgang Goethe-University of Frankfurt/Main
Centre for Biomolecular Magnetic Resonance
and Institute Institute for Biophysical Chemistry
glaubitz@chemie.uni-frankfurt.de
Marie-Curie-Straße 9
D-60439 Frankfurt/Main (Germany)
H.-J. Steinhoff

Multi-frequency EPR spectroscopy and site-directed spin labeling reveal the structure and conformational dynamics of membrane bound protein complexes

X-, Q- and W-band electron paramagnetic resonance (EPR) spectroscopy in combination with site-directed spin labeling (SDSL) and molecular dynamics (MD) simulations has emerged as a powerful method to study the structure and conformational dynamics of membrane proteins. Analyses of the dynamics and accessibility of the spin label side chains as well as the determination of inter-spin distances and of the polarity in the vicinity of the spin label binding sites provide information for restraint modeling of protein structures and conformational changes of the following systems: colicin A (1), the Na⁺/proline transporter PutP (2), and of the halobacterial phototaxis receptor sensory rhodopsin (pSRII) in complex with the transducer pHtrII (3). Light activation of pSRII induces a signal which is transferred across the plasma membrane by a receptor-specific transducer protein (pHtrII) that binds tightly to the photoreceptor. Inter-spin distances determined from pairs of interacting nitroxide spin labels introduced into the pSRII-pHtrII complex lead to a unique structural model of the dimeric complex. Time resolved detection of inter-spin distance changes after light activation reveals conformational changes of pSRII and uncovers the mechanism of the signal transfer from pSRII to the associated transducer pHtrII. The second part of the talk focuses on the Na⁺/proline transporter PutP of Escherichia coli. Four-pulse double electron-electron resonance (DEER) techniques have been applied to study proximity relationships within doubly spin labeled variants of PutP reconstituted in proteoliposomes. Inter-spin distance changes observed between loops 2 and 6 upon Na⁺ binding suggest ligand induced structural alterations of PutP which might be essential for the transport mechanism.

Literature:


Contact:
Prof. Dr. Heinz-Jürgen Steinhoff
University of Osnabrück
Department of Physics
hsteinho@uni-osnabrueck.de
Barbarastraße 7
D-49076 Osnabrück (Germany)
A. Kedrov, Ch. Ziegler, H. Janovjak, W. Kühlbrandt and D.J. Müller

**Observing folding pathways, folding kinetics, and activation of a native antiporter using single-molecule imaging and force spectroscopy**

Mechanisms of membrane protein folding and function, and of malfunction and misfolding build pertinent questions in cell biology and in various neurodegenerative diseases. Using single-molecule force spectroscopy, we directly observe the *in vitro* stepwise folding of a polypeptide into the lipid membrane embedded native Na$^+$/H$^+$ antiporters from *E. coli*. Time-lapse force spectroscopy allowed tracking the folding intermediates corresponding to the Na$^+$-binding site, transmembrane $\alpha$-helices, and polypeptide loops. The folding rates of structural elements ranged from 0.31 s$^{-1}$ to 47 s$^{-1}$. In some cases, however, the folding chain formed stable kinetically trapped non-native structures, which could be assigned to misfolding events of the antiporter. The same time the force spectroscopy data contained detailed information about strength and location of molecular interactions established within native NhaA. While switching NhaA into its functional state we could image functional related conformational changes. These changes were only formed in presence of the ligand Na$^+$. Statistical analysis showed that not all NhaA molecules activated their functional related change at pH 6 but at pH 7. The direct observation of functional activation of an antiporter provides novel insights into their activation mechanisms.

**Literature:**


**Contact:**

Prof. Dr. Daniel J. Müller  
University of Technology Dresden  
Center of Biotechnology  
Daniel.mueller@biotec.tu-dresden.de  
Tatzberg 47-51  
D-01307 Dresden (Germany)
The moving parts of the sodium pump: Conformational coupling to ion transport detected by voltage clamp fluorometry

The method of site-directed fluorescence labeling in combination with voltage-clamp fluorometry was used to analyze spatially resolved protein motions of the Na,K-ATPase expressed in Xenopus oocytes. Despite tremendous progress in structural analysis of P-type ATPases, little is known about the molecular details of conformational changes and the contribution of individual protein segments. A prominent feature of the structure of the SERCA Ca\(^{2+}\)-ATPase – a close homolog of the Na\(^+\)/K\(^+\)-ATPase α subunit – is the 70 Å-long α-helical M5 segment, which links the region of ATP hydrolysis and intermediate phosphorylation with the cation binding sites, suggesting a role in energy transduction. The involvement of other transmembrane helices in conformational changes was also suggested from structural studies, especially for segment M4, which carries cation coordinating residues, too. However, no information is available about the role of the Na\(^+\)/K\(^+\)-ATPase β subunit and its involvement in conformational changes of the functional heterodimer. In order to allow for site-specific attachment of sulfhydryl-specific fluorescent compounds to well-defined positions at the extracellular face of the Na\(^+\)/K\(^+\)-ATPase, cysteine-scanning mutagenesis was carried out within the extracellular loops M5-M6, M3-M4 and at the extracellular end of the β subunit’s single transmembrane domain. Within each of these regions, certain amino acid positions were identified, which upon mutation to a cysteine allowed for site-specific attachment of tetramethylrhodamine-6-maleimide (TMRM) (1,2). Thus, molecular sensor complexes are formed, which exhibit differential fluorescence responses depending on intra- and extracellular ionic conditions or voltage pulses, by which the distribution of the Na\(^+\)/K\(^+\)-ATPase molecules between the principal conformational substates E\(_1\)(P) and E\(_2\)(P) can be modulated. The fluorescence responses show the involvement of individual protein subdomains in conformational changes of the holoenzyme and allow to investigate the influence of electroneutral partial reactions on charge-translocating steps within the catalytic cycle, thereby opening up new ways of carrying out current-independent electrophysiology.

Literature:


Contact:
Dr. Thomas Friedrich
Johann Wolfgang Goethe-University of Frankfurt
Max-Planck-Institute of Biophysics
Department of Biophysical Chemistry
Thomas.Friedrich@mpibp-frankfurt.mpg.de
Max-von-Laue-Straße 3
D-60438 Frankfurt am Main (Germany)
Poster

Poster
R. Abele, J.C. Wolters, Ö. Demirel, and R. Tampé

Selective and ATP-dependent translocation of peptides by the homodimeric ABC transporter TAP-like (ABCB9)

TAP-like (TAPL, ABCB9) belongs to the ATP-binding cassette (ABC) transporter family, which translocates a vast variety of solutes across membranes. The subfamily B of human ABC-transporters with 11 members comprises the multidrug resistance proteins (MDR1, MDR3), the transporter associated with antigen processing (TAP1 and TAP2) and TAPL. This heterogeneous subfamily includes full-size and half-size transporters, the latter must assemble to homodimers or heterodimers. Members of this subfamily are found in different subcellular compartments, such as the plasma membrane, the endoplasmic reticulum, the lysosome, or the inner mitochondrial membrane. The function and the substrate specificity of only five members (MDR1, MDR3, TAP1/2 and BSEP) have been determined, but not these of the others, in particular ABCB9.

Here, we show that TAPL forms a homodimeric complex, which translocates peptides across the membrane. Peptide transport strictly requires ATP hydrolysis. The transport follows Michaelis-Menten kinetics with low affinity and high capacity. Different nucleotides bind and energize the transport with a slight preselection for purine bases. The peptide specificity is very broad, ranging from 6-mer up to at least 59-mer peptides with a preference for 23-mers. Peptides are recognized via their backbone, including the free N- and C-terminus as well as side chain interactions. Although related to the transporter associated with antigen processing (TAP), the TAPL complex differs as far as its interaction partners, transport properties, and substrate specificities are concerned. Furthermore TAPL does not restore major histocompatibility complex (MHC) class I surface expression in TAP deficient cells, thus excluding that TAPL is part of the peptide-loading complex in the classic route of antigen processing via MHC class I molecules.

Contact:
Dr. Rupert Abele
Johann Wolfgang Goethe-University of Frankfurt/Main
Institute of Biochemistry, Biocenter
abele@em.uni-frankfurt.de
Marie-Curie-Straße 9
D-60439 Frankfurt/Main (Germany)
Expression of Channelrhodopsin-2 in Mammalian Cells

Photosensory responses of green algae are mediated by rhodopsins with microbial-type chromophores. The recently detected cDNA sequences from the green alga Chlamydomonas reinhardtii that encode microbial opsin-related proteins were termed Channelopsin-1 and 2 (Chop1/2) by us (Nagel et al., 2002; 2003) and CSRA/B (Sineshchekov et al., 2002) or Acop1/2 (Suzuki et al., 2003) by others. The hydrophobic core region of the proteins shows homology to the light-activated proton pump bacteriorhodopsin. Channelrhodopsin-2 (ChR2 = Chop-2 + retinal) is an ion channel, which is directly switched by light, as shown by expression in *Xenopus laevis* oocytes (Nagel et al., 2003). The action spectrum of ChR2 has its maximum at 460 nm (Sineshchekov et al., 2002; Nagel et al., 2003). We show heterologous expression of Channelopsin-2 in mammalian cells. Whole cell patch clamp studies demonstrate large light-gated ion currents and the capacity of ChR2 to depolarize the membrane by illumination. ChR2 or truncated Channelrhodopsin-2 (ChR2-315), when expressed in mammalian cells, yields light-gated channel activity with no apparent difference from *Xenopus laevis*-expressed ChR2. A fusion protein of ChR2-315 with yellow fluorescent protein (eYFP) induced strong fluorescent labeling of the plasma membrane, indicating its preferential expression there. ChR2-315-eYFP showed the same light-induced activity as ChR2 itself.

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Contact:
Nona Adeishvili
Max-Planck Institute of Biophysics Frankfurt/Main
nona.adeishvili@mpibp-frankfurt.mpg.de
Max-von-Laue Straße 3
D-60438 Frankfurt/Main (Germany)
Expression changes of multi drug resistance genes MDR1 and MRP1 in renal carcinoma cells after methotrexate treatment: A Real time PCR analysis

Cytotoxic drug resistance is a major obstacle in cancer therapy. ABC transporters such as MDR1/ABCB1, MRP1/ABCC1, and MXR/ABCG2 seems to be the important members which cause acquired resistance against multiple cytotoxic drugs in variant cell lines. The presence and significance of MRP1 in cytotoxic drug resistance in human renal carcinoma cells (HRCC) is poorly characterized. Therefore, the aim of the present study was to determine the differential expression of MDR1 and multidrug resistance-associated protein 1 (MRP1) in HRCC after methotrexate treatment. Quantitative reverse transcription-polymerase chain reaction method was optimised to determine the relative RNA levels of MRP1 and MDR1 in HRCC. Both these transporter proteins were detected in renal carcinoma cell line (A498). In methotrexate treated cells the level of MRP1 mRNA was 2.5 fold higher compared for the amount of untreated HRCC. No relatively change was found in the MDR1 expression. These data presume that MRP1 may play an important role in cytotoxic drug resistance in HRCC.

Contact:
Nitin Kumar Agarwal
Georg-August-University of Göttingen
Department of Nephrology
sonupharm@rediffmail.com
Robert Koch Straße 40
D-37075 Göttingen (Germany)
Comparative molecular physiology of the Na\textsuperscript{+}-D-glucose cotransporter SGLT2 in the kidney of the elasmobranch Squalus acanthias

The aim of the current investigation was to clone the SGLT of the early vertebrate Squalus acanthias, to elucidate early evolved functionally important domains of the molecule, and to investigate the distribution in the kidney.

Using primers against conserved regions from mammalian SGLTs, a cDNA was cloned that, based on comparison of amino acid sequence, membrane topology, putative glycosylation and phosphorylation sites, could be shown to belong to the family of slt genes. Its identity to mammalian SGLT2 was higher (68\%) than to SGLT1 (62\%). This classification could be confirmed by kinetic transport analysis in \textit{Xenopus laevis} oocytes.

The elasmobranch gene is most similar to the mammalian genes in the transmembrane helices, suggesting a very early selection process of these domains.

In immunohistochemistry employing an epitope-specific antibody positive immunoreactions were found at the luminal membranes in the early proximal tubule (PI) as well as in the late distal tubule and in the collecting tubule. The large PIIa segment of the proximal tubule showed no reaction.

Thus, at the molecular level SGLT2 in \textit{Squalus acanthias} is quite similar to mammalian transporters. The presence of SGLT2 protein in the late distal tubule segments suggests a second site of D-glucose reabsorption, presumably related to the preceding secretory nephron segment PIIa.

Contact:
Thorsten Althoff
Max-Planck-Institute of Molecular Physiology Dortmund
thorsten.althoff@mpi-dortmund.mpg.de
Otto-Hahn-Straße 11
D-44227 Dortmund (Germany)
Functional identification of Organic-Anion-Transporter 2 (OAT2) as the liver-specific urate transporter

Urate is an intermediate of the purine metabolism in the liver. In birds, reptiles and higher primates including man, urate is the end product of this metabolic pathway and is secreted via the kidneys. Recently, renal human URAT1 (hURAT1) was identified as a key player for the maintenance of high plasma urate levels. Besides hURAT1, other transporters such as OAT1, OAT3, OATv1 or MRP4 are discussed to be involved in renal urate transport, but nothing is known about transport proteins handling urate in the liver. Organic-Anion-Transporter 2 (OAT2) is abundantly expressed in the liver and only weakly expressed in the kidneys. Here we report on the identification of the pig ortholog of OAT2 (pOAT2). Time-dependent uptake of urate into pOAT2-transfected COS 7 cells is higher compared to hURAT1-mediated urate uptake. Further characterizations of pOAT2 yielded the functional hallmarks of OAT2, namely substantial inhibition of urate uptake by 1 mM of probenecid and salicylate, whereas p-aminohippurate (PAH) and glutarate showed only small inhibitory effects. Comparative studies of urate uptake between the pig and rat OAT2 expressed in *Xenopus laevis* oocytes revealed an almost two times higher accumulation of urate by rOAT2. Urate uptake by rOAT2 was saturable with an apparent Km (Kt) of 75 µM. Primary cultures of rat hepatocytes exhibited a time-dependent uptake of urate, which was inhibited by probenecid, consistent with an involvement of OATs in hepatic urate transport.

Contact:
Dr. Andrew Bahn
Georg-August-University of Göttingen
Centre for Physiology and Pathophysiology
Department of Vegetative Physiology and Pathophysiology
abahn@physiol.med.uni-goettingen.de
Humboldtallee 23
D-37073 Göttingen (Germany)
D. Basting

Optimisation of sample preparation conditions of a Small Multidrug Transporter (RV_3065) from M. tuberculosis

The membrane protein Tbsmr (Rv3065) is an efflux transporter from Mycobacterium tuberculosis, with a high degree of homology to the well characterised small multidrug resistance (SMR) protein EmrE, from E. coli. Even though EmrE has been extensively studied, the structure and even the oligomeric state of SMR proteins are still disputed. Here we present preliminary results for Tbsmr: It can be over expressed at levels similar to EmrE and reconstituted into lipid bilayers for solid-state NMR (SSNMR). Crystallisation screens were performed yielding small non-diffracting crystals. Those crystals are not suitable for x-ray diffraction but might offer a suitable sample condition for solid-state NMR studies. We aim at the development of an efficient protocol to obtain optimised sample preparation conditions for structure determination of small membrane proteins by solid-state NMR. We will compare results from crystallisation and reconstitution experiments.

Contact:
Daniel Basting
Johann Wolfgang Goethe-University of Frankfurt/Main
Centre for Biomolecular Magnetic Resonance and
Institute for Biophysical Chemistry
basting@chemie.uni-frankfurt.de
Marie-Curie-Straße 9
D-60439 Frankfurt/Main (Germany)
3D QSAR analysis of di- and tripeptides and β-lactam antibiotics as substrates of the H⁺/peptide cotransporter PEPT2

The H⁺/peptide cotransporter PEPT2 is mainly expressed in the luminal membrane of kidney epithelial cells. The carrier is responsible for the reabsorption of di- and tripeptides as well as peptidomimetics such as many β-lactam antibiotics and several angiotensin converting enzyme inhibitors from the primary filtrate back to the blood. There is increasing interest in this membrane carrier because its transport activity determines - among other factors – the circulatory half-life of peptides and peptide-like drugs in the blood. The three-dimensional structure of the transporter is still unknown and little information exists about the substrate binding site.

We determined the affinity constants (Kᵢ values) of substrates and inhibitors in a competition assay vs. [¹⁴C]Gly-Sar uptake in monolayer cultures of SKPT cells¹. CoMSIA (Comparative Molecular Similarity Indices Analysis) was applied to identify those regions in the substrate structures that are responsible for differences in affinity (features such as steric, electrostatic, lipophilic and hydrogen-bonding properties). A training set containing 30 dipeptides and dipeptide derivatives, 22 tripeptides and 21 β-lactam antibiotics covering a range of affinity constants from 0.3 µM to 9.5 mM was established. We developed a complex 3D QSAR model which is able to explain and predict binding affinities of new PEPT2 substrates. High q² and r² values demonstrate the statistical significance of the model. Finally, a test set of 18 compounds was used to evaluate the model’s predictive power.

Literature:


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The excitatory amino acid transporter (EAAT) 4 is posttranslationally modulated by serum and glucocorticoid inducible kinases and protein kinase B

The serum and glucocorticoid inducible kinase (SGK) 1 is a potent regulator of several membrane ion channels and transporters. In brain tissue the kinase was found to be upregulated by ischemia, neuronal excitation and dehydration. The present study has been performed to elucidate the expression of SGK1 in cerebellar Purkinje cells and to explore whether it influences the colocalized glutamate transporter EAAT4. Intense SGK1 staining was observed in Purkinje cells following 48h of water deprivation. The kinase increased glutamate induced currents in Xenopus oocytes heterologously expressing EAAT4, an effect mimicked by its isoforms SGK2, 3 and PKB. The glutamate induced currents were decreased by the ubiquitin ligase Nedd4-2, an effect partially but not completely reversed by additional coexpression of the SGK kinases or PKB. Also, EAAT4 protein abundance in the cell membrane was stimulated by SGK1 and inhibited by Nedd4-2. In conclusion, SGK1 expression is upregulated by stress in cerebellar Purkinje cells. In oocytes heterologously expressing EAAT4 transporter activity was found to be increased by the respective kinases. The upregulation of SGK1 may serve to stimulate EAAT4 and thus to reduce neuroexcitotoxicity.

Contact:
Dr. Christoph Böhmer
Eberhard Karls University of Tübingen
Department of Physiology I
christoph.boehmer@uni-tuebingen.de
Gmelinstraße 5
D-72076 Tübingen (Germany)
Y. Hagos, J. Steffgen, G. Burckhardt, and B.C. Burckhardt

**Mutational analysis of the flounder renal sodium dicarboxylate cotransporter, fNaDC-3**

The flounder NaDC-3 has a high affinity for succinate and α-ketoglutarate, is located at the basolateral membrane of P II tubule cells, provides the cells with fuels, and maintains the α-ketoglutarate concentration difference driving the uptake of endogenous and exogenous organic anions via the renal organic anion transporter, ROAT. fNaDC-3 is an electroneutral cotransporter translocating 3 sodium ions together with one divalent succinate. This surplus of positive charge results in a potential-dependent inward current within the potential range between -90 and +10 mV. The predicted secondary structure model of fNaDC-3 contains 11 transmembrane (TM) domains with an intracellular N- and an extracellular C-terminus. In the present study, we determined which of the positively charged amino acid residues at or in the 11 TMs may be involved in the interaction with succinate. In a first trial, 12 single and 6 double mutants were created. Most of the mutants showed [$^{14}$C]succinate uptakes or succinate-induced inward currents between half and twice of those evoked by the wildtype (WT). Replacement of RR109,110 by alanine and isoleucine (RR109,110AI) lead to a complete loss of function, suggesting an important role of RR109,110 for targeting to the plasma membrane. K114I, located within TM 4, showed [$^{14}$C]succinate uptake, but, unlike the WT, only a barely detectable inward current. Sodium affinity of K114I was 31.3 ± 3.8 mM which was comparable to that obtained for the WT. The Hill coefficient for K114I was calculated to 1.9 which is less than that of the WT, demonstrating less positive cooperativity of the sodium binding sites. When, at K114, the lysine was replaced by either arginine, glutamic acid, or glutamine to yield K114R, K114E, or K114Q, respectively, the following results were obtained: The currents generated upon application of 1 mM succinate by K114R were comparable in magnitude to those evoked by the WT, whereas K114E and K114Q showed only small or negligible inward currents. In conclusion, the amino acid residue K114 is essential for proper translocation of three sodium ions. Mutation of the basic, positively charged lysine 114 into nonpolar or negatively charged amino acids results in the translocation of two sodium ions and one divalent succinate, i.e. in an electroneutral transport mode.

**Contact:**
**Dr. Birgitta C. Burckhardt**
Georg-August-University of Göttingen
Department of Physiology and Pathological Physiology
bcburckhardt@physiol.med.uni-goettingen.de
Humboldtalle 23
D-37073 Göttingen (Germany)
**A. Burse, S. Discher, J. Kuhn, and W. Boland**

**Sequestration of plant glycosides in leaf beetle larvae**

Phytophagous insects must live with noxious defense compounds of their host plant. Sequestration, i.e. the uptake and storage in specific compartments of such toxins so that they become ineffective, represents one strategy to feed on a formerly toxic host with impunity. Larvae of leaf beetles even use these plant derived compounds for their own defense against predators. The compounds are taken up in the gut tissue and transported via the haemolymph into specialized glands where the deterrent compounds are released in case of attack. Feeding experiments with thioglucosides provided strong evidence that the incorporation of plant glucosides is mediated by transport proteins. In order to characterize the transport proteins, in vitro uptake experiments were performed on dissected gut tissue of leaf beetle larvae using different inhibitors. The inhibition of glucose transport prevented the import of thioglucosides. Based on these results, a GLUT-homologous protein was identified in the gut of *P. cochleariae*. The complete gene was cloned using RACE revealing an open reading frame of 1.5 kb. Quantitative RT-PCR showed the occurrence in all tissues of the larvae including the glands. Expression in the *Xenopus*-oocytes should give information about the physiology of this putative GLUT protein.

**Contact:**
**Dr. Antje Burse**
Max-Planck-Institute for Chemical Ecology Jena
Bioorganic Chemistry
aburse@ice.mpg.de
Hans-Knoell-Straße 8
D-07745 Jena (Germany)
M. Diener, M. Hardt, and A. Sieffjediers

**IP3-receptor subtypes are differentially distributed in rat colonic epithelium**

Inositol-1,4,5-trisphosphate (IP3) is a central second messenger mediating the response to Ca\(^{2+}\)-dependent secretagogues in secretory epithelia. IP3 acts at different subtypes of receptors (IP3R1-3). In order to reveal the distribution of IP3R subtypes in rat colonic epithelium, tissues were cryofixed, stained with antibodies against IP3R1-IP3R3, and visualized with Cy3-coupled anti-mouse IgG.

A monoclonal antibody (KM1112) against IP3R1 did not stain the colonic epithelial cells. In contrast, IP3R2 and IP3R3 were found within the epithelial cells; however, with a different intracellular localization and with a different gradient along the crypt axis. The antibody against IP3R2 (M1083) stained nuclei of the epithelial cells. The signal was distributed all over the nucleus and not restricted to the nuclear envelope as demonstrated by counterstaining with lamin B1. In contrast, an antibody against IP3R3 (Becton Dickinson, Heidelberg) stained the epithelial cells mostly in their apical half. In addition, there was a gradient from the surface region towards the crypt fundus, where the signal strongly decreased. These results demonstrate that the colonic epithelial cells switch their IP3R during differentiation and suggest a nuclear location of IP3R2.

**Contact:**  
Prof. Dr. Martin Diener  
Justus-Liebig-University of Gießen  
Institute of Veterinary Physiology  
Martin.Diener@vetmed.uni-giessen.de  
Frankfurter Straße 100  
D-35392 Gießen (Germany)
Intestinal transport proteins mediate the uptake of plant glucosides in leaf beetle larvae

Leaf beetle larvae of the subtribe Chrysomelina use chemical weapons to defend themselves against predators. For defense they release deterring substances from dorsal glandular reservoirs. Qualitative analyses of defensive secretion revealed three biosynthetic strategies: ancestral species produce repulsing compounds de novo while others depend partly or entirely on plant secondary metabolites, namely glucosides, of their host as precursors for the synthesis of deterring substances. The sequestration of plant glucosides was investigated by feeding experiments with thioanalsogs which are highly similar to the natural substrate and stable against hydrolysis. These studies proved that the transport of plant glucosides across the intestinal tissue to the defensive system is mediated by specialized glucoside-transporting proteins. After a host plant change they may play a crucial role since they select the compounds which can be channeled through the gut tissue to the defensive glands. Thus, transport proteins may contribute directly to the co-evolution of insect and plant.

To determine transporters involved in the uptake of host plant glucosides, in vitro-uptake studies in the presence of different inhibitors were made with dissected gut tissue of Phaedon cochleariae larvae. Different glucose transporters were inhibited and the impact on the uptake of thioglucosidical analogs of defensive secretion precursors was monitored by HPLC/MS. The resulting data gave strong evidence that plant glucosides are taken up by facilitative sugar transporters (GLUT, SLC2A). These investigations allow a closer characterization of the intestinal transport proteins and provide additional information on the molecular mechanisms of the defensive chemistry in leaf beetle larvae.

Contact:
Sabrina Discher
Max Planck Institute for Chemical Ecology Jena
Department of Bioorganic Chemistry
sdischer@ice.mpg.de
Beutenberg Campus
Hans-Knöll-Straße 8
D-07745 Jena (Germany)

Thermodynamics of the ATP hydrolysis cycle of GlcV, the nucleotide binding domain of the sugar ABC transporter of Sulfolobus solfataricus

ATP-binding cassettes (ABC) transporters drive the transport of substrates across the membrane by the hydrolysis of ATP. They have a conserved domain structure, which consist of two membrane-spanning domains (MSDs) that form the transport channel and two cytosolic Nucleotide Binding Domains (NBDs) that energize the transport. Despite the large amount of studies on ABC transporters, both the ATP hydrolysis cycle and the transport mechanism are currently matters of debate. We and others have recently proposed a processive clamp model for ATP hydrolysis in which ATP binding to the NBD monomer results in formation of an NBD dimer followed by hydrolysis of both ATPs, after which the dimer is dissociated. The thermodynamics of different steps in the ATP hydrolysis cycle of GlcV, the NBD of the glucose ABC transporter of the extreme thermophile Sulfolobus solfataricus were studied by isothermal titration calorimetry. In this study, the WT protein which is highly active in ATPase hydrolysis, and two mutants which are blocked at different positions in the ATP hydrolysis cycle were used. The G144A mutant is blocked in dimerization, while the E166A mutant is blocked in ATP hydrolysis and dissociation of the dimer. Binding affinities, binding stoichiometries and binding entropies of ATP, ADP and AMPPNP binding were determined at different temperatures. Based on this the thermodynamic parameters of nucleotide binding, NBD dimerisation and ATP hydrolysis were analysed.

Contact:
Dr. Chris van der Does
Johann Wolfgang Goethe-University of Frankfurt/Main
Institute of Biochemistry, Biocenter
chris.van.der.does@em.uni-frankfurt.de
Marie-Curie-Straße 9
D-60439 Frankfurt/Main (Germany)
ER export of the GABA transporter GAT1 is controlled by two adjacent signals in the proximal part of its carboxyl terminus

Export of the GABA transporter 1 (GAT1) from the endoplasmic reticulum (ER) is controlled by three hydrophobic amino acids (569VMI571) in its C-terminus (Farhan et al. J Biol Chem 2004). Using fluorescence resonance energy transfer (FRET) microscopy we showed that the C-terminus of the rat GABA transporter 1 (GAT1) mediates an interaction with Sec24D. Here we replace the hydrophobic motif (569VMI571) by serines (GAT1-SSS); this led to intracellular retention of the transporter. GAT1-SSS was distributed into a central dense area and peripheral punctate structures. The central dense area was the site of colocalization with Sec24D and ERGIC53. However, the peripheral punctae did not colocalize with the mentioned markers. Thus it is unlikely that these structure represent ER exit sites (ERES). The punctate morphology of GAT1-SSS could be changed to a diffuse reticular staining by co-transfection with a dominant negative Sar1 or GBF1 construct. This indicates that the observed morphology of GAT1-SSS is dependent on assembly of the COPII coat and on subsequent activation of Arf1 on the ER membrane. To determine whether the VMI-motif is the site of interaction with Sec24D we performed FRET microscopy experiments. CFP-GAT1-SSS exhibited a robust FRET with YFP-Sec24D. This interaction could be verified by GST-pulldown assays. In search for a possible interaction site with Sec24D we focused on the 566RL567-motif, which is conserved among all members of the Na+/Cl--dependent neurotransmitter transporter family. Mutation of RL to AS (GAT1-RL/AS) led intracellular retention. Importantly, no FRET could be detected between CFP-GAT1-RL/AS and YFP-Sec24D. Also no interaction could be detected by GST-pulldown. GAT1-RL/AS exhibited a diffuse intracellular staining with a weak signal visible at the plasma membrane. We hypothesized that GAT1-RL/AS has a concentration defect rather than a deficiency in ER export per se. In contrast, the defect in the ER export of GAT1-SSS lies in a later step. We scored surface expression of GAT1-RL/AS and GAT1-SSS 72 h after transfection. About 70% of the GAT1-RL/AS transfected cells exhibited a plasma membrane staining compared with 10% in the case of the GAT1-SSS. Therefore we conclude that the RL-motif mediates an interaction with Sec24D and is responsible for cargo concentration. The VMI-motif on the other hand mediates export from the ER by a yet unidentified mechanism.

Contact:
Dr. Hesso Farhan
Medical University of Vienna
Institute of Pharmacology
hesso.farhan@meduniwien.ac.at
Währinger Straße 13a
A-1090 Wien (Austria)
Role of oatp1a4, mrp2, mdr1, and bcrp in the tissue-selective distribution and hepatobiliary elimination of ouabain in rodents

Pharmacokinetics of the cardiac glycoside ouabain which equals the so called endogenous digitalis shows species specificities. In rodents about 50% of an i.v. ouabain application is secreted into bile, whereas in human, dog, and rabbit the kidney is the major organ of ouabain elimination. Because ouabain is a very hydrophilic molecule, hepatobiliary transport requires the expression of specific carriers in the basolateral and canalicular membrane of hepatocytes.

**Results:** Ouabain uptake by oatp1a4, which is expressed in the basolateral membrane of hepatocytes was sodium-independent with Km of 155µM when tested in *Xenopus laevis* oocytes. These data are in good agreement with the characteristics of ouabain transport in isolated rat hepatocytes [Petzinger and Fischer 1985] and perfused rat livers, showing sodium-independent ouabain transport with Km of 127-360µM. Other hepatic oatp-carriers (oatp1a1, oatp1a5) show only marginal ouabain transport compared to oatp1a4. Human OATP1A2, OATP1B1, and OATP1B3, bovine oatp1a2, and oatp1b4, and rat oatp1b2 do not transport ouabain at concentration of 1µM. Therefore, oatp1a4 is very likely the ouabain carrier in the basolateral membrane of rat hepatocytes. Since a functional homolog of oatp1a4 exists only in mouse but not in man, dog, and cattle, oatp1a4 is a species specific carrier for ouabain uptake in rodent hepatocytes. To determine the efflux site of hepatic ouabain elimination, canalicular ouabain transport was studied in mrp2 deficient TR(-) rats, mdr1a,b (-/-) knockout mice, and bcrp1 (-/-) knockout mice by *in situ* bile duct/gallbladder cannulation. Hepatobiliary excretion of ouabain was about 50% of the applied dose in the wild type animals (Wistar rat, FVB mice). However, in the TR(-) rat biliary ouabain elimination was significantly diminished compared to Wistar rats, and slightly diminished in bcrp1 (-/-) knockout mice. No difference in hepatobiliary elimination of ouabain was observed between mdr1a,b (-/-) and FVB mice.

**Conclusion:** Mrp2 and to a lesser degree bcrp1, but not mdr1, are the main routes of canalicular ouabain efflux in rodents, whereas digoxin is secreted into bile by mdr1.

**Literature:**

**Contact:**
Dr. Joachim Geyer  
Justus-Liebig-University of Gießen  
Institute of Pharmacology and Toxicology  
Joachim.M.Geyer@votmed.uni-gieissen.de  
Frankfurter Straße 107  
D-35392 Gießen (Germany)
Frequency of the nt230(del4) MDR1 mutation in British Herding Breeds from Europe

A subpopulation of Collies and related dog breeds show increased sensitivity to ivermectin and other macrocyclic lactones. The clinical signs of the intoxication are depression, ataxia, somnolence, mydriasis, salivation and in many cases coma and death. For a long time it has been assumed that an abnormal accumulation of ivermectin in the brain may be responsible for the appearance of these neurotoxic signs. This speculation has been supported by studies in mdr1 k.o. mice, which showed 87-fold increased levels of ivermectin in the brain, compared to wild-type mice, indicating that MDR1 plays a crucial role in ivermectin repulse from the brain. A MDR1 deficient genotype was described in ivermectin sensitive collies. This MDR1 mutation involves an exonic 4-bp deletion in the palindromic sequence GATAG (ATAG or GATA) at the nucleotide position 230 of the MDR1 open reading frame and creates a frame shift followed by a premature stop codon at the amino acid position 91. This mutation deletion abolishes the function of MDR1, which is strongly associated with the ivermectin susceptibility in Collie dogs. The aim of this study is to determine the frequency of the nt230(del4) MDR1 mutation in Collies and other breeds from the collie lineage. We have developed a PCR-based diagnostic test to detect the nt230(del4) MDR1 mutation in dogs (*).

Results: A total of 1066 British Sheepdogs have been analyzed from Germany, Austria, Switzerland, Spain and The Netherlands (418 Collies, 296 Border Collies, 172 Australian Shepherds, 101 Shetland Sheepdogs, 35 Wäller, 27 Bearded Collies and 17 Old English Sheepdogs). The highest frequency of the homozygous genotype was for Collies (34.5%), followed by Australian Shepherd (7%), Shetland Sheepdog (4.9%), and Border Collies (0.3%). Wäller and Old English Sheepdog showed only the heterozygous genotype (34.3% and 17.6%, respectively). Only dogs with homozygous MDR1 mutation are affected from the described ivermectin sensitivity, dogs with the heterozygous genotype are carrier of the mutation, but do not respond by clinical signs. Besides ivermectin, other drugs which are well-known substrates of MDR1 are suspicious to induce neurotoxicity after treatment. These include milbemycinoxime, loperamide, cyclosporine, vincristin and vinblastin.

Literature:

Contact:
Dr. Joachim Geyer
Justus-Liebig-University of Gießen
Institute of Pharmacology and Toxicology
Joachim.M.Geyer@vetmed.uni-giessen.de
Frankfurter Straße 107
D-35392 Gießen (Germany)
Solid-State NMR and the multidrug ABC transporter LmrA

The ABC transporter LmrA from *Lactococcus lactis* confers resistance to a wide range of antibiotics and cytotoxic drugs and is a functional homologue of P-glycoprotein. Recently, solid-state NMR methods have shown potential for structural- and non-perturbing, site directed functional studies of proteins as well as ligand bound to them. These experiments require isotopic labelling of protein and/or of ligand selected sites. We have developed a strategy to produce large quantities of LmrA reconstituted at a high density in lipid membranes sufficient for advance NMR studies. This makes the 64 kDa integral membrane protein LmrA and therefore the ABC transporter superfamily accessible to NMR analysis. Here, we present preparatory technique for non-labelled and 2H/13C/15N isotope labelled solid-state NMR studies on ABC transporter LmrA as well as first NMR spectra of isotope labelled LmrA and drug membrane interactions studies.

Contact:
Prof. Dr. Clemens Glaubitz
Johann Wolfgang Goethe-University of Frankfurt/Main
Centre for Biomolecular Magnetic Resonance
Institute for Biophysical Chemistry
glaubitz@em.uni-frankfurt.de
Marie-Curie-Straße 9
D-60439 Frankfurt/Main (Germany)
D. Harder, D. Weitz, and H. Daniel

Cloning and functional characterisation of the first di-/ tripeptide transporters from *E. coli*

In the genome of *E. coli* a family of proton-dependent oligopeptide transporter (POT-family) was identified by sequence analysis and bioinformatics. The POT family is distinct from the ABC-type peptide transporters and shows homology to the proton-dependent peptide transporter DtpT from *Lactococcus lactis* and the mammalian PEPT1 and PEPT2. We have cloned all four yet uncharacterised members of the family (gene names YbgH, YdgR, YhiP and YjdL) and overexpressed them in *E. coli* via the pET vector system. All four membrane proteins were expressed in *E. coli* BL21 cells as proofed by Coomassie stained SDS-PAGE and Western Blot analysis.

The YdgR transport protein was functionally characterised in vivo by transport assays using the fluorescent dipeptide β-Ala-Lys-AMCA. The dipeptide β-Ala-Lys-AMCA is a well defined substrate for PEPT1 and PEPT2 and is also transported by the YdgR protein. Uptake was inhibited by adding an excess of non-labeled Dipeptide Gly-Gln. Transport was completely abolished in the presence of 10 µM CCCP, a selective proton ionophore, indicating a proton-dependent transport mechanism. To determine the substrate specificity of YdgR, inhibition of β-Ala-Lys-AMCA uptake was determined in the presence of amino acids, dipeptides, tripeptides, tetrapeptides, various stereoisomeric peptides composed of L- and D-amino acids and several peptidomimetics like β-lactam antibiotics. The YdgR protein appears to be specific for di- and tripeptides with very similar affinities. Higher affinities were observed for peptides consisting of L-α-amino acids, while dipeptides with D-amino acids in the second position show markedly reduced affinities compared to the same dipeptide carrying the D-amino acid in the first position. Anionic dipeptides seem to have significantly lower affinities than neutral or cationic dipeptides and peptidomimetics like amino-β-lactam antibiotics also displayed good substrate affinities for interaction with the YdgR protein. In conclusion, the YdgR protein from *E. coli* functions as a proton-dependent di- and tripeptide transporter with a substrate recognition pattern comparable to the mammalian peptide transporters PEPT1 and PEPT2.

Contact:
Daniel Harder
Technical University of München
Molecular Nutrition Unict
harder@wzw.tum.de
Am Forum 5
D-85350 Freising-Weihenstephan (Germany)
M. Herget, G. Oancea, S. Schrodt, M. Karas, R. Abele, and R. Tampé

Mapping contact regions in the peptide-binding pocket of the transporter associated with antigen processing (TAP)

The transporter associated with antigen processing (TAP) plays a key role in the adaptive immune response against infected or malignantly transformed cells, by mediating the transport of antigenic peptides into the lumen of the endoplasmic reticulum for subsequent loading of major-histocompatibility complex class I molecules. To identify residues, which physically contact the bound peptide, we attached a chemical protease, Fe-[p-(bromacetamido)benzyl]-EDTA (FeBABE), to each position of a high affinity peptide epitope. We could demonstrate that all peptides modified by the chemical protease bind specifically to TAP. After binding to TAP, the cleavage reaction was initiated generating reactive oxygen species by adding ascorbic acid and hydrogen peroxide. Specific fragments of TAP1 of 48 kDa and 28 kDa were observed if the chemical protease was attached to position six and seven of the epitope. Interestingly, TAP2 was not cleaved by any of these BABE-peptides. By the means of MALDI-TOF-MS, the cleavage site in TAP1 was mapped to residue E290 in the cytosolic loop between TM 2 and TM 3 of the core domain. We finally generated single-cysteine TAP1 mutants to provide direct evidence to a physical contact of the bound peptide to this loop residue by thiol-specific cross-linking. Taken together, these data suggest that the identified loop region may sense the peptide loading and therefore is involved in the crucial crosstalk with the nucleotide-binding domain.

Contact:
Meike Herget
Johann Wolfgang Goethe-University of Frankfurt/Main
Institute of Biochemistry, Biocenter
herget@em.uni-frankfurt.de
Marie-Curie-Straße 9
D-60439 Frankfurt/Main (Germany)
M. Huss

Archazolid and apicularen: Novel V-ATPase inhibitors

**Background:** V-ATPases constitute a ubiquitous family of heteromultimeric, proton translocating proteins. According to their localization in a multitude of eukaryotic membranes, they energize many different transport processes. Since their malfunction is correlated with various diseases in humans, insights into the properties of this enzyme as a drug target and the development of selective inhibitors is one of the future challenges in V-ATPase research.

**Results:** Archazolid A and B, two recently discovered cytotoxic macrolactones produced by the myxobacterium Archangium gephyra, and apicularen A and B, two novel benzolactone enamides produced by different species of the myxobacterium Chondromyces, exert a similar inhibitory efficacy on a wide range of mammalian cell lines as the well established plecomacrolidic type V-ATPase inhibitors concanamycin and bafilomycin. Like the plecomacrolides both new macrolides also prevent the lysosomal acidification in cells and inhibit the V-ATPase purified from the midgut of the tobacco hornworm, Manduca sexta, with IC50 values of 20-60 nM. However, they did not influence azide or vanadate sensitive ATPase activity, respectively, in crude membrane fractions of mouse heart. To confine the binding sites of these new inhibitors we used a semi-synthetic radioactively labelled derivative of concanamycin which exclusively binds to the membrane Vo subunit c. Whereas archazolid A prevented, like the plecomacrolides concanamycin A, bafilomycin A1 and B1, labelling of subunit c by the radioactive I-concanolide A, the benzolactone enamide apicularen A did not compete with the plecomacrolide derivative.

**Conclusion:** The myxobacterial antibiotics archazolid and apicularen are highly efficient and specific novel inhibitors of V-ATPases. While archazolid at least partly shares a common binding site with the plecomacrolides bafilomycin and concanamycin, apicularen adheres to an independent binding site.

**Contact:**
**Dr. Markus Huss**
University of Osnabrück
Department of Biology
huss@biologie.uni-osnabrueck.de
Barbarastrasse 11
D-49076 Osnabrück (Germany)
Cloning of rat Abcb7 and expression analysis of Abcb7 as compared to rat Abcb6

Human ABCB6 and ABCB7 represent related half-transporters belonging to the ATP-binding cassette (ABC) protein superfamily. Both proteins exhibit high sequence homology to Atm1p, a yeast half-transporter involved in iron homeostasis. Since expression of either human ABCB7 or ABCB6 in yeast cells defective for Atm1p has been shown to lead to reversal of the defective phenotype (Csere et al., FEBS Lett. 1998, 441; Mitsuhashi et al., JBC 2000, 275), the question was raised whether ABCB6 and ABCB7 might fulfill similar functions and/or act as partners of heterodimerization to form a functional transporter system. Due to accessibility of rat tissues and primary cells, we concentrated on the rat orthologues of ABCB6 and ABCB7 for further analyses of half-transporter expression. Full-length Rat Abcb6 cDNA had been cloned by us before. Further using rat hepatocytes as an mRNA source and applying an RT-PCR/5´-RACE approach, we cloned rat Abcb7 cDNA whose N-terminus-encoding region differed from a database sequence formerly predicted in silico. The full-length rat Abcb7 cDNA coded for a protein of 752 amino acids. Additionally, we detected a novel splice variant sequence, expected to give rise to a protein lacking 42 amino acids within the C-terminus, but containing an intact ATP-binding cassette. Although Northern blot analyses demonstrated that both rat Abcb6 mRNA and Abcb7 mRNA were expressed in freshly isolated rat hepatocytes and in a variety of tissues, their extent of expression differed: Abcb6 mRNA was most abundant in testis, whereas Abcb7 mRNA was most highly expressed in heart. To compare the subcellular distribution of the half-transporters, we constructed expression vectors harbouring either the Abcb6- or Abcb7-coding sequence in-frame with the coding sequence for a fluorescent protein (enhanced green fluorescent protein (EGFP) or DsRed, respectively), yielding fluorescent fusion proteins in transfected cells. Fluorescence microscopy of transiently transfected LoVo cells revealed that fusion proteins were localized intracellularly, but that the green fluorescence of Abcb6-EGFP and the red fluorescence of Abcb7-DsRed were not strictly co-localized, arguing against obligatory heterodimerization of rat Abcb6 and Abcb7 fusion proteins. Nevertheless, utilization of epitope-tagged Abcb6 and Abcb7 fusion proteins provides a basis for further half-transporter interaction experiments to clarify whether homo- or heterodimerization between the related half-transporters occurs.

Contact:
Ana Jakimenko
Georg-August-University of Göttingen
Institute of Pharmacology and Toxicology
ajakimenko@med.uni-goettingen.de
Robert-Koch-Straße 40
D-37075 Göttingen (Germany)
Structural and biochemical characterisation of the nucleotide binding domain of the ABC transporter Haemolysin B

The ABC-transporter Haemolysin B (HlyB) is a central element of the *E. coli* Haemolysin A (HlyA) secretion machinery, a paradigm of Type I secretion. The transport substrate is translocated across both the inner and outer membrane, via a transport machinery consisting of an ABC protein (HlyB), a membrane fusion protein (HlyD) and an outer membrane protein (TolC). Upon hydrolysis of ATP the substrate toxin HlyA is translocated in an unidirectional manner. To unravel the conversion of chemical energy into mechanical energy we overexpressed, purified and crystallized the nucleotide binding domain (NDB) of HlyB. The crystal structures of the NBD in various nucleotide bound states enabled us to present a detailed and complete picture of the catalytic cycle. Based on these data, we propose a model in which H662, highly conserved among ABC-transporters, acts as a ‘linchpin’, holding together all required parts of the complicated network of dimer formation and ATP hydrolysis. Furthermore biochemical data support this model showing that hydrolysis of ATP is the rate-limiting step of catalysis. The energy of ATP hydrolysis and subsequent dissociation of the dimer is stored in a bending of a distinct helix. By comparing different NBD structures we identified a structural diverse region, which is most likely the region that shuttles information between the NBD and the transmembrane domain. The structural rearrangements upon nucleotide binding and hydrolysis are currently analyzed by NMR. First results reveal large conformational changes upon nucleotide binding. Having overexpressed the inner membrane complex we now can reconstitute the proteins and thus get an insight into the initial steps of substrate translocation.

**Contact:**

**Stefan Jenewein**
Johann Wolfgang Goethe-University of Frankfurt/Main
Institute of Biochemistry, Biocenter
Jenewein@stud.uni-frankfurt.de
Marie-Curie-Straße 9
D-60439 Frankfurt/Main (Germany)
Targeting of enzymes involved in the nonmevalonate pathway of isoprenoid biosynthesis to the plastid-like organelle of malaria parasites

Malaria is caused by unicellular parasites of the genus Plasmodium. The isoprenoid biosynthesis of malaria parasites is achieved by the mevalonate-independent 1-deoxy-D-xylulose 5-phosphate (DOXP) pathway common to most eubacteria and the plastids of plants. According to current evidence, the enzymes of the DOXP pathway are encoded by the nucleus and transported into a non-photosynthetic plastid-like organelle of the parasites. This organelle, the so-called apicoplast, is coated by four membranes reminiscent to the plastids of some algae such as the dinoflagellates and the cryptophytes. Nuclear-encoded apicoplast proteins are characterised by a bipartite targeting sequence consisting of an ER-signal peptide at the extreme N-terminus followed by a plastid transit domain. Targeting appears to proceed via the secretory pathway with sequential removal of the signal peptide and the plastid transit peptide. Our work aims at the characterisation of the processing events occurring during the transport of the enzymes of the DOXP pathway to the apicoplast. In addition, we plan to identify additional apicoplast-resident proteins required as co-factors for the enzymatic activity of the last two enzymes of the DOXP pathway. This will contribute to investigations whether a specialised intra-organelar physicochemical environment may has been a reason why the apicoplast was retained in course of the evolution.

Contact:
Dr. Hassan Jomaa
Justus-Liebig-University of Gießen
Biochemical Institute
hassan.jomaa@biochemie.med.uni-giessen.de
Friedrichstraße 24
D-35392 Gießen (Germany)
A. Kinner, R.K.H. Kinne, and H. Kipp

**SGLT1 trafficking involves Sorting Endosomes but not Recycling Endosomes**

We previously reported (Khouriandi et al., Am. J. Physiol. 287:C1041, 2004) that SGLT1 trafficking is critically involved in the regulation of sodium-dependent D-glucose uptake into Caco-2 cells. In addition, antibody feeding assays using SGLT1-specific antibodies revealed cycling of the transporter between the plasma membrane and intracellular sites. To elucidate mechanisms underlying SGLT1 recycling, we investigated the effect of over-expression of Rab4, Rab5 and Rab11 on SGLT1 distribution in Caco-2 cells. Rab5 controls the trafficking from plasma-membrane to sorting endosomes (SE). Rab4 mediates the direct return from SE to plasma-membrane, while Rab11 participates in the trafficking to plasma-membrane involving in addition recycling endosomes. In cells with increased Rab5a activity the amount of intracellular SGLT1 was reduced, suggesting that increased rates of SGLT1 endocytosis lead to increased degradation. In the Rab5 dependent endocytosis also clathrin seems to play a role as indicated by co-localisation of SGLT1 and clathrin near the membrane. In cells over-expressing defective Rab4 SGLT1 accumulated in Rab4 positive endosomes which probably originated from SE. In contrast, SGLT1 distribution was not altered when Rab11 activity was modulated. Our data suggest a Rab5a and clathrin dependent SGLT1 internalization and a return mechanism via SE. Thus the Rab5a and Rab4 dependent SGLT1 recycling appears to be of major importance as a potential mechanism in the regulation of SGLT1 mediated D-glucose uptake.

**Contact:**
**Dr. Andrea Kinner**
Max-Planck-Institute of Molecular Physiology Dortmund
Laboratory for Molecular Cell Biology
andrea.kinner@mpi-dortmund.mpg.de
Otto-Hahn-Straße 11
D-44227 Dortmund (Germany)
A. Kinner, R.K.H. Kinne, and H. Kipp

The sodium D-glucose cotransporter SGLT1 associates with and is regulated by Rab5a

A considerable amount of the sodium D-glucose cotransporter (SGLT1) in Caco-2 cells, a model for human enterocytes, is located in intracellular vesicles attached to microtubules (Kipp et al., AJP 285: C737, 2003). We, therefore, proposed a role of intracellular SGLT1 in the regulation of sodium-dependent D-glucose uptake. To elucidate the molecular mechanisms of SGLT1 regulation we screened a Caco-2 cDNA library for SGLT1 interacting proteins using the yeast two hybrid system and identified the small GTPase Rab5a, which is essential for early steps in clathrin-coated pit mediated endocytosis. SGLT1-Rab5a association was further confirmed by co-immunoprecipitation from Caco-2 cell lysate. In in vitro binding assays SGLT1 bound to Rab5a wild type protein, to the GTPase defective Rab5aQ79L and also to the GTP binding deficient Rab5aS34N mutant. No interaction of SGLT1 with the homologous protein Rab9 was observed. Also truncated Rab5a, in which the C-terminus of Rab5a was omitted, did not bind in vitro to SGLT1 indicating that the association of Rab5a with SGLT1 is GTP-independent and occurs via the Rab5a C-terminus. Immunofluorescence microscopy revealed that in Caco-2 cells over-expressing wild type Rab5a or constitutively active Rab5a(Q79L) the amount of intracellular SGLT1 was drastically decreased, suggesting an increased rate of endocytosis and degradation of SGLT1. These results suggest an additional role of Rab5a besides its GTP dependent involvement in early endocytosis. The GTP independent association between Rab5a and SGLT1 maybe important for cargo selection of SGLT1 to vesicles undergoing endocytosis. Furthermore, Rab5a dependent endocytosis is critically involved in cellular SGLT1 regulation.

Contact:
Dr. Helmut Kipp
Max-Planck-Institute of Molecular Physiology Dortmund
Laboratory for Molecular Cell Biology
helmut.kipp@mpi-dortmund.mpg.de
Otto-Hahn-Straße 11
D-44227 Dortmund (Germany)
C. Klammt¹, D. Schwarz¹, A. Srivastava², C. Reinhardt², H. Michel², W. Haase², V. Dötsch¹, and F. Bernhard¹.

Functional cell-free expression of G-protein coupled receptors in a preparative scale

¹Institute of Biophysical Chemistry, Johann Wolfgang Goethe-University Frankfurt/Main.
²Max-Planck-Institute for Biophysics Frankfurt/Main

G-protein-coupled receptors (GPCR) represent a large family of eukaryotic transmembrane receptors that are responsible for the transmission of an immense variety of extra-cellular signals. The majority of modern drugs are directed against GPCRs, indicating their importance for the pharmaceutical industry. As for many other membrane proteins, the functional and structural analyses of GPCRs are limited by insufficient production rates in conventional bacterial or eukaryotic expression systems. However, recent advantages in the high level production, labeling and functional reconstitution of integral membrane proteins by cell-free expression techniques have opened new fields for structural studies. We demonstrate the fast and efficient cell-free synthesis of several GPCRs including the human Endothelin B receptor precursor, the rat corticotropin releasing factor receptor 1 precursor and the human vasopressin receptor type 2, in an individual bacterial cell-free coupled transcription translation system. After optimization of the expression system, all GPCRs could be produced in preparative scales, i.e. mg amounts per a single ml of cell-free reaction. Moreover, after modification of our cell-free system by addition of detergents, the GPCRs could be produced instantly as soluble proteins inserted into micelles. The functional folding of the cell-free expressed GPCRs has been analyzed by CD-spectroscopy, by freeze-fracture electron microscopy of reconstituted proteoliposomes, and by binding assays of specific peptide ligands. The results demonstrate that GPCRs can be produced in high amounts and in an active form by cell-free expression and by using bacterial extracts. We further indicate a strong correlation of the specific activities of the produced GPCRs with the applied mode of cell-free expression and with the type of detergent used for their solubilization.

Contact:
Christian Klammt
Johann Wolfgang Goethe-University of Frankfurt/Main
Institute of Biophysical Chemistry
c.klammt@bpc.uni-frankfurt.de
Marie-Curie-Straße 9
D-60439 Frankfurt/Main (Germany)
**Exploring the limits of minimizing the transporter associated with antigen processing (TAP)**

Survival of vertebrates is strongly dependent on the adaptive immune system to protect the organism against invaders or cancer. As an important strategy, the MHC class I-dependent pathway of antigen processing can trigger elimination of affected cells by cytotoxic T lymphocytes (CTL) upon presentation of antigenic peptides at the cell surface. The supply with peptides derived of proteasomal degradation is strongly dependent on the transporter associated with antigen processing (TAP) a heterodimeric ABC-transporter, which translocates peptides into the ER. Peptide-loading onto MHC class I molecules requires the formation of a macromolecular peptide-loading complex comprised of TAP, MHC class I, tapasin, calreticulin, and ERp57. We could show that the transmembrane domains (TMD) of TAP can be subdivided into a core-domain of two times six transmembrane segments (TM), which is sufficient for peptide transport and an N-terminal domain which is essential for binding of the peptide-loading complex adapter protein tapasin. Moreover, we determined the minimal number of TMs needed for the formation of a functional heterodimeric TAP complex on the basis of N-terminal truncation studies. As a result, transport complexes shorter than the core-domain were able to form heterodimers but were deficient in peptide transport and showed a severe reduction in the ability to bind peptides. These data are of particular interest in the light of other ABC transporters, which have less than two times six TMs like e. g. the *E. coli* macrolide antibiotic exporter MacB and the lipoprotein exporter LolC/LolE, respectively.

**Contact:**
**Dr. Joachim Koch**
Johann Wolfgang Goethe-University of Frankfurt/Main
Institute of Biochemistry, Biocenter
joachim.koch@em.uni-frankfurt.de
Marie-Curie-Straße 9,
D-60439 Frankfurt/Main (Germany)
A systematic approach to study Multidrug Transporters of the SMR family – cell free expression and Solid-State NMR

Bacterial multidrug resistance is posing a significant treatment obstacle. Resistance mechanisms such as drug inactivation, target alteration, prevention of drug influx and active drug extrusion have been found to act synergistically. One of the mechanisms, active drug extrusion, can be assigned to membrane-bound efflux pumps. Members of the SMR efflux pump family, which act as proton/drug antiporters, are promising candidates for detailed biochemical and biophysical studies. Using solid-state NMR it is possible to measure chemical shift changes, interatomic distances and angles thus enabling detailed examination of drug extrusion. However, such specific investigations require amino acid selective isotope labelling. Membrane proteins provide a challenge to in-depth biophysical studies mainly due to difficulties involved with expression, purification and refolding.

Here the archaeal SMR protein Hsmr from H. salinarium is under investigation. It can both be expressed in an in vitro prokaryotic coupled transcription and translation system or in vivo in E. coli cells. Cell free expression in the presence of certain detergents provides a suitable hydrophobic environment for the expression of correctly folded membrane proteins. Detergent solubilised proteins are functional as evaluated using established binding assays and can be reconstituted into a liposomes at concentrations required for solid-state NMR experiments. Reconstitution is verified by freeze fracture electron microscopy. Further spectroscopic protein characterisation and first solid-state NMR experiments are reported.

Contact:
Ines Lehner
Johann Wolfgang Goethe-University of Frankfurt/Main
Institute for Biophysical Chemistry
lehner@em.uni-frankfurt.de
Marie-Curie-Straße 9,
D-60439 Frankfurt/Main (Germany)
Molecular anatomy of the non-neuronal cholinergic system of the rat respiratory epithelium

Besides the nervous system, acetylcholine (ACh) is also produced by many non-neuronal cells particular in epithelia. In the nervous system, the cycle of ACh synthesis, release, and reuptake of cleavage products is well characterised. For each of these steps, alternative mechanisms are known. The present study was set up to determine the molecular components of the cholinergic system in the rat respiratory epithelium. Among the 6 known molecular variants of cholineacetyltransferase (ChAT) mRNA exclusively the M-type of cChAT is expressed in the respiratory epithelium while the other types of cChAT and the ChAT isoform being characteristic for peripheral neurons, i.e. pChAT are not expressed. cChAT protein is enriched in the apical portion of ciliated and secretory cells while being more diffusely distributed in the other epithelial cell types. The vesicular acetylcholine transporter (VACHT) protein was detected by immunohistochemistry in secretory cells only, while other epithelial cell types reacted with antisera against organic cation transporters (OCT1-3) which represent alternative release mechanisms for ACh. In ciliated cells, these transporters are located in the apical membrane. Finally, the high-affinity choline transporter CHT1 which was previously considered to be specific for the nervous system, was detected in the apical membrane of the ciliated cells. These data show a complete machinery of ACh synthesis, release and choline reuptake at the luminal front of the ciliated cells, and the molecular identification of its components provides a basis for further experimental and therapeutic intervention.

Contact:
Dr. Katrin Lips
Justus-Liebig-University of Gießen
Institute of Anatomy and Cell Biology
Katrin.S.Lips@anatomie.med.uni-giessen.de
Aulweg 123
D-35385 Gießen (Germany)
Voltage dependence of transient and stationary photocurrents of Proteorhodopsin expressed in Xenopus oocytes

Proteorhodopsin (PR), the first identified true “bacterio”-rhodopsin is a light-driven proton pump from marine bacteria with photocycle similar to bacteriorhodopsin (BR) at neutral pH, including the occurrence of an M-like intermediate. At acidic pH no M is detected spectroscopically or electrically and proton pumping inverts at acidic pH. Using two-electrode voltage-clamping after expression in *Xenopus* oocytes we determined the voltage dependence of stationary photocurrents (I-V curves) at different intra- and extracellular pH. The resulting I-V curves are linear at all pH conditions and the observed photocurrent reversal distinctly depends on membrane potential and pH, shifting by 55 mV each ΔpH unit. Furthermore, we studied the effects of mutations at the putative proton acceptor and donor groups, D97 and E108, respectively. Stationary illumination of mutants D97N and D97T at neutral pH did not produce outward currents but exhibited transient and stationary currents, showing that removal of the primary proton acceptor group abolishes forward proton pumping. Mutant E108G showed much smaller transient and stationary photocurrents than WT, but stationary proton pumping was restored upon azide addition, similar to BR mutant D96G. Furthermore, blue and green laser flashes were applied during or after continuous illumination with green light and the behavior of PR and BR photocurrents was compared under identical conditions. In contrast to BR, blue laser flashes on PR evoked much larger outwardly-directed transient currents upon blue compared to green flashes and transient inward proton translocation was observed with blue and green flashes at acidic extracellular pH. Furthermore, the direction of transient charge translocation inverted at negative membrane potential. These results confirm earlier conclusions drawn by our laboratory that the direction of proton transport by PR can be switched by changes in pH or the membrane potential.

Contact:
Éva Lörinczi
Max-Planck-Institute of Biophysics Frankfurt/Main
eva.lorinczi@mpib-frankfurt.mpg.de
Max-von-Laue Straße 3
D-60438 Frankfurt/Main (Germany)
L. Metzner, G. Kottra, K. Neubert, H. Daniel, and M. Brandsch

Substrates and inhibitors of the proton-coupled amino acid transport system PAT1

The proton-coupled amino acid transporter PAT1, cloned from brain and intestine, mediates the uphill transport of L- and D-proline, L-alanine, glycine, taurine, D-serine, GABA and many other related compounds and drugs. Recently we identified new PAT1 substrates among proline derivatives and proline derived therapeutically drugs\(^1\). In the course of these investigations on substrate specificity we found that L-tryptophan and its derivatives tryptamine, 5-hydroxy-L-tryptophan, serotonin and indole-3-propionic acid strongly inhibit H\(^+\)-dependent L-[\(^3\)H]proline uptake into Caco-2 cells with inhibition constants (K\(_i\)) of 0.9 to 6.1 mM. On the other hand, uptake of L-[\(^3\)H]tryptophan into Caco-2 cells was not inhibited by L-proline. Whereas PAT1 substrates produced significant changes in a membrane potential assay for electrogenic transport in Caco-2 cells, L-tryptophan, tryptamine and 5-hydroxy-L-tryptophan failed to alter membrane voltage. When PAT1 was expressed in *Xenopus laevis* oocytes and analyzed by the two-electrode voltage clamp technique, glycine elicited high inward currents that were dependent on membrane potential but no currents were observed with either L-tryptophan, tryptamine, 5-hydroxy-L-tryptophan or serotonin. Although not transported electrogenically by PAT1, L-tryptophan and its derivatives inhibited glycine-evoked currents dose-dependently\(^2\). We conclude that serotonin, L-tryptophan and tryptamine bind to PAT1 with potencies similar to the prototype substrates, inhibit transport function but are not transported by this carrier protein. They may be considered as the carriers’ naturally occurring inhibitors that alter the transport function of PAT1.

Literature:


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Contact:
Dipl. Pharm. Linda Metzner
Martin-Luther-University of Halle-Wittenberg
Biozentrum
linda.metzner@biozentrum.uni-halle.de
Weinbergweg 22
D-06120 Halle/Saale (Germany)
A novel sugar transporter in *Drosophila melanogaster*

**Keywords:** *Drosophila*, Carbohydrate, Transporter, Melanosome, Albinism

In prokaryotes, fungi and plants, oligosaccharides are transported by well characterized specific transporters, whereas in animals transport is restricted to monosaccharides. Here we report on the existence of the first animal disaccharide transporter. Immunohistochemistry showed that this previously uncharacterized protein is located in melanosome membranes from ovarian follicle cells of *Drosophila melanogaster*. The deduced amino acid sequence exhibits striking similarities with plant sucrose transporters. A typical sucrose binding signature (GRRRP) occurs, like in other well characterized non-animal transporters, between the second and the third transmembrane domains. First experiments with *Saccharomyces cerevisiae* as a heterologous expression system proved that the protein is membrane bound and pointed towards sucrose to be the transported substrate. Knock out studies by other laboratories with the homologous mouse and fish proteins resulting in loss of the protein function led to oculocutaneous albinism, and the analysis of the gene encoding the human homolog indicated that mutations are correlated with a highly increased probability of skin cancer (melanomas). We propose that in *Drosophila* melanosomes this putative disaccharide transporter is necessary for balancing the osmotic equilibrium during the polymerization process of melanin.

**Contact:**

**Heiko Meyer**  
University of Osnabrück  
Department of Biology  
[Meyer@biologie.uni-osnabrueck.de](mailto:Meyer@biologie.uni-osnabrueck.de)  
Barbarastraße 11  
D-49076 Osnabrück (Germany)
Overexpression of proton-dependent peptide transporter PEPT2 in renal proximal tubule cells

OK cells are an immortalized cell line derived from opossum kidney proximal tubule. Polarized OK monolayers are broadly used as a model to study renal reabsorption of amino acids. Transepithelial flux is carried by the action of different transport proteins located at the apical and basolateral membrane. Although a proton-dependent di-/tripeptide influx carried by the renal peptide transporter PEPT2 is shown for membrane vesicles from kidney proximal tubule, this transport activity is not described for wildtype (wt) OK cells.

We have established and started to characterise a stable cell line expressing the renal proton-dependent peptide transporter rPEPT2 (OK-rPEPT2). The pept2 gene was C-terminally fused with a pH-sensitive GFP named “pHluorin”. The overexpression of PEPT2 was proven by fluorescence microscopy and uptake measurements with fluorescent- and radio-labeled substrates. Both the fluorescent-labeled dipeptide b-Ala-Lys-Amca as well as $^{14}$C-labeled dipeptide Gly-Sar serve as good substrates, indicating a functional expression of rPEPT2 in the plasma membrane of the OK cells. Uptake of both dipeptides was inhibited by adding an excess amount of non-labeled Gly-Gln. $^{14}$C-Gly-Sar uptake was increased in OK-rPEPT2 about 30-fold over non-transfected control cells.

In conclusion, the stable cell line OK-rPEPT2 represents an excellent tool to study the substrate specificity of PEPT2. Furthermore OK-rPEPT2 is perfectly suited to investigate the influence of peptide transport on transepithelial amino acid fluxes.

Contact:
Alexander Nickel
Technical University of Munich
Molecular Nutrition Unit
nickel@wzw.tum.de
Am Forum 5
D-85350 Freising-Weihenstephan (Germany)
M. Palmada¹, A. Akel¹, J. Rajamanickam¹, M. Dieter¹, C. Böhmer¹, F.C. Luft², and F. Lang¹

The serum and glucocorticoid inducible kinases 1 and 3 modulate glucose transport

¹ Department of Physiology, Eberhard Karls University of Tübingen, Tübingen, Germany
² Department of Medicine, Charité, Berlin, Germany

The SGK1 kinase enhances ENaC activity by inhibiting the ubiquitin-ligase Nedd4-2, finally leading to retarded channel retrieval from the plasma membrane. Strong expression of SGK1 in cells not expressing ENaC points to further SGK1 functions. The present study was performed to test for regulation of the sodium-dependent (SGLT1) and independent (GLUT1) glucose transporters by SGK1 and/or Nedd4-2. Further, genetic studies investigated whether SGK1 polymorphisms affect body mass index (BMI). To this end, cRNA encoding SGLT1 or GLUT1, SGK1 or SGK3 and/or Nedd4-2 was injected into Xenopus oocytes and glucose transport was quantified from glucose induced current (Iglc) in SGLT1 expressing oocytes or tracer flux uptake in oocytes expressing GLUT1. SGK1 and SGK3 enhanced Iglc while Nedd4-2 inhibited glucose induced current. GLUT1 was similarly stimulated by SGK1 and SGK3, however, transporter activity remained unaffected upon Nedd4-2 coexpression. Besides the functional studies, genetic data revealed that BMI was significantly larger in individuals with the E8CC/CT;I6CC SGK1 polymorphism. Thus, SGK1 may also play an important role in glucose transport regulation and development of obesity.

Contact:
Dr. Monica Palmada
Eberhard Karls University of Tübingen
Department of Physiology
monica.palmada@uni-tuebingen.de
Gmelinstraße 5
D-72076 Tübingen (Germany)
N. Pfleger, D. Basting, and C. Glaubitz

MAS-NMR on 19F-spin-labelled EmrE

EmrE (Ethidium multidrug resistance protein) is a member of a SMR (small multidrug resistance) family with a size of 12 kDa. Hydropathy studies propose four helical segments with the N- and C-termini located in the cytoplasm. The oligomeric state is not known so far. EmrE belongs to the secondary MDR transporters. Studies on mutants indicate that glu 14 is required for transport activity. The substrates of EmrE are composed of an aromatic or hydrophobic part and of hydroxyl groups or cations like tetraphenylphosphonium (TPP), ethidiumbromide, acriflavin, erytromycin and tetracyclin for example. First solid state NMR experiments show interactions of protein and ligand, revealed by a change in the 31P chemical shift of TPP. However, conformational changes during the catalytic cycle as well as investigations of the oligomeric state require long range constraints. For this purpose, 19F labelling is suitable for MAS-NMR experiments because of its high sensitivity (90 % of protons) and a high distance range (0.15-0.2 nm). 19F labeling is achieved by covalently linking trifluoro-acetone to the cystein of the single cys (E84C on a cysless background) protein. Here, we present first 19F-MAS-NMR data of EmrE and discuss the potential of this approach.

Contact:
Nicole Pfleger
Johann Wolfgang Goethe-University of Frankfurt/Main
Center for Biomolecular Magnetic Resonance and
Institute for Biophysical Chemistry
pfleger@chemie.uni-frankfurt.de
Marie-Curie-Straße 9
D-60439 Frankfurt/Main (Germany)
The influence of dicarboxylates and pH on transport by the human Organic Anion Transporter 1 (hOAT1)

Dicarboxylates are important for the function of hOAT1, because they exchange for organic anions at this transporter. The influence of dicarboxylates on the uptake of radiolabeled and/or fluorescent substrates was studied in HEK 293 cells stably transfected with hOAT1 or in X. laevis oocytes expressing wild type hOAT1 or the mutant R466K. In HEK293 cells the relative degree of competitive inhibition of 5 µM fluorescein (FL) uptake was as follows: α-ketoglutarate > succinate > citrate > malate. Transport of radiolabeled p-aminohippurate (PAH) was substantially trans-stimulated by glutarate and PAH preloading. The influence of dicarboxylates on PAH uptake was also measured in X. laevis oocytes expressing hOAT1. The 5 and 6 carbon containing dicarboxylates, glutarate and adipate, respectively, markedly reduced PAH transport, whereas malonate, a 3 carbon containing dicarboxylate, did not affect PAH transport. The replacement of highly conserved arginine with lysine (R466K) resulted in a mutant with markedly reduced transport activity but its interaction with dicarboxylates was qualitatively similar. Finally, lowering pH from 7.4 to 6.0 during transport experiments remarkably increased uptake of FL and glutarate significantly. Our data show that OAT1 interacts with di- and tricarboxylates (minimum length: 4 carbons) and is accelerated at lower pH (organic anion/OH- antiport?).
Internalization of hCAT-1 in response to protein kinase C (PKC) activation

The human cationic amino acid transporter hCAT-1 is probably the major entry route for arginine, lysine, and ornithine in most cells. We have previously demonstrated that the transport activity of hCAT-1 is decreased after PKC activation by phorbol-12-myristate-13-acetate (PMA). We now looked into the mechanism of this down-regulation. In both, *X. laevis* oocytes and U373MG glioblastoma cells, PMA treatment promoted the internalization of hCAT-1.EGFP as visualized by confocal microscopy. Western blot analyses of biotinylated cell surface proteins confirmed that the cell surface expression of hCAT-1.EGFP was significantly reduced upon PMA treatment. The PKC inhibitor BIM I prevented the reduction of both hCAT-1.EGFP-activity, and the internalization of the transporter. Similar results were obtained with hCAT-1 expressed endogenously in DLD-1 colon carcinoma cells. Inhibition of protein synthesis did not augment the PMA effect. In addition, the PMA effect was reverted in washout experiments, while the hCAT-1 protein expression was unchanged, suggesting that the PMA effect is reversible. PKC did not phosphorylate hCAT-1 directly as evidenced by in vivo phosphorylation experiments and mutational analysis, indicating an indirect action of PKC on hCAT-1. Internalization of hCAT-1 in response to external stimuli represents a new regulatory mechanism for this transporter.

Contact:
Dr. Alexander Rotmann
Johannes Gutenberg-University of Mainz
Department of Pharmacology
rotmann@uni-mainz.de
Obere-Zahlbacher-Straße 67
D-55101 Mainz (Germany)
R. Ernst, N. Hanekop, and L. Schmitt

**Purification and reconstitution of the yeast ABC transporter Pdr5p - a translocase for fluorescently labeled phospholipids**

The ABC-transporter Pdr5p from *S. cerevisiae* is a key element of the pleiotrophic drug-resistance (PDR). PDR is phenomenological and functional similar to the human multi drug-resistance. However, it was recently proposed that Pdr5p among other membrane protein is involved in lipid homeostasis. As a prerequisite for a detailed study of this suggestion, we were able to over-express and purify Pdr5p in sufficient quantities. Furthermore, we could show that Pdr5p remained functional in detergent solution as well as in the reconstituted state. After reconstitution in liposomes we were able to show, that Pdr5p is a broad specific translocase for fluorescently labeled phospholipids. In conclusion, these results serve as a starting point to decipher the molecular role and properties of this ABC-transporter.

**Contact:**
**Prof. Dr. Lutz Schmitt**
Heinrich-Heine-University of Düsseldorf
Institute of Biochemistry
lutz.schmitt@uni-duesseldorf.de
Universitätsstraße 1
D-40225 Düsseldorf (Germany)
S. Schröder-Lang¹, P. Hegemann², M. Watanabe³, and G. Nagel¹

Activation of CFTR by light-controlled cAMP increase

¹Max-Planck-Institute of Biophysics, Frankfurt, Germany
²Humboldt-University, Experimental Biophysics, Berlin, Germany
³School of Advanced Sciences, Graduate University, Hayama, Japan


Here we report the functional expression of PACα and PACβ in oocytes of Xenopus laevis in combination with the human epithelial chloride channel CFTR (cystic fibrosis transmembrane conductance regulator). PACα and PACβ show adenylyl cyclase activity which is strongly enhanced upon irradiation with blue light. The cytoplasmic cAMP-increase activates CFTR via the endogenous cAMP-dependent protein kinase (PKA).

We show with this heterologous expression system that both proteins are independent light-activated enzymes.

Contact:
Saskia Schröder-Lang
Max-Planck-Institute of Biophysics Frankfurt/Main
saskia.schroeder-lang@mpibp-frankfurt.mpg.de
Max-von-Laue-Straße 3
D-60438 Frankfurt/Main (Germany)
D. Schwarz\textsuperscript{1}, C. Klammt\textsuperscript{1}, K. Fendler\textsuperscript{2}, V. Dötsch\textsuperscript{1}, and F. Bernhard\textsuperscript{1}

**Evaluation of detergents for the high level and functional expression of α-helical and β-barrel type integral membrane proteins**

\textsuperscript{1}Institute of Biophysical Chemistry, Johann Wolfgang Goethe-University of Frankfurt/Main, Marie-Curie Straße 9, D-60439 Frankfurt/Main (Germany)
\textsuperscript{2}Max-Planck-Institute of Biophysics, Department of Biophysical Chemistry, Max-von-Laue-Strasse 3, D-60438 Frankfurt/Main (Germany)

We previously have shown that integral membrane proteins (IMPs) can be produced in high amounts by using cell-free expression systems based on bacterial extracts. The synthesized IMPs remain non-soluble in the reaction mixture but they can efficiently be re-solubilized simply by adding suitable detergents to the precipitated proteins. While we already could show for the multidrug transporter EmrE that this procedure results in active transporter proteins, the re-solubilization from precipitates might affect the functional refolding of other IMPs. We have therefore evaluated a representative variety of detergents for their suitability as additives for the cell-free expression of soluble IMPs. The cell-free production of three structurally very different IMPs, the bacterial β-barrel type nucleoside transporter Tsx, the α-helical transporter EmrE of *E. coli* and the porcine vasopressin type 2 receptor, has been analysed in presence of the different detergents. The synthesized IMPs were quantified in the non-soluble as well as in the soluble fraction. While only few detergents obviously were detrimental to the cell-free system, the majority was tolerated even at relatively high critical micellar concentrations. We could identify several detergents that resulted in the production of up to four mg of soluble IMPs per one ml of reaction volume. Our results indicate that especially long chain poly-oxo-ethylene derivatives proof to be suitable for the soluble high level expression of a broad range of IMPs. We demonstrate for the first time the preparative scale cell-free expression of a β-barrel type transporter. The activity of the produced Tsx transporter has been analysed by black lipid membrane assays and we present evidence for a strong correlation of the specific transport activity of the purified protein with its mode of expression, i.e. the design of the cell-free expression system.

**Contact:**

**Daniel Schwarz**
Johann Wolfgang Goethe-University of Frankfurt/Main
Institute of Biophysical Chemistry
daniel.schwarz@bpc.uni-frankfurt.de
Marie-Curie-Straße 9
D-60439 Frankfurt/Main (Germany)
Amphetamine-induced monoamine efflux takes two to tango

Amphetamine congeners (e.g. MDMA = “ecstasy”) are substrates for monoamine transporters (i.e. the transporters for serotonin, norepinephrine and dopamine); however, their in vivo-action relies on their ability to promote monoamine efflux. The mechanistic basis for this counter transport remains enigmatic. We tested the hypothesis that outward transport is contingent on the oligomeric nature of neurotransmitter transporters by creating a concatemer of the serotonin transporter (SERT) and the – amphetamine-resistant - GABA-transporter. In cells expressing the concatemer, amphetamine analogues promoted GABA efflux and blunted GABA-influx. In contrast, the natural substrates serotonin and GABA only cause mutual inhibition of influx via the other transporter moiety in the concatemer. GABA efflux through the concatemer that was promoted by amphetamine analogues was blocked by the protein kinase C inhibitors GF109203X and Gö6983. Thus, based on our observations, we propose that, in the presence of amphetamine analogs, monoamine transporters operate as counter-transporters; influx and efflux occur through separate – though coupled - moieties. Influx and efflux are coupled via changes in the ionic gradients, but these do not suffice to account for the action of amphetamines; the activity of a protein kinase C isoform provides a second stimulus that primes the inward facing conformation for outward transport.

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Contact:
Prof. Dr. Harald Sitte
Medical University of Vienna
Institute of Pharmacology
harald.sitte@meduniwien.ac.at
Währinger Straße. 13a
A-1090 Wien (Austria)
Organizers:

Prof. Dr. Gerhard Burckhardt
Georg-August-University of Göttingen
Division of Vegetative Physiology
Department of Physiology and
Pathological Physiology
gburck@wdg.de
Humboldtallee 23
D-37073 Göttingen (Germany)

Prof. Dr. Ernst Petzinger
Justus-Liebig-University of Gießen
Institute of Pharmacology and Toxicology
Ernst.Petzinger@vetmed.uni-giessen.de
Frankfurter Straße 107
D-35392 Gießen (Germany)

Prof. Dr. Robert Tampé
Johann Wolfgang Goethe-University
of Frankfurt/Main
Biocenter
Institute of Biochemistry
tampe@em.uni-frankfurt.de
Marie-Curie-Straße 9
D-60439 Frankfurt/Main (Germany)

Organizing secretary:

Dorothee von Schnakenburg
0641-99-38401
06424-301-107
(during congress)

Scientific administration:

Dr. Jörg Alber
List of speakers:

Prof. Dr. Heinz Bönisch
University of Bonn
Institute of Pharmacology and Toxicology
boenisch@uni-bonn.de
Reuterstraße 2b
D-53113 Bonn (Germany)

Dr. Joachim Geyer
Justus-Liebig-University of Giessen
Institute of Pharmacology and Toxicology
Joachim.M.Geyer@vetmed.uni-giessen.de
Frankfurter Straße, 107
D-35392 Gießen (Germany)

Dr. Ellen I. Closs
Johannes Gutenberg University of Mainz
Department of Pharmacology
Closs@Mail.Uni-Mainz.de
Obere Zahlbacher Straße 67
D-55101 Mainz ( Germany)

Prof. Dr. Clemens Glaubitz
Johann Wolfgang Goethe-University of Frankfurt
Centre for Biomolecular Magnetic Resonance and Institute Institute for Biophysical Chemistry
glaubitz@chemie.uni-frankfurt.de
Marie-Curie-Straße 9
D-60439 Frankfurt/Main ( Germany)

Prof. Dr. Arnold J.M. Driessen
University of Groningen
Department of Molecular Microbiology
Biomolecular Sciences and Biotechnology
Institute & Materials Science Center Plus
a.j.m.driessen@rug.nl
Kerklaan 30
9751 NN HAREN (The Netherlands)

Prof. Dr. Veit Flockerzi
University of Saarland
Institute for experimental and clinical Pharmacology and Toxicology
Faculty of Medicine
Veit.flockerzi.@uniklinik-saarland.de
Gebäude 26
D-66421 Homburg (Germany)

Dr. Alexander Gottschalk
Johann Wolfgang Goethe-University of Frankfurt/Main
Biocenter N210
A.Gottschalk@em.uni-frankfurt.de
Marie-Curie-Straße 9
D-60439 Frankfurt/Main (Germany)

PD Dr. Dirk Gründemann
University of Köln
Department of Pharmacology
University Hospital
dirk.gruendemann@uni-koeln.de
Gleueler Straße 24
D-50931 Köln (Germany)

Prof. Dr. Thomas Friedrich
Johann Wolfgang Goethe-University of Frankfurt
Max-Planck-Institute for Biophysics
Department of Biophysical Chemistry
Thomas.Friedrich@mpibp-frankfurt.mpg.de
Max-von-Laue-Straße 3
D-60438 Frankfurt am Main (Germany)

Prof. Dr. Thomas J. Jentsch
University of Hamburg,
Center for Molecular Neurobiology, ZMNH,
Jentsch@zmnh.uni-hamburg.de
Falkenberg 94
D-20246 Hamburg (Germany)

Prof. Dr. Martin F. Fromm
Friedrich-Alexander-University of Erlangen-Nürnberg
Institute of Clinical and Experimental Pharmacology and Toxicology
fromm@pharmakologie.med.uni-erlangen.de
Fahrstraße 17
D-91054 Erlangen (Germany)

Prof. Dr. Daniel J. Müller
University of Technology Dresden
Center of Biotechnology
Daniel.mueller@biotec.tu-dresden.de
Tatzberg 47-51
D-01307 Dresden (Germany)
Prof. Dr. Michael Niederweis
University of Alabama at Birmingham
Department of Microbiology, BBRB Room 609
mnnieder@uab.edu
Mailbox 24
1530 3rd Ave S
Birmingham, AL 35294 (U.S.A.)

PD Dr. Peter Rehling
Albert-Ludwigs-University of Freiburg
Institute of Biochemistry and Molecularbiology
peter.rehling@biochemie.uni-freiburg.de
Hermann-Herder-Straße 7
D-79104 Freiburg (Germany)

Dr. John Riordan
Mayo Clinic College of Medicine
Department of Biochemistry and Molecular Biology
riordan@mayo.edu
13400 E. Shea Boulevard
Scottsdale, AZ 85259 (U.S.A.)

Prof. Dr. Lutz Schmitt
Heinrich-Heine-University of Düsseldorf
Institute of Biochemistry
lutz.schmitt@uni-duesseldorf.de
Universitätsstraße 1
D-40225 Düsseldorf (Germany)

PD Dr. Blanche Schwappach
Ruprecht-Karls-University of Heidelberg
Centre of Molecular Biology
b.schwappach@zmbh.uni-heidelberg.de
Im Neuenheimer Feld 282
D-69120 Heidelberg (Germany)

Prof. Dr. Heinz-Jürgen Steinhoff
University of Osnabrück
Department of Physics
hsteinho@uni-osnabrueck.de
Barbarastraße 7
D-49076 Osnabrück (Germany)

Dr. Bernhard Ugele
Ludwig-Maximilians-University of München
University Hospital
Department of Gynaecology and Obstetrics
bernhard.ugele@med.uni-muenchen.de
Maistraße 11
D-80337 München (Germany)

List of poster contributors:

Dr. Rupert Abele
Johann Wolfgang Goethe-University of Frankfurt/Main
Institute of Biochemistry, Biocenter
abele@em.uni-frankfurt.de
Marie-Curie-Straße 9
D-60439 Frankfurt/Main (Germany)

Nona Adeishvili
Max-Planck Institute of Biophysics
Frankfurt/Main
nona.adeishvili@mpibp-frankfurt.mpg.de
Max-von-Laue Straße 3
D-60438 Frankfurt/Main (Germany)
Nitin Kumar Agarwal  
Georg-August-University of Göttingen  
Department of Nephrology  
sonupharm@rediffmail.com  
Robert-Koch-Straße 40  
D-37075 Göttingen (Germany)

Dr. Birgitta C. Burckhardt  
Georg-August-University of Göttingen  
Department of Pathology and Pathological Physiology  
bcburckhardt@physiol.med.uni-goettingen.de  
Humboldtallee 23  
D-37073 Göttingen (Germany)

Dr. Antje Burse  
Max-Planck-Institute for Chemical Ecology Jena  
Bioorganic Chemistry  
aburse@ice.mpg.de  
Hans-Knoell-Straße 8  
D-07745 Jena (Germany)

Thorsten Althoff  
Max-Planck-Institute of Molecular Physiology  
Dortmund  
thorsten.althoff@mpi-dortmund.mpg.de  
Otto-Hahn-Straße 11  
D-44227 Dortmund (Germany)

Prof. Dr. Martin Diener  
Justus-Liebig-University of Gießen  
Institute of Veterinary Physiology  
Martin.Diener@vetmed.uni-giessen.de  
Frankfurter Straße 100  
D-35392 Gießen (Germany)

Dr. Andrew Bahn  
Georg-August-University of Göttingen  
Centre for Physiology and Pathophysiology  
Department of Vegetative Physiology and Pathophysiology  
abahn@physiol.med.uni-goettingen.de  
Humboldtallee 23  
D-37073 Göttingen (Germany)

Sabrina Discher  
Max Planck Institute for Chemical Ecology Jena  
Department of Bioorganic Chemistry  
sdischer@ice.mpg.de  
Beutenberg Campus  
Hans-Knöll-Straße 8  
D-07745 Jena (Germany)

Daniel Basting  
Johann Wolfgang Goethe-University of Frankfurt/Main  
Centre for Biomolecular Magnetic Resonance and Institute for Biophysical Chemistry  
basting@chemie.uni-frankfurt.de  
Marie-Curie-Straße 9  
D-60439 Frankfurt/Main (Germany)

Dr. Christoph Böhmer  
Eberhard Karls University of Tübingen  
Department of Physiology I  
christoph.boehmer@uni-tuebingen.de  
Gmelinstrasse 5  
D-72076 Tübingen (Germany)

Dr. Chris van der Does  
Johann Wolfgang Goethe-University of Frankfurt/Main  
Institute of Biochemistry, Biocenter  
chris.van.der.does@em.uni-frankfurt.de  
Marie-Curie-Straße 9,  
D-60439 Frankfurt/Main (Germany)

Dr. Andrew Bahn  
Georg-August-University of Göttingen  
Centre for Physiology and Pathophysiology  
Department of Vegetative Physiology and Pathophysiology  
abahn@physiol.med.uni-goettingen.de  
Humboldtallee 23  
D-37073 Göttingen (Germany)

Dr. Hesso Farhan  
Medical University of Vienna  
Institute of Pharmacology  
hesso.farhan@meduniwien.ac.at  
Währinger Straße 13a  
A-1090 Wien (Austria)
Éva Lörinczi
Max-Planck-Institute of Biophysics
Frankfurt/Main
eva.lorinczi@mpibp-frankfurt.mpg.de
Max-von-Laue Straße 3
D-6043 Frankfurt/Main (Germany)

Ahsan Rizwan
Georg-August-University of Göttingen
Department of Physiology and Pathological Physiology
ahsannaqi@rediffmail.com
Humboldttal 23
D-37073 Göttingen (Germany)

Dipl. Pharm. Linda Metzner
Martin-Luther-University of Halle-Wittenberg
Biozentrum
linda.metzner@biozentrum.uni-halle.de
Weinbergweg 22
D-06120 Halle/Saale (Germany)

Dr. Alexander Rotmann
Johannes Gutenberg-University of Mainz
Department of Pharmacology
rotmann@uni-mainz.de
Obere-Zahlbacher-Straße 67
D-55101 Mainz (Germany)

Heiko Meyer
University of Osnabrück
Department of Biology
Meyer@biologie.uni-osnabrueck.de
Barbarastraße 11
D-49076 Osnabrück (Germany)

Prof. Dr. Lutz Schmitt
Heinrich-Heine-University of Düsseldorf
Institute of Biochemistry
lutz.schmitt@uni-duesseldorf.de
Universitätsstraße 1
D-40225 Düsseldorf (Germany)

Alexander Nickel
Technical University of Munich
Molecular Nutrition Unit
nickel@wzw.tum.de
Am Forum 5
D-85350 Freising-Weihenstephan (Germany)

Saskia Schröder-Lang
Max-Planck-Institute of Biophysics Frankfurt/Main
saskia.schroeder-lang@mpibp-frankfurt.mpg.de
Max-von-Laue-Straße 3
D-60438 Frankfurt/Main (Germany)

Dr. Monica Palmada
Eberhard Karls University of Tübingen
Department of Physiology
monica.palmada@uni-tuebingen.de
Gmelinstraße 5
D-72076 Tübingen (Germany)

Daniel Schwarz
Johann Wolfgang Goethe-University of Frankfurt/Main
Institute of Biophysical Chemistry
daniel.schwarz@bpc.uni-frankfurt.de
Marie-Curie-Straße 9
D-60439 Frankfurt/Main (Germany)

Nicole Pfleger
Johann Wolfgang Goethe-University of Frankfurt/Main
Center for Biomolecular Magnetic Resonance and Institute for Biophysical Chemistry
pfleger@chemie.uni-frankfurt.de
Marie-Curie-Straße 9
D-60439 Frankfurt/Main (Germany)

Prof. Dr. Harald Sitte
Medical University of Vienna
Institute of Pharmacology
harald.sitte@meduniwien.ac.at
Währinger Straße 13a
A-1090 Wien (Austria)