Structure function relationships of plant and non plant nucleoside transporters

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Nucleotide and nucleobase transporters possess crucial importance in order to facilitate uptake of precursors of nucleotide synthesis by salvage pathways in a wide range of eukaryotic organisms. Nucleoside transport across membranes in *A. thaliana* is exclusively mediated by 8 isoforms of equilibrative nucleoside transporters (AtENT1-8). Structural characteristics shared by all AtENT´s (11 TMD´s with cytoplasmatic N-terminus and extracellular C-terminus) will be presented in homology model studies. In *Arabidopsis* ENT1 solely exhibits a prolonged N-terminal cytosolic residue which will be compared with N-termini of non plant homologs like hENT3 (*Homo sapiens*; 27% identity), mENT3 (*Mus musculus*; 27%) and FUN26 (*Saccharomycis cerevisiae*; 23%) all residing in endomembrane structures. Sequence comparison, supported by homology models based on structure of the bacterial Glycerol-3-phosphate transporter, will be used to identify putatively important amino acids for substrate and inhibitor binding. First results from site directed mutagenesis studies of these amino acids will be presented. Furthermore first results of the analysis of *ent3*/*urh3* KO-plants will be shown indicating an altered apoplastic nucleoside composition.

(Re)constructing and Dissecting Membrane Proteins – Understanding Helix-Helix Interactions through Segmental Labelling and Solid-State NMR

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Proteorhodopsin (PR) is a seven-helix-transmembrane protein, which is homolog to bacteriorhodopsin (BR), working as a light-driven proton pump [1]. Although a lot of structural information on PR has been discovered in recent years [5][4], less is known about the functional mechanism of the protein. In this context, one interesting issue is the importance of helix-helix interactions for the function of PR. Helix-helix contacts are generally difficult to predict or to measure but contribute significantly to the functional fold of α-helical membrane proteins. Previous studies showed a specific H-bond formation between H75 (helix B) and D97 (helix C) in PR [2]. Furthermore it has been observed that BR fragment can form spontaneously a retinal binding protein complex [3]. To investigate helix-helix contacts in PR a segmental labelling approach in combination with solid-state NMR studies is applied. Segmental isotopic labelling is used to label only one or both parts of a protein with different isotopes. This method has already been successfully applied to soluble proteins for solution-state NMR [6], but so far not for characterization of membrane proteins. For labelling an intein-based approach is used here. Split-inteins are naturally occurring proteins, which fuse precursors to the full-length proteins in an auto-catalytic process while the intein is spliced out. Two segments of PR (AB- and C-F-helix) are fused with the N- and C-terminal parts of the *Npu* DnaE intein, respectively. Expressing the precursors separately (in *vitro* splicing) or under different promoters in the same cell (in *vivo* splicing) a "ligated PR" is obtained which shows similar characteristics than PRwt. With this method it will be possible to introduce different labels into one protein molecule to investigate interactions between the two parts of proteorhodopsin by solid-state NMR.

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Dissecting mechanisms responsible for increased agonist-induced Ca^{2+} release in fibroblasts deficient in the β3 subunit of voltage-acivated calcium channels

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$Ca_V\beta$ subunits of voltage-activated Ca^{2+} channels are cytoplasmic proteins and play a pivotal role in the trafficking of the pore-forming $Ca_V\alpha1$ subunit to the plasma membrane and modulating the Ca^{2+} current kinetics. We identified $Ca_V\beta2$- and $Ca_V\beta3$-protein expression in primary mouse embryonic fibroblasts (MEFs), skin fibroblasts and cardiac fibroblasts. However, we did not detect any voltage-activated Ca^{2+} influx in these cells. Apparently, $Ca_V\beta$ subunits serve functions in fibroblasts which are unrelated to voltage-activated Ca^{2+} influx. Among the proteins potentially interacting with $Ca_V\beta3$ are the inositol 1,4,5-trisphosphate receptors (IP_3Rs) [1,2] and the phospholipase C gamma1 (PLCγ1). We therefore co-expressed the mouse IP_3R type 1, 2 and 3 and the mouse PLCγ1 cDNAs each of them with the $Ca_V\beta3$ cDNA in COS-7 cells for co-immunoprecipitation. Using a $Ca_V\beta3$ antibody, the β3 protein was precipitated and among the proteins retained by the β3 were the IP_3Rs type 1, 2 and 3 and the PLCγ1 proteins, respectively, and vice versa. IP_3 production was increased in β3-deficient MEFs - compared to wild-type MEFs - under basal conditions. Fura-2 measurements show that agonist (lysophosphatidic acid or bradykinin)-induced IP_3-dependent Ca^{2+} release, was significantly increased in MEFs isolated from β3-deficient mice compared to wild-type, whereas thapsigargin-induced Ca^{2+} release was unaffected. Vice versa expression of $Ca_V\beta3$ in COS-7 cells, which do not express endogenous $Ca_V\beta s$, decreases agonist-induced Ca^{2+} release. Apparently, β3 subunit acts as a brake on IP_3-dependent Ca^{2+} release by directly interacting with the IP_3Rs and modulates the IP_3 basic levels by interacting with the PLCγ1. $Ca_V\beta$ subunits contain two conserved regions, C1 and C2, of ~130 amino acids and ~150 amino acids in length, respectively. The C1 domain shares homology with Src homology domains (SH3) and C2 has minor but detectable similarity to guanylate kinase (GK) domains. Both domains are essential for the regulation of Ca_V channels through the $Ca_V\beta$ subunits. We now constructed $Ca_V\beta3$ deletions ($Ca_V\beta3\Delta SH3$ and $Ca_V\beta3\Delta GK$) to study their impact on agonist-induced Ca^{2+}-release in COS-7 cells and co-immunoprecipitation of IP_3R type 1, 2 and 3 and the mouse PLCγ1. In addition, recombinant Cavβ3 protein was produced in *E. coli*. By introducing the recombinant protein into intact (via patch pipette) and saponin permeabilized cells we want to rescue the $Ca_V\beta3$ effect on Ca^{2+} release in $Ca_V\beta3$-deficient MEFs. As already reported, the increased Ca^{2+}-release in $Ca_V\beta3$-deficient fibroblasts is associated with changes in migration and chemotaxis of these cells resulting in shorter times required for skin wound healing. The experiments now underway, aim to elucidate the molecular mechanism underlying the increased Ca^{2+} release.

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E. coli Sugar Transporters of the Major Facilitator Superfamily - Electrophysiological Investigations on LacY, FucP and XylE

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Sugar-proton symporters of the major facilitator superfamily (MFS), e.g. LacY, FucP and XylE from *Escherichia coli*, potentially share a universal transport mechanism. Fortunately for those three transporters 3D structures are available, showing each a different conformation. Possibly these conformations represent different states during the transport cycle, making these transporters ideal candidates for comparison studies.

Here we analyze the electrogenic steps during the transport cycle of these transporters using solid supported membrane (SSM) based electrophysiology.

Our aim is to develop an overall kinetic model describing the behavior of LacY, FucP and XylE.

Whereas LacY and XylE show two electrogenic steps during the transport cycle, we only observe one electrogenic step for FucP. The main electrogenic step observed for all transporters is assigned to the proton release. Our working hypothesis is that the electrogenic step not observed for FucP is due to a conformational transition upon sugar binding. This conformational transition is not observed for FucP because charged key residues are missing.

In addition there are huge differences in the pH dependence of the transport reactions in the pH range between 4.5 and 9.0. Whereas LacY shows no turnover at highly acidic pH and the turnover increases up to pH 9.0, FucP clearly show a pH optimum at pH 8.0, possibly due to a shift in the pH dependence. In both cases the minimal turnover is less than 10% of the maximal rate. In the case of XylE the pH dependence is not as prominent as in the case of LacY and FucP. The minimal turnover in the observed pH range still is about 50% of the maximal transport rate, which supports our idea of multiple proton binding sites having regulatory effects. Nevertheless, for all transporters the KM of sugar binding is not altered by pH.

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From structure to function: role of accessory proteins of Sec61 in the mammalian translocation machinery

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Directed and efficient protein translocation into the lumen of the mammalian Endoplasmic Reticulum (ER) is mediated by a complex machinery of membrane embedded and associated soluble proteins. The central protein conducting channel formed by the heterotrimeric Sec61 complex has a highly dynamic structure which needs to be regulated. Thus accessory components in close proximity influence the open-closed equilibrium of the channel to allow protein translocation across the membrane while keeping ion flux into the cytosol under control. Hitherto classical crystallographic methods or cryoelectron microscopy single particle analysis are not suitable to resolve the entire machinery and thus structural information is restricted to solubilized parts. How they interact could solely be estimated on basis of classical biochemical approaches as immuneprecipitation and cross-linking whereby their precise localization within the complex remained elusive.

Novel cryoelectron tomography (CET) analysis permits to visualize the whole translocation machinery in a native membrane environment as threedimensional reconstruction of 80S ribosomes attached to functional canine pancreatic ER microsomes *in situ*. By using siRNA mediated gene silencing we attempt to identify complexes around Sec61 involved in nascent chain processing like the Oligosaccharyltransferase and Signalpeptidase and protein translocation itself. The latter process includes translocon-associated protein (TRAP), translocon-associated membrane protein (TRAM), Sec62 and the Hsp40 chaperones Sec63 and ERj1 whose J-domain is implied in binding of the Hsp70 chaperone BiP.

RNA inteference combined with *in vitro* and *in vivo* protein transport experiments revealed a substrate specific necessity for certain accessory components in channel gating. This raises the question of the determining substrate features like the overall length and physical or structural sequence characteristics. So far short precursor proteins seem to be associated with a posttranslational transport route in which Sec62 plays an important role. Besides more hydrophobic and less charged signal sequences result in a necessity for Sec63 and BiP. Also parts within the mature domain or rather helical structures in combination with intrinsically disordered hormone domains were recently described to influence translocation efficiency by a so far unknown mechanism.

Resolving the molecular architecture of the ER translocation machinery by CET will be helpful to interpret results of protein transport experiments for a better understanding of translocation mechanisms.

The lysosomal ABC transporter TAPL: Deciphering the translocation mechanism by fluorescence based assays

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Elucidating the molecular mechanism of ABC transporters is of high interest, because of the linkage of many ABC transporters to human diseases. Therefore, we study solute binding and transport by the human lysosomal ABC transporter TAPL (transporter associated with antigen processing like, ABCB9) as a model for symmetric ABC exporters with the advantage to deal with a hydrophilic solute. TAPL is a homodimeric polypeptide transporter which translocates peptides with differing length (6-59 aa) in an ATP dependent manner from the cytosol into the lysosomal lumen.

For biochemical and biophysical studies, we expressed coreTAPL, fused C-terminally with the YFP derivative mVenus, in the methylotrophic yeast *Pichia pastoris*. 45 mg active coreTAPL per liter cell culture were purified, with a K_M value for peptide translocation in the low µM range for coreTAPL in crude membranes as well as reconstituted in proteoliposomes. Choosing fluorescence anisotropy enabled us to measure peptide binding to TAPL, resulting in a K_D value within the same range as the K_M value. To investigate the transport mechanism in more detail, dual-color fluorescence burst analysis (DCFBA), which is based on fluorescence correlation spectroscopy, was established for this transporter. By this technique the transport of fluorophor labeled peptides into single liposomes was shown for the first time. Moreover, we could demonstrate that TAPL is indeed a primary active transporter which translocates peptides unidirectional. Thus, a 1000-fold accumulation of solute by TAPL transport was obtained which is not limited by electrochemical effects, rather by trans-inhibition. Using DCFBA enabled us to decipher TAPL transport kinetics resulting in a k_{cat} of 16 peptides per min. By comparison of this single particle based method with ensemble measurements, DCFBA turned out to be more accurate since only liposomes containing functional TAPL molecules are considered.

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