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Symposium 8: Epithelial Transport of Ions and Metabolites

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Chair: Stefan Bröer, David Cook

A systems biology approach to understanding the role of peptide transporters in biology

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Cell membrane transporters for di- and tripeptides are found in bacteria, yeast, plants, invertebrates and vertebrates including mammals. They mediate the cellular uptake of essentially all possible di- and tripeptides and numerous pharmacologically active peptidomimetics by a proton-dependent electrogenic symport mechanism.

In mammals, the two di-tripeptide transporters that have been characterized in detail are PEPT1 and PEPT2. PEPT1 mediates as a low affinity but high capacity system the influx of peptides from dietary protein digestion in the gut into intestinal epithelial cells whereas PEPT2 as the high affinity subtype transporter is found in a variety of epithelial cells (i.e. lung, mammary gland, choroid plexus) and prominent expression in renal cells with a role in the reabsorption of filtered peptides. For understanding the biological importance of peptide transporters we follow two lines of research; a gene guided approach by comparing the structure and functions of the same proteins in various organisms (*E. coli, C. elegans*, zebrafish, mice, rabbit, humans) and a technology-driven approach by applying transcriptomics, proteomics and metabolomics for phenotype analysis in animals (*C. elegans* and mice) lacking either one of the peptide transporters.

The cloning and functional characterization of *E. coli* peptide transporters with only a low sequence homology (YGDR) but high functional similarity to mammalian PEPT1 provides new insights into structure-function relationship. Carriers from the various species when studied by electrophysiology after expression in *Xenopus* oocytes show very similar features despite marked sequence differences. Gene deletions followed by analysis of phenotypical consequences have been carried out in *C. elegans* and mice. In the nematode, a deletion of the PEPT1 homologous gene provides clues for the role of the intestinal peptide transporter in delivery of bulk qualities of amino acids for growth and development and for a critical crosstalk with the insulin/IGF receptor pathway. There is also a significant effect on stress-resistance of the animals when lacking PEPT1 (Meissner *et al.*, 2004).

A mouse line lacking a functional PEPT2 protein did not show any obvious phenotypical changes despite impaired transport of model peptides in kidney and choroids plexus (Rubio-Aliaga *et al.*, 2003). However, when kidney tissue samples of KO and WT mice were submitted to gene expression analysis by cDNA microarray, proteome analysis by 2D-SDS-PAGE and peptide mass fingerprinting *via* MALDI-TOF-MS and metabolite fingerprinting *via* GC-MS a variety of metabolic alterations were identified. Pathways of amino acid handling showed impairments and also pathways that process keto acids and carbohydrates. Metabolism of cysteine and moreover of cysteinyl-glycine (Cys-Gly), the break-down product of GSH by γ-GT was identified as altered as well. Analysis of urine samples suggests that PEPT2 in renal cells is primarily responsible for uptake of Cys-Gly from the tubular fluids.

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Na^+-H^+ exchange regulatory factors NHERF-1 and NHERF-2: roles in albumin endocytosis in the proximal tubule

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A key function of the renal proximal tubules is to constitutively reabsorb the several grams of albumin that pass across the glomerular barrier per day. This occurs *via* receptor-mediated endocytosis and requires the formation of a macromolecular complex that involves the scavenger receptor megalin, the Cl^- channel ClC-5 and the Na^+-H^+ exchanger isoform 3 (NHE3). The exact composition of the complex and role of these proteins remains, however, unclear. Patients with Dent's disease (genetic defects in ClC-5) and ClC-5 knockout mice have persistent proteinuria, demonstrating an obligate role for ClC-5 in albumin uptake. We have previously shown that the cytosolic C-terminus of ClC-5 interacts with cofilin and Nedd4-2 to regulate albumin uptake (Hryciw *et al.*, 2003; Hryciw *et al.*, 2004). As ClC-5 contains a potential C-terminal PDZ binding motif, we investigated if ClC-5 interacted with the NHERF-1/2 PDZ scaffolds and the role of this interaction in albumin uptake.

For this study, we used the widely accepted model of renal albumin uptake, the opossum kidney (OK) proximal tubule cell line. Western blotting was used to confirm that these cells expressed NHERF1/2 and electron microscopy was used to confirm subcellular localisation. Co-immunoprecipitation was used to determine whether NHERF1/2 bound to ClC-5 in OK cell lysates. GST-fusion proteins were used to determine which PDZ domain of NHERF-2 bound to ClC-5 and maltose-binding fusion proteins used to identify the binding site for NHERF-2 on the C-terminus of ClC-5. Endogenous NHERF-2 and NHERF-1 were silenced by the use of siRNA transfection plasmids and albumin uptake was measured by standard fluorescent methods. Cell surface biotinylation was also used to monitor changes in ClC-5 under these conditions.

Using electron microscopy we demonstrated that OK cells expressed both NHERF-1 and NHERF-2 with NHERF-1 primarily at the microvilli while NHERF-2 was on intracellular membranes consistent with sites of albumin endocytosis. Co-immunoprecipitation in OK cell lysates showed that NHERF-2 but not NHERF-1 bound to ClC-5 *in vivo*. GST-pulldowns revealed that the C-terminus of ClC-5 bound to NHERF-2 and that this interaction occurred via PDZ-2 of NHERF-2. Further, *in vitro* experiments with maltose-binding protein fusions confirmed that NHERF-2 bound to an internal site on the C-terminus of ClC-5 and not to the terminal PDZ binding motif of ClC-5. Functional analysis of this interaction demonstrated that silencing of NHERF-2 significantly reduced albumin uptake, accompanied by a reduction in cell surface expression of ClC-5. This suggests that NHERF-2 plays a key scaffolding role in the endocytic complex. In contrast, when NHERF-1 was silenced, there was an increase in albumin uptake paralleled by an increase in surface levels of ClC-5.

Our data are consistent with a model in which the efficacy of albumin uptake is dependent on the availability of the components of the macromolecular complex. NHERF-1 is typically responsible for restricting the lateral mobility of NHE3 in the membrane and we propose that knockdown of NHERF-1 may increase the availability of NHE3 to the endocytic complex, resulting in more ClC-5 being recruited into the complex thereby increasing albumin uptake. NHERF-2, on the other hand, plays an integral role in the endocytic complex itself.

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Sulphate ions in mammalian physiology: lessons from sulphate transporter knock-out mice

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Inorganic sulphate (SO_4^{2-}) is the fourth most abundant anion in mammalian plasma and is essential for numerous metabolic and cellular processes (Markovich, 2001). In humans and rodents, sulphate reabsorption is mediated by the Na^+-SO_4^{2-} cotransporter (NaS1) located at the brush border membrane, and Sat-1, a SO_4^{2-}-anion exchanger located on the basolateral membranes of proximal tubular cells. Both NaS1 null (*Nas1*$^{-/-}$) and sat-1 null (*sat-1*$^{-/-}$) mice exhibit hyposulphataemia, highlighting the importance of these transporters in maintaining SO_4^{2-} homeostasis. Since *Nas1*$^{-/-}$ mice exhibit reduced growth and liver abnormalities, including hepatomegaly (Dawson *et al*. 2003), we aimed to investigate the hepatic gene expression profile of *Nas1*$^{-/-}$ mice using oligonucleotide microarrays. The mRNA levels of 130 genes with functional roles in metabolism, cell signalling, cell defence, immune response, cell structure, transcription or protein synthesis were altered (66 induced, 64 down-regulated) in *Nas1*$^{-/-}$ mice when compared to *Nas1*$^{+/+}$ mice. The most up-regulated transcript levels in *Nas1*$^{-/-}$ mice were found for the sulphotransferase genes, *Sult3a1* (~500% increase) and *Sult2a2* (100% increase), whereas the metallothionein-1 gene, *Mt1*, was amongst the most down-regulated genes (70% decrease). Several genes involved in lipid metabolism, including *Scd1*, *Acly*, *Gpam*, *Elov16* and *Acs15*, were found to be up-regulated (≥30% increase) in *Nas1*$^{-/-}$ mice. Increased levels of hepatic lipid (~16% increase), serum cholesterol (~20% increase) and LDL (~100% increase), and reduced hepatic glycogen levels (~50% decrease), were found in *Nas1*$^{-/-}$ mice. In addition, *Nas1*$^{-/-}$ mice have an increased hepatotoxicity to acetaminophen (250-mg/kg i.p.) associated with increased serum ALT activity (>300% increase) and reduced hepatic GSH levels (>60% decrease). *Nas1*$^{-/-}$ mice live longer (~25% increase) than their *Nas1*$^{+/+}$ littermates, and have a decreased incidence (0/7 affected, $P<0.025$) of hepatic tumours, when compared to *Nas1*$^{+/+}$ mice (4/7 affected) at 2 years of age. In summary, the hyposulphataemic *Nas1*$^{-/-}$ mouse provides a previously uncharacterised animal model of increased lifespan and altered hepatic metabolism.

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Regulation of epithelial Na^+ channels

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Body sodium and fluid homeostasis is largely determined by the activity of Na^+ transport proteins that are expressed in the kidney and the GI tract. Among these, the epithelial Na^+ channels (ENaC) play an important role in Na^+ transport by the distal kidney and the distal colon. This is evident from the observations that gain of function mutations of ENaC, as occur in Liddle's syndrome, cause hyperabsorption of Na^+ in the distal collecting duct of the kidney, leading to salt-sensitive hypertension, whereas loss of function mutations, as occur in pseudohypoaldosteronism type I, cause hypotension.

It is well established that the activity of ENaC is tightly regulated. The most important regulators of ENaC are aldosterone and arginine vasopressin which increase activity of the channel during extracellular fluid volume depletion. ENaC activity is also regulated by the concentration of Na^+ in the luminal fluid facing the apical membrane. This regulation is mediated by cytosolic Na^+ concentration which inactivates the channels by a mechanism involving the G protein, G_o, and an ubiquitin-protein ligase, either Nedd4 or Nedd4-2, which ubiquitinates the channels and triggers their endocytosis (Dinudom *et al.*, 1998).

Recent studies have suggested that, in addition to its genomic effects, aldosterone may activate ENaC via a mechanism that involves the serum- and glucocorticoid-stimulated kinase, Sgk. This kinase is believed to phosphorylate Nedd4-2 so as to prevent it binding the channels. Contrary to this belief, we have found in whole-cell patch-clamp studies on mouse mandibular duct cells that inclusion of recombinant, constitutively-active Sgk in the pipette solution does not prevent inactivation of ENaC by increased intracellular Na^+. We found instead that ENaC activity is increased by another protein kinase, the G-protein coupled receptor kinase, Grk2. Our experiments in salivary duct cells further showed that Grk2 phosphorylates the β subunit of ENaC and that this phosphorylation prevents Na^+ feedback inhibition of the channel by preventing the binding of Nedd4/Nedd4-2 to channel (Dinudom *et al.*, 2004)). We then investigated the regulation of ENaC by Grk2 in Fisher Rat Thyroid (FRT) cells, a model epithelium. We found that expression of Grk2 in FRT cells expressing ENaC caused a two-fold increase in the activity of ENaC compared to FRT cells in which ENaC alone is expressed. Conversely transfection of siRNA directed against Grk2 into FRT cells expressing ENaC inhibited ENaC activity. Interestingly, the mechanism by which Grk2 regulates ENaC in FRT cells differed from the mechanism in salivary duct cells. We found in FRT cells that a kinase-dead mutant of Grk2 activated ENaC in a same manner as wild-type Grk2, and that activation of the channels by Grk2 was due to binding of α-subunits of the Gq,11 family of G proteins by the Regulatory G-protein Signalling (RGS) domain of Grk2. The exact identity of the G protein that inhibits ENaC in FRT cells, and the mechanism by which it does so, are currently being investigated.

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Disorders of neutral amino acid resorption in epithelial cells

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Recent successes in the molecular cloning and identification of apical neutral amino acid transporters have shed a new light on inherited neutral amino acidurias, such as Hartnup disorder and iminoglycinuria. Hartnup disorder is caused by mutations in the neutral amino acid transporter B^0AT1 (SLC6A19) (Kleta *et al.*, 2004; Seow *et al.*, 2004). The transporter is found in kidney and intestine, where it is involved in the resorption of all neutral amino acids (Bröer *et al.*, 2004). It belongs to the SLC6 family, comprising transporters for neurotransmitters, osmolytes and creatine. B^0AT1 transports neutral amino acids together with 1 Na^+-ion but in contrast to other members of the SLC6 family is chloride independent. The SLC6 family also contains a number of 'orphan transporters' the physiological function of which has remained elusive. Identification of SLC6A19 as a Na^+-dependent amino acid transporter suggested that orphan neurotransmitter transporters might in fact be amino acid transporters. SLC6A20 turned out to be the long-sought IMINO system, a Na^+ and Cl^--dependent proline transporter (Kowalczuk *et al.*, 2005). SLC6A20 is highly expressed in the kidney and intestine and may play a role in iminoglycinuria, a disorder characterised by hypersecretion of proline and glycine in the urine. Although SLC6A20 transports proline but not glycine, it is considered a candidate for iminoglycinuria because excess of proline in the proximal tubule could compete for glycine uptake by the proline/glycine transporter PAT1 (SLC36A1). Further functional analysis of SLC6 orphan transporters demonstrated that SLC6A15 is a transporter for large neutral amino acids plus proline. The transporter is highly expressed in the brain and kidney. In the kidney it may serve as a high-affinity back-up transporter for selected amino acids in the distal parts of the proximal tubule. Functionally SLC6A15 is related to B^0AT1 and was hence named B^0AT2. It transports neutral amino acids together with 1 Na^+ and is chloride independent. In summary, a new family of Na^+-dependent amino acid transporters has been identified, the members of which are involved in the transport of amino acids in epithelial cells and the nervous system.

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