AuPS/ASB Meeting - Adelaide 2010

Free communications: Membrane transport

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Chair: Brett Cromer

Hot and cold running ion pumps

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Crystal structures of the Na^+,K^+-ATPase from both a warm-blooded animal (pig) (Morth *et al.*, 2007) and a cold-blooded animal (shark) (Shinoda *et al.*, 2009) have recently been published. Although the structures of the enzyme from these two species appear very similar, we have discovered major differences in their kinetics,

From investigations of K^+ occlusion by the phosphoenzyme intermediate of the Na^+,K^+-ATPase and its K^+-stimulated dephosphorylation *via* stopped-flow fluorimetry we have found that, whereas both enzymes appear to have similar rate constants of K^+-occlusion of 370-380 s^{-1}, the two enzymes have very different rate constants of dephosphorylation. For the shark enzyme, dephosphorylation proceeds with a rate constant of only 48 (±2) s^{-1} at 24°C and pH 7.4, whereas for the pig enzyme the rate constant is >365 s^{-1}. The dephosphorylation is, thus, the major rate-determining step of the shark enzyme under saturating concentrations of all substrates. For the pig enzyme, on the other hand, the major rate-determining step under the same conditions is the conformational E2-E1 transition of unphosphorylated enzyme and its associated K^+ release to the cytoplasm. The differences in rate constant of the dephosphorylation reaction of the two enzymes are paralleled by compensating changes to the rate constant for the E2-E1 transition (Kahlid *et al.*, 2010), which explains why the differences in the enzymes' kinetic behaviour have not previously been identified in steady-state kinetic studies of the enzyme's entire reaction cycle.

In mammals, heat generation by the Na^+,K^+-ATPase as a by-product of ion pumping is thought to make approximately a 12% contribution towards the maintenance of body temperature. Therefore, the possibility should be investigated whether under physiological conditions the differences in Na^+,K^+-ATPase kinetics, which we have identified between a warm- and a cold-blooded animal, could in part be responsible for the higher body temperature of warm-blooded animals.

Khalid M, Cornelius F, Clarke RJ. (2010) *Biophysical Journal* **98**: 2290.

Morth JP, Pedersen BP, Toustrup-Jensen MS, Sørensen TL, Petersen J, Andersen JP, Vilsen B, Nissen P. (2007) *Nature* **450**: 1043-9.

Shinoda T, Ogawa H, Cornelius F, Toyoshima C (2009) *Nature* **459**: 446-50.

Mapping the ion translocation pathway in the glutamine transporter SNAT3

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The glutamine transporter SNAT3 plays a pivotal role in the release of glutamine from brain astrocytes, the uptake and release of glutamine from hepatocytes and the uptake of glutamine in epithelial cells of the kidney. SNAT3 has a complex mechanism involving the cotransport of Na+ and the antiport of protons. In addition substrate-dependent and independent ion-conductances are observed. In order to understand the mechanism of the transporter in more detail, we explored the ion translocation pathway by experimental and theoretical approaches.

Until recently, database searches have not revealed homology to any known transporter structure. Comparison of hydropathy plots of the hydantoin permease Mhp1 with the hydropathy plot of SNAT3, however, revealed a significant similarity allowing us to generate a homology model of the transporter. The SNAT3 model suggests an overall topology that is similar to the Mhp1 structure. In this model helix 1 and 6 are lining the translocation pore and a putative Na+-binding site was identified involving residues asparagine 76, methionine 79 in helix 1 and valine 377 and threonine 380 in helix 8.

In line with this model we previously found that mutation of threonine 380 and of asparagine 76 changed the permeability properties of the transporter. Mutation of valine 377 to larger hydrophobic residues resulted in transporters with little activity. Mutation of valine 377 to leucine, however resulted in an active transporter molecule. This conservative exchange abolished the substrate-dependent conductance at pH 8.4, but not at pH 7.4. This is opposite to the change observed in a threonine 380 to alanine substitution, where the conductance at pH 7.4 is abolished. This confirms previous observations that the transporter has different conductance properties at different pH-values. As shown previously mutation of asparagine 76 to aspartate introduces a chloride-conductance that is tightly controlled by pH. We combined this mutation with a truncation of the C-terminal histidine in order to explore whether this residue changes the pH-dependence of the conductance. In this double mutant the anion conductance was still pH-gated, but showed little rectification compared to the N76D mutant.

The results suggest that asparagine 76, valine 377 and threonine 380 line the translocation pore of the glutamine transporter SNAT3. All three residues significantly alter the conductance of the transporter which is consistent with their suggested position close to the substrate and ion binding site of the transporter.

Glutamate transporter loss-of-function mutations cause human dicarboxylic aminoaciduria

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Excitatory amino acid transporter 3 (EAAT3) has been shown to be the major epithelial transporter of glutamate and aspartate in the kidney and intestine in rodents. EAAT3 is also found in the brain where it is responsible for clearing the major excitatory neurotransmitter glutamate from the extracellular space. In this study we describe two mutations in EAAT3 that cause human dicarboxylic aminoaciduria, an autosomal recessive disorder of urinary glutamate and aspartate transport that has been associated with mental retardation. The single point mutation R445W causes an increase in substrate affinity and a significant reduction in transport and cell-surface expression. The non-functional deletion mutation, I395del, exhibits negligable cell surface expression. Our study provides definitive evidence that EAAT3 is the major renal transporter of glutamate and aspartate in humans and implicates EAAT3 in the pathogenesis of neurological disorders.

Determining the physiological state of a membrane protein: investigating the P-gycoprotein crystal structure

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P-glycoprotein is one of the major multi-drug transporters in humans. Expressed primarily in barrier tissues, P-glycoprotein (P-gp) exports a wide range of substrates and is a cellular first defence mechanism against xenotoxins. P-gp is a member of the ABC transporter superfamily, utilising the energy released from Mg^{2+} catalysed ATP binding and hydrolysis between the two nucleotide binding domains (NBDs) to induce a conformational change across the two transmembrane domains (TMDs). This conformational change drives the translocation of substrate across the membrane. Recent studies have crystallized homologues of P-gp in an ATP bound conformation (Dawson & Locher, 2006). Here the two TMDs are in an outwardly splayed conformation and are connected to the tightly coupled NBDs which form a characteristic nucleotide sandwich dimer, with nucleotide analogues sandwiched between them. In 2009 a medium resolution crystal structure of murine P-gp was solved in an alternate conformation, with inwardly facing TMDs and a wide gap separating the two NBDs (Aller *et al.*, 2009). The large conformational differences between the structures gave rise to questions as to whether this nucleotide-free structure represents an alternative conformation in the transport cycle, or whether this structure arose as a crystallization artefact. Here we address these questions *via* two sets of molecular dynamics (MD) simulations: one of P-gp in a cholesterol enriched POPC bilayer, and the second in the crystallographic mother liquor.

Simulations of the crystallographic mother liquor demonstrate that the nucleotide-free structure of P-gp is stabilized primarily by protein-protein contacts in the unit cell crystal lattice. Removal of these contacts is sufficient to destabilize the P-gp crystal structure. The instability arises from an attractive potential between the NBDs, resulting in a salt bridge between D558 and H1228. Experimentally, cholesterol is necessary for the functional activity of P-gp, however MD simulations demonstrate that the presence of cholesterol alone in a POPC membrane is not sufficient to stabilize the P-glycoprotein crystal structure. We are identifying the underlying factors producing the instability of the P-gp crystal structure and examining the effects of different ionic solutions on structural stability of P-gp.

Dawson RJ. Locher KP. (2006) Structure of a bacterial multidrug ABC transporter. *Nature* **443:** 180-5.

Aller SG, Yu J, Ward A, Weng Y, Chittaboina S, Zhuo R, Harrell PM, Trinh YT, Zhang Q, Urbatsch IL, Chang G. (2009) Structure of P-Glycoprotein reveals a molecular basis for poly-specific drug binding. *Science* **323:** 1718-1722.

Acquisition and dissemination of multidrug resistance in cancer *via* microparticles

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Background: A major obstacle to the successful treatment of cancer is the acquisition of multi-drug resistance (MDR), a unique drug resistance in which tumors display cross-resistance to a wide range of unrelated drugs. MDR in cancer cells is typically caused by overexpression of efflux transporters constituting the ATP Binding Cassette Superfamily, of which P-glycoprotein (P-gp) and the Multidrug Resistance Associated protein (MRP1) are the most prominent. These transporters with their remarkable efflux capacity maintain sublethal intracellular drug concentrations, effectively rendering cancer cells treatment unresponsive.

The cellular regulation of these transporters was initially known to occur either pre- or post-transcriptionally. However, we recently discovered a novel non-genetic pathway of MDR acquisition in which microparticles (MPs) provide the vehicle for intercellular transfer of functional P-gp from multidrug resistant donor cells to drug sensitive recipient cancer cells.

Objective: The current study investigated the role of regulatory nucleic acids contained within the MP cargo in the acquisition and regulation of traits in recipient cells.

Methods and Results: MPs isolated from donor MDR cells, VLB_{100} (MDR^+) were co-cultured with drug-sensitive CCRF-CEM (MDR^-) cells for 4 h to allow for MP binding and P-gp transfer. The MP localisation on the cell surface and internalisation in recipient cells was visualised using confocal imaging. The acquisition of fully functional P-gp following MP transfer was established by flow cytometry following direct immunolabeling and by assessing Rhodamine 123 dye exclusion.

Using quantitative RT- PCR the MPs were observed to incorporate and transfer transporter transcripts including those for *ABCB1* and *ABCC1*. We observed a significant 218 fold increase and 30% decrease in *ABCB1* and *ABCC1* mRNA respectively in recipient cells following MP coculture, the resulting phenotype being reflective of that observed in the donor cell population. In examining a potential contribution by regulatory nucleic acids towards this differential profile in recipient cells, we identified *micro-RNAs* as key mediators in this pathway.

Conclusions: These findings serve to further our understanding of the intercellular pathway regulating trait acquisition in cancer cell populations and provide a basis for the development of alternative treatment strategies targeting the emergence of MDR in cancer.

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Microparticles confer multidrug resistance in breast cancer

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Rationale: Despite decades of using cytotoxic drugs, the incidence of cancer relapse is still prevalent. One mechanism contributing to this is multidrug resistance (MDR). MDR occurs when a tumour cell becomes resistant to a number of structurally and functionally unrelated cytotoxic drugs following exposure to a single agent, resulting in a tumour cell that no longer responds to therapy even if drugs of different classes are used. One mechanism contributing to the emergence of MDR is the over-expression of efflux transporters, in particular, P-glycoprotein (P-gp). This transporter maintains a sublethal intracellular drug concentration, effectively rendering cancer cells treatment unresponsive.

Microparticles (MP) are plasma membrane-derived vesicles 0.1-1μm in diameter released by blebbing from various cell types. As such, MP are made up of fragments of the parent cell's plasma membrane and contain its cell surface proteins and cytoplasmic material (Mack *et al.*, 2000). We have previously shown in human acute lymphoblastic leukaemia cells that MP are shed spontaneously from drug-resistant cells, are capable of binding to drug-sensitive cells, and in doing so, transfer functional P-gp to drug-sensitive cells, conferring MDR (Bebawy *et al.*, 2009). Here we demonstrate that this also occurs in solid tumour cells such as the MCF-7 breast cancer cell line. Specifically, we demonstrate that the multidrug resistant MCF-7/Dx cell line spontaneously sheds MP and that these MP carry the multidrug transporter. Furthermore, we demonstrate that MP derived from these cells also transfer transporter transcript and regulatory nucleic acids (microRNAs).

Methods: To determine if MP are shed from drug-resistant cells, the isolated MP population was labelled with FITC-annexin V and analysed by flow cytometry. To determine if MP transferred P-gp to drug-sensitive cells, a co-cultured population of drug-sensitive cells with MP were labelled with FITC-anti-P-gp and analysed by flow cytometry. Transferred P-gp was deemed functional by the daunorubicin dye exclusion assay. Quantitative real time PCR was used to determine the levels of expression of transcripts and microRNAs in donor cells, MP and recipient cells following MP coculture.

Results: Our results show that the multidrug resistant MCF-7/Dx cell line spontaneously sheds MP and that these MP are capable of carrying functional P-gp to the drug-sensitive cell line, rendering recipient cells MDR. We have also determined that MP are capable of incorporating and transferring transporter transcripts and microRNAs, leading to changes in the recipient cells which are reflective of the donor cell phenotype.

Conclusion: The significance of this study is in the elucidation of a non-genetic pathway for the acquisition of P-gp mediated MDR in cancer. This has the potential for translation into clinical outcomes as it provides another avenue by which resistance to chemotherapy can be addressed.

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Mack, M, Kleinschmidt, A, Bruhl, H, Klier, C, Nelson, P.J, Cihak, J, Plachy, J, Stangassinger, M, Erfle, V & Schlondorff, D. (2000) Transfer of the chemokine receptor CCR5 between cells by membrane-derived microparticles: a mechanism for cellular human immunodeficiency virus 1 infection, *Nature Medicine* **6,** 769-75.

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