AuPS Meeting - Melbourne 2008

Symposium: Regulation of Trafficking of Membrane Transporters by Intracellular Signaling Systems

Monday 1 December 2008 − King Theatre

Chair: David Cook

New insights into the formation and function of caveolae

R.G. Parton, Institute for Molecular Bioscience, University of Queensland, Brisbane, Queensland 4072, Australia. (Introduced by D.I. Cook)

Caveolae are flask-shaped pits of the cell surface, an abundant morphological feature of many mammalian cells. Caveolins, the major proteins of caveolae, play a crucial role in the formation of caveolae. Mutations in caveolins are associated with breast cancer and with a number of muscle diseases, including limb girdle muscular dystrophy. We have studied how caveolin-lipid interactions generate the unique architecture of the caveolar domain by studying caveola formation in caveolin-null fibroblasts by light and electron microscopy upon expression of mammalian caveolins (caveolin-1 and caveolin-3), specific mutants of these proteins, or non-mammalian caveolins (from *C. elegans* and the honey bee, *Apis mellifera*) (Kirkham *et al.*, 2008). These studies are being combined with high-resolution analysis of caveolae by electron tomography of isolated plasma membrane sheets and fast frozen cells after freeze substitution (Richter *et al.*, 2008). These studies have adefined the fine structure of caveolae and shown that caveolae are linked by actin filaments to form an interconnected network.

Our recent studies have identified an additional level of regulation of caveola formation. Using a systematic proteomic analysis we have identified a family of evolutionary-conserved coat proteins that work together with caveolins to regulate caveola formation (Hill *et al.*, 2008). Reduction of PTRF-cavin levels in cultured cells causes loss of morphological caveolae with caveolin being released into the bulk membrane. Photobleaching studies show that caveolin is now freely mobile in the plasma membrane in contrast to the immobility of caveolin in control cells. Reduction of PTRF-cavin levels also allows caveolin to be degraded more rapidly. To examine the role of PTRF-cavin in a whole animal system we have utilised the zebrafish embryo as a model organism. Formation of caveolae in the zebrafish correlates temporally with expression of PTRF-cavin in the notochord during zebrafish development. Consistent with this, knockdown of zebrafish PTRF-cavin causes a reduction in the density of caveolae in the notochord (Hill *et al.*, 2008).

We propose that PTRF-cavin family members regulate association of caveolin with caveolae and identify a cellular mechanism to regulate caveolar and non-caveolar functions of caveolins. In addition, we postulate that PTRF-cavin family members can act as molecular sensors of changes in caveola structure or composition.

Hill MM, Bastiani M, Luetterforst R, Kirkham M, Kirkham A, Nixon SJ, Walser P, Abankwa D, Oorschot VM, Martin S, Hancock JF, Parton RG. (2008) *Cell*, **132:** 113-24.

Kirkham M, Nixon SJ, Howes MT, Abi-Rached L, Wakeham DE, Hanzal-Bayer M, Ferguson C, Hill MM, Fernandez-Rojo M, Brown DA, Hancock JF, Brodsky FM, Parton RG. (2008) *Journal of Cell Science*, **121:** 2075-86.

Richter T, Floetenmeyer M, Ferguson C, Galea J, Goh J, Lindsay MR, Morgan GP, Marsh BJ, Parton RG. (2008) *Traffic* **9:** 893-909.

Regulation of receptors, transporters and ion channels by the Nedd4 family of ubiquitin ligases

S. Kumar,[1,6] N. Boase,[1] B. Yang,[2] R. Daly,[3] S. Townley,[1] A. Dinudom,[4] D.I. Cook,[4] P. Poronnik[5] and G.Y. Rychkov,[6] [1]Hanson Institute, IMVS, SA 5000, Australia, [2]Department of Obsterics and Gynecology, University of Iowa, IA 52242, USA, [3]Garvan Institute, Sydney, NSW 2010, Australia, [4]Dicipline of Physiology, Faculty of Medicine, University of Sydney, NSW 2006, Australia, [5]School of Biomedical Sciences, University of Queensland, QL 4067, Australia and [6]Discipline of Physiology, School of Molecular and Biomedical Sciences, University of Adelaide, SA 5005, Australia.

Protein modification by ubiquination controls a number of cellular signalling processes, including protein trafficking and turnover. The process of ubiquitination requires a number of enzymes that include a ubiquitin activating enzyme (E1), ubiquitin conjugating enzymes (E2s) and ubiquitin protein ligases (E3s) (reviewed in Ciechanover, 2005). The specificity of the ubiquitination system is determined by E3s, as they are involved in the transfer of ubiquitin from an E2 to a specific substrate. There are two main types of E3s: the RING type, that act as a large complex containing several proteins, and the HECT type, that act as single chain enzymes that first accept the ubiquitin and then transfer it to the substrate protein. Among HECT ligases, the members of Nedd4 family are characterized by a unique modular domain structure containing a C2 domain, 2-4 WW domains and a C-terminal HECT domain (reviewed in Shearwin-Whyatt *et al.*, 2006). Studies in yeast and mammals suggest that many members of this family are involved in the control of trafficking and endocytosis of membrane associated proteins, including ion channels, transporters and receptors (Ciechanover, 2005). We are studying several members of the Nedd4 family and their physiological functions using biochemical, cellular, physiological and gene knockout (KO) approaches. Our recent data suggest that KO of Nedd4 results in growth retardation and perinatal lethality in mice (Cao *et al.*, 2008). The growth phenotype is due to reduced IGF-I signalling caused by reduced levels of IGF-1R on cell surface. This appears to be caused by increased levels of the adaptor protein Grb10, a known inhibitor of IGF-I signalling (Cao *et al.*, 2008). Thus, Nedd4 is an E3 that controls animal growth. Knockout of Nedd4-2 also resulted in perinatal lethality in most animals, but a few homozygous animals survive for up to 3 weeks. Unlike Nedd4 KOs, Nedd4-2 deficient animals seem to develop normally, but die due to a collapsed lung phenotype. The survivors develop lung infections and die due to severe inflammation of the lungs. These phenotypes are consistent with increased activity of the epithelial sodium channel (ENaC) in the Nedd4-2 KO mice. ENaC has been shown previously to be regulated by Nedd4-2-mediated ubiquitination *in vitro* (Harvey *et al.*, 2001; Kamynina *et al.*, 2001; Fotia *et al.*, 2003). In support of this prediction, we found increased cell surface expression of ENaC in the lung and kidney epithelia. Further studies to delineate the *in vivo* function of Nedd4-2 in regulating ENaC and other channels are currently underway.

Cao XR, Lill NL, Boase N, Shi PP, Croucher D, Shan H, Qu J, Sweezer EM, Place T, Kirby PA, Daly RJ, Kumar S, Yang B. (2008) *Science Signaling*. **In press.**

Ciechanover A (2005) *Nature Reviews Molecular Cell Biology* 6: 79-86.

Fotia A, Dinudom A, Shearwin KE, Koch J-P, Korbmacher C, Cook DI, Kumar S. (2003) *FASEB Journal* **17:** 70-2.

Harvey KF, Dinudom A, Cook DI, Kumar S. (2001) *Journal of Biological Chemistry* **276:** 8597-601.

Kamynina, E, Debonneville, C, Bens, M, Vandewalle, A, Staub, O. (2001) *FASEB Journal* **15:** 204–14.

Shearwin-Whyatt L, Dalton H, Foot N, Kumar S. (2006) *BioEssays* **28:** 617-28.

The role of phospholipids in controlling GLUT4 exocytosis

W.E. Hughes, Garvan Institute, 384 Victoria Street, Darlinghurst, NSW 2010, Australia. (Introduced by Anuwat Dinudom)

Class I phosphatidylinositol 3-kinases (PI3K) generating phosphatidylinositol(3,4,5)trisphosphate, PtdIns(3,4,5)P_3, have a well established role in cellular signalling. This is particularly true in the muscle cell insulin signalling cascade that regulates the uptake and metabolism of glucose to control blood glucose homeostasis. Insulin via the insulin receptor triggers a cascade of protein phosphorylation and translocation events leading to a localized accumulation of PtdIns(3,4,5)P_3 in the plasma membrane. This in turn allows a series of translocation and phosphorylation events that lead to the insertion of the GLUT4 glucose transporter into the plasma membrane.

We have recently established a role for an additional class of PI3K and 3-phosphorylated lipid in this process. Knockdown of the α isoform of class II PI3K (PI3KC2α) in myoblasts reduced insulin-stimulated glucose uptake due to reduced translocation of GLUT4 to the plasma membrane. PI3KC2α appears to represent a component of an novel and independent signalling cascade as the 'classical' class I PI3K cascade appeared unaffected in the knockdown cells. Analysis of PtdIns species identified a lack of insulin-stimulated accumulation of PtdIns3P in the PI3KC2α knockdown clones identifying the *in vivo* product of PI3KC2α, which was previously unclear, and that this lipid plays a role in GLUT4 translocation. Using a variety of state-of-the-art live cell imaging techniques we have endeavoured to identify the molecular step regulated by PI3KC2α generated PtdIns3P.

The physiological roles of sulfate transporters

D. Markovich, School of Biomedical Sciences, University of Queensland, St. Lucia, QLD 4072, Australia.

Sulfate (SO_4^{2-}) is an essential anion involved in many cellular processes. Cells obtain sulfate primarily *via* sulfate transporters that mediate the movement of sulfate across their plasma membranes. Renal sulfate transporters (NaS1 and Sat1) are responsible for regulating serum sulfate levels and sulfate homeostasis. Despite their important roles in sulfate homeostasis, very little is known about the structural identities and sorting mechanisms that control NaS1 and Sat1 expression in cells. NaS1 encodes a Na^+-sulfate cotransporter expressed in renal and intestinal epithelial cells. It contains 595 amino acids with 13 putative transmembrane domains. In the renal proximal tubule, NaS1 is localized to the apical (brush-border) membrane, for which the sorting mechanisms have not been determined. The Sat-1 transporter mediates sulfate/bicarbonate/oxalate anion exchange *in vivo* at the basolateral membrane of the kidney proximal tubule. The aims of this study were to biochemically characterize the NaS1 and Sat1 proteins and identify the sorting mechanisms responsible for their membrane trafficking. For structural characterization of NaS1, a C-terminally hexahistidyl tagged NaS1 construct (NaS1-His) was expressed and metabolically labeled in *Xenopus* oocytes. SDS-PAGE revealed NaS1-His as two bands (50 kD and 60 kD). Treatment with endoglycosidase H led to a small (1–2 KD) shift in the 50 kD (but not the 60kD) band, suggesting complex glycosylation and the presence of a single glycosylation motif. Mutagenesis of a putative N-glycosylation site (N591S) produced a single band (50 kD) that was not shifted by endoglycosidase H, suggestive of a true glycosylation site. Transient transfection of EGFP/NaS1 in renal epithelial cells (OK, LLC-PK1 and MDCK) demonstrated apical membrane expression, which was not affected by tunicamycin. Transfection of the EGFP/NaSi-1 N591S glycosylation mutant still led to apical expression, suggesting that apical sorting was independent of the glycosylation of this site. Treatment with cholesterol depleting compounds (lovastatin and methyl-β-cyclodextrin) was also unable to disrupt apical sorting, suggesting that NaS1 apical trafficking may be independent of membrane lipid rafts. NaS1-His proteins when analyzed by BN-PAGE appeared as a single complex. Dissociation revealed one additional band, indicating a dimeric structure of the complex. Our data demonstrate that NaS1 most likely forms a dimeric protein which is glycosylated at N591, whose sorting to the apical membrane of renal epithelial cells may be independent of lipid rafts and glycosylation. Since Sat1 is not endogenously expressed in any renal cells line, here we transfected Sat1 into two renal cell lines (MDCK and LLC-PK1 cells), which sorted Sat1 exclusively, to the basolateral membrane, as observed *in vivo*. To identify possible sorting determinants, truncations of the Sat1 cytoplasmic C-terminus were generated, fused to enhanced green fluorescence protein (EGFP) or the human IL2R α-chain (Tac) protein and both fusion constructs were transiently transfected into MDCK cells. Confocal microscopy revealed the removal of the last three residues on the Sat1 C-terminus, a putative PDZ domain, had no effect on the basolateral sorting in MDCK cells, nor an effect on sulfate transport in *Xenopus* oocytes. Removal of the last 30 residues led to an intracellular expression for the GFP fusion protein and an apical expression for the Tac fusion protein, suggesting a possible sorting motif lies between the last 3 and 30 residues of the Sat1 C-terminus. Elimination of a dileucine motif at position 677/678 resulted in the loss of basolateral sorting, suggesting this motif is required for Sat1 targeting to the basolateral membrane. This post-translational mechanism may be important for the regulation of sulfate reabsorption by Sat1 in the kidney proximal tubule. Recently, single nucleotide polymorphisms (SNPs) have been identified in the coding regions of human NaS1 and Sat1 genes. These SNPs and their effects on protein sorting may have relevance to the regulation of serum sulfate levels in humans.

Funding support: NHMRC and ARC grants.