



Mark RIDER, Member

Louis HUE, Emeritus Professor
Yu-Chiang LAI, Assistant Member
Didier VERTOMMEN, Assistant Member
Laurent BULTOT, Graduate Student
Yang LIU, Graduate Student
Catheline PLAIDEAU, Graduate Student
Marie-Agnès GUEUNING, Technician (half-time)
Nusrat HUSSAIN, Technician
Liliane MAISIN, Technician (half-time)
Steve CALBERSON, Technician
Vivien O'CONNOR, Secretary (half-time)
Freddy ABRASSART, Technical Staff (part-time)

SIGNAL TRANSDUCTION AND PROTEIN PHOSPHORYLATION

Research in our group focuses on the role of protein phosphorylation in the control of cell function, with special emphasis on the control of metabolism by nutrients, hormones and various stresses. As a model system, we studied 6-phosphofructo-2-kinase (PFK2)/fructose-2,6-bisphosphatase (FBPase-2) and the control of its activity by various protein kinases. This bifunctional enzyme catalyzes the synthesis and degradation of fructose 2,6-bisphosphate, a potent stimulator of glycolysis that was discovered in this Institute by Van Schaftingen, Hue and Hers in 1980. Our previous work on the regulation of heart PFK-2 activity by phosphorylation led to the study of the insulin and AMP-activated protein kinase (AMPK) signalling cascades, which are now our main research interests.

INSULIN SIGNALLING

L. Toussaint, D. Vertommen, L. Hue, M.H. Rider, in collaboration with B. Hemmings, Basel and D. Alessi, Dundee

Activation of heart PFK-2 by insulin

Insulin stimulates heart glycolysis by increasing glucose transport and by activating PFK-2. This in turn leads to a rise in fructose 2,6-bisphosphate (1). Recombinant heart PFK-2 isozyme is a substrate of protein kinases of the insulin signalling pathways, such as protein

kinase B (PKB), also known as Akt, which is believed to mediate most metabolic effects of insulin. Our previous studies suggested that PFK-2 activation by insulin was dependent on PDK1, a protein kinase upstream of insulin-stimulated protein kinases (Fig. 1). We examined the role of SGK3 (serum- and glucocorticoid-regulated protein kinase-3) in insulin-stimulated PFK-2 activation. Co-transfection of HEK 293T cells with SGK3 siRNA did not affect PFK-2 activation, suggesting that this protein kinase is not required for PFK-2 activation by insulin. Therefore, we re-evaluated the role of PKB. In HEK293 cells co-transfected with heart PFK-2 and total PKB siRNA, insulin-induced PFK-2 activation was abrogated. Also in cardiomyocytes treated with the Akti-

1/2 PKB α / β -selective inhibitor, PFK-2 activation by insulin was prevented. Our results with PKB β -knockout mice indicated that this isoform is not required for heart PFK-2 activation by insulin. Moreover, PKB α silencing using the siRNA approach indicated that this PKB isoform is likely to be responsible for heart PFK-2 activation by insulin (manuscript under revision).

Figure 1 summarizes the protein kinases from different signalling pathways that converge to phosphorylate heart PFK-2.

collaboration with P. Courtoy, de Duve Institute, S. Horman, UCL, Brussels, D.G. Hardie, Dundee, K. Sakamoto, Dundee, J. Jenssen, Oslo, K. Storey, Ottawa and B. Viollet, Paris

The AMP-activated protein kinase (AMPK) acts as a sensor of cellular energy status. AMPK is activated by an increase in the AMP/ATP ratio as it occurs during hypoxia or after exposure of cells to inhibitors of the mitochondrial respiratory chain, such as oligomycin. In certain cells, AMPK can also be activated by 5-aminoimidazole-4-carboxamide (AICA)-riboside, which enters cells to be phos-

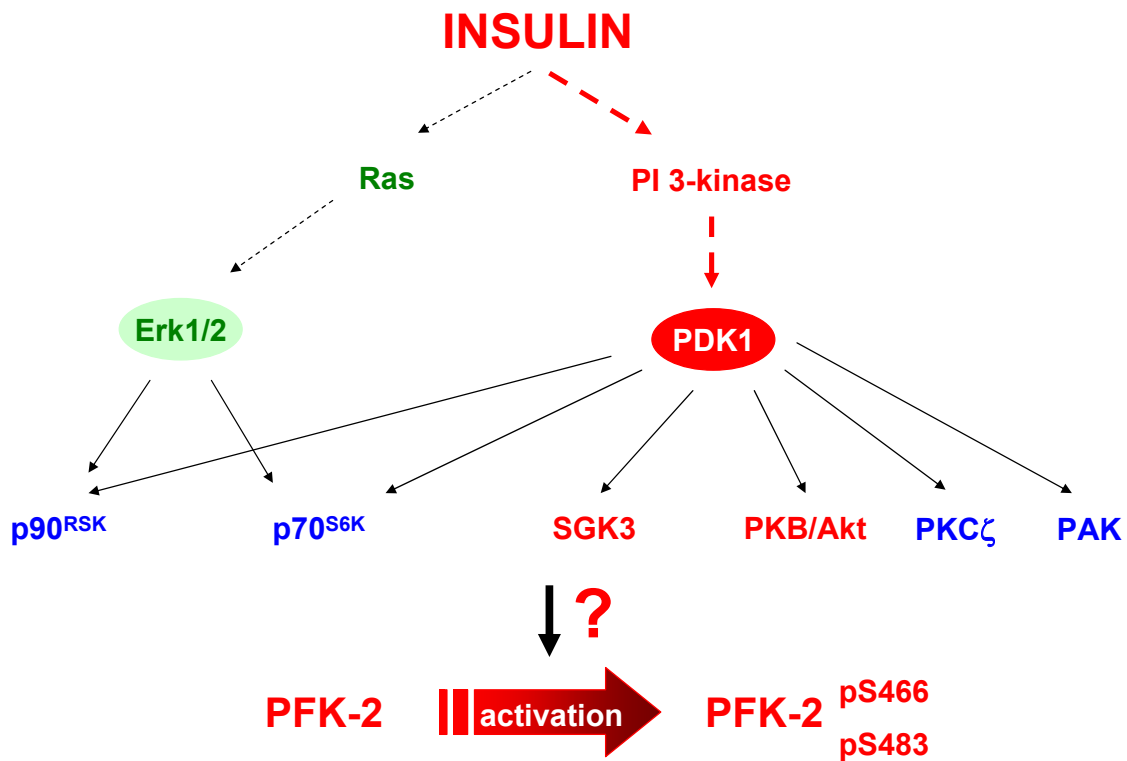


Figure 1. Protein kinases of signalling pathways that converge on heart PFK-2. The numbering of residues refers to the bovine H1 isoform.

AMP-ACTIVATED PROTEIN KINASE

B. Sid, D. Vertommen, L. Miranda, L. Bultot, Y. Liu, C. Plaideau, L. Hue, M.H. Rider, in

phorylated to ZMP, an analogue of AMP, or by the A769662 Abbott compound. For full activation, AMPK α -subunit phosphorylation of its activation loop Thr172 by LKB1 (Peutz-Jegher's protein) or calmodulin-dependent protein kinase kinase- β (CaMKK β) is required. The role of AMPK in the cell is to maintain ATP by stimulating ATP-producing pathways

and inhibiting energy-consuming biosynthetic pathways. We contributed to the development of this concept by the discovery of new substrates of AMPK. We demonstrated that the activation of PFK-2 by AMPK participates in the stimulation of heart glycolysis by ischaemia (2). Similarly, we showed that phosphorylation-induced inactivation of elongation factor 2 (eEF2) by AMPK explains at least in part the inhibition of protein synthesis by anoxia (ref. 3 and see below).

AMPK activation in animals adapting to extreme energy stress

Long-term survival of oxygen deprivation by animals with well-developed anoxia tolerance depends on multiple biochemical adaptations including strong metabolic rate depression. We investigated whether AMPK

could play a regulatory role in the suppression of protein synthesis that occurs when turtles experience anoxic conditions. AMPK activity and the phosphorylation state of ribosomal translation factors (see Fig. 2) were measured in liver, heart, red muscle and white muscle of red-eared slider turtles (*Trachemys scripta elegans*) subjected to 24 h of anoxic submergence. AMPK activity increased 2-fold in white muscle of anoxic turtles compared with aerobic controls but remained unchanged in liver and red muscle, whereas in heart AMPK activity decreased by 40% during anoxia. Eukaryotic elongation factor-2 phosphorylation increased 6- to 8-fold in red and white muscles from anoxic animals but was unchanged in liver and heart. The phosphorylation state of the activating Thr389 site of p70 ribosomal protein S6 kinase was reduced under anoxia in red muscle and heart but was unaffected in liver and white muscle. Exposure to anoxia decreased 40S ri-

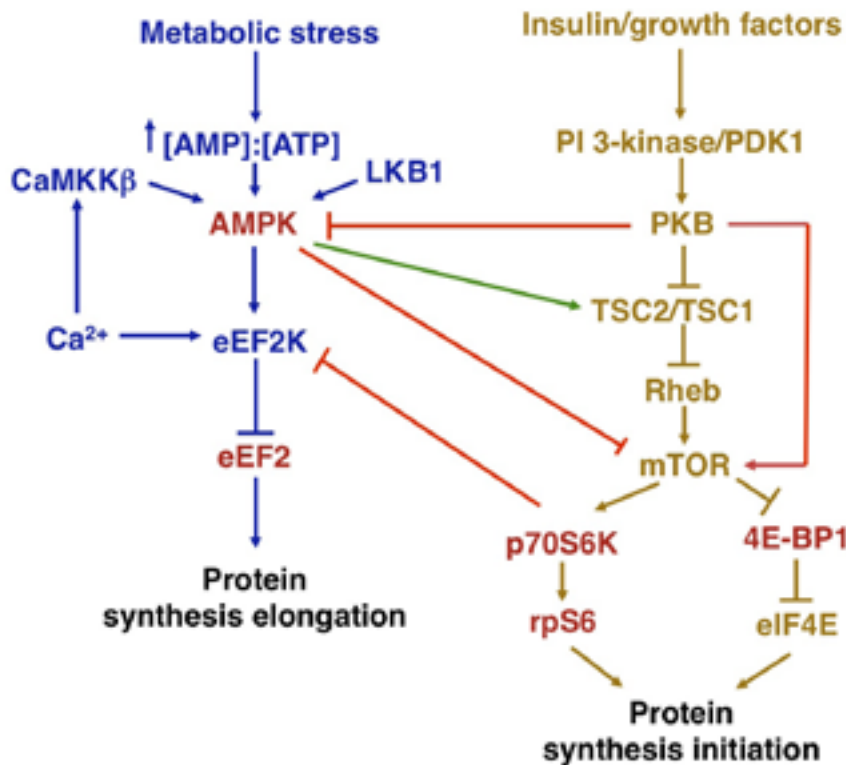


Figure 2. Mechanisms by which AMPK activation inhibits protein synthesis initiation (via a reduction in mTORC1 signalling) and translation elongation (via phosphorylation-induced eEF2K activation).

bosomal protein S6 phosphorylation in heart and promoted eukaryotic initiation factor 4E-binding protein-1 dephosphorylation in red muscle, but surprisingly increased 4E-binding protein-1 phosphorylation in white muscle. The changes in phosphorylation state of translation factors suggest that organ-specific patterns of signalling and response are involved in achieving the anoxia-induced suppression of protein synthesis in turtles (4).

Control of ion transport by AMPK

We studied whether the mechanism of increased $\text{Na}^+\text{-K}^+\text{-2Cl}^-$ (NKCC1) co-transporter activity by osmotic shrinkage involved AMPK activation. AMPK was found to phosphorylate a recombinant GST-dogfish (1-260) NKCC1 fragment at Ser38 and Ser214, corresponding to Ser77 and Ser242 in human NKCC1, respectively. Incubation of human erythrocytes with A769662 AMPK activator increased Ser242 NKCC1 phosphorylation but did not stimulate $^{86}\text{Rb}^+$ uptake. Under hypertonic conditions in human red blood cells (RBCs) incubated with 0.3 M sucrose, NKCC1 activity increased as measured by bumetanide-sensitive $^{86}\text{Rb}^+$ uptake and AMPK was activated. However, there was no effect of AMPK α 1 deletion in mouse RBCs on the increased rate of $^{86}\text{Rb}^+$ uptake induced by hyperosmolarity. AMPK activation by osmotic shrinkage of mouse RBCs was abrogated by the STO-609 CaMKK β inhibitor, but incubation with STO-609 did not affect the increase in $^{86}\text{Rb}^+$ uptake induced by hyperosmolarity. Osmotic shrinkage of human and mouse RBCs led to activation loop phosphorylation of the STE20/SPS1-related proline/alanine-rich kinase (SPAK) at Thr233, which was accompanied by phosphorylation of NKCC1 at Thr203/207/212, one of which (Thr207) is responsible for co-transporter activation. Therefore, phosphorylation-induced activation of NKCC1 by osmotic shrinkage does not involve AMPK and is likely due to SPAK activation (Fig. 3, ref 5).

Role of AMPK in the control of cytoskeletal actin organization

AMPK function is now recognized to extend beyond metabolic regulation to control cell division, cell polarity and cell migration, all of which depend on the actin cytoskeleton. We studied the effects of A769662 on cytoskeletal organization and signalling in epithelial Madin-Darby canine kidney (MDCK) cells. AMPK activation induced disassembly of stress fibers and accumulation of cortical F-actin. In parallel, Rho-kinase downstream targets, namely myosin regulatory light chain (MLC) and cofilin, were phosphorylated. These effects resembled the morphological changes in MDCK cells exposed to hyperosmotic shock, which led to Ca^{2+} -dependent AMPK activation via CaMKK β . Indeed, hypertonicity-induced AMPK activation was markedly reduced by the STO-609 CaMKK β inhibitor, as was the increase in MLC and cofilin phosphorylation. We propose that AMPK links osmotic stress to the reorganization of the actin cytoskeleton (6).

We were unable to confirm published data claiming that MLC can be directly phosphorylated by AMPK. Our results indicate that MLC is not a physiological AMPK substrate to explain energy-dependent changes in cell structure and that this published observation was rather due to commercial AMPK contamination by kinases capable of MLC phosphorylation (7).

MASS SPECTROMETRY

D. Vertommen, S. Calberson, M. Rider in collaboration with C. Sindic, UCL and J.-F. Collet, UCL

The development of mass spectrometry facilities within our laboratory has been an enormous asset to our group and institution. Since the acquisition of our first electrospray mass spectrometer in 1997, the application of mass spectrometry techniques to protein identification, identification of sites of covalent

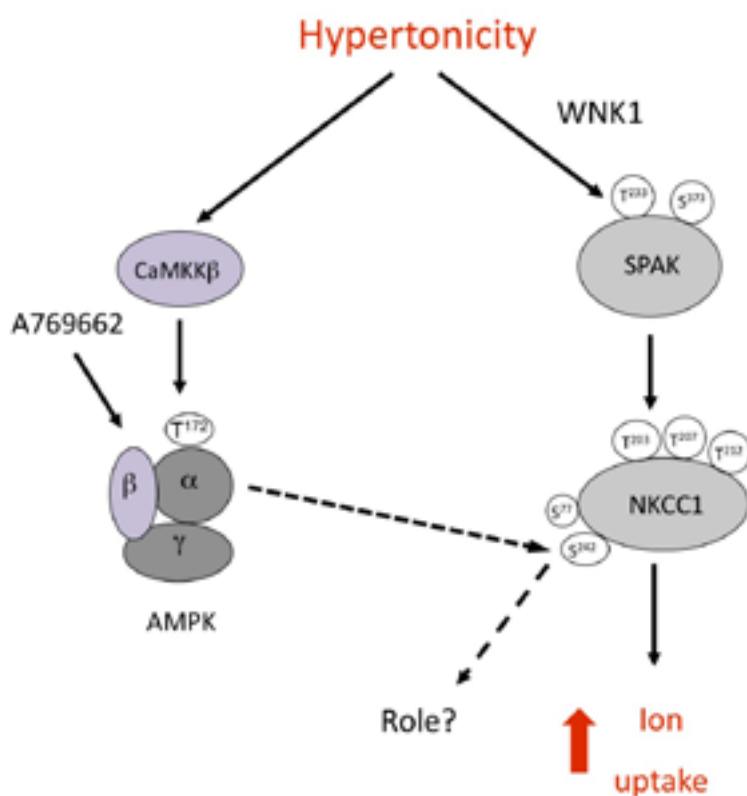


Figure 3. Cell shrinkage by sucrose treatment in RBCs leads to activation of α 1-AMPK and SPAK via the CaMKK β and WNK (with-no-lysine kinase)-1 pathways, respectively. SPAK activation correlates with NKCC1 Thr203/207/212 phosphorylation and is likely responsible for its activation and increased $^{86}\text{Rb}^+$ uptake induced by hyperosmolarity. AMPK activation by hyperosmolarity does not affect NKCC1 activity. Treatment with A769662 activates AMPK in RBCs and increases Ser242 NKCC1 phosphorylation, whose function is at present unknown.

modification and quantification of changes in protein expression has led to well over 30 joint publications. In our own research, it has been paramount in identifying new phosphorylation sites. In 2009 we updated our electrospray machine to the Finnigan LTQ linear ion trap equipped with electron-induced transfer dissociation (ETD) fragmentation and we are now part of the UCL proteomics platform MASS-PROT, open to the scientific community of the de Duve Institute and UCL.

We are continuing our efforts to develop new techniques for the mass spectrometric analysis of proteins. These include a 2D-LC/MS label-free proteomics approach for differential protein expression studies which has now been validated on membrane proteins

(8). For phosphoproteomics to identify new AMPK substrates, a hydrophilic interaction chromatography (HILIC) approach followed by enrichment of phosphopeptides by immobilized metal affinity chromatography (IMAC) and analysis by LC-MS (reviewed in ref. 9) is currently being validated.

We pursue our collaboration with other laboratories within our university and with groups outside and abroad. For example with C. Sindic, the screening of glycoproteins from sera and cerebro-spinal fluid of patients with neurodegenerative diseases is under study to discover new biomarkers. In collaboration with the group of J.-F. Collet, we investigated the mechanism that controls sulfenic acid formation in the periplasm of *E. Coli*. Using a diffe-

rential proteomic approach and targeted chemical modification, we discovered that DsbG and DsbC, two thioredoxin-related proteins, control the global sulfenic acid content of the periplasm and protect single cysteine residues from oxidation. DsbG interacts with the YbiS protein and, along with DsbC, regulates oxidation of its catalytic cysteine residue preventing the irreversible modification to sulfinic and sulfonic acids (10).

SELECTED PUBLICATIONS

1. Rider MH, Bertrand L, Vertommen D, Michels PA, Rousseau GG, Hue L. *6-Phosphofructo-2-kinase/fructose-2,6-bisphosphatase: head-to-head with a bifunctional enzyme that controls glycolysis*. **Biochem J** 2004;381:561-79.
2. Marsin AS, Bertrand L, Rider MH, Deprez J, Beauloye C, Vincent MF, Van den Berghe G, Carling D, Hue L. *Phosphorylation and activation of heart PFK-2 by AMPK has a role in the stimulation of glycolysis during ischaemia*. **Curr Biol** 2000;10:1247-55.
3. Horman S, Browne G, Krause U, Patel J, Vertommen D, Bertrand L, Lavoinnie A, Hue L, Proud C, Rider MH. *Activation of AMP-activated protein kinase leads to the phosphorylation of elongation factor 2 and an inhibition of protein synthesis*. **Curr Biol** 2002;12:1419-23.
4. Rider MH, Hussain N, Dilworth SM, Storey KB. *Phosphorylation of translation factors in response to anoxia in turtles, *Trachemys scripta elegans*: role of the AMP-activated protein kinase and target of rapamycin signalling pathways*. **Mol Cell Biochem** 2009;332:207-13.
5. Sid B, Vertommen D, Viollet B, Rider MH. *Stimulation of human and mouse erythrocyte $\text{Na}^+ \text{-} \text{K}^+ \text{-} 2\text{Cl}^-$ cotransport by osmotic shrinkage does not involve AMP-activated protein kinase, but is associated with STE20/SPS1-related proline/alanine-rich kinase activation*. **J Physiol** 2010; In press.
6. Miranda L, Carpentier C, Platek A, Hussain N, Gueuning M-A, Vertommen D, Ozkan Y, Sid B, Hue L, Courtoy PJ, Rider MH, Horman S. *AMP-activated protein kinase induces actin cytoskeleton reorganization in epithelial cells*. **Biochem Biophys Res Commun** 2010; In press.
8. Vertommen D, Ruiz N, Leverrier P, Silhavy TJ, Collet JF. *Characterization of the role of the Escherichia coli periplasmic chaperone SurA using differential proteomics*. **Proteomics** 2009;9:2432-43.
7. Bultot L, Horman S, Neumann D, Walsh MP, Hue L, Rider MH. *Myosin light chains are not a physiological substrate of AMPK in the control of cell structure changes*. **FEBS Lett** 2009;583:25-8.
9. Rider MH, Waelkens E, Derua R, Vertommen D. *Fulfilling the Krebs and Beavo criteria for studying protein phosphorylation in the era of mass spectrometry-driven kinome research*. **Arch Physiol Biochem** 2009;15:298-310.
10. Depuydt M, Leonard L, Vertommen D, Denoncin K, Morsomme P, Wahni K, Messens J, Carroll K, Collet JF. *A periplasmic reducing system protects single cysteine residues from oxidation*. **Science** 2009;326:1109-11.

Mark Rider

de Duve Institute
Av. Hippocrate 74-75
B - 1200 Brussels

[T] +32 02 764 75 29

[F] +32 02 764 75 07

[E] mark.rider@uclouvain.be

[W] http://www.deduveinstitute.be/control_of_cell_function_by_protein_phosphorylation.php