The AMP-activated protein kinase cascade – a unifying system for energy control

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AMP-activated protein kinase (AMPK) is the downstream component of a protein kinase cascade that acts as an intracellular energy sensor maintaining the energy balance within the cell. This pivotal role of AMPK places it in an ideal position for regulating whole-body energy metabolism, and AMPK might play a part in protecting the body from metabolic diseases such as type 2 diabetes and obesity. Mutations in AMPK cause cardiac hypertrophy and arrhythmia. Recent findings have identified LKB1 – a protein kinase that is mutated in a hereditary form of cancer – as a candidate for the upstream kinase in the AMPK cascade. AMPK could provide a link in human diseases of which the underlying cause is due to defects in energy metabolism.

In the 15 or so years since first being described [1], the AMP-activated protein kinase (AMPK) has come a long way. AMPK not only plays a key role in sensing intracellular ATP levels, but also acts as a crucial component in maintaining the energy balance within cells. AMPK is activated following ATP depletion or, more accurately, a rise in the AMP:ATP ratio within the cell, and responds by adjusting the rates of ATP-consuming (anabolic) and ATP-generating (catabolic) pathways. We now know some of the complex mechanisms by which AMPK is activated by an increase in the AMP:ATP ratio, and many aspects of this have been described in previous reviews; the reader is referred to these for further details [2–4]. Recent results have led to the discovery that AMPK responds to stimuli that do not cause a detectable change in the AMP:ATP ratio, raising the possibility that other signals feed into the system, thus, allowing for a more complex pattern of energy homeostasis. The discovery of naturally occurring mutations in AMPK that cause cardiac hypertrophy provides direct evidence that AMPK has a fundamental role in maintaining normal human physiology. Moreover, a candidate for the upstream kinase in the AMPK cascade has emerged that could implicate AMPK in cancer development. This review focuses on the evidence that suggests that AMPK is a key factor in maintaining energy homeostasis and the recent findings implicating the AMPK cascade in human disease.

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Physiological role of AMPK

Many studies have established that a key function of AMPK is to regulate the energy balance within the cell. One of the first lines of evidence was the finding that AMPK is activated in response to ATP depletion, which causes a concomitant increase in the AMP:ATP ratio [5]. Once activated, AMPK phosphorylates several downstream substrates, the overall effect of which is to switch off ATP-consuming pathways (e.g. fatty acid synthesis and cholesterol synthesis) and to switch on ATP-generating pathways (e.g. fatty acid oxidation and glycolysis) [2–4,6]. In addition to the acute effects of AMPK on energy metabolism, activation of AMPK has longer-term effects, altering both gene expression [7–9] and protein expression [10,11]. Although the physiological consequence of these longer-term effects of AMPK are not fully understood, it seems likely that they are involved in the overall regulation of energy metabolism. New evidence has emerged demonstrating that AMPK is also activated in response to conditions that do not cause a detectable increase in the AMP:ATP ratio. Hyperosmotic stress [12] and the anti-diabetic agent metformin [12,13] both activate AMPK without appearing to alter the intracellular AMP:ATP ratio. It is not clear how these conditions signal activation of AMPK, but they do lead to increased phosphorylation of AMPK by its upstream kinase [12,13]. A major challenge for the future will be to elucidate the mechanism underlying the nucleotide-independent activation of AMPK. A model summarizing the role of AMPK in maintaining the intracellular energy balance is shown in Figure 1.

Much of the previous work on AMPK focused on its regulation of energy levels within individual cells. Recent results, however, suggest that AMPK might have a wider role in regulating whole-body energy metabolism. AMPK is activated in skeletal muscle in response to contraction, resulting in increased glucose uptake [14–16] and fatty acid oxidation [14]. Two adipocyte-derived hormones – leptin and adiponectin, which themselves play key parts in regulating energy homeostasis [17,18] – activate AMPK. Leptin activates AMPK in skeletal muscle, thereby increasing fatty acid oxidation [19], whereas adiponectin activates AMPK in liver and muscle to stimulate glucose usage and fatty acid oxidation, and inhibiting glucose production in liver [20]. AMPK has been shown to

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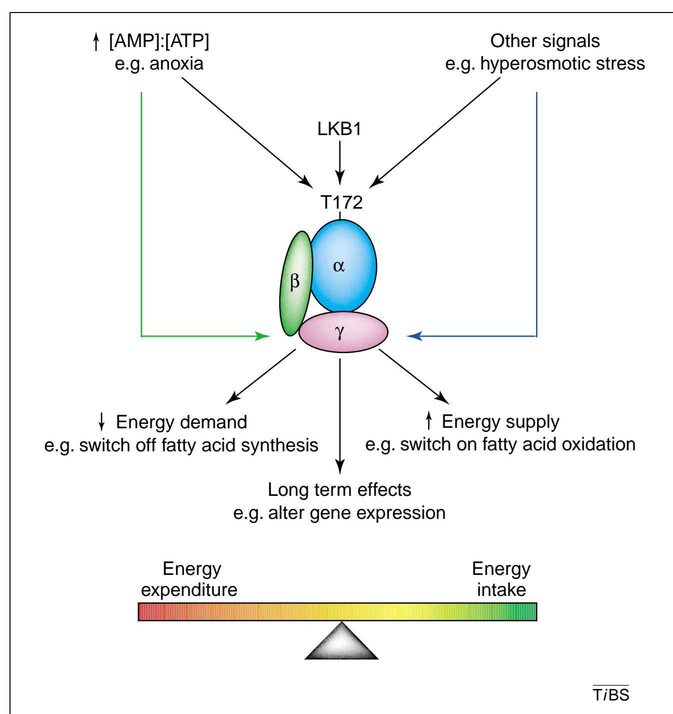

Figure 1. AMP-activated protein kinase (AMPK) maintains the intracellular energy balance. Conditions that cause an increase in the AMP:ATP ratio lead to activation of AMPK through increased phosphorylation on Thr172 (T172) within the α subunit. AMP activates AMPK allosterically (green arrow), and this effect is antagonized by high concentrations of ATP [3,4]. Recent evidence suggests that LKB1 is the upstream kinase in the cascade [48,54,55], although how LKB1 activity is regulated is not known. AMPK is also activated by other factors, such as hyperosmotic stress, which do not alter adenine nucleotide levels [12]. Activation of AMPK by the nucleotide-independent pathway involves phosphorylation of Thr172 by LKB1, but whether this pathway leads to activation of AMPK by other mechanisms (blue arrow) is not known. Once activated, AMPK phosphorylates several downstream substrates resulting in the overall effect of decreasing energy demand by switching off ATP-consuming pathways and increasing energy supply by switching on ATP-generating pathways. AMPK also has longer-term effects, such as altering gene expression. These combined roles of AMPK ensure that the energy status of the cell is finely balanced.

contribute to the regulation of insulin secretion and insulin gene expression in the pancreatic β cell [21], and mice lacking one of the catalytic subunits (α2) of AMPK have impaired insulin sensitivity [22]. In addition to regulating some of the actions of insulin, AMPK itself is regulated by insulin, suggesting a complex interplay between the two. The effects of hormones on AMPK, coupled with the role of AMPK in response to muscle contraction, provide direct evidence that the kinase plays a part in determining whole-body energy metabolism (Figure 2). These findings indicate that AMPK might be important in the development of metabolic diseases, such as type 2 diabetes and obesity.

Structure of AMPK

AMPK is a heterotrimeric complex comprising a catalytic subunit (α) and two regulatory subunits (β and γ). Homologues of all three subunits have been identified in every eukaryotic species examined to date, ranging from mammals, fruitfly (*Drosophila melanogaster*), worm (*Caenorhabditis elegans*), yeast (*Saccharomyces cerevisiae*) and plants. This conservation suggests that formation of the heterotrimeric complex is an essential requirement for at least some of the functions of the kinase. In terms of understanding the regulation of AMPK and also uncovering aspects of its function, one of the most significant findings was that AMPK is structurally and functionally related to a protein kinase complex in *Saccharomyces cerevisiae* termed SNF1 [3]. SNF1 subunits were identified by complementation of yeast mutants that are unable to grow on non-glucose carbon sources, such as sucrose or raffinose [23]. Further studies revealed that SNF1 kinase activity is required for the transcriptional activation of many genes that are repressed in the presence of glucose [23]. In mammals, isoforms of all three subunits, which are encoded by separate genes, have been identified (Table 1). The roles of the different subunits within the AMPK complex provide important clues regarding the physiological functions of the kinase, as well as offering valuable insights into its regulation. Some of the key features of the different subunits are highlighted in Figure 3.

The N-terminal half of the α subunit contains a typical serine/threonine protein kinase catalytic domain, containing features conserved throughout the protein kinase superfamily [24]. Expression studies in mammalian cells have indicated that the C-terminal half of the α subunit contains a region of ~150 amino acid residues at the extreme C terminus that is required for association with the β and γ subunits, whereas a region immediately downstream of the catalytic domain (residues 312–392 in the α1 isoform) appears to have an inhibitory function [25]. The α subunit also contains several residues that can be phosphorylated both *in vitro* and *in vivo*. One of these residues is Thr172 and its phosphorylation is essential for AMPK activity.

It appears that one of the functions of the β subunit is to act as a scaffold for the binding of the α and γ subunits [26]. More recently, however, evidence has emerged that suggests a second, distinct role of the β subunit. A region within the β subunit – corresponding to amino acid residues 72–151 in rat β1 – shares a high degree of sequence similarity to a domain termed an N-isoamylase domain [27,28]. This domain is found in several enzymes that metabolize the α1–6 branch points in α1–4 linked glucans, such as glycogen and starch. The physiological relevance of this domain in AMPKβ is unclear, although previous studies have shown that AMPK phosphorylates glycogen synthase [29] and that activation of AMPK in muscle is suppressed by high glycogen [30]. It is tempting to speculate, therefore, that this domain could be involved in targeting AMPK to glycogen and/or that it provides a mechanism for regulation of AMPK by glycogen but, as yet, the physiological function of this domain remains unknown.

The γ subunit contains four CBS domains, the acronym stemming from cystathionine-β-synthase which itself contains a CBS domain. These domains are found in a wide variety of proteins [31], but their function remains unknown. However, some recent evidence indicates that AMP binding is an important function of the CBS domains in the γ subunits. Studies using 8-azido-AMP – a photoaffinity analogue of AMP – resulted in labelling of the γ subunit, suggesting that the γ subunit binds to the adenosine portion of AMP [32]. A second line of evidence implicating the CBS domains in binding of AMP came from

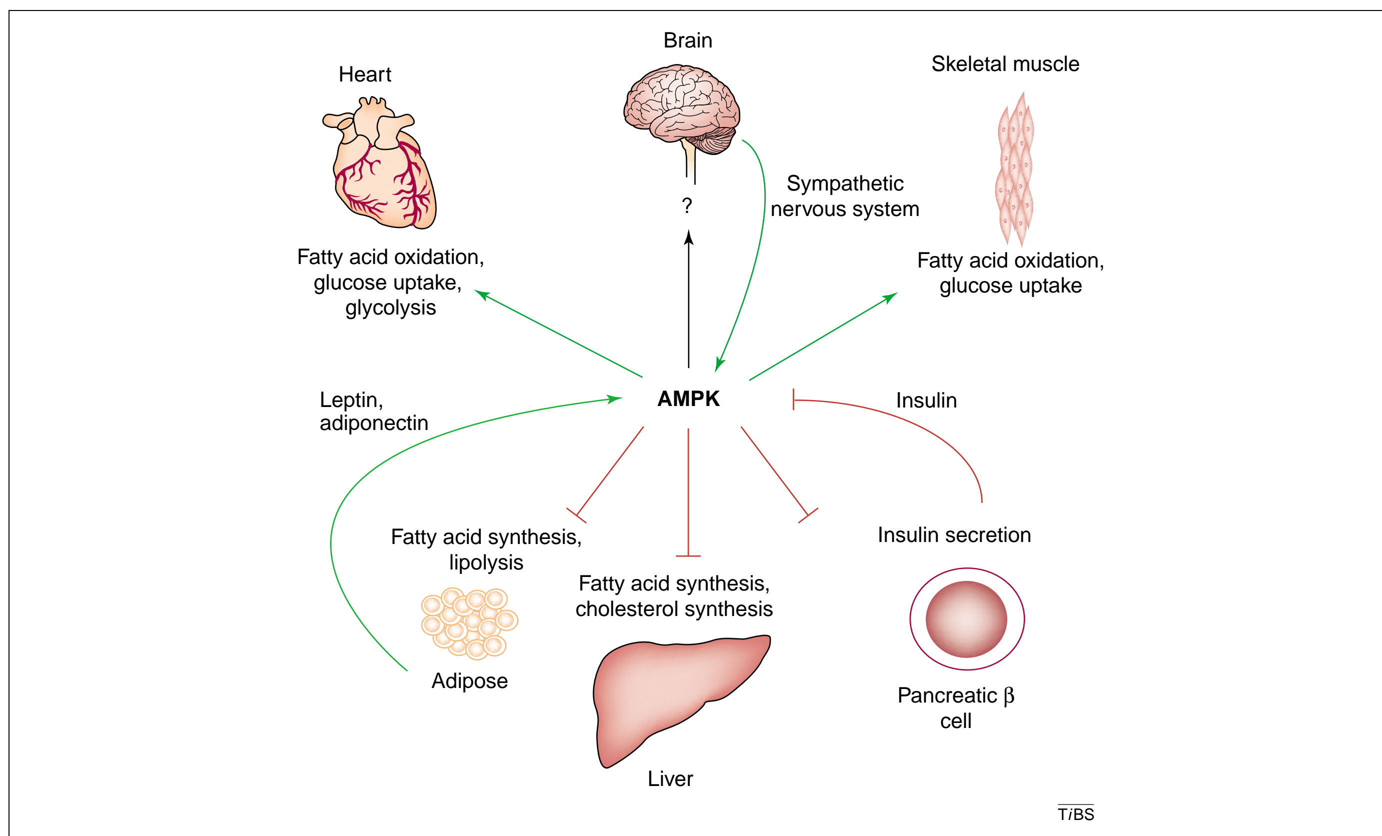

Figure 2. Proposed roles of AMP-activated protein kinase (AMPK) in the control of whole-body energy metabolism. Activation of AMPK stimulates energy-generating pathways (green arrows) in several tissues while inhibiting energy-consuming pathways (red lines). The adipocyte-derived hormones leptin and adiponectin activate AMPK in skeletal muscle, stimulating fatty acid oxidation [19,20]. Activation of AMPK in skeletal muscle by leptin involves the sympathetic nervous system [19]. Adiponectin activates AMPK in liver, thus, increasing fatty acid oxidation and glucose utilization [20]. AMPK inhibits insulin secretion from pancreatic β cells [21], whereas insulin inhibits AMPK activation in heart [46]. It is possible that AMPK plays a part in the central control of energy metabolism mediated via the hypothalamus. A scheme showing the probable effects of AMPK activation on glucose homeostasis has recently been presented [21].

an altogether more unexpected finding, and is described below.

Mutations in AMPK γ-subunit isoforms

The first indication that naturally occurring mutations in AMPK subunits could cause significant physiological effects came from a rather unlikely source. An autosomal dominant allele, identified as RN^-, is common in Hampshire pigs and leads to a marked reduction in meat quality. The major effect of the RN^- allele is a dramatic increase in skeletal muscle glycogen content (~70% increase compared with the wild-type homozygous rn^+ pigs). The RN^- mutation was identified as a missense mutation in AMPK γ3, altering arginine at residue 225 (the numbering used here corresponds to the position of the residue within the predicted full-length sequence of pig γ3 [33]) to a glutamine (R225Q) [34]. Shortly after the discovery of the γ3 mutation, several studies reported the identification of mutations in the γ2 gene from individuals with familial cardiac hypertrophy [35–38]. In most of the patients identified, there were also marked abnormalities in electrical conductance, such as ventricular pre-excitation, similar to the conduction abnormalities observed in a condition called Wolff–Parkinson–White syndrome [36,37]. Two potential polypeptides are predicted from different γ2 mRNA transcripts produced from alternative promoters within the human γ2 gene [37,39]. One polypeptide contains 569 amino acids (designated here as $\gamma 2_L$), whereas the other ($\gamma 2_S$) is predicted to contain the C-terminal 328 residues of $\gamma 2_L$.

Table 1. Summary of the AMPK/SNF1 subunits[a]

Isoform	Human chromosome	Mass (kDa)	Yeast equivalent	Function	Refs
α1	5	63	Snf1	Contain protein kinase catalytic domain and includes	[42,58–60]
α2	1	63		regulatory phosphorylation site (T172) within activation loop.	
β1	12	30	Sip1, Sip2,	C terminus required for bridging of α and γ subunits to form	[26–28,61,62]
β2	1	30	Gal83	complex; contain putative glycogen-binding domain.	
γ1	12	37	Snf4	Contain four CBS domains involved in AMP binding.	[26,32,33–41]
$\gamma 2_L$	7	63		Mutations identified in γ2 (human) and γ3 (pig) lead to distinct	
$\gamma 2_S$	7	38		phenotypes.	
γ3	2	55			

[a]Abbreviations: AMPK, AMP-activated protein kinase; CBS, cystathionine-β-synthase; Sip, Snf1-interacting protein; Snf1, sucrose nonfermenting protein 1.

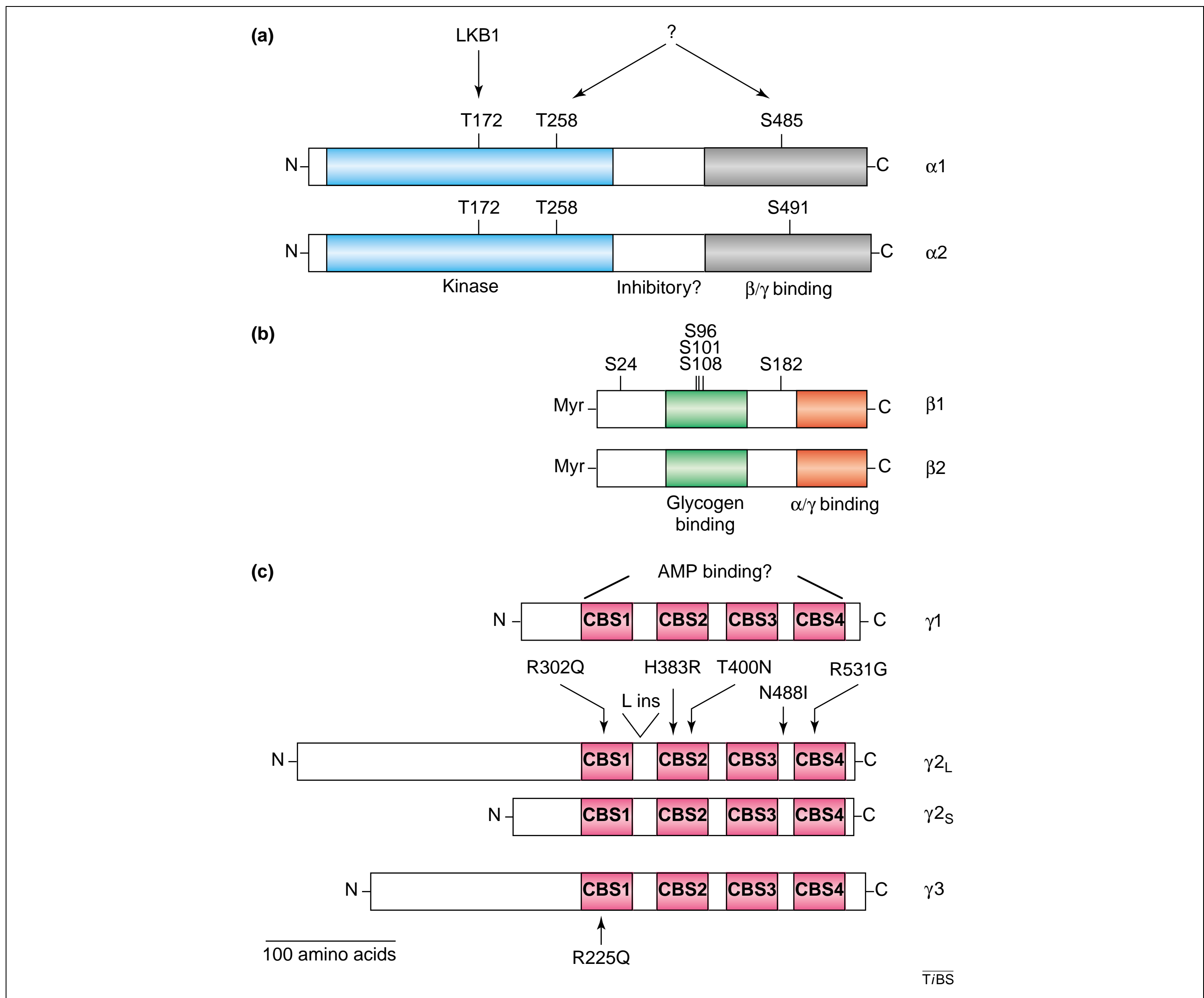

Figure 3. Subunit structures of AMP-activated protein kinase (AMPK). AMPK is composed of three subunits (α, β and γ); there are two isoforms of the α and β subunits, and three γ subunit isoforms [1,6]. **(a)** The C-terminal domain of the α subunit binds to the β and γ subunits. An inhibitory domain is also proposed to lie within the C-terminal half of the α subunit [25]. Three phosphorylation sites have been identified as Thr172 (T172), Thr258 (T258) and Ser485 (α1)/491 (α2) (S485/S491) [44]. LKB1 phosphorylates Thr172 *in vitro* [48], but the identity of the kinase(s) that phosphorylate the other residues is not known. **(b)** The β subunits are myristoylated (Myr) at their N termini and contain multiple phosphorylation sites [44,54]. The C-terminal domain of the β subunit is involved in binding of the α and γ subunits, and a putative glycogen-binding domain has been identified between residues 72–151 in β1 [27,28]. **(c)** The γ subunits contain four copies of a cystathionine-β-synthase (CBS) domain, which appear to be involved in binding AMP [32,40]. The location of the five missense mutations identified within γ2 that cause cardiac abnormalities, together with the missense mutation in γ3 that causes skeletal muscle glycogen accumulation, are indicated by arrows. The mutation resulting in insertion of a leucine residue in γ2 is marked by a V. Two forms of γ2 protein are predicted [37,39], designated $\gamma2_L$ and $\gamma2_S$.

The expression of these different polypeptides has not been examined, so the physiological role of these different polypeptides is unclear. Five missense mutations and one mutation, which causes an in-frame insertion of a leucine residue, have now been identified in γ2 [35–38] (Figure 3). Interestingly, one of the mutations (R302Q; residue number refers to $\gamma2_L$) lies in the equivalent position in the first CBS domain as R225Q in γ3. How do the mutations affect AMPK activity? Unfortunately, the answer to this question does not appear to be straightforward. Three mutations within the CBS domains (R302Q, H383R and R531G) decrease AMPK activity by dramatically reducing the AMP activation of the kinase [40]. These results provide strong evidence that the CBS domains are involved in AMP binding. In the same study, it was found that the leucine insertion mutation had no detectable effect on AMPK activity [40]. In a separate study, indirect evidence was presented indicating that two other mutations (T400N and N488I) caused activation of AMPK [38]. Although these findings are too preliminary to draw firm conclusions, they do raise the possibility that distinct mutations leading to the same disease phenotype could have different effects on AMPK activity. If this is the case, it would suggest that alternative mechanisms can contribute to similar pathogenesis.

At first sight, the phenotypes of the γ2 and γ3 mutations seem to have little, if anything, in common. However, unusual vacuoles – possibly containing glycogen – were found to be present in cardiomyocytes from patients

harbouring two different mutations in γ2 (N488I and T400N) [38]. Furthermore, hearts from transgenic mice overexpressing γ2 and containing the N488I mutation have been found to accumulate high levels of glycogen and display marked hypertrophy coupled with abnormal conduction pathways [41]. These findings suggest that a common mechanism might exist by which mutations in γ2 and γ3 lead to excessive glycogen accumulation in heart and skeletal muscle, respectively. However, it should be noted that glycogen levels have only been studied in two patients [38] and so it is not clear whether this will prove to be a common finding. Another slightly puzzling aspect of the γ mutations is that the phenotypes are restricted to effects either in heart (γ2) or skeletal muscle (γ3). In the case of γ3, this might simply reflect the expression pattern of the protein that is confined almost exclusively to skeletal muscle [32]. However, γ2 is expressed in a wide range of tissues [32,39] raising the possibility that γ2 has a specific function in heart and that changes in this function lead to the disease phenotype. This is an attractive hypothesis, especially given that γ1 accounts for the majority of AMPK activity in all tissues, including heart and skeletal muscle [32].

Regulation of AMPK by phosphorylation

AMPK is phosphorylated and activated by an upstream protein kinase, AMPK kinase (AMPKK). Attempts to isolate AMPKK from tissues succeeded in only partial purification, although this has enabled some biochemical characterization of the kinase, as well as identification of the major regulatory phosphorylation site as Thr172 within the α subunit [42]. Thr172 lies within the activation loop of the kinase catalytic domain, a region where many protein kinases require phosphorylation for their activation [43]. In addition to Thr172, partially purified AMPKK phosphorylates the α subunit on at least two other residues, Thr258 and Ser485 (Ser491 in α2) [44] (Figure 3). The amino acid sequences surrounding Thr258 and Ser485/491 show some similarities, but not with the sequence surrounding Thr172 [44]. It seems likely that Thr258 and Ser485/491 are phosphorylated by a protein kinase (or kinases) that is distinct from the Thr172 kinase. The β subunit is phosphorylated at multiple sites, but the identity of the protein kinases that phosphorylate these residues, or the effect of phosphorylation on AMPK activity, is not known. Phosphorylation of Thr172 appears to be essential for AMPK activity because site-directed mutagenesis of Thr172 to alanine completely abolishes kinase activity [25,45]. Several groups have generated antibodies that specifically recognize the phosphorylated form of Thr172 and these have been used in several studies to demonstrate that the activity of AMPK mirrors the phosphorylation status of Thr172 under all conditions tested. In contrast to Thr172, phosphorylation of either Thr258 or Ser485/491 does not cause any detectable activation of AMPK [44]. Whether phosphorylation of these residues plays a part in the regulation of AMPK other than by direct activation remains unknown. Recently, activation of protein kinase B (PKB; also known as Akt) in heart was found to cause decreased Thr172 phosphorylation of AMPK [46]. It is not known if this effect is the result of direct phosphorylation of AMPK by PKB, but it might explain a previous observation that insulin antagonizes the activation of AMPK in ischaemic heart [47]. One intriguing possibility is that phosphorylation of AMPK at distinct sites (e.g. Thr258 or Ser485/491) could inhibit phosphorylation of Thr172 by AMPKK.

Identification of the upstream kinase in the AMPK cascade

Despite intensive efforts, the molecular identification of AMPKK has remained elusive, which has led to a major research bottleneck in the field. However, through recent advances made in the investigation of the regulation by upstream kinases of SNF1 in yeast this problem seems to be resolved at long last. Three closely related yeast protein kinases – Elm1, Pak1 and Tos3 – have been identified that phosphorylate and activate SNF1 *in vitro* [48,49]. Deletion of all three kinases causes a phenotype that is similar to a *snf1* mutant phenotype and that abolishes SNF1 activity. These results indicate that Elm1, Pak1 and Tos3 have overlapping redundant functions with respect to their ability to activate SNF1. There is also evidence that Elm1, Pak1 and Tos3 have more specific functions, which probably do not involve SNF1, because deletion of the individual kinases results in distinct phenotypes not associated with a *snf1* phenotype [48–50]. On first inspection, identification of Elm1, Pak1 and Tos3 as the upstream kinases in the yeast SNF1 cascade turned out to be something of an anticlimax. The physiological functions of the kinases are obscure and, previously, they were classified as kinases unique to yeast; not an impressive curriculum vitae for the mammalian AMPKK. The amino acid sequences of the catalytic domains of Elm1, Pak1 and Tos3 are most closely related to members of the mammalian Ca^{2+}/calmodulin-dependent protein kinase kinase (CAMKK) subgroup. Earlier studies on AMPKK showed that it was not stimulated by Ca^{2+}/calmodulin [51], which ruled out the AMPKK as a member of the CAMKK group. However, another kinase sharing significant amino acid sequence identity with the three yeast kinases was LKB1 (also known as STK11). Although the physiological role of LKB1 is unclear, inactivating mutations in LKB1 lead to an autosomal dominantly inherited cancer in humans, termed Peutz–Jeghers syndrome [52,53]. Is LKB1 the upstream kinase in the AMPK cascade? A definitive answer to this question is lacking, but recent results strongly suggest that this might be the case. LKB1 phosphorylates and activates AMPK *in vitro* [48] and LKB1 accounts for most of the AMPKK activity detectable in cell extracts [54,55]. Furthermore, AMPK activation is abolished in cells lacking LKB1 expression or following inhibition of LKB1 [56,57]. Identification of AMPKK opens up new avenues of investigation regarding both the regulation of AMPK and the physiological role of LKB1.

The future for AMPK

Currently, research in the AMPK field is enjoying something of a 'golden age' and this seems likely to last for some years. Understanding the role of AMPK in disease is already a high priority and will probably continue to attract significant attention. Identification of LKB1 as a

possible candidate for AMPKK provides an exciting new angle to explore the regulation of AMPK and to investigate potential cross-talk between different signalling pathways. Two new areas that seem ripe for future research are (i) investigating the role of AMPK in cellular senescence and (ii) determining whether AMPK plays a part in the central control of energy balance in the hypothalamus. There is already some evidence that AMPK has a role in cellular senescence [53] and the circumstantial evidence linking AMPK to hypothalamic control of energy metabolism is sufficient to warrant more direct studies. The use of animal models to study the physiological role of AMPK is just beginning to make an impact, and this is another area that looks set to yield significant advances.

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