9
Signaling by AMP-activated Protein Kinase

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Abstract

Intracellular sensors of cellular energy and nutrient status are emerging as key players in the regulation of cell metabolism in health and disease. AMP-activated protein kinase (AMPK) participates in the control of cellular and whole-body energy balance by its exquisite sensitivity to AMP. AMPK is thus able to sense and to react to an increasing AMP:ATP ratio within a complex upstream and downstream signaling network that responds to different energetic and metabolic stresses. The kinase forms heterotrimers with catalytic α- and regulatory β-, and γ-subunits, which exist as different isoforms and splice variants. Global and local cellular ATP:ADP and phosphocreatine:creatine ratios are controlled by the reactions of adenylate kinase and creatine kinase, respectively, which are the first safeguards for keeping cellular ATP:ADP and ATP:AMP ratios high for as long as possible. However, AMPK operates under different metabolic conditions, with different modes of operation and in subsequent time frames. AMP is present at sub-micromolar concentrations in resting cells and increases strongly as a result of the adenylate kinase reaction when ATP is depleted. It is thus an ideal second messenger for reporting cellular energy status to the sensor AMPK. Full activation of AMPK is more complex. In addition to allosteric stimulation by micromolar AMP concentrations, it involves both covalent activation and inhibition via changes in phosphorylation of the Thr172 residue of the α-subunits by different upstream kinases and phosphatases. Such activation depends on different kinds of energy stress as well as on hormones and other signals, including changes in calcium. As a consequence, AMPK aims not only at cellular energy homeostasis but also at whole-body energy balance. Once activated, AMPK induces compensatory measures to maintain cellular ATP:ADP ratios for cell survival. It regulates a large number of downstream targets, shutting down anabolic pathways and stimulating catabolic pathways, thus simultaneously sparing limited energy resources

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and acquiring extra energy, respectively. AMPK acts at two control levels that are responsible for acute and chronic responses: first by directly affecting the activity of key enzymes, e.g., in glucose and fat metabolism, and second by longer-term transcriptional control of key players of these metabolic pathways. However, new evidence indicates that AMPK also participates in the control of non-metabolic processes such as cell proliferation and cell cycle. Lastly, the kinase complex has been proposed as a potential drug target in treatment of type II diabetes, because its activation both stimulates insulin-independent glucose uptake in the periphery and suppresses glucose production by the liver. More recently, the discovery of AMPK activation by the tumor suppressor LKB1 suggests a strong connection between metabolic signaling and cancer.

9.1 Metabolism and Cell Signaling

9.1.1 A Critical Role for Protein Kinase Signaling

The response of a cell to a changing environment is aimed at maintaining cellular homeostasis for as long as possible in order to avoid cellular damage and cell death. Elaborate mechanisms of allosteric control, including metabolic feedback regulation, have evolved to cope with this challenge. On top of this, another layer of cellular regulation via a network of protein kinases and phosphatases has developed. This network integrates signals from extra- and intracellular messengers and executes rapid responses, as well as longer-term adaptations via modification of gene expression profiles. Thus, the cell can respond to the needs of the surrounding tissue as a result of extracellular stimuli sensed by cell surface receptors, as well as to changes in the intracellular milieu via sensor proteins, in order to mount a coordinate response that controls development, proliferation, growth, adhesion, or metabolic function.

A number of signaling pathways mediated by protein kinases, which regulate both metabolism and cell growth and proliferation, have been characterized in recent years. These include the AMP-activated protein kinase (AMPK), protein kinase B (PKB, Akt), and mammalian target-of-rapamycin (mTOR) pathways (for recent reviews, see [1–5]). These constitute so-called signaling hubs, which respond to multiple signals, such as the availability of ATP, glucose, or amino acids, and cause multiple responses in intermediary metabolism, protein synthesis, cell cycle regulation, and transcriptional control. Because of their importance, mutations in these signaling pathways are involved in multiple pathologies, emphasizing the importance of metabolic dysfunction in a number of unrelated diseases, such as cancer and diabetes. In this chapter, we describe the molecular function of AMPK, the protein kinase most relevant to the regulation of cellular bioenergetics.

9.1.2 At the Interface of Energy Metabolism and Protein Kinase Signaling: AMPK as Receptor for Cellular Energy State

Cellular energy metabolism shows a particularly well-developed capacity to maintain homeostasis of the major cellular energy resource, ATP. Especially in the heart, this has been described in great detail as the "homeostasis paradox" [6]. The cellular metabolic network contains multiple regulatory circuits to account for this. Many of them are addressed in other chapters of this volume, including allosteric regulations and feedback loops, micro-compartmentation, metabolic channeling, and similar mechanisms. Two key players in this immediate energy homeostasis control are creatine kinase (CK) and adenylate kinase (AdK) (see Chapters 3, 7, 8, and 11). At the level of protein kinase signaling, AMPK represents a key sensor of the cellular energy state. It detects changes that are accompanied by a rise in AMP and thus an increase in the AMP:ATP ratio, which in resting cells is extremely low [7]. This kinase was characterized during the last decade as an exquisitely sensitive and versatile metabolic sensor and "energy gauge" [8].

One of the classical concepts of regulation in bioenergetics is the "energy charge" model proposed by Atkinson back in 1968 [9]. It proposed that ratios of adenine nucleotides would oppositely regulate key metabolic enzymes, such that a decrease in ATP would stimulate catabolic ATP-generating pathways, while an increase in ATP would stimulate anabolic ATP-consuming pathways. This concept has inspired a generation of biochemists, although it turned out that direct regulation by adenine nucleotides was true only for a small subset of metabolic enzymes. However, it is now clear that many other metabolic enzymes and other proteins are regulated *indirectly* by energy charge, via the AMPK system [10, 11]. As its name suggests, this kinase is activated by AMP, more specifically by a rise in the AMP:ATP ratio, and in addition by multiple upstream kinases, which have added substantial complexity to this signaling pathway. Recent data implicate AMPK signaling as a key player not only in cellular bioenergetics but also in whole-body energy homeostasis, the metabolic syndrome, and cancer. By phosphorylating target proteins, including key metabolic enzymes, AMPK activation increases the flux through catabolic pathways and decreases anabolic processes, as expected from the energy charge hypothesis of Atkinson, thus preserving ATP levels. In addition, by acting on transcription, AMPK also adjusts the expression level of key enzymes and transporters.

A large number of detailed and recent reviews on AMPK signaling are available, e.g., dealing with AMPK in general [1, 8, 12, 13], with specific aspects of upstream and downstream signaling [14, 15], or with AMPK as a drug target for the metabolic syndrome and cancer [16, 17]. Chapter 17 of this volume is dedicated to the role of AMPK in whole-body energy balance and the metabolic syndrome. We will focus here on selected molecular aspects of AMPK signaling that deserve more detailed consideration. First, we discuss why AMP has been chosen

by evolution as an indicator for a challenged cellular energy situation or energy deterioration and how AMP levels in the cell are regulated. After a short description of AMPK structure, we will give an overview of the emerging complexity of mechanisms that activate AMPK as well as of the regulation of its numerous downstream targets.

9.2 Sensing and Signaling of Cellular Energy Stress Situations

9.2.1 Why AMP Represents an Ideal Second Messenger for Reporting Cellular Energy State

ATP is the universal energy currency in all living systems, and the thermodynamic efficiency of biochemical processes that rely on ATP-hydrolysis depends largely on a high local ATP:ADP ratio (at least 100:1) [6]. Therefore, to function economically, healthy cells need to maintain intracellular ratios of ATP:ADP, and concomitantly ATP:AMP, as high as possible. In normal cells, global cellular ATP concentrations ([ATP]) are usually maintained within a narrow range of 3–6 mM, depending on cell type, by the action of CK and AdK preventing a significant decrease in [ATP] under normal cellular workload as depicted in Fig. 9.1 [6]. Both of these kinases display a high affinity for ADP (see below) and have very fast turnover rates. They are therefore able to rephosphorylate and thus very efficiently remove the ADP generated by ATP hydrolysis [6], with CK drawing on the large cellular phosphocreatine (PCr) pool. Tissues and cells with high and fluctuating energy requirements, e.g., skeletal and cardiac muscle, brain, retina and spermatozoa, express high levels of CK [18, 19] and AdK [20, 21]. Assuming that CK and AdK would work at equilibrium in a cell (which may not be true at all, due to compartmentation of these kinases [22]), the AMP:ATP ratio would theoretically vary as the square of the ADP:ATP ratio, as pointed out by Hardie and Hawley [10].

A serious problem in bioenergetics is the fact that although [PCr] and [ATP] can be readily measured in intact cells by chemical methods or even *in vivo* by ^{31}P-NMR, this is more difficult in the case of [ADP] and not possible at all for [AMP] because of their presence in cells at very low concentrations and the limited sensitivity of the methods. Although concentrations can be determined by HPLC or capillary electrophoresis after rapid freezing and acid extraction, these methods ignore cellular compartmentation. In addition, the concentrations of ADP and AMP also may be significantly altered before, during, or after freeze-clamping of the tissues for extraction and analysis. These problems may lead to a deviation of values for [ADP] and [AMP] from the actual *in vivo* situation, e.g., due to mixing of PCr with adenylate pools and rapid equilibration via the CK and AdK reactions, hydrolysis of PCr and ATP, or release of metabolically inactive

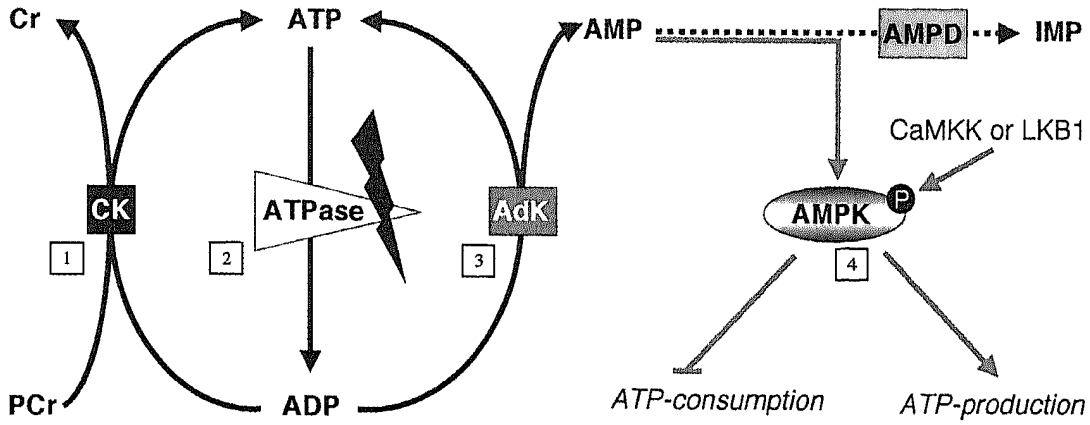

Fig. 9.1 High-energy phosphoryl transfer networks and regulation of cellular energy status. ATP, the universal energy currency, is hydrolyzed by ATPases (2) and the energy is converted, among other purposes, for muscle contraction, ion pumps, and protein synthesis. The cellular ATP:ADP ratio is kept high by the action of two energy-related kinases: creatine kinase (CK) (1) and adenylate kinase (AdK) (3). The ADP generated upon hydrolysis by ATPases is recharged into ATP by the action of CK (1), which draws its energy from a large phosphocreatine (PCr) pool, and by AdK (3), which uses two ADP molecules to regenerate one ATP and to generate one AMP molecule. AMP serves as an indicator for cellular energy stress and stimulates AMPK (4). Phosphorylation by either of the two upstream kinases, i.e., LKB1 or CaMKK, at Thr172 in the α-subunit causes its activation, with AMP inhibiting dephosphorylation. Activated and fully AMP-stimulated AMPK then upregulates catabolic pathways for ATP production and suppresses anabolic pathways that would consume ATP, thus salvaging the cell from irreversible energy deficiency. If AMP concentration is chronically elevated, it becomes deaminated by AMP-deaminase (AMPD) to inosine monophosphate (IMP) and subsequently is dephosphorylated by 5′-nucleotidase to yield inosine. However, inosine can leave the cell, leading to a drop in the total intracellular adenine nucleotide pool. Such a loss of energy-costly building blocks for nucleotide biosynthesis should be avoided if possible.

ADP bound to proteins such as actin [23] (see also [18, 22]). Thus, the "bulk concentrations," if such a parameter exists at all, of ADP and AMP are often calculated from the PCr and ATP concentrations measured directly, assuming complete equilibration of the CK and AdK reactions. Recently, it was possible to measure by *in vivo* ^{31}P-NMR the actual concentration of metabolically active, free ADP (approximately 1.5 mM) in skeletal muscle of an AdK ($^{-/-}$) knockout mouse, after exhaustive stimulation leading to nearly complete PCr depletion [24]. As expected in these animals, the AMP formation during muscle contraction was decreased, resulting in reduced phosphorylation of AMPK [25]. In general, AMP is present in resting cells at extremely low concentrations, probably in the low micromolar range. For example, in normally beating heart muscle, [AMP] has been estimated to be approximately 0.14 μM [26]. Using chemical determination of total metabolite contents by HPLC and assuming that the CK and AdK reactions are in equilibrium, the contents of free AMP and ADP in human skeletal muscle were estimated to be around 7.5 nmol and 57 μmol per kilogram

muscle dry weight at rest and 360 nmol and 260 μmol per kilogram after exhaustive exercise, respectively [27]. Under the same conditions, [PCr] decreased from 86 mmol kg^{-1} dry weight to 23 mmol kg^{-1} dry weight and [ATP] remained almost constant, changing from 22 mmol kg^{-1} to 18 mmol kg^{-1} dry weight [27]. Thus, in these *in vivo* experiments with human subjects, the relative increases in [ADP] and [AMP] from rest to exhaustion were 4.5-fold and 48-fold, respectively, while [ATP] dropped by only 20%.

Similar results are consistently observed in various experimental setups, including ^{31}P-NMR methods (see [28] and references therein), always however, with the caveat that concentrations of ADP and AMP have only been estimated [22]. Nevertheless, within these limitations, the data and the resulting relative nucleotide ratios may indicate a trend that is also followed under the much more complex situation *in vivo*. Thus, globally, AMP would appear at significant concentrations in metabolically stressed cells, the AMP:ATP ratio would change much more than the ADP:ATP ratio, and the relative increase in the AMP:ATP ratio from rest to activation or cellular stress would be by orders of magnitude [7]. Therefore, increasing [AMP] or AMP:ATP ratio would be ideally suited to report changes in the cellular energy state. As shown in Fig. 9.2, the changes in PCr, creatine (Cr), P_i, ATP, ADP, and AMP concentrations can also be visualized graphically as a function of the total "energy-rich" phosphorylated compounds, when considering the fast reactions of AdK and CK in equilibrium [29]. Although this may be an oversimplification (see below), the model reflects the decrease in phospho-compounds that occurs during the transition from rest to increasing workload in skeletal muscle (Fig. 9.2A). The resulting relative changes in the PCr:Cr, ADP:ATP, and AMP:ATP ratios are depicted in Fig. 9.2B. They illustrate that the PCr:Cr ratio falls first, then the ADP:ATP ratio begins to rise, and finally the AMP:ATP ratio rises much more sharply, consistent with the above experimental data. However, even initial ATP consumption, which is almost completely replenished from PCr, is immediately translated into a change in [AMP] by the action of AdK, as shown by plotting adenine nucleotide content on a logarithmic scale (Fig. 9.2C).

Again, although the exact figures for free [ADP] and [AMP] *in vivo* are actually unknown and currently immeasurable [26, 29], the above experimental and theoretical approximations allow some insight into their role in energy signaling. Neither PCr and Cr nor ATP would be well suited as energy status indicators *per se* for all physiological situations, in particular those characterized by a depleted [PCr] pool. PCr and Cr are present in muscle and many other excitable cells at concentrations in the millimolar range and change transiently and in relative terms only by a factor of two- to fivefold; ATP then remains stable for a long time after cell activation (Fig. 9.2A). On the other hand, [ADP] would fluctuate sufficiently to serve as a signaling metabolite but may have some limitations due to its binding to cellular proteins (e.g., F-actin [23]) and a multitude of cellular enzymes. The situation is much different for AMP, although it can also bind to some abundant intracellular enzymes such as glycogen phosphorylase and fructose-1,6-bisphosphatase. As outlined above, [AMP] possesses the typical and

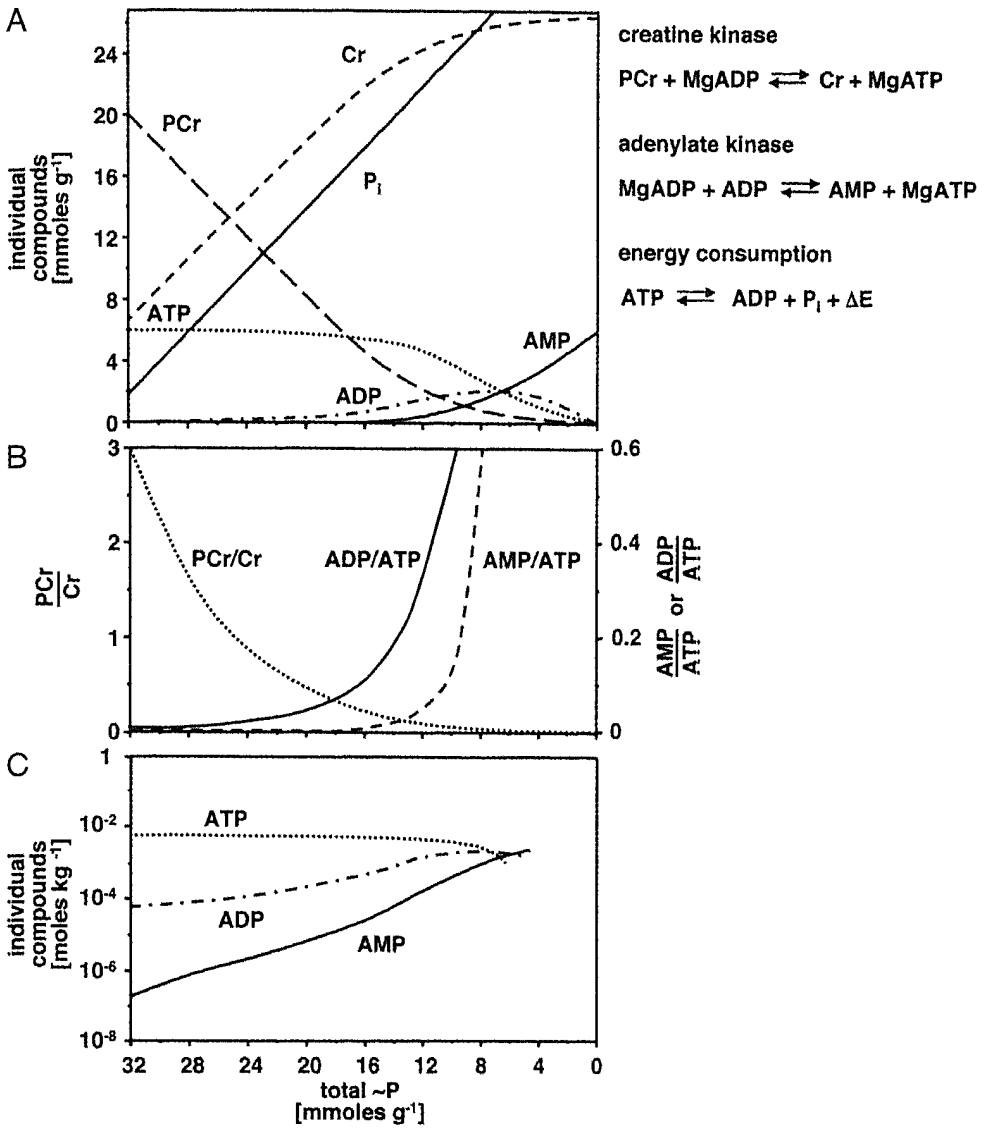

Fig. 9.2 Model calculations for changes in the pool sizes of energy-related metabolites during cell stimulation until exhaustion. (A) Global cellular concentration changes in phosphocreatine [PCr] and adenine nucleotides ([ATP], [ADP] and [AMP]), inorganic phosphate [P_i], and creatine [Cr] calculated from the reactions of CK, AdK, and a generalized ATPase (indicated to the right) at decreasing "high-energy" phosphates, corresponding to a transition from rest to high workload and exhaustion (modified from [29]). At rest, [PCr] and [ATP] are high, while [ADP] is very low and [AMP] is virtually non-measurable. With high-energy phosphate consumption, [ATP] remains constant until more than 80% of the PCr pool is consumed, and only then do [ADP] and [AMP] start to rise dramatically. Note: This simplified model assumes that the CK and AdK reactions work at equilibrium, which may not be true in all *in vivo* situations. In addition, it does not account for specific subcellular localizations of CK and AdK isoforms, which lead to local deviations in metabolite concentrations. (B) Changes in global ratios of PCr:Cr, ADP:ATP, and AMP:ATP as calculated from values shown in (A). Note that the adenine nucleotide ratios do not start to rise markedly until the PCr:Cr ratio has decreased by around 80%. (C) Changes in individual adenine nucleotide concentrations taken from (A) but shown on a logarithmic scale. Note: The relative changes in [AMP], which is present under normal conditions at cell rest at low micromolar concentrations only, can be very large. [AMP] increases from the beginning exponentially over several orders of magnitude, as reflected by the approximately linear increase on the logarithmic scale. This will also transmit into large relative changes in the AMP:ATP ratio, which is the signal sensed by AMPK.

ideal characteristics of an energy signaling metabolite and is well suited as a reporter or "second messenger" for sensing cellular energy stress. Of course, there has to be a corresponding AMP sensor such as a protein kinase, which would respond to changes in [AMP] within the low micromolar concentration range and transmit the signal onwards. AMPK perfectly fulfills all these requirements.

9.2.2 A Closer Look at the Role of Creatine Kinase and Adenylate Kinase in AMPK Activation

With their high affinity for ADP and very fast turnover rates, CK and AdK are able to rephosphorylate ADP and thus efficiently remove the ADP generated by ATP hydrolysis [6] (Fig. 9.1). Removal of free ADP in the cell is essential because this nucleotide has been shown to inhibit several ATPases by product inhibition or allosteric regulation (see [18]). CK and AdK are micro-compartmentalized at various subcellular sites of either ATP utilization (ATPases or ATP-gated processes) or ATP generation (mitochondria and glycolysis), where CK and/or AdK form functionally coupled multi-enzyme complexes to constitute an intricate energy-buffering and high-energy phosphoryl transfer system (see also Chapters 3, 7, 8, and 11) [20, 30–33].

Because it is difficult to know the exact concentrations of free ADP and AMP (see above), it may be helpful to analyze the respective apparent K_m values of enzymes participating in the "energy decay cascade" (see Fig. 9.1) to appreciate preferred pathways and possible bottlenecks for accumulation of a substrate or product. As cells increase their basal rate of ATP hydrolysis, e.g., upon activation, the CK and AdK reactions start to run sequentially or in parallel. It is likely that CK, with a K_m for ADP in the range of 35–80 μM [34–36], first draws on the very large PCr energy pool (15–40 mM, depending on cell type). ATP is regenerated by the CK reaction via transphosphorylation of ADP by PCr. However, if PCr is depleted to any significant extent, free ADP (present under resting conditions at an estimated level of approximately 20 μM) will start to rise (Fig. 9.2). Increasing ADP will then be removed efficiently by the action of AdK, which has an apparent K_m for ADP of 30–40 μM [37, 38], converting two molecules of ADP into 1 ATP and 1 AMP, thus resulting in a rise in AMP [7] (Fig. 9.1). In cells and organs that do not express CK, such as liver, AdK takes over the ATP-buffering function in maintaining a high ATP:ADP ratio and keeping free [ADP] low. This may also explain why the apparent K_m values for ADP of CK and AdK are both low and of the same order of magnitude. Similarly, compensatory mechanisms were observed when the CK system was compromised in muscle, e.g., by the rather specific CK inhibitor DNFB [31], or after CK was ablated in knockout mice [39]. Under the latter conditions, a redistribution of phosphotransfer through glycolytic and AdK networks contributes to energy homeostasis in muscles under genetic and metabolic stress (see Chapter 8).

The appearance in cells of significant [AMP], which is normally estimated to be maintained at low micromolar concentrations [29], presumably takes place only if

much of the cellular PCr is split, i.e., in conditions of relatively severe metabolic stress, such as exhaustive muscle exercise, osmotic shock, oxidative damage, or chronic Ca^{2+} overload (Fig. 9.2). AMP is then sensed as a second messenger by AMPK [10] (Fig. 9.1), which has very low estimated apparent $A_{0.5}$ (the concentration causing half-maximal activation) for AMP of less than 2 μM [40]. Surprisingly, at around 10 μM AMP, the $\alpha 1\beta 1\gamma 1$ and $\alpha 2\beta 2\gamma 1$ complexes of AMPK are already maximally stimulated [40]. If [AMP] should rise very high, it would be converted into IMP by AMP-deaminase (AMPD), which has a rather high apparent K_m for AMP of approximately 1.5 mM [41], or possibly even higher (7 mM) [42]. Interestingly, when muscle was Cr depleted by chronic administration of β-guanidino propionic acid (GPA; known to cause AMPK activation [43]), the kinetic properties of AMPD were changed into a form with a low apparent K_m in the 30 μM range, presumably by phosphorylation [42]. Both AMP and IMP can be dephosphorylated by 5′-nucleotidases to give adenosine and inosine, respectively, which then can leave the cell via equilibrative nucleoside transporters. To prevent an immediate loss of energy-costly purines, the apparent K_m values for AMP and IMP of 5′-nucleotidase, at around 4 mM [44] or 2–5 mM (depending on the free [ADP] [45]), are also high and thus would allow significant accumulation of either AMP or IMP in the cell. Taken together, AMPK with its extremely high affinity for AMP, will respond first to an energy stress signal long before AdK, AMPD, or 5′-nucleotidase, all with significantly higher apparent K_ms for AMP, would start to operate. The K_m values of the enzymes that participate in AMP metabolism would allow a cell or tissue under prolonged energy stress to persistently elevate [AMP] at a level that chronically stimulates AMPK. This would lead to the well-documented long-term effects on gene expression via transcriptional control (see below) [46].

From what has been said so far, it is obvious that CK and AdK are indirectly connected to AMPK signaling. By maintaining cellular [ATP] under cellular energy stress, CK can delay or prevent a strong increase in free [AMP] via AdK and the resulting activation of AMPK [7]. AdK, although also capable of stabilizing cellular [ATP] to a certain degree, will ultimately generate the [AMP] signal that activates AMPK. However, compared to AMPK, both CK and AdK are working in different metabolic areas with completely different mechanisms of operation and in subsequent time frames. CK and AdK are extremely fast enzymes, responding immediately to changes in high-energy phosphate compounds under normal workload that keep local ATP:ADP ratios high and thus regulate energy buffering and energy flux (see Chapters 3, 7, and 11). AMPK signaling involves its activation by upstream kinases and phosphorylation of downstream targets under cellular stress and probably a number of other stimuli (see below). Although AMP could rise early after cell activation (see Fig. 9.2C), cell signaling by AMPK would be expected to set in at a later point in time compared with the action of CK and AdK. There are also obvious tissue-specific differences with respect to the functions and interplays of these kinases. For example, in heart, where phosphorylated metabolites show an astonishing homeostasis at different workloads (different from what is shown in Fig. 9.2 for a tissue such as skeletal muscle),

the CK system provides an exquisite feedback mechanism for energy homeostasis [6], while AMPK may be activated only under more pathological situations such as cardiac ischemia and hypertrophy [47, 48]. By contrast, liver lacks CK (but expresses high levels of AdK), and AMPK plays a prominent regulatory role in this organ [49]. Therefore, the CK/AdK and AMPK systems do not compete with one another but work in a complementary fashion to maintain energy homeostasis.

Some isolated reports have indicated a more direct regulatory crosstalk between the PCr/CK system and AMPK. For example, oral Cr supplementation of rats apparently led to increased glucose uptake, paralleled by increased GLUT4 expression in muscle [50], an event that is also induced after AMPK activation [51]. Using L6 rat skeletal muscle cells, evidence was presented that Cr supplementation led to AMPK phosphorylation [52], which could explain the above *in vivo* finding. In addition, rats that were supplemented with GPA, a strong competitive inhibitor of the creatine transporter [43], showed a marked decrease in total Cr concentration ([PCr] + [Cr]) in muscle that was paralleled by chronic activation of AMPK, indicating some connection between lowered PCr levels or a lowered PCr:Cr ratio and AMPK [53]. Furthermore, evidence was provided for a strong inhibition of AMPK, but not of AMPK kinase, by PCr in muscle that could be alleviated by increasing concentrations of Cr, proposing AMPK as a ratiometric sensor of the PCr:Cr ratio [54]. Conversely, in the same publication, evidence was also provided that AMPK could phosphorylate CK and reduce its activity significantly, that AMPK colocalizes with muscle-type MM-CK at the sarcomeric M-band (see Chapter 7), and that CK and AMPK form a stable complex resistant to high salt [54]. However, a number of laboratories have failed to reproduce some of these experiments. In a detailed investigation, the inhibitory effect of PCr was thrown into doubt by experiments showing that neither PCr nor Cr had a marked direct effect on either AMPK phosphorylation or AMPK activity after activation by LKB1 [55], an upstream AMPK kinase (see below). In addition, PCr and Cr had no effect on LKB1 activity itself. The previously described inhibitory effect of PCr on AMPK may be attributable to the Na^+ salt that was introduced into the AMPK assays along with the PCr preparation, because the disodium salt of PCr had been used [54, 55]. These salt-inhibition experiments have been confirmed with bacterially expressed and highly purified AMPK ([55], Suter and Neumann, unpublished). In addition, PCr as the disodium salt inhibits a number of other protein kinases, suggesting that any effects are nonspecific (Hardie, unpublished). Thus, it now appears that neither PCr nor Cr exerts a direct effect on AMPK or its upstream kinases LKB1 and CaMKK (Suter and Neumann, unpublished). In a human study involving retraining after immobilization with or without Cr supplementation, it was found that retraining activated AMPK to the same extent in both groups; thus, AMPK appears not to be involved in the beneficial effects of oral Cr supplementation during rehabilitation [56].

As to the proposed stable complex between AMPK and CK [54], in a cell *in vivo* this would lead to permanent complex formation of AMPK, a protein of rather low abundance, with the very abundant CK. This would not make much physiological sense for the manifold proposed functions of AMPK [57]. In fact, no direct

interaction between recombinant CK and AMPK isoforms could be detected by surface plasmon resonance spectroscopy (Schlattner et al., unpublished). Concerning the phosphorylation of CK by AMPK, confirmation of the stoichiometry of phosphorylation and identification of the AMPK phosphosite on CK, as well as a demonstration that phosphorylation also occurs *in vivo*, e.g., by using anti-CK phosphosite-specific antibodies, are still lacking, and this issue remains a matter of uncertainty. Therefore, considering the present knowledge, no more than an indirect connection between CK, AdK, and AMPK can be considered an established fact (see Figs. 9.1 and 9.2).

9.2.3
Some Considerations on the Sensing and Signaling Properties of AMPK

Although it is well known that AMPK binds AMP at concentrations in the low micromolar range and is activated by elevated AMP:ATP ratios [12, 58], only recently exact data for allosteric activation of $\alpha 1\beta 1\gamma 1$ and $\alpha 2\beta 2\gamma 1$ AMPK complexes have become available [40]. Using purified recombinant AMPK and a novel HPLC-based AMPK activity *in vitro* assay, it was possible to determine half-maximal and maximal stimulation of AMPK at concentrations as low as approximately 2 μM and 10 μM AMP, respectively [40]. In addition, it was found that AMPK was stimulated by a factor of 1000-fold by the combined action of upstream kinase and AMP [40]. Obviously, AMP and AMPK are extremely well suited to function together as a cellular energy indicator and sensor pair at the physiologically relevant AMP concentrations. AMPK can thus be portrayed as a "ratiometric energy sensor" monitoring the AMP:ATP ratio, which is extremely low at rest and rises exponentially under cellular stress conditions (Fig. 9.2B,C).

Such a paradigm can be very effective for rapid and stringent regulation of cellular processes, as also exemplified in Ca^{2+} signaling, where relatively small changes in the very low resting [Ca^{2+}] during cell activation, in combination with local Ca^{2+} sparks and Ca^{2+} waves, produce strong dynamic and versatile signaling effects [59]. Thus, *in vivo* functioning of AMP/AMPK signaling may be analogous to that observed in Ca^{2+} signaling and Ca^{2+} sensing by Ca^{2+}-responsive proteins. The free cytosolic [Ca^{2+}] corresponds to about 0.05 μM at rest, but increases up to 50 μM after cell activation. Ca^{2+} sensors are able to bind Ca^{2+} specifically at sub-micromolar concentrations, even in the presence of the millimolar concentrations of free Mg^{2+} typically found in eukaryotic cells [60]. AMPK is also able to sense sub-micromolar concentrations of AMP in the presence of millimolar concentrations of ATP [61]. As in Ca^{2+} signaling, a small absolute change in [AMP] can exert a large effect on cell metabolism, transmitted via AMPK. As observed in Ca^{2+} signaling [59] or cyclic AMP signaling [62], AMP/AMPK signaling is also likely to be organized in microdomains in specific subcellular AMP/AMPK compartments, such that metabolic "AMP sparks" and "AMP waves" that are either contained locally or propagated through the cell, respectively, may lead to selective temporal and spatial activation of AMPK and phosphorylation of its downstream targets within a cell.

9.3
Mammalian AMPK Is a Member of an Ancient, Conserved Protein Kinase Family

9.3.1
AMPK in the Eukaryotic Kinome

AMPK is a serine/threonine protein kinase that is not particularly closely related to, but still sometimes is confused with, the cyclic AMP-dependent protein kinase (PKA). In fact, the AMPK system was one of the first protein kinase cascades described back in the 1970s. However, it was not until 1987 that Hardie and Carling showed that AMPK was a multi-substrate kinase that was activated by AMP [63, 64]. With the increasing number of genomes that have been sequenced, two important facts have become apparent. Firstly, AMPK is part of a structurally related family of serine/threonine protein kinases (the AMPK-related kinases [ARKs]) comprising around 14 members [65]. The function of most of these members is not completely clear, although some appear to have a role in cell polarity, cell proliferation, and cell communication (see below). Secondly, AMPK homologues occur in all eukaryotic genomes that have been sequenced to date, from protozoa and yeast to plants and human [66]. AMPK is therefore phylogenetically one of the most ancient eukaryotic protein kinases. It appears to have evolved at a very early stage during the development of eukaryotes and may fulfill basic functions specific to the highly compartmentalized eukaryotic cell.

9.3.2
Structure of AMPK

The basic functional unit of AMPK, from yeast [67] to mammals, seems to be a heterotrimeric complex consisting of a catalytic α-subunit and regulatory β- and γ-subunits, as schematically depicted in Fig. 9.3 (for reviews, see [64, 68]). Further complexity is added by the existence of multiple subunit genes encoding each subunit, giving rise to different isoforms and splice variants. In mammals, there are two α-, two β-, and three γ-subunit genes, with the $\gamma 2$ and $\gamma 3$ genes also giving rise to short and long splice variants.

The α-subunit bears a classical protein kinase catalytic domain, whose X-ray structure has been solved [69], and an inhibitory activation loop involved in its regulation [70, 71]. Different phosphorylation sites that depend on upstream kinases have been mapped, including the "activation loop" or "T-loop" Thr172, which is the key site for AMPK activation by different upstream kinases (see below). The β-subunit seems to interact with both the α- and the γ-subunits [72, 73] and also bears a glycogen-binding domain [72, 74] an N-terminal myristoyl moiety, and some specific phosphorylation sites. While the function of the latter modifications is not clear, the X-ray structure of the glycogen-binding domain has been solved [75], and functions in subcellular targeting to glycogen granules and regulation of glycogen metabolism have been proposed. The γ-subunits have attracted most of the recent interest. These subunits show a large variability, with three isoforms encoded by distinct genes ($\gamma 1$, $\gamma 2$, and $\gamma 3$) and with $\gamma 2$ and $\gamma 3$ exist-

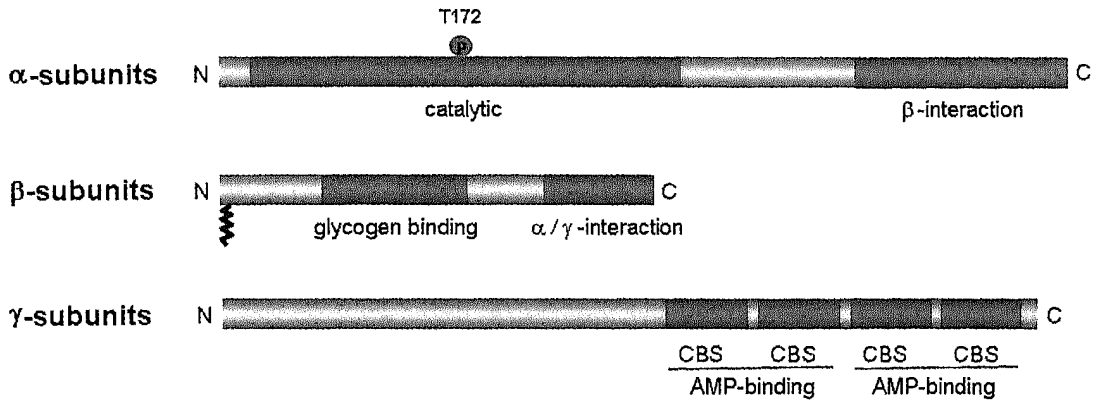

Fig. 9.3 Domain structure of mammalian AMPK. Schematic representation of the domain structure of the three AMPK subunits with selected secondary modifications (activating phosphorylation of the α-subunit T172 residue and N-terminal myristoylation of the β-subunit). Isoforms exist for each subunit, with α1/α2 and β1/β2 showing the most homology, while the γ-subunit isoforms (γ1–3) and their splice variants (γ2/γ3 long and short) differ at their N-terminus (here, a long splice variant is shown).

ing as splice variants, having in their long forms large N-terminal extensions. Most importantly, each γ-subunit contains four so-called cystathionine β-synthetase (CBS) motifs that are organized in tandem pairs to form two AMP-binding sites, the so-called Bateman domains [76]. Several mutations have been mapped in these domains that lead to glycogen storage disorders and a related hereditary heart disease (see below) [77–80].

Although there are crystal structures for the kinase domain of the yeast homologue Snf1 [69], the human α2 subunit (PDB: 2H6D), and a small glycogen-binding domain of rat β1 [75] and human β2 (PDB: 2F15), the molecular structure of the heterotrimeric complex remains unknown. Based on indirect evidence, a current model proposes that the C-terminal domain of the β-subunit binds the C-terminal region of the α-subunit and an unknown region of the γ-subunit [72, 73]. This topology is consistent with a recent X-ray structure of the AMPK orthologue of the yeast *S. pombe* [81]. However, the yeast complex is possibly not activated by AMP, and has been highly truncated for successful crystallization, thus limiting functional and mechanistic insight. Only a structure of full-length heterotrimeric complex at molecular resolution will be able to settle these issues.

9.4 Regulation of AMPK

One important way to transmit extra- and intracellular signals is by reversible phosphorylation of proteins that participate in a cell signaling pathway. Phosphorylation and dephosphorylation of proteins is achieved by protein kinases and phosphatases, respectively. Among the >500 protein kinases encoded by the human genome [82], AMPK plays an important role in the regulation of mam-

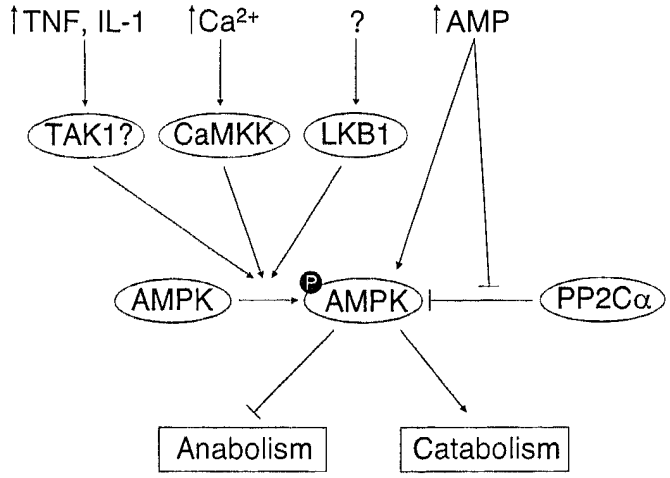

Fig. 9.4 Regulation of AMP-activated protein kinase (AMPK). LKB1 and Ca^{2+}/calmodulin-dependent protein kinase kinase (CaMKK) are capable of phosphorylating and activating AMPK in intact cells or *in vivo*, but the ability of transforming growth factor β–activated protein kinase (TAK1) to activate AMPK so far has been demonstrated only *in vitro*. Protein phosphatase 2Cα (PP2Cα) can dephosphorylate and deactivate AMPK. AMP allosterically activates AMPK and inhibits its dephosphorylation by PP2Cα. Ca^{2+} or tumor necrosis factor (TNF) and interleukin-1 (IL-1) are upstream signals that activate CaMKK and TAK1, respectively, but regulation of LKB1, if any, is not known. After activation, AMPK inhibits anabolic pathways to preserve cellular energy and activates catabolism to replenish ATP.

malian cellular and whole-body energy homeostasis [1, 83]. The requirement of biological systems to maintain high cellular ATP concentrations at all times, even under conditions of high metabolic workload, is reflected by dynamic and stringent control of AMPK activity. Regulation of AMPK activity is an elaborate process involving at least two upstream kinases and at least one protein phosphatase, as well as allosteric mechanisms [1, 8]. The complex regulatory network and metabolic signaling that influence the activity of AMPK is depicted schematically in Fig. 9.4. In general, following activation, AMPK inhibits anabolic pathways, whereas catabolic pathways are activated, although recent studies show that the kinase also regulates processes other than energy metabolism (see Section 9.5).

9.4.1 Allosteric Regulation of AMPK

The molecular mechanism of AMPK activation involves binding of AMP to the regulatory γ-subunit. The γ-subunits contain the two tandem Bateman domains [76], each of which binds one molecule of AMP or ATP (in a mutually exclusive manner) when expressed independently in bacteria [79]. When the tandem Bateman domains are expressed together, they bind two molecules of AMP or ATP with a Hill coefficient of 2, indicating strongly cooperative binding [79]. In the γ2 isoform, which is expressed at particularly high levels in cardiac muscle,

several dominant-acting mutations are associated with heart diseases of varying degrees of severity, involving cardiac hypertrophy, contractile dysfunction, and arrhythmias. These mutations have been found to impair both the binding of AMP to the isolated Bateman domains and the activation of the heterotrimeric complex by AMP [79, 84], proving that the Bateman domains represent the regulatory nucleotide-binding sites of the complex. The disease-causing mutations can occur in either of the tandem domains, suggesting that AMP has to bind to both domains for activation to occur. At least nine different point mutations have now been reported. Five of them (R302Q, H383R, R384T, R531G, and R531Q) convert positively charged side chains of conserved basic residues in the first, second, and fourth CBS motifs (which are probably involved in binding the negatively charged phosphate groups of AMP and ATP) to amino acids with uncharged side chains [79, 84].

Although defects in AMP activation represent a loss-of-function effect, the mutations also cause reduced binding of the inhibitory nucleotide, ATP [79, 84], and this appears to increase the basal activity of the mutant complex [79, 84–86]. This gain-of-function effect probably explains why these mutations are dominant in nature. The primary cause of the heart diseases associated with these mutations appears to be excessive storage of glycogen in the cardiac myocytes [84, 85], possibly because the increased basal activity of the AMPK complex leads to increased basal glucose uptake. In cardiac myocytes, this excessive glycogen storage is harmful and leads to improper development and function of the heart muscle. Interestingly, a similar mutation (R200Q) affecting a conserved basic residue in the first CBS motif of the $\gamma 3$ isoform (which is expressed at the highest levels in skeletal muscle) leads to abnormal deposition of glycogen in skeletal muscle in pigs [77]. The high glycogen content affects meat quality, but muscle function in the pigs does not appear to be adversely affected.

Recent studies in Hardie's laboratory have suggested a novel mechanism to explain allosteric activation by AMP [87]. The second CBS motif in the γ-subunits of all species contains a "pseudosubstrate" sequence that resembles the sequence at target sites for AMPK, except that it has a non-phosphorylatable hydrophobic residue in place of serine or threonine. Evidence was provided that in the absence of AMP, this sequence interacts with and occludes the substrate-binding groove on the kinase domain of the α-subunit [87]. However, binding of AMP prevents this interaction, thus relieving inhibition of the kinase domain. Intriguingly, basic residues in the second CBS motif (H383 and R384 in human $\gamma 2$) proposed to be involved in the interaction of the pseudosubstrate with the substrate-binding groove are identical with those thought to be involved with binding the α phosphate group of AMP. If this model is correct, inhibition of the kinase domain by the pseudosubstrate sequence and binding of AMP would be mutually exclusive, providing an elegant explanation as to how AMP causes allosteric activation [87]. Although the details remain unclear, inhibition of the kinase domain by the pseudosubstrate sequence could act in concert with inhibition by the proposed autoinhibitory domain on the α-subunit itself, because the latter is believed to bind on the opposite face of the kinase domain to the substrate-binding groove [71].

Besides allosterically stimulating AMPK activity, binding of AMP also protects the complex against dephosphorylation of Thr172 by protein phosphatases, especially PP2Cα [40, 58]. It was originally reported that AMP also promoted phosphorylation of AMPK by an upstream kinase [88], later identified as LKB1 [89, 95]. However, this has not been observed in highly purified, reconstituted systems using AMPK and LKB1 complexes expressed in bacteria [40, 90]. This raises the possibility that the effects of AMP to promote phosphorylation observed previously, with preparations of upstream and downstream kinases purified from mammalian cells [88, 89], may have been due to contamination of one of the preparations with PP2C. Protein phosphatase 2Cα has been detected in preparations of AMPK and LKB1 purified from rat liver, and the effect of AMP was shown to imply protection against α-subunit Thr172 dephosphorylation [90]. In any case the net result, i.e., that AMP stimulates phosphorylation of Thr172 and hence activation of AMPK in the intact cell, remains the same irrespective of the exact mechanism.

Two other pairs of metabolites affected by cellular energy or redox status, namely, PCr/Cr [54] and NADH/NAD^+ [91], also have been claimed to be allosteric regulators of AMPK. However, these findings have not been substantiated by more recent studies [40, 55]. Thus, AMP and ATP, exerting their effects both by allosteric regulation and by modulating dephosphorylation, remain the primary regulators of AMPK activity.

9.4.2 Regulation of AMPK by Upstream Kinases

AMPK complexes are active only after phosphorylation at the T-loop Thr172 of the catalytic α-subunit by upstream kinases [92, 93]. There are at least two protein kinases capable of phosphorylating this residue *in vivo*, namely, LKB1 [89, 94, 95] and Ca^{2+}/calmodulin-dependent kinase kinase, especially the β isoform (CaMKKβ) [96–98]. LKB1 is a serine/threonine kinase with a tumor suppressor function. Mutations in the *LKB1* gene (also known as *STK11*) cause an inherited cancer susceptibility termed Peutz-Jeghers cancer syndrome (PJS) and are also observed in some sporadic cancers. In lower eukaryotes such as *Drosophila melanogaster* and *Caenorhabditis elegans*, genetic studies of LKB1 orthologs identified them as playing important roles in cell polarity (reviewed in [2, 99]). Thus, the involvement of LKB1 in the regulation of energy metabolism by activation of AMPK was unexpected and suggested a new connection between AMPK and cancer [17, 100]. Besides phosphorylating AMPK, LKB1 may exert its effects by phosphorylating and activating at least 12 other protein kinases, all members of the AMPK-related kinase family [65, 101]. Interestingly, some of these appear to have roles in the establishment of cell polarity. However, the available evidence suggests that the tumor suppressor function of LKB1 is related to its ability to activate AMPK, at least in part via the ability of the latter to inhibit mTOR signaling linked to cell growth and proliferation [2, 102, 103].

The active form of LKB is a heterotrimeric complex with an armadillo repeat-containing protein, called mouse protein 25 (MO25), and a catalytically inactive protein kinase, a pseudokinase called STRAD, both of which exist as two different isoforms, α and β [104]. In cell-free assays the LKB1–MO25α–STRADα complex is more potent in activating AMPK compared with complexes containing the β isoforms [89]. STRAD contains a pseudokinase domain related to those in conventional serine/threonine protein kinases, but it appears to be catalytically inactive. Interestingly, this domain still binds ATP, although this does not appear to be necessary for its ability to activate LKB1 [105].

While evidence is compelling that LKB1 is indeed the primary upstream kinase for AMPK in many tissues including muscle [106] and liver [107], it remains unclear whether LKB1 is itself regulated. Interestingly, it does not require phosphorylation of its own T-loop residue to be active [105], although binding of LKB1 to STRAD–MO25 is essential [99]. LKB1 is phosphorylated at multiple sites by several kinases [2, 99], but mutation of these sites does not alter the activity of the kinase, and it has therefore been proposed that the LKB1 complex is constitutively active [2]. Because it acts upstream of at least 14 downstream kinases that appear to have distinct functions, it may actually make sense for this to be the case, with any regulatory molecules such as AMP binding to the downstream kinases instead. The idea that LKB1 is constitutively active is also consistent with findings that active complexes were obtained after co-expression of LKB1, MO25α, and STRADα in *E. coli* ([40] and Neumann et al. (2007), Mol. Biotechnol., in press). Because bacteria do not perform most of the post-translational protein modifications seen in higher eukaryotes (such as phosphorylation), this suggests that prior covalent modifications are not required for LKB1 activity. When expressed on its own in mammalian cells, LKB1 is exclusively nuclear, but co-expression with MO25 and STRAD causes it to relocalize from the nucleus to the cytoplasm [104]. The nuclear form that occurs in the absence of MO25 and STRAD appears to be completely inactive, but the question of whether this form has any physiological relevance remains unclear.

CaMKKβ and, to a lesser extent, also CaMKKα are capable of phosphorylating and activating AMPK, thereby linking cytoplasmic Ca^{2+} levels to AMPK activity [96–98]. These CaMKKs were originally identified as the upstream kinases in the Ca^{2+}/calmodulin-dependent protein kinase-I/IV (CaMKI/CaMKIV) signaling pathways [108]. In response to various stimuli, intracellular Ca^{2+} levels increase from around 100 nM to 1–2 μM [108]. One of the key binding proteins that convey signals elicited by elevated Ca^{2+} levels is calmodulin (CaM). Ca^{2+} binding to CaM induces a local conformational change, causing a global structural change that allows the Ca^{2+}–CaM complex to bind and activate several enzymes, including CaMKKs [109]. CaMKKs in turn phosphorylate and activate CaMKI and CaMKIV, which are themselves Ca^{2+}–CaM dependent [109–111].

The tissue distribution of CaMKKs, with the highest levels in brain, suggests that they may play important roles in the nervous system. Indeed, mutant mice lacking CaMKKβ were impaired in some hippocampal-related types of long-term

memory formation [112]. A role for AMPK in regions of the hypothalamus that are involved in the regulation of food intake has been described recently [113]. Whether AMPK has crucial functions in other regions of the brain has not been investigated, but given the ubiquitous expression of AMPK, coupled with the high energy demands and the importance of Ca^{2+}-dependent signaling in excitable cells [114], one would expect the AMPK pathway to be of particular importance in the nervous system. Indeed, several brain-specific potential downstream-targets of AMPK have been recently identified based on a new proteomic screening approach (MudSeeK) (R. Turk et al. (2007), J. Proteome Res., in press). Although CaMKKα is largely restricted to neuronal tissue, CaMKKβ is also expressed in cells of the endothelial/hematopoietic lineage. It has recently been reported that AMPK is activated by thrombin in endothelial cells [115] and by stimulation of the antigen receptor in T cells [116], and in both cases this appeared to involve the Ca^{2+}–CaMKK–AMPK pathway.

Very recently, mammalian transforming growth factor β–activated kinase (TAK1), in complex with its accessory protein TAB1, was identified as a third possible upstream kinase capable of activating AMPK, using expression of a human kinase library in a yeast strain in which the three endogenous upstream kinases had been knocked out [117]. This suggests that TAK1 can activate the yeast AMPK ortholog but does not prove that this is physiologically relevant. A role for TAK1 upstream of AMPK in the heart was also recently suggested [118], although in that case it was proposed that TAK1 was in fact upstream of LKB1, which is not consistent with the data obtained from the yeast system [117]. The role of TAK1 in the AMPK system therefore remains uncertain.

AMPK is also a direct target of Akt/PKB [119, 120] and PKA [121], which appear to phosphorylate the same sites on the catalytic α-subunit, i.e., Ser485 in α1 or Ser491, the equivalent site in α2. Phosphorylation of this site, which also may be catalyzed by AMPK itself in an autophosphorylation reaction, appears to reduce the accessibility of Thr172 to upstream kinases [119, 121]. CaMKK is also phosphorylated and inhibited by PKA [109], while LKB1 is phosphorylated by PKA at its C-terminal Ser428 residue. In the latter case this does not appear to affect its activity or its localization [122]. Nevertheless, these observations suggest that possible crosstalk between the AMPK pathway and other signaling pathways warrants further investigation.

9.4.3 Inactivation of AMPK by Protein Phosphatases

Although much progress has been made in identifying the upstream kinases that activate AMPK by phosphorylating Thr172, much less is known about the protein phosphatases that reverse this process. Protein phosphatase 2Cα (PP2Cα) and protein phosphatase 2A (PP2A) are both capable of dephosphorylating AMPK at Thr172 *in vitro*, whereas protein phosphatase 1 (PP1) is much less effective. However, PP2A does not seem likely to dephosphorylate AMPK *in vivo*, as has been shown by the use of inhibitors of this protein phosphatase family in cultured cells

[58, 123, 124]. Unfortunately, specific inhibitors of PP2Cα have not been described and supportive genetic evidence is lacking, thus leaving open the possibility that protein phosphatases other than or apart from PP2Cα might be responsible for the inactivation of AMPK *in vivo*. However, AMP clearly attenuates the dephosphorylation of AMPK by PP2Cα, which can be attributed to binding of AMP to the regulatory γ-subunit of AMPK rather than direct inhibition of PP2Cα by AMP [40, 58, 90]. Moreover, a recent study showing an inverse relationship between PP2C expression and the phosphorylation state of AMPK at Thr172 supports the conclusion that PP2C plays a major role in AMPK deactivation *in vivo* [125].

9.5 Signaling Downstream of AMPK

9.5.1 Role of AMPK

The role of AMPK as a sensor of cellular energy status was proposed in part on the basis that its first described metabolic targets were in energy-consuming pathways and were inactivated upon phosphorylation, whereas enzymes of catabolic processes were activated by phosphorylation [1, 12, 64, 126]. AMPK is activated in response to hypoxia or ischemia in several tissues and in response to an increase in energy demand during exercise in skeletal muscle. The phosphorylation-induced inactivation of acetyl-CoA carboxylase (ACC), 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR), and glycogen synthase (GS) by AMPK inhibits *de novo* fatty acid, cholesterol, and glycogen synthesis, respectively. AMPK activation also inhibits protein synthesis, a major consumer of ATP, both via the phosphorylation of eukaryotic elongation factor-2 (eEF2) kinase (eEF2K), leading in turn to the phosphorylation and inactivation of eEF2, and via a reduction in mTOR signaling. In contrast, the AMPK-induced increase in glucose uptake in skeletal muscle and 6-phosphofructo-2-kinase activation in cardiac muscle stimulate glycolysis, thereby helping to maintain intracellular ATP levels. ACC phosphorylation (particularly phosphorylation of the ACC2 isoform) is also important in this respect, because its inactivation by AMPK is one factor that determines cytosolic malonyl-CoA concentrations [47]. A decrease in malonyl-CoA stimulates fatty acid oxidation by relieving inhibition of carnitine palmitoyl-CoA acyl transferase-1 (CPT1), via which fatty acids enter mitochondria.

It has recently become apparent that AMPK is also involved in whole-body energy homeostasis, stimulating energy expenditure by promoting fatty acid and glucose oxidation in the periphery, while at the same time inhibiting energy intake via effects on appetite in the hypothalamus [12]. The role of AMPK probably needs to be broadened even further following the discovery of upstream activating AMPK kinases other than LKB1, i.e., CaMKKs [96–98] and perhaps also TAK1 [117]. AMPK activation via these pathways would not necessarily occur only dur-

ing energy stress. While the primary targets for AMPK have been involved mainly in energy homeostasis, AMPK now seems to control non-metabolic processes such as cell growth, progression through the cell cycle, and organization of the cytoskeleton.

9.5.2 The AMPK Recognition Motif

A list of some AMPK targets and their phosphorylation site sequences is given in Table 9.1. The core substrate recognition motif for AMPK is $\phi(X,\beta)XXS/TXXX\phi$, where ϕ is a hydrophobic residue (M, V, L, I, or F), β is a basic residue (R, K, or H), and the parentheses indicate that the order of residues at the P-4 and P-3 positions is not critical [127]. For ACC1 and some other substrates, an amphipathic helix stretching from P-16 to P-5 is also a positive determinant [128]. It can be seen in Table 9.1 that AMPK is predominantly a serine-directed protein kinase and that in some proposed targets the consensus is not strictly adhered to. For example, the site in eEF2K does not contain the basic residue at the −3 position, and in eEF2K, mTOR, and the Ser1176 site of NOS III, the +4 position is not occupied by a hydrophobic amino acid. However, the P+4 site is less critical and NOS III contains a glutamine residue that can substitute for a hydrophobic residue at this position [128]. An interesting possibility is that the various AMPK isoforms might differ slightly in substrate recognition. Without casting doubt on the results published in the literature, some caution needs to be exercised before a target protein can be considered a *bona fide* AMPK substrate (see below). For example, some AMPK targets have been reported on the basis of overexpression of recombinant proteins in eukaryotic cells incubated with pharmacological activators of AMPK, such as AICA riboside, or subjected to treatments that deplete intracellular ATP. These methods are not necessarily completely specific for AMPK, and in any case this approach does not prove that the protein is *directly* phosphorylated by AMPK. Also in some cases, the sites for AMPK phosphorylation have not been identified formally by direct sequencing or mass spectrometry. Instead, this has been performed by searching target protein sequences for the AMPK consensus, then mutating putative phosphorylation sites to negatively charged (to mimic phosphorylation) or non-phosphorylatable residues, and looking at effects on phosphorylation or function. This method is prone to error because mutations can lead to conformational effects at sites remote from the mutation itself.

9.5.3 A Note of Caution: The Krebs–Beavo Criteria

In a classic review of reversible phosphorylation of enzymes, Krebs and Beavo proposed four criteria for establishing that changes in enzyme activity as a result of phosphorylation/dephosphorylation would be of physiological relevance [129]. These criteria can be extended to any changes in protein function resulting from

reversible phosphorylation, but sadly they have been met for only a few AMPK targets and, indeed, for targets of most other protein kinases. The four criteria are as follows:

1. The enzyme (protein) should be phosphorylated stoichiometrically "at a significant rate" in cell-free assays by an appropriate protein kinase and dephosphorylated by a protein phosphatase.
2. The functional properties of the enzyme (protein) should undergo meaningful changes that correlate with the extent of phosphorylation.
3. The enzyme (protein) should be shown to be phosphorylated and dephosphorylated *in vivo* or in an intact cell system with accompanying functional changes. This criterion is easier to satisfy than it was at the time when Krebs and Beavo wrote their review. In those days, to establish that a protein was phosphorylated *in vivo* in response to activation of a kinase, labeling of the ATP pool of isolated cells or organs with millicurie amounts of ^{32}P-labeled inorganic phosphate had to be used. Nowadays, changes in the phosphorylation states of proteins at specific sites can easily be assessed without radioactive labeling by using phosphorylation site–specific antibodies.
4. There should be a correlation between cellular levels of protein kinase effectors and the extent of phosphorylation of the enzyme. When Krebs and Beavo proposed their criteria for establishing physiologically relevant phosphorylation/dephosphorylation of enzymes, this was based mainly on the study of protein phosphorylation in response to changes in cyclic AMP. To verify that AMPK is the physiologically relevant kinase for a particular target, changes in cellular AMPK activity should be correlated with the extent of phosphorylation of the substrate protein at the relevant site. This could be done by incubating cells with a selective AMPK inhibitor, such as "compound C" [130], by transfecting cells with vectors overexpressing constitutively active and/or dominant-negative forms of AMPK [131], by using genetic approaches [132, 133], by knockdown of the AMPK subunits by transfecting small interfering RNAs [134], or by using mouse knockout models [132, 133]. The catalytic subunit knockouts ($\alpha1^{-/-}$ and $\alpha2^{-/-}$) have proved useful in this respect. Unfortunately, a double knockout is embryonic lethal, although mouse embryo fibroblasts in which both catalytic subunit isoforms have been deleted are now available. In addition, tissue-specific, conditional double knockouts are also now being generated.

All of the approaches mentioned under criterion 4 have some drawbacks (e.g., lack of isoform specificity with the dominant-negative approach, incomplete AMPK inhibition using the siRNA approach, or compensatory changes in the expression of other AMPK catalytic subunit isoforms or indeed of other related kinases when using knockdowns or knockouts of AMPK [see below]). The overexpression of a dominant-negative construct may not target endogenous AMPK in the right intracellular compartment, or it may titrate out signaling molecules

Table 9.1 Phosphorylation sites in AMPK substrate proteins. The phosphorylated serine/threonine residue is indicated in red, hydrophobic residues that form the AMPK consensus are in blue and basic residues of the consensus are in green.

Target	Phosphorylation site sequence	Comments A: Activation I: Inactivation or inhibition	Refs.
Metabolic targets			
Ser79 of rat acetyl-CoA carboxylase (ACC1)	HMRSSMSGLHLVK	I. Inhibitory for fatty acid synthesis.	[164]
Ser221 of human acetyl-CoA carboxylase (ACC2)	TMRPSMSGLHLVK	I/A. Inhibits ACC2 but stimulates fatty acid oxidation by lowering [malonyl-CoA]. Site identified by homology with the site in rat ACC1.	[165]
Ser871 of rat 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR)	HMVHNRSKINLQD	I. Inhibitory for cholesterol biosynthesis.	[166]
Ser565 of rat hormone-sensitive lipase (HSL)	SMRRSVSEAALAQ	I. Ser563 upstream of Ser565 is phosphorylated by PKA.	[144]
Ser7 of rabbit muscle glycogen synthase (GS)	PLSRTLSVSSLPG	I. This is the so-called 'site 2' that is also phosphorylated by PKA *in vitro*.	[167]
Ser466 of bovine heart 6-phosphofructo-2-kinase (PFK2FB2)	VRMRRNSFTPLSS	A. Site can also be phosphorylated by PKA, PKCs, and insulin-stimulated protein kinases such as PKB.	[147]
Ser461 of human inducible 6-phosphofructo-2-kinase (PFK2FB3)	PLMRRNSVTPLAS	A. Stimulatory for glycolysis in activated monocytes subjected to hypoxia.	[168]
Transcription factors			
Ser171 of rat transducer of regulated CREB activity (TORC2)	ALNRTSSDSALHT	I. This site is phosphorylated by the AMPK-related Salt-Inducible Kinase-2 (SIK2). Phosphorylation sequesters TORC in the cytoplasm where it cannot co-activate CREB.	[136, 137]

Ser568 of rat carbohydrate-response element-binding protein (ChREBP)	LLRPPESPDAVPE	I. Decreases DNA-binding activity to the promoter of the L-type pyruvate kinase gene.	[169]
Ser304 of human hepatic nuclear factor a (HNF4α)	KIKRLRSQVQVSL	I. Decreases formation of homodimers and DNA binding, and promotes degradation.	[170]
Ser89 of human transcriptional co-activator p300	ELLRSGSSPNLNM	I. Blocks its ability to interact with nuclear receptors such as PPARγ.	[171]
Proteins involved in the control of translation/cell growth			
Thr2446 of the human mammalian target of rapamycin (mTOR)	KRSRTRTDSYSAG	I. Ser2448 downstream of this site can be phosphorylated in response to insulin by PKB or p70S6K.	[159]
Thr1227 of rat tuberous sclerosis complex 2 (TSC2)	TLPRSNTVASFSS	A. This, or the next site, may stabilize the TSC2–TSC1 complex.	[103]
Ser1345 of rat tuberous sclerosis complex 2 (TSC2)	PLSKSSSSPELQT	A. This, or the previous site, may stabilize the TSC2–TSC1 complex.	[103]
Ser398 of human eukaryotic elongation factor 2 kinase (eEF2K)	SLPSSPSSATPHS	A. Inhibits protein synthesis elongation by increasing eEF2 Thr56 phosphorylation.	[172]
Signaling proteins			
Thr494 of human endothelial NO synthase (NOS III)	GITRKKTFKEVAN	A. Increases activity at low [Ca^{2+}/calmodulin].	[148]
Ser1176 of human endothelial NO synthase (NOS III)	SRIRTQSFSLQER	A. This site can be phosphorylated by PKB.	[148]
Ser789 of rat insulin receptor substrate-1 (IRS-1)	HLRLSSSSGRLRY	A. This site is also phosphorylated by Qin-induced kinase (QIK, also known as SIK2).	[173]
Ser621 of human/rat Raf-1	KINRSASEPSLHR	I?	[174]

necessary for the activation of other kinase cascades that converge on AMPK targets. Clearly, where possible, several approaches should be tested in controlled experiments where AMPK activity is monitored in cells, e.g., by measuring phosphorylation of ACC.

9.5.4 The AMPK-related Protein Kinases

Thirteen purified AMPK-related protein kinases (ARKs) in the human kinome, produced by bacterial overexpression, can phosphorylate the AMARA peptide often used to assay AMPK [65], indicating that they have similar specificity and that there may be some overlap between targets for AMPK and ARKs. All the ARKs except maternal embryonic leucine-zipper kinase (MELK) are phosphorylated in their T-loop and activated by LKB1. However, it is not yet known whether CaMKKs can activate any of the ARKs. The AMPK-related kinase Qin-induced kinase (QIK), also known as salt-inducible kinase-2 (SIK2), phosphorylates the same residue in human IRS1 (Ser794) [135] as that phosphorylated by AMPK in rat IRS1 (see Table 9.1) and which increases insulin-stimulated PI 3-kinase activity. Also, SIK2 phosphorylates the Ser171 site in TORC2 [136] that is phosphorylated in response to AMPK activation [137]. The substrate specificities and roles of many of the ARKs remain poorly defined at present [65], although the microtubule affinity-regulating kinases (MARKs) are known to phosphorylate microtubule-associated proteins and are involved in regulating cell polarity [138]. Interestingly, NUAK2 (also known as SNARK, SNF1/AMPK-related kinase), has been reported to be activated, like AMPK, by cellular stresses such as glucose deprivation, ATP depletion, and hyperosmolarity [139]. In rat skeletal muscle, contraction, phenformin treatment, and AICA riboside treatment all increased the activity of the α2 isoform of AMPK, but without increasing the activities of QSK, QIK, MARK2/3, or MARK4 [140].

9.5.5 Multi-site/Hierarchical Phosphorylation and Convergence of Signaling Pathways on AMPK Targets

Virtually all known AMPK targets are multi-site phosphorylated proteins containing either more than one site for AMPK (e.g., ACC, TSC2, and NOS III) or sites for other protein kinases, including PKA (e.g., ACC, glycogen synthase, HSL, heart PFK-2, and eEF2K) and PKB (e.g., heart PFK-2, mTOR, TSC2, and NOS III). Hierarchical phosphorylation of adipocyte HSL by AMPK at Ser565 has an antilipolytic effect [141–143] by decreasing subsequent phosphorylation and activation of HSL by PKA in response to β-agonists [144]. The major regulatory site for PKA was originally thought to be Ser563, adjacent to the AMPK site, although it now appears that Ser559 and/or Ser660 are more important for controlling HSL activity [145]. In muscle cells, but not adipocytes, AMPK activation appears to inhibit adrenaline-induced HSL activation via a decrease in Ser660 rather than

a decrease in Ser563 phosphorylation [146]. AMPK phosphorylation sites are often adjacent to sites phosphorylated by other protein kinases. In other cases, the AMPK site itself is also phosphorylated by a protein kinase from another signaling pathway (see Table 9.1). For example the Ser466 site in heart PFK-2 is phosphorylated not only by AMPK but also by PKA, PKC, and insulin-stimulated protein kinases such as PKB [147]. There are cases where the AMPK and insulin signaling (PKB) pathways converge on the same target at the same site and with the same effects, such as heart PFK-2 [147] and NOS III [148, 149]. Both AMPK activation and insulin also stimulate glucose uptake in muscle via the recruitment of GLUT4 transporters to the plasma membrane. The Rab GTPase-activating protein Akt substrate (AS160) regulates GLUT4 translocation and is phosphorylated at six PKB sites in response to insulin [150]. Moreover, AICA riboside treatment and contraction increased the phosphorylation of AS160 in skeletal muscle [151, 152]; however, the phosphorylation sites for AMPK in AS160 have yet to be identified.

In contrast to the stimulation of glucose uptake, heart PFK-2, and NOS III activation, the insulin and AMPK pathways have opposite effects on protein synthesis. Insulin stimulates protein synthesis by activating the mTOR pathway, which controls translation initiation, and by inactivating eEF2K, allowing elongation to proceed by decreasing eEF2 phosphorylation levels. AMPK activation decreases protein synthesis via eEF2K activation [153] and also by inhibiting mTOR signaling [154], but in some cases the latter effect was evident only after the mTOR pathway had first been stimulated by growth factors/insulin or amino acids [155, 156]. PKB and AMPK have been reported to phosphorylate distinct sites on both TSC2 (Ser939/1086/1088/Thr1422 for PKB [157] and Thr1227/Ser1345 for AMPK [103]) and mTOR (Ser2448 [158] for PKB and Thr2446 [159] for AMPK) with opposing effects. Therefore, the possibility of control via hierarchical phosphorylation by PKB and AMPK of these target proteins obviously needs further investigation.

In conclusion, while the number of papers published on AMPK is still on the rise, the number of *bona fide* target proteins is lagging behind. There are likely to be many more targets to be identified in view of the fact that AMPK is an ancient eukaryotic kinase and that its role extends beyond metabolism and control of cellular and whole-body energy homeostasis.

9.6 Conclusions and Perspectives

Recent years have seen a rapid development in our understanding of the function of AMPK. From a simple "energy sensor" concept, the AMPK signaling pathway has developed into a complex machinery for controlling metabolic stress responses, which integrates signals from within the cell as well as from the cellular environment and the whole organism. In addition to its task in sensing cellular energy status via the high AMP affinity of its allosteric activation mechanism, ad-

ditional activating pathways that involve a growing number of upstream kinases are now being defined. Two of the major drugs widely used for treating type II diabetes, metformin and thiazolidinediones [130, 160], activate AMPK, and their therapeutic effects, particularly in the case of metformin, appear to be largely mediated by AMPK signaling [107]. Furthermore, the identification of a known tumor suppressor, i.e., LKB1 [89, 94, 95], as a critical upstream kinase for AMPK has suggested a role for impaired AMPK in the development of cancer. In fact, activated AMPK negatively regulates proliferation and the cell cycle, mediated by mTOR and p53, respectively [2, 103, 161, 162]. Consequently, AMPK has been proposed as a drug target in the treatment of cancer as well as diabetes [16, 163]. The design of activators and inhibitors of AMPK will be greatly facilitated by a better understanding of the molecular structure of the AMPK complex, with only the X-ray structures of certain individual domains or the truncated heterotrimer of AMPK having been solved to date [69, 75, 81].

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