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THE AMP-ACTIVATED/SNF1 PROTEIN KINASE SUBFAMILY: Metabolic Sensors of the Eukaryotic Cell?

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ABSTRACT

Mammalian AMP-activated protein kinase and yeast SNF1 protein kinase are the central components of kinase cascades that are highly conserved between animals, fungi, and plants. The AMP-activated protein kinase cascade acts as a metabolic sensor or "fuel gauge" that monitors cellular AMP and ATP levels because it is activated by increases in the AMP:ATP ratio. Once activated, the enzyme switches off ATP-consuming anabolic pathways and switches on ATP-producing catabolic pathways, such as fatty acid oxidation. The SNF1 complex in yeast is activated in response to the stress of glucose deprivation. In this case the intracellular signal or signals have not been identified; however, SNF1 activation is associated with depletion of ATP and elevation of AMP. The SNF1 complex acts primarily by inducing expression of genes required for catabolic pathways that generate glucose, probably by triggering phosphorylation of transcription factors. SNF1-related protein kinases in higher plants are likely to be involved in the response of plant cells to environmental and/or nutritional stress.

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CONTENTS

INTRODUCTION

Members of the AMP-activated/SNF1-related protein kinase subfamily are central components of highly conserved protein kinase cascades that now appear to be present in most, if not all, eukaryotic cells. Because the downstream targets of the action of these enzymes are many and varied, they have been discovered and rediscovered several times in different guises and by different approaches. Not until the cloning and sequencing of the DNAs encoding these proteins was it realized that all of the ascribed regulatory functions were carried out by members of the same class of protein kinase. Mammalian AMP-activated protein kinase (AMPK) was discovered through biochemical approaches, which has meant that understanding of its function in vivo has lagged behind insight into its regulation and its specificity for protein substrates in cell-free systems. Conversely, the role of the SNF1 system in the yeast *Saccharomyces cerevisiae* was discovered by genetic approaches, so understanding of its physiological functions in vivo has preceded information about its detailed biochemical properties in vitro. An enhanced level of understanding should be possible by a synthesis of these two approaches, in essence pooling knowledge about the animal and yeast systems. The insights obtained should also provide guidance in investigating

the cellular role of SNF1-related kinases in higher plants, where studies are at a much earlier stage. Even though these investigations are ongoing, it is now possible to present some unifying hypotheses for the mechanisms of regulation and the physiological roles of the AMPK/SNF1 subfamily, which is the main objective of this review. Although the hypotheses presented are unlikely to be correct in every detail, their value is in providing a framework for further experimentation.

EARLY HISTORY OF MAMMALIAN AMP-ACTIVATED PROTEIN KINASE

With hindsight it is now possible to date the first experimental observations of AMP-activated protein kinase (AMPK) to two independent studies in 1973 (1, 2), although it was to be 14 years before it was realized that the phenomena studied were related (3) and 16 years before the kinase was given the name by which it is known today (4). In 1973 Beg and coworkers reported that a microsomal preparation of rat liver 3-hydroxy-3-methylglutaryl–coenzyme A reductase (HMG-CoA reductase), a key regulatory enzyme of cholesterol biosynthesis, was inactivated in a time-dependent manner by incubation with MgATP and a cytosolic fraction (1). The same phenomenon was observed in microsomes from human fibroblasts, where evidence was presented that both ADP and ATP were required (5). The apparent requirement for ADP was almost certainly because AMP was being generated from it via the adenylate kinase reaction. It was later shown that the inactivation was associated with phosphorylation of HMG-CoA reductase (6, 7), and the cytosolic factor became known as HMG-CoA reductase kinase. The other event in 1973 was the report by Carlson & Kim that partially purified rat liver acetyl-CoA carboxylase, the key regulatory enzyme of fatty acid biosynthesis, was phosphorylated and inactivated by a protein kinase that contaminated the preparation (2). The protein kinase responsible remained poorly characterized, but in 1980 Yeh et al reported that the inactivation of acetyl-CoA carboxylase was stimulated by 5′-AMP (8).

In 1985, Ferrer et al found that rat liver HMG-CoA reductase kinase was also stimulated by AMP (9). At the time there appears to have been no suggestion that the acetyl-CoA carboxylase kinase studied bore any relationship to the HMG-CoA reductase kinase(s) studied elsewhere. In the mid-1980s, Carling et al (3) partially purified a protein kinase from rat liver, initially called acetyl-CoA carboxylase kinase-3 (ACK3), which phosphorylated and inactivated acetyl-CoA carboxylase. This activity was stimulated by AMP, which suggested that we had purified the activity observed in cruder fractions by Yeh et al (8). However, ACK3 also inactivated HMG-CoA reductase, and both activities were regulated identically by phosphorylation and by AMP (3).

The acetyl-CoA carboxylase and HMG-CoA reductase kinase activities were later shown to copurify from a rat liver extract and corresponded to the only significant HMG-CoA reductase kinase activity detected (10). Because both HMG-CoA reductase and acetyl-CoA carboxylase appeared to be physiological substrates, the enzyme was renamed AMP-activated protein kinase (AMPK) (4).

EARLY HISTORY OF THE IDENTIFICATION OF THE YEAST SNF1 COMPLEX

For the budding yeast *S. cerevisiae*, glucose is the preferred carbon source, and the expression of genes involved in metabolism of alternative carbon sources, in gluconeogenesis, in respiration, and in peroxisome biogenesis, is repressed when glucose is available. Derepression of these genes is a crucial aspect of the response to glucose limitation. The *SNF1* gene was defined by mutations that prevented growth on glycerol [called *CAT1* (11)], ethanol [called *CCR1* (12)], or sucrose [called *SNF1* (13) for sucrose non-fermenting]. *SNF1* function is required for transcription of glucose-repressed genes, sporulation, glycogen storage, thermotolerance, and peroxisome biogenesis (14; see also 15, 16). Genetic evidence suggests that there are functional interactions between the SNF1 and cyclic AMP-dependent protein kinase pathways (16–18). Mutations in another gene, *SNF4 (CAT3)*, were isolated in similar screens and exhibit genetic behavior similar to that of *snf1* mutations (19, 20).

MOLECULAR CHARACTERIZATION

Purification, Characterization, and Cloning of AMP-Activated Protein Kinase Subunits

Although there were earlier reports of the purification of various acetyl-CoA carboxylase and HMG-CoA reductase kinases that may have been related to AMPK (e.g. see References 21–24), the first convincing identification of the catalytic subunit was by Carling et al in 1989 (10). They labeled a preparation purified 700-fold from rat liver with a reactive ATP analog, [^{14}C]fluorosulfonylbenzoyl adenosine (FSBA), and found that a 63-kDa polypeptide was the only species labeled. Two prominent unlabeled polypeptides of 38 and 35 kDa were also present. The kinase was subsequently purified to homogeneity by two different methods: (*a*) chromatography on an ATP-γ-Sepharose affinity column eluting with AMP (25) and (*b*) chromatography on a peptide substrate affinity column (26, 27). In both cases the final preparation contained stoichiometric amounts of three polypeptides of 63, 38, and 35 kDa. Davies et al (25) provided evidence that these proteins represented the components of a heterotrimeric complex,

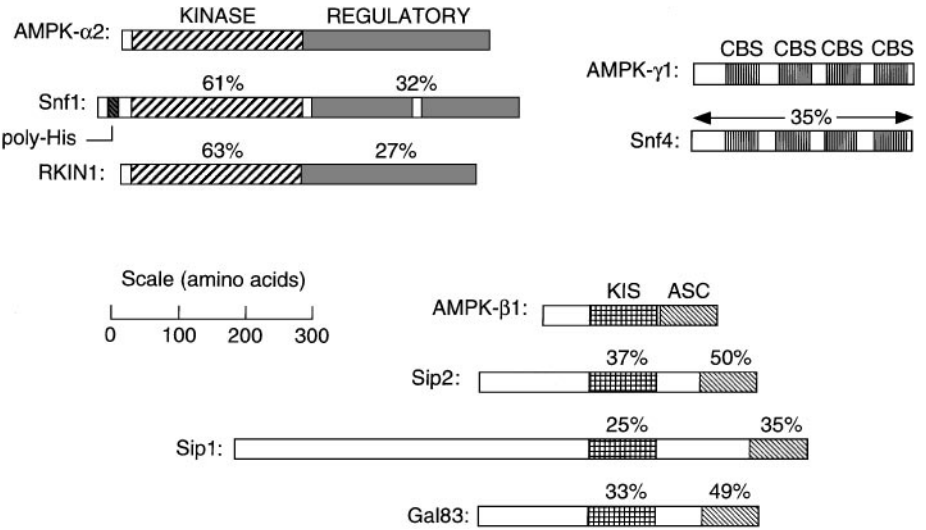

Figure 1 Domain structures of subunits of the AMP-activated protein kinase (AMPK)/SNF1 kinase subfamily. Subunits (*linear bars*) are drawn approximately to scale, with N termini *on the left*. Related domains are represented as *hatched or shaded boxes*, and figures *above these boxes* for the yeast and plant subunits are percent sequence identities with the related domain in the rat subunit shown. Assignment of cystathionine-β-synthase (CBS) domains in the AMPK-γ1/Snf4 sequences as in Reference 36; here only the overall sequence identity of the whole subunits is presented.

which are now referred to as the α (63 kDa), β (38 kDa), and γ (35 kDa) subunits.

Cloning of cDNAs encoding these subunits soon followed, commencing with the catalytic α subunit (28, 29) and then the noncatalytic β and γ subunits (30–32). The most significant, and unexpected, finding to emerge was that all three subunits were closely related to the products of genes previously identified in *S. cerevisiae* (26, 28, 30). The α subunit cDNA encoded a predicted protein of 62 kDa that was 47% identical to the amino acid sequence of the *S. cerevisiae SNF1* gene product, and it contained a typical protein-serine/threonine kinase domain within the N-terminal half. The similarity between AMPK-α and Snf1 extends throughout their lengths, although the C-terminal domains are less closely related than the kinase domains (Figure 1). The AMPK-β cDNA encoded a predicted protein of 30 kDa, significantly smaller than the apparent size (38 kDa) determined by sodium dodecyl sulfate (SDS)–polyacrylamide gel analysis. The reason for this anomaly remains unclear (32). The β sequence contains a predicted myristoylation site at the N terminus, although no covalent

modifications have yet been directly demonstrated at the protein level. The AMPK-γ cDNA encoded a 37-kDa protein. The sequences of the β and γ subunits were, like α, clearly related to products of *S. cerevisiae* genes. The β subunit was related to the products of the *SIP1-SIP2-GAL83* gene family (see below), and the γ subunit was 35% identical to the *SNF4* gene product.

The functions of the β and γ subunits remain unclear, except that coexpression of these subunits with the α subunit is essential for recovery of significant kinase activity (33, 34). Indeed, in CCL13 cells (34) there was no detectable expression of any of the subunit proteins unless all three were coexpressed, suggesting that formation of the ternary complex also stabilizes the kinase. Expression and immunoprecipitation in reticulocyte lysates (32) showed that the α and γ subunits each interact directly with the β subunit, but in this system no stable interaction between α and γ was detected. Similar results were obtained when the interactions were studied using the yeast two-hybrid system (32). These findings suggest that formation of the heterotrimeric complex is mediated, at least in part, by the β subunit. This conclusion is consistent with recent two-hybrid analyses of subunit interactions in the yeast SNF1 system (see below).

In 1996, a second isoform of the α subunit was identified and named (somewhat unconventionally) $\alpha 1$ (35), making the previously characterized isoform, $\alpha 2$. It was originally reported that the $\alpha 1$ isoform accounted for 95% of the AMPK activity measurable in rat liver extracts and that the $\alpha 2$ isoform was virtually inactive in liver (35). Subsequent studies have challenged this finding, however (34). Immunoprecipitation of AMPK from rat liver extracts using isoform-specific antibodies demonstrates that both $\alpha 1$ and $\alpha 2$ contribute approximately equally to total activity. Expression studies in mammalian cells also indicate that both isoforms have comparable specific activities (33, 34). A subtle difference between the two isoforms has been found in their preference for particular peptide substrates (34), raising the possibility that there could be some selectivity for downstream targets in vivo. Additional sequences related to the β and γ subunits have been found in the databases of expressed sequence tags. One of our groups has identified and characterized a second isoform of the β subunit ($\beta 2$, the original form becoming $\beta 1$) (35a) and two additional isoforms ($\gamma 2$, $\gamma 3$) of the $\gamma 1$ subunit (C Thornton, PCF Cheung & DC Carling, unpublished data). The role of each of these different isoforms in mediating the physiological effects of AMPK is unclear. What is certain is that AMPK exists within the cell as a heterogeneous population of complexes composed of different subunit isoforms. A major task that lies ahead is to determine the precise function of each of these complexes in vivo.

Although no obvious domain structure was discerned in the $\gamma 1$ subunit when it was first sequenced, it was recently pointed out that it, along with yeast Snf4,

comprises four repeats of a protein module known as a cystathionine-β-synthase (CBS) domain (36) (Figure 1). This domain is found in various proteins from archaebacteria to eukaryotes, including the enzyme cystathionine-β-synthase itself. Although the functions of CBS domains are not known, point mutations in the CBS domain of cystathionine-β-synthase lead to homocystinuria, and in this case it appears that the mutations affect regulation of the enzyme (37). The enzyme inosine monophosphate (IMP) dehydrogenase, for which the crystal structure has been determined (38), contains a CBS domain with a compact $\beta\alpha\beta\beta\alpha$ structure. Bateman (36) proposed that pairs of CBS domains might associate to form β-barrel structures.

Genetic and Biochemical Characterization of the Yeast SNF1 Complex

The *SNF1 (CAT1)* (39, 40) and *SNF4 (CAT3)* (41, 42) genes were cloned by complementation of the respective mutants. *SNF1* encodes Snf1, a protein of 633 residues, with an N-terminal protein-serine/threonine kinase domain, followed by a long C-terminal extension that is thought to have regulatory functions. It was subsequently found (28) to be closely related in sequence to AMPK-α (Figure 1). Snf1 has a short-segment N-terminal to the kinase domain that is not conserved in the animal homologue. This region contains a stretch of 13 consecutive histidines that are not required for SNF1 function (43) but fortuitously allow facile purification using Ni^{2+}-chelate affinity chromatography (26, 44, 45). *SNF4* encodes a protein of 322 residues that at the time (41, 42) appeared to be unique but was later shown to be closely related to the mammalian AMPK-γ subunits and to contain four repeats of the CBS motif (Figure 1).

Snf1 in yeast extracts is found in high-molecular-mass complexes (46). In the remainder of this review, the term SNF1 is used to refer to these multimeric complexes, whereas Snf1 is reserved for the catalytic subunit encoded by the *SNF1* gene. SNF1 complexes include the product of the *SNF4* gene (Snf4), as judged both by coimmunoprecipitation (42) and purification via Ni^{2+}-chelate affinity chromatography (26, 44, 45). The Snf1/Snf4 association was used to develop the two-hybrid technique for studying protein-protein interactions (47). Snf4 activates the kinase activity of Snf1 both in vivo (42, 43) and in vitro (48). Genetic approaches subsequently identified three additional components of SNF1 complexes: Sip1, Sip2, and Gal83. The *SIP1* and *SIP2* genes were identified in a two-hybrid screen using Snf1 as "bait," and *SIP1* was also recovered as a multicopy suppressor of *snf4* mutants (49, 50). *GAL83* was originally identified by its effects on glucose repression of *GAL* genes (51, 52). Sip1, Sip2, and Gal83 constitute a family of related proteins that appear to serve as alternative members of SNF1 complexes (50). Each of the three proteins

interacts directly and independently with both Snf1 and Snf4, via distinct domains (see Figure 4). A conserved, 80-residue C-terminal region in Sip1, Sip2, and Gal83, designated the *ASC* (association with SNF1 complex) domain (50), binds to Snf4 (45). A conserved internal domain in Sip1, Sip2, and Gal83, designated the *KIS* (kinase interaction sequence) region, mediates interaction with the C-terminal regulatory domain of Snf1 (45). These interactions suggest that one of the functions of each member of the Sip1/Sip2/Gal83 family is to act as a "scaffold," anchoring Snf1 and Snf4 into high-molecular-mass complexes. Neither Sip1, Sip2, nor Gal83 was found in stoichiometric amounts when the active SNF1 complex was purified to homogeneity (26, 30, 44), but this result may be because the purified enzyme is a mixture of different heteromeric complexes. When extracts were prepared from a *sip1sip2gal83* triple mutant, only half of the detectable Snf4 protein copurified with Snf1, in comparison to preparation from wild-type extracts, where copurification of Snf1 and Snf4 was quantitative (45). Although no other genes are closely related to *SIP1, SIP2*, or *GAL83* in the yeast genome, it remains unclear whether other gene products fulfill a similar "scaffold" function to stabilize SNF1 complexes.

Genetic studies have suggested additional roles for Sip1, Sip2, and Gal83 in vivo. Although autophosphorylation activity in an anti-Snf1 immunoprecipitate was greatly reduced in a *gal83*Δ mutant strain (50), the *SIP1*, *SIP2*, and *GAL83* genes are not essential for many of the functions of the SNF1 pathway in vivo. A triple mutant lacking all three genes is defective in sporulation but does not otherwise display an *snf* phenotype (50, 52). It is possible that these proteins optimize the regulatory response to glucose starvation but are not required for minimally adequate function of the kinase pathway. Increased *SIP1* and *GAL83* gene dosage, and a semidominant *GAL83* allele, have distinct effects on the expression of *SUC* and *GAL* genes (49, 50, 52). These findings suggest that these proteins may serve as "adapters" or "targeting subunits" that direct the SNF1 complex to distinct sets of downstream targets, either by binding to the target proteins or by directing the kinase to specific subcellular locations. The N-terminal domains of Sip1, Sip2, and Gal83 are unrelated in sequence (see Figure 4) and may be involved in this proposed targeting function.

Iin the yeast *Kluyveromyces lactis*, a protein called Fog1, which is closely related to Sip1, Sip2, and Gal83 in their KIS and ASC domains, is essential for expression of glucose-repressed genes, as is Fog2, a Snf1 homologue (53).

SNF1-Related Protein Kinases in Higher Plants

Alderson and coworkers (54) cloned and sequenced a cDNA *(RKIN1)* encoding a Snf1 homologue from the higher plant rye (Figure 1). Transformation of an *snf1* mutant strain of yeast with a low-copy *RKIN1* plasmid restored ability to grow on nonfermentable carbon sources (54), showing that Rkin1

is functionally as well as structurally related to Snf1. Snf1 homologues were subsequently cloned from *Arabidopsis thaliana* (55), barley (56, 57), tobacco (58), and several other plant species (NG Halford, personal communication). As expected, the tobacco DNA *(NPK5)* complemented a yeast *snf1* mutant but not an *snf4* mutant.

While these studies were taking place, MacKintosh et al (59) had detected kinase activities in extracts of several mono- and dicotyledonous plants that phosphorylated the *SAMS* peptide—a peptide based on the major AMPK site in rat acetyl-CoA carboxylase, which is a relatively specific substrate for AMPK (60). This activity was purified from cauliflower inflorescences (59, 61) and termed HMG-CoA reductase kinase-A (HRK-A). Although it was not activated by AMP, in many other respects its biochemical properties were very similar to AMPK, and it could even be activated using mammalian AMP-activated protein kinase kinase (AMPKK) and MgATP (59, 61, 62). The results indicated that HRK-A was a higher-plant homologue of AMPK. Although the plant kinase was not purified to homogeneity, the catalytic subunit was identified using [^{14}C]FSBA labeling as a polypeptide of 58 kDa (61), the mass predicted for higher-plant Snf1 homologues. Antibodies raised against a sequence that is conserved in the plant Snf1 homologues cross-reacted with this polypeptide (63). These results strongly suggest that cauliflower HRK-A is encoded by a homologue of rye *RKIN1* and yeast *SNF1*.

As yet, DNAs encoding the higher-plant homologues of the mammalian or yeast γ (Snf4) subunit or β (Sip1/Sip2/Gal83) subunits have not been cloned. There are strong indications that they must exist, however. Tobacco Npk5 interacts with Snf4 in yeast two-hybrid assays (64), and presumably it must be forming a complex with the yeast accessory subunits when it complements the *snf1* mutation in vivo (58). Although no additional subunits could be identified with certainty when HRK-A was purified from cauliflower, the active kinase had an apparent mass by gel filtration of around 200 kDa, similar to that of mammalian AMPK (61).

REGULATION

AMP-Activated Protein Kinase: Studies of Regulation in Vitro

In 1978, Ingebritsen and colleagues (65) reported that AMPK (then termed HMG-CoA reductase kinase) could be inactivated by treatment with a partially purified protein phosphatase and then reactivated by incubation with MgATP. A factor required for the MgATP-dependent reactivation (an upstream kinase?) could be resolved from AMPK on DEAE-Sepharose (66). Fractions containing

AMPK and the putative upstream kinase were also separated by Beg et al (21). These studies indicated that the system was a protein kinase cascade in which an upstream kinase (now termed AMPKK) phosphorylated and activated the downstream kinase (AMPK). Although the concept of a protein kinase cascade is now very familiar, for 10 years the AMPKK/AMPK couple and the cAMP-dependent protein kinase/phosphorylase kinase pair were the only such cascades known to exist.

Regulation of the system by AMP turned out to be remarkably complex. As well as causing allosteric activation of AMPK (8–10), AMP was absolutely required for phosphorylation of AMPK by AMPKK (67, 68). At first it was not clear whether this effect was due to binding of AMP to the enzyme (AMPKK) or to the substrate (AMPK) or to both. An answer to this question became possible with the finding that the upstream and downstream components would interact with the equivalent components in another cascade, namely that involving phosphorylation of calmodulin-dependent kinase I (CaMKI) by calmodulin-dependent kinase I kinase (CaMKK). AMP had no effect on the CaMKK → CaMKI reaction, but activation of CaMKI by AMPKK was stimulated by AMP, as was activation of AMPK by CaMKK. Although these latter two artificial cascades probably have no physiological relevance, the results demonstrated (*a*) that AMPKK is an AMP-activated protein kinase and (*b*) that binding of AMP to AMPK promoted its own phosphorylation (presumably because a conformational change makes the phosphorylation site accessible).

Remarkably, AMP has a fourth effect: It inhibits dephosphorylation and inactivation of AMPK. The kinase can be inactivated by either protein phosphatase-2A (PP2A) or protein phosphatase-2C (PP2C) in vitro (69), but the dephosphorylation in intact rat hepatocytes is not sensitive to okadaic acid (67), indicating that PP2C may be the physiologically relevant protein phosphatase. Inactivation of AMPK by PP2C was almost completely blocked by AMP (70). This effect was due to binding of AMP to AMPK rather than to PP2C because the dephosphorylation of other PP2C substrates was not inhibited. AMP therefore activates the cascade by at least four mechanisms: (*a*) allosteric activation of AMPKK; (*b*) binding to AMPK, making it a better substrate for AMPKK; (*c*) allosteric activation of AMPK; and (*d*) binding to AMPK, making it a worse substrate for PP2C. A simple model to explain these observations, based on the classical Monod/Wyman/Changeux model for allosteric enzymes (71), is presented in Figure 2. AMPK is envisaged as existing in four states, i.e. *R* and *T* conformations, each of which can also exist in phospho and dephospho forms. The dephospho form in the presence or absence or AMP appears to be inactive, but the dephosphorylated *R* state, which is stabilized by AMP binding, is a much better substrate for AMPKK than the *T* state. Phosphorylation of the *R* state

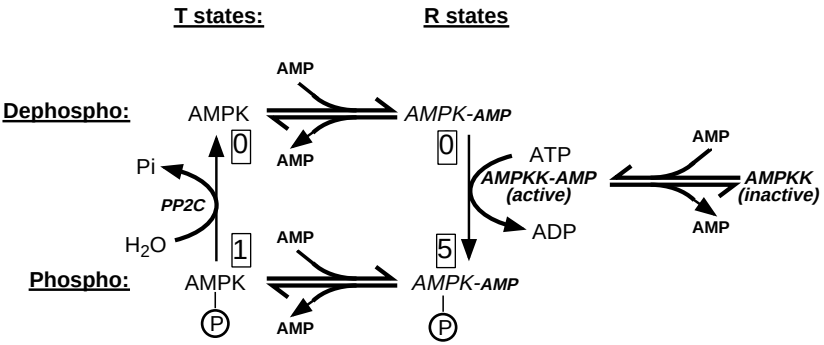

Figure 2 Model for the regulation of AMP-activated protein kinase (AMPK) by AMP and by phosphorylation, based on the classical Monod/Wyman/Changeux model for allosteric enzymes (71). AMPK is proposed to exist in four states, i.e. *R* and *T* states, both of which can exist in phosphorylated and dephosphorylated forms. AMP binding promotes the $T \rightarrow R$ transitions by stabilizing the *R* states. Only the *R* state is a substrate for AMPK kinase (AMPKK), whereas only the *T* state is a substrate for protein phosphatase-2C (PP2C). The figures in *square boxes* indicate the approximate kinase activity of that form, relative to that of the phosphorylated *T* state. Redrawn from Reference 76.

leads to >100-fold activation. Dissociation of AMP from the phosphorylated *R* state leads to partial conversion to the *T* state and a three- to fivefold decrease in activity. More important, in the absence of AMP, the phosphorylation site in this phosphorylated form is exposed to PP2C, leading to complete inactivation. In addition, the upstream kinase is allosterically activated by AMP. There is no evidence that it is regulated by phosphorylation (72).

At least three, if not all, of the mechanisms of activation by AMP are inhibited by high ATP. ATP at 4 mM causes a 10-fold increase in the concentration of AMP required for allosteric activation of AMPK (73) and for inhibition of dephosphorylation (70) (via mechanisms *c* and *d* above). High ATP also inhibits the AMP-dependent reactivation of AMPK by AMPKK (72), although it is not yet clear whether this effect is mediated by binding to AMPK (mechanism *b*), to AMPKK (mechanism *a*), or both. The simplest hypothesis to explain these observations is that AMPK has a single allosteric site that binds both AMP and ATP in a mutually exclusive manner, and that only AMP stabilizes the R conformation. Evidence consistent with this hypothesis includes the effects of the reactive ATP analog, FSBA (24). Incubation of AMPK with FSBA caused irreversible inactivation with a biphasic time course. In the first, rapid phase, FSBA caused inactivation of AMP stimulation, followed by a second slower phase where the basal, AMP-independent activity was lost. This suggests that

FSBA reacts rapidly at the allosteric (AMP/ATP) site and then more slowly at the catalytic (ATP) site. Consistent with that interpretation, both AMP and ATP protected against the first phase of inactivation, whereas only ATP protected against the second (24).

Characterization of AMP-Activated Protein Kinase Kinase

A cDNA encoding the upstream kinase—AMP-activated protein kinase kinase (AMPKK)—is not yet defined in molecular terms, but this enzyme is partially characterized at the protein level. AMPKK has been extensively purified from rat liver using an assay involving its ability to reactivate phosphatase-treated AMPK (72). One intriguing finding was that in the presence of AMPK, AMPKK could be precipitated using an antibody raised against the β subunit of AMPK. This result suggested that the upstream and downstream kinases can form a complex, although they readily dissociate from each other during purification. When AMPKK was coprecipitated with AMPK after incubation with [γ-^{32}P]ATP, a phosphorylated 58-kDa polypeptide was detected. A polypeptide of the same molecular mass was also evident on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) analysis of highly purified AMPKK labeled using either [γ-^{32}P]ATP or [^{14}C]FSBA. These results suggest, but do not prove, that the 58-kDa polypeptide is the catalytic subunit of AMPKK. If this assignment is correct, the molecular masses of the catalytic subunits of AMPKK and AMPK are similar (58 versus 63). The two kinases are also similar in several other physicochemical parameters, i.e. native molecular mass (195,000 Da), Stokes radii, and frictional coefficient (72). Moreover, because both AMPK and AMPKK are activated by AMP (74), it seems likely that they are closely related proteins and that AMPKK is also a heteromeric complex.

The major site on AMPK phosphorylated by AMPKK was found to be Thr-172 (72), located within the so-called activation segment (75), situated between the conserved Asp-Phe-Gly (DFG) and Ala-Pro-Glu (APE) motifs, where many protein kinases require phosphorylation for their activation (Figure 3). Site-directed mutagenesis studies (D Carling, unpublished data) have now confirmed that phosphorylation of T172 is likely to be necessary for activation because a T172A mutant is essentially inactive. It does not appear to be sufficient, however. A T172D mutant, in which the negatively charged aspartic acid might be expected to mimic the introduction of a phosphate group, possesses detectable activity but is still inactivated by protein phosphatase treatment. Therefore at least one other activating phosphorylation site must exist. Because phosphorylation of both residues appears to be essential for activity, it is possible that the second site was missed in the original study (72) because it was not

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AMPKα          DFGLSNMMSDGEFLRTSCGSPNYAAPE
Snf1           DFGLSNIMTDGNFLKTSCGSPNYAAPE
Rkin1          DFGLSNVMHDGHFLKTSCGSLNYAAPE

CAMKI          DFGLSKMEDPGSVLSTACGTPGYVAPE
CaMKIV         DFGLSKIVEHQVLMKTVCGTPGYCAPE
PKA              DFGFAKRVKGRTWTLCGTPEYLAPE
Akt            DFGLCKEGIKDGATMKTFCGTPEYLAPE
PKC            DFGMCKEHMMDGVTTRTFCGTPDYIAPE
Cdc2           DFGLARAFGIPIRVYTHEVVTLWYRSPE
MAPK       DFGLARIADPEHDHTGFLTEYVATRWYRAPE
RSK            DFGFAKQLRAENGLLMTPCYTANFVAPE
MEK1             DFGVSGQLIDSMANSFVGTRSYMSPE
GSK3           DFGSAKQLVRGEPNVSYICSRYYRAPE
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Figure 3 Alignment of the known phosphorylation sites in activation segments (75) of a number of protein kinases. The Asp-Phe-Gly (DFG) and Ala/Ser-Pro-Glu (A/SPE) motifs are in *bold type*, and the sequences are aligned according to the latter. Phosphorylation sites (putative only for Rkin1) are in *bold type and underlined*. AMPKα, rat AMPK-α2; Snf1, *Saccharomyces cerevisiae* Snf1; Rkin1, rye Rkin1; CaMKI, rat calmodulin-dependent protein kinase I; CaMKIV, mouse calmodulin-dependent protein kinase IV; PKA, human cyclic AMP-dependent protein kinase-α catalytic subunit; Akt, human Akt protein kinase; PKC, rat protein kinase C-α; Cdc2, human Cdc2; MAPK, human Erk1 (MAP kinase); RSK, mouse RSK$^{mo\text{-}1}$; MEK1, mouse MAPK/Erk kinase-1 (MAP kinase kinase); GSK3, glycogen synthase kinase 3. For further information see References 75, 150, 151.

completely dephosphorylated prior to rephosphorylation with [γ-^{32}P]ATP and AMPKK.

It had already been shown that mutation of Thr-210 in Snf1 (analogous to Thr-172 in AMPK-α2, as shown in Figure 3) completely abolished SNF1 function in yeast in vivo (46). It remains to be determined whether phosphorylation of an additional site is necessary for the activity of the yeast enzyme.

AMP-Activated Protein Kinase: Regulation in Intact Cells

Given that the AMPK cascade is activated by elevation of AMP and depletion of ATP, what conditions lead to such changes in vivo? Because of the action of adenylate kinase, AMP and ATP concentrations tend to change in

reciprocal directions. Adenylate kinase is a highly active enzyme in apparently all eukaryotic cells. The reaction it catalyzes ($2ADP \leftrightarrow ATP + AMP$) is maintained at close to equilibrium at all times. Because the equilibrium constant ($[ATP][AMP]/[ADP]^2$) is close to one, the AMP:ATP ratio in cells varies approximately as the square of the ADP:ATP ratio; i.e. ($[AMP]/[ATP] \approx ([ADP]/[ATP])^2$). Under optimal conditions, eukaryotic cells typically maintain their ATP:ADP ratio at something on the order of 10:1, so the ATP:AMP ratio is ~100:1. AMP in fully energized cells growing under optimal conditions is therefore extremely low, and AMPK is in the inactive state. But if the cells experience some environmental or nutritional stress such that the ADP:ATP ratio rises, let us say fivefold, the AMP:ATP ratio would rise ~25-fold, which is sufficient to switch on the AMPK cascade. It has been argued that the AMPK system represents a cellular fuel gauge (76) that constantly monitors the energy status of the cell and, if it detects that the energy supply is compromised, AMPK action initiates energy-conserving measures and mobilizes the catabolism of alternative carbon sources if these are available. AMPK therefore protects the cell against environmental and nutritional stresses. An engineering analogy even more apposite than that of a fuel gauge is to the hardware and software systems in laptop computers that monitor the state of the battery charge and, if a problem is detected, request an alternative energy source and activate energy-conserving measures (e.g. dimming the screen).

In yeast, a condition that can dramatically elevate the AMP:ATP ratio is starvation for glucose (see below). In most mammalian cells this situation is much less of a problem because homeostatic mechanisms ensure that even when the animal is starving, blood glucose levels are maintained. Unlike yeast growing in high glucose, most mammalian cells also store glycogen, which acts as a glucose buffer such that short-term glucose deprivation of cells in culture does not usually activate AMPK. However, in mammalian cells, other adverse environmental conditions elevate AMP:ATP and switch on AMPK. Such conditions include treatment of isolated hepatocytes with high fructose (67), with arsenite, or with heat shock (77). High exogenous fructose depletes ATP by trapping phosphate as fructose or triose phosphate esters. A similar mechanism is probably responsible for the depletion of ATP and the inhibition of HMG-CoA reductase and sterol synthesis (indicating activation of AMPK) when hamster fibroblasts are incubated with 2-deoxyglucose (78). Arsenite depletes ATP primarily by inhibiting lipoamide-containing dehydrogenases in the tricarboxylic acid (TCA) cycle. Inhibitors of oxidative phosphorylation such as antimycin A, dinitrophenol, or azide also cause inactivation of acetyl-CoA carboxylase (consistent with activation of AMPK) in Fao hepatoma cells (79). How heat shock causes ATP depletion remains unclear, but almost all of the stresses—e.g. heat shock, hypoxia, arsenite, cadmium ions (77)—that produce

the classical cellular stress response (i.e. elevated synthesis of stress proteins) also cause ATP depletion. As long ago as 1964, Ritossa (80) suggested that depletion of ATP might be a trigger for the cellular stress response. Although it remains unclear whether AMPK has any direct role in induction of the stress proteins, we believe that activation of AMPK is nevertheless a central component of the response of cells to stress. Examples of cellular stress events of great medical importance are heart attacks and strokes that are caused by blockage of blood vessels. Kudo et al have shown that ischemia induced by ligation of coronary arteries in perfused rat hearts is associated with elevation of AMP and depletion of ATP and causes phosphorylation and activation of AMPK (81, 82).

All of the AMPK activating events described above can be regarded as pathological rather than physiological. Under normal circumstances, the most important role for AMPK would likely be in cells wherein energy demands fluctuate highly, such as skeletal muscle. Winder & Hardie (83) have shown that AMPK is activated two- to threefold in quadricep muscles within 5 min of commencement of treadmill exercise of rats. This activation can also be demonstrated in muscles stimulated electrically in situ, and in one study, associated changes in the cellular AMP:ATP ratio were also measured (84, 85). The functions of AMPK activation in contracting muscle are considered later.

It seems reasonable to propose that the primary function of AMPK is to act as a fuel gauge that monitors the levels of the key metabolites AMP and ATP and that protects cells during periods of stress when the cellular fuel status is compromised. Earlier suggestions for the physiological role of AMPK were that it was involved in feedback regulation by lipids (86–88) or in regulation of lipid metabolism by hormones (89, 90). The effects reported were generally smaller than the pronounced effects of cellular stress. It is possible that lipid or hormonal signaling pathways may interact with the AMPK cascade, but this role does not seem to be the primary purpose for which the system evolved.

Genetic Studies on Regulation of the SNF1 Complex

In 1986, the protein kinase activity of Snf1 was demonstrated to be essential for its function in gene derepression (39). It is now clear that Snf1 kinase activity is dramatically elevated on removal of glucose from the medium (44, 48; see next section). The localization of the SNF1 complex does not appear to change. In glucose-sufficient and glucose-starved cells, immunofluorescence studies showed that Snf1 and Snf4 remain in both the nucleus and the cytoplasm (39, 42). The known components of the complex also remain associated with Snf1 regardless of glucose availability: Snf4, Sip1, Sip2, and Gal83 coimmunoprecipitate and copurify with Snf1 when extracts are prepared from cells grown in both high and low glucose (26, 30, 42, 45, 46, 49, 50).

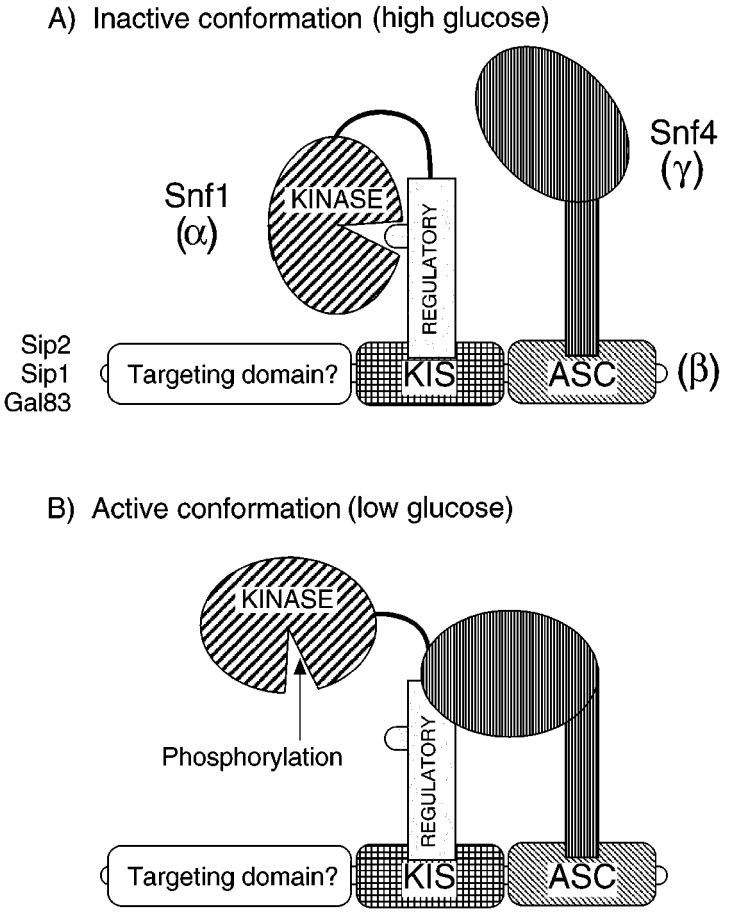

Figure 4 Model for changes in interdomain interactions in the SNF1 complex, based on References 45, 64. In both the inactive and active conformations, the β subunits (Sip1, Sip2, or Gal83) act as a "scaffold" that links Snf1 and Snf4. In the inactive form (repressing conditions, *top*), the kinase domain of Snf1 is inhibited by interaction with the regulatory domain on the same subunit. In response to an unknown signal, this interaction is disrupted (derepressing conditions, *bottom*) and Snf4 interacts with the Snf1 regulatory domain in lieu of the kinase domain. The Snf1 kinase domain is now free to phosphorylate downstream targets.

Although the subunits seem to remain associated, detailed two-hybrid analyses have recently revealed that some of the inter- and intrasubunit interactions are altered by glucose availability (64; see also Figure 4). Snf4 interacts with Snf1 in the two-hybrid system, but only in low-glucose (derepressing) conditions when the kinase would be active. This interaction has been mapped to the C-terminal, regulatory domain of Snf1. Conversely, the regulatory domain of Snf1 interacts with the kinase domain of Snf1 more strongly in high-glucose (repressing) conditions when the kinase is inactive. Snf4 and the Snf1 kinase domains interact with sites on the Snf1 regulatory domain that are distinct but overlapping, and in vitro binding studies and genetic suppressor analyses indicate that both interactions are direct (64). These results are consistent with previous genetic evidence that Snf4 is essential for the function of full-length Snf1 but is not required if a truncated Snf1 is expressed in which the regulatory domain has been deleted (43).

Biochemical Studies on Regulation of the SNF1 Complex

The finding of a close sequence relationship between AMPK-α and Snf1, particularly within the kinase domain, suggested that it might be possible to assay the SNF1 complex using AMPK substrates. This supposition indeed turned out to be correct, and the complex was assayed through use of the *SAMS* peptide. This assay was initially performed using a yeast fraction partially purified by chromatography on DEAE-Sepharose (48), although it was subsequently shown that it was possible to use the assay in a crude extract (44). The method of cell harvesting was found to be critical: The kinase was activated during harvesting if the cells were recovered by a conventional method of centrifugation and resuspension in a homogenization buffer lacking glucose. Rapid filtration and freezing in liquid nitrogen appeared to preserve the in vivo SNF1 activity, and by this method the kinase was found to be activated at least 100-fold within 5 min of glucose removal from the culture medium (44). This situation is reminiscent of the behavior of mammalian AMPK, where rapid freezing of cells is essential to preserve the physiological activation state (91).

The active, phosphorylated form of the SNF1 complex isolated under derepressing conditions could be inactivated by treatment with protein phosphatases and reactivated by adding mammalian AMPKK (44, 48). This result indicated that activation was due to phosphorylation and that the protein kinase cascade is conserved between yeast and mammals. A MgATP-dependent, SNF1-reactivating activity also was observed in partially purified SNF1 and could be resolved from SNF1 on further purification (44). This activity was presumably an upstream protein kinase homologous with AMPKK. Surprisingly, however, a gene encoding a putative upstream kinase has not yet emerged from any of the genetic screens.

The intracellular signals that switch the SNF1 complex on and off remain enigmatic. Snf1 is likely to be the primary target of the high-/low-glucose signal because its overexpression in a *snf1 snf4* double mutant partially restores glucose-repressible gene expression (43). Given that mammalian AMPK is activated by high AMP and low ATP, and given the high degree of conservation between the AMPK and SNF1 complexes, these nucleotides were obvious candidates to be regulators of SNF1. Consistent with this idea, removal of glucose from yeast growing exponentially in a medium containing abundant glucose resulted in large increases in AMP and decreases in ATP, with the AMP:ATP ratio increasing by 200-fold within 5 min (44). This change is perhaps not surprising, given that under growth conditions where high glucose is present, yeast does not store glycogen and uses glucose for ATP production via glycolysis (rather than oxidative phosphorylation). Under a variety of nutritional shifts, such as high to low glucose, low to high glucose, and glucose to sucrose, a reasonable correlation appeared to exist between cellular AMP and ATP levels and SNF1 activity (44). A problem with this hypothesis, however, is the complete failure to find any regulation of SNF1 by these nucleotides in vitro. AMP does not activate homogeneous preparations of SNF1 and does not affect its dephosphorylation by mammalian protein phosphatases. Attempts to demonstrate effects on reactivation by the upstream kinase also have been unsuccessful (44).

Genetic Identification of Elements Upstream of SNF1

Another approach to identify intracellular signals that regulate SNF1 in vivo is to study genes that appear to act upstream of SNF1. Various mutations have been isolated that relieve glucose repression of gene expression and appear to act upstream of Snf1, as judged by genetic interactions with a *snf1* mutation (14, 92). Mutations in three such genes—*HXK2*, *GLC7*, and *REG1*—have been shown to affect the glucose regulation of protein interactions within the SNF1 complex. *HXK2* (*HEX1*) encodes the major hexokinase isoform (PII) that catalyzes the initial step in glucose catabolism. A *hxk2*Δ null mutation affects the two-hybrid interaction of Snf1 and Snf4: Glucose inhibition of this interaction is substantially reduced, indicating a role for hexokinase in regulating SNF1 function (64). *GLC7* is an essential gene that encodes protein phosphatase-1 (PP1) (93), and *REG1* encodes a regulatory/targeting subunit of PP1 that directs its participation in the glucose signaling mechanism (94). Glucose repression is relieved by a specific mutant allele of *GLC7—glc7-T152K* (95)—and by *reg1* deletion. These mutations also relieve inhibition by glucose of the Snf1-Snf4 two-hybrid interaction (64). Thus, PP1-Reg1 acts upstream of the kinase to regulate Snf1-Snf4 interactions in response to the glucose signal. Moreover, Reg1 interacts with the Snf1 kinase domain in two-hybrid analysis,

which implies that it acts on the SNF1 complex directly rather than through a cascade of regulatory events (Ludin, R Jiang & M Carlson, unpublished data).

Model for Regulation of the SNF1 Complex and Its Relevance for Mammalian AMPK

The results of detailed two-hybrid analyses of the SNF1 complex by Jiang & Carlson (45, 64), combined with recent biochemical studies (44, 48), have suggested a new model for the structure and regulation of the SNF1 complex (see Figure 4). Using the mammalian subunit nomenclature, the β subunit (i.e. Sip1, Sip2, or Gal83) forms a scaffold and the γ subunit (Snf4) attaches to β via the conserved C-terminal *ASC* domain. Snf1 associates with the *KIS* segment of β. Thus the unique, N-terminal domains of the β subunits are left free. A reasonable speculation is that these domains are involved in targeting the complex to specific subcellular locations. Under high-glucose (repressing) growth conditions, the regulatory domain of Snf1 interacts with the kinase domain and blocks its activity (64). When glucose is removed from the medium, the interaction between the regulatory and kinase domains of Snf1 is disrupted. The active conformation is stabilized by binding of Snf4 to the regulatory domain of Snf1. It is not clear whether phosphorylation of Snf1 by an upstream kinase is responsible for disruption of the kinase domain–regulatory domain interaction or whether it is a consequence of it.

This model is likely to be broadly applicable to the mammalian AMPK complex. The regions of the mammalian β subunits that are most similar to the Sip1/Sip2/Gal83 family are the *KIS* and *ASC* domains (Figure 1; see also Reference 45). Woods et al have also shown that mammalian α and γ interact poorly when expressed in reticulocyte lysates, whereas α and β and β and γ interact strongly (32). The reticulocyte lysate contains an ATP-regenerating system, which would maintain AMPK in its inactive state. The yeast model (Figure 4) suggests a lack of direct interaction between α and γ under such conditions.

The events that remain unclear, at least in yeast, are the inputs or signals that cause the initial conformational change. In the mammalian system, the signal would be a rise in AMP coupled with a fall in ATP. Because FSBA blocks the allosteric site (24) and [^{14}C]FSBA reacts exclusively with the α subunit (10), the allosteric site is presumed to be on this subunit, presumably in the C-terminal domain. Binding of AMP at this site may either induce the transition to or stabilize the active conformation. According to the yeast model, the α subunit regulatory domain associates with γ in the active conformation. It does not look likely that the activating signal in the yeast system is AMP. However, large increases in AMP (and decreases in ATP) are present in vivo under conditions

where SNF1 is activated. It remains possible that the activating signal in yeast is some other metabolite that varies in concert with AMP.

If the activating signal in yeast is AMP, or some other metabolite whose concentration correlated with it, accommodation of the results with *hxk2* mutants would be straightforward. Because *HXK2* encodes the major isoform of hexokinase, disruption of this gene in cells relying on glycolytic breakdown of glucose would be expected to interfere with ATP production and hence elevate AMP via the adenylate kinase reaction. The simplest explanation of the effects of the *glc7-T152K* and *reg1*Δ mutants is that the Reg1-Glc7 complex is the protein phosphatase that converts SNF1 back to its inactive form. Disruption of this protein phosphatase would cause the SNF1 complex to become constitutively active. Other interpretations are possible, of course. It may be that there are inputs into the SNF1 system other than the putative high-/low-glucose signals, and Reg1-Glc7 may be involved in one of these alternative pathways. Because Snf4 binds to the regulatory domain of Snf1, some of these inputs might be transduced through effects on Snf4.

Regulation of Plant SNF1-Related Kinases

Little is known about regulation of the plant SNF1-related kinases in vitro, and almost nothing is known about their regulation in vivo. Like the yeast system, plant kinases are not allosterically activated by AMP. They are inactivated by protein phosphatases, however, and they can be reactivated by mammalian AMPKK and by a putative upstream kinase in plant extracts that can be removed from the downstream protein kinase on further purification (59). Therefore, plant kinases are probably regulated in a manner similar to that of their animal and yeast counterparts.

DOWNSTREAM TARGETS

The Recognition Motif for Animal, Yeast, and Plant AMPKs

Alignment of six sites phosphorylated by AMPK on protein substrates (96) revealed that the only residues conserved, other than the phosphorylated serine, were hydrophobic residues at P − 5 and P + 4 (i.e. five residues N-terminal, or four residues C-terminal, to the phosphorylated serine) and a single basic residue at P − 3 or P − 4. The influence of these residues was addressed through the use of two series of synthetic peptides. One series (96) consisted of variants of the *SAMS* peptide (HMRSAMSGLHLVKRR). The second series consisted of variants of the *AMARA* peptide (AMARAASAAALARRR) (62), a sequence designed with the key determinants at the P − 5, P − 3, and P + 4 positions but with other residues as alanine. (The basic residues at the C terminus allow

removal from unreacted ATP via binding to phosphocellulose paper, but they are not essential for phosphorylation.) These studies established the recognition motif *Hyd*-(X, *Bas*)-X-X-Ser/Thr-X-X-X-*Hyd*, where *Hyd* carries a bulky hydrophobic side chain (L, M, I, F, or V), *Bas* carries a basic side chain (R > K > H), and the order of amino acids between parentheses is not critical. Synthetic peptide studies show that AMPK will phosphorylate serine or threonine but not tyrosine, although all of the protein substrates found are phosphorylated on serine. The above recognition motif has recently been confirmed by site-directed mutagenesis of a protein substrate (see section below on HMG-CoA reductase).

Using a limited set of the *AMARA* variant peptides (34), the $\alpha 1$ and $\alpha 2$ isoforms of AMPK appeared to recognize the same motif, except that $\alpha 1$ had a less stringent requirement for the hydrophobic residue at the P + 4 position. Both isoforms prefer a hydrophobic residue at this position, but substitution with glycine causes a larger increase in K_m for the $\alpha 2$ isoform than for the $\alpha 1$ isoform (DG Hardie, unpublished data). Michell and coworkers (27) also have examined the specificity of the $\alpha 1$ isoform using a panel of synthetic peptide substrates. They came to the same conclusions regarding the necessity of a hydrophobic residue at P − 5, but they found that a hydrophobic residue at P + 4 was not essential because the peptide with the lowest K_m of those studied had glutamine at this position. Glutamine was not tested as a replacement for the hydrophobic residues in the other studies (62, 96, 97), and it is possible that this uncharged side chain can be accommodated in the substrate-binding pocket on the kinase that recognizes the P + 4 hydrophobic residue. Alternatively, a hydrophobic side chain at P + 4 may not be essential if a sufficient number of other positive determinants are present.

Most, but not all, of the known protein targets for AMPK have a basic residue at the P − 6 position as well as at the P − 3 or P − 4 position. A histidine is present at this position in the *SAMS* peptide, and a lysine is present in the peptide LKKLTRRPSFSAQ , which was found (27) to be an excellent substrate for AMPK. Although the *AMARA* peptide has alanine at P − 6, this position is also the N-terminal residue of the *AMARA* peptide and the α-amino group may provide the necessary positive charge. A basic residue at the P − 6 position may therefore be an additional recognition selectivity determinant; this proposal needs to be studied systematically.

The availability of these panels of peptides allowed the specificity of yeast SNF1 and the higher-plant SNF1-related kinases to be addressed (61, 62, 96). The recognition motifs for the yeast and plant kinases were very similar to those for their animal homologues, although there were subtle differences. Unlike the animal kinase, neither the yeast nor the plant kinases phosphorylated at significant rates peptides containing threonine in place of serine. The yeast

enzyme also had a specific requirement for arginine (not lysine or histidine), which had to be at the P−3 position. The recognition motif for yeast SNF1 (*Hyd*-X-Arg-X-X-Ser-X-X-X-*Hyd*) is therefore more stringent than that of animal or plant kinases.

Targets of Mammalian AMP-Activated Protein Kinase

HMG-COA REDUCTASE HMG-CoA reductase, which catalyzes the key regulatory step in the biosynthesis of isoprenoids and sterols, was one of the first substrates for AMPK to be identified (1), and it remains perhaps the most conclusively established. AMPK phosphorylates a single site in a catalytically active fragment of rat liver HMG-CoA reductase, corresponding to Ser-871 in the full-length sequence in the enzyme from Chinese hamster, and this site is also phosphorylated in the rat liver enzyme in situ (98). This site was subsequently shown to be the only site phosphorylated on full-length HMG-CoA reductase in intact rat hepatocytes when AMPK was activated in response to treatment of the cells with high fructose (99). HMG-CoA reductase is phosphorylated and inactivated, and sterol synthesis dramatically inhibited, when AMPK is activated in intact rat hepatocytes using either arsenite or heat shock (77) or 5-aminoimidazole-4-carboxamide (AICA) riboside (73). Incubation with AICA riboside is a method for activating AMPK in intact cells that may be more specific than stress agents because it does not usually disturb levels of ATP, ADP, or AMP (73, 100). The nucleoside is taken up by mammalian cells and converted to the monophosphorylated form, ZMP, which mimics the effects of AMP on the AMPK system, not only on allosteric activation but also on phosphorylation (73).

Elegant confirmation of the physiological importance of HMG-CoA reductase phosphorylation was obtained when Sato et al (78) showed that deoxyglucose treatment (which depletes intracellular ATP) caused total inhibition of sterol synthesis in cells expressing wild-type HMG-CoA reductase, but that it had no effect on cells expressing a Ser871Ala mutant. Ching et al (97) have studied the effects of point mutations expressed in bacteria on phosphorylation by AMPK in vitro. Mutations at and around the phosphorylation site were used to confirm, using an enzymically active protein, the recognition motif previously established using synthetic peptides (62, 96). Intriguingly, replacement of the histidine at P − 3 with the more preferred arginine residue increased the rate of phosphorylation, as did replacement of the lysine at P + 1 with alanine. A double mutant (arg at P − 3, ala at P + 1) was phosphorylated fivefold more rapidly than the wild type, although it was still functional as HMG-CoA reductase. It was therefore proposed (97) that unlike acetyl-CoA carboxylase, which has almost the optimal combination of determinants, HMG-CoA reductase may

have been selected to be a suboptimal substrate for AMPK. This idea is consistent with findings in isolated rat hepatocytes that a mild heat stress (42°C, which causes only a partial activation of AMPK) inhibits fatty acid but not sterol synthesis, whereas a more severe heat stress (45°C, which causes a much larger activation) inhibits both pathways (77). Conceivably, fatty acid synthesis is a pathway that is completely dispensable in the short term, whereas it may be preferable to maintain at least a low rate of isoprenoid synthesis under moderate stress conditions.

ACETYL-COA CARBOXYLASE AND FATTY ACID SYNTHESIS Acetyl-CoA carboxylase catalyzes the key regulated step in fatty acid synthesis, and AMP-activated protein kinase was codiscovered independently as a factor that regulated this target (2). AMPK phosphorylates three sites (101, 102) corresponding to Ser-79, Ser-1200, and Ser-1215 on rat acetyl-CoA carboxylase (103), although phosphorylation at Ser-79 seemed to be primarily responsible for enzyme inactivation. This conclusion was later confirmed by expression of a Ser79Ala mutant in COS cells (104). Acetyl-CoA carboxylase is generally thought to exert a major degree of control over fatty acid synthesis. Consistent with this view, activation of AMPK by heat shock or arsenite (77) or by AICA riboside (73, 100) causes almost total inhibition of fatty acid synthesis in isolated rat hepatocytes.

ACETYL-COA CARBOXYLASE AND FATTY ACID OXIDATION As well as conserving ATP by switching off fatty acid biosynthesis, phosphorylation of acetyl-CoA carboxylase also appears to switch on an ATP-producing pathway, i.e. fatty acid oxidation. The key regulatory step in fatty acid oxidation is the initial uptake of fatty acids into mitochondria catalyzed by carnitine-palmitoyl transferase I (CPT1). The product of acetyl-CoA carboxylase, malonyl-CoA, is an inhibitor of this enzyme (105). Malonyl-CoA is a particularly potent inhibitor in skeletal and cardiac muscle, where a different isoform of CPT1 is expressed (106). These circumstances suggest a mechanism for activating fatty acid oxidation in response to the demand for ATP, which may be particularly important in muscle. Both skeletal and cardiac muscles express novel isoforms of acetyl-CoA carboxylase [probably corresponding to the products of the ACC-β/ACC-2 gene (107, 108)], and the muscle form has been shown to be susceptible to inactivation by AMPK in vitro (109). These tissues do not express fatty acid synthase or carry out de novo fatty acid synthesis, so acetyl-CoA carboxylase is thought to have a purely regulatory role. In resting muscle, or any other cell where the demand for ATP was low, AMPK would be inactive, acetyl-CoA carboxylase would be active, and some acetyl-CoA would be converted

to malonyl-CoA, thus inhibiting fatty acid oxidation. On the other hand, in working muscle or any cell type where ATP became depleted, AMPK would be activated, acetyl-CoA carboxylase would be inactivated, malonyl-CoA would be reduced, and fatty acid oxidation would increase to generate more ATP. The evidence for this model is compelling. In skeletal muscle of rats exercising on a treadmill, AMPK is activated, acetyl-CoA carboxylase is inactivated, and malonyl-CoA is significantly depressed (83). The same effects are seen in muscles stimulated electrically, and the activation of AMPK was associated with an increase in AMP and decrease in ATP (84). Another group reported that electrical stimulation of rat gastronemius muscle resulted in phosphorylation and inactivation of acetyl-CoA carboxylase and an activation of AMPK that was confined to the $\alpha 2$ isoform (85). Most convincingly of all, perfusion of rat hind-limb muscle with AICA riboside produces the same effects without disturbing AMP or ATP levels, and these effects are accompanied by a large increase in fatty acid oxidation (110).

In cardiac muscle, AMPK is activated by ischemia and remains high during reperfusion, apparently because of increased phosphorylation. This activation of AMPK is accompanied by phosphorylation and inactivation of acetyl-CoA carboxylase activity and decreased levels of malonyl-CoA, which in turn may be responsible for the highly elevated rates of fatty acid oxidation that occur during subsequent reperfusion (81, 82).

Finally, evidence has recently been obtained that AMPK regulates fatty acid oxidation in tissues other than muscle (111). In isolated rat hepatocytes, AICA riboside profoundly depressed acetyl-CoA carboxylase and malonyl-CoA and stimulated fatty acid oxidation twofold. When a permeabilized cell assay for CPT1 was used, only part of the activation could be explained by the reduction of malonyl-CoA concentration, indicating that additional mechanisms may be in operation.

HORMONE-SENSITIVE LIPASE Hormone-sensitive lipase (HSL) is a classical target for protein phosphorylation: It is phosphorylated at Ser-563 by cyclic AMP-dependent protein kinase (PKA) (112). This mechanism is responsible for the activation of triglyceride breakdown in adipose tissue in response to catecholamines or glucagon. HSL also breaks down cholesterol–fatty acid esters, and it is responsible for the major neutral cholesterol esterase activity in most tissues (113). AMPK phosphorylates HSL at Ser-565. This phosphorylation does not cause activation, but it completely prevents phosphorylation by PKA at the neighboring activation site, Ser-563. Conditions that activate AMPK are therefore potentially antilipolytic (114). In support of this supposition, incubation of adipocytes with AICA riboside antagonizes the effects on triglyceride

breakdown of the β-adrenergic agent, isoproterenol, both decreasing the sensitivity to the agonist and decreasing the maximal extent of lipolysis (73, 115). Because oxidation of fatty acids released by lipolysis can be an important source of ATP, inhibition of lipolysis might first appear not to be consistent with the hypothesis that activation of AMPK is concerned with maintaining adequate levels of ATP. However, fatty acids are usually oxidized in cells other than those in which lipolysis occurs. Thus, the function of AMPK is to conserve ATP in the cell in which it is expressed. If lipolysis occurs at a rate faster than that at which free fatty acids can be processed (e.g. by removal in the circulation), fatty acids simply recycle into triglyceride (116), consuming two acid-anhydride bonds of ATP on the way as they are re-esterified with CoA. Recycling of fatty acids into cholesterol esters has also been demonstrated (117). Phosphorylation of HSL therefore could represent an ATP-conserving mechanism, ensuring that the rate at which triglycerides and cholesterol esters are broken down does not exceed the rate at which free fatty acids can be further processed and preventing excessive rates of recycling.

GLYCOGEN SYNTHASE AMPK phosphorylates rabbit muscle glycogen synthase, the key regulatory enzyme of glycogen synthesis, at Ser-7 in vitro (118). Phosphorylation at Ser-7 promotes phosphorylation at Ser-10 by casein kinase-1, and together these phosphorylation events produce a large inactivation of the enzyme (119). Because two acid-anhydride bonds of UTP are consumed for every glucose unit transferred from glucose-6-phosphate to glycogen, phosphorylation of glycogen synthase by AMPK could represent an energy-conserving measure. Ser-7 is known to be phosphorylated in vivo, but several protein kinases other than AMPK also phosphorylate this site in vitro, and it remains unclear whether glycogen synthase is a physiological target for AMPK.

RAF-1 The Raf-1 protein kinase is a key intermediate in the pathway from growth-factor receptors to the mitogen-activated protein kinase (MAP kinase) pathway (120). Raf-1 is recruited to the membrane by interaction of its N-terminal regulatory domain with Ras-GTP, and on subsequent activation (by mechanisms that remain unclear), it phosphorylates MAP kinase kinases that in turn phosphorylate and activate MAP kinases. Raf-1 is phosphorylated in intact cells at several sites, including Ser-259 and Ser-621 (121). The sequence around Ser-621 is an excellent fit to the consensus recognition motif for AMPK, and when a kinase from 3T3 cells was found to phosphorylate a peptide corresponding to this sequence, it quickly became apparent that it was AMPK (122). AMPK was also shown to phosphorylate Ser-621 in an N-terminally truncated Raf-1 expressed in bacteria, and in full-length Raf-1 expressed in insect cells,

although in the latter case there was an additional site, which appeared to be Ser-259.

The function of Ser-621 phosphorylation has been controversial. Mutation of this site to alanine results in total loss of Raf-1 activity, and it was therefore suggested that phosphorylation at this site is essential for activity (121). It was also claimed that phosphorylation at this site was constitutive, because it was partially phosphorylated under basal conditions and did not change in response to growth factors. More recently, it has been reported that overexpression of the catalytic subunit of cAMP-dependent protein kinase results in phosphorylation at Ser-621 and that this is accompanied by inhibition of Raf-1 (123). This report is consistent with recent findings (DG Hardie, unpublished data) that activation of AMPK in isolated rat hepatocytes using fructose or AICA riboside leads to inhibition of Raf-1 and MAP kinase. Although further study of this system is required, phosphorylation of Raf-1 by AMPK could represent a mechanism for inhibiting the transition of cells from the quiescent to the proliferating state when their energy status is compromised.

Targets of Yeast SNF1

ACETYL-COA CARBOXYLASE Yeast acetyl-CoA carboxylase is phosphorylated and inactivated by the purified yeast SNF1 complex in vitro (26). Acetyl-CoA carboxylase is also a physiological target for SNF1, because the enzyme is inactivated under conditions that activate SNF1 (starvation for glucose), but this inactivation of acetyl-CoA carboxylase does not occur in a *snf1*Δ mutant strain (48). The effect survives partial purification, which suggests that it is due to phosphorylation. Acetyl-CoA carboxylase is therefore the first target shown to be conserved between mammalian AMPK and yeast SNF1. A puzzling feature, however, is that the Ser-79 site, whose phosphorylation causes inactivation of the mammalian enzyme, is missing in the yeast sequence. Regulation of yeast acetyl-CoA carboxylase must therefore occur at a different site.

MIG1 Most of the other likely downstream targets for SNF1 have been identified by genetic analysis, which does not normally distinguish whether they are direct substrates for the SNF1 complex or whether the effect is transmitted indirectly via a cascade of other proteins. The best characterized of these potential targets is the Mig1 repressor. Mig1 is a Cys_2His_2 zinc-finger protein (124) that binds to the promoters of several glucose-repressible genes, including the *SUC* and *GAL* genes. Mutation of Mig1, or of its binding sites on promoters, partially relieves glucose repression (124–127). Repression of these genes also requires the Ssn6(Cyc8p)-Tup1 corepressor, which is recruited to different promoters by specific DNA-binding proteins and represses transcription of genes regulated

by glucose and by other signals (128, 129). An LexA-Mig1 fusion protein represses transcription of reporters containing *lexA* operators, and this repression requires Ssn6-Tup1 and is dependent on glucose (130, 131). These data suggest that Mig1 recruits Ssn6-Tup1 to promoters in response to the glucose signal. Mig1 is differentially phosphorylated in response to glucose, with a greater mobility shift in derepressed cells than in glucose-grown cells (130). Recent work has shown that the subcellular localization of Mig1 is also regulated by glucose, the protein being nuclear when glucose is present and transported to the cytoplasm when cells are limited for glucose (132).

Genetic evidence suggests that the SNF1 complex regulates Mig1. A *mig1* mutation suppresses the *snf1* mutant defects in *SUC2* and *GAL1* expression (126, 127), which indicates that SNF1 functions to inhibit repression by Mig1. SNF1 also inhibits transcriptional activation by a hybrid Mig1-VP16 activator in the absence of glucose (133). In an *snf1* mutant, phosphorylation of Mig1 is reduced to a minimal level in both glucose-repressing and -derepressing conditions (M Treitel & M Carlson, unpublished data). Also, Mig1 is localized in the nucleus in *snf1* mutants, even after a shift to glucose-limiting conditions (132).

Expression of Mig1 as a glutathione-S-transferase (GST) fusion in bacteria revealed that Mig1 is an excellent substrate for purified SNF1 in vitro. Mapping of the sites showed that four sites are present (S Smith, SP Davies, DG Hardie & D Carling, unpublished data). Intriguingly, all of the sites lay within or near the regulatory domain 1, which is required for the response to SNF1 (133). Mutation of these sites suggests that some are phosphorylated in vivo, but none of the mutations reduced phosphorylation of Mig1 as completely as did *snf1* mutations. This finding indicates that the SNF1 complex may also act indirectly to affect function of other Mig1 kinase(s) (M Treitel & M Carlson, unpublished data).

SIP3 AND SIP4 Other potential targets for the SNF1 complex include Sip3 and Sip4, which were identified in two-hybrid screens using Snf1 as bait (49, 134, 135). The interaction of Sip4 with the kinase complex is mediated by Gal83 (Vincent and M Carlson, unpublished data). Sip4 has a Cys_6 zinc cluster motif characteristic of DNA-binding proteins of the Gal4 family, and it is a transcriptional activator. Its expression is repressed by glucose, and its activity is regulated by glucose and depends on Snf1. Moreover, Sip4 is differentially phosphorylated in glucose-repressed and -derepressed cells, and phosphorylation requires Snf1. Thus, Sip4 is most likely a target of Snf1. The promoters that are activated by Sip4 remain to be identified. Genetic evidence also supports the idea of a functional relationship between Sip3 and the SNF1

pathway (134). DNA-bound LexA-Sip3 fusion proteins activate transcription of a reporter gene, which suggests that Sip3 acts downstream of Snf1.

CAT8 Cat8 is a zinc cluster protein that is required for derepression of gluconeogenic genes and for growth on nonfermentable carbon sources (136, 137). Its expression is glucose repressible, and on multicopy expression the gene suppresses the growth defect of an *snf1* mutant on ethanol. A Gal4 DNA-binding domain fused to Cat8 mediates glucose-regulated, Snf1-dependent transcriptional activation. Cat8 is differentially phosphorylated in response to glucose availability, and some of the phosphorylation detected in derepressed cells depends on Snf1. Thus, Cat8 also appears to be a downstream target of SNF1.

Targets of Higher-Plant SNF1-Related Protein Kinases

HMG-COA REDUCTASE AND OTHER METABOLIC ENZYMES No physiological targets for plant SNF1-related protein kinases have been identified with certainty. However, bacterial expression of the catalytic domain of an HMG-CoA reductase *(HMG1)* from *Arabidopsis thaliana* showed that it was phosphorylated by cauliflower HRK-A at Ser-577 (equivalent to Ser-871 in the Chinese hamster HMG-CoA reductase). This phosphorylation resulted in complete inactivation of the enzyme (138). Because the kinase recognition motif on *HMG1* is conserved on all of the many higher-plant HMG-CoA reductases for which the DNA has been sequenced (Figure 5), HMG-CoA reductase is likely to be a physiological target for the kinase in plants. Plants synthesize a variety of isoprenoids, and overexpression of HMG-CoA reductase in tobacco indicates that it exerts significant control over the whole pathway, even though other controls operate at later branch points (139).

Of the limited number of other metabolic enzymes known to be regulated by phosphorylation in plants, the phosphorylation sites on two enzymes—sucrose phosphate synthase (SPS) (140) and nitrate reductase (NR) (141, 142)—conform to the AMPK/SNF1 recognition motif (Figure 5). SPS catalyzes a key regulatory step in the synthesis of sucrose in photosynthetic tissues (143): The sucrose is transported to nonphotosynthetic tissues (e.g. roots) and zones of active growth (meristems), where it is the major carbon source. NR is involved in the initial conversion of nitrate to nitrite in the cytoplasm: The nitrite is taken up by chloroplasts and assimilated into amino acids and other nitrogen-containing compounds. These two enzymes are therefore involved in major biosynthetic processes of the plant. Spinach SPS is phosphorylated by an uncharacterized protein kinase in vitro at Ser-158 (140), and this phosphorylation causes inactivation in the presence of appropriate concentrations of the substrate, glucose-6-phosphate, and the inhibitor, phosphate. Spinach NR is

phosphorylated at Ser-543, which causes total inactivation in the presence of 14-3-3 protein (144, 145). Multiple SPS and NR kinases are present in extracts of spinach leaf (146), and at least two of these kinases appear to be members of the SNF1-related kinase family (147; P Donaghy, C Sugden & DG Hardie, unpublished data).

TRANSCRIPTION The expression of a number of genes in plants is repressed by high glucose or sucrose in the cell medium (148). Interestingly, one of them is isocitrate lyase (149), an enzyme of the glyoxylate bypass that is also repressed by glucose in *S. cerevisiae*. Plant SNF1-related kinases are therefore likely to be involved in derepression of these genes, just as SNF1 is in yeast. The transcription factors involved in glucose/sucrose repression have not been identified. Transgenic potato plants have been constructed that express in their tubers DNA encoding the potato *SNF1* homologue in antisense orientation. The SAMS peptide kinase activity in the tubers of some lines was reduced by >90%. In these plants the activity of sucrose synthase was greatly reduced and RNA transcripts were completely missing (NG Halford, personal communication). These results suggest that the potato *SNF1* homologue may be involved in derepression of sucrose synthase in the tuber, a storage organ. Despite its name, sucrose synthase is involved in the degradation of sucrose, which is the carbon source supplied to the nonphotosynthetic "sink" tissues (e.g. the tuber) by the

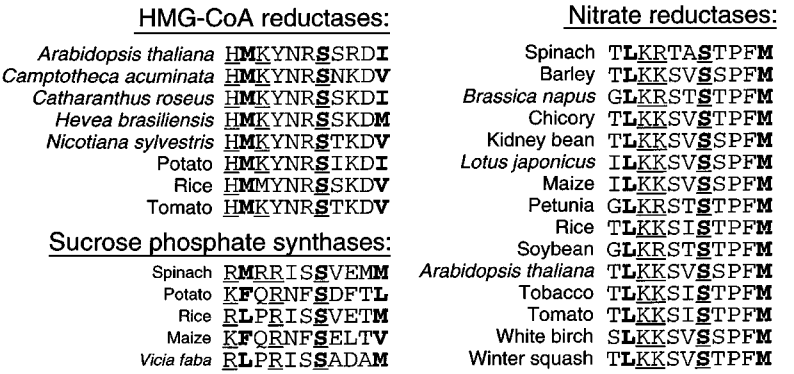

Figure 5 Alignment of sequences around the phosphorylation sites on 3-hydroxy-3-methylglutaryl–coenzyme A (HMG-CoA) reductases, sucrose phosphate synthases, and nitrate reductases from a number of higher plant species. For each enzyme, the phosphorylation site has been established for the species at the top of the list (138, 140–142). Other sequences are from the databases. Residues that may be important in recognition by the plant SNF1-related kinases are highlighted as follows: phosphorylated serine (*bold, underlined*); basic residues at P − 6, P − 3, P − 4 (*underlined*); hydrophobic residues at P − 5, P + 4 (*bold*).

photosynthetic "source" tissues (e.g. the leaves). Sucrose synthase therefore occupies a position in plants that is analogous to the position of the classical glucose-repressed enzyme, invertase, in yeast.

CONCLUSIONS: UNIFYING HYPOTHESES FOR THE REGULATION AND FUNCTION OF THE AMP-ACTIVATED/SNF1 SUBFAMILY

When the sequence similarities between mammalian AMPK subunits and the proteins of the yeast SNF1 complex were first noticed (26, 28), their physiological roles appeared to be different. We believe that this appearance resulted from the very different approaches through which they were discovered. Both can now be regarded as stress response systems, although the type of stress to which they respond may be different. AMPK is activated by environmental stresses such as heat shock, hypoxia, inhibitors of oxidative phosphorylation, and exercise (in muscle), whereas SNF1 is activated by starvation for glucose. A common feature of these stresses is that they involve depletion of cellular ATP and, via the adenylate kinase reaction, elevation of AMP. In the case of the mammalian AMPK cascade, the rise in AMP and fall in ATP are the actual signals that switch on the system. In yeast, direct regulation by AMP does not appear to occur, but many of the other regulatory features of the system are conserved with AMPK. For example, the activation of Snf1 on glucose removal is due to its phosphorylation, because activation can be reversed by protein phosphatase treatment and can be mimicked by treatment with MgATP and the upstream kinase from the mammalian system (AMPKK) or with a putative upstream kinase from yeast (44). Although the yeast cascade has not been found to be directly regulated by AMP in cell-free systems, large increases in cellular AMP and decreases in ATP occur under conditions where SNF1 is activated (44). Therefore a reasonable working hypothesis is that the signal or signals that activate SNF1 are, if not AMP and ATP, some other metabolite(s) that change in concert with them. Although the metabolites to which the yeast SNF1 kinase and the higher-plant SNF1-related kinases respond have not been found, the high degree of similarity to the mammalian AMP-activated protein kinase justifies the subtitle of this review, i.e. that these systems represent metabolic sensors of the cell.

When considering the functions of these systems in terms of downstream events, the mammalian AMPK system can now be seen both to conserve ATP by switching off anabolic pathways (e.g. fatty acid, cholesterol synthesis) and to promote ATP production by switching on an alternative catabolic pathway (i.e. fatty acid oxidation). Although the latter is achieved by direct phosphorylation

of a metabolic enzyme (acetyl-CoA carboxylase) rather than through effects on gene expression, this function is analogous to one of the known functions of the yeast SNF1 system: to derepress genes required for catabolism of alternative carbon sources. Although most of the known downstream targets for SNF1 are transcription factors, SNF1 also directly phosphorylates at least one metabolic enzyme—acetyl-CoA carboxylase (26, 48), one of the classical targets for mammalian AMPK. There is no obvious reason transcription factors should not be important targets for mammalian AMPK; we are actively investigating this area in our laboratories. The genes that may be regulated by AMPK remain a matter for speculation, but a likely place to begin investigations would be with with genes involved in carbon catabolism and maintenance of cellular energy balance.

Studies on the regulation and function of plant SNF1-related kinases are at a much earlier stage. However, if the function of the AMPK/SNF1 subfamily is in the response to cellular stress, members of this subfamily are likely to be playing important roles, because plants are subjected to environmental stress (e.g. lack of water, extremes of temperature) on a regular basis. In plants, reduced carbon is of course normally provided by photosynthesis. An underground tuber in a potato plant receives its carbon fuel in the form of sucrose exported from the leaves. If this amount of carbon fuel were to be reduced (e.g. because of herbivorous animals having grazed the leaves), the tuber would be in a state of starvation, akin to yeast in which glucose had been removed from the medium. It is therefore intriguing that the expression of the sucrose synthase gene (which occupies a metabolic role analogous to yeast invertase) appears to be under the control of an SNF1-related kinase in potato tubers.

To summarize these ideas, we propose the unifying hypothesis that the AMPK, SNF1, and SNF1-related kinase cascades have the overall function of controlling metabolism, gene expression, and perhaps cell proliferation in response to the varying energy status of the cell. These enzymes achieve this regulatory role by directly phosphorylating proteins in the target pathways and indirectly by regulating gene expression. Finally, these cascades appear to be the immediate sensors of metabolic indexes of cellular energy status, such as the levels of AMP and ATP, especially in the case of mammalian AMPK.

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