Expression and regulation of the AMP-activated protein kinase–SNF1 (sucrose non-fermenting 1) kinase complexes in yeast and mammalian cells: studies using chimaeric catalytic subunits

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Mammalian AMP-activated protein kinase (AMPK) and yeast SNF1 (sucrose non-fermenting 1) kinase are members of a highly conserved protein kinase family that plays an important role in energy homoeostasis. AMPK and SNF1 kinase are heterotrimeric complexes consisting of a catalytic subunit and two regulatory subunits. We swapped the C-terminal regulatory domains of the catalytic subunits of AMPK (α) and SNF1 kinase (Snf1) and compared the expression and regulation of these chimaeric proteins with the native catalytic subunits in both mammalian and yeast cells. In mammalian cells, α1–Snf1 yielded a functional kinase complex following co-expression with the yeast regulatory subunits Sip2 and Snf4. Unlike native AMPK, the α1–Snf1 complex was not activated by the stresses that deplete intracellular AMP. Significantly, hyperosmotic stress led to the marked activation of both the α1–Snf1 complex and AMPK, without a detectable change in adenine nucleotide levels, indicating that an alternative, non-AMP-dependent, pathway was responsible for activation. α1–Snf1 was able to restore growth of *snf1* mutant yeast on raffinose and phosphorylated the transcriptional repressor protein Mig1. Co-expression of the AMPK trimeric complex in yeast yielded an activity, increased by low glucose, that was similar to native SNF1 kinase. Importantly, expression of AMPK restored growth of a *snf1* mutant on raffinose. Our results provide clues to the regulation of AMPK and SNF1 kinase and demonstrate that, in mammalian cells, there are at least two pathways that can activate AMPK, namely one that involves an increase in the AMP/ATP ratio and one that is independent of this ratio. In yeast, the glucose signalling pathway is able to activate AMPK, suggesting that the mammalian and yeast kinase pathways are conserved.

Key words: glucose repression, metabolic stress, protein phosphorylation.

INTRODUCTION

Mammalian AMP-activated protein kinase (AMPK) and the yeast sucrose non-fermenting 1 (SNF1) kinase are central components of protein kinase cascades that have been highly conserved throughout evolution [1]. In mammals, AMPK is activated by stresses which deplete ATP and cause a concomitant rise in AMP. The increased AMP/ATP ratio within the cell leads to activation of AMPK via a number of independent mechanisms [1–3]. These include direct allosteric activation and an increase in the phosphorylation and activation of the kinase by an upstream kinase [AMPK kinase (AMPKK)] [3,4]. In yeast, glucose limitation leads to the rapid phosphorylation and activation of SNF1 kinase by an upstream kinase [5,6]. Under these conditions, activation of SNF1 kinase correlates with an increase in the AMP/ATP ratio [6]. However, there is evidence that long-term growth of yeast on non-glucose carbon sources, which requires SNF1 kinase activity, does not lead to an elevated AMP/ATP ratio [7]. Furthermore, unlike AMPK, SNF1 kinase is not directly activated by AMP *in vitro* [5,8]. It is not clear, therefore, whether SNF1 kinase responds directly to a change in adenine nucleotide levels or to some other unidentified signal.

Activation of AMPK leads to a series of metabolic responses, all of which appear to be aimed at restoring the energy balance within the cell. AMPK switches off ATP-consuming anabolic pathways, such as fatty acid and cholesterol synthesis, and switches on ATP-generating pathways, such as fatty acid oxidation and glycolysis [1,2,9]. In addition, AMPK also appears to play a role in regulating gene expression [10–13], which suggests that it may be involved in longer-term adaptations to changing energy demands. SNF1 kinase is essential for the activation of glucose-repressible genes, and *snf1* mutant strains fail to grow on non-glucose carbon sources [14,15]. Although the precise mechanism by which glucose limitation signals the activation of SNF1 kinase, some of the downstream elements in the pathway have been elucidated. SNF1 kinase phosphorylates the transcriptional repressor protein Mig1 [16,17], and this leads to increased export of Mig1 from the nucleus [18]. The subsequent decreased binding of Mig1 to the promoters of glucose-repressible genes relieves transcriptional inhibition by the Ssn6–Tup1 general repressor complex and allows transcription to occur. Like AMPK, SNF1 kinase also appears to play a fairly broad role within the cell and has been implicated in the response to heat shock, salt stress and starvation for other nutrients [19,20].

Both AMPK and SNF1 kinase are heterotrimeric protein complexes, consisting of a catalytic subunit (α) and two regulatory subunits (β and γ). In mammals, isoforms of all three subunits have been identified [21–24], although the physiological significance of these isoforms remains to be established. In yeast, a single gene encodes the catalytic subunit (Snf1) and the γ subunit (Snf4), whereas there are three isoforms of the β subunit (Sip1, Sip2 and Gal83). The β subunit acts as a scaffold protein binding to both the α and γ subunits, and in yeast there is evidence that this binding is regulated by the level of glucose present in the growth medium [25,26]. Recent evidence suggests that, in yeast, the β subunits may be involved in targetting of

Abbreviations used: AMPK, AMP-activated protein kinase; AMPKK, AMPK kinase; SAMS, synthetic peptide substrate with the amino acid sequence HMRSAMSGLHLVKRR; SNF, sucrose non-fermenting; Flag epitope tag, DYKDDDDK; G_{AD}, Gal4–DNA-activation domain; G_{BD}, Gal4–DNA-binding domain.

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SNF1 kinase to different subcellular localizations [27]. On the basis of the results obtained from previous studies, the catalytic subunits can be divided into two separate functional domains: an N-terminal kinase domain [28,29] and a C-terminal regulatory domain, which is involved in binding the regulatory subunits [25,26,28,29]. In the present study we have examined the expression of chimaeric catalytic subunits, formed by swapping the kinase and regulatory domains of α1 and Snf1, in mammalian and yeast cells. Our results reveal clues regarding the regulation of AMPK and SNF1 kinase and provide strong evidence for the existence of an alternative pathway for activating AMPK that does not involve changes in the AMP/ATP ratio within the cell.

MATERIALS AND METHODS

Plasmid construction

All plasmids were constructed using standard molecular-biological protocols and are listed in Table 1. Expression in mammalian cells used the plasmid pCDNA3 (Invitrogen). Yeast expression utilized either pGBT9, pGAD424, pBRIDGE (ClonTech) or pYX212 and pYX222 (R & D Systems). In order to construct α1–Snf1, Snf1 cDNA encoding residues 300–633 was amplified by PCR from *Saccharomyces cerevisiae* cDNA (ClonTech) using a forward oligonucleotide primer containing an *Eco*RV restriction site and a reverse oligonucleotide primer containing a *Xho*I restriction site. The resulting product was digested with *Eco*RV and *Xho*I and ligated into pCDNA3-α1 digested with the same enzymes (rat α1 cDNA contains an internal *Eco*RV site that cleaves in-frame between codons 261 and 262). Snf1–α1 was constructed by amplifying Snf1 cDNA encoding residues 1–299 with a forward primer containing an *Eco*RI site and a Myc epitope tag (amino acid sequence EQKLISEEDL) immediately following the initiating methionine and a reverse primer containing an *Eco*RV site. The product was digested with *Eco*RI and *Eco*RV and ligated into pCDNA3-α1 digested with the same enzymes. cDNA encoding full-length Snf1 cDNA was amplified with a forward primer containing an *Eco*RI site and a Myc epitope tag immediately following the initiating methionine residue and a reverse primer containing an *Xho*I site. The digested product was ligated into the *Eco*RI–*Xho*I sites of pCDNA3. All plasmids were sequenced in order to confirm their authenticity. The inserts from α1–Snf1 and Snf1–α1 were excised from pCDNA3 by digestion with *Eco*RI and *Xho*I and ligated into the *Eco*RI–*Sal*I sites of pGBT9 to allow expression of fusion proteins with the DNA-binding domain of the transcription factor Gal4. Sip2 cDNA was amplified from yeast cDNA (ClonTech) using a forward primer incorporating a Flag epitope tag (DYKDDDDK) immediately following the initiating methionine and ligated into pCDNA3. Snf4 cDNA was amplified from yeast cDNA using a forward primer incorporating a Myc epitope tag immediately following the initiating methionine residue and ligated into pCDNA3. pBRIDGE-γ1-β2, expressing both γ1 and β2, was constructed by cloning a *Sal*I fragment of γ1 cDNA (rat), encoding full-length γ1, into the *Sal*I site of pBRIDGE, allowing expression of γ1 fused to the DNA-binding domain of Gal4. Human β2 cDNA [23] was amplified with oligonucleotide primers each containing a *Not*I restriction site and ligated into pBRIDGE-γ1 digested with *Not*I. The resulting plasmid allows for expression of γ1 (fused to the DNA-binding domain of Gal4) and β2 (when methionine is absent from the growth medium). α1, Snf1, α1–Snf1 and Snf1–α1 cDNAs were excised from pCDNA3 by digestion with *Eco*RI and *Xho*I and cloned into the *Eco*RI–*Xho*I sites of pYX222. In all cases, cDNA inserts were fully sequenced in order to confirm their authenticity.

Yeast strains

S. cerevisiae strains MCY2916 (*MAT*α *his3*, *leu2*, *ura2*, *snf1*Δ*10*) and MCY3913 (*MAT*α *his3*, *leu2*, *ura2*, *ade2*, *lys2*, *trp1*, *snf1*Δ*10*) were generously given by Dr Marian Carlson, Departments of Genetics and Development and Microbiology,

Table 1 Plasmids used in the present study

pCDNA3 (Invitrogen) was used for expression in mammalian cells, pGBT9 and pGAD424 (ClonTech) were used for yeast two-hybrid analysis, and pBRIDGE (ClonTech), pYX212 and pYX222 (R & D Systems) were used for expression in yeast. Where used, epitope tags were placed immediately after the initiating methionine residue.

Vector	Insert	Description	Source or reference
pCDNA3	α1	Full-length α1 (containing an N-terminal Myc tag)	[35]
pYX222			The present study
pCDNA3	α1–Snf1	Residues 2–261 of α1 fused to residues 300–633 of Snf1 (containing an N-terminal Myc tag)	The present study
pGBT9			The present study
pYX222			The present study
pCDNA3	Snf1–α1	Residues 2–299 of Snf1 fused to residues 262–548 of α1 (containing an N-terminal Myc tag)	The present study
pGBT9			The present study
pYX222			The present study
pCDNA3	Snf1	Full-length Snf1 (containing an N-terminal Myc tag)	The present study
pYX222			The present study
pCDNA3	β1	Full-length β1	[45]
pGAD424		Residues 69–270 of β1	[32]
pCDNA3	γ1	Full-length γ1	[45]
pGAD424			[32]
pCDNA3	Sip2	Full-length Sip2 (containing an N-terminal Flag tag)	The present study
pGAD424		Residues 69–415 of Sip2	[46]
pCDNA3	Snf4	Full-length Snf4 (containing an N-terminal Myc tag)	The present study
pGAD424		Full-length Snf4	[46]
pBRIDGE	γ1-β2	Full-length γ1 (from the ADH1 promoter) and full-length β2 (from the Met^{25} promoter)	The present study
pYX212	Mig1	Full-length Mig1 (containing an N-terminal Flag tag)	[16]

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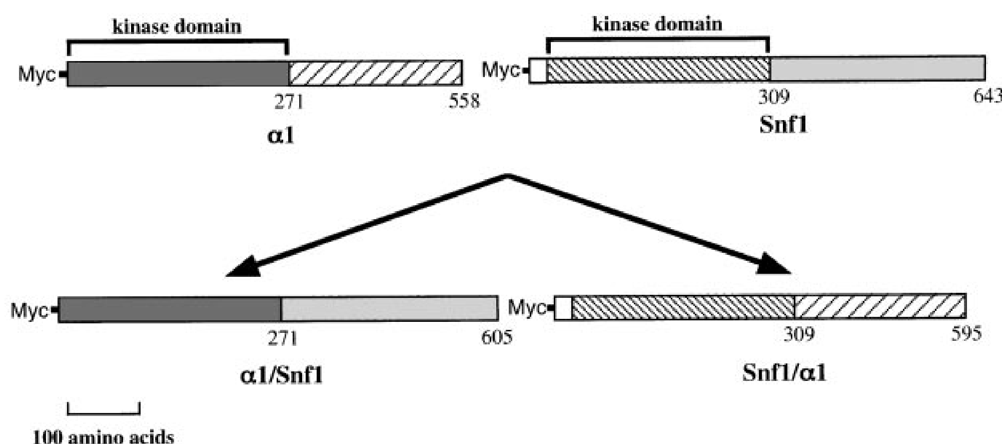

Figure 1 Diagram showing the generation of the chimaeric catalytic subunits used in the present study

Proteins were expressed in mammalian cells from the pCDNA3 vector and in yeast from the pYX222 vector. As indicated, all constructs include an N-terminal Myc epitope tag immediately downstream of the initiating methionine residue to facilitate detection. Numbering of the amino acid residues in the polypeptides includes the ten-residue Myc epitope tag.

Columbia University, New York, NY, U.S.A.). SFY526 (*MATa ura3*, *leu2*, *his3*, *ade2*, *trp1*, *gal4*, *gal80 URA3::GAL1-lacZ*) used for two-hybrid analysis was from ClonTech.

Mammalian cell expression

CCL13 cells (approx. 5.5×10^5 cells per 10-cm-diameter plate) were transfected with plasmid DNA (10 μg plasmid) by calcium phosphate precipitation [30]. Cells were harvested 72 h post-transfection by one of two methods. In the first method, the culture medium was removed and 1 ml of ice-cold lysis buffer [50 mM Tris/HCl (pH 7.5)/50 mM NaF/5 mM sodium pyrophosphate/1 mM EDTA/1 mM dithiothreitol (DTT)/0.1 mM PMSF/10 % (v/v) glycerol/1 % (v/v) Triton X-100] immediately added to the cells. Insoluble material was removed by centrifugation (13000 ***g***, 1 min at 4 °C) and the supernatant fraction used for subsequent analysis. In the second method, which we term slow lysis, following removal of the culture media the cells were briefly rinsed with PBS. Cells were removed from the plate in 1 ml of PBS containing 2 mM EDTA and collected by centrifugation. The resulting cell pellet was re-suspended in 1 ml of lysis buffer and insoluble material removed by centrifugation. In some cases, cells were submitted to oxidative stress by addition of H_2O_2 (final concn. 1 mM), or hyperosmotic stress by addition of sorbitol (final 0.6 M) to the culture medium and incubated for 30 min immediately prior to lysis using the first method described.

Immunoprecipitation of kinase complexes

Kinase complexes were immunoprecipitated from the cell lysates by incubation with 10 μl of a 50 % (w/v) slurry of anti-Myc antibody (clone 9E10) bound to Protein G–Sepharose. The slurry was mixed for 2 h at 4 °C and the immune complex precipitated by centrifugation at 6000 ***g*** for 1 min. The complex was washed twice with lysis buffer and then twice with buffer B [50 mM Hepes (pH 7.5)/1 mM EDTA/1 mM DTT/10 % (v/v) glycerol] prior to assay.

Yeast expression

Yeast strains were transformed using the lithium acetate procedure [31]. MCY2916 was used for studies involving expression of the chimaeras and MCY3913 for studies using expression of AMPK. Positive transformants were recovered by growing them on synthetic complete medium lacking the appropriate nutrients to maintain selection for plasmids. For determining growth on alternative carbon sources, serial dilutions of yeast were spotted on to synthetic complete media lacking the appropriate selection nutrients, and containing either 2 % (w/v) glucose, 2 % raffinose, 2 % galactose or 4 % glycerol, and incubated for 3 days at 30 °C. In order to determine kinase activity, single colonies were grown in liquid culture containing 2 % (w/v) glucose (repressing conditions) at 30 °C until mid-exponential phase [attenuance (D_{600}) 0.5–0.8]. Cells were harvested by brief centrifugation and washed with sterile distilled water before re-suspension in media containing either 2 % glucose or 0.05 % glucose (derepressing conditions). Cells were incubated for a further 30 min and then harvested by rapid filtration on nylon filters (0.45 μm pore size) and immediately frozen in liquid nitrogen. Yeast was homogenized by vortex-mixing with acid-washed glass beads in lysis buffer as previously described [5]. Where indicated, extracts were purified by ion-exchange chromatography on a 1 ml column containing DEAE-Sepharose Fast Flow [5]. Fractions from the column were assayed for kinase activity using the SAMS peptide and the fraction containing the peak of activity used for subsequent analysis.

Interactions using the yeast two-hybrid system

SFY526 yeast was transformed with various combinations of AMPK and SNF1 kinase subunits in pGBT9 and pGAD424 and transformants grown on selective media. Filter-lift assays for blue colour determination, or β-galactosidase activity measurements in liquid culture, were performed as described previously [32]. In some cases the assays were carried out on yeast grown in 2 % raffinose as carbon source rather than 2 % glucose.

SAMS peptide assay

Kinase activity was measured by the incorporation of [^{32}P] phosphate into the SAMS peptide [33]. Assays were carried out at 30 °C for 30 min in the presence or absence of 0.2 mM AMP as stated. Activities were calculated as pmol of phosphate incorporated into the SAMS peptide/min per ml of lysate or per mg of protein.

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Adenine nucleotide analysis

CCL13 cells were grown on 10-cm-diameter plates (approx. 5.5×10^5/plate) and either left untreated or subjected to oxidative or hyperosmotic shock as described above. Following removal of the media, 1 ml of ice-cold perchloric acid (5 %, w/w) was immediately added to the cells. In some cases, cells were harvested by the slow-lysis method (see above) and the cell pellet recovered from the centrifugation step was re-suspended in 1 ml of ice-cold 5 % perchloric acid. Protein precipitated by the perchloric acid was removed by centrifugation (13000 ***g***, 2 min) and the acid was extracted twice from the supernatant fraction with a 10 % excess (v/v) of a 1:1 mixture of tri-n-octylamine and 1,1,2-trichlorotrifluoroethane. Nucleotides were separated by ion-exchange chromatography on a Mini Q column (0.1 ml) attached to a SMART System (Amersham Biosciences). The column was equilibrated in 10 mM potassium phosphate buffer, pH 8, and samples were eluted with a 5 ml linear gradient from 10 mM potassium phosphate to 50 mM potassium phosphate, pH 8, containing 0.2 M NaCl at a flow rate of 0.2 ml/min. Nucleotides were detected by their absorbance at 254 nm and compared with the elution position of standards. Peak areas were quantified using SMART System software.

Western-blot analysis

Proteins were boiled in SDS-sample buffer, resolved by SDS/PAGE and transferred to PVDF membrane. The membrane was blocked by incubation in blocking buffer [10 mM Tris/HCl (pH 7.4)/0.5 M NaCl/0.5 % Tween 20/5 % low-fat-milk powder] for 1 h at room temperature. Membranes were probed with primary antibody for 2–18 h at 4 °C in blocking buffer and then washed extensively with 10 mM Tris/HCl (pH 7.4)/0.5 M NaCl/0.5 % Tween 20. The blots were incubated for 1 h at room temperature with an appropriate secondary antibody (either goat anti-mouse IgG or donkey anti-rabbit IgG), followed by extensive washing. Blots were developed using enhanced chemiluminescence (Boehringer-Mannheim).

RESULTS

Expression of AMPK–SNF1 kinase complexes in mammalian cells

Figure 1 shows schematically the generation of the chimaeric polypeptides used in the present study. cDNAs encoding either AMPKα1, Snf1, the N-terminal catalytic domain (residues 2–261) of AMPKα1 fused to the C-terminal regulatory domain (residues 300–633) of Snf1 (α1–Snf1) or the N-terminal catalytic domain (residues 2–299) of Snf1 fused to the C-terminal regulatory domain (residues 262–548) of AMPKα1 (Snf1–α1) were transfected into mammalian cells. Previous studies have shown that formation of the heterotrimeric complex is essential for significant expression and activity of AMPK [28,29,32]. Consistent with these findings, we were unable to detect any protein expression following transfection of the individual catalytic subunits (results not shown). We therefore co-transfected the various catalytic subunits with different combinations of the regulatory subunits of the AMPK and SNF1 kinase complexes. In this study we used the β1 and γ1 subunits of AMPK and the Sip2 and Snf4 subunits of SNF1 kinase for the co-transfection experiments. Expression of α1 was dependent on the presence of β1 and γ1, whereas expression of α1–Snf1 polypeptide was only detected following co-transfection with Sip2 and Snf4, and Snf1–α1 was detected following co-transfection with β1 and γ1 (Figure 2A). We were unable to detect significant expression of native Snf1 following co-transfection with either the yeast or mammalian subunits (results not shown). The chimaeric polypeptides migrate slightly slower than native α1, as would be expected from their predicted masses. No expression was detected following co-transfection of α1–Snf1 with β1 and γ1 or Snf1–α1 with Sip2 and Snf4 (results not shown). These results indicate that the C-terminal regulatory domain of α1 associates with the mammalian β- and γ-subunits, whereas the regulatory domain of Snf1 associates with the yeast subunits, Sip2 and Snf4. Recombinant kinase complexes were purified from cell lysates by immunoprecipitation with an anti-Myc antibody and activity in the immune complexes was determined using the SAMS peptide assay (Figure 2B). The α1–Snf1 complex yielded significant kinase activity, but, in contrast with the α1 complex, this was unaffected by the presence of AMP in the assay (Figure 2C). We were unable to detect any activity of the Snf1–α1 complex measured in the presence or absence of AMP.

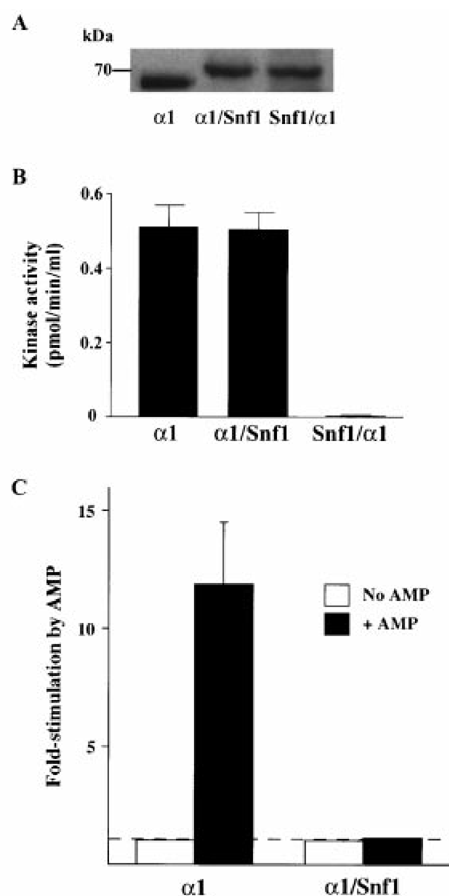

Figure 2 Expression in mammalian cells

CCL13 cells were transformed with plasmids containing cDNA encoding either α1, β1, γ1 or α1–Snf1, Sip2, Snf4 or Snf1–α1, β1, γ1. (**A**) Expression of the Myc-tagged catalytic subunits was determined by Western blotting cell lysates with an anti-Myc antibody. (**B**) Kinase activity in immune complexes isolated by immunoprecipitation with anti-Myc antibody bound to protein G–Sepharose was determined by phosphorylation of the SAMS peptide. Activities are plotted as pmol of phosphate incorporated/min per ml of lysate and are the mean values $\pm$ S.E.M. for four separate transfections. (**C**) Kinase activity was measured in the presence (shaded bars) or absence (open bars) of 0.2 mM AMP. Results are shown as fold-stimulation by AMP and are the means $\pm$ S.E.M. for three independent experiments from separate transfections. Activity in the absence of AMP is indicated by the broken line.

Activation of AMPK–SNF1 kinase complexes

Previous studies have shown that the activity of AMPK correlates closely with the energy status of the cell [1,2]. Depletion of ATP

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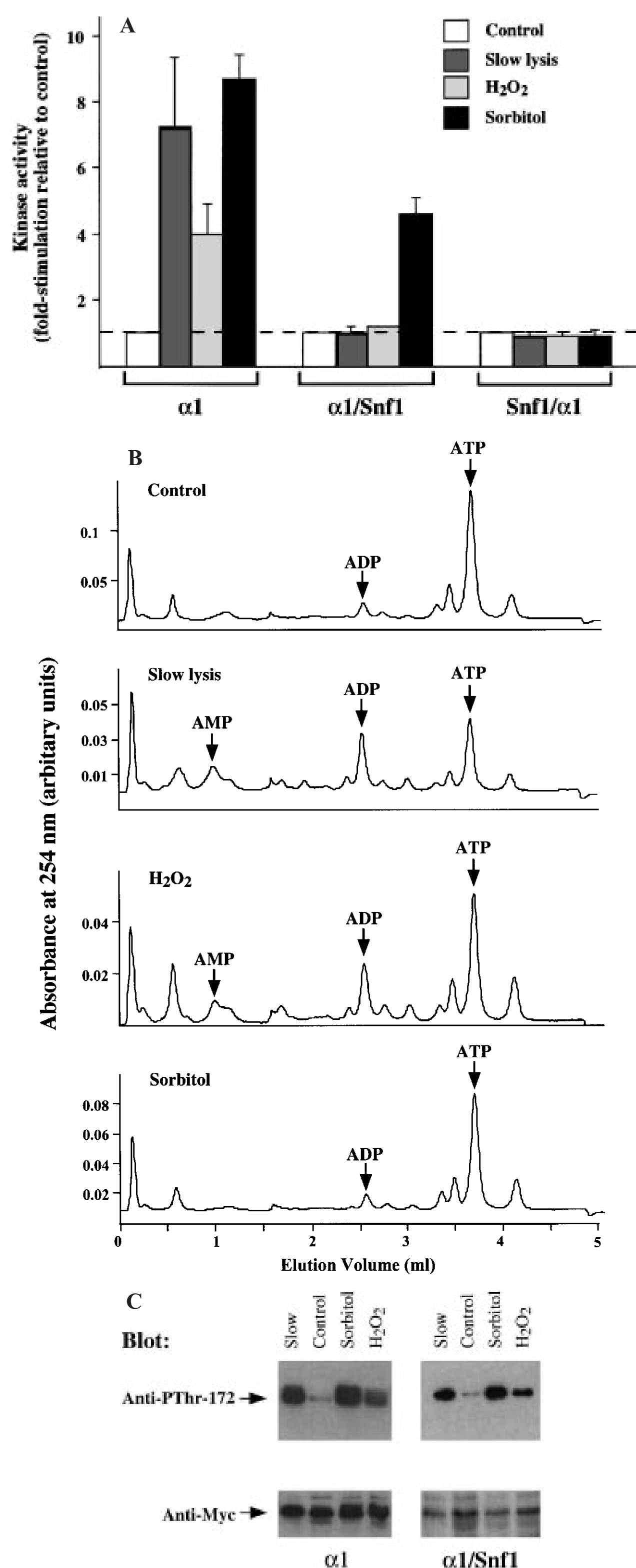

Figure 3 Stress-activation of kinase complexes in mammalian cells

(**A**) CCL13 cells expressing $\alpha 1$, $\beta 1$, $\gamma 1$ or $\alpha 1$–Snf1, Sip2, Snf4 or Snf1–$\alpha 1$, $\beta 1$, $\gamma 1$ were incubated in the absence (Control) or presence of either 1 mM H_2O_2 or 0.6 M sorbitol for 30 min. Cells were lysed directly on the plate by addition of 1 ml of lysis buffer. In some cases, untreated cells were harvested by slow lysis. Kinase activity in immune complexes isolated by immunoprecipitation with anti-Myc antibody bound to Protein G–Sepharose was determined by phosphorylation of the SAMS peptide. Activities are plotted as fold-stimulation relative to the control value and are the means ± S.E.M. for three or four separate transfections. (**B**) Adenine nucleotides in perchloric acid extracts from CCL13 cells were analysed by ion-exchange chromatography. Representative absorbance profiles for each of the cell treatments described above are shown. Peaks of AMP, ADP and ATP are marked by arrows and were identified by co-migration with standards. Areas under the ADP and ATP peaks were integrated and used to calculate the ATP/ADP ratio shown in Table 2. (**C**) The degree of phosphorylation of Thr[172] within the catalytic subunit was determined by Western blotting of cell lysates with an anti-(phospho-Thr[172])-specific antibody (from Cell Signaling Technology). The lower panel shows an equivalent blot probed with an anti-Myc antibody to control for protein loading.

Table 2 ATP/ADP ratio in CCL13 cells

Adenine nucleotides from perchloric acid extracts of CCL13 cells were analysed by ion-exchange chromatography. The areas under the ATP and ADP peaks were integrated and used to calculate the ATP/ADP ratio. In each case the values are the means ± S.E.M. for three or four independent measurements.

Treatment	ATP/ADP ratio
Control	9.08 ± 0.51
Slow lysis	1.72 ± 0.73
Oxidative stress (1 mM H_2O_2)	3.63 ± 0.38
Hyperosmotic stress (0.6 M sorbitol)	8.43 ± 1.02

causes a concomitant rise in AMP, which leads to activation of the kinase [3]. In order to determine whether the chimaeric kinases are regulated in a similar manner, CCL13 cells expressing the various complexes were subjected to different stress treatments that have previously been shown to activate AMPK, and kinase activity in anti-Myc immunoprecipitates determined using the SAMS peptide assay (Figure 3A). In parallel experiments, adenine nucleotide levels in perchloric acid extracts of CCL13 cells were determined by ion-exchange chromatography (Figure 3B and Table 2). The activity of AMPK is affected dramatically by the way in which the cells are treated prior to lysis. Rapid removal of the media followed by the direct addition of lysis buffer yields a relatively low AMPK activity, and this correlates with an ATP/ADP ratio greater than 9. We refer to cells treated under these conditions as 'control'. If the cells are first scraped from the plate and briefly centrifuged before addition of lysis buffer, AMPK activity is greatly increased (see Figure 3A). This increased activity correlates with a significant decrease in the ATP/ADP ratio (Table 2). We were not able to determine accurately the level of AMP, owing to the presence of contaminating peaks eluting from the column in the same area, and we therefore used the ATP/ADP ratio as a measure of the energy charge within the cell. As would be predicted, however, it is clear from the elution profiles that the level of AMP increases significantly as the ATP/ADP ratio falls (Figure 3B, compare control versus slow lysis). In contrast with $\alpha 1$, the activity of either chimaera was unaffected by the method of lysis. Oxidative stress caused by incubation of the cells with 1 mM H_2O_2 for 30 min also led to a significant decrease in the ATP/ADP ratio (3.63 ± 0.38) and a rise in AMP. Under these conditions, $\alpha 1$ activity was increased approx. 4-fold compared with rapidly lysed cells, whereas the activity of both chimaeras remained unchanged. Surprisingly, incubation of the cells in a hyperosmotic medium (by addition of 0.6 M sorbitol) for 30 min, had no significant effect on the ATP/ADP ratio, nor on the level of AMP (Figure 3B). However, under these conditions the activities of both $\alpha 1$ and $\alpha 1$–Snf1 were greatly increased (9-fold and 5-fold respectively), whereas that of Snf1–$\alpha 1$ was unaffected. Taken together, these results suggest that there are at least two pathways leading to activation of AMPK in mammalian cells: one involving a change in adenine nucleotide levels (e.g. oxidative stress), the

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other acting independently of adenine nucleotides (e.g. hyperosmotic stress). AMPK is activated by both pathways, whereas α1–Snf1 is only activated in response to hyperosmotic stress, not in response to stress treatments that decrease the ATP/ADP ratio. Previous studies have shown that phosphorylation of Thr^{172} within the α subunit is critical for activation of AMPK [29,34,35]. We therefore assessed the degree of phosphorylation of Thr^{172} within α1 and α1–Snf1 following the various treatments by Western blotting using a phospho-Thr^{172}-specific antibody [36]. The results in Figure 3(C) show that, in control cells, a very weak signal is detected using the phospho-specific antibody, indicating a low degree of phosphorylation on Thr^{172}, consistent with the low activity of the complexes. Slow lysis, oxidative stress and hyperosmotic shock all significantly increase the phosphorylation state of Thr^{172} in both α1 and α1–Snf1. In the case of α1, the increased phosphorylation of Thr^{172} following these treatments correlates with increased AMPK activity. However, for α1–Snf1 neither slow lysis nor oxidative stress had any effect on activity, indicating that some other event, in addition to phosphorylation of Thr^{172}, is required for activation. The nature of this other step in the activation pathway is not clear at present, but importantly, hyperosmotic shock treatment is able to cause activation of α1–Snf1 in mammalian cells.

Table 3 Interaction of the chimaeric catalytic subunits with the mammalian and yeast regulatory subunits studied using the two-hybrid system

Yeast strain SFY526 was co-transformed with the chimaeric catalytic subunits fused to the Gal4–DNA-binding domain, and the regulatory subunits fused to the Gal4–DNA-activation domain. In the case of Sip2, codons 69–415 were fused to the Gal4–DNA-activation domain [46]. Protein–protein interactions were determined either by colony filter lifts (blue colonies indicate interaction) or by β-galactosidase liquid assays in permeabilized cells. Yeast were grown in the presence of 2% glucose or 2% raffinose (as indicated below). Activities shown are the mean values from four transformants (assays performed in triplicate for each transformant) that varied by less than 20%.

DNA-binding hybrid	DNA-activation hybrid	Colour	β-Galactosidase activity
α1–Snf1	β1	White	46
	γ1	White	41
	Sip2	Blue	411
	Snf4	White	29
	Snf4	Blue	109 (raffinose)
Snf1–α1	β1	Blue	1689
	γ1	Blue	1254
	Sip2	White	17
	Snf4	White	67

Subunit interactions studied using the two-hybrid system

The results from the expression of the chimaeric kinase subunits in CCL13 cells indicated that the C-terminal domain of α1 interacted with the mammalian β and γ subunits, but not with the yeast Sip2 and Snf4 subunits, whereas the opposite was the case for the C-terminal domain of Snf1. In order to examine the interaction of the chimaeras with the AMPK and SNF1 kinase regulatory subunits in more detail, we used the yeast two-hybrid system. The chimaeras were expressed in yeast as fusion proteins with the Gal4 DNA-binding domain (G_{BD}) and the regulatory subunits were expressed as fusion proteins with the Gal4–DNA-activation domain (G_{AD}). Table 3 shows the β-galactosidase activity in yeast transformed with various combinations of the fusion proteins. Transformation of yeast with G_{BD}-α1–Snf1 and G_{AD}-Sip2 or G_{BD}-Snf1–α1 and G_{AD}-β1 and G_{AD}-γ1 resulted in significant β-galactosidase activity, and this correlated with the appearance of blue colonies when grown in the presence of 5-bromo-4-chloroindol-3-yl β-D-galactopyranoside ('X-gal'). In contrast, no interaction was observed between α1–Snf1 and either β1 or γ1, nor between Snf1–α1 and Sip2 or Snf4. These results strengthen the hypothesis that the C-terminal domain of α1 interacts with the mammalian regulatory subunits, but not the yeast subunits. Similarly, the C-terminal domain of Snf1 interacts with the yeast regulatory subunits, but not the mammalian subunits. Consistent with a previous study [25], we found that α1–Snf1 interacted with Snf4 when yeast were grown in the presence of raffinose, but not in the presence of glucose. The interaction of α1–Snf1, or Snf1–α1, with any of the other subunits tested was not affected significantly following growth on raffinose (results not shown).

Expression of the kinase subunits in yeast

We have reported previously that expression of AMPKα2 was unable to rescue a *snf1* deletion strain [37]. We therefore decided to examine whether the chimaeric kinase constructs were able to complement the *snf1* mutation. cDNAs encoding α1, Snf1, α1–Snf1 and Snf1–α1 were cloned into a yeast expression vector (pYX222) under the strong, constitutively active *TPI* promoter and transformed into a *snf1* deletion strain. Transformed yeast was patched on to agar plates containing different carbon sources and incubated at 30 °C for up to 3 days (Figure 4A). As expected, expression of Snf1 was able to rescue growth of the *snf1* mutant on raffinose, galactose and glycerol. Interestingly, expression of the α1–Snf1 chimaera allowed weak growth on raffinose, but not on galactose or glycerol. Expression of either α1 or Snf1–α1 was unable to rescue growth on any of the non-glucose carbon sources tested. These results, together with the data obtained from the interaction study using the two-hybrid system, imply that α1–Snf1 is able to form a functional kinase complex in yeast via association with the endogenous regulatory subunits. In order to test this directly, Snf1- and α1–Snf1-transformed yeast were grown under glucose-repressing conditions (2% glucose) and then transferred to media containing either 2% glucose or 0.05% glucose (glucose derepressing conditions) and incubated for a further 30 min. Yeast extracts were partially purified by ion-exchange chromatography on a DEAE-Sepharose column and kinase activity determined using the SAMS peptide assay. Significant activity was detected following expression of either Snf1 or α1–Snf1 (Figure 4B), whereas no activity could be detected from the untransformed *snf1* strain ([5] and results not shown). For both Snf1 and α1–Snf1 transformants, kinase activity was increased approx. 2-fold following growth in 0.05% glucose compared with 2% glucose. As has been previously reported for native SNF1 [5,8], kinase activity was independent of AMP in the assay (results not shown).

Phosphorylation of Mig1 *in vivo*

SNF1 kinase has been shown to phosphorylate the transcriptional repressor protein, Mig1, both *in vitro* [16] and *in vivo* [17,18,38]. We co-expressed Mig1, containing an N-terminal Flag epitope, with Snf1 or α1–Snf1 in *snf1* mutant yeast. Cell lysates were prepared and the mobility of Mig1 on SDS/PAGE was examined by Western blotting using an anti-Flag antibody. The mobility of Mig1 varies depending on its degree of phosphorylation, and therefore provides a good indicator of the phosphorylation state of Mig1 [39]. Cells grown in glucose-repressing conditions

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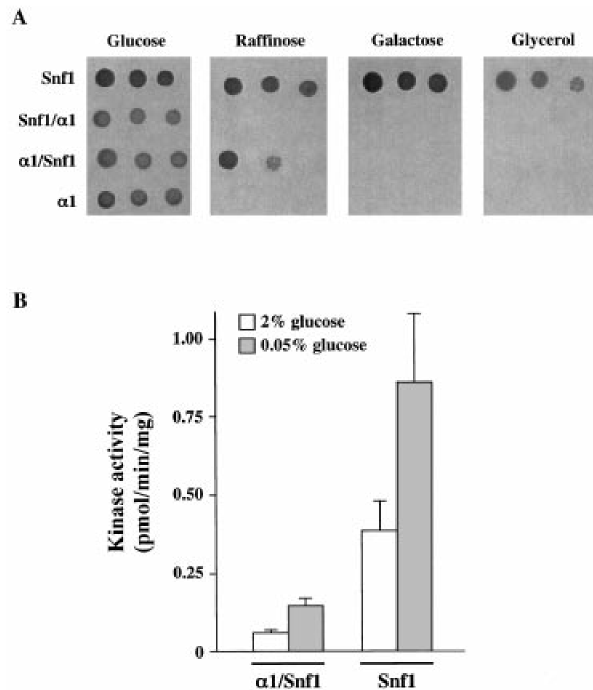

Figure 4 Expression of kinase catalytic subunits in yeast

(**A**) *snf1* mutant yeast were transformed with pYX222 expressing either α1, Snf1, α1–Snf1 or Snf1–α1 and serial dilutions of cells were spotted on to agar plates containing selective media with either 2% glucose, 2% raffinose, 2% galactose or 4% glycerol as the sole carbon source and grown at 30 °C for 3 days. (**B**) *snf1* mutant yeast, transformed with either Snf1 or α1–Snf1, were grown in the presence of 2% glucose (glucose-repressing conditions), spun down and transferred to media containing either 2% glucose or 0.05% glucose (glucose derepressing conditions). After 30 min, yeast were harvested by brief centrifugation, extracts prepared and partially purified by ion-exchange chromatography on DEAE-Sepharose. Kinase activity was measured using the SAMS peptide assay in the absence of AMP. Activities are plotted as pmol/min per mg of protein and are the means ± S.E.M. for four independent experiments.

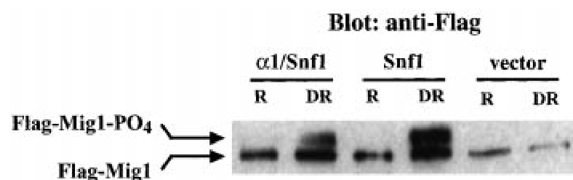

Figure 5 Phosphorylation of Mig1

snf1 mutant yeast were co-transformed with pYX212 harbouring Mig1 containing a Flag epitope tag, and pYX222 vector containing either α1–Snf1, Snf1, or no insert (vector). Yeast was grown at 30 °C in media containing 2% glucose, spun down and transferred to media containing either 2% glucose [glucose repressed (R)] or 0.05% glucose [glucose derepressed (DR)]. After 30 min incubation, yeast were harvested by rapid filtration and yeast extracts prepared. Proteins (25 μg) were separated by SDS/PAGE and the mobility of tagged Mig1 (Flag-Mig1) determined by Western blotting using an anti-Flag monoclonal antibody. Phosphorylated Mig1 (Flag-Mig1-PO_4) migrates with a slightly lower mobility than unphosphorylated Mig1.

exhibited only one form of Mig1 (which migrates at approx. 56 kDa), which is presumably the unphosphorylated form (Figure 5). Following growth in glucose-derepressing conditions a second, slower-migrating form of Mig1 is readily detected in both Snf1- and α1–Snf1-expressing yeast. These results show that α1–Snf1, like native Snf1, is able to phosphorylate Mig1 *in vivo* and that the chimaeric kinase is regulated by the level of available glucose.

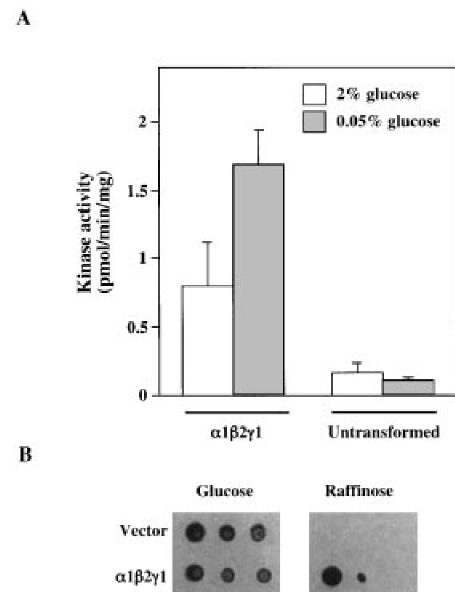

Figure 6 Expression of AMPK in yeast

(**A**) *snf1* yeast were co-transformed with α1, β2 and γ1 and grown in the absence of methionine and the presence of 2% glucose (repressing conditions), spun down and transferred to media containing either 2% glucose or 0.05% glucose (glucose derepressing conditions). After 30 min, yeast were harvested by filtration and extracts prepared. AMPK activity in immune complexes isolated by immunoprecipitation with an anti-γ1 antibody bound to Protein G–Sepharose was determined by phosphorylation of the SAMS peptide. Activities are plotted as pmol/min per mg of protein and are the means ± S.E.M. for four independent experiments. Background activity present in immunoprecipitates isolated from untransformed yeast are shown for comparison. (**B**) *snf1* mutant yeast were co-transformed with α1 (in pYX222), β2 and γ1 (co-expressed in pBRIDGE) or empty vectors (pYX222 and pBRIDGE). Serial dilutions of cells were spotted on to agar plates containing selective media, lacking methionine, with either 2% glucose or 2% raffinose as the sole carbon source and grown for 3 days at 30 °C.

Expression of the heterotrimeric AMPK complex in yeast

Our studies demonstrate that the catalytic α subunit of AMPK is unable to form a functional kinase complex in yeast, since it does not interact with the yeast counterparts of the regulatory β and γ subunits. It remained possible, however, that a functional AMPK kinase complex could be produced in yeast by expressing a combination of the mammalian subunits. We therefore co-transformed *snf1* mutant yeast with α1 (in pYX222), and β2 and γ1 (in pBRIDGE) and measured AMPK activity in the transformants. We were unable to detect significant kinase activity above background following partial purification by ion-exchange chromatography on DEAE-Sepharose (results not shown), and so we assayed AMPK in immune complexes isolated from lysates by immunoprecipitation with anti-γ1-specific antibodies. As can be seen in Figure 6(A), a significant increase in kinase activity was detected following transformation with the three AMPK subunits relative to untransformed yeast. Furthermore, this activity was approx. 2-fold higher in extracts prepared from yeast grown in 0.05% glucose compared with 2% glucose (Figure 6A). These data establish that a functional AMPK complex can be produced in yeast, and that it is regulated by the level of available glucose, analogous to the regulation of the native SNF1 kinase complex. We next addressed whether expression of the AMPK complex was able to rescue the growth of the *snf1* mutant on non-glucose carbon sources. Expression of AMPK allows weak growth on raffinose (Figure 6B), indicating that AMPK is at least partially able to complement for SNF1 kinase function. No

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growth was detected on either galactose- or glycerol-containing media (results not shown).

DISCUSSION

The catalytic subunits of AMPK and SNF1 kinase share 47% amino acid sequence identity [21,37] and contain a typical protein serine/threonine kinase domain within their N-terminal halves [1]. We made chimaeric subunits between AMPKα1 and Snf1 by swapping their kinase domains (from the initiating methionine residue to just after the conserved arginine residue in subdomain XI [40]) and compared their expression and regulation with native α1 and Snf1 in mammalian and yeast cells. In agreement with previous studies on the expression of AMPK in mammalian cells, we were unable to detect expression of the catalytic subunits unless they were co-expressed with the appropriate regulatory subunits. However, we were unable to detect expression of Snf1 in mammalian cells under any of the conditions we employed. We cannot explain this observation at present, but it is possible that other signals or co-factors are necessary for expression of native SNF1 kinase, and that these are not present in mammalian cells. Expression of α1–Snf1 required the presence of Sip2 and Snf4, whereas Snf1–α1 was only expressed in the presence of β1 and γ1. Furthermore, using the two-hybrid system we detected interactions between α1–Snf1 and Sip2 and between Snf1–α1 and β1 and γ1. Taken together these results indicate that α1–Snf1 is able to form a complex with Sip2 and Snf4, whereas Snf1–α1 forms a complex with β1 and γ1. These results support previous studies showing that the C-terminal tail of Snf1 interacts with Sip2 [26] and suggest that a region in the C-terminal tail of α1 is necessary for interaction with β1 and γ1 [29].

Although we were able to detect expression of both chimaeras in mammalian cells, we were unable to measure significant kinase activity with the Snf1–α1 complex following any of the conditions employed during the study. The reason for the lack of catalytic activity of this chimaera remains unresolved, but may be linked to the lack of expression of SNF1 kinase in mammalian cells, as described above. It is possible that activation of SNF1 kinase (and the Snf1–α1 complex) requires phosphorylation at sites that are not phosphorylated by the mammalian upstream kinase or that some further signal, not present in mammalian cells, is required in addition to phosphorylation. In contrast, the α1–Snf1 complex yielded kinase activity measured with the SAMS peptide, although unlike the α1 complex, this activity was not regulated allosterically by AMP. Consistent with this finding, the activity of SNF1 kinase has also been reported to be insensitive to AMP [5,8].

Previous studies have clearly demonstrated that AMPK is activated by a decrease in cellular ATP and a concomitant rise in AMP, i.e. an increase in the AMP/ATP ratio within the cell [1,3,41]. In the present study we found that the method by which the cells are harvested has a dramatic effect on ATP content. Rapidly lysing the cells, by direct addition of lysis buffer, maintains relatively high ATP levels. If the cells are first removed from the plate and briefly centrifuged, the level of ATP is dramatically reduced. The fall in ATP is presumably a consequence of the cells becoming anoxic during the harvesting, although we have not tested this directly. Similar effects have been reported previously for the activation of AMPK during isolation from hepatocytes [42]. Activation is thought to occur as a result of phosphorylation by an upstream kinase and correlates with a large increase in the AMP/ATP ratio. Any method of harvesting leading to cellular stress, such as anoxia during centrifugation, is likely to lead to activation of AMPK by increasing the AMP/ATP ratio. There is also evidence suggesting that SNF1 kinase is activated by a similar mechanism during harvesting of yeast cells [6]. However, unlike the native α1 complex, the activity of the α1–Snf1 complex was not affected by the method of harvesting. In order to examine in more detail this apparent difference in regulation we looked at the effect of other stresses on kinase activity. Oxidative stress, induced by H_2O_2, led to a significant reduction in ATP and a concomitant increase in AMP. This change correlated with an increase in the activity of the α1 complex, but did not alter α1–Snf1 activity. Hyperosmotic stress, following incubation in the presence of 0.6 M sorbitol, caused an increase in both α1 and α1–Snf1 activity. Under these conditions, however, there was no detectable change in adenine nucleotide levels. To our knowledge this is the first demonstration that AMPK can be activated without an increase in the AMP/ATP ratio and raises the possibility that an alternative pathway for activating AMPK exists in mammalian cells. We have previously reported that endogenous AMPK is activated by hyperosmotic stress in muscle cells [43]. Under these conditions there is no detectable change in the AMP/ATP ratio (L. Fryer, A. Patel and D. Carling, unpublished work), indicating that this AMP-independent pathway is a physiologically relevant mechanism in the activation of AMPK.

The finding that the α1–Snf1 complex is activated by hyperosmotic stress, but not by two other stresses which both increase the AMP/ATP ratio (cell harvesting and oxidative stress), provides additional support for the existence of a second, non-AMP dependent pathway for activation of AMPK. Phosphorylation of Thr^{172} within α1 is essential for activation of AMPK [29,35] and recently activation of SNF1 kinase has been shown to require phosphorylation of the equivalent residue in Snf1 (Thr^{210}) [39]. We found that the degree of Thr^{172} phosphorylation in both α1 and α1–Snf1 increased in parallel with an increase in the AMP/ATP ratio, even though the activity of the α1–Snf1 complex was unaffected by a change in this ratio. Thr^{172} phosphorylation also increased following hyperosmotic stress, and under these conditions the activity of both α1 and α1–Snf1 was increased. One explanation for these results is that, in addition to phosphorylation of Thr^{172}, an additional step is required for activation of the α1–Snf1 complex. An increase in the AMP/ATP ratio activates only one step (increased phosphorylation of Thr^{172}), whereas hyperosmotic stress activates both steps. At present the nature of this second step remains unknown, although since all the assays were carried out in immune complexes, it is likely to involve post-translational modification, rather than an allosteric effect. We have previously reported that, in addition to Thr^{172}, AMPKK phosphorylates other sites within both the α and β subunits and that these may be involved in regulating AMPK activity [35]. It is tempting to speculate that phosphorylation of these additional sites may be involved in the response to hyperosmotic stress. In such a model, in which additional phosphorylation sites are implicated, it is possible that multiple upstream kinases are involved in the activation pathways, responding to different signals. Clearly, further work is required to explore this possibility.

Having studied the expression and regulation of the different catalytic subunits in mammalian cells, it was important to investigate their regulation in yeast. Expression of α1–Snf1 was able to rescue the growth of *snf1* mutant yeast on raffinose. Furthermore, the phosphorylation state of Mig1, a downstream substrate of SNF1 kinase in the glucose derepression pathway [44], was increased following growth in low glucose. Consistent with this result, the activity of α1–Snf1 was higher when isolated from yeast grown under derepressing conditions compared with repressing conditions. These findings indicate that α1–Snf1 is

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capable of forming a functional kinase complex in yeast, presumably by associating with the endogenous subunits, and that its activity is regulated by the level of glucose in a manner similar to native Snf1. Expression of α1–Snf1 was unable to rescue growth of *snf1* mutant yeast on either galactose or glycerol. One explanation for this finding is that the α1–Snf1 complexes are unable to phosphorylate the appropriate downstream substrates required for growth on these carbon sources, although at present we have no direct evidence to support this possibility. Alternatively, it is possible that the activity of α1–Snf1 does not reach a sufficient level to allow growth on some carbon sources. This would be consistent with the lower activity of the chimaera, compared with Snf1, as measured *in vitro* (see Figure 4B).

Much of the data gathered to date on AMPK, including the results of the current study, indicate that assembly of a heterotrimeric complex between the catalytic and regulatory subunits is essential for expression of a functionally active kinase. Since expression of either α1 or α2 alone was unable to complement *snf1* mutant yeast, we designed a protocol for the expression of a heterotrimeric AMPK complex in yeast. Using this approach we were able to rescue the growth of a *snf1* deletion strain on raffinose and recover AMPK activity in immune complexes isolated from yeast extracts. Significantly, the activity of AMPK isolated from transformed yeast was dependent on the level of glucose in the growth medium. In order to detect AMPK activity it was necessary to assay the kinase in immunoprecipitates, suggesting that the level of activity was significantly lower than that observed following transformation with either Snf1 or α1–Snf1, whose activity could be detected in partially purified extracts. Nonetheless, these results indicate that AMPK is regulated in yeast in response to the same signal that regulates SNF1 kinase. Furthermore, the finding that expression of AMPK restores growth of a *snf1* mutant on raffinose suggests that AMPK, like the α1–Snf1 chimaera, is also able to phosphorylate Mig1 under glucose derepressing conditions. Consistent with this hypothesis, we have found that AMPK phosphorylates Mig1 *in vitro* at the same sites phosphorylated by SNF1 kinase (F. Smith and D. Carling, unpublished work). A recent study has shown that phosphorylation of Thr^{210} within Snf1 by an upstream kinase is critical for activation of the SNF1 kinase complex [39]. Although the upstream kinases that regulate AMPK and SNF1 kinase remain to be characterized fully, our current work demonstrates that the yeast upstream kinase can phosphorylate and activate AMPK *in vivo*. We reported earlier that a preparation of the mammalian upstream kinase, AMPKK, could activate SNF1 kinase *in vitro* [5]. In addition to functional similarity between AMPK and SNF1 kinase, there appears to be some functional conservation between the upstream kinases. Whether this functional conservation includes the signals used for activation in yeast and mammalian cells is unclear, although it has recently been reported that salt stress increases Thr^{210} phosphorylation Snf1 [39]. Unfortunately, SNF1 kinase activity was not measured in the present study, so it will be interesting to determine whether salt stress activates SNF1 kinase in a manner analogous to activation of AMPK by hyperosmotic stress.

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