

# Investigating the mechanism for AMP activation of the AMP-activated protein kinase cascade

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AMPK (AMP-activated protein kinase) is activated allosterically by AMP and by phosphorylation of Thr<sup>172</sup> within the catalytic  $\alpha$  subunit. Here we show that mutations in the regulatory  $\gamma$  subunit reduce allosteric activation of the kinase by AMP. In addition to its allosteric effect, AMP significantly reduces the dephosphorylation of Thr<sup>172</sup> by PP (protein phosphatase)2C $\alpha$ . Moreover, a mutation in the  $\gamma$  subunit almost completely abolishes the inhibitory effect of AMP on dephosphorylation. We were unable to detect any effect of AMP on Thr<sup>172</sup> phosphorylation by either LKB1 or CaMKK $\beta$  (Ca<sup>2+</sup>/calmodulin-dependent protein kinase kinase  $\beta$ ) using recombinant preparations of the proteins. However, using partially purified AMPK from rat liver, there was an apparent AMP-stimulation of Thr<sup>172</sup> phosphorylation by LKB1, but this was blocked by the addition of NaF, a PP inhibitor. Western blotting of partially purified rat liver AMPK and LKB1 revealed the presence of PP2C $\alpha$  in the preparations. We suggest that pre-

vious studies reporting that AMP promotes phosphorylation of Thr<sup>172</sup> were misinterpreted. A plausible explanation for this effect of AMP is inhibition of dephosphorylation by PP2C $\alpha$ , present in the preparations of the kinases used in the earlier studies. Taken together, our results demonstrate that AMP activates AMPK via two mechanisms: by direct allosteric activation and by protecting Thr<sup>172</sup> from dephosphorylation. On the basis of our new findings, we propose a simple model for the regulation of AMPK in mammalian cells by LKB1 and CaMKK $\beta$ . This model accounts for activation of AMPK by two distinct signals: a Ca<sup>2+</sup>-dependent pathway, mediated by CaMKK $\beta$  and an AMP-dependent pathway, mediated by LKB1.

**Key words:** AMP-activated protein kinase (AMPK), Ca<sup>2+</sup>/calmodulin-dependent protein kinase kinase (CaMKK), energy metabolism, LKB1, metabolic syndrome.

## INTRODUCTION

AMPK (AMP-activated protein kinase) is the downstream component of a protein kinase cascade that plays a key role in energy homeostasis [1,2]. AMPK is a heterotrimeric complex of a catalytic subunit ( $\alpha$ ) and two regulatory subunits ( $\beta$  and  $\gamma$ ). Isoforms of all three subunits have been identified in mammals that allow for the generation of 12 different heterotrimeric complexes [1]. The yeast counterpart of AMPK is the SNF1 (sucrose non-fermenting) complex, composed of Snf1 ( $\alpha$ ), Snf4 ( $\gamma$ ) and either Sip1, Sip2 or Gal83 ( $\beta$  isoforms) [3]. Both AMPK and SNF1 are activated by phosphorylation by upstream kinases. In yeast, three upstream kinases have been identified corresponding to Sak1 (originally referred to as Pak1), Elm1 and Tos3 [4,5]. In mammals, LKB1 and CaMKK $\beta$  (Ca<sup>2+</sup>/calmodulin-dependent protein kinase kinase  $\beta$ ) have been identified as physiological kinases upstream of AMPK [6–11]. More recently, evidence has been reported indicating that Tak1, a member of the MKK (mitogen-activated protein kinase) family, is also capable of phosphorylating and activating AMPK [12].

In addition to activation by phosphorylation, AMPK is allosterically activated by AMP [13]. Several lines of evidence suggest that the  $\gamma$  subunit is involved in the allosteric activation. The  $\gamma$  subunit contains four CBS (cystathionine  $\beta$ -synthase) domains [14]. In human  $\gamma$ 2, naturally occurring mutations in these domains cause cardiac hypertrophy due to massive glycogen accumulation, together with Wolff–Parkinson–White syndrome, a pre-excitation disorder [15–17]. Biochemical studies demonstrate that

some of these mutations interfere with AMP-activation of AMPK [18,19]. Furthermore, the CBS domains have been shown to bind AMP using *in vitro* binding assays [19]. In contrast with AMPK, allosteric activation of SNF1 by AMP has not been demonstrated [20]. As well as allosteric activation, AMP has been proposed to play a role in the phosphorylation of AMPK. Originally, three separate mechanisms were proposed whereby AMP could promote phosphorylation of AMPK. The first mechanism was by direct activation of the upstream kinases by AMP [21]. However, the evidence supporting this mechanism was based on results obtained using partially purified preparations of the upstream kinase from rat liver, which was subsequently shown to be LKB1. Another previous study using highly purified recombinant preparations of LKB1 reveal that AMP does not directly activate LKB1 [8]. Similarly, CaMKK $\beta$  is not directly activated by AMP [7,9]. Two further mechanisms for promotion of phosphorylation by AMP were suggested to be substrate mediated. Binding of AMP to AMPK was proposed to render the kinase a better substrate for phosphorylation by upstream kinases [21], while making it a less attractive substrate for dephosphorylation by protein phosphatases [22]. Although these mechanisms provide an attractive model for AMP activation of AMPK, there is relatively little direct evidence supporting them, and for these studies partially purified preparations of AMPK and the upstream kinases were used. Moreover, recent studies have reported that AMP does not promote phosphorylation of AMPK by CaMKK $\beta$  [7,9]. Since both CaMKK $\beta$  and LKB1 activate AMPK by phosphorylating the same residue within AMPK, it is difficult to envisage a

Abbreviations used: AMPK, AMP-activated protein kinase; AMPKK, AMPK kinase; CaMKK, Ca<sup>2+</sup>/calmodulin-dependent protein kinase kinase; CBS, cystathionine  $\beta$ -synthase; DTT, dithiothreitol; PP, protein phosphatase; Snf1, sucrose non-fermenting 1.

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mechanism that would account for a substrate-mediated effect of AMP that is specific for LKB1. At the time that the present study was in preparation, Suter et al. [23] reported that AMP did not promote phosphorylation of AMPK by a recombinant preparation of LKB1. In view of the apparent discrepancy between earlier studies and more recent findings concerning the effect of AMP on phosphorylation, we decided to revisit the mechanisms for activation of AMPK by AMP using highly purified recombinant proteins. The results of the present study show that AMP allosterically activates AMPK and inhibits dephosphorylation of Thr<sup>172</sup>. Both these effects involve the  $\gamma$  subunit. However, AMP has no effect on phosphorylation of AMPK by either LKB1 or CaMKK $\beta$ . Instead, we present evidence suggesting that previous results indicating that AMP stimulates phosphorylation of AMPK by LKB1 may have been confounded by the presence of endogenous PP (protein phosphatase)2C $\alpha$  in the preparations of the kinases used in the earlier studies.

## MATERIALS AND METHODS

### Materials

BL21-Codon-Plus (DE3)-RIL competent *Escherichia coli* cells were obtained from Novagen. Constructs for bacterial expression of the SNF1 complex (Snf1, Snf4 and Gal83) were a kind gift from Marian Carlson (Columbia University Medical Center, Columbia University, New York, NY, U.S.A.).

### Preparation of recombinant proteins

Recombinant AMPK  $\alpha 1\beta 1\gamma 1$  and  $\alpha 2\beta 1\gamma 1$  complexes [24], CaMKK $\beta$  [9] and PP2C $\alpha$  [25] were expressed in bacteria and purified as described in the respective references. cDNAs encoding *Snf1* and *Snf4* were cloned into multiple cloning sites 1 (SacI/HindIII) and 2 (KpnI/KpnI) respectively, of the pRSF DUET-1 vector (Invitrogen). This allowed for expression of *Snf1* with an N-terminal His<sub>6</sub>-tag. cDNA coding for Gal83 was cloned into the pET DUET-1 vector (Invitrogen) between the BglIII/KpnI sites. These constructs were co-transformed into *E. coli* [BL21-Codon-Plus (DE3)-RIL] and colonies were selected by growth on medium containing the appropriate antibiotic. The SNF1 complex was expressed in *E. coli* following induction with 1 mM isopropyl  $\beta$ -thiogalactopyranoside for 4 h at 25 °C. Cells were sonicated with 1 s pulses separated by 1 s intervals for 2 min at 8 W (Sonics Vibra cell) and insoluble material was removed by centrifugation at 16000 g for 15 min at 4 °C. The supernatant was sterile-filtered and SNF1 complex was purified on a HisTrap HP column using an ÄKTA<sup>TM</sup> purifier (Amersham Biosciences). Following nickel–Sephacryl chromatography, the SNF1 complex was further purified by gel filtration chromatography on a HiLoad 16/60 Superdex 200 column (Amersham Biosciences). Fractions were analysed by SDS/PAGE and those containing the SNF1 complex were pooled and concentrated using a Vivaspinn column (Vivascience). Authenticity of the SNF1 subunits was confirmed by MS of the proteins resolved by SDS/PAGE and subsequently digested with trypsin (results not shown).

Active LKB1 complex was obtained by co-expression of His–LKB1, STRAD $\alpha$  and GST (glutathione S-transferase)–MO25 $\alpha$  using a baculovirus-insect cell expression system (Bac-to-Bac<sup>®</sup> Baculovirus Expression System, Invitrogen). The LKB1 complex was expressed in Sf9 cells and was purified by affinity chromatography on glutathione–Sephacryl. Recombinant protein was stored in 50 mM Hepes, pH 7.4, 200 mM NaCl, 10% (v/v) glycerol and 1 mM Tris(2-carboxyethyl)phosphine hydrochloride at –80 °C.

### Site-directed mutagenesis

Point mutations (R69Q, H150R and R298G) were introduced into the  $\gamma 1$  subunit using the QuikChange<sup>®</sup> site-directed mutagenesis kit (Stratagene) according to the manufacturer's protocol. The introduction of the mutations was verified by DNA sequencing.

### AMPK/SNF1 assays

Recombinant AMPK or SNF1 was incubated in the presence or absence of CaMKK $\beta$  or LKB1 with 200  $\mu$ M ATP, 2.5 mM MgCl<sub>2</sub> and 1 mM DTT (dithiothreitol) for 20 min at 37 °C, and in the presence or absence of 150  $\mu$ M AMP, as stated in the legends to the Figures. In some cases, an aliquot (5  $\mu$ l) of this reaction was taken for Western blot analysis. A separate aliquot (5  $\mu$ l) was removed and diluted in 50 mM Hepes (pH 7.4), and AMPK or SNF1 activity was determined by phosphorylation of the SAMS peptide (His-Met-Arg-Ser-Ala-Met-Ser-Gly-Leu-His-Leu-Val-Lys-Arg-Arg) [26] in the presence or absence of 150  $\mu$ M AMP, as stated in the legends to the Figures. Results shown are presented as specific activity (nmol/min per mg of protein).

### Western blot analysis

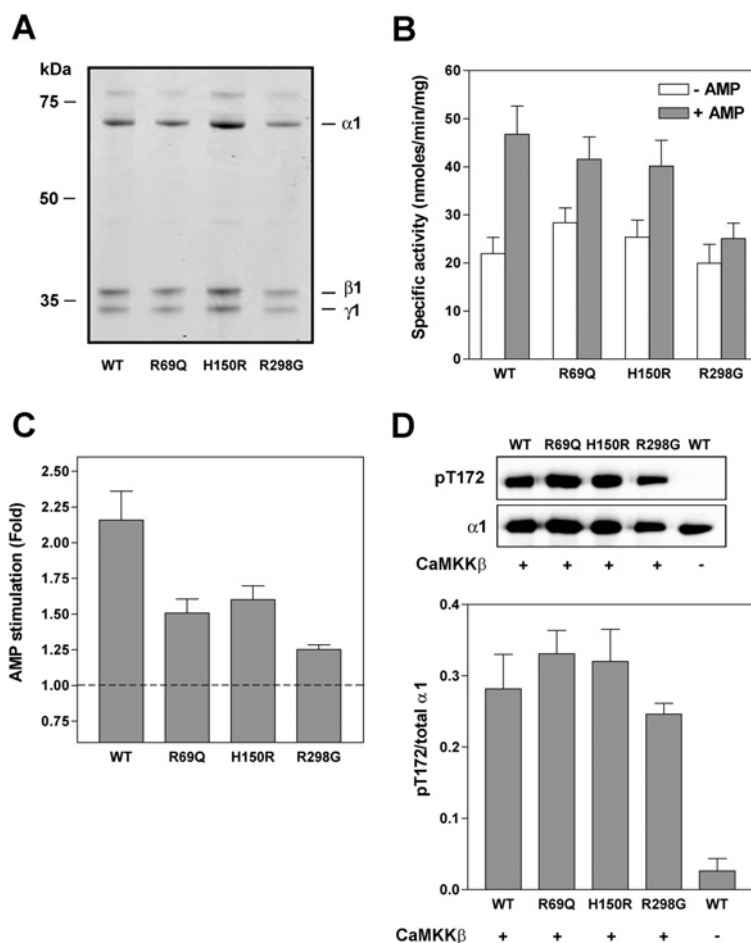
Thr<sup>172</sup> (AMPK) and Thr<sup>210</sup> (SNF1) phosphorylation was determined using rabbit anti-(phospho-Thr<sup>172</sup>) antibody (Cell Signaling). Total AMPK was detected using sheep anti- $\alpha 1$  or anti- $\alpha 2$  antibodies [27]. Total Snf1 was detected using mouse anti-His<sub>6</sub> antibody (Abcam). PP2C $\alpha$  was detected using mouse anti-PP2C $\alpha$  antibody (Acris Antibodies). Primary antibodies were detected using LI-COR IRDye<sup>®</sup> IR Dye secondary antibodies and visualised using an Odyssey Infrared Imager (LI-COR Biotechnology). Quantification of results was performed using Odyssey software and expressed as a ratio of the signal obtained with the phospho-specific antibody relative to the appropriate total antibody.

### Dephosphorylation of AMPK and SNF1

Recombinant AMPK or SNF1 was phosphorylated by CaMKK $\beta$  or LKB1, as described above. An aliquot (5  $\mu$ l) of the phosphorylated AMPK or SNF1 was incubated in 50 mM Hepes, pH 7.4, and 2.5 mM MgCl<sub>2</sub>, in the presence or absence of recombinant PP2C $\alpha$  (26 ng) and the presence or absence of 150  $\mu$ M AMP for 20 min at 37 °C. The reaction was terminated by the addition of gel-loading buffer [50 mM Tris/HCl, pH 7.4, 2.5% (w/v) SDS, 1% (v/v) 2-mercaptoethanol, 10% (v/v) glycerol and 0.05% Bromophenol Blue]. Samples were resolved by SDS/PAGE and subjected to Western blot analysis.

### Rat liver enzymes

Rat liver AMPK was purified up to the DEAE-Sephacryl step as described previously [28], and rat liver LKB1 up to the Q-Sephacryl step as described previously [29]. Rat liver AMPK was dialysed into 50 mM Hepes, 10% (v/v) glycerol, 1 mM EDTA, 1 mM DTT, 1 mM benzamidine, 0.1 mM PMSF and 4  $\mu$ g/ml trypsin inhibitor in order to remove the PP inhibitors present in the purification buffer. Following dialysis, AMPK was incubated in the presence or absence of 2.5 mM MgCl<sub>2</sub>, and the presence or absence of 50 mM NaF, with or without 150  $\mu$ M AMP for 10 min at 37 °C. Aliquots (5  $\mu$ l) were removed for Western blot analysis or for AMPK activity determination using the SAMS peptide assay, measured in the presence of a final concentration of 150  $\mu$ M AMP. In some cases, AMPK was incubated for 10 min at 37 °C in the presence of 200  $\mu$ M ATP, 2.5 mM MgCl<sub>2</sub> and in the presence or



**Figure 1** Effect of mutations within  $\gamma$  subunit on allosteric activation of  $\alpha 1\beta 1\gamma 1$  by AMP

Wild-type (WT) AMPK complex ( $\alpha 1\beta 1\gamma 1$ ) or complexes harbouring point mutations in the  $\gamma$  subunit (R69Q, H150R or R298G) were expressed in *E. coli* and purified by nickel–Sepharose chromatography. **(A)** Proteins ( $\sim 1 \mu\text{g}$ ) were resolved by SDS/PAGE and detected by staining with Coomassie Blue. **(B)** and **(C)** Following phosphorylation by CaMKK $\beta$ , AMP activity of the complexes was determined using the SAMS peptide assay in the presence or absence of  $150 \mu\text{M}$  AMP. Results are the means  $\pm$  S.E.M. for four independent experiments and are shown as the specific activity of the kinase (nmol of phosphate incorporated/min per mg of complex) in **(B)** and the fold stimulation in **(C)**. **(D)** Western blot analysis of AMPK complexes probed with an antibody against either phospho-Thr<sup>172</sup> (pT172) or  $\alpha 1$ . A representative blot is shown in the upper panel and the bottom panel shows the relative quantitation of pT172:total  $\alpha 1$  as means  $\pm$  S.E.M. for four independent experiments. Wild-type AMPK that had not been incubated with CaMKK $\beta$  is included as a negative control.

absence of LKB1 (16 ng), 50 mM NaF or  $150 \mu\text{M}$  AMP (as stated in the legend to the Figure). Aliquots ( $5 \mu\text{l}$ ) of these reactions were removed for AMPK activity determination (measured in the presence of a final concentration of  $150 \mu\text{M}$  AMP).

### Statistical analysis

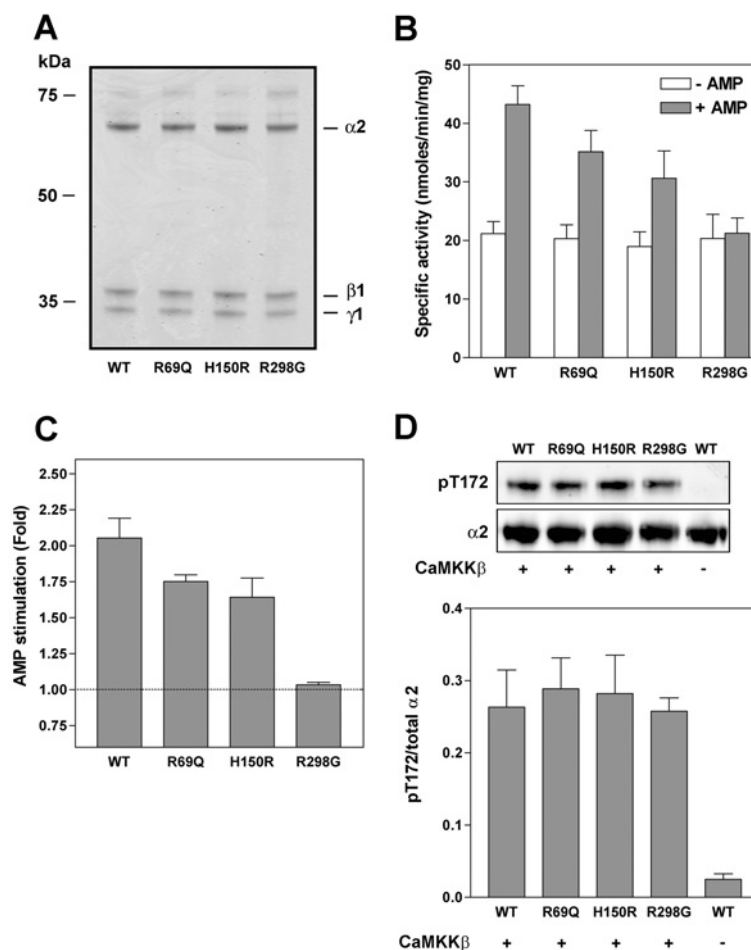
Results are expressed as means  $\pm$  S.E.M. Statistical analysis was carried out using a two-tailed, unpaired Student's *t* test.

## RESULTS

### Effect of $\gamma$ subunit mutations on allosteric activation by AMP

Two previous studies reported that naturally occurring mutations in the  $\gamma 2$ -subunit of AMPK interfere with AMP-binding and allosteric activation of the kinase [18,19]. The residues mutated in  $\gamma 2$  are conserved in all mammalian  $\gamma$  isoforms, suggesting that they may have functionally important roles. In order to investigate more thoroughly the role of these residues in regulation of AMPK by AMP, we expressed AMPK complexes in *E. coli* using a tricistronic expression system [24]. In the present study, we have

used  $\gamma 1$ -containing complexes because, despite extensive efforts, we have been unable to obtain purified preparations of recombinant AMPK complexes containing the  $\gamma 2$  isoform (results not shown). Wild-type AMPK complexes ( $\alpha 1\beta 1\gamma 1$  or  $\alpha 2\beta 1\gamma 1$ ) or complexes harbouring mutations, R69Q (equivalent to R302Q in human  $\gamma 2$ ), H150R (H383R) and R298G (R531G) within the  $\gamma 1$  subunit, were expressed and purified by nickel–Sepharose chromatography. As shown in Figures 1(A) and 2(A), no difference in expression of the various complexes was detected. As reported previously [24], the recombinant AMPK was isolated in an inactive form, but could be activated by phosphorylation using recombinant preparations of the upstream kinases, CaMKK $\beta$  or LKB1. Following phosphorylation by CaMKK $\beta$ , AMP activated the wild-type AMPK complexes 2-fold and this activation was reduced in complexes harbouring mutations in the  $\gamma$  subunit (Figures 1B, 1C, 2B and 2C). The R298G mutation had the most significant effect on AMP activation, completely abolishing activation of the  $\alpha 2$  complex. Mutations in the  $\gamma 1$  subunit did not have a significant effect on the degree of phosphorylation of Thr<sup>172</sup> by CaMKK $\beta$  or LKB1, nor on the specific activity of AMPK measured in the absence of AMP (Figures 1 and 2, and results not shown).



**Figure 2** Effect of mutations within  $\gamma$  subunit on the allosteric activation of  $\alpha 2\beta 1\gamma 1$  by AMP

The same set of experiments as described in the legend for Figure 1 were carried out using  $\alpha 2$  complexes.

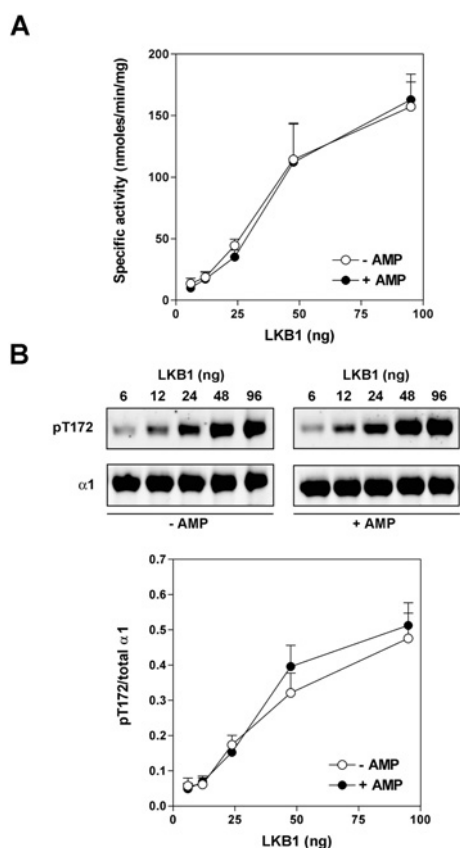
### AMP does not stimulate phosphorylation of AMPK

Previous studies reported that, in addition to allosteric activation, AMP also promotes the phosphorylation of AMPK by CaMKK and LKB1 [6,7,21,30]. In the CaMKK studies, partially purified preparations from pig brain were used [21,30]. In subsequent studies using recombinant CaMKK $\alpha$  and CaMKK $\beta$ , no effect of AMP was observed [7,9]. Taken together these results suggest that the effect of AMP on phosphorylation was mediated by a contaminating factor present in the pig brain preparations of CaMKK. In light of these findings we decided to reinvestigate the effect of AMP on phosphorylation of AMPK by LKB1 using recombinant proteins. Wild-type AMPK complexes were activated following phosphorylation by LKB1, and, under the conditions used, activation was almost linear with the amount of LKB1 added (Figures 3A and 4A). Note that, because AMP is present in one of the activation reactions with LKB1, we added AMP to the SAMS peptide phosphorylation so that the final concentration of AMP is 150  $\mu$ M in this assay, irrespective of whether AMP was present or absent in the LKB1 incubation. Both  $\alpha 1$ - and  $\alpha 2$ -complexes were activated to similar degrees, and activation correlated closely with the extent of phosphorylation of Thr<sup>172</sup> (Figures 3B and 4B). Using this highly purified reconstituted system, we did not detect any effect of AMP on phosphorylation of Thr<sup>172</sup> or on activation of AMPK for either  $\alpha 1$ - or  $\alpha 2$ -complexes. It was reported recently [23] that AMP is generated in the AMPK assay using the SAMS

peptide as a substrate. However, at the end of the phosphorylation reaction with LKB1 and AMPK we were unable to detect AMP in the assay mixture (in the absence of added AMP), as judged by ion-exchange chromatography (results not shown).

### Effect of $\gamma$ subunit mutations on AMP inhibition of dephosphorylation of AMPK

Wild-type AMPK or AMPK harbouring the R298G mutation in  $\gamma 1$  was phosphorylated by CaMKK $\beta$  and subsequently used as a substrate for dephosphorylation by recombinant PP2C $\alpha$ . The phosphorylation state of Thr<sup>172</sup> was assessed by Western blotting using anti-(phospho-Thr<sup>172</sup>) specific antibodies and quantified relative to the level of total  $\alpha$  subunit expression using an IR scanner. As shown in Figure 5, incubation of AMPK with PP2C $\alpha$  alone led to marked dephosphorylation of the  $\alpha$  subunit. Inclusion of AMP in the incubation with PP2C $\alpha$  significantly reduced the dephosphorylation of Thr<sup>172</sup> for the wild-type  $\alpha 1$ - and  $\alpha 2$ -containing complexes (Figure 5). In contrast, AMP had no significant effect on the dephosphorylation of the R298G complexes. The inhibition of dephosphorylation by AMP for the R69Q and H150R complexes was significantly reduced, although to a lesser extent than seen with the R298G complex (results not shown). Similar results were obtained using AMPK phosphorylated by LKB1. These results are consistent with a previous study [22] which reported



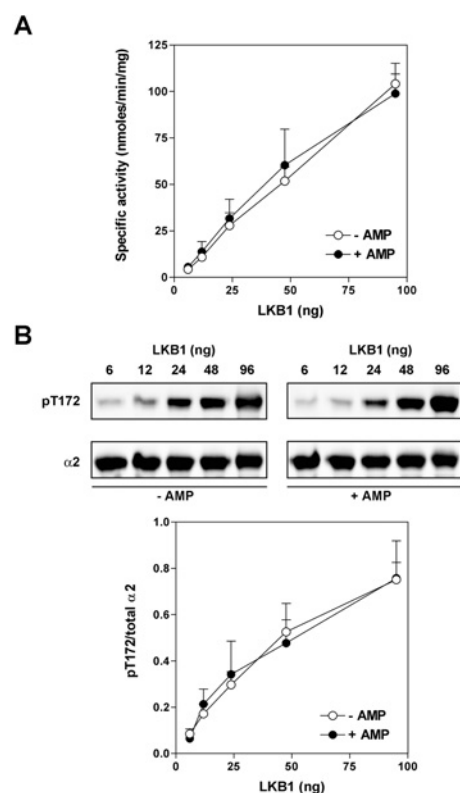
**Figure 3** Effect of AMP on phosphorylation of  $\alpha 1\beta 1\gamma 1$  by LKB1

(A) Wild-type  $\alpha 1\beta 1\gamma 1$  was incubated with MgATP and varying amounts of LKB1 in the presence (closed circles) or absence (open circles) of  $150 \mu\text{M}$  AMP for 10 min at  $37^\circ\text{C}$ . At the end of the incubation an aliquot ( $5 \mu\text{l}$ ) was removed, diluted in buffer and the AMPK activity was determined using the SAMS peptide assay. Note that in the SAMS peptide assay, AMP ( $150 \mu\text{M}$  final concentration) is present irrespective of whether AMP was included in the initial incubation with LKB1. (B) The top panel shows a representative Western blot of phospho-Thr<sup>172</sup> (pT172) and total  $\alpha 1$  of aliquots ( $5 \mu\text{l}$ ) of the reactions described above. The bottom panel shows the relative quantitation of pT172:total  $\alpha 1$  as means  $\pm$  S.E.M for three independent experiments.

that AMP inhibits dephosphorylation of AMPK partially purified from rat liver. Our results extend these previous findings by demonstrating that the effect of AMP on dephosphorylation involves residues that are also involved in the allosteric regulation of AMPK.

### AMP has no effect on yeast SNF1 kinase

In notable contrast with the mammalian enzyme, the yeast counterpart of AMPK, SNF1, has been reported not to be allosterically activated by AMP [20,31], but the effect of AMP on dephosphorylation of SNF1 has not been reported. In order to investigate this, we co-expressed Snf1, Snf4 and Gal83 in *E. coli*. The SNF1 complex was purified by nickel–Sepharose chromatography followed by gel-filtration to near homogeneity (Figure 6A). SNF1 was virtually inactive following purification, but could be activated by phosphorylation with CaMKK $\beta$  *in vitro*. Using radiolabelled ATP, phosphorylation of both the Snf1 and Gal83 subunits was detected. The amino acid sequence surrounding Thr<sup>210</sup> in Snf1, the equivalent site to Thr<sup>172</sup> in AMPK, is highly conserved between yeast and mammals, and the anti-(phospho-Thr<sup>172</sup>) antibody cross-reacted with phosphorylated Snf1 on Western blots (Figure 6B). We were therefore able to measure the dephosphorylation of Thr<sup>210</sup> by PP2C $\alpha$  using the anti-(phospho-



**Figure 4** Effect of AMP on phosphorylation of  $\alpha 2\beta 1\gamma 1$  by LKB1

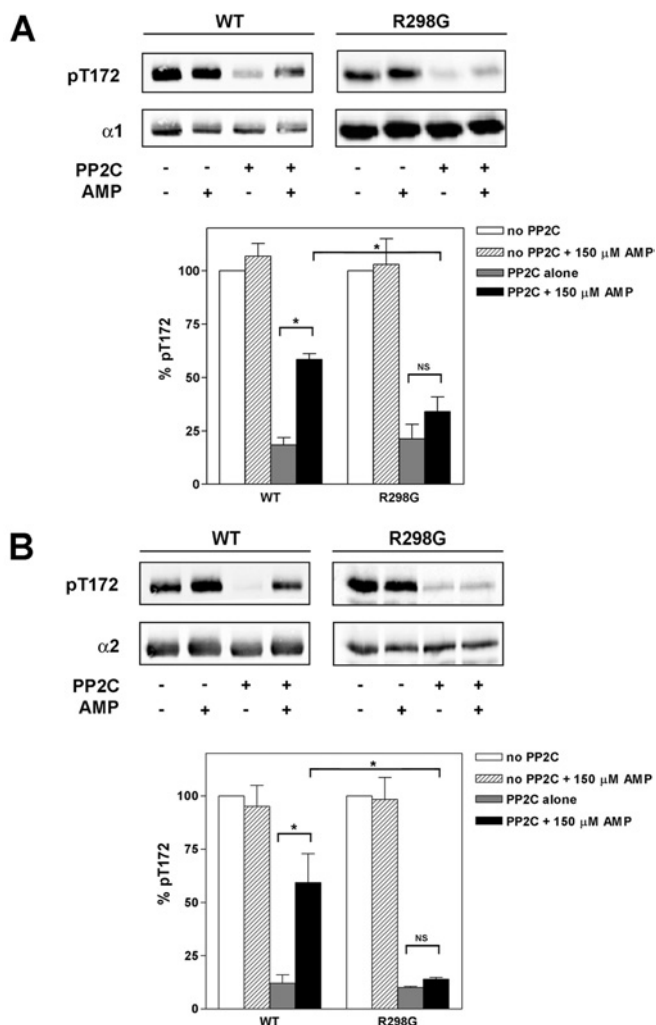
The same set of experiments as described in the legend for Figure 3 were carried out using  $\alpha 2$  complexes.

Thr<sup>172</sup>)-specific antibodies and quantify this relative to the level of total Snf1 expression (detected using an anti-His<sub>6</sub> antibody). The results shown in Figure 6(C) demonstrate that AMP does not inhibit dephosphorylation of Thr<sup>210</sup> in Snf1.

### Partially purified AMPK and LKB1 contain endogenous PP2C $\alpha$

Our results using highly purified recombinant preparations of AMPK, CaMKK $\beta$ , LKB1 and PP2C $\alpha$  reveal that AMP allosterically activates AMPK and inhibits the dephosphorylation of AMPK, but has no effect on phosphorylation by upstream kinases. The latter finding is at odds with several previous studies using partially purified preparations of AMPK and LKB1. One possibility that could explain this apparent discrepancy is that the preparations described in the other reports contain a factor that mediates the effect of AMP on phosphorylation. On the basis of our current results, we reasoned that this factor could in fact be a PP. To test this, we used a partially purified AMPK preparation in an activation assay with recombinant LKB1. AMPK was activated approximately 2-fold by LKB1, and AMP increased activation a further 2-fold (Figure 7A). However, if NaF, a PP inhibitor, was included in the reactions, AMPK activity was increased almost 6-fold by LKB1, and was not further activated by the addition of AMP, i.e. AMP and NaF have similar and non-additive effects on AMPK phosphorylation. Rather than showing an effect of AMP to promote phosphorylation, these results are most consistent with the hypothesis that AMP acts to inhibit the dephosphorylation of AMPK, with the overall effect being an increase in AMPK activity.

In order to determine directly the presence of a PP in a partially purified preparation of rat liver AMPK (purified up to the first



**Figure 5** Effect of AMP on dephosphorylation of recombinant AMPK by PP2C

Following phosphorylation of recombinant AMPK complexes (containing either wild-type  $\gamma 1$  or the R298G mutant) by CaMKK $\beta$ , aliquots (5  $\mu$ l) were removed and incubated in the presence or absence of PP2C (26 ng) and presence or absence of AMP (150  $\mu$ M) for 20 min at 37 °C. At the end of the incubation the reaction mixture was resolved by SDS/PAGE and analysed by Western blotting using anti-(phospho-Thr<sup>172</sup>) (pT172) and anti- $\alpha 1$  (A) or anti-(phospho-Thr<sup>172</sup>) and anti- $\alpha 2$  (B) antibodies. In each case a representative blot is shown in the top panels. In the bottom panels, Thr<sup>172</sup> phosphorylation relative to a control (absence of PP2C and AMP) is shown as the means  $\pm$  S.E.M. for four independent experiments. Significant differences in Thr<sup>172</sup> phosphorylation are denoted by \* $P < 0.05$ ). NS, not significant.

ion-exchange step, as described in [28]) we adopted a shotgun MS approach. The results (not shown) revealed the presence of PP2C $\alpha$ , and this was confirmed by Western blot analysis (Figure 7B). To investigate whether PP2C $\alpha$  activity is present in the AMPK preparation, we incubated AMPK in the presence or absence of MgCl<sub>2</sub>, an essential co-factor for PP2C $\alpha$  [32], and determined AMPK activity and Thr<sup>172</sup> phosphorylation (Figures 7C and 7D). AMPK activity and Thr<sup>172</sup> phosphorylation were significantly reduced only in the presence of MgCl<sub>2</sub> and these decreases were blocked totally by the addition of 50 mM NaF. Consistent with our studies using recombinant AMPK and PP2C $\alpha$ , AMPK activity was significantly inhibited, but did not abolish, these effects. PP2C $\alpha$  was undetectable in more highly purified preparations of AMPK (up to the gel-filtration step, as described in [28]), or following immunoprecipitation. Using these preparations, AMPK activity

was not affected following incubation with MgCl<sub>2</sub>. Furthermore, we did not detect an effect of AMP on activation of immunoprecipitated AMPK by recombinant LKB1 (results not shown). However, AMP stimulation of Thr<sup>172</sup> phosphorylation has been reported using more highly purified preparations of AMPK [6,21]. In these studies, LKB1 was purified from rat liver up to the Q-Sepharose step (as described in [29]), raising the possibility that PP2C $\alpha$  could be present in the preparations. We found that PP2C $\alpha$  was readily detectable by Western blotting in rat liver LKB1 purified to the same stage (Figure 7E).

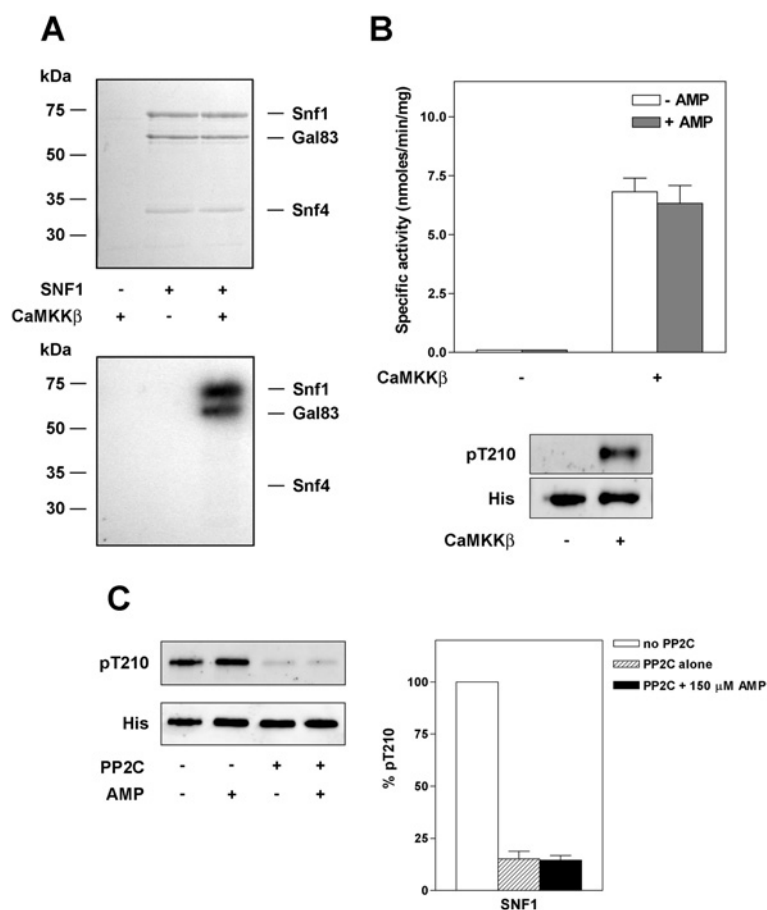
## DISCUSSION

In the present study, we investigated the mechanisms by which AMP leads to activation of AMPK. We took advantage of the use of highly purified recombinant preparations of the components of the AMPK cascade. Wild-type AMPK complexes ( $\alpha 1\beta 1\gamma 1$  and  $\alpha 2\beta 1\gamma 1$ ) expressed in bacteria were activated by phosphorylation using either CaMKK $\beta$  or LKB1, consistent with results reported previously [8,9,24]. AMP allosterically activated both  $\alpha 1$ - and  $\alpha 2$ -containing complexes by approximately 2-fold. The degree of activation of recombinant  $\alpha 1\beta 1\gamma 1$  is slightly greater than that reported previously for  $\alpha 1\gamma 1$  complexes isolated by sequential immunoprecipitation from rat brain (2.1-fold compared with 1.7-fold), whereas activation of recombinant  $\alpha 2\beta 1\gamma 1$  is slightly lower than that reported for  $\alpha 2\gamma 1$  complexes from rat brain (2.1-fold compared with 2.8-fold) [33]. A complication with the measurements using native rat brain AMPK is that these complexes would contain a mixture of  $\beta 1$  and  $\beta 2$  isoforms, and this might account for the minor differences in AMP stimulation observed between the two studies.

The  $\gamma$  subunit isoforms contain four copies of a CBS domain and previous studies [18,19,34] have shown that these domains are involved in AMP binding and subsequent allosteric activation of AMPK. Part of the evidence implicating the CBS domains in regulation of AMPK comes from the identification of naturally occurring mutations in  $\gamma 2$  that cause severe cardiac defects in humans [15–17], and a naturally occurring mutation in  $\gamma 3$  in pig that leads to glycogen accumulation in skeletal muscle [35]. Most of these mutations lie within the CBS domains and interfere with the normal activation of AMPK by AMP [18,19]. For reasons that are unclear, we have been unable to express  $\gamma 2$ - or  $\gamma 3$ -containing complexes in bacteria and so we introduced mutations into the  $\gamma 1$  subunit to mimic three of the equivalent naturally occurring mutations found in  $\gamma 2$ , including the equivalent mutation in pig  $\gamma 3$ . Complexes containing the mutant  $\gamma 1$  subunits were expressed in bacteria and were activated by CaMKK $\beta$  or LKB1, similar to wild-type AMPK. Consistent with previous studies, AMP activation was reduced in the  $\gamma 1$ -mutant complexes, with the R298G mutation having the greatest effect.

Davies et al. [22] reported previously that AMP inhibits dephosphorylation of AMPK. We confirmed that AMP has a significant inhibitory effect on dephosphorylation of recombinant AMPK by PP2C $\alpha$ . While the present paper was in preparation, Suter et al. [23] published results using recombinant AMPK in which they also demonstrated AMP inhibition of dephosphorylation. Importantly, we now show that a mutation in CBS4 of AMPK (R298G) completely abolishes the effect of AMP on dephosphorylation. Furthermore, the R298G mutation almost completely abolishes allosteric activation of AMPK, suggesting that the same mechanism may account for both AMP effects.

SNF1, the yeast counterpart of AMPK, is activated by phosphorylation of Thr<sup>210</sup>, the equivalent residue to Thr<sup>172</sup> in AMPK $\alpha$ . Although SNF1 does not appear to be directly activated by AMP [20,31], there are no reports as to whether AMP has an effect on



**Figure 6** Effect of AMP on recombinant SNF1 complex

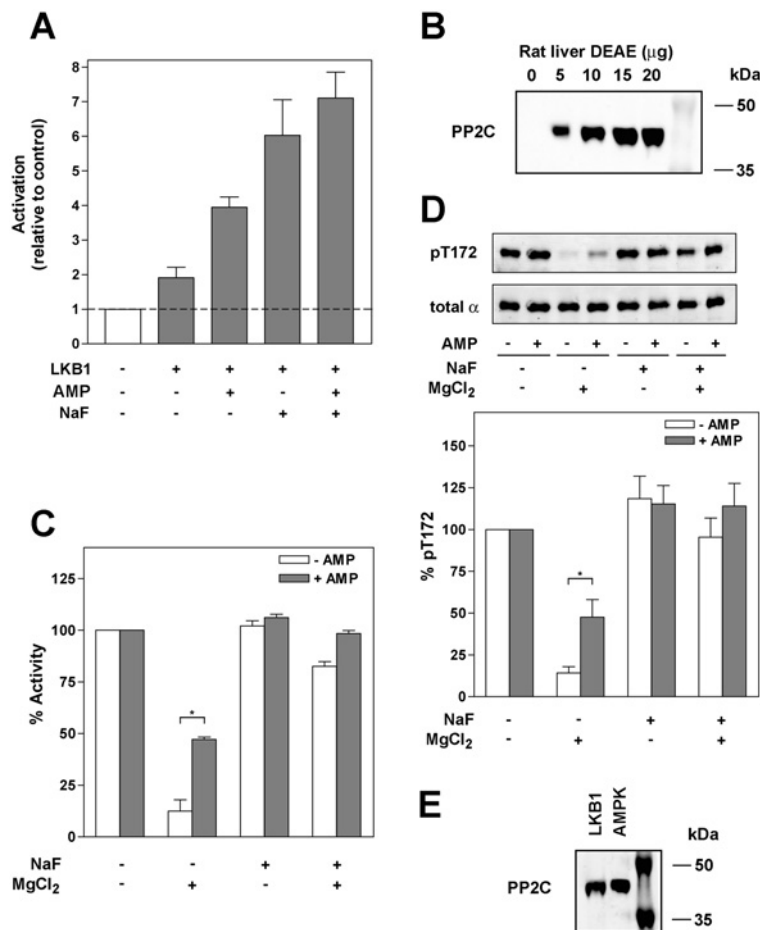
SNF1 (Snf1, Snf4, Gal83) was expressed in *E. coli* and purified on nickel–Sepharose, followed by gel-filtration chromatography. **(A)** Purified SNF1 was incubated with [<sup>32</sup>P]-ATP in the presence or absence of CaMKKβ and resolved by SDS/PAGE. The proteins were visualized by staining with Coomassie Blue (upper panel) and radiolabelled products detected by autoradiography (lower panel). **(B)** SNF1 activity was determined by the SAMS peptide assay in the presence or absence of 150 μM AMP. Activities are plotted as nmol of phosphate incorporated/min per mg of SNF1, and are the means ± S.E.M for three independent experiments. Also shown is a representative blot of the reaction mixture probed with anti-(phospho-Thr<sup>172</sup>) (to detect phospho-Thr<sup>210</sup> in Snf1) or anti-His<sub>6</sub> antibody (to detect total Snf1). **(C)** SNF1 phosphorylated by CaMKKβ was incubated in the presence or absence of PP2C (26 ng) and the presence or absence of 150 μM AMP for 20 min at 37 °C. At the end of the incubation proteins were analysed by Western blotting using either anti-(phospho-Thr<sup>172</sup>) or anti-His<sub>6</sub> antibody as described in the legend for Figure 5. The results are shown as means ± S.E.M. for four independent experiments.

its dephosphorylation. We were able to purify an SNF1 complex following co-expression of Snf1, Snf4 and Gal83 subunits in bacteria. As with AMPK, SNF1 was isolated in an inactive state, but could be activated by phosphorylation *in vitro* using CaMKKβ or LKB1 (results not shown). Consistent with previous studies, we did not detect any allosteric activation of recombinant SNF1 by AMP. In addition, we were unable to detect any effect of AMP on dephosphorylation of Thr<sup>210</sup> by PP2Cα. In yeast, SNF1 is activated following growth under glucose-limiting conditions [36]. The signal transduction pathway linking glucose limitation to SNF1 activation is unclear, but our results suggest that AMP is unlikely to be directly involved. However, the availability of a reconstituted system for SNF1 activation would allow potential activators, such as glucose metabolites, to be investigated.

A previous study [21] reported that phosphorylation of AMPK by preparation of AMPKK (AMPK kinase) from rat liver was stimulated by AMP. Subsequent work has revealed that the identity of AMPKK in these preparations is LKB1 [6]. Using recombinant preparations of AMPK and LKB1, we have, however, been unable to detect any stimulation of Thr<sup>172</sup> phosphorylation by AMP under any conditions. Recently, Suter et al. [23] published results using recombinant AMPK in which they also failed to

detect an effect of AMP on T172 phosphorylation by LKB1 or CaMKKβ [23]. However, the authors were unable to account for the apparent discrepancy between their results and previous studies. In the present study, we provide a plausible explanation for this inconsistency. We reasoned that the partially purified preparations of AMPK and/or LKB1 used in previous studies examining AMP stimulation could contain an additional factor that mediates the effect of AMP on Thr<sup>172</sup> phosphorylation.

In an effort to investigate this further, we began by adopting a shotgun MS approach to determine proteins present in a partially purified preparation of rat liver AMPK (purified up to the first ion-exchange step, as described in [28]). The results (not shown) revealed the presence of PP2Cα, and this was confirmed by Western blot analysis. We present strong evidence that the AMP effect on Thr<sup>172</sup> phosphorylation is due to the presence of PP2Cα, since we only observe AMP stimulation using preparations of AMPK that contain PP2Cα and the effect is abolished by NaF. Based on these findings, we suggest that AMP stimulation of Thr<sup>172</sup> phosphorylation is due to inhibition of PP2Cα present in the preparations of AMPK and/or LKB1 and CaMKK. In support of this hypothesis, we showed that PP2Cα is present in a preparation of rat liver LKB1. Although we cannot rule out other possibilities,



**Figure 7** Inactivation of rat liver AMPK by endogenous PP activity

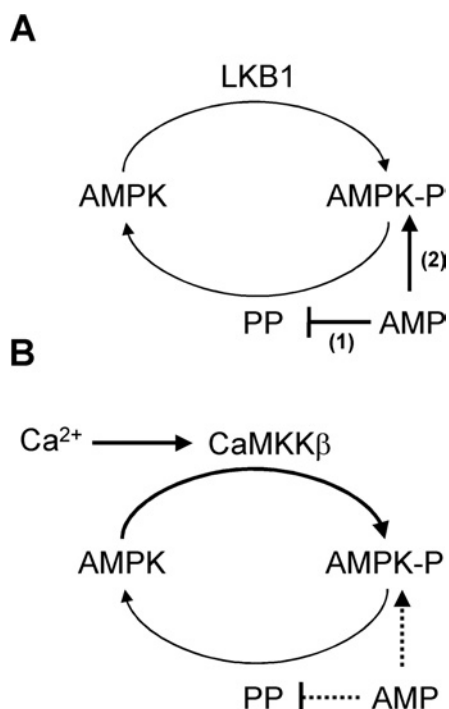
(A) Following dialysis into buffer lacking PP inhibitors, rat liver AMPK was incubated for 10 min at 37°C in the presence of MgATP and the presence or absence of LKB1 (16 ng), AMP (150 μM) and/or NaF (50 mM). Following incubation, AMPK activity was determined. Results shown are plotted relative to AMPK activity in the presence of MgATP alone and are the means ± S.E.M. for three independent experiments. (B) Partially purified rat liver AMPK extract was immunoblotted with an antibody against PP2Cα. (C) Rat liver AMPK was incubated in the presence or absence of MgCl<sub>2</sub> (2.5 mM) and in the presence or absence of either 50 mM NaF, 150 μM AMP or both for 10 min at 37°C. Following incubation, aliquots (5 μl) were removed and AMPK activity was determined using the SAMS peptide assay in the presence of 150 μM AMP (final concentration). (D) In parallel, aliquots (5 μl) were blotted with anti-(phospho-Thr<sup>172</sup>) or a mixture of anti-α1 and -α2 antibodies. A representative blot is shown (upper panel) and the lower panel shows the quantification of the relative level of Thr<sup>172</sup> phosphorylation for three independent experiments (means ± S.E.M.). \*Significant differences ( $P < 0.05$ ) in AMPK activity or Thr<sup>172</sup> phosphorylation. (E) Rat liver AMPK and LKB1, purified up to the Q-Sepharose step (as described in [29]), were probed with an anti-PP2Cα antibody.

this explanation would account for the differences between our studies using defined purified preparations of recombinant proteins and those studies using less well-defined preparations. It is interesting to note that all the studies reporting AMP stimulation of Thr<sup>172</sup> phosphorylation use okadaic acid to inhibit dephosphorylation, but do not include NaF. As PP2Cα is okadaic acid insensitive [37], it would remain active under the conditions of the assay (since PP2Cα would be activated by the Mg<sup>2+</sup> present in the kinase reaction).

Overall, our results lead us to propose a revised model for activation of AMPK. The phosphorylation state of Thr<sup>172</sup> depends on the relative rates of phosphorylation, catalysed by CaMKKβ and LKB1, and dephosphorylation, catalysed by protein phosphatases (Scheme 1). The identity of the protein phosphatase(s) acting on AMPK *in vivo* is unknown; however, the effect of AMP on dephosphorylation is substrate mediated [22] and so AMP would be predicted to inhibit all phosphatases acting on AMPK. Activation of AMPK occurs either by increased phosphorylation or by decreased dephosphorylation. LKB1 appears to be constitutively active and furthermore its activity is not changed by stimuli that

activate AMPK [8,38]. According to our model, increased Thr<sup>172</sup> phosphorylation by LKB1 would occur in response to decreased dephosphorylation following a rise in AMP. In parallel, AMP would allosterically activate AMPK (Scheme 1A). Dephosphorylation and inactivation of AMPK would occur when the concentration of AMP returned to basal levels. Unlike LKB1, the activity of CaMKKβ is subject to regulation within the cell, and is increased in response to signals that raise intracellular Ca<sup>2+</sup> [39,40]. Therefore, signals that increase Ca<sup>2+</sup> activate AMPK as a consequence of increased Thr<sup>172</sup> phosphorylation via increased CaMKKβ activity. Under these conditions there is no requirement for increased AMP levels (Scheme 1B). Consistent with this model, recent studies have reported activation of AMPK by CaMKKβ-mediated signalling without detectable changes in AMP [7,41,42]. A further prediction of our model is that AMPK would be rapidly dephosphorylated following a fall in intracellular Ca<sup>2+</sup>, and this may account for the transient nature of AMPK activation observed in CaMKKβ-mediated responses [7,41,42]. It should be noted that the AMP- and Ca<sup>2+</sup>-signalling pathways for activation of AMPK predicted by our model are not mutually





**Scheme 1** Model for the regulation of AMPK

(A) AMP-dependent activation: under conditions that lead to an increase in AMP, dephosphorylation of AMPK is inhibited (mechanism 1). The identity of the PP responsible for dephosphorylation of AMPK *in vivo* is unknown. Since LKB1 is constitutively active, inhibition of the dephosphorylation reaction leads to an increase in Thr<sup>172</sup> phosphorylation and activation of AMPK. In addition to increasing Thr<sup>172</sup> phosphorylation, AMP allosterically activates AMPK (mechanism 2). (B) Ca<sup>2+</sup>-dependent activation: signals that increase Ca<sup>2+</sup> activate CaMKK $\beta$ , increasing Thr<sup>172</sup> phosphorylation and activation of AMPK, and this can occur without an increase in AMP. However, it is possible that in some situations both Ca<sup>2+</sup> and AMP may increase in parallel, and under these conditions AMP will allosterically activate AMPK and inhibit dephosphorylation (denoted by the dashed lines).

exclusive. It is possible that under some circumstances, where AMP and Ca<sup>2+</sup> rise in concert, both pathways could operate. Under these conditions, both CaMKK $\beta$  and LKB1 would contribute to AMPK activation.

Although our results were obtained with  $\gamma$ 1-containing AMPK complexes, we speculate that the same mechanisms will apply to  $\gamma$ 2- and  $\gamma$ 3-containing complexes. Previous studies have shown that mutations within  $\gamma$ 2 and  $\gamma$ 3 interfere with allosteric activation of AMPK measured *in vitro* [18,19,43], and so it seems likely that these mutations will also interfere with the effect of AMP on dephosphorylation. Two studies have reported that  $\gamma$ 2 mutations decrease AMPK activity in transgenic animal models [44,45], whereas another study reported increased activity [46]. In a study in which  $\gamma$ 3-containing AMPK complexes were overexpressed in COS cells, increased activity was observed in mutant  $\gamma$ 3 relative to the wild-type protein [43]. Despite our understanding of the regulation of AMPK by AMP *in vitro*, elucidating the complex regulation of AMPK both in cells and *in vivo*, and determining the effect of mutations within the  $\gamma$  subunits, is far from complete.

In conclusion, our results demonstrate that AMP activates AMPK via a direct allosteric mechanism and indirectly by inhibiting dephosphorylation of Thr<sup>172</sup>. These effects may be mediated by binding of AMP to the same site within the  $\gamma$  subunit, since both effects are abolished by the same mutation within the  $\gamma$  subunit. We provide evidence that suggests that previous studies reporting activation of Thr<sup>172</sup> phosphorylation by AMP may have been mediated by PP2C $\alpha$  present in the partially purified preparations

of the kinases. On the basis of our results, we propose a model for the regulation of AMPK that accounts for its activation in response to either Ca<sup>2+</sup> signalling or energy depletion, mediated by an increase in AMP.

This work was supported by the Medical Research Council UK, an Integrated Project (LSHM-CT-2004-005272) from the European Commission and a BBSRC-CASE studentship award (to M.J.S.).

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Received 6 October 2006/24 November 2006; accepted 6 December 2006  
Published as BJ Immediate Publication 6 December 2006, doi:10.1042/BJ20061520