The regulation of AMP-activated protein kinase by phosphorylation

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The AMP-activated protein kinase (AMPK) cascade is activated by an increase in the AMP/ATP ratio within the cell. AMPK is regulated allosterically by AMP and by reversible phosphorylation. Threonine-172 within the catalytic subunit (α) of AMPK (Thr^{172}) was identified as the major site phosphorylated by the AMP-activated protein kinase kinase (AMPKK) *in vitro*. We have used site-directed mutagenesis to study the role of phosphorylation of Thr^{172} on AMPK activity. Mutation of Thr^{172} to an aspartic acid residue (T172D) in either α1 or α2 resulted in a kinase complex with approx. 50% the activity of the corresponding wild-type complex. The activity of wild-type AMPK decreased by greater than 90% following treatment with protein phosphatases, whereas the activity of the T172D mutant complex fell by only 10–15%. Mutation of Thr^{172} to an alanine residue (T172A) almost completely abolished kinase activity. These results indicate that phosphorylation of Thr^{172} accounts for most of the activation by AMPKK, but that other sites are involved. In support of this we have shown that AMPKK phosphorylates at least two other sites on the α subunit and one site on the β subunit. Furthermore, we provide evidence that phosphorylation of Thr^{172} may be involved in the sensitivity of the AMPK complex to AMP.

Key words: cell signalling, protein kinase cascade, site-directed mutagenesis, two-dimensional gel electrophoresis.

INTRODUCTION

Mammalian AMP-activated protein kinase (AMPK) belongs to a family of protein kinases that have been highly conserved in animals, plants and yeast [1] and which all play a key role in the regulation of energy homoeostasis [2]. AMPK is activated by stresses which deplete ATP, such as heat shock, hypoxia and ischaemia, which in turn leads to elevation of the AMP/ATP ratio [3]. In addition to allosteric activation by AMP, AMPK is regulated by reversible phosphorylation. AMPK is phosphorylated and activated by an upstream kinase, termed AMPK kinase (AMPKK) [4]. Protein phosphatases 2A and 2C dephosphorylate and inactivate AMPK *in vitro*, although the activity of the latter was shown to be more important in inactivating AMPK in cells [5]. Once activated, AMPK responds by phosphorylating multiple downstream substrates aimed at conserving existing ATP levels. AMPK reduces further ATP expenditure by inhibiting key enzymes in biosynthetic pathways such as acetyl-CoA carboxylase in fatty acid synthesis and 3-hydroxy-3-methyl-CoA reductase in cholesterol synthesis [6]. AMPK also increases the supply of ATP, for example by stimulating the rate of fatty acid oxidation [2,7]. Other metabolic enzymes identified as substrates of AMPK include hormone-sensitive lipase [8], glycogen synthase [9] and creatine kinase [10], leading to the hypothesis that AMPK acts as a kind of cellular 'fuel gauge' [2,7]. In this model AMPK detects changes in the energy charge of the cell and initiates the appropriate responses aimed at conserving ATP and switching on ATP-generating systems. The function of AMPK in protecting the cell against metabolic stress may have evolved from an ancient response system to stress, since homologues of AMPK found in other organisms, such as yeast and plants, appear to play similar roles.

AMPK is a heterotrimeric enzyme, consisting of a catalytic subunit (α) and two regulatory subunits (β and γ). Multiple isoforms of all three subunits have been identified [11–15], raising the possibility that a large family of AMPK complexes exists *in vivo*. Differences in the tissue distribution of the isoforms have been found [13,15,16], suggesting that the different isoforms may have tissue-specific roles. Formation of the trimeric complex is necessary for optimal kinase activity [17,18], and there is evidence that the β subunit acts as a scaffold protein within the complex, interacting with both the α and γ subunits [14]. Recent evidence showed that removal of the C-terminal domain of the α subunit (residues 313–548 within α1) abolished binding of the β and γ subunits [19].

The activity of AMPK within the cell is tightly regulated by the AMP/ATP ratio. This regulation arises via a number of separate mechanisms [20]. First, AMP directly activates both AMPK and its upstream kinase, AMPKK [21]. Secondly, AMP binds to AMPK, making it a better substrate for AMPKK [21]. Thirdly, AMP binds to AMPK, making it a worse substrate for inactivation by protein phosphatases [22]. There is good evidence that some, if not all, of these effects are antagonized by high concentrations of ATP [20]. During the last few years, considerable progress has been made in studying the role of AMPKK in the regulation of AMPK [4,21,23]. Using a partially purified preparation of AMPKK from rat liver, the major site at which it phosphorylates AMPK was identified as threonine-172 within the α subunit (Thr^{172}) [4]. At the time that study was carried out, the existence of the different α isoforms was not known and therefore the AMPK purified from rat liver contained a mixture of both the α1 and α2 isoforms. Although the primary sequence surrounding Thr^{172} is perfectly conserved between the two isoforms [12], there remains the possibility that the regulation of α1 and α2 by phosphorylation is different. Another factor which complicated identification of sites phosphorylated by AMPKK was the high level of autophosphorylation occurring within both the α and β subunits [4].

Abbreviations used: AMPK, AMP-activated protein kinase; AMPKK, AMP-activated protein kinase kinase; DTT, dithiothreitol; SAMS, the synthetic peptide HMRSAMSGLHLVKRR; Thr^{172}, threonine residue 172 within the AMPK α subunit; 2-D, two-dimensional; $\alpha1^{312}$, truncation of α1 at residue 312.

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In the present study we have examined the effect of site-directed mutagenesis of Thr^{172} within both $\alpha1$ and $\alpha2$ in order to address the regulation of AMPK by phosphorylation in more detail. Our results indicate that phosphorylation of Thr^{172} in both isoforms plays the predominant role in regulation of AMPK. Furthermore, we have also shown that Thr^{172} may be involved in the activation of the AMPK complex by AMP. We have used a catalytically inactive kinase mutant that is unable to auto-phosphorylate, to show that, in addition to Thr^{172}, two further sites within the α subunit and an additional site within the β subunit are phosphorylated by AMPKK. These sites may play a more subtle role in modifying AMPK activity.

EXPERIMENTAL

Site-directed mutagenesis

For each mutation, complementary primers spanning the residue to be mutated were synthesized and used to amplify either $\alpha1$ or $\alpha2$ rat cDNA by PCR. In the first round of amplification, two separate reactions were performed. Reaction 1 used a sense primer spanning the site to be mutated, e.g. $\alpha1$TDF (see sequence below) with an antisense primer spanning the stop codon of α, which includes a *Xho*I restriction site. Reaction 2 used the corresponding antisense primer spanning the site to be mutated, e.g. $\alpha1$TDR, with a sense primer spanning the start codon of α, which includes an *EcoR*I restriction site. The products from reactions 1 and 2 were purified by agarose-gel electrophoresis using a Qiagen gel extraction kit. A 1 μl portion of purified product from reactions 1 and 2 were mixed and allowed to undergo five cycles of extension and annealing, in the absence of primers, in order to extend the cDNA template. The primers spanning the α start and stop codons were added and the reaction allowed to undergo a further 20 cycles in order to amplify the full-length cDNA containing the relevant mutation. The product was gel-purified, digested with *EcoR*I and *Xho*I and cloned into pCDNA3. The inserts were sequenced in their entirety in order to confirm the authenticity of the mutation and ensure no other mutations had occurred.

Primer sequences used were as shown in Table 1.

Since the nucleotide sequences of $\alpha1$ and $\alpha2$ are highly conserved at the 5′ end, the same forward primer spanning the start codon could be used for both $\alpha1$ and $\alpha2$. This primer contained a sequence (shown in **bold** below) which encodes a peptide (EQKLISEEDL) derived from c-Myc which is recognized by a monoclonal antibody (clone 9E10) [24].

$\alpha1/\alpha2$ forward primer:
GAATTCGCCATG

GAGCAGAAGCTTATCTCCGAGGAGGACCTC

GGTGGCGGCGAGAAGCAGAAGCACGAC;

$\alpha1$ reverse primer: CTCGAGTTACTGTGCAAGAATTTT

$\alpha2$ reverse primer: CTCGAGTCAACGGGCTAAAGCAGTG

Table 1 Primer sequences used in the present work

Bases encoding the mutations are shown underlined.

AMPK chain	Mutation	Sense/ antisense	Primer name	Sequence
$\alpha1$	T172D	Sense	$\alpha1$TDF	TTTTTAAGAGATAGCTGTGGC
		Antisense	$\alpha1$TDR	CCACAGCTATCTCTTAAAAA
	T172A	Sense	$\alpha1$TAF	TTTTTAAGAGCTAGCTGTGGC
		Antisense	$\alpha1$TAR	GCCACAGCTAGCTCTTAAAAA
	D157A	Sense	$\alpha1$DAF	AAGATAGCCGCCTTCGGTCTTTC
		Antisense	$\alpha1$DAR	GAAAGACCGAAGGCGGCTATCTT
$\alpha2$	T172D	Sense	$\alpha2$TDF	TTTCTACGAGATAGCTGTGGA
		Antisense	$\alpha2$TDR	TCCACAGCTATCTCGTAGAAA
	T172A	Sense	$\alpha2$TAF	TTTCTACGAGCTAGCTGTGGA
		Antisense	$\alpha2$TAR	TCCACAGCTAGCTCGTAGAAA
	D157A	Sense	$\alpha2$DAF	AAGATAGCTGCCTTCGGACTCTC
		Antisense	$\alpha1$DAR	GAGAGTCCGAAGGCAGCTATCTT

Truncation of $\alpha1$

Truncated forms of $\alpha1$ were made by inserting a stop codon immediately after residue 312, as described by Crute et al. [19]. $\alpha1$ cDNA (wild-type and T172D) was amplified using the $\alpha1/\alpha2$ forward primer (described above) and a reverse primer with the sequence CTCGAGTTAGTACAGGCAGCTGAGGACC. The amplified product was digested with *EcoR*I and *Xho*I and cloned into pCDNA3. Inserts were sequenced in their entirety in order to confirm their authenticity and that no mutations had occurred during amplification.

Mammalian cell expression

Plasmid DNA was prepared using a Qiagen Maxi-Prep Kit according to the manufacturer's instructions. CCL13 cells or COS7 cells were transfected with plasmid DNA (10 μg/plasmid) by calcium phosphate precipitation [25]. At 48 h post-transfection the medium was removed and cells washed briefly with PBS, before being scraped from the plate in PBS containing 2 mM EDTA. The cells were collected by brief centrifugation and the cell pellet was lysed by resuspension in 1 ml buffer A [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] containing 1 % Triton X-100. Insoluble material was removed by centrifugation.

Immunoprecipitation of recombinant AMPK complexes

$\alpha1$ and $\alpha2$ complexes were immunoprecipitated from cell lysates by incubation with 10 μl of a 50 % (w/v) slurry of anti-Myc antibody (clone 9E10) bound to Protein A–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 buffer A and then twice with buffer B [50 mM Hepes (pH 7.5)/1 mM EDTA/1 mM DTT/10 % (v/v) glycerol] and used for further analysis. In order to quantify the level of expression of AMPK in the different transfections, 100 μl of lysate was immunoprecipitated with anti-Myc antibody. Immune complexes were resuspended in 40 μl of SDS sample buffer and 5 and 25 μl aliquots of each were analysed by Western blotting using an antibody raised against $\beta1$ [14]. Results from the Western blot were used to estimate the volume of lysate required to give approximately equal amounts of AMPK complex, and this was used in subsequent immuno-precipitations for comparing AMPK activity.

Phosphorylation–dephosphorylation of recombinant AMPK

Immune complexes from lysates which had been equalized for AMPK expression were treated with either recombinant protein phosphatase 2C (0.16 mg/ml) in the presence or absence of 10 mM $MgCl_2$, or protein phosphatase 2A (500 m-units) in the

presence or absence of 200 nM okadaic acid. After incubation for 20 min at 30 °C the immune complex was precipitated by brief centrifugation and washed once with buffer A and twice with buffer B before further treatment or analysis. In some cases AMPK was incubated in the presence of AMP (200 μM), $MgCl_2$ (5 mM), [γ-^{32}P]ATP (200 μM; $\approx$ 400 c.p.m./pmol) and in the presence or absence of AMPKK (200 units) for 30 min at 30 °C. The immune complex was collected by centrifugation and washed extensively with buffer B. AMPK activity in the immune complex was measured by phosphorylation of the synthetic peptide HMRSAMSGLHLVKRR (SAMS) [26]. Activities in the immune complexes were measured by addition of the assay reagents directly to the immunoprecipitate, and the mixture was incubated with shaking at 30 °C. Aliquots (20 μl) of the reaction mixture were taken after 15, 30 and 45 min. Unless stated otherwise, activities were measured in the presence of 200 μM AMP. Activities were calculated as pmol of phosphate incorporated into the SAMS peptide/min per ml of lysate after subtraction of a blank reaction using an immune complex isolated from untransfected cells.

Following the determination of AMPK activity the immune complexes were washed extensively with buffer A. Proteins within the complex were resolved by SDS/PAGE and analysed by Western blotting to confirm that equal amounts of AMPK had been immunoprecipitated. AMPK which had been incubated with [γ-^{32}P]ATP in the presence or absence of AMPKK was resolved by SDS/PAGE and subjected to autoradiography.

Two-dimensional (2-D) gel electrophoresis

Immune complexes were suspended in 120 μl of sample buffer [8 M urea/0.4 % (w/v) DTT/4 % (w/v) CHAPS/0.8 % (v/v) Pharmalyte/4 % (w/v) Bromophenol Blue] and mixed for 40 min at room temperature. The supernatent was placed in a trough of a strip swelling tray (Zeneca Engineering) and the appropriate Immobiline DryStrip (70 mm $\times$ 3 mm $\times$ 0.5 mm; Amersham Pharmacia Biotech) placed gel-side-down, and allowed to swell overnight at room temperature. The DryStrips were removed from the swelling tray and placed into a groove of an Immobilin strip aligner of a Multiphor II flatbed electrophoresis system (Amersham Pharmacia Biotech). The Multiphor II system was programmed to run at 200 V for 1 min, 1000 V for 1 h and then 4000 V for 3 h. The strips were washed twice for 10 min with 12 ml of isoelectric-focusing strip equilibriation buffer [50 mM Tris/HCl (pH 8.8)/6 M urea/30 % (v/v) glycerol/1 % (w/v) DTT/1 % (w/v) SDS]. The strips were then resolved in the second dimension using a Mini Protean II Minigel system (Bio-Rad). Gels were dried, and ^{32}P-labelled spots were detected by autoradiography.

Western blots

Immune complexes were boiled in SDS sample buffer, resolved by SDS/PAGE and transferred to a PVDF membrane. The membrane was blocked by incubation in 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. The membrane was incubated with an anti-β1 antibody (1:20 000 dilution; [14]) in the same buffer for a further 2–18 h at 4 °C. After extensive washing 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 Protein A conjugated with horseradish peroxidase. After further extensive washing the blots were developed using enhanced chemiluminescence (Boehringer Mannheim).

RESULTS

Mutation of Thr^{172} affects AMPK activity

We determined the effect of site-directed mutagenesis of Thr^{172} within both α1 and α2 on the activity of recombinant AMPK expressed in mammalian cells. Previous studies have shown that co-expression of the β and γ subunits is necessary to detect significant AMPK activity following transient transfection of either α1 or α2 in mammalian cells [17,18]. In the present study the α subunit was co-expressed with β1 and γ1. The level of expression of the AMPK complex was quantified by Western blotting. Mutation of Thr^{172} to an aspartate residue resulted in an AMPK complex with 40–50 % the activity of the corresponding wild-type α1 and α2 containing complexes (α1 wild-type: 198 $\pm$ 18 pmol/min per ml, $n = 3$; α2 wild-type: 29.8 $\pm$ 6.1 pmol/min per ml, $n = 4$) (Figures 1A and 1B). Incubation of either wild-type α1 or α2 containing complexes with protein phosphatase 2C reduced activity by over 90 %. In contrast, both α T172D mutants exhibited a marked resistance to treatment with protein phosphatase 2C, with only a small decrease in kinase activity being detected (Figures 1A and 1B). Virtually identical results were obtained if protein phosphatase 2A was used in place of protein phosphatase 2C (results not shown). Mutation of Thr^{172} to an alanine residue, however, resulted in an AMPK complex that was almost completely inactive (approx. 2 % activity of wild-type for α1, 9 % for α2).

Phosphorylation of AMPK by AMPKK

Our results demonstrate that Thr^{172} is a major site involved in the regulation of AMPK by phosphorylation. We did observe a small decrease in activity of the T172D mutant following treatment with protein phosphatase 2C, suggesting that other sites may contribute to the full activity of the AMPK complex. In order to investigate this possibility further, two catalytically inactive mutants (α1 D157A and α1 D157A/T172D) were constructed. Asp^{157} lies in the conserved DFG motif (subdomain VII in protein kinase catalytic subunits), which has been shown to be essential for MgATP binding in all protein kinases [27]. Co-expression of these mutants with β1 and γ1 in CCL13 cells yields a catalytically inactive complex (results not shown) which is unable to undergo autophosphorylation (Figure 2). In contrast, the wild-type complex autophosphorylates on both the α and β subunits in the absence of added AMPKK, with the degree of phosphorylation being increased markedly in the presence of AMPKK. Analysis of the α1 D157A and α1 D157A/T172D complexes by autoradiography following incubation with AMPKK in the presence of [γ-^{32}P]ATP revealed that both the α and β subunits were labelled (Figure 2). In order to determine the number of site(s) phosphorylated within the α and β subunits, the ^{32}P-labelled α1 D157A and α1 D157A/T172D complexes were analysed by 2-D gel electrophoresis (Figure 3). For the α1 D157A mutant, three labelled spots corresponding to the α subunit were detected by autoradiography, indicating that there are three major sites phosphorylated by AMPKK on the catalytic α subunit. By contrast, only two labelled spots corresponding to the α subunit were detected for the α1 D157A/T172D mutant. For both mutants, a single major spot corresponding to the β subunit was detected. The identity of the labelled spots was confirmed by Western blotting using isoform-specific antibodies (results not shown). These results, taken together with the effect of phosphatase treatment on the activity of the T172D mutant, demonstrate that sites other than Thr^{172} are phosphorylated by AMPKK. The finding that the α1 D157A/T172D mutant was

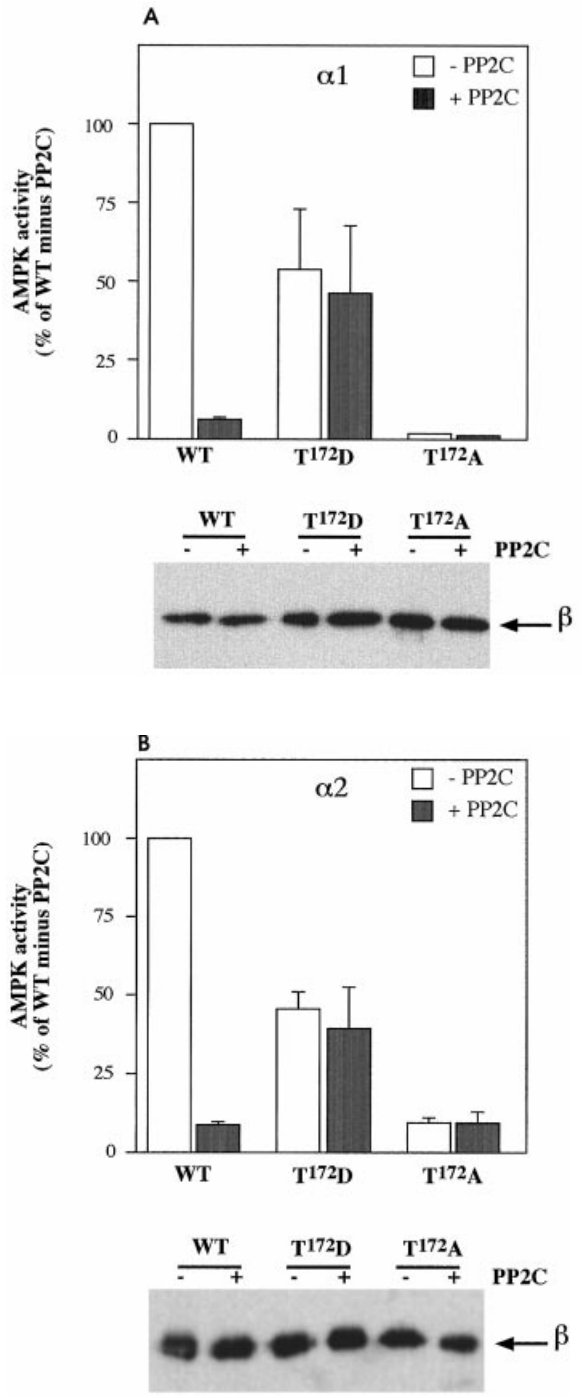

Figure 1 Activity of α Thr [172] mutants

Thr[172] within the α subunit isoforms was mutated to aspartic acid ('T[172]D') or alanine ('T[172]A'), and these mutants were co-expressed with β1 and γ1 in CCL13 cells. AMPK was immunoprecipitated with an anti-Myc antibody and incubated with protein phosphatase 2C ('PP2C'; 0.16 mg/ml) in the presence (black bars) or absence (open bars) of 10 mM $MgCl_2$ for 20 min. Following extensive washing of the immune complex, AMPK activity was determined using the SAMS peptide assay. The activity of the T172D and T172A mutant complexes is expressed as a percentage of wild-type ('WT') activity measured in the absence of active protein phosphatase 2C (no added $MgCl_2$). Results for the α1 isoform are from three independent experiments (**A**), whereas those for α2 are from four independent experiments (**B**). In each case a representative immunoblot of the β subunit present in the immune complexes following determination of AMPK activity is shown.

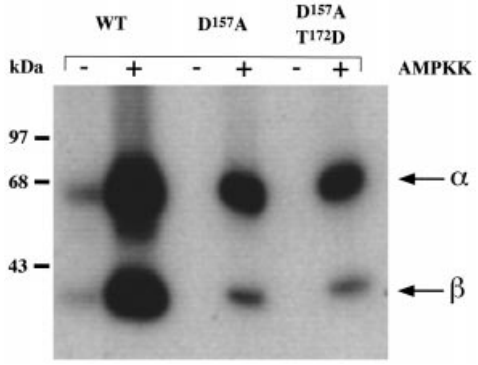

Figure 2 Phosphorylation of AMPK by AMPKK

A catalytically inactive form of α1 ('D[157]A'), in which Asp[157] was mutated to alanine, and α1 harbouring this mutation and a mutation of Thr[172] to aspartic acid ('D[157]A T[172]D'), were co-expressed with β1 and γ1 in CCL13 cells. AMPK complexes were immunoprecipitated from cell lysates and incubated with protein phosphatase 2C. Following quantification by Western-blot analysis of the β subunit, equal amounts of the different complexes were incubated with [γ-^{32}P]ATP in the presence (+) or absence (−) of AMPKK. Proteins were resolved by SDS/PAGE and [^{32}P]phosphate incorporation determined by autoradiography and compared with wild-type ('WT') α1 complexes. Labelled bands corresponding to the α and β subunits are shown by arrows. The migration of marker proteins is shown on the left of the autoradiogram.

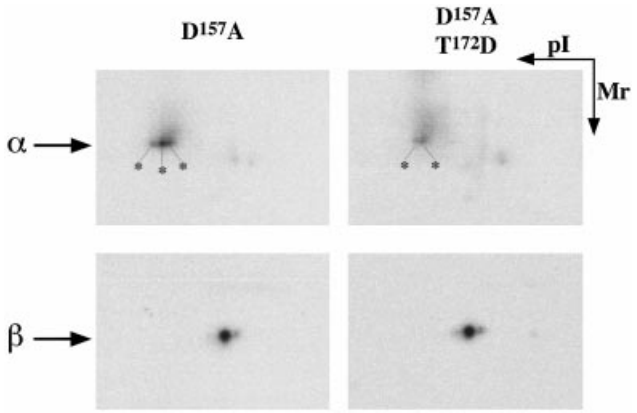

Figure 3 Analysis of phosphorylation sites on AMPK by 2-D gel electrophoresis

α1 D157A ('D157A) and α1 D157A/T172D ('D[157]A T[172]D') complexes were treated with protein phosphatase 2C prior to phosphorylation with AMPKK in the presence of [γ-^{32}P]ATP. The immune complexes were analysed by 2-D gel electrophoresis. The direction of separation by relative molecular mass is indicated by 'Mr'. In order to resolve the α subunit by isoelectric point ('pI'), a pH gradient of 3–10 was used in the first dimension (top panel), whereas a gradient of 4–7 was used for resolution of the β subunit (bottom panel). In both cases the pH gradient is from alkali to acid (left to right). ^{32}P-labelled spots were revealed by autoradiography. The spots corresponding to the α subunit are marked with an asterisk.

still phosphorylated by AMPKK supports our hypothesis that, although Thr[172] is the major regulatory phosphorylation site, it may not be the only site involved in regulating AMPK activity.

AMP-dependence of phosphorylation mutants

We then examined the AMP stimulation of the wild-type and T172D mutants. Wild-type α1 was stimulated 3.0±0.2-fold with a half-maximal effect at 5.7±2.0 μM, whereas wild-type α2 was stimulated 13.3±0.7-fold with a half-maximal effect at 16±

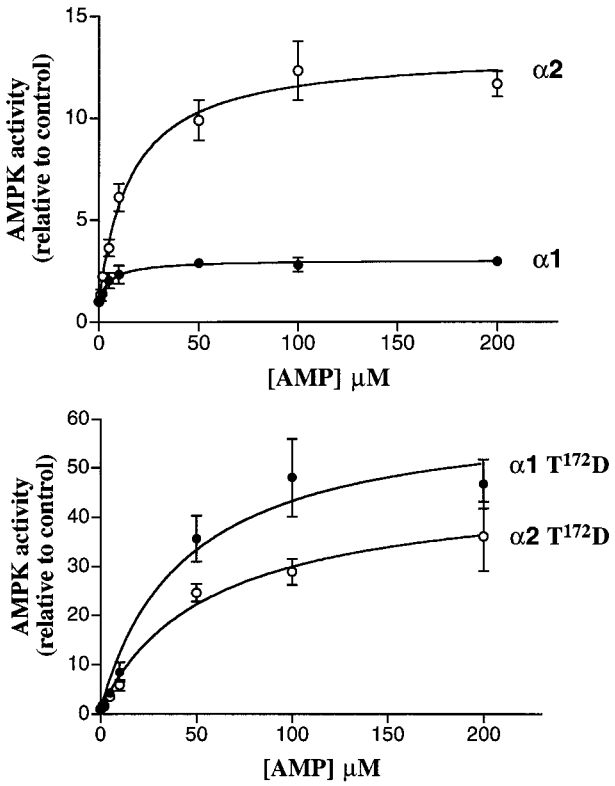

Figure 4 Effect of AMP on the activity of recombinant AMPK

(**A**) The activity of wild-type $\alpha1$ (●) and $\alpha2$ (○) containing immune complexes was determined using the SAMS peptide assay at various concentrations of AMP. Data were fitted to the equation:

$v = b + \{[(s \times b) - b] \times [\text{AMP}]\}/(A_{0.5} + [\text{AMP}])$

where v is the velocity, s is relative stimulation, b is the basal activity and $A_{0.5}$ is the concentration of AMP giving half-maximal activation, by using Prism (Graphpad Prism software). The data plotted are the average values (±S.E.M.) from three to four independent experiments and are expressed relative to the basal activity in the absence of added AMP. The theoretical curves were generated using the curve-fitting program. (**B**) Results obtained as above using T172D ('$T^{172}D$') mutants (●, $\alpha1$; ○, $\alpha2$).

3.5 μM (Figure 4A). The greater extent of AMP stimulation of $\alpha2$ compared with $\alpha1$ that we observe is consistent with the results of a previous study which measured the stimulation of AMPK complexes isolated from rat liver [28]. However, the fold stimulation by AMP for the recombinantly expressed AMPK isoforms is approximately double that seen with the complexes isolated from rat liver. We found that the activity of the T172D-mutant-containing complexes was stimulated to a much higher extent than with the corresponding wild-type subunits. The $\alpha1$ T172D mutant was stimulated 61.6±7.1-fold and the $\alpha2$ T172D mutant 43.4±5.6-fold (Figure 4B). In addition to an increased AMP-dependence, the concentration of AMP required to produce a half-maximal effect was markedly increased in the T172D mutants (43.4±15.4 μM for $\alpha1$ and 57.1±19.3 μM for $\alpha2$) compared with the wild-type complexes.

Truncation of $\alpha1$

Witters and colleagues reported recently the effect of truncating $\alpha1$ on the activity of AMPK [19]. Truncation of $\alpha1$ at residue 312 ($\alpha1^{312}$) yielded a protein that was active in the absence of the β and γ subunits and was insensitive to AMP [19]. It was reported

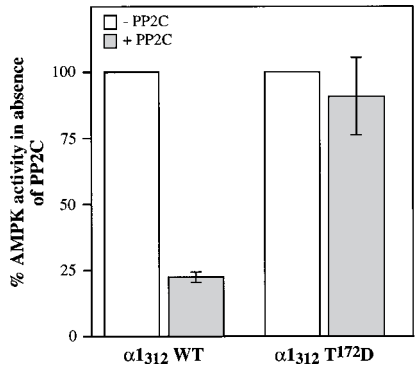

Figure 5 AMPK activity of truncated $\alpha1$ mutants

Wild-type $\alpha1^{312}$ ('$\alpha1_{312}$ WT') and $\alpha1^{312}$ in which Thr^{172} was mutated to an aspartic acid residue ('$\alpha1_{312}$ $T^{172}D$') were immunoprecipitated from CCL13 cell lysates. The immune complexes were incubated with protein phosphatase 2C ('PP2C'; 0.16 mg/ml) in the presence (black bars) or absence (open bars) of 10 mM $MgCl_2$ for 20 min. Following extensive washing of the immune complex, AMPK activity was determined using the SAMS peptide assay. The results are expressed as a percentage of the activity measured in the absence of active protein phosphatase 2C and are the average values (±S.D) from three independent experiments.

that mutation of Thr^{172} to an aspartic acid residue within either $\alpha1^{312}$ or the full-length $\alpha1$ subunit abolished kinase activity. We decided, therefore, to re-examine the effect of mutating Thr^{172} to aspartate within the truncated $\alpha1^{312}$ subunit. Consistent with the results obtained with full-length $\alpha1$, we found that the $\alpha1^{312}$T172D mutant was 33% as active as wild-type $\alpha1^{312}$ ($\alpha1^{312}$T172D: 2.5±0.2 pmol/min per ml; $\alpha1^{312}$: 7.5±1.2 pmol/min per ml, $n = 4$). Furthermore, $\alpha1^{312}$ T172D was almost completely insensitive to treatment with protein phosphatase 2C, whereas the activity of wild-type $\alpha1^{312}$ fell by 80% (Figure 5). The wild-type $\alpha1^{312}$ and $\alpha1^{312}$ T172D mutants were no longer sensitive to AMP and did not associate with the β and γ subunits (results not shown), as was previously reported for the wild-type $\alpha1^{312}$ form [19]. It should be noted, however, that the activities of the truncated $\alpha1$ mutants are markedly lower than those of the corresponding full-length $\alpha1$ complexes. In the study by Crute et al. [19], AMPK was expressed in COS7 (kidney) cells, whereas our results were obtained following expression in CCL13 (liver) cells. We found that the $\alpha1$ T172D mutant complex was approx. 35% as active as wild-type $\alpha1$ following expression in COS7 cells. Consistent with the results obtained in CCL13 cells, the T172D mutant expressed in COS7 was almost completely resistant to treatment with protein phosphatase 2C, whereas the wild-type complex was inactivated by over 90% (results not shown). These results indicate that the cell type used for expression does not markedly affect the activity of the $\alpha1$ T172D mutant.

DISCUSSION

Phosphorylation of AMPK by AMPKK is a critical step in the activation of AMPK in response to depletion of ATP. Thr^{172} within the α subunit of AMPK was identified as the major site phosphorylated by AMPKK *in vitro* [4]. Although the sequence surrounding this site is perfectly conserved in both $\alpha1$ and $\alpha2$, there remained the possibility that the regulation of the two isoforms by phosphorylation could be different. In order to elucidate the exact role of Thr^{172} in the regulation of AMPK activity, we altered this residue by site-directed mutagenesis in $\alpha1$ and $\alpha2$ and measured the activity of the recombinant AMPK. For some proteins replacement of the phosphorylatable residue

by a negatively charged amino acid can mimic the effect of the phosphate group and therefore remove the requirement for phosphorylation at that site [29]. Conclusive evidence that phosphorylation of Thr^{172} plays a major role in determining AMPK activity comes from our finding that mutation of this residue to aspartic acid yields a partially active enzyme that is almost completely resistant to treatment with protein phosphatases. Alternatively, when Thr^{172} was mutated to an alanine residue, which mimics an unphosphorylated residue, AMPK activity was almost totally abolished. Another study recently reported that mutation of Thr^{172} in $\alpha1$ to either an aspartic acid or alanine residue yielded an inactive enzyme complex [19]. Their results did not allow the effect of phosphorylation of Thr^{172} on AMPK activity to be fully evaluated, since it was possible that mutation of Thr^{172} caused inactivation of the kinase by a mechanism distinct from that involving phosphorylation. Our results provide the first direct evidence that phosphorylation of Thr^{172} is necessary and sufficient for AMPK activity. It is difficult to explain the reason for the discrepancy between the previous study and our own. We have ruled out the possibility that the differences in activity were due to the particular cell lines used for expression. It is possible that the form in which the recombinant $\alpha1$ protein is expressed alters the activity of the resulting complex. We used a short epitope tag (Myc), whereas in the previous study $\alpha1$ was expressed as a fusion protein with glutathione S-transferase. Whatever the reason for the lack of activity obtained in the study by Crute et al., our results demonstrate that, for both $\alpha1$ and $\alpha2$, the T172D mutant does retain catalytic activity both before and after treatment with protein phosphatases.

When Thr^{172} was originally identified as the major site phosphorylated within rat liver AMPK by AMPKK, the high level of autophosphorylation may have masked other regulatory phosphorylation sites [4]. As shown in Figure 2, wild-type AMPK is labelled after incubation with [γ-^{32}P]ATP in the absence of added AMPKK. Upon addition of AMPKK, labelling of the α and β subunits within the wild-type complex is markedly increased, owing to incorporation at both the regulatory and autophosphorylation sites (results not shown). Increased labelling of the autophosphorylation sites results from activation of the wild-type AMPK complex by AMPKK. The catalytically inactive double-mutant form of $\alpha1$ ($\alpha1$ D157A/T172D), in which both Thr^{172} and Asp^{157} had been mutated, provided a good reagent with which to analyse whether additional sites were phosphorylated within the AMPK complex. This mutant precludes phosphorylation of Thr^{172} as well as preventing autophosphorylation of the complex, therefore facilitating the identification of additional regulatory phosphorylation sites. Both the α and β subunits in the double-mutant complex were phosphorylated, demonstrating that Thr^{172} is not the only site phosphorylated by the upstream kinase. This is the first evidence to formally demonstrate that AMPKK has protein kinase activity. Until now it had remained possible that AMPKK activated AMPK by promoting autophosphorylation. At present we have no evidence that phosphorylation of these other sites has a direct effect on AMPK activity. It is likely that these additional sites may be involved in fine-tuning the regulation of AMPK. This could occur, for instance, by either changing the subcellular localization, or altering the substrate specificity, of the complex. Identification of these additional sites will help elucidate the exact role of phosphorylation of the AMPK complex in response to cellular stress, e.g. by examining the effect of mutation of these residues on the activity of recombinant AMPK complexes. Ser^{182} within the $\beta1$ subunit has been identified as a potential site for phosphorylation by an as-yet-unidentified protein kinase [30]. Ser^{182} lies within the sequence $LSSS^{182}PPGP$, so it is possible that the kinase responsible for phosphorylation at this site has a Ser–Pro specificity. Thr^{172} within the α subunit lies within the sequence $FLRT^{172}SCGS$, which does not fit this consensus, and so it seems unlikely that the same kinase will phosphorylate both these sites.

It has also emerged from the present study that Thr^{172} may be involved in the activation of AMPK by AMP. Wild-type $\alpha1$ is much less dependent on AMP than $\alpha2$ (3-fold stimulation compared with 13-fold). Mutation of Thr^{172}, however, greatly increased the AMP-dependence of both isoforms and increased the concentration of AMP required for half-maximal stimulation (Figure 4). The AMPK cascade is highly sensitive to AMP [20], but at present there is no information concerning the nature of the AMP-binding site within the trimeric complex. It is an intriguing possibility that, in addition to its role in the regulation of AMPK by phosphorylation, Thr^{172} may participate in the binding of AMP within the complex. It is possible that phosphorylation at this site could affect the sensitivity of the complex to AMP. This could arise either as a direct effect of the negative charge of the phosphate group or by a conformational change of the complex brought about by phosphorylation. Replacement of phosphothreonine with an aspartic acid residue is unlikely to bring about the same conformational change, which could account for the altered sensitivity of the complex to AMP. Without detailed structural information, e.g. from crystallographic studies, the actual mechanism by which AMP binds to the AMPK complex is difficult to verify. However, our observation may provide an important clue in understanding the dual activation of AMPK by AMP and phosphorylation.

It was reported that truncation of wild-type $\alpha1$ at residue 312 ($\alpha1^{312}$) produced a constitutively active form of the kinase [19]. We do not agree with this finding, because wild-type $\alpha1^{312}$ still requires phosphorylation for activity, since it is inactivated by treatment with protein phosphatases. In contrast, the $\alpha1^{312}$ T172D mutant is not inactivated by incubation with protein phosphatases and therefore does possess the properties required to function as a constitutively active from of AMPK. Since the $\alpha1^{312}$ T172D mutant is both insensitive to phosphatases and AMP, it may provide a useful reagent for up-regulating basal AMPK activity and identifying downstream targets. In our opinion, however, the practical value of this mutant may be limited for the following reasons. First, the activity of the truncated proteins is much lower than the corresponding heterotrimeric complexes ($\alpha1^{312}$: 7.5 ± 1.2 pmol/min per ml, $n = 4$; $\alpha1\beta1\gamma1$: 198 ± 18 pmol/min per ml, $n = 3$; $\alpha1^{312}$ T172D: 2.5 ± 0.2 pmol/min per ml; $\alpha1$ T172D$\beta1\gamma1$: 104 ± 34 pmol/min per ml, $n = 3$). This means that very high levels of expression will be required in order to obtain the levels of activity necessary to observe downstream effects. Secondly, the effect of expressing the truncated mutant on cellular responses may be difficult to interpret. In the absence of the regulatory subunits it seems unlikely that the kinase will target to its normal subcellular location or display its true physiological substrate specificity. Any results obtained using the mutant would need to be treated with caution, as the activity of the mutant may not mirror that of the heterotrimeric AMPK complex.

In the present study we have provided direct evidence that Thr^{172} is the major site involved in the regulation of AMPK by phosphorylation. We showed that mutation of Thr^{172} to an aspartic acid residue gave rise to an active complex, whereas mutation to an alanine residue yielded an inactive kinase. Our results suggest that phosphorylation of Thr^{172} may also alter the sensitivity of AMPK to AMP. This raises the possibility that there may be interplay between the primary activating signal, AMP, and phosphorylation. Our findings indicate that phos-

phorylation of Thr^{172} is equally important in the regulation of both $\alpha 1$ and $\alpha 2$. The presence of additional phosphorylation sites in the α and β subunits is likely to add a further degree of complexity to regulation of the AMPK cascade. Differences in the phosphorylation of these other sites may exist between the two α isoforms which could contribute to the diverse role of the AMPK isoform family in response to cellular stress.

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