

Phosphorylation of AMPK by upstream kinases is required for activity in mammalian cells

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ABSTRACT

AMP-activated protein kinase (AMPK) plays a major role in regulating metabolism and has attracted significant attention as a therapeutic target for treating metabolic disorders. AMPK activity is stimulated more than one hundred fold by phosphorylation of threonine 172 (Thr¹⁷²). Binding of AMP to the γ subunit allosterically activates the kinase. Additionally, a number of small molecules e.g. 991, have been identified that bind between the kinase domain and the carbohydrate binding module of the β subunit, stabilising their interaction and leading to activation. It was reported recently that non-phosphorylated Thr¹⁷² AMPK is activated by AMP and A769662. We present here the crystal structure of non-phosphorylated Thr¹⁷² AMPK in complex with AMP and 991. This structure reveals that the activation loop, as well as the complex overall, is similar to the Thr¹⁷² phosphorylated complex. We find that in the presence of AMP and 991 non-phosphorylated Thr¹⁷² AMPK is much less active than the Thr¹⁷² phosphorylated enzyme. In human cells, the basal level of Thr¹⁷² phosphorylation is very low (approximately 1%) but is increased 10-fold by treatment with 2-deoxyglucose. In cells lacking the major Thr¹⁷² kinases, LKB1 and CaMKK β , Thr¹⁷² phosphorylation is almost completely abolished, and AMPK activity is virtually undetectable. Our data show that AMP and 991 binding to non-phosphorylated Thr¹⁷² AMPK can induce an ordered, active-like, conformation of the activation loop explaining how AMPK activity can be measured *in vitro* without Thr¹⁷² phosphorylation. However, in a cellular context phosphorylation of Thr¹⁷² is critical for significant activation of AMPK.

Abbreviations: acetyl-CoA carboxylase, ACC; AMP-activated protein kinase, AMPK; autoinhibitory domain, AID; calcium/calmodulin dependent protein kinase kinase, CaMKK; carbohydrate binding module, CBM; 2-deoxyglucose, 2DG; dimethyl sulphoxide, DMSO; liver kinase B1, LKB1;

Key words: adenine nucleotides; AMP-activated protein kinase (AMPK); metabolic regulation; phosphorylation; signal transduction

INTRODUCTION

AMP-activated protein kinase (AMPK) maintains the energy status of the cell by promoting ATP-producing pathways and inhibiting ATP-utilising pathways [1-3]. By virtue of the metabolic pathways it controls, including glucose and lipid homeostasis, AMPK has emerged as an important therapeutic target for treating metabolic disorders [4, 5]. AMPK is an $\alpha\beta\gamma$ heterotrimer that requires phosphorylation of threonine 172 (Thr¹⁷²) in the activation loop of the kinase domain for maximal activity [6-8]. The negatively charged phosphate group of phospho-Thr¹⁷² (pThr¹⁷²) interacts with residues in the kinase domain, including those from the regulatory α C helix, and a conserved arginine and aspartic acid residue [9, 10]. These interactions lead to stabilisation of the activation loop which in turn optimally positions key residues involved in substrate binding and catalysis [11]. Our previous crystal structures of active AMPK show how phosphorylation of Thr¹⁷² facilitates the formation of a number of these key interactions within the kinase domain that enable effective catalysis [9].

Liver kinase B1 (LKB1) and calcium/calmodulin-dependent protein kinase kinase β (CaMKK β , also known as CaMKK2) have been identified as the two major upstream kinases capable of phosphorylating Thr¹⁷² in mammalian cells [12-17]. AMPK phosphorylated on Thr¹⁷² (hereafter, we refer to phosphorylated Thr¹⁷² as pThr¹⁷² and non-phosphorylated Thr¹⁷² as non-pThr¹⁷²) can be dephosphorylated by protein phosphatases, although it is not known which protein phosphatases carry out this function *in vivo*. In mammalian cells, AMPK is activated by an increase in the AMP:ATP and ADP:ATP ratio, that occur in response to a fall in ATP levels [1, 18]. AMP and ADP binding to the γ subunit of AMPK promote phosphorylation of Thr¹⁷², and AMP and ADP protect pThr¹⁷² from dephosphorylation. As well as regulating the phosphorylation status of Thr¹⁷², AMP, but not ADP, allosterically activates AMPK. In addition to adenine nucleotides, a number of small molecule activators of AMPK have been developed, including A769662 and 991, which both allosterically activate and protect the enzyme from dephosphorylation [9, 19-21]. These small molecule activators bind at an interface formed between the α kinase domain and the carbohydrate binding module (CBM) of the β subunit [9]. We hypothesised that binding to this pocket promotes the interaction of the kinase domain with the regulatory fragment thus protecting the active enzyme from dephosphorylation and inactivation by protein phosphatases.

A recent study reported that binding of AMP and A769662 could synergistically activate AMPK in the absence of phosphorylation [22]. This finding raises the possibility that AMPK activation can bypass the requirement for Thr¹⁷² phosphorylation if AMP is bound at the γ subunit and the kinase domain-CBM interaction is stabilised by binding of a small molecule activator. As a consequence, this would have significant implications for development of therapeutic strategies for activating AMPK in the absence of upstream kinase activity. In this paper we show a crystal structure of non-pThr¹⁷² AMPK in complex with 991. This structure reveals that binding of 991 leads to the ordering of the activation loop of AMPK in the absence of Thr¹⁷² phosphorylation. Although recombinant non-pThr¹⁷² AMPK expressed in bacteria has catalytic activity in the presence of AMP and A769662, it is much less active than the corresponding pThr¹⁷² form. Importantly, in mammalian cells lacking both LKB1 and CaMKK β Thr¹⁷² phosphorylation is almost completely absent, and phosphorylation of acetyl-CoA carboxylase (ACC), a downstream substrate of AMPK is virtually undetectable. Our findings suggest that in mammalian cells Thr¹⁷² phosphorylation is essential for significant AMPK activity despite increased AMP levels and the presence of small molecule activators.

EXPERIMENTAL

Materials and Proteins

Recombinant His-tagged AMPK complexes were expressed in *E. coli* and purified by chromatography on nickel-Sepharose [9]. AMPK was phosphorylated on Thr¹⁷² by overnight incubation with MgATP and CaMKK β , and re-purified as previously described [9]. Subsequent incubation with MgATP and CaMKK β did not increase Thr¹⁷² phosphorylation, demonstrating that Thr¹⁷² is maximally phosphorylated using these conditions. The following antibodies were from Cell Signalling: rabbit anti-AMPK α 1 (#2795), rabbit anti-AMPK α 2 (#2757), mouse anti-AMPK α 1/2 (#2793), rabbit anti-AMPK β 1/2 (#4150), rabbit anti-pACC (#3661), rabbit anti-AMPK γ 1 (#4187) and rabbit anti-pThr¹⁷² (#2535). Mouse anti-vinculin was from Sigma-Aldrich (V9131). Mouse monoclonal anti-CaMKK β antibody was a generous gift from Prof. Grahame Hardie (Dundee University).

AMPK assay

AMPK was assayed using the SAMS peptide assay as described previously [23]. Briefly, purified AMPK was assayed in the presence or absence of AMP (10 μ M), 991 (1 μ M) or both, as indicated in the Figure Legends.

Western blot analysis

Proteins were resolved by SDS-PAGE on 10% polyacrylamide gels (National Diagnostics) and transferred to Immobilon-FL (Millipore) membrane at 4°C. Membranes were probed with primary antibodies at 1:1000 dilution and incubated overnight at 4°C. After extensive washing, membranes were incubated for 30-60 minutes with LI-COR secondary antibodies diluted 1:10000. Blots were imaged on a LI-COR Odyssey CLX. Fluorescence intensity values for individual bands were obtained using Image Studio (LI-COR) to allow quantification of the blots. For estimation of pThr¹⁷² levels in cells, the signal obtained for each sample was within the range obtained for the lowest and highest values determined using the recombinant AMPK standards. For capillary western blotting, cell lysates were diluted in Hepes lysis buffer to 0.4 mg/ml. Samples were prepared and analysed according to manufacturer's instructions (ProteinSimple).

AMPK binding assays

The binding of 991 to AMPK was determined by monitoring the near-UV CD spectra (340-255 nm) as previously reported [9].

Crystallography

Full length AMPK, His- α 2 (human 1-552), β 1 (human 1-270) and γ 1 (human, 1-331) was expressed in *E. coli* and purified using Nickel affinity chromatography and gel filtration as previously described [9]. A stock solution was prepared at 5 mg ml⁻¹ in 50 mM Tris, pH 8.0, 300 mM NaCl and 1 mM TCEP, mixed with a 4-fold molar excess of AMP and one fold of staurosporine and 991 compound. Crystals were grown by vapour diffusion technique at 4°C in hanging drops. Drops were prepared by mixing equal volumes of protein complex with reservoir solution containing 12% polyethylene glycol (PEG3350), 300 mM guanidine in 100 mM piperazine-N,N'-bis(2-ethanesulfonic acid) buffer, pH 7.2. Crystals were first transferred into mother liquid with an additional 25% ethylene glycol, before plunging into liquid nitrogen. Diffraction data were collected on a Pilatus 2M detector (Dectris), Diamond Lightsource, Oxford. Data were integrated using Denzo and scaled with Scalepack. The structure was solved by molecular replacement using Phaser and standard refinement was carried out with Phenix using 4CFE.pdb as search models, with manual model building with COOT [24]. General crystallographic calculations were carried out using the CCP4 package [25]. Figures were created with Pymol (<http://pymol.sourceforge.net/>).

Cell culture

HEK293T and A549 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM, Thermo) supplemented with 10% fetal bovine serum (Sigma-Aldrich). Cells were transferred to serum free DMEM for 2 hours prior to treatment. Cells were incubated with either 0.1 % dimethyl sulphoxide (DMSO, as a vehicle control), 991 (5 μ M), rotenone (10 μ M), phenformin, (2 mM), 2-deoxyglucose (2DG, 12 mM) or 991 (5 μ M) plus 2DG (12 mM) for 60 minutes. Following removal of the cell media, cells were washed rapidly three times with ice-cold phosphate-buffered saline before addition of lysis buffer (50 mM Hepes pH 7.4, 50 mM sodium fluoride, 5 mM sodium pyrophosphate, 1 mM ethylenediaminetetraacetic acid, 10% (v/v) glycerol, 1% (v/v)

Triton X-100, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 4 µg/ml trypsin inhibitor and 0.1 mM benzamidine).

CRISPR mediated deletion of CaMKK β

A549 cells were transfected with plasmids containing Cas9 linked to green fluorescent protein (GFP) via a self-cleaving peptide and guide sequences targeting the first exon of CaMKK β (GCTAGAGACACATGATGACA, GCAGGGCCTCACAGGGCTTC, GGTGGATGCTCAAGGATGAG, GGGCATGGAGTCCTTCATTG, AGCACAGCCCGGCTCACACT; Horizon Discovery, Cambridge, UK). 24 hours post transfection, cells were sorted based on GFP expression and individual colonies analysed by western blotting to determine CaMKK β protein expression.

Nucleotide Analysis

Cells were lysed in perchloric acid (5%), insoluble material removed by centrifugation and the supernatant was extracted twice with an equal volume of 1:1 tri-n-octylamine and 1,1,2-trichlorotrifluoroethane. Adenine nucleotides in the aqueous phase were analysed by capillary electrophoresis using a P/ACE MDQ plus (Sciex) in 100 mM sodium acetate buffer, pH 3.5 containing 0.01% hydroxypropylmethylcellulose on a standard fused-silica capillary. The extract was loaded by pressure injection for 5 seconds at 0.5 psi, and separated at 25 kV. Nucleotide peaks were detected by absorbance at 254 nm and integrated peak areas were automatically calculated. Retention times for AMP, ADP and ATP were confirmed by analyzing nucleotide standards.

RESULTS

Activation of non-phosphorylated Thr¹⁷² AMPK *in vitro*

Recombinant AMPK complexes purified from bacteria are intrinsically non-phosphorylated at Thr¹⁷² and can be phosphorylated by incubation with CaMKK β *in vitro* (**Fig. 1A**). We determined the activity of both $\alpha 1$ and $\alpha 2$ AMPK complexes, and as previously reported by us and others [6-8, 26], found that in the absence of allosteric activators, the activity of the non-pThr¹⁷² complexes was less than 1% of the corresponding pThr¹⁷² complexes (1.41 ± 0.11 vs 882.48 ± 21.43 nmol/min/mg for $\alpha 1\beta 1\gamma 1$ non-pThr¹⁷² vs pThr¹⁷² and 1.37 ± 0.04 vs 189.55 ± 15.89 nmol/min/mg for $\alpha 2\beta 1\gamma 1$ non-pThr¹⁷² vs pThr¹⁷²). The activity of the non-pThr¹⁷² complexes was increased markedly in the presence of the allosteric activators 991 and AMP (see **Table 1** and **Fig. 1B**), confirming allosteric activation of both $\alpha 1\beta 1\gamma 1$ and $\alpha 2\beta 1\gamma 1$, with the $\alpha 1\beta 1\gamma 1$ complex showing the greater response [22, 27]. In our current study we find that the activity of the non-pThr¹⁷² $\alpha 1\beta 1\gamma 1$ complex is approximately 10% of the pThr¹⁷² complex, when assayed in the presence of both 991 and AMP (162.63 ± 7.52 vs 1780.07 ± 6.23 nmol/min/mg), a slightly lower value than that reported in the previous studies [22, 27]. With the $\alpha 2\beta 1\gamma 1$ complex, the activity of the non-pThr¹⁷² enzyme is about 3% of the pThr¹⁷² form (35.44 ± 7.21 vs 1266.77 ± 37.05). Given that 991 stimulates the activity of non-pThr¹⁷² AMPK we examined its binding to non-pThr¹⁷² complexes. As can be seen from the results shown in **Fig. 1C** and **Table 2**, the affinity of the complexes for 991 is not significantly affected by the phosphorylation status of Thr¹⁷² (see below).

Structure of non-phosphorylated Thr¹⁷² AMPK

Since 991 both binds to non-pThr¹⁷² AMPK and stimulates its activity we were interested in determining the conformation adopted by the kinase domain in the non-pThr¹⁷²-991 complex. We obtained the crystal structure of full length human non-pThr¹⁷² $\alpha 2\beta 1\gamma 1$ (**Fig. 2A**), in complex with 991, AMP and staurosporine at a resolution of 2.6 Å (crystallographic statistics are presented in **Table 3**; coordinates deposited in the Protein Databank, PDB ID: 5ISO). The overall arrangement of the heterotrimer is analogous to the structure obtained for the equivalent pThr¹⁷² AMPK complex (**Fig. 2B**) [9]. Significantly, key features are preserved, such as the position of the α -hook region (also referred to as the α -RIM2 [28]) interacting with the γ

subunit at site 3 with AMP bound and the interaction of the C-lobe of the kinase, via its activation loop, with the regulatory fragment of the enzyme (**Fig. 2C**). The different crystal packing and higher resolution diffraction, relative to our earlier complexes, allows us to assign additional regions of the structure. Of particular note, the structure of the auto-inhibitory domain (AID) region is now well resolved (in one of the two molecules in the asymmetric unit) and clearly adopts the three α -helix structure observed for the isolated AID domain [29, 30] (**Fig. 2D**).

Previous studies have shown that Ser¹⁰⁸ within the CBM of the β 1 subunit is an autophosphorylation site [31], and that mutation of Ser¹⁰⁸ to alanine significantly weakens binding of A769662 and 991 to AMPK [9]. We previously reported that pSer¹⁰⁸ in β 1 is involved in a network of electrostatic interactions that play a role in stabilising the interaction between the kinase domain and the CBM [9]. Interestingly, Ser¹⁰⁸ is phosphorylated in the crystal structure of the non-pThr¹⁷² complex (**Fig. 2E**), confirming that AMPK expressed in *E. coli*, although not phosphorylated on Thr¹⁷², is capable of undergoing autophosphorylation on Ser¹⁰⁸, consistent with the findings of a previous study [22]. Thus, another important feature conserved in the non-pThr¹⁷² AMPK structure is the interaction of the CBM with the N-lobe of the kinase domain facilitated by the salt bridge linking pSer¹⁰⁸ on the CBM to the kinase domain. Importantly these interactions preserve the 991 binding site, and the compound makes identical interactions to those observed in the pThr¹⁷² structure. This results in a well ordered CBM and kinase domain N-lobe, and provides an explanation for why 991 binding is not affected by the phosphorylation state of Thr¹⁷² (**Table 2**).

It is clear from the electron density maps that Thr¹⁷² is not phosphorylated in the current structure (**Fig. 3A**), but intriguingly, the activation loop is ordered and adopts a similar conformation to that seen in previous pThr¹⁷² AMPK structures. Consequently, key residues in the regulatory spine and catalytic spine in the non-pThr¹⁷² complex make similar interactions to those observed in the equivalent pThr¹⁷² structure (**Figs. 3A and 3B**). For example, the N-terminus of the activation loop in the present structure makes similar interactions with the regulatory fragment, with the side chain of β His²³⁸ interacting with the main-chain carbonyl of residue α Gly¹⁶⁷ and β His²³³ with the main-chain carbonyl of residue α Ser¹⁶⁵. Ordering of the activation loop to promote these interactions is important for efficient catalysis [32]. Specifically, Phe¹⁵⁸ within the DFG motif adopts an active 'DFG-in' conformation

(**Fig. 3C**) [32]. Notably, in our previous structures [9], the phosphate of pThr¹⁷² made a salt bridge with α Arg¹³⁸ on a loop extending from the α E helix, and a charged hydrogen bond with α Asn¹⁶² from the N-terminal end of the activation loop. These interactions are thought to stabilise the “active conformation”. In the present non-pThr¹⁷² structure, the hydroxyl group of Thr¹⁷² is able to hydrogen bond with the same α Arg¹³⁸ (in one of the copies in the assymetric unit), however, it is not able to make a further stabilising interaction with α Asn¹⁶² (**Fig. 3C**).

Binding of AMP and 991 together with the interactions between the activation loop and the regulatory fragment allow, at least in the crystal, ordering of the activation loop, even in the absence of phosphorylation on Thr¹⁷². In solution this is reflected in the increase in catalytic activity observed for equivalent favourable reaction conditions i.e. the presence of the allosteric activators 991 and AMP, for the non-pThr¹⁷² complex assayed *in vitro*. These findings raise the intriguing issue of whether this activation, in the absence of Thr¹⁷² phosphorylation, could occur in mammalian cells.

Requirement for Thr¹⁷² phosphorylation in cells

The finding that in the presence of allosteric activators non-pThr¹⁷² AMPK complexes have significant activity relative to the pThr¹⁷² complexes raises the important issue of whether non-pThr¹⁷² AMPK is active in cells. We monitored phosphorylation of Thr¹⁷² on AMPK and of its substrate, ACC, in HEK293T cells following treatment with 991, or compounds that lead to changes in adenine nucleotide levels. For these latter treatments we used rotenone and phenformin, both of which inhibit complex I of the electron transport chain, and 2-deoxyglucose (2DG) an inhibitor of glycolysis. The effect of the various treatments on adenine nucleotide levels is shown in **Fig. 4A**. Apart from cells treated with 2DG, the level of AMP was too low to allow accurate determination, and so we calculated the ADP:ATP ratio and used this as a measure of energy stress [33]. Rotenone and phenformin cause a modest, **but not statistically significant**, increase in the ADP:ATP ratio, whereas incubation with 2DG causes a much larger, **statistically significant**, increase in this ratio. **In addition, treatment** with 2DG leads to a clear increase in AMP. As previously reported [34], 991 has no detectable effect on adenine nucleotide levels. All four treatments increase Thr¹⁷² and ACC phosphorylation

(**Figs. 4B, C**). With the exception of 991 treatment, the levels of Thr¹⁷² and ACC phosphorylation are closely correlated in their response to treatment (**Fig. 4D**). A possible explanation for the increased phosphorylation of ACC relative to Thr¹⁷² in response to 991 is that activation of AMPK by 991 involves a significant allosteric component [9]. As a consequence, there is an uncoupling between Thr¹⁷² and ACC phosphorylation, as has been suggested previously for the effect of A769662 in cell-based assays [35, 36]. In order to estimate the level of pThr¹⁷² in cells in response to different treatments we blotted known amounts of recombinant AMPK with either an anti-pThr¹⁷² antibody or an anti- β antibody (raised against a peptide that is completely conserved in both β 1 and β 2) to generate a standard curve. We titrated different amounts of both non-pThr¹⁷² AMPK and AMPK that had been phosphorylated by CaMKK β on the same blot as the HEK293T lysates (**Fig. 4E**). Our initial experiments indicated that the anti- β antibody that we used was less sensitive than the anti-pThr¹⁷² antibody and so we titrated more of the non-phosphorylated complex compared to the phosphorylated form. We noted a slight difference in the migration of pThr¹⁷² in the recombinant AMPK α subunit relative to endogenous AMPK in HEK293T cells, which is likely due to the presence of a hexa-histidine tag engineered on the N-terminus of the recombinant protein. The blots were quantified and the percentage of Thr¹⁷² phosphorylation relative to the total β subunit expression (β 1 plus β 2) in HEK293T cells was determined (**Fig. 4F**). The results we obtained using this method correlate very well with the initial Western blot data shown in **Fig. 4B**. In untreated cells (DMSO control), approximately 1% of total AMPK is phosphorylated on Thr¹⁷². This level increases to just under 10% in the cells treated with 2DG, which gives the greatest increase in pThr¹⁷². These results are consistent with the hypothesis that AMPK exists in a largely inactive, non-phosphorylated Thr¹⁷² form under basal conditions, and that only a relatively small change in pThr¹⁷² is required to alter phosphorylation of downstream targets [37].

Lack of upstream kinases abolishes AMPK activity in cells

The findings above demonstrate that even low levels of pThr¹⁷² are sufficient for significant downstream effects of AMPK (ACC phosphorylation) in cells, but do not address the issue of whether pThr¹⁷² is absolutely required for AMPK signalling in cells. In order to determine this we deleted CaMKK β from A549 cells, which lack

LKB1 expression (**Fig. 5A** and reference [38]) using CRISPR-mediated genome editing. As shown in **Fig. 5A**, deletion of CaMKK β had no detectable effect on the expression levels of ACC and AMPK α or β subunits. AMPK α 1 appears to be the major α isoform expressed in A549 cells, whereas in HEK293T cells, the relative expression of α 2 is higher (**Fig. 5A**). In both A549 and HEK293T cells, β 1 expression is higher than β 2 (**Fig. 5A**). We measured adenine nucleotide levels in the CaMKK β knockout (KO) A549 cells and the parental cells following treatment with 2DG. Incubation with 2DG causes a dramatic increase in the ADP:ATP ratio in both the parental and CaMKK β KO A549 cells, as well as a marked increase in the peak corresponding to AMP (**Fig. 5B**). In the parental cells, which express CaMKK β , 2DG treatment results in a modest increase in phosphorylation of both ACC and Thr¹⁷², whereas 991 causes more robust increases, which are increased further following dual treatment (**Figs. 5C and 5D**). In contrast, Thr¹⁷² and ACC phosphorylation are almost undetectable in the CaMKK β KO A549 cells. However, there is a very low level of Thr¹⁷² and ACC phosphorylation following dual treatment with 991 and 2DG (**Figs. 5C and 5D**). These findings demonstrate that Thr¹⁷² phosphorylation is by far the dominant factor required for detectable AMPK function, as measured by phosphorylation of ACC, even in the presence of a potent small molecule activator and increased cellular AMP levels.

DISCUSSION

It was reported recently that AMPK, particularly $\alpha 1\beta 1\gamma 1$ complexes, can be allosterically activated in the absence of Thr¹⁷² phosphorylation [22, 27]. Here we confirm these findings showing that recombinant non-pThr¹⁷² AMPK is allosterically activated by 991 and AMP in cell-free assays. We solved the structure of the non-pThr¹⁷² AMPK complex which revealed that the activation loop adopts a similar conformation to that observed in pThr¹⁷² AMPK. An important feature of the non-pThr¹⁷² enzyme expressed in *E. coli* is that Ser¹⁰⁸ in $\beta 1$ is phosphorylated, accounting for the finding that 991 binds with a similar affinity to both the non-pThr¹⁷² and pThr¹⁷² forms of AMPK. Thus our structural data show how the binding energy of 991 and AMP are able to induce an active-like conformation in the non-phosphorylated activation loop, accounting for the low, but quantitative, activity of this species *in vitro*. Perhaps more importantly another finding of our current study is that in the absence of LKB1 and CaMKK β , Thr¹⁷² and ACC phosphorylation are virtually undetectable in human cells, with or without allosteric activators. This finding suggests that Thr¹⁷² phosphorylation of AMPK is the critical factor for phosphorylation of its downstream targets in a cellular context.

Activation loop phosphorylation promotes the stabilisation of the active conformation of this loop and the positioning of key residues in the kinase domain. Due to the less stable conformation of kinases lacking such phosphorylation, it is perhaps not surprising that, in general, there are relatively few examples of such structures available in the Protein Databank. However, there are examples of RD kinases that do not require phosphorylation of the activation loop to achieve partial or full activity [39]. In some cases this is achieved by the presence of acidic residues in the activation loop that appear to mimic phosphorylation at these sites [39]. In the case of CDKs, bound cyclins interact with the non-phosphorylated activation loop, allowing it to become ordered, giving rise to partial or full activation of the CDK [40]. In our current study we were able to obtain well-ordered crystals of AMPK in the absence of Thr¹⁷² phosphorylation by having 991 bound. The activation-loop of AMPK makes a number of interactions with the regulatory fragment of the enzyme that enables the loop to become ordered in the absence of it being phosphorylated at Thr¹⁷². We suggest that binding of the activator (991) provides sufficient energy to

stabilise the active-like conformation of the activation loop by driving the binding of the kinase domain, mainly via this loop, onto the regulatory fragment of AMPK.

Around the same time as we solved the crystal structure of the non-pThr¹⁷² complex, a study reported that allosteric activation of $\alpha 1\beta 1\gamma 1$ by A769662 led to significant activity without the requirement for Thr¹⁷² phosphorylation, and addition of AMP increased this activation further [22]. We confirmed these findings with 991, although the degree of activation that we observe is not as great as that reported previously. In our hands, the combined activation of non-pThr¹⁷² $\alpha 1\beta 1\gamma 1$ by AMP and 991 reached about 10% of the activity of pThr¹⁷² AMPK in a cell free assay, demonstrating that full activation of AMPK does require phosphorylation of Thr¹⁷². Thus, the ordering of the activation loop that we see in the crystal structure with AMP and 991 bound supports the finding of significant, but low, kinase activity in the absence of Thr¹⁷² phosphorylation.

As has been suggested previously [22], the ability of allosteric AMPK activators to stimulate non-pThr¹⁷² complexes could be highly significant in terms of potential therapeutic strategies for activating AMPK, particularly those diseases where there is lack of upstream AMPK kinase activity, such as loss of LKB1 in certain cancers [41]. In an attempt to address this important issue, we first estimated the level of Thr¹⁷² phosphorylation in response to either 991 or conditions which interfere with cellular ATP production, and which would therefore be anticipated to increase AMP levels. In HEK293T cells, which express both LKB1 and CaMKK β , the two major Thr¹⁷² kinases, we were only able to detect a significant increase in AMP levels following treatment with the glycolysis inhibitor, 2DG. Neither rotenone nor phenformin treatment caused a detectable increase in AMP, although they did lead to measurable increases in ADP. *Although we were unable to reliably detect AMP under these conditions it remains possible that AMP is increased, but falls below the limit of detection using capillary electrophoresis. In order to resolve this issue, a more sensitive method, such as mass spectrometry, would be required to measure AMP levels.* Consistent with the modest changes in adenine nucleotide levels, the increase in Thr¹⁷² phosphorylation occurred over a fairly narrow range (between 1-10%) following the different treatments. Thus, most of the AMPK within the cell remains in the non-phosphorylated Thr¹⁷² form. This is consistent with a model we proposed previously that we based on the nucleotide binding properties of AMPK in relation to the

physiological concentration of adenine nucleotides in mammalian cells [37]. In this model, the majority of AMPK will have Mg.ATP bound, and will be in the non-phosphorylated Thr¹⁷² form. Small (2-3 fold) changes in AMP and/or ADP levels lead to similar fold increases in pThr¹⁷², consistent with our experimental observations in HEK293T cells. It is important to note, however, that although the proportion of AMPK in the pThr¹⁷² form is low, following treatment with 2DG the relative increase in Thr¹⁷² phosphorylation is substantial (10-fold). There was a good correlation between the increase in phosphorylation of Thr¹⁷² and ACC for rotenone, phenformin and 2DG. In contrast, 991 treatment, which caused the smallest increase in Thr¹⁷² phosphorylation out of the conditions used, resulted in a disproportionate increase in ACC phosphorylation. A potential explanation for this finding is that 991, in addition to increasing Thr¹⁷² phosphorylation, allosterically activates AMPK [9]. As has been noted previously, judging AMPK activation in cells solely on Thr¹⁷² may be misleading, and monitoring downstream substrate phosphorylation provides a more accurate reflection of AMPK activity in cells [35].

A previous study performed a similar analysis for estimating the degree of pThr¹⁷² in HEK293 cells [36]. In this earlier study, a much higher level of basal Thr¹⁷² phosphorylation (approximately 27%) was reported. The reason for the apparent difference in the studies is unclear, but may be related to the use of different cell lines (HEK293 versus HEK293T cells). In HEK293 cells $\alpha 1$ has been reported to account for approximately 95% of total AMPK activity [42], whereas in the HEK293T cells used in our current study, we find that $\alpha 2$ is the predominantly expressed α isoform (Fig. 5A). Interestingly, a recent study reported that $\alpha 2$ -containing AMPK complexes are significantly more sensitive to dephosphorylation than $\alpha 1$ -containing complexes [43]. This difference in phosphatase sensitivity could account, at least in part, for the difference in basal Thr¹⁷² phosphorylation between HEK293 and HEK293T cells.

The allosteric activation of the non-pThr¹⁷² AMPK complex reported in the current study and previously by others [22, 27] has potentially significant implications for therapeutic targeting of AMPK. In order to determine whether AMPK is capable of signalling in the absence of Thr¹⁷² phosphorylation in human cells we generated A549 cells lacking the two major Thr¹⁷² kinases, CaMKK β and LKB1. A549 cells are a human lung adenocarcinoma cell line that lack endogenous expression of LKB1

[38, 44]. By deleting CaMKK β from these cells using the CRISPR-Cas9 system, we were able to establish a human cell line lacking the major AMPK Thr¹⁷² kinases. Similar to lung tissue [45], A549 cells express predominantly the $\alpha 1$ and $\beta 1$ isoforms, making them an appropriate cell line for investigating this mechanism, since it is the non-pThr172 $\alpha 1 \beta 1 \gamma 1$ complex that is allosterically activated to the greatest extent. Interestingly, a previous study used a pharmacogenetic approach to investigate the role of CaMKK isoforms on downstream signalling in A549 cells, concluding that CaMKK β is the major CaMKK isoform responsible for Thr172 phosphorylation in these cells [46].

In A549 cells, treatment with 2DG caused a modest increase in phosphorylation of ACC and Thr¹⁷² compared to 991 treatment (Fig. 5). The modest effect of 2DG treatment is almost certainly due to the lack of LKB1 expression in these cells, since LKB1 has been shown to play the major role in nucleotide-mediated activation of AMPK [12, 47]. Dual treatment with 991 and 2DG caused a greater increase in ACC and Thr¹⁷² phosphorylation than either treatment alone. In contrast to these results, phosphorylation of ACC and Thr¹⁷² was essentially undetectable in the CaMKK β KO A549 cells following any of the treatments. We confirmed that AMP and ADP levels were increased in CaMKK β KO A549 cells following 2DG treatment. A very low signal for ACC phosphorylation was evident following the dual treatment of the CaMKK β KO A549 cells, and this correlated with a similarly weak signal for Thr¹⁷² phosphorylation. These findings suggest that a low level of Thr¹⁷² kinase activity remains in the CaMKK β KO cells, and that this results in a low level of downstream substrate phosphorylation. Whatever the source of this residual activity, our results clearly show that in the absence of appreciable Thr¹⁷² phosphorylation, cellular AMPK has negligible ability to phosphorylate its downstream substrate. Taken together, these findings demonstrate that in the absence of CaMKK β and LKB1, Thr¹⁷² phosphorylation is virtually abolished and ACC phosphorylation prevented. At first sight, this appears to be at odds with the cell-free data, which shows non-pThr¹⁷² AMPK is capable of substrate phosphorylation *in vitro*. However, the cell-free assays are performed at high substrate concentration, which will be different within the cell. In addition, phosphorylation of downstream targets within a cellular context are dynamic, due to the action of protein phosphatases, which is not the case in a cell-free assay.

Furthermore, additional factors are present within the cell, that are not present in the cell-free studies, that influence the activity of the non-pThr¹⁷² complex. For example, glycogen has been shown to inhibit AMPK activation *in vivo* [48]. In addition, AMPK has been shown to interact with other proteins within the cell, and these interactions could impact on AMPK activity, as well as sub-cellular localisation. For example, recent studies by Lin and colleagues have revealed that under certain conditions, AMPK activation by LKB1 requires the interaction of LKB1 with axin on the surface of the lysosome [49-51]. Cellular AMPK activity is therefore controlled by a combination of factors that are not recapitulated in the cell-free assays. Whatever the reason for the differences, the requirement of developing strategies for activating AMPK that bypass the requirement for Thr¹⁷² phosphorylation is unsubstantiated. Moreover, we are not aware of any pathology where both CaMKK β and LKB1 are inactive and so the requirement for activation of the non-pThr¹⁷² form has not been proven.

Author Contribution

Robin Willows, Matthew J. Sanders, Bing Xiao, Bhakti R. Patel and Stephen R. Martin carried out the experimental work. All authors helped conceive and design the experiments, and contributed to the writing of the manuscript.

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Figure Legends

Figure 1. Allosteric activation of non-phosphorylated Thr¹⁷² AMPK.

(A) AMPK $\alpha 1\beta 1\gamma 1$ and $\alpha 2\beta 1\gamma 1$ complexes were expressed in *E. coli*, purified and analysed by Western blotting using either an anti-pThr¹⁷² antibody or an anti- β antibody. Recombinant complexes that had been phosphorylated with CaMKK β ($\alpha 1\beta 1\gamma 1$ (P) and $\alpha 2\beta 1\gamma 1$ (P)) are shown alongside the non-phosphorylated proteins. (B) AMPK activity of the non-phosphorylated complexes was measured using the SAMS peptide assay in the absence or presence of 991 (1 μ M), AMP (10 μ M) or both 991 and AMP. Results shown are plotted as nmol/min/mg and are the means $\pm$ S.E.M. of 3 independent experiments. (C) Comparison of circular dichroism signal change measured at 306 nm for non-pThr172 (open symbols) and pThr172 (closed symbols) $\alpha 1\beta 1\gamma 1$ complexes as a function of 991/AMPK.

Figure 2. Structure of non-phosphorylated Thr¹⁷² AMPK bound to 991, AMP and staurosporine.

(A) Bar diagram indicating the three subunits that make up the complex, showing the location of specific regions within the subunits (AID: autoinhibitory domain; CBM: carbohydrate binding module; CBS: cystathionine- β -synthase domain), as well as the location of Thr¹⁷² in the α subunit and Ser¹⁰⁸ in the β subunit. (B) Cartoon representation of full-length human $\alpha 2\beta 1\gamma 1$ in complex with 991. The domains of the three subunits are coloured according to (A). The orientation of the figure is similar to our earlier papers, so that the kinase domain is ‘upside-down’ with respect to the classical kinase orientation. 991, which binds at the interface of the kinase domain and CBM, is shown in stick representation, with its carbon atoms coloured magenta. The three AMP molecules bound in the $\gamma 1$ subunit, and staurosporine bound to the kinase domain, are also shown. The α -hook (also known as α RIM2) region of $\alpha 2$ interacting at site 3 in the $\gamma 1$ subunit is shown in blue. Thr¹⁷² is shown in space-filling representation. (C) Detailed view of the α -hook interaction at site 3 with AMP bound. (D) Comparison of the autoinhibitory domain (AID) region from the current structure (cyan) with the isolated AID of human $\alpha 1$ (PDB ID:4RED; grey). (E) 991 binding site at the interface of the CBM of $\beta 1$ and the kinase domain, showing electrostatic interactions with pSer¹⁰⁸. The coordinates of the non-pThr¹⁷² AMPK structure have been deposited in the Protein Databank; PDB ID: 5ISO.

Figure 3. Comparison of phosphorylated and non-phosphorylated Thr¹⁷² structures.

(A) Omit maps for activation loop residues from non-phosphorylated and phosphorylated Thr¹⁷² structures. (B) Overlay of non-pThr¹⁷² activation loop (shown in red) and pThr¹⁷² (grey). The “DFG-in” conformation is similar in both structures (highlighted in pink for the non-pThr¹⁷² structure). (C) Detailed view showing interaction between the activation loop and residues from the regulatory core of AMPK in the non-pThr¹⁷² complex.

Figure 4. Quantification of Thr¹⁷² phosphorylation in HEK293T cells.

HEK293T cells were incubated with either 991 (5 μ M), rotenone (10 μ M), phenformin (2 mM) or 2-deoxyglucose (12 mM, 2DG), for 60 minutes. (A) ADP and ATP levels in perchloric acid extracts was determined by capillary electrophoresis, and the ratio of ADP:ATP is shown (mean $\pm$ S.E.M. of 3 independent experiments). (B) A representative capillary western blot of HEK293T cell lysates probed with anti-pACC, anti-pThr¹⁷² and pan- β antibody is shown. For each condition, samples were analysed in triplicate. Fold changes in pThr¹⁷²:total β subunit expression and pACC:total β subunit expression, relative to DMSO control are shown (mean $\pm$ S.E.M. for 3 independent experiments). (C) The ratio of pACC:total β expression is plotted against the ratio of pThr¹⁷²: total β expression. (D) HEK293T cell lysates (samples run in triplicate) were blotted with either anti-pThr¹⁷² antibody or pan- β antibody alongside varying amounts of purified, recombinant AMPK, either non-phosphorylated (α 1 β 1 γ 1) or phosphorylated by CaMKK β (α 1 β 1 γ 1(P)) as indicated on the Figure. Blots were quantified using the LICOR-imaging system and the percentage of Thr¹⁷² phosphorylation relative to total AMPK plotted in (E). Results shown are the mean $\pm$ S.E.M. of 3 independent experiments.

Figure 5. Effect of deletion of CaMKK β on Thr¹⁷² and ACC phosphorylation in A549 cells.

The CRISPR-Cas 9 system was used to delete CaMKK β in A549 cells (which lack endogenous LKB1 expression). (A) Western blot analysis of the parental A549 cells and the CaMKK β KO cell line with antibodies against ACC, AMPK α 1, α 2, and pan- α . For comparison, HEK293T cell lysate is included. Vinculin expression is shown as a

total protein loading control. In all cases, duplicate samples from independent cell preparations are analysed. (B) Nucleotide content of perchloric acid extracts of parental or CaMKK β KO A549 cells, incubated with or without 12 mM 2DG for 60 minutes was determined by capillary electrophoresis. In each case, a representative trace showing UV absorbance at 254 nm is shown and the migration of adenine nucleotide standards is indicated on the traces. (C) Parental and CaMKK β KO A549 cells were treated with either 991 (5 μ M), 2-deoxyglucose (12 mM, 2DG), or both 991 and 2DG, for 60 minutes. Cell lysates were resolved by capillary electrophoresis and the levels of ACC and Thr¹⁷² phosphorylation, together with AMPK β subunit expression, were determined. In each case, a representative blot with 2 independent samples is shown. (D) Quantification of the blots shown in (C) determined by chemiluminescence. Results are shown as mean $\pm$ S.E.M for 3 independent experiments and are plotted as arbitrary units (AU).

Table 1. Activity of non-pThr¹⁷² AMPK complexes

AMPK complex/activators	Specific Activity (nmol/min/mg)	Activation (fold)
$\alpha 1\beta 1\gamma 1$	1.41 (0.11)	1
$\alpha 1\beta 1\gamma 1 + 991$	63.71 (7.52)	45.7 (6.0)
$\alpha 1\beta 1\gamma 1 + \text{AMP}$	5.21 (0.63)	3.7 (0.4)
$\alpha 1\beta 1\gamma 1 + 991 + \text{AMP}$	162.63 (7.52)	116.6 (17.5)
$\alpha 2\beta 1\gamma 1$	1.37 (0.04)	1
$\alpha 2\beta 1\gamma 1 + 991$	14.37 (1.28)	10.5 (1.4)
$\alpha 2\beta 1\gamma 1 + \text{AMP}$	3.14 (0.25)	2.4 (0.12)
$\alpha 2\beta 1\gamma 1 + 991 + \text{AMP}$	35.44 (7.21)	25.6 (4.8)

AMPK complexes were expressed in *E. coli* and activity of the purified complexes measured using the SAMS peptide assay in the absence or presence of the allosteric activators, 991 (1 μM), AMP (10 μM) or both activators together. Fold-activation relative to activity measured in the absence of activator is also shown. In all cases, results shown are the mean ($\pm$ S.E.M.) determined from at least three independent experiments. In all cases, allosteric activation causes a statistically significant increase in AMPK activity compared to the control value ($p < 0.05$).

Table 2. Equilibrium dissociation constants for 991 binding to AMPK complexes.

AMPK complex	CD (306 nm) K_d (μ M)
$\alpha 1\beta 1\gamma 1$	0.13 ± 0.09
$\alpha 1\beta 1\gamma 1(P)$	0.08 ± 0.02
$\alpha 2\beta 1\gamma 1$	0.17 ± 0.03
$\alpha 2\beta 1\gamma 1(P)$	0.09 ± 0.02

AMPK complexes were expressed in *E. coli* and dissociation constants (K_d) for 991 were determined using circular dichroism (CD). The K_d values are reported as the mean ($\pm$ S.E.M) determined from at least 3 independent experiments. The values for the phosphorylated Thr¹⁷² complexes (P) are taken for a previous study (Xiao et al. 2013).

Table 3. Data collection and refinement statistics (Molecular replacement).

	Non-pThr ¹⁷² complex with 991/AMP bound PDB ID: 5ISO
Data collection	
Space group	P1212
Cell dimensions	
<i>a, b, c</i> (Å)	75.42 129.30 139.28
α, β, γ (°)	90 92.73 90
Resolution (Å)	19.97-2.63 (2.72-2.63) *
R_{sym} or R_{merge}	0.03 (0.40)
$I/\sigma I$	14.69 (2.20)
Completeness (%)	99.12(99.85)
Redundancy	3.4 (1.9)
Refinement	
Resolution (Å)	19.97-2.63 (2.72-2.63)
No. reflections	78451 (7875)
$R_{\text{work}}/ R_{\text{free}}$	0.256/0.189
No. atoms	15048
Protein	14835
Ligand/ion	132
Water	81
B-factors	82.50
Protein	82.70
Ligand/ion	62.80
Water	73.70
<i>r.m.s deviations</i>	
Bond lengths (Å)	0.009
Bond angles (°)	1.22

*Highest resolution shell is shown in parenthesis.

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Figure 1

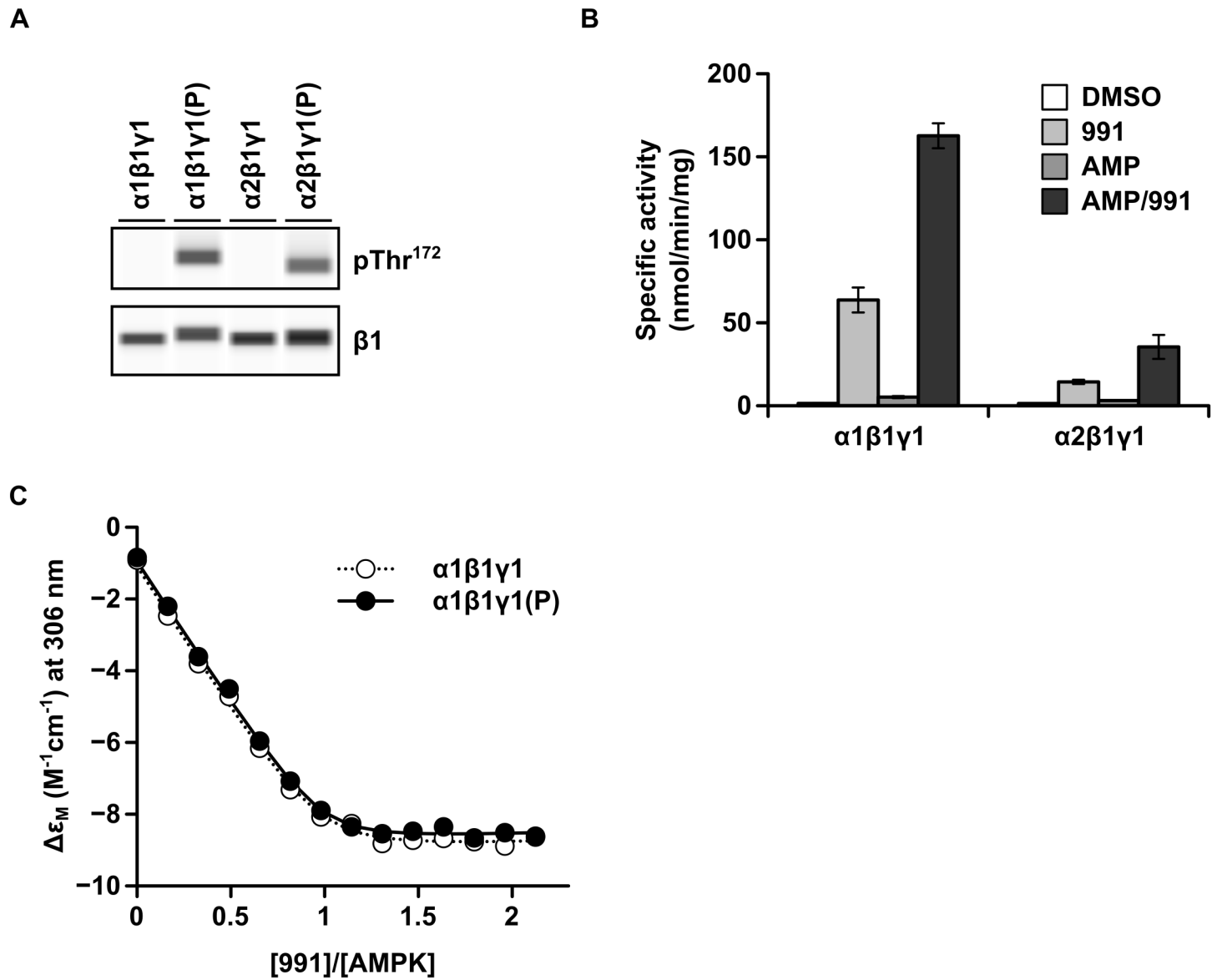


Figure 2

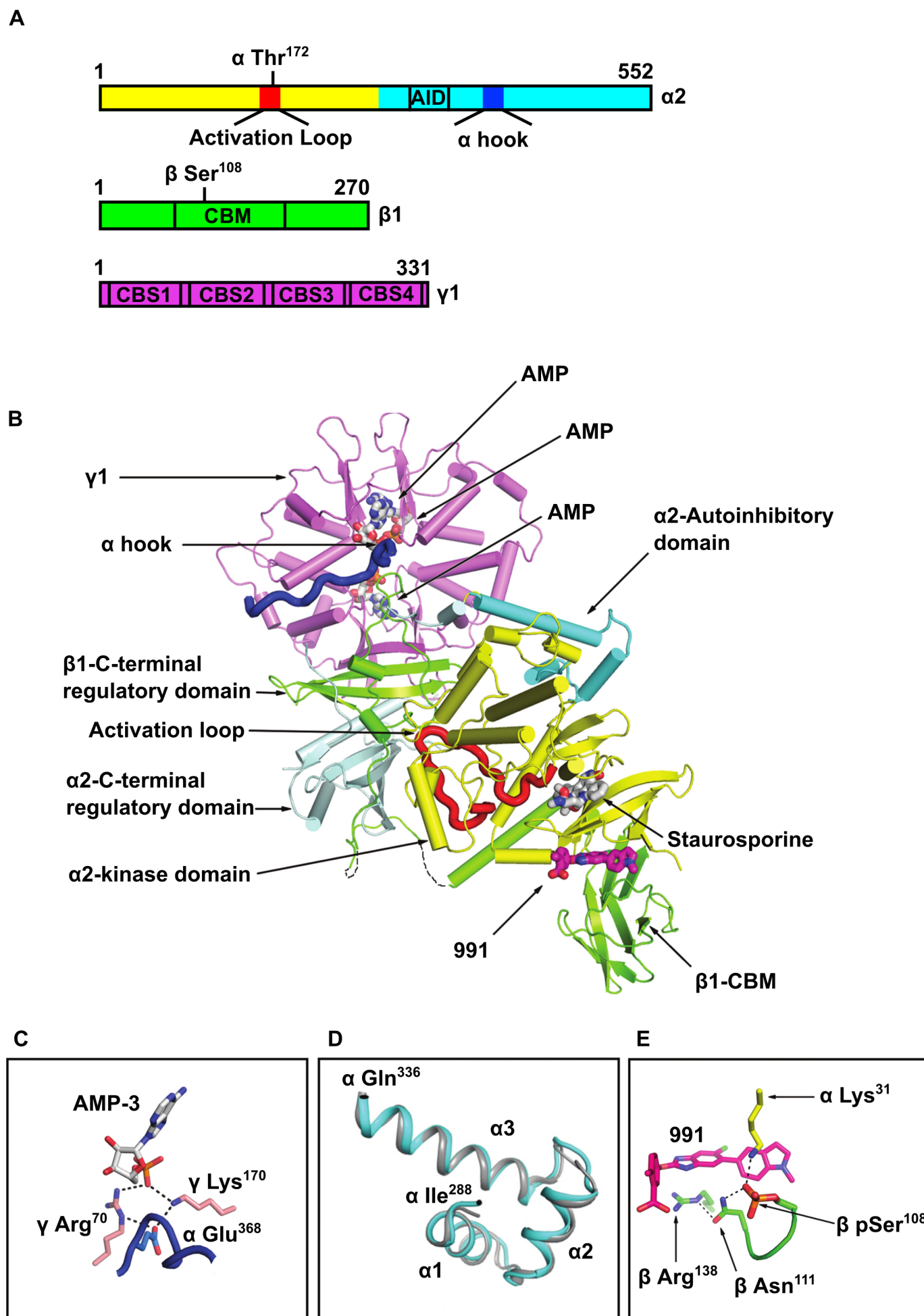
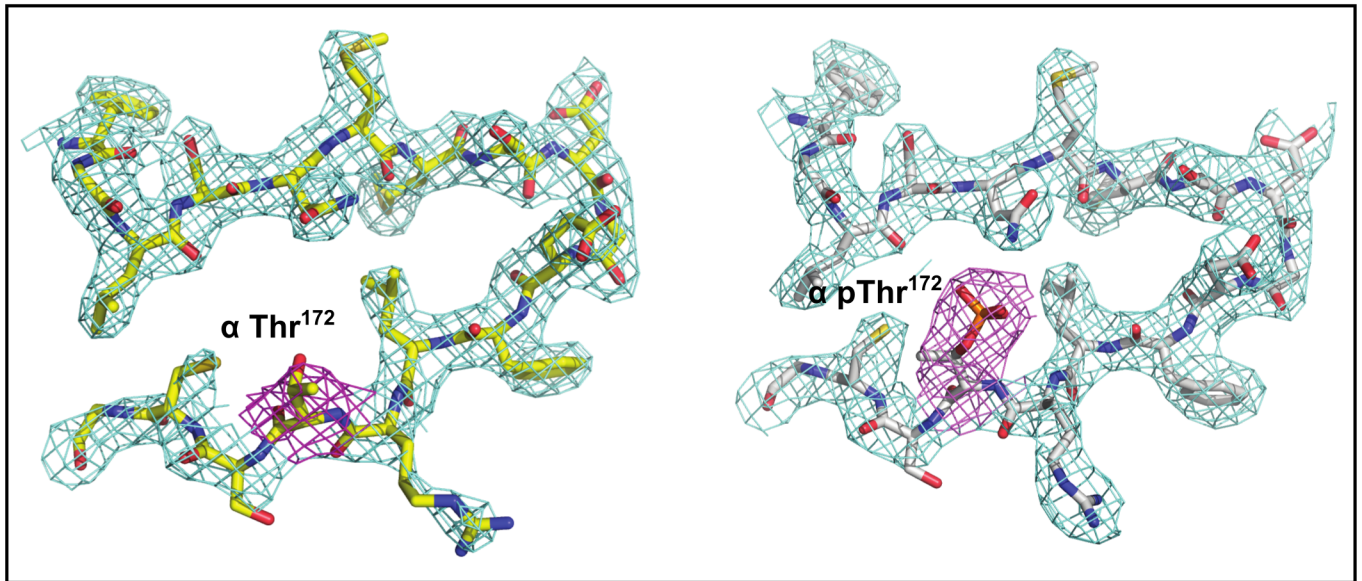
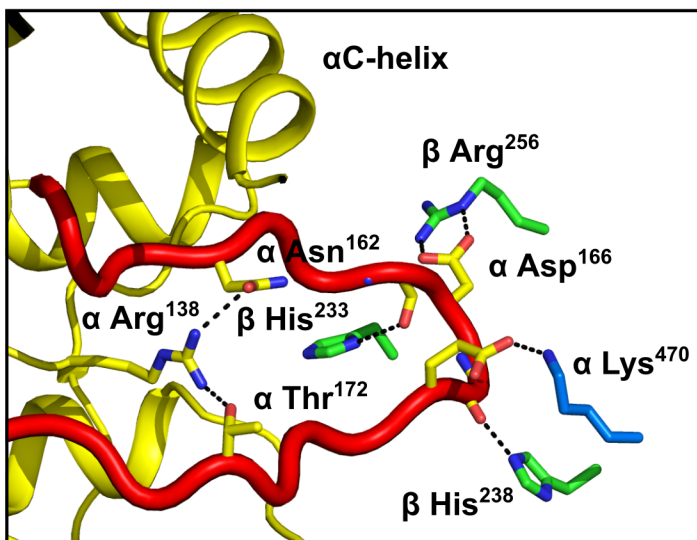


Figure 3

A



B



C

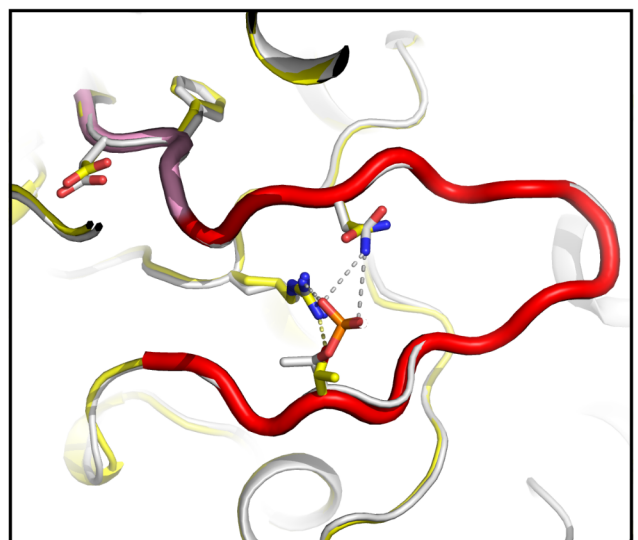
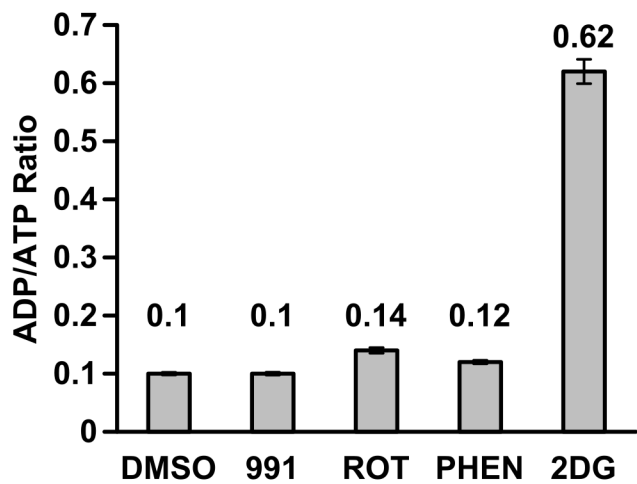


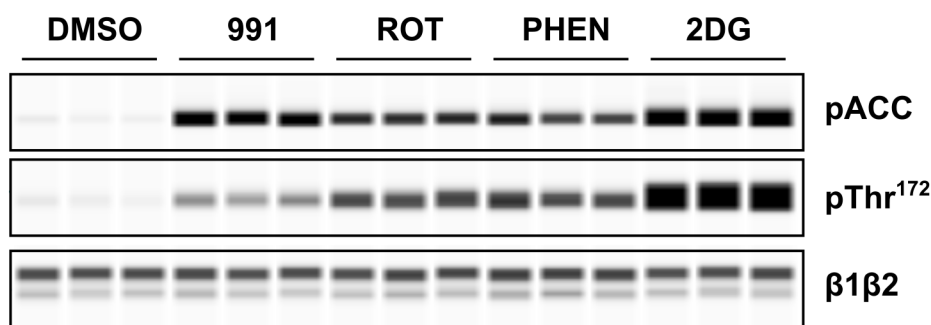
Figure 4

A

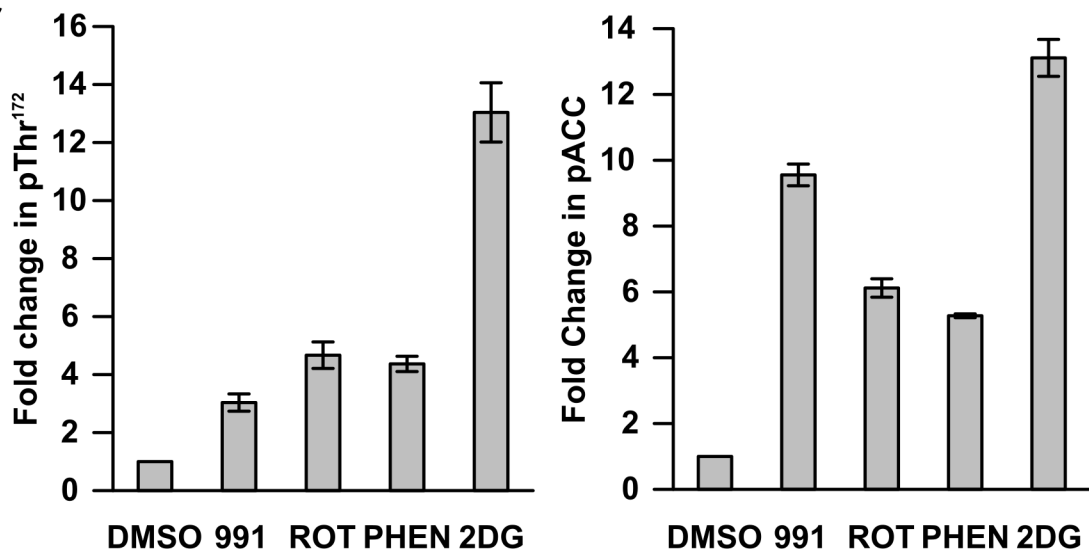


Willows et al. AMPK phosphorylation is critical for activity

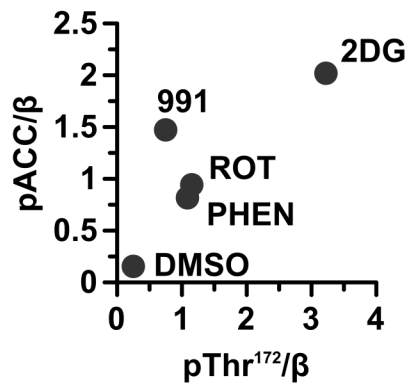
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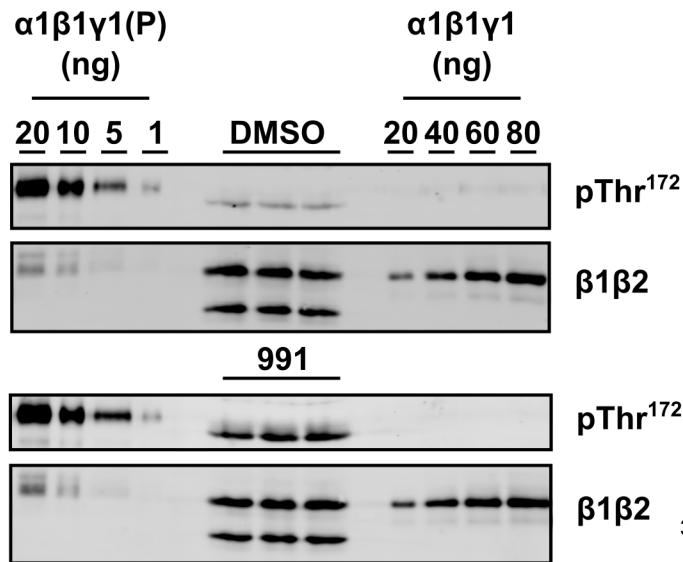
C



D



E



F

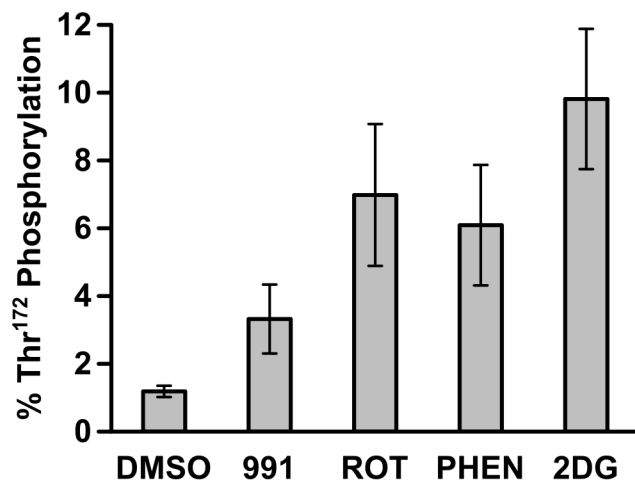


Figure 5

Willows et al. AMPK phosphorylation is critical for activity

