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

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Abbreviations used: AMP-activated protein kinase, AMPK; Ca²⁺/calmodulin dependent protein kinase kinase, CaMKK; CBS, cystathionine-β-synthase; protein phosphatase, PP;

Abstract

AMP-activated protein kinase (AMPK) is activated allosterically by AMP and by phosphorylation of threonine residue 172 (T172) within the catalytic subunit (α). Here we show that mutations in the γ regulatory subunit reduce allosteric activation of the kinase by AMP. In addition to its allosteric effect, AMP significantly reduces the dephosphorylation of T172 by protein phosphatase 2C α (PP2C α). Moreover, a mutation in the γ subunit almost completely abolishes the inhibitory effect of AMP on dephosphorylation. We were unable to detect any effect of AMP on T172 phosphorylation by either LKB1 or Ca²⁺/calmodulin-dependent protein kinase kinase (CaMKK) β using recombinant preparations of the proteins. However, using partially purified AMPK from rat liver there was an apparent AMP-stimulation of T172 phosphorylation by LKB1, but this was blocked by the addition of NaF, a protein phosphatase inhibitor. Western blotting of partially purified rat liver AMPK and LKB1 revealed the presence of PP2C α in the preparations. We suggest that previous studies reporting that AMP promotes phosphorylation of T172 were misinterpreted. A plausible explanation for this effect of AMP is inhibition of dephosphorylation by PP2C α , present in the preparations of the kinases used in the earlier studies. Taken together, our results demonstrate that AMP activates AMPK via two mechanisms: direct allosteric activation and by protecting T172 from dephosphorylation. Based on our new findings, we propose a simple model for the regulation of AMPK in mammalian cells by LKB1 and CaMKK β . This model accounts for activation of AMPK by two distinct signals: a Ca²⁺-dependent pathway, mediated by CaMKK β and an AMP-dependent pathway, mediated by LKB1.

INTRODUCTION

AMP-activated protein kinase (AMPK) 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 (α) and two regulatory subunits (β and γ). 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 (α), Snf4 (γ) and either Sip1, Sip2 or Gal83 (β isoforms) [3]. Both AMPK and SNF1 are activated by phosphorylation by upstream kinases. In yeast, 3 upstream kinases have been identified corresponding to Sak1 (originally referred to as Pak1), Elm1 and Tos3 [4, 5]. In mammals, LKB1 and CaMKK β (Ca²⁺-calmodulin dependent protein kinase kinase) have been identified as physiological kinases upstream of AMPK [6-11]. More recently, evidence has been reported indicating that Tak1, a member of the mitogen activated protein kinase kinase 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 γ subunit is involved in the allosteric activation. The γ subunit contains four CBS (cystathionine- β -synthase) domains [14]. In human γ 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 in *in vitro* binding assays [19]. In contrast to 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 would promote phosphorylation of AMPK. The first mechanism was 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. More recent studies using highly purified, recombinant preparations of LKB1 reveal that AMP does not directly activate LKB1 [8]. Similarly, CaMKK β 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], whilst making it a worse 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 β [7, 9]. Since both CaMKK β and LKB1 activate AMPK by phosphorylating the same residue within AMPK it is difficult to envisage a mechanism that would account for a substrate-mediated effect of AMP that is specific for LKB1. While this manuscript was in preparation, Suter et al. reported that AMP did not promote phosphorylation of AMPK by a recombinant preparation of LKB1 [23]. 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. We report here that AMP allosterically activates AMPK and inhibits dephosphorylation of T172. Both these effects involve the γ subunit. However, AMP has no effect on phosphorylation of AMPK by either LKB1 or CaMKK β . 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 protein phosphatase 2C α (PP2C α) in the preparations of the kinases used in the earlier studies.

MATERIALS AND METHODS

Materials

BL21-Codon-Plus (DE3)-RIL competent 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).

Preparation of recombinant proteins

Recombinant AMPK $\alpha 1\beta 1\gamma 1$ and $\alpha 2\beta 1\gamma 1$ complexes [24], CaMKK β [9] and PP2C α [25] were expressed in bacteria and purified as described previously. cDNAs encoding Snf1 and Snf4 were cloned into multiple cloning site 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 hexa-histidine tag. cDNA coding for Gal83 was cloned into the pET DUET-1 vector (Invitrogen) at 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- β -thiogalactopyranoside for 4 h at 25°C. Cells were sonicated and insoluble material removed by centrifugation. The supernatant was sterile-filtered and SNF1 complex was purified on a HisTrap HP column using an ÄKTATM purifier (Amersham Biosciences). Following nickel-Sepharose 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 Vivaspin column (Vivascience). Authenticity of the SNF1 subunits was confirmed by mass spectrometry of the proteins resolved by SDS-PAGE and following digestion with trypsin (data not shown).

Active LKB1 complex was obtained by co-expression of His-LKB1, STRAD α and GST-MO25 α using a baculovirus-insect cell expression system (Bac-Bac Baculovirus Expression System, Invitrogen). The LKB1 complex was expressed in Sf9 cells and was purified by chromatography on glutathione-Sepharose. Recombinant protein was stored in 50 mM Hepes, pH 7.4, 200 mM NaCl, 10% 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 γ 1 subunit using the QuickChange 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 β or LKB1 with 200 μ M ATP, 2.5 mM MgCl₂ and 1 mM dithiothreitol for 20 min at 37°C, and in the presence or absence of 150 μ M AMP, as stated in the figure legends. In some cases, an aliquot of this reaction was taken for Western blot analysis. A separate aliquot was removed and diluted in 50 mM HEPES (pH 7.4) and AMPK or SNF1 activity was determined by phosphorylation of the SAMS peptide [26] in the presence or absence of 150 μ M AMP, as stated in the figure legends. Results shown are presented as specific activity (nmol/min/mg).

Western blot analysis

T172 (AMPK) and T210 (SNF1) phosphorylation was determined using rabbit anti-pT172 antibody (Cell Signaling). Total AMPK was detected using sheep anti- α 1 or anti- α 2 antibodies [27]. Total Snf1 was detected using mouse anti-His antibody (Abcam). PP2C was detected using mouse anti-PP2C α antibody (Acris Antibodies). Primary antibodies were detected using LI-COR IRDye[®] Infrared 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 β or LKB1, as described above. An aliquot of the phosphorylated AMPK or SNF1 was incubated in 50 mM HEPES, pH 7.4, 2.5 mM MgCl₂, in the presence or absence of recombinant PP2C α (26 ng) and the presence or absence of 150 μ M AMP for 20 min at 37°C. The reaction was terminated by the addition of gel-loading buffer. Samples were resolved by SDS-PAGE and subjected to Western blot analysis.

Rat liver enzymes

Rat liver AMPK was purified up to the DEAE-Sepharose step [28] and rat liver LKB1 up to the Q-Sepharose step [29]. Rat liver AMPK was dialysed into 50 mM HEPES, 10% glycerol, 1 mM EDTA, 1 mM dithiothreitol, 1 mM benzamidine, 0.1 mM phenylmethane sulphonyl fluoride, 4 µg/ml trypsin inhibitor in order to remove the protein phosphatase inhibitors present in the purification buffer. Following dialysis, AMPK was incubated in the presence or absence of 2.5 mM MgCl₂, and the presence or absence of 50 mM NaF, plus or minus 150 µM AMP for 10 mins at 37°C. Aliquots 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 µM AMP. In some cases, AMPK was incubated for 10 min at 37°C in the presence of 200 µM ATP, 2.5 mM MgCl₂ and the presence or absence of LKB1 (16 ng), 50 mM NaF or 150 µM AMP (as stated in the figure legend). Aliquots of these reactions were removed for AMPK activity determination (measured in the presence of a final concentration of 150 µM AMP).

Statistical analysis

Results are expressed as mean values ±SEM. Statistical analysis was carried out using a two-tailed, unpaired Student's t test.

RESULTS

Effect of γ 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 γ 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 this 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 (data not shown). Wild type AMPK complexes ($\alpha 1\beta 1\gamma 1$ or $\alpha 2\beta 1\gamma 1$) or complexes harbouring substitution of 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 can be seen in Figs. 1A and 2A, no difference in expression of the various complexes was detected. As previously reported [24], the recombinant AMPK was isolated in an inactive form, but could be activated by phosphorylation using recombinant preparations of the upstream kinases, CaMKK β or LKB1. Following phosphorylation by CaMKK β , AMP activated the wild-type AMPK complexes 2-fold and this activation was reduced in complexes harbouring mutations in the γ subunit (Figs. 1B, C and 2B, C). 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 T172 by CaMKK β or LKB1, nor on the specific activity of AMPK measured in the absence of AMP (Figs. 1 and 2, and data not shown).

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 α and CaMKK β , 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 (Fig. 3A and 4A). Note that because AMP is present in one of the activation reactions with LKB1, we added AMP to the SAMS phosphorylation so that the final concentration of AMP is 150 μ M in this assay, irrespective of whether AMP was present or absent in the LKB1 incubation. Both α 1- and α 2-complexes were activated to similar degrees and activation correlated closely with the extent of phosphorylation of T172 (Fig. 3B and 4B). Using this highly purified, reconstituted system, we did not detect any effect of AMP on phosphorylation of T172 or on activation of AMPK for either α 1- or α 2-complexes. It was reported recently that AMP is generated in the AMPK assay using the SAMS peptide as a substrate [23]. 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 (data not shown).

Effect of γ mutations on AMP inhibition of dephosphorylation of AMPK

Wild-type AMPK or AMPK harbouring the R298G mutation in γ 1 was phosphorylated by CaMKK β and subsequently used as a substrate for dephosphorylation by recombinant PP2C α . The phosphorylation state of T172 was assessed by Western blotting using anti-pT172 specific antibodies and quantified relative to the level of total α expression using an infra-red scanner. As seen in Fig 5, incubation of AMPK with PP2C α alone led to marked dephosphorylation of the α subunit. Inclusion of AMP in the incubation with PP2C α significantly reduced the dephosphorylation of T172 for the wild-type α 1- and α 2-containing complexes (Fig. 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, though to a lesser extent than seen with the R298G complex (data not shown). Similar results were obtained using AMPK phosphorylated by LKB1. These results are consistent with a previous study that reported that AMP inhibits dephosphorylation of AMPK partially purified from rat liver [22]. Our results extend the 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 to 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 (Fig. 6A). SNF1 was virtually inactive following purification, but could be activated by phosphorylation with CaMKK β *in vitro*. Using radiolabelled ATP phosphorylation of both the Snf1 and Gal83 subunits was detected. The amino acid sequence surrounding threonine 210 (T210) in Snf1, the equivalent site to T172 in AMPK, is highly conserved between yeast and mammals, and the anti-pT172 antibody cross-reacted with phosphorylated Snf1 on Western blots (Fig. 6B). We were therefore able to measure the dephosphorylation of T210 by PP2C α using the anti-pT172 specific antibodies and quantify this relative to the level of total Snf1 expression using an anti-His antibody. The results shown in Fig. 6C demonstrate that AMP does not inhibit dephosphorylation of T210 in Snf1.

Partially purified AMPK and LKB1 contain endogenous PP2C α

Our results using highly purified, recombinant preparations of AMPK, CaMKK β , LKB1 and PP2C α 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 these preparations contain a factor that mediates the effect of AMP on phosphorylation. Based on our current results we reasoned that this factor could in fact be a protein phosphatase. 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 (Fig. 7A). However, if NaF, a protein phosphatase 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 data 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 protein phosphatase in a partially purified preparation of rat liver AMPK (purified up to the first ion-exchange step [28]) we adopted a shotgun mass spectrometry approach. The results (not shown) revealed the presence of PP2C α , and this was confirmed by Western blot analysis (Fig. 7B). To investigate whether PP2C α activity is present in the AMPK preparation we incubated AMPK in the presence or absence MgCl₂, an essential co-factor for PP2C α [32], and determined AMPK activity and T172 phosphorylation (Fig. 7C, D). AMPK activity and T172 phosphorylation were significantly reduced only in the presence of MgCl₂ and these decreases were totally blocked by the inclusion of 50 mM NaF. Consistent with our studies using recombinant AMPK and PP2C α , AMP significantly inhibited, but did not abolish, these effects. PP2C α was undetectable in more highly purified preparations of AMPK (up to the gel-filtration step [28]), or following immunoprecipitation. Using these preparations, AMPK activity was not affected following incubation with MgCl₂. Furthermore, we did not detect an effect of AMP on activation of immunoprecipitated AMPK by recombinant LKB1 (data not shown). However, AMP-stimulation of T172 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 [29], raising the possibility that PP2C α could be present in the preparations. We found that PP2C α was readily detectable by Western blotting in rat liver LKB1 purified to the same stage (Fig. 7E).

DISCUSSION

In this 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 β or LKB1, as has been 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 *vs* 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 *vs* 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 γ subunit isoforms contain 4 copies of a CBS (cystathionine- β -synthase) domain and previous studies have shown that these domains are involved in AMP-binding and subsequent allosteric activation of AMPK [18, 19, 34]. 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 substitutions in 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 β 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.* reported previously that AMP inhibits dephosphorylation of AMPK [22]. We confirmed that AMP has a significant inhibitory effect on dephosphorylation of recombinant AMPK by PP2C α . Whilst our manuscript was in preparation, Suter *et al.*

published results using recombinant AMPK in which they also demonstrated AMP inhibition of dephosphorylation [23]. 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 T210, the equivalent residue to T172 in AMPK α . 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 its dephosphorylation. We were able to purify a 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 CaMKK β or LKB1 (data not shown). Consistent with the 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 T210 by PP2C α . 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.

Previous studies reported that phosphorylation of AMPK by preparations of an AMPK kinase (AMPKK) from rat liver was stimulated by AMP [21]. Subsequent work has revealed that the identity of AMPKK in these preparations is LKB1 [6]. Using recombinant preparations of AMPK and LKB1, however, we have been unable to detect any stimulation of T172 phosphorylation by AMP under any conditions. Recently, Suter *et al.* published results using recombinant AMPK in which they also failed to detect an effect of AMP on T172 phosphorylation by LKB1 or CaMKK β [23]. However, the authors were unable to account for the apparent discrepancy between their results and previous studies. Here 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 T172 phosphorylation.

In an effort to investigate this further, we began by adopting a shotgun mass spectrometry approach to determine proteins present in a partially purified preparation of rat

liver AMPK (purified up to the first ion-exchange step [28]). The results (not shown) revealed the presence of PP2C α , and this was confirmed by Western blot analysis. We present strong evidence that the AMP effect on T172 phosphorylation is due to the presence of PP2C α since we only observe AMP-stimulation using preparations of AMPK that contain PP2C α and the effect is abolished by NaF. Based on these findings we suggest that AMP-stimulation of T172 phosphorylation is due to inhibition of PP2C α present in the preparations of AMPK and/or LKB1 and CaMKK. In support of this hypothesis, we showed that PP2C α is present in a preparation of rat liver LKB1. Whilst we cannot rule out other possibilities, 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 T172 phosphorylation use okadaic acid to inhibit dephosphorylation, but do not include NaF. Since PP2C α is okadaic acid insensitive [37], it would remain active under the conditions of the assay (since PP2C α would be activated by the Mg²⁺ present in the kinase reaction).

Overall, our results lead us to propose a revised model for activation of AMPK. The phosphorylation state of T172 depends on the relative rates of phosphorylation, catalysed by CaMKK β and LKB1, and dephosphorylation, catalysed by protein phosphatases (Fig. 8). 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 T172 phosphorylation by LKB1 would occur in response to decreased dephosphorylation following a rise in AMP. In parallel, AMP would allosterically activate AMPK (Fig. 8A). Dephosphorylation and inactivation of AMPK would occur when the concentration of AMP returned to basal levels. Unlike LKB1, the activity of CaMKK β is subject to regulation within the cell, and is increased in response to signals that raise intracellular Ca²⁺ [39, 40]. Therefore, signals that increase Ca²⁺ activate AMPK as a consequence of increased T172 phosphorylation via increased CaMKK β activity. Under these conditions there is no requirement for increased AMP levels (Fig. 8B). Consistent with this model, recent studies have reported activation of AMPK by CaMKK β -

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^{2+} , and this may account for the transient nature of AMPK activation observed in CaMKK β -mediated responses [7, 41, 42]. It should be noted that the AMP- and Ca^{2+} -signalling pathways for activation of AMPK predicted by our model are not mutually exclusive. It is possible that under some circumstances, where AMP and Ca^{2+} rise in concert, both pathways could operate. Under these conditions, both CaMKK β and LKB1 would contribute to AMPK activation.

Although our results were obtained with γ 1-containing AMPK complexes we speculate that the same mechanisms will apply to γ 2- and γ 3-containing complexes. Previous studies have shown that mutations within γ 2 and γ 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 γ 2 mutations decrease AMPK activity in transgenic animal models [44, 45], whereas another study reported increased activity [46]. In a study in which γ 3-containing AMPK complexes were over-expressed in COS cells, increased activity was observed in mutant γ 3 relative to wild-type [43]. Despite our understanding of the regulation of AMPK by AMP *in vitro*, elucidating the complex regulation of AMPK in cells and *in vivo*, and determining the effect of mutations within the γ 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 T172. These effects may be mediated by binding of AMP to the same site within the γ subunit, since both effects are abolished by the same mutation within the γ subunit. We provide evidence that suggests that previous studies reporting activation of T172 phosphorylation by AMP may have been mediated by PP2C α present in the partially purified preparations of the kinases. Based on our results we propose a model for regulation of AMPK that accounts for its activation in response to either Ca^{2+} -signalling or energy depletion, mediated by an increase in AMP.

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FIGURE LEGENDS

Figure 1. Effect of mutations within γ subunit on the 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 γ subunit (R69Q, H150R or R298G) were expressed in *E. coli* and purified by nickel-Sepharose chromatography. (A) Proteins (~1 μ g) were resolved by SDS-PAGE and detected by staining with Coomassie. (B, C) Following phosphorylation by CaMKK β , AMPK activity of the complexes was determined using the SAMS peptide assay in the presence or absence of 150 μ M AMP. Results are the average \pm S.E.M. for 4 independent experiments and are shown as the specific activity of the kinase (nmole phosphate incorporated/min/mg complex) in (B) and the fold-stimulation in (C). (D) Western blot analysis of AMPK complexes probed with an antibody against either phospho-T172 (pT172) or $\alpha 1$. A representative blot is shown in the upper panel and a graph showing relative quantitation of pT172:total $\alpha 1$ from 4 independent experiments (average \pm S.E.M.) is shown below. Wild-type AMPK that had not been incubated with CaMKK β is included as a negative control.

Figure 2. Effect of mutations within γ subunit on the allosteric activation of $\alpha 2\beta 1\gamma 1$ by AMP. The same set of experiments as described for Figure 1 were carried out using $\alpha 2$ complexes.

Figure 3. Effect of AMP on the 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 symbols) or absence (open symbols) of 150 μ M AMP for 10 min at 37° C. At the end of the incubation an aliquot was removed, diluted in buffer and AMPK activity determined using the SAMS assay. Note that in the SAMS assay, AMP (150 μ M final concentration) is present irrespective of whether AMP was included in the initial incubation with LKB1. (B) A representative Western blot of pT172 and total $\alpha 1$ of aliquots of the reactions described above is shown. A graph of the relative quantitation of pT172:total $\alpha 1$ from 3 independent experiments (average \pm S.E.M.) is also shown.

Figure 4. Effect of AMP on the phosphorylation of $\alpha 2\beta 1\gamma 1$ by LKB1. The same set of experiments as described for Figure 3 were carried out using $\alpha 2$ complexes.

Figure 5. Effect of AMP on the dephosphorylation of recombinant AMPK by PP2C. Following phosphorylation of recombinant AMPK complexes (containing either wild type or R298G γ 1) by CaMKK β , aliquots were removed and incubated in the presence or absence of PP2C (26 ng) and presence or absence of AMP (150 μ 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-pT172 and anti- α 1 (A) or anti-pT172 and anti- α 2 (B) antibodies. In each case a representative blot is shown. A graph of pT172 phosphorylation relative to a control (absence of PP2C and AMP) is shown (average \pm S.E.M. of 4 independent experiments). Significant differences in T172 phosphorylation are denoted by * ($p < 0.05$). Non-significant results are denoted NS.

Figure 6. Effect of AMP on recombinant SNF1 complex. SNF1 (Snf1, Snf4, Gal83) was expressed in *E. coli* and purified by nickel-Sepharose, followed by gel-filtration chromatography. (A) Purified SNF1 was incubated with 32 P-ATP in the presence or absence of CaMKK β and resolved by SDS-PAGE. Protein was visualised by staining with Coomassie (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 μ M AMP. Activities are plotted as nmole phosphate incorporated per min per mg SNF1 and are the average \pm S.E.M from 3 independent experiments. Also shown is a representative blot of the reaction mixture probed with anti-pT172 (to detect pT210 in Snf1) or anti-His antibody (to detect total Snf1). (C) SNF1 phosphorylated by CaMKK β was incubated in the presence or absence of PP2C (26 ng) and the presence or absence of 150 μ M AMP for 20 min at 37°C. At the end of the incubation proteins were analysed by Western blotting using either anti-pT172 or anti-His antibody as described for Figure 5. Results shown are the average \pm S.E.M. from 4 independent experiments.

Figure 7. Inactivation of rat liver AMPK by endogenous protein phosphatase activity. (A) Following dialysis into buffer lacking protein phosphatase 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 μ M) and/or NaF (50 mM). Following the incubation, AMPK

activity was determined. Results shown are plotted relative to AMPK activity in the presence of MgATP alone and are the mean \pm S.E.M. of three independent experiments. (B) Partially purified rat liver AMPK extract was blotted with an antibody against PP2C α . (C) Rat liver AMPK was incubated in the presence or absence of MgCl₂ (2.5 mM) and in the presence or absence of either 50 mM NaF, 150 μ M AMP or both for 10 min at 37°C. Following this incubation, aliquots were removed and AMPK activity determined using the SAMS assay in the presence of 150 μ M AMP (final concentration). (D) In parallel, aliquots were blotted with anti-pT172 or a mixture of α 1 and α 2 antibodies. A representative blot is shown and a graph of the quantification of the relative T172 phosphorylation from 3 independent experiments (\pm S.E.M.). Significant differences in AMPK activity or T172 phosphorylation are denoted by * ($p < 0.05$). (E) Rat liver AMPK and LKB1, purified up to the Q-Sepharose step, were probed with an anti-PP2C α antibody.

Figure 8. 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 protein phosphatase (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 T172 phosphorylation and activation of AMPK. In addition to increasing T172 phosphorylation, AMP allosterically activates AMPK (mechanism 2). (B) Ca²⁺-dependent activation: Signals that increase Ca²⁺ activate CaMKK β , increasing T172 phosphorylation and activation of AMPK and this can occur without an increase in AMP. However, it is possible that in some situations both Ca²⁺ and AMP may increase in parallel, and under these conditions AMP will allosterically activate AMPK and inhibit dephosphorylation (denoted by the dashed lines).

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Figure 1

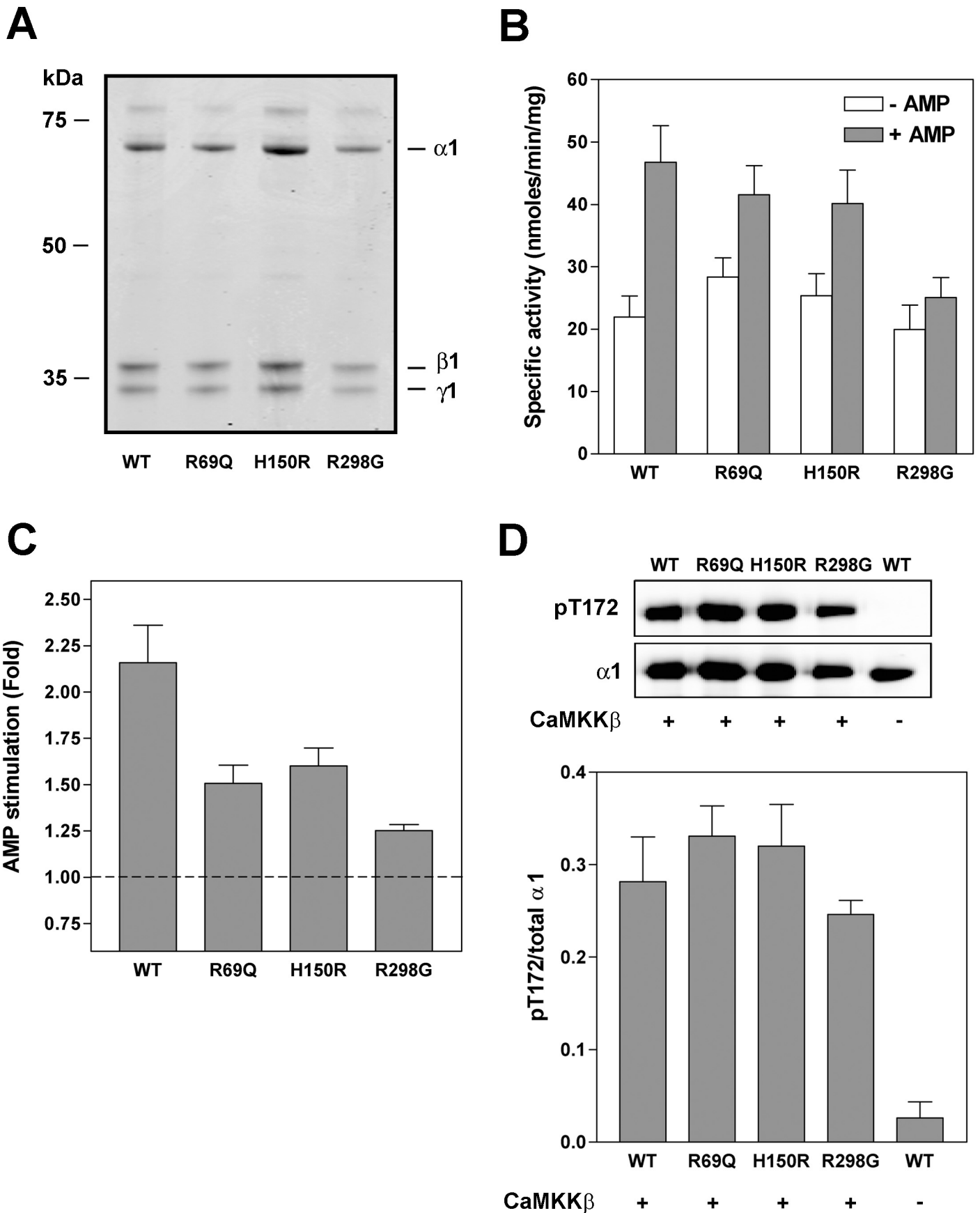


Figure 2

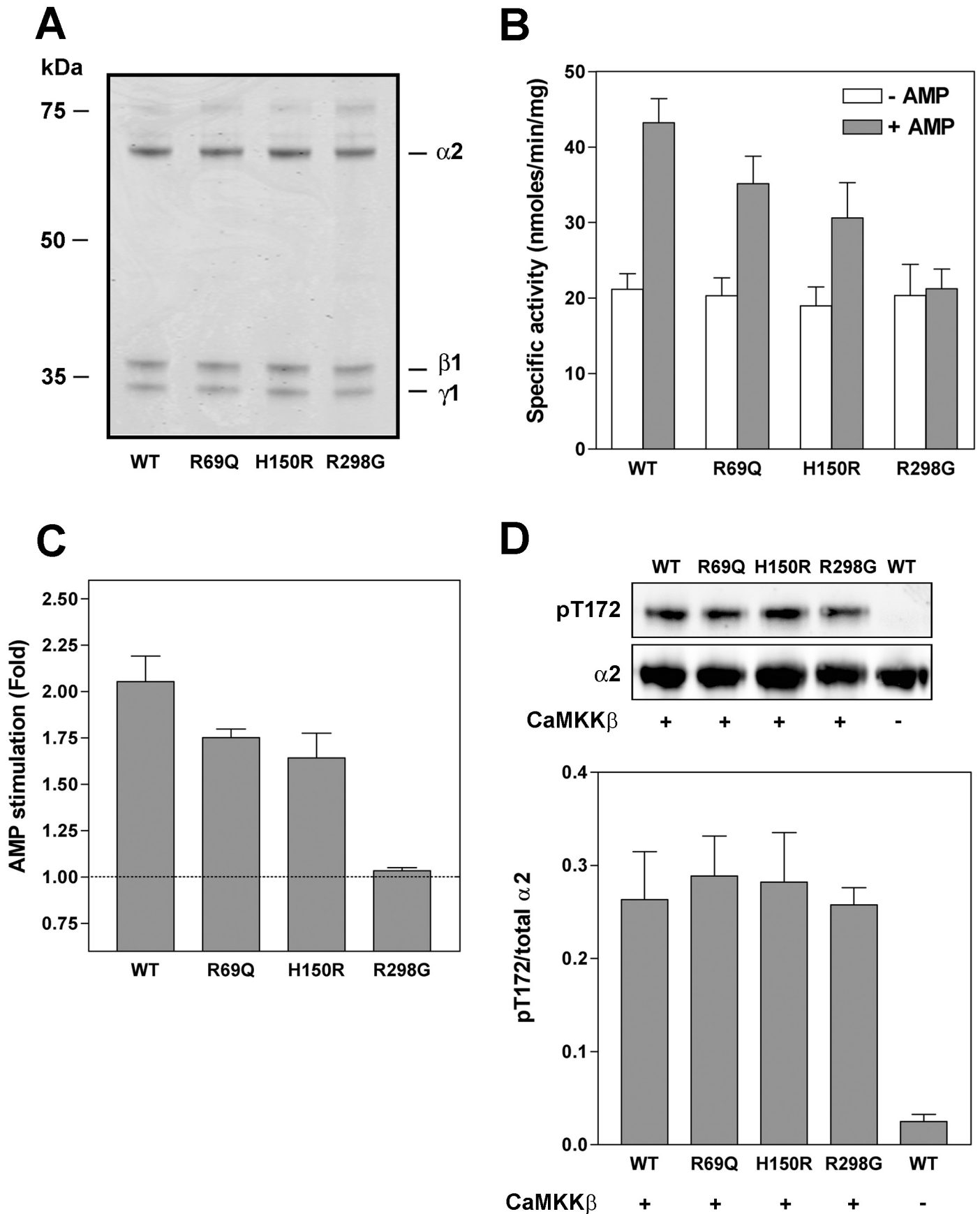
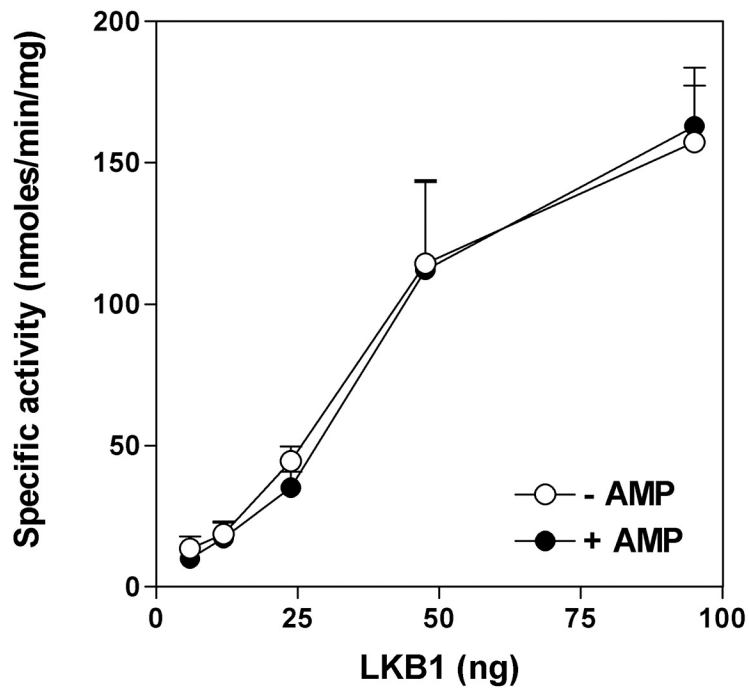


Figure 3

A



B

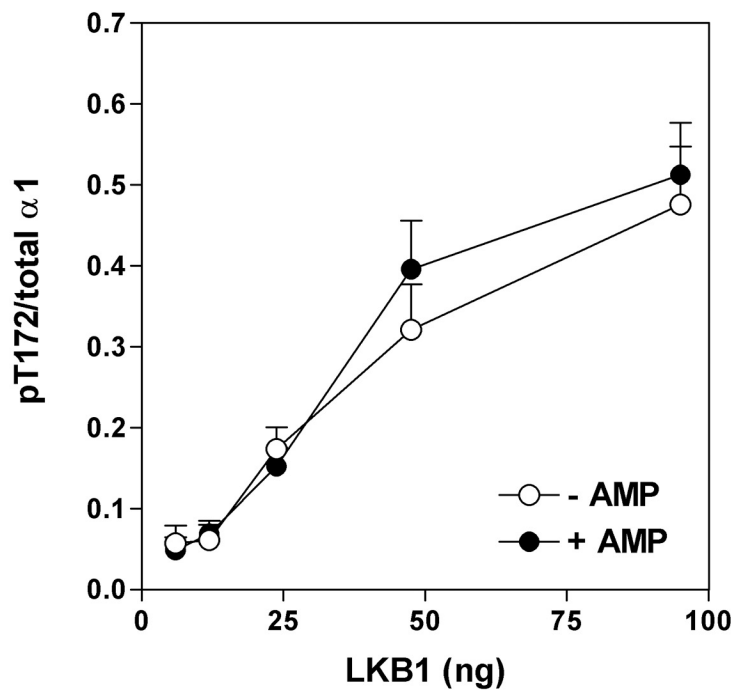
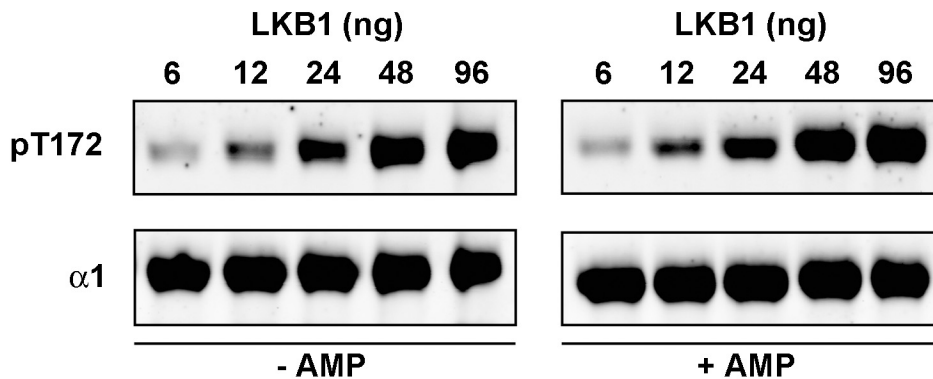
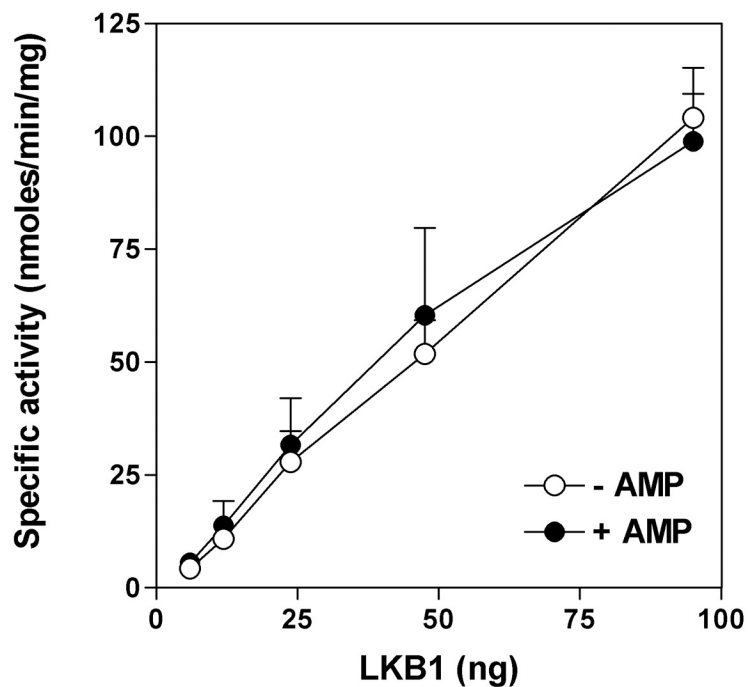


Figure 4

A



B

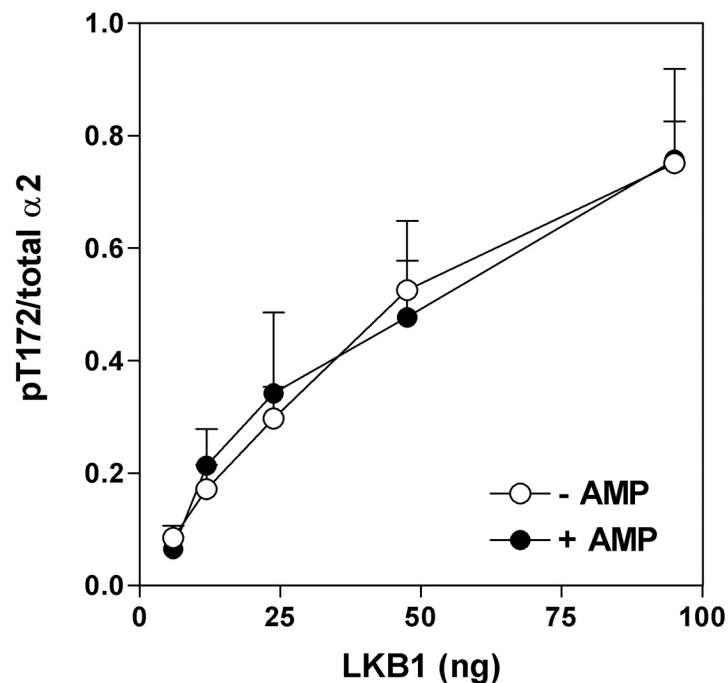
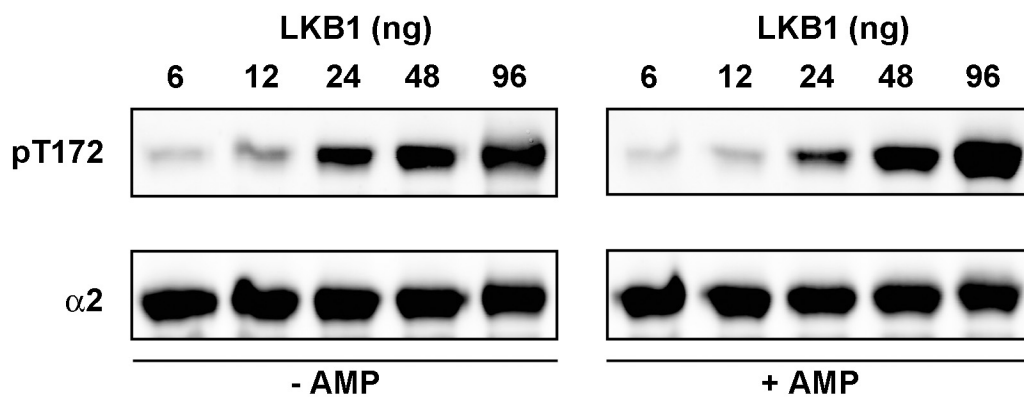
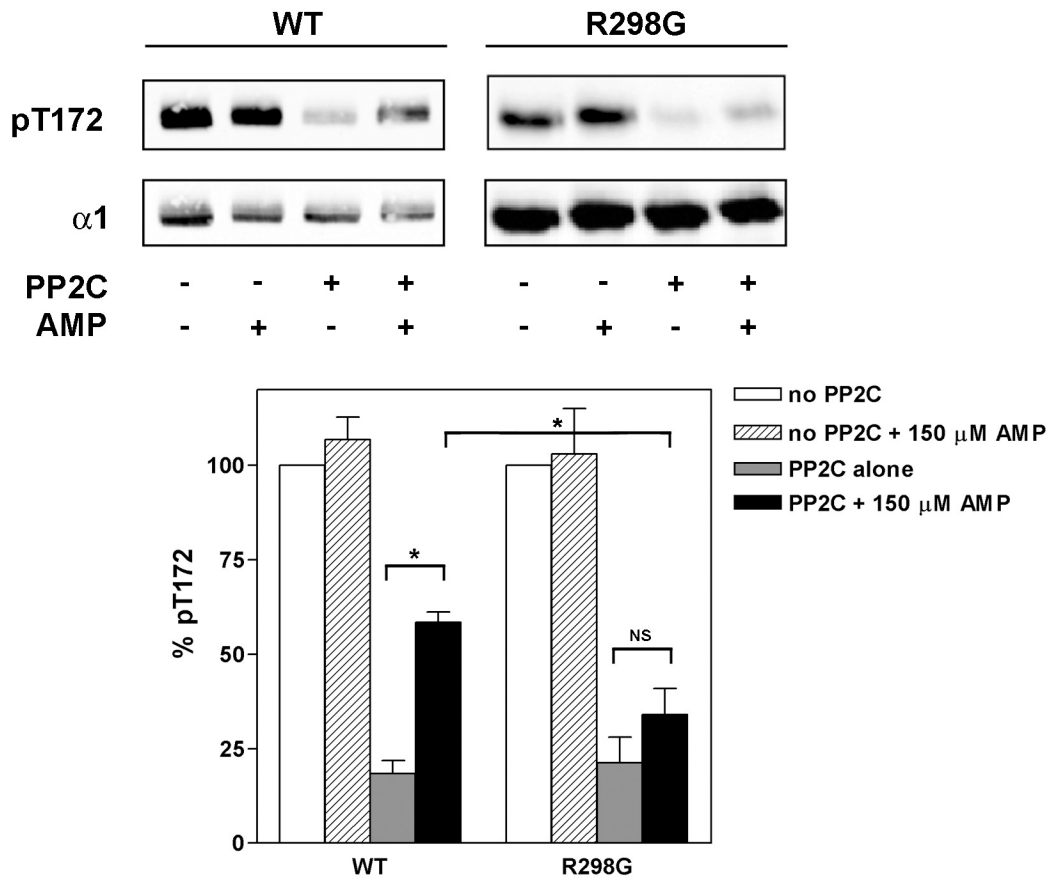


Figure 5

A



B

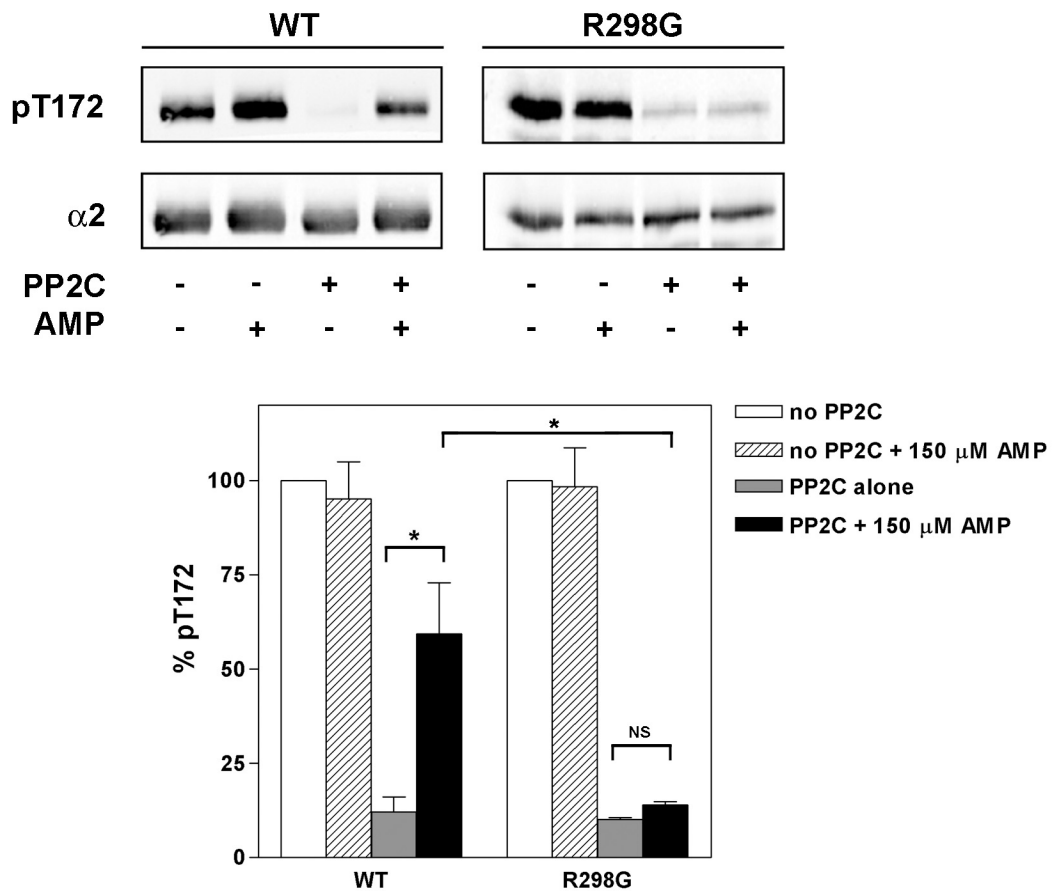


Figure 6

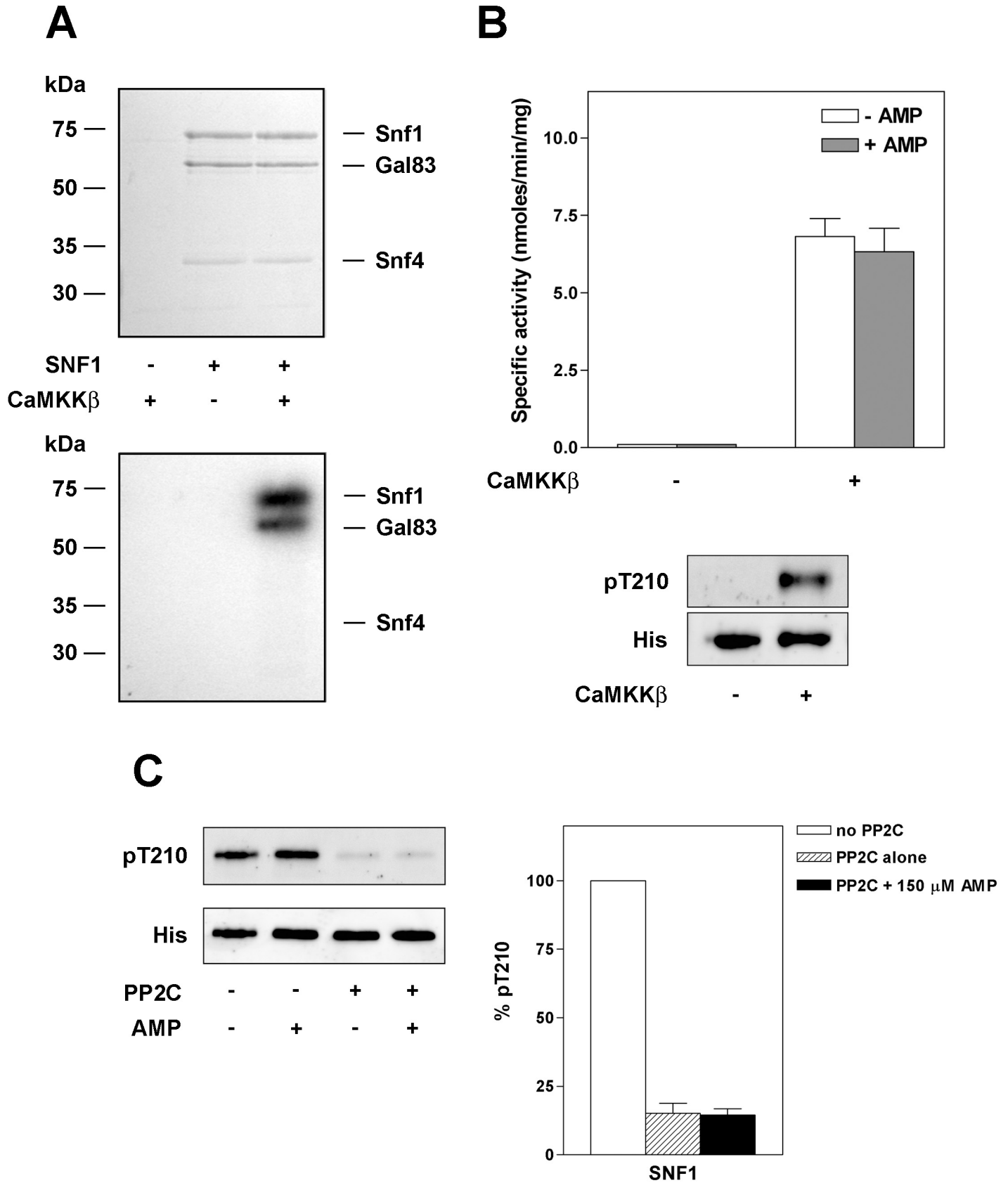


Figure 7

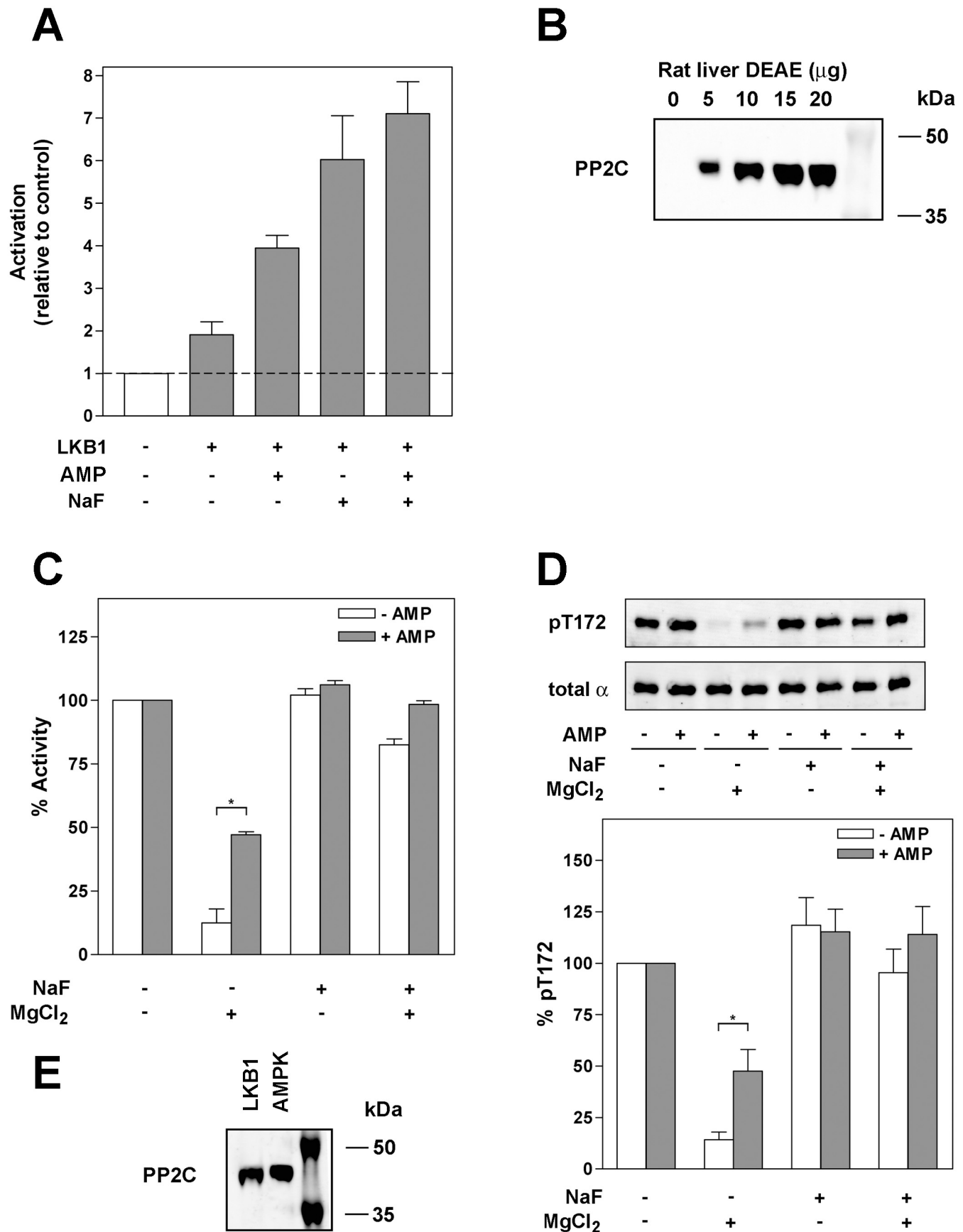
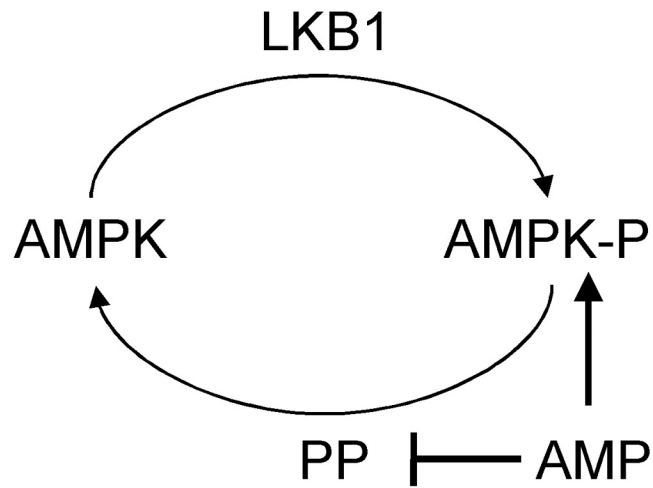


Figure 8

A



B

