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Small Molecule Drug A-769662 and AMP Synergistically Activate Naive AMPK Independent of Upstream Kinase Signaling

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SUMMARY

The AMP-activated protein kinase (AMPK) is a metabolic stress-sensing αβγ heterotrimer responsible for energy homeostasis, making it a therapeutic target for metabolic diseases such as type 2 diabetes and obesity. AMPK signaling is triggered by phosphorylation on the AMPK α subunit activation loop Thr172 by upstream kinases. Dephosphorylated, naive AMPK is thought to be catalytically inactive and insensitive to allosteric regulation by AMP and direct AMPK-activating drugs such as A-769662. Here we show that A-769662 activates AMPK independently of α-Thr172 phosphorylation, provided β-Ser108 is phosphorylated. Although neither A-769662 nor AMP individually stimulate the activity of dephosphorylated AMPK, together they stimulate >1,000-fold, bypassing the requirement for β-Ser108 phosphorylation. Consequently A-769662 and AMP together activate naive AMPK entirely allosterically and independently of upstream kinase signaling. These findings have important implications for development of AMPK-targeting therapeutics and point to possible combinatorial therapeutic strategies based on AMP and AMPK drugs.

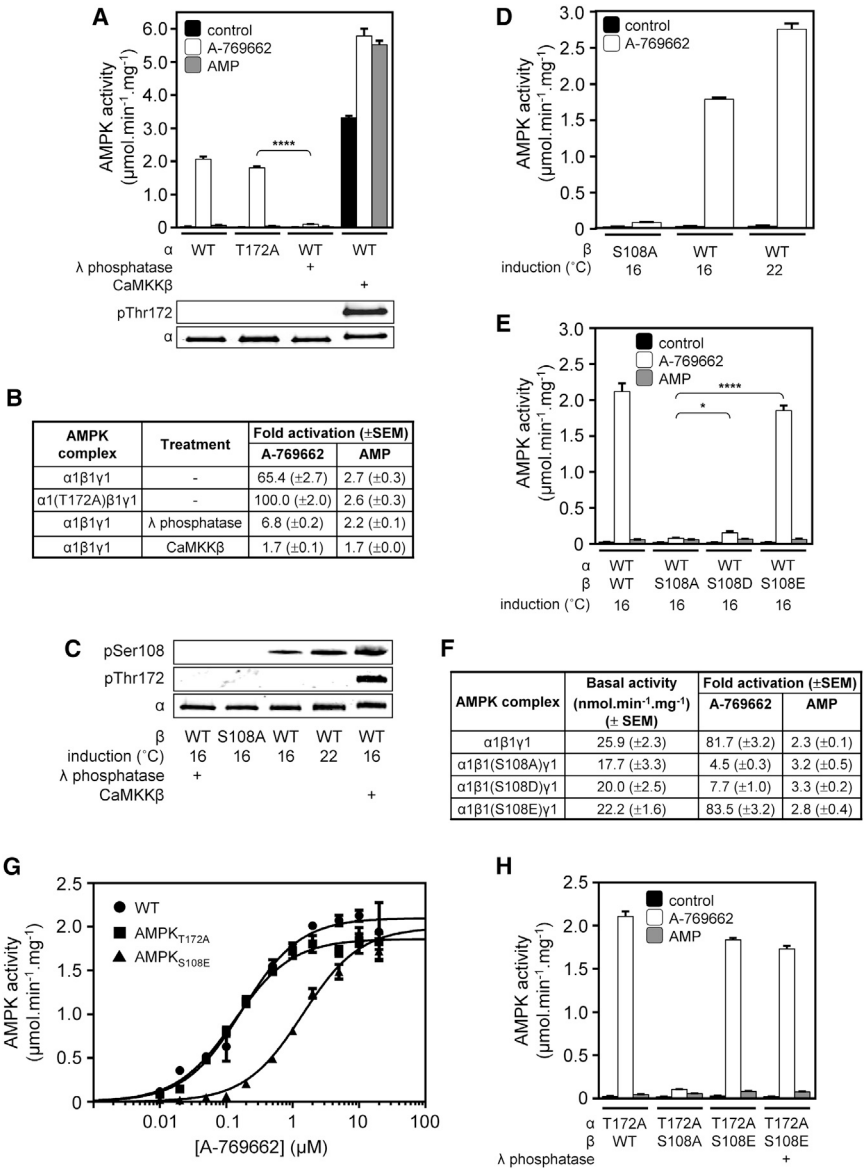
INTRODUCTION

The AMP-activated protein kinase (AMPK) is a metabolic stress-sensing serine-threonine kinase that is activated by depletion of cellular adenylate charge (reduced ATP/ADP + AMP), triggering AMPK phosphorylation on the α subunit activation loop Thr172 by upstream kinases LKB1 or Ca^{2+}/calmodulin-dependent protein kinase β (CaMKKβ) (Hardie, 2007; Oakhill et al., 2012). Dephosphorylated, naive AMPK is thought to be catalytically inactive. Once activated, AMPK redirects cellular metabolism away from anabolic pathways to energy-generating catabolic pathways by phosphorylating key enzymes in all branches of metabolism, resulting in ATP production and restoration of cellular energy balance.

The AMPK heterotrimer is composed of a catalytic α subunit and regulatory β- and γ subunits (Hardie, 2007; Oakhill et al., 2012). AMPK γ has three functional nucleotide-binding sites (sites 1, 3, and 4) that bind ATP, ADP, and AMP interchangeably (Xiao et al., 2007, 2011; Oakhill et al., 2011; Chen et al., 2012). AMP binding triggers Thr172 phosphorylation by LKB1 (Oakhill et al., 2010; Zhang et al., 2013; Gowans et al., 2013). Both AMP and ADP stimulate phosphorylation of Thr172 by CaMKKβ (Oakhill et al., 2010; Oakhill et al., 2011), although this has recently been challenged (Gowans et al., 2013). AMP and, to a lesser extent, ADP are thought to sustain AMPK signaling by suppressing dephosphorylation of phosphorylated Thr172 (pThr172) by protein phosphatases (Oakhill et al., 2010, 2011; Xiao et al., 2011; Gowans et al., 2013). Once phosphorylated on Thr172, AMPK is further allosterically activated up to 13-fold by AMP (Gowans et al., 2013).

The metabolic dimensions of diseases including type 2 diabetes, obesity, cardiovascular disease, and cancer have encouraged efforts to develop direct activating drugs for AMPK. An increasing number of direct AMPK-activating drugs such as the thienopyridone derivative A-769662 have been identified (Cool et al., 2006; Giordanetto and Karis, 2012), but little progress has been made in elucidating their mechanism of action. We previously showed that A-769662 preferentially activates AMPK complexes containing the β1-isoform, and a requirement for the β subunit carbohydrate binding module (CBM) as well as the autophosphorylation site Ser108 within the β-CBM has been demonstrated (Mitchelhill et al., 1997; Warden et al., 2001; Sanders et al., 2007; Scott et al., 2008). The structural basis for β-CBM dependence has recently been provided with the report of a crystal structure for AMPK complexed with A-769662 (Xiao et al., 2013), in which a drug binding site is positioned between the β-CBM and α subunit kinase domain small lobe. Although not directly involved in drug binding, phosphorylated β-Ser108 forms hydrogen bonds with α-Thr21 and α-Lys31 at the domain interface and most likely contributes to the stability of the binding pocket.

Studies examining the mechanism of AMPK activation by A-769662 in vitro have used enzyme phosphorylated on Thr172 (Sanders et al., 2007; Göransson et al., 2007; Scott



AMPK complex	Treatment	Fold activation (±SEM)	
		A-769662	AMP
α1β1γ1	-	65.4 (±2.7)	2.7 (±0.3)
α1(T172A)β1γ1	-	100.0 (±2.0)	2.6 (±0.3)
α1β1γ1	λ phosphatase	6.8 (±0.2)	2.2 (±0.1)
α1β1γ1	CaMKKβ	1.7 (±0.1)	1.7 (±0.0)

AMPK complex	Basal activity (nmol.min-1.mg-1) (± SEM)	Fold activation (±SEM)	
		A-769662	AMP
α1β1γ1	25.9 (±2.3)	81.7 (±3.2)	2.3 (±0.1)
α1β1(S108A)γ1	17.7 (±3.3)	4.5 (±0.3)	3.2 (±0.5)
α1β1(S108D)γ1	20.0 (±2.5)	7.7 (±1.0)	3.3 (±0.2)
α1β1(S108E)γ1	22.2 (±1.6)	83.5 (±3.2)	2.8 (±0.4)

Figure 1. Phosphorylation of β-Ser108 Is Sufficient for A-769662 Allosteric Activation of AMPK

For direct activation, activities of AMPK (His_6-α1β1γ1) expressed in *E. coli* were measured by SAMS assay following incubation with A-769662 (20 μM, open bars) or AMP (200 μM, gray bars). WT or mutant α- and β subunits are indicated, along with enzyme pretreatment and induction temperature. The data are presented as means ± SEM (n = 4).

(A) A-769662 allosteric activation of AMPK is independent of α-Thr172 phosphorylation. A significant difference in fold activation with A-769662 between $AMPK_{T172A}$ and λ phosphatase-treated, WT AMPK is shown (****$p < 0.0001$). Immunoblot shows α-pThr172 levels for each preparation.

(B) Fold activation, calculated from data in (A).

(C) Immunoblot showing relative changes in β-pSer108 and α-pThr172 as a result of induction temperature and enzyme pretreatment.

(D) A-769662-stimulated activity of AMPK is commensurate with the extent of β-Ser108 phosphorylation. Activities of untreated AMPK preparations immunoblotted in (C) were measured with A-769662.

(E) Sensitivity to A-769662 allosteric activation is restored following substitution of β-Ser108 for Glu. Significant differences in fold activation with A-769662 between β mutants S108A and S108D (*$p < 0.05$) or S108E (****$p < 0.0001$) are shown.

(F) Fold activation, calculated from data in (E).

(G) A-769662 dose-response curves for direct activation of AMPK (WT and indicated mutants). The data are presented as means ± SEM (n = 4).

(H) Autophosphorylated residues other than β-Ser108 do not contribute to A-769662 allosteric activation of AMPK.

et al., 2008); however, the requirement for phosphorylation has not been examined in detail. Several reports have shown that incubation of isolated tissue, hepatocytes, and cultured cell lines with A-769662 (≤100 μM) triggered AMPK signaling with little or no detectable change in pThr172 but with significant phosphorylation of the downstream AMPK substrate acetyl-CoA carboxylase 1 (ACC1) at position Ser79 (Göransson et al., 2007; Foretz et al., 2010; Santidrián et al., 2010; Kim et al., 2011; Gowans et al., 2013) (Figure S1 available online). Göransson et al. (2007) demonstrated this convincingly using LKB1-deficient HeLa cells pretreated with the CaMKKβ inhibitor STO-609, leading them to raise the possibility that A-769662 activates nonphosphorylated AMPK. However, A-769662 did not activate a purified rat liver AMPK preparation pretreated with protein phosphatase-2Cα (PP2Cα), indicating that prior phosphorylation of AMPK was required (Göransson et al., 2007). Although a dependence on Ser108 phosphorylation for A-769662 activation had not been unequivocally demonstrated, we reasoned that dephosphorylation of phosphorylated Ser108 (pSer108) by PP2Cα may account for the loss of A-769662 sensitivity in the AMPK preparation used in the Göransson study (Göransson et al., 2007).

RESULTS AND DISCUSSION

A-769662 Activates AMPK Independently of α-Thr172 Phosphorylation

To examine the requirement for phosphorylation of AMPK in A-769662 allosteric activation, we initially used purified, recombinant AMPK (α1β1γ1) expressed in *Escherichia coli* (Figure S2A), which is not phosphorylated on Thr172 and is therefore catalytically inactive (Figure 1A) (Neumann et al., 2003). We found that A-769662 activated this AMPK preparation approximately 65-fold (stimulated specific activity: 2.1 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ enzyme) and also activated >100-fold an AMPK mutant in which Thr172 was substituted to Ala ($AMPK_{T172A}$) (Figures 1A and 1B; Figure S2B), demonstrating that activity in the

presence of the drug was independent of Thr172 phosphorylation. On the other hand, A-769662 sensitivity was lost following treatment of wild-type (WT) AMPK with lambda protein phosphatase (λ phosphatase), indicating that phosphorylation at another site(s) was required for drug activation (Figures 1A and 1B; Figure S2B).

Phosphorylation of β-Ser108 Is Required for A-769662 Activation of AMPK

We found recombinant AMPK expressed in *E. coli* grown in Luria Bertani broth was autophosphorylated on both α- and β subunits (Oakhill et al., 2010) when WT α, but not the kinase dead (KD) α mutant D141A ($AMPK_{KD}$), was expressed (Figure S2B). β-Ser108 was one of the residues phosphorylated in WT AMPK as shown by immunoblot and tandem mass spectrometry (MS) analysis (Figure 1C; Figure S2C), and an AMPK mutant in which Ser108 was exchanged to Ala ($AMPK_{S108A}$) was insensitive to A-769662 as reported previously (Figure 1D) (Sanders et al., 2007). Comparison of time-of-flight (TOF) mass graphs of β subunits from WT AMPK and $AMPK_{S108A}$ revealed that pSer108 accounted for the dominant singularly phosphorylated species in *E. coli*-expressed AMPK (Figure S2B). The extent of Ser108 autophosphorylation varied with expression conditions and increased 1.5-fold from ~60% to ~90% in AMPK expressed in *E. coli* at 22°C compared with 16°C (Figure 1C; Figure S2B). This was accompanied by a corresponding increase in activation by A-769662 (93-fold) to yield a specific activity of 2.8 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ enzyme (Figure 1D), comparable to the basal specific activity of AMPK fully phosphorylated on Thr172 by pretreatment with CaMKKβ (3.3 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ enzyme) (Figure 1A) (Davies et al., 1994; Michell et al., 1996; Woods et al., 2003a; Suter et al., 2006).

To examine possible contributions of other phosphorylated residues in our bacterial AMPK preparations, we exchanged Ser108 for negatively charged residues Glu or Asp with the intention of mimicking phosphorylation. We found that sensitivity to A-769662 allosteric activation was substantially restored upon substitution with Glu108 (stimulated specific activity: 1.9 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ enzyme), which was a more effective phosphomimetic than Asp108 (Figures 1E and 1F; Figure S3). The concentration of A-769662 giving half-maximal activation ($K_{0.5}$) of $AMPK_{S108E}$ was 1.42 ± 0.13 μM, which was 9- and 11-fold higher than $K_{0.5}$ for A-769662 allosteric activation of WT AMPK (0.18 ± 0.02 μM) and $AMPK_{T172A}$ (0.13 ± 0.01 μM), respectively (Figure 1G). Pretreatment of $AMPK_{T172A/S108E}$ double mutant (Figure S2A) with λ phosphatase produced dephosphorylated AMPK (Figure S3) that retained full activation by A-769662 (Figure 1H), demonstrating that no other autophosphorylated residues in *E. coli*-expressed AMPK contribute to drug activation. Combined, our results demonstrate that phosphorylation on β1-Ser108 alone is sufficient to render AMPK sensitive to allosteric activation by A-769662 and that this stimulation entirely compensates for Thr172 phosphorylation as an AMPK regulatory mechanism.

Dynamic Regulation of β-Ser108 Phosphorylation

We examined the mechanism by which Ser108 is autophosphorylated by incubating $AMPK_{S108A}$ with CaMKKβ in the presence of either λ phosphatase-treated, dephosphorylated WT AMPK or $AMPK_{KD}$. We reasoned that Thr172 could be phosphorylated in both $AMPK_{S108A}$ and $AMPK_{KD}$ complexes; however, activation would be confined to $AMPK_{S108A}$; therefore $AMPK_{KD}$ Ser108 could only be phosphorylated via a *trans* event. S108A mutation did not diminish the ability of CaMKKβ to phosphorylate Thr172, but as expected Ser108 autophosphorylation was restricted to the WT complex (Figure 2A, left). We detected pSer108 in the $AMPK_{S108A}$/WT mixture following incubation with CaMKKβ; however, we were unable to detect pSer108 when $AMPK_{S108A}$ was coincubated with $AMPK_{KD}$ (Figure 2A, right). These observations are consistent with Ser108 *cis*-autophosphorylation.

We next investigated cellular regulation of β-Ser108 phosphorylation using COS7 cells transiently transfected to express glutathione S-transferase (GST)-tagged AMPK. Basal levels of AMPK phosphorylation were determined by isolating complexes from cells incubated in fresh medium (25 mM glucose) for 2 hr and immunoblotting for pThr172 and pSer108. Immunoblots were calibrated against standards of known pThr172 and pSer108 stoichiometry that were generated by mixing, in defined proportions, equimolar bacterial AMPK preparations that had either been fully dephosphorylated by λ phosphatase (0% phosphorylation as determined by TOF mass spectrometry) or maximally phosphorylated by CaMKKβ (assumed 100% phosphorylation on Thr172 and Ser108) (Figures 1C and 2B; Figure S2B). From densitometry analysis of immunoreactive bands and subsequent comparison with these standards, we measured basal pThr172 and pSer108 stoichiometry in isolated AMPK complexes from COS7 cells at 3.6 ± 0.2% and 6.3 ± 0.2%, respectively (mean ± SEM, n = 4) (Figure 2B). Incubation with ionomycin, a Ca^{2+} ionophore that triggers CaMKKβ signaling and AMPK activation, resulted in a 2.2-fold increase in pThr172 above basal levels and a similar (2.5-fold) increase in pSer108 (Figure 2B). $AMPK_{KD}$ Thr172 was phosphorylated in response to ionomycin to a similar extent (2.1-fold) as WT (Figure 2C). However, we did not detect pSer108 in this mutant either under basal conditions or following ionomycin incubation, even though endogenous AMPK in these cells was also activated by ionomycin (Figure 2C). Our results indicate that Ser108 is mainly a *cis*-autophosphorylation site.

Subsequent incubation of ionomycin-treated, WT AMPK-transfected COS7 cells with STO-609, a specific inhibitor of CaMKKβ, led to rapid dephosphorylation of pThr172 within 2 min. In contrast, pSer108 was dephosphorylated at a slower rate and remained substantially elevated relative to pThr172 at all measured time points to 15 min (Figure 2B). A reduced rate of pSer108 dephosphorylation relative to that of pThr172 was reflected in vitro using the phosphatase PP2Cα, which has previously been used to dephosphorylate pThr172 (Figure 2D) (Woods et al., 2003b; Suter et al., 2006; Oakhill et al., 2010; Gowans et al., 2013). Thus, basal and ionomycin-stimulated levels of β-Ser108 autophosphorylation are intrinsically linked to those of α-pThr172; however, regulatory disconnectivity is achieved at the dephosphorylation step; pSer108 and pThr172 are dephosphorylated by either distinct phosphatases or alternatively a common phosphatase(s) with markedly different kinetics.

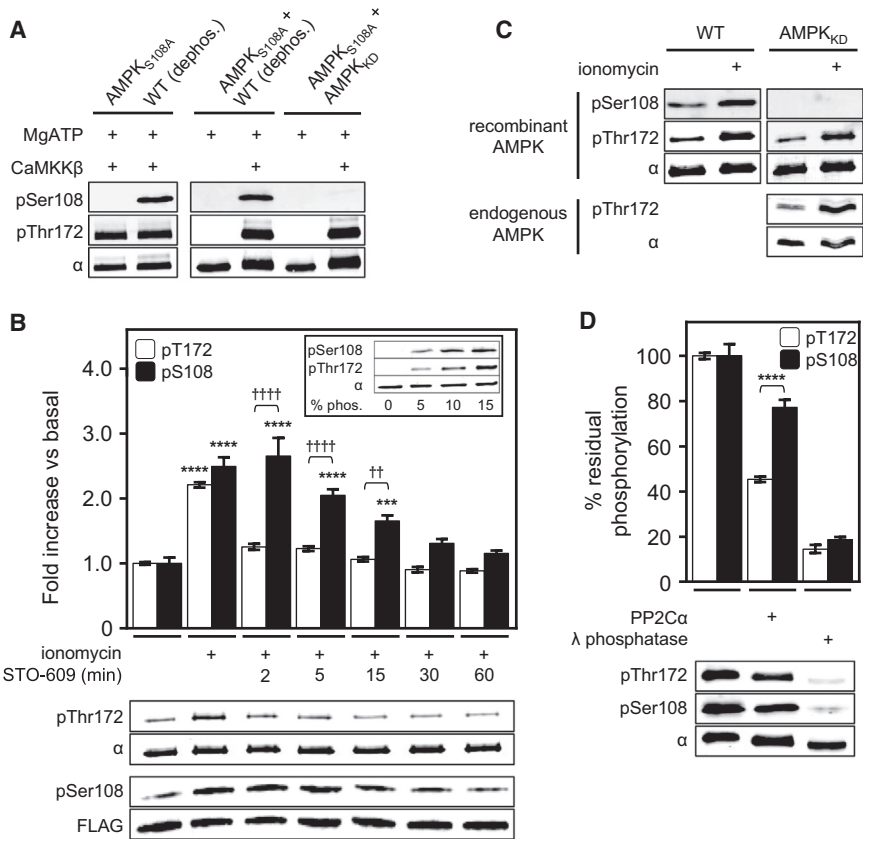


Figure 2. Regulation of β-Ser108 Phosphorylation

(A) In vitro phosphorylation of bacterial-expressed AMPK by CaMKKβ. 400 ng of total AMPK (either $AMPK_{S108A}$ or λ phosphatase-treated WT (dephos.) individually or 1:1 mixtures of $AMPK_{S108A}$ + WT (dephos.) or $AMPK_{S108A}$ + $AMPK_{KD}$) was incubated with 2 mM $MgCl_2$, 200 μM ATP ± 50 ng of CaMKKβ for 30 min at 32°C. Assays were terminated by addition of SDS sample buffer and immunoblotted for β-pSer108, α-pThr172, and total α. A representative immunoblot from three independent experiments is shown.

(B) α-Thr172 and β-Ser108 phosphorylation (normalized against α subunit and FLAG (β subunit), respectively) were measured by immunoblot in AMPK isolated from transfected COS7 cells, following initial incubation ± ionomycin and subsequent incubation ± STO-609. The data are presented as means ± SEM (n = 4). Statistical analysis was performed by two-way ANOVA. Significant differences between basal and treated levels of α-pThr172 and β-pSer108 (***$p < 0.001$; ****$p < 0.0001$) and between fold increases in α-pThr712 and β-pSer108 at each time point (††$p < 0.01$; ††††$p < 0.0001$) are indicated. A representative immunoblot is shown. Inset: bacterial-expressed AMPK, maximally phosphorylated by CaMKKβ, was mixed with dephosphorylated AMPK to generate standards of known α-pThr172 and β-pSer108 stoichiometry (% phos).

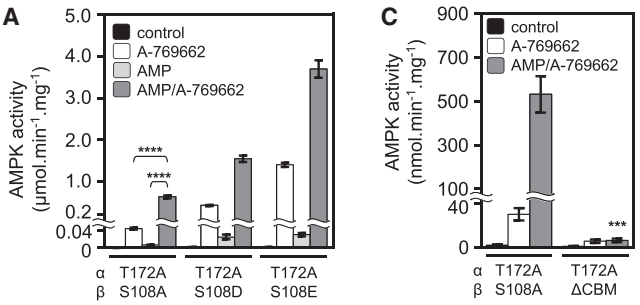
(C) COS7 cells transfected to express WT AMPK or $AMPK_{KD}$ were incubated with ionomycin. Isolated recombinant AMPK and endogenous AMPK from lysates were immunoblotted for β-pSer108 and/or α-pThr172. A representative immunoblot from three independent experiments is shown.

(D) In vitro α-pThr172 and β-pSer108 dephosphorylation by PP2Cα and λ phosphatase. The data are presented as means ± SEM (n = 6). Significant difference between percentage of residual α-pThr172 and β-pSer108 following PP2Cα treatment is indicated (****$p < 0.0001$). A representative immunoblot is shown.

AMP and A-769662 Synergistically Activate Dephosphorylated, Naive AMPK

We reasoned that A-769662 mediates pThr172-independent activation of AMPK by inducing a conformational change upon binding that leads to reorientation of the α activation loop. A similar mechanism regulates cyclin-dependent kinase 2 (CDK2) in the absence of activation loop phosphorylation, although in this case loop reorientation is mediated by direct interaction with cyclinA (Jeffrey et al., 1995). Subsequent phosphorylation of CDK2 Thr160 (analogous to AMPK α-Thr172) by CDK-activating kinase results in further rearrangement of the activation loop, largely driven by burial of the phosphorylated Thr160 side chain, which moves >6 Å from its unphosphorylated position (Russo et al., 1996). Strikingly, the recent finding that A-769662 binds to the AMPK α-catalytic domain small lobe revealed proximity between a drug binding site and activation loop (Xiao et al., 2013). However, in this structure, the activation loop adopts a constrained conformation, almost superimposable to phosphorylated CDK2, because the crystallized AMPK construct was phosphorylated with CaMKKβ during preparation. Unfortunately the current AMPK structures do not reveal the mechanism of pThr172-independent allosteric regulation by A-769662. The drug-binding signal may be transduced through the α-C helix (residues 60-70) in the kinase domain small lobe, which contacts the activation loop in the Thr172-phosphorylated conformation (Xiao et al., 2011) and is reorientated by hydrophobic interaction with a short α helix C-terminal to the CBM, termed the C-interacting helix, that is recruited to the αC helix following A-769662 binding (Xiao et al., 2013). Indeed, A-769662 induced a substantial reduction in solvent accessibility of both the α-C helix and the region encompassing pSer108, as recently determined by hydrogen/deuterium exchange mass spectrometry (Landgraf et al., 2013). Alternatively, stabilization of the unphosphorylated activation loop may be facilitated by AMPK elements that were not resolved in the A-769662-complexed AMPK structure but may be recruited to the region upon drug binding.

We investigated whether AMP further stimulated A-769662-activated $AMPK_{T172A}$. To measure combined allosteric effects we expressed AMPK complexes in COS7 cells containing WT γ, T172A-mutated GST-α fusion and either S108A-, S108D-, or S108E-mutated β-Myc fusions. Recombinant AMPK expressed in mammalian cells is isolated in the more fully modified form (e.g., myristoylated on the β subunit) compared with enzyme expressed in bacteria, which is an important consideration when investigating AMPK regulatory mechanisms in vitro. Complexes isolated using glutathione Sepharose were devoid of contaminating endogenous AMPK α and β, which both possess higher electrophoretic mobility than their respective recombinant tagged counterparts (Figure S4). Compared with low but consistently measureable basal activities of recombinant AMPK



B

β-Ser108 mutant	Basal activity (nmol.min^{-1}.mg^{-1}) (±SEM)	Fold activation (±SEM)		
		A-769662	AMP	AMP/A-769662
Ala	0.6 (±0.4)	78.3 (±3.6)	12.2 (±0.4)	1049.9 (±63.6)
Asp	1.6 (±0.9)	241.0 (±10.3)	15.3 (±3.3)	928.2 (±46.1)
Glu	1.5 (±0.9)	916.7 (±343.2)	20.3 (±2.6)	2452.9 (±141.1)

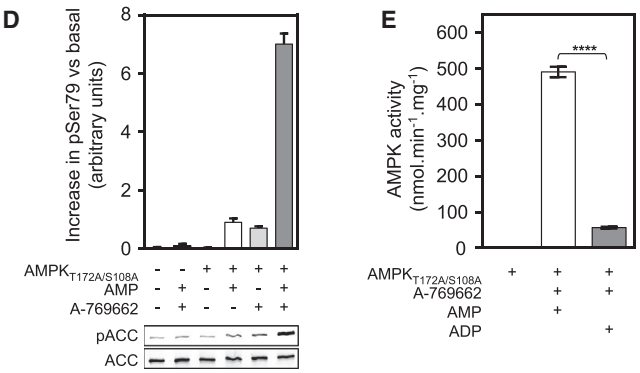


Figure 3. AMP and A-769662 Synergistically Activate AMPK Independently of α-Thr172 and β-Ser108 Phosphorylation

For direct activation, activities of AMPK (GST-α1β1γ1) expressed in COS7 cells were measured by SAMS assay unless stated, using 100 μM AMP/ADP and 20 μM A-769662 as indicated. WT or mutant α- and β subunits are indicated. The data are presented as means ± SEM (n = 4).

(A) AMPK activities with A-769662 (open bars), AMP (light gray bars), or AMP and A-769662 (dark gray bars). Significant differences in fold activation of $AMPK_{T172A/S108A}$ between individual incubations and coincubation are shown (****$p < 0.0001$).

(B) Fold activation, calculated from data in (A).

(C) AMP/A-769662 synergistic activation of AMPK is CBM dependent. Activities of $AMPK_{T172A/S108A}$ and truncated complex $AMPK_{T172A/\Delta CBM}$ were measured with A-769662 (open bars) or AMP and A-769662 (dark gray bars). A significant difference in fold activation with AMP/A-769662 coincubation of $AMPK_{T172A/S108A}$ is shown (***$p < 0.001$).

(D) AMP/A-769662 coincubation stimulates phosphorylation of ACC1-Ser79. ACC1 was phosphorylated by $AMPK_{T172A/S108A}$ with A-769662 (open bars), AMP (light gray bars), or AMP and A-769662 (dark gray bars). Increases in pSer79 compared to basal (untreated control) are presented. A representative immunoblot is shown.

(E) ADP and A-769662 do not synergistically activate AMPK. A significant difference in fold activation between AMP/A-769662 and ADP/A-769662 coincubations is shown (****$p < 0.0001$).

expressed in bacteria (Figure 1F), complexes purified from COS7 cells displayed negligible basal activities (Figures 3A and 3B). This confirmed that endogenous α, which is partially phosphorylated on Thr172 in the basal state, was excluded from our COS7 cell preparations.

Incubation with A-769662 alone stimulated the activities of COS7 cell-expressed $AMPK_{T172A/S108D}$ (~240-fold) and $AMPK_{T172A/S108E}$ (~900-fold), which were increased following incubation with both AMP and A-769662 a further 2- to 4-fold (Figures 3A and 3B). $AMPK_{T172A/S108A}$ has a low basal activity that was stimulated 78-fold by A-769662 alone to give a specific activity of only 0.04 μmol•min^{-1}•mg^{-1} enzyme. In contrast, AMP/A-769662 coincubation dramatically activated $AMPK_{T172A/S108A}$ (>1,000-fold) to yield a specific activity of 0.6 μmol•min^{-1}•mg^{-1} enzyme (Figures 3A and 3B). Despite lacking a requirement for pSer108, AMP/A-769662 activation nevertheless is entirely β-CBM dependent, because deletion of the β N-terminal 145 residues (ΔCBM) rendered AMPK insensitive to synergistic activation (Figure 3C). Similarly AMPK containing the β2 isoform is insensitive to activation by A-769662 (Scott et al., 2008), and bacterial-expressed β2-AMPK, which is not extensively autophosphorylated on the β subunit (Figure S3), was also insensitive to synergistic activation by AMP and A-769662 (data not shown).

It was of interest to test whether AMP/A-769662-activated AMPK was capable of phosphorylating a native AMPK substrate, because we had previously observed with Ca^{2+}/calmodulin-dependent protein kinase I that certain peptide substrates do not require kinase activation loop phosphorylation (Hook et al., 1999). Incubation of $AMPK_{T172A/S108A}$ with both AMP and A-769662 resulted in a >140-fold increase in phosphorylation of HeLa cell-derived ACC1, relative to an AMPK-untreated control (Figure 3D). ADP does not allosterically activate Thr172-phosphorylated AMPK (Xiao et al., 2011; Oakhill et al., 2011), nor did it activate $AMPK_{T172A/S108A}$ when coincubated with A-769662 (Figure 3E). Additionally, there was no detectable autophosphorylation of α- or β subunits in λ phosphatase-treated WT AMPK following AMP/A-769662 coincubation in the presence of MgATP (Figure S3), demonstrating that AMP/A-769662 synergistic activation of AMPK in vitro does not involve autophosphorylation and occurs exclusively via an allosteric mechanism. Synergistic regulation of AMPK has previously been demonstrated in hepatocytes coincubated with 5-aminoimidazole-4-carboxamide 1-β-D-ribofuranoside (AICAR), a pro-drug metabolized to the AMP analog ZMP, and A-769662 (Foretz et al., 2010; Ducommun et al., 2014); however, in both cases increased AMPK activity was attributed to the combined suppressive effects of both ligands on pThr172 dephosphorylation.

To further investigate the mechanism of pThr172-independent AMP/A-769662 synergy, we measured activity of λ phosphatase-treated, COS7 cell-expressed AMPK over a range of A-769662 concentrations at constant AMP concentration. $K_{0.5}$ for A-769662 stimulation in the presence of 100 μM AMP was 0.37 ± 0.04 μM (Figure 4A), which is comparable to $K_{0.5}$ for A-769662 allosteric activation of Thr172-phosphorylated WT AMPK (0.15 μM) (Scott et al., 2008). Thus, A-769662 stimulation through both pSer108-dependent and pSer108-independent mechanisms seems likely to involve the same drug binding site on AMPK. Ser108 is largely unphosphorylated in the basal state (Figure 2B) as previously shown for Thr172 (Gowans et al., 2013), indicating that the putative drug binding site in the absence of AMP is transient and highly regulated. We previously proposed that A-769662 may mimic an intracellular metabolite (Scott et al., 2008), and our findings here extend the possibility that sensitivity to an alternate allosteric ligand is mediated either

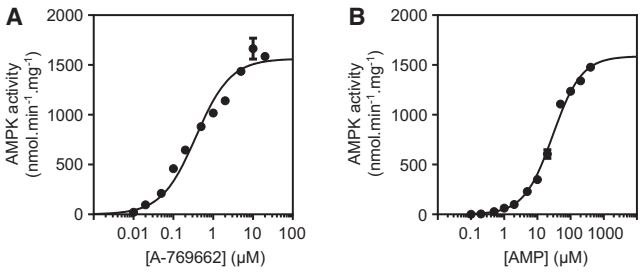


Figure 4. A-769662/AMP Dose Response for AMPK Activation
A-769662 (A) and AMP (B) dose-response curves at fixed AMP (100 μM) or A-769662 (20 μM), respectively, for direct activation of λ phosphatase-treated, COS7 cell-expressed AMPK. The data are presented as means ± SEM (n = 4).

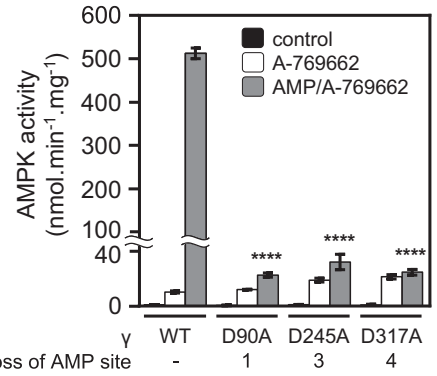


Figure 5. γ-Nucleotide-Binding Sites 1, 3, and 4 Are Required for AMP/A-769662 Synergistic Activation of AMPK
For direct activation, activities of $AMPK_{T172A/S108A}$ expressed in COS7 cells with γ mutants as indicated were measured by SAMS assay using A-769662 (20 μM) (open bars) or AMP (100 μM) and A-769662 (20 μM) together (dark gray bars). The data are presented as means ± SEM (n = 4). Significant differences in AMP/A-769662 fold activation between WT γ and γ-Asp mutants are shown (****p < 0.0001).

through autophosphorylation cycling or even by a β-Ser108 upstream kinase.

At 200 μM ATP and with constant A-769662 concentration (20 μM), activation of λ phosphatase-treated, COS7 cell-expressed AMPK by AMP gave $K_{0.5}$ = 29.2 ± 1.5 μM (V_{max} = 1.6 μmol•min^{-1}•mg^{-1} enzyme, 980-fold activation) (Figure 4B), comparable to $K_{0.5}$ for AMP stimulation of Thr172 phosphorylation by CaMKKβ at 200 μM ATP (33 ± 10 μM) (Oakhill et al., 2010). We also examined the role of individual γ adenine nucleotide-binding sites in mediating synergistic AMP/A-769662 activation by coexpressing $AMPK_{T172A/S108A}$ with γ mutants in which Asp residues in nucleotide site 1 (Asp90), 3 (Asp245), or 4 (Asp317) were individually exchanged with Ala. We previously employed these mutants to show that γ sites 3 and 4 predominantly mediate allosteric activation by AMP, whereas all three sites contribute to AMP regulation of the Thr172 phosphorylation step (Oakhill et al., 2010). The γ residues Asp90, Asp245, and Asp317 form critical hydrogen bonds with ribose hydroxyl groups (Figure S5A) (Xiao et al., 2007); however, the absolute requirement of this bonding network for nucleotide binding has recently been questioned (Mayer et al., 2011). We extended our previous observations using 2′-deoxy AMP (dAMP), which lacks the ribose 2′-hydroxyl forming hydrogen bonds with γ-site Asp side chains. dAMP was substantially less effective than AMP at regulating WT AMPK through either direct activation (Figure S5B) or stimulation of CaMKKβ-mediated phosphorylation of Thr172 (Figure S5C). Using T172A/S108A/γ-Asp mutants, we found that disruption of sites 1, 3, and 4 each led to a substantial reduction in AMP/A-769662 activation (Figure 5).

Several important ramifications arise from a purely allosteric AMPK regulatory mechanism involving AMP in combination with a drug, not least of which is the potential for AMPK activation independent of upstream kinases and prior Ser108 autophosphorylation cycles (Figure 6). Because the specific activity of naive AMPK synergistically regulated by AMP and A-769662 approaches that achieved by phosphorylation of Thr172, the mechanism provides an effective activation pathway in circumstances where there is genetic loss of upstream kinase, such as loss of LKB1 in a variety of cancer models (Shackelford and Shaw, 2009). In human liver where β2 is the dominant AMPK β isoform (Wu et al., 2013), the mechanism may also allow for synergy between drugs that bind at the A-769662 site and activate β2-AMPK complexes (e.g., pyrrolopyridone derivatives (Mirguet et al., 2013) and compound 991 (Xiao et al., 2013)) and agents that increase cellular AMP, such as metformin, which is extensively used to treat type 2 diabetics. The interdependence of drug activation with AMP highlights the possibility of exploiting the mechanism in designing ultrasensitive drug screening strategies for AMPK. Nevertheless, it is an ironic twist that AMPK activation by A-769662 cannot transcend natural metabolic stress regulation. Thus, A-769662 could combine with AMP to activate naive, dephosphorylated AMPK, or A-769662 alone may activate enzyme autophosphorylated on β-Ser108 from a previous activation cycle involving upstream kinase signaling; either way the outcome is dictated by AMP.

SIGNIFICANCE

AMPK is a critical regulator of cellular and systemic energy homeostasis whose regulation by ligands and signaling pathways has been intensively studied for three decades. The metabolic dimensions of diseases, including type 2 diabetes, obesity, cardiovascular disease, and cancer, have encouraged efforts to elucidate these regulatory mechanisms and develop direct activating drugs for AMPK. We have demonstrated two allosteric mechanisms that are independent of activation loop phosphorylation, previously thought to be an absolute requirement to initiate AMPK signaling. Our findings provide significant advances to aid development of AMPK-targeting therapeutics, both in a mechanistic sense and in the context of screening strategies. β-Ser108 is currently regarded as an autophosphorylation site; however, this does not preclude the existence of upstream kinases for this site that could potentially be exploited with the aim of increasing AMPK drug potency. Additionally, synergistic activation of AMPK by drugs and AMP constitutes a roadmap for combinatorial therapies. Importantly, our findings demonstrate that cell-based studies require observation of AMPK substrate phosphorylation, in

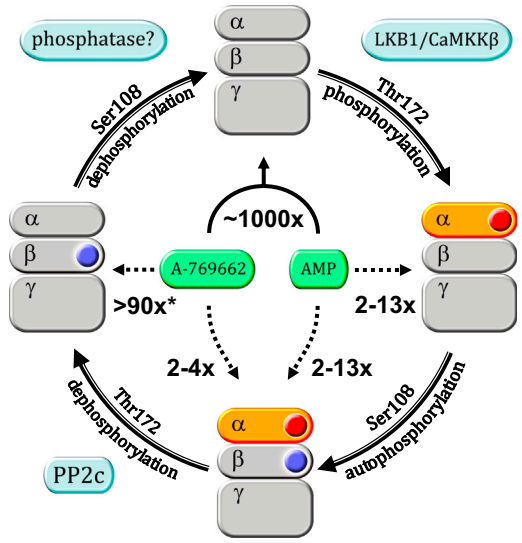


Figure 6. Hypothetical Model of Allosteric Regulation of AMPK by AMP and A-769662

AMP and A-769662 synergistic (solid arrow) and individual (dashed arrows) direct activation of AMPK in various α-Thr172 and β-Ser108 phosphorylation states, in the context of the AMPK activation cycle in response to cellular energy charge depletion. AMP-stimulated α-Thr172 phosphorylation (red button) by upstream kinases results in basal AMPK activity (orange) and β-Ser108 autophosphorylation (blue button). Rapid α-pThr172 dephosphorylation results in β-Ser108-phosphorylated AMPK that is sensitive to drug alone. In vitro fold activation by AMP and/or A-769662 of AMPK in each phosphorylation state is indicated. *90-fold activation by A-769662 is most likely an underestimation because bacterial-expressed AMPK used to generate the data consistently displayed higher basal activity than α-Thr172 dephosphorylated AMPK expressed in COS7 cells.

addition to α-Thr172 phosphorylation, as a complimentary indicator of AMPK activity.

EXPERIMENTAL PROCEDURES

Protein Production

All mutants were generated using QuikChange Site-Directed Mutagenesis Kits (Stratagene), and constructs were sequence verified. Expressed constructs were mass verified by TOF mass spectrometry.

Heterotrimeric human AMPK His_6-α1β1γ1 (WT and indicated mutants and truncations) was expressed in *E. coli* strain Rosetta (DE3) using the pET DUET expression system (Novagen). cDNAs for α1 and γ1 were sequentially inserted into pET DUET multiple cloning sites (MCS) 1 (NcoI/EcoRI) and 2 (MfeI/XhoI), respectively, resulting in incorporation of an N-terminal hexahistidine tag onto α1. cDNA for β1 was inserted into pET RSF DUET MCS1 (NcoI/BamHI). AMPK myristoylated on the β subunit residue Gly2 was generated by coexpression with N-myristoyltransferase (inserted into pET RSF DUET MCS2 (BglII/XhoI)) as described previously (Oakhill et al., 2010). Expression cultures were grown in Luria Bertani broth and induced at 16°C or 22°C with 0.25 mM isopropyl β-D-thiogalactopyranoside, prior to overnight incubation. Cells were ruptured using a precooled EmulsiFlex-C5 homogenizer (Avestin) and AMPK purified using nickel Sepharose and size exclusion chromatography. Final storage buffer (Buffer A) consisted of 50 mM Tris•HCl, pH 7.4, 150 mM NaCl, 10% glycerol, and 2 mM tris(2-carboxyethyl)phosphine. Dephosphorylated AMPK (100 μg) was generated by incubation with 400 units of λ phosphatase (New England Biolabs), 2 mM $MnCl_2$ (2 hr, 22°C). α-Thr172 phosphorylated AMPK (100 μg) was generated by incubation with 2.5 μg of CaMKKβ (produced in Sf21 cells as a C-terminal FLAG fusion as previously described (Oakhill et al., 2010)), 2 mM $MgCl_2$, 200 μM ATP (2 hr, 22°C). We were unable to detect further increases in Thr172 phosphorylation with extended CaMKKβ incubation. λ phosphatase- and CaMKKβ-treated AMPK was repurified on nickel Sepharose and buffer exchanged into Buffer A using PD10 desalt columns (GE Life Sciences).

Heterotrimeric human AMPK GST-α1β1γ1 (WT and indicated mutants and truncations) was expressed in COS7 cells cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum as described previously (Oakhill et al., 2010). Briefly, AMPK complexes were expressed following simultaneous triple transfection of cells with constructs for α1 (pDEST27, N-terminal GST fusion), β1 (pcDNA3, C-terminal Myc, or FLAG fusion), and γ1 (pMT2, N-terminal hemagglutinin [HA] fusion), prepared as liposomes with FuGENE HD (Promega) according to the manufacturer's protocols. Lysates were harvested 48 hr post-transfection in 50 mM Tris-HCl, pH 7.4, 50 mM NaCl, 10% glycerol, 1 mM EDTA, 1 mM EGTA, 5 mM sodium pyrophosphate, 50 mM NaF, 1% Triton X-100, cOmplete protease inhibitor cocktail (Roche) and clarified by centrifugation (14,000 rpm, 3 min). AMPK was isolated from COS7 cell lysates on glutathione Sepharose 4B (GE Life Sciences), extensively washed with buffer A, and either assayed while immobilized or eluted with buffer A + 20 mM glutathione. AMPK for α-Thr172 phosphorylation and dose-response assays was dephosphorylated with λ phosphatase (2 mM $MnCl_2$, 2 hr, 22°C) prior to elution. For the CBM dependency assay, α1β1(S108A)γ1 and α1β1(146-270)γ1 complexes were isolated from COS7 cell lysates on anti-c-Myc affinity gel (Sigma). All AMPK preparations were quantitated by comparative α subunit immunoblot standardized with a bacterial-expressed, purified AMPK preparation of known concentration, determined by BCA protein assay (Pierce).

Endogenous ACC1 was isolated from cultured HeLa cells previously incubated for 2 hr with fresh DMEM. Lysates prepared in 50 mM Tris•HCl, pH 7.4, 50 mM NaCl, 1% Triton, 1 mM EDTA, 1 mM EGTA, 10% glycerol, 50 mM NaF, 5 mM sodium pyrophosphate, and protease inhibitor cocktail (Roche) were incubated with streptavidin Sepharose (GE Life Sciences, 2 h) prior to washing with PBS.

SAMS Activity Assay

AMPK activity was determined by phosphorylation of the SAMS peptide using 100 μM SAMS, 200 μM [γ-^{32}P]ATP, 5 mM $MgCl_2$, and the indicated ligands in a 25 μl reaction volume at 30°C. Reactions were terminated after 10 min by spotting 15 μl onto P81 phosphocellulose paper (Whatman) and washing in 1% phosphoric acid (Glass et al., 1978). Radioactivity was quantified by scintillation counting.

Immunoblotting

α-pThr172 and total α1 were detected by immunoblot as previously described (Oakhill et al., 2010). FLAG, β-pSer108, and ACC1-pSer79 were detected using antibodies from Epitomics or Cell Signaling, followed by incubation with anti-rabbit or anti-mouse IgG 2° antibody fluorescently labeled with IR680 or IR800 (LI-COR Biosciences). Total ACC was detected using streptavidin fluorescently labeled with IR680. Immunoblots were visualized on an Odyssey membrane imaging system (LI-COR Biosciences).

COS7 Cell Incubations

COS7 cells were transiently transfected to express AMPK (GST-α1(WT or kinase dead/β1-FLAG/HA-γ1) for 48 hr as described above. Cells were incubated for 2 hr in fresh medium prior to addition of ionomycin (2.5 μM final concentration) or DMSO for 15 min, followed by addition of STO-609 (2 μM final concentration). Lysates were prepared at indicated time points for a further 60 min as described above. AMPK was isolated on glutathione Sepharose 4B and immunoblotted for α-pThr172, total α, β-pSer108, and FLAG.

AMPK α-pThr172 and β-pSer108 Dephosphorylation Assay

Assays were conducted as previously described (Oakhill et al., 2010). 200 ng of CaMKKβ-treated, *E. coli*-expressed AMPK was incubated with either 100 ng of protein phosphatase PP2cα (produced as a GST fusion in Sf21 cells as described previously (Oakhill et al., 2010)) + 2 mM $MgCl_2$ or 20 units λ phosphatase + 2 mM $MnCl_2$ for 15 min at 32°C. Assays were terminated by addition of SDS sample buffer and immunoblotted for α-pThr172, β-pSer108, and total α.

AMPK α-Thr172 Phosphorylation Assay

Assays were conducted as previously described (Oakhill et al., 2010). 200 ng of dephosphorylated, COS7 cell-expressed AMPK was incubated with 2 mM $MgCl_2$, 200 μM ATP, 8 ng CaMKKβ, and the indicated ligands for 10 min at 32°C. Assays were terminated by addition of SDS sample buffer and immunoblotted for α-pThr172 and total α.

ACC Phosphorylation Assay

ACC1 immobilized on streptavidin Sepharose was incubated with 2 mM $MgCl_2$, 200 μM ATP, 125 ng of COS7 cell-expressed α1(T172A)β1(S108A)γ1 AMPK double mutant, and the indicated ligands for 15 min at 32°C with agitation. Assays were terminated by addition of SDS sample buffer and immunoblotted for ACC-pSer79 and total ACC.

Tandem Mass Spectrometry Analysis

Samples were digested with trypsin according to the manufacturer's protocols and analyzed by reversed-phase nHPLC-ESI-MS/MS using an UltiMate 3000 Nano LC HPLC system (Dionex) directly connected to a Triple-TOF 5600 mass spectrometer (AB SCIEX) in direct injection mode. Peptide mixtures were resolved on an analytical nanocapillary HPLC column (100 μm inner diameter [i.d.] × 15 cm) packed with C_{18} Acclaim PepMap100 (3 μm particle size, 100 Å pore size) using a 1%–75% elution gradient of 98% acetonitrile/2% of 0.1% formic acid (v/v) in water at a flow rate of 250 nl/min. Mass spectrometric data were analyzed using the database search engine ProteinPilot using the Paragon algorithm.

TOF Mass Spectrometry Analysis

Samples used for activity measurements were analyzed by reversed-phase HPLC-ESI-MS using an UltiMate 3000 HPLC system directly connected to a Triple-TOF 5600 mass spectrometer. AMPK subunits were resolved on an Aquapore RP-300 column (1 mm i.d. × 10 cm, Applied Biosystems) packed with C_8 (7 μm particle size) using an elution gradient of 20%–55% acetonitrile at 150 μl/min. The MS was set to intact protein mode, and the mass range was set to 600–1500 m/z. Mass determination was performed using BioAnalyst Software.

Statistical Analysis

The data are presented as mean values ± SEM of at least three independent experiments. The unpaired two-tailed Student's t test was used for all comparisons unless stated.

SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures and can be found with this article online at http://dx.doi.org/10.1016/j.chembiol.2014.03.006.

AUTHOR CONTRIBUTIONS

J.W.S., N.L., T.A.D., M.T.O., Z.-P.C., S.G., C.G.L., and J.S.O. prepared reagents and conducted experiments. S.M.A.I. performed mass spectrometry studies. G.R.S. provided conceptual input. J.S.O. led the team and, together with J.W.S and B.E.K., designed the experiments and wrote the manuscript.

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