Structures of AMP-activated protein kinase bound to novel pharmacological activators in phosphorylated, non-phosphorylated, and nucleotide-free states

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Edited by Wolfgang Peti

AMP-activated protein kinase (AMPK) is an attractive therapeutic target for managing metabolic diseases. A class of pharmacological activators, including Merck 991, binds the AMPK ADaM site, which forms the interaction surface between the kinase domain (KD) of the α-subunit and the carbohydrate-binding module (CBM) of the β-subunit. Here, we report the development of two new 991-derivative compounds, R734 and R739, which potently activate AMPK in a variety of cell types, including B2-specific skeletal muscle cells. Surprisingly, we found that they have only minor effects on direct kinase activity of the recombinant αβγ isoenzyme yet robustly enhance protection against activation loop dephosphorylation. This mode of activation is reminiscent of that of ADP, which activates AMPK by binding to the nucleotide-binding sites in the γ-subunit, more than 60 Å away from the ADaM site. To understand the mechanisms of full and partial AMPK activation, we determined the crystal structures of fully active phosphorylated AMPK αβγ bound to AMP and R734/R739 as well as partially active nonphosphorylated AMPK bound to R734 and AMP and phosphorylated AMPK bound to R734 in the absence of added nucleotides at <3-Å resolution. These structures and associated analyses identified a novel conformational state of the AMPK autoinhibitory domain associated with partial kinase activity and provide new insights into phosphorylation-dependent activation loop stabilization in AMPK.

AMPK2 is a three-subunit protein kinase that consists of two alternative kinase domain-containing α-subunits (α1 and α2), two alternative carbohydrate-binding module-containing β-subunits (β1 and β2), and three alternative AMP/ADP/ATP-binding γ-subunits (γ1, γ2, and γ3) (1–3). In humans, the β1 isofrom is only present in a fraction of AMPK complexes in liver but not immunologically detectable in skeletal muscle, whose predominant isofrom is αβγ (4–6). AMPK senses the energy state of cells by competitive binding of AMP, ADP, and ATP to three separate sites in its γ-subunit. Upon energy stress, i.e. increases in the ratio of AMP and ADP to ATP, AMPK is activated ~100-fold by activation loop phosphorylation and an additional 2–10-fold by direct allosteric kinase activation (7). The activated enzyme phosphorylates key metabolic and regulatory proteins to stimulate ATP-generating catabolic pathways and inhibit ATP-consuming anabolic pathways and cellular programs (8–10). AMPK is an attractive potential target for the treatment of metabolic diseases, most prominently type 2 diabetes (9, 11, 12). Currently, metformin is the frontline drug for the treatment of type 2 diabetes. Metformin is taken up predominantly by the liver where it generates AMPK-activating energy stress by mildly inhibiting oxidative phosphorylation. In addition, it has been reported that at low pharmacological levels AMPK increases phosphorylation through LKB1 by inducing AMPK heterotrimer formation (13) and formation of an LKB1–*2 The abbreviations used are: AMPK, AMP-activated protein kinase; ADaM site, allosteric drug and metabolite-binding site; AICAR, 5-aminoimidazole-4-carboxamide ribonucleotide; α-CTD, C-terminal domain of the α-subunit; AID, autoinhibitory domain; αRIM, α-regulatory subunit interaction motif; β-CTD, C-terminal domain of the β-subunit; CBM, carbohydrate-binding module; CBS, cystathionine β-synthetase; HDX, hydrogen/deuterium exchange; LKB1, liver kinase B1; KD, kinase domain; m.s.d., root mean square deviation; ZMP, 5-amino-4-imidazolecarboxamide ribonucleotide; pAMPK, phosphorylated AMPK; ACC, acetyl-CoA carboxylase; pACC, phospho-acetyl-CoA carboxylase; %D, percentage of deuterium; PP2Cα, protein phosphatase 2C α.

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AMPK in complex with novel pharmacological activators

AMPK complex at the lysosomal membrane (14) without changing the AMP:ATP ratio (15, 16). Together, metformin potently activates AMPK, which mediates a subset of its effects (17, 18). In addition, acute activation of AMPK in skeletal muscle, which in humans is $\beta_2$-isomorph-specific, is mediated by exercise, but not by metformin, and induces robust and acute glucose uptake in rodents and primates, indicating that AMPK activation in muscle has important additional benefits (5, 19, 20).

Although indirect AMPK activators, like metformin or AMP analogs like ZMP (the intracellular phosphorylation product of AICAR), activate both $\beta_1$- and $\beta_2$-containing AMPK isoforms, they are not specific for AMPK. In contrast, ADaM site-binding pharmacological AMPK activators such as A769662 and especially 991 are specific but do not (A769662) (21) or relatively weakly (991) (22, 23) activate $\beta_2$-containing complexes. Recently, both Merck (MK-9722) and Pfizer (PF-739) developed 991-derivative pan-AMPK activators (i.e. activators with reduced $\beta_1$ specificity), and both compounds efficiently reduced blood glucose levels by insulin-independently stimulating muscle to take up glucose (5, 19). Another AMPK activator, SC4, efficiently activates $\beta_2$ complexes that also contain the $\alpha_2$-subunit but only poorly activates complexes that contain the $\alpha_1$-subunit (24).

The ADaM site is located at the interface between the kinase domain of the $\alpha$-subunit and the carbohydrate-binding module (CBM) of the $\beta$-subunit. Phosphorylation (or a phosphomimetic mutation) of CBM Ser-108 is either required or greatly increases binding of ADaM site ligands (22, 25). ADaM site binders stabilize the interface, which in turn conformationally stabilizes the highly dynamic CBM and shifts the equilibrium toward the active conformation of the kinase domain (22). Consequently, ADaM site activators, activation loop phosphorylation, and AMP-mediated direct allosteric AMPK activation can all collaborate, and ADaM site activators and AMP can partially (26) and possibly fully (25) activate AMPK in the absence of activation loop phosphorylation. To explore the mechanisms of full and partial AMPK activation, we have solved high-resolution crystal structures of phosphorylated $\alpha,\beta,\gamma_1$ AMPK bound to AMP and the two new AMPK ADaM site binders R734 and R739, phosphorylated $\alpha,\beta,\gamma_1$ AMPK bound to R734 in the absence of AMP, and nonphosphorylated $\alpha,\beta,\gamma_1$ AMPK with a CBM phosphomimetic mutant (CBM S108D) bound to AMP and R734, which have provided unanticipated insight into the conformational flexibility of AMPK.

Results

991-derivative compounds activate AMPK in cells

An important component of the KD–CBM interaction and its stabilization by ADaM site ligands is AMPK phosphorylation at Ser-108 in the CBM, which mediates interaction with two lysines (Lys-29 and Lys-31) in the KD (22, 27, 28). These interactions are stabilized by ADaM site compounds 991 and A769662, which form charge interactions with Lys-29 in $\beta_1$-containing complexes (22, 27). We generated two derivatives of 991 in which we replaced the Lys-29–binding, negatively charged carboxyl group of the 991 o-methylbenzoic acid moiety against uncharged N-hydroxylamide (R734) and the 1-methylindole heterocyclic ring against 2-methylbiphenyl (R739) (Fig. 1). These compounds activated endogenous AMPK in all cell lines tested, including $\beta_2$-specific LHCN-M2 human skeletal muscle cells, with low μM AC2X values (the concentration that increases activity 2-fold; Fig. 2, A and B). Both compounds also efficiently inhibited proliferation of A549 and H1299 human lung cancer cell lines (Fig. 2A). The ability of these compounds to activate AMPK in human skeletal muscle cells, which do not contain detectable amounts of AMPK $\beta_1$ complexes (5), suggests that these compounds can activate both $\beta_1$- and $\beta_2$-containing AMPK complexes in cells.

R734 and R739 separate isotype-selectively direct kinase activation from protection against activation loop dephosphorylation

Next, we analyzed the effects of R734 and R739 (Fig. 3A) and 991 (Fig. S1) on the direct kinase activity of bacterially produced, phosphorylated AMPK $\alpha,\beta_1,\gamma_1$ and $\alpha,\beta_2,\gamma_1$. Both compounds consistently activated AMPK $\alpha,\beta_1,\gamma_1$ with similar EC90 values (265 and 257 nM, respectively), although -fold activation was at least twice as high for R734 than R739 (Fig. 3A). In contrast, activation of AMPK $\alpha,\beta_2,\gamma_1$ was weak and varied depending on ligand, substrate, protein preparation, and construct variations, resulting from mild activation to mild inhibition.

In contrast to their variable effects on the direct kinase activity of AMPK $\alpha,\beta_2,\gamma_1$, both compounds robustly increased protection against activation loop dephosphorylation of both AMPK $\alpha,\beta_1,\gamma_1$ and $\alpha,\beta_2,\gamma_1$ (Fig. 3B). As a control, both compounds did not inhibit phosphatase activity (Fig. S2). Therefore, R739 and, to a lesser degree, R734 can functionally separate two mechanisms of AMPK activation (direct kinase activation and protection against activation loop dephosphorylation) selectively for the $\alpha_1,\beta_2,\gamma_1$ isoform. Therefore, AMPK $\alpha_1,\beta_2,\gamma_1$ activation by R739 in cells may occur predominantly or exclusively by increasing net activation loop phosphorylation.
R734 and R739 strongly increase net AMPK activation loop phosphorylation in cells

To understand how R734 and R739 affect activation loop phosphorylation and downstream signaling in cells, we tested their effects on AMPK activation loop phosphorylation and phosphorylation of the AMPK targets Raptor and acetyl-CoA carboxylase (ACC) in parallel with 991 and A769662. We incubated HepG2 cells with increasing doses of the four compounds, lysed cells, and analyzed lysates by immunoblotting. As shown in Fig. 4, the increase in phosphorylation of Raptor and ACC in the presence of R734 and R739 was similar to that of 991 and stronger than that of A769662 (note that A769662 does not activate AMPK/Thr-174/Thr-172 phosphorylation is clearly stronger in the presence of R734 than in the presence of 991 and A769662. The level of AMPK activity, measured by downstream target phosphorylation, depends on the combination of activation loop phosphorylation and direct, allosteric activation. 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phorylation but different levels of AMPK activation loop phosphorylation is therefore consistent with the inability of R739 to directly activate AMPK/H9251/H9252/H9253.

Crystal structure determination of phosphorylated AMPK in complex with AMP and R734 or R739

To gain insight into the structural basis of full and partial AMPK activation by R734 and R739, we first cocrystallized both compounds with fully active phosphorylated AMPK/H9251/H9252/H9253, the kinase domain–stabilizing ATP-competitive kinase inhibitor staurosporine, and AMP (Fig. S3). This allowed us to determine their structures at 2.9- and 2.7-Å resolution, respectively (Fig. 5, A and B; structure statistics in Table 1). As expected, both structures show the hallmarks of fully active AMPK, including a fully aligned regulatory spine and fully resolved activation loop (Fig. S4A), as well as formation of a helix in the CBM linker (C-interacting helix; Fig. 5, A and B) that packs against and stabilizes the regulatory C helix of the kinase domain, a hallmark for AMPK activated by ADaM site agonists (22, 27). Both structures are highly similar to each other (r.m.s.d. value of 0.47 Å over 845 residues) as well as to AMPK/H9251/H9252/H9253 bound to the parent compound 991 (r.m.s.d. values of 1.66 Å over 806 residues (R734) and 1.69 Å over 818 residues (R739)).

Although R734 and R739 lacked the 991 carboxyl group that forms an ionic interaction with Lys-29 in the KD, they unexpectedly made more extensive polar interactions with KD and CBM than 991 (R734: KD, Gly-28, Asn-48, and Asp-88; CBM, Arg-83; R739: KD, Gly-19, Gly-28, Lys-31, Asn-48, and Asp-88; CBM, Arg-83; 991: KD, Lys-29 and Asp-88) (Fig. 5, C–E). These additional interactions might further stabilize the CBM/KD interface and kinase domain active conformation.

R734 weakly synergizes with AMP for AMPK activation

Nonphosphorylated AMPK has at least 100-fold lower catalytic activity than AMPK in which activation loop Thr-174 (human AMPK/H9251; Thr-172 in human/H9252 and rat/H9251 and/H9252) is phosphorylated. Surprisingly, Scott et al. (25) demonstrated that AMPK/H9251/H9252/H9253, which lacks activation loop phosphorylation but retains phosphorylation of Ser-108 in the CBM, can be fully activated by a combination of A769662 and AMP, implying that the AMPK kinase domain can adopt a fully active conformation in the absence of activation loop phosphorylation. 991 can also synergize with AMP to activate nonphosphorylated AMPK but only to about 10% of the activity of phosphorylated AMPK (26). In our hands, Escherichia coli–produced, nonphosphorylated AMPK/H9251/H9252/H9253 carrying a CBM S108D mutation, which partially mimics the effect of Ser-108 phosphorylation (25), was activated ~20-fold by A769662, ~3-fold by AMP, and ~33-fold by the combination of A769662 and AMP (Fig. 6A). R734 activated the same protein ~5.5-fold and
AMPK in complex with novel pharmacological activators

in combination with AMP −10-fold (Fig. 6A), suggesting a marginal synergism that is insufficient to fully compensate for activation loop phosphorylation.

Crystal structure determination of nonphosphorylated AMPK in complex with AMP and R734

Because the requirement of activation loop phosphorylation for full AMPK activity can be partially overcome by a combination of R734 and AMP (Fig. 6A), we wanted to explore the structure of nonphosphorylated AMPK $\alpha_\beta_\gamma$ in the presence of R734, staurosporine, and AMP. The phosphorylation-mimicking CBM S108D mutation allowed stabilization of the interaction with R734 in the absence of AMPK phosphorylation. The purified protein readily crystallized (Fig. S3) and allowed us to determine its structure at 2.65-Å resolution (Fig. 6B). Although the overall complex structure was well-defined by strong electron density, the regions of the AID and the following RIM domains, particularly the residues from Lys-296 through Glu-338, show relatively weak density and high B factors. To correctly build this region of the structure, we calculated a $2F_o-F_c$ composite omit map with 3000 K simulated annealing to reduce model bias (Fig. 7, A and B, and Fig. S5, A–C). We surprisingly found that the AID conformation is largely different from those resolved in previously published AMPK structures (22, 24, 28) (Fig. 7C and Fig. S5, D–H). Although the overall omit map density is relatively weak, we observed reliable main chain density of the AID $\alpha_1$ helix from Met-289 through Leu-295, of the $\alpha_2$ helix from Leu-313
through Asn-317, and of the α3 helix from Tyr-326 through Met-335. Many large side chains also showed confident density in the composite omit map contoured at 0.7 to 1. Examples are Met-289, Ile-290, and Glu-297 of α1; Asn-315 and Arg-316 of α2; and Tyr-326, His-327, Ile-329, Ile-330, and Arg-333 of α3. Further support for model building of the AID came from electrostatic interactions between AID residues and surrounding residues with clear density, such as Arg-316 of α2 that electrostatically interacts with Glu-281, Arg-333 of α3 that interacts with Glu-293, and Arg-334 of α3 that interacts with Asp-282.

In addition to the unique conformation of the AID, two other features stood out. First, despite the relatively low kinase activity, the catalytic center adopted a fully active conformation with a fully resolved activation loop (Fig. S4, B and C) and a CBM linker with resolved C-interacting helix (Fig. 6B). Second, the modified benzyl ring of R734, which is connected through a freely rotatable single bond, adopted a 180° rotated configuration in the ligand-binding pocket, pointing toward the CBM instead of the KD (Fig. S7). Although the remaining parts of R734 and R739 had strong electron density, the density of the

**Figure 5. Structures of phosphorylated AMPK α,β,γ in complex with R734/R739, AMP, and staurosporine.** A and B, structure overviews. C–E, Close-ups of R734 (C), R739 (D), and 991-occupied (E) ADaM sites. Dashed lines, polar interactions. The CBM is colored cyan, the KD is green, and the ATP-binding G-loop of the KD is dark green.
N-hydroxylamide group of the benzyl ring was relatively weak in all structures, indicating that the pocket can adopt the ring in different orientations (Fig. S7). The density indicates that in phosphorylated AMPK the orientation of the N-hydroxylamide group has a weak preference toward the KD, allowing the N-hydroxylamide to interact with the ATP-binding G-loop (Fig. 5, C and D), whereas in nonphosphorylated AMPK it preferentially pointed toward the CBM at the positively charged pocket entrance (Fig. S7).

The autoinhibitory domain of nonphosphorylated AMPK–R734–AMP adopts a unique γ-subunit–bound conformation

The AID of AMPK is in a dynamic equilibrium between a KD-bound (inactive, ATP-induced) state and a γ-subunit–
bound (active, AMP-induced) state (28–30). Due to its dynamic nature, the AID has relatively poor density in all structures of AMPK and could not be modeled in the majority of structures of fully active AMPK, including the structures of phosphorylated AMPK bound to R734–AMP and R739–AMP. In the structure of nonphosphorylated AMPK α1β1γ1–R734–AMP in the presence of R734, staurosporine, and AMP, the AID is bound to the γ-subunit in a conformation in which it is rotated relative to the structures of the more active phosphorylated AMPK complexes (pdb code 4CFE), pAMPK α1β1γ1–AMP (pdb code 4XER), and AMPK α1β1γ1–991–AMP (pdb code 5ISO) (Fig. 7C and Fig. S5, D–G). As a consequence, the AID helix α1 adopts a position that overlaps with the position of α3 in phosphorylated, AMP-bound AMPK (Fig. 7C and Fig. S5, D–G). In contrast to the different sets of AID residues involved in γ-subunit binding, the AID-binding residues within the well-resolved γ-subunit are largely identical for both conformations. Importantly, this conformation is not due to crystal packing as the AID is not involved in any substantial interactions with neighboring molecules in the crystals lattice (Fig. S6). To test whether this conformation affects the activity of AMPK, we introduced single and double mutations into Met-289 and Glu-293 of the short AID helix. These residues are modeled at the AID/γ-subunit interface in nonphosphorylated AMPK (Fig. 7A) but make no interactions in any structure of phosphorylated AMPK (n = 3; error bars, S.D.). ns, not significant (p > 0.05); **, p < 0.01; ***, p < 0.001; ****, p < 0.0001 (two-way analysis of variance with Bonferroni post-test).

Figure 7. The AID of nonphosphorylated AMPK α1β1γ1–R734–AMP adopts a unique γ-subunit-bound conformation. A, cartoon representation of the AID (brown), its immediate flanking residues (yellow), and adjacent β- (cyan) and γ-subunit (magenta) regions with AID side chains shown in thin stick presentation. Key interface residues are shown in the inset. B, 2Fobs – Fcalc composite omit map of the AID contoured at 1.0 σ in the same orientation as in A. A subset of AID residues with well-resolved densities is labeled. Residues 298–313 were not resolved. C, structural overlay of nonphosphorylated AMPK α1β1γ1 (AID, green; γ-subunit, magenta) and phosphorylated AMPK α1β1γ1 (pdb code 4RER; yellow) to highlight the extent of the AID rotation indicated by the arrow. D, AID mutational analysis. Met-289 and Glu-293 of the AID α1 helix interact with the γ-subunit in the structure of nonphosphorylated AMPK α1β1γ1–R734–AMP but make no interactions in any structure of phosphorylated AMPK (n = 3; error bars, S.D.). ns, not significant (p > 0.05); **, p < 0.01; ***, p < 0.001; ****, p < 0.0001 (two-way analysis of variance with Bonferroni post-test).
suggesting that the AID conformation seen in the structure of nonphosphorylated AMPK is less potent in shifting the equilibrium from the KD-bound state to the γ-subunit–bound state. In contrast to activity-compromising mutations, activity increases cannot be explained by protein destabilization and are therefore highly reliable indicators for the disruption of inhibitory interactions.

Recently, Willows et al. (26) determined the structure of nonphosphorylated AMPK α2β1γ1 in complex with 991, AMP, and staurosporine. 991 activates AMPK α2β1γ1 in conjunction with AMP much more potently than R734 and R739 (26-fold compared with 10-fold activation of AMPK α2β1γ1 by R734 and AMP), and the AID adopted a structure very similar to that seen in phosphorylated AMPK. Together, this suggests that the AID, besides the known active and inactive conformations, can also adopt a novel conformation that is associated with intermediate kinase activity.

**Nonphosphorylated AMPK–R734–AMP adopts a stable activation loop conformation in crystals but requires phosphorylation for stabilization in solution**

Although nonphosphorylated AMPK in the presence of R734 and AMP only moderately activates AMPK, the crystal structure revealed all hallmarks of an active kinase conformation, including a completely resolved activation loop (Fig. S4, B and C), similar to nonphosphorylated AMPK α2β1γ1−991 (26). AMPK belongs to the family of arginine-aspartate (RD) kinases, which are frequently regulated by activation loop phosphorylation. In RD kinases, the negatively charged phosphate rearranges the activation loop by binding to a positively charged pocket in the KD, which in turn arranges the arginine (R) and adjacent aspartate (D) of the catalytic loop to position the substrate phospho-acceptor site (Fig. S4A). AMPK is unusual in that the activation loop is directly stabilized by interaction with the stable core structure of AMPK, consisting of the β-subunit–interacting C terminus of the α-subunit (α-CTD) and the α/γ-subunit–interacting C terminus of the β-subunit (β-CTD; Fig. 8) (26, 31).

The fact that the nonphosphorylated activation loop has a stable, active conformation in the crystal structures of AMPK α2β1γ1−991 (26) and AMPK α2β1γ1−R734 (Fig. S4 and Fig. 8, B and C), even though these complexes are only partially active, suggests that they may represent a minor fraction of total conformations that is captured in the crystallization snapshot due to their reduced dynamics. Moreover, the conformation could further be influenced by binding of the ATP-competitive inhibitor staurosporine, which stabilizes AMPK in a largely closed (substrate-bound–mimicking) conformation. We therefore used hydrogen/deuterium exchange MS (HDX-MS) to analyze the conformational landscape of the activation loop in solution. As shown in Fig. 9 and Figs. S8–S10, in solution the activation loop, as well as the activation loop–interacting segment of the β-subunit (β-subunit shown in Figs. S8–S10), indeed became strongly stabilized by phosphorylation and moderately stabilized by staurosporine, clearly indicating that the crystal structure represents a minor conformation snapshot.

In addition, binding of staurosporine, which induces the closed (substrate-bound) conformation of the kinase domain, stabilizes the AID (Fig. 9D). This is consistent with the proposed inability of the AID to bind the closed kinase domain conformation (28, 30, 32), which further shifts the equilibrium to the more stable AMP-induced AID–γ-subunit interaction.

**Nucleotide occupancy in pAMPK–R734 in the absence of added nucleotides**

Several structures of holo-AMPK have been solved in the AMP-bound state, but none have been solved in the apo or
AMPK in complex with novel pharmacological activators

Figure 10. AMP is bound at CBS4 in the structure of phosphorylated AMPK \(\alpha_1\beta_1\gamma_1-R734\) in the absence of added nucleotides. A, structure overview. B, composite omit map contoured at 1 \(\sigma\) around the three AMP-binding sites. The omit map is overlaid with the transparent cartoon structure of the heteromeric complex and a stick model of AMP at CBS4. For orientation, it is also overlaid with stick models (white) of the three AMP molecules of pAMPK in the presence of added AMP (PDB code 6C9F; see Fig. S4).

ATP-bound state. AMP binding at CBS3 is thought to be critical for allosteric AMPK regulation because it structurally stabilizes the interaction between the AMP-sensing \(\alpha\)RIM2 and AMP-bound CBS3 (29, 31). This interaction in turn shifts the AID/\(\alpha\)RIM1 equilibrium from the inhibitory KD-bound conformation to the \(\gamma\)-subunit–bound conformation as a mechanism for direct allosteric kinase activation (28–31). To gain insight into nucleotide occupancy and the conformation of AMPK with unoccupied CBS3, we determined the crystal structure of phosphorylated AMPK \(\alpha_1\beta_1\gamma_1-R734\) in the absence of added AMP. As shown in Fig. 10A, the overall structure is very similar to that of pAMPK–R734–AMP. Although we see density at \(\alpha\)RIM2, the density was weak. The composite omit map in Fig. 10B and Fig. S11 revealed that AMP was stably bound at CBS4 as predicted by previous biochemical analysis (33, 34). We further detected very weak AMP density at CBS3 (too weak to build an AMP in the model), indicating very low occupancy, whereas no reliable density could be seen at CBS1 (Fig. 10B and Fig. S11). These results confirm the stable binding of AMP at CBS4 as well as the recent realignment of CBS3 and CBS1 as the exchangeable higher- and lower-affinity nucleotide binding-sites, respectively (35).

Discussion

An ADaM site ligand can isotype-selectively separate direct AMPK activation and protection against activation loop dephosphorylation

AMPK is a central signaling hub that phosphorylates and regulates numerous targets. Consequently, AMPK dysregulation is associated with a spectrum of metabolic diseases, spanning from diabetes and obesity to cancer, inflammation, and cardiometabolic and neurodegenerative diseases. The pleiotropic effects of AMPK’s known physiological activators, AMP and ADP, have made it challenging to clearly attribute complex effects to AMPK activity states and to therapeutically regulate AMPK activity in different tissues with high selectivity. In 2006, Abbot Laboratories developed compound A769662 (36), the first example of a group of small molecules that activate AMPK with high specificity by binding the ADaM site at the KD/CBM interface (22). A limitation of this class of compounds is their preference for AMPK complexes that contain the \(\beta_1\)-subunit and phosphorylated CBM Ser-108.

The ADaM sites formed by \(\beta_1\)- and \(\beta_2\)-subunits differ only by two CBM residues (defined as residues within 5 Å to the KD), Thr-106 and Asn-111 in \(\beta_1\) complexes, which correspond to Ile-106 and Asp-111 in \(\beta_2\) complexes, respectively (see Fig. S12). Consistently, the high-affinity \(\beta_2\) ADaM site compound SC4 forms a hydrogen bond with \(\beta_2\) Asp-111 but not with \(\beta_1\) Asn-111, providing a structural rationale for its elevated \(\beta_2\) selectivity (37). \(\beta_3\) Asp-111 and \(\beta_4\) Asn-111 form different polar interaction networks with phospho-Ser-108, which in turn forms strong ionic interactions with Lys-29 and Lys-31 of the KD to stabilize the ADaM site. The carboxyl group of 991 further increases stability of this network by direct interaction with KD Lys-29 (Fig. 5E), suggesting that it may contribute to isoform selectivity. In addition, removal of the carboxyl group reduces uptake by hepatocyte organic anion transport proteins and would therefore be expected to decrease the liver cell:muscle cell uptake ratio.

The 991 derivatives R734 and R739 lack the carboxyl group but still activate recombinant \(\beta_1\) complexes to a larger degree than \(\beta_2\) complexes. Strikingly, R739 failed to allosterically activate E. coli–purified AMPK \(\alpha_1\beta_2\gamma_1\) but robustly decreased activation loop dephosphorylation, similar to ADP, which binds the nucleotide-binding sites in the \(\gamma\)-subunit more than 60 Å away from the ADaM site. Therefore, these two activities can be pharmacologically separated by both ADaM site–binding and by \(\gamma\)-subunit–binding compounds. Direct allosteric activation stabilizes the active kinase conformation (in the case of ADaM site agonists by stabilizing the regulatory \(\alpha C\) helix of the KD
(22)). In contrast, protection against activation loop dephosphorylation is likely determined by the conformation of the linker between the CBM of the β-subunit at the ADAM site and the C-terminal scaffolding domain of the β-subunit at the γ-subunit (22, 31). This suggests that R739 may be unable to induce formation of a properly positioned αC-stabilizing helix in the CBM linker of the α1β1γ1 complex but may sufficiently reposition the linker to shield the activation loop against phosphatases. Details of how ligands change the conformation of the linker remain unknown in the absence of a structure of holo-AMPK in the inactive, ATP-bound state.

The AID adopts a conformation in partially active AMPK that is associated with reduced activity

Activation loop phosphorylation catalytically activates E. coli–produced AMPK ~100-fold (38, 39), and AMP activates it an additional 2-fold (in mammalian cells up to 10-fold (7)). The combination of R734 and AMP only partially activated nonphosphorylated AMPK (~10-fold), but the crystal structure of AMPK α1β1γ1 showed the hallmarks of an active kinase, including a fully resolved activation loop. This is reminiscent of the recently reported structure of nonphosphorylated AMPK α1β1γ1 bound to 991 and AMP (26). Although 991 activates nonphosphorylated AMPK more potently than R734, it fails to fully activate the kinase; however, the kinase domain also adopted an active conformation with fully resolved activation loop. Our HDX-MS analysis demonstrated that this conformation in AMPK α1β1γ1 represents a minor fraction that likely crystallizes more easily due to its higher order and that, for the majority of AMPK, phosphorylation is required for full activation loop stabilization. Consistently, phosphorylation is also required for full activity of AMPK bound by 991 and AMP in mammalian cells (26).

A surprising finding was that the AID adopted a novel conformation in which its α1 helix, rather than its α3 helix, interacted with the γ-subunit. The AID can therefore interact with at least three different surfaces within AMPK: (i) the KD as seen in the inactive, ATP-bound and nonphosphorylated conformation; (ii) the γ-subunit via AID α3 in the fully active phosphorylated and AMP-bound conformation; and (iii) the γ-subunit via AID α1 in this structure of partially active, nonphosphorylated and AMP-bound AMPK. To probe for the biological relevance of this conformation, we tested the effect of mutations in AID α3 at the AID/γ interface on the kinase activity of largely or fully active, phosphorylated AMPK. The fact that these mutations increased the kinase activity suggests that the partially active conformation competes predominantly with the fully active conformation, not the inactive conformation.

The AID in this structure was better resolved than in the corresponding structure of fully active phosphorylated AMPK. However, the mutations designed to disrupt the AID α1/γ-subunit interface increased AMPK activity, implying that this conformation is more stable but less active than the competing AID conformation seen in all active AMPK structures with resolved AID. The presence of the AID either in an unresolved state or in at least three different, distinct conformations as well as the high B factor of the AID in all resolved structures illustrates the highly dynamic nature of the AID in the context of holo-AMPK, consistent with its key regulatory switch role in allosteric activation by adenine nucleotides.

Experimental procedures

Chemical synthesis

Synthesis schemes for compounds 991, R734, and R739 are provided in the supporting information.

DNA construct for crystallization

Human AMPK Hisα1(13–550)-β1(76–272)-γ1(24–327) with deletion of the serine- and threonine-rich (ST) loop in the α-subunit (amino acids 476–529) and S108D mutation in the β-subunit was cloned into a tricistronic vector. Mutations were introduced by QuickChange site directed mutagenesis (Stratagene) or standard PCR-based methods. All constructs and mutations were sequence-verified.

AMPK expression and purification

AMPK expression plasmids were transformed into E. coli BL21 (DE3). Cells were grown in LB medium to an A₆₀₀ of ~1 at 28 °C and induced with 100 μM isopropyl β-D-thiogalactopyranoside at 16 °C overnight. Cell pellets were resuspended in 25 mM Tris, pH 8.0, 300 mM NaCl, 25 mM imidazole, 10% glycerol, 5 mM β-mercaptoethanol and lysed using a French press with pressure set to 900 pascals. Lysates were cleared by centrifugation for 30 min at 20,000 × g, passed over a 10-ml HisTrap HP column (GE Healthcare), and eluted with 25 mM Tris, pH 8.0, 300 mM NaCl, 500 mM imidazole, 10% glycerol, 5 mM β-mercaptoethanol. The eluted AMPK was further purified by size-exclusion chromatography through a HiLoad 26/60 Superdex 200 column (GE Healthcare) in 25 mM Tris, pH 8.0, 300 mM NaCl, 5 mM MgCl₂, 1 mM EDTA, 10% glycerol, 2 mM DTT. AMPK was phosphorylated by incubation with Ca²⁺/calmodulin-dependent protein kinase kinase β at a 1:0.02 molar ratio in 0.2 mM AMP, 0.2 mM ATP, 2 mM CaCl₂, 10 mM DTT, 1 μM calmodulin at room temperature overnight (16 h). The phosphorylated AMPK was repurified by size-exclusion chromatography through a HiLoad 26/60 Superdex 200 column in 25 mM Tris, pH 8.0, 300 mM NaCl, 5 mM MgCl₂, 1 mM EDTA, 10% glycerol, 2 mM DTT. The protein eluted from the gel filtration column at a volume corresponding to the size of a monomeric complex at a purity ≥95% as judged by SDS-PAGE (Fig. S3).

Crystallization

Purified nonphosphorylated and phosphorylated AMPK fractions were incubated with a 3-fold molar excess of staurosporine for 3 h at 4 °C, spin-concentrated to ~7 mg/ml, and incubated with R734 or R739 at molar ratios of protein:compound of 1:5 in the presence and absence of 5 mM AMP. Crystals of both R734-bound and R739-bound phosphorylated AMPK in complex with AMP and staurosporine were grown at ~20 °C in sitting drops containing 0.3 μl of purified protein at ~7 mg/ml and 0.3 μl of reservoir solution consisting of 0.1 M trisodium acetate, pH 5.6, 0.2 M ammonium acetate, 15% (w/v) PEG 4000. Crystals of phosphorylated R734-bound AMPK in the absence of added nucleotides were grown at ~20 °C in sitting drops containing 0.3 μl of purified protein at ~7 mg/ml
AMPK in complex with novel pharmacological activators

and 0.3 μl of reservoir solution consisting of 0.1 M triammonium citrate, pH 7.0, 12% (w/v) PEG 3350. Crystals of nonphosphorylated R734-bound AMPK in complex with AMP were grown at ~20 °C in sitting drops containing 0.3 μl of purified protein at ~7 mg/ml and 0.3 μl of reservoir solution consisting of 0.2 M magnesium formate.

Structure determination and refinement

X-ray diffraction data were collected at 21-ID of the Life Science Collaborative Access Team at the Advanced Photon Source. The observed reflections were reduced, merged using MOSFLM, and scaled with SCALA of the CCP4 package (40). The structures were solved by molecular replacement performed using the CCP4 program Phaser (41) with the structure of AMPK in complex with 991 (22) (PDB code 4CFE) as an initial search model. The program Coot (42) was used to manually fit the protein model. Model refinement was performed with the PHENIX program package (43). The statistics of data collection and the model refinement are summarized in Table S1.

Cell-based phospho-acetyl-CoA carboxylase (pACC) ELISAs

HepG2 cells (200,000 cells/well seeded a day before and starved for 2 h in low-glucose medium), LHCN-M2 cells (20,000 cells/well differentiated for 5 days), and A549 cells (20,000 cells/well seeded a day before) in 96-well flat-bottom tissue culture plates were incubated for 2 h at 37 °C in the medium containing serial dilutions of the test samples. The reaction was stopped using 100 μl of 4% formaldehyde in phosphate-buffered saline (PBS). Cells were washed and incubated for 1 h at room temperature on a shaker in blocking buffer (1:1000) for 1 h that room temperature. The stained cells were washed and incubated with 200 μl of horseradish peroxidase–conjugated secondary antibody diluted in blocking buffer (1:1000) for 1 h at room temperature. The stained cells were washed, and chemiluminescent reagent was added. Chemiluminescence was read using a Victor plate reader (PerkinElmer Life Sciences) within 5 min. The EC_{50} determination was executed using Prism software version 7 (GraphPad, Inc.). Means of EC_{50} values acquired from multiple experiments (n = 2) are shown in Fig. 2A.

Proliferation assay

Cells were plated in ViewPlate96 96-well plates (PerkinElmer Life Sciences) in duplicate replicates. The compound dilutions for the six-point screens (from 10 to 0.41 M, 3-fold dilution) were performed manually. Following incubation with the compound for 48 h, cells were fixed with 2.0% paraformaldehyde (Sigma-Aldrich) in PBS (Ca^{2+}/Mg^{2+}-free) for 30 min, washed with PBS, stained for 60 min with a 6 ng/ml solution of 4,6-diamidino-2-phenylindole dihydrochloride (Molecular Probes Inc., Eugene, OR) in PBS. Cells were stored at 4 °C in PBS for at least 16 h before imaging to allow stain to equilibrate. A Zeiss Axiovert S100 inverted fluorescence microscope, equipped with a Plan-NEOFLUAR 10× objective (Carl Zeiss Inc., Thornwood, NY) and a Hamamatsu Lightningcure 200 mercury-xenon light source with an Omega Optical XF57 quad filter (Hamamatsu Photonics, Japan), was used for capturing images. Nine images per well were taken, in an adjacent grid pattern, in each well of the 96-well plates of treated tumor cells. Images were analyzed in a 12-bit format using segmentation and morphological routines contained in the Image Pro software package (Media Cybernetics Inc., Bethesda, MD). The EC_{50} curve fitting was executed with MathLab software version 6.5 (Math-Works Inc., Natick, MA). For cell cycle analysis, DNA content of each nucleus in the sample images was plotted and smoothed using the Lowess method (48). Apoptosis was assessed by manual inspection of the cell cycle profile and fragmented nuclei as assessed by the imaging analysis. Endoreduplication was assessed by manual inspection based upon enlarged nuclei and the presence of the 8N population.

AMPK [γ-32P]ATP kinase assay

10 nM AMPK was incubated with 50 μM biotin-SAMS peptide (biotin-HMRSAMSGLHLVKRR; synthesized by Peptide 2.0 Inc., Chantilly, VA), 2 mM DTT, and 0.25 μM of [γ-32P]ATP/15-μl reaction in kinase buffer (25 mM Tris, pH 7.4, 12 mM MgCl_2, 1 mM Na_2VO_4, 5 mM NaF) for 20 min at room temperature in the presence or absence of various concentrations of R734, R739, or 991 for activity dose-response curve determination (Fig. 3A and Fig. S1) or in the presence or absence of 20 μM R734, 20 μM A769662, or 200 μM AMP for single-point kinase activity determination (Figs. 6A and 7D). Reactions were terminated by addition of 0.5 volume of 7.5 M guanidine hydrochloride solution in water, and reactions were spotted on a SAM 2° Biotin Capture Membrane (Promega). The membrane was washed once for 30 s with 2 M NaCl, three times for 2 min with 2 M NaCl, four times for 2 min with 2 M NaCl in 1% H_3PO_4, and two times for 30 s with deionized water to remove unbound reaction components. After drying the membrane at room temperature for 30 – 60 min, signals were quantitated by phosphorimaging analysis.

AMPK α-pThr-172 phosphatase protection assay

5 μM phosphorylated AMPK protein was incubated with either 4 μM H6GST-PP2Cα in 25 mM Tris, pH 8.0, 300 mM NaCl, 5 mM MgCl_2, 1 mM EDTA, 10% glycerol, 2 mM DTT or 100 units of λ-phosphatase (New England Biolabs) in 50 mM HEPES, pH 7.5, 100 mM NaCl, 2 mM DTT, 0.01% (v/v) Brij 35, 1 mM MnCl_2 in the presence or absence of 20 μM R734, 20 μM R739, 20 μM A769662, or 200 μM AMP in a total volume of 20 μl for 30 or 60 min at room temperature. Reactions were stopped by addition of SDS sample buffer, separated by SDS-PAGE, and immunoblotted with an AMPKα, pThr-174–specific antibody (Abcam) following standard protocols.

HDX detected by mass spectrometry

Differential HDX-MS experiments were conducted as described previously with a few modifications (44). Peptide identification—Peptides were identified using tandem MS (MS/MS) with an Orbitrap mass spectrometer (Q Exactive, Thermo Fisher). Product ion spectra were acquired in data-dependent mode with the top five most abundant ions.
selected for the product ion analysis per scan event. The MS/MS data files were submitted to Mascot (Matrix Science) for peptide identification. Peptides included in the HDX analysis peptide set had a Mascot score greater than 20, and the MS/MS spectra were verified by manual inspection. The Mascot search was repeated against a decoy (reverse) sequence, and ambiguous identifications were ruled out and not included in the HDX peptide set.

**HDX-MS analysis**—Protein (10 μM) was incubated with the respective ligands at a 1:10 protein:ligand molar ratio for 1 h at room temperature. Next, 5 μl of sample was diluted into 20 μl of D_2O buffer (20 mM Tris–HCl, pH 7.4, 150 mM NaCl, 2 mM DTT) and incubated for various time points (0, 10, 60, 300, 900, and 3,600 s) at 4 °C. The deuterium exchange was then slowed by mixing with 25 μl of cold (4 °C) 3 M urea, 1% TFA. Quenched samples were immediately injected into the HDX platform. Upon injection, samples were passed through an immobilized pepsin column (2 mm × 2 cm) at 200 μl min⁻¹, and the digested peptides were captured on a 2-mm × 1-cm C_6 trap column (Agilent) and desalted. Peptides were separated across a 2.1-mm × 5-cm C_18 column (1.9 μl of Hypersil Gold, Thermo Fisher) with a gradient of 4–40% CH_3CN, 0.3% formic acid over 5 min. Sample handling, protein digestion, and peptide separation were conducted at 4 °C. Mass spectrometric data were acquired using an Orbitrap mass spectrometer (Exacto, Thermo Fisher). HDX analyses were performed in triplicate with single preparations of each protein–ligand complex. The intensity weighted mean m/z centroid value of each peptide envelope was calculated and subsequently converted into a percentage of deuterium incorporation. This was accomplished by determining the observed averages of the undeuterated and fully deuterated spectra using the conventional formula described elsewhere (45). Statistical significance for the differential HDX data was determined by an unpaired t test for each time point, a procedure that is integrated into the HDX Workbench software (44). Corrections for back-exchange were made on the basis of an estimated 70% deuterium recovery and accounting for the known 80% deuterium content of the deuterium exchange buffer.

**Data rendering**—Deuterium uptake for each peptide was calculated as the average of %D for all on-exchange time points, and the difference in average %D values between the apo and ligand-bound samples is presented as a heat map with a color code given at the bottom of each figure (warm colors for deprotection and cool colors for protection). Peptides are colored by the software automatically to display significant differences, determined either by a >5% difference (less or more protection) in average deuterium uptake between the two states or by using the results of unpaired t tests at each time point (p value <0.05 for any two time points or a p value <0.01 for any single time point). Peptides with nonsignificant changes between the two states are colored gray. The exchange at the first two residues for any given peptide is not colored. Each peptide bar in the heat map view displays the average Δ%D values, associated standard deviation, and the charge state.

**Immunoblot analysis of compound-stimulated Raptor, ACC, and AMPK phosphorylation in cells**

HepG2 cells were cultured, treated, and lysed following a published protocol (46). Specifically, cells were incubated for 1 h with increasing doses of R734, R739, 991, and A769662 in Krebs–HEPES buffer (20 mM Na-HEPES, pH 7.4, 118 mM NaCl, 3.5 mM KCl, 1.3 mM CaCl_2, 1.2 mM MgSO_4, 1.2 mM KH_2PO_4, 10 mM glucose, 0.1% (w/v) BSA) and lysed in 50 mM Tris–HCl, pH 7.4, 50 mM NaF, 5 mM sodium pyrophosphate, 1 mM EDTA, 1 mM EGTA, 250 mM mannitol, 1% (v/v) Triton X-100, 1 mM DTT, 1 mM benzamidine, 0.1 mM phenylmethylsulfonyl fluoride, 5 μg/ml soybean trypsin inhibitor. Cell lysates were separated by SDS-PAGE and immunoblotted with an AMPK α_1/α_2 pThr-174/Thr-172–specific antibody (Abcam), an AMPK α_1 antibody (Y365) (Abcam), a Raptor pSer-792–specific antibody (Cell Signaling Technology), a Raptor antibody (24C12) (Cell Signaling Technology), an ACC pSer-79–specific antibody (Cell Signaling Technology), an ACC antibody (Cell Signaling Technology), and a β-actin antibody (Abcam), respectively, following standard protocols.

**Colorimetric phosphatase assay**

20 μM phosphorylated AMPK substrate peptide (GSTKM-APTLVDLGYKK where pT is phosphothreonine) (47) was incubated with H_2O or 20 μM R734, 20 μM R739, 4 μM 991, or 20 μM A769662 in the presence and absence of 4 μM H6GST-PP2Cα in a total volume of 200 μl of 10 mM Tris, pH 7.4, 150 mM NaCl for 30 min at room temperature. Phosphate release from pSer-175 from the phosphopeptide was determined by colorimetric assay (BioVision) and quantitated by the phosphate standard curve shown in Fig. S2A.

**Author contributions**—Y. Y., X. E. Z., S. J. S., J. S. B., Y. H., P. R. G., H. E. X., and K. M. formal analysis; Y. Y., X. E. Z., S. J. N., S. J. S., J. S. B., Y. H., and K. M. validation; Y. Y., X. E. Z., S. J. N., S. J. S., Y. L., J. S. B., Y. H., and P. R. G. investigation; Y. Y., S. J. N., S. J. S., Y. L., J. S. B., Y. H., and P. R. G. methodology; Y. Y., X. E. Z., S. J. N., S. J. S., Y. L., J. S. B., Y. H., P. R. G., and H. E. X. writing-review and editing; X. E. Z., S. J. S., and K. M. data curation; S. J. S., J. S. B., Y. H., and K. M. conceptualization; S. J. S., P. R. G., and K. M. resources; S. J. S., J. S. B., Y. H., P. R. G., H. E. X., and K. M. supervision; S. J. S., J. S. B., Y. H., P. R. G., H. E. X., and K. M. funding acquisition; S. J. S. and P. R. G. project administration; K. M. visualization; K. M. writing-original draft.

**Acknowledgments**—We thank Michelle Martin for administrative support and staff members of the Life Science Collaborative Access Team of the Advanced Photon Source (APS) for assistance in data collection at the beam lines of sector 21, which is in part funded by the Michigan Economic Development Corporation and the Michigan Technology Tri-Corridor under Grant 08SP1000817. Use of APS was supported by the Office of Science of the United States Department of Energy under Contract DE-AC02-06CH11357.

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