Published in final edited form as:

Science. 2021 July 23; 373(6553): 413–419. doi:10.1126/science.abe7565.

Structure of an AMPK complex in an inactive, ATP-bound state

Yan Yan¹, Somnath Mukherjee^{2,†}, Kaleeckal G. Harikumar^{3,†}, Timothy S. Strutzenberg^{4,†}, X. Edward Zhou^{1,†}, Kelly Suino-Powell¹, Ting-Hai Xu^{1,5}, Ryan D. Sheldon⁶, Jared Lamp⁷, Joseph S. Brunzelle⁸, Katarzyna Radziwon², Abigail Ellis⁶, Scott J. Novick⁴, Irving E. Vega⁷, Russell G. Jones⁶, Laurence J. Miller³, H. Eric Xu⁹, Patrick R. Griffin⁴, Anthony A. Kossiakoff^{2,10}, Karsten Melcher^{1,*}

¹Department of Structural Biology, Van Andel Institute, Grand Rapids, MI 49503, USA.

²Department of Biochemistry and Molecular Biology, University of Chicago, Chicago, IL 60637, USA.

³Department of Molecular Pharmacology and Experimental Therapeutics, Mayo Clinic, Scottsdale, AZ 85259, USA.

⁴Department of Molecular Medicine, The Scripps Research Institute, Scripps Florida, Jupiter, FL 33458, USA.

⁵Center for Epigenetics, Van Andel Institute, Grand Rapids, MI 49503, USA.

⁶Metabolic and Nutritional Programming, Center for Cancer and Cell Biology, Van Andel Institute, Grand Rapids, MI 49503, USA.

⁷Integrated Mass Spectrometry Unit, Department of Translational Neuroscience, Michigan State University College of Human Medicine, Grand Rapids Research Center, Grand Rapids, MI 49503, USA.

⁸Life Sciences Collaborative Access Team, Northwestern University Synchrotron Research Center, Northwestern University, Argonne, IL 60439, USA.

⁹Center for Structure and Function of Drug Targets, The CAS Key Laboratory of Receptor Research, Shanghai Institute of Materia Medica, Chinese Academy of Sciences (CAS), Shanghai 201203, China.

¹⁰Institute of Biophysical Dynamics, University of Chicago, Chicago, IL 60637, USA.

*Corresponding author. karsten.melcher@vai.org.

†These authors contributed equally to this work.

Author contributions: K.M. conceived of the project. I.E.V., R.G.J., L.J.M., H.E.X., P.R.G., A.A.K., and K.M. designed the experiments. Y.Y., S.M., K.G.H., T.S.S., X.E.Z., K.S.-P., T.-H.X., R.D.S., J.L., J.S.B., K.R., A.E., and S.J.N. performed and/or interpreted the experiments. Y.Y. and K.M. wrote the paper, with support from all authors.

Competing interests: R.G.J. holds an adjunct professorship at McGill University, serves on the scientific advisory board of Immunomet Therapeutics, and is a scientific consultant for Agios Pharmaceuticals.

Data and materials availability: Crystal structure data were deposited in the Protein Data Bank (PDB) under ID 7JII. Cryo-EM data were deposited in the Electron Microscopy Data Bank (EMD) under accession number EMD-22336 and in PDB under ID 7JHG (AMPK-CpdC-ATPγS-Fab-nanobody); EMD-22337 and PDB ID 7JHH (AMPK-ATPγS-Fab-nanobody); and EMD-23708 and PDB ID 7M74 (AMPK-CpdC-ATPγS-Fab-nanobody, nonphosphorylated sample). The XL-MS raw data and search results have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD019017. All other data needed to evaluate the conclusions in the paper are presented in the main text or supplementary materials.

Abstract

Adenosine monophosphate (AMP)-activated protein kinase (AMPK) regulates metabolism in response to the cellular energy states. Under energy stress, AMP stabilizes the active AMPK conformation, in which the kinase activation loop (AL) is protected from protein phosphatases, thus keeping the AL in its active, phosphorylated state. At low AMP:ATP (adenosine triphosphate) ratios, ATP inhibits AMPK by increasing AL dynamics and accessibility. We developed conformation-specific antibodies to trap ATP-bound AMPK in a fully inactive, dynamic state and determined its structure at 3.5-angstrom resolution using cryo-electron microscopy. A 180° rotation and 100-angstrom displacement of the kinase domain fully exposes the AL. On the basis of the structure and supporting biophysical data, we propose a multistep mechanism explaining how adenine nucleotides and pharmacological agonists modulate AMPK activity by altering AL phosphorylation and accessibility.

Adenosine monophosphate-activated protein kinase (AMPK) is the primary energy sensor and regulator of energy homeostasis in eukaryotes (1-5). Upon activation by energy stress, AMPK reprograms metabolism by phosphorylating and modulating the activities of metabolic enzymes and key regulators of metabolism, growth, and proliferation (6-9). Deregulation of AMPK is associated with metabolic diseases, and AMPK is a pharmacological target for the treatment of diabetes, obesity, cancer, and cardiometabolic disease (10-12).

AMPK is a heterotrimeric protein kinase that is assembled from an α subunit ($\alpha 1$ or $\alpha 2$) containing the kinase domain (KD) and regulatory β subunits ($\beta 1$ or $\beta 2$) and γ subunits ($\gamma 1$, $\gamma 2$, or $\gamma 3$) (13) (Fig. 1A). AMPK senses the cellular energy state by competitive binding of adenosine monophosphate (AMP), adenosine diphosphate (ADP), and adenosine triphosphate (ATP) to three sites in its γ subunit, of which site 3 (CBS3) is the primary sensor (14, 15) (Fig. 1A). In addition, pharmacological compounds including Abbot A769662 and Merck 991 bind to a distant site called the allosteric drug and metabolite (ADaM) site, which is at the interface between the KD and the carbohydrate-binding module (CBM) of the β subunit (16, 17) (Fig. 1A). AMPK is activated about 100-fold by phosphorylation of a residue (Thr¹⁷⁴ in human $\alpha 1$ and Thr¹⁷² in human $\alpha 2$) in its kinase activation loop (AL) and up to 10-fold by direct allosteric kinase activation (18-22). In reconstituted systems, AMP, ADP, and ADaM agonists all increase net AL phosphorylation by stabilizing a conformation that limits phosphatase access to the AL (14, 19, 20, 23, 24), although AL protection by ADaM agonists has been less clear in a cellular context (23, 25). In addition, binding of AMP, but not ADP or ADaM ligands, further increases AL phosphorylation by stabilizing an interaction with AMPK's main upstream kinase, the tumor suppressor LKB1 (22, 26-28), and possibly with other upstream kinases (21). Under energy excess, ATP competes with AMP and ADP at one or more of the allosteric sites and inhibits AMPK.

Modulation of AL accessibility by adenine nucleotides was identified as a major mechanism of AMPK regulation more than 20 years ago (19), but how adenine nucleotides and ADaM agonists induce conformational changes that modulate AL accessibility is unknown because the structure of the AMPK complex in its highly dynamic, ATP-bound state has yet to

be resolved. Equally important, how agonists can restrict access to protein phosphatases without impeding access to AL kinases has remained enigmatic.

Conformational trapping of inactive-state AMPK by protein engineering and conformation-selective Fabs

AMP binding stabilizes, and ATP binding destabilizes, an interaction between the γ subunit and the α linker that is required for AMPK activation (29). To determine the structure of ATP-bound AMPK in the phosphorylated, more-AL-accessible state, we stabilized the phosphorylated AMPK (pAMPK) construct shown in Fig. 1A through (i) adenosine-5'-(γ -thio)-triphosphate (ATP γ S), which we found to more potently inhibit the interaction between the γ subunit and the α linker than ATP (fig. S1A); (ii) a nonflexibly linked maltose-binding protein (MBP) tag; (iii) the AMPK inhibitor compound C (CpdC) (30); and (iv) an E199A/E200A surface entropy reduction mutation in the β subunit (see materials and methods in the supplementary materials). These modifications did not change AMPK modulation by AMP and ATP (fig. S1, B to D) and allowed us to solve its crystal structure at a modest resolution of 5.4 Å (fig. S1E and table S1). Although the resolution was insufficient to gain detailed mechanistic insight, it showed that the KD was associated with the β subunit as in active AMPK and that all three binding-competent CBS sites were bound by adenine nucleotides. A difference density map with the phosphate group omitted from the model suggested that AL residue T174 was phosphorylated (fig. S1E).

The resolution could not be improved through further optimization, indicating the need for more potent trapping of the dynamic complex in a distinct, inhibitory conformation. To do so, we generated Fabs specific for the inactive conformation of the AMPK crystallization construct (Fig. 1B and fig. S2). We also included a Fab-binding nanobody (31) to eliminate flexibility between the Fab's constant and variable domains. Together, these measures allowed us to determine a cryo-electron microscopy (cryo-EM) structure at 3.48-Å nominal resolution from 286,895 particles used in the three-dimensional reconstruction (Fig. 1, C to E; fig. S3; and table S2). Because CpdC is a nonphysiological AMPK inhibitor, we also determined the structure in its absence at a nominal resolution of 3.92 Å (fig. S4 and table S2). The two cryo-EM structures were very similar [root mean square deviation (RMSD) of 1.48 Å; fig. S6], indicating that CpdC did not change the overall domain organization or complex conformation of the inhibited AMPK.

Structures of Fab-stabilized, ATP-bound AMPK

The cryo-EM structures allowed an unambiguous assignment of the KD, core domain, and amino acid residues 180 through 203 of the β linker, as well as of the MBP tag, Fab, and nanobody in the EM density map (Fig. 1, D and E). We observed a conformation that is very different from both active-state pAMPK and the crystal structure of inactive ATP γ S/CpdC-bound pAMPK (Fig. 1F). Although the core complex remained largely superimposable with that of active-state AMPK (RMSD = 2.1 Å), the KD was completely dissociated from the β subunit by a rotation of $\sim 180^\circ$ and a translation of ~ 100 Å (Fig. 1, D to F, and movie S1). In this conformation, the KD associated weakly with the γ subunit (see fig. S7 for

details and mutational analysis) in an orientation in which the AL became fully accessible to phosphatases and upstream kinases.

AL residues 169 through 177 showed relatively weak density compared with the core domains in both structures, but the lack of the characteristic strong phosphate density [as seen for AMP in the same structure (compare fig. S6 with Fig. 1G)] suggested that residue T174 was not phosphorylated. This conclusion was supported by the lack of countercharges around the buried T174 to balance a heavily negatively charged phosphate group. To confirm this interpretation, we also determined the structure of AMPK in complex with Fab and CpdC from a protein preparation that was not phosphorylated (figs. S5 and S8). As seen in fig. S8, the structure from the nonphosphorylated sample was superimposable with the other two cryo-EM structures, with the same interfaces between subunits, including an identical interface between KD and the γ subunit. Using mass spectrometry, we confirmed that T174 in AMPK used for Fab selection and structure determination was incompletely phosphorylated (5.3% nonphosphorylated; table S3). We expect that preclearing with phosphorylated active-state AMPK during Fab selection enriched for Fabs binding to the nonphosphorylated target.

The KD in the cryo-EM structures adopted an inhibitory conformation showing the hallmarks of inactive kinase domains (fig. S9). Moreover, ATP destabilizes the interaction between the α linker and CBS3 (29). Consistently, the α linker was disordered in the structures, and the position of the resolved flanking sequences of the α linker and preceding autoinhibitory domain (AID) suggests that the α linker was indeed displaced (fig. S10). Further, we saw no density for the CBM, consistent with double electron-electron resonance spectra indicating that the CBM can dissociate from the KD in the absence of ADaM ligands and adopt multiple alternative positions, especially in nonphosphorylated AMPK (32).

In this conformation, the CBM, AID, and α linker are unresolved and completely or partially dissociated, and the KD makes only limited interactions with the γ subunit [515-Å² buried interface compared with buried interfaces of 1677 Å² with β subunit and α -CTD in the KD-associated conformation (4RER)]. This state is in equilibrium with the more stable active AMPK conformation. The Fab shifts this equilibrium by binding a surface in nonphosphorylated AMPK that largely overlaps the AL-containing surface in pAMPK that is protected by β linker and core AMPK (fig. S11). This mutually exclusive binding is the basis for the Fab's conformational selectivity and stabilization of the inactive conformation.

An important question in the AMPK field is which adenine nucleotides bind to which individual CBS sites. Unexpectedly, although AMP and ADP were not added for the ATP-complex formation, we observed AMP-occupied CBS4 and ADP-occupied CBS1 (Fig. 1G; see detailed discussion in the supplementary materials and fig. S12). Only CBS3, the nucleotide sensor site, was occupied by ATP γ S.

In contrast to the cryo-EM structures, in the crystal structure in the absence of Fab, the AL was phosphorylated, as expected, and the KD remained associated with the β subunit (fig. S1E). On the basis of these results, we hypothesized that allosteric ATP binding of phosphorylated AMPK may partially destabilize AL protection in a KD-associated

conformation, whereas AL dephosphorylation may lead to KD displacement and full inhibition.

Dephosphorylation is a switch for KD displacement

We tested whether AL dephosphorylation indeed leads to KD displacement in the context of full-length AMPK in solution and in the absence of Fab and nanobody. First, we scrutinized our previously published hydrogen-deuterium exchange mass spectrometry (HDX-MS) data (33). We overlaid all resolved peptides (Fig. 2A, colored gray) having phosphorylation-induced reduction in deuterium exchange (i.e., that have increased interaction and/or stability in pAMPK) onto the structures of KD-associated and KD-displaced AMPK. These peptides were found in three regions: (i) the AL-containing surface of the KD that is protected in KD-associated conformation but is solvent-accessible in the KD-displaced conformation; (ii) the β -CTD surface that interacts with the AL in the KD-associated conformation; and (iii) the γ subunit surface that interacts with the α linker, which is dissociated from the γ subunit in KD-displaced AMPK. In contrast, no such changes occurred in purified pAMPK upon the shift from energy stress to energy excess nucleotide levels (fig. S13) (32). These data therefore support that the KD-displaced conformation is primarily adopted by fully inactive, dephosphorylated AMPK.

Next we probed the AMPK conformation using AlphaScreen luminescence proximity sensors (29). This assay measures the proximity between a hexahistidine (His6) tag introduced at the N terminus of the KD and a biotin tag introduced at the N terminus of the γ subunit (Fig. 2B). AMP, ATP, and ATP γ S had only small effects on tag distances in purified phosphorylated AMPK. In contrast, the luminescence signal was more than threefold stronger (i.e., the distance between the tags shorter) in non-phosphorylated than in phosphorylated AMPK (Fig. 2B). This is in agreement with the difference in distances seen in the structures of KD-associated versus KD-displaced AMPK and further supports dephosphorylation-induced KD displacement.

The assay also provided insight into AL rephosphorylation. AMP stimulates AL rephosphorylation by inducing an interaction of AMPK with LKB1-bound axin (22). How AMP can stimulate a conformation in which the AL is protected against dephosphorylation and yet is accessible to upstream kinases had been puzzling. Our data indicate that the AL is protected only in phosphorylated AMPK, the substrate for dephosphorylation; it is accessible in nonphosphorylated AMPK. As in our previous study (29), incubation of nonphosphorylated AMPK with AMP caused a strong and highly significant increase in the AlphaScreen luminescence proximity signal indicative of an AMP-induced movement of the N termini of the KD and the γ subunit toward each other (Fig. 2B). While the details of this induced conformational change are unclear, we speculate that they mediate the effects of AMP-induced binding of AMPK to LKB1-bound axin.

We further probed the protein structures of phosphorylated, AMP-bound AMPK (fully active) and ATP γ S-bound, nonphosphorylated AMPK (fully inactive) by cross-linking coupled with mass spectrometry (XL-MS), using the MS-cleavable cross-linker disuccinimidyl sulfoxide (DSSO) (34). The maximum Ca–Ca distance between any

two stably positioned lysine residues cross-linked by DSSO is ~ 30 Å (35). Because of the distance constraints, cross-linked residues are often located in flexible loops, whose movements allow extended cross-linking distances.

We identified seven cross-links that (i) could be reliably identified (cross-linking scores >50); (ii) were statistically significant ($P < 0.05$); (iii) could be mapped onto the structures of both pAMPK/AMP and non-pAMPK/ATP γ S (i.e., the lysine residues of both peptides were resolved or bordered resolved regions); and (iv) had C α –C α distances that differed in both structures (Fig. 2, C and D; see data S1 for full data). The two cross-links enriched in pAMPK/AMP were between residues that are ~ 19 Å ($\alpha 1$ -K71– $\alpha 1$ -K476) and 30.1 Å ($\alpha 1$ -K53– $\beta 2$ -K203) apart in the structure of pAMPK/(AMP+staurosporine) but that would be ~ 65 and 87.4 Å apart, respectively, in the structure of AMPK/(ATP γ S+CpdC) (Fig. 2C). Similarly, the cross-links enriched in AMPK/ATP γ S ($\alpha 1$ -K71– $\gamma 1$ -K234, $\alpha 1$ -K6– $\gamma 1$ -K234, $\alpha 1$ -K62– $\gamma 1$ -K234, $\alpha 1$ -K71– $\gamma 1$ -K58, and $\alpha 1$ -K116– $\gamma 1$ -K100) were only compatible with the KD-displaced structure (distances between 24.7 and 46.3 Å, with distances >30 Å involving loop residues); these would be between 56.6 and >100 Å apart in the KD-associated structure (Fig. 2D). Together, the XL-MS data confirmed that AMP-bound phosphorylated AMPK preferentially adopted the KD-associated conformation and that ATP γ S-bound, nonphosphorylated AMPK preferentially adopted the KD-dissociated conformation. The XL-MS analysis also provided insight on the positions of the unresolved N terminus of the β subunit and the position(s) of the CBM (fig. S14 and additional data analysis section of the supplementary materials).

ATP binding stimulates AL dephosphorylation by destabilizing an interaction between AL and the β linker

To understand how AMPK ligands change AL accessibility in pAMPK, we first identified the AL-protecting elements. The AMPK core consisting of the γ subunit and the α - and β -CTDs was previously implicated in AL protection (14), and only the core and the β linker are in the vicinity of the AL and have the potential to shield it (fig. S15, A and B) (14, 29). Whereas pT174 was rapidly dephosphorylated by only 100 nM human protein phosphatase 2C α (PP2C) in the isolated KD, similar rates of dephosphorylation required about threefold higher concentration of PP2C for a truncated AMPK complex that contains the core but lacks the CBM and β linker (CBM– β linker) and an ~ 260 -fold higher concentration for full-length AMPK (fig. S16). This result indicated that the core complex contributes to restricting AL access but that most of the protection is due to the β linker. Energy excess nucleotide levels (4.5 mM ATP, 400 μ M ADP, 40 μ M AMP) slightly reduced AL protection, although the decrease was not statistically significant, whereas energy stress levels (3.8 mM ATP, 1 mM ADP, 0.3 mM AMP) moderately, and compound 991 strongly, increased AL protection for full-length AMPK. In contrast, none of the ligands affected AL protection in the isolated KD or in AMPK (CBM– β linker), consistent with a previous finding (16) (fig. S16). Therefore, both adenine nucleotides and ADaM site ligands likely regulate pT172 or pT174 access through modulation of the β linker–AL interaction.

Next we sought to identify the connections between ligand binding and the AL- β linker interaction. Both AMP and 991 allosterically stabilize the active KD conformation (3, 33) thereby ordering the inherently flexible AL (36) and its interaction with the β linker. To test whether the active KD conformation contributes to pT174 protection, we stabilized the conformation by an unrelated approach: binding of the catalytic cosubstrate Mg^{2+} -ATP or the high-affinity, ATP-competitive kinase inhibitor staurosporine (fig. S17A). Both reduced AL dephosphorylation, suggesting that the allosteric kinase activation by AMP and 991 contributed to the increase in AL protection.

In addition, we hypothesized that both ligands stabilize the β linker by stabilizing its flanking domains, the CBM and the β -CTD (Fig. 3A). ADaM ligands, and to a lesser degree CBM S108 phosphorylation, stabilize the CBM conformation and position and induce formation of a helix, called the C-interacting helix, at the N terminus of the β linker (Fig. 3A) (16, 17, 29). Confirming our hypothesis, replacement of the C-interacting helix with an equally long, flexible linker (GSAGSAGSAGS), and possibly to a lesser degree the CBM S108A mutation, reduced AL protection by 991 while having little effect on protection in the absence of ligands or in the presence of AMP (Fig. 3B and fig. S17, B and C).

AMP is sensed through formation of a CBS3-AMP- α linker interaction through α -E364 (fig. S15B, inset) that is required for both direct AMPK activation and protection against AL dephosphorylation (14, 29, 37). The E364-adjacent R365 binds an inducible pocket (R365 pocket) in the β -CTD C terminal to the β linker (fig. S15B, inset) (37). Mutation of either R365 or the R365 pocket reduced AL protection by AMP yet had little effect on protection in the absence of ligands or in the presence of 991 (Fig. 3B and fig. S17, B and C). This result strongly supports an important role for the R365 pocket in AMP-mediated AL protection. In contrast, mutation of the AL-interacting core residue β 2-H233 reduced AL protection, as previously reported (14), regardless of the presence or absence of AMP or 991 (Fig. 3B and fig. S17, B and C). Stabilization by the H233 interaction is thus not regulated by AMPK agonists. These results are confirmed in the context of full-length wild-type AMPK by analyzing our previously published HDX-MS data (33, 38, 39), which showed that AMP, ADaM ligand A769662, and AMPK phosphorylation induced stabilization of the β linker flanking regions (fig. S18).

Showing further agreement with our previously reported results, the α linker is dissociated from its position at the γ subunit in ATP-bound AMPK (fig. S19), and the R365 pocket moved up to 6 Å relative to its position in AMP-bound AMPK (fig. S19A). In addition, the lack of density for the CBM in all three structures of ATP-bound AMPK, together with our XL-MS data (see supplementary materials) and previous double electron-electron resonance spectra (32), indicates that the CBM can dissociate from the KD in the absence of ADaM ligands and adopt multiple alternative positions, especially in nonphosphorylated AMPK. Finally, the resolved part of the β linker moved outward by up to 9 Å (fig. S19B). Collectively, our results thus indicate that a series of connected conformational changes link ATP binding and the absence of ADaM agonists to an α linker movement, CBM dissociation, and destabilization of the β linker, which then promotes dephosphorylation. Dephosphorylation in turn destabilizes KD association, which depends on phosphate-

mediated charge interactions that stabilize the AL and the CBM–KD interaction (fig. S20, A and B).

Live-cell time course of energy excess–induced conformational changes

Our luminescence proximity, HDX-MS, and XL-MS assays provided three independent lines of support for the KD-displaced conformation seen in our cryo-EM structures. Because these experiments used recombinant, purified proteins, we developed a bioluminescence resonance energy transfer (BRET) conformational sensor to monitor changes in the position of the KD in cells. We genetically fused yellow fluorescent protein (YFP) to the N terminus of the KD and *Renilla* luciferase (Rlu) to either the C terminus of β 2 or the C terminus of the α -CTD. For both combinations, Rlu and YFP move closer together when the KD adopts the displaced conformation (50 and 56 Å, respectively) relative to the KD-associated conformation (Fig. 4A).

To determine the time course of AMPK inactivation, we first pretreated COS-1 cells expressing these two sets of BRET conformational sensors with 5 mM metformin, which activates AMPK by producing energy stress (30, 40). Because COS-1 cells are highly dependent on glycolysis to generate ATP, we induced the transition to energy excess by replacing metformin with 25 mM glucose. We followed AMPK inactivation over 2 hours by monitoring, in parallel, AMPK conformation by BRET (Fig. 4B); AL dephosphorylation by immunoblotting (Fig. 4C and fig. S22); changes in the AMP/ATP and ADP/ATP ratios by liquid chromatography–mass spectrometry (Fig. 4, D and E); and AMPK activity by immunoblotting of the AMPK substrate acetyl–coenzyme A carboxylase (ACC) (Fig. 4F). At 15 min after glucose addition, the AMP/ATP and ADP/ATP ratios, AL phosphorylation, and ACC phosphorylation had already dropped drastically and remained at low levels (Fig. 4, C to F). Simultaneously, BRET ratios for both BRET combinations approximately doubled and reached a maximum of about a 2.5-fold increase after 30 min of glucose treatment (Fig. 4B), indicating a closer distance between KD and core AMPK upon AMPK inactivation by energy excess. This indicated that energy excess and AL dephosphorylation were coupled to the conformational change of the KD–AMPK core interaction. The delay in reaching maximum BRET ratios relative to AMPK dephosphorylation, while in part due to a small delay of pelleting cells for immunoblotting (see methods), is consistent with our data suggesting that, predominantly, phosphorylation precedes KD displacement in cells. Together, this illustrates a multistep model of AMPK inactivation and reactivation (fig. S21). In the fully active state, AMPK is bound by AMP and/or an ADaM agonist and both the KD and CBM are phosphorylated. AMP stabilizes the β linker through the CBS3/ α linker/R365 pocket network and ADaM ligands through the KD/CBM/C-interacting helix network. Under conditions of energy excess, ATP replaces AMP at CBS3. ATP weakens the interaction network, thereby destabilizing the β linker and the KD active conformation, resulting in increased AL access to phosphatases. Partial destabilization of AL– β linker and CBM–KD interactions increases AL and CBM dephosphorylation leading to KD and CBM displacement and full inhibition. Upon energy stress, AMP or ADP replaces ATP at CBS3 again. This induces a conformational change in AMPK that facilitates AL rephosphorylation by LKB1 and CBM phosphorylation by autophosphorylation. The detailed mechanism of AL accessibility regulation revealed in this study provides a structural framework for the

rational development of therapeutics that selectively modulate AL accessibility, and it may serve as a paradigm for other signaling kinases that are regulated through changes in AL protection.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

We thank D. Wu (Guangzhou Institute for Biomedicine and Health, China) for the CaMKK β expression plasmid described in (29). Cryo-EM data were collected at the David Van Andel Advanced Cryo-Electron Microscopy Suite at the Van Andel Institute. We are grateful to X. Meng for cryo-EM data collection and EM technical support and to D. Nadziejka for language editing of the manuscript. We also thank 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.

Funding:

This work was supported in part by NIH grants R01 GM117372 (A.A.K.) and R01 GM129436 (K.M. and P.R.G.) and by the Van Andel Institute (R.G.J. and K.M). Use of the Advanced Photon Source (APS), an Office of Science User Facility operated for the US Department of Energy (DOE) Office of Science by Argonne National Laboratory, was supported by the US DOE under contract DE-AC02-06CH11357.

REFERENCES AND NOTES

1. Garcia D, Shaw RJ, Mol. Cell66, 789–800 (2017). [PubMed: 28622524]
2. Hardie DG, Genes Dev. 25, 1895–1908 (2011). [PubMed: 21937710]
3. Hardie DG, J. R. Soc. Interface15, 20170774 (2018). [PubMed: 29343628]
4. Steinberg GR, Kemp BE, Physiol. Rev89, 1025–1078 (2009). [PubMed: 19584320]
5. Yuan HX, Xiong Y, Guan KL, Mol. Cell49, 379–387 (2013). [PubMed: 23395268]
6. Dzamko Net al., J. Biol. Chem285, 115–122 (2010). [PubMed: 19892703]
7. Egan DFet al., Science331, 456–461 (2011). [PubMed: 21205641]
8. Akman HOet al., Pediatr. Res62, 499–504 (2007). [PubMed: 17667862]
9. Marin TLet al., BMC Syst. Biol9, 13 (2015). [PubMed: 25890336]
10. Fogarty S, Hardie DG, Biochim. Biophys. Acta1804, 581–591 (2010). [PubMed: 19778642]
11. Wong AK, Howie J, Petrie JR, Lang CC, Clin. Sci116, 607–620 (2009).
12. Rutter GA, Leclerc I, Mol. Cell. Endocrinol297, 41–49 (2009). [PubMed: 18611432]
13. Ross FA, MacKintosh C, Hardie DG, FEBS J. 283, 2987–3001 (2016). [PubMed: 26934201]
14. Xiao Bet al., Nature472, 230–233 (2011). [PubMed: 21399626]
15. Xin FJ, Wang J, Zhao RQ, Wang ZX, Wu JW, Cell Res. 23, 1237–1240 (2013). [PubMed: 23999859]
16. Xiao Bet al., Nat. Commun4, 3017 (2013). [PubMed: 24352254]
17. Calabrese MFet al., Structure22, 1161–1172 (2014). [PubMed: 25066137]
18. Carling D, Clarke PR, Zammit VA, Hardie DG, Eur. J. Biochem186, 129–136 (1989). [PubMed: 2598924]
19. Davies SP, Helps NR, Cohen PTW, Hardie DG, FEBS Lett. 377, 421–425 (1995). [PubMed: 8549768]
20. Gowans GJ, Hawley SA, Ross FA, Hardie DG, Cell Metab. 18, 556–566 (2013). [PubMed: 24093679]
21. Oakhill JSet al., Science332, 1433–1435 (2011). [PubMed: 21680840]
22. Zhang YLet al., Cell Metab. 18, 546–555 (2013). [PubMed: 24093678]
23. Göransson Oet al., J. Biol. Chem282, 32549–32560 (2007). [PubMed: 17855357]
24. Sanders MJet al., J. Biol. Chem282, 32539–32548 (2007). [PubMed: 17728241]

25. Scott JW et al., Chem. Biol21, 619–627 (2014). [PubMed: 24746562]
26. Chen Jet al., eLife6, e31268 (2017). [PubMed: 29027899]
27. Puustinen Pet al., Autophagy16, 1871–1888 (2020). [PubMed: 31983282]
28. Jia Jet al., Mol. Cell77, 951–969.e9 (2020). [PubMed: 31995728]
29. Li Xet al., Cell Res. 25, 50–66 (2015). [PubMed: 25412657]
30. Zhou Get al., J. Clin. Invest108, 1167–1174 (2001). [PubMed: 11602624]
31. Ereño-Orbea Jet al., J. Mol. Biol430, 322–336 (2018). [PubMed: 29277294]
32. Gu Xet al., J. Biol. Chem293, 16994–17007 (2018). [PubMed: 30206123]
33. Yan Yet al., J. Biol. Chem294, 953–967 (2019). [PubMed: 30478170]
34. Kao Aet al., Mol. Cell. Proteomics10, M110.002212 (2011).
35. Wang Xet al., J. Biol. Chem292, 16310–16320 (2017). [PubMed: 28821611]
36. Nolen B, Taylor S, Ghosh G, Mol. Cell15, 661–675 (2004). [PubMed: 15350212]
37. Chen Let al., Nature498, E8–E10 (2013). [PubMed: 23765502]
38. Gu Xet al., J. Biol. Chem292, 12653–12666 (2017). [PubMed: 28615457]
39. Landgraf RRet al., Structure21, 1942–1953 (2013). [PubMed: 24076403]
40. Shaw RJet al., Science310, 1642–1646 (2005). [PubMed: 16308421]

Fig. 1. Overall structure of the ATP γ S-bound AMPK/CpdC/Fab/nanobody complex.

(A) Diagram of the AMPK construct and its domain architecture, using the same color code as in (D) to (G). It lacks flexible termini and the ST loop ($\alpha 1$ [477–528]) (white). These loops impede structure determination, are unresolved in all AMPK structures, and are not required for AMPK structural integrity or regulation by adenine nucleotides or ADaM site ligands (14, 17, 29, 35). Segments with patterned shading are not resolved. ADaM, allosteric drug and metabolite site (brown oval); AID, autoinhibitory domain; AL, activation loop; CBM, carbohydrate-binding module; CBS1, CBS2, and CBS4, adenine nucleotide binding sites; CTD, C-terminal domain; KD, kinase domain; PPases, protein phosphatases; p, phosphorylation site in the AL (T174) and the CBM (S108). Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr. (B) Fab13 binding to active (AMP+staurosporine) and inactive (ATP+CpdC) AMPK. A_{450} , absorbance at 450-nm wavelength. (C) SDS–polyacrylamide gel electrophoresis analysis of the purified AMPK $\alpha 1\beta 2\gamma 1$ complex bound to Fab and nanobody. (D) Cryo-EM density map. (E) Atomic model. (F) Side-by-side structural

alignment of the inactive, Fab-stabilized AMPK cryo-EM structure (left) with the crystal structures of active pAMPK [Protein Data Bank (PDB) ID 4RER; middle] and Fab-free ATP γ S- and CpdC-bound pAMPK (right). MBP, Fab, and nanobody were omitted. (**G**) Nucleotide occupancy at CBS1, CBS3, and CBS4 overlaid with EM density (shown as mesh) contoured at 7.5σ (PyMOL).

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Fig. 2. In vitro analysis of AMPK conformations.

(A) Phosphorylation-induced HDX-MS changes overlaid on the structures of pAMPK/AMP/staurosporine (PDB ID 4RER) (left) and AMPK/ATP/CpdC/Fab (right). Peptides with reduced deuterium exchange in phosphorylated AMPK relative to non-phosphorylated AMPK are overlaid in gray. T174 is shown as spheres. (B) (Top) Cartoons of His6- and biotin-tagged AMPK. (Bottom) AlphaScreen assay ($n = 3$ technical replicates, error bars = SEM; one-way analysis of variance (ANOVA): *** $P < 0.001$; **** $P < 0.0001$; ns, not significant). (C and D) XL-MS analysis. Cross-linking distances that are compatible with the indicated structure are shown in blue, those that are incompatible, in orange. (C) Cross-links that are enriched in phosphorylated AMPK/AMP overlaid onto the KD-associated (PDB ID 4RER) and KD-displaced (cryo-EM) structures. (D) Cross-links that are enriched in nonphosphorylated AMPK/ATPγS overlaid onto the structures. Superscript number 1 indicates a loop residue. Superscript number 2 indicates a residue that borders a resolved region, so the residue–residue distance is only estimated.

Fig. 3. Elements at the termini of the β linker mediate the effects of compound 991 and AMP on AL accessibility.

(A) Schematic presentation of the AMPK β linker with flanking elements. AL-int., AL-interacting. (B) PP2C α protection assays. Full-length wild-type and mutant AMPK $\alpha 1\beta 1\gamma 1$ were incubated with PP2C for the indicated amount of time, and T174 phosphorylation determined by immunoblotting (fig. S17B); $n = 3$ biological repeats, error bars = SEM; ** $P < 0.01$, *** $P < 0.001$ (cumulative robust linear regression of each time course relative to the corresponding wild-type time course; with ordinary regression, the P values for R365E/AMP and H233A/AMP change from ** to *, while all other values remain the same).

Fig. 4. Cell-based BRET-immunoblot-MS analysis.

(Left) Construct combination 1: YFP- α 1/ β 2-Rlu/ γ 1. (Right) Construct combination 2: YFP- α 1-Rlu/ β 2/ γ 1. (A) BRET constructs in the context of the KD-associated and KD-displaced AMPK structures. (B) BRET ratios. (C) AL phosphorylation ratios determined by immunoblotting. pT174 phosphorylation (P) and total expression (total) were quantified with untreated sample set as 1.0. Representative immunoblots are shown below the bar graphs. (D and E) AMP/ATP (D) and ADP/ATP (E) ratios relative to ratios under energy stress (met = 1.0). (F) ACC phosphorylation ratios determined by immunoblotting and quantified with untreated sample set as 1.0. Representative immunoblots are shown below the bar graphs. Met: 5 mM metformin. $n = 3$ biological replicates, error bars = SEM, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ (against 0 min glucose; one-way ANOVA). Uncropped blots are shown in fig. S22.