THE EMBO JOURNAL

Manuscript EMBO-2009-71173

PKA phosphorylates and inactivates AMPK to promote efficient lipolysis

Nabil Djouder, Roland D. Tuerk, Marianne Suter, Paolo Salvioni, Ramon F. Thali, Roland Scholz, Kari Vaahtomeri, Yolanda Auchli, Helene Rechsteiner, René A. Brunisholz, Benoit Viollet, Tomi P. Mäkelä, Theo Wallimann, Dietbert Neumann, Wilhelm Krek

Corresponding author: Wilhelm Krek, ETH Zurich

Review timeline:

Submission date:	22 April 2009
Editorial Decision:	29 May 2009
Revision received:	22 September 2009
Editorial Decision:	06 October 2009
Revision received:	22 October 2009
Accepted:	26 October 2009

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 29 May 2009

Thank you for submitting your manuscript for consideration to The EMBO Journal. I do apologize for the slight delay in getting back to you with a decision that was caused by one late incoming report. As you will see from the enclosed assessments, the referees appreciate the potentially interesting regulatory phosphorylation of AMPK by PKA. However, all three of them also request further delineation of this mechanism in-vitro (ref#1) and confirmation of its physiological significance in-vivo (refs #2 and #3). The second point seems of major importance, as your observations contradict some earlier reports and it would be useful to aim at solving this issue both experimentally and by careful discussion of the related literature. Apart from this, all referees encourage to offer you the chance to address their critiques using a single round of thorough revisions. I also have to remind you that it is usually EMBO_J policy to allow a single round of amendments only, which means that the decision on acceptance or rejection entirely depends on the content of the final version of your manuscript that will be assessed involving some of the initial referees.

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely,

Editor

The EMBO Journal

REFEREE REPORTS

Referee #1 (Remarks to the Author):

The authors of this interesting study provide quite persuasive evidence that PKA can directly phosphorylate and inhibit the AMPK pathway to promote lipolysis in fat cell lines. This is mediated by direct phosphorylation of AMPK at Ser173 by PKA, which is the residue that follows from the site targetted by the activating LKB1/CAMKK enzymes. My feeling is that this study is of sufficient importance to merit publication in the EMBO Journal. Below I outline some relatively straightforward points that the authors should address in a revised version of the manuscript:

1a. Phosphorylation of Ser173 by PKA is concluded to inhibit LKB1 mediated phosphorylation of Thr172 by LKB1. This conclusion is derived from the use of a phosphospecific antibody that recognises Thr172. It is very likely that epitope recognition of the Thr172 antibody will be abolished if Ser173 is also phosphorylated as the +1 residue in any phosphospecific antibody plays a key role in antibody recognition. Tinkering with the +1 residue of a phosphoantibody normally inhibits epitope recognition. Therefore, many of the effects seen with reduced Thr172 antibody recognition following PKA activation could actually be due to phosphorylation of the adjacent Ser173 residue without necessarily resulting in dephosphorylation of Thr172. To address this point the authors would need to study how single and double phosphorylation of a Thr172 Ser173-containing peptide effects recognition by the Thr172 antibody.

1b. Similarly recognition of phosphorylated Ser173 with the phospho Ser173 antibody could be markedly affected by adjacent Thr172 phosphorylation.

To address the above points the authors could do in vitro phosphorylations of the heterotrimeric AMPK complex in the presence or absence of LKB1 and/or PKA and use mass spectrometry to directly demonstrate whether it is possible to phosphorylate both Thr172 and Ser173 residues. This procedure should reveal unambiguously that, once AMPK is quantitatively phosphorylated at Ser173 by PKA, then it is no longer phosphorylatable at Thr172 by LKB1.

2. Is this mechanism a regulation of AMPK by PKA ubiquitous and seen in cells other than adiposities?

3. Is the association observed between PKA and AMPK influenced by agonists that elevate cyclic AMP levels in cells?

4. Does PKA also prevent activation of AMPK in response to stimuli that elevate calcium and induce CAMKK to phosphorylate AMPK?

5. Many of the western blots presented in the figures are hopelessly small, making it impossible to properly evaluate their quality. Could the revised version have much larger figures to enable reviewers to inspect the quality of these more accurately?

Referee #2 (Remarks to the Author):

In this manuscript, the authors demonstrate that PKA phosphorylates the AMPK alpha1 catalytic subunit at Ser173 both in vitro and in vivo. This phosphorylation by PKA interferes with the phosphorylation of AMPK by the upstream activating kinase LKB1 at the nearby Thr172 in vitro and hence its activation. This is a potentially important regulatory mechanism by which PKA inhibits AMPK activity. However, the contribution of Ser173 phosphorylation to the inhibition of AMPK activity by PKA and its biological function require further demonstration and clarification.

Major Comments:

1) In Figures 6D and 6E, the authors show that AMPKa1 S173C mutant is refractory to the inhibition of T172 phosphorylation by PKA in adiopocytes, and that expression of S173C mutant moderately inhibits the release of NEFA induced by Iso treatment, compared to expression of WT AMPKa1. However, the authors only examined the phosphorylation of AMPK Thr 172 from the pool of transfected myc-tagged AMPK, but not the overall AMPK activity from total cell lysates, such as phosphorylation levels of AMPK Thr172, ACC Ser79 and HSL Ser 565 (as shown in Figure 6A). These experiments will answer the question whether expression of AMPKa1 S173C is sufficient to, or to what extent, render adipocytes insensitive to the inhibition of AMPK and downstream signaling by PKA upon FSK or ISO treatment. This information is very critical because previously PKA has been suggested to regulate two upstream kinases of AMPK, LKB1 and CAMKK. On the same note, the author may also address this aspect of literature in the discussion.

2) In Figure 7A, the authors show that long-term ISO treatment leads to an increase of AMPK phosphorylation at T172. However, the effect of short-term ISO treatment (i.e. 30 min) on AMPK T172 phosphorylation is inconsistent with the data shown in Fig 1A. In addition, the authors did not provide a clear explanation on why the level of AMPK T173 phosphorylation decreases upon long-term ISO treatment. This should at least be addressed in the discussion.

3) It would be interesting to examine whether this regulatory mechanism by which PKA inhibits AMPK activity is only specific to adipocytes, or it is a more general phenomena.

Minor Comments:

1) The quality of several western blotting figures (i.e. 1C, 1D, 6D and 7E) could be improved.

2) Fig 3A: it appears there is a slight mobility shift of AMPK upon PKA phosphorylation. Is this reproducible and real? If it is, the authors may be able to take advantage of this shift to estimate the stoichiometry of phosphorylation in cell lysates.

3) Fig 4C: it is important to compare AMPK D157A with D157A/S173C double mutant in this experiment to examine whether mutation of S173C has any effect on T172 phosphorylation by LKB1 at all. Similarly, AMPK D157A mutant is needed as a positive control in Fig. 4A experiment to show that LKB1 protein used is indeed active.

Referee #3 (Remarks to the Author):

In this study, Djouder et al. examined the effect of PKA on AMPK activity in vitro and in primary murine adipocytes. The authors provide evidence that PKA phosphorylates AMPKα at multiple sites such as Ser485, which was reported previously (Hurley et al. JBC 2006)(this work should be cited), and the novel site Ser173. Their data also indicate that in cell free in vitro assays, phosphorylation of the Ser173 residue by PKA appears to impede on the phosphorylation of the Thr172 site by LKB1, an important event for the activation of AMPK. Experiments conducted in cultured adipocytes show that cAMP-inducing agents phosphorylate AMPKα at Ser173 and that this event negatively correlates with P-AMPK Thr172 levels. The authors conclude that PKA inhibits AMPK activity in adipocytes through phosphorylation at Ser173 and that this favours the lipolytic response to cAMP-inducing agents.

The major criticism of this manuscript is that most experiments were conducted in cell free in vitro assays and that it is not clear that the phenomena described occur in vivo. In addition, the in vivo experiments showing that cAMP-inducing agents inhibit AMPK phosphorylation are contradictory to what has been reported by several laboratories (Moule and Denton 1998; Yin, Mu et al. 2003; Daval, Diot-Dupuy et al. 2005; Koh, Hirshman et al. 2007 ; Gauthier, Miyoshi et al. 2008; Lobo, Wiczer et al. 2009; Omar, Zmuda-Trzebiatowska et al. 2009) and this is not sufficiently discussed. On that matter, the authors partly explain this discrepancy by stating on page 13 that: 'However, these studies investigated single time point of isoproterenol stimulation, usually 60 minutes of isoproterenol treatment, rather than a time course.' However, most of these studies did show earlier time-points and even time-courses of treatment of adipocytes and found increased activity and

phosphorylation of AMPK with cAMP-inducing agents. The authors need to comment on that.

Other comments:

1. in vitro and in vivo experiments with a phosphomimetic mutant of AMPK Ser173 would be useful to confirm the inhibitory effect of this site on Thr172 phosphorylation.

2. In figure 1 and 7, AMPK activity assay of immunoprecipitated AMPK from adipocytes treated with the different agents would be useful to confirm the results. Also, immunoblots of the downstream target of AMPK P-ACC Ser79 would be useful.

3. Bar graphs of the densitometric analysis of the immunoblots should be shown. In addition, for most of the experiments, the n value and number of experiments is not stated.

4. The experiments performed in cultured adipocytes treated with orlistat or triacsin C are confirmatory of previous reports (Gauthier, Miyoshi et al. 2008; Omar, Zmuda-Trzebiatowska et al. 2009) and in keeping with Lobo et al. findings (Lobo, Wiczer et al. 2009). This should be mentioned.

5. The authors state that cAMP-inducing agents inhibit AMPK activity in adipocytes through PKA in the short-term, and that longer-term treatments activate AMPK because of energy depletion. Measurements of energy state would be useful. Also, the results shown in figure 7 doesn't support that conclusion since isoproterenol clearly increased AMPK and ACC phosphorylation at 30 min which contradicts what is shown in figure 1. What is the explanation for that?

6. The figures are too small, especially the western-blots, which make it difficult to read and interpret.

7. The time of incubation of adipocytes with the agents should be made clearer in the different sections of the manuscript.

8. NEFA can be re-esterified and re-incorporated into TAGs, thus glycerol measurements provide a better assessment of lipolysis since glycerol kinase activity is very low in adipocytes. Also, NEFA measurements with the WACO colorimetric kit are not reliable when triacsin C is used in experiments since the kit reagents contain and uses acyl-CoA synthetase in their reactions.

9. In Figure 4E, why isn't Thr172 phosphorylation blocked when PKi is added with LKB1?

10. The title is too broad and not supported by the current data.

Minor comments:

1. It should be mentioned that Perilipin and ATGL are also major players in the regulation of lipolysis by beta-adrenergic-PKA pathway.

2. In the introduction, the authors wrote: 'Energy stress leads to an increase in the AMP:ATP ratio and AMP binds directly to the AMPKα1 subunit, thereby inducing a conformational change (Riek et al, 2008) that protects the α1subunit from dephosphorylation at the critical activation loop threonine (Thr-172) by upstream kinases (Anderson et al, 2008; Carling et al, 2008; Sanders et al, 2007; Suter et al, 2006).' The wording make it seems like upstream kinases dephosphorylate AMPK.

3. Forskolin is more well known as an activator of adenylyl kinase.

4. In figure 6B, the results show that basal lipolysis was blocked by PKi, not stimulated-lipolysis.

References:

Daval, M., F. Diot-Dupuy, et al. (2005). "Anti-lipolytic Action of AMP-activated Protein Kinase in Rodent Adipocytes

10.1074/jbc.M414222200." J. Biol. Chem. 280(26): 25250-25257.
Gauthier, M.-S., H. Miyoshi, et al. (2008). "AMP-activated Protein Kinase Is Activated as a Consequence of Lipolysis in the Adipocyte: POTENTIAL MECHANISM AND PHYSIOLOGICAL RELEVANCE
10.1074/jbc.M708177200." J. Biol. Chem. 283(24): 16514-16524.
Koh, H.-J., M. F. Hirshman, et al. (2007). "Adrenaline is a critical mediator of acute exercise-induced AMP-activated protein kinase activation in adipocytes
10.1042/BJ20061479." Biochem J 403(3): 473-481.
Lobo, S., B. M. Wiczer, et al. (2009). "Functional analysis of long-chain acyl-coa synthetase 1 in 3T3-L1 adipocytes
10.1074/jbc.M109.017244." J. Biol. Chem.: M109.017244.
Moule, S. K. and R. M. Denton (1998). "The activation of p38 MAPK by the [beta]-adrenergic agonist isoproterenol in rat epididymal fat cells." FEBS Letters 439(3): 287-290.
Omar, B., E. Zmuda-Trzebiatowska, et al. (2009). "Regulation of AMP-activated protein kinase by cAMP in adipocytes: Roles for phosphodiesterases, protein kinase B, protein kinase A, Epac and lipolysis." Cellular Signalling 21(5): 760-766.
Yin, W., J. Mu, et al. (2003). "Role of AMP-activated Protein Kinase in Cyclic AMP-dependent Lipolysis In 3T3-L1 Adipocytes
10.1074/jbc.M308484200." J. Biol. Chem. 278(44): 43074-43080.

1st Revision - Authors' response 22 September 2009

We thank all three reviewers for their insightful comments. We have added a number of new experiments and addressed all issues raised, which we believe improved the manuscript significantly. A detailed response is given below.

Reviewer 1

Points 1a and 1b:

In Figure 3A, we show that PKA phosphorylates AMPKα1(D157A/S485A/S497A) at Ser-173 as evidenced by ^{32}P-incorporation and recognition by anti-phospho Ser-173 antibodies. Conversely, when AMPK(D157A/S485A/S497A) complexes were incubated with LKB1 prior to the addition of PKA, phosphorylation of AMPKα1(D157A/S485A/S497A) by PKA still occurred to a similar extend as indicated by similar ^{32}P-incorporation. However, the anti-phospho Ser-173 antibody failed to recognize the phospho-Ser173 epitope in this setting (Figure 3A, lane 4). We note that the AMPKα1 species used in this experiment as substrate is mutated at all potential PKA phosphorylation sites except for Ser-173. These results indicate that Thr-172 phosphorylation of AMPKα1 by LKB1 does not preclude subsequent PKA phosphorylation of AMPKα1 at Ser-173 *in vitro* and that in this setting the anti-phospho Thr-172 antibody appears to recognize phosphorylation at Thr-172 normally if Ser-173 is phosphorylated. Moreover, they further suggest that the anti-phospho Ser-173 antibody can recognize the Ser-173 epitope only when AMPKα1 is not phosphorylated at Thr-172.

In reciprocal experiments, LKB1 phosphorylated AMPK (D157A) complexes on Thr-172 as evidenced by ^{32}P-incorporation and recognition of AMPKα1(D157A) by anti-phospho Thr-172 antibodies (Figure 3B, lane 2). Importantly, when AMPK(D157A) complexes were incubated with PKA prior to the addition of LKB1, Ser-173 phosphorylation occurred but autoradiography of *in vitro* kinase assays demonstrate that subsequent phosphorylation of AMPKα1(D157A) at Thr-172 by LKB1 was blocked (Figure 3B, lane 4). These results suggest that PKA-mediated phosphorylation of AMPKα1 at Ser-173 efficiently interferes with subsequent LKB1 phosphorylation of AMPKα1 at Thr-172.

In short, when AMPKα1 is first phosphorylated by PKA, LKB1 cannot phosphorylate AMPKα1 at Thr-172 and our phospho Ser-173 antibody recognizes such phosphorylated species. When AMPKα1 is first phosphorylated by LKB1, PKA can phosphorylate AMPKα1 at Ser-173 but our phospho Ser-173 cannot recognize such doubly phosphorylated species. However, the Thr-172 antibody does recognize its epitope. We are thankful to the reviewer to raise this issue as this characterization is important for interpreting our results.

Point 2:

The regulatory mechanism of PKA-mediated inhibition of AMPK activation is not restricted to adipocytes but extends also to other cell types. We explored the effect of forskolin on AMPK activation in U2-OS osteosarcoma and HepG2 hepatoma cells. We show that PKA stimulating agents inhibit glucose starvation-induced Thr-172 phosphorylation of AMPKα1 in U2-OS and HepG2 cells, while promoting AMPKα1 Ser-173 phosphorylation in a time-dependent manner (Supplementary Figure S5A and S5B, respectively). Additionally, depletion of both PKAα and β subunits by siRNAs in U2-OS cells lead to a significant increase of Thr-172 phosphorylation of AMPKα1 in glucose starved cells (Supplementary Figure S5C). Finally, like in adipocytes, endogenous AMPKα1 immunoprecipitates derived from U2-OS cells contain also PKAα (Supplementary Figure S5D). Therefore, the crosstalk between PKA and AMPK reported here likely extends to other cell types supporting the view that it represents an important regulatory mechanism in distinct contexts.

Point 3:

Agents that elevate cAMP (isoproterenol) or calcium (ionomycin) to stimulate PKA and CaMKK, respectively did not affect complex formation of PKA and AMPK kinases as now shown in Supplementary Figure S1C.

Point 4:

Activation of Ca^{2+} signaling by ionomycin did not interfere with Thr-172 phosphorylation of AMPKα1 in adipocytes (Supplementary Figure S1B).

Point 5:

We apologize for the small Western blots in the first version of the manuscript. We believe that this maybe the result of problems with the conversion of certain data files. We have now increased the size of the blots and changed the way of file conversion to provide better quality of the figures.

Reviewer 2

Major comments

Point 1:

We agree with the reviewer that clarification of the contribution of Ser-173 phosphorylation to the control and release of FFA from lipid stores in adipocytes is critical. To address this point, we initiated a collaboration with Tomi Mäkelä and Bennoit Viollet (that are now included as authors), who provided us with mouse embryonic fibroblasts (MEFs) generated from AMPKα1 and AMPKα2 double KO mouse and immortalized by stably expressing a mutant p53 species (see Materials and methods). We used these MEFs to differentiate the cells into adipocytes. The major advantage of this cell system over the one we have been initially using is that adipocytes derived in this way do not contain any endogenous AMPKα species, thus allowing one to reintroduce wild-type and mutant species and assess their contribution to stimulated lipolysis. This cell system is therefore superior over the one we have been using which relied on overexpression of AMPKα species in the presence of endogenous protein.

AMPK-/- MEFs were infected either with control adenovirus or recombinant adenoviruses producing myc-tagged species of AMPKα1(wt) or a (S173C) mutant derivative (Supplementary Figure 6). Importantly, isoproterenol stimulation failed to suppress Thr-172 phosphorylation in cells expressing the myc-AMPKα1(S173C) mutant, suggesting that phosphorylation of AMPKα1 at Ser-173 is inhibitory to Thr-172 phosphorylation. Furthermore, the failure of isoproterenol to suppress phosphorylation of myc-AMPKα1(S173C) at Thr-172 translated into a failure to induce phosphorylation of HSL at Ser-660 (a PKA site) and to suppress phosphorylation of HSL at Ser-565

(a AMPK site) (Figure 6C). ACC phosphorylation has also been assessed in this context and the results obtained are fully in line with our hypothesis that Ser-173 phosphorylation of AMPKα1 is critical to suppress Thr-172 phosphorylation in response to lipolytic stimuli (Figure 6C). As a consequence, we also observed significant less release of glycerol from myc-AMPKα1(S173C) expressing adipocytes as compared to adipocytes expressing myc-AMPKα1(wt) (Figure 6D). These results suggest that PKA-mediated phosphorylation of AMPKα1 at Ser-173 is critical for efficient lipolysis in vivo. As suggested by the reviewer, we have now also included a section in the discussion about this finding in the context of recent literature.

Point 2:

Differences between Figure 7A and Figure 1A relate to the exposure time of the immunoblots. Longer exposure of blots of Figure 1A would have led to detection of a weak phosphorylation signal of AMPKα1 at Thr-172 after short-term isoproterenol treatment. Likewise, shorter exposure of immunoblots of Figure 7A would have revealed weaker signals of phosphorylated Thr-172 after short term isoproterenol treatment. We now show that 30 minutes isoproterenol treatment did not affect Thr-172 phosphorylation of AMPKα1 nor ACC phosphorylation. Thus, in the revised manuscript we have included the new experiment in Figure 7A to clarify this issue.

Moreover, as suggested by the reviewer, we have included in the results and discussion sections a paragraph on the finding that phosphorylation of Ser-173 of AMPKα1 decreased upon long term isoproterenol treatment in the context of the ability of the antibody to recognize single (phospho Ser-173) and not doubly (phospho Ser-173 and phospho Thr-172) phosphorylated species of AMPKα1.

Point 3:

The regulatory mechanism of PKA-mediated inhibition of AMPK activation is not restricted to adipocytes but extends also to other cell types. We explored the effect of forskolin on AMPK activation in U2-OS osteosarcoma and HepG2 hepatoma cells. We show that PKA stimulating agents inhibit glucose starvation-induced Thr-172 phosphorylation of AMPKα1 in U2-OS and HepG2 cells, while promoting AMPKα1 Ser-173 phosphorylation in a time-dependent manner (Supplementary Figure S5A and S5B, respectively). Additionally, depletion of both PKAα and β subunits by siRNAs in U2-OS cells lead to a significant increase of Thr-172 phosphorylation of AMPKα1 in glucose starved cells (Supplementary Figure S5C). Finally, like in adipocytes, endogenous AMPKα1 immunoprecipitates derived from U2-OS cells contain also PKAα (Supplementary Figure S5D). Therefore, the crosstalk between PKA and AMPK reported here likely extends to other cell types supporting the view that it represents an important regulatory mechanism in distinct contexts.

Minor comments

Point 1:

We apologize for the small Western blots and apparent low quality in the first version of the manuscript. We believe that this maybe the result of problems with the conversion of certain data files. We have now increased the size of the blots and changed the way of file conversion to provide better quality of the figures.

Point 2:

Upon treatment of AMPK with PKA an electrophoretic shift of the α-subunit is observed, which is indeed reproducible and consistently seen. However, as shown in the Figure 1 'referee-only supplementary material', the mobility shift of the AMPK α-subunit is similarly observed upon AMPK activation, i.e. after LKB1-dependent Thr-172 phosphorylation. Therefore the electrophoretic mobility shift alone cannot be used to estimate the stoichiometry of phosphorylation by either kinase (LKB1 or PKA).

Point 3:

We thank the reviewer of pointing this out. In a new experiment, AMPK(D157A) was analyzed side by side with AMPK(D157A/S173C) double mutant to test whether the mutant S173C has an effect on Thr-172 phosphorylation of AMPKα1 by LKB1 (new Figure 4C). Likewise, in Figure 4A, in a new experiment, we added AMPKα1 wt as a positive control to show that LKB1 kinase is indeed active and phosphorylates Thr-172 of AMPKα1 wt.

Reviewer 3

Specific comment:

The reviewer criticized that most experiments were conducted in vitro and that in vivo evidence is lacking. We performed several additional experiments to complement our existing experiments. Most importantly, we used a new cell system to evaluate the role of Ser-173 phosphorylation in a physiologic context.

We initiated a collaboration with Tomi Mäkelä and Bennoit Viollet (that are now included as authors), who provided us with mouse embryonic fibroblasts (MEFs) generated from AMPKα1 and AMPKα2 double KO mouse and immortalized by stably expressing a mutant p53 species (see Materials and methods). We used these MEFs to differentiate the cells into adipocytes. The major advantage of this cell system over the one we have been initially using is that adipocytes derived in this way do not contain any endogenous AMPKα species, thus allowing one to reintroduce wild-type and mutant species and assess their contribution to stimulated lipolysis. This cell system is therefore superior over the one we have been using which relied on overexpression of AMPKα species in the presence of endogenous protein.

AMPK-/- MEFs were infected either with control adenovirus or recombinant adenoviruses producing myc-tagged species of AMPKα1(wt) or a (S173C) mutant derivative (Supplementary Figure 6). Importantly, isoproterenol stimulation failed to suppress Thr-172 phosphorylation in cells expressing the myc-AMPKα1(S173C) mutant, suggesting that phosphorylation of AMPKα1 at Ser-173 is inhibitory to Thr-172 phosphorylation. Furthermore, the failure of isoproterenol to suppress phosphorylation of myc-AMPKα1(S173C) at Thr-172 translated into a failure to induce phosphorylation of HSL at Ser-660 (a PKA site) and to suppress phosphorylation of HSL at Ser-565 (a AMPK site) (Figure 6C). ACC phosphorylation has also been assessed in this context and the results obtained are fully in line with our hypothesis that Ser-173 phosphorylation of AMPKα1 is critical to suppress Thr-172 phosphorylation in response to lipolytic stimuli (Figure 6C). As a consequence, we also observed significant less release of glycerol from myc-AMPKα1(S173C) expressing adipocytes as compared to adipocytes expressing myc-AMPKα1(wt) (Figure 6D). These results suggest that PKA-mediated phosphorylation of AMPKα1 at Ser-173 is critical for efficient lipolysis in vivo. Therefore, we have now demonstrated the physiological relevance of phosphorylation of Ser-173 of AMPKα1 on NEFA release. In addition, we showed that Ser-173 phosphorylation occurs concomitant with decreased Thr-172 phosphorylation in response to cAMP elevating agents not only in adipocytes but also in other cell lines, in particular U2-OS osteosarcoma and HepG2 hepatoma cells (Suppl. Figure S5).

As suggested by the reviewer, we also discussed our findings in the context of the literature and refer also to the work of Hurley et al. JBC 2006.

Other comments

Point 1:

We addressed this point by using the phosphomimetic mutant S173D of AMPKα1(D157A). In an *in vitro* kinase assay, this mutant did not affect phosphorylation at Thr-172 by LKB1 (see Figure 2 of the "referee-only supplementary material'). Because of this experimental evidence, we did not use the phosphomimetic mutant for over-expression experiments in cell culture.

Point 2:

We recognize the importance of that experiment and we provide a SAMS based-AMPK activity assay of immunoprecipitated AMPKα1 from adipocytes after short-term and long-term isoproterenol treatment (Supplementary Figure 1SA and S7B, respectively). We confirmed that AMPKα1 activity is suppressed in a short-term isoproterenol treatment and recovered after long-term treatment. Moreover, immunoblots of phosphorylation of the acetyl-CoA carboxylase (ACC), a downstream target of AMPKα1 is now included in all Western blots of adipocytes, providing another direct readout of AMPKα1 activity in these cells.

Point 3:

We appreciate the suggestion but believe that bar graphs of densitometric analysis of Western blots would not add additional information in particular also since the effects we are reporting here are not minor. In several experiments we provide time-courses of the behaviour of phosphorylated species of kinases and substrates under investigation. In addition, the primary data shown in this manuscript are always representative examples of experiments that have been done multiple times but at a minimum 3 times. We have now indicated this in the Figure legends.

Point 4:

The experiments shown in Figures 7C-F were performed with Orlistat or Triacsin C and are indeed confirmatory of previous reports. Importantly, we view them now in the context of the behaviour of the phospho Ser-173 phosphorylation as a new regulatory mechanism of AMPKα1. As requested, we discussed this in the revised version of the manuscript.

Point 5:

We included measurement of ATP in adipocytes treated with isoproterenol in a time dependent manner (Supplementary Figure S7A). Our new results show that increased phosphorylation of AMPKα1 at Thr-172 elicited by long-term isoproterenol treatment (Figure 7A) is accompanied by a drop of ATP levels, thus being consistent with the notion that energy depletion in adipocytes is occurring, which in turn is consistent with increased AMPKα1 phosphorylation at Thr-172 at this time.

Differences between Figure 7A and Figure 1A relate to the exposure time of the immunoblots. Longer exposure of blots of Figure 1A would have led to detection of a weak phosphorylation signal of AMPKα1 at Thr-172 after short-term isoproterenol treatment. Likewise, shorter exposure of immunoblots of Figure 7A would have revealed weaker signals of phosphorylated Thr-172 after short term isoproterenol treatment. We now show that 30 minutes isoproterenol treatment did not affect Thr-172 phosphorylation of AMPKα1 nor ACC phosphorylation. Thus, in the revised manuscript we have included the new experiment in Figure 7A to clarify this issue.

Point 6:

We apologize for the small Western blots and apparent low quality in the first version of the manuscript. We believe that this maybe the result of problems with the conversion of certain data files. We have now increased the size of the blots and changed the way of file conversion to provide better quality of the figures.

Point 7:

We agree that the incubation time of adipocytes with different reagents used in this report should be made clearer. We included incubation time and concentration of reagents where missing.

Point 8:

We fully agree with the reviewer and thus measured glycerol release instead of NEFAs specifically for the experiment shown in Figure 7F where cells were treated with Triacsin C and obtained the same results. We also measure glycerol release in the new Figure 6D.

Point 9:

Our results show that phosphorylation of AMPKα1 at Thr-172 is not blocked when PKi was added together with LKB1 (Figure 4E, lanes 7 and 10). PKi is an inhibitor of PKA and it does not inhibit LKB1. Thus, addition of PKi blocks Ser-173 phosphorylation and therefore allows the phosphorylation of AMPKα1 at Thr-172 by LKB1.

Point 10:

We believe that with the additional data provided, in particular the in vivo results with AMPK-deficient adipocytes, the title is warranted.

Minor comments

Point 1:

We have now mentioned in the text that perilipin and ATGL are also critical components in the regulation of lipolysis by stimulation of β-adrenergic stimuli.

Point 2:

We agree with the reviewer and the wording has now been changed.

Point 3:

Forskolin is known as an activator of adenylyl cyclase (not as an activator of adenylyl kinase as mentioned by the reviewer). We provide this information in the text.

Point 4:

We thank the reviewer for pointing this out. We have changed the wording in the text accordingly.

Additional Supporting Figures

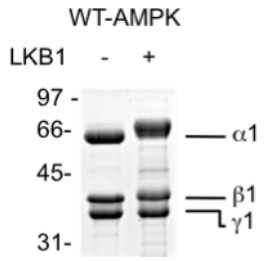

Figure 1: Thr-172 phosphorylation of AMPKa1 induces an electrophoretic mobility shift. WT-AMPK (α1β1γ1) was incubated with/without LKB1 and ATP as indicated. Following incubation with LKB1 the electrophoretic mobility of the AMPK α-subunit is reduced compared to non-activated wild type AMPK.

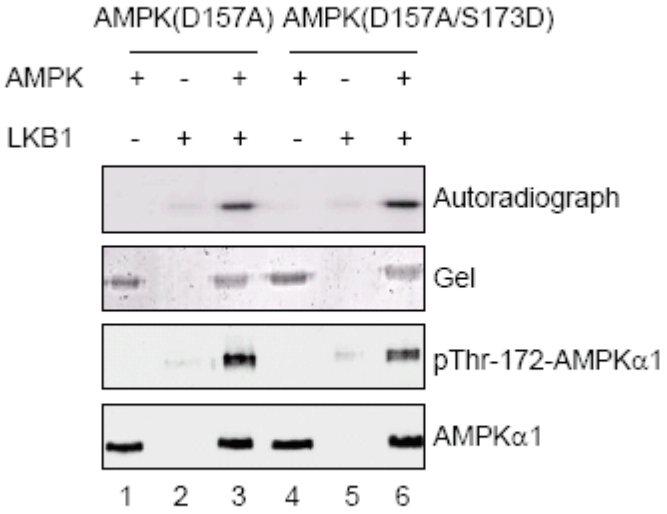

Figure 2: AMPK(D157A) (lanes 1 to 3) and AMPK(D157A/S173D) (lane 4 to 6) complexes were either incubated with LKB1 or not, in the presence of [γ-^{32}P]ATP. AMPKα1(D157A) and AMPKα1(D157A/S173D) protein phosphorylation assessed by autoradiography (upper panel) and immunoblotting (lower panel).

2nd Editorial Decision 06 October 2009

Your revised manuscript has now been re-assessed by two of the original referee's, their comments are enclosed below.
As you will see from the remarks ref#3 requests a few modifications to put your results into the context of published data and indicates small inconsistencies in results and data presentation. Together with repetition of some western blots (as mentioned by referee#1 already during initial review), we kindly ask you to revise and modify the manuscript accordingly and provide us with the ultimate version as soon as possible.
Please include a cover letter with an itemised list of all changes made, in response to these comments when you send us your revision.

Thank you for the opportunity to consider your work for publication. I look forward to your revised manuscript.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS:

Referee #2 (Remarks to the Author):

The authors have satisfactorily addressed all the comments raised previously. There are only two additional minor comments here:

- FKS, instead of FSK, was used as an abbreviation for forskolin at some places.

- The quality of some western blots (as mentioned in previous comments), not just the digital files, could be improved by repeating the experiments.

Referee #3 (Remarks to the Author):

1- The authors still need to discuss the fact that their finding that adipocyte AMPK activity is inhibited by short-term incubation with cAMP-inducing agents is contrary to what has been reported by several laboratories. Indeed, various published reports show that adipocyte AMPK activity and phosphorylation at Thr172 are increased as soon as 3 min after treatment with cAMP-inducing agents (Koh et al. : 15min; Moule & Denton: 10 min; Yin et al. : 3min; Omar et al.: 10 min; Lobo et al. 30 min; etc).
2- In Fig 7A, it still appears that Thr172 phophorylation of AMPK is increased after 30 min of iso treatment as compared to control, which is inconsistent with what is shown in Fig 1A.
3- In supplementary figure 1A, in order to assess the effect of isoproterenol on AMPK activity, the results should be presented as percentage of untreated controls not as percentage of AICAR treated.
4- In supplementary Fig6, it appears that, according to oil red O staining, the different cells do not accumulate equal amounts of lipids and that S173C mutants have less lipid accumulation. Could that be the reason why they exhibit lower isoproterenol-stimulated lipolysis?

Minor comments:
-The cell signaling antibody for P-AMPK Thr172 detects both alpha1 and alpha2 subunits, not just alpha1. This should be corrected in the results section as well as in the figures.

2nd Revision - Authors' response 22 October 2009

Thank you for having our manuscript re-assessed. Please find enclosed a second revised version of the manuscript by Djouder et al. entitled 'PKA Phosphorylates and Inactivates AMPK to Promote Efficient Lipolysis'.

In response to the remaining issues raised by reviewers 2 and 3, we have made the following changes:

Reviewer 2

1. We have introduced the abbreviation FSK throughout the manuscript, supplementary information and in the Figure labels.
2. We have repeated the immunoblots for Figure 1C and Figure 1D. Originally, also the quality of Figure 6D was criticized by this reviewer. We note that for the first Revision, we replaced Figure 6D by new western blots (Figure 6C, revision 1). We strongly believe that the western blots are of very high quality, in particular because all our experiments are performed with extracts derived from white adipose tissue isolated from mice and not with extracts from transformed cell lines.

Reviewer 3

1. We have now included a detailed and balanced discussion on AMPK's role in adipocytes and the timing of its activation vis-a-vis the inhibitory action of PKA with reference to the various papers existing in the field and mentioned by the reviewer. We cite a series of recent papers that all point to a requirement for ongoing lipolysis for AMPK activation. In these papers (that are mentioned in the discussion section) various functional test have been done with, for example, advanced tools such as adipocytes derived from AMPK knock out mice or siRNA experiments to downregulate endogenous components. All these papers come to the conclusion that AMPK activity is anti-lipolytic, consistent with our findings and our model. There are only two papers that

suggest that AMPK activity promotes lipolysis but in these papers the authors have either used overexpression of a putative dominant-inhibitory mutant of AMPK in 3T3 L1 cells or applied one pharmacological inhibitor of AMPK at high dose and based on those single results, concluded on a lipolytic effect of AMPK. This is now also mentioned in our discussion. Finally, we have included a discussion on the issue of timing of AMPK activation following a PKA-activating signal. Review of all data of the papers mentioned by the reviewers and now cited show that AMPK activity rises within the first 15-30 min. Evaluation of the paper by Yin et al., reporting AMPK activation after 3 min following isoproterenol addition reveals little if any robust increase in AMPK Thr-172 phosphorylation at that time (see Yin et al., 2003; JBC 278; page 43077 Figure 3, panel a). In this Figure a robust increase in AMPK Thr-172 phosphorylation is seen after 30 min, consistent with other reports. Moreover, different reports use different concentrations of PKA activating signals and different cell systems (e. g. 3T3 L1 cell line versus primary adipocytes from mice) and thus it is difficult to make a direct comparison. Lastly, we cite a very recent paper by Martin et al., JBC 2009 that demonstrates that, in adipocytes, within seconds after application of PKA activating signals (and not minutes), cAMP rises and HSL phosphorylation on the PKA site (but not on the AMPK site) is detectable. Thus, PKA becomes active very rapidly to proceed with inhibition of AMPK as proposed in our model.

2. This issue has been originally brought up by reviewer 2 and 3. In response to this query we answered the following: *Differences between Figure 7A and Figure 1A relate to the exposure time of the immunoblots. Longer exposure of blots of Figure 1A would have led to detection of a weak phosphorylation signal of AMPKa1 at Thr-172 after short-term isoproterenol treatment. Likewise, shorter exposure of immunoblots of Figure 7A would have revealed weaker signals of phosphorylated Thr-172 after short term isoproterenol treatment. We now show that 30 minutes isoproterenol treatment did not affect Thr-172 phosphorylation of AMPKa1 nor ACC phosphorylation. Thus, in the revised manuscript we have included the new experiment in Figure 7A to clarify this issue.* Reviewer 2 stated that we have satisfactorily addressed the issue. Reviewer 3 brings up this issue again without referring to our response. Possibly, he/she has overlooked our response.

3. In the revised version 2 of our ms we now present AMPK activity as percentage of untreated control as suggested by the reviewer.

4. It is important to note that the experiment presented in Figure 6 was done by first differentiating MEFs derived from AMPKα1 and α2 double ko mice into adipocytes and then infecting them with the indicated recombinant viruses. This was stated in the Methods section and the Figure legend. The reason for choosing this strategy is simple: we wanted to prevent any possibly interference of wild-type AMPK versus S173 mutant protein expression with the differentiation process. Since the experiment was performed as stated above, and for each infection the same differentiated cells were used, lipid accumulation is identical in each setting. If we would have performed the experiment the other way round, then the question of reviewer 3 would be a relevant one.

5. Minor comment: We thank reviewer 3 for pointing out this issue. We have corrected this in the ms, supplementary material and figure labels.

We have also moved part of the Materials and Methods section to Supplementary information in order to meet the 55,000 character limit.

I hope that our response and revisions meet your satisfaction and am looking forward to hearing from you.

