Defining the Mechanism of Activation of AMP-activated Protein Kinase by the Small Molecule A-769662, a Member of the Thienopyridone Family*  

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AMP-activated protein kinase (AMPK) plays a key role in maintaining energy homeostasis. Activation of AMPK in peripheral tissues has been shown to alleviate the symptoms of metabolic diseases, such as type 2 diabetes, and consequently AMPK is a target for treatment of these diseases. Recently, a small molecule activator (A-769662) of AMPK was identified that had beneficial effects on metabolism in ob/ob mice. Here we show that A-769662 activates AMPK both allosterically and by inhibiting dephosphorylation of AMPK on Thr-172, similar to the effects of AMP. A-769662 activates AMPK harboring a mutation in the γ subunit that abolishes activation by AMP. An AMPK complex lacking the glycogen binding domain of the β subunit abolishes the allosteric effect of A-769662 but not the allosteric activation by AMP. Moreover, mutation of serine 108 to alanine, an autophosphorylation site within the glycogen binding domain of the β1 subunit, almost completely abolishes activation of AMPK by A-769662 in cells and in vitro, while only partially reducing activation by AMP. Based on our results we propose a model for activation of AMPK by A-769662. Importantly, this model may provide clues for understanding the mechanism by which AMP leads to activation of AMPK, which in turn may help in the identification of other AMPK activators.

Maintaining energy balance is a key process at both the level of the individual cell and the whole body. In mammals, defects in energy homeostasis underlie the development of metabolic diseases, including type 2 diabetes and obesity, the incidence of which is increasing at a significant rate in humans. Understanding the molecular basis for energy balance is a prerequisite for developing new strategies, including pharmacological intervention, for combating the rise in these metabolic diseases. An important component in the regulation of energy homeostasis that has emerged over the last few years is the AMP-activated protein kinase (AMPK)* pathway. AMPK is a heterotrimeric protein kinase complex that acts as an energy sensor, responding to a rise in AMP levels by increasing ATP-generating pathways and reducing ATP-consuming pathways (1–3). Initially, AMPK was considered primarily as a gauge of energy status at the cellular level (4), and consistent with this idea, orthologues of AMPK have been identified in single cell eukaryotes, such as Saccharomyces cerevisiae (1). Accumulating evidence indicates, however, that in mammals AMPK regulates whole body energy homeostasis acting in metabolic tissues in response to nutrient and hormonal signals. For instance, the adipokines leptin and adiponectin activate AMPK stimulating fatty acid oxidation in liver and muscle (5, 6), while suppressing hepatic glucose production (6, 7). In addition to its peripheral effects, AMPK has been implicated in the central control of energy balance. Activation of AMPK in the hypothalamus has been reported to stimulate food intake, whereas inhibition leads to reduced food intake (8–11). However, a recent study has reported that in mice a genetic deletion of AMPK in pro-opiomelanocortin neurons leads to an obese phenotype (12), conflicting with the results of previous studies.

Many of the downstream effects of AMPK are predicted to be beneficial in treating, and potentially preventing, aspects of metabolic diseases. Consistent with this hypothesis, 5-aminimidazole-4-carboxamide riboside, a compound that results in activation of AMPK in cells and in vivo, improved insulin sensitivity in animal models of insulin resistance (13–15). Furthermore, metformin, which has been used for nearly 50 years as an anti-diabetic drug and is currently estimated to be used by over 120 million people, activates AMPK via an indirect mechanism (16). It was reported recently that the glucose lowering effect of metformin requires hepatic expression of LKB1, an upstream kinase in the AMPK pathway, providing further evidence that in liver metformin acts via activation of AMPK (17). Taken together, these results indicate that activation of AMPK may provide an effective means for treatment of metabolic disorders.

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6 The abbreviations used are: AMPK, AMP-activated protein kinase; CaMKK, Ca2+/calmodulin-dependent protein kinase kinase; GBD, glycogen binding domain.
Activation of AMPK requires phosphorylation on Thr-172 within the α subunit (18, 19). To date, four upstream kinases have been identified that phosphorylate Thr-172 as follows: LKB1 (20, 21), CaMKKα/β (22–24), and transforming growth factor β-activated kinase-1 (25). In addition to phosphorylation, AMPK is allosterically activated by AMP. It was originally
proposed that AMP promoted phosphorylation of Thr-172 by the upstream kinases (26), although subsequent studies have demonstrated that this is not the case (27, 28). Nonetheless, AMP does increase the phosphorylation of AMPK, but this occurs by inhibiting dephosphorylation of Thr-172 through a substrate-mediated effect (27). The mechanism by which AMP inhibits Thr-172 dephosphorylation is not known, but mutations in the γ subunit that reduce the allosteric activation by AMP also reduce the effect of AMP on dephosphorylation (27).

Recently, Cool et al. (29) described the identification of A-769662, a small molecule activator of AMPK. A-769662 was shown to directly activate AMPK, and in vivo administration of the compound in ob/ob mice lowered plasma glucose by 40%, reduced body weight gain, and significantly decreased both plasma and liver triglyceride levels (29). These findings further support the hypothesis that activation of the AMPK pathway in vivo is a viable approach for treatment of metabolic diseases in humans.

In this study, we have investigated the mechanism of activation of AMPK by A-769662, the first small molecule direct pharmacological activator of AMPK to be identified (29). Elucidating this mechanism may help in the design of more potent AMPK activators and may help our efforts to understand the mechanism of activation of AMPK by AMP. We show that like AMP, A-769662 inhibited dephosphorylation of Thr-172, as well as allosterically activating AMPK. However, we present data demonstrating that A-769662 activates AMPK through a mechanism that is distinct from AMP activation.

**EXPERIMENTAL PROCEDURES**

**Materials**—STO-609 was from Tocris (Ellisville, MO). BL21-Codon-Plus (DE3)-RIL competent cells were obtained from Novagen.

**Site-directed Mutagenesis**—The point mutations (S24A, S108A S108D, and S182A) were introduced into the β1 subunit using the QuikChange® site-directed mutagenesis kit (Stratagene) according to the manufacturer’s protocol. Mutations were verified by DNA sequencing.

**AMPK/SNF1 Assays**—Recombinant AMPK complexes and SNF1 were expressed in *Escherichia coli* and purified as described previously (27). AMPK and SNF1 activity were determined by phosphorylation of the SAMS peptide (30) in the presence or absence of 150 μM AMP and/or 10 μM A-769662, as stated in the figure legends. Results shown are presented as specific activity (nmol/min/mg of protein).

**Western Blot Analysis**—Phosphorylation of Thr-172 (AMPK) and Thr-210 (SNF1) were determined using a rabbit anti-phospho-Thr-172 specific antibody (Cell Signaling). Total AMPK was detected using sheep anti-AMPK antibody (Abcam). Primary antibodies were detected using LI-COR IRDye® infrared dye secondary antibodies and visualized using an Odyssey Infrared Imager (LI-COR Biotechnology). Quantification of results was performed using Odyssey software and expressed as a ratio of the signal obtained with the phospho-specific antibody relative to the appropriate total antibody.

**Dephosphorylation of AMPK and SNF1**—Recombinant AMPK or SNF1 complexes were phosphorylated by CaMKKβ as described previously (27). An aliquot of the phosphorylated AMPK or SNF1 was incubated in 50 mM HEpes, pH 7.4, 2.5 mM MgCl₂ in the presence or absence of recombinant PP2Ca (26 ng) and the presence or absence of 150 μM AMP and/or 10 μM A-769662 for 20 min at 37 °C. Reactions were terminated by the addition of SDS-gel loading buffer. Samples were resolved by SDS-PAGE and subjected to Western blot analysis.

**Rat Liver AMPK**—Rat liver AMPK was purified up to the DEAE-Sepharose step (32) and treated as reported previously (27). Partially purified AMPK was incubated in the presence or absence of 2.5 mM MgCl₂ and the presence or absence of 150 μM AMP and/or 10 μM A-769662 for 10 min at 37 °C. Aliquots were removed for Western blot analysis.

**Mammalian Cell Culture**—HEK293 and CCL13 cells were grown at 37 °C with 5% CO₂ in Dulbecco’s modified Eagle’s medium (4500 mg/liter glucose with sodium pyruvate and pyridoxine) supplemented with 10% fetal calf serum and 1 mM glutamine. Cells were transfected into serum-free media for 2 h prior to treatment with 1 mM H₂O₂ (for 15 min) or varying concentrations of A-769662 for 1 h. Cells were washed briefly with ice-cold phosphate-buffered saline before rapid lysis in ice-cold buffer (50 mM HEPES, pH 7.4, containing protease inhibitor mixture (Roche Applied Science), 1 mM EDTA, 10% glycerol (v/v), 50 mM NaF, and 1% (v/v) Triton X-100). Insoluble material was removed by centrifugation at 10,000 × g for 10 min at 4 °C, and the supernatant was used for subsequent analysis.

**Transient Transfection of HEK293 Cells**—Plasmid DNA was prepared using a Qiagen maxiprep kit according to the manufacturer’s instructions. Cells were transfected by Ca²⁺-phosphate precipitation with 10 μg of each plasmid encoding myc-α1 and FLAG-γ1 subunits (33) and either wild-type β1 or β1 harboring the S108A mutation. cDNA encoding the β1 subunit was cloned into a vector to allow expression of a

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**FIGURE 1.** A-769662 allosterically activates AMPK and protects against dephosphorylation of Thr-172. A recombinant αβ γ1, αβ γ1, or SNF1 (SNF1, Snf4, and Gal83) complexes were activated by phosphorylation using recombinant CaMKKβ (29) for 20 min at 37 °C. Activity was determined using the SAMS peptide assay in the absence (white) or presence of 150 μM AMP (gray), 10 μM A-769662 (hatched), or both (black). Results shown are the mean ± S.E. for four independent experiments and are plotted as nmol/min/mg β. Active recombinant complexes were incubated in the presence of 2.5 mM MgCl₂ and in the presence or absence of recombinant PP2Ca, 150 μM AMP, and/or 10 μM A-769662 for 20 min at 37 °C. Reactions were terminated in SDS sample buffer and subjected to Western blot analysis using anti-phospho-Thr-172 (pThr-172) and either anti-α1, α2, or His antibodies. Bar charts showing Thr-172 phosphorylation relative to control (absence of PP2Ca, AMP, and A-769662) are shown and are the means ± S.E. for three independent experiments. In each case, a representative blot is shown below the graphs. C, partially purified rat liver AMPK was incubated in the presence or absence of 2.5 mM MgCl₂ plus or minus 150 μM AMP and/or 10 μM A-769662 for 15 min at 37 °C. Reactions were terminated in SDS sample buffer and subjected to Western blot analysis using anti-phospho-Thr-172 and a mixture of anti-α1 and α2 antibodies. A representative blot is shown alongside a graph showing the quantification of the relative level of Thr-172 phosphorylation for three independent experiments ± S.E. A-769662 inhibited Thr-172 dephosphorylation to a significantly greater extent than AMP (p < 0.05).
Small Molecule Activation of AMPK

**RESULTS**

**Activation of AMPK by A-769662**—The small molecule AMPK activator A-769662 was synthesized following identification of a related thienopyridone (A-592107) in a screen of over 700,000 compounds using partially purified AMPK from rat liver (29). In that study, A-769662 was shown to directly activate AMPK, even though its structure shows no obvious similarity with AMP. We have investigated the mechanism of activation of AMPK by A-769662 because elucidating this mechanism might help in the design of more potent activators of AMPK. Furthermore, defining the mechanism of activation by A-769662 may help in our efforts to understand the mechanism of activation of AMPK by AMP. Fig. 1A shows that A-769662 activated highly purified preparations of recombinant αβγ and αβγ AMPK complexes (α1, 1.8 ± 0.16-fold; α2, 1.9 ± 0.25-fold; n = 4). In addition, A-769662 inhibited dephosphorylation of Thr-172 (Fig. 1B). In both cases, the maximal effects of A-769662 were similar to those obtained with AMP. By using our recombinant preparations of AMPK we saw no evidence for additivity between A-769662 and AMP. By using our recombinant preparations of AMPK we saw no evidence for additivity between A-769662 and AMP, in contrast to a previous study (29). A-769662, like AMP, had no direct effect on activating yeast SNF1 (Fig. 1A) or on dephosphorylation of Thr-210, the equivalent residue to Thr-172 in AMPK (Fig. 1B). A-769662 inhibited dephosphorylation of Thr-172 using AMPK isolated from rat liver, and in this case, A-769662 had a significantly greater effect than AMP (Fig. 1C).

Activation of AMPK requires phosphorylation of Thr-172 by an upstream kinase, and under most of the conditions examined to date either LKB1 or CaMKKβ appear to mediate this phosphorylation. To assess which upstream kinase is involved in activation of AMPK by A-769662, we measured activation in cells that either express or lack LKB1 (Fig. 2). In HEK293 cells, which express LKB1, maximal activation of AMPK by A-769662 was observed at 200 μM concentration and was similar to the activation elicited by treatment with 1 mM H₂O₂ (Fig. 2A). Similarly, A-769662 also induced a dose-dependent activation of AMPK in CCL13 cells (data not shown), which do not express LKB1 (21). Prior incubation of CCL13 cells with STO-609, to inhibit CaMKK, almost completely abolished AMPK

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**FIGURE 2. A-769662 activates endogenous AMPK in LKB1-expressing (HEK293) and LKB1-deficient (CCL13) cells.** 

A, HEK293 cells were treated with increasing concentrations of A-769662 for 1 h or 1 mM H₂O₂ for 15 min. Endogenous AMPK was immunoprecipitated from 200 μg of total protein using an anti-pan-AMPK antibody, and activity in the immune complexes was measured using the SAMS peptide assay in the presence of 150 μM AMP. Ctrl, control. CCL13 cells (B) and HEK293 cells (C) were incubated in the presence or absence of STO-609 (10 μg/ml) for 2 h and then treated with either A-769662 (200 μM) for 1 h or H₂O₂ (1 mM) for 15 min, and AMPK activity was determined as above. Results are shown as AMPK activity (μmol/min/mg lysate) and are the mean ± S.E. of three independent experiments. D, recombinant α1β1γ1 and α2β1γ1 were incubated in the presence or absence of limiting amounts of CaMKKβ or LKB1 plus or minus 150 μM AMP or 10 μM A-769662 for 10 min at 37 °C. Samples were subjected to Western blot analysis using anti-Thr(P)-172 and anti-α antibodies.

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**A-769662**

fused protein with green fluorescent protein (N-terminal of β1). 30 h post-transfection cells were harvested as described above.

**Immunoprecipitation of AMPK from Mammalian Cells**—Endogenous AMPK was immunoprecipitated using a rabbit anti-pan-β antibody (34). Recombinant AMPK from transfected cells was immunoprecipitated using an anti-Myc antibody (Upstate). Immune complexes were washed extensively, and AMPK activity was determined by the SAMS assay in the presence of 150 μM AMP. Ctrl, control. CCL13 cells (B) and HEK293 cells (C) were incubated in the presence or absence of STO-609 (10 μg/ml) for 2 h and then treated with either A-769662 (200 μM) for 1 h or H₂O₂ (1 mM) for 15 min, and AMPK activity was determined as above. Results are shown as AMPK activity (μmol/min/mg lysate) and are the mean ± S.E. of three independent experiments. D, recombinant α1β1γ1 and α2β1γ1 were incubated in the presence or absence of limiting amounts of CaMKKβ or LKB1 plus or minus 150 μM AMP or 10 μM A-769662 for 10 min at 37 °C. Samples were subjected to Western blot analysis using anti-Thr(P)-172 and anti-α antibodies.

**Statistical Analysis**—Results are expressed as mean values ± S.E. Statistical analysis was carried out using a two-tailed, unpaired Student’s t test.
activation by either A-769662 or \( \text{H}_2\text{O}_2 \) (Fig. B). In HEK293 cells STO-609 did not have any significant effect on activation by either A-769662 or \( \text{H}_2\text{O}_2 \) (Fig. 2C). These results suggest that A-769662 can activate AMPK via both LKB1 and CaMKK pathways. As was reported previously (29), we did not detect a change in adenine nucleotide levels following treatment with A-769662 (data not shown). As shown in Fig. 2D, A-769662 has no direct effect on phosphorylation of AMPK by either LKB1 or CaMKKβ in a cell-free assay. The simplest interpretation from these results is that A-769662 activates AMPK in intact cells by inhibiting dephosphorylation of Thr-172.

Identification of Residues within AMPK Involved in Activation by A-769662—We reported previously that mutations in \( \alpha_1 \)-containing complexes, activation of the mutant by A-769662 is reduced relative to the wild-type complex (wild-type, 1.9 ± 0.3-fold; R298G mutant, 1.4 ± 0.1-fold; \( n = 4 \)). A-769662 inhibits dephosphorylation of Thr-172 in the mutant \( \gamma_1 \)-containing complexes to a similar degree as seen in the wild-type complexes (cf. Figs. 1B and 3B). Consistent with our previous study, AMP had no effect on the mutant \( \gamma_1 \) complexes (27).

To investigate the mechanism of action of A-769662 further, we examined its effect on the isolated catalytic domain of the \( \alpha_1 \) subunit (residues 1–312). This fragment does not bind the \( \beta \) or \( \gamma \) subunits but is active following phosphorylation of Thr-172, although it is not activated allosterically by AMP (18). A-769662 had no direct effect on the activity of the isolated kinase domain (Fig. 4A) or on dephosphorylation of Thr-172 (data not shown). These findings exclude the possibility that A-769662 acts directly on the catalytic domain. Rather, these results suggest that like AMP, A-769662 exerts its effect by binding to a regulatory region within the AMPK complex.

We next measured the effect of A-769662 on an AMPK complex containing a truncated \( \beta \) subunit lacking the N-terminal 185 residues (\( \beta_1-(186–270) \)). Previous studies have shown that this C-terminal region of the \( \beta \) subunit is sufficient to bind to the \( \alpha \) and \( \gamma \) subunits, yielding a functional AMPK complex that is activated by AMP (36, 37). We expressed this complex (\( \alpha_1 \beta_1-(186–270) \)-\( \gamma_1 \)) in bacteria, activated it by phosphorylation with CaMKKβ, and measured its activity in the presence and absence of A-769662. Although activation by AMP was maintained, as reported previously (36, 37), allosteric activation by A-769662 was almost completely abolished (Fig. 4B).

The \( \beta_1 \) subunit has been shown to undergo post-translational modification \textit{in vivo}, and three phosphorylation sites in \( \beta_1 \) have been identified in AMPK isolated from rat liver (38). All three phosphorylation sites (serine 24, serine 108, and serine 182) lie within the region deleted in the \( \alpha_1 \beta_1-(186–270) \)-\( \gamma_1 \) complex. To investigate whether any of these sites are involved in the activation of AMPK by A-769662, we expressed AMPK complexes harboring serine to alanine substitutions for each of the three sites and assayed AMPK in
FIGURE 4. Activation of AMPK by A-769662 involves Ser-108 in the β1 subunit. A–D, recombinant proteins were expressed in E. coli, purified by nickel-Sepharose chromatography, and activated by phosphorylation with recombinant CaMKKβ for 20 min at 37 °C. In each case, AMPK activity was determined using the SAMS peptide assay in the absence (white) or presence of 150 μM AMP (gray), 10 μM A-769662 (hatched), or both (black). Results are plotted as specific activity (nmol/min/mg) and are the mean ± S.E. for four independent experiments. E, relative fold stimulation by AMP or A-769662 for the recombinant AMPK complexes is shown (means ± S.E. for four independent experiments). F, α1β1(S108A)γ1 mutant was used in a dephosphorylation assay, as described for Fig. 1B. Results are the mean ± S.E. for three independent experiments, and a representative blot is shown below the graph.
the presence and absence of the compound. As shown in Fig. 4C, mutation of either serine 24 or serine 182 to an alanine residue had no significant effect on allosteric activation by either A-769662 or AMP. In marked contrast, mutation of Ser-108 to alanine almost completely abolished allosteric activation by A-769662, while having no significant effect on AMP. In addition, mutation of Ser-108 to an aspartic acid residue had no significant effect on allosteric activation by A-769662 or H2O2 (Fig. 5). The basal activity of AMPK was reduced by ~45% in the S108A mutant complex, similar to that reported in a previous study (39). H2O2 activated the S108A mutant complex by a similar degree as the wild type, although as with the basal condition, the specific activity of the S108A mutant was ~45% that of the wild-type complex (Fig. 5A). In contrast, activation by A-769662 was virtually abolished. Thr-172 phosphorylation was not increased by A-769662 in the S108A complex, whereas oxidative stress caused a significant increase in phosphorylation in this mutant relative to basal levels, consistent with the increase in AMPK activity.

**DISCUSSION**

In this study we examined the mechanism of activation of AMPK by a recently discovered small molecule AMPK activator, A-769662. Like AMP, A-769662 inhibits dephosphorylation of Thr-172 as well as providing modest allosteric activation. Importantly, however, A-769662 activates AMPK harboring a mutation within the γ subunit that abolishes activation by AMP. This result strongly suggests that the mechanisms of activation by AMP and A-769662 are distinct. In addition, we found that mutation of Ser-108 to alanine within the β1 subunit completely abolished activation by A-769662, while only partially reducing AMP activation, providing further evidence that the mechanisms are different. Previously, Ser-108 was shown to undergo autophosphorylation (38, 40), and it was reported that mutation of Ser-108 to alanine reduced AMPK activation by ~60% relative to the wild type, following expression in COS cells (39). In the same study it was reported that the reduction in activity was not correlated with reduced Thr-172 phosphorylation but might be explained by changes in subunit interaction in the AMPK heterotrimer (39). In this study, we
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also observed a marked reduction in the activity of AMPK harboring the S108A mutation compared with wild type, in both basal and H$_2$O$_2$-treated cells. However, in contrast to the study by Warden et al. (39), the reduction in activity that we observed correlated closely with decreased phosphorylation of Thr-172 in the S108A mutant complex compared with wild-type AMPK (~25% decrease in basal phosphorylation and ~40% decrease in H$_2$O$_2$-treated cells). Our results suggest that in addition to being essential for activation by A-769662, Ser-108 in $\beta$ is also involved in the endogenous cellular activation of AMPK by other signaling pathways. Consistent with this possibility, we found that the S108A mutation reduced the inhibitory effect of AMP on dephosphorylation of Thr-172 in vitro.

A key step in the activation of AMPK is phosphorylation of Thr-172 within the $\alpha$ subunit, because in the absence of this phosphorylation, AMPK is inactive (18, 19). To date, four upstream kinases have been identified that phosphorylate Thr-172 in vitro, although based on the current evidence available, LKB1 and CaMKK$\beta$ account for the majority of the upstream kinase activity detectable in cells and in vivo (17, 20–22, 24, 41–44). A-769662 activated AMPK both in cells that express LKB1 and that lack LKB1, without altering adenine nucleotide levels (see Ref. 29 and data not shown). In cells lacking LKB1, activation of AMPK by A-769662 was almost completely blocked by the CaMKK inhibitor, STO-609, suggesting that in these cells CaMKK$\beta$ is responsible for Thr-172 phosphorylation. In contrast, STO-609 had no effect on AMPK activation by A-769662 in cell expressing LKB1. In cell-free assays, A-769662 had no direct effect on the ability of either LKB1 or CaMKK$\beta$ to phosphorylate AMPK. The simplest interpretation of these results is that in cells, similar to its effects in vitro, A-769662 inhibits dephosphorylation of Thr-172, and acts independently of the upstream kinase involved in the signaling pathway. This is analogous to the mechanism that we proposed recently for activation of AMPK by AMP (27). An additional finding to emerge from our present study is that to account for the activation of AMPK by A-769662 in CCL13 cells, which lack LKB1, there must be sufficient basal activity of CaMKK$\beta$ to phosphorylate AMPK. An alternative explanation would be that another Thr-172 kinase, which is also inhibited by STO-609, is present in these cells. Previous studies have shown that although CaMKK$\beta$ activity is increased by a rise in intracellular calcium, there is detectable activity under basal conditions (45), making it the most likely candidate for phosphorylation of Thr-172 in our system.

Based on our results, we propose a model for activation of AMPK by A-769662 (Fig. 6). In this model, binding of A-769662 to AMPK stabilizes a conformation that is resistant to dephosphorylation of Thr-172. This conformation requires phosphorylation of Ser-108 within $\beta$ because mutation of this residue to alanine completely abolishes activation of AMPK by A-769662. A model that we favor is that phospho-Ser-108 interacts with another region of the AMPK heterotrimer, and this interaction is promoted by A-769662. At present, we do not know the binding site for A-769662 within the AMPK complex. It is interesting to note, however, that serine 108 lies within a region of the $\beta$ subunit that has been termed a glycogen binding domain (GBD) due to the fact that it shares significant amino acid sequence similarity with a number of other proteins involved in the metabolism of glucans, such as starch and glycogen (36, 46). Although the function of the GBD in AMPK remains enigmatic, it has been shown to bind directly to the cyclic sugar $\beta$-cyclodextrin (47). Therefore, it is conceivable that the GBD could bind other molecules, such as A-769662. Another key question raised by our model is what is the nature of the interaction between phospho-Ser-108 and the AMPK complex. An intriguing possibility stems from the recently solved structure of a fragment of the fission yeast homologue of AMPK, containing the $\gamma$ subunit and the C-terminal regions of the $\alpha$ and $\beta$ subunits (48). In this structure, which lacked the GBD of $\beta$, it was reported that the $\gamma$ subunit binds one molecule of either AMP or ATP. Remarkably, no major structural changes attributable to bound nucleotide were detected (48). Therefore, the difference in nucleotide binding seems likely to be associated with the difference in charge between the phosphate groups on AMP and ATP. An attractive hypothesis for the activation mechanism would be that in the AMP-bound form a binding site is available for a phosphate group(s) from phosphorylated residues within the $\alpha$ and/or $\beta$ subunits that would stabilize a particular conformation of the complex. In the ATP-bound form, this interaction would be significantly weakened. In our model we would predict that A-769662 stabilizes interaction of phospho-Ser-108 with the $\gamma$ subunit, even in the presence of ATP. In this conformation, dephosphorylation of Thr-172 is inhibited, possibly via simple steric hindrance caused by a rearrangement of the $\beta$ subunit (as depicted in Fig. 6). Although the mechanism for activation of AMPK by A-769662 is distinct from that of AMP, it is possible that the
model we have proposed may provide clues for understanding the mechanism of activation by AMP. Mutation of serine 108 caused a partial reduction on the effect of AMP on dephosphorylation of Thr-172 in vitro. Furthermore, this mutation also reduced AMPK activity and Thr-172 dephosphorylation of Thr-172 in vivo [14]. It is conceivable that other phosphorylation sites within the AMPK-122, seven other phosphorylation sites within the α and β subunits have been identified [40], and further studies are needed to address this issue.

In general terms, activation of AMPK leads to an increase in catabolic pathways and a decrease in anabolic pathways (3). The effects of activation of AMPK in peripheral tissues, such as liver and skeletal muscle, include stimulation of fatty acid oxidation, increased glucose uptake and glycolysis, inhibition of fatty acid synthesis and cholesterol synthesis, and inhibition of hepatic gluconeogenesis. All of these effects would be beneficial in treating aspects of the metabolic syndrome (9). Characterization of the mechanism of action of A-769662 may help in identifying other small molecule activators of AMPK, as well as providing important clues regarding the physiological activation of AMPK by AMP.

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