Cell PRESS
Chemistry & Biology
Article

Thienopyridone Drugs Are Selective Activators of AMP-Activated Protein Kinase β1-Containing Complexes

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DOI 10.1016/j.chembiol.2008.10.005

SUMMARY

The AMP-activated protein kinase (AMPK) is an αβγ heterotrimer that plays a pivotal role in regulating cellular and whole-body metabolism. Activation of AMPK reverses many of the metabolic defects associated with obesity and type 2 diabetes, and therefore AMPK is considered a promising target for drugs to treat these diseases. Recently, the thienopyridone A769662 has been reported to directly activate AMPK by an unexpected mechanism. Here we show that A769662 activates AMPK by a mechanism involving the β subunit carbohydrate-binding module and residues from the γ subunit but not the AMP-binding sites. Furthermore, A769662 exclusively activates AMPK heterotrimers containing the β1 subunit. Our findings highlight the regulatory role played by the β subunit in modulating AMPK activity and the possibility of developing isoform specific therapeutic activators of this important metabolic regulator.

INTRODUCTION

The AMP-activated protein kinase (AMPK) is a key regulator of cellular and whole-body energy balances that coordinates metabolism in response to changes in energy supply and demand. At the cellular level, AMPK protects against stresses that lower energy charge by switching metabolism in favor of ATP production while inhibiting ATP consumption. AMPK regulates virtually every branch of cellular metabolism including fatty acid and sterol synthesis, lipid oxidation, protein synthesis, and carbohydrate metabolism. It has also become evident that AMPK plays a pivotal role in regulating whole-body energy homeostasis, integrating hormonal and nutritional signals in multiple tissues to regulate food intake and body weight.

AMPK functions as a heterotrimer comprising an α catalytic subunit and regulatory β and γ subunits. The α subunit consists of an N-terminal catalytic domain and a C-terminal domain involved in complex formation (Crute et al., 1998). The conserved C-terminal domain of the β subunit functions as a scaffold binding the α and γ subunits (Iseli et al., 2005; Townley and Shapiro, 2007). The β subunits also contain an internal carbohydrate-binding module (CBM; previously termed GBD) that is similar to domains found in other carbohydrate-binding proteins such as glycogen branching enzyme and isoamylase (Hudson et al., 2003; Polekhina et al., 2003). The CBM serves to associate AMPK complexes with glycogen particles in vitro and in intact cells; however, the functional significance of this interaction remains poorly understood. The γ subunits of AMPK contain four tandem repeats of a sequence known as CBS motifs that function in pairs to form discrete structural units termed Bateman domains, which are the allosteric binding sites for AMP and ATP (Scott et al., 2004). AMPK is principally activated by phosphorylation of Thr172 within the activation loop of the α catalytic domain by multiple protein kinases including LKB1, Ca^{2+}/calmodulin-dependent protein kinase kinase β (CaMKKβ), and possibly transforming growth factor β kinase (TAK1) (reviewed in Hardie, 2007). Binding of AMP to the γ subunits activates AMPK by two distinct mechanisms involving direct allosteric activation and inhibition of dephosphorylation by protein phosphatases (Sanders et al., 2007b).

Activation of AMPK in vivo elicits a number of metabolic responses that would be expected to counteract the physiological abnormalities associated with obesity and type 2 diabetes, and consequently AMPK is regarded as one of the most promising targets for new drugs to tackle the growing epidemic of metabolic diseases. As proof of concept, pharmacological activation of AMPK with AICAR (which is converted in cells to the AMP mimetic, ZMP) in animal models of insulin resistance reverses many of the hallmarks indicative of the metabolic syndrome (Bergeron et al., 2001; Song et al., 2002). Moreover, AMPK is activated indirectly by two classes of glucose-lowering drugs, the biguanides and thiazolidindiones, which are prescribed worldwide (Fryer et al., 2002; Zhou et al., 2001). As part of ongoing efforts to identify new activators of AMPK, it was reported that a small-molecule thienopyridone, A769662, directly activates AMPK (Cool et al., 2006). Two recent studies demonstrated that A769662 mimics both effects of AMP on the AMPK system but via an AMP-independent mechanism, suggesting that A769662 binds to an alternate allosteric site (Goransson et al., 2007; Sanders et al., 2007a). Furthermore, the β subunit and in particular Ser108 within the CBM, but not the glycogen-binding site, were found to be critical for activation. Despite these advances in our understanding, the binding site for A769662 remains to be elucidated.

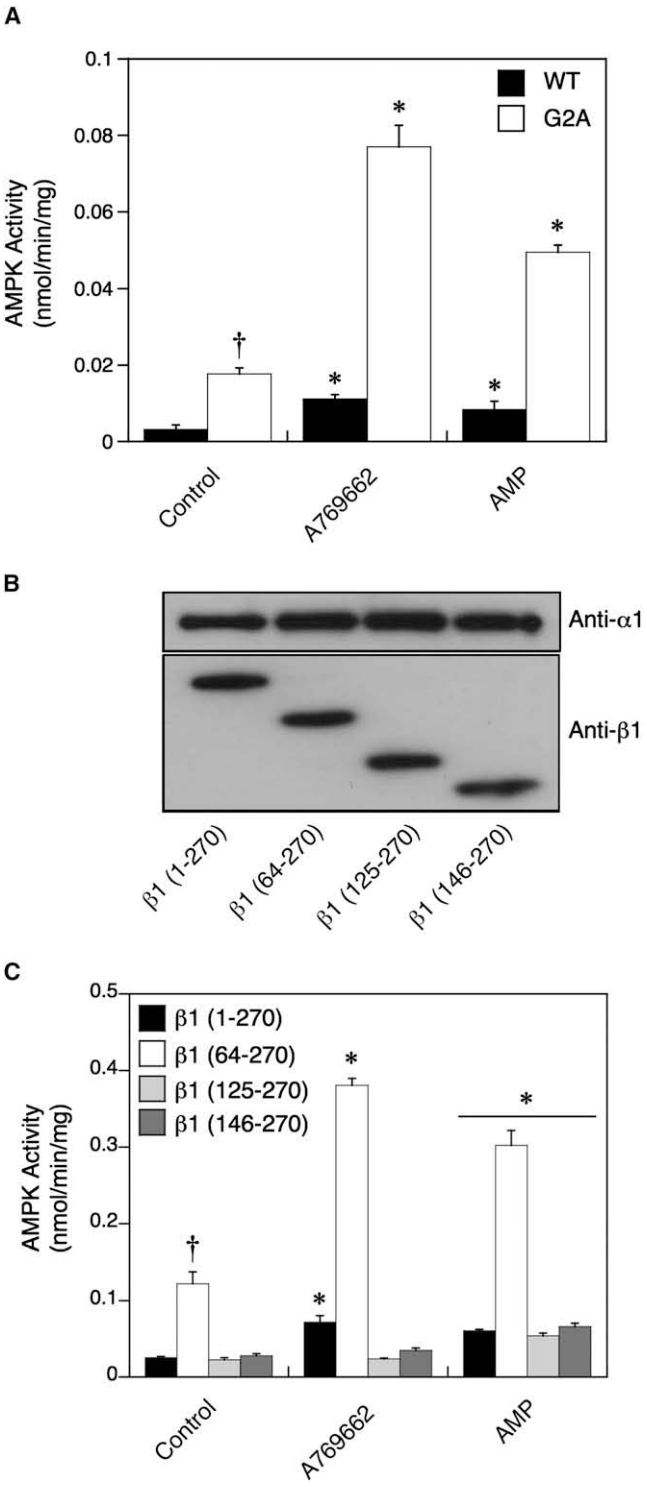

Figure 1. The β1 Subunit Carbohydrate-Binding Module Is Essential for Activation by A769662

(A) The effect of β1 subunit myristoylation on activation of AMPK by A769662 was determined using the nonmyristoylated β1 G2A mutant. AMPK activity was measured using the SAMS assay in the absence or presence of either 20 μM A769662 or 100 μM AMP and shown as the mean ± SEM for duplicate experiments.

(B) Analysis of expression of β1 truncated complexes by western blot. Ten microliters of AMPK immobilized on glutathione-Sepharose beads was separated on SDS-PAGE. Membranes were probed with rabbit polyclonal anti-α1 and rabbit monoclonal anti-β1 antibodies, and protein was detected using protein G-coupled horseradish peroxidase and enhanced chemiluminescence.

(C) The effect of β1 subunit deletions on activation of AMPK was measured using the SAMS assay in the absence or presence of either 20 μM A769662 or 100 μM AMP and results are shown as the mean ± SEM for duplicate experiments. * denotes significantly different from control ($p < 0.05$). † denotes significantly different from wild-type control ($p < 0.02$).

In the present study, we have investigated the activation of AMPK by A769662 using recombinant enzyme preparations and intact cells derived from wild-type and AMPK β1 null mice in order to gain further insights into its mechanism of action and its effects on cellular metabolism. We report that activation by A769662 involves an interaction between the β subunit CBM and residues from the γ subunit that are not involved in AMP binding. Unexpectedly, we found that A769662 exclusively activates AMPK complexes containing the β1 subunit isoform and, using hepatocytes generated from AMPK β1 null mice, found that the glucose-lowering effect of A769662 in these cells occurs independently of AMPK activation.

RESULTS

Defining the Region of the β1 Subunit Required for Activation by A769662

Previous studies showed that an AMPK complex lacking residues 1–185 of the β1 subunit is no longer activated by A769662, suggesting that either the uncharacterized N-terminal region of the β subunit, the CBM, or both are required for activation (Sanders et al., 2007a). The N-terminal extension of the β1 subunit is myristoylated on Gly2, and removal of the myristoyl group by point mutation activates AMPK (Warden et al., 2001); therefore, we initially investigated whether A769662 activates AMPK by a mechanism involving relieving inhibition caused by myristoylation. Figure 1A shows that the Gly2Ala mutant has approximately 4-fold increased basal activity compared with wild-type but is still activated by A769662 and AMP. These findings demonstrate that A769662 actions are independent of β1 myristoylation. Next, we generated a series of N-terminal deletions to more accurately define the region of the β1 subunit that is required for activation by A769662. Deletions were generated by PCR and coexpressed with GST-α1 and γ1 subunits in COS7 cells. Protein was corrected for expression by immunoblotting with an anti-α1 antibody and densitometry. We also used a monoclonal C-terminal β1 antibody to confirm that all truncations were successfully expressed (Figure 1B). Truncation of the first 64 residues substantially increased basal activity, but A769662-mediated activation was unaffected. Further deletions removing either half or the complete CBM abolished the effect of the drug (Figure 1C). Although deletion of the β1 residues 1–63 caused substantial activation, this effect was not retained in further C-terminal deletions (125–270 and 146–270).

A769662 Does Not Bind Directly to the Carbohydrate-Binding Module

Given that the CBM is necessary for activation by A769662, we speculated that A769662 might bind to a novel binding site on

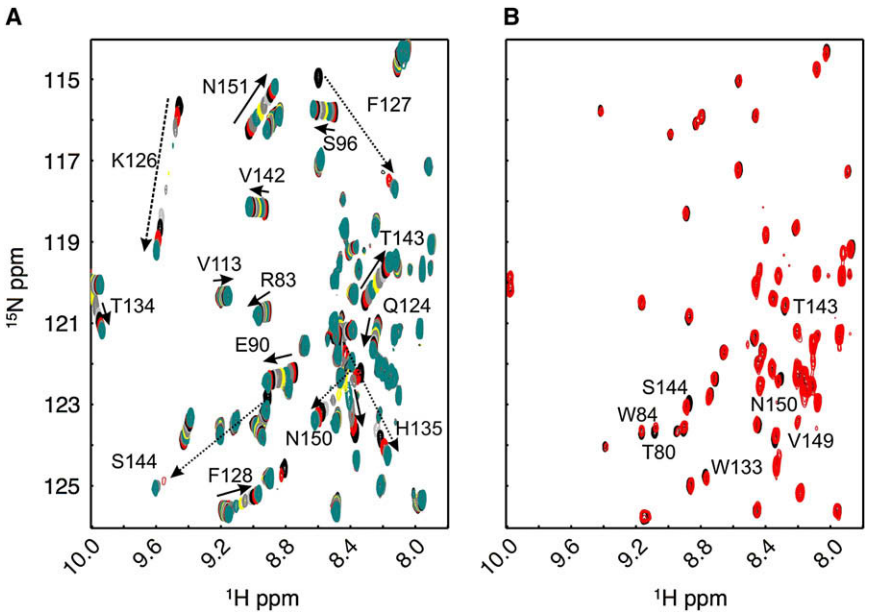

Figure 2. The Carbohydrate-Binding Module Is Not Sufficient to Bind A769662

(A) Overlayed two-dimensional ^{1}H-^{15}N HSQC spectra showing chemical shift changes in the nuclei of β1-CBM after the addition of 10 mM maltoheptaose. Labeled residues are those that showed significant chemical shift upon titration of ligand.

(B) Same as (A), except spectra were collected in the absence and presence of 5 mM A769662.

the CBM distinct from the carbohydrate-binding site. We explored this possibility using nuclear magnetic resonance (NMR) spectroscopy, which has been used previously to study the nature of sugar binding to the carbohydrate-binding site (Koay et al., 2007). A number of ^{1}H-^{15}N HSQC spectra were collected using labeled β1-CBM (0.2 mM) in the presence and absence of either 10 mM maltoheptaose or 5 mM A769662 in order to determine binding. Under these conditions, we detected significant changes in the chemical shift pattern in the presence of maltoheptaose (Figure 2A), such as Lys126 known to be involved in sugar binding from the crystal structure (Polekhina et al., 2005); however, there were negligible changes in the chemical shift distribution after the addition of A769662 (Figure 2B). Based on these results, we conclude that the CBM alone does not contain the A769662-binding site.

Identification of Residues in the γ Subunit Involved in Activation by A769662

In order to further understand the molecular mechanism of activation by A769662, we next investigated whether other regions of the AMPK complex in close proximity to the CBM influence A769662 activation. The crystal structure of the core fragment of the *Saccharomyces cerevisiae* AMPK ortholog (Protein Data Bank ID code 2QLV) shows a direct interaction between the carbohydrate-binding loop of the CBM and the γ subunit helix α2B (Amodeo et al., 2007). We therefore investigated whether residues from the γ subunit that are predicted to interact with the β1-CBM modulate A769662 activation. Because the crystal structure of the mammalian αβγ core complex does not include the CBM, we constructed a homology model for this region based on the mammalian heterotrimer using the *S. cerevisiae* structure as a template for positioning the CBM, and the resulting model for β1:CBM/β1:190–272/γ1:23–326 is shown in Figure 3A. Three residues (Phe175, Leu178, and Phe179) in helix α2B of γ1 form a hydrophobic patch, which is positioned to interact with the sugar-binding loop from β1-CBM (Figure 3B). In addition, γ1-Arg171 is positioned to enable hydrogen bonding with backbone Ser144 and Gln145 oxygens within the β1-CBM. Because A769662 is relatively lipophilic by virtue of the two aromatic rings attached to its thienopyridone core, this prompted us to investigate whether the γ1 residues that form this hydrophobic patch influence activation by the drug. We therefore generated a series of γ1 point mutants (Arg171Gln, Phe175-Tyr, Phe175Leu, Leu178Ala, Phe179Tyr, and Phe179Leu) that were coexpressed with GST-α1 and β1 in COS7 cells. Expression levels were assessed by western blot using an anti-α1 antibody, and protein levels were corrected for differences in expression by densitometry. Several studies have shown that mutation of residues in the AMP-binding sites render AMPK insensitive to the stimulatory effects of AMP while at the same time increasing basal activity in the absence of AMP (Burwinkel et al., 2005; Hamilton et al., 2001). We therefore initially tested the effect of the γ1 mutants on basal activity and the Thr172 phosphorylation status independently of any effect on allosteric activation. Figure 4A shows that the Phe175Tyr, Phe175Leu, and Phe179Tyr mutations increased phosphorylation of Thr172 and AMPK activity. In contrast, the Leu178Ala and Phe179Leu mutations reduced Thr172 phosphorylation and reduced AMPK activity, whereas Arg171Gln had no effect on basal phosphorylation (Figure 4A). We next measured allosteric activation of the γ1 mutants with either A769662 or AMP and found that all the mutants could be activated with either agonist to varying extents. The Phe175Tyr, Phe175Leu, and Phe179Tyr mutants all had reduced allosteric activation by both A769662 and AMP, whereas the Leu178Ala and Phe179Leu mutants had a similar fold activation to wild-type AMPK (Figure 4B). Although the Arg171Gln mutant had a similar activation with A769662 as wild-type enzyme, it had reduced stimulation with AMP. As it was apparent that the γ1 mutants with increased basal phosphorylation had reduced allosteric activation, we decided to measure allosteric activation of the AMPK α1β1γ1 complex at various time points after phosphorylation with CaMKKβ. Figure 4C shows that phosphorylation of AMPK with CaMKKβ for longer than 10 min causes a decrease in the ability of both A769662 and AMP to allosterically activate AMPK. This suggests that a higher degree of phosphorylation of AMPK is important in regulating sensitivity to allosteric activation; therefore, in order to study activation of the γ1 mutants by A769662 and AMP independently of their different phosphorylation states, the mutants were first dephosphorylated with λ phosphatase for 60 min and then reactivated with CaMKKβ for 10 min in order to obtain

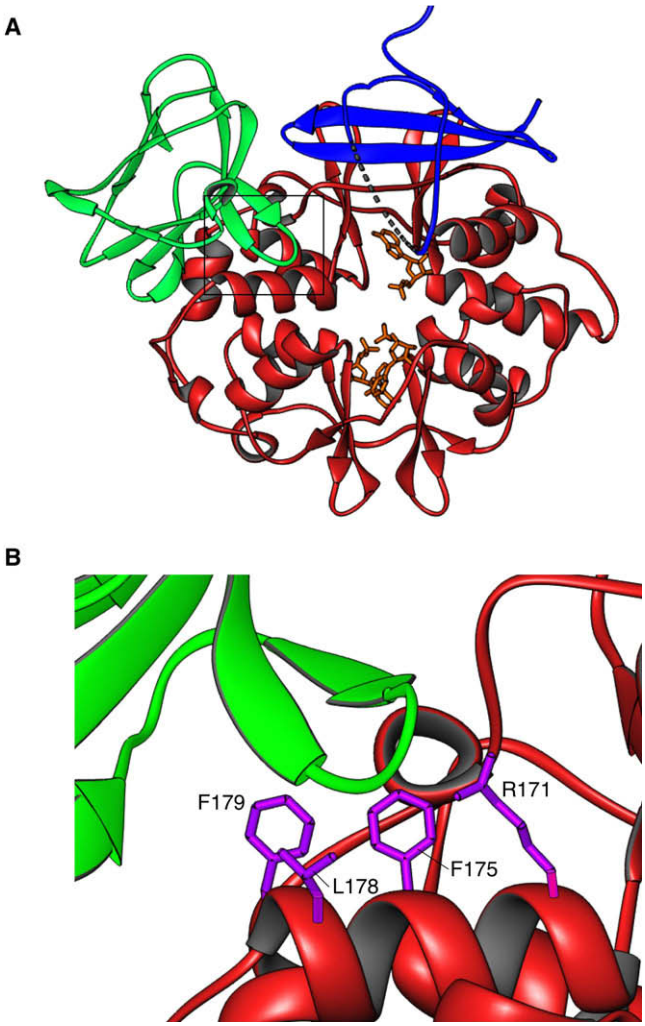

Figure 3. Structural Models of β1-CBM and the γ1 Subunit

(A) Structural model of β1:CBM (green)/β1:190–272 (blue)/γ1:23–326 (red) based on the *S. cerevisiae* Snf4/Sip2 complex structure. AMP nucleotides evident in the structure of the regulatory fragment of mammalian AMPK are in orange.

(B) Boxed region encompassing the γ1 helix α2B is shown in more detail, along with residues selected for mutation analysis in magenta.

similar Thr172 phosphorylation and activities in the absence of the activators (Figure 4D). Dephosphorylation and reactivation with CaMKKβ restores the A769662 and AMP dependence of the Phe175Tyr, Phe175Leu, and Phe179Tyr mutants to similar levels as wild-type AMPK (Figure 4D). Puzzlingly, the Phe179Leu mutant was partially resistant to phosphorylation by CaMKKβ, even up to incubation times of 60 min (data not shown); however, it is activated 2.7- and 2.8-fold by A769662 and AMP, respectively. As the CaMKKβ-treated γ1 mutants have similar dependence to A769662 and AMP as wild-type AMPK, with the exception of Arg171Gln, which still had reduced AMP stimulation, we next determined whether they had different sensitivities to A769662 and AMP by measuring activation over a range of concentrations. Two mutants, Leu178Ala and Phe179Leu, increased the concentration of A769662 required to give half-maximal activation (Figure 4E; see Table S1 available online), whereas the Arg171Gln and to a lesser extent the Phe175Leu mutant increased the dose dependence for AMP activation (Figure 4F; Table S1).

A769662 Specifically Activates AMPK Complexes Containing the β1 Subunit

Because AMPK subunit isoform composition is important in determining AMP sensitivity and dependence, we explored whether activation of AMPK by A769662 is influenced by the presence of a particular subunit isoform. We expressed all 12 αβγ combinations of AMPK complex in COS7 cells and measured activity in the presence of either 20 μM A769662 or 100 μM AMP. The fold activation of AMPK by either AMP or A769662 varied according to the subunit isoform composition. Figure 5A shows that only AMPK complexes containing the β1 subunit were allosterically activated by A769662 and that complexes containing the β2 subunit were completely insensitive to the drug. To test whether activation of AMPK by A769662 is specific to β1 complexes in intact cells, we utilized isolated hepatocytes from β1 null mice and measured AMPK activity in response to A769662 treatment. Mice with a germline deletion of the β1 subunit gene were generated as described (Figure S1). We confirmed by western blotting the presence of the β1 subunit in hepatocytes from wild-type mice but not in those obtained from β1 null mice (Figure 5B). In the β1 null hepatocytes, α1 and α2 expression was decreased; however, there was no change in the expression of the β2 subunit (Figure 5B). Similarly, α1 and α2 activity was decreased by 55% and 44% in the β1 null hepatocytes, respectively. Both α complexes were activated in wild-type hepatocytes treated with 100 μM A769662 or 2 mM AICAR; however, activation by A769662 was completely abolished in the hepatocytes lacking the β1 subunit (Figure 5C), supporting our in vitro findings. AICAR was still able to activate both α1 and α2 complexes from the β1 null hepatocytes, although to a lesser extent than in wild-type cells. We also analyzed phosphorylation of acetyl-CoA carboxylase (ACC), a downstream substrate of AMPK, in response to A769662 in the β1 null hepatocytes. Figure 5D shows that ACC phosphorylation was increased by treatment with either 2 mM AICAR or 100 μM A769662 in the wild-type cells; however, the effect of A769662 was lost in the β1 null hepatocytes, which is consistent with the AMPK activity data. Unlike A769662, AICAR increased ACC phosphorylation in the β1 null hepatocytes, although to a lower extent than that observed in wild-type cells. Previous studies have shown that A769662 lowers plasma glucose in obese mice, and this may be mediated primarily by activation of AMPK in the liver (Cool et al., 2006); therefore, we tested whether A769662 decreased glucose production in the β1 null hepatocytes. Wild-type and β1 null cells were incubated with either 2 mM AICAR or 100 μM A769662, and glucose production was measured at various time points up to 120 min. Figure 5E shows that basal glucose production in the β1 null hepatocytes was similar to wild-type cells. Glucose production in wild-type hepatocytes was decreased by 30% and 90% in response to A769662 and AICAR, respectively; however, A769662 lowered glucose production in the β1 null hepatocytes despite being unable to activate AMPK in these cells (Figure 5F). Our findings indicate that A769662 can also regulate hepatic glucose output by an AMPK-independent mechanism.

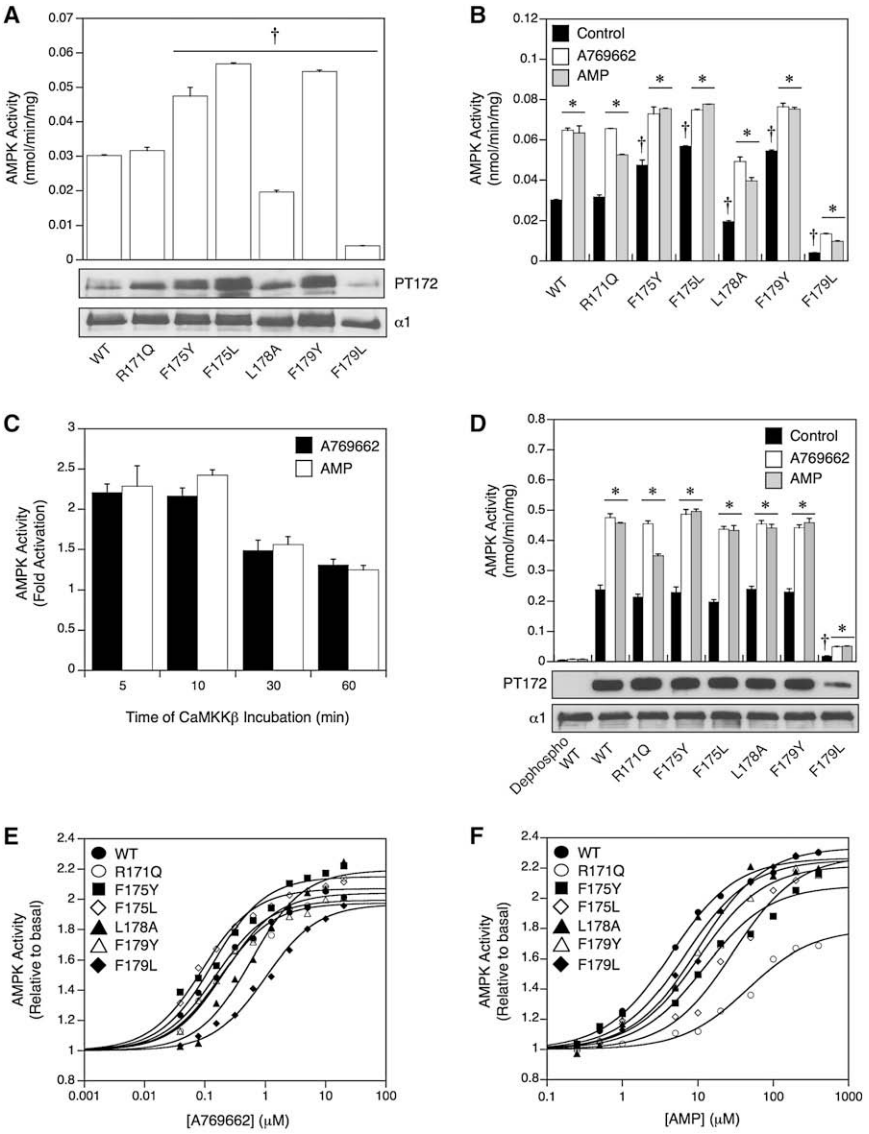

Figure 4. Activation of AMPK by A769662 Involves the γ Subunit

(A) Basal activity of AMPK γ1 subunit mutants was determined using the SAMS assay and by western blot using anti-phosphothreonine 172 and anti-α1 antibodies. Results are shown as the mean ± SEM for duplicate experiments.

(B) Activation of γ1 subunit mutants was determined using the SAMS assay in the presence or absence of 20 μM A769662 or 100 μM AMP. Results are shown as the mean ± SEM for duplicate experiments.

(C) Activation of the AMPK α1β1γ1 complex with either 20 μM A769662 or 100 μM AMP measured at different time points of incubation with CaMKKβ and 200 μM MgATP. AMPK activity was determined using the SAMS assay and results are shown as the mean ± SEM for duplicate experiments.

(D) Activation of γ1 subunit mutants with either 20 μM A769662 or 100 μM AMP after 10 min incubation with CaMKKβ and 200 μM MgATP. AMPK activity was determined using the SAMS assay and results are shown as the mean ± SEM for duplicate experiments.

(E) Activation of γ1 subunit mutants measured over a range of concentrations (0–20 μM) of A769662. The results were fitted to the following equation: Activity = Basal + (((Fold Stimulation × Basal) − Basal) × ([AMP]/($A_{0.5}$ + [AMP])).

(F) Same as for (E), except measured over a range of concentrations (0–100 μM) of AMP. * denotes significantly different from control ($p < 0.02$). † denotes significantly different from wild-type control ($p < 0.02$).

A769662 Is Unable to Stimulate Glucose Uptake in Skeletal Muscle

We examined the effect of A769662 and AICAR on AMPK activity and glucose uptake in extensor digitorum longus (EDL) muscle isolated from wild-type or β1 null mice. Figure 6A shows that expression of the α1 subunit was significantly decreased in the β1 null muscle, whereas there was no change in α2 subunit expression and no compensatory increase in the expression of the β2 subunit. There was no change in α2-associated activity between wild-type and β1 null muscle; however, α1 activity was decreased by 53% (Figure 6B). Incubation with A769662 increased α1 activity in the wild-type muscle; however, activation was abolished in the β1 null muscle whereas there was no activation of α2 complexes by A769662 in either wild-type or β1 null muscle. AICAR activated both α1 and α2 complexes in EDL muscle; however, activation of α1 was lower in the β1 null muscle but there was no reduction in the activation of α2 complexes (Figure 6C). Phosphorylation of ACC was increased in response to either A769662 or AICAR; however, only the effect of A769662 was abolished in the β1 null muscle (Figure 6D). We next investigated the ability of A769662 and AICAR to stimulate glucose uptake in both wild-type and β1 null EDL muscle. A769662 was unable to stimulate glucose transport in either wild-type or β1 null muscle (Figure 6E). In contrast, AICAR stimulated glucose uptake to the same extent in both wild-type and β1 null EDL muscle (Figure 6F), suggesting that β1 complexes do not participate in regulating glucose transport.

DISCUSSION

In this study, we have investigated activation of AMPK by A769662 and AMP and provided new insights into the mechanisms of allosteric regulation, in particular the role of the β subunit and the CBM in regulating kinase activity. The β subunits have divergent N-terminal sequences of poorly defined function, and the β1 subunit has several posttranslational modifications within this region including myristoylation at Gly2 and autophosphorylation of Ser24/Ser25 (Mitchelhill et al., 1997). The Gly2Ala mutant had increased activity but A769662 was able to further activate this mutant, indicating that activation does not involve simply relieving inhibition caused by the myristoyl group. Deletion of the β1 sequence comprising residues 1–63 also resulted

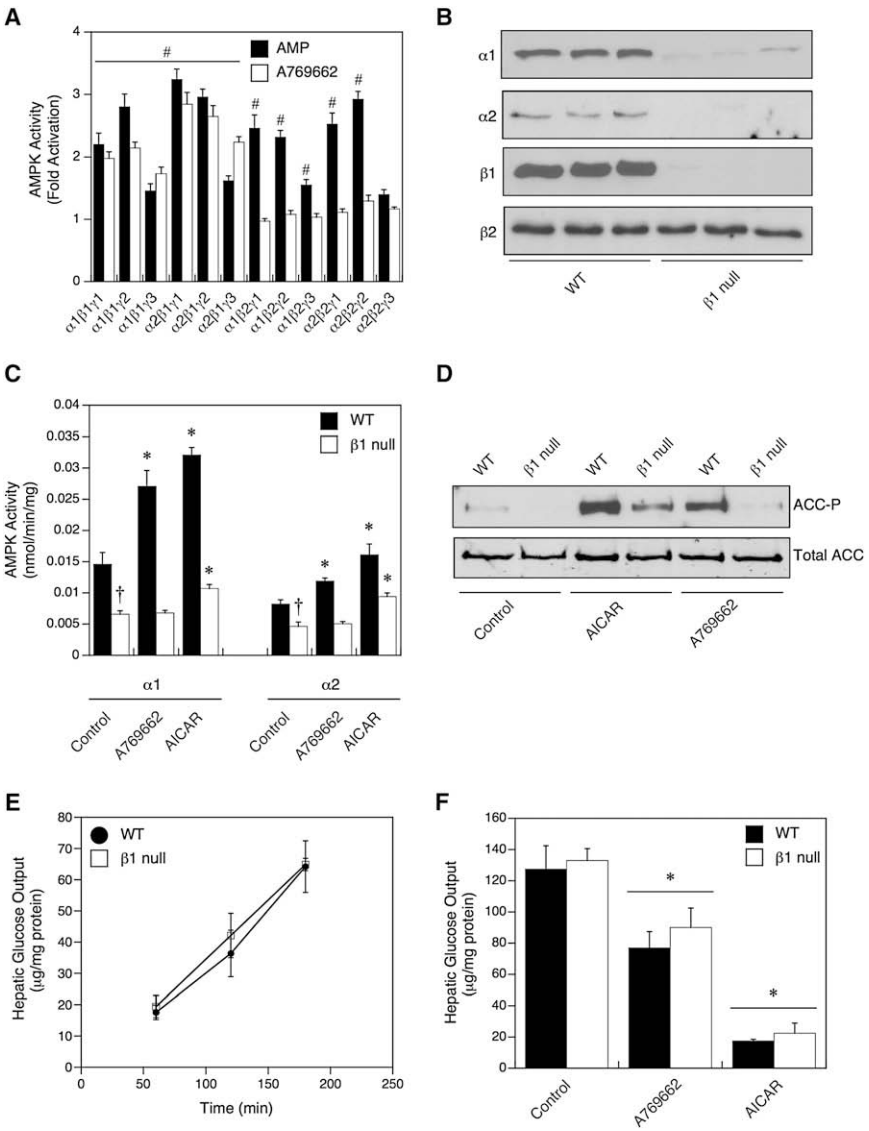

Figure 5. A769662 Selectively Activates AMPK Complexes Containing the β1 Subunit

(A) Activity of AMPK complexes was measured using the SAMS assay with either 20 μM A769662 or 100 μM AMP and shown as the mean ± SEM for duplicate experiments. # denotes significantly different from control ($p < 0.05$).

(B) Western blot analysis of AMPK subunits in isolated hepatocytes from wild-type and β1 null mice. Twenty micrograms of extract was analyzed except for the β2 blot, where 200 μg was used due to low expression of the β2 subunit.

(C) AMPK α1 or α2 complexes were immunoprecipitated from 100 μg of lysate from wild-type or β1 null hepatocytes incubated with either 100 μM A769662 or 2 mM AICAR. AMPK activity was measured in the immunoprecipitates using the SAMS assay and data are shown as mean ± SEM (n = 8).

(D) Western blot analysis of ACC phosphorylation in wild-type and β1 null hepatocytes treated with either 100 μM A769662 or 2 mM AICAR. Phospho-ACC was detected using a rabbit phospho-specific-ACC antibody and total ACC was detected using a streptavidin fluorescent dye conjugate.

(E) Basal glucose output of wild-type and β1 null hepatocytes measured at various time points (60, 120, and 180 min). Data are shown as mean ± SEM (n = 6).

(F) Glucose output in wild-type and β1 null hepatocytes after treatment with 100 μM A769662 or 2 mM AICAR. Data are shown as mean glucose output ± SEM (n = 6) from two independent experiments after 120 min. * denotes significantly different from control in the same genotype ($p < 0.05$). † denotes significantly different from wild-type control ($p < 0.05$).

in further activation of AMPK, but again this fragment was still activated by A769662. Our results indicate that β1 N-terminal residues either have a direct autoinhibitory role or a tethering function to secure autoinhibition indirectly. Only truncations removing either half or the complete CBM essentially abolished activation by A769662, further supporting a role for this domain in the regulation of AMPK. Importantly, deletion of the CBM had no effect on activation of AMPK by AMP, indicating that the loss of A769662 sensitivity was unlikely to be due to gross functional changes in the enzyme. Our findings are in agreement with Sanders et al., who also reported that the CBM is essential for A769662-mediated activation using an α1β1(186–270)γ1 construct (Sanders et al., 2007a). Their and our findings further reinforce the view that A769662 binds to a site that is distinct from the AMP-binding sites. Our data also suggest that it is unlikely that A769662 binds to either the carbohydrate-binding site or an alternate site located within the CBM, as we were unable to detect significant binding of the compound to isolated CBM using NMR, despite clearly observing changes in the chemical shift pattern for maltoheptaose binding to the carbohydrate-binding site. There was a small change in chemical shift stemming from Ser144 and Val149 in the presence of A769662; however, mutation of these residues to alanine had no effect on activation by the drug (data not shown).

Although the CBM plays a crucial role in activation by A769662, our studies also suggest a role for the γ subunit that is independent of the three known AMP-binding sites. From our homology modeling and mutagenesis studies, we have identified a hydrophobic patch on the γ subunit that is situated proximal to the carbohydrate-binding loop of β1-CBM and showed that this structural feature is important in regulating phosphorylation of Thr172 and activation by A769662 and AMP. Mutation of conserved hydrophobic residues within the patch had varying effects on allosteric activation in addition to alterations in kinase activity. Both the Leu178Ala and Phe179Leu mutations showed decreased basal activity whereas Phe175Tyr, Phe175Leu, and Phe179Tyr exhibited increased activity, and all of these effects on kinase activity were associated with altered Thr172

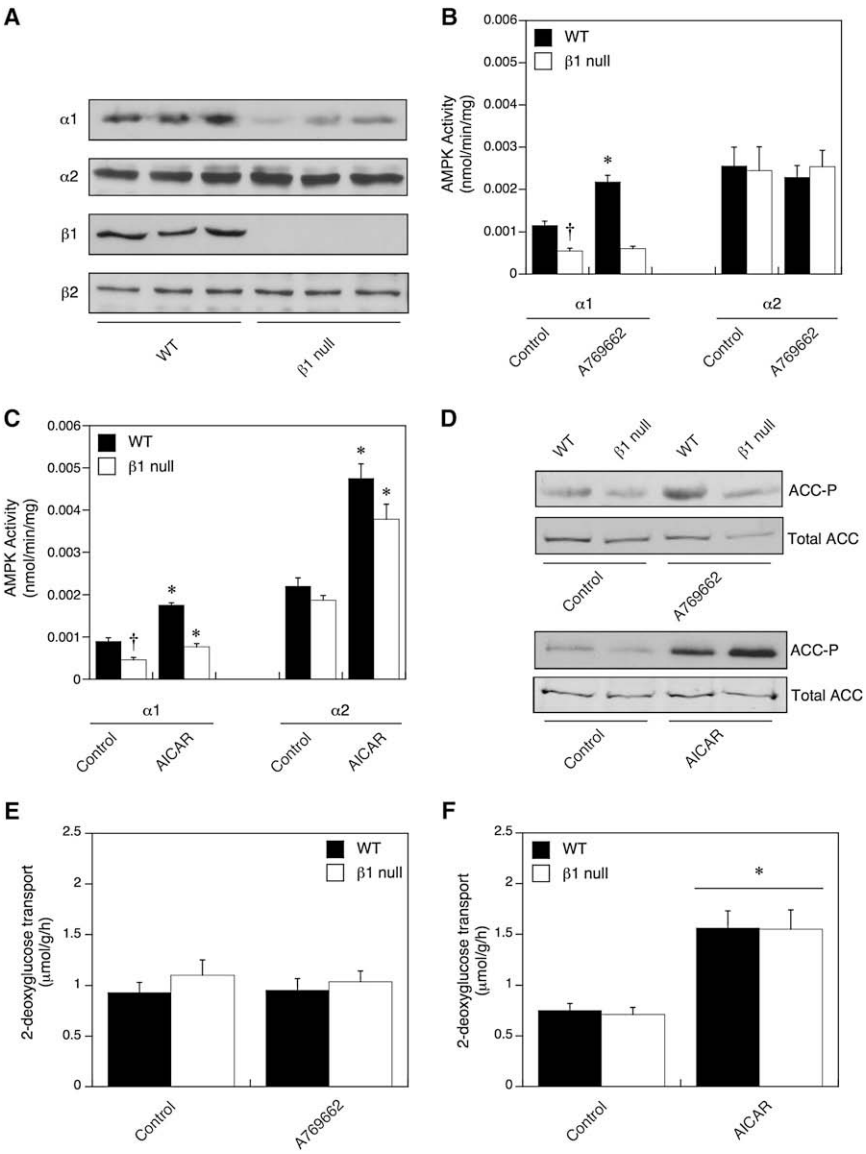

Figure 6. A769662 Does Not Stimulate Glucose Uptake in Skeletal Muscle

(A) Western blot analysis of AMPK subunits in EDL muscle (50–80 μg) from wild-type and β1 null mice.

(B) AMPK α1 or α2 complexes were immunoprecipitated from 100 μg of EDL muscle lysate from wild-type or β1 null mice incubated with 100 μM A769662. AMPK activity was detected in the immunoprecipitates using the SAMS assay and data are shown as mean ± SEM (n = 8).

(C) AMPK α1 or α2 complexes were immunoprecipitated from 100 μg of EDL muscle lysate from wild-type or β1 null mice incubated with 2 mM AICAR. AMPK activity was detected in the immunoprecipitates using the SAMS assay and data are shown as mean ± SEM (n = 8).

(D) Western blot analysis of ACC phosphorylation in wild-type and β1 null EDL muscle treated with either 100 μM A769662 or 2 mM AICAR. Phospho-ACC was detected using a rabbit phospho-specific-ACC antibody and total ACC was detected using a streptavidin fluorescent dye conjugate.

(E) Glucose uptake in EDL muscle from wild-type or β1 null mice incubated with 100 μM A769662. Data are shown as mean ± SEM (n = 8).

(F) Glucose uptake in EDL muscle from wild-type or β1 null mice incubated with 2 mM AICAR. Data are shown as mean ± SEM (n = 8). * denotes significantly different from basal in the same genotype ($p < 0.05$). † denotes significantly different from wild-type control ($p < 0.05$).

phosphorylation. Although the Phe179Tyr mutation is activating, substitution with leucine produced the opposite effect, highlighting the critical nature of this region in switching kinase activity between active and inactive states. In support of our findings, mutation of Asn177 in Snf4, which is equivalent to Phe179 in γ1, increases the activity of the SNF1 complex even under high glucose conditions by increasing phosphorylation of Thr210 (the residue corresponding to Thr172 in AMPK) (Momcilovic et al., 2008). The Phe179Leu mutant also led to a 6.6-fold increase in the half-maximal concentration for activation by A769662, whereas on the other hand substitution with tyrosine had no effect on this parameter. This indicates that the Phe179 aromatic side chain is an important structural element, at least for the mammalian kinase, and this residue may be located near or have connectivity with the A769662-binding site. The Leu178Ala mutant also caused a decrease in AMPK activity as well as causing a 3.8-fold increase in the half-maximal concentration for A769662 activation. It is interesting to note that the Leu178Ala and Phe179Leu mutants not only increased the half-maximal concentration for A769662 but also were the only two mutants that showed decreased Thr172 phosphorylation and kinase activity. The low basal activity of the Leu178Ala mutant may be explained by our finding that this mutant exhibits some resistance to phosphorylation by CaMKKβ, and it is possible that this may also apply to phosphorylation by other upstream kinases such as LKB1. A possible explanation for the effect of the Phe179Leu mutant is that A769662 mimics AMP in protecting against Thr172 dephosphorylation by phosphatases (Sanders et al., 2007a), so the decreased level of phosphorylation of the Phe179Leu mutant may be associated with increased susceptibility to dephosphorylation. Because the Phe179Leu mutant alters the half-maximal concentration for A769662 but does not have a marked effect on AMP sensitivity, it may imply that another intracellular metabolite besides AMP can protect against AMPK Thr172 dephosphorylation and may bind to the A769662 site. The Arg171Gln and Phe175Leu mutants had striking effects on the half-maximal concentration for AMP activation without affecting A769662 dose dependence, which was unexpected considering that neither residue is directly involved in nucleotide binding (Xiao et al., 2007). Arg171 is located within the putative nucleotide-binding site that is unoccupied in the mammalian γ1 structure; however, in the recent *Schizosaccharomyces pombe* core structure, ADP

occupies CBS site 2 by virtue of a contribution from Asp250 in the β subunit (Jin et al., 2007), and therefore it is possible that this site becomes occupied by a similar mechanism in the intact AMPK heterotrimer. It was noticeable that the γ mutants with increased basal activity had lower A769662 and AMP dependence, suggesting that a higher degree of phosphorylation renders AMPK relatively insensitive to allosteric activation. Indeed, AMPK that we extensively treated with CaMKKβ had reduced allosteric activation by both A769662 and AMP. Previous studies have shown that extensively autophosphorylated AMPK is less AMP dependent (Suter et al., 2006), and this may constitute a negative feedback mechanism where allosteric regulation of AMPK is dampened after heightened activity. There is evidence to suggest that this mechanism operates in vivo, as studies with ischemic hearts found that AMPK activity increased rapidly with the onset of ischemia but then decreased even though the AMP/ATP ratio remained elevated (Beauloye et al., 2001). Interestingly, the fact that increased phosphorylation reduced allosteric activation by both A769662 and AMP to the same extent lends further support to the idea that both molecules use a convergent mechanism to activate AMPK.

Activation of AMPK by AMP is strongly influenced by the γ subunit isoform present in the complex; for example, complexes containing the γ2 subunit have the greatest AMP dependence, whereas those containing the γ3 subunit have the least (Cheung et al., 2000). This prompted us to test whether activation of AMPK by A769662 was sensitive to the presence of a particular subunit isoform, and therefore we analyzed the effects of A769662 against all 12 possible permutations of the AMPK αβγ complex. Only β1-containing heterotrimers were activated by A769662, as all β2 complexes were insensitive irrespective of their α or γ subunit isoform partners. A769662 also selectively activated β1 complexes in intact cells, as activation of both α1 and α2 complexes was abolished in hepatocytes lacking the β1 subunit. Activation with AICAR was only partially reduced in the β1 null hepatocytes, demonstrating that AICAR is still able to activate the remaining β2 complexes. Previous studies using hepatocytes lacking both α subunit isoforms showed that increased phosphorylation of ACC by A769662 is completely dependent on the expression of the α catalytic subunits (Goransson et al., 2007). This is supported by our finding that phosphorylation of ACC in response to A769662 is completely abolished in the β1 null hepatocytes. It is unclear at present why AMPK complexes containing the β2 subunit are insensitive to A769662, as there is very little sequence variation between the β isoforms within the CBM, which is an essential component for activation by A769662. Intriguingly, one of the few regions of sequence divergence between β1 and β2 within the CBM occurs in the sequence surrounding the Ser108 autophosphorylation site, which has been shown to be critical for A769662 activation (Sanders et al., 2007a). It has yet to be determined whether the corresponding residue Ser107 on the β2 subunit is autophosphorylated, and this may potentially explain why β2 complexes are A769662 insensitive. Not only is there variation in the sequences surrounding Ser107/Ser108, but these residues also point in opposite directions in the β1/β2 CBM crystal structures; therefore, it is possible that these subtle differences impact on the ability of Ser107 on β2 to be autophosphorylated.

An important in vivo effect of A769662 is to lower plasma glucose, and it is thought that this is mediated primarily by activation of AMPK in the liver. Activation of AMPK decreases hepatic glucose production by inhibiting transcription of key enzymes in the gluconeogenic pathway such as phosphoenolpyruvate kinase and glucose-6-phosphatase, and mice treated with A769662 have decreased expression of these enzymes (Cool et al., 2006). It was therefore surprising that A769662 was as effective at lowering glucose output in the β1 null hepatocytes as in wild-type cells. There was no alteration in the ability of AICAR to lower glucose production in the β1 null hepatocytes either, indicating that A769662 and AICAR may decrease hepatic glucose output in part by an AMPK-independent mechanism. Interestingly, there was no change in basal glucose output in the β1 null hepatocytes despite the fact that both α1 and α2 activity were significantly decreased. Liver-specific deletion of the α2 subunit leads to increased hepatic glucose production (Andreelli et al., 2006), and therefore it was surprising that there was no change in glucose production in the β1 null cells given that α2 activity was decreased. It is possible that the remaining α2/β2 complexes are sufficient to regulate basal and AICAR-stimulated glucose output. It is well established that AICAR is able to affect other AMP-sensitive enzymes such as fructose 1,6-bisphosphatase and glycogen phosphorylase, both of which can alter hepatic glucose metabolism through gluconeogenesis and glycogenolysis, respectively (Longnus et al., 2003; Vincent et al., 1991). A769662 has been shown to inhibit fructose 1,6-bisphosphatase but only at concentrations in the millimolar range (Cool et al., 2006), and therefore the mechanism underlying the ability of A769662 to lower glucose output in the β1 null hepatocytes remains unclear. One interesting possibility is that A769662 activates phosphofructokinase 1 (PFK1), which plays a key role in controlling the rate of glycolysis and is activated allosterically by AMP. Consistent with this idea, activation of PFK1 in liver has been shown to lower glucose production by increasing the rate of glycolysis (Wu et al., 2005). It is now beginning to emerge that A769662 can interfere with other biological pathways unrelated to AMPK, such as the recently described inhibition of nonproteolytic components of the 26S proteosome by A769662, indicating the possibility of multiple off-target effects (Moreno et al., 2008).

Activation of AMPK in skeletal muscle is associated with several important metabolic responses including stimulation of fatty acid uptake/oxidation and increased glucose transport, which provided the impetus to study the effects of A769662 in this tissue. A769662 selectively activated α1 complexes in EDL muscle whereas there was no activation of α2 complexes; however, A769662 failed to activate α1 complexes in EDL muscle lacking the β1 subunit. These findings confirm that selective activation of β1 complexes by A769662 occurs beyond the liver and includes skeletal muscle. The expression and activity of only α1 complexes was reduced in the β1 null EDL muscle, indicating that the β1 subunit likely exists in complex exclusively with the α1 subunit isoform in EDL. A769662 was unable to stimulate glucose uptake either in the presence or absence of the β1 subunit; however, this may not be that surprising given that the α2 subunit is essential for upregulation of glucose transport and that A769662 was unable to activate α2 complexes in EDL (Jorgensen et al., 2004). Activation of α2 complexes and stimulation of glucose transport by AICAR were unaffected in β1 null muscle and, taken together with data from α2 null mice, suggest that AMPK complexes containing α2/β2 subunits are responsible

for mediating the AICAR effect on glucose transport. Interestingly, recent studies have identified a role for AMPK complexes containing the α1 subunit in the stimulation of glucose uptake in response to twitch contraction in skeletal muscle (Jensen et al., 2008). These findings suggest that activation of α1 complexes by A769662 and AICAR may not be sufficient to regulate glucose uptake and that additional stimuli that only occur during twitch contraction are also required.

SIGNIFICANCE

AMPK is a key regulator of cellular and systemic energy metabolism and is an attractive drug target for treatment of metabolic diseases, particularly obesity and type 2 diabetes, and therefore elucidating the mechanisms underlying AMPK regulation may aid the development of better therapeutic activators of AMPK. We have demonstrated that the thienopyridone A769662 activates AMPK by a novel mechanism that is independent of the AMP-binding sites but dependent on the β subunit carbohydrate-binding module and γ subunit. Our results provide compelling evidence that the β subunit of AMPK plays an important role in autoregulation of the αβγ complex in addition to its known scaffold function to anchor the α and γ subunits. The revelation that activation of AMPK by A769662 is β1 specific in one sense limits its use as a universal AMPK-activating drug but highlights an unexpected opportunity of being able to develop isoform specific activators that can target AMPK in specific tissues such as skeletal muscle.

EXPERIMENTAL PROCEDURES

Animals

All experimental protocols involving animals were approved by the St. Vincent's Hospital Animal Ethics Committee.

Isolation and Culture of Hepatocytes

Isolated hepatocytes from wild-type and β1 null mice were prepared by the collagenase perfusion method (Seglen, 1976). Briefly, catheters were inserted into the hepatic portal vein and the livers were perfused with HEPES buffered saline solution for 10 min, followed by buffer containing collagenase (1.2 mg/ml) for a further 15 min. Hepatocytes were plated at 10^6 cells per well in collagen-coated six-well dishes in DMEM supplemented with 10% fetal bovine serum, glutamine, and antibiotics. The cells were initially cultured for 4 hr in high insulin, after which the cells were transferred into low-insulin media and grown overnight. Cells were incubated with either 100 μM A769662 (prepared by CreaGen) or 2 mM AICAR (Toronto Research Chemicals) for 3 or 1 hr, respectively. Following the treatments, the media were changed and glucose was measured at various time points using the glucose oxidase/peroxidase-coupled colorimetric assay (Sigma) and corrected for protein content.

Muscle Incubations and Glucose Uptake

Extensor digitorum longus (EDL) muscles were dissected from anesthetized mice (6 mg of pentobarbital/100 g body weight) and transferred to incubation flasks containing 2 ml of essential buffer (Krebs-Henseleit buffer with 2 mM pyruvate, 8 mM mannitol, 0.1% BSA), gassed with 95% O_2/5% CO_2, and maintained at 30°C as previously described (Steinberg et al., 2006). For all experiments, muscles were preincubated for 15 min with essential buffer, after which they were incubated with either 100 μM A769662 or 2 mM AICAR for 3 or 1 hr, respectively. 2-deoxy-D-glucose (2DG) uptake was measured over 10 min by replacing existing incubation buffer with buffer containing 0.50 μCi/ml 2-deoxy-D-[2,6-^{3}H]glucose (GE Healthcare), 1 mM 2-deoxy-D-glucose (Sigma), and 0.20 μCi/ml D-[1-^{14}C]mannitol (GE Healthcare). Following incubation with 2DG, muscles were snap-frozen and lysed in buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 50 mM NaF, 5 mM NaPPi, 1 mM EDTA, 1 mM EGTA, and 1% (v/v) Triton X-100 and radioactivity was quantified by liquid scintillation counting while remaining lysates were utilized for AMPK activity assays.

Nuclear Magnetic Resonance

AMPK β1-CBM was expressed and isotopically labeled as described previously. Briefly, β1-CBM was expressed as a His-tag fusion in BL21 *Escherichia coli* grown in 1 L of minimal media supplemented with $^{15}NH_4Cl$ in a Braun Biostat fermentor. The β1-CBM was purified by Ni^{2+} Sepharose and S200 gel-filtration chromatography (GE Healthcare) and exchanged into NMR buffer (25 mM Na_2HPO_4-NaH_2PO_4 [pH 7.5], 0.02% sodium azide, 10% D_2O). NMR spectra were collected using a Bruker 800 MHz AVANCE spectrometer. For maltoheptaose (Sigma), ^1H-^{15}N heteronuclear single quantum correlation (HSQC) spectra were collected at various titration points ranging from 0 to 10 mM at 800 MHz at 25°C. NMR spectra were collected for β1-CBM in the absence or presence of 5 mM A769662 using the same conditions as for maltoheptaose.

Expression of Recombinant AMPK and Mutant Variants

COS7 cells were grown in DMEM with 10% fetal calf serum at 37°C/5% CO_2. Cells were transfected at 60% confluency with 1 μg of the various plasmids expressing AMPK subunits (pcDNA3 GST-α1, GST-α2, β1, and β2, and pMT2 γ1, γ2, and γ3) and deletion/point mutants using FuGene 6 (Roche Diagnostics) according to the manufacturer's instructions. Transfected cells were harvested after 36 hr by washing with ice-cold PBS followed by rapid lysis in situ using 1 ml of lysis buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 50 mM NaF, 1 mM NaPPi, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1% [v/v] Triton X-100). Cellular debris was removed by centrifugation and total protein was determined using the Bradford protein assay (Bio-Rad). AMPK was isolated from 50 μg of lysate using 10 μl of a 50% slurry of glutathione-Sepharose (GE Healthcare) pre-equilibrated with lysis buffer, followed by successive washes in lysis buffer containing 1 M NaCl and finally into assay buffer (50 mM HEPES [pH 7.4], 1 mM DTT, 0.02% Brij-35). Samples were adjusted for protein expression by immunoblotting for the α subunit and densitometry.

AMPK Assays

AMPK complexes were purified either by glutathione-Sepharose pull-down (see above) or immunoprecipitation. For immunoprecipitation experiments, 5 μl of protein A Sepharose (Sigma) complexed with 5 μg of the appropriate antibody was incubated with 100 μg of lysate for 2 hr at 4°C followed by successive washing with lysis buffer containing 1 M NaCl and finally into assay buffer, after which activity was determined by phosphorylation of the SAMS peptide using 100 μM SAMS, 200 μM [γ-^{32}P]ATP, and 5 mM $MgCl_2$ in a standard 25 μl volume assay at 30°C. Reactions were terminated after 10 min by spotting 15 μl onto P81 phosphocellulose paper (Whatman) and washing in 1% phosphoric acid. Radioactivity was quantified by scintillation counting.

λ Phosphatase and CaMKKβ Treatments

AMPK immobilized on glutathione-Sepharose (5 μl) was incubated with 200 ng of λ phosphatase in 20 μl of dephosphorylation buffer (50 mM HEPES [pH 7.4], 1 mM DTT, 2 mM $MnCl_2$) for 60 min at 30°C, followed by successive washes in 50 mM HEPES (pH 7.4), 1 mM DTT, 1 M NaCl and finally into assay buffer. Fifty nanograms of CaMKKβ and 200 μM ATP, 5 mM $MgCl_2$ was added to a final volume of 25 μl and incubated at 30°C for 10 min. The CaMKKβ reaction was terminated by washing the beads into 50 mM HEPES (pH 7.4), 50 mM EDTA, 1 mM DTT, followed by successive washes in 50 mM HEPES (pH 7.4), 1 mM DTT, 1 M NaCl, after which they were washed into assay buffer.

SUPPLEMENTAL DATA

Supplemental Data include Supplemental Experimental Procedures, one figure, one table, and Supplemental References and can be found with this article online at http://www.chembiol.com/cgi/content/full/15/11/1220/DC1/.

ACKNOWLEDGMENTS

This study was supported by grants from the Australian Research Council (B.E.K.), National Health and Medical Research Council (B.E.K., G.R.S.),

Danish Research Council of Health and Diseases (S.B.J.), and National Heart Foundation (B.E.K.). G.R.S. is an NHMRC Senior Research Fellow and B.E.K. is an ARC Federation Fellow.

Received: May 8, 2008
Revised: September 9, 2008
Accepted: October 2, 2008
Published: November 21, 2008

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