AMP-activated protein kinase (AMPK) action in skeletal muscle via direct phosphorylation of PGC-1α

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Activation of AMP-activated kinase (AMPK) in skeletal muscle increases glucose uptake, fatty acid oxidation, and mitochondrial biogenesis by increasing gene expression in these pathways. However, the transcriptional components that are directly targeted by AMPK are still elusive. The peroxisome-proliferator-activated receptor γ coactivator 1α (PGC-1α) has emerged as a master regulator of mitochondrial biogenesis; furthermore, it has been shown that PGC-1α gene expression is induced by exercise and by chemical activation of AMPK in skeletal muscle. Using primary muscle cells and mice deficient in PGC-1α, we found that the effects of AMPK on gene expression of glucose transporter 4, mitochondrial genes, and PGC-1α itself are almost entirely dependent on the function of PGC-1α protein. Furthermore, AMPK phosphorylates PGC-1α directly both *in vitro* and in cells. These direct phosphorylations of the PGC-1α protein at threonine-177 and serine-538 are required for the PGC-1α-dependent induction of the PGC-1α promoter. These data indicate that AMPK phosphorylation of PGC-1α initiates many of the important gene regulatory functions of AMPK in skeletal muscle.

mitochondria | respiration

Organisms at all levels of the evolutionary scale have found ways to translate changes in environmental conditions into fine metabolic adjustments. The cellular energy charge is determined by a combination of catabolic and anabolic reactions. Because the cellular concentrations of AMP change more dramatically than that of ATP or ADP, AMP is a key monitor of the cellular energy status (1). The major molecular sensor for AMP level in cells is AMP-activated protein kinase (AMPK), an enzyme that is activated by cellular stresses that result in ATP depletion. Stimuli of AMPK are generally either processes that inhibit ATP production (e.g., metabolic poisons, hypoxia, or glucose deprivation) or accelerate ATP consumption (e.g., rapid contraction in muscle) (2–5). AMPK is also activated by the adipokines leptin and adiponectin, important regulators of whole-body energy metabolism (2–5). Furthermore, the finding that AMPK is an indirect target of metformin (6–9), a widely used antidiabetic drug, has led to growing interest in AMPK as a potential target for the treatment of type 2 diabetes. The stimulation of AMPK results in the repression of many anabolic processes (such as fatty acid and cholesterol synthesis, gluconeogenesis) and activation of several catabolic processes (such as fatty acid uptake and oxidation, glucose uptake) (2–5). In skeletal muscle, the metabolic changes induced by AMPK activation are either acute, through direct phosphorylation of metabolic enzymes, or chronic, through control of gene expression (10). Activation of AMPK increases glucose uptake acutely because of increased translocation of glucose transporter 4 (GLUT4) to the plasma membrane (11). Moreover, fatty acid oxidation is acutely increased by phosphorylation of acetyl-CoA-carboxylase-2 (ACC-2) leading to a decrease in malonyl-CoA, an inhibitor of carnitine *O*-palmitoyltransferase-1. Consequently, fatty acid transport into the mitochondria is increased (12, 13).

Much of the progress in knowledge about the downstream targets of AMPK has come from the use of the chemical compound 5-aminoimidazole-4-carboxamide 1-β-D-ribofuranoside 5-aminoimidazole-4-carboxamide riboside (AICAR) (14), an AMP analog. Several *in vivo* studies using AICAR to activate AMPK chronically determined that mitochondrial enzymes [e.g., cytochrome *c*, uncoupling protein 3 (UCP-3)] (15–18) and proteins involved in glucose uptake (GLUT4) (18–20) are increased at the transcriptional level in skeletal muscle. Interestingly, these genes are also downstream targets of proliferator-activated receptor γ coactivator 1α (PGC-1α), a highly regulated coactivator of nuclear receptors and many other transcription factors outside of the nuclear receptor family (21). PGC-1α is a key player in the oxidative metabolism of brown fat and muscle by increasing mitochondrial biogenesis and augmenting the expression of enzymes of the electron transport system and uncoupling proteins (22, 23). In addition, ectopic expression of PGC-1α in muscle cells increases expression of GLUT4, resulting in increased glucose uptake (24).

PGC-1α is expressed preferentially in oxidative muscle fibers, and transgenic mice ectopically expressing PGC-1α in muscle tissue show conversion of type IIb (glycolytic) fibers into mitochondria-rich type IIa and I fibers (25). Interestingly, PGC-1α expression and PGC-1α-responsive genes involved in oxidative phosphorylation are down-regulated in skeletal muscle of human type 2 diabetics (26, 27). Because PGC-1α gene expression in muscle is increased *in vivo* with exercise and AICAR and metformin treatment (28–31, 33, 34), these results suggest the important role of PGC-1α in whole-body energy metabolism; they further suggest that PGC-1α is likely a very important downstream target of AMPK.

The mechanisms by which activated AMPK induces gene expression are not yet clear. Because different subunits of AMPK are preferentially located in the nucleus (35), it has been proposed that it regulates gene expression by directly phosphorylating certain transcription factors (36). Indeed, AMPK has been shown previously to phosphorylate transcription factors and coactivators such as p53, p300, TRIP6, and TORC2 (36–39). We show here that many effects of activated AMPK on gene expression in skeletal muscle, including the inductions of the PGC-1α, GLUT4, and mitochondrial genes, require the presence of the PGC-1α protein. Furthermore, AMPK directly phosphorylates PGC-1α, and this phosphorylation mediates an increase in PGC-1α protein action on the PGC-1α promoter.

Results

PGC-1α Protein Is Required for AMPK Action on Gene Expression and Mitochondrial Function. We investigated whether PGC-1α is required for the effects of AMPK activation on gene expression in

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Abbreviations: ACC, acetyl-CoA-carboxylase; AICAR, 5-aminoimidazole-4-carboxamide 1-β-D-ribofuranoside; AMPK, AMP-activated protein kinase; 8-BrAMP, 8-bromo-AMP; GLUT4, glucose transporter 4; PGC-1α, peroxisome-proliferator-activated receptor γ coactivator 1α; UCP, uncoupling protein; PDK4, pyruvate dehydrogenase kinase isoenzyme 4.

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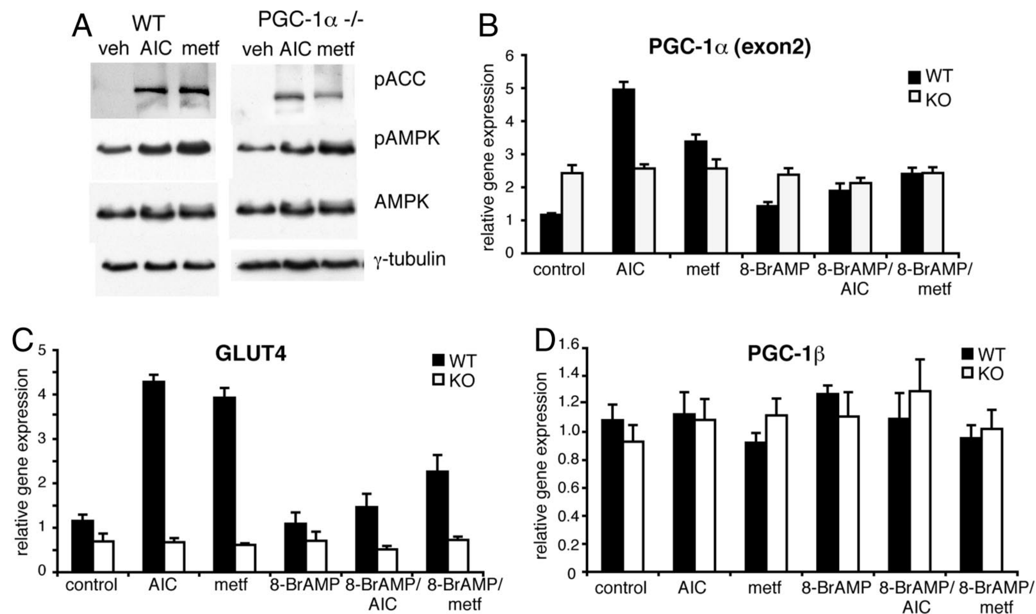

Fig. 1. AMPK-driven increase in PGC-1α and GLUT4 gene expression requires PGC-1α protein. (*A*) AMPK is activated in AICAR and metformin-treated primary myotubes. Comparison of AMPKα protein phosphorylated at threonine-172 (pT172) with total levels of AMPKα protein in WT and PGC-1α knockout (KO) primary myotubes treated with vehicle, AICAR, or metformin for 1 h is shown. *A* also shows the levels of ACC protein phosphorylated at serine-79: loading control (γ-tubulin). (*B*) AICAR and metformin treatments elevate the expression of PGC-1α in WT but not in PGC-1α −/− cells. The inhibitor of AMPK, 8-BrAMP, blocks this increase. (*C*) mRNA levels of GLUT4. (*D*) The relative gene expression of PGC-1β does not change under the same conditions. Primary myotubes were treated with vehicle, 500 μM AICAR, 1 mM metformin, 1 mM 8-BrAMP, 8-BrAMP/AICAR, and 8-BrAMP/metformin in DMEM/0.5% BSA for 16 h. The relative PGC-1α mRNA levels were determined with primers in exon2, which is present in WT and PGC-1α −/− cells, by using semiquantitative PCR.

catabolic pathways. We could not use the full-body PGC-1α −/− because AMPK is constitutively activated in the skeletal muscle of these mice, probably the result of a central nervous system-linked hyperactivity (40). Therefore, primary muscle cells isolated from wild-type (WT) and PGC-1α −/− mice (40) were differentiated into myotubes and subsequently treated with 500 μM AICAR, 1 mM metformin, or vehicle for 16 h. That AMPK was activated under these conditions in both cell types was shown by Western blotting with anti-pACC and anti-pAMPKα antibodies (Fig. 1*A*). These treatments induced a significant increase in the expression of mRNAs encoding PGC-1α [measured within exon 2, which is still present in the PGC-1α −/− (ref. 40)], GLUT4, and several mitochondrial target genes of AMPK, such as cytochrome *c*, UCP-2 and UCP-3 (Figs. 1 *B* and *C* and 2 *A*–*C*). Strikingly, cells lacking PGC-1α showed a complete ablation of this induction (Figs. 1 *B* and *C* and 2 *A*–*C*). Additionally, these increases in gene expression were almost completely blocked in cells that were preincubated with the AMPK inhibitor 8-BrAMP

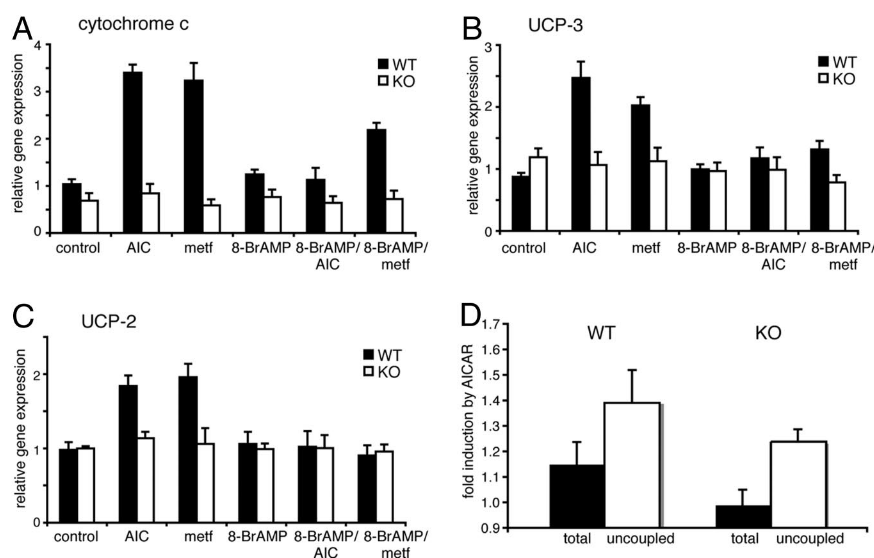

Fig. 2. AMPK-driven increase in expression of mitochondrial genes and in respiration requires PGC-1α. Primary myotubes were treated as in Fig. 1, and mRNA levels of cytochrome *c* (*A*), UCP-3 (*B*), and UCP-2 (*C*) were determined by using semiquantitative PCR. KO, knockout. (*D*) Oxygen consumption was measured in WT and PGC-1α −/− primary myotubes treated with AICAR in DMEM/5% HS for 2 days. At day 3, cells were shifted to DMEM/0.5% BSA and treated for an additional 16 h before oxygen consumption was measured as described in *Materials and Methods*.

(1 mM) (41) (Figs. 1 *B* and *C* and 2 *A–C*). The expression of PGC-1β did not change upon AMPK activation, showing that active AMPK specifically induces PGC-1α (Fig. 1*D*). This finding indicates that PGC-1α is absolutely required for the induction of GLUT4 and mitochondrial genes via AMPK activation.

To determine whether this PGC-1α-mediated increase in mitochondrial genes stimulated by AMPK is reflected in mitochondrial function, we measured respiration in WT and PGC-1α −/− myotubes. As shown in Fig. 2*E*, WT cells had a 15% increase in total respiration and a 40% increase in uncoupled respiration upon AICAR treatment. However, total respiration did not increase with AICAR treatment of the PGC-1α −/− cells; the effect in uncoupled respiration was reduced to 24%. These data show that PGC-1α is required for the positive effects of AMPK activation on mitochondrial function in muscle cells.

PGC-1α Protein Is Required for AMPK Action on Gene Expression of PGC-1α, GLUT4, and Cytochrome *c in Vivo*. Mice lacking PGC-1α specifically in skeletal muscle have been developed (42). We tested whether AMPK was also constitutively activated in the skeletal muscle of these mice as in the full-body knockout (data not shown). Because this hypothesis was not the case, we could use these mice to determine whether AMPK action on the expression of the PGC-1α gene and some of its key target genes *in vivo* also requires PGC-1α. We treated WT and skeletal muscle-specific PGC-1α −/− mice with 250 mg/kg AICAR for 6 h. AMPK was activated in both genotypes, as determined by Western blotting with anti-pACC antibodies (Fig. 3*A*). AICAR increased the expression of PGC-1α mRNA (exon 2) and GLUT4 mRNA ≈2.5-fold in the muscle of WT mice, whereas PGC-1β gene expression did not change (Fig. 3 *B* and *C*). Cytochrome *c* mRNA increased 1.7-fold (Fig. 3*B*). The mice lacking PGC-1α in skeletal muscle completely failed to induce PGC-1α (exon 2), cytochrome *c*, and GLUT4 mRNA in response to AICAR (Fig. 3 *B* and *C*). Interestingly, UCP-3 mRNA was induced ≈4-fold in skeletal muscle of both genotypes (Fig. 3*B*), indicating that this gene did not require PGC-1α for AMPK action. In addition to GLUT4, we examined two other genes involved in glucose metabolism [hexokinase and pyruvate dehydrogenase kinase isoenzyme 4 (PDK4)] that were shown to be induced by AMPK (20, 30, 43). Hexokinase expression did not change in our experimental conditions, in either WT or in the skeletal muscle-specific PGC-1α −/− mice (Fig. 3*C*). In contrast, gene expression of the PGC-1α target PDK4 (44, 45) was slightly induced in WT and in the skeletal muscle knockouts (Fig. 3*C*). These data indicate that AMPK activation *in vivo* induces PGC-1α, GLUT4, and cytochrome *c* in a PGC-1α-dependent way. However, an alternative pathway clearly exists *in vivo* for the AMPK-mediated induction of UCP-3 and PDK4.

AMPK Directly Phosphorylates PGC-1α Protein on Threonine-177 and Serine-538. PGC-1α protein is involved in the induction of the PGC-1α gene in a feed-forward loop in skeletal muscle (46). Thus, the data above suggest that AMPK could directly activate the PGC-1α protein, perhaps by a direct phosphorylation. We first asked whether AMPK and PGC-1α interact directly. As shown in Fig. 4*A*, coimmunoprecipitation experiments indicate that AMPK and PGC-1α form a complex in cells. We next tested whether AMPK directly phosphorylates PGC-1α in cells. As shown in Fig. 4*B* (*Left*), AICAR stimulated an increased phosphorylation of the PGC-1α protein in primary myotubes. AMPK also phosphorylates PGC-1α *in vitro*, as shown in Fig. 4*C*. Using full-length protein and various fragments purified from bacteria, this phosphorylation was robust and increased by the presence of AMP. Fragments of PGC-1α from amino acids 1–190 and 395–565 were phosphorylated, whereas those encoding amino acids 200–400 and 551–797 were not. Mass spectrometry analysis indicated that phosphorylations occurred on threonine-177 and serine-538. Mutations of these sites completely ablated these AMPK-mediated phosphorylations *in vitro* (Fig. 4*D*) and in primary myotubes (Fig. 4*B Right*).

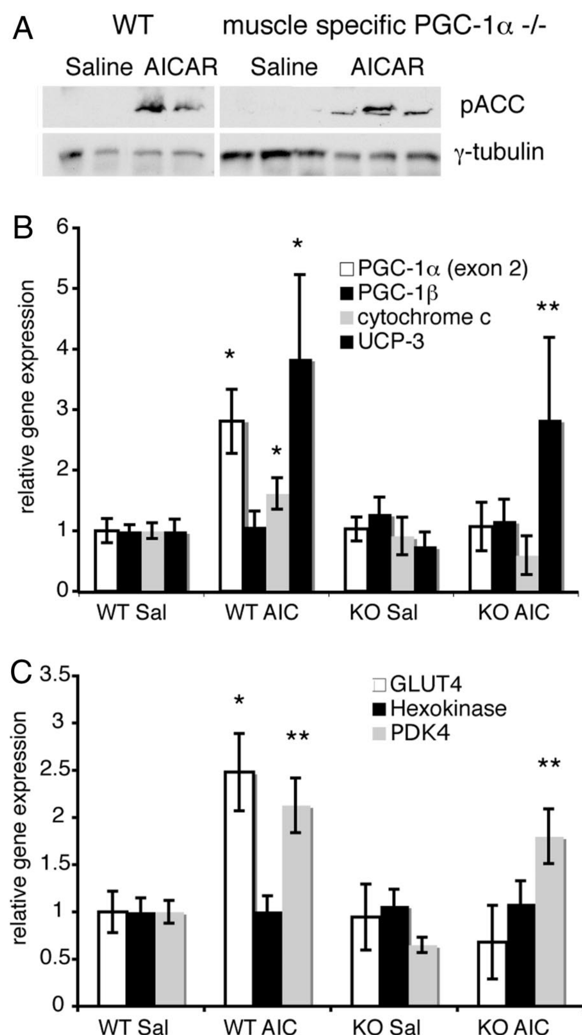

Fig. 3. AMPK-driven increase in PGC-1α, GLUT4, and cytochrome *c* gene expression requires PGC-1α protein *in vivo*. (*A*) AMPK is activated in skeletal muscle of AICAR-injected mice. Levels of ACC protein phosphorylated at serine-79 in gastrocnemeus muscle from WT and PGC-1α muscle-specific knockout (KO) mice, injected with saline or AICAR are shown. (*B*) Injection of AICAR induces the mRNA expression of PGC-1α and cytochrome *c* but not PGC-1β in the skeletal muscle of WT mice (*, $P < 0.01$); this induction does not occur in the skeletal muscle of the muscle-specific PGC-1α −/− mice. UCP-3 gene expression is also increased in the muscle-specific PGC-1α −/− mice (**, $P < 0.05$). (*C*) Injection of AICAR induces the mRNA expression of GLUT4 (*, $P < 0.01$) and PDK4 (**, $P < 0.1$) but not of hexokinase in the skeletal muscle of WT mice. PDK4 gene expression increases also in the muscle-specific PGC-1α −/− mice. Female mice were injected with either saline or 250 mg/kg AICAR. Skeletal muscle was harvested after 6 h, and gene expression was measured by using semiquantitative PCR (n = 5–7).

Phosphorylation by AMPK Increases PGC-1α-Dependent Activation of Its Own Promoter. Finally, we asked whether the induction of the PGC-1α promoter by AMPK requires AMPK-mediated phosphorylation of the PGC-1α protein. Fig. 4*E* illustrates that, as shown previously (46), the PGC-1α protein has a significant effect on the 2-kb PGC-1α promoter. This effect is greatly augmented by AICAR treatment of cells. A PGC-1α protein with alanine replacements at the two direct AMPK sites (threonine-177 and serine-538) is completely resistant to this effect of AICAR. These data strongly suggest that direct phosphorylation of the PGC-1α

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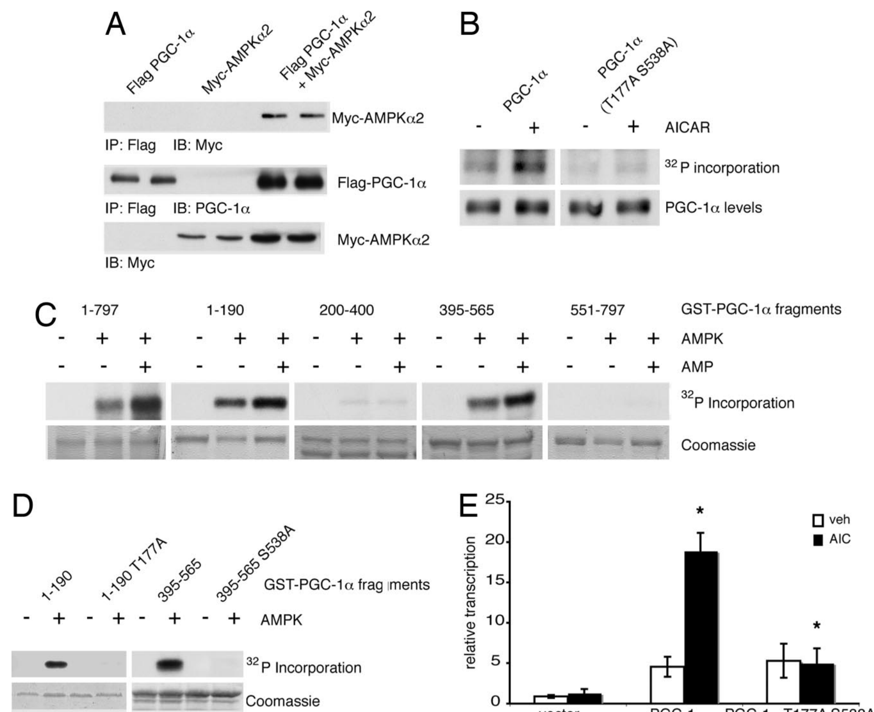

Fig. 4. AMPK phosphorylates PGC-1α at threonine-177 and serine-538 *in vitro* and in cells. (*A*) PGC-1α interacts with AMPKα2 in cells. Expression vectors for FLAG-PGC-1α and Myc-AMPKα2 were transfected into BOSC cells, as indicated. Coimmunoprecipitation was performed as described in *Materials and Methods*. (*B*) Primary PGC-1α −/− myotubes stably expressing PGC-1α and PGC-1α T177A/S538A, respectively, were treated with vehicle or AICAR for 1 h in the presence of ^{32}P. (*C*) Purified recombinant GST-PGC-1α fragments (full-length 1–797, 1–190, 200–400, 395–565, 551–797) were incubated with purified AMPK, and phosphorylation was determined by incorporation of [γ-^{32}P]ATP. (*D*) Mass spectrometry identified threonine-177 and serine-538 as phosphorylated residues. The GST-PGC-1α fragment containing amino acid 1–190 T177A and the GST-PGC-1α fragment containing amino acids 395–565 S538A are not phosphorylated by AMPK. (*E*) The phosphorylation of PGC-1α protein by AMPK is required for elevated PGC-1α-dependent activity of the PGC-1α promoter. C2C12 muscle cells were transfected with a 2-kb PGC-1α promoter construct and expression plasmids for PGC-1α or PGC-1α T177A/S538A, respectively. After transfection, cells were differentiated for 1 day and treated with AICAR for 7.5 h before reporter gene levels were determined (*, $P < 0.01$).

coactivator by AMPK initiates a cascade of gene expression that controls many mitochondrial target genes and respiration and genes of glucose and oxidative metabolism in muscle.

Discussion

AMPK and PGC-1α have both been shown to play important roles in energy homeostasis. AMPK is an important sensor of decreased energy charge in cells and subsequently acts to increase catabolic reactions and decrease anabolic reactions. PGC-1α is a critical regulator of transcription of many genes of energy homeostasis and is particularly involved in fuel oxidation and mitochondrial biology. A functional relationship between these two proteins is therefore not surprising. Earlier work showed that activated AMPK increases PGC-1α gene expression in cultured muscle cells, in umbilical vein endothelial cells, in fat, and in skeletal muscle (30–33, 47–53). Moreover, the use of RNAi against PGC-1α has suggested that PGC-1α mediates certain of these effects of AMPK, particularly in fatty acid oxidation (29). In this work, we demonstrate two important points: first, that AMPK requires PGC-1α for many of its most important effects on GLUT4 and mitochondrial gene expression in skeletal muscle, both in culture and *in vivo*. Second, AMPK binds to and activates PGC-1α in muscle by direct phosphorylation on two critical residues, threonine-177 and serine-538.

Previous work has demonstrated that PGC-1α can function as a regulator of its own gene expression in muscle, in a feed-forward loop (46). Therefore, it was possible that the AMPK-phosphorylated PGC-1α was involved in the induction of the PGC-1α gene, with many subsequent effects of AMPK secondary to this induction of PGC-1α. As shown in Fig. 4, work with the isolated PGC-1α promoter supports this idea. AMPK robustly increases the action of PGC-1α on the 2-kb promoter. Mutation of the two AMPK phosphorylation sites in the PGC-1α protein completely ablated the effect of AICAR on this promoter. These data also suggest that the phosphorylation of PGC-1α will likely affect the action of this protein on other promoters, either positively or negatively. It is worth noting that PGC-1α is a potent activator of gluconeogenic gene expression in the liver, whereas AMPK activators such as AICAR and metformin suppress gluconeogenic gene expression. It has been shown in liver that activated AMPK prevents the nuclear import of TORC2, the transcriptional coactivator of CREB, and therefore blocks the fasting-induced induction of PGC-1α (39); it remains to be determined whether the phosphorylation at threonine-177 and serine-538 occurs in liver and whether these phosphorylations are activating or inhibiting in this tissue.

Mechanistically, these AMPK-mediated phosphorylations could modulate the ability of PGC-1α to dock on certain transcription factors or affect the binding or function of other cofactors in the PGC-1α coactivator complex. The modulation of PGC-1α docking on certain transcription factors by AMPK might provide a simple explanation by which this enzyme could activate certain PGC-1α functions in muscle (such as GLUT4 gene expression) while inhibiting PGC-1α functions in liver (such as gluconeogenic gene expression). Recently, another example of posttranslational regulation of PGC-1α in skeletal muscle metabolism has been demonstrated. Fasting-induced deacetylation of PGC-1α by SIRT1 activates gene expression of mitochondrial fatty acid oxidation genes (54). In summary, our work

demonstrates that posttranslational modifications of PGC-1α by AMPK play an important role in integrating environmental changes into the corresponding metabolic adjustments. More specific research into mechanisms affected by the AMPK-mediated phosphorylation of PGC-1α is warranted.

Materials and Methods

Reagents. AICAR was obtained from Calbiochem (San Diego, CA) for animal experiments or Toronto Research Chemicals (Toronto, ON, Canada) for cell experiments. 8-Bromo AMP and metformin were purchased from Sigma (St. Louis, MO). Basic FGF was obtained from Invitrogen (Carlsbad, CA). Antibodies against pACC, pAMPK, AMPK, and γ-tubulin were purchased from Cell Signaling Technology (Danvers, MA). Active AMPK was obtained from Upstate Biotechnology (Lake Placid, NY). The 2-kb PGC-1α promoter has been described previously (46). PGC-1α mutant constructs encoding PGC-1α T177A/S538A were generated with a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA).

Cell Culture, Transfection, Reporter Gene Assays, and Coimmunoprecipitations. C2C12 myotubes were grown in DMEM supplemented with 10% FBS and differentiated into myotubes in DMEM supplemented with 2% horse serum. Primary muscle cells were isolated from PGC-1α WT and −/− mice as described previously (55). Myoblast were cultured in F10/Ham's medium supplemented with 20% FBS and basic FGF. For differentiation into myotubes, cells were shifted to DMEM supplemented with 5% HS for 2 days. The myotubes were treated with 500 μM AICAR or 1 mM metformin in DMEM supplemented with 0.5% BSA for 16 h. For the inhibitor studies, the myotubes were pretreated for 30 min with 1 mM 8-BrAMP. Reporter gene assays were performed in a C2C12 muscle cell line. Myoblast were transfected with SuperFect (Qiagen, Valencia, CA) and subsequently differentiated for 36 h before treatment with 500 μM AICAR for 7.5 h. Firefly luciferase activity was measured and normalized to *Renilla* luciferase expression (dual luciferase reporter assay system; Promega, Madison, WI). Empty pGL3basic reporter gene vector and pCDNA.3 vector served as the control for the PGC-1α 2-kb promoter and PGC-1α constructs, respectively. For coimmunoprecipitation experiments, cells were transformed with the corresponding plasmids [pCMV-Myc-AMPKα2; gift from P. Sanz (ref. 38) and pCMV-FLAG-PGC-1α] with Superfect (Qiagen). Forty-eight hours after transfection, cells were lysed [50 mM Tris·HCl, pH 7.8/137 mM NaCl/1 mM EDTA/0.2% sarkosyl/1% Triton X-100/1 mM DTT/10% (vol/vol) glycerol], and 500 μM total protein was subjected to immunoprecipitation with an M2 agarose anti-FLAG resin (Sigma) for 2 h at 4°C. Proteins were separated by SDS/PAGE and transferred to PVDF membrane. PGC-1α was detected with anti-PGC-1α antibodies (56), and the Myc-AMPKα2 was detected with anti-c-Myc (A14) antibodies (Santa Cruz Biotechnology, Santa Cruz, CA).

Phosphorylation Analyses *in Vitro* and *in Vivo*. GST-PGC-1α fragments, WT and mutants, were expressed in bacteria (BL21 strain; Invitrogen) and purified by using glutathione–Sepharose beads (GE Healthcare, Piscataway, NJ). Recombinant proteins were used as a substrate for *in vitro* phosphorylation reaction with activated AMPK, according to the manufacturer's instructions (Upstate Biotechnology). After the phosphorylation reactions, the glutathione beads were washed extensively, and eluted proteins were analyzed by SDS/PAGE and autoradiography. Protein levels were monitored by Coomassie blue staining. For *in vivo* phosphorylation, primary PGC-1α −/− myotubes were infected with retroviruses expressing a FLAG PGC-1α WT or mutant protein, respectively. Stable cell lines were established by selection with puromycin (pMSCVpuro; Clontech, Mountain View, CA). Cells were differentiated and treated with 250 μM AICAR and labeled for 1 h with $^{32}P_i$ (Amersham Biosciences, Piscataway, NJ). Cells were harvested and lysed [50 mM Tris·HCl, pH 7.8/137 mM NaCl/1 mM EDTA/0.2% sarkosyl/1% Triton X-100/1 mM DTT/10% (vol/vol) glycerol] and subjected to immunoprecipitation with an M2 agarose anti-FLAG resin (Sigma) for 2 h at 4°C. Immunoprecipitates were subjected to SDS/PAGE and transferred to a PVDF membrane; ^{32}P incorporation was visualized by autoradiography. PGC-1α protein levels were analyzed by Western blotting with M2 anti-FLAG antibodies (Sigma).

Oxygen Consumption Measurements. Primary muscle cells from PGC-1α WT and −/− mice were differentiated for 2 days in DMEM supplemented with 5% HS with or without 500 μM AICAR. Myotubes were treated for another 16 h with AICAR in DMEM supplemented with 0.5% BSA. Four days after differentiation, cells were washed with PBS at room temperature and trypsinized from the plates. After centrifugation, cells were resuspended in PBS and transferred to a 1-ml Clark-type oxygen electrode chamber. After recording the basal respiration rate, the uncoupled respiration was measured in the presence of the ATP synthase inhibitor, oligomycin (2.5 μg/ml). Rates of oxygen consumption were normalized to cell counts. No nonmitochondrial respiration was detected in the cells.

Analysis of Gene Expression. Total RNA was isolated from cells by using TRIzol (Invitrogen). For real-time PCR analysis, RNA was treated with DNase and subsequently reverse-transcribed by using iSCRIPT (Bio-Rad, Hercules, CA). Using semiquantitative PCR (Applied Biosystems, Foster City, CA) and SYBR Green (Applied Biosystems), mRNA levels were first normalized to TATA box-binding protein mRNA, and then relative mRNA levels were determined by using the $\Delta\Delta C$t.

Animal Experiments. All animal experiments were performed according to a protocol approved by the Institutional Animal Care and Use Committee. Mice with a muscle-specific mutation in PGC-1α are described elsewhere (42). Female mice, 5–7 weeks old, were injected i.p. with 250 mg/kg AICAR in sterile 0.9% NaCl or with 0.9% NaCl. Mice were killed 6 h later, and the gastrocnemius muscle was harvested for RNA analysis and Western analysis.

Statistical Analysis. Results are expressed as ±SD for cell experiments and ±SEM for animal experiments. Two-tailed Student's *t* tests were used to determine *P* values.

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