AMP-activated protein kinase phosphorylates transcription factors of the CREB family

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AMP-activated protein kinase (AMPK) has been identified as a regulator of gene transcription and mitochondrial proteins of oxidative metabolism as well as hexokinase expression in skeletal muscle. In mice, muscle-specific knockout of LKB1, a component of the upstream kinase of AMPK, prevents contraction- and 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR)-induced activation of AMPK in skeletal muscle, and the increase in hexokinase II protein that is normally observed with chronic AICAR activation of AMPK. Since previous reports show a cAMP response element in the promoter region of the hexokinase II gene, we hypothesized that the cAMP-response element (CRE) binding protein (CREB) family of transcription factors could be targets of AMPK. Using radioisotopic kinase assays, we found that recombinant and rat liver and muscle AMPK phosphorylated CREB1 at the same site as cAMP-dependent protein kinase (PKA). AMPK was also found to phosphorylate activating transcription factor 1 (ATF1), CRE modulator (CREM), and CREB-like 2 (CREBL2), but not ATF2. Treatment of muscle with AICAR increased luciferase activity approximately 2-fold more than 20 years ago, acetyl-CoA carboxylase (ACC) and 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase were identified as phosphorylation targets for the newly discovered AMP-activated protein kinase (AMPK) (11). Since that time, an increasing number of cellular proteins have been identified as direct downstream targets (49). In liver, phosphorylation of ACC and HMG-CoA reductase by AMPK results in decreased fatty acid and cholesterol synthesis, in keeping with this kinase’s general role of enhancing ATP production and reducing ATP utilization during times of energy stress. Specific transcription factors (e.g., p300, HNF4-α, ChREBP, TORC2) are phosphorylated by AMPK in liver, which results in decreased expression of lipogenic and gluconeogenic enzymes (11, 49). In skeletal muscle, activation of AMPK in response to contraction is important in stimulation of glucose uptake and fatty acid oxidation. Phosphorylation/activation of ACC in muscle results in a decrease in malonyl-CoA, relieving inhibition of carnitine acyl transferase and allowing fatty acyl-CoA to enter the mitochondrial matrix where oxidation occurs (31, 37, 52). Although activation of AMPK using 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) results in GLUT4 translocation and stimulation of glucose uptake into muscle, the specific signaling pathway is unknown (13, 28). In addition to acute effects on glucose uptake and fatty acid oxidation, recurrent activation of AMPK results in increases in expression of mitochondrial proteins, hexokinase, and GLUT4, thus increasing capacity of the muscle to produce ATP in response to contraction (16, 19, 35, 54). The increase in GLUT4 is thought to be due in part to direct phosphorylation of a transcription factor, GLUT4 enhancing factor (GEF), and also to stimulation of nuclear localization of myocyte enhancing factor (MEF2) (17). Both of these transcription factors have binding sites on the GLUT4 promoter. Recently, peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α) has been identified as one target of AMPK in the Thr177 isomer of the coactivator responsible in GLUT4 promoter (20). In addition, PGC-1α, the coactivator responsible in part for regulating expression of many mitochondrial enzyme genes, has a CRE in the promoter region of its gene (10, 14, 15, 23). A complex of LKB1, STRAD, and MO25 proteins serves as the major upstream kinase for AMPK in skeletal muscle (26, 39, 48). Expression of PGC-1α, cytochrome c, and citrate synthase each was diminished in red quadriceps muscle of muscle-specific LKB1 knockout (MLKB1-KO) mice (48).

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We hypothesized first that induction of hexokinase II by chronic AICAR injection would be prevented in mice lacking LKB1/AMPK signaling in their muscles. A previous study designed to develop AMPK assays using relatively small synthetic peptides with sequence homology to CREB suggested that CREB could be phosphorylated by AMPK (29). In addition, intracerebroventricular (icv) injection of AICAR induced a large increase in phospho-CREB in the arcuate nucleus, detected by immunohistochemistry (25). These observations along with scanning of peptide sequences for AMPK recognition motifs led to the hypothesis that CREB and other members of the CREB family of proteins are direct downstream targets for AMPK and that activation of AMPK would result in an increase in phospho-CREB in skeletal muscle and other tissues.

MATERIALS AND METHODS

Animal care and generation of LKB1-KO mice. Experimental procedures were approved by the Institutional Animal Care and Use Committee of Brigham Young University. Mice were bred and housed in a temperature-controlled (21–22°C) room with a 12:12-h light-dark cycle, with free access to standard chow and water. Muscle-specific knockout of LKB1 (LKB1-KO) was achieved by cross-breeding LKB1 conditional mice (3) (provided by R. DePinho and N. Bardeesy, Dana-Farber Cancer Institute, Boston, MA) in which the LKB1 allele contains loxP sites in introns 2 and 6 with MCK-Cre transgenic mice (4) (provided by C. R. Kahn, Joslin Diabetes Center, Boston, MA) in which Cre recombinase is expressed constitutively in skeletal muscle and heart under the creatine kinase promoter. In the resultant LKB1-KO mice, the skeletal muscle-specific expression of Cre results in Cre-mediated recombination between loxP sites and deletes exons of the LKB1 gene between the two sites. Presence of the floxed LKB1 and the MCK-Cre genes was assessed by PCR ear-nip analysis as described previously (48).

Treatment of wild-type and MLKB1-KO mice with AICAR. Wild-type and MLKB1-KO mice were given a subcutaneous injection (0.5 mg/g body wt) of AICAR 5 days in succession and were anesthetized (pentobarbital sodium, 45 mg/kg ip) for tissue removal 1 h after the final injection. The white region of the quadriceps was removed for Western blot.

Phosphorylation of CREB in incubated epitrochlearis muscles. Wistar strain rats (95–130 g body wt) were anesthetized with pentobarbital sodium (45 mg/kg ip). After rats had been under anesthesia for 45 min to 1 h, the epitrochlearis muscles were quickly removed and incubated in cell culture medium (DMEM) for 60 min at 37°C in the presence or absence of 2 mM AICAR. During the incubation period, the flasks were gassed continuously with 95% oxygen-5% carbon dioxide. Muscles were frozen in liquid nitrogen. Homogenates were prepared in medium containing 50 mM Tris, 250 mM mannitol, 50 mM NaF, 5 mM sodium pyrophosphate, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 1 mM DTT, 1 mM benzamidine, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), and 5 μg/ml soybean trypsin inhibitor, pH 7.4. Homogenates were freeze-thawed three times to ensure rupture of the nuclei and then centrifuged at 1,200 × g to remove particulate matter before blotting. Western blots for phosphorylated AMPK (Thr172, Cell Signaling no. 2531, Danvers, MA), phosphorylated ACC (Cell Signaling no. 3661), and phospho-CREB (pSer133) (Upstate, Charlottesville, VA) were run on the supernatants using standard blotting procedures as described previously (48).

In an additional experiment, LKB1, phospho-AMPK, and phospho-CREB were quantitated by Western blot in gastrocnemius, tibialis anterior, and heart muscle from wild-type and MLKB1-KO mice (n = 6 per genotype), using similar procedures as outlined above, except that the mouse homogenates were clarified by centrifugation at 5,000 g.

Effect of AICAR on HEK-293 cells transfected with a CREB-driven luciferase reporter. The HEK-293/CREB-luc cell line was obtained from Panomics (Redwood City, CA). This line is stably transfected with a luciferase reporter construct that has a CRE in its promoter. Cells were cultured in DMEM supplemented with 10% FBS, 10,000 U penicillin and streptomycin/ml, and 100 μg hygromycin B/ml in a humidified incubator at 37°C in 5% CO2. Cells were then transfected to 96-well plates (–5 × 104 cells/well) with AMPK, phospho-ACC, phospho-CREB, and phospho-ATF1 (Cell Signaling no. 9104), total AMPK, total ACC, total CREB, and phosphorylated CREB-related proteins, ATF1, ATF2, and CREM. In a control experiment, LKB1-
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STRAD-MO25 was added to the reaction mix with each of the recombinant proteins but in the absence of rAMPK. No phosphorylation of any of the proteins was noted unless AMPK was present. In some experiments, phosphorylation of CREB and other CREB-related proteins was determined using Western blotting with an antibody specific for the phospho-Ser133 site (Millipore, Billerica, MA). The identical or very similar amino acid sequence in phospho-CREB1, phospho-ATF1, and phospho-CREM is each detected with this antibody. CREB1 lacks a second AMPK site present in CREB.

To determine if AMPK phosphorylates the same site as PKA, a competition assay was performed. For the first reactions, AMPK and PKA were allowed (separately) to phosphorylate CREB1 for 60 min in the presence of 0.2 mM ATP (unlabeled) and [γ-32P]ATP (labeled ATP), as described above. In the next reactions, AMPK was allowed to phosphorylate CREB1 for 30 min using only unlabeled ATP (0.2 mM). Then PKA was added to this reaction along with labeled ATP, and the reaction was allowed to proceed for another 30 min. This process was then reversed in another tube, with PKA added first in the absence of label and AMPK added second along with the label. If PKA and AMPK phosphorylate the same site on CREB1, then prior phosphorylation (with unlabeled phosphate) of CREB1 by either PKA or AMPK would prevent the subsequent incorporation of labeled phosphate into CREB1 by the other kinase since the serine targets would all be occupied by unlabeled phosphate. On the other hand, if the two kinases did not phosphorylate the same site, or if one of the kinases phosphorylated an additional site, significant incorporation of labeled ATP would occur after preincubation with the other kinase and unlabeled ATP.

To confirm that CREB1 could be phosphorylated by native AMPK as well as by the rAMPK, CREB1 was added to the phosphorylation mix indicated above along with the AMPK isolated from liver or muscle. Autoradiography was used to determine incorporation of label into the recombinant CREB1.

**Phosphorylation of an artificial peptide having the sequence surrounding Ser133 of CREB.** A peptide (CREB-Ser133) with the sequence ILSRRPSYRKILRR was prepared by BioPeptide (San Diego, CA). This peptide has an amino acid sequence identical to the one previously phosphorylated as indicated above. After a 10-min incubation at 30°C, an aliquot of the reaction mix was spotted on a 1-cm-square P81 filter paper. The labeled ATP was washed out by six washes with 1% phosphoric acid. The papers were immersed briefly in acetone, dried, and added to scintillation vials. After addition of Ecolite scintillation cocktail, radioactivity was determined in a liquid scintillation counter. The Km for CREB-Ser133 was also determined by measurement of activity at concentrations ranging from 4 μM to 100 μM CREB-Ser133. The Km was calculated using the enzyme kinetics module of Sigma Plot (Aspire Software International, Ashburn, VA).

**RESULTS**

The increase in white quadriceps hexokinase that occurs with 5 days of chronic AICAR injections is prevented when LKB1 is not expressed in muscle (Fig. 1B). Although phospho-AMPK did not appear to be increased 1 h following the final injection with AICAR, phospho-ACC was markedly increased in wild-type but not in MLKB1-KO mice (Fig. 1A). This provides evidence for the ZMP-triggered allosteric activation of phospho-AMPK. The MLKB1-KO mice, however, had much less phospho-AMPK and phospho-ACC. ZMP derived from phosphorylation of AICAR would not be expected to activate the nonphosphorylated AMPK. rAMPK has no activity in the presence or absence of 0.2 mM AMP or 0.2 mM ZMP unless first phosphorylated by LKB1-STRAD-MO25 (47). CREB phosphorylation was lower in gastrocnemius, tibialis anterior, and heart muscles (Fig. 2) from a separate group of MLKB1-KO mice (in which AMPK phosphorylation is virtually eliminated), suggesting that AMPK may play a role in maintaining basal levels of CREB phosphorylation. CREB phosphorylation was not significantly altered in red and white quadriceps muscles from MLKB1-KO mice (data not shown).

In incubated rat epitrochlearis muscles, phospho-AMPK is significantly increased in response to 60 min incubation with AICAR to activate AMPK (Fig. 3A). This is accompanied by a concurrent increase in phosphorylation of the downstream target, ACC (Fig. 3B). Under these conditions, phospho-CREB is also significantly increased over muscles incubated with medium only, without AICAR (Fig. 3C).

In the HEK-293 cells transfected with the luciferase reporter gene, bioluminescence increased ~13-fold in response to 10 μM forskolin over the course of a 4-h incubation (data not shown). AICAR also stimulated transcription of the CREB-driven luciferase gene with a progressive increase in activity over the course of the 4-h period (Fig. 4A). After 24 h an approximate threefold increase occurred in luciferase activity in response to AICAR compared with controls. Compound C completely blocked this increase in luciferase expression triggered by incubation with AICAR (Fig. 4B). Phosphorylation of AMPK (Fig. 4C), ACC (Fig. 4D), and CREB (Fig. 4E) were all increased after 24 h of incubation with AICAR as well, and all of these effects were likewise blocked by compound C.

Figure 5 shows that both AMPK and PKA phosphorylate CREB1 with similar stoichiometry. Figure 6A indicates both PKA and AMPK compete for phosphorylation at the same site on CREB1. When CREB1 is first phosphorylated with PKA with 0.2 mM ATP in the absence of radioactively labeled ATP, little phosphorylation is observed with subsequent addition of labeled ATP and AMPK. Likewise, when AMPK is added first with 0.2 mM ATP and without label, Ser119 is already ester-

Table 1. Amino acid sequences of putative AMPK target sites of CREB-related proteins

<table>
<thead>
<tr>
<th>Name</th>
<th>Phosphorylation Site</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>CREB</td>
<td>Ser98</td>
<td>LKRLFSGTQI</td>
</tr>
<tr>
<td></td>
<td>Ser133</td>
<td>LSRRPSYRKI</td>
</tr>
<tr>
<td>CREB1</td>
<td>Ser119</td>
<td>LSRRPSYRKI</td>
</tr>
<tr>
<td>ATF1</td>
<td>Ser63</td>
<td>LARRPSYRKI</td>
</tr>
<tr>
<td></td>
<td>Ser267</td>
<td>LKDLYSNKSIV</td>
</tr>
<tr>
<td>ATF2</td>
<td>No consensus sites</td>
<td></td>
</tr>
<tr>
<td>CREM</td>
<td>Ser71</td>
<td>LSRRPSYRKI</td>
</tr>
<tr>
<td></td>
<td>Ser192</td>
<td>VKCLESRVAV</td>
</tr>
<tr>
<td>CREBL2</td>
<td>No consensus sites</td>
<td></td>
</tr>
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AMPK, AMP-activated protein kinase; CREB, cAMP-response element (CRE) binding protein; CREM, CRE modulator; CREBL2, CREB-like-2; ATF1 and ATF2, activating transcription factor 1 and 2, respectively.
ified with cold phosphate so that subsequent incubation with PKA + labeled ATP shows little incorporation of labeled phosphate into the protein. Additional evidence that AMPK and PKA phosphorylate the identical site of CREB1 is provided by the Western blot data using a specific antibody for Ser133 phospho-CREB (Fig. 6B). After incubation with the activated rAMPK or PKA, the phospho-serine-specific antibody detected similar amounts of phosphorylation by the two kinases. Similar results were observed for ATF1 and CREM, indicating that these two transcription factors are also phosphorylated at the same site by AMPK and PKA (data not shown).

Figure 7 provides evidence that CREB1 can be phosphorylated by native AMPK isolated and partially purified from rat liver and rat skeletal muscle. The phenomenon is not restricted to the recombinant kinase.

Figure 8 demonstrates that AMPK phosphorylates two preparations of CREB: recombinant CREB1 and recombinant CREB fused with maltose binding protein. ATF1, CREM, and CREBL were all found to be phosphorylated by AMPK. We noted two distinct bands in the CREBL2. It is not clear if this represents dimer formation. Recombinant ATF2 was not found to be phosphorylated by AMPK (data not shown).

Figure 9 shows phosphorylation of synthetic peptides by rAMPK. At concentrations of 200 μM, all peptides were phosphorylated by rAMPK, although the CREB-Ser133 peptide was phosphorylated to a lesser extent than either preparation of SAMS or AMARA (Fig. 9A). The $K_m$ for CREB-Ser133 was determined to be 11.2 ± 2.0 μM (Fig. 9B).

**DISCUSSION**

The failure of AICAR to induce an increase in hexokinase II expression in the MLKB1-KO mice provided the first clue that the CREB family of transcription factors may be targets for AMPK. Since the hexokinase II gene has a CRE element in its promoter, we hypothesized that AMPK could be stimulating transcription of this gene by phosphorylating CREB or ATF1.

Known target proteins of AMPK have amino acid sequences surrounding the phosphorylated serine residue with the following characteristics: 1) an amino acid with a bulky hydrophobic R-group (M, L, I, F, V) at position P (phospho-serine) minus 5; 2) an amino acid with a bulky hydrophobic R-group at P plus 4; 3) an amino acid with a basic R-group (R, K, H) at either P minus 4 or P minus 3 (49). Table 1 shows the sequences in the proteins we investigated, demonstrating that all except CREBL2 have one or two putative sites that could be phosphorylated by AMPK. Note that CREB (Ser133), CREB1 (Ser119), and CREM (Ser71 in this variant and Ser120 in full-length human CREM) all have identical sequences surrounding one AMPK recognition site. ATF1 (Ser63) is identical except for an alanine in place of serine in the P minus 4 position.
In these experiments, CREB1 was found to be phosphorylated at the same site by both PKA and AMPK evidenced by competition for the same site in the labeled ATP assays and by the Western blots using the antibody targeting Ser133 of CREB. This antibody also detected phosphorylation of ATF1 and CREM by AMPK. The sequence recognition motif for PKA is -XRRXSX- (X/Arbitrary amino acid), which is present for CREB, CREB1, ATF1, and CREM within the AMPK targeting site (8, 42). The synthetic peptide CREB-Ser133 was found to be a good substrate for AMPK. The $K_m$ for this peptide was relatively low ($11.2 \pm 2 \mu M$) and comparable to reported values for SAMS peptide (26 $\mu M$) (41) and that reported for the best model substrate, a peptide based on rat ACC1 (5 $\mu M$) (40).

CREB has previously been identified as a target for a number of different kinases, including protein kinase C (PKC), Akt, MSK-1, RSK2, p70S6K, MAPKAP-K2, and CaMKII and -IV (8, 30, 42). These CREB-related transcription factors have the capacity therefore to integrate a number of upstream regulatory signals. In the present experiment, it is possible that one or more kinases in addition to AMPK may have been activated by AICAR. For example, atypical forms of PKC have been reported to be activated with AICAR treatment, and evidence is presented that this activation is downstream from AMPK activation (6). Thus AMPK may not only phosphorylate CREB directly but also may activate other kinases capable of phosphorylating CREB.

Phosphorylation of CREB, ATF1, and CREM has previously been demonstrated to be important for enhancement of transcriptional activity. Although phosphorylation is not essential for binding to the CRE on promoter regions of genes, recruitment of essential coactivators (CREB binding protein or CBP, and p300) to the CREB-CRE complex is greatly enhanced by the phosphorylation (8, 30, 42). Each of these factors may bind as homo- or heterodimers to the palindromic CRE motif (TGACGTCA). Single-motif CREs also exist (GTCA). CBP and p300 interact with TFIIB, TBP, and an RNA helicase and also have histone acetyltransferase activity (42, 43). The net effect of phosphorylation of CREB, ATF1, and CREM is an increase in rate of transcription of the target gene.

A large number of genes have been found to have CRE elements in their promoters. Transcription factors of the CREB family influence expression of a vast number of proteins involved in many physiological processes. These include metabolic regulation, neurotransmitter and neurotransmitter receptor synthesis, memory, long-term potentiation, expression of growth factors, immune regulation, structural protein expression, cell cycle regulation, DNA repair, and transport (30). Those of particular importance to muscle include genes for lactate dehydrogenase, cytochrome c, amino-levulinate synthase, carnitine palmitoyl-transferase, phosphoenolpyruvate carboxykinase, hexokinase II, pyruvate dehydrogenase, and PGC-1, to name a few (30).

Evidence that phosphorylation of CREB by AMPK may be an important cellular regulatory mechanism is supported by the experiment with the HEK-293 cells transfected with the CREB-driven luciferase reporter. The well-known activator of AMPK, AICAR, stimulates a significant increase in both CREB phosphorylation and transcription of the reporter gene resulting in accumulation of the luciferase product. The AMPK inhibitor, compound C, completely inhibits the 24-h response to AICAR in the HEK-293 cells. The presence of LKB1 signaling is necessary for induction of an increase in hexokinase II expression in muscle by chronic injection with AICAR for 5 days. These observations, coupled with a previous report indicating reduced expression of PGC-1, cytochrome c, and citrate synthase in skeletal muscles and heart of MLKB1-KO mice compared with wild type suggest a physiological role for

**Fig. 3.** Effect of 2 mM AICAR on phosphorylation of AMP-activated protein kinase (AMPK) (A), ACC (B), and CREB (C) in incubated rat epitrochlearis muscle. Muscles were removed from male Wistar rats and incubated for 1 h in DMEM without (control) or with 2 mM AICAR under 95% oxygen-5% carbon dioxide. After freezing the muscles, homogenates were prepared for Western blots. Values are means ± SE. Representative Western blot images are shown for each protein. *Significantly different from control, $P < 0.05$; $n = 8$. 

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the LKB1/AMPK signaling pathway in controlling CREB phosphorylation and gene expression in skeletal muscle.

Additional evidence for a physiological role of AMPK phosphorylation of CREB comes from studies on the arcuate nucleus in the hypothalamus (25). C75, a fatty acid synthetase blocker, reduced AMPK phosphorylation in the hypothalamus and reduced food intake. Infusion of AICAR reversed this effect on food intake. Blocking the action of AMPK using compound C also caused a marked reduction in food intake. Infusion of AICAR (icv) into the hypothalamus caused a marked increase in phospho-CREB. The immunoreactivity of phospho-CREB fluctuated similarly to phospho-AMPK in response to C75 and fasting. It was hypothesized that AMPK phosphorylates and activates CREB, which then increases neuropeptide Y (NPY) expression. The increase in NPY then triggers the increase in food intake. More recent studies in our laboratory show a concurrent increase in AMPK activation along with an increase in phospho-CREB in response to ex-

Fig. 4. Effect of 1 mM AICAR and 20 μM compound C (CC) on HEK-293 cells stably transfected with a CREB-driven luciferase reporter gene. A: luciferase activity after cells were incubated in serum-free DMEM (control) or serum-free DMEM + AICAR for the times indicated. Control, serum free media + vehicle (DMSO); AIC, 1 mM AICAR; CC, 20 μM compound C. C: AMPK phosphorylation after incubation of cells for 24 h with the indicated treatments. For all experiments, values are means ± SE for 6 incubations per treatment group. Representative images are shown for the Western blotting data. *Significantly different from control at same time point, P < 0.05.
Experimental hyperthyroidism in the rat (unpublished data from our laboratory). These hyperthyroid rats also show increases in protein expression of several genes with CREs in their promoters. AMPK is clearly activated in muscle in response to contraction, as the free concentration of AMP increases (50, 52, 53). In other tissues, any energy challenge, such as hypoxia or substrate deficiency, can activate AMPK (5, 11, 24, 49). The system is also subject to regulation by hormones, including IL-6, leptin, and adiponectin (21, 32, 33, 56). Since several CREB proteins are clearly downstream targets for AMPK as shown from our in vitro experiments, AMPK must be added to the list of kinases that can regulate the CREB family of transcription factors.

Because of the large number of kinases that can phosphorylate CREB, it may prove difficult to consistently demonstrate changes in CREB phosphorylation state in a tissue by removal or activation of a specific kinase, such as AMPK. When the influence of one of these kinases is removed, others may compensate under many circumstances. For instance, we observed reduced CREB phosphorylation in the gastrocnemius, tibialis anterior, and heart muscles from MLKB1-KO mice, but not in the quadriceps muscles. Likewise, it was only after several unsuccessful trials that we were able to define conditions where an increase in phospho-CREB could be seen in incubated muscle in response to AMPK activation with AICAR. First we found it necessary to use serum-free and albumin-free medium with no hormones added. Before collection of the epitrochlearis, rats were anesthetized for 45 min to 1 h to allow stress of the injection of anesthetic to subside. Rats in the 95–130 g range of body weight were utilized so that the epitrochlearis muscles were small and could be oxygenated adequately by continuous gassing of the incubation flask with 95% oxygen-5% CO₂. Without these stringent conditions, we found the response to AMPK activation with AICAR to be somewhat variable with respect to phospho-CREB content, although trends were noted with less stringent conditions.

Little data is available regarding effect of muscle contraction on phosphorylation of CREB. One study on human subjects exercising one leg for 1 h demonstrated an increase in phospho-CREB in the nonexercising leg 1 h postexercise, but not in the contracting muscle, although there appeared to be a trend toward an increase at the end of exercise in the contracting muscle (51). Subjects were working at ~70% of maximal O₂ uptake. A more recent one-leg training study reports phospho-CREB to be elevated 15 h postexercise in muscle biopsies from both nontrained and 3-wk trained muscle (36).

We have previously reported increases in mitochondrial enzyme and hexokinase II expression in muscle in response to
chemical activation of AMPK with AICAR (16, 54). PGC-1α is now well-established as being important in control of expression of mitochondrial oxidative enzymes (2, 9, 10, 23). Genes for PGC-1α, hexokinase II, and other muscle proteins also have CREB-regulated elements in their promoters. A CRE has been demonstrated to be essential for nerve stimulation-induced increase in PGC-1α promoter activity in mouse tibialis anterior (1, 57). AMPK phosphorylation of CREB may therefore be responsible in part for increasing these proteins in response to AICAR and possibly other physiological means of AMPK activation.

Recent studies have identified additional levels of regulation of CREB by the LKB1 signaling pathway (22). LKB1 is the upstream kinase of salt-inducible kinase (SIK), which represses CREB by phosphorylating the transducer of regulated CREB activity (TORC). TORC upregulates CREB activity by a mechanism independent of Ser133 phosphorylation. When phosphorylated by SIK, TORC is exported from the nucleus, and its stimulatory effect on CREB is lost. In liver, AMPK can also phosphorylate TORC directly, which induces retention in the cytoplasm (7, 27). Currently, it is not clear how direct phosphorylation/activation of the CREB proteins by AMPK is balanced by the parallel negative signals mediated by SIK or under what conditions the positive signal predominates. In skeletal muscle, however, SIK immunoreactivity has been reported to be undetectable (38), but TORC has been reported to be a major stimulator of PGC-1α gene expression and mitochondrial biogenesis in mouse primary muscle cultures (55).

In summary, induction of an increase in hexokinase II with chronic AICAR injection is prevented in the MLKB1-KO mice. HEK-293 cells stably transfected with a CREB-driven luciferase reporter show an increase in CREB phosphorylation and luciferase expression on treatment with AICAR. These increases are blocked with an AMPK inhibitor. When AMPK is activated in incubated epitrochlearis with AICAR, an increase in phospho-CREB was observed after 1 h of incubation. The recombinant transcription factors rCREB, rATF1, and rCREM are all phosphorylated by recombinant AMPK. CREB1 can be phosphorylated by both PKA and AMPK at the same phosphorylation site. CREB1 is also phosphorylated by native AMPK isolated from liver and skeletal muscle. A synthetic peptide with sequence identical to the site surrounding the phospho-Ser133 site of CREB1 specifically inhibits AMPK phosphorylation of CREB1 in vitro.

**Fig. 7.** Autoradiogram demonstrating phosphorylation of CREB1 by AMPK isolated from skeletal muscle (SM AMPK) or isolated from rat liver (Liv AMPK) or by recombinant AMPK (rAMPK). Representative of 5 determinations for liver.

**Fig. 8.** Autoradiogram showing phosphorylation of CREB1, CREB maltose binding protein fusion protein (CREB-MBP), activating transcription factor 1 (ATF1), CRE modulator (CREM), and CREB-like 2 (CREBL2). Representative of 5 autoradiograms.

**Fig. 9.** A: AMPK phosphorylation of SAMS (two vendors, Z and B), AMARA, and CREB-Ser133 (CREB-S133) (sequence = ILSRRPSYRKILRR) at a peptide concentration of 200 μM (n = 4). The reaction mix contained 0.2 mM ATP, radiolabeled ATP, and 0.2 mM AMP in the phosphorylation buffer. B: determination of the Km for CREB-S133. The rate of phosphorylation was determined for concentrations of the peptide (n = 3 for each concentration) ranging from 0 to 100 μM. Data were fitted to the Michaelis-Menten equation using the enzyme kinetics module of SigmaPlot. The Km was determined to be 11.2 ± 2 μM.
ing Ser133 in CREB is phosphorylated with a $K_m$ lower than that reported for SAMS peptide. We conclude that the LKB1/AMPK signaling system exhibits the capacity to regulate the CREB family of transcription factors by phosphorylation.

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