

# The Ca<sup>2+</sup>/Calmodulin-dependent Protein Kinase Kinases Are AMP-activated Protein Kinase Kinases\*<sup>§</sup>

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The AMP-activated protein kinase (AMPK) is an important regulator of cellular metabolism in response to metabolic stress and to other regulatory signals. AMPK activity is absolutely dependent upon phosphorylation of AMPK $\alpha$ Thr-172 in its activation loop by one or more AMPK kinases (AMPKKs). The tumor suppressor kinase, LKB1, is a major AMPKK present in a variety of tissues and cells, but several lines of evidence point to the existence of other AMPKKs. We have employed three cell lines deficient in LKB1 to study AMPK regulation and phosphorylation, HeLa, A549, and murine embryo fibroblasts derived from LKB1<sup>-/-</sup> mice. In HeLa and A549 cells, mannitol, 2-deoxyglucose, and ionomycin, but not 5-aminoimidazole-4-carboxamide-1- $\beta$ -D-ribofuranoside (AICAR), treatment activates AMPK by  $\alpha$ Thr-172 phosphorylation. These responses, as well as the downstream effects of AMPK on the phosphorylation of acetyl-CoA carboxylase, are largely inhibited by the Ca<sup>2+</sup>/calmodulin-dependent protein kinase kinase (CaMKK) inhibitor, STO-609. AMPKK activity in HeLa cell lysates measured *in vitro* is totally inhibited by STO-609 with an IC<sub>50</sub> comparable with that of the known CaMKK isoforms, CaMKK $\alpha$  and CaMKK $\beta$ . Furthermore, 2-deoxyglucose and ionomycin-stimulated AMPK activity,  $\alpha$ Thr-172 phosphorylation, and acetyl-CoA carboxylase phosphorylation are substantially reduced in HeLa cells transfected with small interfering RNAs specific for CaMKK $\alpha$  and CaMKK $\beta$ . Lastly, the activation of AMPK in response to ionomycin and 2-deoxyglucose is not impaired in LKB1<sup>-/-</sup> murine embryo fibroblasts. These data indicate that the CaMKKs function in intact cells as AMPKKs, predicting wider roles for these kinases in regulating AMPK activity *in vivo*.

olic stress (1). AMPK is a serine/threonine protein kinase and a member of the Snf1/AMPK protein kinase family (1). It is an  $\alpha\beta\gamma$  heterotrimeric protein, consisting of an  $\alpha$  catalytic subunit, a  $\beta$  subunit important both for enzyme activity and for targeting, and a  $\gamma$  regulatory subunit, which binds the allosteric activator, AMP. The activity of AMPK absolutely requires phosphorylation of the  $\alpha$  subunit on Thr-172 in its activation loop by one or more upstream kinases (AMPKK) (1).

The major breakthrough in identifying AMPK upstream kinases came from the study of the regulation of the AMPK ortholog, Snf1, in *Saccharomyces cerevisiae*, in which Pak1 was shown to act as a Snf1p kinase kinase (2). Subsequently, it was shown that three closely related kinases, Pak1p, Tos3p, and Elm1p, needed to be deleted to generate the Snf1<sup>-</sup> phenotype (3, 4). Sequence comparison revealed that the human LKB1 tumor suppressor kinase was the most closely related mammalian kinase. LKB1 was subsequently identified by several groups as being an important upstream kinase active on AMPK (5–7).

Several lines of evidence point to the presence of non-LKB1 AMPKKs. Multiple AMPKK activities are separable during chromatography of extracts from rodent heart (8, 9). Murine fibroblasts obtained from LKB1<sup>-/-</sup> embryos by two different groups still demonstrate residual AMPK172 $\alpha$  phosphorylation and AMPK activity, albeit not as responsive to the usual activators of AMPK, such as the nucleoside AICAR (5, 7). Partially purified Ca<sup>2+</sup>/CaM-dependent protein kinase kinase (CaMKK) from pig brain has been shown to be active *in vitro* on AMPK, but it was concluded that the kinetics of phosphorylation by CaMKK were weaker than those for a partially purified AMPKK and that CaMKKs were unlikely to function as AMPKKs in intact cells and tissues (10). Although this view has been widely accepted (28), Nath *et al.* (2) suggested that CaMKK $\beta$  may be an AMPKK based on homology with yeast PAK1. Recently, CaMKK $\alpha$  has been shown directly to function as a Snf1-activating kinase in yeast cells lacking the three Snf-activating kinases, Pak1, Tos3, and Elm1 (29).

The protein products of the CaMKK gene family, CaMKK $\alpha$  and CaMKK $\beta$ , show significant homology to LKB1 and to the three aforementioned yeast kinases (3–5). In the present study, we have investigated the possibility that one or both CaMKKs might serve as AMPKKs to regulate AMPK in cell lines lacking expression of LKB1.

The AMP-activated protein kinase (AMPK)<sup>1</sup> regulates many aspects of cellular metabolism, especially in response to meta-

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<sup>§</sup> The on-line version of this article (available at <http://www.jbc.org>) contains a supplemental figure showing AMPK activity and phosphorylation in A549 cells.

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<sup>1</sup> The abbreviations used are: AMPK, AMP-activated protein kinase;

AMPKK, AMPK kinase; MEF, mouse embryo fibroblast; ACC, acetyl-CoA carboxylase; CaM, calmodulin; CaMKK, CaM-dependent protein kinase kinase; siRNA, small interfering RNA; HRP, horseradish peroxidase; ANOVA, analysis of variance; 2-DG, 2-deoxyglucose; MAP, mitogen-activated protein; AICAR, 5-aminoimidazole-4-carboxamide-1- $\beta$ -D-ribofuranoside.

## EXPERIMENTAL PROCEDURES

**Cell Culture and Incubations**—Panc-1, AsPC-1, and COS cells were purchased from ATCC. Mouse embryo fibroblasts (MEFs) from LKB1<sup>+/+</sup> and LKB1<sup>-/-</sup> mice and HeLa cells were kindly provided by Reuben Shaw (Harvard University). These cell lines were grown in

Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. A549 cells (ATCC) were grown in Ham's F-12 medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. All cell lines were maintained at 37 °C in a humidified atmosphere containing 5% CO<sub>2</sub>. Cells were incubated in 6-well plates after various additions (see the figure legends) prior to extraction for immunoblotting and analysis of AMPK activity.

**Preparation of Cell Extracts**—Cell extracts were prepared by three different methods. For analysis of AMPK activity, digitonin lysis followed by ammonium sulfate precipitation was employed, as in Ref. 11. For immunoblotting of total cellular protein, cells were lysed either in a Triton X-100-containing buffer, as in Ref. 12, or in an SDS-containing buffer (2×) and lysed directly on the plate with boiling SDS buffer (1% SDS, 100 mM NaCl, 10 mM Tris-HCl, pH 7.5). These extracts were then sheared with a 25-gauge needle and boiled for 5 min. Protein concentration in all extracts was determined with a BCA assay (Pierce), according to the manufacturer's protocol.

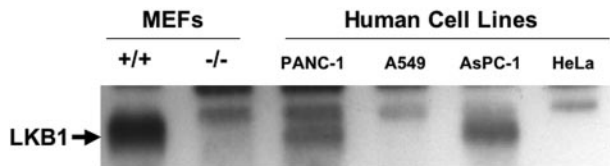


FIG. 1. **Human cell lines lacking LKB1.** SDS lysates were prepared from wild-type (+/+) or LKB1 null (-/-) MEFs and from four human cancer cell lines PANC-1, A549, AsPC-1, and HeLa, as described under "Experimental Procedures." Equal amounts of protein from each (20 μg/lane) were subjected to SDS-PAGE followed by immunoblot analysis using an antibody directed against LKB1.

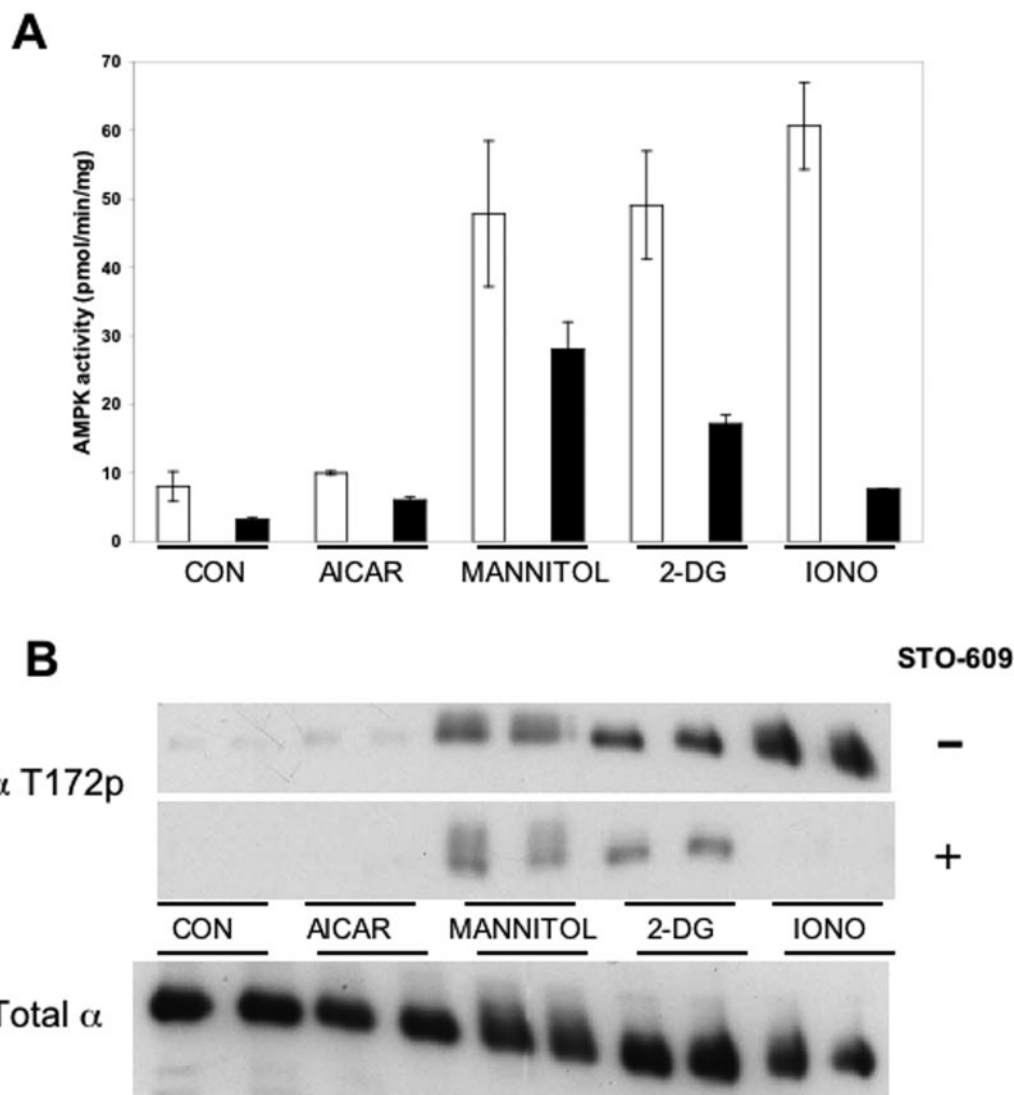


FIG. 2. **AMPK activity and phosphorylation in HeLa cells.** HeLa cells were incubated in serum-free Dulbecco's modified Eagle's medium with either STO-609 (1 μg/ml) or an equivalent volume of its diluent, Me<sub>2</sub>SO (1:2000), for 6 h followed by treatment with one of the following reagents: AICAR (2 mM, 2 h), mannitol (0.6 M, 15 min), 2-DG (50 mM, 15 min), and ionomycin (*Iono*) (1 μM, 5 min). In these and the other cell experiments in this study, cells were plated in 6-well plates. Cells extracts were then prepared by the digitonin lysis method, as described under "Experimental Procedures." Each extract (*n*) represents a pooling of three wells incubated under identical conditions. *A*, extracts (*n* = 3 at each condition) were assayed for AMPK activity, as described under "Experimental Procedures." *Open bars* represent treatment with Me<sub>2</sub>SO, and *shaded bars* represent treatment with STO-609. Data are expressed as mean ± S.D. as pmol of <sup>32</sup>P incorporation into the SAMS peptide per minute per mg of protein. As determined by ANOVA analysis, the stimulations by mannitol, 2-DG, and ionomycin and the inhibition of these effects by STO-609 are significant at *p* < 0.0001; there is no statistically significant difference between control (*CON*) and AICAR samples either in the presence or in the absence of STO-609. For the entire data set, *F*(4,20) = 26.4, *p* < 0.001 for the effects of STO-609. *B*, representative immunoblots of duplicate extracts from each incubation condition are shown developed with antibodies directed against either AMPKαT172p (*top* and *middle*) or total AMPKα (*bottom*).

**Enzyme Activities and Immunoblotting**—AMPK activity against the SAMS peptide was determined at a saturating concentration of AMP, as in Ref. 11. AMPKK activity in Triton X-100 cell lysates was determined by phosphorylation of a recombinant AMPK $\alpha$  protein, as in Ref. 12. Cell extracts were examined by immunoblotting, as in Ref. 12, employing a panel of different antibodies/reagents. These included anti-AMPK total  $\alpha$  (reactive against  $\alpha$  and  $\alpha_2$ ), anti-AMPK $\alpha$ T172p, anti-ACCS79p, streptavidin-HRP (13), anti-CaMKK $\alpha/\beta$  (C-terminal; BD Transduction Laboratories catalog number 610544), and anti-LKB1 (a kind gift from Reuben Shaw and Ronald DePhino (Harvard University)) (7).

**RNA Interference**—siRNA oligonucleotides against a scrambled, non-targeting sequence as a negative control (D-001206-13-20) and CaMKK $\beta$  (human CaMKK2, SIGENOME SMARTpool reagent M-004842-00-0050, accession number NM\_006549) were obtained from Dharmacon Research (Lafayette, CO). siRNA oligonucleotides designed against CaMKK $\alpha$  (siRNA Gene Silencers, human) were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). HeLa cells were plated at a density of  $5 \times 10^5$  cells/well in a 6-well plate and allowed to adhere overnight. Cells were transfected with a non-targeting control siRNA pool (200 nM), CaMKK $\beta$  siRNA pool (100 nM), or CaMKK $\alpha$  siRNA (200 nM). Preliminary studies established that the latter two were employed at their maximally effective concentrations (data not shown). Transfection was carried out using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. At 48 h after transfection, cells were either harvested or treated with AMPK activators and then harvested.

**Preparation of LKB1/STRAD/Mo25 Complex**—COS cells were triply transfected with cDNAs expressing glutathione *S*-transferase-tagged LKB1 and FLAG-tagged STRAD and Mo25 (generous gifts from Reuben Shaw (Harvard University) and Dario Alessi (University of Dundee)) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Cells were lysed in buffer containing 1% Triton X-100, as described above, 48 h after transfection. Cleared supernatants were aliquoted into microcentrifuge tubes (1 ml/tube) and incubated with 100  $\mu$ l of 50% glutathione bead slurry (Sigma) for 2 h at 4 °C. Lysates were spun down briefly (20 s, 14,000 rpm) to gently pellet beads. The supernatant was carefully removed, and beads were washed once with buffer containing 1% Triton X-100 and then subsequently washed two times with 4 $\times$  assay buffer (0.4 M HEPES, 0.6 M NaCl). Beads were then resuspended in 4 $\times$  assay buffer containing 20 mM glutathione (Sigma) to elute the enzyme complex. Following a 20-min incubation on ice, samples are spun down for 1–2 min at 14,000 rpm to firmly pellet beads, and the supernatant, containing the purified LKB1/STRAD/Mo25 complex, was removed and used immediately in the *in vitro* kinase assays.

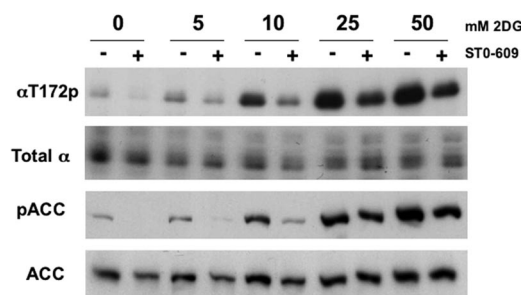
**Statistical Analysis**—Statistical analysis of experimental data were performed by a factorial ANOVA with multiple comparisons, using the least significant difference by the STATISTICA software package.

## RESULTS AND DISCUSSION

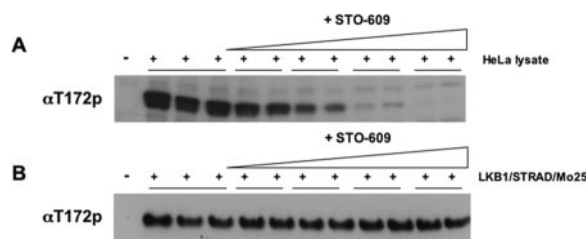
In an effort to identify AMPKKs distinct from LKB1, two human cell lines that are deficient in LKB1 were selected for study, the cervical carcinoma-derived HeLa and the lung adenocarcinoma-derived A549 lines. LKB1 is not detectable in these two lines nor in LKB1<sup>-/-</sup> mouse embryo fibroblasts by immunoblotting (Fig. 1). We did detect expression of LKB1 in other human cancer lines including PANC-1 and AsPC-1, the latter previously thought to be LKB1-negative (14).

HeLa and A549 cells were stimulated with a variety of agents known to activate AMPK by increasing phosphorylation of AMPK $\alpha$ T172 and with ionomycin, the latter to increase intracellular Ca<sup>2+</sup>. Mannitol, 2-deoxyglucose (2-DG), and ionomycin, but not AICAR, markedly stimulate both AMPK activity (Fig. 2A) and  $\alpha$ T172 phosphorylation (Fig. 2B, top panel) in HeLa cells. In A549 cells, mannitol and 2-deoxyglucose (2-DG), but not AICAR (data not shown), also stimulated AMPK activity and phosphorylation (Supplemental Fig. 1). The inability of AICAR to stimulate AMPK in HeLa cells and in LKB1<sup>-/-</sup> mouse embryo fibroblasts has been observed previously (5, 7).

The above experiments indicate the presence of AMPKK(s) in HeLa and A549 cells distinct from LKB1. Two other mammalian protein kinases have a significant homology to mammalian LKB1 and to the LKB1 orthologs in *S. cerevisiae*, namely Ca<sup>2+</sup>/CaM-dependent protein kinase kinase  $\alpha$



**FIG. 3. STO-609 inhibits AMPK and ACC phosphorylation in response to 2-deoxyglucose in HeLa cells.** HeLa cells, preincubated with either STO-609 (1  $\mu$ g/ml) or an equivalent volume of Me<sub>2</sub>SO for 6 h, were treated with increasing concentrations of 2-DG (5, 10, 25, 50 mM) for 15 min. Cell extracts were prepared by the digitonin lysis method, as described under "Experimental Procedures." Duplicate samples, matched for protein, from each incubation condition were pooled and subjected to SDS-PAGE followed by immunoblot analysis using antibodies AMPK $\alpha$ T172p, total AMPK $\alpha$  (Total  $\alpha$ ), or ACCS79p. Total ACC was detected by blotting with streptavidin-HRP. pACC, phosphorylated ACC.

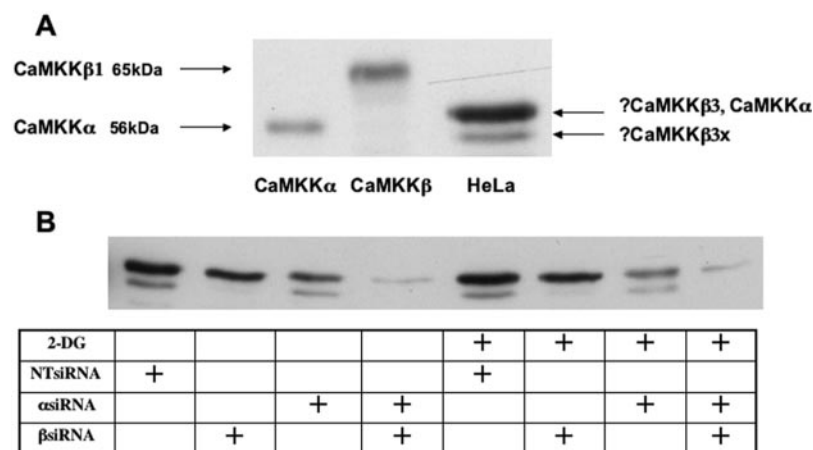


**FIG. 4. STO-609 inhibition of *in vitro* AMPK activity.** Triton X-100 lysates from unstimulated HeLa cells (A) or purified LKB1/Strad/Mo25 (B) were incubated, as in Ref. 12, in the absence (–) or presence (+); duplicate incubations) of recombinant AMPK $\alpha$  along with ATP/Mg<sup>2+</sup> for 20 min at 30 °C after a 10-min preincubation in the presence of increasing concentrations of STO-609 (0, 0.01, 0.1, 1, 10  $\mu$ g/ml). Reactions were stopped by the addition of 4 $\times$  Laemmli sample buffer, and samples were boiled for 5 min. Proteins were separated on 9% SDS-PAGE and probed with an antibody directed against AMPK $\alpha$ T172p to detect phosphorylation of the recombinant AMPK $\alpha$ .

(CaMKK $\alpha$ ) and Ca<sup>2+</sup>/CaM-dependent protein kinase kinase  $\beta$  (CaMKK $\beta$ ) (15–20). A CaMKK preparation from pig brain (isolated prior to the recognition that there were two isoforms of the enzyme) has previously been shown to phosphorylate and activate AMPK *in vitro*; this phosphorylation is enhanced by the binding of AMP to the AMPK heterotrimer (10). Since both CaMKK $\alpha$  and CaMKK $\beta$  are expressed in HeLa cells (but not in murine embryonic fibroblasts),<sup>21</sup> they were chosen for study of the possible roles for these enzymes (21).

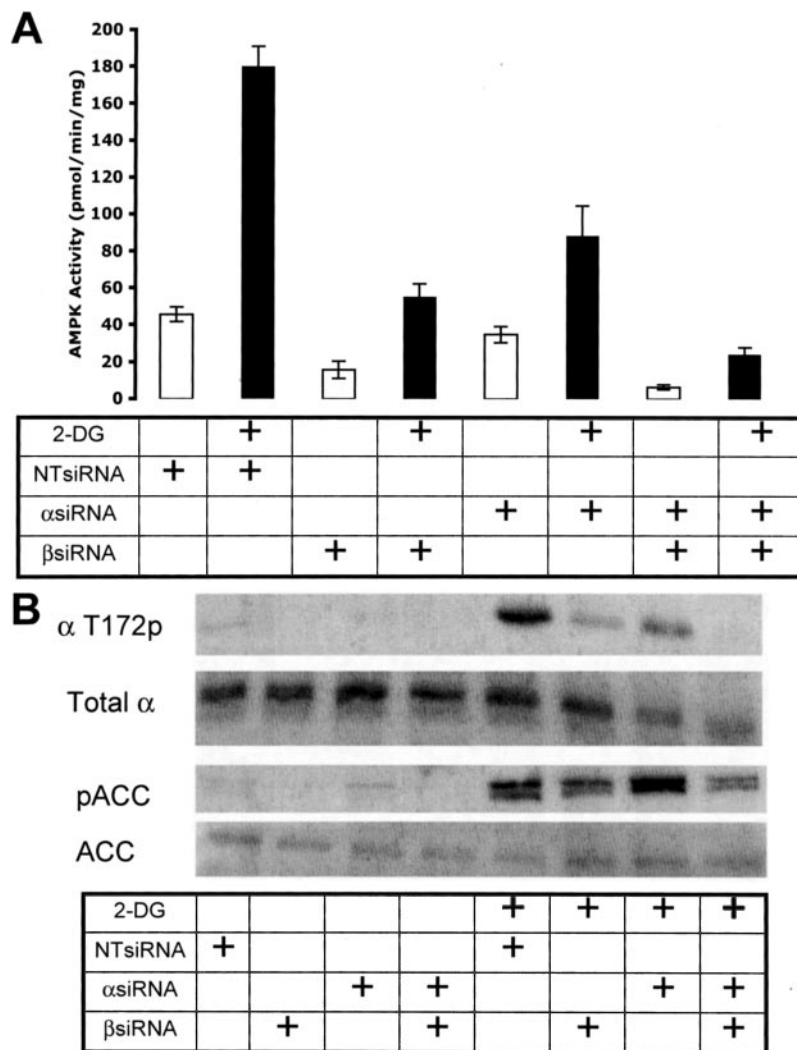
To test the hypothesis that one or both CaMKKs in HeLa cells functions as an AMPKK, HeLa cells were incubated under basal conditions or were stimulated with mannitol, 2-DG, or ionomycin in the presence of the CaMKK inhibitor, STO-609 (22, 23). At a concentration of 1  $\mu$ g/ml, STO-609 significantly inhibits AMPK activation and  $\alpha$ T172 phosphorylation in response to all three agents in HeLa cells (Fig. 2). The degree of inhibition of STO-609 was greatest in the ionomycin-stimulated cells (Fig. 2A). Since STO-609 did not, however, totally block the activation and phosphorylation of AMPK in response to mannitol and 2-DG, this might suggest that these cells possess residual LKB1 (although undetectable by immunoblot) or perhaps another AMPKK. STO-609 also inhibited AMPK activation in response to mannitol and 2-DG in A549 cells (Supplemental Fig. 1). Dose-dependent activation by 2-DG of AMPK  $\alpha$ T172 phosphorylation and phosphorylation of a down-

<sup>21</sup> R. L. Hurley, K. A. Anderson, J. M. Franzone, B. E. Kemp, A. R. Means, and L. A. Witters, unpublished observations.



**FIG. 5. CaMKK siRNA effects on immunoreactive HeLa CaMKKs.** A, shown is an immunoblot of a HeLa cell Triton X-100 extract (*right-hand lane*) probed with an antibody that recognizes C-terminal sequences common to CaMKKα and CaMKKβ (BD Transduction Laboratories; catalog number 610544). The two *left-hand lanes* represent CaMKK standards obtained following transfection of HeLa cells with expression plasmids encoding CaMKKα (a kind gift of Thomas Soderling) and CaMKKβ1. B, in parallel with the experiments shown in Fig. 6, HeLa cells were transfected with siRNAs against a non-targeting (NT) sequence, CaMKKα (indicated by αsiRNA), or CaMKKβ (indicated by βsiRNA) individually or together for 48 h. Cells were then incubated in the presence or absence of 50 mM 2-DG for 15 min and lysed in buffer containing Triton X-100, as described under “Experimental Procedures.” Shown is a representative immunoblot in which duplicate protein-matched samples were pooled and subjected to SDS-PAGE followed by immunoblotting with the above antibody.

**FIG. 6. siRNA knockdown of CaMKKs blocks AMPK activation by 2-deoxyglucose.** HeLa cells were transfected with siRNAs against a non-targeting (NT) sequence, CaMKKα (indicated by αsiRNA), or CaMKKβ (indicated by βsiRNA) followed by incubation for 48 h. Cells were then incubated in the presence or absence of 2-DG (50 mM) for 15 min and harvested by digitonin lysis. For each condition shown, transfections and subsequent incubations were carried out in quadruplicate wells. A, cell extracts prepared by digitonin lysis ( $n = 4$  at each condition) were assayed for AMPK activity, as described under “Experimental Procedures.” *Open bars* represent control incubations, and *shaded bars* represent treatment with 2-DG. Data are expressed as mean  $\pm$  S.D. as pmol of <sup>32</sup>P incorporation into the SAMS peptide per minute per mg of protein. As determined by ANOVA analysis, the stimulations under all conditions by 2-DG are significant at  $p < 0.0001$ . The inhibition of AMPK activity by siRNA under basal conditions is statistically significant for CaMKKβ siRNA and the combined siRNAs ( $p < 0.0001$ ), whereas the inhibition of 2-DG-stimulated AMPK activity is significant at  $p < 0.001$  for all siRNAs combinations tested. For the entire data set,  $F(3,24) = 81.44$ ,  $p < 0.0001$  for the effects of 2-DG. B, shown is a representative immunoblot in which duplicate protein-matched samples from each condition were pooled and subjected to SDS-PAGE followed by immunoblot analysis using antibodies AMPKαT172p, total AMPKα (*total α*), or ACCS79p. Total ACC was detected by blotting with streptavidin-HRP. *pACC*, phosphorylated ACC.



stream target of activated AMPK, acetyl-CoA carboxylase (ACC), were significantly inhibited by STO-609 in HeLa cells at 2-DG concentrations of  $\leq 10$  mM (Fig. 3). The IC<sub>50</sub> for STO-609 inhibition of 2-DG-stimulated αT172 phosphorylation in HeLa

cells was  $\sim 0.7$  μg/ml (data not shown). In other experiments (data not shown), we have found that STO-609 can inhibit the *in vitro* activity of purified AMPK against the SAMS peptide, although the IC<sub>50</sub> is 20-fold higher than that for the CaMKKs.

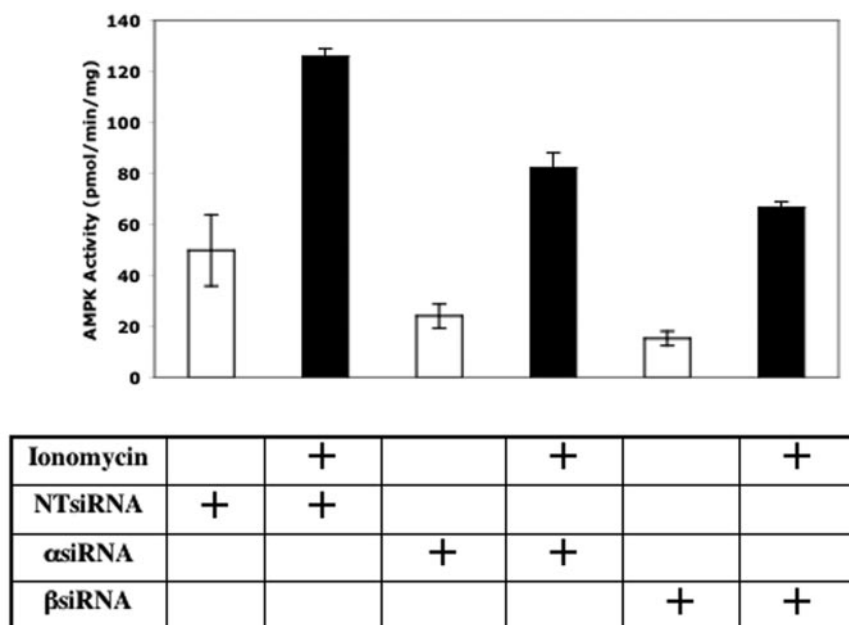


FIG. 7. **siRNA knockdown of CaMKKs reduces AMPK activation by ionomycin.** HeLa cells were transfected with siRNAs against a non-targeting (NT) sequence, CaMKK $\alpha$  (indicated as  $\alpha$ siRNA), or CaMKK $\beta$  (indicated as  $\beta$ siRNA) and incubated for 48 h. Cells were then incubated in the presence or absence of 1  $\mu$ M ionomycin for 5 min and harvested by digitonin lysis. Extracts were assayed against the SAMS peptide as described under "Experimental Procedures," to assess AMPK kinase activity. These results ( $n = 4$  separate pooled lysates at each condition) are expressed as mean  $\pm$  standard deviation of AMPK activity expressed as pmol of <sup>32</sup>P incorporation into the SAMS peptide per minute per mg of protein. *Open bars* represent basal activity, and *shaded bars* represent ionomycin-stimulated activity. As determined by ANOVA analysis, the inhibition of basal AMPK activity by both siRNAs is significant at  $p < 0.001$ , and that of the inhibition of the stimulation by ionomycin by each siRNA is significant at  $p < 0.0001$ . For the entire data set,  $F(2,18) = 7.17$ ,  $p < 0.005$  for the effects of ionomycin.

Thus, we cannot exclude the possibility that some fraction of the STO-609 inhibition of ACC phosphorylation in intact cells is due to the direct inhibition of AMPK, although the concentrations we employed in these intact cells studies was 1  $\mu$ g/ml. However,  $\alpha$ T172 is not an AMPK auto-phosphorylation site, so any effects of STO-609 cannot be accounted for by inhibition of auto-regulation.

CaMKK $\alpha$  activity is largely dependent upon Ca<sup>2+</sup> and CaM, whereas CaMKK $\beta$  has substantial constitutive activity in its absence, although it can be stimulated by a rise in Ca<sup>2+</sup> (15–17). Ionomycin increases intracellular Ca<sup>2+</sup> via an initial phase representing mobilization of intracellular stores and a sustained component representing Ca<sup>2+</sup> influx (24). In HeLa cells, the marked increase in AMPK activity and  $\alpha$ T172 phosphorylation in response to ionomycin (Fig. 2) might thus be due to a Ca<sup>2+</sup> stimulation of either of the CaMKKs. In support of this hypothesis, STO-609 completely blocks the ability of ionomycin to elicit these changes in AMPK (Fig. 2).

AMPK activity, measured in HeLa cell lysates against a recombinant AMPK $\alpha$  *in vitro*, was completely inhibited by STO-609 (Fig. 4A). The IC<sub>50</sub> for this *in vitro* STO-609 inhibition was  $\sim 0.02$   $\mu$ g/ml (data not shown). This IC<sub>50</sub> value is comparable with that reported for the inhibition of recombinant CaMKK $\alpha$  (0.12  $\mu$ g/ml) and CaMKK $\beta$  (0.04  $\mu$ g/ml) phosphorylation of a Ca<sup>2+</sup>/calmodulin-dependent kinase I (CaMKI) fusion protein *in vitro* (22). The IC<sub>50</sub> of STO-609 against several other protein kinases (CaM kinases I, II, and IV, myosin light chain kinase, protein kinase C, cAMP-dependent protein kinase, and p42 MAP kinase) exceeds 10  $\mu$ g/ml, establishing (in part) its specificity and potency as a CaMKK inhibitor (22). Additionally we have demonstrated that at these concentrations, STO-609 does not inhibit the activity of a LKB1/STRAD/Mo25 heterotrimer against the recombinant AMPK $\alpha$  *in vitro* (Fig. 4B), suggesting the potential utility of this inhibitor in establishing the relative roles of the CaMKKs and LKB1 as AMPKs in various tissues or cell preparations. STO-609, whereas specific for the

CaMKKs, however cannot clearly distinguish between the two CaMKK isoforms, CaMKK $\alpha$  and CaMKK $\beta$ .

HeLa cells have been reported to express both CaMKK $\alpha$  and CaMKK $\beta$  mRNAs (21). The CaMKK $\beta$  gene encodes several isoforms generated through differential usage of polyadenylation sites and/or as a result of alternative splicing of the internal exons 14 and/or 16 (21, 25), although the characteristics of the protein products (gel mobility, kinase activity, and tissue distribution) have not been fully delineated. In HeLa cells, the  $\beta$ 1 and  $\beta$ 2 isoforms are absent; mRNAs encoding two novel CaMKK isoforms (CaMKK $\beta$ 3 and CaMKK $\beta$ 3x) generated through alternative splicing have been noted (21). As probed with a monoclonal antibody that recognizes a C-terminal sequence common to CaMKK $\alpha$  and CaMKK $\beta$ , HeLa cell extracts display two immunoreactive species, migrating similarly to a CaMKK $\alpha$  standard and at the predicted molecular masses of CaMKK $\beta$ 3 (60 kDa) and CaMKK $\beta$ 3x (55 kDa) (Fig. 5A) (21).

Given the possibility that the effects of STO-609 to inhibit AMPK activity were not entirely mediated by specific chemical inhibition of the CaMKKs, we employed RNA interference as an independent way to inhibit their activity. As compared with transfected non-targeting siRNA, CaMKK $\beta$ -specific siRNA substantially decreased basal and 2-DG-stimulated AMPK activity and  $\alpha$ T172 phosphorylation, whereas CaMKK $\alpha$ -specific siRNA had smaller, but still apparent, effects on these 2-DG-stimulated AMPK alterations (Fig. 6A). No significant effects of the individual siRNAs on 2-DG-stimulated ACC phosphorylation are seen, and this is likely explained by the residual 2-DG-stimulated AMPK activity (Fig. 6B). We have noted that the phosphorylation of ACC in other systems is very sensitive to even small changes in AMPK activity (27). The combination of both CaMKK siRNAs nearly eliminated both basal and stimulated AMPK activity (Fig. 6A) and  $\alpha$ T172 and ACC phosphorylation (Fig. 6B). Both CaMKK $\alpha$ - and CaMKK $\beta$ -specific siRNAs also significantly reduced ionomycin-stimulated AMPK activity (Fig. 7). CaMKK $\beta$ -specific siRNA eliminated

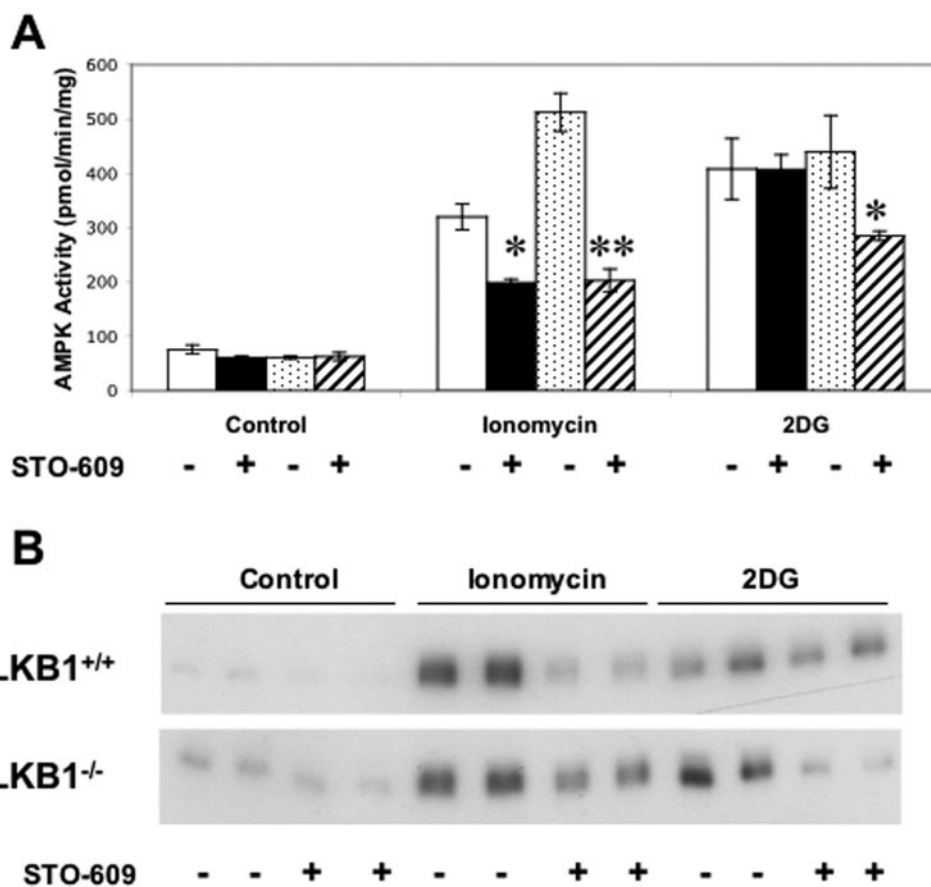


FIG. 8. AMPK activation and phosphorylation by ionomycin and 2-deoxyglucose in LKB1<sup>-/-</sup> murine embryo fibroblasts. LKB1<sup>+/+</sup> and LKB1<sup>-/-</sup> fibroblasts were stimulated either with ionomycin (1  $\mu$ M; 5 min) or with 2-DG (50 mM; 15 min) in the presence and absence of STO-609 (1  $\mu$ g/ml; 6 h preincubation before addition of activators). Extracts from  $n = 3$  separate wells at each incubation condition were then assayed for AMPK activity (A) or immunoblotted with anti- $\alpha$ T172p antibody (B). The activity results are expressed as mean  $\pm$  standard deviation of AMPK activity expressed as pmol of <sup>32</sup>P incorporation into the SAMS peptide per minute per mg of protein. Open and filled bars, LKB1<sup>+/+</sup> cells; stippled and cross-hatched bars, LKB1<sup>-/-</sup> cells. As determined by ANOVA analysis, \* =  $p < 0.05$  and \*\* =  $p < 0.001$  as compared with the absence of STO-609 at the stimulated condition. For the entire data set,  $F(11,24) = 45.72$ ,  $p < 0.0001$  the stimulatory effects. The immunoblots in B represent replicates at each incubation condition in LKB1<sup>+/+</sup> (upper panel) and LKB1<sup>-/-</sup> cells (lower panel) with equivalent protein load per lane (12  $\mu$ g); total AMPK $\alpha$  content was unaltered during these incubations (data not shown).

the 55-kDa band and diminished the 60-kDa band observed on immunoblotting, whereas CaMKK $\alpha$ -specific siRNA diminished slightly the 60-kDa band observed (Fig. 5B). This observation indicates that the upper band in HeLa extracts is a mixture of CaMKK $\beta$ 3 and CaMKK $\alpha$  and that the lower band is CaMKK $\beta$ 3x. We have been unable to confirm the specificity of a commercially available CaMKK $\alpha$  antibody (Santa Cruz Biotechnology, Inc.) to verify more precisely the composition of the 60-kDa band. The individual siRNAs have negligible effects on either ACC or total AMPK $\alpha$ , although total AMPK $\alpha$  is slightly diminished in the presence of both (Fig. 6B). The combined siRNAs eliminate nearly all immunoreactivity of the CaMKK bands (Fig. 5B), coincident with a near abrogation of AMPK activity and phosphorylation (Fig. 6).

To examine the relative roles of LKB1 and the CaMKKs in a cultured cell line in which all three kinases are expressed, LKB1<sup>+/+</sup> MEFs were stimulated with ionomycin and 2-DG in the presence and absence of STO-609. Basal and stimulated AMPK activity was then compared with that observed in LKB1<sup>-/-</sup> cells (Fig. 8). We and others have previously found that AICAR, hydrogen peroxide, and phenformin fail to activate AMPK in LKB1<sup>-/-</sup> MEFs (5, 7). Activation of AMPK (Fig. 8A) and  $\alpha$ T172 phosphorylation (Fig. 8B) in response to ionomycin and 2-DG is not impaired in LKB1<sup>-/-</sup> MEFs as compared with LKB1<sup>+/+</sup> cells. Indeed, the response to ionomycin appeared to be enhanced in the absence of LKB-1 expression.

STO-609 partially blocks the response to ionomycin in both cell types, whereas its ability to inhibit 2-DG-stimulated AMPK activation is only observed in the LKB1<sup>-/-</sup> MEFs. These data revealed that ionomycin-induced AMPK activation is mediated largely through one or both CaMKKs, whereas the 2-DG response may be contributed to by either LKB1 or the CaMKKs. In the wild-type cells, however, any contribution of CaMKKs might be redundant to that of LKB1. We cannot, of course, exclude the involvement of yet-to-be characterized AMPKs in either cell line.

The data reported herein provide compelling evidence that both CaMKKs function as AMPKs in the cell lines studied, making them attractive candidates for non-LKB1 AMPKs in other tissues and cell lines. Although initially characterized and isolated from neuronal tissue, both CaMKK $\alpha$  and CaMKK $\beta$  have a wide expression in rodent tissues (16, 17, 26), suggesting important roles for each in the regulation of AMPK activity *in vivo*. The data further indicate that both cellular calcium and AMP may play separate or interdependent roles in the regulation of AMPK activity.

With the recognition of three mammalian AMPKs, future investigation will be required to characterize in detail their relative contributions to AMPK regulation in other individual tissues and cell types. Although LKB1 may be the predominant AMPK regulating AMPK responses to contraction and phenformin in murine skeletal muscle (30), it seems quite likely,

given variable tissue expression of these AMPKKs, that there may be considerable heterogeneity in the regulation of AMPK under both basal and stimulated conditions, perhaps dependent on the nature of the stimulus. In addition, recent data from yeast indicate that the different  $\beta$  subunit-containing isoforms of the Snf1 kinase display stress-dependent preferences for Pak1, Tos3, and Elm1 upstream kinases (31). Thus, it might be anticipated that different AMPK heterotrimers composed of varying combinations of different catalytic  $\alpha$  subunits ( $\alpha 1$ ,  $\alpha 2$ ) and non-catalytic subunits ( $\beta 1$  and  $\beta 2$ ;  $\gamma 1$ ,  $\gamma 2$ , and  $\gamma 3$ ) might be differentially regulated by the three mammalian AMPKKs, increasing the complexity of overall regulation of AMPK activity *in vivo*.

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