Akt Activates the Mammalian Target of Rapamycin by Regulating Cellular ATP Level and AMPK Activity*§

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The serine/threonine protein kinase Akt, also known as protein kinase B, a downstream effector of phosphoinositide-3-kinase, has emerged as a critical mediator of the mammalian target of rapamycin (mTOR). However, the mechanism by which Akt activates mTOR is not fully understood. The known pathway by which Akt activates mTOR is via direct phosphorylation and inhibition of tuberous sclerosis complex 2 (TSC2), which is a negative regulator of mTOR. Here we establish an additional pathway by which Akt inhibits TSC2 and activates mTOR. We provide for the first time genetic evidence that Akt regulates intracellular ATP level and demonstrate that Akt is a negative regulator of the AMP-activated protein kinase (AMPK), which is an activator of TSC2. We show that in Akt1/Akt2 DKO cells AMP/ATP ratio is markedly elevated with concomitant increase in AMPK activity, whereas in cells expressing activated Akt there is a dramatic decrease in AMP/ATP ratio and a decline in AMPK activity. Currently, the Akt-mediated phosphorylation of TSC2 and the inhibition of AMPK-mediated phosphorylation of TSC2 are viewed as two separate pathways, which activate mTOR. Our results demonstrate that Akt lies upstream of these two pathways and induces full inhibition of TSC2 and activation of mTOR both through direct phosphorylation and by inhibition of AMPK-mediated phosphorylation of TSC2. We propose that the activation of mTOR by Akt-mediated cellular energy and inhibition of AMPK is the predominant pathway by which Akt activates mTOR in vivo.

The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1 and S2.

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§ The abbreviations used are: mTOR, mammalian target of rapamycin; S6K1, S6 kinase 1; 4E-BP, 4E-binding protein; TSC1, -2, tuberous sclerosis complexes 1 and 2; AMPK, AMP-activated protein kinase; CA-AMPK, constitutively active AMPK; HA, hemagglutinin; MMEM, Drosophila’s modified Eagle’s medium; FBS, fetal bovine serum; MEF, mouse embryonic fibroblast; DN, dominant negative; HPLC, high performance liquid chromatography; MOPS, 4-morpholinepropanesulfonic acid; ACC, acetyl-CoA carboxylase; p-ACC, phosphorylated ACC; WT, wild type; 5-TG, 5-thiogulosose; 2-DOG, 2-deoxyglycose; TSC2(520,530); TSC1(519,520,525,527); TSC2(519,520,525,527); DKO, double knock-out cells; AICAR, 5-aminoimidazole-4-carboxamide.
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concomitant decrease in the AMP/ATP ratio and inactivation of AMPK. We suggest that the activation of mTOR by Akt via inhibition of AMPK could be more relevant at the organismal level where cells do not always have access to nutrients for energy metabolism. This pathway by which Akt activates mTOR may also explain recent results showing that in Drosophila, TSC2 mutated in all Akt phosphorylation sites can still rescue the lethality and cell growth defect of TSC2 null mutant (14). Thus, Akt-phosphorylation mutants of TSC2 can still be activated by AMPK and be inhibited by Akt.

EXPERIMENTAL PROCEDURES

Plasmids, Retroviral Vectors, Antibodies, and Reagents—The plasmid pcDNA3-HA-4E-BP1 has been previously described (18). The expression vector for the Myc-tagged and activated AMPK, pcDNA3-Myc-AMPKa1312Thr172D, was obtained from D. Carling (19). The retroviral vectors pBabe-Puro-mAkt and pBabe-eGFP-mAkt have been previously described (20, 21). The retroviral vectors pBabe-Puro-DN-AMPK and pBabe-eGFP-DN-AMPK were constructed using previously described dominant negative AMPK (22). The rat Myc-tagged AMPKα2-K45R was excised from pcDNA3-Myc-AMPKα2-K45R (22) and inserted into EcoRI site of pBabe-puro and pBabe-eGFP. The retroviral vector pLPX-Alpha-TSC2(5939D/S1086D/S1088E/T1422E) was constructed by inserting HA-tagged TSC2(5939D/S1086D/S1088E/T1422E) from pcDNA3-HA-TSC2(5939D/S1086D/S1088E/T1422E) (obtained from K. Inoki and K-L Guan (10)) into the NotI site of pLPXAnti. Anti-phospho-ACC-S79, anti-Akt, anti-phospho-Akt-S473, anti-phospho-4E-BP-S65, anti-S6K1, anti-phospho-S6K1-T389, and anti-phospho-TSC2-T1462 antibodies were from Cell Signaling Technology (Beverley MA). Anti-AMPK, anti-phospho-AMPK-a-T172, anti-S6, anti-phospho-S6-525/236, and anti-phospho-Ser/Thr Akt substrate were from Cell Signaling Technology (Beverley MA). Anti-AMPK, anti-phospho-AMPK-α-T172, anti-S6, anti-phospho-S6-S235/236, and anti-phospho-Ser/Thr Akt substrate were from Cell Signaling Technology (Beverley MA). Anti-TSC2 (C-20) was from Santa Cruz Biotechnology (Santa Cruz, CA), anti-β-actin was from Sigma, anti-4E-BP1 was previously described (18), and horseradish peroxidase-labeled goat anti-rabbit and horseradish peroxidase-labeled rabbit anti-mouse was from Zymed Laboratories Inc. (San Francisco, CA). 5-Thio-d-glucose was from ICN Biochemical Inc.; AICAR was from Toronto Research Chemical Inc.; and 2-deoxy-d-glucose, rotenone, and all other chemicals were from Sigma.

Cell Culture, Retrovirus Infection, and Transfection—All cell lines were cultured in Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal bovine serum (FBS). In certain experiments DMEM lacking glucose was supplemented with 5.5 mM glucose and dialyzed FBS. Primary mouse fibroblasts were isolated and cultured as previously described (23). Immunized MEFs were generated by infection with pBabe-Puro-GSE56 expressing a dominant negative form of p53 (24) followed by selection with puromycin to generate polyclonal cell lines. For transient transfection of HEK293-mAkt cells, 1 × 106 cells per 6-cm plate were plated in DMEM with 10% FBS and transfected with increasing concentrations of pcDNA3-Myc-AMPKa1312Thr172D (10, 15, and 20 μg of DNA) and 2.5 μg of pcDNA-4EBP1-HA per 1 × 106 cells using Lipofecta
temine 2000 (Invitrogen). For serum or insulin stimulation of WT or Akt1/Akt DKO cells, 1.5 × 106 primary MEFs (passage 3) were plated in 15-cm plates in DMEM containing 10% FBS and deprived of serum for 24 h. Cells were stimulated with either insulin (1 μg/ml), or serum for 60 min and intracellular ATP, ADP, and AMP concentrations were analyzed by HPLC (see below). For immunoblot analysis cells were stimulated with 10% FBS and 20% FBS for 30 and 60 min. For ATP depletion experiments, Rat1a, TSC2+/−/−, MEFS and TSC2−/−/− MEFS (1 × 106 per 10-cm plate) were plated in DMEM (5.5 mM glucose) with 10% FBS (dialyzed) and deprived of serum for 24 h, stimulated with insulin (1 μg/ml) for 30 min, and either further stimulated with insulin (1 μg/ml) for 30 min or ATP-depleted by using different concentrations of 5-thio-
d-glucose, 2-deoxy-d-glucose, or rotenone. Rat1a-mAkt cells (1 × 106 per 10-cm plate) expressing activated Akt were deprived of serum for 24 h in DMEM (5.5 mM glucose) and treated with different concentrations of 5-thioglu
cose, rotenone, or AICAR.

Immunoblotting and Immunoprecipitation—For Immunoblotting cell lysates were prepared in 100–200 μl of lysis buffer (20 mM Tris HCl (pH 7.5), 100 mM KCl, 20 mM β-glycerol phosphate, 1 mM dithiothreitol, 0.25 mM Na3VO4, 10 mM NaF, 1 mM EDTA, 1 mM EGTA, 1 mM phenylethyl
dium, fluoride, 10 mM sodium pyrophosphate, 10 mM okadaic acid, and complete protease inhibitor mixture (Roche Applied Science) by thaw-freeze-cycle extraction. Proteins in cell lysates were separated on 15% SDS-PAGE for 4E-BP1, on 10% SDS-PAGE for other proteins, and 6% or 8% SDS-PAGE for TSC2 and mTOR. Proteins were then transferred to nitrocellulose membranes (0.2 mm, Schleicher & Schuell, Dot Scientific, Inc., Burton). The phosphospecific antibody was always used in the first round of immunoblotting. After stripping the membrane with stripping buffer (62 mM Tris HCl (pH 6.8), 100 mM β-mercaptoethanol, 2% SDS) the membrane was then probed using antibodies that recognize the total amount of a specific protein of interest. For immunoprecipitations, cell lysates were prepared in 1.0 ml of lysis buffer (20 mM Tris HCl (pH 7.5), 150 mM NaCl, 1 mM MgCl2, 1 mM dithio
treitol, 50 mM β-glycerol phosphate, 50 mM NaF, 1 mM phenylethyl
dium, fluoride, 10 mM sodium pyrophosphate, 10 mM okadaic acid, and complete protease inhibitor mixture) and boiled in 2X La
mml sample buffer. The entire sample was used to separate the proteins via 6% SDS-PAGE and transferred to a nitrocellulose membrane for immuno
blotting with anti-Akt-pS/T substrate (Cell Signaling Technology) and anti-TSC2 (Santa Cruz Biotechnology).

Adenine Nucleotides Analysis—Cultured cells were quickly harvested into phosphate-buffered saline and immediately centrifuged for 2 min at 1000 × g (4 °C). Pellets were resuspended in 150 μl of perchloric acid, 4% v/v, and incubated on ice for 30 min. Within 1 h the lysates were adjusted to pH 6–8 using a solution of 2 M KOH/0.3 M MOPS and incubated for 30 min on ice. Precipitated salt was separated from the liquid phase by centrifugation at 13,000 × g for 10 min. Aliquots of samples were stored at −80 °C. Adenine nucleotide measurements were conducted using HPLC (HPLC-Pro Star from Varian, Walnut Creek, CA) with a Spherisorb column (ODS II, 5 mm, 0.46 × 25 cm, Z22.697-1, Sigma). The nucleotides analyzed, detected spectropho

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**RESULTS**

Akt Maintains the Intracellular Level of ATP and Regulates AMPK Activity—Akt was shown to phosphorylate and inactivate TSC2 (9, 10, 13), thereby activating mTOR. mTOR activity, as measured by 4E-BP1 phosphorylation following serum stimulation (see Ref. 15 and Fig. 1A), is impaired in mouse embryonic fibroblasts (MEFs) deficient for Akt1 and Akt2. However, this impairment did not correlate with a decrease in TSC2 phosphorylation (see Ref. 15 and Fig. 1B). These results suggest that the remaining Akt3 activity in Akt1/Akt2 double knock-out (DKO) cells (Fig. 1A) is sufficient to substantially phosphorylate TSC2 and that TSC2 phosphorylation may not be sufficient for Akt to fully activate mTOR. Thus, these results prompted us to investigate whether there is an additional function of Akt that is required to fully activate mTOR and that is impaired in Akt1/Akt2 DKO cells.

mTOR activity is also dependent on intracellular ATP level and AMPK activity (16, 17). Thus, we sought to determine whether Akt regulates intracellular ATP levels that could affect mTOR activity. We first determined the intracellular ATP level in Akt1/Akt2 DKO cells. Basal ATP level was lower in serum-deprived Akt1/Akt2 DKO cells compared with WT cells (Fig. 2A). Following insulin or serum stimulation, the ATP level was increased but it was still retained markedly reduced in DKO cells, 2- to 3-fold lower than WT. Likewise, in cells expressing activated myristoylated Akt (Rat1a-mAkt), intracellular ATP was markedly higher (about 3-fold) than that measured in control cells (Fig. 2B). These results show that Akt mediates the insulin- and serum-dependent increase in intracellular ATP level.

AMPK is the sensor of ATP level in cells (27, 28). AMPK activation inhibits mTOR activity (17, 29) via direct phosphorylation of TSC2 (17). AMPK activity is dependent on the cellular AMP/ATP ratio (27, 28). We found that, in the presence of growth factors, this ratio was markedly higher (about 2- to 3-fold) in Akt1/Akt2 DKO cells compared with WT cells (Fig. 2C), consistent with the lower ATP level in Akt1/Akt2 DKO cells (Fig. 2A). Indeed, AMPK activity, as measured by its phosphorylation at Thr172 (30) and by the phosphorylation of the AMPK target acetyl-CoA carboxylase (ACC) at Ser-79 (31), was significantly higher in Akt1/Akt2 DKO cells compared with WT cells in the presence of serum (Fig. 2D). Moreover, introduction of a conditionally active Akt into Akt1/Akt2 DKO cells and modulating its activity decreased AMP/ATP ratio in Akt-dependent manner (supplemental Fig. S1).

In the absence of serum, the AMP/ATP ratio in WT and Akt1/Akt2 DKO cells was comparable (Fig. 2C). Although p-ACC was substantially increased in WT cells following serum deprivation, it did not reach the level observed in DKO cells (Fig. 2D). One possible explanation for this apparent discrepancy between AMPK activity and AMP/ATP ratio is that Akt has an additional impact on AMPK, which is less dependent on AMP/ATP ratio (see “Discussion”). Consistent with the results observed in the Akt1/Akt2 DKO cells, in Rat1a cells expressing activated Akt (Rat1a-mAkt) the AMP/ATP ratio was about 3-fold lower than in control cells (Fig. 2E). When control Rat1a cells were deprived of serum, the AMP/ATP ratio markedly increased, concomitant with an increase in AMPK activity (Fig. 2, E and F). Although the AMP/ATP ratio also increased in serum-deprived Rat1a-mAkt cells, this ratio was comparable to the ratio in control cells in the presence of serum, and thus AMPK activity was not markedly increased in these cells (Fig. 2, E and F). The increase in the AMP/ATP ratio and AMPK activity in control cells correlated with a decrease in mTOR activity, as determined by S6K1 and 4E-BP1 phosphorylation, and by 4E-BP1-mobility shift (Fig. 2F). Thus, cells that maintained an AMP/ATP ratio below a certain threshold level also had high mTOR activity (Fig. 2, E and F). Taken together these results provide genetic evidence and demonstrate that Akt is a regulator of energy metabolism, which is required to maintain low AMPK activity in the cells.

ATP Depletion and Activation of AMPK Attenuate the Ability of Akt to Activate mTOR—To determine whether the Akt-mediated increase in the intracellular level of ATP and the decrease in the AMP/ATP ratio are required for Akt to activate mTOR, we first used inhibitors of glycolysis and oxidative phosphorylation to deplete ATP in cells expressing activated Akt. In Rat1a-mAkt cells, mTOR is constitutively active even in the absence of growth factors, as determined by 4E-BP1 phosphorylation and mobility shift (18) (Figs. 2F and 3A). However, ATP depletion by inhibition of glycolysis (using the glucose analogue 5-thiogluucose, 5-TG) or the inhibition of oxidative phosphorylation (using rotenone) inhibited this Akt-mediated 4E-BP1 phosphorylation, as determined by p-4E-BP1 and by mobility shift, with no significant effect on either Akt activity or TSC2 phosphorylation by Akt (Fig. 3A). However, AMPK activity was elevated as measured by ACC phosphorylation (Fig. 3A, right panel). Addition of 5-TG impaired the 4E-BP1 phosphorylation, induced by insulin in Rat1a cells, more strongly than the 4E-BP1 phosphorylation mediated by activated Akt in Rat1a-mAkt cells in the absence of insulin (Fig. 3A, A and B). Similarly, 5-TG had a more profound effect in Akt1/Akt2 DKO cells than in WT cells (supplemental Fig. S2). Higher concentrations of 5-TG were required to impair mTOR activity in cells expressing activated Akt, which is also correlated with the more dramatic effect of 5-TG on AMPK activity in control cells (Fig. 3B, right panel). This is correlated with the more substantial decline in ATP level in control cells in comparison with its decline in activated Akt expressing cells following addition of 5-TG (Fig. 3C). This could be due to increased glucose uptake and glycolysis in cells expressing activated Akt (see “Discussion”). We note that Akt-mediated mTOR activity also could be inhibited by adding a high level of 2-deoxyglucose (2-DOG, 100 mM) to cells expressing activated Akt (data not shown). However, this concentration of 2-DOG can also induce osmotic stress; moreover,
5-TG is probably a more effective inhibitor because, unlike 2-DOG, it is a competitive inhibitor that cannot be phosphorylated. Therefore, we used 5-TG for our experiments with cells expressing activated Akt to show that, even in these cells that have higher ATP level and lower AMP/ATP ratio, it is possible to decrease mTOR activity if ATP is depleted. Thus, these results suggest that the ability of Akt to mediate mTOR activity is dependent on its ability to increase the intracellular ATP level, which subsequently down-regulates AMPK.

To further assess the possibility that AMPK is a downstream effector of Akt leading to mTOR activation, we first exposed serum-deprived Rat1a-mAkt cells to AICAR, which activates AMPK and impairs insulin-mediated S6K1 phosphorylation. As shown in Fig. 4A, exposure of insulin-stimulated Rat1a cells, to increasing concentrations of AICAR increased AMPK activity, as measured by AMPK and ACC phosphorylation, with a concomitant decrease in 4E-BP1 and S6 phosphorylation. Similar results were obtained in Rat1a-mAkt cells, in which mTOR was constitutively activated, although higher concentrations of AICAR were required. Thus, AICAR impairs the constitutive activation of mTOR in Rat1a-mAkt cells. We then examined whether an activated form of AMPK can alleviate the ability of Akt to activate mTOR as measured by 4E-BP1 phosphorylation.

For this purpose, HA-tagged 4E-BP1 was transiently co-transfected along with increasing amounts of an activated form of AMPK (CA-AMPK) into HEK293 cells stably expressing mAkt. As we previously showed, in contrast to control HEK293 cells, 4E-BP1 in mAkt-expressing cells was constitutively phosphorylated even in the absence of insulin stimulation (18) (Fig. 4C, lane 1). However, 4E-BP1 phosphorylation was impaired following expression of CA-AMPK (Fig. 4C, lanes 2–4). These results indicate that the Akt-mediated increase in the ATP level and the decrease in AMP/ATP ratio is required for Akt to fully activate mTOR.

Dominant Negative AMPK Restores mTOR Activity in Akt1/Akt2 DKO Cells—If the ability of Akt to activate mTOR is dependent on its ability to increase ATP level and to inhibit AMPK kinase activity, then it is expected that the inhibition of AMPK activity in Akt1/Akt2 DKO cells would restore the impaired mTOR activity in these cells. To explore this possibility, we utilized WT and Akt1/Akt2 DKO MEFs immortalized by the expression of a dominant-negative form of p53 using retroviral infection (see “Experimental Procedures”). As in the primary cells, mTOR activity was impaired in immortalized Akt1/Akt2 DKO cells (Fig. 5). The immortalized WT and DKO cells were infected with retrovirus expressing dominant negative (DN) form of p53.
AMPK to generate polyclonal cell lines stably expressing DN-AMPK. DN-AMPK markedly decreased AMPK activity, as measured by ACC phosphorylation, and restored mTOR activity in the DKO cells as determined by the phosphorylation of S6K1, S6, and 4E-BP1 (Fig. 5). These results clearly demonstrate that mTOR activity in Akt1/Akt2 DKO cells is impaired because of the inability to sufficiently increase the intracellular ATP level via insulin and growth factors and to sufficiently decrease AMPK activity in these cells.
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The Ability of Akt to Activate mTOR by Inhibiting AMPK Is Dependent on TSC2—TSC2 is phosphorylated and activated by AMPK, establishing one potential mechanism by which ATP and AMPK regulate mTOR activity (17). We thus examined TSC2−/−/− p53−/− MEFs in comparison with TSC2+/−/+ p53−/− MEFs and found that, consistent with previous results (17), ATP depletion had only a moderate effect on mTOR activity in TSC2−/− cells under these conditions, suggesting that the ATP level regulates mTOR activity mostly through TSC2 (Fig. 6A). In addition, we found that the AMP/ATP ratio and AMPK activity were markedly higher in TSC2−/− cells compared with TSC2+/− cells (Fig. 6, B and C, lanes 1 and 3). Thus, mTOR is constitutively activated in TSC2-deficient cells despite the high AMP/ATP ratio and AMPK activity. The higher AMP/ATP ratio and the higher AMPK activity in TSC2 null cells could be due to reduced Akt activity (Fig. 6A, lanes 1 and 2 and lanes 5 and 6) via a negative feedback loop mechanism (12, 25). Indeed, when we stably expressed activated Akt in TSC2 KO cells the AMP/ATP ratio was restored with a concomitant decrease in AMPK activity, similar to that in TSC+−/− cells (Fig. 6, B and C). These results further establish a role for Akt in regulating AMPK activity. The decrease in AMPK activity in TSC2 KO cells expressing activated Akt did not significantly increase mTOR activity (Fig. 6C), further supporting previous results that TSC2 is the major target for AMPK upstream of mTOR (17).

The results presented thus far strongly suggest that the phosphorylation of TSC2 by Akt is not sufficient to fully activate mTOR and that the inhibition of AMPK activity by Akt is also required for mTOR activation. To further assess this interpretation we utilized the Akt-phosphomimetic mutant of rat TSC2, TSC2(S939D/S1086D/S1088E/T1422E), in which four residues phosphorylated by Akt are substituted with acidic residues (10). HA-TSC2(S939D/S1086D/S1088E/T1422E) was cloned into a retroviral vector that we introduced into TSC2−/−/− cells to generate a clonal cell line (Fig. 7A). TSC2(S939D/S1086D/S1088E/T1422E) rendered mTOR activity in TSC2−/−/− cells more sensitive to ATP depletion (Fig. 7B, lanes 2, 5, and 8). Thus, the phosphorylation of TSC2 by Akt is not sufficient to render mTOR activity resistant to ATP depletion, indicating that the activation of TSC2 via its phosphorylation by AMPK is dominant over the inhibition of TSC2 via its phosphorylation by Akt. However, when mAkt was co-expressed with TSC2(S939D/S1086D/S1088E/T1422E), it renders mTOR activity resistant to ATP depletion back to what was observed in the parental TSC2−/−/− cells (Fig. 7B, lanes 3, 6, and 9). These results demonstrate that Akt leads to the activation of mTOR through both direct phosphorylation and inactivation of TSC2 and through inhibition of AMPK activity. The
phosphorylation of TSC2 by Akt is not sufficient to fully activate mTOR, and Akt-mediated intracellular ATP level and the subsequent reduction in AMPK activity in conjunction with the direct phosphorylation of TSC2 by Akt is required to fully inhibit TSC2 and fully activate mTOR (Fig. 7C). It should be noted, however, that although mTOR activity in TSC2−/− cells is relatively resistant to ATP depletion, it is still sensitive to ATP depletion, as determined by the phosphorylation of S6K1 (Fig. 7A). This observation suggests that the effect of ATP depletion on mTOR activity is not exclusively mediated by TSC2 and that ATP level and AMPK also can regulate mTOR activity through other unknown mechanisms.

**DISCUSSION**

Our present work provides genetic evidence and establishes that the serine/threonine kinase Akt is a key regulator of energy metabolism that inhibits AMPK. Akt-deficient cells have reduced ATP levels and elevated AMPK activity, whereas cells expressing activated Akt have markedly elevated ATP levels and reduced AMPK activity. The effect of Akt on the generation of ATP occurs via an increase in glycolysis and oxidative phosphorylation (32). Although the one or more exact mechanisms by which Akt affects these processes are not known, Akt can affect glycolysis through multiple mechanisms, including glucose transporters expression and translocation (33–36), and the increased activity and expression of glycolytic enzymes (32, 37, 38). The ability of Akt to increase glycolysis also could ultimately affect oxidative phosphorylation in the mitochondria by increasing the availability of substrates for oxidative phosphorylation. The effect of Akt on ATP level causes a concomitant reduction in the AMP/ATP ratio and therefore reduces AMPK activity. AMPK is a heterotrimeric complex comprising a catalytic subunit (\(\alpha\)) and two regulatory subunits (\(\beta\) and \(\gamma\)). AMP causes allosteric changes that activate AMPK and promote phosphorylation of Thr-172 in the activation loop of the \(\alpha\) subunit by AMPK kinase, recently identified as LKB1. This phosphorylation is required for full activation of AMPK (reviewed in Ref. 39). As shown by our present work, Akt not only decreases the phosphorylation of Thr-172 as well as the in vivo activity of AMPK as measured by the phosphorylation of ACC, but it is also required for the inhibition of AMPK activity by growth factors. We attributed this effect of Akt to its ability to regulate the intracellular AMP/ATP ratio. However, as a kinase, Akt also could potentially affect AMPK activity via phosphorylation of AMPK itself or its upstream regulator, LKB1. Indeed, Thr-366 in LKB1 lies within a consensus for the optimal phosphorylation motif for Akt. Although it
was reported that LKB1 is a poor substrate for Akt in vitro (40, 41), it is still conceivable that Akt phosphorylates LKB1 in vivo under certain physiological conditions.

AMPK impairs the induction of mTOR activity by growth factors (29) and directly phosphorylates and activates TSC2 thereby inhibiting mTOR activity (17). Here we show that in order for Akt to fully inhibit TSC2 and to activate mTOR it needs to directly phosphorylate TSC2 and to inhibit AMPK preventing it from activating TSC2. We showed that in cells deficient for Akt1 and Akt2, mTOR activity is impaired without a substantial effect on TSC2 phosphorylation by Akt. However, AMPK activity is elevated in these cells, suggesting that the residual Akt activity in Akt1/Akt2-deficient cells mediated by Akt3 is sufficient to phosphorylate TSC2 but insufficient to maintain normal ATP levels, thus leading to AMPK activation. Indeed, expression of DN-AMPK in Akt1/Akt2-deficient cells restores mTOR activity. Furthermore, expression of an Akt-phosphomimetic mutant of TSC2 in TSC2-deficient cells (in which mTOR activity is relatively refractive to ATP depletion) restores sensitivity of mTOR to ATP depletion. These data imply that TSC2 phosphorylation by Akt does not prevent the activation of TSC2 by AMPK. However, expression of activated Akt together with the Akt-phosphomimetic TSC2 mutant reverses the sensitivity of mTOR activity to ATP depletion to the same as observed in TSC2-deficient cells. In addition, AMPK activity is elevated in TSC2-deficient cells, and the expression of activated Akt inhibits the elevated AMPK activity in these cells. Taken together these results clearly demonstrate that Akt, in addition to inhibiting TSC2 via direct phosphorylation, also inhibits TSC2 and activates mTOR through the inhibition of AMPK. This establishes an alternative mechanism for the activation of mTOR by growth factors and Akt. This alternative pathway by which Akt activates mTOR can explain, at least in part, why in Drosophila a TSC2, which is mutated in all Akt phosphorylation sites, can still rescue the lethality and cell growth defect of TSC2 null mutant (14). Thus, TSC2 mutants that are not directly phosphorylated by Akt can still be activated by AMPK and inhibited by Akt. This could be particularly of importance at the organismal level and in tumors where cells do not always have access to excess of nutrients for energy metabolism, raising the possibility that at the organismal level the predominant effect of Akt on TSC2 is via the inhibition of AMPK.

mRNA translation and ribosomal biogenesis, two processes that are mediated by mTOR, consume high levels of cellular energy. Thus the high consumption of ATP in TSC2-deficient cells together with the reduced Akt activity, due to a negative feedback loop (12, 25), could
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Abstract

AMP-activated protein kinase (AMPK) is activated by elevated AMP and/or reduced ATP, and its activation stimulates the phosphorylation of Thr-2448 by Akt and thus can either inhibit mTOR activity (42), and we have not observed any reduction in Thr-2448 phosphorylation following ATP depletion. However, thus far it has not been demonstrated that Thr-2448 phosphorylation by Akt has any impact on mTOR activity (42), and we have not observed any reduction in Thr-2448 phosphorylation following ATP depletion. Clearly more experiments are required to delineate the additional mechanism(s) by which AMPK and/or Akt affect mTOR activity.

Both TSC2 and LKB1 appear to act as tumor suppressors, and their deficiency leads to the development of benign tumors and hamartomas (44–46). It is therefore possible that LKB1 exerts its tumor suppressor activity by activating AMPK and inhibiting TSC2 (47, 48). Akt is frequently activated in human cancers mainly through the inactivation of the tumor suppressor activity of LKB1 (Fig. 7C). Thus, phosphatase and tensin homolog deleted on chromosome ten (PTEN) deficiency should also affect mTOR activity in a TSC2-independent manner.

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