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AMPK Phosphorylation of Raptor Mediates a Metabolic Checkpoint

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SUMMARY

AMPK is a highly conserved sensor of cellular energy status that is activated under conditions of low intracellular ATP. AMPK responds to energy stress by suppressing cell growth and biosynthetic processes, in part through its inhibition of the rapamycin-sensitive mTOR (mTORC1) pathway. AMPK phosphorylation of the TSC2 tumor suppressor contributes to suppression of mTORC1; however, TSC2-deficient cells remain responsive to energy stress. Using a proteomic and bioinformatics approach, we sought to identify additional substrates of AMPK that mediate its effects on growth control. We report here that AMPK directly phosphorylates the mTOR binding partner raptor on two well-conserved serine residues, and this phosphorylation induces 14-3-3 binding to raptor. The phosphorylation of raptor by AMPK is required for the inhibition of mTORC1 and cell-cycle arrest induced by energy stress. These findings uncover a conserved effector of AMPK that mediates its role as a metabolic checkpoint coordinating cell growth with energy status.

INTRODUCTION

The AMP-activated protein kinase (AMPK) is a highly conserved heterotrimeric kinase complex composed of a catalytic (α) subunit and two regulatory (β and γ) subunits. AMPK is activated under conditions of energy stress, when intracellular ATP levels decline and intracellular AMP increases, as occurs during nutrient deprivation or hypoxia (Hardie, 2007). Upon energy stress, AMP directly binds to tandem repeats of crystathionine-β-synthase (CBS) domains in the AMPK γ subunit. Binding of AMP is thought to prevent dephosphorylation of the critical activation loop threonine in the α subunit (Hardie, 2007). The phosphorylation of the activation loop threonine is absolutely required for AMPK activation. Biochemical and genetic analyses in worms, flies, and mice have revealed that the serine/threonine kinase LKB1 represents the major kinase phosphorylating the AMPK activation loop under conditions of energy stress across metazoans (Apfeld et al., 2004; Shaw et al., 2005; Sakamoto et al., 2005; Mirouse et al., 2007; Lee et al., 2007b).

LKB1 was originally identified as a human tumor suppressor gene mutated in Peutz-Jeghers syndrome (PJS), an autosomal dominant inherited cancer disorder (Hemminki et al., 1998). In addition, LKB1 mutations occur in a large percentage (30%–40%) of sporadic non-small-cell lung cancers (NSCLC) (Sanchez-Cespedes et al., 2002; Ji et al., 2007). PJS shares a number of clinical features with Cowden's Disease, which is caused by inactivating mutations in the PTEN tumor suppressor. This phenotypic overlap suggested that LKB1-dependent signaling might negatively regulate some aspect of PI3-kinase (PI3K) signaling, analogous to PTEN function. However, while classic PI3K/Akt signaling is not elevated in LKB1-deficient cells, mammalian target of rapamycin (mTOR) signaling is uniquely hyperactivated in LKB1-deficient murine embryonic fibroblasts (MEFs) and liver (Corradetti et al., 2004; Shaw et al., 2004b, 2005). Similarly, mTOR signaling is hyperactivated in hamartomas from LKB1-heterozygous mice (Shaw et al., 2004b) and in LKB1-deficient human lung carcinomas (Carretero et al., 2007).

mTOR is a highly conserved nutrient-responsive regulator of cell growth found in all eukaryotes (Wullschleger et al., 2006). Whereas AMPK is active under nutrient-poor conditions and inactive under nutrient-rich conditions, mTOR is activated in the inverse pattern. In higher eukaryotes, mTOR activation requires positive signals from both nutrients (glucose, amino acids) and growth factors. mTOR, like its budding yeast orthologs, is found in two biochemically and functionally distinct signaling complexes (Wullschleger et al., 2006). The mTORC1 complex is nutrient sensitive, acutely inhibited by rapamycin, and functions as a master regulator of cell growth, angiogenesis, and metabolism (Sabatini, 2006). mTORC1 is composed of four known subunits: mTOR, mLST8/Gbl, PRAS40, and the WD40 repeat-containing subunit raptor (Sabatini, 2006; Sancak et al., 2007; Vander Haar et al., 2007). Raptor acts as a scaffold to recruit downstream substrates such as 4EBP1 and ribosomal S6 kinase (p70S6K1) to the mTORC1 complex (Nojima et al., 2003; Schalm et al., 2003).

Genetic studies in *Drosophila* and mammalian cells identified the tuberous sclerosis complex (TSC) tumor suppressors as critical upstream inhibitors of the mTORC1 complex. TSC2 (also known as tuberin) contains a GTPase activating protein (GAP) domain at its carboxyl terminus that inactivates the Rheb GTPase, which has been shown to associate with and directly activate the mTORC1 complex in vitro (Sancak et al., 2007). Loss of TSC1 or TSC2 therefore leads to hyperactivation of mTORC1. Phosphorylation of TSC1 and TSC2 serves as an integration point for a wide variety of environmental signals that

regulate mTORC1 (Sabatini, 2006). Mitogen-activated kinases including Akt, Erk, and Rsk directly phosphorylate TSC2, leading to its inactivation by an unknown mechanism. In addition, another Akt substrate, PRAS40, was recently shown to bind and inhibit the mTORC1 complex. Upon phosphorylation by Akt, PRAS40 no longer inhibits mTORC1 (Sancak et al., 2007; Vander Haar et al., 2007).

In addition to these growth stimulatory cues that activate mTORC1, the complex is rapidly inactivated by a wide variety of cell stresses, thereby ensuring that cells do not continue to grow under unfavorable conditions. One of the unique aspects of the mTORC1 complex is that unlike many of the aforementioned growth factor activated kinases, it is dependent on nutrient availability for its kinase activity. Withdrawal of glucose, amino acids, or oxygen leads to rapid suppression of mTORC1 activity (Shaw and Cantley, 2006). Upon LKB1- and AMP-dependent activation of AMPK by nutrient loss, AMPK directly phosphorylates the TSC2 tumor suppressor on conserved serine sites distinct from those targeted by other kinases, which constitutes one mechanism through which glucose and oxygen control mTORC1 activation (Inoki et al., 2003; Corradetti et al., 2004; Shaw et al., 2004b; Liu et al., 2006).

We have found that cells lacking TSC2 remain responsive to energy stress, albeit less so than wild-type cells, suggesting that additional AMPK substrates may directly or indirectly modulate mTORC1 activity. Moreover, the relationship between glucose inactivation of AMPK and stimulation of TOR is conserved across all eukaryotes, including several that lack TSC2 orthologs such as *C. elegans* and *S. cerevisiae*. This suggests that either additional mechanisms exist to coordinate the kinase activity of these two master regulators of cell growth and metabolism, or AMPK must target additional conserved components of the pathway. Here, we find that the critical mTOR binding partner raptor is a direct substrate of AMPK, and that phosphorylation of raptor by AMPK is required for suppression of mTORC1 activity by energy stress. Further, we report that raptor phosphorylation is necessary for the full engagement of an AMPK-mediated metabolic checkpoint. These findings have broad implications for the control of cell growth by nutrients in a number of cellular and organismal contexts.

RESULTS

Peptide Library Identification of the Optimal Substrate Motif for AMPK

In an effort to find substrates of AMPK that may mediate its effects on growth and metabolic control, we determined its consensus phosphorylation motif with the aim of identifying proteins that carry optimal phosphorylation sequences. We utilized a positional scanning peptide library (PSPL) technique in which radiolabeled kinase assays are performed on a spatially arrayed set of peptide mixtures. Each peptide contains one fixed amino acid at a given position relative to a centrally fixed phosphoacceptor (an even mixture of serine or threonine) and degenerate amino acid mixtures at all flanking positions (Hutti et al., 2004). From the relative amount of phosphate incorporated into each peptide mixture, one obtains a quantitative measure of the selectivity for, and against, each individual amino acid residue at each position (Turk et al., 2006). We and our colleagues have previously used this technique to successfully identify optimal substrate motifs for a number of mammalian kinases, including CK2, Erk, PKA, Akt, Pim, Pak, MAP3K, and IKK family kinases (Hutti et al., 2004, 2007; Bullock et al., 2005; Bunkoczi et al., 2007; Rennefahrt et al., 2007).

PSPL profiling revealed that AMPK is a highly selective kinase, strongly preferring basic residues in the −3 and −4 positions relative to the phosphoacceptor site. In addition, hydrophobic residues including leucine and methionine were strongly selected in the −5 position and the +4 position consistent with previous studies of the optimal peptide substrates for AMPK based on mutagenesis and molecular modeling (Scott et al., 2002; Towler and Hardie, 2007). In addition, strong selection for polar residues in the +3 position was noted, with asparagine and aspartate being the most highly selected.

Comparing the optimal motif we identified from the peptide library screen to all known well-established in vivo substrates of AMPK shows excellent concordance (Figure 1B). Each of these substrates contains not only the required basic residue in −3 or −4, and hydrophobic residues in −5 and +4, but they also exhibit strong bias toward the secondary selections for serine and valine in the −2 position and polar residues in the +3 position. The strong selectivity for particular residues in at least four out of the nine flanking residues analyzed makes AMPK one of the most selective mammalian kinases we have examined thus far (out of ~60 kinases profiled to date; B.E.T., unpublished data). The high degree of selectivity at multiple residues substantially reduces the odds that any protein will contain serine residues within this sequence context by random chance, especially when examined for evolutionary conservation. This suggests that proteins that do carry this signature sequence are likely to be authentic substrates of AMPK or related kinases. Thus we used our optimal AMPK substrate motifs to mine protein databases to search for matching sequences—using bioinformatics tools including Scansite (http://scansite.mit.edu) and Prosite (http://ca.expasy.org/prosite/). We focused our efforts on those candidate substrates bearing optimal AMPK motifs in which the target serine and its critical flanking residues that dictate AMPK-dependent substrate specificity were conserved broadly throughout eukaryotes.

Raptor Is an AMPK Substrate

We first examined potential AMPK substrates that might underlie the ability of AMPK and its upstream kinase LKB1 to regulate cell growth and tumorigenesis. A number of recent studies have revealed that a key effector of AMPK signaling in the control of cell growth is the suppression of the mTORC1 signaling complex. We and others previously reported that the effect of LKB1 and AMPK to regulate mTORC1 is at least in part via direct phosphorylation of the TSC2 tumor suppressor by AMPK (Corradetti et al., 2004; Shaw et al., 2004b). Indeed Ser1387 of human TSC2 conforms perfectly to the AMPK optimal motif we obtained with our peptide library analysis, and this residue and its flanking sequences are conserved across vertebrates and to *Drosophila* (Figure 1B, data not shown).

However, two pieces of data suggested that TSC2 could not be the only substrate of AMPK to regulate mTORC1 signaling.

Figure 1. Peptide Library Profiling the Optimal Substrate Motif for AMPK and Comparison with Known and Candidate In Vivo Phosphorylation Sites

(A) A spatially arrayed PSPL was subjected to in vitro phosphorylation with active AMPKα1 and radiolabeled ATP. Each peptide contained one residue fixed at one of nine positions relative to the centrally fixed phosphoacceptor (an equal mix of serine and threonine). Aliquots of each reaction were spotted onto avidin membrane, which was washed, dried, and exposed to a phosphor storage screen, providing the array of spots shown in the figure. AMPK displayed strong selectivity at the −5, −4, −3, −2, +3, and +4 positions.

(B) The optimal and secondary selections taken from triplicate analyses as in (A) are displayed. AMPK phosphorylation sites in the best established in vivo substrates of AMPK conform to the substrate motif derived from the peptide library data. All substrates shown were isolated in bioinformatics searches for proteins containing a conserved AMPK phosphorylation motif. These same searches yielded two predicted AMPK sites in raptor.

(C) The predicted AMPK sites in raptor are highly conserved across evolution.

First, the inverse regulation of TOR and AMPK by glucose levels is found throughout all eukaryotes examined thus far, including *C. elegans* and *S. cerevisiae*, although a TSC2 ortholog is not found in either of those species. Second, while performing further experiments to examine the role of TSC2 in regulating energy stress, we found that while TSC2 is needed for rapid suppression of mTORC1 by the AMP-mimetic AICAR and the mitochondrial complex I inhibitor phenformin, mTORC1 is still potently inhibited by both of these AMPK activators in *TSC2*$^{-/-}$ MEFs (Figure 2A). Similar findings have been made by others using glycolytic inhibitors (e.g., 2-DG) in TSC2-deficient cells (Hahn-Windgassen et al., 2005).

Our bioinformatics analysis revealed that the mTOR binding partner raptor contains two conserved serine sites that match the AMPK consensus motif (serine 722 and serine 792 of human raptor). Importantly, the critical residues flanking raptor Ser792 which were found in the peptide library studies to be important for recognition by AMPK are highly conserved through *Drosophila*, *C. elegans*, and Dictyostelium, as well as in both budding and fission yeast (Figure 1C). Such a high degree of conservation is rare among phosphorylation sites. For example, of the ten best established AMPK substrates shown in Figure 1B, only two of them are conserved across eukaryotes (ACC1 Ser1216 and HMG CoR Ser862). Moreover, half of the known AMPK substrate proteins, including TSC2, have no orthologs in primitive eukaryotes. The striking conservation in the candidate AMPK sites in raptor suggested it could represent an ancestral AMPK target that dictates the responsiveness of TOR to nutrients across eukaryotes.

To test the possibility that raptor is an AMPK substrate, we first examined whether we could detect phosphorylation of raptor in cultured cells using phosphomotif antibodies. These antibodies broadly recognize phosphorylated serine or threonine residues found within a specific sequence motif (Zhang et al., 2002). Interestingly, we found that the "14-3-3 motif" antibody, which was generated against peptides bearing R-X-X-pS or R-X-X-X-pS sequences, recognized raptor in HEK293 cells. Coexpression with wild-type and kinase-dead LKB1 led to an increase and decrease, respectively, in reactivity of raptor to the antibody (Figure 2B). Moreover, cotransfection with a truncated constitutively active allele of AMPKα1 resulted in a dramatic increase in raptor phosphorylation, and activation of endogenous AMPK through the

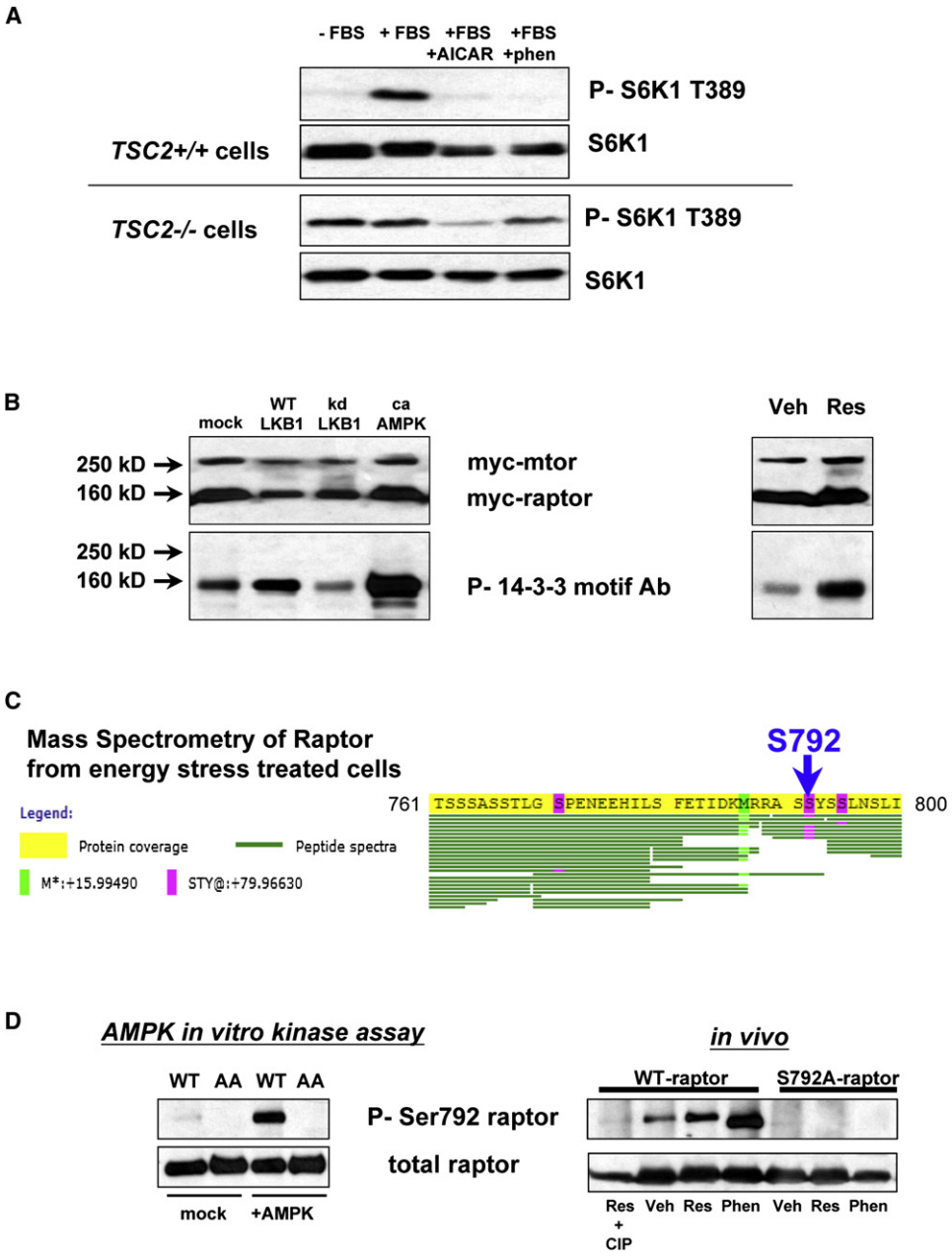

Figure 2. Raptor Is Phosphorylated In Vitro and In Vivo by AMPK

(A) mTORC1 signaling in TSC2-deficient cells remains responsive to energy stress. *TSC2*$^{+/+}$ or *TSC2*$^{-/-}$ matched MEFs were serum starved overnight (−ser) and replaced with fresh media containing 10% fetal bovine serum (+FBS) or serum-containing media with 2 mM AICAR or 5 mM phenformin. Cells were lysed 1 hr after media replacement. Lysates were immunoblotted for the mTORC1-dependent Thr389 phosphorylation in p70 S6K1 and for total S6K1 protein.

(B) Overexpressed raptor is phosphorylated in HEK293 cells in an LKB1- and AMPK-dependent manner. (Left panel) myc-tagged mTOR and myc-tagged raptor were coexpressed in HEK293 cells with empty vector, wild-type LKB1, kinase-dead LKB1, or a constitutively active AMPKα1 allele (1-312 truncation). Raptor phosphorylation was detected using the phospho-14-3-3 motif antibody. (Right panel) HEK293 cells expressing mTOR and raptor were treated with 50 μM resveratrol for 30 min and phosphorylation of raptor was detected with the phospho-14-3-3 motif antibody.

(C) Raptor is phosphorylated at a high level on Ser792 following resveratrol treatment. Mass spectrometry was performed on raptor protein purified from resveratrol-treated HEK293 cells as in (B). Coomassie-stained raptor protein was isolated from an SDS-polyacrylamide gel and subjected to chymotryptic digestion prior to analysis by LC-MS/MS. Amino acids 761–800 of human raptor are shown here. Each recovered peptide is illustrated by a single green line. Phosphorylated residues are shown in magenta.

(D) A phosphospecific antibody against Ser792 of raptor recognizes raptor phosphorylated in vitro by AMPK (left) as well as wild-type, but not S792A mutant, raptor (right) following treatment with 50 μM resveratrol or 5 mM phenformin in HEK293 cells.

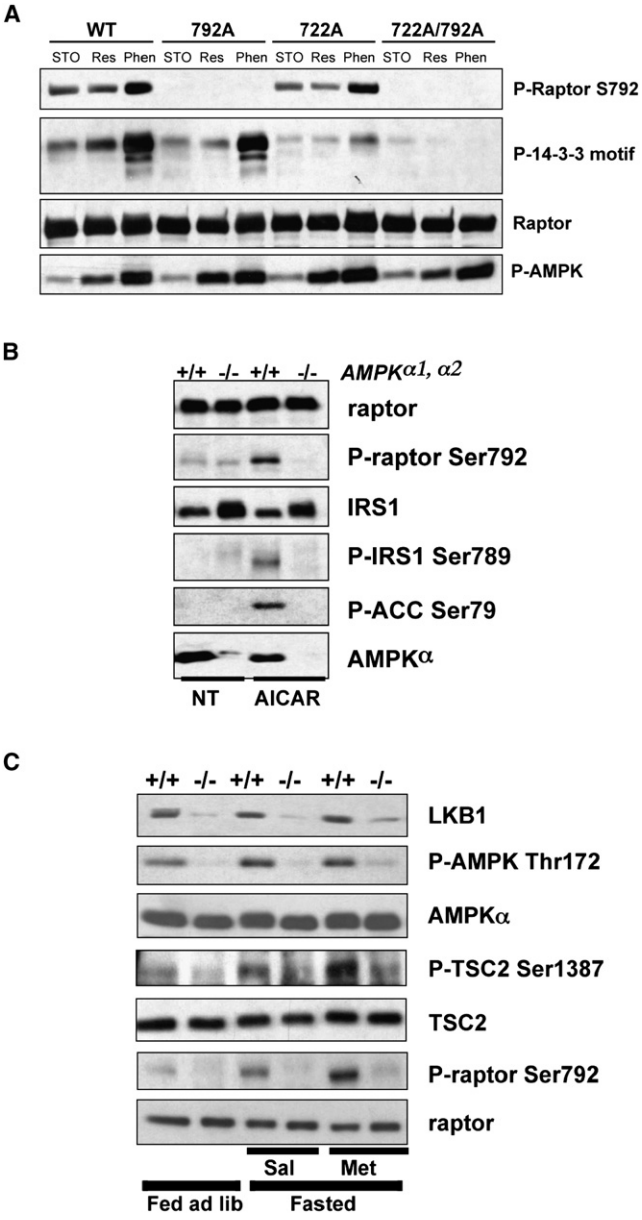

Figure 3. Raptor Ser722 and Ser792 Are Phosphorylated by AMPK in Cultured MEFs and in Murine Liver in an AMPK- and LKB1-Dependent Manner

(A) Both Ser722 and Ser792 are phosphorylated in an AMPK-dependent manner in HEK293 cells. HEK293 cells were transfected with wild-type, S722A, S792A, or the double mutant S722A/S792A raptor allele and treated as indicated. Raptor was immunoprecipitated and immunoblotted with the phospho-Ser792, phospho-14-3-3 motif, or anti-Myc epitope tag antibody. Phospho-ACC was immunoblotted from the total cell extracts to illustrate the degree of AMPK activation in the cells.

(B) Endogenous raptor is phosphorylated at Ser792 in wild-type, but not AMPK-deficient (*AMPK*$\alpha 1^{-/-}$ $\alpha 2^{-/-}$), immortalized MEFs. MEFs were treated with 2 mM AICAR for 1 hr (AICAR) or left untreated (NT), and total cell extracts were immunoblotted with the indicated antibodies.

(C) Endogenous raptor is phosphorylated at Ser792 in wild-type, but not LKB1-deficient, murine liver following fasting and metformin treatment. Eight-week-old mice were either fed ad libidum (ad lib) or fasted 18 hr and treated with either 250 mg/kg metformin in saline (Met) or saline alone (Sal) for 1 hr. Total cell extracts made from harvested livers were immunoblotted with the indicated antibodies.

use of the polyphenol compound resveratrol also stimulated acute phosphorylation of raptor (Figure 2B).

Tandem mass spectrometry was then used to identify the specific sites of phosphorylation of raptor in cultured cells. Epitope-tagged raptor was cotransfected with mTOR in HEK293T cells. Cells were either untreated, or treated with either resveratrol or phenformin, both of which potently activate AMPK in HEK293T cells. Mass spectrometry (MS) of chymotryptic fragments of raptor from resveratrol- and phenformin-treated cells revealed that the Ser792 site was phosphorylated at high stoichiometry in both samples, with 5 of the 7 peptides identified containing this serine residue being phosphorylated with either treatment (Figure 2C), unlike the untreated sample that revealed 2 of 9 peptides bearing phosphate at raptor Ser792 (data not shown). The region flanking the candidate Ser722 site was not well represented in our mass spectrometry analysis despite repeated attempts, including digestion with alternative proteases (see complete MS analysis of raptor phosphorylation sites in Figure S1 available online). Notably, during the course of this study, two large-scale analyses of phosphoproteins from rat and mouse liver revealed phosphorylation of endogenous raptor at Ser722, suggesting that it is indeed a bona fide phosphorylation site in vivo (Moser and White, 2006; Villen et al., 2007).

A phosphospecific antibody against the Ser792 site in human raptor was generated, and its specificity was assessed using epitope-tagged wild-type or S792A mutant raptor overexpressed in HEK293T cells, under conditions analogous to those employed for the mass spectrometry (Figure 2D). In addition, we examined whether purified AMPK could directly phosphorylate raptor at Ser792 in vitro. Active AMPK rapidly and potently induced raptor Ser792 phosphorylation in vitro (Figure 2D). Similarly, employing nonphosphorylatable mutants, we mapped the sites recognized by the 14-3-3 motif antibody. As expected, we found that reactivity to the phospho-Ser792 antibody was unaffected in the S722A mutant. Surprisingly, the AMPK-induced reactivity of raptor with the 14-3-3 motif antibody was minimally affected in the S792A mutant but was dramatically reduced in the S722A mutant (Figure 3A). Reactivity was completely abolished in the S722A/S792A double mutant (henceforth referred to as the "AA mutant"). These results suggest that AMPK activation can induce phosphorylation of both Ser722 and Ser792.

To determine whether AMPK is the physiological kinase for phosphorylation of endogenous raptor at Ser792, immortalized wild-type or AMPK-deficient MEFs (bearing a targeted disruption of both AMPK α genes) were treated with the AMP-mimetic AICAR, followed by immunoblotting for phospho-Ser792 raptor. As controls, we also examined phosphorylation of two well-established AMPK substrates (ACC1/2 Ser79 and IRS1 Ser789). As seen in Figure 3B, raptor Ser792 is phosphorylated following AICAR treatment in wild-type, but not AMPK null, MEFs, precisely paralleling phospho-ACC and phospho-IRS1, thereby indicating that raptor Ser792 is a bona fide AMPK site in vivo. To further define the physiological conditions under which raptor

Ser792 phosphorylation is modulated by AMPK, we examined raptor Ser792 phosphorylation in liver extracts from wild-type or LKB1-liver-specific KO mice fed ad libitum, fasted, or treated with the biguanide diabetes therapeutic metformin. We have previously shown that metformin rapidly activates AMPK in murine liver in a manner completely dependent on LKB1 (Shaw et al., 2005). Here we observed that raptor Ser792 phosphorylation in murine liver was slightly potentiated in fasted mice and was dramatically increased by metformin treatment in a manner completely dependent on intact LKB1 (Figure 3C). These results were further extended in isolated primary hepatocytes from wild-type and LKB1-deficient liver (Figure S2). Taken altogether, these results indicate that endogenous raptor Ser792 is phosphorylated in multiple mammalian tissue types in an LKB1- and AMPK-dependent manner following energy stress.

Raptor Phosphorylation Is Required for Inhibition of mTORC1 by AMPK

To examine the requirement for raptor phosphorylation in the regulation of mTORC1 activity by energy stress, we utilized the nonphosphorylatable AA mutant in which both Ser722 and Ser792 were replaced by alanine. To assess the physiological role of raptor Ser722 and Ser792 phosphorylation in mTORC1 regulation, we replaced the endogenous raptor by creating cell lines stably expressing low levels of human wild-type or AA raptor using retroviral expression, followed by subsequent knockdown of the endogenous murine raptor utilizing a lentivirally expressed shRNA that does not target the human raptor sequence. In this manner, we replaced endogenous raptor with human wild-type or AA mutant in three murine cell lines: C2C12 myoblasts, $TSC2^{+/+}$ $p53^{-/-}$ MEFs, and $TSC2^{-/-}$ $p53^{-/-}$ MEFs. In murine raptor lentiviral shRNA infected cultures lacking reconstitution with human raptor, we observed functional suppression of raptor levels and mTORC1 signaling (see Figure S3). We then examined the requirement for raptor Ser722 and Ser792 phosphorylation in mTORC1 suppression following AICAR or phenformin treatment in these stable cell lines. Mutation of these sites prevented AMPK agonists from fully suppressing mTORC1, both in cells with normal mTOR signaling (C2C12, $TSC2^{+/+}$ MEFs) (Figure 4A and Figure S4) and in cells lacking TSC2 (Figure 4B and Figure S4). Despite the elevated basal levels of mTORC1 activity in $TSC2^{-/-}$ MEFs, AICAR or phenformin treatment potently suppressed mTORC1 signaling, an effect that was almost fully abolished by reconstitution with the AA raptor allele. In AA raptor mutant expressing $TSC2^{-/-}$ MEFs, mTORC1 activity levels were dramatically elevated compared to $TSC2^{-/-}$ MEFs expressing wild-type raptor at all time points following AICAR treatment (Figure 4B). Similar results were seen with phenformin, which activates AMPK via distinct mechanism (AICAR is an AMP mimetic; phenformin is a mitochondrial complex I inhibitor); notably each may have additional distinct effects on signaling independent of the LKB1/AMPK pathway. Altogether our data demonstrate that raptor phosphorylation on Ser722/Ser792 is required for full mTORC1 suppression by AMPK agonists in all cell types we examined. Furthermore, these findings indicate that TSC2 and raptor represent the major targets of AMPK required for the suppression of mTORC1 in mouse embryonic fibroblasts.

AMPK Phosphorylation of Raptor Induces 14-3-3 Binding

We next considered the mechanism by which AMPK-mediated raptor phosphorylation leads to inactivation of the mTORC1 kinase complex in vivo. We investigated the possibility that phosphorylation of raptor leads to the specific association or dissociation of proteins with the mTORC1 complex. Mass spectrometry was utilized to identify proteins coimmunoprecipitating with overexpressed wild-type raptor following phenformin treatment in HEK293 cells. Among the proteins identified coprecipitating with raptor were two isoforms of 14-3-3 (Figure S5). A common mechanism for phosphorylation-based inactivation of target proteins is through direct phosphorylation-dependent binding to the 14-3-3 family of proteins (Bridges and Moorhead, 2005). As AMPK-mediated phosphorylation of raptor also created an epitope recognized by the 14-3-3 binding motif antibody (Figures 2B and 3A), we more closely examined the possibility that phosphorylation of serine 722 and 792 may induce 14-3-3 binding to raptor. Exhaustive peptide library screening and proteomic analyses have revealed that 14-3-3 proteins generally interact with R-X-X-pS/pT-X-P or R-X-X-X-pS/pT-X-P target sequences. Raptor Ser722 and Ser792 both contain the required upstream arginine residue; however, neither site contains a proline residue in the +2 position, although several well-established 14-3-3 binding sites also lack proline at +2 (Cbl, IRS-1, PRAS40). Moreover, both Ser722 and Ser792 in raptor have residues at +1 and +2 that arose as secondary selections in peptide library experiments (Yaffe et al., 1997; Rittinger et al., 1999).

We first examined whether 14-3-3 bound to raptor when coexpressed, in an AICAR- or Ser722/Ser792-dependent manner. Coexpressed GST-14-3-3, but not GST alone, immunoprecipitated with wild-type, but not AA mutant raptor, when cells were subjected to AICAR (Figure 5A). In addition, recombinant GST-14-3-3 protein fixed to beads precipitated wild-type, but not AA mutant, raptor in lysates from the MEF stable cell lines treated with AICAR or phenformin (Figure 5B). Furthermore, endogenous raptor coprecipitated with recombinant 14-3-3 protein from wild-type, but not LKB1-deficient, MEFs following treatment (Figure 5C). Finally, consistent with the original mass spectrometry data (Figure S5), endogenous 14-3-3 ζ and γ isoforms coimmunoprecipitated with wild-type, but not AA mutant, raptor in a phenformin-dependent manner (Figure 5D). However, it is worth noting that little specificity has been demonstrated for 14-3-3 isoforms other than 14-3-3 σ, and many of the isoforms can form heterodimers with each other (Gardino et al., 2006; Wilker et al., 2005). Thus, we expect the 14-3-3 isoforms that bind AMPK-phosphorylated raptor may vary between cell types based on expression levels.

AMPK Phosphorylation of Raptor Regulates mTORC1 IP-Kinase Activity

14-3-3 has been shown to regulate its best studied binding partners through three distinct mechanisms, each involving allosteric conformational changes that (1) induce changes in protein catalytic activity, (2) trigger a disruption of existing protein-protein interactions, or (3) cause changes in subcellular localization. Analyses of crystal structures of 14-3-3 isoforms bound to phosphopeptides suggest that 14-3-3 regulates the activity of many

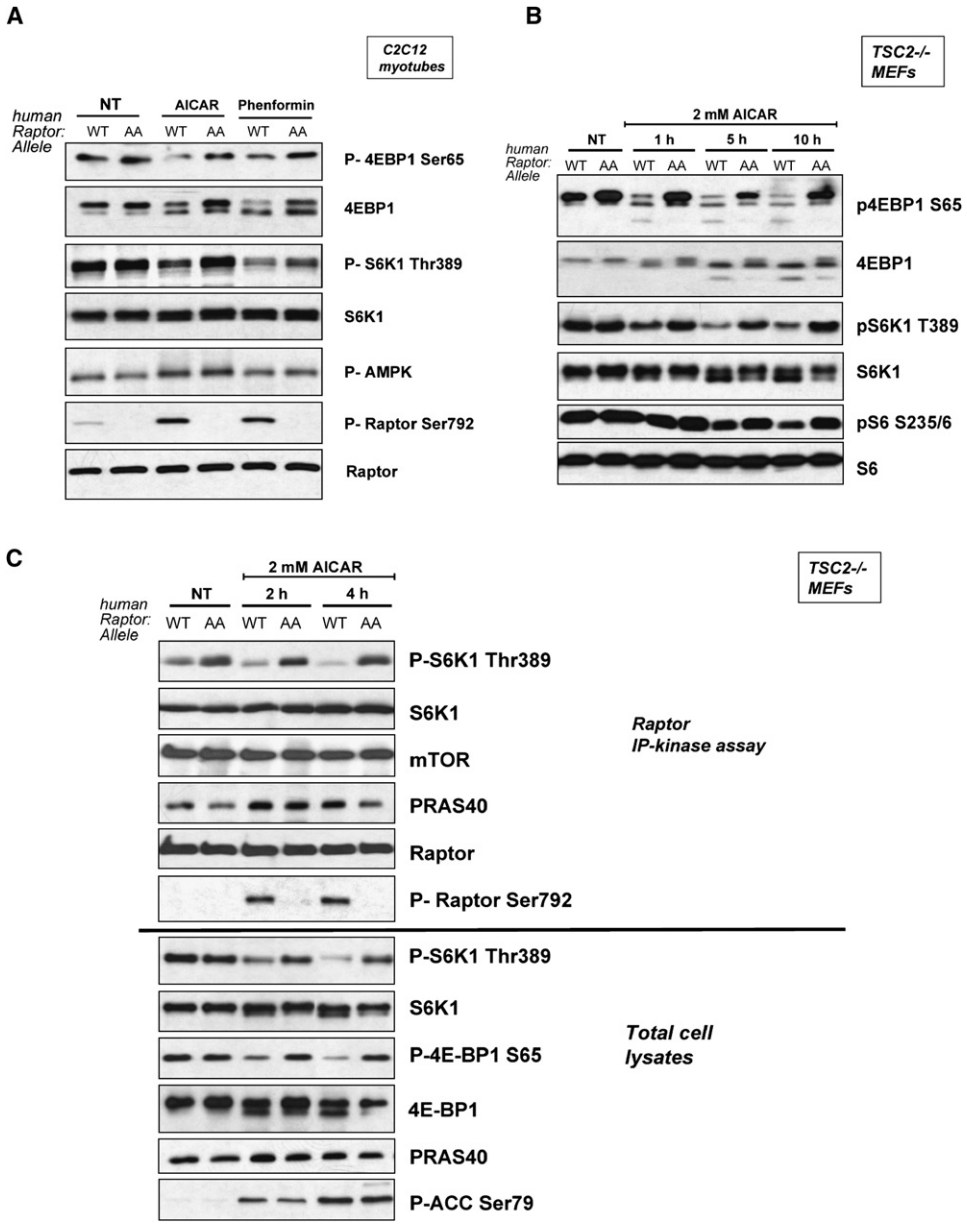

Figure 4. Phosphorylation of Ser722 and Ser792 Is Required to Inhibit mTORC1 Following Energy Stress in a Variety of Cell Types

(A) C2C12 cells in which endogenous raptor has been knocked down were stably reconstituted with human wild-type or AA raptor (see Figure S3), and were treated with 1 mM AICAR or 1 mM phenformin for 1 hr as indicated. Total cell extracts were immunoblotted with indicated antibodies to examine mTORC1 signaling.

(B) *TSC2*$^{-/-}$, *p53*$^{-/-}$, raptor knockdown MEFs stably reconstituted with wild-type or AA raptor were treated with 2 mM AICAR as indicated and immunoblotted with indicated antibodies to examine mTORC1 signaling.

(C) *TSC2*$^{-/-}$ *p53*$^{-/-}$, raptor knockdown MEFs stably reconstituted with wild-type or AA raptor were treated with 2 mM AICAR as indicated. Raptor was immunoprecipitated in CHAPS buffer and assayed for mTORC1 kinase activity using purified S6K1 as a substrate as previously described (Sancak et al., 2007). (Top) IP-kinase assays were immunoblotted for phosphorylation of purified S6K1 substrate using phospho-Thr389 S6K1 antibody as well as for level of immunoprecipitated raptor, mTOR, and PRAS40. (Bottom) Five percent of the total cell extracts that raptor was immunoprecipitated from were immunoblotted with indicated antibodies.

of its binding partners via allosteric stabilization of unfavorable states ("the molecular anvil" hypothesis) (Yaffe, 2002). We first examined whether we could detect suppression of mTORC1 IP-kinase activity by immunoprecipitating raptor from AICAR-treated MEFs. Using conditions that were recently reported to reconstitute insulin-dependent stimulation of mTORC1 IP-kinase activity (Sancak et al., 2007), we found that raptor immunoprecipitates from AICAR-treated cells showed a time-and

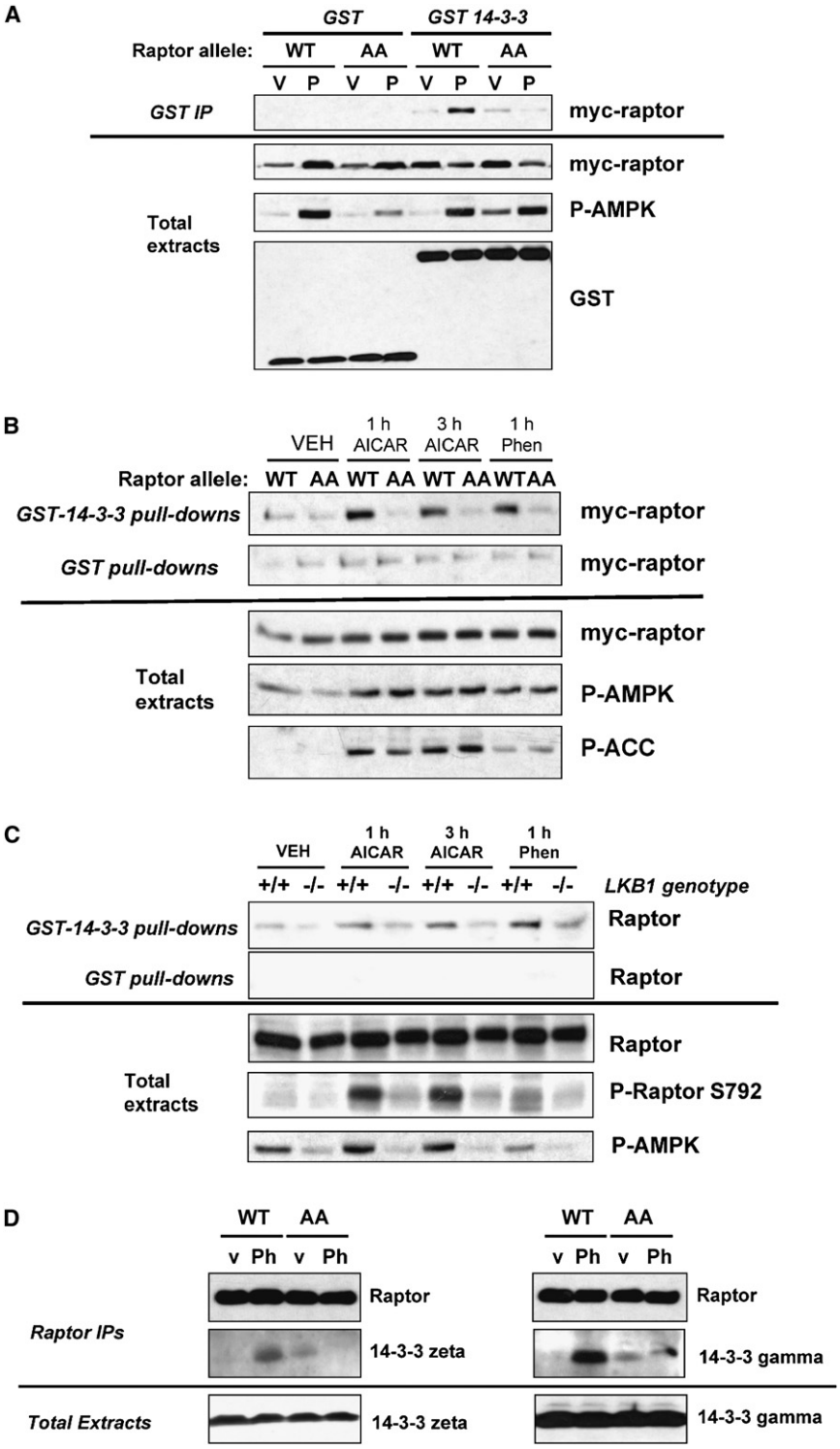

Figure 5. AMPK Phosphorylation of Raptor Induces 14-3-3 Association

(A) Wild-type, but not AA mutant, raptor complexes with 14-3-3 only under energy stress conditions. HEK293 cells were cotransfected with pEBG or pEBG-14-3-3 with wild-type or AA mutant raptor, and then complexes were precipitated on glutathione beads. Beads or total cell extracts were immunoblotted with the indicated antibodies. Cells were treated with V, vehicle (DMEM) or P, 5 mM phenformin for 1 hr.

(B) Wild-type, but not AA mutant, raptor precipitates with recombinant GST-14-3-3 protein in extracts from energy stress treated *TSC2*$^{-/-}$ MEFs stably reconstituted with human raptor alleles. GST protein pulldowns or total cell extracts were immunoblotted with the indicated antibodies.

(C) Endogenous raptor binds to immobilized recombinant GST-14-3-3 protein, but not recombinant GST protein, from extracts of cells treated with energy stress in an LKB1-dependent manner. GST protein pull-downs or total cell extracts were immunoblotted with the indicated antibodies.

(D) Myc-tagged raptor immunoprecipitates from phenformin (Ph)- or vehicle (v)-treated cells were eluted with myc peptide and immunoblotted for endogenous 14-3-3 isoforms as indicated.

dose-dependent suppression of mTORC1 kinase activity toward purified S6K1 protein that paralleled the inhibition of mTORC1 signaling by AMPK activation in vivo (Figure 4C). We subsequently examined the IP-kinase activity of raptor complexes containing the S722A/S792A double mutant. As seen in Figure 4C, mTORC1 complexes containing AA raptor were refractory to the inhibition of kinase activity seen in mTORC1 complexes containing wild-type raptor. Critically, the amount of mTOR found in association with raptor was not affected by mutation of raptor Ser722/Ser792 or by AMPK activation (Figure 4C). These data indicate that immunoprecipitates containing the same amount of complexed mTOR and raptor show differences in mTORC1 kinase activity dependent on Ser722/Ser792 phosphorylation by AMPK.

We further examined the association of endogenous mTOR and endogenous raptor from wild-type, LKB1-deficient, and AMPKα-deficient MEFs. The amount of raptor and mTOR coimmunoprecipitating was constant in all contexts examined (Figure S6). We also examined whether AICAR induced changes in the amount of endogenous PRAS40 coimmunoprecipitating with raptor. As seen in Figure 4C, AICAR treatment induced greater immunoprecipitation of PRAS40 with raptor, which was modestly suppressed in cells expressing the nonphosphorylatable raptor. However, the levels of PRAS40 immunoprecipitating with raptor do not strictly correlate with mTORC1 IP-kinase activity or with raptor phosphorylation, suggesting that PRAS40 association is not the key event dictating the impact of raptor phosphorylation on mTORC1 IP kinase activity. AMPK phosphorylation of raptor

may lead to changes in the amount of both 14-3-3 and PRAS40 bound, which collectively act to suppress raptor-associated mTOR kinase activity. Finally, the subcellular localization of each of the raptor alleles with and without AMPK activation in the reconstituted C2C12 myoblasts, *TSC2*$^{+/+}$ MEFs, or *TSC2*$^{-/-}$ MEFs was unchanged (e.g., Figure S7).

AMPK Phosphorylation of Raptor Engages a Metabolic Checkpoint and Prevents Apoptosis

Activation of AMPK by energy stress causes a metabolic checkpoint, in which cells with intact AMPK signaling undergo cell-cycle arrest, while those cells defective for AMPK activation (e.g., LKB1 deficient) or key components of the AMPK pathway (e.g., TSC2 deficient or p53 deficient) continue cycling and subsequently undergo apoptosis (Inoki et al., 2003; Corradetti et al., 2004; Shaw et al., 2004a, 2004b; Jones et al., 2005; Buzzai et al., 2007). A failure to downregulate mTORC1 under conditions of energy stress preferentially induces cells to undergo accelerated apoptosis.

We therefore wished to determine whether phosphorylation of raptor by AMPK is required for full activation of this metabolic checkpoint, and whether the inability to phosphorylate raptor would affect the ability of cells to undergo growth arrest or apoptosis following energy stress. To exclude effects of AMPK regulation of TSC2 and p53 in this process, we utilized MEFs lacking both genes that were suppressed for endogenous raptor and reconstituted with human wild-type raptor or AA raptor (as discussed previously—see Figure 4). Importantly, under standard growth conditions these cells grew at comparable rates and displayed no differences in viability or proliferation. We examined the response of these cells to several AMPK activating agents, analyzing their DNA content and cell-cycle profile by propidium iodide and flourescence-activated cell sorting (FACS). MEFs lacking TSC2 and p53 but expressing wild-type raptor undergo a significant growth arrest in the G1 and S phases of the cell cycle following treatment with AICAR, depending on the time point examined (Figure 6A, Figure S9, and data not shown). This was most readily observed as a decrease in the fraction of cells progressing into G2/M as quantified by DNA content (Figure 6A and Figure S8). Cells expressing wild-type raptor undergo a significant arrest (13% in G2/M as compared to 22% in the untreated cells), whereas the cells expressing AA raptor do not. Consistent with engagement of a cell-cycle checkpoint following energy stress, the reduction in the cycling G2/M peak in AICAR- treated cells expressing wild-type raptor was paralleled by a decrease in the levels of the mitotic marker phospho-histone H3 Ser10, as detected by immunoblotting (Figure 6C). In parallel cultures expressing the nonphosphorylatable AA mutant raptor, the cells continued cycling, as observed by a complete absence of reduction in the G2/M population and a similar lack of suppression of phospho-histone H3 levels by AICAR (Figures 6A–6C). The suppression of mitotic cells was also observed using the phospho-histone H3 Ser10 antibody for immunocytochemistry on AICAR- and phenformin-treated cell populations (Figure 6B). The percentage of wild-type raptor-expressing cells arresting prior to G2/M and the percentage of AA raptor-expressing cells failing to arrest were concordant in the DNA content FACS analysis, phospho-histone H3 immunocytochemistry, and phospho-histone H3 immunoblotting. By all three assays, AICAR and phenformin led to a similar suppression of mitotic cells in cells expressing wild-type raptor, but not the AA mutant, raptor.

In addition to AMPK phosphorylation dictating cell-cycle arrest, profound effects on apoptosis were observed at later times following energy stress. Previously, in cells lacking LKB1, AMPK, or TSC2 function, inappropriate hyperactivation of mTORC1 was found to promote apoptosis under conditions of energy stress and rapamycin treatment led to suppression of the apoptotic response (Corradetti et al., 2004; Shaw et al., 2004b; Jones et al., 2005; Lee et al., 2007a). *TSC2*$^{+/+}$ MEFs expressing human AA raptor underwent a modest increase in apoptosis in response to prolonged (48 hr) treatment with phenformin compared to identical cells expressing human wild-type raptor (48 hr). Strikingly, in cells lacking TSC2 that express the AA raptor mutant, and are thereby severely attenuated in their ability to downregulate mTORC1 following energy stress (see Figure 4B), the percentage of cells undergoing apoptosis following phenformin more than doubled when compared to cells lacking TSC2 and expressing the human wild-type raptor (Figure 6D).

To ensure that this differential apoptosis was due to signals coming from AMPK signaling and not a gain-of-function effect of the AA mutant, we examined whether the AA raptor mutant sensitized cells to apoptosis in a manner dependent on intact upstream AMPK signals. To test this hypothesis, we utilized A549 lung adenocarcinoma cell lines, which bear LKB1 missense mutations and are null for LKB1 protein expression. A549 cell lines stably reconstituted with wild-type or kinase-dead LKB1 were subsequently infected with retroviruses expressing wild-type or AA raptor. Stable cell lines expressing each raptor allele in combination with each LKB1 allele were then treated with phenformin, and as before apoptotic rates were quantified using Annexin V FACS sorting. As seen in Figure 6E, wild-type, but not AA mutant, raptor conferred protection from phenformin-induced apoptosis only in cells expressing wild-type LKB1. In cells expressing kinase-dead LKB1 and hence unable to activate AMPK, we observed no difference in the percentage of cells undergoing cell death between those expressing wild-type and those expressing AA mutant raptor. This observation suggests that the survival signal requires both wild-type LKB1 and wild-type raptor, consistent with the maximal suppression of mTORC1 in these cells. Furthermore, the extent of apoptosis observed from overexpressing the AA raptor mutant in cells with wild-type LKB1 (42%) was equivalent to the degree of apoptosis in cells expressing kinase-dead LKB1 (42%–48%). These results indicate, at least in A549 cells, that raptor phosphorylation is a key control point in the response to energy stress, and other targets of LKB1/AMPK signals such as TSC2 or p53 are not sufficient to induce effective growth arrest and prevent apoptosis in these cells. Taken together with the cell-cycle analysis, these data suggest that cells unable to inhibit mTORC1 through LKB1-AMPK-raptor signaling continue to proliferate inappropriately under energy stress conditions, ultimately leading to increased rates of apoptosis.

DISCUSSION

A fundamental requirement of all cells is that they couple the availability of nutrients to signals emanating from growth factors

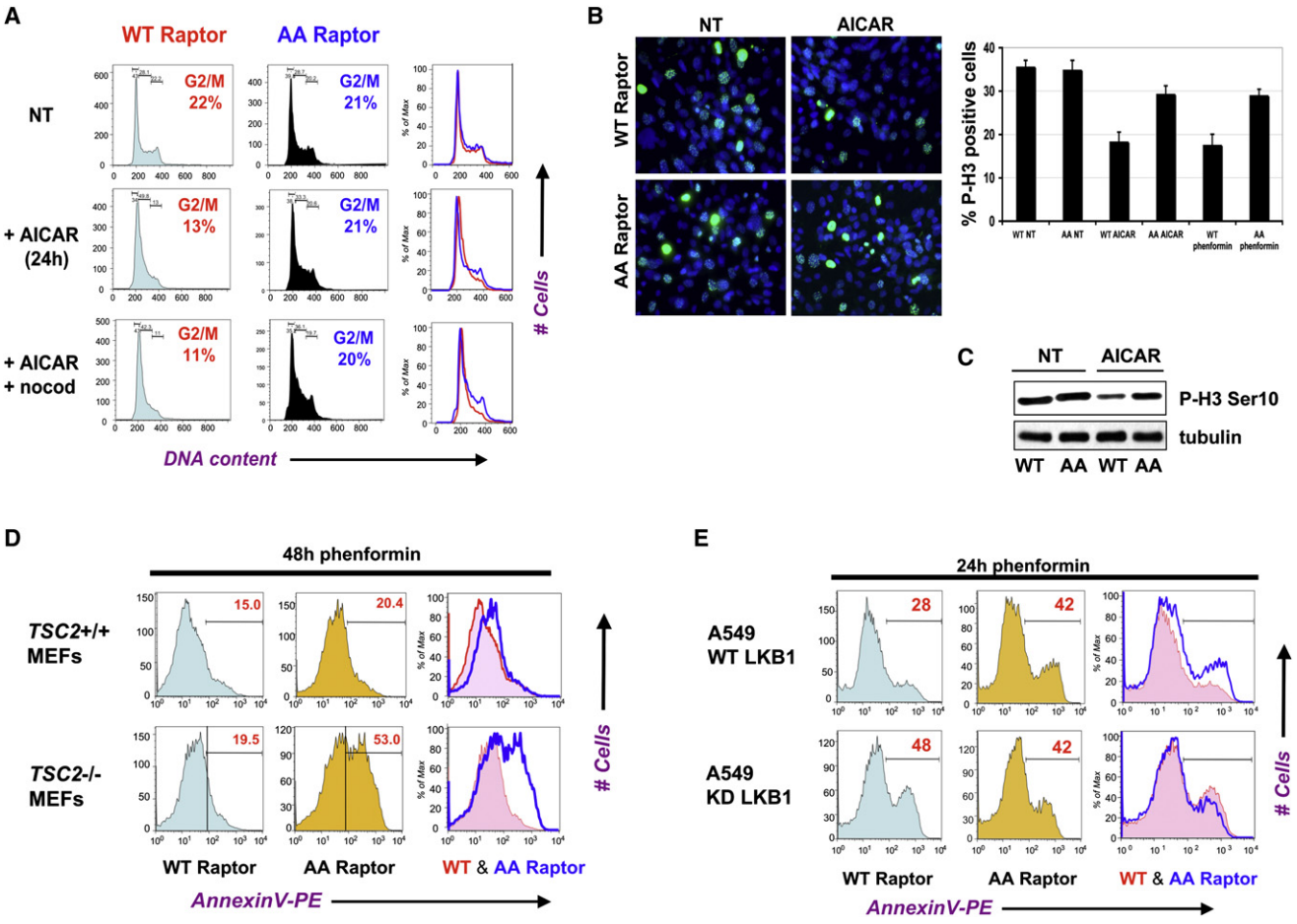

Figure 6. Phosphorylation of Raptor at Ser722 and Ser792 Dictates a Metabolic Checkpoint Controlling Growth Arrest and Apoptosis in Response to Energy Stress

(A) *TSC2*$^{-/-}$, *p53*$^{-/-}$ MEFs expressing wild-type raptor undergo G1/S arrest following AICAR treatment, while those expressing AA mutant raptor do not. Cells were left untreated (NT) or treated with 2 mM AICAR, or treated with 2 mM AICAR and 3 hr later exposed to nocodazole (+nocod) to arrest any cycling cells in G2/M. At 24 hr after AICAR treatment, all cells were fixed and analyzed for DNA content using propidium iodide and FACS analysis. The percentage of cells in the G2/M phase of the cell cycle is highlighted in each population.

(B) Cells analogous to those in (A) were plated on coverslips and the next day left untreated (NT) or treated with 2 mM AICAR and fixed 18 hr later. Cells were processed for phospho-histone H3 Ser10 immunocytochemistry to visualize the cells actively going through mitosis at the time the cells were fixed. DAPI was used as a nuclear counterstain. Histogram quantifies phospho-histone H3 immunocytochemistry on indicated cells treated with 5 mM phenformin, or 2 mM AICAR for 18 hr. At least 300 cells were scored for each condition. Error bars indicate standard deviation.

(C) Cell extracts from parallel plates to the ones analyzed in (A) were immunoblotted for phospho-histone H3 Ser10 as a marker of the percentage of cells in mitosis.

(D) *TSC2*$^{+/+}$, *p53*$^{-/-}$ MEFs or *TSC2*$^{-/-}$, *p53*$^{-/-}$ MEFs expressing AA mutant raptor undergo apoptosis to a greater extent than those expressing WT raptor at later time points following energy stress treatment. Cell populations of indicated genotypes were treated with 5 mM phenformin, and at 48 hr the percentage of cells undergoing apoptosis was quantified using Annexin V staining and FACS analysis. Histograms of cells expressing wild-type raptor (red trace) and AA raptor (blue trace) are overlaid in the rightmost panel. The percentage of apoptotic cells in the Annexin V-positive population is indicated in red at the upper right hand corner of each histogram.

(E) Upstream AMPK signals from LKB1 are needed for the protective effect of WT raptor on apoptosis following energy stress. A549 human lung adenocarcinoma cells, which are null for LKB1, were stably reconstituted with wild-type LKB1 (WT) or mutant kinase-dead K78I (KD) LKB1-expressing retroviruses. These cells were subsequently stably infected with retroviruses expressing wild-type or AA raptor. Each of the four resulting populations was treated with 5 mM phenformin and analyzed for apoptosis as above.

to drive proliferation only when nutrients are in sufficient abundance to guarantee successful cell division. We show here that the direct phosphorylation of the mTOR binding subunit raptor by AMPK under conditions in which ATP levels are low represents a biochemical mechanism by which eukaryotic cells couple their nutrient status to a central regulator of cell growth and proliferation.

Taken together with previous studies, the findings reported here suggest that energy stress results in LKB-dependent activation of AMPK, which directly phosphorylates both TSC2 and

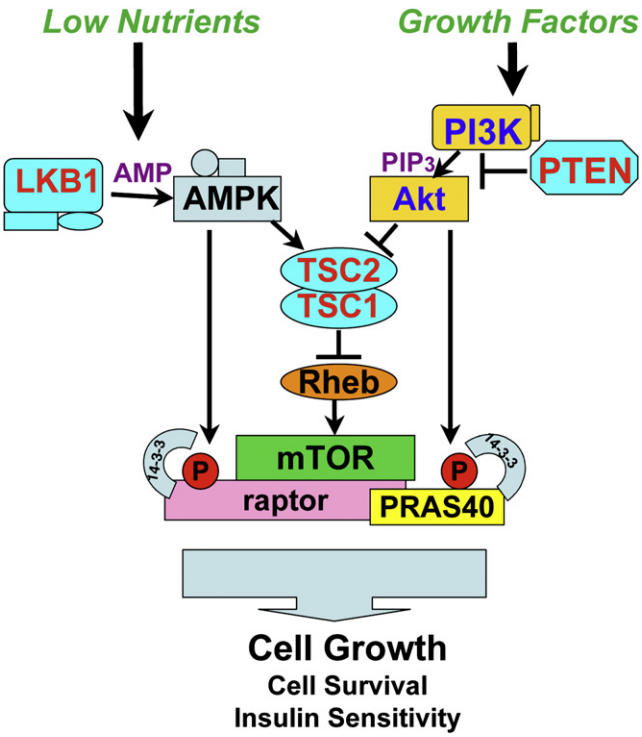

Figure 7. Nutrients and Growth Factors Control mTORC1 Activity through Common and Unique Downstream Targets

AMPK and Akt both converge to phosphorylate distinct sites in TSC2. In addition, AMPK directly phosphorylates raptor and Akt directly phosphorylates PRAS40 to regulate the activity of the mTORC1 complex through separate means. Strikingly, both AMPK-mediated suppression of raptor and Akt-mediated suppression of PRAS40 involve the phosphorylation sites in each protein binding to 14-3-3, resulting in the inactivation of those targets. Inherited mutations in LKB1, TSC1. TSC2, and PTEN all result in hamartoma syndromes in humans, indicating that hyperactivation of mTORC1 is a common biochemical mechanism underlying these genetic disorders.

raptor to inhibit mTORC1 activity by a dual-pronged mechanism (Figure 7). It recently has become apparent that Akt-mediated activation of mTORC1 is also controlled via phosphorylation of two substrates by Akt: TSC2 and an mTORC1 inhibitor, PRAS40 (Sancak et al., 2007; Vander Haar et al., 2007). In parallel opposing pathways, AMPK-mediated phosphorylation of raptor induces 14-3-3 binding and inhibition of mTORC1, while Akt-mediated phosphorylation of PRAS40 induces its binding to 14-3-3 and activation of mTORC1.

We have demonstrated here that the AMPK phosphorylation sites in raptor play a key role in the function of AMPK as a metabolic checkpoint. This metabolic checkpoint is fully analogous to the DNA damage checkpoint, with kinases serving as sensors of the stress (here ATP loss), and then initiating a response to correct the pathological damage from the stress (stimulating creation of ATP) and halting cell-cycle progression while the damage is being corrected. This metabolic checkpoint function of AMPK has been shown to be critical in a variety of cell types under conditions of low glucose, hypoxia, or following acute treatments with mitochondrial inhibitors, glycolytic inhibitors, or AMP-mimetics (Inoki et al., 2003; Corradetti et al., 2004; Shaw et al., 2004a, 2004b; Jones et al., 2005; Liu et al., 2006; Buzzai et al., 2007; Lee et al., 2007a). Inactivation of mTORC1 has previously been demonstrated to be critical for the ability of AMPK to enforce a metabolic checkpoint (Inoki et al., 2003; Shaw et al., 2004b). When mTORC1 cannot be inactivated under energy stress conditions, we show here that cells continue through the cell cycle and ultimately undergo apoptosis.

Recent evidence suggests the AMPK-mediated metabolic checkpoint on cell growth is widely conserved across eukaryotes. Hyperactivation of AMPK suppressed cell proliferation in both *Drosophila* and Dictyostelium mutants with defective mitochondrial function (Mandal et al., 2005; Bokko et al., 2007). In *C. elegans*, AMPK (aak-2) and LKB1 (par-4) orthologs are required for the extended cell-cycle arrest of germ cells in dauer worms (Narbonne and Roy, 2006) as well as the arrest of L1 stage V lineage cells under starvation conditions (Baugh and Sternberg, 2006). In both lineages, AMPK or LKB1 loss causes inappropriate proliferation under nutrient-poor conditions. In addition, AMPK activation is required in *C. elegans* for lifespan extension by daf-2, heat shock, and glycolytic inhibitors (Apfeld et al., 2004; Schulz et al., 2007). In budding yeast (SNF1) and *Arabidopsis* (KIN10/11), AMPK orthologs play key roles in regulating growth and lifespan in response to diverse nutrient and environmental stresses (Ashrafi et al., 2000; Baena-Gonzalez et al., 2007; Hong and Carlson, 2007; Thelander et al., 2004). Given these conserved functions for AMPK, it will be interesting to determine whether the predicted AMPK phosphorylation sites in raptor orthologs in lower organisms play a role in these nutrient-dependent controls on cell growth, aging, and stress response.

Taken altogether, our findings indicate that AMPK utilizes multiple targets in mammalian cells to effectively suppress mTORC1 signaling. The integral role that raptor plays in mTORC1 function and the remarkable conservation of the AMPK sites across eukaryotes suggest that raptor phosphorylation by AMPK orthologs may be an ancestral mechanism for coupling cell growth to nutrient status. The phosphorylation of raptor by AMPK could also play a physiological role in other mammalian processes that both AMPK and mTORC1 regulate, including autophagy, angiogenesis, insulin sensitivity, mitochondrial metabolism, and specific transcriptional responses. In addition, the existence of this direct regulation of mTORC1 by AMPK suggests that widely used diabetes therapeutics such as metformin, which act through AMPK activation, or environmental factors such as exercise and diet that contribute to physiological AMPK activation, may modulate tumorigenesis through this distinct signaling route. The direct phosphorylation and inhibition of raptor function by AMPK also suggest a possible therapeutic window for the use of AMPK agonists to treat tumors arising in patients with tuberous sclerosis complex or tumors exhibiting hyperactivation of mTOR via other genetic lesions.

As the response to a shortage of environmental nutrients and resultant loss in cellular energy represents one of the most fundamental pathological events of all organisms, we anticipate that further investigation of the downstream targets of AMPK will provide great insight into the emerging nexus of cancer, diabetes, and lifespan extension controlled by this ancestral signaling pathway.

EXPERIMENTAL PROCEDURES

Materials

Antibodies to phospho-AMPK (T172), AMPK α, phospho-ACC (S79), ACC, raptor, mTOR, PRAS40, phospho-S6K1(T389), S6K1, phospho-ribosomal protein S6 (S235/236), ribosomal protein S6, eIF4E, phospho-Akt (S473), phospho-Erk (T202/Y204), phospho 4E-BP1(S65), 4E-BP1, phospho IRS1 (S789), 14-3-3 ζ, 14-3-3 γ, Myc epitope (9B11), Myc epitope polyclonal, and phospho-14-3-3 substrate motif antibodies were obtained from Cell Signaling Technology. Anti-IRS1 and anti-phospho histone H3 (S10), and active recombinant AMPK were obtained from Millipore. Anti-Flag antibodies (M2 monoclonal and Flag polyclonal), phenformin, resveratrol, and AMP were obtained from Sigma. HA probe polyclonal and mTOR (N19) antibodies and protein A/G Sepharose were obtained from Santa Cruz Biotechnology. AICAR was obtained from Toronto Research Chemicals. Glutathione-Sepharose was obtained from Amersham Pharmacia. Colloidal blue stain and Supersensitive ECL kits were obtained from Pierce.

Additional Experimental Procedures can be found in the Supplemental Data available online.

SUPPLEMENTAL DATA

Supplemental Data include eight figures, Supplemental Experimental Procedures, and Supplemental References and can be found with this article online at http://www.molecule.org/cgi/content/full/30/2/214/DC1/.

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