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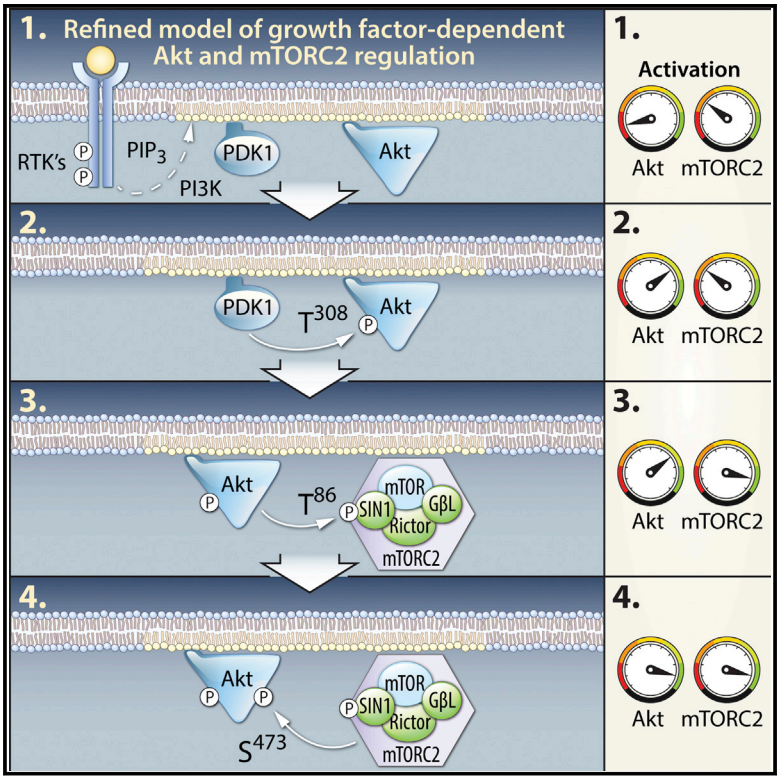
A Positive Feedback Loop between Akt and mTORC2 via SIN1 Phosphorylation

Graphical Abstract



Highlights

- Akt, but not S6K, is the major kinase for SIN1 T86 in diverse cell lines and conditions
- PDK1-mediated phosphorylation of Akt T308 is required for SIN1 phosphorylation
- SIN1 phosphorylation mediates a positive feedback loop between Akt and mTORC2

Authors

Guang Yang, Danielle S. Murashige, Sean J. Humphrey, David E. James

Correspondence

david.james@sydney.edu.au

In Brief

The mechanistic target of rapamycin complex 2 (mTORC2) responds to growth factors through a poorly defined mechanism that requires PI3K. Yang et al. reveal that Akt phosphorylates SIN1, a key component of mTORC2, at Threonine 86, in diverse cells and conditions, enhancing mTORC2 activity.

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A Positive Feedback Loop between Akt and mTORC2 via SIN1 Phosphorylation

Guang Yang,[1,3,4] Danielle S. Murashige,[1,4,5] Sean J. Humphrey,[1,2] and David E. James[1,3,*]
[1]Diabetes and Obesity Program, Garvan Institute of Medical Research, Darlinghurst, NSW 2010, Australia
[2]Department of Proteomics and Signal Transduction, Max Planck Institute for Biochemistry, Martinsried 82152, Germany
[3]The Charles Perkins Centre, The School of Molecular Bioscience, Sydney Medical School, The University of Sydney, Sydney, NSW 2006, Australia
[4]Co-first author
[5]Present address: Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA 19104, USA
*Correspondence: david.james@sydney.edu.au
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SUMMARY

The mechanistic target of rapamycin complex 2 (mTORC2) regulates cell survival and cytoskeletal organization by phosphorylating its AGC kinase substrates; however, little is known about the regulation of mTORC2 itself. It was previously reported that Akt phosphorylates the mTORC2 subunit SIN1 at T86, activating mTORC2 through a positive feedback loop, though another study reported that S6K phosphorylates SIN1 at the same site, inhibiting mTORC2 activity. We performed extensive analysis of SIN1 phosphorylation upon inhibition of Akt, S6K, and mTOR under diverse cellular contexts, and we found that, in all cell lines and conditions studied, Akt is the major kinase responsible for SIN1 phosphorylation. These findings refine the activation mechanism of the Akt-mTORC2 signaling branch as follows: PDK1 phosphorylates Akt at T308, increasing Akt kinase activity. Akt phosphorylates SIN1 at T86, enhancing mTORC2 kinase activity, which leads to phosphorylation of Akt S473 by mTORC2, thereby catalyzing full activation of Akt.

INTRODUCTION

The mechanistic target of rapamycin (mTOR) is an essential regulator of many major cellular functions, such as metabolism, growth, proliferation, and survival. In executing this role, mTOR participates in two distinct complexes called mTOR complex 1 (mTORC1) and 2 (mTORC2), enabling it to signal to distinct sets of substrates in response to growth factors (GFs), stress, nutrient availability, and other stimuli. Dysregulation of mTOR is thus a common feature of many diseases, including cancer, obesity, and type 2 diabetes (Laplante and Sabatini, 2012).

Both mTOR complexes contain the catalytic mTOR subunit, GβL, DEPTOR, and the Tti1/Tel2 complex, while Rictor, SIN1, and Protor1/2 are specific to mTORC2 (Bar-Peled and Sabatini, 2014). Unlike mTORC1, the function and regulation of mTORC2 is less well defined. Once known as the elusive PDK2, mTORC2 is best known for its role as the kinase responsible for activating AGC kinases including Akt, SGK, and PKC, via phosphorylation of their hydrophobic motif (HM)/turn motif (TM) sites to regulate cell survival and cytoskeletal organization (Facchinetti et al., 2008; García-Martínez and Alessi, 2008; Ikenoue et al., 2008; Sarbassov et al., 2005). Loss of mTORC2 in fat and skeletal muscle leads to impaired GF-stimulated GLUT4 translocation, glucose transport, and glucose tolerance (Kumar et al., 2008, 2010), while disruption of mTORC2 in liver results in constitutive gluconeogenesis, impaired glycolysis, and lipogenesis (Hagiwara et al., 2012; Lamming et al., 2014; Yuan et al., 2012), implicating an important role for mTORC2 in the regulation of glucose and lipid metabolism.

Although it is widely appreciated that mTORC2 is a crucial node in GF signaling in diverse cells and tissues, the regulatory mechanisms governing mTORC2 activity remain unclear. One mechanism for PI3K-dependent regulation of mTORC2 involves insulin-induced binding of the complex to ribosomes (Zinzalla et al., 2011). Another involves post-translational modifications of the mTORC2 complex member Rictor (Dibble et al., 2009). Rac1 is also reported to be a regulator of both mTORC1 and mTORC2 in response to GF stimulation (Saci et al., 2011). One of the confounding factors in mTORC2 regulation concerns its subcellular localization. The original models for regulation of Akt posited that upon GF stimulation Akt translocates to the plasma membrane (PM) together with its upstream regulatory kinases, PDK1 and mTORC2. While it remains unclear whether mTORC2 is PM localized, there is evidence suggesting that mTORC2 is found at the PM in different cell types (Berchtold and Walther, 2009; Saci et al., 2011; Schroder et al., 2007). More recently, mTORC2 also has been localized to mitochondria-associated ER membranes (MAMs), where it is thought to play an important role in Akt-mediated phosphorylation of mitochondrial proteins (Betz et al., 2013), and as mentioned it also has been found at ribosomes (Zinzalla et al., 2011). Thus, it seems that mTORC2 has a complex cellular distribution, and that pools of mTORC2 may be found in different locations throughout the cell. Future studies will be needed to establish the molecular basis and function of this differential targeting.

We recently showed that the mTORC2 subunit SIN1 is a physiological Akt substrate in 3T3-L1 adipocytes and HEK293 cells,

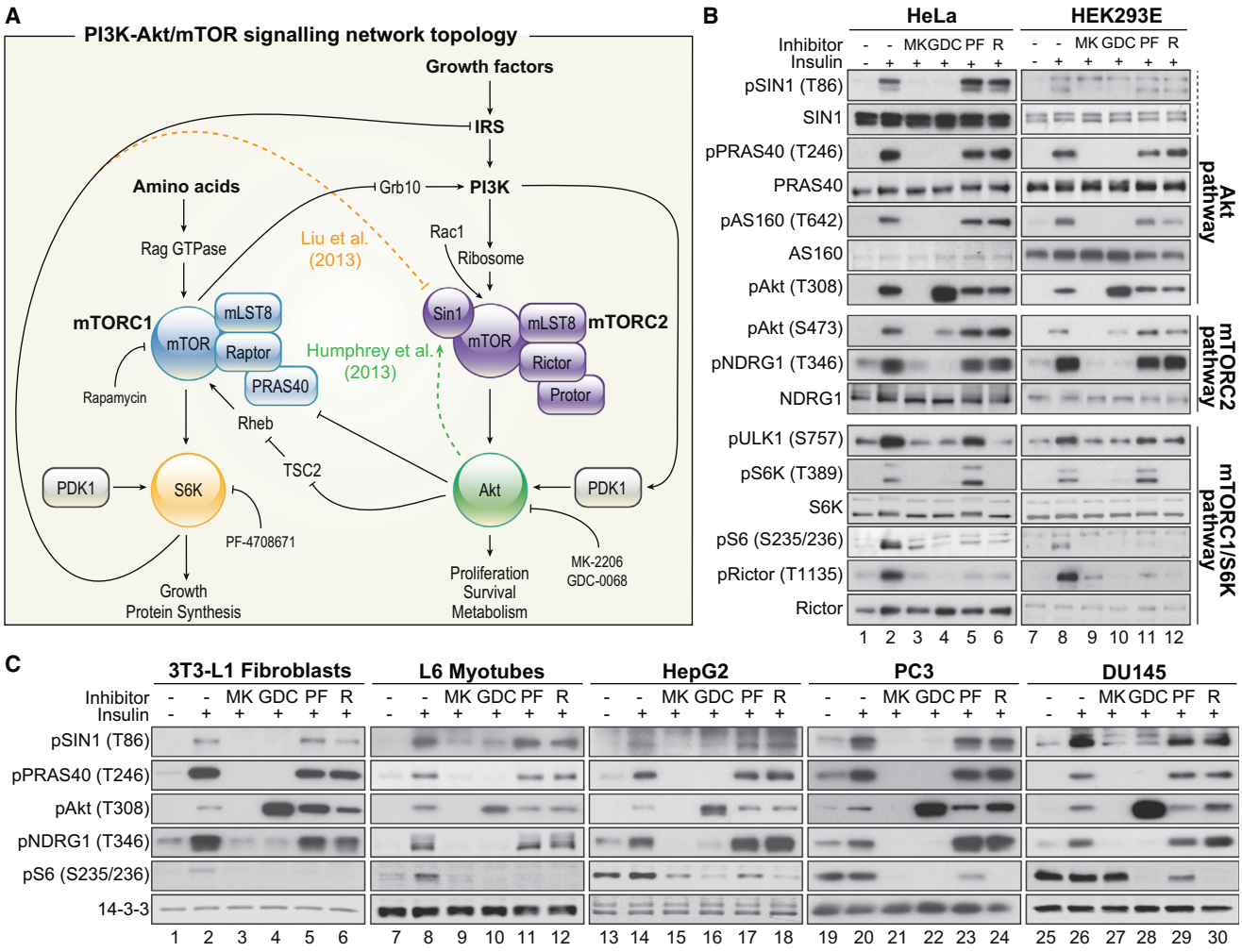

Figure 1. Insulin-Induced Phosphorylation of SIN1 T86 Is Blocked by Akt Inhibitors, but Not by mTORC1 or S6K Inhibitors, in Different Cell Lines

(A) Schematic shows the PI3K-Akt/mTOR signaling network.

(B) HeLa and HEK293E cells were serum starved for 1.5 hr, treated with MK-2206 (MK, 10 μM), GDC-0068 (GDC, 10 μM), PF-4708671 (PF, 10 μM), or rapamycin (R, 100 nM) for a further 30 min, followed by insulin (100 nM, 10 min). Samples were analyzed by western blotting (n = 3 biological replicates).

(C) Cell lines were serum starved and treated with inhibitors and insulin as in (B) (n = 2 biological replicates).

Phospho-SIN1 antibody validation and quantitative analysis of (B) are shown in Figure S1.

and that phosphorylation of SIN1 T86 by Akt positively regulates mTORC2 signaling in vivo and mTORC2 kinase activity in vitro (Figure 1A). This revealed a new mechanism for GF-regulated mTORC2 activity (Humphrey et al., 2013). Subsequently, Liu et al. also described GF-dependent phosphorylation of SIN1 at T86, as well as at another site, T398 (Liu et al., 2013), which was not identified in our study or in other large-scale phosphoproteomics studies to date (Hornbeck et al., 2012). The authors confirmed our findings in 3T3-L1 adipocytes, but reported that in HeLa cells, S6K, but not Akt, is the principal kinase responsible for SIN1 T86 phosphorylation. It was suggested that these differences may reflect cell-type- or context-specific kinase-substrate specificity (HeLa versus 3T3-L1 adipocytes) (Liu et al., 2013). Moreover, this study reported that phosphorylation of SIN1 inhibits mTORC2 activity, indicative of a negative feedback loop between mTORC1 and mTORC2 independent of IRS-1 and Grb10 (Figure 1A). Here we report a comprehensive analysis of kinase selectivity toward SIN1 T86 in a range of cell types under diverse experimental conditions. These data provide evidence that Akt is the major physiological kinase responsible for SIN1 phosphorylation at T86 in diverse cellular contexts, supporting the previously identified positive regulatory loop between Akt and mTORC2.

RESULTS AND DISCUSSION

Insulin-Induced SIN1 T86 Phosphorylation Is Blocked by Inhibitors of Akt, but Not S6K, in Diverse Cell Lines

To explore the role of Akt versus S6K as the SIN1 T86 kinase in a context-dependent manner, we performed small-molecule

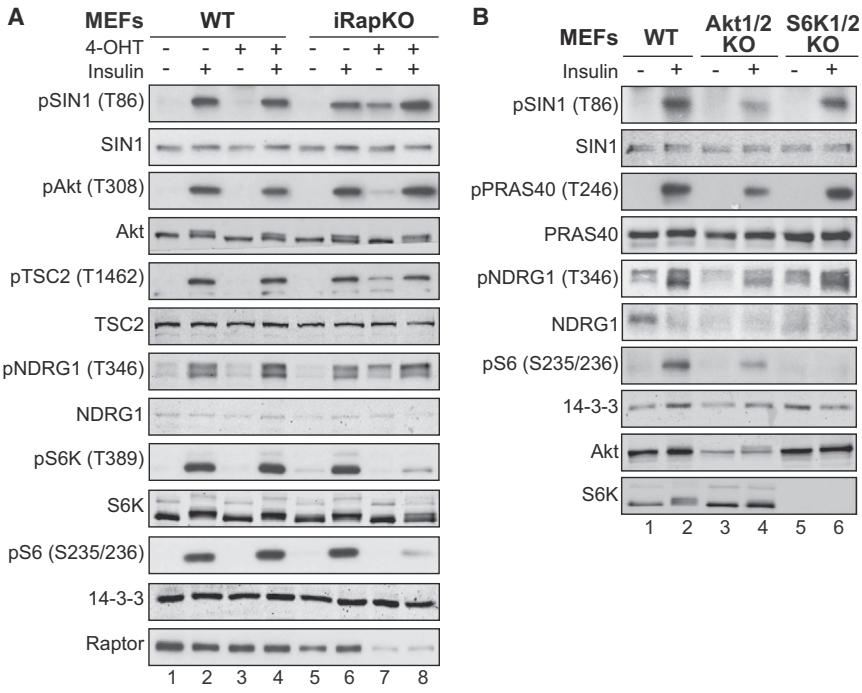

Figure 2. Insulin-Induced Phosphorylation of SIN1 T86 Is Blocked in Akt1/2, but Not in Raptor and S6K1/2, Knockout MEFs

(A) Control or iRapKO MEFs were treated with or without 1 μM 4-Hydroxytamoxifen (4-OHT) for 3 days to induce Raptor KO. Treated cells were serum starved for 2 hr followed by insulin (100 nM, 10 min), and samples were analyzed by western blotting.

(B) Akt1/2 double knockout (Akt1/2 DKO), S6K1/2 double knockout (S6K1/2 DKO), and control MEF cells were serum starved for 2 hr followed by insulin (100 nM, 10 min), and samples were analyzed by western blotting.

n = 3 biological replicates. Quantitative analysis is shown in Figure S2.

inhibitor treatments in numerous cell lines derived from different species and tissues of origin. We also studied the effects of two different classes of Akt inhibitor as follows: (1) MK-2206, an allosteric inhibitor; and (2) GDC-0068, an ATP-competitive inhibitor. As next-generation inhibitors, both demonstrate improved potency and specificity compared with earlier Akt inhibitors (Lin et al., 2013; Tan et al., 2011). SIN1 T86 phosphorylation was blocked by both Akt inhibitors in HeLa and HEK293 cells (Figure 1B; Figure S1); but, in contrast to previous studies (Liu et al., 2013), neither mTORC1 nor S6K inhibitors (rapamycin and PF-4708671, respectively) had any effect on SIN1 T86 phosphorylation in HeLa cells, despite potently inhibiting insulin-stimulated S6 and Rictor phosphorylation (Figure 1B; Figure S1C). Overall, the pattern of SIN1 pT86 mirrored that of Akt substrates (PRAS40 pT246 and AS160 pT642) and mTORC2 activity (Akt pS473 and NDRG1 pT346), but was in stark contrast to mTORC1 substrates (ULK1 pS757 and S6K pT389) and S6K substrates (S6 pS235/236 and Rictor pT1135). We observed similar patterns in 3T3-L1 fibroblasts, L6 Myotubes, and three cancer cell lines (HepG2, PC3, and DU145) (Figure 1C), suggesting that SIN1 pT86 is not dependent on mTORC1 or S6K activity in different cell types, rather it is an ubiquitous and highly conserved Akt substrate.

Insulin-Induced Phosphorylation of SIN1 T86 Is Blocked in Akt1/2, but Not in Raptor and S6K1/2, Knockout MEFs

We next utilized a genetic approach to explore the role of mTORC1/S6K on SIN1 T86 phosphorylation, using 4-Hydroxytamoxifen (4-OHT)-inducible Raptor knockout (iRapKO) mouse embryonic fibroblasts (MEFs). Since Raptor is an essential component of mTORC1, but not mTORC2, its deletion selectively disrupts the mTORC1/S6K pathway. Consistent with previous studies, insulin-stimulated S6K activity was almost completely abolished in iRapKO cells, while Akt signaling was elevated following serum starvation (Akt pT308 and TSC2 pT1462; Figure 2A; Figure S2A; Cybulski et al., 2012). This is likely due to elimination of the mTORC1/S6K-mediated negative feedback to IRS1 (Harrington et al., 2004; Shah et al., 2004) and Grb10 (Hsu et al., 2011; Yu et al., 2011), resulting in elevated PI3K-Akt signaling. Notably, SIN1 pT86 was increased in the 4-OHT-induced iRapKO cells, consistent with it being an Akt substrate as opposed to a substrate of mTORC1/S6K.

We next performed similar studies in Akt1/2 and S6K1/2 double-knockout (DKO) MEFs. As expected, insulin-stimulated phosphorylation of the S6K substrate S6 pS235/236 was completely abolished in S6K1/2 DKO MEFs. In contrast, SIN1 pT86 was unaffected. Moreover, insulin-induced phosphorylation of SIN1 was attenuated to a similar extent as other Akt substrates in Akt1/2 DKO cells (Figure 2B; Figure S2B), albeit not completely, due to functional compensation by Akt3 (Liu et al., 2006). Collectively, these data indicate that genetic ablation of S6K has little effect on SIN1 T86 phosphorylation, but that pT86 is dependent on Akt activity.

Akt Is the Major SIN1 T86 Kinase under Diverse Conditions

Since mTORC1 is involved in multiple GF-signaling pathways, we next investigated phosphorylation of SIN1 T86 in response to other stimuli. Epidermal growth factor (EGF) and serum robustly increased SIN1 pT86, as well as the Akt substrate AS160 pT642 and the SGK substrate NDRG1 pT346, in HEK cells. Phosphorylation at each of these sites was blocked by MK-2206, but not by the S6K inhibitor PF-4708671, while S6K substrates (S6 pS235/236 and Rictor pT1135) were potently blocked by PF-4708671, demonstrating its efficacy in the assay (Figure 3A; Figure S3A).

Phorbol myristate acetate (PMA), which promotes mTORC1 activity independently of the PI3K/Akt pathway (Aeder et al., 2004), activated both S6K and to a lesser extent Akt (Figure 3B). While PMA-induced S6K activity was blocked by S6K

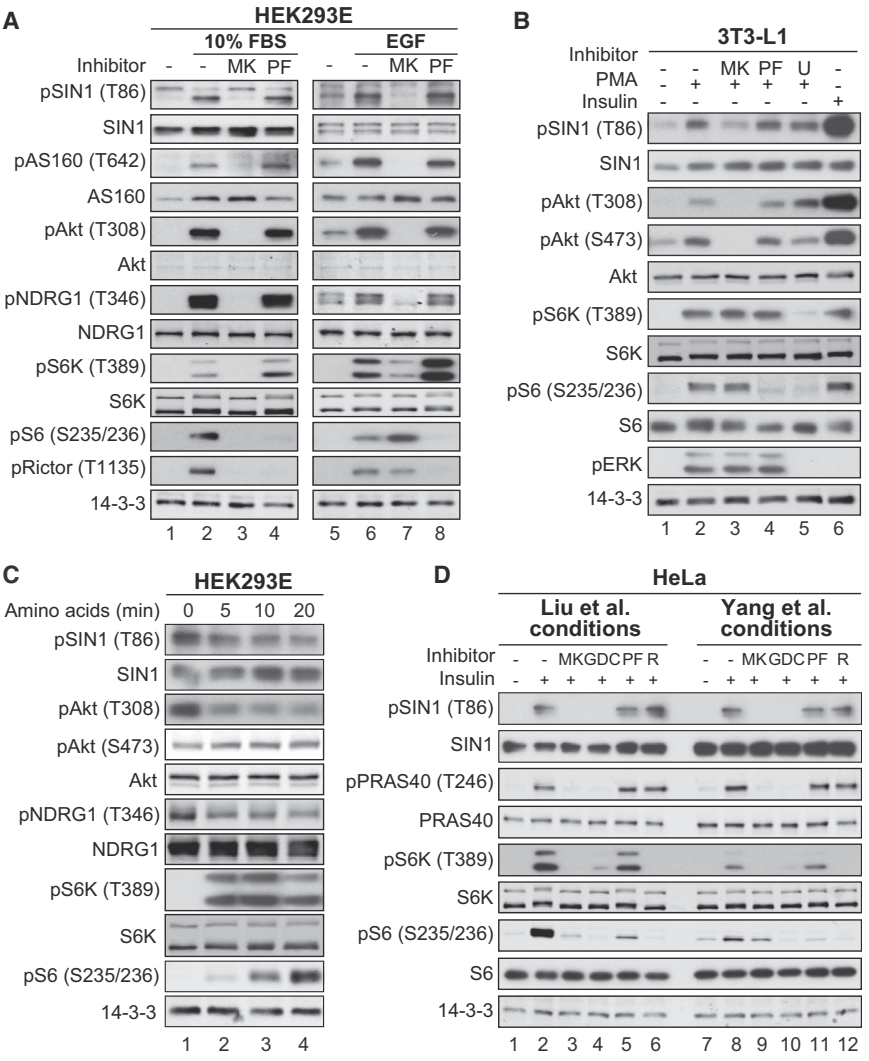

Figure 3. Akt Is the Major SIN1 Kinase under Diverse Cellular Conditions

(A) HEK293 cells were serum starved for 1.5 hr, treated with indicated inhibitors for a further 30 min, followed by EGF (100 ng/ml, 30 min) or 10% fetal bovine serum (30 min). Samples were analyzed by western blotting.

(B) 3T3-L1 fibroblast cells were serum starved for 24 hr before treatment with PMA (250 ng/ml) together with inhibitors (MK-2206, 10 μM; PF, 10 μM; and U0126, 10 μM) for 20 min or insulin (100 nM) for 10 min. Samples were analyzed by western blotting.

(C) HEK293E cells were transferred to amino acid-free KRPH medium containing dialyzed serum and then treated with amino acids for the indicated times.

(D) HeLa cells were treated as in Liu et al. (2013) (Liu et al. conditions, left) or as in Humphrey et al. (2013) (Yang et al. conditions, right). Specifically, cells were serum starved for 24 hr and treated with the indicated inhibitors simultaneously with insulin (100 nM) for 30 min (left), or they were serum starved for 1.5 hr and treated with the indicated inhibitors for a further 30 min, followed by insulin (100 nM) for 10 min (right). Samples were analyzed by immunoblotting.

n = 3 (A and C) or 2 (B and D) biological replicates. Quantitative analysis of (A) and (C) is shown in Figure S3.

(PF-4708671) and MEK inhibitors (U0126), neither of these compounds blocked PMA-induced phosphorylation of SIN1 (Figure 3B). Conversely, the Akt inhibitor (MK-2206), which had no effect on PMA-induced S6K activity, completely blocked PMA-induced Akt and SIN1 pT86 phosphorylation (Figure 3B).

Nutrients, particularly amino acids, are essential for mTORC1 activity. Even in the presence of serum, amino acid withdrawal inhibits mTORC1 and S6K activity, and this is restored by the re-addition of amino acids (Hara et al., 1998). We found that amino acid withdrawal completely blocked the activity of mTORC1 and S6K, while SIN1 pT86 was not impaired (Figure 3C). In contrast, acute re-stimulation with amino acids moderately inhibited Akt phosphorylation and activity, presumably due to negative feedback by mTORC1/S6K on IRS1 and Grb10 (Hsu et al., 2011; Yu et al., 2011). Analogous to other Akt substrates, SIN1 pT86 was blunted following amino acid re-stimulation (Figure 3C; Figure S3B). These data suggest that, in the context of amino acid sensing under normal growth conditions, SIN1 pT86 is not dependent on mTORC1 or S6K activity, but mirrors Akt substrate phosphorylation.

Two major differences between our study and that of Liu et al. (Humphrey et al., 2013; Liu et al., 2013) is that they used longer starvation durations (12–24 hr compared with our 1.5–2 hr) and they added inhibitory compounds simultaneously with insulin for 30 min, whereas we pre-incubated cells with inhibitor compounds for 30 min prior to insulin stimulation. We replicated these various conditions and found that, irrespective of starvation duration or timing of the addition of inhibitors and GFs, SIN1 pT86 was blocked by both Akt inhibitors, but was unaffected by S6K and mTORC1 inhibitors, while pS6K levels were almost completely abolished by both compounds (Figure 3D).

Temporal Analysis of GF-Mediated SIN1 T86 Phosphorylation

We previously showed that, in 3T3-L1 adipocytes, Akt is activated very rapidly (<1 min) following insulin addition, whereas the mTORC1/S6K pathway is activated more slowly (≥5 min), and SIN1 pT86 temporal phosphorylation mirrored that of Akt substrates (Humphrey et al., 2013). Indeed, this observation suggested to us that SIN1 T86 is likely a substrate of Akt and not S6K. Thus, we further investigated the temporal relationship between Akt versus S6K vis-à-vis SIN1 phosphorylation. Cells were first stimulated with insulin for 10 min to activate the Akt pathway and rapamycin was then added. We reasoned that if S6K is a

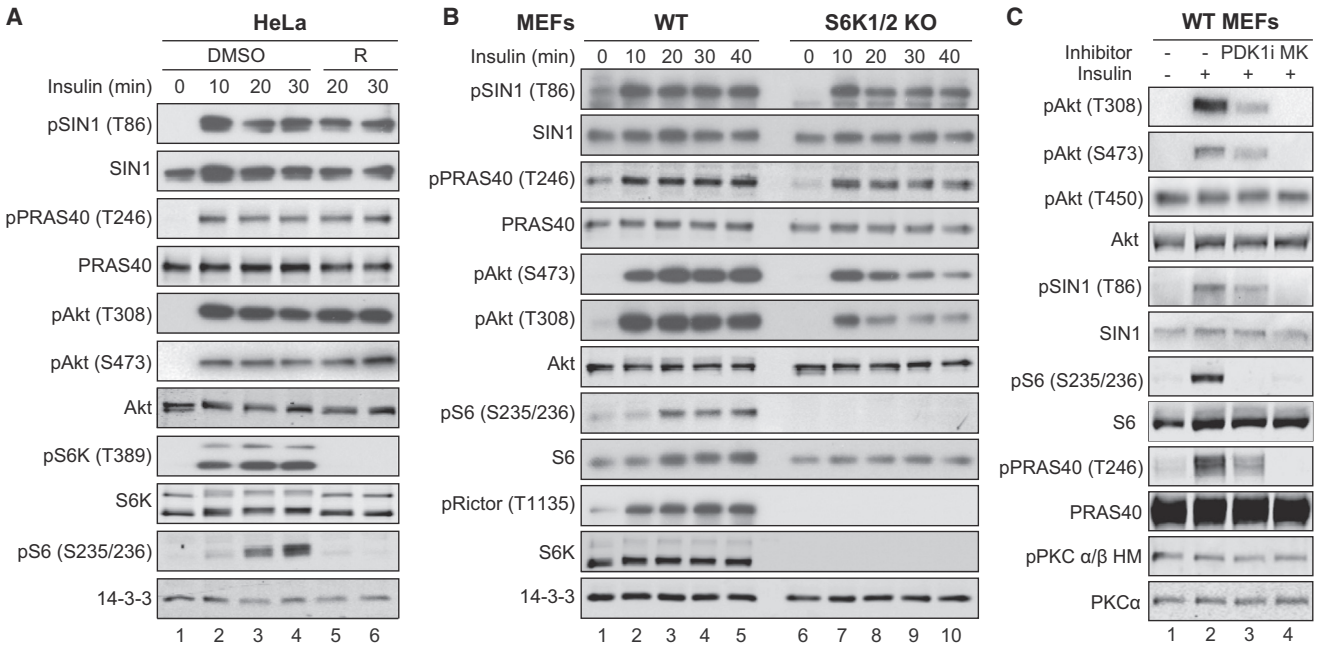

Figure 4. Temporal Analysis of SIN1 T86 Phosphorylation

(A) HeLa cells were serum starved for 24 hr, stimulated with insulin (100 nM), and harvested at the indicated time points, or they were stimulated with insulin (100 nM) for 10 min and subsequently rapamycin was added (R, 100 nM) for a further 10 or 20 min.

(B) WT and S6K1/2 DKO MEFs were serum starved in DMEM for 24 hr before adding insulin (100 nM). Cells were harvested at the indicated time points and samples were analyzed by immunoblotting.

(C) WT MEFs were serum starved for 1.5 hr, treated with GSK2334470 (PDK1i, 1 μM) and MK-2206 (MK, 10 μM) for a further 30 min, followed by insulin (100 nM) for 10 min. Samples were analyzed by immunoblotting.

n = 3 (B and C) or 2 (A) biological replicates. Quantitative analysis of (B) and (C) is shown in Figure S4.

late-acting SIN1 T86 kinase, then SIN1 pT86 should become attenuated under these conditions. Rather, we found that late inhibition of S6K caused complete blockade of pS6 phosphorylation, but SIN1 pT86 was unimpaired, arguing against this hypothesis (Figure 4A). If S6K were a late SIN1 kinase, one would have expected attenuation of SIN1 T86 phosphorylation relative to other Akt substrates at later but not earlier time points, but this was not evident. Moreover, time-course studies in wild-type (WT) and S6K1/2 DKO MEFs showed that phosphorylation of S6 and Rictor were completely blocked in S6K1/2 DKO MEFs, whereas SIN1 pT86 persisted (Figure 4B; Figure S4A). Although a moderate decrease in SIN1 pT86 was observed at 20 min and later time points relative to 10 min, phospho-Akt (both pS473 and pT308) and phospho-PRAS40 displayed the same decrease, indicating that the temporal pattern of SIN1 phosphorylation again mimicked Akt and its substrates in both cell types (Figure 4B; Figure S4A).

Acute Inhibition of PDK1 Impairs GF-Dependent mTORC2 Activity

In our current model, Akt phosphorylation at T308 by PDK1 is required for SIN1 T86 phosphorylation, and this in turn enhances mTORC2 activity via an as yet undefined mechanism. Therefore, disruption of PDK1 activity should inhibit both SIN1 pT86 and mTORC2 activity. We observed a significant inhibition of SIN1 pT86 in PDK1−/− cells compared with their WT controls, suggesting that PDK1 and Akt pT308 are indeed important for SIN1 T86 phosphorylation (Figure S4B). However, unlike pT308, elevated Akt pS473 was observed in PDK1−/− cells compared with PDK1+/+ cells (Figure S4B; Williams et al., 2000). Cells lacking PDK1 exhibit increased PI3K activity and this may explain elevated Akt pS473 in these cells (Williams et al., 2000). Chronic activation of PI3K could result in long-term compensatory mechanisms activating mTORC2 via alternate pathways, such as the PI3K-ribosome-mTORC2 pathway (Zinzalla et al., 2011). This hypothesis is supported by a time-course study using PDK1 inhibitors, in which acute inhibition of PDK1 resulted in transient inhibition of Akt pS473 (1–2 hr), and this inhibitory effect disappeared gradually during prolonged pharmacological inhibition of PDK1 (Nagashima et al., 2011).

To confirm this result, we acutely inhibited PDK1 using a different PDK1 inhibitor, GSK2334470. In agreement with the previous study, after 30 min of treatment, 75% of Akt pT308 phosphorylation was blocked, while SIN1 pT86 and Akt pS473 were inhibited by 57% and 33%, respectively (Figure 4C; Figure S4C). This moderate but significant inhibition of Akt pS473 demonstrates that mTORC2 activity is at least partially PDK1-dependent, while the remaining effect of GF-dependent mTORC2 activity may depend on other factors, including translocation to the PM and PIP3 binding. Furthermore, Newton and colleagues showed that neither kinase-dead nor a T308A Akt mutant became phosphorylated on S473 in response to

stimulation, supporting our model (Toker and Newton, 2000). Also consistent with our proposal, Hemmings and colleagues (Andjelković et al., 1997) showed that targeting of kinase-dead Akt to the PM was sufficient to trigger phosphorylation at T308, but not S473. However, stimulation of these cells with IGF1 resulted in a 2-fold increase in pT308 and a 5-fold increase in pS473. These data are consistent with the notion that translocation of Akt to the membrane is sufficient for the most part for T308 phosphorylation (PDK1 displays constitutive activity toward Akt), while this is not the case for S473 phosphorylation, because the latter also requires GF-dependent activation of mTORC2.

Interestingly, both genetic and pharmacological inhibition of PDK1 had no effect on the phosphorylation of two other mTORC2 substrates, Akt T450 (TM) and PKCα S657 (HM) (Figure 4C; Figure S4B). Both of these sites are phosphorylated by mTORC2, but are not regulated by GF stimuli, although they are important for Akt and PKCα function, respectively (Facchinetti et al., 2008; Guertin et al., 2006; Hauge et al., 2007; Ikenoue et al., 2008). Sabatini and colleagues have shown that there are five isoforms of SIN1, and that these isoforms can form three distinct forms of mTORC2 (Frias et al., 2006). Importantly, only two of these mTORC2 isoforms are responsive to insulin. The mTORC2 containing the short isoform of SIN1, which lacks a pleckstrin homology (PH) domain, has constitutive activity (Frias et al., 2006). This finding could explain the distinct effects of PDK1 activity toward different mTORC2 substrates. The activity of PDK1 as well as Akt and SIN1 T86 phosphorylation are each responsive to GFs, and it is therefore reasonable that SIN1 pT86 enhances the acute GF-dependent component of mTORC2 activity (as measured by Akt pS473), but not the GF-insensitive component of mTORC2 activity (as measured by Akt pT450 and PKCα pS657). Future studies examining these possibilities are likely to be very fruitful.

Conclusions

Collectively, our results reveal that Akt is the predominant SIN1 T86 kinase under a wide range of cellular contexts. Furthermore, we have been unable to observe a discernible role for the mTORC1/S6K pathway in this biological process despite earlier reports. While we cannot exclude the possibility that under conditions of hyperactive S6K (such as in the case of TSC2 deletion) (Gao et al., 2002) that this kinase could phosphorylate substrates outside of its normal physiological reach, including SIN1 T86, our data reveal that this does not appear to be a feature of the cellular architecture under normal conditions. Importantly, the finding that Akt phosphorylates and positively regulates mTORC2 kinase activity, combined with the fact that complete disruption of the mTORC2 sites in Akt affects some, but not all, Akt substrates (Hagiwara et al., 2012; Jacinto et al., 2006) is inconsistent with the canonical model of Akt signaling in which mTORC2 is positioned simply upstream of Akt. Further studies are required to unravel the true biological function of this pathway as well as the physiological role of Akt S473 phosphorylation in vivo.

EXPERIMENTAL PROCEDURES

Amino Acid and Inhibitor Treatment

For amino acid treatment, subconfluent cells were incubated for 2 hr in Krebs-Ringer-phosphate-HEPES (KRPH) buffer containing dialyzed serum and treated with a mixture of amino acids corresponding to the concentrations present in DMEM for the indicated times.

For inhibitor assays, subconfluent cells were incubated for 1.5 hr in serum-free media and treated for 30 min with 10 μM MK-2206, 10 μM GDC-0068, 10 μM PF-4708671, 100 nM rapamycin, 1 μM GSK-2334470, or vehicle (DMSO), followed by 100 nM insulin for 10 min.

Immunoblotting

Cells were rinsed twice with ice-cold PBS, solubilized in 2% SDS in PBS, sonicated, and spun at 15,000 × *g* for 15 min. Protein content was determined by bicinchoninic acid (BCA) assay. Proteins were separated by SDS-PAGE, transferred to polyvinylidene fluoride (PVDF) membranes, and immunoblotted as described in the Supplemental Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and four figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2015.07.016.

AUTHOR CONTRIBUTIONS

G.Y. and D.E.J. designed the studies with assistance from S.J.H. G.Y. and D.S.M. performed the experiments. G.Y., S.J.H., and D.E.J. wrote the manuscript with assistance from D.S.M. All authors proofread the manuscript.

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