

The mammalian target of rapamycin complex 2 controls folding and stability of Akt and protein kinase C

Valeria Facchinetti^{1,*}, Weiming Ouyang²,
Hua Wei³, Nelyn Soto³, Adam Lazorchak²,
Christine Gould⁴, Carolyn Lowry³,
Alexandra C Newton⁴, Yuxin Mao⁵,
Robert Q Miao⁶, William C Sessa⁶,
Jun Qin⁷, Pumin Zhang⁸, Bing Su^{1,2,*}
and Estela Jacinto^{3,*}

¹Department of Immunology, The University of Texas, MD Anderson Cancer Center, Houston, TX, USA, ²Department of Immunobiology and Vascular Biology and Transplantation Program, New Haven, CT, USA, ³Department of Physiology and Biophysics, UMDNJ-Robert Wood Johnson Medical School, Piscataway, NJ, USA, ⁴Department of Pharmacology, University of California at San Diego, La Jolla, CA, USA, ⁵Department of Cell Biology, Yale School of Medicine, New Haven, CT, USA, ⁶Department of Pharmacology, Yale School of Medicine, New Haven, CT, USA, ⁷Verna and Mars McLean Department of Biochemistry and Molecular Biology, Baylor College of Medicine, Houston, TX, USA and ⁸Department of Physiology, Baylor College of Medicine, Houston, TX, USA

The target of rapamycin (TOR), as part of the rapamycin-sensitive TOR complex 1 (TORC1), regulates various aspects of protein synthesis. Whether TOR functions in this process as part of TORC2 remains to be elucidated. Here, we demonstrate that mTOR, SIN1 and rictor, components of mammalian (m)TORC2, are required for phosphorylation of Akt and conventional protein kinase C (PKC) at the turn motif (TM) site. This TORC2 function is growth factor independent and conserved from yeast to mammals. TM site phosphorylation facilitates carboxyl-terminal folding and stabilizes newly synthesized Akt and PKC by interacting with conserved basic residues in the kinase domain. Without TM site phosphorylation, Akt becomes protected by the molecular chaperone Hsp90 from ubiquitination-mediated proteasome degradation. Finally, we demonstrate that mTORC2 independently controls the Akt TM and HM sites *in vivo* and can directly phosphorylate both sites *in vitro*. Our studies uncover a novel function of the TOR pathway in regulating protein folding and stability, processes that are most likely linked to the functions of TOR in protein synthesis.

The EMBO Journal advance online publication, 19 June 2008;
doi:10.1038/emboj.2008.120

*Corresponding authors. E Jacinto, Department of Physiology and Biophysics, UMDNJ-Robert Wood Johnson Medical School, 675 Hoes Lane, Piscataway, NJ 8854, USA. Tel.: +1 732 235 4476; Fax: +1 732 235 5038; E-mail: jacintes@umdnj.edu or B Su, Department of Immunobiology, Yale University School of Medicine, 10 Amistad Street, New Haven, CT 06520, USA. Tel.: +1 203 737 2463; Fax: +1 203 737 2293; E-mail: bing.su@yale.edu or V Facchinetti, Department of Immunology, The University of Texas, MD Anderson Cancer Center, 1515 Holcombe Blvd, Houston, TX 77030, USA. Tel.: +1 713 794 3069; Fax: +1 713 563 3276; E-mail: vfacchin@mdanderson.org

Received: 30 January 2008; accepted: 28 May 2008

Subject Categories: signal transduction

Keywords: Akt; mTOR; PKC; protein folding; turn motif

Introduction

The target of rapamycin (TOR) is an evolutionarily conserved Ser/Thr protein kinase that forms two distinct protein complexes that regulate cell growth in response to a variety of environmental cues (Wullschleger *et al*, 2006). In mammals, in addition to mammalian TOR (mTOR), the conserved components of these complexes consist of mLST8 and raptor for mTOR complex 1 (mTORC1), and mLST8, rictor and SIN1 for mTORC2. In the presence of growth-promoting signals such as nutrients and growth factors, mTOR controls growth by upregulation of protein synthesis (Mamane *et al*, 2006; Wullschleger *et al*, 2006). TORC1 regulates translation initiation by phosphorylating the translational regulator and AGC (protein kinases A/G/C) kinase family member, S6K (yeast SCH9), at the conserved hydrophobic motif (HM) site (Mamane *et al*, 2006; Jacinto and Lorberg, 2008). Mammalian and yeast TOR also regulates the biogenesis of ribosomes, which constitute the basic protein synthesis machinery (Martin *et al*, 2006). The highly conserved function of TOR in protein synthesis, primarily attributed to TORC1, is inhibited by the immunosuppressive and potential anti-cancer drug rapamycin. The function of TORC2 is less well-defined, but in both yeast and mammals, TORC2 is involved in actin cytoskeleton reorganization and cell survival (Loewith *et al*, 2002; Jacinto *et al*, 2004, 2006; Sarbassov *et al*, 2004). These TORC2 functions may also be linked to the regulation of other AGC kinases (Sarbassov *et al*, 2004; Kamada *et al*, 2005; Jacinto *et al*, 2006). Mammalian (m)TORC2 is required for phosphorylation of the HM site of the AGC kinases, Akt and protein kinase C- α (PKC α) (Hresko and Mueckler, 2005; Sarbassov *et al*, 2005; Jacinto *et al*, 2006; Shiota *et al*, 2006; Yang *et al*, 2006). Phosphorylation of the HM site increases their catalytic activity (Newton, 2003) and may also affect kinase interaction with substrates (Holz *et al*, 2005; Jacinto *et al*, 2006). The HM is part of the carboxyl-terminal (C) tail of AGC kinases. This kinase family is characterized by the presence of a unique interaction between the C-tail and the catalytic domain to further regulate kinase activity (Kannan *et al*, 2007). The mammalian AGC kinases become optimally activated by phosphorylation at the activation loop (A-loop) in the catalytic domain by PDK1 and at the HM site of the carboxyl-terminus (Newton, 2003). Accumulating evidence indicates that mTOR, either as part of mTORC1 or mTORC2 mediates phosphorylation of several AGC kinases at the HM site (Jacinto and Lorberg, 2008). HM phosphorylation of S6K and Akt is mediated by mTORC1 and mTORC2, respectively, and occurs in response to growth stimuli (Hara *et al*, 1998; Hresko and Mueckler, 2005;

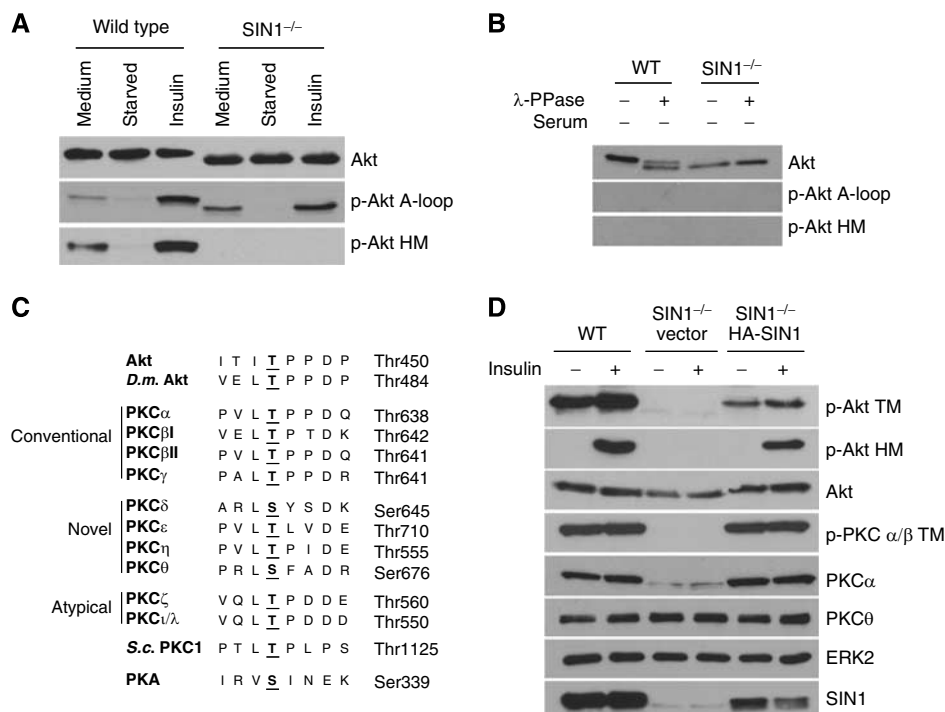


Figure 1 Identification of the TM phosphorylation in Akt and PKC α/β as a novel SIN1-regulated, growth factor-independent event. (A) Akt from either starved or growth factor-stimulated SIN1-deficient cells migrates faster than its counterpart in wild-type cells on an SDS-PAGE gel. Wild-type and SIN1^{-/-} MEFs were grown in complete medium (medium), or starved of serum overnight and amino acids for 1 h (starved), or starved then restimulated with insulin for 30 min as indicated. Total cellular extracts were prepared for immunoblot analysis for total Akt and phosphorylated Akt at Thr308 in the activation loop (p-Akt A-loop) and at Ser473 in the HM (p-Akt HM). (B) The faster migrating Akt band in SIN1-deficient cells is due to a defective phosphorylation unrelated to the A-loop or HM site phosphorylation. Wild-type and SIN1^{-/-} MEFs were starved as described in 1A. Total cellular extracts were prepared and treated with or without lambda protein phosphatase (λ -PPase) for 30 min at 30 °C before analysis of total Akt and phospho-Akt as described in panel A. (C) Sequence alignment of the TM region of various members of AGC kinases in yeast, *Drosophila* and mammals. Underlined residue indicates the phosphorylation site in the TM. Numbering is based on human sequences except as indicated; *D.m.*, *Drosophila melanogaster*; *S.c.*, *Saccharomyces cerevisiae*. (D) TM phosphorylation of Akt and PKC α/β is defective in SIN1^{-/-} cells. SIN1^{-/-} MEFs were infected with a control (SIN1^{-/-} vector) or HA-SIN1 (SIN1^{-/-} HA-SIN1) retroviral expression vectors as indicated. Total cell extracts were prepared from starved or starved then insulin stimulated wild-type MEFs or retroviral-infected cells as indicated and analysed by immunoblotting for total Akt, PKC α and phospho-Akt and phospho-PKC α/β . ERK2 and PKC θ levels were used as loading control.

Sarbasov *et al*, 2005; Jacinto *et al*, 2006). HM phosphorylation of PKC α and the yeast YPK1/2, both shown to be mediated by TORC2, does not seem to be responsive to growth stimuli, however (Kamada *et al*, 2005; Guertin *et al*, 2006).

Another highly conserved region in the carboxyl-terminus of AGC kinases is the turn motif (TM). Most AGC kinases are phosphorylated at a conserved residue in this motif (Hauge *et al*, 2007). Structural and *in vitro* studies done on PKC and PKA suggest that phosphorylation at the TM site stabilizes the kinase core (Bornancin and Parker, 1996; Yonemoto *et al*, 1997). Whether this is also true for Akt is unclear (Alessi *et al*, 1996; Bellacosa *et al*, 1998; Tokar and Newton, 2000). The nature of the TM kinase and the biological significance of this phosphorylation are poorly understood. Here, we address the importance of TM phosphorylation *in vivo*. We demonstrate that phosphorylation of Akt and conventional (c)PKC TM site is mediated by mTORC2 and is crucial for proper carboxyl-terminal folding and stability of Akt and cPKC. In the absence of TM site phosphorylation, undegraded Akt and cPKC depend on Hsp90 for stability. Our results suggest that mTORC2 controls both the growth factor-dependent HM phosphorylation and the growth factor-independent TM phosphorylation of Akt. Our findings reveal a novel function for mTORC2 in protein folding that may be closely

linked to the conserved function of mTORC1 in protein synthesis.

Results

The TM phosphorylation in Akt and PKC α/β is a novel SIN1-controlled, growth factor-independent event

We recently found that in SIN1-deficient murine embryonic fibroblasts (MEFs) in which mTORC2 is disrupted, Akt remains active and the A-loop site (Thr308) is phosphorylated normally in a growth factor-dependent manner (Jacinto *et al*, 2006). However, the growth factor-induced HM phosphorylation of Akt at Ser473 was abolished and this defect correlated with decreased cell survival upon stress induction (Jacinto *et al*, 2006). Careful examination of Akt from SIN1^{-/-} cells indicated an additional post-translational modification that could not be explained by the lack of Akt HM site phosphorylation. Akt from SIN1^{-/-} cells had faster electrophoretic mobility than in wild-type cells even without A-loop and HM phosphorylation, suggesting that this mobility shift is not due to their absence (Figure 1A). This mobility shift was not altered using different growth conditions. To determine if this modification is phosphorylation, wild-type cellular extracts were phosphatase-treated. To exclude phosphorylation of the

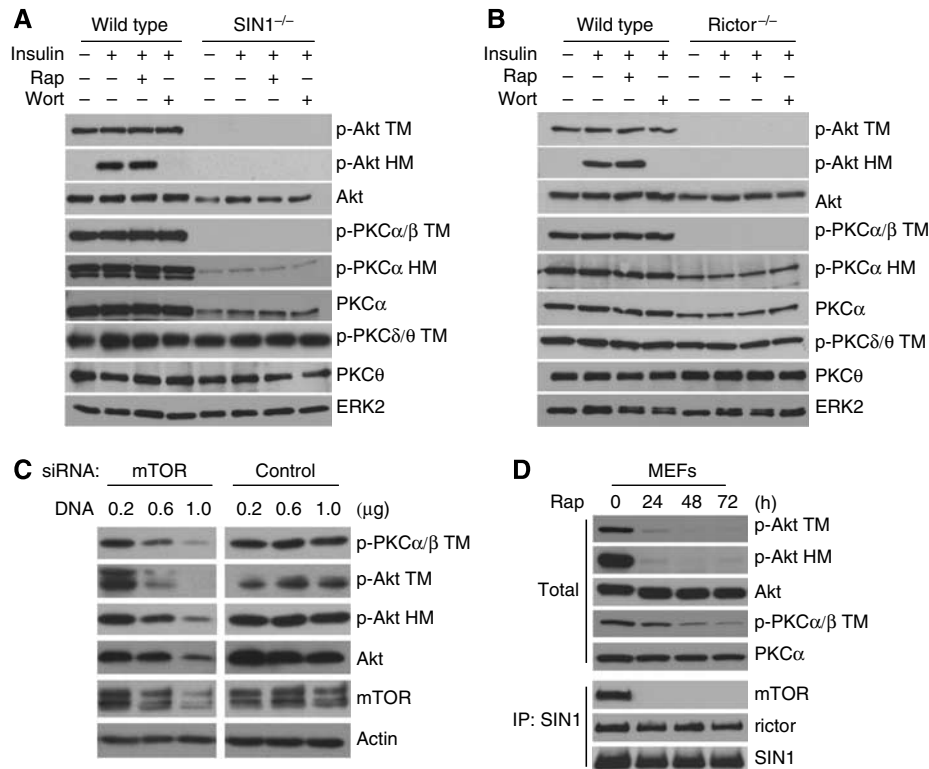


Figure 2 The TORC2 components are essential for Akt and PKC α/β TM phosphorylation. (A) The TM phosphorylation of Akt and conventional PKC is insensitive to short-term treatment with rapamycin and wortmannin. WT and SIN1^{-/-} MEFs were starved as described in Figure 1A, then treated with vehicle (-), rapamycin (50 nM) or wortmannin (100 nM) for 30 min, followed by insulin stimulation for another 30 min as indicated. Phosphorylation and total protein levels were determined by immunoblotting as described in Figure 1B. (B) The mTORC2 component rictor is essential for the Akt and PKC α/β TM site phosphorylation. WT and rictor^{-/-} MEFs were starved, stimulated and analysed for phosphorylation and total protein levels by immunoblotting as described in panel A. ERK2 was assayed as a loading control. (C) Knockdown of mTOR expression attenuates the Akt and PKC α/β TM phosphorylation. NIH3T3 cells were transfected with varying amounts (0.2, 0.6 and 1.0 μ g) of either vector control or mTOR-siRNA-expressing plasmid DNA as indicated. Total cell extracts were prepared and phosphorylation was analysed by immunoblotting. All lanes from each antibody-blotted panel come from the same blot. (D) Disruption of mTORC2 by prolonged rapamycin treatment decreases TM phosphorylation. Wild-type MEFs were incubated with rapamycin (Rap; 100 nM) for the indicated number of hours (h). SIN1 was immunoprecipitated and associated mTOR and rictor were detected by immunoblotting. Total extracts were analysed for phosphorylation and for total protein levels by immunoblotting.

A-loop and HM sites that normally occurs in the presence of serum, extracts from starved cells were used. As a control, cellular extracts from SIN1^{-/-} cells were also phosphatase-treated. In the absence of Thr308 and Ser473 phosphorylation, phosphatase treatment led to increased Akt mobility in wild-type cells that paralleled the migration pattern in SIN1^{-/-} cells (Figure 1B). Thus, defective phosphorylation accounts for the increased Akt mobility in SIN1^{-/-} cells.

In addition to the A-loop and HM site phosphorylation, Akt is phosphorylated at other sites, although their significance is less well understood (Alessi *et al*, 1996; Bellacosa *et al*, 1998). By mass spectrometry, we found that phosphorylation of Ser124, a reported constitutively phosphorylated site in Akt (Alessi *et al*, 1996), was not abolished in SIN1^{-/-} cells (data not shown). We therefore examined the TM site, Thr450, which was also shown to be constitutively phosphorylated (Alessi *et al*, 1996; Bellacosa *et al*, 1998) and highly conserved from yeast to mammals (Figure 1C) (Newton, 2003; Jacinto and Lorberg, 2008).

We examined the phosphorylation of the TM site in Akt and cPKC, which display very similar sequence around the TM. Indeed, we found that TM site phosphorylation of Akt and PKC α/β are abolished in SIN1^{-/-} cells (Figure 1D and Supplementary Figure 1). The phospho-PKC α/β TM

(Ser638/641) antibody also recognized a 60 kDa protein, which we confirmed to be Akt (Supplementary Figure 1). Unlike Akt HM phosphorylation, which is induced upon insulin stimulation, TM phosphorylation is present even under starved conditions. Upon re-expression of SIN1, the phosphorylation of the TM sites of both Akt and PKC α/β in SIN1^{-/-} MEFs was restored (Figure 1D). These results reveal that SIN1 is essential for both Akt and PKC α/β TM site phosphorylation and provide the first genetic evidence for a mechanism that controls TM site phosphorylation.

The TM phosphorylation of Akt and PKC α/β requires mTORC2 components

Turn motif phosphorylation of Akt and PKC α/β is insensitive to various growth factors and stress inducers such as tunicamycin, heat shock, etoposide, peroxide, UV and gamma irradiation (data not shown). Pharmacological inhibition of the PI3K pathway (by wortmannin) or mTORC1 (by rapamycin) up to 8 h also had no significant effect on the TM site phosphorylation (Figure 2A and data not shown). Likewise, the PI3K inhibitor Ly294002 and MAPK inhibitors U0126, SB203580, SP600125, CDK inhibitor roscovitine, cyclosporin A and FK506 had no obvious effect on TM phosphorylation (data not shown). Taken together, these results suggest that

the TM phosphorylation is stable and insensitive to transient inhibition of a number of kinases including PI3K, MAPKs, CDKs and mTORC1. The TM site phosphorylation of novel PKC δ/θ and PKA was not abolished in SIN1^{-/-} cells (Figure 2A and data not shown); it is noteworthy that these kinases do not have the consensus Thr-Pro-sequence, which is found in Akt (*Drosophila* and mammals), mammalian cPKC and yeast PKC1 (Figure 1C) in their TM. These results suggest that the SIN1-mediated TM site phosphorylation is specific to a subset of the AGC family kinases.

To further determine if TM phosphorylation is mTORC2 dependent, we examined if rictor also mediates the TM phosphorylation of Akt and PKC α/β II. Using rictor^{-/-} cells, essentially the same defects in Akt and PKC α/β II TM phosphorylation were observed similar to SIN1^{-/-} cells (Figure 2B). TM phosphorylation of novel PKC δ/θ and PKA was also not affected in rictor^{-/-} cells (Figure 2B and data not shown), further suggesting that mTORC2 components specifically mediate TM phosphorylation of Akt and cPKC.

Next, we silenced mTOR expression with siRNA and found that knockdown of mTOR decreased the TM site phosphorylation of both Akt and PKC α/β II (Figure 2C). As it was reported that prolonged rapamycin treatment could inhibit the assembly of mTORC2 in many cell types (Sarbasov *et al*, 2006), we examined if TM phosphorylation is affected under this condition. Treatment of MEFs, PC3 and Jurkat cells with rapamycin for 24 h or longer, which dissociated mTOR binding from SIN1, caused a dramatic decrease in TM phosphorylation of Akt and PKC α/β II (Figure 2D and Supplementary Figure 2). The faster Akt migration in SDS-PAGE, which accompanies the loss of TM phosphorylation, is also evident upon prolonged rapamycin treatment (Figure 2D). HM phosphorylation of Akt was also diminished under these conditions. As previously reported for the HM site (Sarbasov *et al*, 2006), the TM phosphorylation of Akt in HEK293T and HeLa cells was also resistant to the prolonged rapamycin treatment most likely due to the remaining intact mTORC2 found in these cells (Supplementary Figure 2). Taken together, these results further indicate that mTORC2 is required for TM phosphorylation.

The TM phosphorylation mediated by TORC2 is highly conserved

As Akt and PKC have orthologues in lower organisms, we examined if the TORC2-dependent TM site phosphorylation is conserved. We conditionally depleted the SIN1 orthologue AVO1 in *Saccharomyces cerevisiae* (Loewith *et al*, 2002). TM phosphorylation of PKC1 was significantly decreased upon depletion of AVO1, but not of KOG1, a TORC1 component (Supplementary Figure 3A). These results indicate that similar to mammalian PKC α/β II, the TM phosphorylation of yeast PKC1 requires TORC2. To further address the conserved function of TORC2 in Akt and PKC TM site phosphorylation, we also knocked down *Drosophila* SIN1 (dSIN1) by siRNA. Knockdown of dSIN1, but not dRaptor, a key component of dTORC1, decreased the phosphorylation of dAkt at Thr484, the equivalent TM site in dAkt (Supplementary Figure 3B), further demonstrating that the TORC2-dependent TM phosphorylation of dAkt is also conserved in *Drosophila*. Collectively, these data demonstrate that SIN1-mediated TM phosphorylation is conserved in yeast, flies and mammals.

Protein levels of Akt and PKC α are diminished in the absence of SIN1 or rictor

Previous studies indicated that TM site phosphorylation of cPKC is important for protein maturation, and in its absence, cPKC α was unstable *in vitro* (Bornancin and Parker, 1996; Edwards *et al*, 1999). As the mTOR function in protein synthesis is evolutionarily conserved, we postulated that mTORC2 may regulate events that are closely linked to protein synthesis, such as folding and stabilization of newly synthesized proteins. Since improperly folded proteins are known to be unstable and prone to degradation (Hartl and Hayer-Hartl, 2002), we determined the protein levels of Akt and PKC α in several independently generated MEF cell lines from either wild-type or from the SIN1^{-/-} littermate embryos. To appreciate subtle changes in protein expression, we loaded less but equal amounts of lysates from wild-type and SIN1^{-/-} cells. Compared with the wild-type cell lines where the levels of Akt and PKC α were highly uniform, the levels of Akt and PKC α in all the SIN1^{-/-} cell lines varied considerably but were consistently reduced compared with wild-type cells (Figure 3A). A significant decrease in PKC α protein level was also observed in rictor^{-/-} cells (Figure 2B) consistent with previous studies (Guertin *et al*, 2006), suggesting that the decreased Akt and cPKC protein level is an mTORC2-related phenotype.

To further examine whether the absence of TM phosphorylation may lead to degradation, we determined if Akt in SIN1^{-/-} cells could have increased ubiquitination, a mechanism that leads to proteasome turnover. Indeed, there was increased ubiquitination in SIN1^{-/-} cells of exogenous Akt, which was more evident in the presence of the proteasome inhibitor MG132 (Figure 3B). Endogenous Akt ubiquitination also became more pronounced in SIN1^{-/-} cells upon MG132 pretreatment (Figure 3C). Taken together, these results indicate that the lack of TM phosphorylation predisposes Akt towards degradation through ubiquitination and proteasome-mediated turnover.

The maturation of PKC α is defective in the absence of SIN1

We next examined if maturation of cPKC, which was reported to require TM phosphorylation (Edwards *et al*, 1999), is defective in SIN1^{-/-} cells. By pulse-chase analysis, we found that, whereas newly synthesized PKC α from wild-type cells underwent maturation as depicted by the appearance of a slower migrating band with a half-life time of 10 min, PKC α maturation in SIN1^{-/-} cells was severely impaired with a much slower kinetics (Figure 3D). The slower migrating form of PKC α corresponds to phosphorylated TM and HM sites, while the fast-migrating species is unphosphorylated at the carboxyl-terminus (Keranen *et al*, 1995). The TM phosphorylation of PKC α was also proposed to induce a closed conformation, such that in its absence, PKC α was rapidly degraded *in vitro* (Bornancin and Parker, 1996). To further investigate how lack of TM phosphorylation renders Akt and PKC α unstable *in vivo*, we determined the half-life of Akt and PKC α in wild-type and SIN1^{-/-} cells. Surprisingly, we found that upon inhibition of translation using cycloheximide, Akt and PKC α in either SIN1^{-/-} or rictor^{-/-} cells had a half-life comparable with wild-type cells (Supplementary Figure 4). These results led us to speculate that some of the TM site unphosphorylated Akt

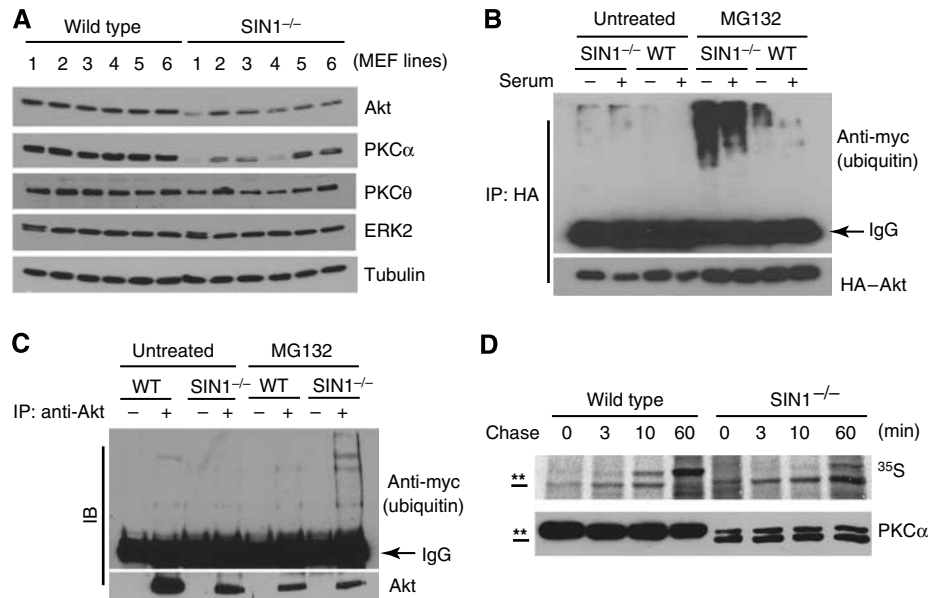


Figure 3 The TM site phosphorylation is required for Akt and PKC α / β II stability and PKC α maturation. (A) Protein levels of Akt and PKC α are diminished in different SIN1^{-/-} MEF lines. Individual WT and SIN1^{-/-} MEFs established from six different embryos (1–6) were analysed by immunoblotting for Akt, PKC α and PKC θ levels. ERK2 and tubulin expression was assayed as a loading control. (B, C) Akt is degraded through the ubiquitination pathway in SIN1^{-/-} cells. WT and SIN1^{-/-} MEFs were transfected with myc-ubiquitin or co-transfected with myc-ubiquitin and HA-Akt-expressing plasmid DNA. After 36 h, transfected cells were either untreated or treated with MG132 (10 μ M) for another 12 h before harvest. Exogenous HA-Akt (B) or endogenous Akt (C) were immunoprecipitated and further analysed for ubiquitination by immunoblotting with an anti-myc antibody. Immunoprecipitated Akt or HA-Akt levels were also determined. (D) Defective PKC α maturation in SIN1^{-/-} MEFs. WT and SIN1^{-/-} MEFs were metabolically labelled with [³⁵S] methionine/cysteine for 7 min and chased at different time points (min) as indicated before harvesting. Endogenous PKC α was immunoprecipitated from detergent-solubilized lysates with an anti-PKC α antibody, separated by SDS-PAGE, and visualized by autoradiography (top panel). Newly synthesized, unphosphorylated PKC α is indicated by the *dash* (-); phosphorylated and mature PKC α is indicated by a double asterisk (**). The bottom panel shows an immunoblot analysis of total PKC α .

and PKC α may elude the proteasome pathway and are protected in cells under normal growing conditions.

Akt and PKC α in SIN1^{-/-} cells are protected by Hsp90

As heat shock proteins such as Hsp90 can protect newly synthesized and/or improperly folded kinases (Caplan *et al*, 2007), we examined if disrupting Hsp90 function would increase susceptibility of Akt and PKC α to proteasome degradation in SIN1^{-/-} cells. We used three different strategies to disrupt Hsp90 function. First, we used a well-characterized pharmacological inhibitor of Hsp90, 17-(Allylamino)-17-demethoxygeldanamycin (17-AAG). Although the 17-AAG treatment of wild-type cells had no significant effect on Akt and PKC α levels for up to 8 h, there was a dramatic decrease in Akt and PKC α levels in SIN1^{-/-} and rictor^{-/-} cells as early as 2 h following treatment (Figure 4A and 4B; data not shown). Akt and PKC α protein levels were rescued upon expression of HA-SIN1 in SIN1^{-/-} MEFs, indicating that the 17-AAG-induced reduction is specific to the absence of SIN1 (Figure 4A). Second, we used a cell-permeable peptide inhibitor of Hsp90 that could disrupt Hsp90 interaction with Akt (Miao *et al*, 2008). Third, we used a dominant-negative Hsp90 mutant to inhibit the Hsp90 pathway (Miao *et al*, 2008). The inhibitory peptide or expression of the dominant-negative Hsp90 both significantly decreased Akt levels in the SIN1^{-/-} MEFs (Supplementary Figure 5). As Akt degradation is mediated through the ubiquitin pathway (Figure 3B and C), we would anticipate that Hsp90 inhibition should further augment Akt ubiquitination in SIN1^{-/-} cells. Indeed, Akt ubiquitination was significantly increased in SIN1^{-/-} cells

upon 17-AAG treatment (Figure 4C). Taken together, these results demonstrate that Akt and PKC α from SIN1^{-/-} cells, which lack TM phosphorylation, are highly dependent on Hsp90 for stability and to prevent proteasome-mediated degradation.

To further address how the absence of TM phosphorylation may affect Akt and PKC α stability, we induced protein destabilization and misfolding by heat shock treatment. We reasoned that under sustained heat stress, the Hsp90 pathway becomes overwhelmed and thus may lose its ability to rescue Akt/cPKC in SIN1^{-/-} cells. Heat shock attenuated Akt and PKC α levels significantly in SIN1^{-/-} cells but only marginally in wild-type cells (Figure 4D). We also observed that both the endogenous Akt and exogenous HA-Akt bound more Hsp90 upon heat shock in SIN1^{-/-} cells (Supplementary Figure 6). Despite increased Hsp90 binding, endogenous Akt underwent degradation when heat shock was sustained (Supplementary Figure 6). These results further suggest that the TM site phosphorylation-defective Akt that escaped proteasome degradation was protected by Hsp90 in SIN1^{-/-} cells under normal growing conditions.

We then asked what would happen to protein levels of Akt and PKC α in wild-type cells if we inhibit both mTOR and Hsp90. Combined 17-AAG and prolonged rapamycin treatment led to a considerable decrease in Akt protein level at longer time points (Figure 4E). Remarkably, combined treatment with 17-AAG, rapamycin and knockdown of mTOR expression diminished Akt expression to similar levels seen in 17-AAG-treated SIN1^{-/-} cells (Figure 4E). The expression of PKC α was also significantly decreased by combined Hsp90

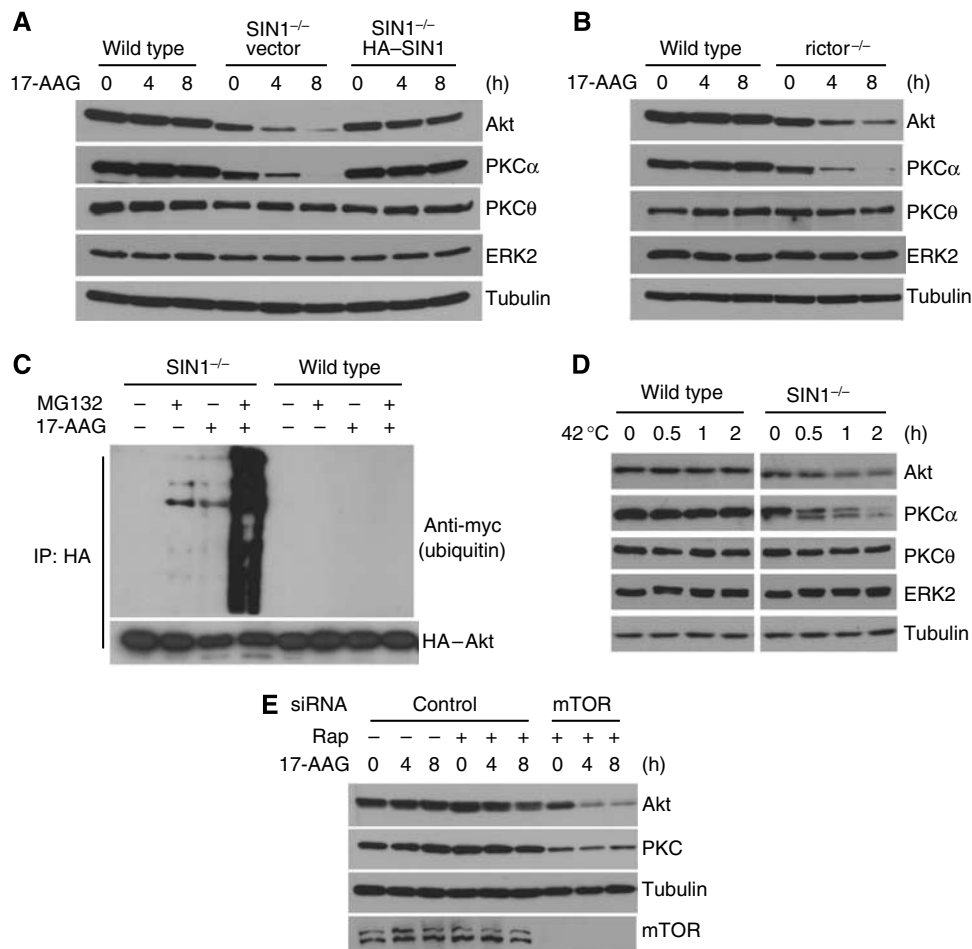


Figure 4 The remaining Akt and PKC α in SIN1^{-/-} cells are protected from degradation by the molecular chaperone Hsp90. (A, B) Inhibition of Hsp90 with 17-AAG destabilizes Akt and PKC α dramatically in SIN1^{-/-} or rictor^{-/-} cells but not in wild-type cells. Normal-growing wild-type, SIN1^{-/-} or HA-SIN1-reconstituted SIN1^{-/-} MEFs (A) or rictor^{-/-} MEFs (B) were either untreated (0) or treated with 17-AAG (1 μ M) for 4 or 8 h before harvest, followed by immunoblotting analysis for Akt, PKC α and PKC θ levels. ERK2 and tubulin expression was assessed as a loading control. (C) Inhibition of Hsp90 robustly augmented Akt ubiquitination in SIN1^{-/-} cells. Wild-type and SIN1^{-/-} MEFs were co-transfected and treated as in 3B with the addition of 17-AAG (1 μ M), alone or with MG132 for another 6 h before harvesting. HA-Akt was immunoprecipitated and bound ubiquitin was detected as in 3B. (D) Heat shock destabilizes Akt and PKC α more pronouncedly in SIN1^{-/-} cells than in wild-type cells. Wild-type and SIN1^{-/-} MEFs, grown at 37 °C, were shifted to a 42 °C incubator for the indicated time periods as indicated before harvesting. Protein levels were determined by immunoblotting. All lanes from each antibody-blotted panel come from the same blot. (E) Combined inhibition of mTOR and Hsp90 diminishes Akt and PKC α protein levels. Wild-type cells were transfected with vector (control) or mTOR siRNA. Two days after transfection, some of the samples were additionally treated with rapamycin (100 nM) for 36 h, followed by treatment with 17-AAG (1 μ M) for 4 or 8 h. Total protein levels were determined by immunoblotting.

and mTOR inhibition/knockdown. These results suggest that when mTORC2-mediated TM phosphorylation is disrupted, Hsp90 becomes essential for Akt and cPKC stability.

The TM phosphorylation is specifically required for Akt and PKC β II protein stability

To further investigate if it is the specific lack of TM phosphorylation in Akt that caused instability of Akt, we determined how mutation of Thr450 to Ala could affect Akt stability in wild-type cells. Expression of Thr450Ala Akt in wild-type MEFs phenocopied the wild-type Akt in SIN1^{-/-} cells by displaying an augmented susceptibility to the 17-AAG-induced degradation (Figure 5A). In sharp contrast, wild-type Akt or Ser473Ala mutant Akt remained stable under the same conditions. Consistently, the TM site mutants were highly ubiquitinated in wild-type cells resembling that of Akt in the SIN1^{-/-} cells (Figure 5B). Furthermore, the 17-AAG-induced and ubiquitin-mediated Akt degradation

could be inhibited by the proteasome inhibitor MG132 (Supplementary Figure 7). As compensating phosphorylation by adjacent Ser/Thr sites was reported to occur in the absence of TM site phosphorylation in PKC β II (Edwards *et al*, 1999), we also examined the effect of combined mutation of Thr450 with the adjacent Thr443 to alanine in Akt. Although total HA-Akt levels were comparable in the single versus double mutant (Figure 5A), we observed more pronounced ubiquitination of the double mutant (Figure 5B). Similar to Akt from SIN1^{-/-} cells (Figure 5C), the TM site mutants also bound more Hsp90 than wild-type Akt (Figure 5D). Thus, the lack of TM phosphorylation specifically renders Akt unstable and dependent on Hsp90 for protection.

We also examined if mutation of the TM site in PKC β II can confer instability upon inhibition of Hsp90. As adjacent Ser/Thr sites can compensate for the absence of phosphorylation at the TM site, we used a PKC β II TM site mutant that contains

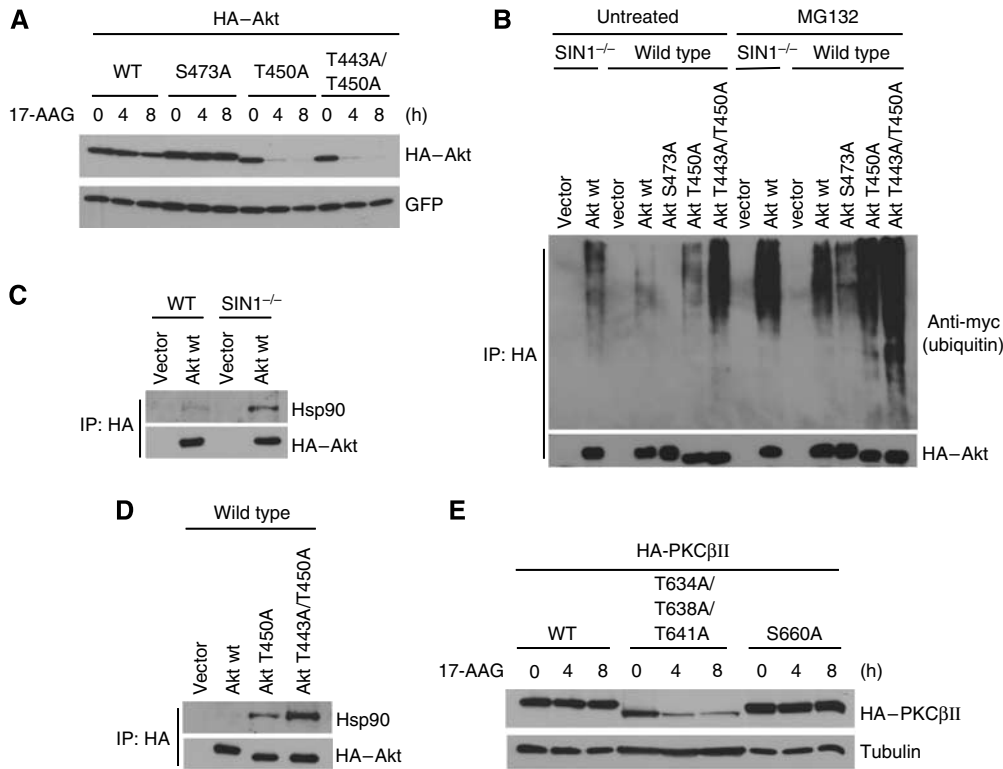


Figure 5 The TM, but not HM, phosphorylation is essential for protein stability of Akt and PKC α . **(A)** Mutation of the TM, but not the HM, phosphorylation site destabilizes Akt. Wild-type MEFs were stably infected with retroviral expression vectors for HA-tagged wild-type Akt (WT) or mutant Akt at the HM site (S473A), or TM sites (T450A and T443A/T450A). Cells were either untreated (0) or treated with 1 μ M 17-AAG for 4 or 8 h before harvest, and the protein levels of HA-Akt were determined by immunoblotting. Infection efficiency was normalized by analysing expression of GFP, which is co-expressed with the genes of interest in an IRES expression cassette (pMIGW). **(B)** Mutation of the TM, but not HM, phosphorylation site augments Akt ubiquitination. Wild-type or *SIN1*^{-/-} MEFs that were stably infected with either wild-type HA-Akt (Akt wt) or HA-Akt HM site mutant (Akt S473A) or TM site mutants (Akt T450A, Akt T443A/T450A) were transfected with myc-ubiquitin. Cells were treated and processed as in 3B. **(C, D)** The absence of TM phosphorylation increases Akt binding to Hsp90. Cellular extracts from wild-type (WT) or *SIN1*^{-/-} MEFs, both stably infected with HA-Akt (C) and from wild-type MEFs stably infected with HA-Akt wt or TM mutants (D), were subjected to immunoprecipitation using HA antibody. Co-immunoprecipitated Hsp90 were detected by immunoblotting. **(E)** TM, but not HM, site mutation destabilizes conventional PKC β II. Wild-type or mutant HA-PKC (Thr634/638/641 triple Ala or Ser660Ala) were transfected into HeLa cells. After 36 h, cells were left untreated or treated with 17-AAG for the indicated time (h). Total protein expression was detected by immunoblotting.

adjacent Ala mutations at the compensating sites (Edwards *et al*, 1999). Upon 17-AAG treatment, protein levels of the TM mutant, but not the HM mutant, Ser660Ala, were decreased (Figure 5E). Taken together, these results indicate that phosphorylation at the TM is specifically required for protein stability and that Hsp90 can stabilize the unphosphorylated Akt and cPKC.

Complementary interactions between a conserved basic patch at the catalytic domain and the phospho-threonine in the TM have an important function in the stabilization of Akt and cPKC

Why did the lack of TM site phosphorylation cause an instability specifically to Akt and cPKC in *SIN1*^{-/-} and rictor^{-/-} cells? Is there a structural basis for this TM site phosphorylation-mediated Akt and cPKC stabilization? The TM region is localized in a mobile loop and undetected in the previous crystal structure of PKB β (Akt2) (Yang *et al*, 2002). However, electrostatic surface potential calculation revealed that an area at the catalytic domain close to the missing TM loop (marked by a square in Figure 6A and B) contains intensive positive charges. This basic patch is mainly contributed by K165, R184, R224 (human Akt2 numbering). Interestingly, these residues are conserved in several AGC

kinases including the conventional and atypical PKCs, but only partially conserved in PKA and novel PKCs (Figure 6C and data not shown).

Structural modelling of the TM loop with phosphorylated T451 (human Akt2 numbering, and equivalent to T450 in human Akt1 used throughout this study) showed that the negatively charged phosphate group forms strong electrostatic interactions with the conserved basic patch in the N-lobe of the catalytic domain (Figure 6A and B). It is likely that either during or immediately following Akt translation, the newly synthesized Akt is phosphorylated at the TM site, which may tether the entire TM loop to the basic patch through a 'knot'-like interaction as described above (Figure 6B), and consequently stabilizes the C-terminal tail. In the absence of TM phosphorylation, the C-terminal tail might be more flexible, hence more accessible by the protein degradation machinery, as we have shown in the above analysis. We would therefore expect that abrogation of the interactions between the conserved basic patch and phosphorylated TM site should result in destabilization of Akt protein.

To test our model, we mutated these basic residues to methionine and determined the effect on Akt protein stability. We found that despite the presence of TM site phosphoryla-

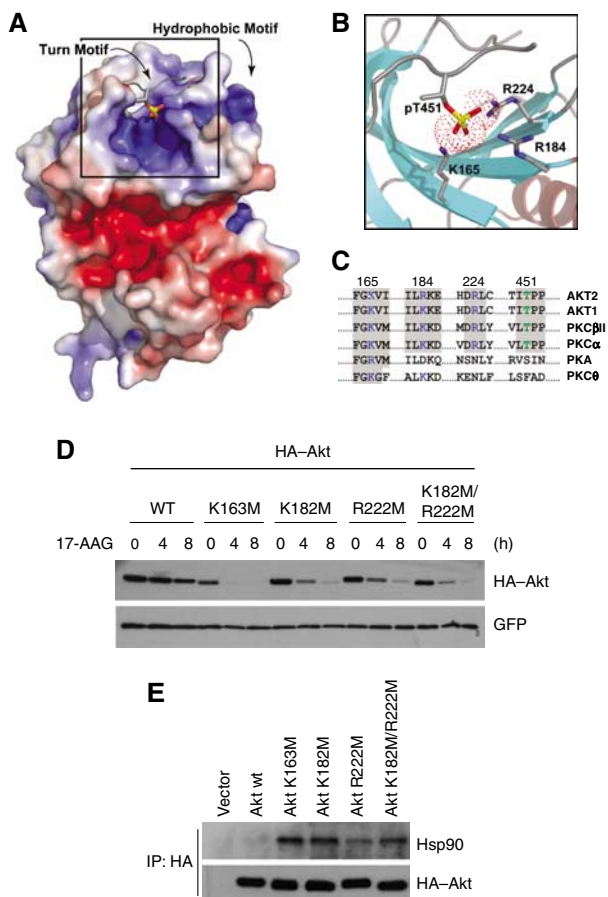


Figure 6 The TM site phosphorylation mediates proper carboxyl-terminal folding and stabilization of Akt structure, by interacting with three conserved basic residues at the catalytic domain. (A) Structural modelling of the TM phosphorylation. Molecular surface of Akt2 (PDB ID: 1O6K), coloured blue (basic) and red (acidic). The TM peptide that is missing in 1O6K (with pT451) was modelled to the structure and energy minimized by simulated annealing with CNS programme (Brunger *et al*, 1998). pT451 is shown in sticks. Note the top of the N-lobe is highly positively charged, which is ideal for accommodating a phosphorylated threonine. (B) Electrostatic interactions between pT451 from the TM and K165, R184, R224 in the N-lobe. (C) Sequence alignment of residues surrounding K165, R184, R224 and T451 for selected human AGC kinases. Note that T451 is not conserved in PKA and PKC θ , and the R/Ks, particularly R224, are also not conserved in these two enzymes. (D, E) Mutation of each of the conserved basic residues in Akt1 that are predicted to interact with the TM site phosphate led to (D) Akt destabilization upon inhibition of Hsp90 and (E) increased binding of Hsp90. Wild-type MEFs were stably infected with HA-Akt (wild-type or methionine mutants) using retroviral vectors. (D) Before harvest, cells were treated with 17-AAG (1 μ M) for the indicated time. Infection efficiency was normalized by analysing expression of GFP (co-expressed under the control of the same promoter). HA-Akt levels were analysed by immunoblotting with HA antibody. (E) Extracts from each cell line were immunoprecipitated using HA antibody. Co-immunoprecipitated Hsp90 was detected by immunoblotting.

tion (Supplementary Figure 8), the K163M, K182M or R222M mutants (human Akt1 numbering) expressed in wild-type cells displayed a very similar stability defect as the Akt TM mutant (Figure 6D). In the presence of 17AAG, all these Akt mutants, but not wild-type Akt or Akt S473A mutant, exhibited dramatic degradation in wild-type cells comparable with that of wild-type Akt in SIN1^{-/-} cells (Figures 5A and 6D). Additionally, each of these mutants bound more Hsp90

compared with wild-type Akt (Figure 6E). These results strongly suggest that the complementary charge-charge interactions between these conserved basic residues at the catalytic domain and the phospho-threonine in the TM have an important function in the folding and stability of Akt, and most likely conventional PKC as well, which displays both sequence and structure conservations to Akt.

The Akt TM phosphorylation is controlled independently of its HM phosphorylation

Turn motif site phosphorylation occurs in starved cells when neither A-loop nor HM sites are phosphorylated, suggesting that TM phosphorylation is not dependent on phosphorylation of either of these two sites. However, as phosphorylation of both the TM and HM sites is defective in the SIN1^{-/-} cells, one possibility for abolished HM phosphorylation in stimulated cells could be due to the lack of TM phosphorylation. We therefore determined if the Akt HM phosphorylation could occur in wild-type cells expressing TM site mutant Akt. In the Akt TM site mutants, HM phosphorylation was still induced by growth factors, similar to wild-type Akt (Figure 7A). These results demonstrate that the TM and HM phosphorylation of Akt are two independent events.

A potential function of SIN1 is to bring Akt to mTOR or prolong their interaction to allow TM or HM phosphorylation, as SIN1 and Akt can interact (Jacinto *et al*, 2006). Akt activation also requires membrane translocation (Bellacosa *et al*, 1998). We then determined if constitutive membrane targeting of Akt may override the requirement for the mTORC2-mediated HM and TM phosphorylation. We expressed a myristylated form of Akt (Myr-Akt) and examined if it can rescue the TM or HM phosphorylation in SIN1^{-/-} cells. Interestingly, we found that membrane targeting of Akt in SIN1^{-/-} cells is clearly unable to restore TM phosphorylation but could partially rescue HM phosphorylation (Figure 7B). As mTORC2 assembly is disrupted in SIN1^{-/-} cells, we asked whether autophosphorylation may account for the observed HM phosphorylation of Myr-Akt in SIN1^{-/-} cells (Toker and Newton, 2000). Upon expression of a kinase inactive allele of Akt (Myr-Akt-KD) in SIN1^{-/-} cells, very little HM phosphorylation was observed, indicating that in the absence of SIN1, the HM site of Myr-Akt undergoes autophosphorylation (Figure 7B). This residual autophosphorylation is resistant to prolonged rapamycin treatment (Figure 7C). In contrast, the majority of HM phosphorylation of both wild-type Akt and Myr-Akt can be inhibited by prolonged rapamycin treatment in wild-type cells (Figure 7C). As expected, non-myristylated Akt-KD mutant expressed in SIN1^{-/-} cells displayed no phosphorylation at the TM and HM sites (data not shown). These results confirm that HM phosphorylation mainly occurs through mTORC2, although residual autophosphorylation could occur when Akt is artificially targeted to the membrane.

mTORC2 phosphorylates the TM and HM sites of Akt *in vitro*

Finally, we addressed if mTORC2 can phosphorylate the TM site *in vitro*. We used dephosphorylated GST-Akt1 from HEK293 cells (see Materials and Methods) as the substrate for *in vitro* kinase assays. We immunoprecipitated mTORC2 with an anti-SIN1 antibody and used this as the kinase source. In the presence of SIN1 immunoprecipitates, TM

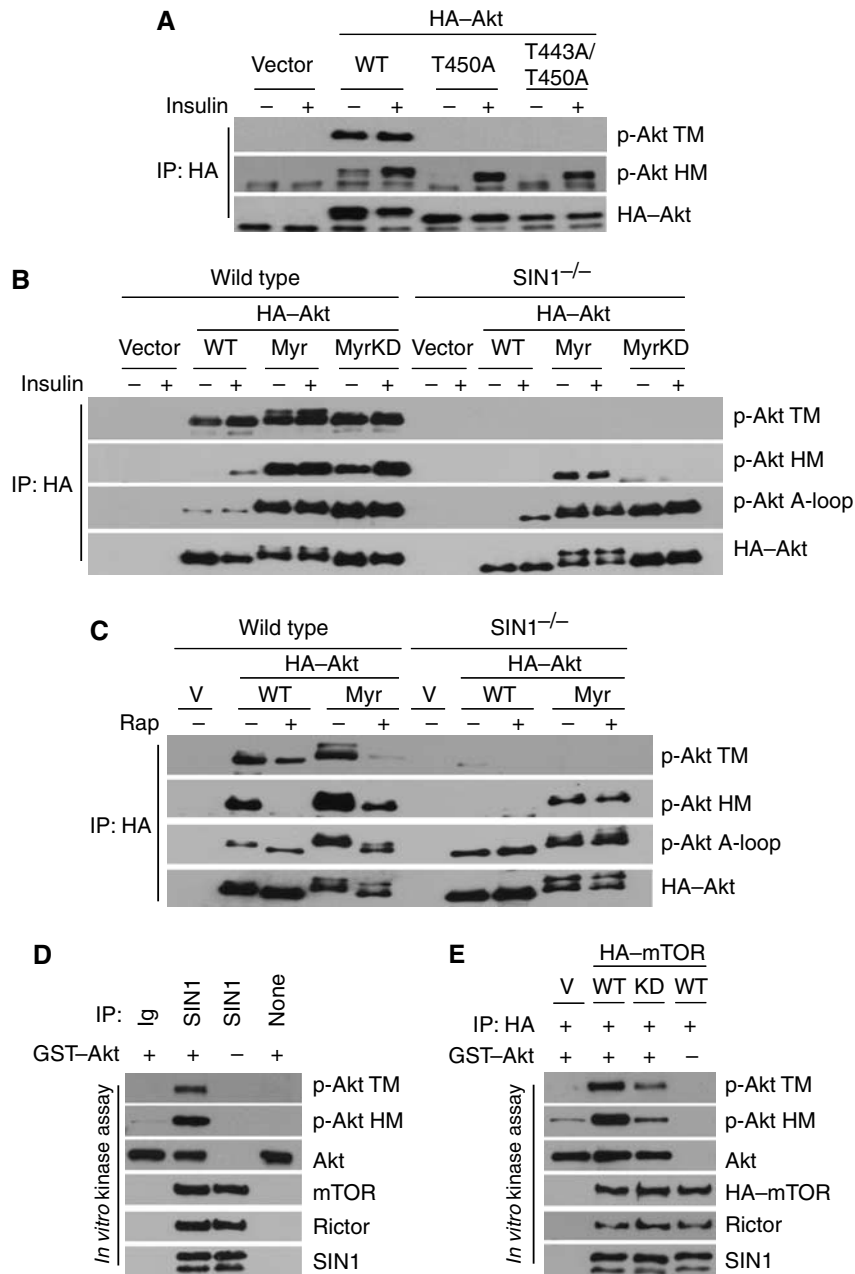


Figure 7 Phosphorylation at the TM and HM sites is independently regulated by mTORC2 and requires mTOR kinase activity. **(A)** The TM site phosphorylation is not a prerequisite for the HM site phosphorylation of Akt. Wild-type MEFs were stably infected with a control vector (Vector) or with retroviral expression vectors for HA-Akt (WT) or mutant (T450A or T443A/T450A) to express comparable and close to physiological levels. Cells were either starved overnight (-), or starved then restimulated with insulin for 30 min before harvest. HA-Akt WT or mutants were immunoprecipitated using an anti-HA antibody, and analysed for phosphorylation and immunoprecipitated levels by immunoblotting. **(B)** Membrane-targeted Akt is not phosphorylated at the TM site but undergoes partial phosphorylation at the HM site. Wild-type or *SIN1*^{-/-} MEFs were stably infected with a control vector (Vector) or vectors expressing HA-Akt (WT), myristylated Akt (Myr) or Myr-Akt-kinase dead (MyrKD) as indicated. Cells were starved, stimulated and processed as in A. **(C)** TM and HM site phosphorylation in wild-type cells is strictly dependent on *SIN1*/mTORC2. Wild-type and stably infected *SIN1*^{-/-} MEF cells as described in panel B were treated with rapamycin (100 nM) for 36 h before processing as described in panel A. **(D)** A *SIN1*-associated kinase phosphorylates Akt at HM and TM sites *in vitro*. HeLa cells were immunoprecipitated with control Ig or *SIN1* antibody. Immunoprecipitates were used to phosphorylate GST-Akt *in vitro*. Phosphorylation of GST-Akt and amounts of immunoprecipitated mTORC2 components were analysed by immunoblotting. **(E)** mTOR kinase activity is required for Akt phosphorylation at HM and TM sites. HeLa cells were transfected with a control vector (V) or with expression vector for HA-tagged mTOR (WT) or a kinase-dead mutant form of HA-mTOR (KD). HA-mTOR (WT and KD) was immunoprecipitated with an anti-HA antibody and used to phosphorylate GST-Akt *in vitro*. Phosphorylation of Akt and amounts of immunoprecipitated mTORC2 components were analysed by immunoblotting.

phosphorylation of GST-Akt1 was observed (Figure 7D, lane 2). No TM phosphorylation was detected in the absence of either *SIN1* immunoprecipitates (Figure 7D, lanes 1 and 4) or

GST-Akt1 (lane 3). Confirming previous studies (Sarbasov *et al*, 2005; Yang *et al*, 2006), the HM site of Akt can be robustly phosphorylated by a *SIN1*-associated kinase

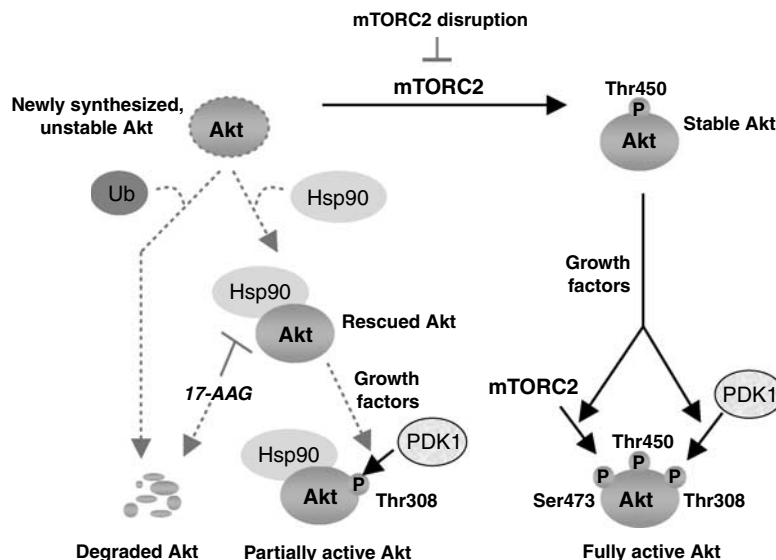


Figure 8 Model for Akt regulation by mTORC2. mTORC2 phosphorylates newly synthesized Akt at the TM (Thr450) site to facilitate carboxyl-terminal folding and to stabilize Akt. In the presence of growth factors, Akt is further phosphorylated at the HM (Ser473) and A-loop (Thr308) sites by mTORC2 and PDK1, respectively, leading to full activation of Akt. When mTORC2 is disrupted genetically or by prolonged rapamycin treatment, Akt is unstable due to lack of TM site phosphorylation. However, in the absence of TM phosphorylation, Akt becomes protected by Hsp90 from ubiquitination-mediated proteasome degradation. Rapid degradation of Akt occurs upon simultaneous inhibition of mTORC2 and Hsp90.

(Figure 7D). The HM site appears to be more efficiently phosphorylated than the TM site, suggesting that the assay conditions used are more favourable towards HM site phosphorylation. These results indicate that a SIN1-associated kinase(s) can phosphorylate these two distinct Akt sites.

As mTOR, but not SIN1 or rictor, possesses kinase activity, we then analysed if mTOR can directly phosphorylate the Akt HM and TM sites. We compared the kinase activity of HA-tagged mTOR with a kinase-dead mutant of HA-mTOR (KD). Whereas both wild-type and KD mTOR pulled down similar amounts of SIN1 and rictor, wild-type mTOR, in contrast to KD mTOR, can more efficiently phosphorylate the HM and TM sites (Figure 7E). Taken together, these results provide convincing evidence that Akt TM and HM phosphorylation are mediated and likely undergo direct phosphorylation *in vivo* by mTORC2.

Discussion

Early studies on PKC and PKA have suggested that phosphorylation at the TM is important for their stabilization (Bornancin and Parker, 1996; Yonemoto *et al*, 1997; Edwards *et al*, 1999; Messerschmidt *et al*, 2005; Grodsky *et al*, 2006). The regulatory mechanism and biological significance of the TM site phosphorylation in Akt and cPKC were previously only speculative. The function of this phosphorylation in Akt, one of the most widely studied AGC kinases due to its oncogenic potential, has been overlooked because of its apparently constitutive nature (Alessi *et al*, 1996; Bellacosa *et al*, 1998). We have now shown that phosphorylation of this site is strictly dependent on the integrity of mTORC2 and is essential for the folding and stability of Akt and cPKC. Without TM phosphorylation, Akt and cPKC are partially protected by Hsp90 for stability. Lack of TM phosphorylation of endogenous Akt and cPKC has not been observed *in vivo* before. Thus, our present study reveals a new and unique regulatory mechanism for a subset

of the AGC family kinases by mTORC2 (see Akt regulation as modelled in Figure 8).

Using molecular remodelling and *in vivo* studies, we demonstrate that the interaction of the phosphate on the TM site with the three highly conserved basic residues in the catalytic region functions to fold and stabilize Akt (Figure 6). A similar structural model was recently proposed, but TM phosphorylation was suggested to synergize with HM phosphorylation to enhance catalytic activity of a number of AGC kinases (Hauge *et al*, 2007). This is in contrast to our findings that demonstrate an important function of TM phosphorylation primarily for protein stability. Our results concur with previous studies wherein TM site mutation of Akt and PKC did not affect their *in vitro* catalytic activity (Bornancin and Parker, 1996; Bellacosa *et al*, 1998; Toker and Newton, 2000) (data not shown). Further studies should reveal how regulation of Akt stability can affect its activity towards its numerous cellular targets.

The TM and HM phosphorylation of Akt clearly require mTORC2, but necessitate different temporal and spatial conditions. Whereas TM phosphorylation remains stable when growth stimuli are withdrawn, HM phosphorylation requires growth factors. Growth factor stimulation is critical for PI3K activation that leads to Akt recruitment to the membrane where it undergoes HM phosphorylation. Interestingly, artificial membrane targeting of Akt in mTORC2-disrupted cells could partially rescue HM, but not TM, phosphorylation, highlighting the importance of Akt membrane localization for HM phosphorylation. This partial rescue of HM phosphorylation in SIN1^{-/-} cells was not through mTOR but was actually due to autophosphorylation (Figure 7B and C). However, the observed mTORC2-independent autophosphorylation in this case was minimal (Figure 7B), and it is unlikely that under physiological conditions it could bypass the requirement for mTORC2. In wild-type cells, the majority of HM and TM phosphorylation of Myr-Akt can be inhibited by prolonged rapamycin treatment, further indicating that HM

and TM site phosphorylation are primarily mediated by mTORC2. This possibility is further supported by the evidence that mTOR (as part of mTORC2) could exert an effect as a direct kinase for the TM and HM sites (Figure 7D–E).

The consensus sequence surrounding the TM (Thr-Pro-Pro-Asp) of Akt and cPKC is distinct from the HM (Phe-Ser-Tyr). The TM consensus sequence is also present in dAkt and partially conserved in the budding yeast PKC1. In addition to a function of budding yeast TORC2 in regulating PKC1 activity through the Rho GTPases, we show here that it may also mediate TM site phosphorylation (Supplementary Figure 3). TORC2 also controls TM phosphorylation of the *S. cerevisiae* AGC kinase YPK2 *in vitro* (Kamada *et al*, 2005) and the *S. pombe* Gad8p (Matsuo *et al*, 2003). Our study demonstrates that mTOR kinase activity is required for TM phosphorylation. Thus, mTOR has likely preserved its primitive, non-growth factor-inducible function in mediating TM phosphorylation from yeast to mammals. Although we could not rule out the existence of another Pro-directed kinase for TM site phosphorylation, pharmacological inhibition of other known Pro-directed kinases such as MAPKs and CDKs did not significantly inhibit TM phosphorylation (data not shown). Supporting mTOR as a kinase for a Thr-Pro motif, the phosphorylation sites in the bona fide mTOR substrate, 4E-BP, are also followed by a Pro (Gingras *et al*, 2001).

How can mTORC2 similarly mediate phosphorylation of the Akt TM and HM sites that do not respond to the same cellular stimulus? One model would be that the proper localization and/or conformation of Akt, and likely PKC as well, determines when it will be regulated or phosphorylated by mTORC2. A similar model was proposed for the PDK1 phosphorylation of the activation loop of several AGC kinases that are regulated by various stimuli (Biondi, 2004). If mTOR were the kinase for both sites, this would imply that mTOR intrinsic activity does not have to be regulated by these various signals. This also implies that mTORC2 may be distinctly regulated in different cellular compartments. Identification of specific components that may regulate mTORC2 in these compartments would provide a mechanism for this divergent regulation. The immediate appearance (visible at 3 min) of mature, phosphorylated PKC in our pulse-chase experiment hints that the TM site phosphorylation may occur during or immediately following translation (Figure 3D). Therefore, although mTORC2-mediated HM phosphorylation occurs post-translationally as a result of Akt localization to the membrane, it is possible that mTORC2-mediated TM site phosphorylation of Akt and cPKC may occur at the translational machinery.

Modulation of folding and stability of AGC kinases by mTOR and molecular chaperones is perhaps a fundamental mechanism for AGC kinase function. Hsp90 and Hsp70 can prolong the kinase activity of Akt and PKC β II, respectively (Sato *et al*, 2000; Gao and Newton, 2002). Whether TM site phosphorylation (Ser371) of the mTORC1-regulated S6K controls folding and stability of this kinase would need to be evaluated, but this site is also mitogen-induced and can be phosphorylated by mTOR *in vitro* (Moser *et al*, 1997; Saitoh *et al*, 2002). The co-regulation of protein synthesis and folding is highly conserved among prokaryotes and eukaryotes and involves association of chaperones with the protein synthesis machinery (Hartl and Hayer-Hartl, 2002).

The TOR complexes may have evolved to co-regulate the protein synthesis machinery and co/post-translational folding of eukaryotic AGC kinases. As TM site phosphorylation may stabilize the structure of the majority of AGC kinases (Hauge *et al*, 2007), future studies should uncover the exact mechanism for the target specificity by mTORC2.

Finally, given that Akt, PKC, Hsp90 and mTOR are tapped as important drug targets, our findings provide rationale for how the control of protein stability of Akt and PKC by the mTOR pathway and molecular chaperones could be exploited to develop more specific and efficacious drugs against cancer and other growth-related diseases. Combination therapy that target mTOR and the chaperone pathways should lead to more efficient growth inhibition of cancer cells.

Materials and methods

Plasmid constructs, antibodies, reagents

HA-tagged Akt constructs were obtained from D Alessi (University of Dundee) (wt, S473A, Myr-Akt) and A Tokar (Harvard University) (T450A, T443A/T450A). Wild-type or mutant HA-Akt ORFs were each subcloned into the retroviral vector pMIGW at the BglII and ERI sites. GST-Akt was obtained from D Sarbassov (UTMDACC). HA-Akt K to M mutants and Myr-Akt-KD (K179M) were constructed by using a PCR-directed mutagenesis method. HA-PKC β IIAAA mutant was obtained by subcloning PKC β IIAAA (Edwards *et al*, 1999) into pCI-HA vector. pMIGW-HA-SIN1 and siRNA constructs were prepared as described previously (Jacinto *et al*, 2004, 2006). Myc-ubiquitin was obtained from X Feng (Baylor). HA-mTOR WT and KD were prepared as described previously (Dennis *et al*, 2001). All other reagents were obtained as described in Supplementary data.

Cell culture, stimulation and harvest

Murine embryonic fibroblasts were cultured, stimulated and harvested as described previously (Jacinto *et al*, 2006). Rictor $^{-/-}$ MEFs were obtained from KL Guan (UCSD). MEFs were infected with retrovirus expressing either HA-Akt or HA-SIN1 as described previously (Jacinto *et al*, 2006) and in Supplementary data.

Kinase assay

GST-Akt1 was prepared as described in Supplementary data. Kinase assay reactions were performed following the methods described previously (Sarbassov *et al*, 2005) and in Supplementary data.

Pulse-chase analysis

Murine embryonic fibroblasts were starved for 30 min in DMEM lacking methionine and cysteine, then pulsed for 7 min with 35 S-methionine/cysteine labelling mix. Cells were chased with normal DMEM supplemented with 5 mM methionine and cysteine. PKC α was immunoprecipitated with PKC α monoclonal antibody from BD Transduction (San Jose, CA, USA), then fractionated by SDS-PAGE.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

Acknowledgements

We thank Chang-Chih Wu, Drs Dario Alessi, Alex Tokar, Xin-Hua Feng, Guy Werlen and Hans-Peter Schmitz for sharing plasmids and reagents; Dos Sarbassov for advise on mTORC2 kinase assays; Kun-Liang Guan and Qian Yang for rictor $^{-/-}$ cells and *Drosophila* siRNA reagents; Terri Goss Kinzy, Anja Lorberg and Arthur Horwich for helpful discussions and critical reading of the manuscript. This work was supported in part by the American Heart Association, New Jersey Commission on Cancer Research and American Cancer Society RSG0721601TBE (to EJ), and by grants HL070225 (NIH) (to BS) and Nuclear Receptor Signaling Atlas (DK62434) (to JQ).

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