mTOR Complex 2 Targets Akt for Proteasomal Degradation via Phosphorylation at the Hydrophobic Motif*

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The protein kinase Akt (also known as protein kinase B) is a critical signaling hub downstream of various cellular stimuli such as growth factors that control cell survival, growth, and proliferation. The activity of Akt is tightly regulated, and the aberrant activation of Akt is associated with diverse human diseases including cancer. Although it is well documented that the mammalian target of rapamycin complex 2 (mTORC2)-dependent phosphorylation of the Akt hydrophobic motif (Ser-473 in Akt1) is essential for full Akt activation, it remains unclear whether this phosphorylation has additional roles in regulating Akt activity. In this study, we found that abolishing Akt Ser-473 phosphorylation stabilizes Akt following agonist stimulation. The Akt Ser-473 phosphorylation promotes a Lys-48-linked polyubiquitination of Akt, resulting in its rapid proteasomal degradation. Moreover, blockade of this proteasomal degradation pathway prolongs agonist-induced Akt activation. These data reveal that mTORC2 plays a central role in regulating the Akt protein life cycle by first stabilizing Akt protein folding through the turn motif phosphorylation and then by promoting Akt protein degradation through the hydrophobic motif phosphorylation. Taken together, this study reveals that the Akt Ser-473 phosphorylation-dependent ubiquitination and degradation is an important negative feedback regulation that specifically terminates Akt activation.

The kinase Akt, also called protein kinase B, is a central signaling molecule that links multiple signaling pathways to control cell growth, proliferation, metabolism, and survival (1, 2). Aberrant loss or gain of Akt activity has been linked to diverse human diseases, including cancers and metabolic disorders (1). For instance, various types of human cancers have been found harboring hyperactivated Akt (3). Conversely, activation of Akt is reduced in human patients suffering from type 2 diabetes (4). Thus, understanding the regulatory mechanisms of Akt activity becomes crucial and indispensable for elucidating the complex regulation of cellular homeostasis as well as for developing effective Akt-targeting therapeutic strategies.

Akt is a member of the AGC (cAMP-dependent, cGMP-dependent, and protein kinase C) kinase family (5, 6). The mammalian genome contains three Akt genes that encode Akt1, Akt2, and Akt3, respectively. Akt1 and Akt2 are expressed in multiple tissues, whereas Akt3 is predominantly expressed in the brain (7–11). Akt (hereafter Akt1 unless specified) contains three conserved phosphorylation sites, which are Thr-308 in the activation loop, Thr-450 in the turn motif (TM),7 and Ser-473 in the hydrophobic motif (HM). Upon growth factor stimulation, the PI3K produces phosphatidylinositol 3,4,5-trisphosphate that recruits Akt and PDK1 to plasma membrane through their pleckstrin homology domain. At the plasma membrane, PDK1 phosphorylates Akt at Thr-308 to activate Akt (12). In addition to the Thr-308 phosphorylation, Akt is phosphorylated at Ser-473 in the HM, which further enhances Akt activity (1, 13). Recently, the mTOR complex 2 (mTORC2) comprising mTOR, Rictor, mLST8, and Sin1 has been identified as the kinase responsible for the Akt Ser-473 phosphorylation (14–16). It is believed that the mTORC2 kinase activity toward Akt Ser-473 phosphorylation is also dependent on PI3K activation, although the underlying molecular mechanism remains elusive (14, 17, 18). Studies from our group and another group showed more recently that Akt Thr-450 phosphorylation in the TM is also mediated by mTORC2 and that TM phosphorylation is essential for stabilizing the newly synthesized Akt by facilitating its proper folding (17, 19, 20).

Under physiological conditions, Akt activity is tightly regulated to maintain its functional homeostasis. Therefore, multiple mechanisms are evolved to deactivate Akt and negatively regulate the Akt-mediated cellular activities. One well-documented mechanism to suppress Akt signaling is the elimination of the PI3K product phosphatidylinositol 3,4,5-trisphosphate by phophatase and tensin homolog (PTEN) and inositol polyphosphate-5-phosphatase D (Inpp5d). PTEN and Inpp5d

7 The abbreviations used are: TM, turn motif; HM, hydrophobic motif; MEF, mouse embryonic fibroblast; IGF, insulin growth factor; CHX, cycloheximide; IP, immunoprecipitation; CHAPS, 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonic acid; mTORC, mammalian target of rapamycin complex.

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are lipid phosphatases that are able to hydrolyze phosphatidylinositol 3,4,5-trisphosphate into phosphatidylinositol 4,5-bisphosphate and phosphatidylinositol 3,4-bisphosphate, respectively, thereby preventing Akt phosphorylation at the activation loop and HM sites (1, 21–23). In addition to the elimination of the upstream activator, Akt may be directly inactivated by the phosphatases protein serine/threonine phosphatase 2A and PH domain and leucine rich repeat protein phosphatase, which dephosphorylate Thr-308 (24) and Ser-473 (25), respectively. Moreover, Akt activity can be attenuated via binding to pseudokinase proteins that serve as the endogenous Akt inhibitors, such as tribbles-related protein 3 (Trb3) and c-Jun NH2-terminal kinase (JNK)-interacting protein 1 (JIP1) (26, 27).

Ubiquitination-mediated degradation is a well established negative regulatory mechanism to switch off diverse cellular activities. Previous studies showed that the lack of Akt Thr-450 phosphorylation in Sin1-deficient mouse embryonic fibroblast (MEF) cells caused Akt ubiquitination and degradation (17). Interestingly, it has been well documented that phosphorylation of numerous cellular targets, including serine/threonine kinases, initiates the ubiquitination-dependent degradation. Whether Akt utilizes phosphorylation-dependent ubiquitination and degradation as a potential mechanism to terminate its activity has not been fully studied.

In this study, we report that the Sin1/mTORC2-mediated Akt HM site phosphorylation targets Akt for Lys-48-linked polyubiquitination and rapid proteasomal degradation. An Akt S473A mutant, which lacks HM site phosphorylation, is much more stable than the wild-type Akt. Consistently, Akt is resistant to growth factor-induced degradation in Sin1-deficient MEF cells where the Akt phosphorylation at Ser-473 is abrogated. More importantly, insulin growth factor-1 (IGF-1) treatment, which rapidly induces Ser-473 phosphorylation, promotes the Lys-48 ubiquitination and degradation of the activated Akt. Blocking the proteasomal degradation pathway led to a more sustained IGF-1-induced Akt activation. Taken together, our study unveils a previously unknown function of Akt HM site phosphorylation, which promotes Akt ubiquitination and degradation, thus preventing Akt hyperactivation.

**EXPERIMENTAL PROCEDURES**

**Antibodies and Reagents**—Anti-phospho-Akt Ser-473, anti-phospho-Akt Thr-450, and anti-Akt antibodies were purchased from Cell Signaling. Anti-GFP antibody was from Covance. Anti-tubulin, anti-FLAG, anti-HA, and anti-Myc antibodies were from Cell Signaling. Anti-GAPDH antibody has been described previously (28). LY294002, MG132, and cycloheximide (CHX) were from Sigma-Aldrich. Stable cell lines were established by fluorescence-activated cell sorting and analyzed 7 days after infection.

**Reverse Transcript-PCR**—RNA was extracted with the RNeasy kit (Qiagen). One mg of total RNA from each sample was used as a template for cDNA synthesis with a Quantitect reverse transcriptase kit (Qiagen). An equal volume of cDNA product was used in the PCR using the PrimeSTAR® HS DNA polymerase (Takara Bio). PCR amplification was performed using the following primers (Invitrogen): human Akt gene forward, 5′-CAACTTCTCTGTGGCGCAGTGC-3′; and reverse, 5′-TGTTTGTGAAAGGGCCAGCGAC-3′; and mouse GAPDH forward, 5′-GTGTCTACCCCAAAATGT-3′; and reverse, 5′-CTGATCATACCTGGCGATTTC-3′. The PCR conditions were set according to the standard protocol. The PCR products were resolved on an agarose gel and quantitated using a Bio-Rad Chemidoc XRS image station (Bio-Rad).

**Immunoblot and Co-immunoprecipitation**—Immunoblot was performed following standard procedures. The cells were lysed in M2 lysis buffer: 20 mM Tris (pH 7.0), 0.5% Nonidet P-40, 250 mM NaCl, 3 mM EDTA, 3 mM EGTA, 2 mM dithiothreitol, phosphatase inhibitor mixture (Pierce), and the protease inhibitor mixture (Roche Applied Science). An equal amount of protein was resolved on a SDS-PAGE gel, transferred onto PVDF membrane (Bio-Rad), blocked with 5% nonfat milk, then probed with appropriate first and second antibodies, and developed with enhanced chemiluminescence method (Millipore) using Kodak BioMax MR film (Kodak). The co-immunoprecipitation (IP) assay was performed following the established protocol with minor modifications (14, 17). Briefly, the treated cells were lysed for 1 h in the IP lysis buffer: 40 mM HEPES (pH 7.4), 2 mM EDTA, 0.3% CHAPS, phosphatase inhibitor mixture (Pierce), and protease inhibitor mixture. The lysate containing the same amount of HA-Akt or total protein were pre-cleared with 20 μl of protein A/G-agarose beads (10% slurry) (Santa Cruz) for 1 h. The post-cleared lysates were then incubated with 30 μl of protein A/G-agarose beads and 1 μg of antibody overnight at 4 °C. After incubation, the beads were extensively washed with lysis buffer five times, and the immunoprecipitated proteins were boiled for 5 min in sample buffer (Bio-Rad), further characterized by immunoblotting, and normalized to the control samples after quantitation using a
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Image] image processing program (National Institutes of Health).

Protein Synthesis Analysis—Murine embryonic fibroblasts were starved for 30 min in DMEM lacking methionine and cysteine and then pulsed for the indicated time duration shown in the figure legend with [35S]methionine/cysteine labeling mix. HA-Akt was immunoprecipitated with an anti-HA antibody, then separated on a SDS-PAGE, and visualized by autorography.

RESULTS

Akt Ser-473 Phosphorylation Regulates the Stability of Akt Protein—In our previous study, we revealed a critical role of the mTORC2-mediated Akt TM site phosphorylation in regulating the stability of newly synthesized Akt protein. Interestingly, abolishment of Akt Ser-473 phosphorylation, a process that is also mediated by mTORC2 by mutating Akt Ser-473 to an Ala (S473A), did not destabilize Akt (17). In contrast, the S473A mutation appeared to stabilize Akt protein because expression of the Akt S473A mutant protein was markedly increased as compared with either Akt T450A mutant or wild-type Akt (data not shown). This raised an interesting possibility that the HM site phosphorylation may be a negative regulator of Akt protein stability.

To systematically investigate whether Akt Ser-473 phosphorylation regulates Akt protein stability, we transiently expressed wild-type Akt and Akt S473A and Akt T450A mutants in 293T cells using a pMIGW expression vector (17). This expression vector co-expresses a GFP protein driven by an IRES expression cassette that is used as an internal control for normalization of transfection and expression efficiencies. As shown in Fig. 1A, Akt S473A and Akt T450A mutants were not phosphorylated at Ser-473 and Thr-450, respectively. Consistent with previous reports that Thr-450 phosphorylation is required for proper Akt folding and stability (14, 17), T450A mutation resulted in reduced expression of this mutant Akt protein (Fig. 1A). In contrast, S473A mutation led to a marked increase in Akt expression. In fact, Akt expression was not only higher than the Akt T450A mutant but also higher than the wild-type Akt (Fig. 1A). Using the same approach, we further showed that the Akt S473A mutant exhibited a higher expression level than wild-type Akt in wild-type MEF cells and HeLa cells (Fig. 1B and data not shown). Increased expression of the Akt S473A mutant protein is not due to enhanced transcription because the mRNA level of the S473A mutant was comparable with that of the wild-type Akt (Fig. 1C). Collectively, these results suggest that Akt Ser-473 phosphorylation may destabilize Akt. We further questioned whether such a destabilization is due to the consequences of augmented Akt kinase activity. In contrast to a Akt S473A mutant that is unable to be phosphorylated and activated at Ser-473, mutating Ser-473 to Asp (Akt S473D) partially mimics the constitutively activated Akt without being phosphorylated at Ser-473 (29). We tested whether the S473D mutation destabilizes Akt. Interestingly, the S473D mutation also stabilized Akt, showing a similar effect as S473A mutation (Fig. 1D). This finding suggests that it is not the electrostatic effect but the phosphorylated residue on Akt Ser-473 that is the key in regulating Akt stability upon its activation.

To determine whether the above observed Akt stability regulation by Ser-473 phosphorylation is physiologically relevant, we treated the cells expressing wild-type Akt or Akt S473A mutant with IGF-1. As expected, IGF-1 treatment induced Ser-473 phosphorylation of wild-type Akt but not Akt S473A mutant. Importantly, the IGF-1 treatment also led to reduced expression of wild-type Akt but not Akt S473A mutant (Fig. 1E). Taken together, these data suggest that Ser-473 phosphorylation may negatively regulate Akt stability.

The mTORC2-mediated Ser-473 Phosphorylation Controls Akt Stability—To verify that the augmented expression of Akt S473A mutant is mainly due to an increase in stability, we next determined the rate of protein synthesis and the half-life of wild-type Akt and Akt S473A mutant by using a metabolic labeling assay. We found that the protein synthesis rates of wild-type Akt and Akt S473A mutant were comparable (Fig. 2A). However, using a pulse-chase assay in the presence of protein synthesis inhibitor CHX, we found that the wild-type Akt protein degraded much faster than the Akt S473A mutant (Fig. 2B), further suggesting that Akt was stabilized in the absence of HM site Ser-473 phosphorylation.

Akt Ser-473 is phosphorylated by mTORC2 (14). Disruption of mTORC2 by genetic deletion of Sin1 or Rictor, the crucial components of mTORC2, has been shown to abolish the Akt Ser-473 phosphorylation (14, 30). If Akt Ser-473 phosphorylation indeed destabilizes Akt, Akt should be stabilized in Sin1/ mTORC2-deficient cells as compared with wild-type cells. However, mTORC2 also mediates Thr-450 phosphorylation at the TM, which plays an essential role in proper folding and stability of newly synthesized Akt protein (17, 19). To exclude the confounding effects of Akt Thr-450 phosphorylation on Akt protein stability from our analysis, we expressed an Akt T450A mutant in wild-type and Sin1-deficient MEFs and compared its stability in both cell types. As shown in Fig. 2C, the Akt T450A mutant could be still phosphorylated at Ser-473 in wild-type MEFs but not in Sin1-deficient MEFs. As predicted, more Akt T450A mutant protein was accumulated in the Sin1-deficient cells than in wild-type cells (Fig. 2C). Consistently, the Akt T450A protein showed a slower degradation rate in the Sin1-deficient cells than in wild-type cells (Fig. 2D).

To further confirm that the increased Akt T450A stability in Sin1-deficient MEFs was due to the lack of Ser-473 phosphorylation, we next determined the protein stability of wild-type Akt, Akt T450A, and Akt T450A/S473A double mutant in 293T cells. As expected, the expression of Akt bearing the only T450A mutation was lower than wild-type Akt, whereas the Akt T450A/S473A double mutant exhibited an even higher expression compared with wild-type Akt (Fig. 2E). These data suggest that phosphorylation at Akt Ser-473 regulates Akt protein stability in a manner that is independent of Akt Thr-450 phosphorylation.

Ser-473 Phosphorylation Targets Akt for Lys-48-linked Polyubiquitination and Proteasomal Degradation—Phosphorylation-directed protein ubiquitination and degradation is a general negative regulation of protein stability and activity. We thus determined whether Ser-473 phosphorylation might also promote Akt turnover via the ubiquitination-mediated proteasomal degradation process. MG132, a proteasome inhibitor,
was able to stabilize wild-type Akt but not Akt S473A mutant (Fig. 3A), suggesting that wild-type Akt was degraded via the ubiquitination-proteasome pathway, which was blocked by S473A mutation. We next examined ubiquitination of wild-type Akt and Akt S473A mutant directly by co-expressing Akt and a Myc-tagged ubiquitin in 293T cells. As shown in Fig. 3B, wild-type Akt but not Akt S473A mutant was heavily ubiquitinated in this assay.

The polyubiquitin chain can be conjugated through different lysine residues (Lys-6, Lys-11, Lys-27, Lys-29, Lys-33, Lys-48,}

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**FIGURE 1.** Defective Ser-473 phosphorylation increases Akt protein amount. **A**, 293T cells were transfected with plasmids expressing HA-tagged WT Akt and S473A and T450A Akt mutants. Twenty-four hours after transfection, the cell lysates were prepared. The cell lysate containing a comparable amount of total HA-Akt were used for immunoprecipitation with an anti-HA antibody. Equivalent amounts of immunoprecipitated HA-Akt protein were loaded on the gel and analyzed by immunoblotting for Ser-473 and Thr-450 phosphorylation. Equal amounts of whole cell lysate (WCL) were loaded on the gel and subjected to immunoblotting for the transfected Akt expression with an anti-HA antibody. GFP expression was determined by immunoblotting and used as the internal control. The expression levels of Akt and GFP were quantitated by densitometry, and the ratios of HA-Akt/GFP were presented in the bottom panel (the WT HA-Akt/GFP ratio was set as 1). **B**, MEF cells were infected with pMIGW vector-based retroviruses expressing either HA-tagged WT Akt or Akt S473A (S473A) mutant. The cell lysates were analyzed for HA-Akt and GFP expression as described for A. The HA-Akt/GFP ratios were quantified and presented in the right panel as described for A. **C**, total RNA were prepared from the MEF cells stably expressing HA-tagged human WT or Akt S473A (S473A) mutant described above in B and used to determine the mRNA levels of the ectopically expressed human Akt by RT-PCR and normalized to the levels of endogenous GAPDH mRNA. **D**, 293T cells were transfected with plasmids expressing HA-tagged WT Akt and S473A and S473D Akt mutants. Twenty-four hours after transfection, the cell lysates were prepared. Equal amounts of whole cell lysates were subject to immunoblotting to analyze HA-Akt and GFP. The HA-Akt/GFP ratios were quantified as described for A. **E**, 293T cells were transfected with plasmids expressing HA-tagged WT Akt or Akt S473A mutant. Twenty-four hours after transfection, the cells were treated with IGF-1 (20 ng/ml) for 6 h, and the cell lysates were analyzed by immunoprecipitation and immunoblotting as described for A. The HA-Akt/GFP ratio was quantified and presented in the bottom panel as described for A.
and Lys-63), among which the Lys-48-linked polyubiquitin chain has been well established for directing the targeting proteins for degradation (31, 32). Recently, it was reported that Akt activity could be regulated by Lys-63-dependent ubiquitination, and the Akt Ser-473 phosphorylation was implicated in this process (33). Because Lys-63-dependent ubiquitination is not associated with protein degradation, we then determined whether Akt was modified by Lys-48 polyubiquitination. We co-expressed a FLAG-Akt with either a HA-tagged wild-type ubiquitin or a Lys-48 only ubiquitin mutant (ubiquitin can only be added to the Lys-48 site) in 293T cells and found that Akt was indeed modified by Lys-48-linked polyubiquitination (Fig. 3C).

FIGURE 2. Akt hydrophobic motif site phosphorylation controls Akt protein stability. A, MEF cells were pulse-labeled with [35S]methionine/cysteine-containing medium for the indicated time before being lysed. The 35S-labeled HA-Akt was immunoprecipitated from lysates, separated by a SDS-PAGE gel, and visualized by autoradiography. The 35S signal in each lane was normalized to the wild-type Akt with 10 min of pulse labeling, which is arbitrarily set to value 1. B, 293T cells were transfected with plasmids expressing HA-tagged Akt and Akt S473A mutant. Twenty-four hours after transfection, CHX (10 μM) was added to the cultured cells for the indicated time points before being lysed for immunoblotting analyses for HA-Akt and GFP expression. The HA-Akt/GFP ratio was quantified and presented in the lower panel as described for Fig. 1A (the ratio of time 0 is set to 1). C, WT and Sin1−/− MEFs were transfected with plasmid expressing HA-tagged Akt T450A mutant. The transfected cells were lysed 24 h later, and the lysates were subjected to IP and immunoblotting as described in Fig. 1A. D, WT and Sin1−/− MEFs stably expressing HA-tagged Akt T450A were either untreated (time 0) or treated with CHX (10 μM) for the indicated time points before being analyzed for HA-Akt T450A and GFP expression by immunoblotting with anti-HA or anti-GFP antibodies as indicated. The HA-Akt/GFP ratio was quantified and presented in the right panel as described for Fig. 1A (the ratio at time 0 was set to 1). E, 293T cells were transfected with HA-tagged WT Akt and Akt T450A and Akt T450A/S473A mutants. Twenty-four hours later, the cell lysates were prepared and subjected to IP and immunoblotting analyses as described for Fig. 1A, WCL, whole cell lysate.
augment the Lys-48-linked ubiquitination. As expected, IGF-1 treatment induced not only Akt Ser-473 phosphorylation but also extensive Lys-48-dependent ubiquitination, both of which were blocked by the pretreatment of the cells with the PI3K inhibitor LY294002, indicating that these processes were dependent on PI3K (Fig. 3D). Collectively, these data suggest that Akt Ser-473 phosphorylation triggers Lys-48-polysubstitution, leading to subsequent Akt degradation via the proteasomal pathway.

The Akt HM Site Phosphorylation Mediates Endogenous Akt Degradation upon Activation—To further determine whether the regulation of Akt stability by Ser-473 phosphorylation is physiologically significant, we examined the turnover of endogenous Akt proteins in 293T cells upon IGF-1 stimulation in the presence of CHX. As shown in Fig. 4A, IGF-1 treatment induced Akt Ser-473 phosphorylation and a clear reduction of Akt protein at the 3- and 6-h time points after the treatment. Moreover, the Akt protein degradation was completely blocked by the pretreatment of cells with LY294002, suggesting that the degradation is associated with PI3K activation and Akt Ser-473 phosphorylation (Fig. 4A). To further corroborate that the mTORC2-mediated Ser-473 phosphorylation is required for Akt protein degradation, we compared the effect of IGF-1 treatment on Akt stability in wild-type and Sin1-deficient cells. As expected, the IGF-1 treatment induced Akt phosphorylation at Ser-473 in wild-type but not Sin1-deficient MEFs (Fig. 4B). In
Akt activity is regulated by phosphorylation at the multiple sites. Previously, we showed that the newly synthesized Akt is phosphorylated at Thr-450 in the TM by mTORC2, which facilitates its proper folding and enhances its stability (17). In this study, we unraveled a previously unidentified mechanism of negative regulation of Akt that is mediated by the Sin1/mTORC2-dependent phosphorylation of Ser-473 in the HM. Phosphorylation of Akt at Ser-473 induces a Lys-48-linked polyubiquitination, resulting in Akt degradation via the proteasomal pathway. We further show that HM phosphorylation-mediated Akt degradation functions to attenuate endogenous Akt signaling in response to the IGF-1 stimulation, and we show that suppression of the proteasomal degradation pathway.

**DISCUSSION**

Akt activity is regulated by phosphorylation at the multiple sites. Previously, we showed that the newly synthesized Akt is phosphorylated at Thr-450 in the TM by mTORC2, which facilitates its proper folding and enhances its stability (17). In this study, we unraveled a previously unidentified mechanism of negative regulation of Akt that is mediated by the Sin1/mTORC2-dependent phosphorylation of Ser-473 in the HM. Phosphorylation of Akt at Ser-473 induces a Lys-48-linked polyubiquitination, resulting in Akt degradation via the proteasomal pathway. We further show that HM phosphorylation-mediated Akt degradation functions to attenuate endogenous Akt signaling in response to the IGF-1 stimulation, and we show that suppression of the proteasomal degradation pathway.
leads to an overabundance of active Akt in response to growth factor stimulation (Fig. 4C). Although several previous studies suggested that the Akt activity could be negatively controlled by the proteasomal pathway (34–36), the regulatory mechanism of such regulation is unclear. Our current study reveals that Akt HM phosphorylation links the activation of Akt with the subsequent proteasome-dependent degradation of Akt. Therefore, our study for the first time reveals that Sin1/mTORC2-mediated Akt HM phosphorylation not only positively regulates Akt signaling but also selectively targets active Akt for destruction via the proteasome.

Although different mechanisms are proposed to negatively regulate the Akt activity following its activation, the negative regulatory mechanism discovered in our study has multiple implications. First, it is well known that Akt is crucial for numerous cellular functions that may not operate at the same place and/or at the same time. This unique negative regulatory mode would allow cells to specifically terminate one branch of Akt signal without affecting the total pool of Akt protein that is required for various other cellular functions. In fact, Akt belongs to a class of cellular proteins with a long half-life, indicating that a steady cellular pool of Akt protein is essential for cell survival and growth. However, because unchecked Akt activation is harmful and in many cases may lead to cellular transformation, it is crucial to tightly and also specifically control the pool of Akt proteins that are active and capable of signaling.

Being an important Akt activation marker, Ser-473 phosphorylation may be selected as an effective strategy to turn off the unwanted Akt activity. Based on these analyses, we propose a simple model illustrating the role of the mTORC2-dependent HM site phosphorylation of Akt in the negative regulation of Akt activity in Fig. 5.

Full activation of Akt requires phosphorylation at both the A-loop site (Thr-308) and HM (Ser-473) site (1). Interestingly, the Thr-308-phosphorylated Akt has been recently shown to be ubiquitinated and targeted for proteasomal degradation (34). Our study reveals that the Ser-473-phosphorylated Akt is also subjected to proteasomal regulation. It is unclear at the moment whether these two types of negative Akt regulation are controlled by the same cellular mechanism or use similar regulators. However, it seems unlikely that these two processes are co-regulated because we showed previously that the Akt HM site phosphorylation in Sin1/mTORC2-deficient cells was abolished completely, but the Akt Thr-308 phosphorylation was not affected at all (14). Our finding that the Ser-473 phosphorylation-dependent Akt degradation was abolished in Sin1-deficient cells suggests that Akt Thr-308 phosphorylation may utilize different regulators to control Akt stability. Our data also indicate that regulation of Akt activity and stability by Thr-308 phosphorylation is not dependent on Sin1 and mTORC2 activity.

We showed previously that the Sin1/mTORC2-mediated Thr-450 phosphorylation at the TM site protects newly synthesized Akt from degradation (17). In this study, we show that Sin1/mTORC2 is capable of targeting Akt for degradation via Ser-473 phosphorylation. It is intriguing that mTORC2 exhibits its opposite roles in controlling Akt stability. However, this may be explained by the functions of mTORC2 in different places and at different times. To regulate the stability of newly synthesized Akt, mTORC2 phosphorylates Akt at Thr-450 immediately following Akt translation. This phosphorylation, which is independent of growth factor stimulation and requires no PI3K signaling, protects Akt from degradation by facilitating its proper folding (17). Indeed, we recently found that this regulation is coupled with new protein synthesis and occurs at the active translating ribosomes (37). The Thr-450-phosphorylated Akt constitutes the total pool of Akt, which is ready to be activated. In response to growth factor stimulation or elevated PI3K signals, a small fraction of the Thr-450-phosphorylated Akt will be recruited from the cytosolic Akt pool onto the plasma membrane, where it is phosphorylated by mTORC2 at Ser-473 for full activation. As a negative feedback regulator, Ser-473 phosphorylation promotes Akt ubiquitination and degradation as illustrated in our model (Fig. 5). Therefore, the pro-degradation function of mTORC2 is growth factor-dependent and restricted to the activated pool of Akt, whereas pro-stabilization function of mTORC2 is growth factor-independent.

Phospho-HM directed Akt degradation may play an important physiologic role in regulating the activity of the Akt signaling pathway. Our studies utilizing IGF-1 indicate that Akt HM phosphorylation may serve as a feedback loop to limit the over-

**FIGURE 5.** The mTORC2-mediated Akt HM site phosphorylation directs the active Akt for Lys-48 polyubiquitination and proteasomal degradation. Newly synthesized Akt is phosphorylated by mTORC2 at Thr-450 in the TM, which constitutes the total Akt pool. In response to growth factor stimulation (IGF-1), Akt translocates to the plasma membrane where it is activated by phosphorylation at the A-loop (Thr-308) and hydrophobic motif (Ser-473) by PDK1 and mTORC2, respectively. Akt is subsequently inactivated by phosphatases or is targeted for polyubiquitination and degradation via the proteasomal pathway.
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all strength of Akt signaling. Alternatively, phospho-HM-directed Akt degradation may function as a buffer to prevent Akt signaling in response to weak signaling inputs or signaling pathway noise. For example, this mechanism of Akt regulation could play an important role in the immune system where it is advantageous to limit weak or transient immune activating signals to prevent autoimmunity. Because these types of signals typically originate from weak reactions to self or harmless environmental antigens, it would be beneficial to prevent these stimuli from triggering full activation of the mitogenic pathways downstream of Akt. Finally, aberrant Akt activity is associated with diverse human diseases such as cancer and neurodegeneration (38, 39); our finding that activated Akt is eliminated via the ubiquitination-proteasomal pathway may shed new light on our understanding of human diseases and reveal novel strategies for therapy.

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