Evidence for direct activation of mTORC2 kinase activity by phosphatidylinositol 3,4,5-trisphosphate
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Mammalian target of rapamycin complex 2 (mTORC2) plays important roles in signal transduction by regulating an array of downstream effectors including protein kinase AKT. However, its regulation by upstream regulators remains poorly characterized. Although phosphatidylinositol 3,4,5-trisphosphate [PtdIns(3,4,5)P₃] is known to regulate the phosphorylation of AKT Ser473, the hydrophobic motif (HM) site, by mTORC2, it is not clear whether PtdIns(3,4,5)P₃ can directly regulate mTORC2 kinase activity. Here, we used two membrane-docked AKT mutant proteins, one with and the other without the pleckstrin homology (PH) domain, as substrates for mTORC2 to dissect the roles of PtdIns(3,4,5)P₃ in AKT HM phosphorylation in cultured cells and in vitro kinase assays. In HEK293T cells, insulin and constitutively active mutants of small GTPase H-Ras and PI3K could induce HM phosphorylation of both AKT mutants, which was blocked by the PI3K inhibitor LY294002. Importantly, PtdIns(3,4,5)P₃ was able to stimulate the phosphorylation of both AKT mutants by immunoprecipitated mTOR2 complexes in an in vitro kinase assay. In both in vivo and in vitro assays, the AKT mutant containing the PH domain appeared to be a better substrate than the one without the PH domain. Therefore, these results suggest that PtdIns(3,4,5)P₃ can regulate HM phosphorylation by mTORC2 via multiple mechanisms. One of the mechanisms is to directly stimulate the kinase activity of mTORC2.

INTRODUCTION
Mammalian target of rapamycin (mTOR) is an evolutionarily conserved Ser/Thr protein kinase that plays an integral role in coordinating cell growth and division in response to growth factors, nutrients, and other micro-environmental changes of the cell [1, 2]. This kinase is found in mTORC1 and mTORC2, two structurally and functionally distinct complexes, which are also conserved from yeasts to mammals. In addition to shared mTOR and mLST8, distinct components of these two complexes have been reported: Raptor and PRAS40 for mTORC1 [3-7], and Rictor, SIN1 and PRR5/PRR5L for mTORC2 [8-12]. Two mTOR complexes have distinct cellular functions and are regulated differently. The mTORC1 activity is sensitive to inhibition by rapamycin, which regulates diverse cellular processes, including protein synthesis, ribosome biogenesis, transcription and autophagy, some of which are regulated through its direct substrates S6 kinases (S6K) and the eIF-4E binding protein 1 [13-16]. The mTORC2 activity is resistant to rapamycin, at least in short-term treatment [17, 18]. It phosphorylates the hydrophobic motif
(HM) sites of several AGC kinases, including AKT, PKC and SGK1, to activate their kinase activities [19-23]. Studies using cells derived from Rictor, mSin1, or mLST8 knockout mice show that intact mTORC2 is necessary for AKT HM phosphorylation [12, 24-26]. In addition, mTORC2 phosphorylates the Turn Motif (TM) site of AKT and regulates AKT stability, but this phosphorylation is independent of growth factor regulation [19, 21]. Moreover, mTORC2 can also regulate actin cytoskeleton reorganization through yet to be characterized mechanisms [10, 22, 27, 28]. Many extracellular stimuli including growth factors, G protein-coupled receptor ligands and cytokines stimulate AKT HM phosphorylation in a PI3K-dependent manner. The PI3K-mediated regulation of AKT HM phosphorylation was initially attributed to PtdIns(3,4,5)P3-mediated membrane translocation and conformational changes of AKT. However, recent evidence suggests that the mTORC2 kinase can be directly regulated. The mTORC2 protein immunoprecipitated from growth factor-treated cells shows higher kinase activity in an in vitro kinase assay, and growth factors was initially reported to stimulate the phosphorylation of Ser2481 of mTOR presented in mTORC2, but not mTORC1 [29]. However, more recently insulin was shown to stimulate the phosphorylation of Ser2481 of mTOR associated with both mTORC1 and 2 in a wortmannin-dependent manner [30]. Furthermore, mTORC2 phosphorylates SGK1 in response to growth factors even though SGK1 lacks a PH domain and is activated independently of membrane recruitment. Nevertheless, it is not known how mTORC2 kinase activity is regulated.

In this report, we characterized the role of PI3K and its product PtdIns(3,4,5)P3 in regulation of AKT HM phosphorylation using two membrane-docked AKT mutant proteins. We found that PtdIns(3,4,5)P3 regulates AKT HM phosphorylation via at least three mechanisms. We confirmed that membrane translocation and conformation changes may contribute to PtdIns(3,4,5)P3-stimulated AKT HM phosphorylation, but importantly, we, for the first time, demonstrated that PtdIns(3,4,5)P3 could directly stimulate the intrinsic mTORC2 kinase activity.

**EXPERIMENTAL PROCEDURES**

**Materials.** Reagents were obtained from the following sources: protein A/G-PLUS-Sepharose and the antibody to Ras from SantaCruz; the antibodies to mTOR, Phospho-mTOR (Ser2481), and AKT, Phospho-Akt (Ser473) from Cell Signaling Technology; the Lipofectamine/Plus transfection reagent and Insulin from Invitrogen; LY294002 from ALEXIS Biochemicals; 1,2-Dioleoyl-sn-Glycero3 [Phospho-L-Serine], 1,2-dioleoyl-sn-glycero-3-phospho-(1'-myo-inositol-3',4',5'-trisphosphate) [18:1 P(3,4,5)P3 ], and 1,2-dioleoyl-sn-glycero-3-phospho-(1'-myo-inositol-4',5'-bisphosphate) [18:1 P(4,5)P2] from Avanti Polar Lipid.

**Cell Culture and Stimulation.** HEK293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 4.5 g/liter glucose supplemented with 10% fetal bovine serum (FBS). Transient transfection was performed using the Lipofectamine/Plus reagent. Three hours post transfection, cells were changed to serum free DMEM medium.
for overnight. Before insulin (2 μg/ml) or LY294002 (10 μM) treatment, the cells were incubated with phosphate buffered saline for 60 min.

**Preparation of GST-fusion protein.**
GST-AKT(KT) and GST-DPH-AKT(KT) were expressed in *E. coli* BL21 (DE3). After isopropyl-1-thio-β-D-galactopyranoside (100 μM) induction at 22°C for 24 h, proteins were extracted with a buffer containing 20 mM Tris-HCl, pH 7.5, 50 mM NaCl, 0.1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, and 0.5 mg/ml lysozyme. After high speed centrifugation, the supernatant was loaded on glutathione-agarose beads and eluted with the buffer containing 20 mM Tris-HCl, pH 7.5, 50 mM NaCl, 0.1% Triton X-100, 5% glycerol, and 5mM reduced-glutathione. GST-PKCα was acquired from SignalChem.

**Immunoprecipitation and Kinase Assays.**
Immunoprecipitation of mTOR complexes and kinase assays were performed as previously described [23]. HEK293T cell extracts harvested from a 10 cm plate were used for each immunoprecipitation condition. The cells were lysed on ice for 20 min in the lysis buffer (40 mM HEPES pH7.5, 120 mM NaCl, 0.3% CHAPS, 1 mM EDTA, 10 mM pyrophosphate, 10 mM glycerophosphate, 50 mM NaF, and EDTA-free protease inhibitors). After centrifugation, the supernatant was incubated with the RICTOR Antibody at 4°C for 90 min, followed by incubation with protein A/G-PLUS-agarose for another hour. Immunocomplexes were washed four times in the lysis buffer and twice with mTORC2 kinase buffer (25 mM HEPES pH7.5, 100mM potassium acetate, 2 mM MgCl₂). For the kinase assay reaction, immunocomplexes were incubated in a final volume of 30 μl for 30 min at 37°C in the mTORC2 kinase buffer containing 500 ng of GST-fusion substrates and 500 μM ATP. The reaction was terminated by adding 10μl of 4× SDS sample buffer, followed by Western analysis.

**RESULT AND DISCUSSION**

The ultimate goal of this study is to examine the effect of PtdIns(3,4,5)P₃ on mTORC2 kinase activity. Although the HM site of Akt (Ser473) is an excellent substrate for mTORC2 kinase, simple examination of HM phosphorylation of the full-length AKT may not allow us to assess the effect of PtdIns(3,4,5)P₃ on mTORC2 kinase activity. This is because PtdIns(3,4,5)P₃ has a role in AKT recruitment to plasma membranes, where mTORC2 phosphorylates AKT HM. To eliminate this membrane translocation effect on AKT, we attached the myristylation signal to AKT. To avoid possible autophosphorylation of AKT HM, we also mutated Lys179, which is involved in ATP binding, and Thr308, which contributes to kinase activation after its phosphorylation by PDK1. The resultant AKT mutant, named as Myr-AKT(KT) (Fig. 1A), was expressed in HEK293T cells that were stimulated with insulin and/or treated with a PI3K inhibitor LY294002. The phosphorylation of Myr-AKT(KT) HM detected by an phospho-Thr473-AKT antibody was enhanced by insulin and inhibited by LY294002 (Fig. 1B). This result suggests that PtdIns(3,4,5)P₃ has a role more than substrate translocation in regulating HM phosphorylation.

The PH domain of AKT binds to
PtdIns(3,4,5)P$_3$ and is involved in
PtdIns(3,4,5)P$_3$-mediated AKT translocation.
In addition to this translocation role, the PH
domain, upon interaction with PtdIns(3,4,5)P$_3$,
can induce Akt conformational change [31],
which may make the AKT a better substrate
for phosphorylation by mTORC2. To
eliminate any possible effect of the PH
domain, we generated the Myr-$\Delta$PH-AKT(KT)
mutant that lacks the PH domain (Fig. 1A).
When this mutant was tested, the
phospho-Thr473-AKT antibody detected
weaker HM phosphorylation of this mutant
than that of Myr-AKT(KT) (Fig. 1B). These
results suggest that the AKT PH domain may
render AKT a better substrate for HM
phosphorylation. Nonetheless, insulin could
still induce HM phosphorylation on
Myr-$\Delta$PH-AKT(KT), which was inhibited by
LY294002 (Fig. 1B). We also examined the
effects of insulin and LY294002 treatment on
Ser450 phosphorylation of both AKT
substrates. Consistent with previous
findings [19, 21], phospho-Ser450 contents
were not affected by either treatment (Fig. 1B).
Thus, these results together suggest that
insulin can regulate AKT HM phosphorylation
independently of the PH domain, but in a
PI3K-dependent manner.

The Ras protein is one of the signaling
molecules for growth factors including insulin
and has been implicated in the regulation of
mTORC2 [32, 33]. We examined the role of
Ras in HM phosphorylation of
Myr-$\Delta$PH-AKT(KT). Expression of a
constitutive active mutant of H-Ras (G12V)
strongly induced HM phosphorylation of
Myr-$\Delta$PH-AKT(KT) (Fig. 1C), whereas
expression of a dominant negative mutant of
H-Ras (S17N) markedly blocked
insulin-induced HM phosphorylation (Fig. 1C).
Because Ras can activate PI3K [34-36], we
tested the effect of LY294002 on active
Ras-mediated HM phosphorylation.
LY294002 almost completely blocked
Ras-G12V-induced HM phosphorylation (Fig.
1D). To more directly demonstrate that PI3K
can induce HM phosphorylation of
Myr-$\Delta$PH-AKT(KT), a constitutive active PI3K
(PI3K-CAXX) [37] was expressed, and it
increased the HM phosphorylation (Fig. 1D).
These results together support a conclusion
that insulin and its downstream signaling
molecule Ras may stimulate AKT HM
phosphorylation via PI3K.

Given that mTORC2 is an upstream
kinase for phosphorylation of AKT HM, we
tested if LY294002 could affect
mTOR-Ser2481 phosphorylation. This
phosphorylation is stimulated by insulin, and
its phosphorylation in mTORC2 may require
the intact complex [29] even though the
phosphorylation also occurs in mTORC1 [30].
We found that insulin could induce the
phosphorylation in HEK293T cells, which
could be inhibited by LY294002 (Fig. 2A). In
addition, expression of the constitutive active
PI3K or H-Ras in HEK293T cells increased
mTOR-Ser2481 phosphorylation, which could
also be blocked by LY294002 (Fig. 2B).
These results are consistent with the
conclusion that Ras and PI3K may be
upstream regulators of mTOR in response to
insulin.

To more directly assess the roles of
PI3K in insulin-mediated regulation of
mTORC2 kinase activity, we
immunoprecipitated mTORC2 using a RICTOR antibody from serum/nutrient-starved cells stimulated with insulin and treated with/without LY294002 and carried out an in vitro kinase assay using recombinant GSK-AKT(KT) prepared from E. coli as the substrate. The immunocomplexes pulled down from cells treated with insulin caused significantly greater HM phosphorylation of GST-AKT(KT) than those from non-treated cells (Fig. 3A). In addition, treatment of cells with LY294002 prior to insulin stimulation could block insulin-mediated increases in HM phosphorylation by immunoprecipitated mTOR (Fig. 3A). We also tested another known mTORC2 substrate, PKCα.

Immunoprecipitated mTORC2 stimulated phosphorylation of recombinant GST-PKCa at Ser-638, which was blocked by LY294002 treatment (Fig. 3A). Because LY294002 inhibits PI3K activity by competitively inhibiting ATP binding to the catalytic subunit of PI3K, there is a possibility that LY294002 might directly inhibit ATP binding to mTOR. To exclude this possibility, we treated the immunocomplexes pulled down by the mTOR antibody with LY294002 before the substrate and ATP were added to initiate the kinase assay. Direct treatment of immunoprecipitated mTOR by LY294002 did not inhibit its phosphorylation of GST-AKT(KT) at HM (Fig. 3B). Therefore, these results indicate that insulin can stimulate mTORC2 kinase activity via PI3K.

To determine whether the PI3K product, PtdIns(3,4,5)P3, can directly regulate mTOR-mediated HM phosphorylation in vitro, we tested the effects of lipid vesicles containing PtdIns(3,4,5)P3 in the in vitro kinase assays with immunoprecipitated mTORC2. In addition to recombinant GST-AKT(KT) and GST-PKCa, GST-ΔPH-AKT(KT) was also used as a substrate. The immunocomplexes pulled down by the RICTOR antibody from serum/nutrient-starved HEK293T cells were incubated with or without phosphatidyethanolamine (PE) vesicles containing 0, 5 and 25 ng/ml of PtdIns(3,4,5)P3 before the kinase assay was initiated by adding the substrates and ATP. HM phosphorylation of GST-ΔPH-AKT(KT), GST-AKT(KT) (Fig. 4A) and GST-PKCa (Fig. 4B) was significantly increased in the samples treated with PtdIns(3,4,5)P3-containing PE vesicles compared to those treated with the PE vesicle alone. PE vesicle itself or the one containing PtdIns(4,5)P2 had little effect on the phosphorylation (Fig. 4A). In addition, the increase in HM phosphorylation was dependent on the PtdIns(3,4,5)P3 dose. Moreover, PtdIns(3,4,5)P3-containing PE vesicles induced significantly stronger HM phosphorylation of GST-AKT(KT) than that of GST-ΔPH-AKT(KT), while HM phosphorylation of GST-AKT(KT) and GST-ΔPH-AKT(KT) remained the same in the absence of PtdIns(3,4,5)P3 regardless of the presence of PE vesicles (Fig. 4A). These results indicate that PtdIns(3,4,5)P3 can directly activate the kinase activity of immunoprecipitated mTORC2. In addition, they confirm that PtdIns(3,4,5)P3 has additional roles in mTORC2-mediated HM phosphorylation probably via its interaction with the PH domain of the substrate.

In this report, we used two AKT mutants as probes to investigate the role of PI3K and PtdIns(3,4,5)P3 in their regulation of
mTORC2-mediated HM phosphorylation and found that PtdIns(3,4,5)P$_3$ regulates the phosphorylation via at least three different mechanisms as illustrated in Fig. 4B. Previous studies have demonstrated that the PH domain at AKT N-terminus has an important role in membrane translocation of the AKT molecule and is hence required for ligand-stimulated HM phosphorylation. Our studies indicate that the PH domain may have an additional role, i.e., its presence may make a better substrate for HM phosphorylation. Although we postulate that this may be the result of a conformational change induced upon the interaction of the PH domain with PtdIns(3,4,5)P$_3$, other possible explanations may exist. Although Ras has been implicated in the regulation of mTORC2, our results suggest that it may primarily act through PI3K and PtdIns(3,4,5)P$_3$. In addition to the effects mediated by the PH domain, our in vitro kinase assay results clearly demonstrated that PtdIns(3,4,5)P$_3$ directly regulates the kinase activity of mTORC2. However, we do not know the precise mechanisms by which PtdIns(3,4,5)P$_3$ regulates mTORC2 kinase activity. PtdIns(3,4,5)P$_3$, may directly regulate the kinase activity of mTOR or one of the components of the complex. Several of the mTORC2 components contain PH domains and may subject to regulation by PtdIns(3,4,5)P$_3$. Further work is needed to characterize these detailed mechanisms.
FIGURE LEGENDS

Figure 1. Insulin and Ras regulate membrane-docked HM phosphorylation via PI3K. A) Schematic representation of Akt mutants. B) Effects of LY294002 (LY) on insulin-induced HM phosphorylation of Myr-AKT(KT) and Myr-ΔPH-AKT(KT) expressed in HEK293T cells. The transfected cells were incubated with serum-free medium for overnight and HBSS for 1 hour before they were stimulated with insulin (2 μg/ml) for 30 min with or without pretreatment of LY294002 (15 min). NS, non-specific band; Endo p473-AKT, endogenous p-473AKT. C-D) The roles of Ras and PI3K in the regulation of HM phosphorylation of Myr-ΔPH-AKT(KT). HEK293 cells were cotransfected with Myr-ΔPH-AKT(KT) and H-Ras-G12V, H-Ras-S17N or PI3K-CAAX (PI3K*). All of the experiments were repeated at least three times. Quantification of the phosphorylated Akt contents normalized against total Akt contents from all of these independent experiments are shown in the bar charts. Data are presented as means ± SD (Student’s t-Test). Representative blots are also shown.

Figure 2. Insulin, Ras and PI3K increase mTORC2-Ser2481 phosphorylation. A) HEK293 cells were treated as described in Fig. 1B followed by Western analysis. B) HEK293 cells were transfected with plasmids encoding LacZ, PI3K-CAAX or Ras-G12V, followed by LY294002 treatment and Western analysis. Data analysis and presentation are as described in Fig. 1.

Figure 3. Insulin stimulates mTORC2 kinase activity via PI3K. A) HEK293 cells were treated as in Fig. 1B and lysed. The cell lysates were used for immunoprecipitation (IP) with a control antibody (IgG) or RICTOR antibody. Immunocomplexes were used to phosphorylate GST-AKT(KT) or GST-PKCα in an in vitro kinase assay. The kinase assay mixtures were subjected to Western analysis by an antibody to phospho-Ser473-AKT, phospho- pan PKC (it recognizes the phospho-HM site of PKCα), RICTOR, AKT, or mTOR. B) Cells were stimulated with insulin, and their lysates were subjected to immunoprecipitation with an mTOR antibody. The immunocomplexes were then treated with LY294002 for 15 min before they were used in the in vitro kinase assay to phosphorylate GST-AKT(KT). Data analysis and presentation are as described in Fig. 1.

Figure 4. PIP3 can directly activate mTORC2 kinase activity. A) HEK293 cells were lysed and their lysates were subjected to immunoprecipitation with the RICTOR antibody. The immunocomplexes were incubated with PE (100ng/ul) vesicles containing 0, 25 ng/ml of PtdIns(4,5)P2, 5 or 25 ng/ml of PtdIns(3,4,5)P3 and GST-ΔPH-AKT(KT), GST-AKT(KT) or GST-PKCα in an in vitro kinase assay. Data analysis and presentation are as described in Fig. 1. B) A model for the regulation of AKT HM phosphorylation by Insulin/Ras/PI3K signaling. PtdIns(3,4,5)P3, whose contents are elevated upon insulin stimulation may regulate the HM phosphorylation via at least three mechanisms: direct stimulation of the mTORC2 kinase activity, recruitment of AKT to the plasma membrane, and making AKT a better substrate. The latter two mechanisms appear to be mediated by the N-terminal PH domain.
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Figure 1

A. Schematic representation of Myr-AKT(KT) and Myr-ΔPH-AKT(KT) constructs. Myristylation tag, kinase domain, and PH domain are indicated.

B. Western blot analysis of p473-AKT-HA and Myr-AKT(HA) in the presence of Insulin, LY (10 μM), and RasG12V.

C. Western blot analysis of p473-ΔPH-AKT-HA and Myr-ΔPH-AKT(KT)-HA in the presence of Insulin, RasG12V, RasS17N, PI3K*, and LY (10 μM) and 30 μM).

D. Western blot analysis of p473-ΔPH-AKT-HA and Myr-ΔPH-AKT(KT)-HA in the presence of RasG12V, PI3K*, and LY (10 μM) and 30 μM).

Statistical significance is indicated by p<0.01 and p<0.05.
Figure 2

A

Insulin: - - + +
LY (10 μM): - + - +
p2481-mTOR- mTOR- p473-AKT- AKT- β-catenin-

B

LacZ PI3K+ RasG12V
LY (10 μM): - + - + - M +
p2481-mTOR- mTOR- p473-AKT- AKT-

p<0.01 p<0.01 p<0.01 p<0.01
Figure 3

A

IP: IgG Anti-RICTOR
Insulin: - - + +
LY: - - - +
p-GST-PKCα -
GST-PKCα -
p473-GST-AKT -
GST-AKT(KT) -
mTOR -
RICTOR -
RICTOR -
mTOR -
β-catenin -

Kinase assay

Cell lysate

B

IP: IgG Anti-mTOR
Insulin: - - + +
LY (μM): 0 0 2 10 0 2 10
p473-GST-AKT -
GST-AKT(KT) -
mTOR -

p473/AKT (fold)

p < 0.01 p < 0.05
p > 0.5

p473/AKT (fold)
Figure 4

A

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C

Insulin → Ras/PI3K → PtdIns(3,4,5)_3 → mTORC2 kinase activity improvement → AKT membrane translocation → AKT HM phosphorylation