Published in final edited form as:

Int J Biochem Cell Biol. 2015 July ; 64: 195–201. doi:10.1016/j.biocel.2015.04.007.

Threonine 34 phosphorylation by phosphoinositide-dependent protein kinase 1 facilitates dissociation of Akt from the plasma membrane

Bill X. Huang, Rachel Lee, Mohammed Akbar, and Hee-Yong Kim

Laboratory of Molecular Signaling, National Institute of Alcohol Abuse and Alcoholism, National Institutes of Health, Bethesda, MD 20892-9410

Abstract

Akt is a key mediator of cell proliferation, survival and metabolism. After translocation to the membrane and phosphorylation at T308 and S473, the activated Akt dissociates from the plasma membrane to cytoplasm, which is an important step to phosphorylate its downstream targets. In addition to its central role in regulating the kinase activity, phosphorylation of T308 in the kinase loop has been reported to be necessary for this dissociation process. However, it is not clear whether the membrane detachment requires further mechanisms. In the present report, we demonstrate that membrane dissociation of Akt requires phosphoinositide-dependent protein kinase 1 (PDK1) which directly phosphorylates not only T308 but also T34 in the pleckstrin homology (PH) domain. Like T308, T34 was phosphorylated in a phosphatidylinositol 3,4,5-trisphosphate- and phosphatidylserine-dependent manner. Phosphorylation of T34 also occurred in cells following growth factor stimulation, concurrently with T308 phosphorylation. Moreover, when T34 was mutated to aspartic acid (T34D) to mimic its phosphorylation, Akt-membrane association assessed by surface plasmon resonance spectroscopy was significantly reduced. In cells, this mutation impaired the IGF-induced Akt membrane translocation and subsequent phosphorylation at T308 and S473. Taken together, our results demonstrate that T34 phosphorylation by PDK1 promotes the membrane dissociation of activated Akt for its downstream action through attenuating membrane binding affinity. This membrane dissociation mechanism offers a new insight for Akt activation process and provides a potential new target for controlling the Akt-dependent cellular processes.

Keywords

Protein kinase B; Threonine 34; phosphorylation; membrane translocation; Akt activation; PDK1

© 2015 Published by Elsevier Ltd.

Correspondence should be addressed to: Hee-Yong Kim, Ph.D., Laboratory of Molecular Signaling, National Institute of Alcohol Abuse and Alcoholism, National Institutes of Health, 5625 Fishers Lane, Rm. 3N-07, Bethesda, MD 20892-9410, 301-402-8746 (Tel), 301-594-0035 (Fax), hykim@nih.gov.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

1. Introduction

Akt, also called protein kinase B, is a serine/threonine kinase that plays a central role in regulating key cellular processes including cell proliferation, survival and metabolism (Brazil and Hemmings, 2001). Three isoforms of Akt (Akt1, Akt2, and Akt 3) identified in mammals share a similar structural organization consisting of an N-terminal pleckstrin homology (PH) domain, a central kinase domain (KD), and a C-terminal regulatory domain (RD) (Bellacosa et al., 1991, Thomas et al., 2002). Akt is a downstream target of phosphatidylinositol 3 kinases (PI3K) (Manning and Cantley, 2007). After growth factor stimulation, PI3K converts phosphatidylinositol 4,5 bispophosphate to phosphatidylinositol 3,4,5 trisphosphate (PIP₃) that triggers the activation of Akt (Bellacosa et al., 1998).

Akt activation is tightly regulated by stepwise biochemical events including membrane translocation, phosphorylation and membrane dissociation (Alessi and Cohen, 1998, Downward, 1998). Cytosolic Akt is recruited to the plasma membrane through interacting with not only PIP₃ but also constitutive phosphatidylserine (PS) at the plasma membrane (Huang et al., 2011). This membrane interaction causes Akt conformational change to unfold the PH and regulatory domains from the kinase domain, exposing T308 at the kinase domain and S473 of regulatory domain, respectively (Alessi et al., 1996, Huang et al., 2011). These residues are phosphorylated by PDK1 and mammalian target of rapamycin complex 2 (mTORC2) respectively (Alessi et al., 1996, Andjelkovic et al., 1997, Stokoe et al., 1997, Sarbassov et al., 2005). The phosphorylated Akt then dissociates from the membrane to phosphorylate a numbers of cytosolic or nuclear proteins, such as caspase-9, BAD, glycogen synthase kinase 3 β and FKHR, thereby regulating downstream cellular processes (Brunet et al., 1999, Cardone et al., 1998, Cross et al., 1995, Datta et al., 1997). While the membrane recruitment and phosphorylation of Akt at T308 and S473 have been studied extensively at the molecular level, less study has been carried out to understand how the active Akt dissociates from the plasma membrane to interact with its target proteins in the cytoplasm and nucleus (Ananthanarayanan et al., 2007).

In a previous study, we demonstrated that Akt undergoes conformational changes in each step of the activation process (Huang and Kim, 2006). Of note, upon phosphorylation by PDK1 and mTORC2, the membrane-induced open PH-KD configuration returns to the folded structure, while the open RD-KD arrangement remains open until substrate binding. Based on this observation, we proposed that the phosphorylation-induced change in the PH-KD interdomain conformation initiates the dissociation of the active Akt from the membrane (Huang and Kim, 2006). This postulation is consistent with the importance of PH-KD interaction for T308 phosphorylation by PDK1 (Calleja et al., 2007), as well as the necessity of the T308 phosphorylation for the membrane detachment of Akt as demonstrated in NIH3T3 cells using fluorescence imaging combined with site mutation (Ananthanarayanan et al., 2007).

Akt PH domain contains membrane binding elements including a PIP₃ binding pocket that is well defined by a complex hydrogen bonding network, and positively charged PS binding residues. Alteration of the hydrogen bonding environment or positively charged amino acid residues has been shown to influence the Akt-membrane association. For example,

substitution of glutamic acid at amino acid 17 with lysine (E17K) results in pathological localization of Akt to the membrane (Carpten et al., 2007), whereas mutation of K20A or R15A disrupts Akt membrane translocation (Akbar et al., 2005). Presumably with a similar mechanism, Powell et al. have observed that phosphorylation of T34 near the PIP₃ binding pocket by PKC ζ , inhibits the binding of the PH domain to PIP₃ in L6 muscle cells (Powell et al., 2003). These structural and functional evidences prompt us to hypothesize that phosphorylation of T34 may contribute to the dissociation of the active Akt from the plasma membrane, in addition to the T308 phosphorylation which is thought to instigate a PH-KD interaction that masks the PIP₃-binding pocket (Ananthanarayanan et al., 2007). In view of the fact that T308 phosphorylation is modulated by the accessibility of T308 to PDK1 through opening the PH-KD configuration (Huang et al., 2011), we further hypothesized that this interdomain structural change might concurrently bring the contact of T34 with PDK1 leading to a possible phosphorylation event. In the present study we tested these propositions using biochemical, molecular and cell biological approaches, in an attempt to ascertain a novel role of T34 phosphorylation in Akt dissociation from the plasma membrane.

2. Materials and Methods

2.1. Materials

Inactive Akt1, active Akt, active PDK1 and ATP/Mg²⁺ cocktail were purchased from Upstate Cell Signaling Solutions. Mitogen-activated protein kinase-activated protein kinase 2 (MAPKAPK2) was purchased from Calbiochem. 1-stearoyl-2-docosaheptaenoyl-*sn*-glycero-3-phospho-L-serine (18:0,22:6-PS), 1-stearoyl-2-docosaheptaenoyl-*sn*-glycero-3-phosphoethanolamine (18:0, 22:6-PE), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (16:0, 18:1-PC), and 1-stearoyl-2-arachidonoyl-*sn*-glycero-3-phosphoinositol-3,4,5-trisphosphate (18:0, 20:4-PIP₃) were purchased from Avanti Polar Lipids. Sequencing grade modified trypsin was purchased from Promega. Anti-phospho-Akt (T308) (Cat#: 2965), anti-phospho-Akt (S473) (Cat#: 9271), anti-Akt (Cat#: 9272), anti-protein kinase C zeta (PKC ζ) (Cat#: 9368), anti-phospho-PKC ζ (T410) (Cat#: 9378), anti-phospho-PDK1 (S241) (Cat#: 3061), anti-PDK1 (Cat#: 3062), and anti-GAPDH (Cat#: 2118) antibodies were purchased from Cell Signaling Technology. Anti-phospho-Akt (T34) (Cat#: 23509) was obtained from Abcam. A dilution of 1:1000 was used in western blotting for all of these antibodies. Insulin-like growth factor-1 (IGF-1) was purchased from PeproTech. PBS without Ca²⁺ was purchased from Quality Biological, Inc. Other reagents were purchased from Sigma or Quality Biological, Inc.

2.2. Preparation of multilamellar and unilamellar vesicles

PE (18:0, 22:6), PC (16:0, 18:1), PS (18:0, 22:6), and PIP₃ (18:0, 20:4) dissolved in methanol/chloroform were mixed at desired proportions. After drying with a N₂ stream, the lipid mixture was redissolved in 2 mL cyclohexane containing 75 μ M BHT (2,6-di-tert-butyl-p-cresol), frozen with dry ice and lyophilized under vacuum. Multilamellar vesicles were prepared by resuspending the lyophilized phospholipids in 1 mL of PBS (pH 7.4) containing 50 μ M DTPA (diethylenetriamine pentaacetic acid). Unilamellar vesicles were prepared by extruding the multilamellar vesicles through a 0.1 μ m polycarbonate membrane

(Corning, Inc.) with an Avanti mini-extruder (Avanti Polar Lipids). All of the above procedures were carried out in an argon box except the drying step.

2.3. *In vitro* phosphorylation of Akt

Two μL of inactive Akt at $0.1 \mu\text{g}/\mu\text{L}$ reconstituted in HEPES buffer was incubated with $15 \mu\text{L}$ unilamellar vesicles (PE/PC/PS/PIP₃: 50/19.8/30/0.2), $3 \mu\text{L}$ PDK1 at $0.005 \mu\text{g}/\mu\text{L}$ or/and $0.5 \mu\text{L}$ MAPKAPK2 at $0.1 \mu\text{g}/\mu\text{L}$, and $5 \mu\text{L}$ ATP(0.5 mM)/Mg²⁺ (50 mM) cocktail at 30°C for 45 min. The mixture was subjected to western blot analysis using anti-phospho-Akt or anti-Akt antibodies with an enhanced chemiluminescent substrate (Thermo Fisher Scientific). Blots were visualized by a Gel Logic 440 imaging system (Kodak) Separately the mixture was subjected to in solution digestion using trypsin for mass spectrometric analysis. Alternatively, reduction by DTT and alkylation by iodoacetamide were performed prior to the tryptic digestion.

2.4. Separation of membrane-bound Akt from free enzyme

The unilamellar vesicles used in the aforementioned phosphorylation was replaced by multilamellar vesicles with various lipid compositions. After the *in vitro* phosphorylation, samples were centrifuged at $14,000 \text{ g}$ for 30 min at 20°C to separate the vesicle-bound Akt from the free enzyme (Corbalan-Garcia et al., 2003). The supernatants and pellets were subjected to SDS-PAGE separation followed by western blot analysis against phospho-Akt or Akt antibodies.

2.5. Biomolecular interaction analysis

Biomolecular interaction analysis based on surface plasmon resonance (SPR) was used to determine the binding affinity of Akt to the membrane. SPR measurements were performed on a Biacore X instrument. A CM5 sensor chip was used for the binding of the GFP-PH domain to liposomes, using a protocol described previously (Huang et al., 2011). Briefly, Anti-GFP antibody ($25 \mu\text{g}/\text{mL}$ in 10 mM sodium acetate, $\text{pH } 5.0$) was immobilized onto the chip by amine coupling. Cell lysate containing the GFP-PH domain (WT or T34 mutants) of Akt expressed in Neuro 2A cells was manually injected into the experimental cell until 15–20 RU of GFP-tagged protein was captured onto the antibody surface, whereas no lysate was injected into the control cell. Thirty μL unilamellar vesicles (PE/PC/PS/PIP₃: 50/19.8/30/0.2) were injected into both flow cells and all sensorgrams were recorded against the control. The anti-GFP surface was regenerated by injecting 10 mM sodium acetate ($\text{pH } 4.0$) and radioimmuno-precipitation assay (RIPA) buffer subsequently. Alternatively, an L1 sensor chip was used for the binding of recombinant full-length Akt to the membrane (Huang et al., 2011). In this case, $35 \mu\text{L}$ unilamellar vesicles (PE/PC/PS/PIP₃: 50/19.8/30/0.2) or control liposomes (PE/PC: 50/50) were injected into the experimental and control cells respectively. The lipid coating in both cells was stabilized by repeatedly injecting 50 mM NaOH until a steady signal was obtained. Akt at 250 nM was injected into both cells. The regeneration of the sensor surface was achieved by completely removing the protein and liposomes with injection of 40% isopropanol in 50 mM NaOH. HEPES buffer was used as the running buffer.

2.6. GFP tagging and point mutation of Akt

cDNA of human Akt (GenBank accession no. NM_005163) or Akt-PH domain was cloned into pEGFP-C1 vector (Takara Bio Inc.) to prepare EGFP-Akt (full length) or EGFP-Akt-PH at Veritas Laboratory. QuickChange II Site-Directed Mutagenesis kit (Agilent Technologies) was used for the point mutation in accordance to manufacturer's instruction. The forward/reverse primers for T34 wild type, T34A and T34D PH domains are as follows.

T34 (WT): 5'-CTC AAG AAT GAT GGC ACC TTC ATT GGC TAC AAG
GAG-3'/5'-CTC CTT GTA GCC AAT GAA GGT GCC ATC ATT CTT GAG-3'

T34A: 5'-CTC AAG AAT GAT GGC GCC TTC ATT GGC TAC AAG GAG-3'/5'-
CTC CTT GTA GCC AAT GAA GGC GCC ATC ATT CTT GAG-3'

T34D: 5'-CTC AAG AAT GAT GGC GAC TTC ATT GGC TAC AAG GAG-3'/5'-
CTC CTT GTA GCC AAT GAA GTC GCC ATC ATT CTT GAG-3'

After transformation, colonies were picked and amplified in LB medium. The plasmid DNA was purified with a Qiaprep Spin Miniprep kit (QIAGEN). An ABI BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems) was used to confirm the mutation of the purified plasmids. Positive clones were selected for maxiprep using a Qiagen Maxi prep kit.

2.7. Cell culture, transfection, and IGF stimulation

Neuro 2A (mouse neuroblastoma) cells purchased from American Tissue Culture Collection were maintained in Dulbecco's Modified Eagle Medium (DMEM) (Sigma) containing 5% fetal bovine serum in a humidified atmosphere with 5% CO₂ at 37 °C. Cells were transfected with the GFP-tagged Akt-PH domain or GFP-Akt (full-length) using Fugene-6 transfection reagent (Roche) for 36 h. After overnight serum starvation with serum-free DMEM, cells were stimulated with IGF-1 (10 ng/mL) and subjected to western blotting or microscopic analysis. The western blot bands were visualized by a Gel Logic 440 imaging system and quantified with 1D imaging analysis software (Kodak).

2.8. Microscopic study of GFP-Akt membrane translocation

Microscopic monitoring of Akt membrane translocation was achieved by using an inverted IX81 Olympus microscope with a GFP filter and a 60X oil objective. The cells that were serum-starved overnight were maintained at 35°C with a Delta T4 culture dish controller (Bioptechs). After taking the initial image, the serum-free medium was replaced with IGF-containing buffer. The time series of microscopic images were recorded with a Photometrics Coolsnap FX camera (Roper Scientific) and analyzed by Microsuite software (Soft imaging systems). The relative increase of fluorescence intensity in the plasma membrane over the cytosol ($R = (\text{plasma membrane fluorescence} - \text{cytosolic fluorescence})/\text{cytosolic fluorescence}$) was quantified by MetaMorph software using three to five cells per condition.

2.9. Nano-HPLC/electrospray ionization (ESI) mass spectrometry (MS)

Tryptic peptides of Akt samples obtained upon *in vitro* phosphorylation were desalted with a C-18 ZipTip microcolumn (Millipore) prior to HPLC/ESI-MS analysis which was performed on an Agilent ion trap mass spectrometer (XCT) equipped with an Agilent 1100 nanoflow

HPLC system. Peptides were loaded onto a C18 enrichment column (Zorbax 300SB C18, 0.3 mm x 5mm, Agilent) and separated by a Zorbax 300 SB C18 column (75 μ m x 150 mm, 3.5 μ m) using a mobile phase that contained solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in acetonitrile) at a flow rate of 300 nL/min. The mobile phase composition was held initially at 3% B for 5 min, and gradually changed to 15%, 45% and 85% B at 8, 50, and 55 min, respectively, and held at 85% for 5 min. The data-dependent automatic scan mode ("Auto-MS²") was used. The capillary voltage was set to 1750 V. The mass spectrometer was scanned from 300 to 2000 m/z range.

2.10. Statistical analysis

Data are representative of three independent experiments and presented as mean $\pm$ SE.

Statistical analysis was performed using the unpaired two-tailed student's *t*-test.

3. Results

3.1. Release of phosphorylated Akt from membrane depends on the phosphorylation by PDK1

For the inner leaflet plasma membrane surface where Akt interacts with phospholipids, we used multilamellar vesicles containing PE(18:0, 22:6)/PC(16:0,18:1)/PS(18:0, 22:6)/PIP₃(18:0, 20:4) at a ratio 50:19.8:30:0.2 (PE/PC/PS/PIP₃) which approximates a lipid composition in the inner leaflet of the neuronal plasma membrane under a stimulation condition (Huang et al., 2011, Huang and Kim, 2012). To evaluate the dissociation of active Akt from the plasma membrane, Akt was first phosphorylated by PDK1 and/or MAPKAPK2 in the presence of the multilamellar vesicles and the liposome-bound Akt (pellet) was separated from those released to the supernatant by centrifugation (Corbalan-Garcia et al., 2003). As shown in Fig. 1A, MAPKAPK2, which can serve as a substitute for the physiologic mTORC2 for *in vitro* studies (Alessi et al., 1996), phosphorylated S473 in a PIP₃- and PS-dependent manner (Huang et al., 2011). However, S473 phosphorylation detected in supernatant from the samples incubated with MAPKAPK2 alone was negligible (Fig. 1A). When PDK1 was used together with MAPKAPK2, phosphorylation of S473 Akt was clearly detected in the supernatant (Fig. 1A). On the other hand, the PIP₃- and PS-dependent phosphorylation of T308 by PDK1 was detected in the supernatant regardless of the presence of MAPKAPK2 (Fig. 1B). These data indicated that the detachment of the active Akt from the membrane is not affected by S473 phosphorylation but relies on the phosphorylation event performed by PDK1. It was apparent that both PIP₃ and PDK1 were required for the release of the phosphorylated Akt from the membrane (e.g. Fig. 1A, last two lanes). Given that PIP₃ is also critical for the function of PDK1 (Stokoe et al., 1997), the data further supported the decisive role of PDK1 in the Akt dissociation from the plasma membrane.

3.2. T34 is phosphorylated by PDK1 in a PS- and PIP₃- dependent manner

Previous study demonstrated that PDK1 is associated with Akt in the cytoplasm but phosphorylation of T308 occurs only after both proteins co-translocate to the plasma membrane (Calleja et al., 2007). It is now clear that this is due to the Akt-membrane interaction that causes the unfolding of the PH domain from the kinase domain exposing

T308 to PDK1 (Huang et al., 2011). We hypothesized that the open conformation may also bring the contact of PDK1 with phosphorylatable residues in the PH domain, resulting in additional phosphorylation events. Therefore, we incubated Akt with unilamellar vesicles (PE/PC/PS/PIP₃, 50:19.8:30:0.2) and ATP/Mg²⁺ in the presence or absence of active PDK1 and evaluated the modification of Akt by phosphorylation using tandem mass spectrometry (MS/MS) coupled with high performance liquid chromatography. The HPLC-MS/MS analysis was performed after the Akt samples were digested by trypsin. This MS-based screening approach revealed that T34 in the PH domain was phosphorylated by PDK1, in addition to T308 of which phosphorylation is critical for Akt kinase activity. As shown in Fig. 2A, a peptide peak with m/z of 547.75 emerged at 33.0 min when active PDK1 was used regardless of the presence of MAKAPK2. The MS/MS analysis indicated that this peak originated from peptide N[30-39]K, with modification of phosphorylation at T34 (Fig. 2A, right). We next confirmed the MS result by western blot analysis with the antibody specific to phosphorylation of T34. As depicted in Fig. 2B using the same samples for the aforementioned Akt release study (Fig. 1), the phosphorylation of T34 increased dose-dependently with PIP₃ and PS, exactly as was the case for T308 phosphorylation (Fig. 1). These data strongly suggest that both T34 and T308 phosphorylation utilize the same conformation-based mechanism, by which both PIP₃ and PS jointly regulate the open configuration of the PH-KD (Huang et al., 2011) to expose these threonine residues for phosphorylation by PDK1. It is worthwhile to note that T34 reportedly can be phosphorylated by atypical PKC ζ and PKC ζ itself can be activated through phosphorylation of T410 by PDK1 (Chou et al., 1998, Hirai and Chida, 2003). Interestingly, our western blot analysis clearly indicated that phosphorylated PKC ζ (pT410) or total PKC ζ was not present in the *in vitro* system tested (Fig. 2B). Instead, constitutively active PDK1 was detected as expected (Fig. 2B). The data ruled out the probability of the co-existence of PKC ζ in the purified enzymes (Akt and PDK1) used in the study, indicating that PDK1 directly phosphorylates T34.

3.3. Phosphorylation of T34 accompanies Akt activation by growth factor-stimulation

If T34 phosphorylation is involved in the Akt activation process, it must be a physiological event that responds to external stimuli such as growth factors. To examine whether phosphorylation of T34 occurs in the cellular context, we treated Neuro 2A cells with insulin-like growth factor 1. We found that T34 was concomitantly phosphorylated with T308 and S473 in an IGF-dependent manner (Fig. 2C).

3.4. T34 phosphorylation attenuates Akt-membrane binding affinity

To assess the molecular basis of the effect of Akt phosphorylation on membrane dissociation, we evaluated the membrane binding affinity of purified Akt at different phosphorylation stages. To this end, biomolecular interaction analysis based on surface plasmon resonance (SPR) was performed. As shown in Fig. 3, fully active Akt where Akt was phosphorylated at T308 and S473 showed significantly less binding to the unilamellar vesicles mimicking the plasma membrane upon stimulation (PE/PC/PS/PIP₃, 50:19.8:30:0.2), compared to unphosphorylated or inactive Akt. Notably, the phosphorylation of T34 was also detected in the active Akt molecules, along with phosphorylation of T308 and S473. These data are in well-agreement with the results

obtained by the Akt-membrane release assay using centrifugation mentioned above (Fig. 1). Since T308 phosphorylation has been previously shown to be important for Akt membrane detachment (Ananthanarayanan et al., 2007), we focused on examining the effect of T34 phosphorylation on the membrane binding using SPR combined with site-specific mutation. The GFP-tagged WT or T34 mutants were expressed in Neuro 2A cells, captured by anti-GFP antibody immobilized on the sensor chip, and made to interact with the liposomes. Mutation of T34 to aspartic acid (T34D), which mimics the phosphorylation status of T34, decreased the binding of the PH domain to the vesicles considerably, compared to the WT (Fig 3). The unphosphorylatable mutant T34A slightly enhanced the PH domain binding to the vesicles, presumably due to a more favorable conformation (Powell et al., 2003). These data strongly suggested that the phosphorylation of T34 adversely influenced the affinity of Akt to the plasma membrane, thus promoting the release of the activated Akt from the membrane.

3.5. T34 phosphorylation impairs Akt membrane translocation and Akt activation

Akt-membrane interaction is the basis for the cellular Akt translocation to and release from the plasma membrane where Akt is phosphorylated and thereby activated. Therefore we examined the effect of the T34 mutation on the membrane translocation and phosphorylation in living cells. We first evaluated the IGF-induced Akt translocation in Neuro 2A cells overexpressing GFP-tagged T34D or T34A PH domain mutants by using quantitative time-lapse fluorescence microscopy. In comparison to WT, T34D, the mutant mimicking T34 phosphorylation, showed considerably inhibited membrane translocation of the PH domain, while T34A, the non-phosphorylatable mutant, showed slightly enhanced translocation (Fig. 4). These results were consistent with the *in vitro* binding assay data shown in Fig. 3B. Subsequent examination of the IGF-triggered GFP-Akt phosphorylation indicated that phosphorylation at both T308 and S473 was significantly reduced in the T34D mutant, in comparison to the WT or T34A (Fig. 5A&B). The IGF-dependent phosphorylation of the endogenous Akt at both T308 and S473 did not change regardless of the mutation (Fig. 5A), indicating that the PIP₃ production or PIK3 activity was not affected by the expression of these mutants as expected. Both *in vitro* and *in vivo* data clearly indicated that T34 phosphorylation attenuates membrane binding affinity, which facilitates the dissociation of Akt from the plasma membrane.

4. Discussion

A key issue that remains less clear in Akt activation process concerns the mechanism by which active Akt is released from the plasma membrane for its downstream function, although it has been shown that the release depends on the phosphorylation of T308 at the activation loop of the kinase domain (Ananthanarayanan et al., 2007). Using multidisciplinary approaches, we demonstrated here that Akt phosphorylation by PDK1 plays a significant role in dissociating activated Akt from the membrane. We found that PDK1 phosphorylates not only T308 but also T34 during activation, and the Akt-membrane interaction becomes significantly reduced after T34 phosphorylation. Decreased membrane association due to T34 phosphorylation provides a mechanism for the facilitated detachment of Akt from the plasma membrane after activation.

The function and regulation of Akt depend on its phosphorylation. It is well established that IGF-induced phosphorylation of T308 by PDK1 and S473 by mTORC2 are required for full kinase activity of Akt (Alessi et al., 1996). In addition, Akt is constitutively phosphorylated at T450 in the turn motif of the regulatory domain (Bellacosa et al., 1998). The T450 phosphorylation is thought to be important for correct folding thus the stability of Akt (Liao and Hung, 2010). Very recently, phosphorylation of S477 and T479 at the extreme carboxyl terminus by cyclin-dependent kinase 2/cyclin A or mTORC2 has been identified (Liu et al., 2014). This newly identified phosphorylation event has been shown to influence cell-cycle progression through promoting Akt S473 phosphorylation. Compared to these phosphorylation events, the phosphorylation of Akt at T34 is less studied and its role in Akt signaling remains largely unknown. The T34 phosphorylation is shown to be phosphorylated by PKC ζ which is activated by lipid ceramide in L6 muscle cells (Powell et al., 2003). In addition to ceramide stimulation, PKC ζ can be phosphorylated and activated by PDK1 in a PIP₃-dependent manner *in vitro* or in cell lines such as HEK 293 and embryonic stem cells (Balendran et al., 2000, Le Good et al., 1998). A mechanism for the PKC ζ activation under these circumstances is that PKC ζ interacts with PIP₃, releasing the autoinhibitory domain from the activation loop that allows T410 of the loop phosphorylated by PDK1 (Hirai and Chida, 2003). Interestingly, our mass spectrometric and western blot data showed that PDK1 directly phosphorylates T34 in the absence of active PKC ζ (Fig. 2A&B). Moreover, T34 phosphorylation occurs in an IGF-dependent manner (Fig. 2C) in Neuro 2A cells where the phosphorylation of PKC ζ did not appear to respond to IGF stimulation (Fig. 2C). Although further studies are needed to uncover detailed mechanism for the PKC ζ signaling, it is possible that phosphorylation by PKC ζ depends on types of cells or tissues. Nevertheless, our *in vitro* and in cell data indicate that T34 is the direct substrate of PDK1.

Membrane PIP₃ and PS have been shown to jointly regulate the PH-KD interdomain conformation that controls the accessibility of T308 for phosphorylation by PDK1 (Huang et al., 2011). Our *in vitro* data showed that T34 phosphorylation depends on not only PIP₃ but also membrane PS (Fig. 2B), similar to T308 phosphorylation (Fig. 1). *In vivo*, T34 phosphorylation also occurs concurrently with T308 phosphorylation (Fig. 2C) upon IGF stimulation. These data strongly suggest that the membrane-induced open PH-KD conformation enables the contact of PDK1 with not only T308 of the loop region but also T34 of the PH domain at the same time. Our data also indicate the sequence T³⁴FIGYKER can be the substrate of PDK1 in addition to the consensus motif (i.e. T³⁰⁸FCGTPEY). Other kinases such as Akt itself also display a similar ability to phosphorylate some variant sequences apart from the consensus motif (Huang and Kim, 2013, Vandermoere et al., 2007).

Although ceramide-induced T34 phosphorylation has been reported to inhibit Akt activity (Powell et al., 2003), the effect of T34 phosphorylation on Akt dissociation from the plasma membrane has not been reported. We demonstrated that the mutation of T34 to its phosphorylation mimic T34D impaired the Akt-membrane association (Fig. 3). In Neuro 2A cells, this mutation impaired the Akt membrane translocation and subsequent IGF-induced phosphorylation at T308 and S473 (Fig. 4 and 5). Because T34 is located near the PIP₃ binding pocket, it is conceivable that the T34 modification by phosphorylation alters the PH

domain configuration, either by partially masking the PIP₃ binding pocket or changing the hydrogen bonding, and thus reduces the affinity to the membrane. Further investigation such as conformational probing may help delineate the molecular basis by which T34 phosphorylation adversely affects association of Akt with the membrane.

In conclusion, our *in vitro* and *in vivo* data identified the Akt-membrane interaction regulated through T34 phosphorylation by PDK1 as a molecular mechanism for the dissociation of activated Akt from the plasma membrane for downstream signaling. These results imply a new target for controlling Akt-dependent cell proliferation, survival and metabolism.

Abbreviations

Akt	protein kinase B
IGF	insulin-like growth factor
KD	kinase domain
MAPKAPK2	mitogen-activated protein kinase-activated protein kinase 2
MS	mass spectrometry
PDK	phosphoinositide-dependent protein kinase
PH	pleckstrin homology
PIK3	phosphatidylinositol 3 kinases
PIP₃	phosphatidylinositol 3,4,5-trisphosphate
PKCζ	protein kinase C zeta
PS	phosphatidylserine
RD	regulatory domain
SPR	surface plasmon resonance
mTORC2	mammalian target of rapamycin complex 2

References

- Akbar M, Calderon F, Wen Z, Kim HY. Docosahexaenoic acid: a positive modulator of Akt signaling in neuronal survival. *Proc Natl Acad Sci U S A*. 2005; 102:10858–10863. [PubMed: 16040805]
- Alessi DR, Andjelkovic M, Caudwell B, Cron P, Morrice N, Cohen P, Hemmings BA. Mechanism of activation of protein kinase B by insulin and IGF-1. *Embo Journal*. 1996; 15:6541–6551. [PubMed: 8978681]
- Alessi DR, Cohen P. Mechanism of activation and function of protein kinase B. *Current Opinion in Genetics & Development*. 1998; 8:55–62. [PubMed: 9529606]
- Ananthanarayanan B, Fosbrink M, Rahdar M, Zhang J. Live-cell molecular analysis of Akt activation reveals roles for activation loop phosphorylation. *Journal of Biological Chemistry*. 2007; 282:36634–36641. [PubMed: 17928291]
- Andjelkovic M, Alessi DR, Meier R, Fernandez A, Lamb NJC, Frech M, Cron P, Cohen P, Lucocq JM, Hemmings BA. Role of translocation in the activation and function of protein kinase B. *Journal of Biological Chemistry*. 1997; 272:31515–31524. [PubMed: 9395488]

- Balendran A, Hare GR, Kieloch A, Williams MR, Alessi DR. Further evidence that 3-phosphoinositide-dependent protein kinase-1 (PDK1) is required for the stability and phosphorylation of protein kinase C (PKC) isoforms. *FEBS Lett.* 2000; 484:217–223. [PubMed: 11078882]
- Bellacosa A, Chan TO, Ahmed NN, Datta K, Malstrom S, Stokoe D, McCormick F, Feng JN, Tsichlis P. Akt activation by growth factors is a multiple-step process: the role of the PH domain. *Oncogene.* 1998; 17:313–325. [PubMed: 9690513]
- Bellacosa A, Testa JR, Staal SP, Tsichlis PN. A Retroviral Oncogene, Akt, Encoding a Serine-Threonine Kinase Containing an Sh2-Like Region. *Science.* 1991; 254:274–277. [PubMed: 1833819]
- Brazil DP, Hemmings BA. Ten years of protein kinase B signalling: a hard Akt to follow. *Trends Biochem Sci.* 2001; 26:657–664. [PubMed: 11701324]
- Brunet A, Bonni A, Zigmond MJ, Lin MZ, Juo P, Hu LS, Anderson MJ, Arden KC, Blenis J, Greenberg ME. Akt promotes cell survival by phosphorylating and inhibiting a forkhead transcription factor. *Cell.* 1999; 96:857–868. [PubMed: 10102273]
- Calleja V, Alcor D, Laguerre M, Park J, Vojnovic B, Hemmings BA, Downward J, Parker PJ, Larijani B. Intramolecular and intermolecular interactions of protein kinase B define its activation in vivo. *Plos Biology.* 2007; 5:780–791.
- Cardone MH, Roy N, Stennicke HR, Salvesen GS, Franke TF, Stanbridge E, Frisch S, Reed JC. Regulation of cell death protease caspase-9 by phosphorylation. *Science.* 1998; 282:1318–1321. [PubMed: 9812896]
- Carpten JD, Faber AL, Horn C, Donoho GP, Briggs SL, Robbins CM, Hostetter G, Boguslawski S, Moses TY, Savage S, Uhlik M, Lin AM, Du J, Qian YW, Zeckner DJ, Tucker-Kellogg G, Touchman J, Patel K, Mousses S, Bittner M, Schevitz R, Lai MHT, Blanchard KL, Thomas JE. A transforming mutation in the pleckstrin homology domain of AKT1 in cancer. *Nature.* 2007; 448:439–U431. [PubMed: 17611497]
- Chou MM, Hou WM, Johnson J, Graham LK, Lee MH, Chen CS, Newton AC, Schaffhausen BS, Toker A. Regulation of protein kinase C zeta by PI 3-kinase and PDK-1. *Current Biology.* 1998; 8:1069–1077. [PubMed: 9768361]
- Corbalan-Garcia S, Garcia-Garcia J, Rodriguez-Alfaro JA, Gomez-Fernandez JC. A new phosphatidylinositol 4,5-bisphosphate-binding site located in the C2 domain of protein kinase Calpha. *Journal of Biological Chemistry.* 2003; 278:4972–4980. [PubMed: 12426311]
- Cross DA, Alessi DR, Cohen P, Andjelkovich M, Hemmings BA. Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. *Nature.* 1995; 378:785–789. [PubMed: 8524413]
- Datta SR, Dudek H, Tao X, Masters S, Fu HA, Gotoh Y, Greenberg ME. Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. *Cell.* 1997; 91:231–241. [PubMed: 9346240]
- Downward J. Mechanisms and consequences of activation of protein kinase B/Akt. *Current Opinion in Cell Biology.* 1998; 10:262–267. [PubMed: 9561851]
- Hirai T, Chida K. Protein kinase C zeta (PKC zeta): Activation mechanisms and cellular functions (vol 133, pg 1, 2003). *Journal of Biochemistry.* 2003; 133:395–395.
- Huang BX, Akbar M, Kevala K, Kim HY. Phosphatidylserine is a critical modulator for Akt activation. *J Cell Biol.* 2011; 192:979–992. [PubMed: 21402788]
- Huang BX, Kim HY. Interdomain conformational changes in Akt activation revealed by chemical cross-linking and tandem mass spectrometry. *Mol Cell Proteomics.* 2006; 5:1045–1053. [PubMed: 16531397]
- Huang BX, Kim HY. Effects of ethanol on conformational changes of Akt studied by chemical cross-linking, mass spectrometry, and (18)O labeling. *ACS Chem Biol.* 2012; 7:387–394. [PubMed: 22129086]
- Huang BX, Kim HY. Effective identification of Akt interacting proteins by two-step chemical crosslinking, co-immunoprecipitation and mass spectrometry. *PLoS One.* 2013; 8:e61430. [PubMed: 23613850]

- Le Good JA, Ziegler WH, Parekh DB, Alessi DR, Cohen P, Parker PJ. Protein kinase C isoforms controlled by phosphoinositide 3-kinase through the protein kinase PDK1. *Science*. 1998; 281:2042–2045. [PubMed: 9748166]
- Liao Y, Hung MC. Physiological regulation of Akt activity and stability. *American Journal of Translational Research*. 2010; 2:19–42. [PubMed: 20182580]
- Liu PD, Begley M, Michowski W, Inuzuka H, Ginzberg M, Gao DM, Tsou PL, Gan WJ, Papa A, Kim BM, Wan LX, Singh A, Zhai B, Yuan M, Wang ZW, Gygi SP, Lee TH, Lu KP, Tokar A, Pandolfi PP, Asara JM, Kirschner MW, Sicinski P, Cantley L, Wei WY. Cell-cycle-regulated activation of Akt kinase by phosphorylation at its carboxyl terminus. *Nature*. 2014; 508:541. [PubMed: 24670654]
- Manning BD, Cantley LC. AKT/PKB signaling: navigating downstream. *Cell*. 2007; 129:1261–1274. [PubMed: 17604717]
- Powell DJ, Hajdich E, Kular G, Hundal HS. Ceramide disables 3-phosphoinositide binding to the pleckstrin homology domain of protein kinase B (PKB)/Akt by a PKC zeta-dependent mechanism. *Molecular and Cellular Biology*. 2003; 23:7794–7808. [PubMed: 14560023]
- Sarbassov DD, Guertin DA, Ali SM, Sabatini DM. Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. *Science*. 2005; 307:1098–1101. [PubMed: 15718470]
- Stokoe D, Stephens LR, Copeland T, Gaffney PRJ, Reese CB, Painter GF, Holmes AB, McCormick F, Hawkins PT. Dual role of phosphatidylinositol-3,4,5-trisphosphate in the activation of protein kinase B. *Science*. 1997; 277:567–570. [PubMed: 9228007]
- Thomas CC, Deak M, Alessi DR, van Aalten DMF. High-resolution structure of the pleckstrin homology domain of protein kinase B/Akt bound to phosphatidylinositol (3,4,5)-trisphosphate. *Current Biology*. 2002; 12:1256–1262. [PubMed: 12176338]
- Vandermoere F, El Yazidi-Belkoura I, Demont Y, Slomianny C, Antol J, Lemoine J, Hondermarck H. Proteomics exploration reveals that actin is a signaling target of the kinase Akt. *Mol Cell Proteomics*. 2007; 6:114–124. [PubMed: 17088265]

Fig. 1.

Effect of PIP₃- and PS-dependent-phosphorylation of Akt by PDK1 or MAPKAPK2 on the dissociation of phosphorylated Akt from the membrane. The T308 phosphorylation by PDK1 (B), but not S473 phosphorylation by MAPKAPK2 (A), results in the release of phosphorylated Akt from the membrane. The membrane dissociation assay was performed using liposomes containing PE(50%)/PC(50-PS-PIP₃%)/PS(0-30%)/PIP₃(0-1%) to mimic the plasma membrane. Akt in the pellet or supernatant (sup) represents the membrane bound- or released- Akt, respectively.

Fig. 2.

Phosphorylation of Akt at T34 by PDK1 or growth factor stimulation. A. Mass spectrometric detection of Akt phosphorylation at T34 by PDK1. An Akt peptide peak with m/z of 547.75 eluting at 33.0 min chromatographically was detected from the Akt sample after PDK1 treatment (left). MS/MS analysis reveals that this peak corresponds to the Akt peptide phosphorylated at T34 (right). B. Western blot analysis indicates the PIP₃- and PS-dependent phosphorylation of T34 by PDK1. C. IGF-induced phosphorylation of T34 in Neuro 2A cells. Lysate from IGF-activated Neuro 2 cells was used as positive control (Ctl) for PKC ζ .

Fig. 3.

Akt-membrane interaction measured by surface plasmon resonance (SPR). A. SPR sensorgrams showing that the binding of full-length Akt to unilamellar liposomes (PE/PC/PS/PIP₃: 50/19.8/30/0.2) is lower for the active form compared to the inactive form. In addition to phosphorylation at T308 and S473, phosphorylation of T34 was detected by western blotting in the active Akt. B. Sensorgrams showing the effect of T34 mutation on the membrane binding of the PH domain of Akt. The GFP-PH domain (WT or T34 mutants) was captured by anti-GFP antibody immobilized on a CM5 chip followed by the injection of the unilamellar liposomes (inset).

Fig. 4.

Effect of T34 mutation on the membrane translocation of Akt PH domain. The PH domain translocation was significantly impaired by T34D mutation. Neuro 2A cells expressing GFP-tagged PH domain were stimulated with 10 ng/mg IGF and the translocation was recorded by time-lapse fluorescence microscopy. Showing are the representative micrographs and time course of translocation by using averaged R values ($R = (\text{plasma membrane fluorescence} - \text{cytosolic fluorescence}) / \text{cytosolic fluorescence}$). Data represent three independent experiments.

Fig. 5.

IGF-induced Akt phosphorylation impaired by T34D mutation. A. The time course of IGF-induced Akt phosphorylation at T308 and S473 in Neuro 2A cells expressing full-length GFP-Akt WT or T34 mutants. B. Quantitative data for T308 and S473 phosphorylation of GFP-Akt normalized to total GFP-Akt. Data are presented as mean $\pm$ SE (n=3). *, $p < 0.05$; **, $p < 0.01$. ns, not significant.