Activation of the serine/threonine protein kinase Akt/PKB is a multi-step process involving membrane recruitment, phosphorylation, and membrane detachment. To investigate this process in the cellular context, we employed a live-cell fluorescence imaging approach to examine conformational changes of Akt and its membrane association. A fluorescence resonance energy transfer-based reporter of Akt action (ReAkt) reveals a conformational change that is critically dependent on the existence of a phosphorylatable threonine 308 in the activation loop, because mutations to either aspartate or alanine abolished the change. Furthermore, a mutant carrying a phosphorylation mimic at this position showed diminished membrane association, suggesting that this phosphorylation plays an important role of promoting the dissociation of activated Akt from the membrane. In addition, the membrane-associating pleckstrin homology domain was found to associate with the catalytic domain when Thr\(^{308}\) is phosphorylated, suggesting such an interdomain interaction as a mechanism by which phosphorylation within the catalytic domain can affect membrane association. These studies uncover new regulatory roles of this critical phosphorylation event of Akt for ensuring its proper activation and function.

Serine/threonine kinase Akt, also known as protein kinase B, plays important roles in cellular processes such as cell growth, metabolism, proliferation, and survival (1, 2). Its activation can be initiated by various extracellular signals that turn on phosphoinositides (13). Phosphatidylinositol-dependent kinase 1 is known to phosphorylate Akt at threonine 308 (Thr\(^{308}\) in Akt-1) in the activation loop of the catalytic/kinase domain (7, 8). On the other hand, various kinases have been suggested to phosphorylate serine 473 (Ser\(^{473}\)) located in the C-terminal regulatory hydrophobic motif, including the most recently identified mTOR protein kinase complex containing GBL and rictor (reviewed in Ref. 9).

Although Akt activation has been elaborately dissected through various PH domain and phosphorylation site mutants (10), some important steps in the activation process are still not clearly understood. Specifically, the mechanism by which active Akt molecules dissociate from the membrane remains to be elucidated (11). Such membrane dissociation is critical for Akt to gain access to various substrates in different subcellular locations by translocating to cytosol and nucleus (4, 12). Our previous studies have shown that nuclear Akt activity accumulates, whereas there are still high levels of 3-phosphoinositides present at the plasma membrane, suggesting departure of Akt from the membrane occurs prior to massive degradation of 3-phosphoinositides (13).

We hypothesize that a conformational change generated within Akt during its activation facilitates its dissociation from the membrane despite the presence of high levels of 3'-phosphoinositides. Here we employed fluorescence resonance energy transfer (FRET)-based fluorescence imaging to reveal the conformational changes within full-length Akt and further utilized fluorescence-based membrane recruitment assay to evaluate its membrane association. Taking advantage of the availability of known mutations that affect phosphoinositide binding and individual phosphorylation events of Akt, we set out to investigate the conformational changes and functional effects associated with critical phosphorylation events in Akt activation.

**EXPERIMENTAL PROCEDURES**

ReAktion and Mutant Construction—The cDNA of enhanced cyan fluorescent protein and citrine were fused to human Akt-1/protein kinase B-\(\alpha\) by PCR with Sphl and BglIII incorporated at the 5' and 3' ends of Akt-1 and subcloned into a modified pcDNA3 vector (Invitrogen). All of the point mutations were generated by either QuickChange method (Stratagene) or overlap extension PCR. For plasma membrane targeting, GCIKSRKD was added to the N terminus. The plasma
membrane-targeted (pm) catalytic domain (residues 117-480) and the PH domain (residues 1-116) were cloned by PCR, and the HA tag was fused to the PH domain.

Cell Culture and Imaging—HEK-293, NIH3T3 cells were plated onto sterilized glass coverslips in 35-mm dishes and grown to 30–90% confluency in Dulbecco’s modified Eagle’s medium (10% fetal bovine serum at 37 °C with 5% CO2). The cells were transfected with Lipofectamine 2000 (Invitrogen) or calcium phosphate.

For live-cell imaging, the cells were maintained in buffer in the dark at room temperature and imaged on a Zeiss Axiovert 200 m microscope with a cooled charge-coupled device camera MicroMAX BFT512 (Roper Scientific, Trenton, NJ) controlled by METAFLUOR 6.2 software (Universal Imaging, Downingtown, PA) as described before (13). Briefly, dual emission ratio imaging used a 420DF20 excitation filter, a 450DRLP dichroic mirror, and two emission filters (475DF40 for CFP and 535DF25 for YFP) alternated by a filter changer Lambda 10-2 (Sutter Instruments, Novato, CA). Fluorescence images were background-corrected by subtracting autofluorescence intensity of untransfected cells (or background with no cells) from the emission intensities of cells expressing fluorescent reporters. The ratios of yellow-to-cyan emissions were then calculated at different time points.

Western Blotting and Immunoprecipitation—The cells were lysed with RIPA lysis buffer containing EDTA-free protease inhibitor mixture (Roche Applied Science). Protein concentration in the lysate was determined using BCA protein quantitation assay (Pierce).

For Western blotting, 10–30 μg of total protein were solubilized in SDS sample buffer by boiling and fractionated in a polyacrylamide gel. Protein was transferred to a nitrocellulose membrane and subjected to primary and secondary antibody incubation. Antibodies used in Western blotting were anti-Akt, anti-Thr(P)308 (Cell Signaling Technology), and horseradish peroxidase-labeled goat anti-rabbit IgG secondary antibody (Pierce). The membranes were developed using horseradish peroxidase-based chemiluminescent substrate (Pierce).

For co-immunoprecipitation, 100–200 μg of lysate was immunoprecipitated with mouse monoclonal anti-HA antibody (Cell Signaling Technology) or an anti-Akt antibody (Cell Signaling Technology) overnight at 4 °C, and the resulting immune complexes were captured by incubating with protein A/G beads for 3 h (Santa Cruz Biotechnology). Following three washes with RIPA, the beads were dissolved in Laemmli buffer and boiled for 5 min, and proteins were separated by SDS gel electrophoresis and probed with anti-Akt, anti-Thr(P)308, or anti-HA antibodies, respectively.

FIGURE 1. ReAktion reports the membrane translocation and conformational change of Akt during activation. A, schematic representation of the ReAktion construct. B, YFP fluorescence images of a representative NIH3T3 cell expressing ReAktion showing its membrane translocation upon PDGF stimulation (second panel) and returning to cytoplasm following PI3K inhibition by LY294002 (third panel). C, anti-Akt Western blot analysis of lysates from ReAktion-expressing NIH3T3 cells. D, representative pseudocolor images depicting the changes in emission ratios (yellow/cyan) upon drug addition. E, graphical representation of a representative emission ratio (yellow/cyan) time course of ReAktion in NIH3T3 cells treated with PDGF. F, same as E, except NIH3T3 cells were treated with LY294002 after PDGF stimulation to inhibit PI3K. G, images of a representative NIH3T3 cell expressing ΔPH-ReAktion before and after PDGF addition, showing no translocation. Graphical representation of an emission ratio (yellow/cyan) time course of ΔPH-ReAktion in NIH3T3 cells treated with PDGF. H, images of a representative NIH3T3 cell expressing R23A/R25A ReAktion before and after PDGF addition. Graphical representation of a representative emission ratio (yellow/cyan) time course of R23A/R25A ReAktion in NIH3T3 cells stimulated with PDGF.
Roles of Akt Activation Loop Phosphorylation

RESULTS

Reporter for Akt Action (ReAktion) Reports Akt Translocation and Conformational Change—To detect the conformational change within full-length Akt in living cells, we constructed a FRET-based reporter of Akt action (ReAktion), where full-length Akt-1 is fused to donor CFP at the N terminus and acceptor YFP at the C terminus (Fig. 1A). When ReAktion was expressed in NIH3T3 cells, uniform distribution of fluorescence was observed (Fig. 1B, first panel), and no proteolysis was detected (Fig. 1C). Stimulation of endogenous PDGF receptor resulted in the translocation of ReAktion to the plasma membrane (Fig. 1B, second panel), similarly to endogenous Akt detected by immunofluorescence (not shown). In addition, ReAktion generated an increase in the ratio of yellow-to-cyan emissions (Fig. 1D), which was detectable within several seconds and reached a plateau of 5.5 ± 1.7% (average ± S.D.) (n = 8) within 3–4 min (t½ = 1.65 ± 0.09 min) (Fig. 1E).

To verify that the FRET response and the translocation event were dependent on PI3K activity, PI3K inhibitor LY294002 was added after the response reached a plateau. LY294002 indeed reversed the FRET response (Fig. 1F) as the levels of 3’ phosphoinositides decreased (13). The fluorescence of ReAktion also returned to the cytoplasm (Fig. 1B, third panel). Hence, ReAktion was capable of displaying the same characteristics of Akt upon upstream PI3K activation, consistent with previous observations that tagging Akt at both termini does not change its translocation or phosphorylation (14, 15). In addition to the translocation event, a change in FRET between two fluorophores flanking the full-length Akt was also observed, indicating a conformational change within Akt during its activation.

Previous studies showed that the PH domain is responsible for binding to 3’ phosphoinositides, thus driving Akt to the membrane (4, 10). We therefore constructed a PH domain deletion mutant of ReAktion, ΔPH-ReAktion, and the R23A/R25A ReAktion construct where two key residues involved in binding to anionic lipid head-group (16) were mutated to alanines. Both constructs showed neither translocation nor a FRET change (Fig. 1, G and H), indicating that phosphoinositide binding by the PH domain, as the first step during Akt activation, is required for conferring the conformational change associated with this activation process.

The two events observed during Akt activation, translocation, and conformation change can be separated by taking advantage of the targetability of FRET-based reporters. ReAktion was targeted to the plasma membrane (Fig. 2B) by attaching an N-terminal Lyn targeting sequence (Fig. 2A). This construct, plasma membrane-targeted ReAktion (pm ReAktion), upon PDGF stimulation showed an increase in emission ratio (yellow-to-cyan) of 7.1 ± 3.1% (n = 4), which could be reversed by LY294002 (Fig. 2, B and C and Table 1). Intracellular FRET efficiencies were measured by acceptor photobleaching to be 11 ± 1 and 15 ± 1% (n = 4), before and after PDGF stimulation, respectively. These experiments further demonstrated that a conformational change occurred within Akt during its activation following 3’ phosphoinositide production and eliminated the possibility that the FRET change observed with ReAktion was a mere consequence of the translocation event.

| TABLE 1 |
| Responses of ReAktion phosphorylation mutants in NIH3T3 cells upon PDGF stimulation |

| ReAktion construct and mutants | FRET change (plasma membrane-targeted)* | Translocation (untargeted) |
|-------------------------------|------------------------------------------|---------------------------|
| ReAktion                      | 7.1 ± 3.1% (n = 4)                       | Yes (n = 8)               |
| T308A/S473A                   | None (n = 4)                            | Yes (n = 4)               |
| T308D/S473D                   | None (n = 4)                            | No (n = 4)                |
| T308A                         | None (n = 4)                            | Yes (n = 2)               |
| T308D                         | None (n = 4)                            | No (n = 4)                |
| S473A                         | 11.2 ± 0.8% (n = 4)                      | Yes (n = 5)               |
| S473D                         | 13.4 ± 3.0% (n = 4)                      | Yes (n = 4)               |

* FRET changes are represented by the average percentage changes in emission ratio (yellow/cyan).

Thr308 Phosphorylation and Conformational Change—Phosphorylation at Thr308 and Ser473 leads to full activation of Akt (6). To evaluate the contributions of these phosphorylation events to the conformational change of Akt, we mutated pm ReAktion to mimic constitutively phosphorylated (T308D/S473D) or to generate a nonphosphorylatable mutant (T308A/S473A). Interestingly, both types of double mutations, T308D/S473D and T308A/S473A, abolished the ability of plasma membrane targeted ReAktion to generate a conformational change (Fig. 3, A and B, and Table 1). Both mutants showed no sign of misfolding with uniform distribution of fluorescence from both N-terminal CFP and C-terminal YFP. The fact that Thr308 and Ser473 mutations abolished the observed conformational change suggested that phosphorylation events play an important role in the observed conformational change.

Next we mutated individual phosphorylation sites to evaluate the contributions of either phosphorylation event. Mutation of Thr308 to either Ala or Asp in membrane attached pm ReAktion...
generated mutants that did not show any emission ratio change upon PDGF stimulation or LY294002 treatment, whereas variants with Ser473 mutations, i.e. S473D and S473A, responded to PDGF stimulation in a similar fashion as previously seen with pm ReAktion (Fig. 3, C and D, and Table 1). Thus, mutating Thr308 to either a phosphorylation mimic Asp or a nonphosphorylatable Ala abolished the FRET change, independent of mutations at the Ser473 position. The mutational analysis collectively showed that eliminating the process of Thr308 phosphorylation severely hindered the conformational change undergone by the protein, suggesting that phosphorylation at Thr308 critically contributes to the conformational change.

It is known that phosphorylation at Thr308 is a critical step in Akt activation (6). Such PDGF-induced phosphorylation of

ReAktion was seen in parallel with endogenous Akt in NIH3T3 cells (Fig. 4A). However, previously published studies reported that the plasma membrane-bound Akt molecule is a constitutively active form of Akt, thus capable of mimicking the oncogenicity of viral v-Akt, which is also attached to the membrane through myristoylation of Gag (viral group-specific antigen) protein at the N terminus (17). To determine whether the observed FRET response correlated with an increase in Thr308 phosphorylation in plasma membrane targeted ReAktion, we probed lysates expressing pm ReAktion with anti-Thr(P)308 antibody. Western blot analysis showed basal phosphorylation at Thr308 in pm ReAktion, which was further enhanced upon PDGF stimulation (Fig. 4B). This result was in accordance with the observed stimulant-induced increase in Thr308 phosphorylation in the plasma membrane-targeted Akt (18). These experiments show that the conformational change that occurs within Akt during the activation process not only requires a phosphorylatable Thr308 but also correlates with its phosphorylation.

**Thr308 Phosphorylation and Membrane Association**—To determine the consequence of this conformational change to Akt protein, particularly on its membrane association, we evaluated the membrane recruitment of various mutants by monitoring the translocation of untargeted reporters from the cytosol to membrane upon stimulation. The effects of phosphorylation were evaluated by examining ReAktion variants with double mutations at both Thr308 and Ser473 sites. As shown in Table 1, nonphosphorylatable mutant T308A/S473A ReAktion was able to translocate from cytosol to plasma membrane upon PDGF stimulation. By contrast, the mutant mimicking the constitutively phosphorylated form, T308D/S473D ReAktion, showed no translocation (Table 1).

When contributions from individual phosphorylation events were evaluated, S473A and S473D ReAktion both showed clear translocation (Table 1). T308A ReAktion upon stimulation also showed translocation to the membrane, consistent with a previous report (10). On the other hand T308D ReAktion, which mimics monophosphorylated Akt molecule, showed no translocation. This membrane translocation assay provides direct evaluation of the association of Akt with the plasma membrane containing 3’ phosphoinositides. Reduced membrane association for T308D ReAktion in comparison with wt and T308A

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**FIGURE 3.** The responses of plasma membrane-targeted ReAktion mutants. Shown are graphical representations of the emission ratio (yellow/cyan) time courses of pm ReAktion mutants T308A/S473A (A), T308D/S473D (B), T308D (C), and S473D (D) in NIH3T3 cells treated with PDGF. Insets show representative images of cells expressing plasma membrane targeted ReAktion mutants. The targeted mutants remain appropriately localized to the plasma membrane after PDGF stimulation.

**FIGURE 4.** PDGF stimulated increases in Thr308 phosphorylation. A, lysates from NIH3T3 cells expressing ReAktion probed with anti-Thr(P)308 and anti-Akt antibodies. B, Western blot analysis of pm ReAktion using anti-Thr(P)308 and anti-Akt antibodies.
Roles of Akt Activation Loop Phosphorylation

To understand how phosphorylation of Thr\(^{308}\) in the catalytic domain translates to a loss of membrane association of the catalytic domain during activation.

To further eliminate the possibility of disruption of Ser\(^{473}\) phosphorylation status at Ser\(^{473}\), showed clear translocation, and the same was true for the unphosphorylatable CFP-T308A/S473A construct (Fig. 5). Consistent with the data obtained in vitro, the catalytic domain was found to associate with the PH domain and a mutant plasma membrane catalytic domain carrying the T308A mutation (Fig. 6D). The T308A mutation in the catalytic domain abolished IGF-1-induced association with the PH domain, demonstrating the requirement of phosphorylation of Thr\(^{308}\) for the interdomain communication between the PH domain and catalytic domain. These experiments identified a novel association between PH domain and catalytic domain in the presence of a phosphorylated Thr\(^{308}\), providing a possible mechanism for modulating the properties of the PH domain following changes in the catalytic domain during activation.

DISCUSSION

New Approaches for Examining Akt Activation—FRET-based live-cell imaging has found increasingly widespread use in the investigation of molecular mechanisms of cellular processes because of its capability of monitoring dynamic molecular events in real time and space (20, 21). In this study, an approach combining mutational analysis with live-cell fluorescence imaging was used to dissect how various regulatory elements contribute to the conformational changes of Akt and its association with the plasma membrane. Compared with complementary in vitro approaches that evaluate Akt conformational changes by the C-terminal YFP fusion, we omitted YFP and only fused CFP to the N terminus of Akt and some of the Akt mutants to monitor their membrane translocation. Our wild type positive control construct CFP-Akt showed translocation to the membrane upon stimulation as observed in previous studies (Fig. 5) (11). CFP-S473D, which mimics single phosphorylation at Ser\(^{473}\), showed clear translocation, and the same was true for the unphosphorylatable CFP-T308A/S473A construct (Fig. 5). Consistent with the data obtained using dual fluorophore-labeled constructs (Table 1), the CFP-T308D/S473D construct showed no discernible membrane translocation (Fig. 5).

Thr\(^{308}\) (in Akt-1) is located in an activation segment or loop that plays a central role in regulating the catalytic activity of many kinases (19). The aforementioned data suggest that, in addition to the role in regulating kinase activity, Thr\(^{308}\) phosphorylation may also reduce the membrane binding affinity of Akt, possibly facilitating its detachment from the membrane despite the presence of high levels of 3’ phosphoinositides.

Association between PH Domain and Catalytic Domain—An implication of the aforementioned data is that phosphorylation at Thr\(^{308}\) in Akt reduces its membrane association. Yet, how does phosphorylation of Thr\(^{308}\), located in the catalytic domain, affect membrane association, a function specifically assigned to the N-terminal PH domain? We hypothesize that a conformational change induced by Thr\(^{308}\) phosphorylation relies on some interdomain communication between the PH domain and catalytic domain to generate such functional effects.

To understand how phosphorylation of Thr\(^{308}\) in the catalytic domain translates to a loss of membrane association of N-terminal PH domain, we tested whether there exists any direct or indirect interaction between these two domains of Akt in cells. HEK293 cells were co-transfected with HA-tagged PH domain and a plasma membrane-targeted catalytic domain (pm catalytic domain) and stimulated with insulin-like growth factor (IGF). Co-immunoprecipitation experiments were carried out with an anti-HA antibody, and the immunoprecipitates were probed for the catalytic domain. As shown in Fig. 6A, the catalytic domain was found associated with PH domain and appeared as a distinct band in the co-immunoprecipitate.

To determine whether the interaction between the PH domain and catalytic domain is dependent on the phosphorylation of Thr\(^{308}\), HEK293 cells expressing HA-PH and pm catalytic domain were subject to different treatments. Lysates were then prepared from cells that were unstimulated, pretreated with LY294002, stimulated with IGF-1 to activate PI3K, or stimulated with IGF-1 in the presence of LY294002 to block PI3K activity, respectively. The catalytic domain was immunoprecipitated from cell lysates with a monoclonal anti-Akt antibody. The immunoprecipitates were next probed with an anti-HA antibody (Fig. 6B). Conversely, the HA-PH domain was immunoprecipitated with an anti-HA antibody and then probed with anti-Akt antibody and anti-Thr(P)\(^{308}\) antibody (Fig. 6C). The catalytic domain was found to co-immunoprecipitate with the HA-PH domain from cells stimulated with IGF-1, and to a lesser degree, from the unstimulated cells (Fig. 6, B and C). More importantly, the association was correlated with phosphorylation of Thr\(^{308}\), with diminished association and Thr\(^{308}\) phosphorylation in the LY294002-treated samples (Fig. 6C).

To verify that phosphorylation of Thr\(^{308}\) was required for association of the catalytic domain with the PH domain, the co-immunoprecipitation experiment was performed using cells expressing the PH domain and a mutant plasma membrane catalytic domain carrying the T308A mutation (Fig. 6D). The T308A mutation in the catalytic domain abolished IGF-1-induced association with the PH domain, demonstrating the requirement of phosphorylation of Thr\(^{308}\) for the interdomain communication between the PH domain and catalytic domain. These experiments identified a novel association between PH domain and catalytic domain in the presence of a phosphorylated Thr\(^{308}\), providing a possible mechanism for modulating the properties of the PH domain following changes in the catalytic domain during activation.

FIGURE 5. Membrane translocation of CFP-Akt constructs. NIH3T3 expressing CFP-Akt and phosphorylation mutants were stimulated with PDGF. These representative CFP fluorescence images show translocation for CFP-Akt, CFP-S473D, and CFP-T308A/S473A constructs and no translocation for CFP-T308D/S473D. The arrows indicate translocation of CFP-Akt, CFP-S473D, and CFP-T308A/S473A to the plasma membrane.

ReAktion suggests that Thr\(^{308}\) phosphorylation plays a role in modulating the membrane association.

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fluorophores upon stimulation. In contrast, ReAktion and plasma membrane-targeted ReAktion both showed a gain of FRET upon stimulation. This reversal of the sign in the FRET change is likely due to the difference in the actual fluorophores and in the linkers connecting the fluorophores to Akt, thereby resulting in a difference in the distance and relative orientation between the FRET pairs (23). Since both distance and orientation contribute to FRET, the increase in the energy transfer between the CFP and YFP in ReAktion clearly indicates a conformational change, although the exact nature of the change may not be directly deduced from the sign of the FRET change. In this study, combining mutational analysis with live-cell imaging using ReAktion, we demonstrated that the conformational change observed during Akt activation requires a phosphorylatable Thr<sup>308</sup>. This initial finding further led to a series of experiments that unraveled the new regulatory roles of Thr<sup>308</sup> phosphorylation.

Membrane recruitment assay was used to evaluate membrane association of various mutants in a series of experiments. Direct visualization of membrane departure of Akt requires synchronized action of a significant number of Akt molecules and can be achieved when massive degradation of 3′ phosphoinositides occurs upon inhibition of PI3K in NIH3T3 cells (Fig. 1B, third panel). In the absence of PI3K inhibitors, the levels of 3′ phosphoinositides remain high for an extended period of time after PDGF stimulation of NIH3T3 cells (13, 24). It can be envisioned that at a given time, a subpopulation of already activated Akt molecules depart from the membrane, and some, upon deactivation, travel back to get reactivated. Visualization of such trafficking of individual Akt molecules may be achieved with single-molecule imaging in future studies. Of note, the kinetics of Akt activation and patterns of trafficking can be cell type-specific. In fact, ligation of B cell antigen receptor was shown to induce a transient localization of Akt to the plasma membrane and a sustained membrane localization of Akt domain itself, which led to the speculation that a molecular mechanism exists to cause active Akt to dissociate from the plasma membrane in B cells despite the continued generation of 3′ phosphoinositides (11). In this study, a series of experiments based on the membrane recruitment assay revealed critical involvement of Thr<sup>308</sup> phosphorylation in regulation of dissociation of active Akt from the plasma membrane.

**Effects of Thr<sup>308</sup> Phosphorylation—Thr<sup>308</sup> (in Akt-1) is located in an activation segment or loop that is housed in the kinase domain of all AGC kinases (25). This activation loop plays a central role in regulating the catalytic activity of the kinases, where phosphorylation of a threonine residue between the invariant DFG and APE motifs is required for enzyme activation (19, 26). By analogy, Thr<sup>308</sup> present in the activation segment of Akt is crucial for its activity, and its phosphorylation alone increases the activity of Akt-1 by 10-fold (6).

Does this phosphorylation have any other functional consequence? By monitoring membrane translocation of various ReAktion mutants, a permanently phosphorylated Thr<sup>308</sup> was found to prevent the membrane translocation of the protein upon stimulation, which indicated reduced association with the plasma membrane containing 3′ phosphoinositides. Hence, Thr<sup>308</sup> phosphorylation not only enhances Akt activity, but...
Roles of Akt Activation Loop Phosphorylation

![Diagram of Akt activation](image)

**FIGURE 7. A model of Akt activation.**

- **A**, inactive Akt molecule remains in the cytosol. Stimulation leads to PI3K activation and generation of 3'-phosphoinositides at the plasma membrane. Akt molecule is recruited to the plasma membrane through specific binding of 3'-phosphoinositides by PH domain (Step 1). The blue circle depicts the phosphoinositide-binding pocket in the PH domain. **B**, Akt undergoes phosphorylation at Ser473 and Thr308 (Step 2). Phosphorylated Thr308 and Ser473 are represented by red ovals. Phosphorylation in the activation loop at Thr308 leads to a conformational change within Akt (shown by an intermediate), resulting in the loss of membrane binding affinity of N-terminal PH domain. **C**, active Akt detaches from the membrane (Step 3) to phosphorylate downstream substrates in other subcellular locations such as nucleus. In active Akt, the PH domain is blocked from membrane association by direct interdomain interaction between the PH domain and catalytic domain or with the aid of other protein partners.

may also reduce the membrane binding affinity of Akt, facilitating its detachment from the membrane despite the presence of high levels of 3'-phosphoinositides. On the other hand, phosphorylation of Ser473 does not appear to have such a significant effect. Of note, recent studies showed that Akt with defective Ser473 phosphorylation affected only a subset of substrate targets in vivo, suggesting that Ser473 phosphorylation is dispensable to some extent (27). In light of the demonstration that Thr308 phosphorylation is the second phosphorylation event (28), we propose that this phosphorylation enables the final step of departure of Akt from the plasma membrane after it is fully activated.

**Communication between Domains**—Different domains of Akt are known to have distinct functions. For instance, PH domain is involved in binding to 3'-phosphoinositides and association with the plasma membrane, and the catalytic domain is responsible for the kinase activity. However, our experiments suggest that these domains do not function completely independent of one another, and there exists a functional "communication" between them. A phosphorylation event in the catalytic domain (at Thr308) may affect the membrane association via the PH domain. To understand the molecular basis for such "communication," we performed co-immunoprecipitation experiments and detected a novel association between the PH domain and catalytic domain, which is correlated with the phosphorylation state of Thr308.

Such interaction could be direct or via other interaction partners. Recent studies suggest that individual PH domains, in addition to binding phosphoinositides, may interact with protein binding partners to play more specific regulatory roles (29). PH domain has also been shown to be involved in interacting with Tc11 (T-cell leukemia-1), a protein involved in Akt regulation (30, 31). Involvement of potential interacting partners and the effect of different phosphorylation events on such interdomain interaction will be subjects of further studies.

**New Regulatory Roles of Activation Loop Phosphorylation**—A model of Akt activation is shown in Fig. 7, where new regulatory roles of Thr308 phosphorylation are depicted. Phosphorylation at Thr308 triggers a conformational change within active Akt, which alters the membrane binding properties of the PH domain via the association and functional "communication" between the PH domain and catalytic domain. The reduced membrane association may result from shielding of the phosphoinositide-binding region of the PH domain by direct interaction with the catalytic domain or via recruitment of other interaction partners. Future studies will look into the nature of this new mode of dynamic regulation of PH domain function.

A direct functional effect of Thr308 phosphorylation is therefore reduced membrane affinity of Akt despite high levels of 3'-phosphoinositides, which facilitates dissociation of active Akt from the membrane to phosphorylate its target substrates in other cellular compartments. Akt molecules that have been inactivated by phosphatases could also be recruited to these vacant phosphatidylinositol 3,4,5-trisphosphate-rich membrane-binding sites and get reactivated until lipid phosphatases degrade 3'-phosphoinositides. Thus, this mechanism for the departure of Akt from the membrane prior to massive degradation of phosphoinositides would ensure appropriate temporal control and accumulation of Akt activity in various subcellular locations, which would not be solely dependent on the levels of lipid second messengers. Illustrated here are novel roles of activation loop phosphorylation in regulating the spatial and temporal distribution of the activity of a kinase, the generality of which awaits further investigation. The live-cell
fluorescence-based molecular approach used in this study should also be generally applicable to facilitate such investigations in the native cellular environment to delineate molecular mechanisms of cell signaling.

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