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Alternative Activation Mechanisms of Protein Kinase B Trigger Distinct Downstream Signaling Responses*

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Background: Protein Kinase B (PKB) phosphorylates many targets to induce various cellular responses.
Results: Whereas some regulatory features of PKB control intrinsic kinase activity, others regulate substrate specificity.
Conclusion: PKB activation in the cytosol or via membrane targeting trigger distinct downstream signals.
Significance: These data are important to develop therapeutics that selectively target specific branches of PKB signaling.

Protein kinase B (PKB/Akt) is an important mediator of signals that control various cellular processes including cell survival, growth, proliferation, and metabolism. PKB promotes these processes by phosphorylating many cellular targets, which trigger distinct downstream signaling events. However, how PKB is able to selectively target its substrates to induce specific cellular functions remains elusive. Here we perform a systematic study to dissect mechanisms that regulate intrinsic kinase activity *versus* mechanisms that specifically regulate activity toward specific substrates. We demonstrate that activation loop phosphorylation and the C-terminal hydrophobic motif are essential for high PKB activity in general. On the other hand, we identify membrane targeting, which for decades has been regarded as an essential step in PKB activation, as a mechanism mainly affecting substrate selectivity. Further, we show that PKB activity in cells can be triggered independently of PI3K by initial hydrophobic motif phosphorylation, presumably through a mechanism analogous to other AGC kinases. Importantly, different modes of PKB activation result in phosphorylation of distinct downstream targets. Our data indicate that specific mechanisms have evolved for signaling nodes, like PKB, to select between various downstream events. Targeting such mechanisms selectively could facilitate the development of therapeutics that might limit toxic side effects.

Protein kinase B (PKB, also known as Akt)[5] is a member of the AGC kinase family that mediates signals induced by growth factors and cytokines (1–3). By phosphorylating a large number of downstream targets, PKB controls several important cellular responses, including cell growth, survival, proliferation, or metabolism (4). Three isoforms exist in mammals: PKBα/Akt1, PKBβ/Akt2, and PKBγ/Akt3. Mouse studies indicate that all isoforms are important for animal growth; PKBα is particularly implicated in placental development and adipocyte differentiation, PKBβ is particularly implicated in glucose metabolism and adipogenesis, and PKBγ is particularly implicated in postnatal brain development (reviewed in Refs. 5 and 6). Activating mutations, amplifications, and overexpression of PKB is found in a number of malignancies, and in general activation of the PI3K-PKB signaling pathway is one of the most frequent alterations in cancer (reviewed in Refs. 7 and 8). Conversely, inactivation of PKB causes type II diabetes, as well as cardiovascular and neurological diseases (7).

All PKB isoforms share a common domain structure that includes a N-terminal pleckstrin homology (PH) domain, a serine/threonine kinase domain, and a C-terminal hydrophobic motif (HM), typical of AGC kinases (see Fig. 1*A* and Table 1). Following growth factor stimulation, the PH domain targets PKB to the plasma membrane via binding to the lipid phosphatidylinositol 3,4,5-trisphosphate (PIP3), which is generated by type I PI3K. Membrane targeting is a crucial step in PI3K-induced PKB activation (9) and induces phosphorylation on two important phosphorylation sites, Thr-308 in PKBα (Thr-309 in PKBβ) within the activation loop of the kinase domain and Ser-473 (Ser-474 in PKBβ) in the HM. Phosphorylation at both sites induces full catalytic activity of PKB (10). The upstream kinases are PDK1 for Thr-308 (11) and mTORC2, as well as DNA-PK for Ser-473 (12–14). PIP3 binding to the PH domain of PKB has a dual effect in inducing efficient phosphorylation of Thr-308 by PDK1. First, the PH domain appears to

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[5] The abbreviations used are: PKB, protein kinase B; PH, pleckstrin homology; HM, hydrophobic motif; PIP3, phosphatidylinositol 3,4,5-trisphosphate; PIF, PDK1-interacting fragment; IGF1, insulin-like growth factor 1, TCEP, tris(2-carboxyethyl)phosphine; SPR, surface plasmon resonance.

sequester the Thr-308 phosphorylation site, and PIP3 binding exposes the site for efficient phosphorylation (11, 15). Second, PKB and PDK1, which also contains a PIP3 binding PH domain, colocalize at PIP3-rich membranes for efficient Thr-308 phosphorylation (16). Phosphorylation of Ser-473 in the HM has been shown to enhance PKB activity by promoting the HM to dock to a hydrophobic pocket within the kinase N-lobe of PKB (17). Structural studies indicate that both a productive active site and the hydrophobic pocket are not formed in unphosphorylated PKB (18), but Thr-308 phosphorylation and HM docking synergize to stabilize the helices αB and αC in the kinase N-lobe, which is required for an active kinase conformation (17).

PDK1 phosphorylates and activates many AGC kinases. However, because most AGC kinases do not have a PIP3 binding PH domain, they employ a different mechanism for PDK1 colocalization. They first undergo phosphorylation at their HM, which enables them to interact with the hydrophobic pocket in PDK1, also referred to as the PDK1-interacting fragment (PIF) pocket. This mechanism (hereafter referred to as the PIF pocket mechanism) was shown to be required for efficient phosphorylation of the activation loop of several AGC kinases but not for PKB (19–22). Only recently it was suggested that in scenarios where PDK1 efficiency is reduced the PIF pocket mechanism could be utilized for Thr-308 phosphorylation of PKB (23, 24). Further, PKB has been shown to be activated in the nucleus upon DNA damage by DNA-PK (25), which phosphorylates the HM of PKB and hence might also promote the PIF pocket mechanism.

An important feature of PKB signaling is the large number of identified cellular substrates (4). Presumably, phosphorylation of specific subsets of PKB substrates is responsible for triggering distinct cellular processes. A fundamental unresolved question is how PKB is regulated to selectively phosphorylate its substrates to achieve a desired cellular response. One model is that different threshold levels of PKB activity trigger distinct responses (24). In particular it was suggested that low levels of PKB signaling support cell survival and proliferation, whereas more specialized functions such as neuron differentiation and T-cell trafficking require higher PKB activity (26, 27). However, specific regulatory features, such as Ser-473 phosphorylation, have also been implicated in substrate specificity (28, 29).

In this study, we address the question which of the regulatory mechanisms of PKB control intrinsic kinase activity and which affect various substrates differentially to provide substrate specificity. To this end, we use highly purified PKB forms in well defined states, containing or lacking the important regulatory elements. We show that activation loop phosphorylation by PDK1 and the presence of the HM are essential to promote an active kinase conformation. On the other hand, phosphorylation of the HM, the PH domain, and PIP3 binding to the PH domain mainly affect substrate selectivity. Our data further indicate that PIF pocket-mediated phosphorylation of PKB can occur in the absence of PI3K activity and PIP3 binding. This leads to a model where PKB activation either via PI3K or the PIF pocket mechanism results in phosphorylation of distinct subsets of PKB substrates, hence triggering different cellular responses.

Experimental Procedures

Generation of Purified PKB Proteins—PKBβ proteins were expressed as His_6 tag fusions in *Escherichia coli* BL21 (DE3). Initial purification was performed by nickel-chelating affinity purification, followed by protease cleavage to remove the tag. Proteins were further purified by ion exchange chromatography using a Source 15S cation exchange column (PH-containing constructs) or a source 15Q anion exchange column (kinase constructs) (GE Healthcare). In a final step, proteins were purified by size exclusion chromatography using a Superdex 200 column (GE Healthcare). To obtain PKB proteins phosphorylated on Thr-309, purified PKB proteins were incubated at 20 μM at RT for 4 h with 1 μM of purified PDK1 kinase domain (human PDK1 76–360), 2 mM ATP, 4 mM $MgCl_2$, 20 mM HEPES, pH 7.0, 200 mM NaCl, 5% glycerol, and 2 mM TCEP. Stoichiometric and specific single phosphorylation was verified by intact mass spectrometry for PKB 1–481 S474D on a maXis QQ-TOF instrument (Bruker) (see Fig. 1*B*), and equal phosphorylation levels for all constructs was verified by Western blot.

Kinase Activity—An enzyme-coupled kinase assay was employed to determine ATP turnover as described before (30). In brief, kinase reactions contained 1 μM of unphosphorylated PKB or 0.1 μM of phosphorylated PKB, 20 mM HEPES, pH 7.0, 100 mM NaCl, 2 mM TCEP, 10 mM $MgCl_2$, 100 mM phosphoenolpyruvate, 0.28 mM NADH, 0.08 units/μl pyruvate kinase, 0.1 units/μl lactate dehydrogenase, and 50 μM of the peptide substrate ARKRERTYSFGHHA (Bio-Synthesis Inc.). For results reported in Fig. 1*E*, reactions were performed in presence or absence of 10 μM soluble PIP3 containing eight carbon acyl chains (diC8-PIP3; Echelon Biosciences). Reactions were initiated by addition of 0.5 mM ATP. Absorbance at 340 nm was monitored for 20 min, and the initial slope was used to calculate initial velocities.

In Vitro Thr-309 Phosphorylation Monitored by ELISA—PKB phosphorylation time courses were performed at room temperature using 400 nM purified PKB protein, 100 nM PDK1 kinase domain (PDK1 76–360), 1 mM ATP, and 2 mM $MgCl_2$. Reactions were performed in the presence or absence of 10 μM diC8-PIP3 (Echelon Biosciences) or 200 μM lipid vesicles containing either 10% (mol/mol) PIP3 (16:0; Echelon Biosciences) and 90% (mol/mol) phosphatidylcholine (PC, from chicken egg; Avanti Polar Lipids) (resulting in 10 μM PIP3 on the outer leaflet of vesicles) or 100% PC. Reactions were stopped by addition of 30 mM EDTA. Thr-309 phosphorylation levels were quantified by a standard ELISA method using a specific anti-phospho-AKT (Thr-308) antibody (Cell Signaling) as a primary antibody, a HRP-conjugated anti-rabbit immunoglobulin antibody (Dako) as a secondary antibody, and TMB (3,3′,5,5′-tetramethylbenzidine) (Calbiochem) for detection. Following addition of 90 mM sulfuric acid, absorbance was recorded at 450 nm.

PIP3 Binding Assays by Fluorescence Polarization—Affinities of PKB to soluble PIP3 was determined using a fluorescence polarization assay adapted from Ref. 31. The purified PKB proteins (at 18.5 nM, 39 nM, 78.1 nM, 312.5 nM, 625 nM, and 1.25 μM) were added to 12.5 nM of BODIPY-TMR-labeled diC6-PIP_3 (Echelon Biosciences) in buffer containing 20 mM HEPES, pH

7.5, 150 mM NaCl, 1 mM TCEP and incubated for 20 min at room temperature. Fluorescence anisotropy was measured at a excitation wavelength of 544 nm and a emission of 570 nm. Binding curves and dissociation constants were determined by fitting a one-site binding model.

SPR Measurements—PIP3 vesicles were prepared containing 10% (mol/mol) PIP3 (16:0; Echelon Biosciences) and 90% (mol/mol) PC (from chicken egg; Avanti Polar Lipids) at a final total lipid concentration of 1.5 mM. Organic solvent was removed by rotary evaporation for 2 h at 40 °C. The lipid film resuspended in SPR running buffer (20 mM HEPES, pH 7.5, 150 mM NaCl, 1 mM TCEP) and subjected to six cycles of freeze-thawing. To obtain unilamellar vesicles, the lipid sample was passed 15 times through a membrane with 100-nm pore size using a mini extruder (Avanti Polar Lipids). SPR experiments were performed on a Biacore X100 instrument (GE Healthcare). The L1 sensor chip was coated by injecting PIP3 vesicles for 15 min at a flow rate of 2 μl/min, followed by two 1-min injections of 100 mM NaOH solution at 10 μl/min to remove loosely bound vesicles. PKB proteins were injected at 500 nM (which was determined not to result in a maximal response) for 2 min at a flow rate of 30 μl/min in running buffer. Dissociation was monitored for 10 min. The following equation was fitted to the first 20 s of the association phase: $RU = R_{max}*(1 - \exp(-k_{obs}*t))$, where RU = SPR response, R_{max} = maximal RU at steady state, t = time from start of injection, and k_{obs} = apparent kinetic association constant. Fitted average R_{max} and k_{obs} values from multiple injections are shown in Table 2. Dissociation phases did not follow a single exponential behavior, and reliable k_{off} values could not be determined.

Substrate Phosphorylation in Cell Extracts—HeLa cells were cultured in serum-free medium, DMEM for 16 h. Cells were pelleted, washed with PBS, and resuspended in kinase lysis buffer (20 mM HEPES, pH 7.4, 5 mM $MgCl_2$ and 0.5 mM EGTA, 100 μM ATP, 1 mM DTT) and one-third volume of glass beads (particle size, 425–600 μm) was added. For lysis, samples were vortexed twice at maximum speed for 30 s and subsequently centrifuged for 10 min at 4 °C at 13,000 rpm, and the supernatant was collected. 1 μg of unphosphorylated or phosphorylated PKB protein was incubated with the soluble cell lysates at 30 °C and stopped after 5 min by addition of Laemmli sample buffer. Samples were immunoblotted with the primary antibodies indicated in Fig. 4 (phosphosites of pPKB antibodies corresponds to PKBα numbering) and developed by chemiluminescence.

To monitor phosphorylation of PKB targets in presence of PIP3, lipid vesicles were added to soluble cell lysates to obtain a final PIP3 concentration of 1 μM on the outer leaflet of vesicles. PIP3 vesicles were prepared by mixing phospholipids (Avanti Polar Lipids) to obtain 6% PIP3 (18:1) in 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine. Solvent was removed on a rotary evaporator, and lipid films were hydrated with 20 mM HEPES, pH 7.0, 200 mM NaCl, and 2 mM TCEP and sonicated until resuspended.

Substrate Phosphorylation with Purified Substrates—FOXO3a and GSK3β were expressed as GST fusions in *E. coli* Rosetta2(DE3)pLys and purified using glutathione-Sepharose beads (GE Healthcare). TSC2 was purified from starved HeLa cells by immunoprecipitation using protein G coupled to Sepharose beads (GE Healthcare). The purified substrates were incubated for 5 min at 30 °C with 200 nM of Thr-309 phosphorylated PKB in 20 mM HEPES, pH 7.0, 150 mM NaCl, 5% glycerol, 2 mM TCEP, 1 mM ATP, and 2 mM $MgCl_2$. PIP3-containing vesicles (prepared as above) were incubated 10 min with the PKB proteins prior to phosphorylation reactions. Substrate phosphorylations were monitored by immunoblotting using phospho-specific antibodies.

Cellular PKB Activation in Presence of Selective PI3K, DNA-PK, or PKB Inhibitors—HeLa and HCT-116 cells were cultured in serum-free medium, DMEM for 16 h. The cells were then treated for 2 h with 5 μM of the PI3K inhibitor (ETP46321) (32, 33), the DNA-PK inhibitor (NU7441) (34), or the PKBα/β inhibitor VIII (Sigma). To induce DNA damage, pretreated cells were irradiated with UV (1.5 s, 30V) and put back into culture with the inhibitors for 1 h. Alternatively, PKB activation was induced by adding 100 ng/ml insulin-like growth factor 1 (IGF1) to the media 30 min before harvesting the cells. Cells were harvested and lysed as described above.

Results

Thr-309 Phosphorylation and the HM Are Essential for Catalytic Activity of PKBβ—We generated various forms of PKBβ (Fig. 1*A*), which were highly purified by three consecutive chromatographic steps (see "Experimental Procedures"). Thr-309 phosphorylated PKB was generated by *in vitro* phosphorylation with purified PDK1 kinase domain. Stoichiometric phosphorylation was verified by intact mass spectrometry (Fig. 1*B*). Ser-474 phosphorylation was mimicked using a S474D mutation, as previously described (10, 17). We first tested how the various regulatory elements contribute to the intrinsic catalytic activity of PKB by measuring ATP turnover with a peptide substrate. We found that both Thr-309 phosphorylation and the HM were required for significant levels of activity (Fig. 1*C*). As reflected by determined K_{cat} values, the S474D mutation increased and the presence of the PH domain decreased catalytic turnover (Fig. 1*D* and Table 1). On the other hand, the PH domain did not significantly affect the K_m for the peptide substrate used, whereas the S474D mutation increased K_m values ~2-fold. As a consequence the S474D mutation did not significantly alter the catalytic efficiency (K_{cat}/K_m) (Table 1). Further, the presence of diC8-PIP3 did not significantly affect the activity of any PKB form tested (Fig. 1*E*). Together, these data confirm that Thr-309 phosphorylation and the HM are the main determinants of intrinsic PKBβ activity.

PIP3 Binding and the HM Enhance Thr-309 Phosphorylation in Solution—Because Thr-309 phosphorylation by PDK1 is one of the critical events triggering PKB activity (Fig. 1*C*), we next tested which of the regulatory features of PKB affect the phosphorylation efficiency by monitoring Thr(P)-309 levels of PKB upon incubation with the purified PDK1 kinase domain (PDK1 76–360), lacking the PIP3 binding PH domain. In line with previous reports (11, 15), our studies confirmed that the PH domain of PKB negatively regulates Thr-309 phosphorylation *in vitro* (Fig. 2, *A* and *B*). Interestingly, binding of diC8-PIP3 or PIP3 vesicles to the PH domain restored the Thr-309 phosphorylation rate to similar levels as observed for PKB lacking the PH

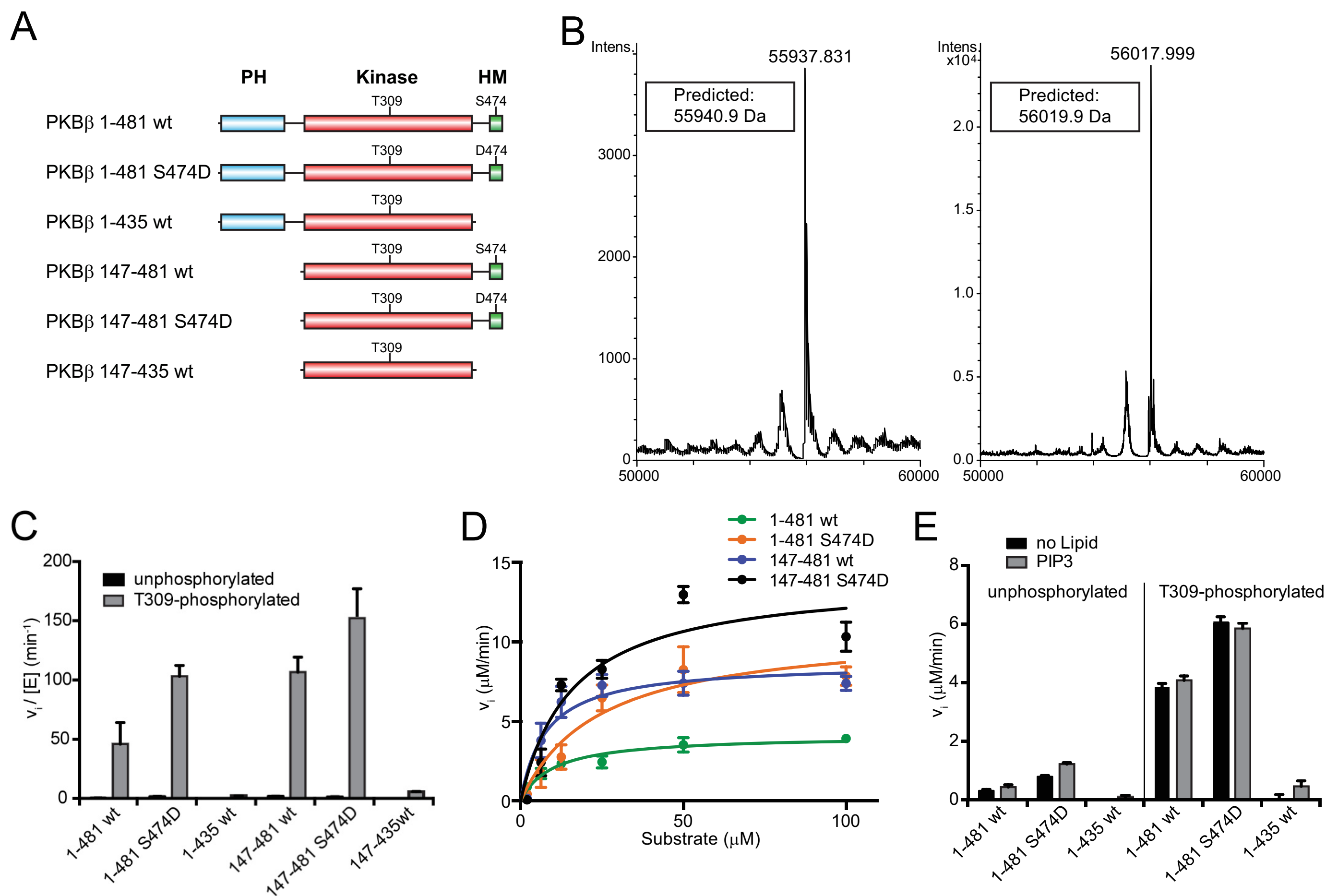

FIGURE 1. **Catalytic activity of purified PKB proteins.** *A*, schematic domain structure of the purified PKB forms generated. Thr-309 phosphorylated forms were generated by incubation with purified PDK1, whereas Ser(P)-474 was mimicked by the S474D mutation. *B*, intact mass analysis of unphosphorylated (*left*) and PDK1 phosphorylated (*right*) PKB is shown for PKBβ 1–481 S474D. The electrospray mass spectrometry analysis indicates stoichiometric addition of a single phosphate during the PDK1 phosphorylation reaction. Experimentally determined and predicted molecular weights are indicated. *C*, catalytic turnover of PKB proteins was determined with a peptide substrate at a concentration of 50 μM. Plotted are initial velocities (v_i) divided by the enzyme concentration ([E]), which was 0.1 μM for phosphorylated and 1 μM for unphosphorylated PKB to ensure sufficient signal. *D*, substrate titration of phosphorylated PKB forms. The *curves* were fitted with the Michaelis-Menten equation using Prism (GraphPad Software). Derived kinetic parameters are reported in Table 1. *E*, PKB activity in presence or absence of diC8-PIP3. *C–E*, unphosphorylated PKB is assayed at 1 μM; phosphorylated forms are assayed at 0.1 μM. The *error bars* represent S.D. from three independent experiments.

TABLE 1
Enzyme kinetic parameters of Thr-309 phosphorylated PKBβ

The parameters V_{max} and K_m were derived by fitting the data shown in Fig. 1*D* to the Michaelis-Menten equation. K_{cat} is calculated as follows: $K_{cat} = V_{max}/[E]$. [E] = Enzyme concentration = 0.1 μM.

	1–481 WT	1–481 S474D	147–481 WT	147–481 S474D
V_{max} (μM/min)	4.1 ± 0.2	10.8 ± 1.1	8.7 ± 0.6	14.0 ± 1.4
K_m (μM)	10.0 ± 1.9	24.0 ± 6.5	7.8 ± 2.0	15.9 ± 4.8
K_{cat} (min^{-1})	41.3	108.1	87.4	140.4
K_{cat}/K_m (min^{-1} μM^{-1})	4.1	4.5	11.2	8.8

domain. Further, we found that the presence of the HM was essential for efficient Thr-309 phosphorylation by PDK1. The S474D mutation in the HM further increased Thr-309 phosphorylation rates. Although PDK1 reactions at longer time points appear nonlinear (Fig. 2*A*), reactions at 5 min exhibit a linear dependence with varying PDK1 concentrations (Fig. 2*C*). These data indicate that the HM is essential for both catalytic activity and Thr-309 phosphorylation in solution, whereas PIP3 binding only promotes Thr-309 phosphorylation, but not activity. We note that the HM requirement for efficient Thr-309 phosphorylation likely only applies to a scenario where PKB is phosphorylated in the absence of membrane-mediated colocalization with PDK1.

The Activation State of PKB Affects Binding to PIP3 Membranes, but Not Soluble PIP3—The fact that PIP3 binding to the PH domain of PKB releases a negative regulatory effect on PDK1 phosphorylation suggests that PIP3 induces a conformational change in PKB, which exposes Thr-309 for more efficient phosphorylation. A potential scenario could be that PIP3 is competing with a protective intramolecular interaction between the PH and kinase domain. As a consequence, one would expect differences in PIP3 affinity between different PKB forms. To test this, we first determined PKB affinities to fluorescently labeled soluble diC6-PIP3. Surprisingly, all PKB forms exhibited a similar affinity for PIP3, with K_D values in the range of 40–100 nM (Fig. 3, *A–D*). As expected, PKB lacking the PH domain did not interact with diC6-PIP3. Next, we measured interactions of PKB to PIP3-containing vesicles by SPR. Although we found no significant difference in the apparent kinetic association parameter k_{obs} (Table 2), the steady state

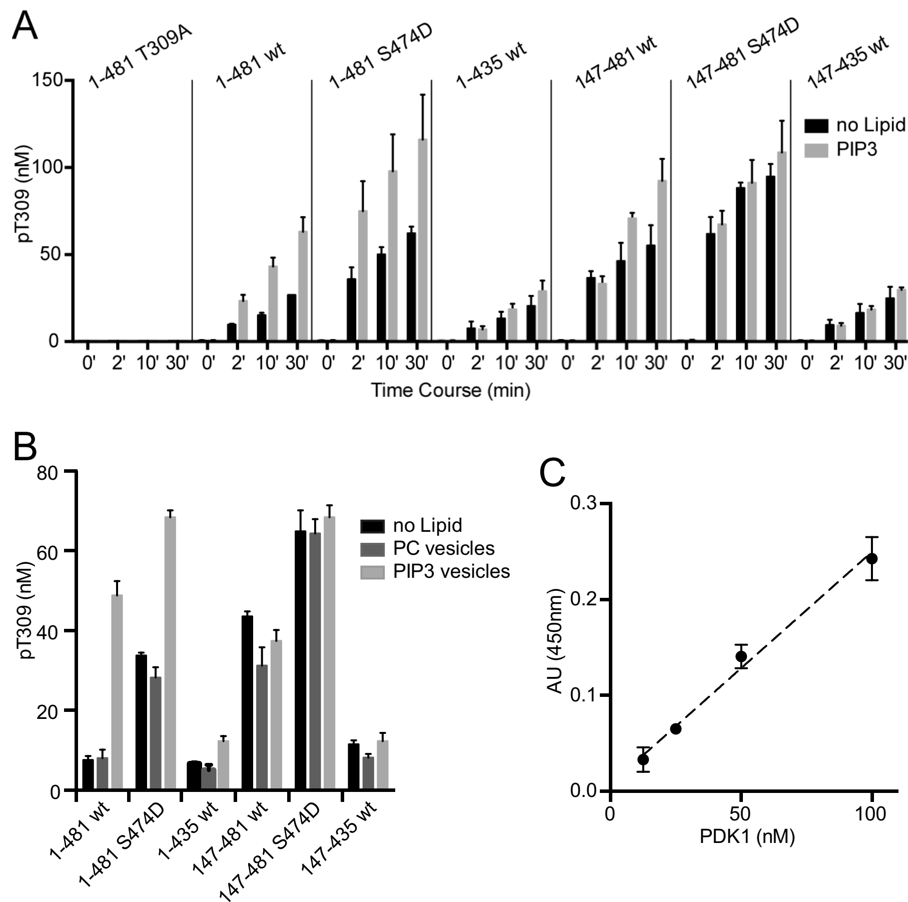

FIGURE 2. **PDK1 mediated Thr-309 phosphorylation of PKB.** *A*, the efficiency of Thr-309 phosphorylation of PKB proteins by the PDK1 kinase domain (PDK1 76–360) in the presence or absence of diC8-PIP3 was measured at the indicated time points using an ELISA method. The total PKB concentration in the reactions is 400 nM, indicating incomplete reactions also after 30 min. *B*, Thr-309 phosphorylation of PKB in presence or absence of PIP3-containing or PC vesicles was measured after 5 min of incubation with the PDK1 kinase domain. *C*, Thr-309 phosphorylation at 5 min of the fastest reaction with PKB 147–481 S474D behaves linear with varying PDK1 concentrations. *A–C*, the *error bars* represent S.D. from three independent experiments.

SPR response (R_{max}) was consistently lower for Thr-309 phosphorylated PKB forms (Table 2 and Fig. 3*E*). On the other hand, the S474D mutation did not affect the level of PKB association at binding equilibrium. Together, these data suggest that in all tested PKB forms, the PIP3 binding site is accessible to a similar extent for soluble PIP3, but steric effects caused by PIP3 embedment in a lipid bilayer lowers the affinity for the PKB conformation adopted upon Thr-309 phosphorylation.

The PH Domain, PIP3 Binding, and Ser-474 Phosphorylation Affect Substrate Specificity—To test how the regulatory elements affect phosphorylation of different cellular targets, we first monitored the ability of the different purified PKB forms to phosphorylate the PKB substrates FOXO3a, GSK3, and TSC2 in cell extracts. In agreement with activity studies using a peptide substrate (Fig. 1*C*), Thr-309 phosphorylation was required to phosphorylate all tested cellular substrates (Fig. 4*A*). Equally, PKB containing the PH and kinase domain but lacking the HM did not phosphorylate any of the tested targets. Interestingly, we found that some regulatory elements have variable effects on different substrates. Whereas FOXO3a was readily phosphorylated by all full-length PKB forms, GSK3 phosphorylation required the S474D phosphomimetic mutation, and TSC2 phosphorylation did not occur with any full-length PKB form. Strikingly, removal of the PH domain resulted in phosphorylation of all tested substrates, suggesting an inhibitory effect of the PH domain on PKB activity in cell extracts toward the cellular targets TSC2 and GSK3 (Fig. 4*A*). To test whether substrate specific effects might be caused indirectly by components in the cell lysates, we performed phosphorylations with purified substrates (Fig. 4*B*). This confirmed that FOXO3a is efficiently phosphorylated by all PKB forms tested. We also confirmed that the S474D mutation is required for GSK3 phosphorylation. Removal of the PH domain in PKBwt increased GSK3 phosphorylation as in cell extracts; however, the S474D mutation further enhanced GSK3 phosphorylation by PKB lacking the PH domain. Interestingly, in contrast to experiments in cell extracts, we did not observe an increase in TSC2 phosphorylation upon removal of the PH domain of PKB.

To test whether PIP3 binding to the PH domain of PKB reverts some of the inhibitory effects observed for the PH domain, we monitored PKB phosphorylation of cellular substrates in the presence of PIP3-containing lipid vesicles. Phosphorylation studies with cell extracts demonstrated that PIP3 did not affect phosphorylation of the substrates FOXO3a and GSK3 (Fig. 4*C*). However, PIP3 vesicles did revert the inhibitory effect of the PH domain on TSC2 phosphorylation observed in cell extracts (Fig. 4, *A* and *C*). Experiments with purified substrates confirmed that FOXO3a phosphorylation is insensitive

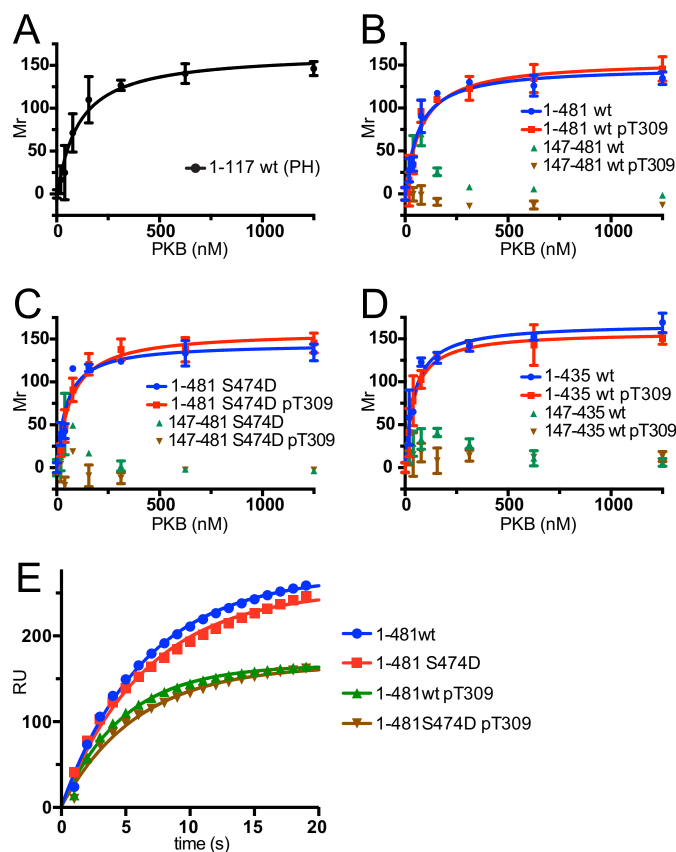

FIGURE 3. **PKB binding to PIP3.** *A–D*, PIP3 affinities of PKB proteins were determined using a fluorescence polarization assay. A nonlinear one-site binding model was fitted to the data points of PH containing PKB proteins using Prism (GraphPad Software). The resulting K_D values are 99.1 ± 27.3 nM for the isolated PH domain (PKBβ1–117) (*A*), 61.0 ± 13.5 and 71.9 ± 18.6 nM for unphosphorylated or Thr-309-phosphorylated PKBβ1–481wt, respectively (*B*); 44.7 ± 10.5 and 63.6 ± 12.6 nM for unphosphorylated or phosphorylated PKBβ1–481 S474D, respectively (*C*); and 43.6 ± 8.5 and 41.1 ± 9.2 nM for unphosphorylated or Thr-309-phosphorylated PKBβ1–435wt, respectively (*D*). PKB lacking the PH domain does not exhibit significant PIP3 binding. The *error bars* represent S.D. from three independent experiments. *E*, association of the indicated PKB forms to PIP3-containing vesicles was measured by SPR. The first 20 s of the association phases are fitted with the following equation: RU = R_{max}*(1 − exp(−k_{obs}*t)); RU = SPR response; t = time from injection. Average R_{max} and k_{obs} values from multiple injections are given in Table 2. Shown are representative curves.

TABLE 2
Parameters for PKB association to PIP3 containing vesicles by SPR

The parameters R_{max} (maximal SPR response at steady state) and k_{obs} (apparent kinetic association constant) were derived by fitting the SPR association curves shown in Fig. 3*E* to the following equation: RU = R_{max}*(1 − exp(−k_{obs}*t)). RU = SPR response; t = time from start of injection in seconds. Errors are given as standard deviation from independent protein injections (n = 7 for unphosphorylated 1–481 WT and 1–481 S474D and n = 4 for Thr-309 phosphorylated samples).

	1–481 WT	1–481 S474D	1–481 WT Thr(P)-309	1–481 S474D Thr(P)-309
k_{obs} (s^{-1})	0.15 ± 0.01	0.15 ± 0.01	0.21 ± 0.02	0.16 ± 0.01
R_{max} (RU)	262 ± 16	250 ± 15	162 ± 13	169 ± 10

to the presence of PIP3 (Fig. 4*D*). However, for GSK3 this was only the case for phosphorylation with S474D mutant PKB. On the other hand, GSK3 phosphorylation by PKBwt and phosphorylation of TSC2 by any full-length PKB form was strongly enhanced by the presence of PIP3 containing vesicles. In summary, whereas FOXO3a only requires Thr-309 phosphorylation of PKB, TSC2 phosphorylation requires in addition PIP3 binding to PKB, and for GSK3 phosphorylation is promoted by PIP3 binding or the S474D mutation. These results indicate that Ser-474 phosphorylation and PIP3 binding to the PH domain are specific mechanisms providing substrate selectivity.

Growth Factor- or DNA Damage-induced PKB Activation Promote Phosphorylation of Distinct Downstream Targets—In contrast to other AGC kinases, PKB has previously been shown not to require HM docking to the PDK1 PIF pocket for Thr-309 phosphorylation (19, 21, 22), which was attributed to the fact that PKB and PDK1 can colocalize on PIP3-rich membranes via their PH domains. However, because we find that efficient phosphorylation of Thr-309 in solution requires an intact HM (Fig. 2), we considered that the PIF pocket mechanism might be important for Thr-309 phosphorylation of PKB, particularly in the absence of PIP3 mediated membrane targeting. The PIF pocket mechanism requires initial phosphorylation of the HM, which in PKB is accomplished by DNA-PK and the mTORC2 complex (12–14). Because PKB was shown to be activated in the nucleus by DNA-PK upon DNA damage (25), we wondered whether this might occur via the PIF pocket mechanism and hence would not require PI3K activity. To test this, we induced PKB activation by UV irradiation of HeLa cells previously treated with the highly specific PI3K inhibitor ETP-46321 (32, 33). Indeed, ETP-46321 treatment had little effect on Thr-308 phosphorylation induced by UV irradiation of HeLa cells (Fig. 5*A*) but fully inhibited PKB activation in the colon carcinoma HCT116 cell line (Fig. 5*B*). On the other hand, the specific DNA-PK inhibitor NU7441 (34) potently inhibited PKB phosphorylation induced by DNA damage but had little effect on cells stimulated by IGF1. Remarkably, PI3K-independent PKB activation via DNA damage resulted in phosphorylation of the downstream targets FOXO3a and GSK3, but not TSC2. We verified that these substrates are targeted by PKB upon UV or IGF treatment of HeLa cells by using the specific PKB α/β inhibitor VIII (Fig. 5, *C* and *D*). These data suggest that PKB activation via membrane targeting or PIF pocket mechanism triggers distinct downstream signaling events.

Discussion

PKB regulation occurs on multiple levels and several sequential steps are required to switch PKB from its inactive to the active state. Numerous previous studies have identified a canonical PKB activation mechanism, which involves growth factor-mediated PI3K activation, PIP3 enrichment in the plasma membrane, PKB membrane targeting, and phosphorylation (Fig. 6, *path 1*). In this study, we take a systematic approach to dissect events that are essential for switching the PKB kinase into an active conformation and events that provide selective activity toward specific substrates.

Using both a peptide substrate and the cellular targets FOXO3a, GSK3, and TSC2, we establish that phosphorylation on Thr-309 but not Ser-474 is strictly required for PKBβ activity (Figs. 1, *C* and *D*, and 4, *A* and *B*, and Table 1). Further, the HM, even if unphosphorylated, is required for significant PKB activity (Fig. 1*C*). We propose that also the unphosphorylated HM is able to synergize with Thr(P)-309 to stabilize the αB and αC helices and form a productive active site (17), albeit with

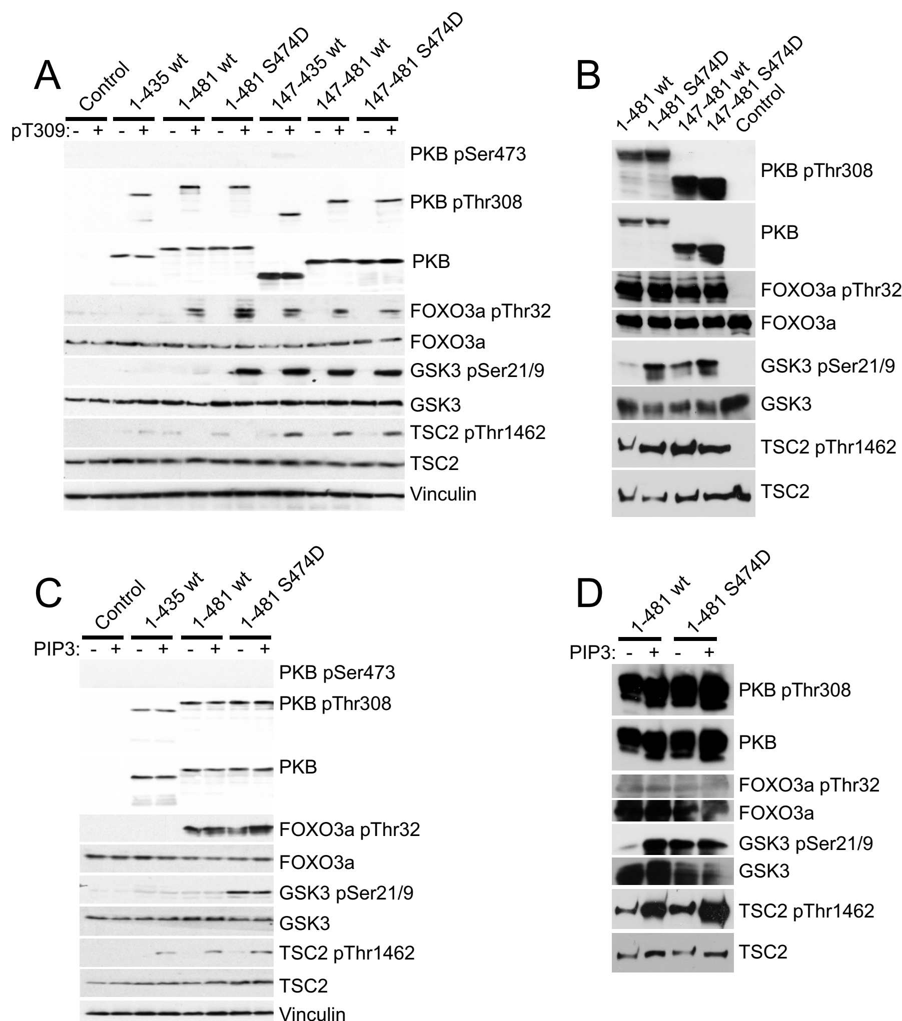

FIGURE 4. **Phosphorylation of PKB downstream targets with purified PKB proteins.** *A*, purified PKB proteins were added to soluble cell extracts from unstimulated HeLa cells. Phosphorylation of FOXO3a, GSK3, and TSC2 at the indicated sites was monitored using phospho-specific antibodies. *B*, GST fused FOXO3a and GSK3 were expressed and pulled down from *E. coli*, and TSC2 was immunoprecipitated from unstimulated HeLa cell lysates. The purified substrates were phosphorylated by the indicated phosphorylated PKB proteins. *C*, phosphorylation of FOXO3a, GSK3, and TSC2 in unstimulated HeLa cell extracts by selected PKB forms was monitored in presence or absence of PIP3-containing vesicles. *D*, the purified substrates (as in *B*) were phosphorylated by the indicated phospho-PKB forms in presence or absence of PIP3 vesicles. *A–C*, in control experiments no exogenous PKB protein was added.

lower efficiency than the phosphorylated HM. This is supported by the crystal structure of the PKBβ kinase domain bound to the S474D-HM, which shows significant interactions between the HM and the kinase N-lobe that do not involve Asp-474 (17), such as interactions involving Phe-470, Phe-473, Glu-472, and Tyr-475 in the HM. Further, it should be noted that protein kinase A does not require HM phosphorylation for N-lobe docking (35).

Our and other studies indicate that Thr-309 phosphorylation by PDK1 is the crucial step in PKB activation. In accordance with previous studies (11, 15), we find that the PH domain prevents and PIP3 binding enables efficient Thr-309 phosphorylation by PDK1. Because we use PDK1 lacking the PH domain, there is no colocalization with PKB (16), and we conclude that binding to PIP3 by itself fully removes the Thr-309 protecting effect of the PH domain, because binding to soluble or membrane-embedded PIP3 has a similar effect as deleting the PH domain (Fig. 2, *A* and *B*). A plausible scenario is that PIP3 binding to inactive PKB competes with a Thr-309 sequestering intramolecular PH-kinase interaction to expose Thr-309 for efficient phosphorylation. A possible PH-kinase conformation in inactive PKBα is observed in a crystal structure where the PH and kinase domain of PKBα are bound to the allosteric PKB α/β inhibitor VIII (36). In this structure, the PIP3 binding site in the PH domain is buried, and the conformation of the Thr-309 phosphorylated activation loop not accessible, providing a plausible mode for a PIP3 competitive PH release that promotes Thr-309 phosphorylation. However, our binding data does not support this model, because we find similar affinities for soluble PIP3 (Fig. 3, *A–D*), and similar association kinetics to PIP3 membranes (Fig. 3*E* and Table 2) independent of the Thr-309 or Ser-474 phosphorylation states. Because soluble PIP3 also accelerates the Thr-309 phosphorylation, the PIP3 binding site is possibly accessible in either conformation, but PIP3 binding induces conformational changes within the PH domain, which alters the PH-kinase interaction to expose Thr-

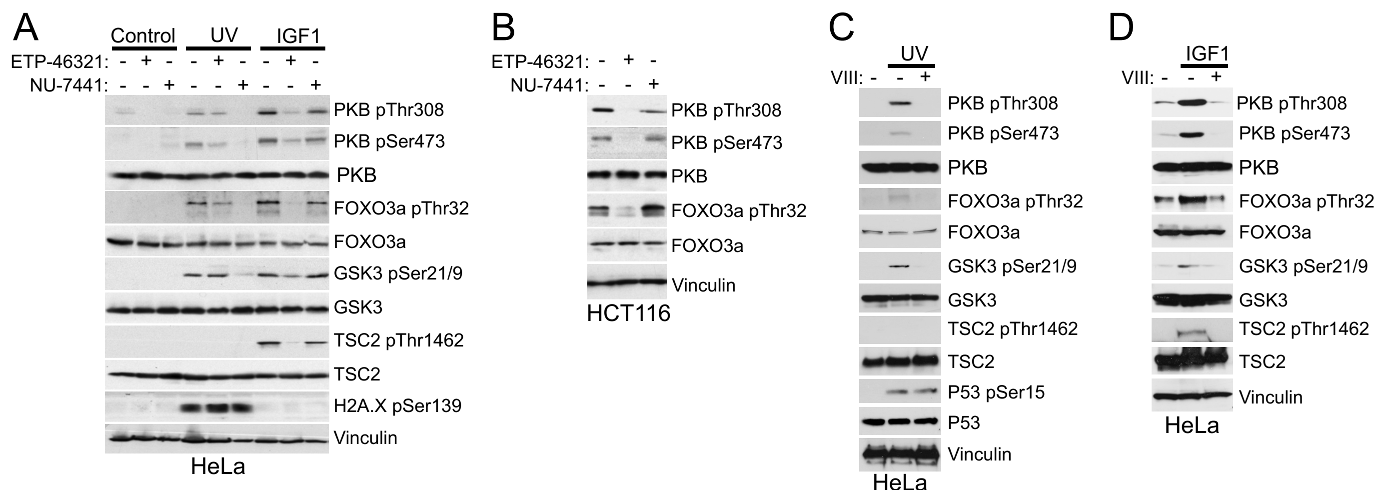

FIGURE 5. **PI3K activity is required for TSC2, but not FOXO3a or GSK3 phosphorylation.** *A*, PKB phosphorylation was induced in HeLa cells by UV radiation or IGF1 stimulation. Phosphorylation of the PKB downstream targets FOXO3a, GSK3, and TSC2 was monitored in absence or presence of specific inhibitors for PI3K (ETP-46321) or DNA-PK (NU-7441). *B*, the potency of the PI3K inhibitor ETP-46321 in our experimental setup was validated on the PI3K addicted HCT116 cell line. *C* and *D*, to verify that phosphorylation of FOXO3a, GSK3, and TSC2 is mediated by PKB, UV-stimulated (*C*) or IGF1-stimulated (*D*) cells were treated with the specific PKBα/β inhibitor VIII.

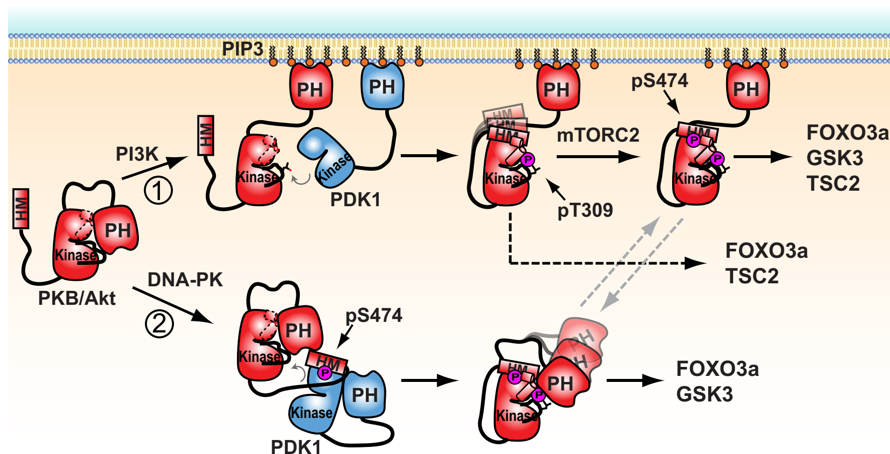

FIGURE 6. **Model for alternative PKB activation mechanisms that do (*path 1*) or do not (*path 2*) require membrane targeting.** In the inactive state of PKB (*left*), the PH domain sequesters the Thr-309 phosphorylation site in the kinase domain. *Path 1*, canonical PKB activation occurs via PI3K-induced PIP3 generation and corecruitment of PKB and PDK1 to PIP3-enriched membranes, resulting in Thr-309 phosphorylation. The unphosphorylated HM interacts weakly with the PKB kinase N-lobe. PI3K also activates mTORC2, which phosphorylates Ser-474 in PKB. *Path 2*, alternatively, we propose that upon DNA damage, phosphorylation of Ser-474 by DNA-PK can initiate PKB activation via the PIF pocket mechanism. This results in doubly phosphorylated PKB, which is not membrane-bound. Once Thr-309 is phosphorylated, helices αB and αC are stabilized (indicated by the switch from *dashed* to *solid cylinders*). This results in formation of a stable hydrophobic pocket in PKB, which likely causes the PKB-HM to switch to an intramolecular interaction. Our results indicate that the two pathways, or lack of Ser-474 phosphorylation, can result in phosphorylation of different sets of downstream targets. *Gray dashed arrows* indicate potential membrane release or membrane targeting of activated PKB.

308/Thr-309. Indeed, PIP3 binding has been found to induce substantial conformational changes within the PH domain (37). Because in the structure reported by Wu *et al.* (36) inhibitor VIII occupies a substantial portion of the interdomain interface, it remains to be determined whether a similar conformation occurs natively in absence of the inhibitor.

Phosphorylated PKB was proposed to engage in distinct PH-kinase interactions, which are responsible for release of PKB from the plasma membrane (38–41). Indeed, we find that Thr-309 phosphorylation reduces PKB association to PIP3-containing membranes (Fig. 3*E* and Table 2). The fact that we do not observe this difference with soluble PIP3 suggests that PH interactions with the Thr-309 phosphorylated PKB kinase sterically hinder an efficient interaction with PIP3 embedded in a lipid bilayer. The observation that the PH domain negatively affects catalytic turnover (K_{cat}) of phosphorylated PKB (Fig. 1, *C* and *D*) further supports the view that the PH domain also interacts with the phosphorylated kinase domain. Importantly, the PH domain variably affects phosphorylation of cellular targets, indicating an important role for the PH domain in substrate selectivity. Although the PH domain does not affect phosphorylation of FOXO3a, it does inhibit phosphorylation of GSK3 by PKBwt (Fig. 4, *A* and *B*). Further, PIP3 binding to the PH domain of PKBwt removes this inhibition toward purified GSK3 (Fig. 4*D*) but also activates PKB for phosphorylation of TSC2 (Fig. 4, *C* and *D*). Whereas, in the case of GSK3, PIP3 binding reverts a direct inhibition by the PH domain (Fig. 4*B*), this does not appear to be the case for TSC2. For TSC2 the PH-mediated inhibition likely requires additional partners, because it is only observed in cell extracts, but not with puri-

fied TSC2 (Fig. 4, *A* and *B*). Because for TSC2 PIP3 binding does not mimic PH removal (Fig. 4, *B* and *D*), the PIP3 bound PH domain might actively be involved in potentiating the TSC2 phosphorylation.

Strikingly, the S474D phosphomimetic mutation in full-length PKB strongly enhances phosphorylation of GSK3, but not FOXO3a or TSC2 (Fig. 4, *A–D*). Furthermore, the S474D mutation eliminates the requirement of PIP3 for efficient GSK3 phosphorylation. Indeed, HM phosphorylation has previously been reported to affect substrate selectivity of PKB (28, 29). In these studies Ser-473 phosphorylation of PKBα was prevented in mouse embryonic fibroblasts by knocking out the mTORC2 components RICTOR, mLST8, or SIN1. Consistent with our results, it was found that TSC2 phosphorylation is not affected by Ser-473 phosphorylation. However, contrary to our finding, it was reported that the absence of HM phosphorylation significantly reduces FOXO3a phosphorylation, whereas GSK3 phosphorylation is affected to a lesser extent. The exact reason for this discrepancy is unclear but likely is due to the different approaches employed. Possibly, nonfunctional mTORC2 could have secondary effects, which are not directly related to the lack of Ser-473 phosphorylation in PKB. Regardless, it is unclear whether a singly Thr-308/Thr-309 phosphorylated PKB species reaches significant levels in the cell, because mTORC2 is activated simultaneously with PI3K upon growth factor or insulin stimulation (42, 43). It could, however, be an important consideration when applying mTOR inhibitors in therapy.

If PI3K is active in the cell, PIP3 induced colocalization of PKB, and PDK1 is likely the reason that PKB, in contrast to other AGC kinases, does not require HM attachment to PDK1 in a PIF pocket mechanism (19–22). However, recent studies suggest that upon challenging efficient PKB activation using PDK1 inhibitors or PDK1 mutations preventing membrane targeting, the PIF pocket mechanism is utilized (23, 24). Whether the PIF pocket mechanism contributes to PKB activation or not likely depends on the sequence of the phosphorylation events. If the HM is phosphorylated first, the PIF pocket mechanism is likely to occur. Delaying Thr-309 phosphorylation by PDK1 inhibitors or PDK1 mutations therefore enhances the PIF pocket mechanism (23, 24). Our finding that the S474D-HM significantly increases the Thr-309 phosphorylation in solution (Fig. 2) prompts us to hypothesize that the PIF pocket mechanism could be dominant in cases where PKB is not recruited to the membrane (Fig. 6, *path 2*). Such a scenario could, for instance, occur upon DNA damage, where PKB was reported to be activated in the nucleus by DNA-PK (25), which is known to phosphorylate the HM of PKB. Indeed, we find that selective inhibition of PI3K does not inhibit PKB activation upon DNA damage, whereas PI3K inhibition fully abolishes IGF1 induced PKB activity (Fig. 5). Importantly, whether PKB activation is triggered by PI3K or not appears to have important consequences on substrate selectivity. In accordance with the PIP3 requirement for TSC2 phosphorylation, we find *in vitro* (Fig. 4, *C* and *D*) IGF1-induced membrane targeting is required for phosphorylation of TSC2, but not for FOXO3a and GSK3 (Fig. 5*A*).

The DNA damage-induced PKB activation, presumably by the PIF mechanism, is significantly weaker than the growth factor-induced response (Fig. 5*A*). Indeed, it has been proposed that different threshold levels of PKB activity might regulate substrate selectivity (24, 27). Consistent with our results in Fig. 5*A*, it was reported that FOXO and GSK3 are phosphorylated at low PKB activity resulting from PDK1 mutations that prevent its membrane targeting, whereas TSC2 phosphorylation requires higher PKB activity induced by membrane targeted wild-type PDK1 (26). Although a threshold mechanism might be operating in the cell, the data we present in Fig. 4 demonstrates that Ser-474 phosphorylation and PIP3 binding of PKB are specific and direct mechanisms to selectively phosphorylate GSK3 and TSC2 respectively, at equal levels of active PKB.

In conclusion, our study provides important new insights on PKB regulatory mechanisms that either control intrinsic kinase activity or modulate PKB activity toward specific substrates. Importantly, we find that two distinct pathways of PKB activation result in phosphorylation of different subsets of PKB substrates (Fig. 6). These findings could have important implications when designing therapeutic strategies targeting PKB. As also proposed in Ref. 23, for some pathogenic conditions it could be necessary to apply therapeutic approaches that target all possible activation mechanisms employed by PKB. On the other hand, other therapies might favor selective targeting of specific PKB responses to prevent excessive toxicity. Our data suggest that the latter could be achieved by strategies that specifically prevent either PKB membrane targeting or PKB association with PDK1 via the PIF pocket mechanism.

Author Contributions—D. B. cloned and purified all proteins and performed and analyzed experiments shown in Figs. 1 and 3 (*A–D*) and Table 1. M.-A. F performed and analyzed experiments in Figs. 4 (*A* and *C*) and 5 (*A* and *B*). J. V. V. performed and analyzed experiments shown in Figs. 4 (*B* and *D*) and 5*C*. C. M. S. performed and analyzed experiments shown in Fig. 3*E* and Table 2. J. Y. designed, performed, and analyzed experiments shown in Fig. 2*A*. J. P. contributed to experiments shown in Fig. 5*B*. R. C.-O. designed and analyzed experiments shown in Fig. 3*E* and Table 2. N. D. designed and analyzed experiments shown in Figs. 4 and 5. D. L. conceived and coordinated the study, wrote the paper, and performed and analyzed experiments shown in Fig. 2 (*B* and *C*). All authors reviewed and approved the final version of the manuscript.

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