Mechanism of Phosphorylation of Protein Kinase B/Akt by a Constitutively Active 3-Phosphoinositide-dependent Protein Kinase-1*

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Phosphorylation of Thr<sup>308</sup> in the activation loop and Ser<sup>473</sup> at the carboxyl terminus is essential for protein kinase B (PKB/Akt) activation. However, the biochemical mechanism of the phosphorylation remains to be characterized. Here we show that expression of a constitutively active mutant of mouse 3-phosphoinositide-dependent protein kinase-1 (PDK1<sup>A280V</sup>) in Chinese hamster ovary cells overexpressing the insulin receptor was sufficient to induce PKB phosphorylation at Thr<sup>308</sup> to approximately the same extent as insulin stimulation. Phosphorylation of PKB by PDK1<sup>A280V</sup> was not affected by treatment of cells with inhibitors of phosphatidylinositol 3-kinase or by deletion of the pleckstrin homology (PH) domain of PKB. C<sub><small>2</small></sub>-ceramide, a cell-permeable, indirect inhibitor of PKB phosphorylation, did not inhibit PDK1<sup>A280V</sup>-catalyzed PKB phosphorylation in cells and had no effect on PDK1 activity in vitro. On the other hand, co-expression of full-length protein kinase C-related kinase-1 (PRK1/PKN) or 2 (PRK2) inhibited PDK1<sup>A280V</sup>-mediated PKB phosphorylation. Replacing alanine at position 280 with valine or deletion of the PH domain enhanced PDK1 autophosphorylation in vitro. However, deletion of the PH domain of PDK1<sup>A280V</sup> significantly reduced PDK1<sup>A280V</sup>-mediated phosphorylation of PKB in cells. In resting cells, PDK1<sup>A280V</sup> localized in the cytosol and at the plasma membrane. However, PDK1<sup>A280V</sup> lacking the PH domain localized predominantly in the cytosol. Taken together, our findings suggest that the wild-type PDK1 may not be constitutively active in cells. In addition, activation of PDK1 is sufficient to phosphorylate PKB at Thr<sup>308</sup> in the cytosol. Furthermore, the PH domain of PDK1 may play both positive and negative roles in regulating the in vivo function of the enzyme. Finally, unlike the carboxyl-terminal fragment of PRK2, which has been shown to bind PDK1 and allow the enzyme to phosphorylate PKB at both Thr<sup>308</sup> and Ser<sup>473</sup>, full-length PRK2 and its related kinase PRK1/PKN may both play negative roles in PKB-mediated downstream biological events.

Protein kinase B (PKB or Akt)<sup>1</sup> is a Ser/Thr protein kinase that has been shown to play an important role in the regulation of a wide spectrum of cellular signaling events, including insulin-stimulated glucose transporter GLUT4 membrane translocation, anti-apoptosis, protein synthesis, and glycogen metabolism (1).

Phosphorylation of Thr<sup>308</sup> in the activation loop and Ser<sup>473</sup> at the carboxyl terminus is essential for PKB activation (2, 3). Although it has been shown that phosphorylation of PKB at Thr<sup>308</sup> is catalyzed by 3-phosphoinositide-dependent protein kinase-1 (PDK1) (4, 5), the effector of PKB Ser<sup>473</sup> phosphorylation remains controversial. Because PDK1 does not phosphorylate PKB at Ser<sup>473</sup>, it has been suggested that phosphorylation at this site is catalyzed by a distinct but yet to be characterized enzyme termed PDK2 (6). On the other hand, it has been shown that PDK1, through an interaction with a PDK1-interacting fragment (PIF) derived from the carboxyl terminus of PRK2, can switch its substrate specificity to phosphorylate PKB at Ser<sup>473</sup> in vitro (7). Alternatively, it has recently been reported that phosphorylation of the wild-type but not the kinase-inactive PKB at Thr<sup>308</sup> triggers phosphorylation at Ser<sup>473</sup>, suggesting that phosphorylation of PKB at Ser<sup>473</sup> occurs through an autophosphorylation mechanism (8).

The current model of PKB activation suggests that the enzyme undergoes mitogen-stimulated translocation to the plasma membrane, which is mediated by the binding of phosphatidylinositol 3-kinase (PI3K) products, namely PtdIns(3,4,5)P<sub>3</sub> and PtdIns(3,4)P<sub>2</sub>, to the PH domain of PKB. This binding also leads to a conformational change that exposes Thr<sup>308</sup> and Ser<sup>473</sup> to the membrane-associated and constitutively activated upstream kinases PDK1 and PDK2, respectively. This model is supported by the finding that myristoylation/palmitoylation of PKB is sufficient to induce phosphorylation of the enzyme at Thr<sup>308</sup> and Ser<sup>473</sup> (9). In addition, PDK1 purified from cells is constitutively active, and treatment of cells with mitogen does not stimulate PDK1 activity in vitro (4). However, there are some data suggesting that the PH domain of PKB is not necessary for PKB phosphorylation and activation. First, it has been shown that PKB constructs lacking the PH domain can still be activated by insulin and insulin-like growth factor-1 (10, 11). In addition, the activity of a membrane-targeted PKB by myristoylation/palmitoylation is still sensitive to the PI3K inhibitor LY294002 (9). These data suggest that activation of a PKB upstream kinase plays an important role in the phosphorylation and activation of PKB.
In the current study, we examined the phosphorylation mechanism of PKB using a constitutively active mutant of mouse PDK1, PDK1A280V, in which the alanine at position 280 was replaced with valine. We have found that ectopic expression of PDK1A280V resulted in a marked increase in PKB phosphorylation at Thr308 to an extent similar to that induced by insulin stimulation. The PDK1A280V-mediated phosphorylation of PKB was not affected by treatment of cells with the PI3K inhibitors wortmannin and LY294002, nor by ceramide, a compound known to indirectly inhibit PKB phosphorylation (12). Deletion of the PH domain of PDK1 had little effect on PDK1A280V-mediated phosphorylation of PKB. On the other hand, deletion of the PH domain of PDK1A280V inhibited the phosphorylation of PKB in cells. Taken together, our data suggest that wild-type PDK1 may not be constitutively active in cells. In addition, the PH domain of PDK1 may play both a positive and a negative role in regulating the function of the enzyme in cells. Finally, we have found that overexpression of the full-length PRK1/PKN or PRK2 inhibited PDK1A280V-mediated phosphorylation of PKB, suggesting that these enzymes play negative roles in PKB-mediated downstream signaling.

EXPERIMENTAL PROCEDURES

Cell Lines, cDNAs, and Antibodies—A Chinese hamster ovary (CHO) cell line overexpressing the human insulin receptor (CHO/IR) and the cDNA encoding mouse PKBα were gifts from Dr. Richard A. Roth. cDNA encoding mouse PDK1 and a polyclonal antibody against the protein were described previously (13). The cDNA encoding the FLAG-tagged PRK1/PKN, PRK2, and the carboxyl-terminal fragment of PDK1/PRK1 (AFS-PDK1) were gifts of Drs. Hideyuki Mukai and Yoshitaka Ono and have been described previously (14). Anti-PKB and anti-phospho-PKB antibodies (Thr308 and Ser473) were obtained from New England BioLabs. Monoclonal antibody to the FLAG-tag was purchased from IBI-Kodak. Polyclonal and monoclonal anti-Myc antibodies were from BABCO and Santa Cruz Biotechnologies, Inc., respectively. The anti-GST-polyclonal antibody was generated by immunizing a rabbit with GST and was affinity-purified.

Site-directed Mutagenesis—Myec-tagged PDK1A280V, PKB T308A, and PKB K77A were generated by single-stranded site-directed mutagenesis according to the protocol as described by Kunkel (15) using customized primers. All site-directed mutagenesis products were confirmed by restriction mapping and DNA sequencing. The kinase inactive mutant PDK1K159E was described previously (15).

Generation of Flag-tagged and PH Domain-deleted Mutants of PDK1 and PDK1A280V—cDNAs encoding mouse PDK1 or PDK1A280V with a truncation in the PH domain (PDK1ΔPH and PDK1A280VΔPH); residues 1–459) were generated by PCR, using either PDK1 or PDK1A280V cDNA as a template. The PCR primers used were: 5'-GCGAGTTGGA-TGTGGGCTGCA-TGGCC-3' and 5'-GCTCGAGCTGCA-TGAGGGT-3', with the added restriction sites underlined. After restriction digestion with EcoRI and XhoI, the cDNA fragments were subcloned into the mammalian expression vector pcDNA3-Myc-His-A (Invitrogen, San Diego, CA), in-frame with a sequence encoding a Myc-tag at the 3'-end.

Generation of Flag-tagged PH Domain-truncated Mutant of PDK1—cDNAs encoding the PH domain-truncated mutant of PKB were amplified by PCR using human PKBα-cDNA as a template. The forward PCR primers for full-length and the PH domain-deleted PKB were: 5'-CCGGAATTCCTGACATGAAAGGTGTTG-3' and 5'-CCGGAAT-TCCATGACATGAAAGGTGTTG-3', respectively. The reverse primer for both constructs was 5'-CTAGCTCAGAGATGAGAAGACTGGAAG-3', with the added restriction sites underlined. The cDNA fragments were subcloned into the EcoRI/XbaI sites of pFLAG-MVC2 vector (Sigma), in-frame with the sequence encoding a Flag-tag at the 5'-end.

Cell Culture, Immunoprecipitation, and Western Blot—CHO/IR cells were transfected with PTK2 medium (Life Technologies) supplemented with 10% newborn calf serum and 1% penicillin/streptomycin. Transfections of CHO/IR cells were normally performed in 60-mm plates with 2 μg of each recombinant plasmid, using LipofectAMINE reagent according to the manufacturer's protocol (Life Technologies). Twenty-four hours after transfection, cells were serum-starved for 2–4 h, treated with or without 10 nM insulin, and lysed in 120–400 μl of Buffer A (50 mM Hepes, pH 7.6, 150 mM NaCl, 1% Triton X-100, 10 mM NaF, 20 mM sodium pyrophosphate, 20 mM β-glycerophosphate, 1 mM sodium orthovanadate, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 μM microcystin-LR, and 1 mM phenylmethylsulfonyl fluoride). The homogenate was centrifuged (10,000 g, 4 °C, 10 min), and the supernatants were used for immunoprecipitation or Western blot experiments. For immunoprecipitation studies, cell lysates were incubated with specific antibodies bound to protein G beads (Amersham Pharmacia Biotech) for 4–6 h at 4 °C with gentle rotation. After incubation, immunoprecipitates were washed extensively with ice-cold Buffer B (50 mM Hepes, pH 7.6, 150 mM NaCl, and 0.1% Triton X-100). Proteins bound to the beads were eluted by heating at 95 °C for 4 min in SDS-PAGE sample loading buffer. The eluted proteins were separated by SDS-PAGE, transferred to nitrocellulose membranes, and detected by specific antibodies. The expression of various proteins was detected by Western blot of cell lysates with antibodies specific to the Myc or FLAG-tag or to the protein. Quantitation of the relative increase in PKB phosphorylation (expressed as percentage of maximum phosphorylation) was performed by analyzing Western blots using the National Institutes of Health IMAGE 1.58 program and was normalized for the amount of PKB expression in each experiment. All experiments were repeated independently at least three times to ensure the consistency of the results.

PDK1 Autophosphorylation—CHO/IR cells overexpressing Myc-tagged wild-type PDK1, PDK1A280V, or PH domain-deleted mutants of PDK1 or PDK1A280V were serum-starved for 4 h, treated with or without 10 nM insulin for 5 min, and lysed in Buffer A. Cell lysates were centrifuged at 10,000 × g for 10 min at 4 °C, and the proteins in the supernatants were immunoprecipitated with antibody to the Myc-tag. After washing the immunocomplexes twice with Buffer B and once with Buffer C (50 mM Tris-HCl, pH 7.5, 5 mM MgCl2, 1 mM Na3VO4, 1 mM sodium pyrophosphate, 10 mM NaF, and 1 mM phenylmethylsulfonyl fluoride), autophosphorylation of PDK1 was initiated with the addition of 30 μl of Buffer C plus 2 μCi of [γ-32P]ATP and incubated for 10 min at 30 °C. Bound proteins were eluted by heating at 95 °C for 4 min in SDS-PAGE sample buffer. Proteins were separated by SDS-PAGE and blotted to a nitrocellulose membrane. Phosphorylation of PDK1 was visualized by autoradiography and quantitated using the NIH IMAGE 1.58 program.

Confocal Immunofluorescence Microscopy—CHO/IR cells were seeded in 24-well plates containing coverslips and transfected with plasmids encoding Myc-tagged PDK1, PDK1A280V, or the PH domain-deleted mutant forms of PDK1 or PDK1A280V using LipofectAMINE (Life Technologies). 24 h after transfection, cells were serum-starved for 1 h, treated with or without 10 nM insulin for 10 min, and then fixed in phosphate-buffered saline containing 4% paraformaldehyde for 20 min at room temperature. Fixed cells were permeabilized and blocked with phosphate-buffered saline containing 0.05% Triton X-100 and 10% normal goat serum. The expression of Myc-tagged full-length or the PH domain-deleted mutants of PDK1 or PDK1A280V was detected by incubation with the anti-Myc-antibody, followed by a rhodamine-conjugated secondary antibody (KPL). Images were captured by an Olympus Fluoview confocal fluorescence microscope.

RESULTS

Overexpression of PDK1A280V Is Sufficient to Phosphorylate PKB at Thr308 in Vivo—To characterize the biochemical mechanism by which PKB is phosphorylated and activated by PDK1, we generated a mutant mouse PDK1 in which the alanine at position 280 was replaced with valine (PDK1A280V). Mutation at the equivalent position in the Caenorhabditis elegans homologue of mammalian PDK1 has been shown to increase PDK1 kinase activity and bypass the requirement for AGE1/PI3K in transducing upstream signals to PKB (16). To test whether PDK1A280V constitutively phosphorylated PKB in cells, we co-transfected CHO/IR cells with PKB and either wild-type, A280V, or the kinase-defective mutant (PDK1K114G) (13) of PKB. Western blots using phosphospecific antibodies directed against either Thr308 or Ser473 of PKB showed little PKB autophosphorylation under basal conditions or in cells co-expressing wild-type PDK1 or the kinase-defective mutant PDK1K114G (Fig. 1A, top and second panels, lanes 1, 2, and 4). On the other hand, co-expression of PDK1A280V with PKB resulted in a marked increase in PKB phosphorylation at Thr308 to a level approximately equal to that attained by insulin treatment (Fig. 1A, top panel, lane 3 versus lane 5). Co-expressing wild-type PDK1 or PDK1A280V with the kinase-defective mutant PDK1K114G did not result in a further increase in PKB phosphorylation (Fig. 1A, top panel, lanes 2 and 3).
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Fig. 1. Overexpression of PDK1A280V is sufficient to phosphorylate PKB at Thr308 in CHO/IR cells. A, the effect of PDK1A280V overexpression on PKB phosphorylation. Plasmid encoding Myc-PKB was co-transfected into CHO/IR cells in 60-mm plates with various constructs as indicated. Quiescent cells were stimulated with (+) or without (−) 10 nm insulin for 5 min and lysed in 120 μl of lysis buffer. Cell lysates (8 μl) were directly analyzed for PKB phosphorylation at Thr308 (top panel) and Ser473 (second panel) using phospho-specific antibodies. The expression of Myc-tagged PKB and PDK1 was confirmed by Western blot using antibodies against PKB (third panel) and PDK1 (bottom panel), respectively. B, overexpression of PKN and PRK2 inhibits PKB phosphorylation. CHO/IR cells were transfected with various constructs as indicated. Quiescent cells were stimulated with or without insulin and lysed as described in A. Cell lysates were directly analyzed by Western blot using antibodies against PKB (third panel) and PDK1 (fourth panel), respectively. C, effect of the AF3-PKN and the PIF fragment on PKB phosphorylation by PDK1A280V. Quiescent cells were co-transfected with various plasmids as indicated. Quiescent cells were stimulated with or without insulin and lysed as described in A. Cell lysates were analyzed by Western blot with phospho-specific antibodies to PKB-Thr308 (top panel) or PKB-Ser473 (second panel). The expression of Myc-PKB, Myc-PDK1, FLAG-PKN-AF3, and GST-PIF was confirmed by Western blot using antibodies to PKB (third panel), PDK1 (fourth panel), FLAG (fifth panel), or GST (bottom panel), respectively. All experiments were performed at least three times with similar results.


tantly, a modest increase of phosphorylation at Ser473 was also observed (Fig. 1A, second panel, lane 3 versus 1). Overexpression of PDK1A280V did not stimulate the phosphorylation of MAP kinase (data not shown).

Overexpression of Full-length PRK1/PKN or PRK2 Inhibits PDK1A280V-mediated PKB Phosphorylation—A recent study has shown that PDK1 can phosphorylate Ser473 of PKB upon binding the PDK1-interactive fragment (PIF) derived from PRK2 (7). To test whether PDK1A280V was able to phosphorylate PKB at Ser473, we co-expressed Myc-tagged PKB and Myc-tagged PDK1A280V in CHO/IR cells together with either FLAG-tagged PRK2 or PRK1 (also named PKN), a PRK2-related protein that has recently been found to interact with PDK1 in intact cells (17). Our results showed that overexpression of PRK1/PKN significantly inhibited PDK1A280V-stimulated PKB phosphorylation at Thr308 (Fig. 1B, top panel, lane 4 versus lane 3) and Ser473 (Fig. 1B, second panel, lane 4 versus lane 3). A lesser inhibition of PKB phosphorylation was observed in cells expressing PRK2 (Fig. 1B, top and second panels, lane 5 versus lane 3), probably due to lesser expression of this protein compared with PKN (Fig. 1B, bottom panel, lane 5 versus lane 4). These findings suggest that full-length PKN and PRK2 play negative roles in PKB-mediated downstream events.

It is possible that the interaction of full-length PRK1/PKN or PRK2 with PDK1 induced a conformational change that was different from that induced by the PIF fragment. To test this possibility, we co-expressed Myc-tagged PKB in CHO/IR cells together with either a control plasmid (Fig. 1C, lanes 1 and 2), Myc-tagged PDK1A280V (Fig. 1C, lane 3), Myc-tagged PDK1A280V plus the FLAG-tagged carboxyl terminus of PRK1/PKN (AF3-PKN) (14) (Fig. 1C, lane 4), or Myc-tagged PDK1A280V plus the GST-PIF fusion protein (Fig. 1C, lane 5). Overexpression of either AF3-PKN or GST-PIF had no significant effect on the expression levels of PKB (Fig. 1C, third panel) or PDK1 (Fig. 1C, fourth panel). However, overexpression of these proteins significantly inhibited PDK1A280V-induced PKB phosphorylation at Thr308 (Fig. 1C, top panel, lane 3 versus lane 4 or 5). Although co-expression of PDK1A280V and AF3-PKN did not significantly stimulate PKB phosphorylation at Ser473 (Fig. 1C, second panel, lane 3 versus lane 4), co-expression of PDK1A280V and the GST-PIF fusion protein led to a 3-fold increase in PKB phosphorylation at this site (Fig. 1C, second panel, lane 3 versus lane 5). These results are in agreement with the recent finding that binding of PIF fragment enables PDK1 to phosphorylate PKB at Ser473 (7).

Phosphorylation of PKB at Thr308 by PDK1A280V Is Independent of PI3K Activation—To test whether PI3K is required for PDK1A280V-mediated phosphorylation of PKB, we co-transfected PKB into CHO/IR cells together with either a pcDNA control plasmid or a plasmid encoding PDK1A280V and examined PKB phosphorylation in the presence or absence of PI3K inhibitors. In the absence of PDK1A280V, insulin treatment led to a significant increase in PKB phosphorylation at Thr308 (Fig. 2A, top panel, lane 1 versus lane 2). This insulin-stimulated phosphorylation of PKB at Thr308 was blocked by either wortmannin (Fig. 2A, top panel, lane 3) or LY294002 (Fig. 2A, top panel, lane 4). In agreement with our earlier findings (Fig. 1A, top panel), co-expression of PDK1A280V with PKB resulted in a marked increase in PKB phosphorylation at Thr308 (Fig. 2A, top panel, lane 5), which was only slightly enhanced after insulin treatment (Fig. 2A, top panel, lane 6). However, neither wortmannin nor LY294002 inhibited the PDK1A280V-stimulated PKB phosphorylation at Thr308 (Fig. 2A, top panel, lane 5 versus lanes 7 or 8).

Overexpression of PDK1A280V Prevents Ceramide-induced Inhibition of PKB Phosphorylation—To further understand the
mechanism of PKB phosphorylation, we investigated the phosphorylation of PKB by PDK1 in the presence or absence of C3-ceramide, a cell-permeable reagent that indirectly inhibits PKB phosphorylation and activity (12). Consistent with the results of others (18, 19), we found that treatment of CHO/IR cells with ceramide inhibited PKB phosphorylation at both Thr308 (Fig. 2, A, top panel, lane 1 versus lanes 2 and 3) and Ser473 (Fig. 2B, A, second panel, lane 1 versus lanes 2 and 3) but had no significant effect on the autophosphorylation of the IR and the tyrosine phosphorylation of IR substrate-1 (IRS-1) (data not shown). The inhibition of PKB phosphorylation by ceramide was partially prevented by overexpression of PDK1 and almost completely blocked by overexpression of PDK1A280V (Fig. 2B, A, top panel, lanes 4–9). In addition, we have found that ceramide did not inhibit PDK1 activity in vitro (data not shown).

Phosphorylation at Thr308 and the Kinase Activity of PKB Are Important for PKB Phosphorylation at Ser473—It has recently been shown that phosphorylation by PDK1 in vitro triggers the phosphorylation at Ser473 in kinase-active, but not thermally inactivated PKB (8). To test whether phosphorylation of PKB at Thr308 is sufficient to initiate the phosphorylation of PKB at Ser473 in intact cells, we co-expressed Myc-tagged wild-type PKB, PKB179A, or a kinase-inactive PKB179A in CHO/IR cells together with either a control plasmid or Myc-tagged PDK1A280V. Cells were treated with or without insulin, and the phosphorylation of PKB at Thr308 and Ser473 was examined. Stimulation of cells with insulin (Fig. 3, top panel, lane 1 versus lane 2) or overexpression of PDK1A280V (Fig. 3, top panel, lane 1 versus lane 3) led to a marked increase in the phosphorylation at Thr308 of wild-type PKB. As expected, the PKB205 mutant was not phosphorylated in the activation loop under these conditions (Fig. 3, top panel, lane 1 versus lane 3). Inactivation of the kinase by replacing the ATP-binding site lysine residue (Lys1472) with alanine resulted in a 70% decrease in the insulin- or PDK1A280V-stimulated phosphorylation at Thr308 (Fig. 3, top panel, lane 2 and 3 versus 8 and 9). However, replacing Ser473 with alanine had essentially no effect on PKB phosphorylation at Thr308 (data not shown). Consistent with the findings of others (8), a mutation at Thr308 inhibited the insulin-stimulated phosphorylation at Ser473 (Fig. 3, second panel, lane 2 versus lane 3). However, quantitation analysis revealed that approximately 46% of Ser473 phosphorylation remained for this kinase-inactive mutant. Together, these findings suggest that phosphorylation at Thr308 and the kinase activity of PKB play an important role in the phosphorylation of the enzyme at Ser473. However, because PKB179A was still able to undergo insulin-stimulated phosphorylation at Ser473 in cells, a mechanism other than or in addition to autophosphorylation may play a role in the insulin-stimulated phosphorylation of PKB at this site.

The PH Domain of PKB Is Not Essential for PDK1A280V-Catalyzed Phosphorylation of PKB at Thr308—To test whether the PH domain of PKB is required for PDK1A280V-catalyzed phosphorylation of PKB, we co-transfected CHO/IR cells with PDK1A280V and either the wild-type or a PKB mutant in which the PH domain residues 1–102 was deleted. Western blot studies showed that insulin treatment led to an increase in PKB phosphorylation at Thr308 (Fig. 4A, A, top panel, lane 1 versus lane 2). This insulin-stimulated phosphorylation was abolished for the PH domain-deleted mutant PKBΔPH (Fig. 4A, A, top panel, lane 5). On the other hand, only a partial inhibition of the PDK1A280V-mediated phosphorylation was observed for PKBΔPH compared with that of the full-length enzyme (Fig. 4A, A, top panel, lane 3 versus lane 6). Because the
Deletion of the PH Domain of PDK1A280V Inhibits PDK1A280V-catalyzed Phosphorylation of PKB at Thr308—The PH domain of PDK1 has been shown to bind phosphoinositides and be necessary for PDK1-mediated phosphorylation and activation of PKB (20, 21). To test whether the PH domain is required for PDK1A280V-mediated phosphorylation of PKB at Thr308, we generated mutants of PDK1 and PDK1A280V in which the PH domains were deleted. Co-expression of PKB with wild-type PDK1 or PDK1(ΔPH) had no significant effect on basal PKB phosphorylation at Thr308 (Fig. 4B, top panel, lanes 1 and 2). Conversely, co-expression of PDK1A280V with PKB markedly increased the phosphorylation of PKB at this site (Fig. 4B, top panel, lane 3). The PDK1A280V-stimulated phosphorylation of PKB at Thr308 was markedly decreased by deletion of the PH domain of PDK1A280V (Fig. 4B, top panel, lane 3 versus lane 4). In addition, overexpression of the PDK1(ΔPH) or PDK1A280V(ΔPH) inhibited insulin-stimulated phosphorylation of PKB at Thr308 (Fig. 4B, top panel, lane 5 versus 6 and lane 7 versus 8). These findings indicate that the PH domain of PDK1 is important for the enzyme to interact with and phosphorylate PKB in cells.

One explanation for the inhibition of PKB phosphorylation by PDK1(ΔPH) or PDK1A280V(ΔPH) might be that removal of the PH domain rendered the enzyme inactive. To test this possibility, we examined the autophosphorylation of wild-type and PH-domain-deleted mutants of PDK1 and PDK1A280V. Wild-type PDK1 and PDK1A280V isolated from both serum-starved and insulin-treated cells were autophosphorylated in vitro (Fig. 4C). Deletion of the PH domain or replacing alanine at 280 with valine led to approximately a 2- and 3-fold increase in the autophosphorylation of PDK1, respectively. However, deletion of the PH domain did not further stimulate the autokinase activity of PDK1A280V (Fig. 4C). Therefore, deletion of the PH domain increased, rather than inhibited, PDK1 catalytic activity per se.

Deletion of the PH Domain of PDK1A280V Prevents Its Plasma Membrane Translocation—The finding that deletion of the PH domain did not affect PDK1A280V autophosphorylation in vitro but prevented the enzyme from phosphorylating PKB in cells suggested that the PH domain plays a role in the interaction of PDK1A280V with its substrates. To test this idea, we examined the cellular localization of PDK1, PDK1A280V, and their PH-domain-deleted mutants by confocal immunofluorescence studies. In unstimulated CHO/IR cells, PDK1A280V localized both at the plasma membrane and in the cytosol in a punctate manner (Fig. 5, a and b). Plasma membrane association of PDK1A280V was also observed in cells treated with insulin (Fig. 5, c and d). On the other hand, no significant plasma membrane immunofluorescence staining was observed for PDK1A280V(ΔPH) before or after insulin treatment (Fig. 5, e–h). Under serum-starved conditions, wild-type PDK1 (Fig. 5, i and j) and PDK1(ΔPH) (Fig. 5, m and n) resided predominantly in the perinuclear region. Although insulin treatment led to a decrease in the perinuclear staining of the wild-type PDK1 (Fig. 5, k and l), it had no significant effect on the intracellular translocation of PDK1(ΔPH) (Fig. 5, o and p). These findings are consistent with an earlier report that the PH domain is important for PDK1 translocation to the plasma membrane (20).

**DISCUSSION**

Phosphorylation of PKB at Thr308 in the activation loop by PDK1 is considered essential for PKB activation. However, the biochemical and cellular mechanisms by which PKB is phosphorylated by upstream kinases remain unclear.

In the present studies, we have examined the biochemical mechanism of PKB phosphorylation by using a constitutively active mutant of PDK1. Expression of the gain-of-function mu-
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Fig. 5. The PH domain is important for PDK1 subcellular localization. CHOK1IR cells expressing Myc-tagged full-length PDK1 or PDK1Δ280V or the PH domain-deleted mutants of these enzymes were serum-starved for 1 h and treated with or without 10 nM insulin for 10 min. Cells were then fixed and labeled with the anti-Myc antibody, followed by rhodamine-labeled second antibody. The confocal images presented here represent projections of 2-series scans. Representative fields are shown of immunofluorescence (anti-Myc) and the corresponding differential interference contrast (DIC) images. Data are representative of three independent experiments with similar results.

Phosphorylation of PKB by a Constitutively Active PDK1 substrates under non-stimulatory conditions. After growth factor stimulation, the binding of phospholipids to the PH domain may induce a conformational change that relieves the inhibitory blockage so that PDK1 becomes fully active. In addition to activating PDK1, the binding of phospholipids may also promote the plasma membrane translocation of the enzyme. Thus, the PH domain may function as a switch to regulate PDK1-mediated biological events in cells.

Until recently, it has been suggested that PDK1 is constitutively active in cells and that phosphorylation and activation of its downstream substrates is mediated mainly by translocation and conformational changes of its substrates (24, 25). However, our results suggest that PDK1 can be activated above its basal level both in vitro and in cells. Consistent with this, a mutation at Ala280 or deletion of the PH domain significantly increased PDK1 autophosphorylation in vitro (Fig. 4C). In addition, overexpression of PDK1Δ280V, but not wild-type PDK1, was able to phosphorylate PKB at Thr308 to the same extent as that stimulated by insulin (Fig. 1A). Thus, although PDK1 is constitutively active in vitro, its activation may be tightly regulated in cells. This idea is consistent with a recent finding that PDK1 is activated by sphingosine both in vitro and in vivo (26). Moreover, it has also recently been shown that treatment of cells with the phosphatase inhibitors okadaic acid and calyculin A inhibited PKB phosphorylation at Thr308 (27), suggesting that phosphorylation plays a role in regulating PDK1 activity in cells. Consistent with the findings of others (28), we have found that phosphorylation of Ser244 in the activation loop of mouse PDK1 is essential for the autophosphorylation and kinase activity of the enzyme.  

Ceramide has been shown to be a potent inhibitor for PKB phosphorylation in cells (18, 19). Because C₃-ceramide does not inhibit insulin-stimulated PI3K activation in cells and has no

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effect on PKB catalytic activity in vitro, it has been hypothe-
sized that its action may be due to the inhibition of an imme-
diate upstream kinase for PKB (12). However, our data showed
that ceramide-induced inhibition could be blocked by PDK1^{A280V} (Fig. 2B). In addition, ceramide did not inhibit
PDK1 activity in vitro (data not shown). These findings suggest
that the inhibition of PKB by ceramide is not mediated by
direct inhibition of PDK1. These results are consistent with
the findings that the inhibition of PKB phosphorylation by cer-
amide in cells is due to the activation of a protein phosphatase (27, 29) rather than by the inhibition of the PDK upstream kinase
PDK1.

It has recently been suggested that phosphorylation of PKB at
the PDK2 site is mediated by autophosphorylation (8). This
conclusion is supported by the findings that incubation of puri-
ﬁed PKB with PDK1 led to phosphorylation at both Thr^{308}
and Ser^{473}, and phosphorylation at Ser^{473} requires prior phos-
phorylation at Thr^{308} and the intrinsic catalytic activity of
the enzyme (8). However, whether phosphorylation of PKB at
Thr^{308} is sufﬁcient to trigger autophosphorylation at Ser^{473} in
intact cells is unknown. In our studies, we found that expres-
sion of a constitutively active PDK1 induced marked phos-
phorylation of PKB at Thr^{308} and to a lesser extent Ser^{473} (Fig. 1).
Because PDK1 does not directly phosphorylate PKB at Ser^{473},
these ﬁndings suggest that phosphorylation of Thr^{308} by PDK1
is able to induce PKB autophosphorylation at Ser^{473}. This idea
is consistent with a recent ﬁnding that phosphorylation of Ser^{473}
may occur through an autophosphorylation mechanism
(8). However, it should be pointed out that, in our studies, the
PDK1^{A280V}-induced PKB phosphorylation at Ser^{473} is normally
less than 20% of that stimulated by insulin. In addition, it has
previously been shown that a kinase-inactive, membrane-tar-
geted mutant PKB can be phosphorylated at both Thr^{308}
and Ser^{473} in unstimulated cells (9). Consistent with these results,
we have found that a disruption of the kinase activity of the
enzyme inhibited PKB phosphorylation at both Thr^{308}
and Ser^{473} (Fig. 3). In addition, inactivation of PDK1 did not com-
pletely inhibit insulin-stimulated PKB phosphorylation at Ser^{473} (Fig. 3).
Therefore, it remains to be established whether the growth factor-stimulated stoichiometric phosphorylation of PKB at this site is mediated by autophosphorylation, by a distinct kinase, or by both.

It has recently been shown that, upon binding to the PIF
fragment derived from PRK2, PDK1 is capable of phosphory-
lating PKB at Ser^{473} (7). In agreement with this ﬁnding, we
found that co-expression of PDK1^{A280V} with the PIF fragment
led to an increase of PKB phosphorylation at Ser^{473} (Fig. 1C,
second panel, lane 3 versus 5). However, we also found that
co-expression of full-length PRK2, its isoform PRK1/PKN, or
the carboxyl terminus of PRK1/PKN (PKN-AF3) inhibited
PDK1^{A280V}-mediated PKB phosphorylation at both Thr^{308} and
Ser^{473} (Fig. 1, B and C). These ﬁndings suggest the full-length
PRK1/PKN and PRK2 play negative roles in PDK1-mediated
phosphorylation of PKB in cells.

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Mechanism of Phosphorylation of Protein Kinase B/Akt by a Constitutively Active 3-Phosphoinositide-dependent Protein Kinase-1

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