Regulation of 3-Phosphoinositide-dependent Protein Kinase-1 (PDK1) by Src Involves Tyrosine Phosphorylation of PDK1 and Src Homology 2 Domain Binding*

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3-Phosphoinositide-dependent protein kinase-1 (PDK1) appears to play a central regulatory role in many cell signalings between phosphoinositide-3 kinase and various intracellular serine/threonine kinases. In resting cells, PDK1 is known to be constitutively active and is further activated by tyrosine phosphorylation (Tyr9 and Tyr373/376) following the treatment of the cell with insulin or pervanadate. However, little is known about the mechanisms for this additional activation of PDK1. Here, we report that the SH2 domain of Src, Crk, and GAP recognized tyrosine-phosphorylated PDK1 in vitro. Destabilization of PDK1 induced by geldanamycin (a Hsp90 inhibitor) was partially blocked in HEK 293 cells expressing PDK1-Y9F. Co-expression of Hsp90 enhanced PDK1-Src complex formation and led to further increased PDK1 activity toward PKB and SGK. Immunohistochemical analysis with anti-phospho-Tyr9 antibodies showed that the level of Tyr9 phosphorylation was markedly increased in tumor samples compared with normal. Taken together, these data suggest that phosphorylation of PDK1 on Tyr9, distinct from Tyr373/376, is important for PDK1/Src complex formation, leading to PDK1 activation. Furthermore, Tyr9 phosphorylation is critical for the stabilization of both PDK1 and the PDK1/Src complex via Hsp90-mediated protection of PDK1 degradation.

One of the key features of multicellular organisms is that all cells are able to adjust to changes in the surrounding environment. A diverse set of environmental cues utilize intracellular protein phosphorylation-dephosphorylation cascades to rapidly and reversibly transduce their signals from their plasma membrane receptors to the cytoplasm and the nucleus. 3-Phosphoinositide-dependent protein kinase-1 (PDK1) was originally identified as an upstream kinase for protein kinase B (PKB/Akt) (1) and is recognized as a master protein kinase for regulating in many cell-signaling pathways (2–5). Targets of PDK1 include many of the AGC family of protein kinases, including protein kinase B (PKB/Akt), p70 ribosomal protein S6 kinase (p70(S6K)), cyclic AMP-dependent protein kinase, protein kinase C, serum and glucocorticoid-inducible kinase (SGK), p90 ribosomal protein S6 kinase (RSK), and p21-activated kinase-1 (PAK1) (4). However, the generation of PDK1-ablated or PDK1-hypomorphic (~10% of PDK1 expression) mice revealed that most of the PDK1 substrates identified in vitro were not physiological targets for PDK1 in vivo, with the exception of PKB, p70(S6K), and RSK (6, 7). PDK1(−/−) mice die at embryonic day 9.5 with multiple abnormalities, whereas hypomorphic PDK1 mice are viable (6). Nevertheless, these mice are 40–50% smaller than control animals due to small cell size, but not cell number, providing genetic evidence that PDK1 is essential for mouse embryonic development and regulates cell size (6).

PDK1 possesses an N-terminal kinase domain and a C-terminal pleckstrin homology domain (8, 9). Phosphorylation of PKB by PDK1 is dependent upon prior activation by phosphoinositide 3-kinase and the production of the second messengers, phosphatidylinositol 3,4,5-trisphosphate and phosphatidylinositol 3,4-bisphosphate, which bind to the pleckstrin homology domains of PDK1 and PKB. These lipids do not activate either PKB or PDK1 but instead recruit and co-localize these kinases at the plasma membrane, allowing PDK1 to activate PKB (3).

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3 The abbreviations used are: PDK1, 3-phosphoinositide-dependent protein kinase-1; PKB, protein kinase B; SH2, Src homology 2; SH3, Src homology 3; siRNA, small interfering RNA; GST, glutathione S-transferase; 17-AAG, 17-allylamino-17-demethoxygeldanamycin; PMA, phorbol 12-myristate 13-acetate; WT, wild type.
Although the importance of PDK1 in insulin signaling has been well characterized, the regulatory mechanism of PDK1 activity remains controversial. At the plasma membrane, PDK1 adopts the appropriate conformation for optimal activation (10, 11). A previous report demonstrated that phosphorylation of Ser\(^{241}\) in the activation loop was necessary for PDK1 activity (12). There is convincing evidence that PDK1 undergoes tyrosine phosphorylation in response to several growth factors (13–15). Studies with the tyrosine phosphatase inhibitor pervanadate indicate that full activation of PDK1 requires phosphorylation at Tyr\(^{373/376}\) in HEK 293 cells (13). It has also been suggested that in HEK 293 cells, insulin-mediated regulation of PDK1 activity is accomplished by an Src family kinase (13, 15). However, the molecular mechanism of how Src kinases contribute to tyrosine phosphorylation-mediated PDK1 activation has not yet been elucidated.

In the current study, we have investigated the molecular basis for PDK1 regulation by using the SH2-containing proteins, Src, phosphoinositide 3-kinase, and Grb2. We show that the SH2 domain of Src, Crk, and GAP recognize tyrosine-phosphorylated PDK1 in vitro. Using deletion mutants of Src, we confirm that the SH2 domain of Src is responsible for the interaction between PDK1 and Src in HEK 293 cells. Using site-directed mutants of PDK1, we show that although conversion of Tyr\(^9\) to Phe completely disrupted PDK1-Src complex formation, mutation of Tyr\(^{373/376}\) to Phe led to moderate binding of PDK1 and Src. Based on results from phosphopeptide binding assays, phosphorylation of PDK1 on Tyr\(^9\) appears to act as a docking site for the binding of Src, leading to PDK1 activation. Consistent with previous reports (16, 17) geldanamycin, an Hsp90 inhibitor, destabilized PDK1 protein levels. This destabilization was partially blocked in HEK 293 cells expressing PDK1-Y9F. Co-expression of Hsp90 enhanced PDK1-Src complex formation and led to further increases in PDK1 activity, resulting in the activation and phosphorylation of AGC protein kinases. Furthermore, knockdown of Hsp90 by siRNA inhibited PDK1 activity and PDK1/Src binding. With the help of anti-phospho-Tyr\(^9\) antibodies, these events were evaluated in the various cancer tissues. Thus, these data show that PDK1 activity is further regulated by the SH2 protein Src through the formation of a Src-PDK1-Hsp90 complex.

**EXPERIMENTAL PROCEDURES**

Reagents—Anti-Myc 9E10 and anti-HA 12CA5 monoclonal antibody were produced from hybridomas. Anti-PDK1, anti-PKB, and anti-phospho-SGK (Thr\(^{250}\)) antibody were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Anti-GST and anti-Hsp90 antibody were from Stressgen. Anti-FLAG and anti-actin antibodies were from Sigma. Anti-phospho-PKB (Thr\(^{308}\)) antibody was from Cell Signaling. Anti-phospho-PDK1 (Tyr\(^9\) or Tyr\(^{373/376}\)) antibody has been described previously (13). Horseradish peroxidase-conjugated anti-mouse and anti-rabbit IgG antibodies were from Calbiochem, and horseradish peroxidase-conjugated anti-mouse IgG antibody was obtained from Sigma. Sodium orthovanadate (Na\(_3\)VO\(_4\)) was purchased from Sigma.

Construction of Expression Vectors—Myc-tagged full-length PDK1 (Myc-PDK1) and the phosphorylation site mutants (Myc-PDK1-Y9F and Myc-PDK1-Y373F/Y376F) were the same as reported previously (13). The collection of GST-SH2 protein expression constructs has been previously described (18). HA-tagged c-Src constructs (HA-Src) were prepared by amplifying the cDNA of HEK 293 cells with primers 5’-CG GGA TCC ATG GGG AGC AGC AAG and 3’-G GAA TTC CTA TAG GTT CTC TCC. The PCR products were subcloned between BamHI and EcoRI sites of the mammalian expression vector, pcDNA3-HA. The deletion mutants of Src were constructed in pcDNA3-HA expression vector by a standard PCR-cloning strategy (HA-Src-ΔSH2 and HA-Src-ΔSH3). The mutants at Lys\(^{295}\) (kinase-deficient HA-Src KD K295M) and Tyr\(^{527}\) (constitutively active HA-Src-CA Y527F) were created by using the QuikChange \(^2\)M site-directed mutagenesis kit (Stratagene) as described by the manufacturer with pcDNA3 HA-Src WT as template. The FLAG-Hsp90 construct has been described previously (19). FLAG-Hsp40 and FLAG-Hsp27 constructs in pTag2 were a kind gift from Dr. Y. M. Kim. All constructs were confirmed by automated DNA sequencing.

**Regulation of PDK1 Activity by Src**

Cell Culture and Stimulation—Stable cell lines expressing the Myc-tagged PDK1 WT, PDK1-Y9F, or PDK1-Y373F/Y376F were derived from HEK 293 cells and the same as reported previously (13). The cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin (Invitrogen), and 1 μg/ml puromycin and transfected using jetPEI (Qi-biochem) or Lipofectamine (Invitrogen) reagent by following the instructions provided by the manufacturer. The transfection mixture was removed after 24 h of incubation, and cells were serum-starved for 16 h before stimulation for 15 min with 0.1 mM pervanadate prepared with 0.2 mM H\(_2\)O\(_2\) (20) or for the indicated time with 5 μg/ml Ghandanamycin (Calbiochem), 1 μM 17-allylamino-17-demethoxygeldanamycin (17-AAG; Calbiochem), or 0.2 μM phorbol 12-myristate 13-acetate (PMA; Calbiochem). Pretreatment with 1 μM lactacystin (Alexis), 1 μM PSI (Alexis) or 5 μM MG132 (Calbiochem) were done for 1 h before cell stimulation. For RNA interference, human Hsp90α/β siRNA and a scrambled RNA duplex as a control were obtained from Santa Cruz Biotechnology. HEK 293 cells were transfected with the Hsp90α/β siRNA or a control siRNA using Lipofectamine (Invitrogen).

Expression of GST Fusion Protein in Bacterial System and GST Pull-down Assay—The GST-SH2 fusion protein was isolated from BL21DE3 cells transformed with GST-SH2 expression construct. Briefly, bacteria were initially grown at 37°C for 2 h (A\(_{600}\) = 0.5–0.7) and subsequently induced with 0.5 mM isopropyl-thio-β-d-galactopyranoside (Promega) for 6 h at 30°C. Cells were lysed using a sonicator (Beckman) in lysis buffer (0.5% Nonidet P-40, 50 mM Tris-HCl (pH 8), 120 mM NaCl, 7 mM dithiothreitol, and 0.1 mM phenylmethylsulfonyl fluoride). The GST-SH2 fusion proteins were purified on glutathione-agarose beads (Amersham Biosciences) as described previously (21). For GST pull-down assays, 2 μg of the indicated purified GST fusion proteins were incubated with 500 μg of lysates overexpressing Myc-PDK1 WT for 2 h at 4°C, following which the beads were washed three times in wash buffer (100 mM NaCl, 1 mM EDTA, 10 mM Tris-HCl (pH 8.0), 0.5% Nonidet
Regulation of PDK1 Activity by Src

P-40, and 0.05% SDS). After removing the supernatant in the final wash, samples were resuspended in 25 μl of 5 × SDS sample buffer and boiled for 5 min, and the proteins retained on the beads were resolved by SDS-PAGE. The bound PDK1 was detected by anti-PDK1 antibody. Binding was compared with that of 10% of the lysates added to the binding reactions. The incubated GST fusion proteins were determined by Coomassie Brilliant Blue staining solution (0.25% Coomassie Blue G-250, 10% methanol, and 10% acetic acid).

Immunoprecipitation and in Vitro Kinase Assay for PDK1—HEK 293 cells were placed on ice and extracted with lysis buffer containing 50 mM Tris-HCl, pH 7.5, 1% (v/v) Nonidet P-40, 120 mM NaCl, 25 mM sodium fluoride, 40 mM β-glycerol phosphate, 0.1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, and 2 μM microcystin-LR. Lysates were centrifuged for 15 min at 12,000 × g, and Myc-PDK1 protein was immunoprecipitated from 500 μg of cell-free extracts with anti-Myc 9E10 monoclonal antibody immobilized on protein G-Sepharose (Amersham Biosciences). The immune complexes were washed once with lysis buffer containing 0.5 M NaCl, followed by lysis buffer and finally with kinase assay buffer (50 mM Tris–HCl, pH 7.5, 0.1% (v/v) 2-mercaptoethanol).

In vitro kinase assays were performed for 60 min at 30 °C in a 50-μl reaction volume containing 30 μl of immunoprecipitates in kinase buffer, 100 μM Suntide (RRKDGATMKTFCGTPE) as substrate, 10 mM MgCl2, 1 μM protein kinase A inhibitor peptide (Alexis), and 100 μM [γ-32P]ATP (1,000–2,000 cpm/pmol; Amersham Biosciences). Reactions were stopped by adding EDTA to a final concentration of 50 mM and processed as described previously (13). For detection of endogenous complexes, endogenous Src was immunoprecipitated from 4 mg of HEK 293 cell-free extracts with 1 μg of anti-Src antibody. Protein concentrations were determined by the method of Bradford (Bio-Rad) using bovine serum albumin as a standard.

Preparation of Crude Plasma Membrane Fractions—HEK 293 cells were treated as described and then placed on ice. After washing once in ice-cold PBS, cells were scraped in 500 μl of ice-cold fractionation buffer containing 20 mM HEPES-NaOH, pH 7.4, 250 mM sucrose, 25 mM sodium fluoride, 1 mM sodium pyrophosphate, 0.1 mM sodium orthovanadate, 2 μM microcystin LR, 1 mM phenylmethylsulfonyl fluoride, and 1 mM benzamidine and then homogenized by passing with a 1-ml Dounce homogenizer by passing 15 times. Homogenates were centrifuged at 3,000 × g for 10 min to separate the cytosolic fraction (supernatant) from organelles (pellet). The resulting supernatant centrifuged at 106,000 × g for 60 min in a Beckman TLA 100.3 rotor to separate cytosolic S100 fraction (supernatant) from plasma membrane P100 fraction (pellet). The P100 pellet was resuspended in fractionation buffer containing 1% (v/v) Nonidet P-40, and the protein concentration was determined. A defined concentration of protein from each fraction was then used for kinase assay and immunoblotting.

Immunohistochemistry—We purchased a human cancer tissue array slide with paraffin sections from BioMax. Histostain-Plus kits (Zymed Laboratories Inc.) were used in accordance with the manufacturer’s instructions for the immunohistochemistry of tissue array (22). Briefly, paraffin sections were deparaffinized with xylene and rehydrated in a graded series of ethanol. The slide was submersed in peroxidase quenching solution for 10 min. After it was washed twice with PBS for 5 min, it was added with 2 drops of Reagent A for blocking and incubated for 30 min. Following two washes with PBS, the primary antibody, anti-Tyr(P)9 antibody, was applied at 4 °C overnight. Then biotinylated secondary antibody, Reagent B, was added after rinsing with PBS. It was incubated at room temperature for 1 h. It was rinsed with PBS and dropped with enzyme-conjugated Reagent C. After it was washed with PBS, DAB chromogen, and a mixture of Reagent D1, D2, and D3, it was dropped, and signals were observed with a florescence microscope. Then the reaction was stopped with distilled water, and pictures were taken with a florescence microscope.

Statistical Analysis—Quantification of Western blot analysis was done by using the Tina version 2.1 program (Raytest Isotopemgerate). Briefly, the relative intensity (area × density) of bands of interest was quantitated by a densitometer. The background value from a blank band was subtracted. The results were calculated as the ratio change compared with the corresponding control bands. Data are presented as means ± S.D. of the three independent experiments. The results were analyzed by Student’s t test (SPSS version 12.0 software, SPSS Inc.). p < 0.05 (*) was considered significant, and p < 0.01 (**) was highly significant compared with corresponding control values.

RESULTS

In Vitro Association of Tyrosine-phosphorylated PDK1 with SH2 Domain-containing Proteins—It has been reported that PDK1 becomes phosphorylated following stimulation of cells with pervanadate or insulin (13). Additionally, coexpressed v-Src or IR induced tyrosine phosphorylation of PDK1 in HEK 293 cells or L6 myoblasts (13, 14, 23). These findings prompted us to investigate the possible interaction between tyrosine-phosphorylated PDK1 and proteins containing an SH2-phosphorylated domain. SH2 domains of Src, Crk, and GAP bind to tyrosine-phosphorylated PDK1 in vitro. HEK 293 cells overexpressing Myc-PDK1 were treated with 100 μM pervanadate for 15 min. Cell extracts were mixed with GST-SH2 proteins (Src, Lck, Fyn2, phospholipase Cγ, p85, Crk, Sy p, GAP, Nck, Grb2, and Vav) that had been produced and purified on glutathione-Sepharose beads from Escherichia coli. Following recovery of fusion protein on glutathione-Sepharose beads, the bound PDK1 was detected by Western blot analysis using an anti-PDK1 antibody (top). Input of each GST-SH2 protein was also detected by anti-GST antibody (bottom). N and C, the N-terminal and C-terminal SH2 domain, respectively. Similar results were obtained in three separate experiments.

FIGURE 1. SH2 domains of Src, Crk, and GAP bind to tyrosine-phosphorylated PDK1 in vitro. HEK 293 cells overexpressing Myc-PDK1 were treated with 100 μM pervanadate for 15 min. Cell extracts were mixed with GST-SH2 proteins (Src, Lck, Fyn2, phospholipase Cγ, p85, Crk, Sy p, GAP, Nck, Grb2, and Vav) that had been produced and purified on glutathione-Sepharose beads from Escherichia coli. Following recovery of fusion protein on glutathione-Sepharose beads, the bound PDK1 was detected by Western blot analysis using an anti-PDK1 antibody (top). Input of each GST-SH2 protein was also detected by anti-GST antibody (bottom). N and C, the N-terminal and C-terminal SH2 domain, respectively. Similar results were obtained in three separate experiments.
Phosphorylation of PDK1 on Tyr^9 mediates the association with Src. A, for sequential binding, HEK 293 cells were transiently transfected with HA-Src WT alone and treated with 100 μM pervanadate for 15 min. First, HA-Src WT was immunoprecipitated (IP) from 500 μg of cell extracts using anti-HA 12CA5 monoclonal antibody. Immunoprecipitated HA-Src was then incubated with pervanadate-treated lysates from Myc-PDK1 WT cells. Immunoprecipitation was then repeated using anti-HA antibody (Seq-bind), for co-binding, lysates overexpressing either HA-Src or Myc-PDK1 were mixed and incubated, and bound PDK1 was then recovered on HA-Src immuno-precipitates (Co-bind). For co-expression, HA-Src WT was transiently transfected into HEK 293 cells stably overexpressing Myc-PDK1 WT, Y9F, or Y373F/Y376F (Co-expression). The cells were treated with 100 μM pervanadate for 15 min. Bound Myc-PDK1 on HA-Src immunoprecipitates were detected using anti-Myc 9E10 monoclonal antibody (middle). For all conditions, changes in the tyrosine phosphorylation of bound-PDK1 were detected by Western blot analysis with anti-Tyr(P)9 or anti-Tyr(P)^373/376 antibodies. Input of each PDK1 mutant protein was also detected using anti-Myc antibody (bottom). The data are representative of three independent experiments. B, purified GST-SH2 proteins (Src, Fyn2, and Crk) were employed (13). Briefly, synthetic phosphopeptide beads were prepared, immobilized phosphopeptides corresponding to the sequences surrounding Tyr(P)^9 or Tyr(P)^373/376 from human PDK1. Bound proteins were detected by Western blot analysis using an anti-GST antibody. Input of each GST-SH2 proteins was also detected (bottom). Similar results were obtained in three separate experiments.

Regulation of PDK1 Activity by Src

Binding of Src to PDK1 Requires Phosphorylation of Tyr^9—HEK 293 cells were used to investigate the regulation of PDK1 by the phosphoinositide 3-kinase signaling pathway. As previously reported, three tyrosine residues (Tyr^9 and Tyr^373/376) on PDK1 are phosphorylated in response to pervanadate in vivo (13). To further confirm an interaction between the Src SH2-domain and tyrosine-phosphorylated PDK1, we examined the ability of wild type HA-Src to bind to a Myc-tagged PDK1 in vitro and in vivo. No interaction between Src and PDK1 was detected when lysates from HEK 293 cells overexpressing HA-Src or Myc-PDK1 were mixed (Fig. 2A, lanes 2 and 3). However, tyrosine-phosphorylated PDK1 interacted with HA-Src in vivo when both are coexpressed in HEK 293 cells (Fig. 2A, lane 4, Co-expression), suggesting that binding of PDK1 to Src is indirect and may require additional protein molecules to facilitate this interaction in vivo. Coexpressed Y9F-PDK1 disrupted the formation of Src-PDK1 complex, whereas co-expression of Y373F/Y376F-PDK1 results in partial inhibition of complex formation (Fig. 2A, lanes 5 and 6). Furthermore, Tyr^9 phosphorylation was still detected in bound Y373F/Y376F-PDK1, whereas Tyr^373/376 phosphorylation was completely blocked in bound Y9F-PDK1, indicating that Tyr^9 phosphorylation is important for Src-PDK1 complex formation (Fig. 2A, lanes 5 and 6).

To further evaluate the binding specificity of this interaction, synthetic phosphopeptide beads were employed (13). Briefly, synthetic phosphopeptide corresponding to the 14 or 17 amino acids surrounding Tyr(P)^9 or Tyr(P)^373/376, respectively, were prepared, immobilized on Affi-Gel 15, and then incubated with the selected GST-SH2 proteins (Src, Fyn2, and Crk). As illustrated in Fig. 2B, the recovery of bound GST-SH2 domain on phosphopeptide beads revealed that there was strong interaction between Src/Crk and Tyr(P)^9 peptide, consistent with previous results (Fig. 1). However, no interaction with p373/376 peptide was observed. Taken together, these results indicated that the formation of Src-
Regulation of PDK1 Activity by Src

PDK1 complex requires Tyr9 phosphorylation of PDK1 and the help of additional molecules.

SH2 Domain of Src Is Responsible for the Binding to Myc-PDK1 in HEK 293 Cells—Previous authors have demonstrated that SH3 domains are involved in mediating protein-protein interactions together with SH2 domains in a cooperative manner (25, 26). To investigate the role of each domain from Src on the formation of Src-PDK complexes, SH2 or SH3 deletion mutants, a kinase-dead mutant, and a constitutive active Src on the formation of Src-PDK complexes, SH2 or SH3 deletion mutants, a kinase-dead mutant, and a constitutive active Src were prepared (Fig. 3A). To investigate the role of each domain from Src on the formation of Src-PDK complexes, SH2 or SH3 deletion mutants, a kinase-dead mutant, and a constitutive active Src were prepared (Fig. 3A). To eliminate difficulties with levels or protein expression in a transient transfection system, Myc-PDK1-stable HEK 293 cells were used. Coexpression of these mutants in HEK 293 cells stably overexpressing Myc-PDK1 revealed that the SH2 domain of Src was required for the association of Src and PDK1 (Fig. 3B, lane 5). Of note, binding of the SH3 deletion mutant of Src to Myc-PDK1 was significantly reduced compared with Src WT (Fig. 3B, lanes 2 and 6). Strikingly, a constitutive active version of Src formed a tighter complex with Myc-PDK1 compared with Src WT (Fig. 3B, lanes 2 and 4). In contrast, there was almost no interaction between kinase-dead Src and Myc-PDK1, indicating that the interaction between Src and PDK1 requires the SH2 domain and Src catalytic activity of Src.

Hsp90 Inhibitors Promote PDK1 Destabilization, Leading to Disruption of Hsp90/PDK1 Complexes—Our data to date suggest that PDK1 binding to Src requires additional protein molecules (Fig. 2A). Previous authors have demonstrated that inhibition of PDK1 binding to Hsp90 triggers proteasome-dependent degradation of PDK1 (17). This allowed us to investigate the possible involvement of Hsp90 in modulating PDK1-Src complex formation. First, we tested whether geldanamycin, an Hsp90 inhibitor, altered PDK1 stability in our experimental setting. Treatment of HEK 293 cells stably expressing PDK1 WT, PDK1-Y9F, or PDK1-Y373F/Y376F with geldanamycin showed that PDK1 stability was affected in PDK1 WT cell line (Fig. 4A, lanes 1–3), consistent with previous reports (17). In contrast, PDK1 stability was not affected in the PDK1-Y9F cell line (Fig. 4A, lanes 4–6), whereas levels of PDK1 were reduced by 30% at 24 h in the PDK1-Y373F/Y376F cell line (Fig. 4A, lanes 7–9). We then monitored the levels of PDK1-Hsp90 complex in the above experiment. When we normalized the amount of Hsp90 co-immunoprecipitating to the total amount of Hsp90 in each cell line, the association of Hsp90 with PDK1 WT or PDK1-Y373F/Y376F was reduced upon treatment of cells with geldanamycin, whereas the interaction between PDK1 and Hsp90 was almost unchanged (Fig. 4C and D). In addition, decreased binding of PDK1-Y9F/Hsp90 compared with PDK1 WT/Hsp90 was observed when the expression level of PDK1 WT and PDK1-Y9F were normalized (Fig. 4E). However, the interaction between PDK1 WT, Y9F, and Y373F/Y376F and Hsp90 was not affected upon treatment of cells with geldanamycin (Fig. 4E). Treatment of cells with 17-AAG, a second Hsp90 inhibitor, gave a similar result (data not shown). The above results suggest that Tyr9 phosphorylation of PDK1 is critical for Hsp90-mediated PDK1 stabilization and PDK1-Hsp90 complex formation.

Hsp90 Inhibitor-induced Destabilization of PDK1 Is Mediated by a Proteasome-dependent Pathway—It has been previously proposed that PDK1 degradation by Hsp90 inhibitors is...
Regulation of PDK1 Activity by Src

FIGURE 5. The effect of proteasome inhibitors on 17-AAG-mediated PDK1 destabilization. Myc-PDK1 WT cells were pretreated with 1 μM lactacystin, 1 μM PSI, or 5 nM MG132 for 1 h before the treatment of cells with 1 μM 17-AAG for 24 h. A, total cell lysates were further fractionated into 1% (v/v) Nonidet P-40 soluble and insoluble fraction. Equal amounts of protein lysates from soluble and insoluble fraction were analyzed by immunoblotting using anti-PDK1 antibodies (top two panels). Control for equal loading was checked by immunoblotting with anti-actin antibodies (bottom two panels), B, statistical differences of soluble and insoluble PDK1 were determined by comparing values for actin at each lane. The results are mean ± S.D. of three independent experiments. *, p < 0.05; **, p < 0.01.

mediated by proteasome-dependent pathway in MCF7 and HEK 293T cells (17, 27). To analyze this finding in our experiments, PDK1 WT cells were cultured in medium containing 17-AAG with proteasome inhibitors (lactacystin, PSI, or MG132). Consistent with previous data (17, 27), PDK1 degradation was partially inhibited by proteasome inhibitors (Fig. 5A) but not by other protease inhibitors (benzoylloxycarbonyl-VAD and NH4Cl; data not shown). It has been reported that binding to Hsp90 prevents some signaling proteins from self-association or from becoming insoluble (28, 29). We also examined the amounts of PDK1 in detergent-insoluble fractions recovered from centrifuge precipitates, since proteasome inhibitors could not completely protect PDK1 from degradation (Fig. 5A). Semiquantification of soluble PDK1 levels revealed that proteasome inhibitors increased the level of soluble PDK1 by 2-fold (Fig. 5B, top). Furthermore, the analysis of detergent-insoluble fraction recovered from centrifuge precipitates indicated that the level of insoluble PDK1 was significantly increased in the presence of proteasome inhibitors (lactacystin and PSI; Fig. 5, A and B). In contrast to previous reports (17), there was little effect on the levels of insoluble PDK1 in the presence of MG-132 in our experimental settings (Fig. 5, A and B, lanes 7 and 8).

Subcellular Localization of PDK1 Is Modified by Src-CA and Hsp90—It has been suggested that PDK1 can translocate to plasma membrane following treatment of cells with pervanadate or insulin (13). To evaluate the involvement of Src and Hsp90 in this process, we first monitored the subcellular localization of PDK1 and Src following treatment of cells with pervanadate (Fig. 6, A and B). Consistent with previous reports, PDK1 translocated to plasma membrane in the presence of pervanadate. Interestingly, significant amounts of Src were also translocated to plasma membrane under these conditions (Fig. 6, A and B). Levels of the cytosolic marker protein α-tubulin and membrane marker protein caveolin-1 indicated that no significant contamination of membrane or cytosolic fraction (Fig. 6A, bottom). To investigate the effects of Src-CA and Hsp90 overexpression on PDK1 localization, we lysed cotransfected Myc-PDK1 WT cells in Hepes/sucrose buffer, facilitating the preparation of cytosolic and membrane fraction for immunoblotting. Subcellular fractionation of HEK 293 cells revealed that overexpression of either Src-CA or Hsp90 led to membrane translocation of PDK1 in serum-starved conditions (Fig. 6, C and D). These results clearly suggested that Src-CA and Hsp90 play an important role in regulating the subcellular localization of PDK1.

Role of Hsp90 in PDK1/Src Association and Src-mediated PDK1 Activation—To evaluate the direct involvement of Hsp90 in PDK1/Src association in vitro, Myc-PDK1 WT and HA-Src CA (constitutively active) were separately prepared. Previous data demonstrated that no interaction between Myc-PDK1 WT and HA-Src was observed when protein lysates from transfected cells were mixed in vitro (Fig. 2A). Strikingly, co-expressed Hsp90, but not Hsp40 or Hsp27, enhanced PDK1-Src complex formation in vitro (Fig. 7A, lane 3, upper panel). Endogenous Src was immunoprecipitated from HEK 293 cells and the resulting immunoprecipitates were assayed for the presence of PDK1 or Hsp90. Endogenous PDK1 and Hsp90 co-immunoprecipitated with Src only in the presence of pervanadate, indicating that these proteins form an endogenous complex in vivo (Fig. 7B). We then analyzed the role of Hsp90 in the formation of PDK1-Src complex in vivo. The recovery of bound Src in Myc-PDK1 immunoprecipitates revealed that the level of PDK1-Src complex was dramatically increased in HEK 293 cells coexpressing Hsp90 compared with control cells in the presence of pervanadate (Fig. 8A, bottom). To check whether increased PDK1-Src complex formation directly affected PDK1 activation, the kinase activity of immunoprecipitated Myc-PDK1 was assayed by using Suntide (13) as a substrate. As shown in Fig. 8, B and C, Hsp90 expression increased the overall kinase activity of PDK1 by 1.5–2-fold as well as increasing tyrosine phosphorylation of PDK1 on Tyr9 and Tyr373/375 residue. To further confirm Hsp90-induced PDK1 activation, we transfected HEK 293 cells with siRNA targeting Hsp90 (Fig. 8D, top). PDK1 activity from the membrane fraction of Hsp90-siRNA-transfected cells was markedly reduced, whereas negligible changes in PDK1 activity were observed in control siRNA-transfected cells (Fig. 8D). Consequently, PDK1-Src complex formation was also decreased under these conditions, potentially due to decreased PDK1 stability (Fig. 8, E and F). Next, we examined whether these changes in phosphorylation were relevant to downstream tar-
Regulation of PDK1 Activity by Src

for the indicated times, Hsp90 expression is increased together with PDK1 levels (Fig. 9, A and B). IkB degradation was also detected to monitor the PMA activity in these cells. The binding of PDK1 to Src was also increased potentially due to increased PDK1 stability by Hsp90 (Fig. 9B, bottom). As shown in Fig. 2, the importance of Tyr<sup>9</sup> phosphorylation in PDK1 activation was apparent. Also, there is increasing evidence that PDK1 is involved in the cancer progression and invasion (32–34). Therefore, we employed the multi-organ human tissue array from normal and diseased tissue to screen the possible involvement of Tyr<sup>9</sup> phosphorylation on PDK1 in various cancers. Immunohistochemical analysis with anti-phospho-Tyr<sup>9</sup> antibodies showed that the level of Tyr<sup>9</sup> phosphorylation is markedly increased in diseased tissue compared with normal tissue from lung, liver, colon, and breast (Fig. 9C). To further evaluate these findings, total cell lysates from normal and cancerous tissues from four patients who underwent surgery for malignant colon cancer were analyzed by Western blotting with anti-PDK1 or anti-Hsp90 antibodies. As shown in Fig. 9B, colon cancer tissues expressed PDK1 at a significantly higher level than the normal tissues in all samples. This observation was also true for Hsp90 expression (Fig. 9D, middle), suggesting that Hsp90-mediated PDK1 stabilization leads to an increase in Tyr<sup>9</sup> phosphorylation.

**DISCUSSION**

PDK1 is well characterized as a master kinase that activates various intracellular serine/threonine kinases, such as PKB, p70<sup>S6K</sup>, protein kinase C, SGK, and RSK in many different signaling pathways (1, 21, 35–37). Once activated, these enzymes mediate many of the diverse effects of insulin and other growth factors on cells by phosphorylating key regulatory proteins that play crucial roles controlling processes such as cellular survival, proliferation, protein synthesis, and gene expression (for a review, see Refs. 3 and 4). All of these enzymes require phosphorylation by PDK1 on conserved Ser/Thr residue in the activation loop of their kinase domains (1, 21, 35–37). Therefore, regulation of PDK1 activity may be a critical “hub” for the control of multiple diverse signaling pathways. It is thought that...

**FIGURE 6. Effects of Src-CA or Hsp90 overexpression on subcellular localization of PDK1.** A, Myc-PDK1 WT cells were either untreated (Un) or treated with pervanadate (Van) for 15 min, and the cytosolic fraction (Cyt) and plasma membrane fraction (Mem) were prepared as described “Experimental Procedures.” Changes in the distribution of PDK1 and Src were detected by Western blot analysis with anti-Myc and anti-HA antibodies, respectively. α-Tubulin for the cytosolic marker protein and caveolin-1 for membrane marker protein were used for monitoring the cross-contamination. B, the relative density was obtained by the densitometry of the corresponding immunoblot data. Statistical differences of each fraction were determined by comparing values for the amount of total cell lysates (Tot) at each lane. The results are mean ± S.D. of three independent experiments. *, p < 0.05; **, p < 0.01.

Cyt and Mem were detected to monitor the cross-contamination. C, Myc-PDK1 WT cells were transfected with HA-Src CA or FLAG-Hsp90 expression vectors. Cells were serum-starved for 16 h prior to subcellular fractionation. D, bands of PDK1 were further analyzed by densitometry. The ratio of PDK1 amount between membrane fraction and cytosolic fraction from control vector-transfected cells was set as 1. Statistical differences of each ratio were determined by comparing the ratio from control vector-transfected cells. The results are mean ± S.D. of three independent experiments. *, p < 0.05; **, p < 0.01.

gets of PDK1 in these cells. PDK1 has been shown to phosphorylate PKB at residue Thr<sup>308</sup> and SGK at the Ser<sup>256</sup> residue (1, 21, 30). Overexpression of Hsp90 triggered a significant increase in phosphorylation of both PKB and SGK (Fig. 8, G and H). Taken together, these results strongly suggest that Hsp90 facilitates the stabilization of PDK1 and PDK1-Src complex formation, leading to the activation of downstream targets of PDK1 in vitro and in vivo.

Hsp90 and PDK1 Expression in Physiological and Disease Conditions—Recently, it was revealed that the promoter of the hsp90α gene, which encodes the inducible form of Hsp90, is regulated by NF-κB activity (31). Therefore, we employed PMA to induce the Hsp90 expression via NF-κB signaling under physiological conditions, since all of the experiments for PDK1 stability were performed with Hsp90 inhibitors (Figs. 4 and 5). Upon treatment of cells with PMA and activation of PDK1 in cancerous tissue.
Regulation of PDK1 Activity by Src

PDK1 exists in an active form in quiescent cells, since insulin stimulation does not significantly increase the PDK1 activity (9, 36). This notion was supported by the identification of phosphorylation sites on PDK1 in vivo (12). This report suggested that PDK1 was constitutively phosphorylated in vivo on several serine residues, and phosphorylation of serine 241 was critical for PDK1 activity. This phosphorylation was also not affected by treatment of cells with agonists, such as IGF-1 (12). However, there is growing evidence that PDK1 activity can be further increased following stimulation with pervanadate, hydrogen peroxide, and insulin in adipocytes, HEK 293 cells, and A20 lymphoma cells (13–15). Three tyrosine residues on PDK1 were identified in this activation process in vivo using mass spectrometry and site-directed mutagenesis: Tyr9, Tyr373, and Tyr376 (13, 15). Furthermore, it was proposed that the Src family kinases, Abl, RET/PTC, and Pyk2 were potential candidates for PDK1 activation (13–15, 38, 39). This observation was further supported by the recent report showing that PDK1 binds directly to the insulin receptor, leading to tyrosine phosphorylation and activation of PDK1, mediating the metabolic effects of insulin (23).

Here we provide clear evidence that PDK1 directly interacts with the SH2 domain of Src, Crk, and GAP in vitro (Fig. 1). We also show that this interaction between PDK1 and Src is mediated by Tyr9 phosphorylation of PDK1 but not by Tyr373/376 phosphorylation. In vivo, the association of PDK1 and Src is enhanced by Hsp90 overexpression, resulting in an increase in PDK1 activity towards PKB and SGK. Taken together, these data suggest that PDK1 activation by Src is mediated by SH2-domain of Src and Tyr9 phosphorylation of PDK1, and this event is assisted by Hsp90 via the stabilization of each protein and nucleating protein complex formation. Our current model for the regulation of PDK1 is outlined in Fig. 10.

There is now considerable evidence that PDK1 becomes tyrosine-phosphorylated and further activated in different cell types. There appears to be an order in the phosphorylation events of PDK1 on Tyr9 and Tyr373/376, since phosphorylation on Tyr9 has an influence on Tyr373/376 phosphorylation (13). Tyr9 phosphorylation occurred 5 min earlier than Tyr373/376 phosphorylation when the cells were stimulated with pervanadate (13). Several tyrosine kinases were proposed as upstream regulators of PDK1 in these cells. They can be further divided into two groups: Tyr9 kinases and Tyr373/376 kinases. Based on previous reports, Src and Abl were proposed as Tyr373/376 kinases (13–15), whereas RET/PTC, Pyk2, and IR were candidates for the Tyr9 phosphorylation (23, 38, 39). Among these upstream kinases for PDK1, Pyk2 and IR have been shown to directly interact with PDK1, permitting Tyr9 phosphorylation (23, 38). These observations prompted us to investigate the putative interaction between Src and PDK1, as previously suggested (13). We demonstrated that tyrosine-phosphorylated PDK1 directly interacts with Src in vitro and in vivo (Figs. 1–3). This association specifically required Tyr9 phosphorylation of PDK1 (Fig. 2). Furthermore, the SH2 domain of Src is required for this interaction (Fig. 3), providing novel biochemical evidence that PDK1 and Src can form a complex via phospho-Tyr9 of PDK1 and the SH2 domain of Src-mediated interaction. Interestingly, similar to the previous reports (25, 26), the SH3 domain of Src is also involved in the PDK1-Src complex formation together with SH2 domains in a cooperative manner (Fig. 3B, lane 6).

It has been previously suggested that Src is involved in TRANCE-mediated PKB activation in osteoclasts and dendritic cells (40) and in epidermal growth factor-mediated PKB activation in COS1 cells (41). An important role for Src in H2O2-induced activation of PKB was demonstrated in CHO cells overexpressing the human insulin receptor (42). In these studies, H2O2-stimulated PKB phosphorylation, which was blocked by PP2, a highly specific inhibitor of Src activity. Recently, Azar and co-workers (43) have implicated Src in H2O2-evoked PKB phosphorylation in A10 VSMC, with an intermediary role for Pyk2 in this process. One report also showed that elevated PKB activity is observed in v-Src-transformed NIH3T3 cells, accompanied by increased tyrosine phosphorylation of PKB (44). However, no significant data demonstrating that PKB is the direct target for Src were presented. Therefore, the previous data support the notion that PDK1 could serve as the intermediate molecule between PKB and Src-Pyk2 in different signaling pathways, since Src-Pyk2 has been suggested to be an upstream kinase for PDK1 (13–15, 38). It is, of course, interesting to ask if PDK1 activity induced by growth factors is significantly reduced in SYF cells lacking Src, Yes, and Fyn or if PDK1 activation could be restored by introducing c-Src back into these cells.

As shown in Fig. 2, PDK1 does not bind to Src in vitro. This led us to speculate that additional protein molecules are involved in this association. It has been shown that Hsp90 can directly bind and stabilize PKB activity by preventing PP2A-mediated dephosphorylation (45). Hsp90 can also recruit PKB...
Regulation of PDK1 Activity by Src

FIGURE 8. Coexpressed Hsp90 enhances Src-mediated PDK1 activation as well as PDK1-Src interaction. Myc-PDK1 WT cells were cotransfected with HA-Src WT and FLAG-Hsp90, followed by treatment with 100 μM pervanadate for 15 min. A, bound HA-Src in Myc-PDK1 immunoprecipitates was analyzed using anti-HA antibody (bottom). Protein expression was also detected using anti-Myc, anti-HA, and anti-Hsp90 antibodies. B, changes in the tyrosine phosphorylation of immunoprecipitated PDK1 were detected by Western blot analysis with anti-Tyr(P)9 or anti-Tyr(P)373/376 antibodies (top two panels). Bands of interest were further analyzed by densitometry. Data were normalized to actin. The results are mean ± S.D. of three independent experiments. *, p < 0.05; **, p < 0.01. C, the immune complexes were assayed for kinase activity using Suntide peptide as substrate. Activity of PDK1 immunoprecipitated from untreated cells was set at 1. Kinase activity is plotted relative to untreated cells. D, HEK 293 cells were transfected with Hsp90 siRNA or control siRNA (con siRNA). The activity of PDK1 immunoprecipitated from the membrane fraction of untreated cells was set at 1. Kinase activity is plotted as the average ± S.D. of three independent experiments. *, p < 0.05; **, p < 0.01. Total amount of Hsp90 was detected by immunoblotting with anti-Hsp90 antibody. E, HEK 293 cells were transfected with Hsp90 siRNA or control siRNA (con siRNA). Bound Myc-PDK1 in HA-Src immunoprecipitates was analyzed using anti-Myc antibody (bottom). Protein expression was also detected using anti-Hsp90, anti-Myc, anti-HA, and anti-actin antibodies. F, statistical differences of bound Myc-PDK1 in HA-Src immunoprecipitates were determined by comparing values from untreated cells. The results are mean ± S.D. of three independent experiments. *, p < 0.05; **, p < 0.01. G, phosphorylation of downstream target for PDK1 was analyzed by immuno- blotting with anti-phospho-PKB (Thr308) or anti-phospho-SGK (Thr256) antibodies. The total amount of each protein was also monitored by anti-PKB or anti-actin antibodies. H, statistical differences of phospho-PKB and phospho-SGK were determined by comparing values for PKB and actin at each lane. The results are mean ± S.D. of three independent experiments. *, p < 0.05; **, p < 0.01.

to the eNOS complex during NO-dependent angiogenic processes (46, 47). Furthermore, it has also been suggested that Hsp90 acts on PDK1 in a similar manner to PKB (16, 17, 27, 48, 49). However, the mode of Hsp90 action on PKB and PDK1 is somewhat different. Hsp90 inhibitors did not inhibit PKB-Hsp90 binding or PKB activity (45, 50, 51), whereas Hsp90 inhibitors suppress PDK1-Hsp90 complex formation, leading to PDK1 destabilization without directly inhibiting PDK1 activity (Fig. 4) (17). We also observed that Y9F-PDK1 is resistant to PDK1 destabilization and PDK1-Hsp90 complex formation, induced by an Hsp90 inhibitor, suggesting that Tyr9 phosphorylation of PDK1 is important for this process (Fig. 4). However, Tyr9 phosphorylation of PDK1 appears to have no effects on kinase activity itself, since Y9F-PDK1 activity was similar to wild type activity (13).

Consistent with previous reports (17), we also confirmed that PDK1 destabilization is mediated by proteasomal degradation (Fig. 5). However, proteasome inhibitors could not completely protect PDK1 from degradation (Fig. 5A). We monitored PDK1 protein level in detergent-insoluble fractions, which can be an explanation for the role of Hsp90 (28, 29). The level of insoluble PDK1 was significantly increased in the presence of proteasome inhibitors (lactacystin and PSI; Fig. 5, A and B). In contrast to previous reports (17), there was little effect on the levels of insoluble PDK1 in the presence of MG-132 in our experimental setting (Fig. 5, A and B, lanes 7 and 8). Furthermore, it has been reported that insoluble PDK1 was dephosphorylated on Ser241 (inactive PDK1) (17). Therefore, Hsp90 stabilizes PKB activity by preventing PP2A-mediated dephosphorylation and by stabilizing PDK1 via Hsp90 protein complex formation. Indeed, we found that only Hsp90, not Hsp40 and Hsp27, facilitate PDK1-Src complex formation in vitro (Fig. 7A). Overexpression of
Regulation of PDK1 Activity by Src

Hsp90 enhanced PDK1-Src complex formation by ~3-fold (Fig. 8A) with concomitant changes in PDK1 kinase activity (Fig. 8C). These results were further confirmed by siRNA-mediated knockdown of Hsp90 (Fig. 8, D–F), indicating that Hsp90 is an important molecule that is required for the association of PDK1 and Src and PDK1 activation in vivo.

Although growth factor-stimulated translocation of PKB to the plasma membrane has been well characterized (3, 52), it is controversial whether or not PDK1 translocates in the presence of growth factors (53–57). However, it has been demonstrated that PDK1 is recruited to the plasma membrane with a concomitant increase in kinase activity and tyrosine phosphorylation when cells were exposed to insulin (13). In this regard, overexpression of either Src-CA or Hsp90 led to membrane translocation of PDK1 in serum-starved conditions (Fig. 6, C and D), suggesting that Src-CA and Hsp90 play an important role in regulating PDK1 kinase activity through subcellular localization of PDK1.

Unlike other AGC kinases that are substrates for PDK1, direct in vitro or in vivo association of PKB with PDK1 has not been demonstrated (55). By employing Foester resonance energy transfer technology, it was recently shown that PDK1 is complexed to its substrate, PKB, in the cytoplasm (58). In this study, PKB activation is regulated by intramolecular and intermolecular interactions, caused by upstream signals and its concomitant translocation to plasma membrane (58). Our data suggest that Src and Hsp90 are also components of this protein complex and regulate the balance of PKB and PDK1 activity, since protein-protein interactions have previously been demonstrated to modulate PDK1 activity (59–63). In the presence of PRK2-interacting fragment, PDK1 activity toward a synthetic peptide substrate was increased up to 4-fold (60), indicating that PDK1 may depend on protein-protein interactions to stabilize the catalytically active conformation of the enzyme. Indeed, it has been shown that phosphorylation of a hydrophobic motif containing an PRK2-interacting fragment-like sequence serves as a docking site for PDK1 and increases the PDK1 activity toward its substrates (64). Similarly, our results showed that overexpression of Hsp90 enhanced Src-mediated PDK1 activation toward PKB and SGK (Fig. 8, G and H) as well as protein-protein interaction (Fig. 8A).

To our knowledge, this work provides the first evidence in support of the involvement of Hsp90 in Src-induced phosphorylation of PDK1. Hsp90 has reemerged as an important molecule that is required for stability and function of signaling proteins, including PDK1 and PKB, that are frequently mutated and overexpressed in human disease. Several groups, including ours, suggested that Hsp90 was overexpressed in a wide variety of cancer cells and in virally transformed cells (Fig. 9D) (19, 65–67). Indeed, Tyr9 phosphorylation of PDK1 was increased in diseased tissue from lung, liver, colon, and breast (Fig. 9C), which is similar to the observations of the previous reports (32–
Regulation of PDK1 Activity by Src

34. Furthermore, the treatments of cells with PMA, which is a physiological inducer for Hsp90 expression via NF-κB signaling (31), led to increased PDK1 stability by Hsp90 and to increased PDK1-Src complex formation (Fig. 9, A and B). Taken together, these data suggest that a fuller appreciation of the complex role played by Hsp90 in regulating phosphoinositide 3-kinase/PDK1/PKB signaling pathways will better inform the clinical utility of Hsp90 inhibitors.

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