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Identification of Tyrosine Phosphorylation Sites on 3-Phosphoinositide-dependent Protein Kinase-1 and Their Role in Regulating Kinase Activity*

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3-Phosphoinositide-dependent protein kinase-1 (PDK1) plays a central role in signal transduction pathways that activate phosphoinositide 3-kinase. Despite its key role as an upstream activator of enzymes such as protein kinase B and p70 ribosomal protein S6 kinase, the regulatory mechanisms controlling PDK1 activity are poorly understood. PDK1 has been reported to be constitutively active in resting cells and not further activated by growth factor stimulation (Casamayor, A., Morrice, N. A., and Alessi, D. R. (1999) *Biochem. J.* 342, 287–292). Here, we report that PDK1 becomes tyrosine-phosphorylated and translocates to the plasma membrane in response to pervanadate and insulin. Following pervanadate treatment, PDK1 kinase activity increased 1.5- to 3-fold whereas the activity of PDK1 associated with the plasma membrane increased ~6-fold. The activity of PDK1 localized to the plasma membrane was also increased by insulin treatment. Three tyrosine phosphorylation sites of PDK1 (Tyr-9 and Tyr-373/376) were identified using *in vivo* labeling and mass spectrometry. Using site-directed mutants, we show that, although phosphorylation on Tyr-373/376 is important for PDK1 activity, phosphorylation on Tyr-9 has no effect on the activity of the kinase. Both of these residues can be phosphorylated by v-Src tyrosine kinase *in vitro*, and co-expression of v-Src leads to tyrosine phosphorylation and activation of PDK1. Thus, these data suggest that PDK1 activity is regulated by reversible phosphorylation, possibly by a member of the Src kinase family.

3-Phosphoinositide-dependent protein kinase-1 (PDK1)[1] appears to play a central regulatory role in many cell-signaling pathways (1–12). Several substrates of PDK1 have so far been identified, including protein kinase B (PKB), p70 ribosomal protein S6 kinase (p70S6K), cyclic AMP-dependent protein kinase (PKA), protein kinase C (PKC), serum and glucocorticoid-inducible kinase (SGK), p90 ribosomal protein S6 kinase (RSK), and p21-activated kinase-1 (1, 2, 9, 13–31). However, most of these putative PDK1 substrates were identified using *in vitro* phosphorylation experiments, and not all the substrate proteins appear to be physiological targets for PDK1 *in vivo*. Experiments with embryonic stem (ES) cells ablated for both alleles of PDK1 revealed that PKB, p70S6K, and RSK are no longer phosphorylated or activated by agonists that are potent stimuli in normal ES cells, providing *in vivo* evidence that these three enzymes are dependent on PDK1 for activation (32). The regulation of the other potential substrates of PDK1 is apparently unaffected in the knock-out cell line (32).

PKB, p70S6K, RSK, and SGK, members of the AGC family of protein kinases, are apparently maintained in the cytoplasm in an inactive state. Upon stimulation of receptor tyrosine kinases, phosphoinositide 3-kinase (PI3K) becomes activated, generating the phospholipid second messengers, PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2. These lipids then mediate the phosphorylation and activation of PDK1 targets through diverse mechanisms (1, 2, 13, 14, 27–30, 33). In the case of PKB, this activation appears to be facilitated by lipid binding to pleckstrin homology (PH) domains that mediate the recruitment of both PKB and PDK1 to the plasma membrane, thereby promoting phosphorylation of PKB by PDK1 and the as-yet-unidentified Ser-473 kinase (34–46). In the case of SGK/RSK/p70S6K, the precise mechanism for stimulatory phosphorylation of the activation loop by PDK1 is less well understood. It is possible that there is a mechanism for inducing transient association of PDK1 and SGK/RSK/p70S6K. Indeed, PDK1 and SGK are able to form a stable complex (27), and phosphorylation of a hydrophobic motif on RSK serves as a docking site for PDK1 (47).

Although a role for PDK1 in insulin signaling is well established, the mechanism of regulation of PDK1 activity remains controversial. Previously, it was thought that PDK1 is constitutively active in resting cells and not further activated by growth factor stimulation (48). In addition, the subcellular localization of PDK1 also appears to be growth factor-insensitive (49), although others have reported growth factor-dependent translocation of PDK1 to the plasma membrane (42, 50).

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[1] The abbreviations used are: PDK1, 3-phosphoinositide-dependent protein kinase-1; PKB, protein kinase B; p70S6K, p70 ribosomal protein S6 kinase; PKA, cyclic AMP-dependent protein kinase; PKC, protein kinase C; SGK, serum and glucocorticoid-inducible kinase; RSK, p90 ribosomal protein S6 kinase; ES cells, embryonic stem cells; PI3K, phosphoinositide 3-kinase; PtdIns, phosphatidylinositol; PH, pleckstrin homology; HEK, human embryonic kidney; IGF-1, insulin-like growth factor-1; TC-PTP, T-cell protein-tyrosine phosphatase; IR, insulin receptor; PMSF, phenylmethylsulfonyl fluoride; DTT, dithiothreitol; Suntide, RRKDGATMK**T**FCGTPE; PP2Ac, protein phosphatase 2A catalytic subunit; PAGE, polyacrylamide gel electrophoresis; PP1, 4-amino-5-(4-methylphenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine; pS, phosphoserine; pT, phosphothreonine; pY, phosphotyrosine; HPLC, high performance liquid chromatography; MS, mass spectrometry; MS/MS, tandem mass spectrometry; ESI, electrospray ionization; CID, collision-induced dissociation; PIF, PDK1-interacting fragment; SH2, Src homology-2; Lambda-PP, Lambda protein phosphatase; PCR, polymerase chain reaction; aa, amino acid(s); AGC, second messenger regulated subfamily of protein kinases.

Several serine sites (Ser-25, Ser-241, Ser-393/396, Ser-410) are phosphorylated on PDK1 in unstimulated human embryonic kidney (HEK) 293 cells, as well as insulin-like growth factor-1 (IGF-1)-stimulated cells (48). However, only phosphorylation on the activation loop Ser-241 (equivalent to Thr-308 of PKB) is necessary for PDK1 activity (48). Furthermore, this study examined the effect of IGF-1 stimulation on PDK1 activity and was unable to find any activation (48). Although physiologically relevant, signal transduction events elicited by IGF-1 may be transient, and/or too small in magnitude for detection in this experimental paradigm.

To further probe the regulation of PDK1, we have utilized pervanadate, an inhibitor of protein-tyrosine phosphatases that apparently mimics insulin action, to stimulate cells (51). Pervanadate stimulates PKB activity to a much higher extent than insulin or IGF-1 (52), and through this amplification of signal intensity, we hoped to observe changes in PDK1 activity and/or subcellular localization previously not detectable with insulin stimulation. Indeed, stimulation of cells with pervanadate resulted in a significant increase in PDK1 activity and translocation to the plasma membrane, together with an induction of tyrosine phosphorylation. During the course of our experiments, work from two independent groups (50, 53) also reported that PDK1 becomes tyrosine-phosphorylated following stimulation with pervanadate or hydrogen peroxide in adipocytes, HEK 293 cells, and A20 lymphoma cells. In this report, we identify three tyrosine phosphorylation sites on PDK1 using *in vivo* labeling and mass spectrometry: Tyr-9 and Tyr-373/376. Using site-directed mutants, we show that although phosphorylation on Tyr-373/376 is important for PDK1 activity, phosphorylation on Tyr-9 has no effect on the activity of the kinase. Both sites can be phosphorylated by v-Src tyrosine kinase *in vitro*, and co-expression of v-Src leads to tyrosine phosphorylation and activation of PDK1. Thus, these data show that PDK1 activity is regulated by reversible phosphorylation, possibly by a Src family kinase.

EXPERIMENTAL PROCEDURES

Reagents—Purified recombinant T-cell protein-tyrosine phosphatase (TC-PTP (54)) and v-Src were kindly provided by Dr. Nick Tonks (Cold Spring Harbor, NY) and Dr. Doriano Fabbro (Novartis, Switzerland), respectively. Monoclonal anti-Src and anti-insulin receptor (IR) antibody were gifts from Dr. Kurt Ballmer-Hofer (Paul Scherrer Institute, Switzerland) and Dr. Kenneth Siddle (University of Cambridge, UK), respectively. Anti-Myc 9E10 and anti-phosphotyrosine 4G10 monoclonal antibody were from commercial sources.

Construction of Expression Vectors—The Myc-tagged full-length PDK1 (Myc-PDK1) was generated by a two-step PCR using Myc-PDK1-Δ50 lacking the first 50 amino acids (14) as a template with the following primers (1st round PCR, 5′-CCC GGT ACC ACC ATG GCT TAC CCA TAC GAT GTT CCA GAT TAC GCT TCG ACC GTC AAA ACC GAG GCT GCT CGA; 2nd round PCR, 5′-CCC GGT ACC ACC ATG GCT TAC CCA TAC GAT GTT CCA GAT TAC GCT TCG ACC and 3′-CCC GGT ACC ACC ATG GCT TAC CCA TAC GAT GTT CCA GAT) and subcloned into pCMV5 vector. The PH domain deletion mutant of PDK1 was generated by a standard PCR-cloning strategy (Myc-ΔPH-PDK1). The mutants at Tyr-9 (Myc-PDK1 Y9F), Tyr-373/376 (Myc-PDK1 Y373/376F), and both Tyr-9 and Tyr-373/376 (Myc-PDK1 FFF) were created by using the QuikChange site-directed mutagenesis kit (Stratagene) as described by the manufacturer with pCMV5 Myc-PDK1 as template. v-Src and kinase-dead mutants of v-Src constructs in pcDNA3.1 (v-Src and v-Src-KD) were a gift from Dr. Monilola Olayioye (FMI, Switzerland). Wild type Fyn (Fyn-WT) and insulin-receptor tyrosine kinase (IR-WT) expression constructs have been described previously (55, 56). All constructs were confirmed by automated DNA sequencing. Sequences of the mutagenic oligonucleotides are available upon request.

Cell Culture and Stimulation—HEK 293 cells were maintained and transfected using a modified calcium phosphate method as previously described (27). The transfection mixture was removed after 16-h incubation, and cells were serum-starved for 24 h before stimulation for 15 min with 100 nM insulin (Roche Molecular Biochemicals), 50 ng/ml IGF-1 (Life Technologies), 100 nM calyculin-A (Alexis), or 100 μM pervanadate prepared with 0.2 mM H_2O_2 (51), or for 1 h with 1 μM okadaic acid (Alexis). Pretreatment with 50 μM LY294002 (Alexis) or 10 μM 4-amino-5-(4-methylphenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine (PP1, Alexis) was for 15 or 30 min before cell stimulation, respectively.

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 (PMSF), 1 mM benzamidine, and 2 μM microcystin-LR. Lysates were centrifuged for 15 min at 12,000 × *g* and the Myc-PDK1 protein was immunoprecipitated from 200–400 μg of cell-free extracts with the anti-Myc 9E10 monoclonal antibody immobilized on protein G-Sepharose (Amersham Pharmacia Biotech). 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 $MgCl_2$, 1 μM protein kinase A inhibitor peptide (Bachem), and 100 μM [γ-^{32}P]ATP (Amersham Pharmacia Biotech; 1000–2000 cpm/pmol). Reactions were stopped by adding EDTA to a final concentration of 50 mM and processed as described previously (52). Protein concentrations were determined by the method of Bradford (Bio-Rad) using bovine serum albumin as a standard.

Production of Antibodies against PDK1—Polyclonal antisera that recognize specific phosphorylation sites were raised against the following peptides: RTTSQLYDAVPIQS (Tyr-9; 3–16 aa), VLCSCPSPSMVRTQ (Ser-25; 19–32 aa), KQARANSFVGTAQY (Ser-241; 235–248 aa), EDDEDCYGNYDNLLSQF (Tyr-373/376; 367–383 aa), VSSSSSSHSLSASDTG (Ser-393/396; 388–403 aa), LPQRSGSNIEQYIH (Ser-410; 404–417 aa), where the phosphorylated amino acids are underlined. An antisera against the C-terminal region of PDK1 was generated by injecting with the following peptide: KIQEVWRQRYQSHPDAAVQ (538–556 aa). In addition, antisera were prepared by simultaneously immunizing with the four phosphoserine peptides and the C-terminal peptide. All peptides were coupled with Keyhole-Limpet hemocyanin and injected into rabbits. After purification by Protein A-Sepharose (Amersham Pharmacia Biotech) chromatography, some antibodies were affinity-purified using antigenic peptides coupled to Affi-Gel 10 or 15 (Bio-Rad). All procedures were performed at 4 °C.

Protein Phosphatase and Protein Tyrosine Kinase Treatment—For protein phosphatase treatment, immunoprecipitates of Myc-PDK1 from the pervanadate-treated HEK 293 cells were incubated with 25 ng of purified recombinant TC-PTP (54) in 45 μl of buffer containing 50 mM Tris-HCl (pH 7.5), 1 mM dithiothreitol (DTT), 0.1 mM EDTA, and 0.1% bovine serum albumin, or 25 ng of purified recombinant protein phosphatase 2A catalytic subunit (PP2Ac) purified from the baculovirus-infected Sf21 cells (57) in 45 μl of buffer containing 50 mM Tris-HCl (pH 7.5), 1% 2-mercaptoethanol, 1 mM $MnCl_2$, 1 mM benzamidine, and 0.5 mM PMSF, or 400 units of recombinant Lambda protein phosphatase (Lambda-PP, New England BioLabs) in 45 μl of buffer containing 50 mM Tris-HCl (pH 7.5), 0.1 mM Na_2EDTA, 5 mM DTT, 0.01% Brij 35, and 2 mM $MnCl_2$ for 30 min at 30 °C. The reactions were stopped by addition of 0.1 mM sodium orthovanadate for TC-PTP and Lambda-PP or 1 μM okadaic acid for PP2Ac. The immune complexes were washed three times with 50 mM Tris-HCl (pH 7.5), containing 1 mM benzamidine, 0.5 mM PMSF, 0.1 mM sodium orthovanadate, and 1 μM okadaic acid, and then analyzed for PDK1 activity, or by immunoblot analysis.

For protein-tyrosine kinase treatment, immunoprecipitated Myc-PDK1 from untreated HEK 293 cells was prepared as above. *In vitro* phosphorylation of PDK1 were performed with 250 ng of purified v-Src in 100 μl of Src kinase buffer containing 25 mM Tris-HCl (pH 7.5), 30 mM $MgCl_2$, 0.5 mM EGTA, 60 μM sodium orthovanadate, 0.5 mM DTT, 10 mM $MnCl_2$, 1 μM protein kinase A inhibitor peptide (Bachem), and 100 μM [γ-^{32}P]ATP (1000–2000 cpm/pmol; Amersham Pharmacia Biotech) for 60 min at 30 °C. Reactions were stopped by addition of SDS-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer, and PDK1 was purified by 12% preparative SDS-PAGE.

Immunoblot Analysis—HEK 293 cell extracts and immunoprecipitates were resolved by 7.8% SDS-PAGE, and transferred to Immobilon-P membranes (Millipore). The filters were blocked for 30 min in 1 × phosphate-buffered saline (140 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4 \cdot 2H_2O$, 1.5 mM KH_2PO_4, pH 7.4), containing 5% skimmed milk, 0.5% Triton X-100, and 0.5% Tween 20, followed by a 2-h incubation with the anti-Myc 9E10 or anti-phosphotyrosine 4G10 monoclonal

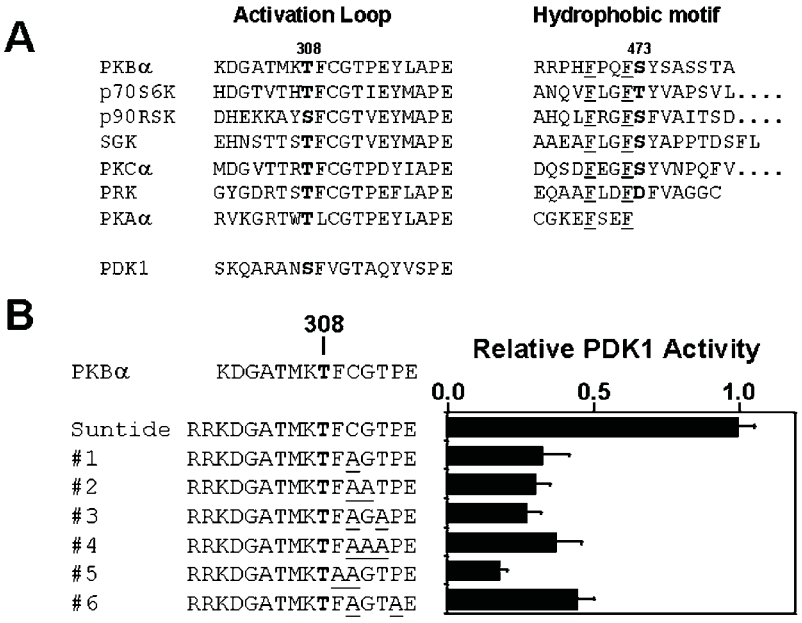

FIG. 1. **Development of *in vitro* PDK1 kinase assay.** *A*, sequence alignment of the activation loop and hydrophobic sequence motifs of second-messenger regulated kinases. Phospho-acceptor residues are in *boldface*. Shown are the human sequences for PKBα (P31749), p70S6K (AAA36410), RSK (P17252), SGK (AAD41091), PKCα (P17252), PRK1 (AAC50209), PKAα (P17612), and PDK1 (AAC51825). *B*, immunoprecipitated Myc-PDK1 was prepared from quiescent HEK 293 cells. Peptides were tested for phosphorylation by Myc-PDK1 as described under "Experimental Procedures." The extent of phosphorylation of each peptide is expressed as a percentage relative to the original PKB peptide containing residue Thr-308 (*Suntide*, RRKDGATMKTFCGTPE). The threonine phospho-acceptor (*position 0*) is in *boldface*, and residues mutated to Ala are *underlined*.

antibody diluted 1000-fold, or the anti-phospho-site-specific polyclonal antisera in the same blocking solution. The secondary antibody was alkaline phosphatase-conjugated anti-mouse IgG or anti-rabbit IgG (Sigma Chemical Co.), diluted 2500-fold in the blocking buffer. The detection and quantitation of PDK1 expression was carried out by using the alkaline phosphatase color development reagents from Bio-Rad.

Preparation of Crude Plasma Membrane Fraction—HEK 293 cells were treated as described, and then placed on ice. After washing once in ice-cold phosphate-buffered saline, 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 PMSF, and 1 mM benzamidine and then homogenized by passing through a 26-gauge needle 10 times. Homogenates were centrifuged at 14,000 $\times$ *g* for 10 min to separate the cytosolic fraction (supernatant) from organelles (pellet). The resulting pellet was resuspended in 1 ml of fractionation buffer and layered onto 10 ml of sucrose cushion (20 mM HEPES-NaOH, pH 7.4, 1.15 M sucrose) and centrifuged at 77,000 $\times$ *g* for 60 min in a Beckman SW41 rotor. The diffuse band at the interface of the sucrose solutions was collected (1 ml), mixed with 1 ml of fractionation buffer, and centrifuged at 100,000 $\times$ *g* for 20 min to pellet crude plasma membrane fraction. The pellet was resuspended in fractionation buffer, and the protein concentration was determined.

In Vivo Labeling of 293 Cells Expressing Wild Type Myc-PDK1 and Phosphoamino Acid Analysis—HEK 293 cells transiently transfected with wild type Myc-PDK1 were serum-starved for 24 h, and then incubated for 4 h with $^{32}P_i$ (1 mCi/10-cm plate, Amersham Pharmacia Biotech) in phosphate-free Dulbecco's modified Eagle's medium. Cells were stimulated with buffer or 100 μM pervanadate for 15 min (4 $\times$ 10-cm plates per each condition). Lysates were prepared and Myc-PDK1-immunoprecipitated as described above, and then resolved by 12% preparative SDS-PAGE. After staining with Coomassie Blue and autoradiography, the ^{32}P-labeled band corresponding to Myc-PDK1 were excised from the gel, reduced with 10 mM DTT, alkylated with 100 mM iodoacetamide, and cleaved with 1 μg of trypsin (Promega, sequencing grade). After the trypsin cleavage the gel pieces were dried and 1 μg of Asp-N (Roche Molecular Biochemicals, sequencing grade) was added in 40 μl of 50 mM sodium phosphate buffer (pH 8.0) containing 10% acetonitrile and incubated for 6 h at 37 °C. The peptides were then extracted (58) and processed as described below. Phosphoamino acids were identified following hydrolysis in 6 M HCl containing 0.1 mg/ml bovine serum albumin at 110 °C for 60 min. The hydrolysate was separated by thin-layer electrophoresis at pH 3.5 to resolve phosphoserine (pS), phosphothreonine (pT), and phosphotyrosine (pY) (59), and radioactivity was detected using a PhosphorImager (Molecular Dynamics).

Mapping of the Phosphorylation Sites by Mass Spectrometry and Edman Degradation—Peptides were analyzed by high performance liquid chromatography (HPLC) interfaced with electrospray ionization mass spectrometry (ESI-MS) using a Rheos 4000 chromatograph. This chromatograph was equipped with a 1- x 250-mm Vydac (Hesperia, CA) C18 column and interfaced with a Sciex API 300 mass spectrometer (PE Sciex, Toronto, Ontario, Canada) operated in the single quadrupole. The mass range from 300 to 2400 Da was scanned with a step size of 0.3 Da and a dwell time of 3.6 s per scan. The HPLC column was equilibrated in 95% solvent A (2% CH_3CN, 0.05% trifluoroacetic acid in H_2O), 5% solvent B (80% CH_3CN, 0.045% trifluoroacetic acid in H_2O), and a linear gradient was developed from 5 to 50% solvent B in 60 min at a flow rate of 50 μl/min. The phosphopeptides were detected by Cerenkov counting of the HPLC fractions and were then further analyzed by NanoESI-MS (60). Masses of the phosphopeptides were determined by the 79-Da precursor-ion scanning in the negative ion mode (61). Low energy collision induced dissociation (CID) tandem MS (MS/MS) of the peptides was performed by setting the resolution of the first quadrupole to 500 for the selected mass and the resolution of the third quadrupole to 600 over the entire mass range. The mass spectra were acquired on an Sciex API 300 triple quadrupole mass spectrometer equipped with a NanoESI source (Protana, Odense, Denmark). Also, each phosphopeptide was subjected to solid-phase Edman degradation using an automated model 477A sequencer (Applied Biosystems). Fractions from each cycle of Edman degradation were lyophilized, redissolved in 50% acetonitrile, and spotted onto a thin-layer chromatography plate, before exposure to a PhosphorImager screen.

RESULTS

Development of an in Vitro Kinase Assay for PDK1—To investigate the regulation of PDK1 we developed an *in vitro* kinase assay using synthetic peptides encompassing the Thr-308 phosphorylation site of PKBα, with two additional arginines located at the N terminus to facilitate the assay (*Suntide*, Fig. 1*B*). The selection of this peptide was based on the conservation of phosphorylation sites in different members of the AGC family kinase (Fig. 1*A*). To optimize the assay, wild type Myc-PDK1 was immunoprecipitated from transiently transfected HEK 293 cell extracts with anti-Myc 9E10 monoclonal antibody, and the activity was measured with variants of Sun-

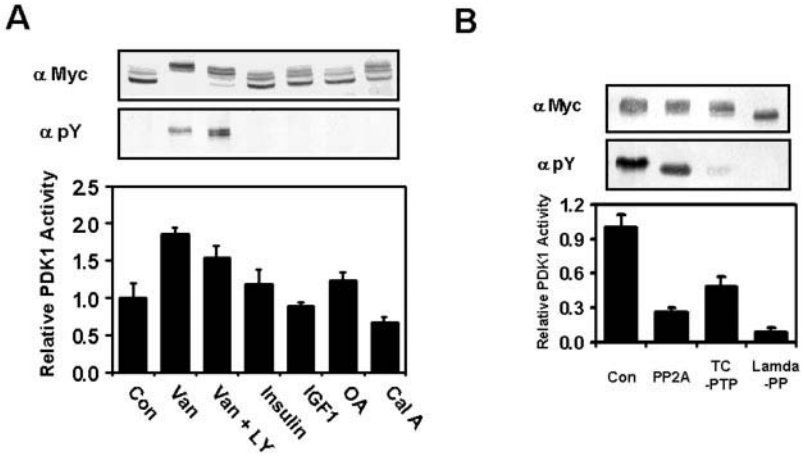

FIG. 2. **Pervanadate-mediated activation of PDK1 in HEK 293 cells and its reversal by incubation with protein phosphatases.** *A*, HEK 293 cells overexpressing Myc-PDK1 were treated with 100 μM pervanadate for 15 min (*Van*), pretreated with 50 μM LY294002 for 15 min followed by 100 μM pervanadate for 15 min (*Van+LY*), or treated with 100 nM insulin (*Insulin*), 50 ng/ml IGF-1 (*IGF-1*), 1 μM okadaic acid (*OA*), or 100 nM calyculin-A (*Cal A*). Myc-PDK1 was immunoprecipitated from cell extracts with anti-Myc 9E10 monoclonal antibody, and the immune complexes were assayed for kinase activity using Suntide as substrate. Kinase activity is the average ± standard deviation (S.D.) of three independent experiments. Activity of PDK1 immunoprecipitated from untreated cells was taken as 1. PDK1 migration following the above treatments was determined by immunoblotting 20 μg of cell lysate protein with anti-Myc 9E10 monoclonal antibody (*top panel*). Tyrosine phosphorylation of immunoprecipitated PDK1 was monitored by immunoblotting with anti-phosphotyrosine 4G10 monoclonal antibody. *B*, Myc-PDK1 was immunoprecipitated from extracts of cells stimulated with 100 μM pervanadate for 15 min. The immune complexes were incubated with PP2Ac, TC-PTP, Lambda-PP, or buffer (*Con*) alone for 30 min, and then PDK1 activity was measured. Relative kinase activity is the average (±S.D.) of four experiments with duplicate immunoprecipitates. Activity of stimulated PDK1 was taken as 1. Differences in the migration of PDK1 and tyrosine phosphorylation were monitored by immunoblotting with anti-Myc 9E10 monoclonal antibody or anti-phosphotyrosine 4G10 monoclonal antibody, respectively.

tide (Fig. 1*B*). The results indicated that a peptide corresponding to the original amino acid sequence of PKBα was the best substrate for PDK1 from the range of peptides tested. By comparing the phosphorylation of Suntide with the variant peptides, we concluded that phenylalanine at position +1 and cysteine at position +2 from the phospho-acceptor site are required for PDK1 activity (Fig. 1). In subsequent experiments, Suntide (100 μM) was used to determine PDK1 activity. Similar results were obtained in terms of PDK1 activity using T256tide (RRENGTTSTFCGTPE), corresponding to the Thr-256 activation loop phosphorylation site of SGK (data not shown).

Regulation of PDK1 Activity by Reversible Protein Phosphorylation—HEK 293 cells were used to investigate the regulation of PDK1 by the PI3K signaling pathway. When cells were treated with pervanadate, a well-characterized inhibitor of protein-tyrosine phosphatases that mimics insulin signaling (51, 52, 62), immunoprecipitated Myc-PDK1 activity was increased 1.5- to 2.5-fold in three different experiments (Fig. 2*A*). Consistent with a previous report (48), the activity of Myc-PDK1 was not significantly increased by other stimuli, including insulin, IGF-1, and okadaic acid (Fig. 2*A*). Interestingly, in untreated cells, Myc-PDK1 migrated as a triplet during 7.8% SDS-PAGE and Western blot analysis, and a marked shift in electrophoretic mobility was observed following pervanadate treatment (Fig. 2*A*). Two approaches were taken to determine if the mobility shift was due to phosphorylation on tyrosine or serine/threonine residues: immunoblotting with anti-phosphotyrosine 4G10 monoclonal antibody and treatment with tyrosine- or serine/threonine-specific phosphatases. Interestingly, Myc-PDK1 from pervanadate-treated cells was tyrosine-phosphorylated, and this phosphorylation was not significantly blocked by pretreatment with the PI3K inhibitor, LY294002 (Fig. 2*A*, *middle panel*, *lanes 2* and *3*). The slight change in PDK1 mobility is potentially due to changes in Ser/Thr phosphorylation of PDK1. Treatments that did not increase PDK1 kinase activity also did not induce its tyrosine phosphorylation, suggesting that there is a correlation between the activity changes of PDK1 and its tyrosine phosphorylation status. To directly assess the influence of phosphorylation on PDK1 activity, the *in vitro* effects of protein phosphatases were examined. Myc-PDK1 immunoprecipitated from pervanadate-stimulated HEK 293 cells was incubated with either PP2Ac (a serine/threonine-specific phosphatase), TC-PTP (a tyrosine-specific phosphatase), or Lambda-PP (a dual specific phosphatase). Dephosphorylation of activated PDK1 *in vitro* by PP2Ac, TC-PTP, or Lambda-PP resulted in ~70%, ~ 50%, or ~96% reductions in kinase activity, respectively (Fig. 2*B*). A decrease in tyrosine phosphorylation was observed with TC-PTP and Lambda-PP, whereas treatment with Lambda-PP abolished both serine/threonine and tyrosine phosphorylation causing a marked increase in electrophoretic mobility (Fig. 2*B*). The results show that dephosphorylation of serine/threonine residues by PP2Ac, or tyrosine residues by TC-PTP leads to partial loss of PDK1 activity, whereas removal of both serine/threonine and tyrosine phosphorylation with Lambda-PP leads to a complete loss of PDK1 activity. These results strongly suggest that both pS/pT and pY residues control the activity of PDK1.

Pervanadate-activated PDK1 Localizes to the Plasma Membrane of HEK 293 Cells—To further characterize the effect of pervanadate, we next examined its effect on the subcellular localization of PDK1, as compared with treatment with insulin. Subcellular fractionation of cells revealed a marked recruitment of PDK1 (~5-fold) to the plasma membrane following pervanadate treatment, where it was activated and tyrosine-phosphorylated (Fig. 3). In the case of insulin treatment, a significant increase (~3.8-fold) in PDK1 recruitment to the plasma membrane was observed, where a corresponding increase in activity and tyrosine phosphorylation could also be observed (Fig. 3). This result may explain why significant changes in PDK1 activity from total cell extracts following insulin stimulation were not seen (Fig. 2*A*).

Identification of Phosphorylated Residues of PDK1 from Unstimulated and Stimulated HEK 293 Cells—We observed that PDK1 became activated *via* tyrosine phosphorylation upon treatment of cells with pervanadate (Fig. 2*A*). These findings prompted us to map the phosphorylation sites that appear to be

involved in PDK1 activation/regulation. Myc-PDK1 was isolated from cells that had been metabolically labeled with $^{32}P_i$ prior to pervanadate treatment, and equal amounts of immunoprecipitated proteins from pervanadate-treated or untreated cells were analyzed by SDS-PAGE. Autoradiography of the gel showed that Myc-PDK1 from pervanadate-treated cells was more heavily phosphorylated than that from untreated cells (Fig. 4*A*). Phosphoamino acid analysis of total protein revealed that this 2.5-fold increase of ^{32}P incorporation into PDK1 from pervanadate-treated cells was due to tyrosine phosphorylation (Fig. 4*B*). The bands corresponding to Myc-PDK1 were excised and digested sequentially with trypsin and Asp-N, because the sizes of tryptic peptides were incompatible with HPLC-MS analysis (data not shown). The resultant mixture of peptides was separated on a C_{18} column by HPLC. The UV trace, as well as the peptide masses detected, were very similar between pervanadate-treated and untreated material (data not shown), whereas the abundance of phosphopeptides derived from pervanadate-treated cells in the radioactivity profile was markedly increased compared with control cells (Fig. 4, *C* and *D*). Fractions with higher radioactivity were further analyzed by NanoESI-MS/MS with the 79-Da precursor scan in the negative mode (61). The NanoESI-MS/MS measures the mass: charge ratio (*m/z*) of each peptide in the mixture that, upon fragmentation, liberates a species with a *m/z* of 79 Da (corresponding to a single phosphate group). By using this approach, we can selectively detect phosphopeptides among other peptides in each fraction. An example of the specificity is shown for fraction 7 from pervanadate-treated cells (Fig. 4*D*). Approximately 20 peptides were detected by NanoESI-MS in the positive ion full scan mode (Fig. 5*A*); however, only one phosphopeptide was detected in the 79-Da precursor scan (Fig. 5*B*). The observed *m/z* of the peak by NanoESI-MS/MS (789.9 Da) in the 79-Da precursor scan accounted for the PDK1-derived peptide (4–9 aa; TTSQLY), which was 80 Da heavier than would be expected for the non-phosphorylated form (Table I). To precisely locate the phosphorylation site of this phosphopeptide, CID tandem MS was performed (63). The fragment ions y_1 to y_3 as well as b_3 to b_5 all proved that Tyr-9 is phosphorylated (Fig. 5*C*). To investigate this further, we also analyzed the fractions by phosphoamino acid analysis as well as by phosphate-release experiments using Edman degradation. The results for fraction 7 are shown in Fig. 5*D*. The radiolabeled phosphopeptide gave rise exclusively to phosphotyrosine upon acid hydrolysis (Fig. 5*D*, *top panel*), and released radioactivity in the sixth cycle of Edman degradation, confirming that Tyr-9 is phosphorylated (Fig. 5*D*, *lower panel*).

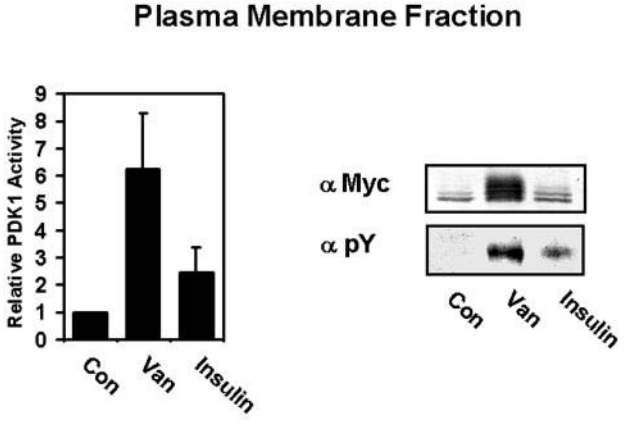

FIG. 3. **Subcellular distribution of PDK1 protein and activity following pervanadate stimulation in HEK 293 cells.** HEK 293 cells were treated with 100 μM pervanadate (*Van*) or 100 nM insulin (*Insulin*) for 15 min, and the plasma membrane fractions were prepared as described under "Experimental Procedures." Changes in the distribution of PDK1 were detected by Western blot analysis with anti-Myc 9E10 monoclonal antibody (*top panel*). PDK1 was immunoprecipitated with anti-Myc 9E10 monoclonal antibody and analyzed for levels of tyrosine phosphorylation by immunoblotting with anti-phosphotyrosine 4G10 monoclonal antibody (*lower panel*), or assayed for kinase activity. Activity of immunoprecipitated Myc-PDK1 is the average (±S.D.) of four independent experiments with duplicate immunoprecipitates. Activity of PDK1 immunoprecipitated from unstimulated cells was taken as 1.

Using this approach, we analyzed all ^{32}P-containing fractions, and the phosphopeptides detected are summarized in Table I. The phosphopeptide detected in fraction 15 with a mass of 1226.6 Da was identified by NanoESI-MS/MS as a peptide comprising amino acids 408–417 (SGSNIEQYIH). The

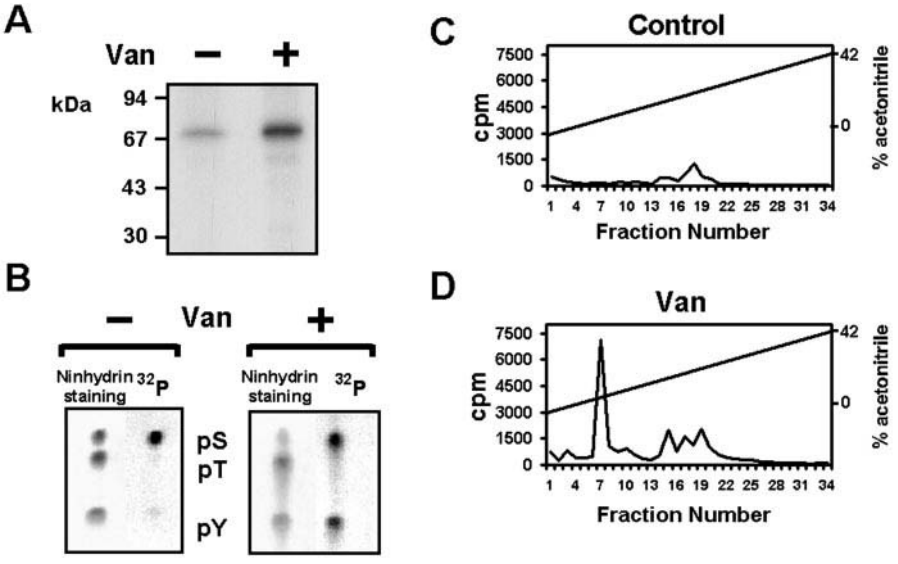

FIG. 4. **Analysis of ^{32}P-labeled PDK1 from unstimulated and pervanadate-stimulated HEK 293 cells.** HEK 293 cells expressing Myc-PDK1 were metabolically labeled with $^{32}P_i$ prior to pervanadate treatment. *A*, immunoprecipitated PDK1 was resolved by a 12% preparative SDS-PAGE and then stained with Coomassie Blue. ^{32}P-Labeled PDK1 was visualized by autoradiography. *B*, extracted protein from both conditions was hydrolyzed in 6 M HCl at 110 °C for 60 min. The hydrolysate was separated by thin-layer electrophoresis at pH 3.5 to resolve pS, pT, and pY (59), and radioactivity was detected using a PhosphorImager. *C* and *D*, the ^{32}P-labeled bands corresponding to Myc-PDK1 were excised from the gel, reduced with 10 mM DTT, alkylated with 100 mM iodoacetamide, and cleaved first with trypsin followed by Asp-N digestion. Extracted peptides were then separated and analyzed by HPLC-MS. The phospho-peptides were detected by Cerenkov counting of the HPLC fractions.

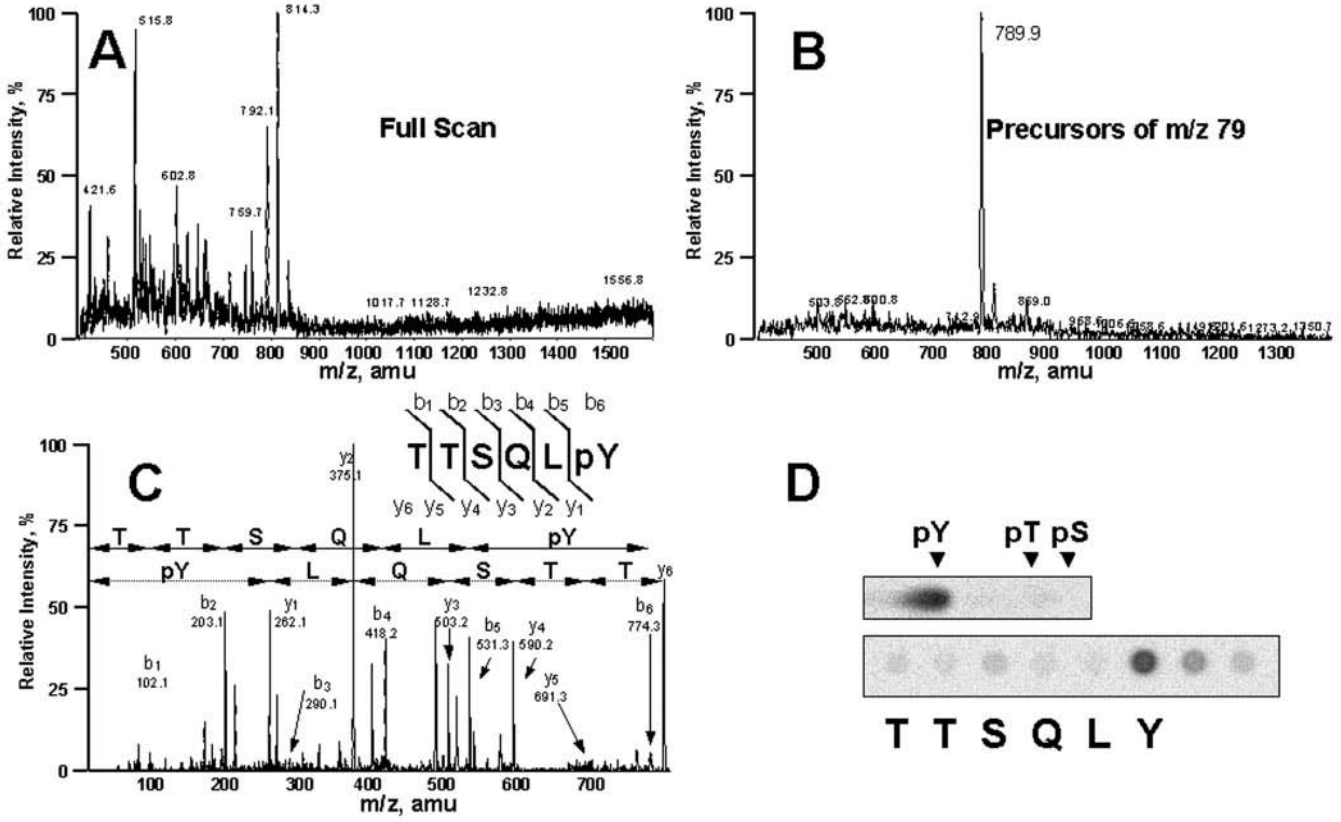

FIG. 5. **Identification of phosphotyrosine-9 in pervanadate-treated PDK1.** *A*, the full scan mass spectrum of fraction 7 in positive mode was acquired; *B*, the masses of the phosphopeptides were identified by 79-Da precursor-ion scanning in the negative ion mode (61). *C*, tandem mass spectrum derived by collision-induced dissociation of the $(M + H)^+$ precursor, *m/z* 792.9. Fragment ions in the spectrum represent many single-event preferential cleavages of peptide bonds resulting in the sequence information recorded from both the N and C termini of the peptide simultaneously. The single-letter code for the amino acids is shown. *D*, phosphoamino acid analysis (*upper panel*) and phosphate release (*lower panel*) of [^{32}P]phosphopeptide from fraction 7. Positions of ninhydrin-stained phosphoamino acid standards are shown *above the upper panel*. ^{32}P release by Edman degradation was detected by spotting sequencer fractions onto thin-layer chromatography plates, before exposure to a PhosphorImager.

TABLE I

Summary of phospho-peptide analysis of pervanadate-treated PDK1 by phosphate release and mass-spectrometry

As shown in Figs. 4 and 5, HPLC fractions with higher radioactivity detected by Cerenkov counting were further analyzed by NanoESI-MS.79-Da precursor-ion scanning in the negative ion mode, CID tandem MS/MS, and phosphoamino acid analysis as described under "Experimental Procedures." For molecular weights below 1600 Da, the monoisotopic mass (M), and for molecular weights above 1600 Da the average isotopic mass (A) of the unprotonated peptide, was calculated. Amino acids underlined are phosphorylated.

Fraction	Peptide	Mass		Position in PDK1
		Predicted	Detected	
		Da		
7	TTSQL<u>Y</u>	711.3^{M}	790.9 (+80)	Tyr-9
15	SG<u>S</u>NIEQYIH	1146.5^{M}	1226.6 (+80)	Ser-410
16	DAVPIQSSVVLCSCP<u>S</u>PSM$_{OX}$VR	2305.7^{A}	2384.2 (+79)	Ser-25
17	DAVPIQSSVVLCSCP<u>S</u>PSMVR	2289.7^{A}	2369.2 (+80)	Ser-25
18	LTAYLPAMSEDDEDC<u>Y</u>GN<u>Y</u>	2227.4^{A}	2306.2 (+79)	Tyr-373 or Tyr-376
19	AN<u>S</u>FVGTAQYVSPELLTEK	2054.3^{A}	2134.2 (+80)	Ser-241

fragment ions detected (y_1 through y_7) all indicated that the C-terminal tyrosine residue was not phosphorylated, leaving Ser-408 or Ser-410 as possible phosphorylation sites in this peptide. The phosphate release experiment of this peptide clearly showed that Ser-410, not Ser-408, is phosphorylated (Table I, and data not shown). Fractions 16 and 17 contained the same phosphopeptide (10–30 aa; DAVPIQSSVVLCSCP-<u>S</u>PSMVR) with Ser-25 phosphorylated. The earlier eluting peptide in fraction 16 was ~16 Da heavier and contained an additional oxidized Met-28 in comparison with the otherwise identical peptide in fraction 17.

Fraction 18 contained the phosphopeptide corresponding to residues 358–376 of PDK1. The observed mass of 2306 Da by NanoESI-MS/MS was in agreement with the calculated mass of the doubly digested phosphopeptide comprising residues 358–376 (LTAYLPAMSEDDE DC<u>Y</u>GN<u>Y</u>) and phosphorylated at one residue. The CID tandem MS clearly showed that the phosphate is located either on Tyr-373 or Tyr-376, but not at both residues, based on the observed mass (2307.2 Da). Because we detected the ions y_2 (*m/z* 376), y_3 (*m/z* 433), b_{16} (*m/z* 1875), which showed that Tyr-373 is phosphorylated, as well as the ions y_2 (*m/z* 296), y_3 (*m/z* 353), b_{16} (*m/z* 1955), which indicated that Tyr-376 is phosphorylated, we conclude that this fraction probably contains a mixture of the peptide either phosphorylated at Tyr-373 or Tyr-376.

Fraction 19 contained a phosphopeptide comprising amino acids 239 to 257 of PDK1 (AN<u>S</u>FVGTAQYVSPELLTEK) with Ser-241 phosphorylated. Ser-241 lies in the activation loop of

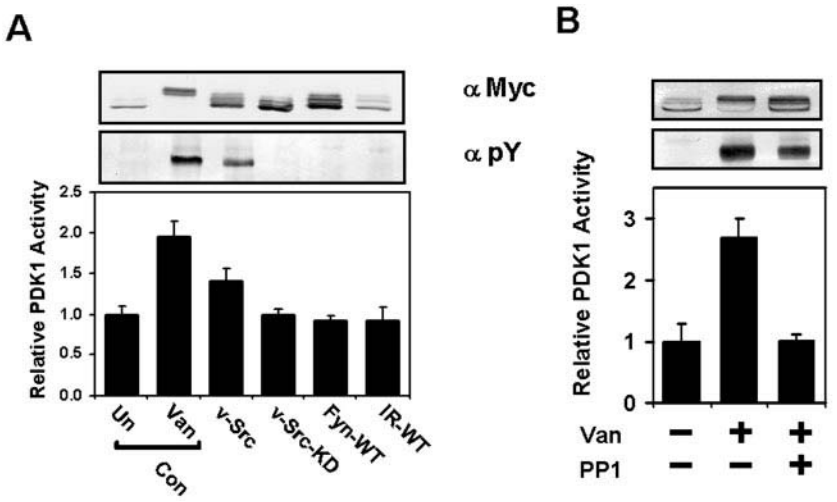

FIG. 6. **Effect of v-Src kinase on PDK1 activity and phosphorylation on tyrosine residue.** *A*, v-Src, kinase-deficient v-Src-KD, wild type Fyn-WT, wild type IR-WT, or pcDNA3.1 vector was transiently cotransfected into HEK 293 cells with Myc-PDK1. Immunoprecipitated Myc-PDK1 activity was assayed using Suntide as substrate, and PDK1 migration and tyrosine phosphorylation as detected by immunoblot analysis is shown in the *top two panels*. Kinase activity is the average (±S.D.) of three experiments with duplicate immunoprecipitates. Activity of PDK1 immunoprecipitated from pcDNA3.1 vector-transfected cells was taken as 1. The expression from each construct was observed to be equal by immunoblot analysis (*top panel*). *B*, cells were pretreated with 10 μM PP1 for 30 min prior to stimulation for 15 min with 100 μM pervanadate, immunoprecipitated Myc-PDK1 activity was assayed, and PDK1 migration and tyrosine phosphorylation on PDK1 were monitored. Activity of PDK1 immunoprecipitated from untreated cells was taken as 1.

the PDK1 kinase domain equivalent to the phosphorylation site of its substrate. This site appears to be constitutively phosphorylated even in quiescent HEK 293 cells, because we only observed the phosphorylated form of this peptide in both stimulated and non-stimulated cells.

Analysis of PDK1 from unstimulated cells did not allow detection of any tyrosine phosphorylation by phosphoamino acid analysis (Fig. 5*C*) or mass spectrometry (not shown). However, we were able to detect all the pS sites (Ser-25, Ser-241, and Ser-410) identified from pervanadate-treated PDK1 (Table I). Of note, we were unable to detect phosphorylation of PDK1 on the previously reported site, Ser-393/396 (48). The reason that phosphorylation of Ser-393/396 was not detectable in our system may be due to low stoichiometry of this site or to the limits of our detection system.

We attempted to estimate the ratio of the phosphorylated *versus* non-phosphorylated peptides by comparing the extracted ion chromatograms of the HPLC-MS data under each condition. The Ser-241 phosphopeptide can be detected only in the phosphorylated form, from stimulated as well as unstimulated cells. Because we detected some incorporated ^{32}P in Ser-241 by Cerenkov counting and phosphate-release experiments using Edman degradation, there seems to be some turnover of the phosphorylation at this site (data not shown). In contrast, the Tyr-9-containing peptide (4–9 aa; TTSQLY) is not phosphorylated in non-stimulated cells. Upon stimulation, ~75% of the peptide detected was phosphorylated on Tyr-9. Due to the low levels of the other phosphopeptides, no estimation of the stoichiometry can be made from the HPLC-MS data (data not shown).

Co-expressed v-Src Kinase Activates PDK1 in HEK 293 Cells—Having identified the sites on PDK1 that become tyrosine-phosphorylated upon pervanadate stimulation, we wished to identify the tyrosine kinase involved in PDK1 activation. Src family tyrosine kinases have been implicated in insulin signaling (64, 65). To address this issue, Myc-PDK1 was cotransfected with v-Src kinase (v-Src or v-Src-KD), Fyn kinase (Fyn-WT), or IR tyrosine kinase (IR-WT) (55, 56) in HEK 293 cells, and the kinase activity and tyrosine phosphorylation were examined (Fig. 6*A*). Co-expression of v-Src caused up to a 2-fold activation of Myc-PDK1 in three independent experiments, accompanied by tyrosine phosphorylation without any stimulation of cells (Fig. 6*A*, *lane 3*). As a control, co-expression of kinase-deficient v-Src caused no activation or tyrosine phosphorylation of Myc-PDK1 (Fig. 6*A*, *lane 4*). Under the same conditions, co-expressed Fyn or IR did not lead to any observed activity changes or tyrosine phosphorylation of PDK1 (Fig. 6*A*). These results suggest that PDK1 can be tyrosine-phosphorylated by v-Src, and this phosphorylation increases PDK1 activity *in vivo*. To further confirm the involvement of v-Src in tyrosine phosphorylation of PDK1 *in vivo*, cells were pretreated with 10 μM PP1, an inhibitor of Src family kinases (66), prior to pervanadate stimulation. As previously observed (*cf.* Fig. 2*A*), Myc-PDK1 activity was elevated 3-fold by pervanadate treatment, concomitant with a change in electrophoretic mobility and tyrosine phosphorylation (Fig. 6*B*, *lanes 1* and *2*). Pervanadate-stimulated PDK1 activation was completely blocked by pretreatment of cells with PP1, and an intermediate electrophoretic mobility between control and pervanadate-treated PDK1 was observed (Fig. 6*B*, *lane 3*). However, PP1 failed to completely abolish pervanadate-induced tyrosine phosphorylation of PDK1, suggesting that a second tyrosine kinase is involved (Fig. 6*B*). Taken together, these data strongly suggest that Src, or a Src family member, acts as an upstream kinase to regulate PDK1 activity *in vivo*.

Identification of in Vitro Phosphorylation Sites on PDK1 by v-Src—Based on the observations that v-Src co-expression increased the kinase activity and tyrosine phosphorylation of PDK1, and PP1 pretreatment blocked the activation of PDK1 together with decreased tyrosine phosphorylation, we attempted to identify the regulatory sites on PDK1 that are phosphorylated by v-Src *in vitro*. Myc-PDK1 immunoprecipitated from HEK 293 cells was phosphorylated with [γ-^{32}P]ATP using purified v-Src (250 ng) *in vitro* and then subjected to SDS-PAGE. Phosphoamino acid analysis of total protein revealed that phosphopeptides containing predominantly pY and a modest increase in pS were found in v-Src-treated PDK1 only (Fig. 7, *A* and *B*). The protein band corresponding to Myc-PDK1 (~20 μg) was excised and digested sequentially with trypsin and Asp-N. The resultant mixture of peptides was purified by HPLC and analyzed by NanoESI-MS/MS and phosphate release analysis using the same conditions as described above. As

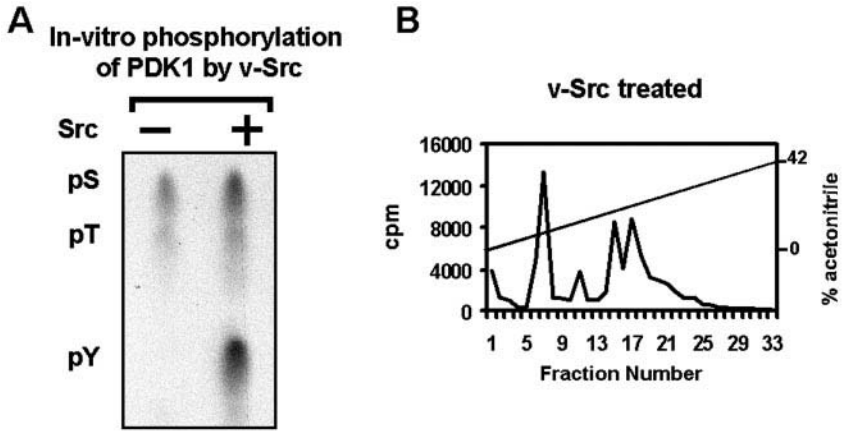

FIG. 7. ***In vitro* phosphorylation of PDK1 by v-Src kinase.** *A*, immunoprecipitated Myc-PDK1 from HEK 293 cells was labeled with [γ-^{32}P]ATP by purified v-Src and isolated by SDS-PAGE after *in vitro* kinase reaction. Myc-PDK1 was excised and digested with trypsin, followed by Asp-N. The resultant mixture of peptides from both v-Src-treated and control samples were extracted, dried, and hydrolyzed in 6 M HCl at 110 °C for 60 min. The hydrolysate was separated by thin-layer electrophoresis at pH 3.5 to resolve pS, pT, and pY (59), and radioactivity was detected using a PhosphorImager. *B*, the double-digested peptides were fractionated and collected by HPLC under the same conditions as the earlier experiment (Fig. 4).

TABLE II

Summary of phospho-peptide analysis of Src-treated PDK1 by phosphate release and mass-spectrometry.

^{32}P-labeled PDK-1 phosphorylated by v-Src were isolated and digested with trypsin followed by Asp-N. HPLC fractions with higher radioactivity detected by Cerenkov counting were then further analyzed as described under "Experimental Procedures." For molecular weights below 1600 Da, the monoisotopic mass (M), and for molecular weights above 1600 Da the average isotopic mass (A) of the unprotonated peptide, was calculated. Amino acids underlined are phosphorylated.

Fraction	Peptide	Mass		Position in PDK1
		Predicted	Detected	
		Da		
7	TTSQL<u>Y</u>	711.3^{M}	790.9 (+80)	Tyr-9
11	TQ<u>T</u>ESSTPPGIPGGSR	1570.7^{M}	1650.8 (+80)	Thr-33
15	SG<u>S</u>NIEQYIH	1146.5^{M}	1226.6 (+80)	Ser-410
16	LTAYLPAM$_{OX}$SEDDEDC<u>Y</u>GNY	2243.4^{A}	2323.2 (+80)	Tyr-373
17/18	NFK<u>T</u>FFVHTPNR	1506.8^{M}	1585.8 (+79)	Thr-513
19/20	AN<u>S</u>FVGTAQYVSPELLTEK	2054.3^{A}	2134.2 (+80)	Ser-241
21	QLLLTEGPHL<u>Y</u>YV	1544.8^{M}	1624.8 (+80)	Tyr-485

shown in Fig. 7*B*, the radioactivity profile of v-Src-treated PDK1 was similar to that of pervanadate-treated PDK1 (*cf.* Fig. 4*D*). Seven phosphopeptides were identified, four of which were also found in PDK1 isolated from pervanadate-stimulated cells (Table II). As previously described, phosphorylated Tyr-9 was detected as well as phosphorylated Tyr-373. However, ions that corresponded to phosphorylated Tyr-376 were not found by CID tandem MS in v-Src-treated PDK1. Phosphorylated Ser-241 was also observed, confirming that PDK1 autophosphorylates on this site (48) and suggesting that there is either some turnover or a pool of PDK1 that is not phosphorylated on the activation loop in unstimulated cells. Phosphorylated Ser-410 was also found, consistent with a previous report that Ser-410 is an autophosphorylation site (48). Three additional phosphorylation sites were found in v-Src-treated PDK1 that were not found in PDK1 from pervanadate-stimulated cells: Thr-33, Thr-513, and Tyr-485. Although we did not detect any other kinases in the immunoprecipitate, we cannot exclude the possibility that phosphorylation on these sites results from a co-immunoprecipitating kinase. One possibility is that Thr-33 or Thr-513 may be additional *in vitro* autophosphorylation sites of PDK1, and Tyr-485 may be an *in vitro* v-Src phosphorylation site. Together with identification of the *in vivo* phosphorylation sites, our results indicate that phosphorylation on Tyr-9 and Tyr-373 contribute to v-Src-mediated PDK1 activation.

Mutation of the Tyrosine Phosphorylation Site of PDK1 Abolishes Its Activation in HEK 293 Cells—Because Tyr-9 and Tyr-373/376 were identified as inducible phosphorylation sites following pervanadate stimulation, we next investigated the role of these sites in PDK1 regulation by site-directed mutagenesis. PDK1 mutants were transfected into HEK 293 cells, and the effect of pervanadate treatment on PDK1 activity and tyrosine phosphorylation was examined (Fig. 8). Conversion of Tyr-9 to Phe caused a slight reduction in pervanadate-stimulated tyrosine phosphorylation of PDK1 but had almost no effect on kinase activity (Fig. 8). In contrast, mutation of Tyr-373/376 to Phe led to a reduction in basal activity and, in addition, to a decrease in pervanadate-stimulated activity and tyrosine phosphorylation (Fig. 8). Constructs with triple mutations were also generated, but this protein was very poorly expressed or unstable when transfected into HEK 293 cells, and thus could not be analyzed further (data not shown).

Inhibition of v-Src Reduces Pervanadate-stimulated Tyr-373/376 Phosphorylation but Not Tyr-9 Phosphorylation—To further decipher the roles of Tyr-9 and Tyr-373/376 in PDK1 regulation, we generated phospho-specific antibodies against pY9 and pY373/376, which allowed us to monitor phosphorylation of the PDK1 mutants. For these experiments HEK 293 cells were transiently transfected with wild type Myc-PDK1 or the mutants (Myc-PDK1 Y9F or Myc-PDK1 Y373/376F) and pretreated with 10 μM PP1 for 30 min prior to stimulation for 15 min with 100 μM pervanadate. Interestingly, phosphorylation of Tyr-373/376 was dramatically reduced in the presence of PP1, whereas phosphorylation on Tyr-9 was not significantly affected (Fig. 9, *left panel*). These results suggest that the decrease in tyrosine phosphorylation and activation of PDK1 in the presence of PP1 (Fig. 6*B*) was due to inhibition of phosphorylation on Tyr-373/376, and not Tyr-9. Similar results were

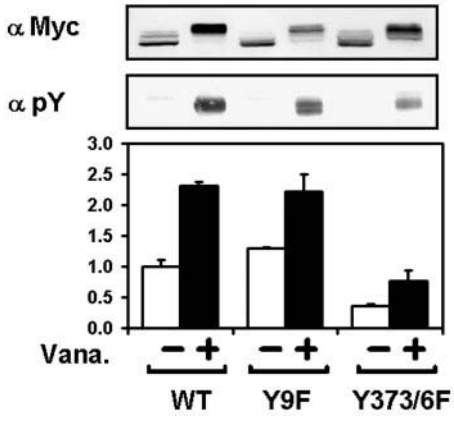

FIG. 8. **Effect of PDK1 phosphorylation mutants on activation by pervanadate.** HEK 293 cells were transiently transfected with wild type Myc-PDK1 or the mutants (Myc-PDK1 Y9F or Myc-PDK1 Y373/376F). Cells were treated with 100 μM pervanadate or buffer for 15 min. Immunoprecipitated PDK1 from cell extracts was assayed for kinase activity, after correcting PDK1 expression levels for each DNA construct. Kinase activity is the average (±S.D.) of three independent experiments with duplicate immunoprecipitates. The results are expressed as -fold activation relative to the specific activity of wild type Myc-PDK1 from unstimulated HEK 293 cells. Equal amounts of extracts were immunoblotted with anti-Myc 9E10 monoclonal antibody to check expression levels of different PDK1 mutants in HEK 293 cells (*top panel*). Tyrosine phosphorylation of PDK1 was monitored by immunoblot analysis with anti-phosphotyrosine 4G10 monoclonal antibody after immunoprecipitation with anti-Myc 9E10 monoclonal antibody (*middle panel*).

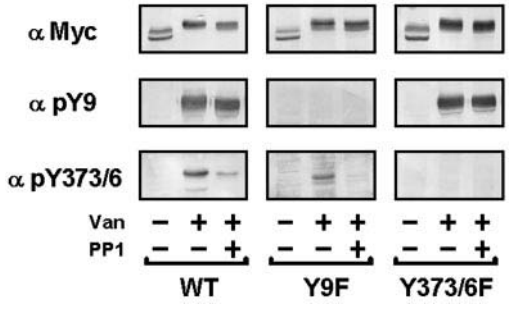

FIG. 9. **Analysis of PDK1 phosphorylation during pervanadate-mediated activation using anti-phosphotyrosine specific antibodies.** As described under "Experimental Procedures," anti-phospho-site-specific antibodies against pY9 and pY373/376 were generated and characterized by using Y9F and Y373/376F mutants of PDK1. Cells expressing wild type Myc-PDK1 or the mutants (Myc-PDK1 Y9F or Myc-PDK1 Y373/376F) were pretreated with 10 μM PP1 for 30 min prior to stimulation for 15 min with 100 μM pervanadate. Differences in the migration of PDK1 and tyrosine phosphorylation events were monitored by immunoblotting with anti-Myc 9E10 monoclonal antibody, anti-pY9, or anti-pY373/376 polyclonal antisera, respectively.

observed with the tyrosine mutants (Fig. 9). In the case of the Y9F mutant, pervanadate-stimulated phosphorylation on Tyr-373/376 was completely blocked in the presence of PP1, suggesting that phosphorylation on Tyr-9 has an influence on Tyr-373/376 phosphorylation.

Translocation to the Plasma Membrane Is Required for Tyr-373/376 Phosphorylation—We have observed that insulin stimulated PDK1 tyrosine phosphorylation only at the plasma membrane and that phosphorylation on Tyr-373/376 likely mediates pervanadate-induced activation of PDK1. To further examine the relationship between the subcellular location of PDK1 and its phosphorylation and activation, we determined the subcellular distribution and phosphorylation of PDK1 and its mutants following pervanadate or insulin stimulation. Plasma membrane and cytosolic fractions were prepared from cells expressing wild type, Y9F, Y373/376F, or ΔPH-PDK1,

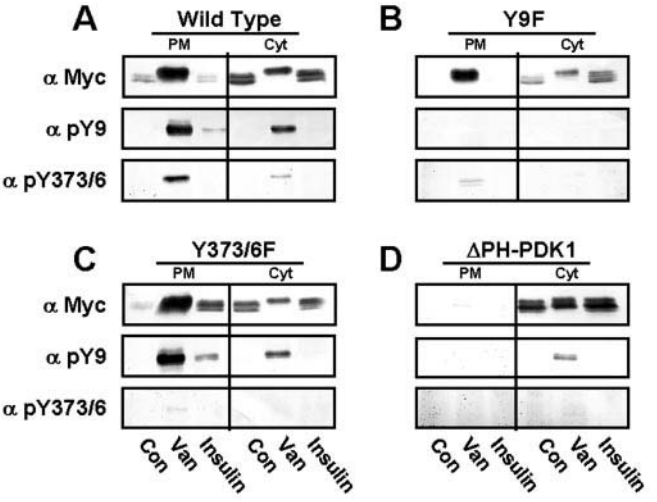

FIG. 10. **Effect of PDK1 phosphorylation mutants and PH domain deletion mutant on the subcellular localization of PDK1 in the presence of pervanadate or insulin.** Cells expressing wild type Myc-PDK1 or the mutants (Myc-PDK1 Y9F, Myc-PDK1 Y373/376F, or Myc-ΔPH-PDK1) were treated with 100 μM pervanadate (*Van*) or 100 nM insulin (*Insulin*) for 15 min, and plasma membrane fraction (*PM*) and cytosolic fraction (*Cyt*) were prepared as described in earlier experiments (Fig. 3). Differences in the migration of PDK1 and tyrosine phosphorylation events were monitored by immunoblotting with anti-Myc 9E10 monoclonal antibody, anti-pY9, or anti-pY373/376 polyclonal antisera, respectively.

which were treated with pervanadate or insulin, and then analyzed for the level of PDK1 or phosphorylated PDK1, by Western blotting (Fig. 10). In agreement with previous data (Fig. 3), wild type PDK1 translocated to the plasma membrane upon stimulation with pervanadate, where phosphorylation of Tyr-9 and Tyr-373/376 was detected (Fig. 10*A*). A low level of phosphorylation on both Tyr-9 and Tyr-373/376 of PDK1 could also be detected in the cytosol fraction (Fig. 10*A*). Mutation of Tyr-9 to Phe led to decreased Tyr-373/376 phosphorylation in both the plasma membrane and cytosolic fractions (Fig. 10*B*). In contrast, Tyr-9 phosphorylation appears to be unaffected in the Y373/376F mutant (Fig. 10*C*), suggesting that Tyr-9 phosphorylation facilitates phosphorylation on Tyr-373/376. Finally, we examined the localization and phosphorylation of a PDK1 mutant lacking the PH domain (ΔPH-PDK1, Fig. 10*D*). Consistent with previous data (67), deletion of the PDK1 PH domain results in its inability to translocate to the plasma membrane (Fig. 10*D*). Interestingly, pervanadate stimulation also failed to induce phosphorylation of ΔPH-PDK1 on Tyr-373/376, and Tyr-9 phosphorylation was severely reduced (Fig. 10*D*). Taken together, these data suggest that pervanadate-stimulated translocation to the plasma membrane is required for PDK1 phosphorylation on Tyr-373/376 and that Tyr-9 phosphorylation plays a role in mediating this phosphorylation.

Phosphorylation Events on PDK1 during Pervanadate-mediated Activation—It has been previously reported that insulin stimulation does not increase serine phosphorylation or the catalytic activity of PDK1 (48). Because we were able to observe an increase in tyrosine phosphorylation, specifically at Tyr-9 and Tyr-373/376 of PDK1, together with an increase in kinase activity, following pervanadate treatment of cells, we were interested in determining if serine phosphorylation was also increased under these conditions. To this end, we produced antibodies specific for PDK1 phosphorylated on Ser-25 and Ser-241 and monitored the level of PDK1 phosphorylation following a time course of pervanadate stimulation, together with the pY9- and pY373/376-specific antibodies described above (Fig. 11). Phosphospecific antibodies for pS410 and pS393/396

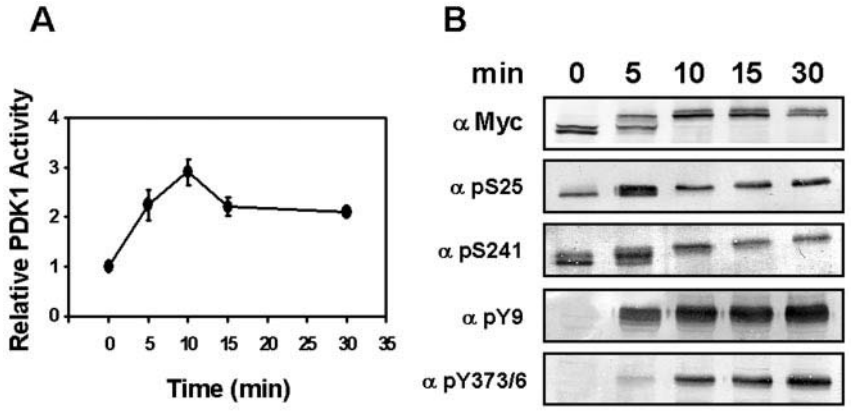

FIG. 11. **Total phosphorylation events on PDK1 during pervanadate-mediated activation.** *A*, time course of PDK1 activation following pervanadate stimulation of HEK 293 cells. Kinase activity is the average (±S.D.) of three independent experiments with duplicate immunoprecipitates. Activity of PDK1 immunoprecipitated from unstimulated cells was taken as 1. *B*, difference in the migration of PDK1 and total phosphorylation events were monitored by immunoblotting with anti-Myc 9E10 monoclonal antibody, anti-pS25, anti-pS241, anti-pY9, or anti-pY373/376 polyclonal antisera, respectively.

were also prepared; however, the quality of these antibodies was not high enough for use in the current study (data not shown). As shown in Fig. 11, PDK1 activation (2.5-fold) occurred within 5 min of pervanadate treatment, and kinase activity remained high for at least 30 min. The time course of PDK1 activation and the concomitant gel shift promoted by pervanadate correlated most closely with Tyr-373/376 phosphorylation (Fig. 11). Tyr-9 phosphorylation preceded Tyr-373/376 phosphorylation, consistent with the hypothesis that Tyr-9 phosphorylation facilitates phosphorylation on Tyr-373/376. In agreement with a previous study (48), both Ser-25 and Ser-241 were phosphorylated in unstimulated cells, and phosphorylation on these sites was modestly increased following pervanadate treatment (Fig. 11).

DISCUSSION

PDK1 is a key upstream activator of second-messenger regulated protein kinases such as PKB, p70S6K, PKC, SGK, and RSK, which are involved in a number of important intracellular processes, including cellular survival, proliferation, protein synthesis, and gene expression (for a review see Refs. 10, 12). All of these kinases require phosphorylation by PDK1 on a conserved Ser/Thr residue in the activation loop of their kinase domains (1, 2, 9, 13–30, 33). This places PDK1 in a pivotal position to regulate many different signaling pathways. Two major questions remain outstanding: 1) How is the phosphorylation of each substrate by PDK1 tightly regulated, distinct from other substrates? and 2) Why is PDK1 partially active in the cytoplasm of cells?

Here we provide evidence that activation of PDK1 in response to pervanadate correlates with tyrosine phosphorylation on residues Tyr-9 and Tyr-373/376. We also show that Src kinase mediates this phosphorylation and increases PDK1 activity *in vivo*. Furthermore, activation of cells by both pervanadate and insulin causes translocation of PDK1 to the plasma membrane. Taken together, these data suggest that tyrosine phosphorylation appears to regulate the intrinsic catalytic activity of PDK1 in a substrate-targeted manner in the plasma membrane. Our current model for the regulation of PDK1 is summarized in Fig. 12*B*.

Initial reports suggested that insulin stimulation did not significantly increase the activity of PDK1 when assayed using PKB or p70S6K protein *in vitro* (14, 68). This led to the idea that PDK1 existed in an active and phosphorylated form in quiescent cells and was not further activated following treatment of cell with agonists that activate PI3K (48). Surprisingly, we observed a 2- to 3-fold increase in PDK1 activity in response to pervanadate, using an optimized peptide substrate to measure *in vitro* activity (Figs. 2*A* and 6*B*). Consistent with our observations, two groups demonstrated that the catalytic activity of PDK1 toward PKB and SGK was increased by treatment of cells with pervanadate or hydrogen peroxide (50, 53). Interestingly, tyrosine phosphorylation and catalytic activity of PDK1 correlated well in these reports (50, 53) as well as in our experiments (Fig. 11). Both we and others failed to detect any change in overall PDK1 activity or tyrosine phosphorylation in response to insulin or IGF-1 when assayed using PDK1 isolated from total cell extracts. In our hands, however, insulin induced an increase in kinase activity and tyrosine phosphorylation of PDK1 localized in the plasma membrane fraction of cells (Fig. 3). A role for tyrosine phosphorylation in PDK1 activity is further supported by our observation that dephosphorylation of pY reduces pervanadate-induced PDK1 activity. Other reports have suggested that protein phosphatase treatment failed to inactivate PDK1, with residue Ser-241 in the catalytic domain being particularly resistant to phosphatase treatment (1, 48). However, we have found that dephosphorylation of either pS/pT by PP2Ac, or pY by TC-PTP, leads to a partial loss of PDK1 activity, whereas removal of both pS/T and pY with Lambda-PP completely abolishes PDK1 activity (Fig. 2*B*). Thus, it is possible that PDK1 becomes tyrosine-phosphorylated at the plasma membrane but can be rapidly dephosphorylated by protein-tyrosine phosphatases in the cytosol. In addition, the results suggest that PDK1 could be regulated by PP2A through dephosphorylation of Ser-241.

In terms of the modulation of PDK1 activity by reversible phosphorylation, five pS sites have been identified on PDK1 *in vivo*, but only one of these sites, Ser-241 in the activation loop of PDK1, is essential for activity (48). It seems likely that PDK1 autophosphorylates itself on this residue (48). In this regard, it is striking that tyrosine phosphorylation causes an increase in catalytic activity of PDK1 in the presence of pervanadate or hydrogen peroxide (Fig. 2*A* (50, 53)). In the current study, three tyrosine residues on PDK1 that are phosphorylated in response to pervanadate *in vivo* were identified (Table I). Tyr-9 is located at the N terminus of PDK1, whereas Tyr-373/376 are present in the linker region between the catalytic domain and PH domain (Fig. 12*A*). These three tyrosines are not conserved in PDK1 homologues from *Drosophila melanogaster* (68, 69), *Saccharomyces cerevisiae* (70), *Schizosaccharomyces pombe* (71), or *Caenorhabditis elegans* (72). This suggests that regulation of PDK1 by tyrosine phosphorylation may be a relatively recent event during evolution.

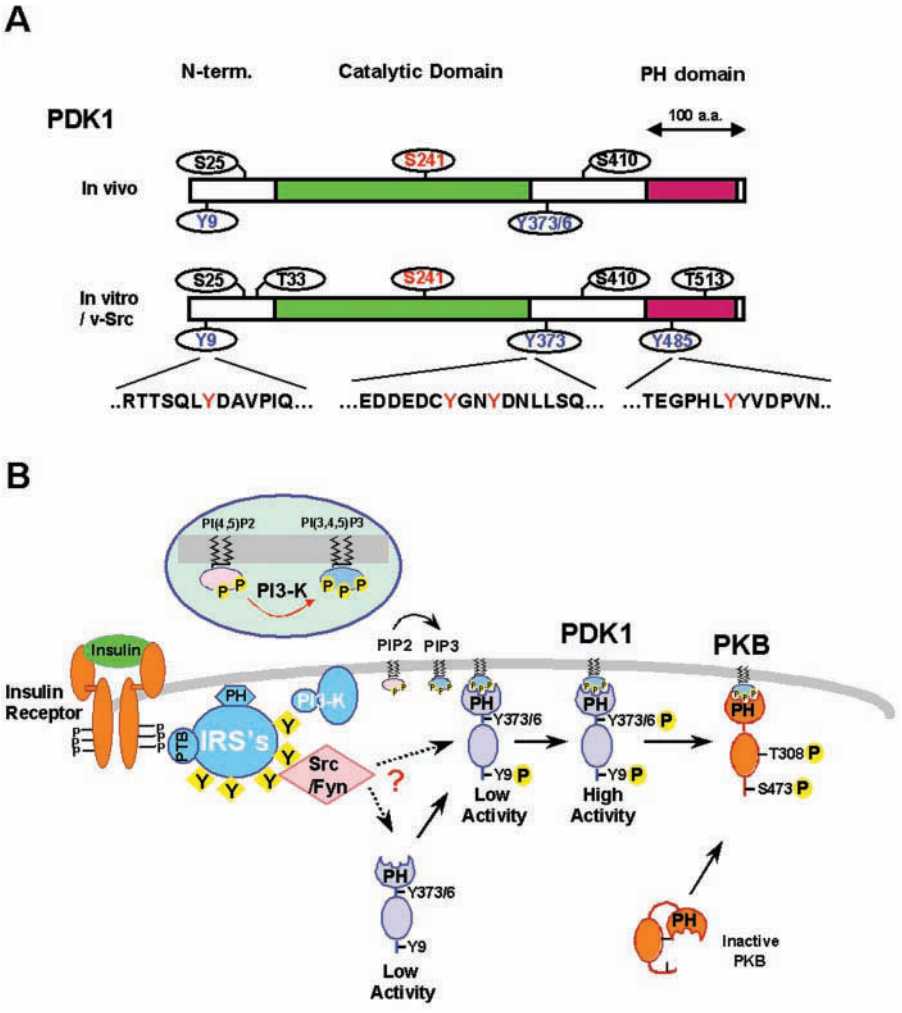

FIG. 12. **Possible model for recruitment and coordinated activation of PDK1 by tyrosine phosphorylation.** *A*, summary of phosphorylation sites on PDK1. *B*, PDK1 is recruited to the plasma membrane following growth factor signaling *via* sequential tyrosine phosphorylation. First, tyrosine phosphorylation on Tyr-9 by a cytoplasmic protein-tyrosine kinase (not inhibited by PP1) promotes translocation of PDK1 to the plasma membrane together with PH domain in a coordinated manner. Then PDK1 becomes phosphorylated and activated by Src family kinase *via* Tyr-373 phosphorylation.

As previously reported (50, 53), PDK1 is activated and tyrosine-phosphorylated by the v-Src tyrosine kinase *in vitro* and *in vivo* (Figs. 7*A* and 8). Co-expression of v-Src leads to tyrosine phosphorylation of PDK1 without growth factor stimulation, as well as an increase in PDK1 activity (Fig. 7*A*). Tyr-9 and Tyr-373 are also phosphorylated by v-Src *in vitro* (Fig. 8 and Table II). These results are supported by the observation that pretreatment of cells with PP1, an inhibitor of Src family kinases, prior to pervanadate stimulation, partially blocked the tyrosine phosphorylation of PDK1 and reduced the kinase activity back to basal level (Fig. 7*B*). This partial inhibition of phosphorylation on tyrosine residues by the inhibitor PP1 indicates that another tyrosine kinase is involved in the tyrosine phosphorylation of PDK1. PDK1 phosphospecific antibodies allowed us to establish that the inhibition of kinase activity in the presence of PP1 is due to an effect on Tyr-373/376 phosphorylation rather than Tyr-9 phosphorylation (Fig. 9). Consistent with these results, extensive mutational analysis of these sites indicated that Tyr-373/376 is important for PDK1 catalytic activity, whereas conversion of Tyr-9 to Phe had little effect on the kinase (Fig. 9). In agreement with the prediction of Tyr-373 as a phosphorylation site of Src kinase (50, 73), pY373, but not pY376, was detected in v-Src-treated PDK1, indicating that Tyr-373 is likely to be the authentic regulatory phosphorylation site *in vivo*. Furthermore, examination of pY sequences on PDK1 revealed that Tyr-9 is in a consensus sequence for the Src homology-2 (SH2) binding motif of Src (74–76). Thus, it is possible that PDK1 phosphorylated on Tyr-9 is recruited to Src by its SH2 domain, leading to Tyr-373 phosphorylation of PDK1 at the plasma membrane and PDK1 activation.

Because PDK1 regulates multiple substrates in the cell, it is of interest to ascertain how substrate specificity is maintained by PDK1. One hypothesis is that the catalytic activity of PDK1 is modulated by the conformation of the substrate (11, 77). Evidence for this has emerged from studies on the PDK1-interacting fragment (PIF) of protein kinase C-related kinase-2 (78), where the hydrophobic motif of the substrate is crucial for recognition by PDK1. Apparently, this hydrophobic motif is conserved between PDK1 substrates (Fig. 1*A*). A series of studies by Alessi and colleagues (78–81) has revealed a preference of PDK1 for a negatively charged residue in the phospho-acceptor position of this motif and demonstrated the importance of flanking hydrophobic residues. Furthermore, the interaction between the hydrophobic motif of PKA and PDK1 appears to be mediated by the hydrophobic pocket in the kinase domain of PDK1 (82). Similar results were reported with RSK2 (47), and these authors suggest that PDK1 is recruited by the hydrophobic motif of RSK2 in a phosphorylation-dependent manner, leading to stimulation of PDK1 kinase activity by autophosphorylation. Similar observations are also reported with the hydrophobic motif of other kinases (*e.g.* PKC isoforms and PAK) (23, 80, 83–85). These data raise the question as to whether the interaction of PDK1 with its substrates actually modulates the catalytic activity of PDK1. *In vitro* studies have shown that the activity of PDK1 increases 4-fold in the presence of PIF (82) or 5-fold in the presence of phosphorylated Ser-386 peptide (RSK2) (47). Indeed, preincubation of PDK1

with PIF or phosphorylated Ser-473 peptide (PKB) caused an ~5-fold increase in PDK1 activity.[2] Therefore, the docking of the hydrophobic motif of the substrate to the hydrophobic pocket of PDK1 permits efficient phosphorylation of the substrates by potentially stabilizing and enhancing PDK1 activity.

Recent genetic studies with *C. elegans* has provided hints that PDK1 activity can be increased above basal levels by the substitution of Ala-277 to Val in the catalytic domain (72). Furthermore, inactivating mutants of PDK1 from *S. cerevisiae* (70) and *S. pombe* (71) indicated that mutations in the catalytic domain or linker region led to defective PDK1 in these signaling pathways. These results converge on the idea that a conformational change of the catalytic domain, introduced by mutations, leads to active or inactive PDK1, suggesting the importance of structural conformation for catalytic activity. It is possible that tyrosine phosphorylation of PDK1 also causes a conformational change in the catalytic domain similar to that observed in the activated *C. elegans* PDK1 mutant, even though the phosphotyrosine sites important for catalytic activity are located in the linker region between the catalytic domain and PH domain in mammalian PDK1 used in our experiments.

Another important regulation mechanism for PDK1 activity is cellular localization. Although growth factor-stimulated translocation of PKB to the plasma membrane has been well characterized (34–46, 86, 87), it is controversial whether or not PDK1 translocates in the presence of growth factors. Although PDK1 was initially identified as a kinase that phosphorylates PKB in a 3-phosphoinositide-dependent manner (1, 2, 68, 88), PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 binding to the PH domain of PDK1 did not directly induce any change in catalytic activity of PDK1 (49, 88). It has been demonstrated that PDK1 selectively binds PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 with 20-fold higher affinity than PKB (49). Four reports have provided evidence that supports a PH domain-dependent relocalization of PDK1 from the cytosol to the plasma membrane (42, 46, 67, 89). In this study, we also made the observation that a fraction of PDK1 is translocated to the plasma membrane in the presence of pervanadate or insulin (Fig. 3). Using immunogold labeling and electron microscopy, IGF-1-stimulated translocation of PDK1 in HEK 293 or porcine aortic endothelial cells was also observed, which was interpreted as insignificant because only a small proportion of PDK1 was detected in the plasma membrane (49). It is hard to imagine that the PH domain of PDK1, which binds PtdIns(3,4,5)P_3 and PtdIns(3,4)P_2 with high affinity, does not facilitate the translocation of PDK1 to the plasma membrane in response to ligands that activate PI3K. In this regard, insulin-stimulated translocation of PDK1 was consistently observed in our experiments, accompanied by an increase in PDK1 activity and tyrosine phosphorylation in this fraction, suggesting that PDK1 indeed translocates to the plasma membrane (Fig. 3). Moreover, membrane translocation of PDK1 is required for ligand-induced tyrosine phosphorylation, as demonstrated by the severely reduced level of tyrosine phosphorylation of the ΔPH-PDK1 mutant (Fig. 10*D*).

In summary, we propose a model where growth factor stimulation leads to PDK1 recruitment to the plasma membrane in a PH domain-dependent manner, followed by sequential tyrosine phosphorylation on Tyr-9 and Tyr-373. Phosphorylation on Tyr-9 by a PP1-insensitive protein kinase promotes phosphorylation on Tyr-373 by a Src family kinase at the plasma membrane, thus facilitating PDK1 signaling to its substrates at the plasma membrane (Fig. 12*B*). The complexity of PDK1 regulation emphasizes its crucial role as a "master-regulator" of protein kinases. The potential role of the other pS sites on PDK1 remains to be examined. Future experiments will determine further regulatory elements involved in this pathway.

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