Oxidative Stress and Vanadate Induce Tyrosine Phosphorylation of Phosphoinositide-Dependent Kinase 1 (PDK1)

Nagendra Prasad, Robert S. Topping, Dongmei Zhou, and Stuart J. Decker*

Department of Cell Biology, Parke-Davis Pharmaceutical Research Division, Ann Arbor, Michigan 48105

Received February 18, 2000; Revised Manuscript Received April 7, 2000

ABSTRACT: Phosphoinositide-dependent kinase (PDK1) regulates a number of pathways involved in responses to stress and in growth factor signaling; however, little is known concerning the mechanisms governing the activity of PDK1. In this report, we find that oxidative stress (H_2O_2) and vanadate induce tyrosine phosphorylation of PDK1. These effects of H_2O_2 and vanadate were found in 293T cells and CH310T1/2 cells expressing exogenous PDK1 and in A20 lymphoma cells expressing endogenous PDK1. Exogenously expressed PDK1 was also tyrosine-phosphorylated in response to NGF treatment of 293T expressing TrkA. H_2O_2 induced a more rapid tyrosine phosphorylation of PDK1 relative to vanadate, and only vanadate-induced tyrosine phosphorylation of PDK1 was sensitive to pretreatment of cells with wortmannin. In vitro, PDK1 could be tyrosine-phosphorylated by both the c-Src and Abl tyrosine kinases. Both H_2O_2 and vanadate treatments increased the activity of PDK1 when the serum/glucocorticoid regulated kinase (SGK) was used as substrate. Vanadate treatment appeared to bypass the requirement for phosphatidylinositol 3,4,5-trisphosphate when Akt was used as substrate for PDK1. Tyrosine phosphorylation of PDK1 by the Abl tyrosine kinase also increased the activity of PDK1 toward SGK and Akt. These data suggest a novel mechanism through which PDK1 activity may be regulated.

Reactive oxygen species (ROS) such as H_2O_2 and superoxide are believed to mediate oxidative stress through regulation of specific signaling pathways (*1*). ROS may function as second messengers affecting redox-sensitive enzymes, including protein kinases and phosphatases (*2*). In particular, H_2O_2 has been shown to induce tyrosine phosphorylation of many proteins involved in growth factor and cytokine signaling, perhaps through inhibition of protein tyrosine phosphatases (*3*−*5*). STAT3 (*6*), p72syk (*7*), lck (*8*), and a number of tyrosine kinase growth factor receptors (*9*−*11*) have been reported to be tyrosine-phosphorylated in response to H_2O_2. In addition, treatment of cells with a variety of agonists including EGF (*12*), PDGF (*13*), FGF (*14*), IL-1 (*15*, *16*), and TNF-α (*15*) has been shown to increase ROS levels. ROS also stimulate the activities of serine/threonine kinases such as JNK (*17*), extracellular-regulated protein kinase (*18*−*20*), Akt/PKB (*21*, *22*), and p70 S6 kinase (*23*).

Phosphatidylinositol 3-kinase (PI 3-kinase) has emerged as a key regulator of cell survival. In response to growth factors and other survival signals, activated PI 3-kinase generates important second messengers, including phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P3),[1] which mediates membrane localization and activation of effectors containing PH domains such as Akt/PKB, *p*hosphoinositide-*d*ependent *k*inase 1 (PDK1), and Btk family tyrosine kinases (*24*). Membrane localization is thought to facilitate regulatory phosphorylation of Akt on threonine 308 and serine 473 (*25*). PDK1 has been shown to be the kinase responsible for phosphorylation of threonine 308 of Akt (*26*). Substrates for Akt include a transcription factor, forkhead (*27*), an enzyme regulating glucose metabolism, glycogen synthase kinase-3 (*28*), and a pro-apoptotic protein BAD (*29*). In addition, Akt has recently been reported to activate NF-kB via activation of an upstream regulatory kinase (*30*−*32*).

PDK1 appears to play a central regulatory role in several cell-signaling pathways (*33*). Substrates for PDK1 include Akt (*26*), serum/glucocorticoid regulated kinase (SGK) (*34*, *35*), p70S6 kinase (*36*), p90RSK (*37*), cAMP-dependent protein kinase (PKA) (*38*), and protein kinase C-δ and PKC-ζ (*39*). However, little is known about regulation of PDK1. The PH domain of PDK1 binds PtdIns(3,4,5)P3 and targets PDK1 to the plasma membrane (*40*). PDK1 phosphorylates Akt/PKB and PKC-ζ and PKC-δ in a PI 3-kinase-dependent manner; however, no such requirement was observed with other substrates. PDK1 has consensus phosphorylation sites for PKC, PKA, casein kinase I/II, CaMII, GSK3, p70S6 kinase, p34cdc2, and for tyrosine kinases, making it likely that PDK1 is regulated by phosphorylation in response to external signals. Recently, autophosphorylation of PDK1 at serine 241 was shown to be essential for its activity, while several other phosphorylated residues were identified with no significant effect on its enzymatic function (*41*). Generally, little is known of the mechanisms through which PDK1 phosphorylation and activity are regulated.

Here we report that PDK1 is tyrosine-phosphorylated in response to treatment of cells with H_2O_2 and vanadate and

* Address correspondence to this author at Parke-Davis Pharmaceutical Research Division, 2800 Plymouth Rd., Ann Arbor, MI 48105. Phone: 734-622-5945. Fax: 734-622-5668. E-mail: stuart.decker@wl.com.

[1] Abbreviations: PDK1, phosphoinositide-dependent kinase 1; PtdIns(3,4,5)P3, phosphatidylinositol 3,4,5-trisphosphate; NGF, nerve growth factor; SGK, serum/glucocorticoid regulated kinase.

10.1021/bi000387i CCC: $19.00
Published on Web 05/16/2000

that tyrosine phosphorylation of PDK1 correlates with increased enzymatic activity.

EXPERIMENTAL PROCEDURES

Materials. A human PDK1 cDNA with an N-terminal myc epitope was a generous gift from P. Hawkins, Babraham Institute. Anti-myc (clone 9E10) monoclonal antibody and protein A/G plus agarose were from Santa Cruz Biotech; monoclonal anti-phosphotyrosine (clone 4G10), sheep polyclonal anti-PDK1 antibodies used for immunoprecipitation, and anti-sheep IgG were from UBI (06-637). Anti-PDK1 monoclonal antibody used for immunoblot analysis was from Transduction Laboratories (P78020). Akt antibody and antibody specific for phosphorylated threonine 308 and phosphorylated serine 473 of Akt and anti-rabbit IgG were from New England Biolabs. HRP-conjugated anti-mouse IgG was from BioRad.

Cell Culture. 293T cells and CH310T1/2 cells were grown in DMEM containing10% heat-inactivated FBS. A20 lymphoma cells were grown in RPMI 1640 medium containing 10% FBS. For transient transfection of myc-PDK1, 293T cells were cultured in 60 mm dishes and transfected with 3 μg of pcDNA3-myc-PDK1 using a calcium phosphate protocol. Cells were used 48 h after transfection. Stable populations of CH310T1/2 fibroblasts were established using pBABE-myc-PDK1 retrovirus.

Immunoprecipitation and Western Blot Analyses. Transiently transfected 293T cells or stable pools of CH310T1/2 cells were treated as indicated prior to lysis in HNTG buffer (50 mM Hepes, pH 7.4, 150 mM NaCl, 1% Triton X-100, 10% glycerol, 1 mM EGTA, 1 mM EDTA, 10 mM sodium pyrophosphate, 100 mM sodium fluoride, 0.2 mM sodium orthovanadate, 1 mM PMSF, and protease inhibitor cocktail) (Boehringer Mannheim). Lysates were precleared with protein A/G agarose beads for 30 min, centrifuged at 14000*g* for 10 min, and immunoprecipitated with specified antibodies and protein A/G beads. Beads were washed 4× with lysis buffer and resuspended in 2× sample buffer. Proteins were analyzed on SDS gels, transferred to nitrocellulose, and probed with the appropriate antibodies. Blots were developed with chemiluminescence reagents.

Assay of PDK1 Activity. For assays using serum/glucocorticosteroid-induced protein kinase (SGK) as substrate, anti-myc immune complexes from 293T cells transiently transfected with myc-PDK1 were washed 4 times in HNTG buffer and twice in kinase assay buffer (50 mM Tris-HCl, pH 7.5, 0.1 mM EGTA, 0.1 mM EDTA, 0.1% mercaptoethanol, 0.1 mM sodium orthovanadate). Beads were then incubated at 30 °C for 10 min with 250 ng of recombinant SGK (UBI, 14332) in assay buffer containing 2 mM $MgCl_2$, 20 μM ATP, 1 μCi of [γ-^{32}P]ATP (3000 Ci/mL), and 2.5 μM protein kinase A inhibitor peptide. Reactions were stopped by placing the tubes on ice and adding sample buffer. Samples were boiled for 3 min and analyzed on SDS gels. Gels were dried, and autoradiography was performed using Kodak BioMax films. For experiments to determine the effects of YOP treatment on PDK1 activity, anti-myc immune complexes were washed twice with YOP phosphatase buffer (NEB) followed by incubation with plus/minus 25 units of tyrosine phosphatase YOP for 30 min at 30 °C. Reactions were stopped by adding 10 mM sodium vanadate. Immune complexes were then washed 3 times in kinase assay buffer and normalized for the amount of PDK1 by anti-PDK1 blot, and equal amounts of PDK1 were assayed for activity using SGK as substrate.

For activity assays using Akt as substrate, similarly washed immune complexes were incubated for 20 min at 30 °C with 500 ng of his-tagged Akt (expressed in SF-9 insect cells and purified on a metal ion affinity column in 1.5× assay buffer containing 2 mM $MgCl_2$, 0.5 mM $MnCl_2$, 100 μM ATP, and 2.5 μM PKI with or without phospholipid vesicles. Phospholipid vesicles were prepared by drying appropriate amounts of each lipid, resuspension in assay buffer, and sonication for 15 min on ice in a bath sonicator. Final concentrations of phospholipids were 100 μM L-α-phosphatidyl-L-serine, dioleoyl, 100 μM L-α-phosphatidylcholine, β-arachidonoyl-γ-stearoyl (C20:4, [cis]-5,8,11,14/C18:0), and 10 μM PtdIns(3,4,5)P3. Reactions were stopped by placing the tubes on ice and by adding sample buffer. Samples were analyzed on SDS gels followed by immunoblotting with anti-Akt phospho-Thr308.

In Vitro Phosphorylation of PDK1 Using Recombinant Src and Abl. One hundred units of Abl tyrosine kinase (NEB) was incubated with 500 ng of recombinant his-tagged PDK1 (expressed in SF-9 insect cells and purified on a metal ion affinity column) in the presence of 50 mM Tris-HCl, pH 7.5, 0.01% Brij-35, 1 mM EGTA, 20 mM $MgCl_2$, 10 mM $MnCl_2$, 2 mM DTT, 0.1 mM sodium orthovanadate, 200 μM ATP at 30 °C for 15 min. For phosphorylation of PDK1 by c-Src, 3 units of recombinant c-Src (Upstate Biotechnology 14117) and 500 ng of PDK1 were combined in 30 μL of 10 mM Hepes, pH 7.4, 10 mM $MgCl_2$, 10 mM $MnCl_2$, 1 mM DTT, 1 mM EGTA, and 2.5 μM PKA inhibitor at room temperature for 1 min. Reactions were terminated by adding SDS sample buffer. Samples were analyzed by SDS−PAGE followed by immunoblotting with anti-phosphotyrosine.

To measure the effects of tyrosine phosphorylation of PDK1 by Abl tyrosine kinase on PDK1 activity, Abl tyrosine kinase and PDK1 were incubated at room temperature overnight as described above. Reactions were stopped by placing the tubes on ice and diluting 4× with assay buffer to 10 ng/μL PDK1 when using Akt as substrate or 40× with assay buffer to 1 ng/μL PDK1 when using SGK as substrate. Final reaction conditions for PDK1 phosphorylation of Akt were 25 mM Tris, pH 7.5, 5 mM β-glycerophosphate, 2 mM DTT, 10 mM $MgCl_2$, 0.1 mM sodium orthovanadate, 1 μL of Abl−PDK1 mix, and 7.5 μM ATP containing 2.5 μCi of [γ-^{32}P]ATP (3000 Ci/mL), and reactions were incubated at 30 °C for 15 min. Conditions using SGK as substrate were 50 mM Tris, pH 7.5, 2 mM $MgCl_2$, 0.1 mM EGTA, 0.1 mM EDTA, 0.1% β-mercaptoethanol, 0.1 mM sodium orthovanadate, 1 μL of Abl−PDK1 mix, and 20 μM ATP containing 1.0 μCi of [γ-^{32}P]ATP (3000Ci/mL) at 30 °C for 10 min.

Phosphoamino acid analyses of anti-myc immunoprecipitates from ^{32}P-orthophosphate-labeled 293T cells expressing myc-PDK1 were performed as described previously (*42*).

RESULTS

Tyrosine Phosphorylation of PDK1. Reactive oxygen species such as hydrogen peroxide (H_2O_2) activate the Akt serine/threonine kinase; however, little is known concerning

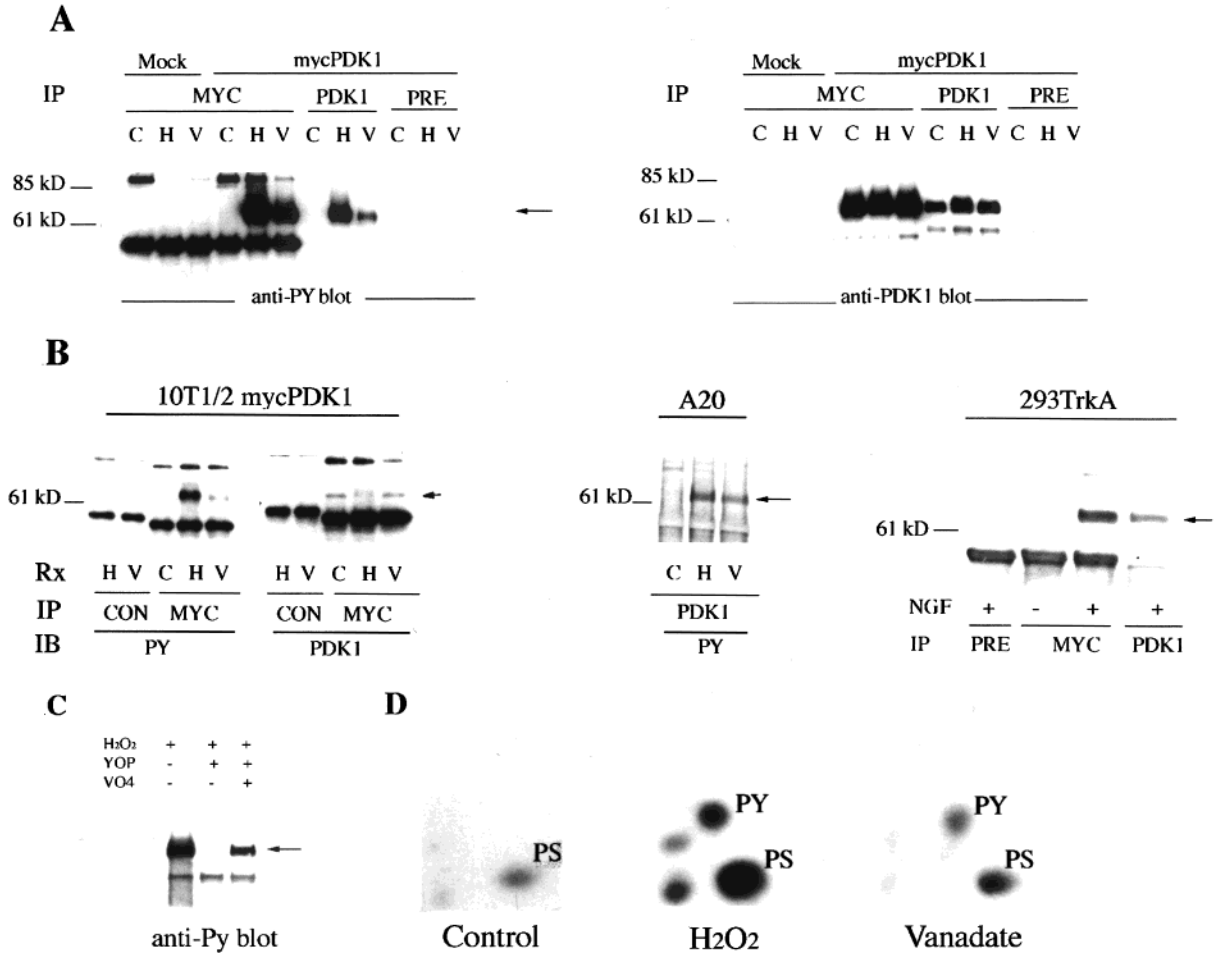

FIGURE 1: Tyrosine phosphorylation of PDK1. In panel A, 293T cells were mock-transfected or transfected with myc-PDK1 vector and treated with either vehicle (C), 5 mM H_2O_2 for 5 min (H), or 1 mM sodium vanadate for 15 min (V). Cells were lysed and lysates immunoprecipitated with anti-myc (MYC), anti-PDK1 (PDK), or preimmune serum (PRE). Precipitated proteins were resolved on SDS gels, transferred to nitrocellulose membranes, and immunoblotted with either anti-phosphotyrosine or anti-PDK1 as indicated. For panel B, samples were prepared as above except that CH310T1/2 cells expressing myc-PDK1, A20 cells, or 293-Trk cells treated with NGF for 10 min were used. In panel C, 293T cells transfected with myc-PDK1 vector were treated with 5 mM H_2O_2 for 5 min and lysates immunoprecipitated with anti-myc. Washed immunoprecipitates were treated with vehicle, with 50 units of YOP tyrosine phosphatase, or with YOP plus 1 mM vanadate for 20 min at room temperature. Proteins were resolved on an SDS gel and immunoblotted with anti-phosphotyrosine. In panel D, 293T cells transfected with myc-PDK1 were labeled with ^{32}P-orthophosphate and treated with vehicle (Control), with 5 mM H_2O_2 for 5 min, or with 1 mM vanadate for 15 min. Labeled myc-PDK 1 was immunoprecipitated and prepared for phosphoamino acid analysis as described under Experimental Procedures. Positions of phosphotyrosine (PY) and phosphoserine (PS) standards are indicated. All experiments were performed at least twice, and representative data are shown.

the effects of oxidant treatment of cells on the regulation of PDK1, the activating kinase for Akt. Since hydrogen peroxide has been reported to stimulate tyrosine phosphorylation of signaling proteins in a number of cellular systems (*3*, *7*−*11*, *43*−*49*), we investigated the effects of H_2O_2 on tyrosine phosphorylation of PDK1. Initial experiments were performed using 293T cells transiently expressing myc-tagged PDK1. Since H_2O_2 is thought to act as an inhibitor of tyrosine phosphatases in some instances (*3*−*5*), we also examined the effects of sodium vanadate (a known inhibitor of tyrosine phosphatases) on tyrosine phosphorylation of PDK1. As shown in Figure 1A, strong bands of approximately $M_r = 65\,000$ were detected with anti-phosphotyrosine in anti-myc immunoprecipitates from H_2O_2 and sodium vanadate treated cells. The $M_r = 65\,000$ bands were also seen in anti-PDK1 immunoprecipitates. Bands of similar gel mobility were immunoprecipitated with anti-myc from CH310T1/2 fibroblasts stably expressing myc-PDK1 and with PDK1 antibodies from A20 cells treated with H_2O_2 or vanadate (Figure 1B). Transiently expressed myc-PDK1 also appeared to be tyrosine-phosphorylated in response to NGF treatment of 293T cells expressing TrkA (Figure 1B). However, we were unable to observe tyrosine phosphorylation of PDK1 in response to factors such as EGF, PDGF, insulin, and TNF-α or in response to UV light or heat shock (data not shown). A protein band of approximately $M_r = 85\,000-90\,000$ in anti-Py blots was considered nonspecific since it appeared in anti-myc immunoprecipitates from mock-transfected 293T cells (Figure 1A) and control antibody immunoprecipitates from CH310T1/2 fibroblasts stably expressing myc-PDK1 (Figure 1B). Several approaches were taken to confirm that the anti-phosphotyrosine reactivity truly represented tyrosine phosphorylation of PDK1. Myc immunoprecipitates from myc-PDK1 expressing CH310T1/2 cells treated with H_2O_2 were incubated with the YOP bacterial tyrosine phosphatase or YOP in the presence of vanadate prior to immunoblotting with anti-phosphotyrosine. YOP treatment completely eliminated the anti-phosphotyrosine signal, and this effect was substantially reversed by vanadate (Figure 1C). In addition, cells were in vivo labeled with ^{32}P-

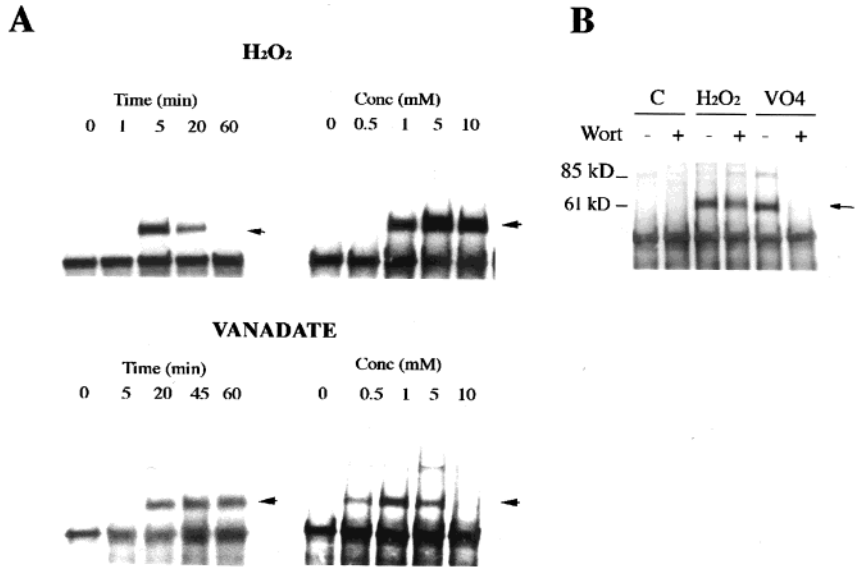

FIGURE 2: Time courses, dose responses, and effects of wortmannin on PDK1 tyrosine phosphorylation. (A) For time course experiments, CH310T1/2 cells expressing myc-PDK1 were treated with 5 mM H_2O_2 or with 1 mM vanadate for the indicated times. For dose response experiments, cells were treated for 5 min with various concentrations of H_2O_2 or for 15 min with concentrations of vanadate. Cells were lysed, and proteins were immunoprecipitated with anti-myc and immunoblotted with anti-phosphotyrosine as in Figure 1. (B) CH310T1/2 cells expressing myc-PDK1 were treated with 100 nM wortmannin for 30 min prior to addition of vehicle (C), 5 mM H_2O_2 for 5 min (H_2O_2), or 1 mM vanadate for 15 min (VO4). Cells were lysed, and proteins were immunoprecipitated with anti-myc and immunoblotted with anti-phosphotyrosine as in Figure 1.

orthophosphate and ^{32}P-labeled myc-PDK1 isolated by immunoprecipitation and analyzed for phospho-amino acid content (Figure 1D). Both H_2O_2 and vanadate treatment resulted in the appearance of a prominent spot comigrating with the phosphotyrosine standard in thin-layer analyses of digests. These treatments also increased the levels of phosphoserine in PDK1.

In Figure 2A, dose responses and time courses of tyrosine phosphorylation of PDK1 following H_2O_2 and vanadate treatment are shown. H_2O_2 treatment of CH310T1/2 cells expressing myc-PDK1 resulted in rapid tyrosine phosphorylation of PDK1 peaking at about 5 min with maximal response occurring at about 5 mM H_2O_2. The vanadate response was maximal at 1 mM vanadate and appeared 20 min posttreatment, and the maximal response was maintained up to 60 min. Since both H_2O_2 (*22*, *35*) and vanadate (*50*, *51*) have been reported to stimulate PI 3-kinase activity, the effects of the PI 3-kinase inhibitor wortmannin on tyrosine phosphorylation of PDK1 were examined (Figure 2B). Surprisingly, vanadate-stimulated tyrosine phosphorylation of PDK1 was sensitive to wortmannin treatment, whereas H_2O_2-dependent tyrosine phosphorylation was unaffected.

In Vitro Tyrosine Phosphorylation of PDK1. To further establish PDK1 as a substrate for tyrosine kinases, in vitro experiments were performed using the c-Src and Abl tyrosine kinases to phosphorylate PDK1 (Figure 3). Incubation of both c-Src and Abl proteins with recombinant PDK1 caused rapid and readily detectable tyrosine phosphorylation of PDK1.

Effects of Tyrosine Phosphorylation on PDK1 Activity. We next sought to determine if tyrosine phosphorylation of PDK1 correlated with a change in PDK1 activity. Following treatment of cells with H_2O_2 or vanadate, PDK1 was immunoprecipitated and PDK1 activity assayed using Akt or SGK as substrate (Figure 4). For Akt assays, reactions were performed in the presence or absence of PtdIns(3,4,5)-P3 (Figure 4A). Phosphorylation of Akt by PDK1 from

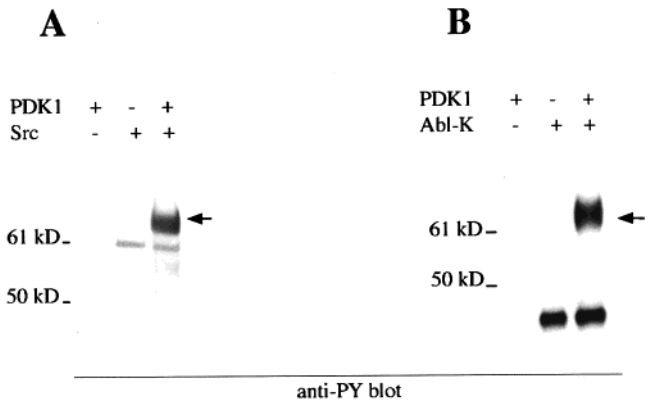

FIGURE 3: In vitro tyrosine phosphorylation of PDK1 by c-Src and Abl tyrosine kinases. (A) Reactions contained 500 ng of PDK1 alone, 3 units of c-Src tyrosine kinase alone, or PDK1 plus c-Src and were initiated by the addition of 200 μM ATP. (B) Reactions contained 500 ng of PDK1, 100 units of Abl tyrosine kinase, or PDK1 plus Abl and were initiated by the addition of 200 μM ATP. Incubations were for 15 min at room temperature. Samples were run on SDS gels, transferred to nitrocellulose, and immunoblotted with anti-phosphotyrosine. Assay conditions were as described under Experimental Procedures.

control and H_2O_2-treated cells was stimulated 2−3-fold by inclusion of PtdIns(3,4,5)P3. However, PDK1 activity from vanadate-treated cells in the absence of PtdIns(3,4,5)P3 was consistently elevated 2−3-fold relative to PDK1 from control cells and was not further stimulated by PtdIns(3,4,5)P3. PDK1 activity toward SGK (which is not dependent on phosphatidylinositides) (*34*, *35*) was stimulated 2−4-fold by both H_2O_2 and vanadate treatments. To determine whether tyrosine phosphorylation of PDK1 directly affects PDK1 activity, PDK1 immunoprecipitated from cells exposed to H_2O_2 or vanadate was treated with the bacterial tyrosine phosphatase YOP. As shown in Figure 4C, YOP treatment inhibited both H_2O_2 and vanadate-stimulated PDK1 activity. In vitro tyrosine phosphorylation of PDK1 by Abl tyrosine

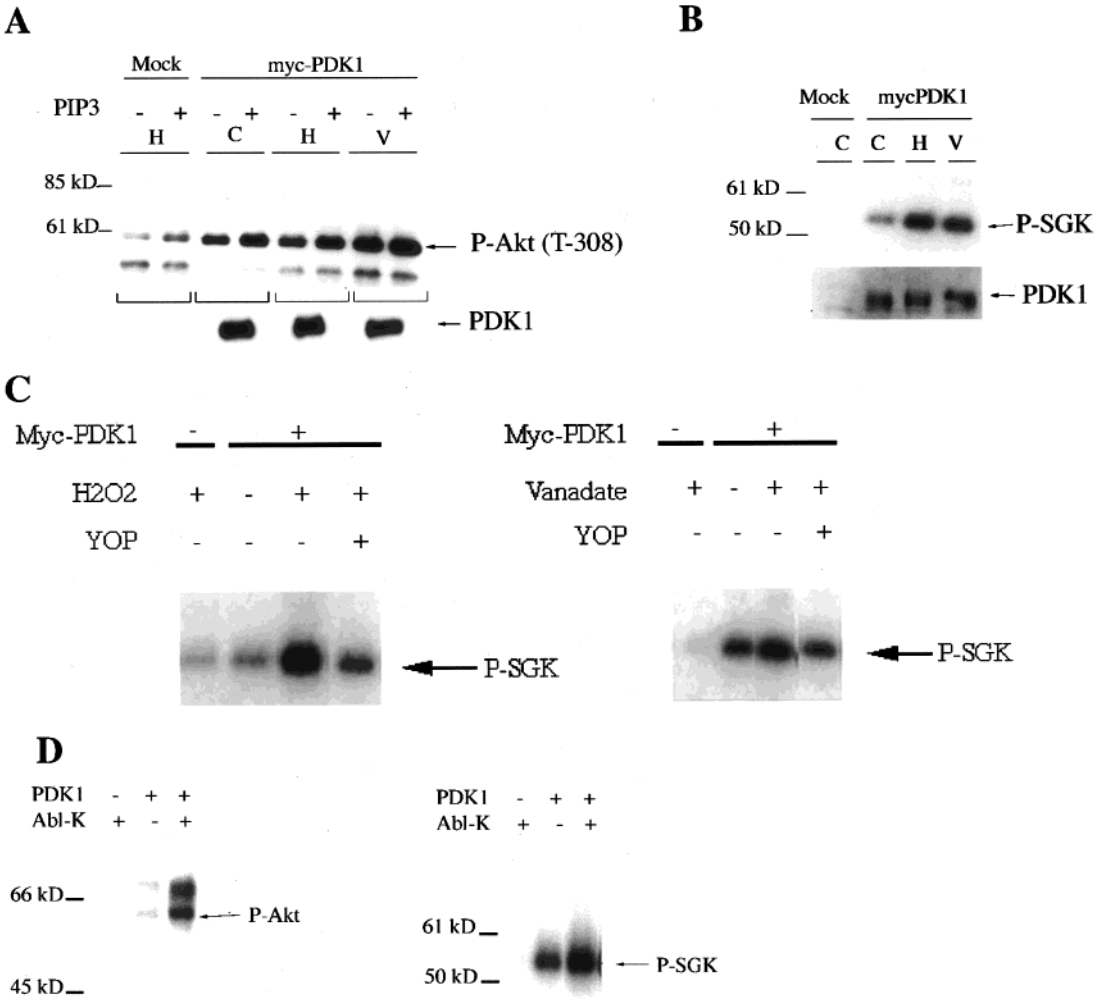

FIGURE 4: Effects of tyrosine phosphorylation on PDK1 activity. In (A), PDK1 activity from 293T cells transfected with myc-PDK1 was assayed. Cells were treated with vehicle (C), with 5 mM H_2O_2 for 5 min (H), or with 1 mM vanadate (V) for 15 min prior to lysis and immunoprecipitation with anti-myc. Washed immunoprecipitates were incubated with 0.5 μg of Akt and 100 μM ATP for 20 min at room temperature in the presence or absence of PtdIns(3,4,5)P3. Samples were run on SDS gels, transferred to nitrocellulose, and immunoblotted with anti-phospho-Akt (Thr308). PDK1 levels in immunoprecipitates from the various treatments were measured (bottom panel) prior to the final plus/minus PIP3 aliquoting step to ensure normalization among treatments. In (B), cells were treated and prepared as in (A) except that SGK was used as substrate and PtdIns(3,4,5)P3 was not used. Conditions for both assays were as described under Experimental Procedures. In (C), the effects of YOP treatment on PDK1 activity are shown. PDK1 activity from 293T cells transfected with myc-PDK1 was assayed using SGK as substrate as described above except that immunoprecipitates were preincubated with plus/minus 25 units of bacterial tyrosine phosphatase, YOP, for 30 min at 30 °C. The arrows indicate the positions of the phosphorylated SGK. In (D), the effects of Abl phosphorylation on the activity of PDK1 are shown. Substrates (Akt or SGK) were incubated in reaction buffer with [γ-^{32}P]ATP for 15 min at room temperature by themselves or with PDK1 which had been treated with Abl buffer or with Abl tyrosine kinase as described under Experimental Procedures. The arrows indicate the positions of the phosphorylated substrates.

kinase increased PDK1 activity toward both Akt and SGK (Figure 4D). The increased activity toward Akt was independent of PtdIns(3,4,5)P3.

DISCUSSION

In this study, we describe the novel finding that PDK1 becomes tyrosine-phosphorylated in response to treatment of cells with H_2O_2 and vanadate. These effects were seen in several cell types including 293T cells, CH310T1/2 cells, and A20 cells. Tyrosine phosphorylation of PDK1 in response to both H_2O_2 and vanadate treatment correlated with an increase in the enzymatic activity of PDK1. In addition, in vitro tyrosine phosphorylation of recombinant PDK1 by the Abl tyrosine kinase also stimulated PDK1 activity, indicating that tyrosine phosphorylation of PDK1 alone was sufficient to affect PDK1 activity. An increase in serine phosphorylation of PDK1 from H_2O_2- and vanadate-treated cells also occurred which could contribute to the modulation of PDK1 activity seen in vivo. It was intriguing that NGF stimulated tyrosine phosphorylation of PDK1 when transfected into 293 cells expressing TrkA. However, we were unable to detect tyrosine phosphorylation of PDK1 in response to addition of growth factors or cytokines to cells expressing endogenous levels of PDK1 and endogenous levels of receptors. Environmental stress such as heat shock and UV light also failed to induce tyrosine phosphorylation of PDK1. This could be due to a failure to use the appropriate agonists or cell types or perhaps tyrosine phosphorylation of PDK1 is specific for oxidative stress.

This is the first report in which the activity of PDK1 has been shown to be regulated in response to extracellular stimuli. PDK1 has previously been shown to be constitutively phosphorylated in vivo on several serine residues, and phosphorylation of serine 241 has been shown to critical for

PDK1 activity. Neither phosphorylation of these sites nor PDK1 activity is regulated by treatment of cells with agonists such as IGF-1 (*41*). Regulation of PDK1 is thought to occur primarily through translocation of the active enzyme to the plasma membrane mediated by binding of its PH domain to PtdIns(3,4,5)P3 generated by PI 3-kinase (*40*). Tyrosine phosphorylation of PDK1 may represent another level of PDK1 regulation perhaps affecting PDK1 activity, substrate specificity, or intracellular localization. The apparent PtdIns-(3,4,5)P3-independent phosphorylation of Akt by PDK1 from vanadate-treated cells represents a subtle mechanism through which differential regulation of Akt might be achieved.

Although both H_2O_2 and vanadate modulate PDK1 activity in some contexts, their effects are different. For example, tyrosine phosphorylation of PDK1 in response to vanadate treatment is inhibited by wortmannin, but H_2O_2-stimulated tyrosine phosphorylation is not. Tyrosine phosphorylation of PDK1 by H_2O_2 also appears to be of somewhat shorter duration compared to the effects of vanadate. These data suggest that H_2O_2 and vanadate stimulate tyrosine phosphorylation of PDK1 through different mechanisms. Vanadate-dependent tyrosine phosphorylation appears to result from activation of a tyrosine kinase (or inhibition of a tyrosine phosphatase) which functions downstream of PI 3-kinase. Activation of the tyrosine kinase, which phosphorylates PDK1 in response to H_2O_2, appears not to require PI 3-kinase. The effect of vanadate, but not H_2O_2, to cause PtdIns(3,4,5)P3 independent phosphorylation of Thr-308 of Akt further highlights the differences in signaling capacity of PDK1 from cells treated with H_2O_2 or vanadate. These results are somewhat unexpected since both vanadate and H_2O_2 are best characterized as inhibitors of tyrosine phosphatases (*5*, *49*). However, since both have rather pleiotrophic effects on tyrosine phosphorylation, it is possible that different subsets of tyrosine phosphatases are inactivated by the two agents. Alternatively, vanadate and H_2O_2 may act through as yet unknown mechanisms that are unrelated to their effects on tyrosine phosphatases.

Activation of PDK1 by tyrosine phosphorylation may play a role in influencing the survival in response to oxidative stress and perhaps other stimuli. A number of other protein kinases in critical signaling pathways including map kinase (*18*−*20*), JNK/SAPK (*17*, *19*, *52*), p38 (*18*, *19*), p70 S6 kinase (*23*), and Akt (*21*, *22*) are activated following H_2O_2 treatment of cells. In fact, PKC isoforms have been shown to become tyrosine-phosphorylated and activated following H_2O_2 treatment of COS-7 cells (*43*). The orchestrated activities of these enzymes may affect the balance between apoptosis and survival in response to oxidant injury. Since PDK1 directly regulates Akt [a critical mediator of cell survival (*53*, *54*)] and p70 S6 kinase [an important regulator of protein synthesis (*55*)] and of several PKC isoforms, it lies at a potentially critical juncture where various signals may be integrated and appropriate responses coordinated.

ACKNOWLEDGMENT

We express our appreciation to Phillip Hawkins for providing a myc-PDK1 construct and Alan Saltiel for invaluable assistance.

REFERENCES

1. Remacle, J., Raes, M., Toussaint, O., Renard, P., and Rao, G. (1995) *Mutat. Res. 316*(3), 103−122.
2. Rhee, S. G. (1999) *Exp. Mol. Med. 31*(2), 53−59.
3. Denu, J. M., and Tanner, K. G. (1998) *Biochemistry 37*(16), 5633−5642.
4. Sullivan, S. G., Chiu, D. T., Errasfa, M., Wang, J. M., Qi, J. S., and Stern, A. (1994) *Free Radical Biol. Med. 16*(3), 399−403.
5. Hecht, D., and Zick, Y. (1992) *Biochem. Biophys. Res. Commun. 188*(2), 773−779.
6. Carballo, M., Conde, M., El Bekay, R., Martin-Nieto, J., Camacho, M. J., Monteseirin, J., Conde, J., Bedoya, F. J., and Sobrino, F. (1999) *J. Biol. Chem. 274*(25), 17580−17586.
7. Schieven, G. L., Kirihara, J. M., Burg, D. L., Geahlen, R. L., and Ledbetter, J. A. (1993) *J. Biol. Chem. 268*(22), 16688−16692.
8. Hardwick, J. S., and Sefton, B. M. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*(10), 4527−4531.
9. Koshio, O., Akanuma, Y., and Kasuga, M. (1988) *Biochem. J. 250*(1), 95−101.
10. Gamou, S., and Shimizu, N. (1995) *FEBS Lett. 357*(2), 161−164.
11. Gonzalez-Rubio, M., Voit, S., Rodriguez-Puyol, D., Weber, M., and Marx, M. (1996) *Kidney Int. 50*(1), 164−173.
12. Bae, Y. S., Kang, S. W., Seo, M. S., Baines, I. C., Tekle, E., Chock, P. B., and Rhee, S. G. (1997) *J. Biol. Chem. 272*(1), 217−221.
13. Sundaresan, M., Yu, Z. X., Ferrans, V. J., Irani, K., and Finkel, T. (1995) *Science 270*(5234), 296−299.
14. Krieger-Brauer, H. I., and Kather, H. (1995) *Biochem. J. 307*(Pt 2), 549−556.
15. Meier, B., Radeke, H. H., Selle, S., Younes, M., Sies, H., Resch, K., and Habermehl, G. G. (1989) *Biochem. J. 263*(2), 539−545.
16. Lo, Y. Y., Conquer, J. A., Grinstein, S., and Cruz, T. F. (1998) *J. Cell Biochem. 69*(1), 19−29.
17. Lo, Y. Y. C., Wong, J. M. S., and Cruz, T. F. (1996) *J. Biol. Chem. 271*(26), 15703−15707.
18. Zhang, J., Jin, N., Liu, Y., and Rhoades, R. A. (1998) *Am. J. Respir. Cell Mol. Biol. 19*(2), 324−332.
19. Bhat, N. R., and Zhang, P. (1999) *J. Neurochem. 72*(1), 112−119.
20. Wang, X., Martindale, J. L., Liu, Y., and Holbrook, N. J. (1998) *Biochem. J. 333*(Pt 2), 291−300.
21. Konishi, H., Matsuzaki, H., Tanaka, M., Takemura, Y., Kuroda, S., Ono, Y., and Kikkawa, U. (1997) *FEBS Lett. 410*(2−3), 493−498.
22. Shaw, M., Cohen, P., and Alessi, D. R. (1998) *Biochem. J. 336*(Pt 1), 241−246.
23. Bae, G. U., Seo, D. W., Kwon, H. K., Lee, H. Y., Hong, S., Lee, Z. W., Ha, K. S., Lee, H. W., and Han, J. W. (1999) *J. Biol. Chem. 274*(46), 32596−32602.
24. Downward, J. (1998) *Curr. Opin. Cell Biol. 10*(2), 262−267.
25. Alessi, D. R., Andjelkovic, M., Caudwell, B., Cron, P., Morrice, N., Cohen, P., and Hemmings, B. A. (1996) *EMBO J. 15*(23), 6541−6551.
26. Alessi, D. R., James, S. R., Downes, C. P., Holmes, A. B., Gaffney, P. R., Reese, C. B., and Cohen, P. (1997) *Curr. Biol. 7*(4), 261−269.
27. Brunet, A., Bonni, A., Zigmond, M. J., Lin, M. Z., Juo, P., Hu, L. S., Anderson, M. J., Arden, K. C., Blenis, J., and Greenberg, M. E. (1999) *Cell 96*(6), 857−868.
28. Cross, D. A., Alessi, D. R., Cohen, P., Andjelkovich, M., and Hemmings, B. A. (1995) *Nature 378*(6559), 785−789.
29. Datta, S. R., Dudek, H., Tao, X., Masters, S., Fu, H., Gotoh, Y., and Greenberg, M. E. (1997) *Cell 91*(2), 231−241.
30. Kane, L. P., Shapiro, V. S., Stokoe, D., and Weiss, A. (1999) *Curr. Biol. 9*(11), 601−604.
31. Ozes, O. N., Mayo, L. D., Gustin, J. A., Pfeffer, S. R., Pfeffer, L. M., and Donner, D. B. (1999) *Nature 401*(6748), 82−85.
32. Romashkova, J. A., and Makarov, S. S. (1999) *Nature 401*(6748), 86−90.

33. Belham, C., Wu, S., and Avruch, J. (1999) *Curr. Biol. 9*(3), R93−96.
34. Kobayashi, T., and Cohen, P. (1999) *Biochem. J. 339*(Pt 2), 319−328.
35. Park, J., Leong, M. L., Buse, P., Maiyar, A. C., Firestone, G. L., and Hemmings, B. A. (1999) *EMBO J. 18*(11), 3024−3033.
36. Pullen, N., Dennis, P. B., Andjelkovic, M., Dufner, A., Kozma, S. C., Hemmings, B. A., and Thomas, G. (1998) *Science 279*(5351), 707−710.
37. Jensen, C. J., Buch, M. B., Krag, T. O., Hemmings, B. A., Gammeltoft, S., and Frodin, M. (1999) *J. Biol. Chem. 274*(38), 27168−27176.
38. Cheng, X., Ma, Y., Moore, M., Hemmings, B. A., and Taylor, S. S. (1998) *Proc. Natl. Acad. Sci. U.S.A. 95*(17), 9849−9854.
39. Le Good, J. A., Ziegler, W. H., Parekh, D. B., Alessi, D. R., Cohen, P., and Parker, P. J. (1998) *Science 281*(5385), 2042−2045.
40. Currie, R. A., Walker, K. S., Gray, A., Deak, M., Casamayor, A., Downes, C. P., Cohen, P., Alessi, D. R., and Lucocq, J. (1999) *Biochem. J. 337*(Pt 3), 575−583.
41. Casamayor, A., Morrice, N. A., and Alessi, D. R. (1999) *Biochem. J. 342*(Pt 2), 287−292.
42. Decker, S. J. (1984) *Mol. Cell Biol. 4*(9), 1718−1724.
43. Konishi, H., Tanaka, M., Takemura, Y., Matsuzaki, H., Ono, Y., Kikkawa, U., and Nishizuka, Y. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*(21), 11233−11237.
44. Vepa, S., Scribner, W. M., and Natarajan, V. (1997) *Free Radical Biol. Med. 22*(1−2), 25−35.
45. Suzuki, Y., Ohsugi, K., and Ono, Y. (1996) *Immunology 87*(3), 396−401.
46. Nagai, K., Inazu, T., and Yamamura, H. (1994) *J. Biochem. (Tokyo) 116*(5), 1176−1181.
47. Nakamura, K., Hori, T., Sato, N., Sugie, K., Kawakami, T., and Yodoi, J. (1993) *Oncogene 8*(11), 3133−3139.
48. Schieven, G. L., Kirihara, J. M., Myers, D. E., Ledbetter, J. A., and Uckun, F. M. (1993) *Blood 82*(4), 1212−1220.
49. Heffetz, D., Bushkin, I., Dror, R., and Zick, Y. (1990) *J. Biol. Chem. 265*(5), 2896−2902.
50. Ida, M., Imai, K., Hashimoto, S., and Kawashima, H. (1996) *Biochem. Pharmacol. 51*(8), 1061−1067.
51. Molero, J. C., Martinez, C., Andres, A., Satrustegui, J., and Carrascosa, J. M. (1998) *FEBS Lett. 425*(2), 298−304.
52. Tournier, C., Thomas, G., Pierre, J., Jacquemin, C., Pierre, M., and Saunier, B. (1997) *Eur. J. Biochem. 244*(2), 587−595.
53. O'Connor, R. (1998) *Adv. Biochem. Eng. Biotechnol. 62*, 137−166.
54. Nunez, G., and del Peso, L. (1998) *Curr. Opin. Neurobiol. 8*(5), 613−618.
55. Kawasome, H., Papst, P., Webb, S., Keller, G. M., Johnson, G. L., Gelfand, E. W., and Terada, N. (1998) *Proc. Natl. Acad. Sci. U.S.A. 95*(9), 5033−5038.

BI000387I