

## p21-activated Kinase (PAK1) Is Phosphorylated and Activated by 3-Phosphoinositide-dependent Kinase-1 (PDK1)\*

Received for publication, July 24, 2000, and in revised form, September 18, 2000  
Published, JBC Papers in Press, September 19, 2000, DOI 10.1074/jbc.M006553200

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In this study, we show that phosphorylated 3-phosphoinositide-dependent kinase 1 (PDK1) phosphorylates p21-activated kinase 1 (PAK1) in the presence of sphingosine. We identify threonine 423, a conserved threonine in the activation loop of kinase subdomain VIII, as the PDK1 phosphorylation site on PAK1. Threonine 423 is a previously identified PAK1 autophosphorylation site that lies within a PAK consensus phosphorylation sequence. After pretreatment with phosphatases, autophosphorylation of PAK1 occurred at all major sites except threonine 423. A phosphothreonine 423-specific antibody detected phosphorylation of recombinant, catalytically inactive PAK1 after incubation with wild-type PAK1, indicating phosphorylation of threonine 423 occurs by an intermolecular mechanism. The biological significance of PDK1 phosphorylation of PAK1 at threonine 423 *in vitro* is supported by the observation that these two proteins interact *in vivo* and that PDK1-phosphorylated PAK1 has an increased activity toward substrate. An increase of phosphorylation of catalytically inactive PAK1 was observed in COS-7 cells expressing wild-type, but not catalytically inactive, PDK1 upon elevation of intracellular sphingosine levels. PDK1 phosphorylation of PAK1 was not blocked by pretreatment with wortmannin or when PDK1 was mutated to prevent phosphatidylinositol binding, indicating this process is independent of phosphatidylinositol 3-kinase activity. The data presented here provide evidence for a novel mechanism for PAK1 regulation and activation.

PAK1,<sup>1</sup> a member of the PAK/Ste20 family of protein ki-

\* This work was supported in part by National Institutes of Health Grants GM 39434 (to G. M. B.) and GM43154 (to A. C. N.), a grant from the Krebsforschung Schweiz (to B. A. H.), and Breast Cancer Research Program of the University of California Grant BCRP 3PB-0062. This is publication 13439-IMM from the Scripps Research Institute. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

<sup>‡</sup> Recipient of an Arthritis Foundation postdoctoral fellowship.

<sup>¶¶</sup> Recipient of United States Army Breast Cancer Research Program Grant DAMD 17-98-1-8151.

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<sup>1</sup> The abbreviations used are: PAK, p21-activated kinase; PDK, 3-phosphoinositide-dependent kinase; PBD, p21 binding domain; AID, autoinhibitory domain; PKC, protein kinase C; PtdIns, phosphatidylinositol; GTP $\gamma$ S, guanosine 5'-3-O-(thio)triphosphate; PDGF, platelet-derived growth factor; WT, wild-type; GST, glutathione S-transferase; PH, pleckstrin homology; PBS, phosphate-buffered saline; DMEM, Dulbecco's modified Eagle's medium; Pipes, piperazine-N,N'-bis(2-ethane-

nases, contains two major functional domains, an amino-terminal regulatory domain and a carboxyl-terminal kinase domain (1). These kinases were discovered by their ability to selectively bind the Rho GTPase family members Cdc42 and Rac (2). Like all low molecular weight GTPases, Cdc42 and Rac exist in an inactive, GDP-bound state and an active, GTP-bound state (3). PAK1 specifically binds the GTP-bound form of Cdc42 or Rac (3), resulting in a conformational change that leads to autophosphorylation on several serine and/or threonine residues and an increase in activity toward substrates (4). Recently, the ability of a membrane-targeted PAK1 to become activated in a Cdc42- or Rac-independent manner suggested that alternate mechanisms for PAK1 activation must exist (5). Certain sphingolipids, such as sphingosine, but not ceramide or sphingosine 1-phosphate, were subsequently shown to activate PAK1, suggesting PAKs also transduce lipid signals (5). In addition to the kinase domain, PAK1 also contains a p21 binding domain (PBD), which binds active Cdc42 and Rac, an autoinhibitory domain (AID), four proline-rich regions, and an acidic domain. The first proline-rich region interacts with the adapter protein Nck (6), and the fourth proline-rich region interacts with the putative guanine nucleotide exchange factor/regulatory protein PIX (7).

PAKs play an important role in diverse cellular processes, including cytoskeletal rearrangements, and growth/apoptotic signal transduction through mitogen-activated protein kinases (8–16). Some aspects of cytoskeletal rearrangement, such as neurite outgrowth (17), appear to be independent of PAK kinase activity, suggesting PAKs may also act as intermolecular scaffolds. However, accumulating evidence indicates that PAK phosphorylates key cytoskeletal components. PAK1 phosphorylates and down-regulates myosin light chain kinase activity (18) and phosphorylates and activates LIM kinase (19). PAK kinase activity has been shown to induce activation of c-Jun NH<sub>2</sub>-terminal kinase/p38 kinase pathways (11, 12), and in several cell types, a catalytically inactive PAK1 construct was shown to block signal-induced c-Jun NH<sub>2</sub>-terminal kinase/p38 activation (9, 11). PAKs also play a regulatory role in apoptosis, phosphorylating and inactivating the pro-apoptotic protein Bad (20, 21). Additionally, PAK2 is cleaved and activated by caspases 3 and 7 (22). PAKs phosphorylate the p47<sup>phox</sup> component of the phagocyte NADPH oxidase *in vitro* at physiologically relevant sites (23),<sup>2</sup> suggesting an important regulatory role in the inflammatory response. The requirement for PAK kinase activity in such diverse processes underscores the importance of understanding the molecular basis for regulation of PAK activity.

sulfonic acid); PP1 and PP2A, protein phosphatases 1 and 2A.

<sup>2</sup> U. G. Knaus and C. C. King, unpublished data.

The kinetics of PAK autophosphorylation in human neutrophils stimulated with the chemoattractant f-Met-Leu-Phe (fMLP) is rapid. Maximum autophosphorylation occurs within 30 s to 1 min and decreases to basal levels after 5 min (23). In marked contrast to the rapid (30 s) and transient activation (<5 min) in stimulated neutrophils, PAK activation *in vitro* is slow (15–20 min) and sustained (7). These observations suggest that additional mechanisms of PAK regulation exist *in vivo*. Recent data suggest that PAK family members may be regulated by phosphorylation. The yeast PAK homolog, Ste20, was shown to be phosphorylated by the yeast cell cycle-dependent kinase Cdc28 when complexed with Cln2 (24). PAK activity has also been shown to be down-regulated after phosphorylation by the neuron-specific p35/cyclin-dependent kinase 5 complex (25). Field *et al.* (21) recently showed that PAK1 was a substrate for Akt kinase. Additionally, we recently showed that kinase subdomain VIII of PAK1 could be phosphorylated *in vitro* by PDK1, suggesting phosphorylation events within the catalytic domain may regulate PAK1 activity (26).

3-Phosphoinositide-dependent kinase-1 (PDK1) contains an amino-terminal kinase domain and a carboxyl-terminal pleckstrin homology (PH) domain. PDK1 appears to be conserved throughout evolution, with homologs in yeast, plants, *Drosophila*, rodent, and human (27–31). Although the PDK1 PH domain binds the products of the phosphatidylinositol 3-kinase (PtdIns 3-kinase) reaction, phosphatidylinositol 3,4-bisphosphate and phosphatidylinositol 3,4,5-trisphosphate (PtdIns 3,4,5-P<sub>3</sub>), with low nanomolar affinity (32), binding of these lipids does not alter PDK1 activity. Rather, binding of these lipids is necessary to localize PDK1 to the plasma membrane. The enzyme has been reported to have a low level of kinase activity (27, 33). Recently, our laboratory showed that sphingosine, another biologically active lipid, activated PDK1 toward a variety of substrates (26). It is well established that PDK1 phosphorylates the activation loop (kinase subdomain VIII) of AGC kinase family members p70<sup>S6</sup> kinase, Akt, protein kinase A (cAMP-dependent protein kinase), various protein kinase C (PKC) isoforms, and serum- and glucocorticoid-inducible kinase (26, 31, 33–37).

Until recently, the ability of PDK1 to phosphorylate substrates has been thought to be modulated by localization of the enzyme to the membrane through the carboxyl-terminal PH domain. PDK1 plays a regulatory role in phosphorylating substrates whose activation loops are accessible in quiescent cells, such as cAMP-dependent protein kinase and PKC $\beta$ II. Indeed, heterologous phosphorylation of PKC $\beta$ II by PDK1 is absolutely required to make PKC $\beta$ II catalytically competent (36). Other defined PDK1 substrates appear to have their activation loop masked by regulatory domains in resting cells. Upon cell stimulation, the activation loop is exposed by a conformational change, allowing PDK1 access to its substrate. For example, p70<sup>S6</sup> kinase must undergo multiple regulatory phosphorylations by proline-directed protein kinases before PDK1 can phosphorylate the activation loop of p70<sup>S6</sup> kinase (38). Another example is the well defined mechanism of Akt activation: binding of PtdIns 3,4,5-P<sub>3</sub> to the PH domain of Akt precedes PDK1 phosphorylation of the activation loop (31).

Regulatory phosphorylation(s) and/or conformational changes incurred by the binding of regulatory molecules have been hypothesized to expose the activation loop of PDK1 substrates in stimulated cells. Although PAKs are not members of the AGC kinase family, they show many similarities to PDK1 substrates. First, the kinase domain interacts with an AID that requires binding of Cdc42-GTP, Rac-GTP, or sphingosine to relieve this interaction (39). Additionally, sequence alignment of the activation loop of PAK1 with kinase substrates of PDK1

shows a high degree of homology (26). In this report, we show that PAK1 phosphorylates other PAK1 molecules at the activation loop threonine 423 by an intermolecular autophosphorylation mechanism. Pretreatment of PAK1 with phosphatase enzymes prevented phosphorylation of threonine 423, but had a slight effect on the ability of PAK1 to autophosphorylate at other sites. PDK1 phosphorylates PAK1 in the presence of sphingosine specifically at threonine 423. Support for the biological significance of this phosphorylation is strengthened by the observation that these two proteins interact *in vivo* and that PDK1-phosphorylated PAK1 has an increased activity toward substrate. Finally, we show that wild-type PDK1, but not catalytically inactive PDK1, phosphorylates PAK1 in NIH3T3 cells. Phosphorylation of PAK1 by PDK1 is independent of PtdIns 3-kinase activity. Wortmannin, a PtdIns 3-kinase inhibitor, and mutation of PDK1 to prevent phosphatidylinositol lipid binding did not alter the ability of PDK1 to phosphorylate PAK1. These studies suggest PAK1 activity is controlled, in part, by phosphorylation of the activation loop by PDK1 and that this may be an important regulatory step for activation *in vivo*.

#### MATERIALS AND METHODS

**cDNA Constructs and Plasmids**—PAK1 WT constructs were prepared in the pCMV6M vector as described previously (8). PDK1 constructs were prepared in the pCMV5M vector as described (33). Both vectors use a cytomegalovirus promoter and contain an amino-terminal 9E10 Myc epitope tag. PAK1 K299A, PAK1 K299A/T423A, PDK1 K111Q, PDK1 K111Q/S241A, and PDK1 R474A mutations were made by polymerase chain reaction amplification using a QuickChange kit (Stratagene) according to the manufacturer's instructions, followed by sequencing to confirm mutations. The primers for PAK1 K299A were as follows: forward primer, 5'-GGACAGGAGGTGGCCATTGCGCAGATG-AATCTTCAG-3'; reverse primer, GCTGAAGATTCATCTGCGCAATGGCCACCACAGTCC-3'. PAK1 K299A/T423A was synthesized using PAK1 K299A as a template. The primers for PAK1 T423A were as follows: forward primer, 5'-CAGAGCAAACGGAGCGCCATGGTAGGA-ACCC-3'; reverse primer, 5'-GGGGTTCTTACCATTGGCGCTCCGTT-TGCTCT-3'. The primers for PDK1 K111Q were as follows: forward primer, 5'-CCAGAGAATATGCGATTCAAATCTGGAGAAGCG-3'; reverse primer, 5'-CGCTTCTCCAGAATTTGAATCGCATATTCTCTGg-3'. PDK1 K111Q/S241A was synthesized using PDK1 K111Q as a template. The primers for PDK1 S241A were as follows: forward primer, 5'-GCCAGGGCCAACGCATTCGTGGGAACAGCG-3', reverse primer, 5'-CGCTGTTCCCAAGCAATGCGTTGGCCCTGGC-3'. The primers for PDK1 R474A were as follows: forward primer, 5'-CGGAG-GGGTTATTTGCAAGACGAGCAGCTGTTGCTC-3'; reverse primer, 5'-GAGCAACAGCTGTGCTCGTCTTGCAAATAAACCCCTT-3'. Underlined base pairs represent the mutated amino acids. GST-PAK1 K299A, prepared as described above, was cloned into the pGEX4T3 vector at the *Bam*HI/*Eco*RI site. GST-PAK1 kinase subdomain VIII was prepared as described (26). GST-Bad and GST-p47<sup>phox</sup> were prepared as described previously (20, 40). The GST moiety on the p47<sup>phox</sup> peptide was cleaved with thrombin. His-PKC $\beta$ II was prepared according to the QiaExpressionist protocol.

**Cell Culture and Transfections**—COS-7 cells and HeLa cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Life Technologies, Inc.) containing 10% heat-inactivated calf serum, 10 mM Hepes, pH 7.0, 2 mM glutamine, 100 units/ml penicillin G, and 100  $\mu$ g/ml streptomycin at 37 °C in 10% CO<sub>2</sub>. NIH3T3 cells, which contained a tetracycline-regulated transactivator (41) inducibly expressing PAK1 K299R (42), were maintained in DMEM containing 5% heat-inactivated fetal calf serum and 5% heat-inactivated newborn calf serum with 10 mM Hepes, pH 7.0, 2 mM glutamine, 100 units/ml penicillin G, 100  $\mu$ g/ml streptomycin, 2  $\mu$ g/ml puromycin, 2.5 mM histidinol, and 0.5  $\mu$ g/ml tetracycline at 37 °C in 10% CO<sub>2</sub>. PAK1 K299R expression was induced by incubation in tetracycline-free complete medium for at least 12 h.

For single transfections, COS-7 cells or HeLa cells (seeded at  $1.2 \times 10^6$  cells/100-mm dish) were grown to 70–80% confluence and then transfected with 5  $\mu$ g of the plasmid DNA using the LipofectAMINE transfection protocol (Life Technologies, Inc.). The cells were allowed to express the protein for 30–48 h after transfection, then washed with phosphate-buffered saline (PBS). Lysis buffer (150 mM NaCl, 25 mM

Tris-HCl, pH 7.5, 1 mM EDTA, 0.1 mM EGTA, 5 mM MgCl<sub>2</sub>, 1 mM dithiothreitol, 10% glycerol, 2% Nonidet P-40, 50 IU/ml aprotinin, 2 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride) was added to each plate, and the COS-7 cells were scraped into Eppendorf tubes at 4 °C. After 5 min at 4 °C, the lysates were centrifuged at 14,000 rpm for 10 min and the supernatants were collected. For *in vivo* phosphorylation of PAK1 by PDK1, NIH3T3 cells (seeded at 0.2 × 10<sup>6</sup> cells/35-mm dish) were transfected with 1–2 μg of PDK1 WT, K111Q, or R474A using the LipofectAMINE transfection protocol. After 6 h in transfection medium (DMEM with 250 μg of tetracycline), the medium was replaced with complete NIH3T3 medium without tetracycline to express PAK1 K299R. Cells were starved for 18 h in DMEM alone, stimulated, and lysed as described above. When used, PDGF-BB (Upstate Biotechnology, Inc.) was at 50 ng/ml.

**Immunoprecipitations**—PAK1 or PDK1 constructs were immunoprecipitated from cells by incubating lysates with the anti-Myc (9E10) monoclonal antibody in binding buffer (100 mM KCl, 3 mM NaCl, 3.5 mM MgCl<sub>2</sub>, 10 mM Pipes, pH 7.3, 1.0 mM dithiothreitol, and 1.0 mM phenylmethylsulfonyl fluoride) overnight at 4 °C, followed by a 1 h incubation with bovine serum albumin (Sigma)-coated protein G-Sepharose beads (Amersham Pharmacia Biotech). The beads were pelleted by centrifugation, and the pellet was washed three times in binding buffer containing 1% Nonidet-40, three times in binding buffer alone, and three times in kinase buffer (50 mM Hepes, pH 7.5, 10 mM MgCl<sub>2</sub>, 2 mM MnCl<sub>2</sub>, and 0.2 mM dithiothreitol). PAK1 was immunoprecipitated from NIH3T3 cells using either a rabbit polyclonal antibody (R2124) raised against amino acids 174–306 of a histidine-tagged PAK1 (23) or with the monoclonal hemagglutinin antibody (BabCo, Richmond, CA). Samples were incubated for 2 h with lysis buffer containing 1% Nonidet P-40 at 4 °C followed by a 1-h incubation with bovine serum albumin-coated protein A-agarose beads (Sigma).

**Kinase Assay**—Immunoprecipitated PAK1 or PDK1 constructs were incubated in kinase buffer (50 mM Hepes, pH 7.5, 10 mM MgCl<sub>2</sub>, 2 mM MnCl<sub>2</sub>, and 0.2 mM dithiothreitol) containing 20 μM ATP and 5 μCi of [γ-<sup>32</sup>P]ATP (ICN). When added, Cdc42-GDP or Cdc42-GTPγS was at 1.5 μg and sphingosine was 400 μM, unless otherwise indicated. After 30 min at 30 °C, Laemmli sample buffer was added to each sample at 4 °C to stop the reaction. Samples were separated on SDS-polyacrylamide gels, followed by transfer to nitrocellulose for immunoblotting and/or autoradiography. Radiolabeled phosphate incorporation was measured by PhosphorImager analysis (Molecular Dynamics, Inc., Sunnyvale, CA).

**Tryptic digestion and phosphopeptide mapping**—Kinase assays were performed to induce autophosphorylation of PAK1 alone or after treatment with protein phosphatases. PAKs were separated on 6.5% polyacrylamide gels, transblotted on to nitrocellulose, and detected by autoradiography. Each band was excised from the nitrocellulose and resuspended in ammonium bicarbonate, pH 8.3 (50 mM), containing trypsin (1.5 μg) for 14 h at 37 °C. The tryptic peptides were extracted, centrifuged at 14,000 rpm to remove debris, and lyophilized. Two-dimensional phosphopeptide mapping of the tryptic peptides was performed according to the procedure of Boyle *et al.* (43). The lyophilized samples were resuspended in 2 μl of pH 1.9 electrophoresis buffer (2.2% formic acid, 8% acetic acid), spotted onto 100-μm cellulose coated plates (EM Science, Gibbstown, NJ) in 0.5-μl aliquots, and electrophoresed for 40 min at 1300 V in a Multiphor II horizontal electrophoresis unit (Amersham Pharmacia Biotech) in pH 1.9 electrophoresis buffer. Plates were air-dried and chromatographed in buffer containing 62.5% isobutyric acid, 1.9% *n*-butanol, 4.8% pyridine, and 2.9% glacial acetic acid until the mobile phase was 3 cm from the top of the plate. <sup>32</sup>P-Labeled tryptic peptides were detected by autoradiography on Kodak X-AR film for up to 12 h.

**Immunofluorescence**—NIH3T3 cells containing a stably transfected PAK1 K299R construct under the control of a tetracycline promoter were seeded at 0.12 × 10<sup>6</sup> cell/35-mm on 1-mm coverslips (Corning, Big Flats, NY). Cells were transfected with either 1.0 μg of wild-type PDK1 or PDK1 K111Q/S241A using LipofectAMINE (Life Technologies, Inc.) and allowed to express the PDK1 protein in the presence of tetracycline for 18 h. Cells were serum-starved in the presence or absence of tetracycline for an additional 18 h prior to PDGF stimulation. After stimulation with 50 ng/ml PDGF for 10 min, the cells were fixed with 4% formaldehyde for 30 min, washed three times with PBS, and permeabilized in PBS containing 0.5% Triton X-100 for 20 min. Cells were washed three times in PBS containing 3% bovine serum albumin and then blocked in PBS containing 3% bovine serum albumin for 1 h at 25 °C. The anti-activation loop antibody (1:250) and anti-Myc antibody (1:500) were added to the cells in PBS containing 3% bovine serum albumin for 1 h at 25 °C, washed four times with PBS, then incubated

1 h at 25 °C with Alexa 488 goat anti-rabbit (green) and Alexa 546 goat anti-mouse antibodies (red) (each at 1:500, Molecular Probes, Eugene, OR). Cells were mounted on coverslips with Prolong (Molecular Probes) mounting medium and visualized with an Olympus epifluorescence microscope. Fluorescent micrographs were taken with a Princeton MicroMAX cooled CCD camera and edited in Adobe Photoshop (Adobe Corp., San Jose, CA).

## RESULTS

**Intermolecular Phosphorylation of PAK1 at Threonine 423**—The amino acid sequence immediately upstream of threonine 423 was examined and found to form a consensus PAK1 phosphorylation sequence of Lys-Arg-Ser-Thr (44), suggesting PAKs might undergo intermolecular phosphorylations. This observation, coupled with data showing PDK1 phosphorylation of PAK1 kinase subdomain VIII, led us to ask whether threonine 423 was a true PAK1 autophosphorylation site. To test this, PAK1 was incubated with protein phosphatase 2A (PP2A) in order to remove any preexisting phosphates. At the concentration of PP2A used, <sup>32</sup>P-labeled PAK1 is completely dephosphorylated (data not shown). Samples were stimulated with Cdc42-GTPγS or sphingosine in the presence of 10 μM microcystin LR to inhibit any remaining phosphatase, and phosphate incorporation was assessed by two-dimensional phosphopeptide mapping (Fig. 1A). Untreated PAK1 was heavily phosphorylated at threonine 423 (*spots 1 and 2*) when Cdc42-GTPγS or sphingosine was used as the stimulus (*panels A and D*). Pretreatment with PP2A decreased phosphate incorporation at threonine 423 by 90% as measured by PhosphorImager analysis (*panels B and E*). Pretreatment of PAK1 with PP2A also changed the intensity of the major phosphorylation sites, but had no effect on the distribution of the spots. To test whether the spots labeled *1 and 2* represented the peptide containing threonine 423, we ran two-dimensional phosphopeptide maps on Cdc42-GTPγS or sphingosine-stimulated PAK1 in which threonine 423 was mutated to alanine. Two spots were missing from the maps of the PAK1 T423A sample (*panels C and F*). However, it was possible that Thr-423 was required for activity and that *spots 1 and 2* represent additional phosphorylation sites. To test this, we mapped the autophosphorylation sites of a constitutively active PAK1 in which threonine 423 was mutated to a glutamic acid (PAK1 T423E; Fig. 1A, *panel G*). If spots *1 and 2* represented different or novel sites whose phosphorylation was dependent on activation and threonine 423 phosphorylation, it would be predicted that this site(s) would be phosphorylated in the two-dimensional phosphopeptide map of the constitutively active PAK1 T423E mutant. The observation that *spots 1 and 2* are not present in maps with PAK1 T423E strongly suggest that these spots represent differentially cleaved threonine 423 containing peptides. Additional data supporting differential cleavage come from mass spectroscopy analysis of PAK1, which yielded peaks of 1926 Da (serine 422 through arginine 438) and 2053 Da (serine 422 through lysine 439) (data not shown). Based on the above data, we conclude these spots represent differential tryptic cleavage products of the peptide STMGTPTYMAPEVVTRK. Similar results were obtained when protein phosphatase 1 (PP1) was used to dephosphorylate PAK1 (data not shown). Further studies to determine whether threonine 423 was an autophosphorylation site were performed. Phosphatase-treated PAK1 WT was incubated with sphingosine, and phosphate incorporation was assessed by autoradiography and immunoblotting and an activation loop antibody (Fig. 1B, see below). Pretreatment of PAK1 with phosphatase did not prevent PAK1 autophosphorylation upon restimulation with sphingosine (Fig. 1B, *top*). In the absence of phosphatase, PAK1 was able to efficiently phosphorylate threonine 423, while pretreatment with PP1 or PP2A prevented phosphorylation of PAK1 at thre-

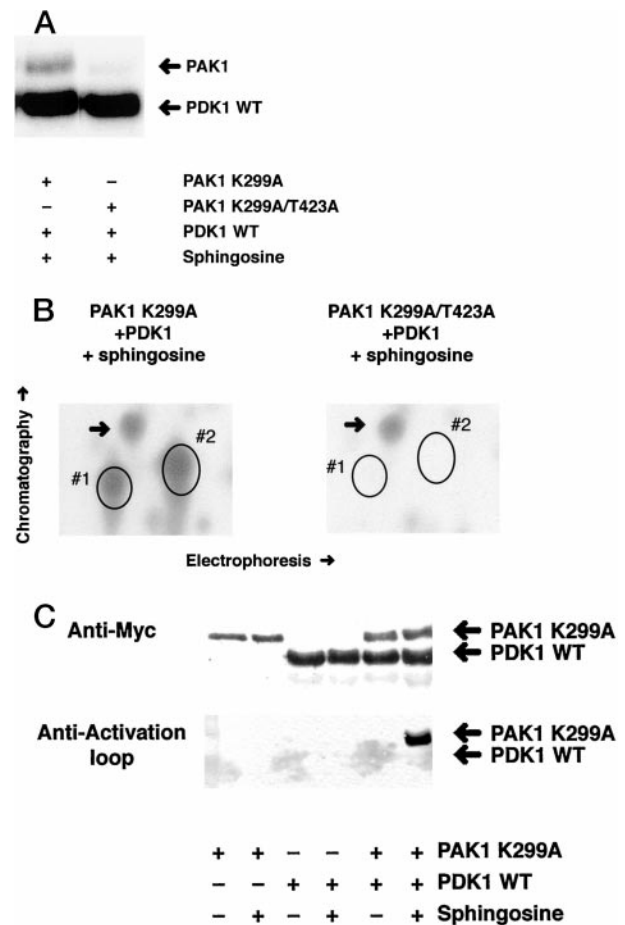


Next, we wanted to determine whether PAK1 could phosphorylate threonine 423 of other PAK1 molecules. The activation loop antibody was used to detect threonine 423 phosphorylation on catalytically inactive PAK1 (GST-PAK1 K299A) incubated with Cdc42-GTP $\gamma$ S or sphingosine in the absence or presence of immunoprecipitated PAK1 WT (Fig. 2B). Consistent with previous results, GST-PAK1 K299A was unable to autophosphorylate in the presence of Cdc42-GTP $\gamma$ S or sphingosine. However, GST-PAK1 K299A was strongly phosphorylated at threonine 423 in the presence of PAK1 WT, indicating that PAK1 transphosphorylates other PAK1 molecules at threonine 423. Overall, these results indicate that threonine 423 is an inefficient autophosphorylation site that becomes phosphorylated primarily by an intermolecular mechanism.

**PDK1 Phosphorylates PAK1 at Threonine 423**—Previous results from our laboratory indicated that PDK1 phosphorylates kinase subdomain VIII of PAK1 (26). The similarity in amino acid sequence between the activation loop of PAK1 and other PDK1 substrates suggests that PDK1 might phosphorylate PAK1 at threonine 423 (26). We used *in vitro* kinase assays to determine whether PDK1 could phosphorylate catalytically inactive PAK1 (PAK1 K299A) at threonine 423. Fig. 3A shows that PDK1 phosphorylates PAK1 K299A in the presence of sphingosine, but that this phosphorylation was decreased by 90% in PAK1 K299A/T423A, suggesting that threonine 423 is the primary site of PDK1 phosphorylation on PAK1. As reported previously, no PAK1 phosphorylation by PDK1 was observed in the presence of Cdc42-GTP $\gamma$ S (26). Two spots overlapping with spots identified in Fig. 1 as threonine 423 autophosphorylation sites were observed (Fig. 3B). The spots disappeared when threonine 423 was mutated to an alanine. At least one other PDK1 phosphorylation site on PAK1 was observed. The identity and significance of this site is currently under investigation. *In vitro* kinase assays were used to detect threonine 423 phosphorylation of PAK1 by PDK1 (Fig. 3C). The antibody failed to detect a significant amount of phosphorylated threonine 423 on PAK1 K299A alone or in the presence of sphingosine. Additionally, the antibody did not cross react with PDK1 alone or in the presence of sphingosine. Phosphorylation of threonine 423 only occurred in the presence of PDK1 WT and sphingosine. Taken together, these data identify threonine 423 of PAK1 as the primary site of phosphorylation by PDK1.

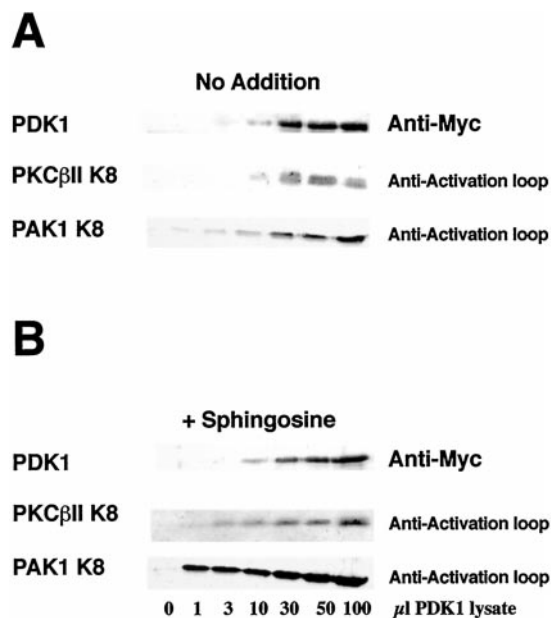
To address whether PAK1 kinase subdomain VIII is a good PDK1 substrate, we compared the ability of recombinant PKC $\beta$ II kinase subdomain VIII (His-PKC $\beta$ II K8), a well described PDK1 substrate (36), or PAK1 kinase subdomain VIII (GST-PAK1 K8) to be phosphorylated by PDK1 (Fig. 4). Potential conformational influences induced by activators such as sphingosine are eliminated using isolated domains of these kinases. In the absence of sphingosine, phosphorylated His-PKC $\beta$ II K8 and GST-PAK1 K8 was easily visualized, even at concentrations of PDK1 not detected by immunoblotting (Fig. 4A). Stimulation of PDK1 with sphingosine (Fig. 4B) increased phosphate incorporation into His-PKC $\beta$ II K8 and GST-PAK1 K8 significantly. Although we did not determine the exact amount of PDK1 immunoprecipitated from the COS-7 lysate, the concentration of PDK1 present in small amounts of lysate, undetectable by immunoblot, was sufficient to strongly phosphorylate GST-PAK1 K8. Therefore, we conclude that stoichiometric amounts of PDK1 are sufficient to phosphorylate both His-PKC $\beta$ II K8 and GST-PAK1 K8 in the presence or absence of sphingosine.

**Phosphorylation of PAK1 at Threonine 423 by PDK1 Is Associated with an Increase in Phosphorylation of PAK1 Substrates**—Phosphorylation of Akt by PDK1 at threonine 308 increases the catalytic activity of Akt toward substrate (38).

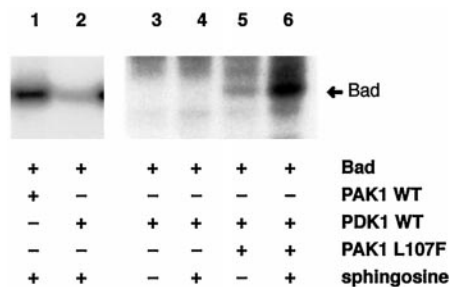


**FIG. 3. PDK1 phosphorylates PAK1 at threonine 423 and other sites.** A, immunoprecipitated Myc-PAK1 was incubated with Myc-PAK1 K299A or Myc-PAK1 K299A/T423A in the presence of 400  $\mu$ M sphingosine and 5  $\mu$ Ci of [ $\gamma$ - $^{32}$ P]ATP for 30 min at 30  $^{\circ}$ C. Samples were separated on a 6.5% SDS-polyacrylamide gel and transblotted to nitrocellulose, and detected by autoradiography. B, immunoprecipitated Myc-PDK1 was incubated with Myc-PAK1 K299A or Myc-PAK1 K299A/T423A in the presence of 400  $\mu$ M sphingosine and 30  $\mu$ Ci of [ $\gamma$ - $^{32}$ P]ATP for 30 min at 30  $^{\circ}$ C. Samples were separated on a 6.5% SDS-polyacrylamide gel and transblotted to nitrocellulose, and two-dimensional phosphopeptide mapping was performed as described under "Materials and Methods." The arrow represents a second PDK1 phosphorylation site on PAK1. C, Myc-PDK1 or Myc-PAK1 K299A were immunoprecipitated from lysates of COS-7 cells and incubated in an *in vitro* kinase assay in the presence or absence of sphingosine. Expression levels of Myc-PDK1 and Myc-PAK1 K299A were equal as detected by immunoblotting a portion of the total reaction mixture with the anti-Myc antibody (top panel). The remainder of the samples was immunoblotted with the activation loop antibody to detect phosphorylation at threonine 423.

Therefore, we postulated that phosphorylation of PAK1 at threonine 423 by PDK1 should result in an increased PAK1 activity toward substrate. Consistent with other reports, we found Bad, a regulator of apoptosis, was phosphorylated by PAK1 (Fig. 5, lane 1) (20, 21), but PDK1 was unable to phosphorylate Bad (Fig. 5, lane 2). Because sphingosine is an activator of both PAK1 and PDK1, it was necessary to uncouple sphingosine activation from either PAK1 or PDK1. Previously, it was shown that mutation of leucine to phenylalanine at position 107 in PAK1 disrupted the interaction of the autoinhibitory domain of PAK1 with the kinase domain (45), resulting in a protein that was not regulated by sphingosine. Although this construct has been reported to be constitutively active, we wanted to determine whether preincubation of PAK1 L107F with protein phosphatase 1 and 2A followed by incubation with sphingosine-stimulated PDK1 would increase phosphate incorporation into Bad. Pretreatment of PAK1 L107F with phosphatase resulted



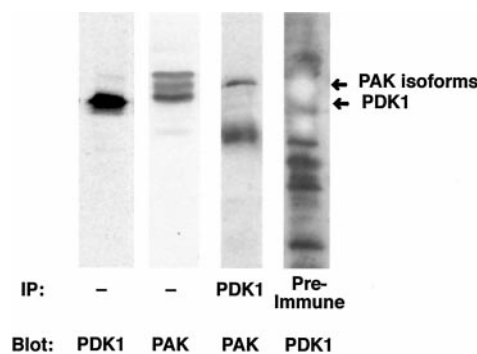
**FIG. 4. Substoichiometric amounts of PDK1 phosphorylate kinase subdomain VIII of PKC $\beta$ II and PAK1.** *In vitro* kinase assays compared the ability of increasing concentrations of PDK1 to phosphorylate His-PKC $\beta$ II K8 or GST-PAK1 K8 in the presence or absence of sphingosine. Increasing volumes (0, 1, 3, 10, 30, 50, and 100  $\mu$ l) of COS-7 lysate overexpressing Myc-PDK1 were immunoprecipitated and incubated with His-PKC $\beta$ II (1  $\mu$ g) or GST-PAK1 kinase subdomain VIII (1  $\mu$ g) in the absence (A) or presence (B) of sphingosine for 30 min at 30 °C. The results shown are representative of four experiments performed.



**FIG. 5. PAK1 activity is increased by phosphorylation at threonine 423 by PDK1.** Myc-PAK1 L107F was immunoprecipitated from lysates of COS-7 cells and incubated 30 min at 30 °C with PP1 (1 unit/ $\mu$ l) and PP2A (1 unit/ $\mu$ l). PAK1 L107F activity toward Bad (1  $\mu$ g) was measured by an *in vitro* kinase assay in the presence of 10  $\mu$ M microcystin, sphingosine, 5  $\mu$ Ci of [ $\gamma$ - $^{32}$ P]ATP in the presence or absence of PDK1. Samples were detected by autoradiography. The results shown are representative of two experiments performed.

in a strong decrease in the ability of this protein to phosphorylate Bad (data not shown). The addition of PDK1 in the presence or absence of sphingosine to the incubation mixtures containing Bad did not alter the phosphorylation state of Bad (Fig. 5, lanes 3 and 4). A strong increase in phosphate incorporation into Bad was seen when sphingosine stimulated PDK1 was added to phosphatase-treated PAK1 L107F (Fig. 5, lane 6). An increase in Bad phosphorylation was seen even with unstimulated PDK1, suggesting that the open conformation of PAK1 allows PDK1 access to the phosphorylation site (Fig. 5, lane 5).

**Interaction of PDK1 and PAK1 *In Vivo***—*In vitro* experiments clearly established that PDK1 phosphorylates PAK1 at threonine 423 and this results in an increase in PAK1 activity toward substrate. We next wanted to determine whether PAK1 and PDK1 could interact *in vivo*. Fig. 6 shows that HeLa cells contain significant endogenous amounts of both PDK1 (lane 1)

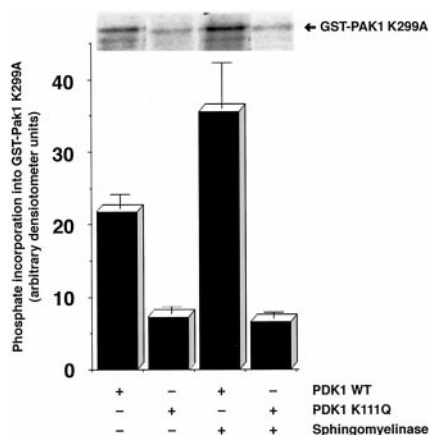


**FIG. 6. PDK1 and PAK1 interact *in vivo*.** HeLa lysate (75  $\mu$ g) was tested for endogenous levels of PDK1 (lane 1) or PAKs (lane 2). Subsequently, 200  $\mu$ g of HeLa lysate was incubated with anti-PDK1 (1:40; lane 3; Upstate Biotechnology, Inc., Lake Placid, NY) or PAK 2124 preimmune serum (1:40, lane 4), or for 6 h at 4 °C. Protein A (Repligen, Needham, MA) or protein G beads (Amersham Pharmacia Biotech, Uppsala, Sweden) were added to each mixture and incubated for another 1 h at 4 °C, then washed three times in detergent-containing buffer. Samples were separated on 10% SDS-polyacrylamide gels, transferred to nitrocellulose, and detected with anti-PDK1 antibodies (lanes 1 and 4), or anti-PAK antibodies (lanes 2 and 3).

and multiple PAK isoforms (lane 2). PDK1 was immunoprecipitated from HeLa cell lysates, and the immunoprecipitates probed with an antibody raised to PAK1 (lane 3). This antibody detects three PAK isoforms in HeLa lysates. One immunoreactive species, which appears to be PAK1 based on molecular weight, co-immunoprecipitated with PDK1. Similar results were seen when lysates were immunoprecipitated with PAK1 antibodies and immunoblotted with anti-PDK1. No PDK1 was detected when PAK preimmune serum was used for immunoprecipitation (lane 4). Similar results were obtained in COS-7 and 293 cells, suggesting that the interaction of PAK and PDK1 was not specific to HeLa cells. Although PAK1 and PDK1 co-immunoprecipitate *in vivo*, the interaction could not be established *in vitro* using purified PAK1 and PDK1, suggesting that the two proteins do not directly interact (data not shown).

**Elevated Sphingosine Levels Increase PDK1 Activity in COS-7 Cells**—*In vitro* experiments established that sphingosine activated PDK1 (26). We next wanted to determine whether transfected PDK1 could become activated in COS-7 cells treated for 30 min with 1 unit/ml sphingomyelinase, which catalyzes the breakdown of sphingomyelin to form ceramide and subsequently sphingosine. Serum-starved COS-7 cells expressing Myc-tagged PDK1 wild-type (WT) or catalytically inactive PDK1 (Myc PDK1 K111Q) were treated with or without sphingomyelinase. PDK1 was immunoprecipitated and *in vitro* kinase assays were performed using GST-PAK1 K299A as the substrate (Fig. 7). PDK1 WT phosphorylated significantly more GST-PAK1 K299A than PDK1 K111Q in the absence of stimulus, suggesting that the transfected PDK1 WT was partially activated. PDK1 WT transfected cells showed an increased level of GST-PAK1 K299A phosphorylation in cells incubated with sphingomyelinase, but not by PDK1 K111Q. This suggests that elevated sphingosine levels are able to activate PDK1 in cells. This is consistent with lipid stimulation of PDK1 *in vitro* and with our previous observation that elevated sphingosine levels increase phosphorylation of the activation loop of Akt by PDK1 (26).

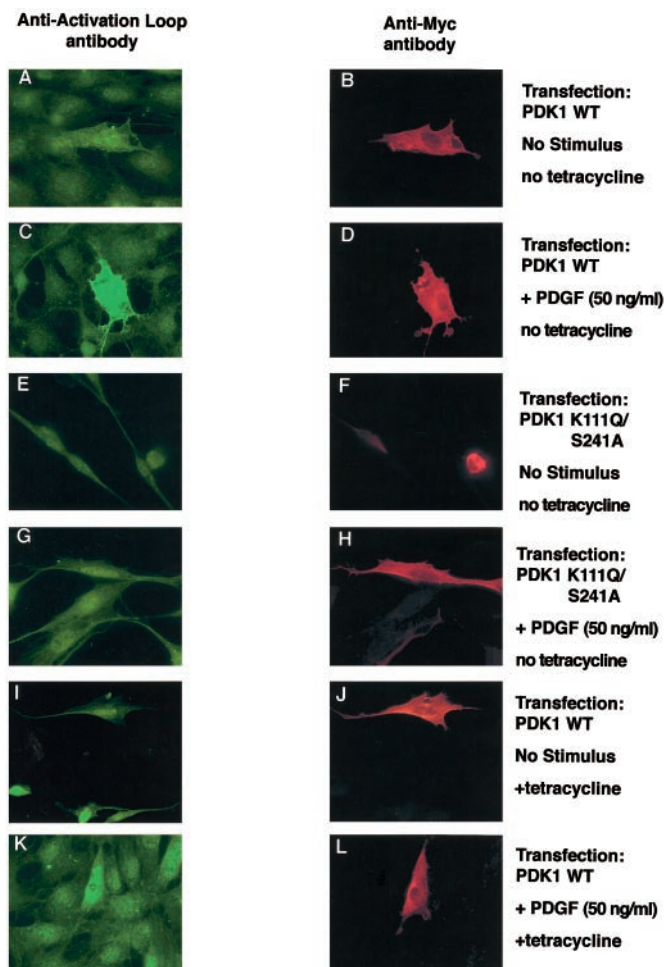
**PDK1 Phosphorylates Catalytically Inactive PAK1 in PDGF-stimulated Cells**—Immunofluorescence and biochemical studies were performed to determine whether PDK1 phosphorylated catalytically inactive PAK1 (PAK1 K299R) in response to PDGF, a stimulus previously shown to increase intracellular sphingosine levels (46–48). Duplicate sets of NIH3T3 fibroblasts containing a stably transfected hemagglutinin-tagged



**FIG. 7. Elevation of cellular sphingosine levels activates PDK1.** COS-7 cells were transfected with PDK1 WT or PDK1 K111Q and allowed to express for 24 h in DMEM with 10% FBS, after which the cells were starved for 18 h in DMEM alone. The cells were incubated alone or stimulated with 1 unit/ml sphingomyelinase for 30 min, and PDK1 was immunoprecipitated. *In vitro* kinase assays were performed on the immunoprecipitated samples in the presence of 1  $\mu$ g of GST-PAK1 K299A and [ $\gamma$ - $^{32}$ P]ATP. Data represent the mean  $\pm$  S.E. of three separate experiments. *Inset* shows a representative sample from this set of experiments.

PAK1 K299R vector, under the control of a tetracycline promoter (42), were mock transfected, or transfected with either wild-type PDK1 or catalytically-inactive PDK1 (PDK1 K111Q/S241A) (Fig. 8). Transfection of the cells did not change the uniform expression of PAK1 as detected by immunoblotting (Fig. 9, lower panel). Transfection of PDK1 WT into NIH3T3 cells in the absence of tetracycline (*i.e.* PAK1 K299R is expressed) resulted in a strong increase in activation loop antibody staining in PDGF-stimulated cells compared with unstimulated cells (Fig. 8, A–D). Transfection of catalytically inactive PDK1 (PDK1 K111Q/S241A) into NIH3T3 cells in the presence or absence of tetracycline did not alter the amount of activation loop antibody staining of NIH3T3 cells in the presence or absence of PDGF (Fig. 8, E–H). Staining with the activation loop antibody in mock transfected cells was minimal in both unstimulated and PDGF-stimulated cells. Transfection of PDK1 WT into NIH3T3 cells in the presence of tetracycline (*i.e.* no PAK1 K299R expression) did not result in an increase in activation loop antibody staining in the absence of PDGF. However, a slight increase in activation loop antibody staining in PDGF-stimulated cells was observed (Fig. 8, I–L). The increase in fluorescence seen in the presence of PDGF can most likely be attributed to an increase in phosphorylation of endogenous proteins such as protein kinase C, protein kinase A, and Akt. Taken together, these data suggest that PAK1 is specifically phosphorylated at threonine 423 in the presence of PDGF in a reaction that requires catalytically active PDK1.

We further analyzed the ability of transfected PDK1 to phosphorylate PAK1 K299R in NIH3T3 cells by *in vitro* kinase assay. For these studies, PDK1 WT, PDK1 K111Q, or a PDK1 construct with an inactivating PH domain mutation (PDK1 R474A) were transfected into NIH3T3 cells stably expressing PAK1 K299R. PDK1 R474A was used in these studies to determine whether the activation of PDK1 toward PAK1 was dependent upon PtdIns 3,4,5- $P_3$  production by PI 3-kinase. PAK1 was not phosphorylated in the absence of PDGF (lanes 1–4), but upon stimulation with PDGF there was an increase in PAK1 phosphorylation in cells transfected with PDK1 WT or PDK1 R474A (lanes 6 and 7), but not with vector alone or with catalytically inactive PDK1 K111Q (lanes 5 and 7). Pretreatment of cells for 15 min with wortmannin did not significantly lower the incorporation of labeled phosphate into PAK1. In

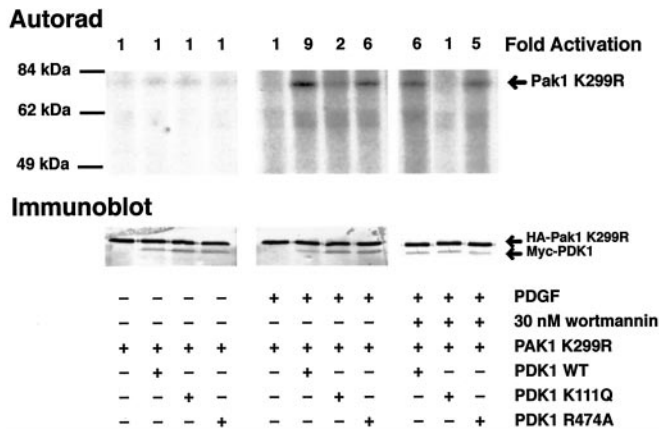


**FIG. 8. PDK1-dependent staining of the activation loop antibody into PDGF-stimulated NIH3T3 cells containing PAK1 K299R.** NIH3T3 cells were prepared for immunofluorescence as described under "Materials and Methods." Transfection of PDK1 WT into NIH3T3 cells in the absence of tetracycline (PAK1 K299R expressed) had little effect on the distribution of anti-activation loop antibody in unstimulated cells (panels A and B). However, when the cells were stimulated with PDGF, cells expressing Myc-PDK1 displayed a strong increase in anti-activation loop antibody staining (panels C and D). This increase was not observed when catalytically inactive PDK1 (PDK1 K111Q/S241A) was transfected into resting or PDGF-stimulated cells (panels E and F and panels G and H, respectively). Transfection of PDK1 in the presence of tetracycline (no PAK1 K299R expression) had little effect on the distribution of anti-activation loop antibody in unstimulated cells (panels I and J). Cells expressing Myc-PDK1 stimulated with PDGF, displayed an increased staining in anti-activation loop antibody (panels K and L). This most likely represents staining of endogenous proteins recognized by the activation loop antibody.

control experiments, expression of PDK1 WT in NIH3T3 stably transfected with an empty vector did not result in phosphorylation of a band corresponding to PAK1 (data not shown). These results indicate that both PDGF stimulation of NIH3T3 cells and a catalytically competent PDK1 are required for PAK1 to become phosphorylated. Both PDK1 R474A or wortmannin-treated cells expressing PDK1 WT were able to phosphorylate PAK1 as efficiently as PDK1 WT alone, indicating that neither phosphoinositides nor membrane localization are required for PAK1 phosphorylation by PDK1.

#### DISCUSSION

Until recently, the regulation of PAK activity has been thought to occur primarily through the binding of activated GTPases or sphingosine to the amino-terminal regulatory domain. Reports of Ste20 phosphorylation by Cln2/Cdc28 (24) and



**FIG. 9. Catalytically competent PDK1 constructs phosphorylate PAK1 K299R after PDGF stimulation.** The indicated PDK1 constructs were expressed in NIH3T3 cells containing a stably transfected PAK1 K299R construct under the control of an inducible tetracycline promoter. Identical sets of constructs were allowed to express in the presence of medium containing 10% fetal bovine serum (no tetracycline) for 36 h, serum-starved for 18 h, then stimulated for 10 min with 50 ng/ml PDGF. Cells were lysed, incubated with 5  $\mu$ Ci of [ $\gamma$ - $^{32}$ P]ATP for 30 min at 30 °C in the presence of 1  $\mu$ M microcystin, and then immunoprecipitated with a mixture of anti-PAK 2124 (1:40) and anti-hemagglutinin (1:100) antibodies, after which an *in vitro* kinase assay was performed (*upper panel*). Phosphorylation was quantitated by PhosphorImager analysis. A portion of the lysate was removed and immunoblotted to determine expression levels (*lower panel*). When added, wortmannin was at 30 nM. This experiment is representative of three performed.

PAK1 phosphorylation by p35/cdk5 (25) and Akt (21) raised the possibility that kinases also regulate PAK activity. In this study, we present data indicating that PDK1 phosphorylates threonine 423 in the activation loop of PAK1 in the presence of sphingosine. PDK1 interacts with PAK1 *in vivo* and phosphorylation of PAK1 by PDK1 increases PAK1 kinase activity. This process was not blocked by wortmannin, and occurred normally in a PDK1 mutant that cannot bind PtdIns lipids, indicating that the process is PtdIns 3-kinase independent. Although PAK1 and PDK1 interact *in vivo* in a variety of cell lines, we were unable to see a direct interaction between the two purified proteins. This is consistent with the observation that PAK1 does not contain a PDK1 binding motif (49). The results suggest that another protein(s) is required to link PAK1 and PDK1.

PDK1 phosphorylates PAK1 on threonine 423, an amino acid previously suggested to be an autophosphorylation site. Our laboratory and others have observed that recombinant or immunoprecipitated PAK1 is autophosphorylated at multiple sites, including threonine 423, when activated by Rho GTPases or sphingosine (4, 7). Our data support the hypothesis that threonine 423 is in fact an inefficient PAK1 autophosphorylation site, and that threonine 423 phosphorylation may result from the activation and phosphorylation of a localized PAK1 population by PDK1. Our data suggest that a fraction of PAK1 is phosphorylated at threonine 423 prior to stimulation. First, the activation loop antibody (Fig. 2A) recognized a portion of immunoprecipitated PAK1 that was phosphorylated at threonine 423 in unstimulated COS-7 cell lysates. No PAK1 was detected with this antibody after treatment with phosphatase, indicating that there was no cross-reactivity with dephosphorylated PAK1. Examination of threonine 423 by matrix-assisted laser desorption/ionization mass spectroscopy detected both phosphorylated and dephosphorylated forms of PAK1 in lysates from COS-7 cells, providing further evidence for a fraction of threonine 423 phosphorylated PAK1.<sup>3</sup> Second, threonine

423 falls within a PAK1 consensus phosphorylation sequence of Lys-Arg-X-(Ser/Thr) (49). Upon stimulation, immunoprecipitated PAK1 phosphorylated recombinant PAK1 K299A at threonine 423. This provides additional evidence that PAK1 can transphosphorylate other PAK1 molecules (39).

Activation of PDK1 and PtdIns 3-kinase activation have been linked (50). PDK1 has a carboxyl-terminal PH domain and has been shown to bind and localize to the membrane in response to increased levels of PtdIns 3,4,5- $P_3$ . However, PtdIns 3,4,5- $P_3$  has not been shown to activate PDK1 itself; instead, PtdIns 3,4,5- $P_3$  appears to act on PDK1 substrates. PAK1 K299A is phosphorylated in PDGF stimulated NIH3T3 cells transfected with wild-type PDK1 or PDK1 R474A, a mutation in the PH domain previously shown to prevent binding of PtdIns 3,4,5- $P_3$  (51). These data, coupled with the observation that *in vivo* PAK1 phosphorylation is independent of wortmannin in NIH3T3 cells, suggest that PDK1 can phosphorylate certain substrates in the absence of PtdIns 3-kinase activation.

PDK1 was shown to phosphorylate a catalytically inactive PAK1 in NIH3T3 cells when the cells were stimulated with PDGF, a stimulant previously shown to elevate intracellular sphingosine levels. Elevation of intracellular sphingosine by PDGF has been shown to modulate processes including  $Ca^{2+}$  mobilization (45), activation of phospholipase D (52), and tyrosine phosphorylation of p125<sup>FAK</sup> and paxillin (53). We propose that the elevation of intracellular sphingosine by PDGF can result in the activation of both PDK1 and PAK1 in a PtdIns 3-kinase independent manner. Although it is possible that PDK1 activation by PDGF may occur through additional mechanisms, our experiments with sphingomyelinase indicate that elevation of intracellular sphingosine represent at least one mechanism by which PDK1 becomes activated.

Until recently, PAK1 was viewed solely as an effector of Rho family GTPases. The demonstration that sphingosine also regulated PAK1 activity suggested that PAK1 might also act as a transducer of lipid signals. Both Cdc42-GTP $\gamma$ S and sphingosine disrupt the interaction between the AID and kinase domain of PAK1 (39). However, we observed that only sphingosine was effective for PDK1-induced phosphorylation of PAK1 (26). We propose that sphingosine disrupts the interaction of the AID and kinase domain such that the PAK1 activation loop is exposed to PDK1. The Cdc42-GTP $\gamma$ S-induced disruption of the PAK1 AID/kinase domain interaction does not lead to phosphorylation by PDK1; one possibility is that sphingosine-activated PAK1 may be regulated and signal differently than GTPase-stimulated PAK1. Another is that other unidentified kinases and/or regulatory phosphorylations may be required for GTPase signaling. However, we have previously shown that Cdc42-GTP $\gamma$ S, but not Cdc42-GDP, can down-regulate basal PDK1 activity (26). It is possible that activated GTPases may specifically inhibit PAK1 phosphorylation by PDK1. The interplay between PDK1 and Rho family GTPases is currently under investigation.

**Acknowledgments**—We thank Martine R. Wayne, Wesley D. Scott, Justine E. Lu, and Sara Ocon for technical assistance and Toni Lestell for secretarial assistance.

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<sup>3</sup> C. C. King and G. M. Bokoch, unpublished results.

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