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Sphingosine Is a Novel Activator of 3-Phosphoinositide-dependent Kinase 1*

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3-Phosphoinositide-dependent kinase 1 (PDK1) has previously been shown to phosphorylate the activation loop of several AGC kinase family members. In this study, we show that p21-activated kinase 1, the activity of which is regulated by the GTP-bound form of Cdc42 and Rac and by sphingosine, is phosphorylated by PDK1. Phosphorylation of p21-activated kinase 1 by PDK1 occurred only in the presence of sphingosine, which increased PDK1 autophosphorylation 25-fold. Sphingosine increased PDK1 autophosphorylation in a concentration-dependent manner and significantly increased phosphate incorporation into known PDK1 substrates. Studies on the lipid requirement for PDK1 activation found that both sphingosine isoforms and stearylamine also increased PDK1 autophosphorylation. However, C_{10}-sphingosine, octylamine, and stearic acid were unable to increase PDK1 autophosphorylation, indicating that both a positive charge and a lipid tail containing at least a C_{10}-carbon backbone were required for PDK1 activation. Three PDK1 autophosphorylation sites were identified after stimulation with sphingosine in a serine-rich region located between the kinase domain and the pleckstrin homology domain using two-dimensional phosphopeptide maps and matrix assisted laser desorption/ionization mass spectroscopy. Increased phosphorylation of endogenous Akt at threonine 308 was observed in COS-7 cells expressing wild type PDK1, but not catalytically inactive PDK1, when cellular sphingosine levels were elevated by treatment with sphingomyelinase. Sphingosine thus appears to be a true PDK1 activator.

3-Phosphoinositide-dependent kinase 1 (PDK1)[1] is a recently described kinase containing a pleckstrin homology domain (1). The pleckstrin homology domain has been demonstrated to bind the lipid products of the phosphatidylinositol 3-OH-kinase reaction, phosphatidylinositol 3,4 bisphosphate, and phosphatidylinositol 3,4,5 trisphosphate (PtdIns 3,4,5 P_3), with low nanomolar affinity, and it redistributes PDK1 to the membrane without increasing PDK1 autophosphorylation or incorporation of phosphate into substrate (2). PDK1 activity has been described as constitutive and has been demonstrated to phosphorylate a conserved threonine in kinase subdomain VIII of AGC family kinase family members, including Akt, $p70^{S6}$ kinase, protein kinase A (PKA), and a variety of PKC isoforms (1, 3–7). Phosphorylation of the conserved threonine by PDK1 has proven to be a crucial step in activating these enzymes (1).

p21 activated kinase 1 (PAK1) becomes active in the presence of either the GTP-bound form of the Rho family GTPases Cdc42 or Rac1, or the positively charged lipid sphingosine (8, 9). Activation of PAK1 is characterized by autophosphorylation of 7–8 serine/threonine amino acids and an increase in substrate phosphorylation (10). Although it is not an AGC kinase family member, PAK1 contains a conserved threonine in kinase subdomain VIII equivalent to the PDK1 phosphorylation site in AGC kinase family members. Although this site has been reported to be a PAK1 autophosphorylation site, we wanted to determine whether this site in a catalytically inactive PAK1 construct could serve as a PDK1 phosphorylation site.

In this study, we show that PDK1 phosphorylates PAK1 in the presence of sphingosine but not of Cdc42-GTPγS. Incubation of wild type PDK1, but not catalytically inactive PDK1, with sphingosine increased both autophosphorylation and the incorporation of phosphate into PDK1 substrates *in vitro* and *in vivo* dramatically, indicating that the enzymatic activity of PDK1 can be modified by sphingosine and related lipids. A closer examination of the lipid requirement for PDK1 activation indicates that a positively charged head group and a hydrophobic tail with at least a C_{10}-carbon backbone is required for activation of PDK1. Our data also identify a serine-rich region between the amino-terminal kinase domain and the carboxyl-terminal pleckstrin homology domain as the region of sphingosine-stimulated autophosphorylation.

EXPERIMENTAL PROCEDURES

Materials—All lipids used in these studies were from Sigma except *sn*-1,2, dipalmitoyl PtdIns bis-4,5 phosphate, *sn*-1,2, dipalmitoyl PtdIns bis-3,4 phosphate, and *sn*-1,2, dipalmitoyl PtdIns bis-3,4,5 phosphate, which were purchased from Matreya. The phospho-Akt threonine 308

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[1] The abbreviations used are: PDK1, 3-phosphoinositide-dependent kinase 1; PAK1, p21-activated kinase 1; GTPγS, guanosine 5′-3-*O*-(thio)triphosphate; PtdIns 3,4,5 P_3, phosphatidylinositol 3,4,5-trisphosphate; MALDI, matrix assisted laser desorption/ionization; MS, mass spectroscopy; PKA, protein kinase A; IGF-1, insulin-like growth factor-1; GST, glutathione *S*-transferase; Pipes, piperazine-*N*,*N*′-bis-(2-ethanesulfonic acid).

antibody was from New England Biolabs (Beverly, MA). All other reagents were of the highest grade available from Sigma.

cDNA Constructs and Cell Culture—PAK1 constructs were prepared in the pCMV6m vector as described previously (11). PDK1 constructs were prepared in the pCMV5m vector as described (3). Both vectors use a cytomegalovirus promoter and contain an amino-terminal 9E10 Myc epitope tag. Kinase subdomain VIII of PAK1 and Akt was amplified by polymerase chain reaction. (The primers for PAK1 kinase subdomain VIII were as follows: forward primer, 5′-CGG ATT TCT AAA ACG GAG CAC CAT GGT AAG-3′; reverse primer, 5′-GCC GGT CGA CTT ACA CAA CCT CTG GTG CCA TCC-3′. The primers for Akt kinase subdomain VIII were as follows: forward primer, 5′-GCC GCG GAT CCG AGG GGA TCA AGG ATG GT-3′; reverse primer, 5′ CGC CGG AAT TCG CCG TAG TCG TTG TCC TC-3′. Underlined base pairs indicate restriction sites.). The PAK1 kinase subdomain VIII polymerase chain reaction product was cut with *Bam*HI-*Sal*I and inserted into *Bam*HI-*Sal*I cut pGEX-2T vector (Amersham Pharmacia Biotech). The Akt kinase subdomain VIII polymerase chain reaction product was cut with *Bam*HI-*Eco*RI and inserted into *Bam*HI-*Eco*RI cut pGEX-4T3 vector (Amersham Pharmacia Biotech). The GST-PAK1 kinase subdomain VIII and GST-Akt kinase subdomain VIII were expressed in DH10B cells and purified according to the standard protocol (GST gene fusion system, 3rd edition, Amersham Pharmacia Biotech).

COS-7 cells were maintained in Dulbecco's modified Eagle's medium (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 μg/ml streptomycin at 37 °C in 10% CO_2. For transient transfection experiments, COS-7 cells (seeded at 1.2×10^6 cells/100-mm dish) were grown to 80–90% confluence and then incubated with 5 μg of the plasmid DNA using the LipofectAMINE transfection protocol (Life Technologies, Inc.). The cells were allowed to express the protein for 30–48 h posttransfection and then washed with phosphate-buffered saline and lysed, or they were starved and incubated with Me_2SO, sphingomyelinase (1 unit/ml), or insulin-like growth factor-1 (IGF-1) (Life Technologies, Inc.). Lysis buffer (150 mm NaCl, 25 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.1 mM EGTA, 5 mM $MgCl_2$, 1 mM dithiothreitol, 10% glycerol, 2% Nonidet P-40, 50 IU/ml aprotinin, 2 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, and 10 μM okadaic acid) 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.

Kinase Assays—COS-7 lysates containing various PDK1 or PAK1 constructs were incubated with the anti-Myc (9E10) monoclonal antibody in binding buffer (100 mM KCl, 3 mM NaCl, 3.5 mM $MgCl_2$, 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 a 1:1 slurry of 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 P-40, three times in binding buffer alone, and three times in kinase buffer (50 mM Hepes, pH 7.5, 10 mM $MgCl_2$, 2 mM $MnCl_2$, and 0.2 mM dithiothreitol). The immobilized PDK1 and/or PAK1 were incubated in kinase buffer containing 0.5 μCi of [^{32}P]ATP in a 20 μM ATP solution for 30 min at 30 °C. A 4× solution of Laemmli sample buffer was added to stop the reaction, and the samples were boiled for 5 min. Proteins were separated on SDS-polyacrylamide gels and either transferred to nitrocellulose for immunoblotting or dried. Radioactive bands were detected by autoradiography using Eastman Kodak Co. AR film.

Lipid Preparation—Lipids, prepared as 15 mM stocks in $CHCl_3$ or ethanol, were dried under nitrogen and resuspended by sonication in 25 mM Tris-HCl, pH 7.5. Mixed lipid vesicles were prepared by adding the appropriate amount of lipid to a single tube prior to drying the sample under nitrogen and sonication.

MALDI Mass Spectroscopy—Immunoprecipitated PDK1 was incubated alone or with sphingosine (400 μM) and ATP for 30 min at 30 °C. Excised gel pieces containing autophosphorylated PDK1 were subjected to an in-gel trypsin digestion as described in Ref. 12. The PDK1 peptides were eluted from the gel with acetonitrile and lyophilized. Aliquots of 1 μl of the peptides were mixed with 1 μl of the matrix, a saturated solution of 2,5-dihydroxybenzoic acid, on the sample plate and air dried prior to MS analysis. Samples were analyzed in a 1.14-m MALDI/time of flight mass spectrometer with dynamic extraction (Dynamo ThermoBioAnalysis, BioMolecular Instruments, Santa Fe, NM). Peptides were analyzed using the ProFound data base at Rockefeller University. Calibration was external and based on a mixture of angiotension (MH^+ = 1297.5), renin tetradecapeptide (MH^+ = 1760.0), and insulin (MH^+ = 5734.6). Spectra shown are representative of four obtained.

TABLE I
Sequence alignment of PAK1 kinase subdomian VIII with known PDK1 substrates

The phosphorylated threonine residue is shown in boldface type.

Kinase	Amino acid sequence																	
	420																	436
PAK-1	K	R	S	**T**	M	V	G	T	P	Y	W	M	A	P	E	V	V	
PKA	R	T	W	**T**	L	C	G	T	P	E	Y	L	A	P	E	I	I	
PKCβII	T	T	K	**T**	F	C	G	T	P	D	Y	I	A	P	E	I	I	
AKT	T	M	K	**T**	F	C	G	T	P	E	Y	L	A	P	E	V	L	
P70^{S6}	V	T	H	**T**	F	C	G	T	I	E	Y	M	A	P	E	I	L	

RESULTS

Sphingosine Increases PDK1 Autophosphorylation and Substrate Phosphorylation—PDK1 phosphorylates a conserved threonine residue in kinase subdomain VIII of AGC kinase family members. Although PAK1 is not an AGC kinase family member, it too contains the conserved threonine at position 423 in kinase subdomain VIII and has a high degree of sequence similarity in the carboxyl-terminal flanking region (Table I). We wanted to determine whether PDK1 could phosphorylate PAK1. Fig. 1 shows that catalytically inactive PAK1 (PAK1 K299A) did not autophosphorylate alone or in the presence of the known PAK1 activators Cdc42-GTPγS or sphingosine. Wild type PDK1 incorporated a basal level of [^{32}P]phosphate, consistent with previous reports (3, 13), but was unable to phosphorylate PAK1 K299A. Incubation of PDK1 and PAK1 K299A with sphingosine increased PDK1 autophosphorylation 25-fold over incubation with [γ-^{32}P]ATP alone, as measured by PhosphorImager analysis. An increase in PDK1 autophosphorylation was accompanied by a band shift in the immunoblot (Fig. 1, *Myc antibody*). Incubation of PDK1 with sphingosine also led to a significant incorporation of phosphate into PAK1 K299A. Substoichiometric amounts of PDK1 have subsequently been shown both *in vitro* and *in vivo* to phosphorylate PAK1 at threonine 423 in kinase subdomain VIII.[2] Cdc42-GDP had no effect on PDK1 autophosphorylation, but interestingly, autophosphorylation was decreased significantly in the presence of Cdc42-GTPγS, suggesting that PDK1 activity may be negatively regulated by Cdc42-GTPγS. PDK1 incubated with GDP- or GTPγS-loaded Cdc42 was unable to phosphorylate PAK1 K299A. The significance of the effect of Cdc42 on PDK1 activity is further strengthened by the observation that the two proteins physically associate.[3]

The ability of sphingosine to stimulate PDK1 autophosphorylation and substrate phosphorylation was further examined. Fig. 2*A* shows the results of incubating increasing concentrations of sphingosine with immunoprecipitated PDK1 in the presence of substrate (GST-Akt kinase subdomain VIII). Low sphingosine concentrations were sufficient to induce a 2–4-fold increase in both PDK1 autophosphorylation and substrate phosphorylation. PDK1 autophosphorylation reached a maximum at approximately 100 μM sphingosine and had an apparent ED_{50} of 20 μM. Autophosphorylation of PDK1 in the presence of sphingosine was unchanged in the presence or absence of substrate (data not shown). Substrate phosphorylation by PDK1 also increased in the presence of increasing sphingosine concentrations and reached a maximum at 100 μM. These data suggest that sphingosine has a direct effect on PDK1. We next wanted to determine whether sphingosine-stimulated PDK1 increased phosphate incorporation into a variety of PDK1 substrates. PDK1 has previously been shown to phosphorylate threonine 197 in the activation loop of PKA, threonine 500 in the activation loop of protein kinase CβII (PKCβII), and thre-

[2] C. C. King and G. M. Bokoch, manuscript in preparation.
[3] C. C. King and G. M. Bokoch, unpublished observations.

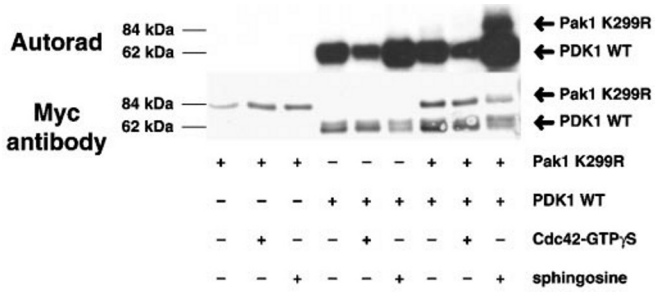

FIG. 1. **PDK1 WT phosphorylates PAK1 K299A in the presence of sphingosine.** COS-7 lysates overexpressing Myc-tagged PAK1 K299A or PDK1 were incubated overnight at 4 °C with the anti-Myc (9E10) monoclonal antibody, followed by a 1-h incubation with a 1:1 slurry of bovine serum albumin (Sigma)-coated 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 P-40, three times in binding buffer alone, and three times in kinase buffer. *In vitro* kinase activity of PAK1 K299A or PDK1 immunoprecipitates was measured in kinase buffer containing 20 μM ATP and 5 μCi of [γ-^{32}P]ATP (ICN) for 30 min at 30 °C. When added, Cdc42-GTPγS was at 1.5 μg, and sphingosine was 400 μM. The samples were separated on 12% SDS-polyacrylamide gels and then transferred to nitrocellulose for immunoblotting and autoradiography. Radiolabeled phosphate incorporation was measured by PhosphorImager analysis (Molecular Dynamics, Inc., Sunnyvale, CA). Results shown are representative of five separate experiments. The *bottom blot* shows the levels of PAK1 K299A or PDK1 WT as determined by immunoblotting with the anti-Myc monoclonal antibody.

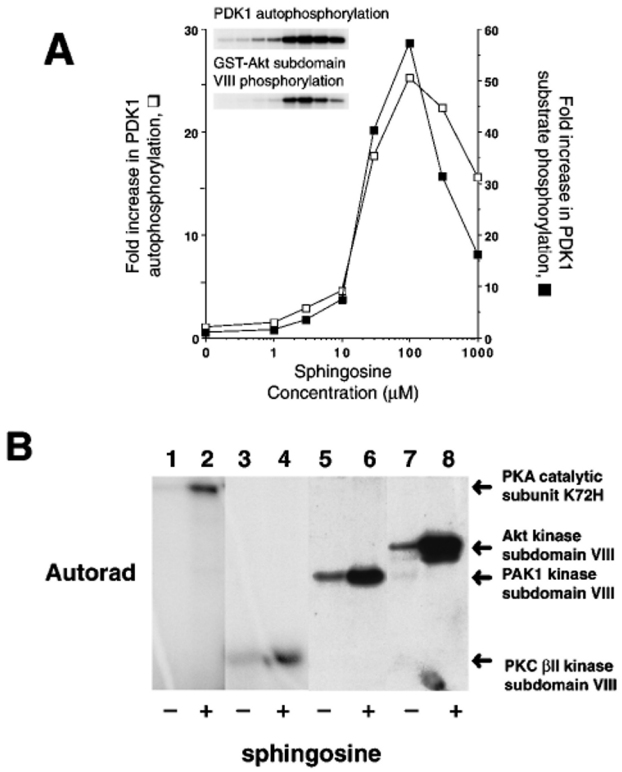

FIG. 2. **Sphingosine specifically increases phosphate incorporation into PDK1 and PDK1 substrates.** *A,* autophosphorylation of immunoprecipitated Myc-PDK1 was measured in the presence of increasing sphingosine concentrations and 1 μg of GST-Akt kinase subdomain VIII by an *in vitro* kinase assay. The data shown are representative of three separate experiments as measured by PhosphorImager analysis. *Inset,* autoradiographs of PDK1 autophosphorylation (*top*) and phosphorylation of GST-Akt kinase subdomain VIII alone (*bottom*) or in the presence of 1, 3, 10, 30, 100, 300, or 1000 μM sphingosine (*lanes* from *left* to *right*). *B,* immunoprecipitated Myc-PDK1 was incubated with approximately 1 μg of PKA (K72H), 1 μg of His-PKCβII activation loop, 1 μg of GST-PAK1 kinase subdomain VIII, or 1 μg of GST-Akt kinase subdomain VIII in the presence or absence of 400 μM sphingosine in an *in vitro* kinase assay. Samples were then separated on a 13% SDS-polyacrylamide gel, dried, and measured by PhosphorImager analysis.

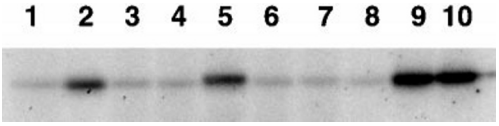

FIG. 3. **PDK1 activation by lipids requires a positive charge and a hydrophobic tail.** *In vitro* kinase assays were performed on immunoprecipitated Myc-PDK1 alone (*lane 1*) or in the presence of 400 μM sphingosine (*lane 2*), sphingosine 1-phosphate (*lane 3*), C_{10}-sphingosine (*lane 4*), stearylamine (*lane 5*), stearic acid (*lane 6*), or octylamine (*lane 7*). When added, phospholipid vesicles contained 100 μM phosphatidylcholine and 100 μM phosphatidylserine alone or 10 μM *sn*-1,2, dipalmitoyl PtdIns bis-4,5-phosphate, 10 μM *sn*-1,2, dipalmitoyl PtdIns bis-3,4-phosphate or 10 μM *sn*-1,2-dipalmitoyl PtdIns tris-3,4,5-phosphate (*lane 8*), 10 μM *sn*-1,2-dipalmitoyl PtdIns tris-3,4,5-phosphate and sphingosine (*lane 9*) or sphingosine alone (*lane 10*). This experiment is representative of at least three performed.

onine 308 in the activation loop of Akt (1, 5, 6). In Fig. 2*B,* immunoprecipitated PDK1 WT was incubated in the presence or absence of sphingosine with either the catalytically inactive kinase domain of PKA (PKA-K72H) (*lanes 1* and *2*), the activation loop of PKCβII (*lanes 3* and *4*), the activation loop of PAK (*lanes 5* and *6*) or the activation loop of Akt (*lanes 7* and *8*). A significant increase in [^{32}P]phosphate incorporation into PKA K72H and into the activation loops of PKCβII, PAK1, and Akt was observed in the presence of PDK1 and sphingosine over samples incubated with PDK1 alone, indicating that sphingosine serves as a true PDK1 activator. To confirm PDK1 phosphorylation of PKCβII was due to phosphate incorporation at threonine 500 in the activation loop and not some other serine or threonine, we used an antibody raised specifically to phosphothreonine at amino acid residue 500 in the activation loop of PKCβII. This site is analogous to threonine 197 in PKA and threonine 423 in PAK1. Previously (5), this antibody was shown to cross-react with phosphothreonine 197 in PKA. Immunoblots with this antibody showed an increase in phosphate incorporation specifically at the activation loop threonine in both PKCβII and PKA after sphingosine stimulation of PDK1. Additionally, we have established that this antibody exclusively recognizes phosphothreonine 423 in PAK1.[3] A commercially available phospho-Akt threonine 308 antibody was used to confirm that PDK1 specifically phosphorylated the activation loop threonine (data not shown).

To better understand the role of sphingosine in PDK1 activation, we studied sphingosine and related sphingolipids for their ability to activate PDK1. The data in Fig. 3 and Table II show the effect of sphingosine and a variety of sphingosine-related compounds on PDK1 autophosphorylation. As previously shown, PDK1 alone incorporated a small amount of [^{32}P]phosphate. Phosphate incorporation was greatly enhanced in the presence of sphingosine, but not in the presence of C_2- or C_8-ceramide or sphingosine 1-phosphate. In both of these compounds, the free amine group is either conjugated to another lipid (ceramide) or the charge is offset by the presence of a negatively charged phosphate group (sphingosine 1-phosphate). A variety of sphingosine-derived lipids, each of which contains a positive charge in the head group, were able to stimulate PDK1 autophosphorylation (Table II), suggesting that the positive charge and not the stereochemistry play an important role in sphingosine activation of PDK1. Stearylamine, but not stearic acid, was also as efficient as sphingosine at activating PDK1 autophosphorylation, providing further evidence that a positive charge was important for PDK1 activation. Interestingly, octylamine, a positively charged lipid that contains a C_8-carbon backbone, was not able to activate PDK1 autophosphorylation. Furthermore, a C_{10}-sphingosine molecule was also

TABLE II
Effect of lipids on PDK1 autophosphorylation

Lipid	Increase in PDK1 Autophosphorylation[a]	*n*
Sphingosine	Yes	35
C_{10}-sphingosine	No	3
Sphingosine-1-phosphate	No	7
C_2-ceramide	No	5
C_8-ceramide	No	5
D-Erythro-sphingosine	Yes	3
L-Erythro-sphingosine	Yes	3
D-Threo-sphingosine	Yes	3
D-Threo-sphingosine	Yes	3
D,L-Dihydrosphingosine	Yes	3
D,L-Erythro-dihydrosphingosine	Yes	3
D,L-Threo-dihydrosphingosine	Yes	3
Phytosphingosine	Yes	3
Octylamine	No	7
Stearylamine	Yes	7
Stearic acid	No	7
Ptd Cho/Ser[b]	No	3
Ptd Cho/Ser + sphingosine	Yes	3
PtdIns 3,4 P_2	No	4
PtdIns 4,5 P_2	No	4
PtdIns 3,4,5 P_3	No	4
PtdIns 3,4,5 P_3 + sphingosine	Yes	3

[a] An increase in PDK1 autophosphorylation was defined as an 8-fold increase in autophosphorylation over PDK1 autophosphorylation in the presence of ATP alone as measured by PhosphorImager analysis.
[b] Ptd Cho/Ser, phosphatidylcholine and serine.

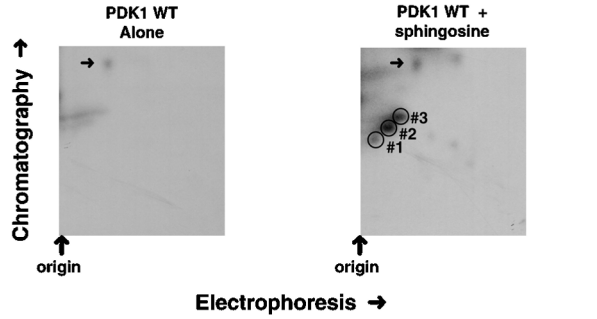

FIG. 4. **PDK1 autophosphorylates on three novel sites in the presence of sphingosine.** Immunoprecipitated PDK1 was incubated alone or sphingosine (400 μM) and [γ-^{32}P]ATP for 30 min at 30 °C. Autophosphorylated PDK1 was separated on a 6.5% polyacrylamide gel, transferred to nitrocellulose, and detected 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.* (24). 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 coated cellulose 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. ^{32}P-Labeled tryptic peptides were detected by autoradiography on Kodak X-AR film for up to 12 h.

unable to induce PDK1 autophosphorylation, suggesting that a hydrophobic tail was also required for PDK1 activation. Catalytically inactive PDK1 (PDK1 K111Q) did not incorporate phosphate alone or in the presence of sphingosine or any other sphingosine-derived lipids. This observation virtually eliminates the possibility that a sphingosine-activated kinase was co-immunoprecipitated with PDK1 and was responsible for the incorporation of phosphate into PDK1.

PDK1 contains a carboxyl-terminal pleckstrin homology domain that has been shown to bind PtdIns 3,4,5 P_3. Binding has been shown to localize PDK1 to the membrane, but does not activate the enzyme (2). PtdIns 3,4,5 P_3-dependent activation of PDK1 has recently been described in the presence of a PDK1-interacting factor (14). We wanted to test whether sphingosine stimulation of PDK1 in the presence of PtdIns 3,4,5 P_3-containing lipid vesicles could enhance PDK1 autophosphorylation (Fig. 3 and Table II). Consistent with previously reported data, PtdIns 3,4,5 P_3-containing vesicles were unable to induce PDK1 autophosphorylation or substrate phosphorylation over incubation with ATP alone. Phosphatidylserine or phosphatidylcholine lipid vesicles containing sphingosine in the presence or absence of PtdIns 3,4,5 P_3 stimulated PDK1 autophosphorylation consistently more than sphingosine alone. The increase in PDK1 autophosphorylation was modest (~2-fold), suggesting that there is little or no added effect of PIP_3 on PDK1 activation by sphingosine.

Sphingosine-stimulated PDK1 Autophosphorylates in a Serine-rich Region—We used two-dimensional phosphopeptide mapping and MALDI mass spectroscopy to identify the sites of sphingosine-stimulated PDK1 autophosphorylation. Two-dimensional phosphopeptide maps of PDK1 were performed alone or in the presence of sphingosine. PDK1 incubated alone incorporated phosphate at one site (Fig. 4, *left panel, arrow*), consistent with the basal phosphorylation previously observed. PDK1 incubated with sphingosine resulted in the appearance of three new phosphorylation sites. The diagonal arrangement of the spots suggested a single peptide was phosphorylated to various extents. MALDI mass spectroscopy was used to determine which peptide contained the PDK1 autophosphorylation sites. Fig. 5 shows the mass spectrograms of PDK1 with ATP alone (*top panel*) or with sphingosine and ATP (*bottom panel*). Using this method, we identified over 85% of expected PDK1 tryptic fragments. The undetected peptides primarily consisted of tryptic fragments with a mass less than 820 Da. It was unlikely that any of these peptides contained the sites of autophosphorylation because none of these peptides contained three or more serine and/or threonine amino acids. The peaks detected in the presence or absence of sphingosine were identical, except for the peak at 5375 Da. Within the sequence of PDK1, there is one tryptic fragment positioned between the amino-terminal kinase domain and the carboxyl-terminal pleckstrin homology domain with a mass of 5375 Da that is serine-rich and contains 13 possible phosphorylation sites. In the presence of sphingosine, three peaks appear, each separated by 80 Da, the mass of a phosphate group. No other reasonable combination of differential tryptic cleavages produced peptides within 20 Da of this peak, providing further evidence supporting this peptide as the site of autophosphorylation. These data strongly indicate that the serine-rich region located between the amino-terminal kinase domain and the carboxyl-terminal pleckstrin homology domain is the site of sphingosine-stimulated PDK1 autophosphorylation. Attempts to determine which amino acids are autophosphorylated by MS/MS analysis were unsuccessful, due to the large mass of the peptide to be analyzed. Mutational analysis and MS/MS on enzymatically digested PDK1 are currently being pursued.

Elevated Intracellular Sphingosine Levels Increase PDK1 Activity in Vivo—Our data indicated that sphingosine, at concentrations between 1 and 1000 μM, increased PDK1 autophosphorylation *in vitro* (Fig. 2). We wanted to determine whether elevation of intracellular sphingosine levels were sufficient to activate PDK1 substrate phosphorylation *in vivo*. Previously, transfected PDK1 was shown to increase Akt activity toward substrates by specifically phosphorylating threonine 308 in the activation loop (15). We examined the phosphorylation state of threonine 308 in endogenous Akt after treatment of cells with

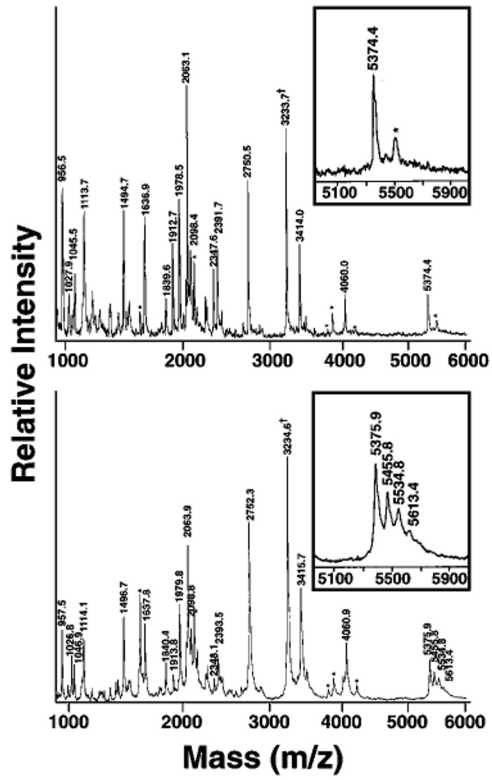

FIG. 5. **MALDI mass spectroscopy indicates that PDK1 autophosphorylation occurs in a serine-rich region.** Immunoprecipitated PDK1 was incubated alone or with sphingosine (400 μM) and ATP for 30 min at 30 °C. Excised gel pieces containing autophosphorylated PDK1 were subjected to an in-gel trypsin digestion as described under "Experimental Procedures." Labeled peaks represent the average of all peaks from four experiments. Peaks marked with an *asterisk* appeared in at least one spectra but did not align with calculated PDK1 masses. The peak marked with a *cross* is the tryptic digest representing the carboxyl-terminal amino acids of the Myc tag and the amino-terminal amino acids of PDK1. The region encompassing the mass range of 5100–5900 Da represented the only significant difference in the spectra in the presence or absence of sphingosine (*top* and *bottom insets*). In the *top inset,* only one peak corresponding to a PDK1 mass could be identified. The other peak (*) in the inset did not correspond to any PDK1 mass. In the *bottom inset,* three new peaks appeared after sphingosine stimulation, each separated from the original peptide (5375.9) by a multiple of 80 Da.

a known PDK1 activator, IGF-1 (16), or with sphingomyelinase, an enzyme that has previously been shown to increase intracellular sphingosine levels (9). Serum-starved COS-7cells, expressing either PDK1 WT or the catalytically inactive PDK1 (PDK1 K111Q) were either left untreated or stimulated with sphingomyelinase or IGF-1. Fig. 6 (*top panel*) shows a Western blot of Akt phosphorylation at threonine 308. Unstimulated cells incorporate a basal level of phosphate at threonine 308. Incubation of cells with sphingomyelinase increased threonine 308 phosphorylation 4-fold in cells transfected with PDK1 WT, compared with 6-fold in cells stimulated with IGF-1. Cells transfected with PDK1 K111Q also showed a basal incorporation of phosphate at threonine 308 in unstimulated cells. Phosphorylation was not increased in cells treated with sphingomyelinase and was increased to a reduced extent (~2-fold) in cells stimulated with IGF-1. Fig. 6 (*middle panel*) shows a Western blot of endogenous Akt in the PDK1-transfected cells in the presence or absence of stimuli, indicating protein levels were identical in each condition. Fig. 6 (*bottom panel*) shows a Western blot of Myc-tagged PDK1 in each of the reactions, indicating equal expression under each condition. Taken together, these data indicate that elevation of intracellular sphingosine levels is sufficient to stimulate PDK1 to phosphorylate threonine 308 of Akt.

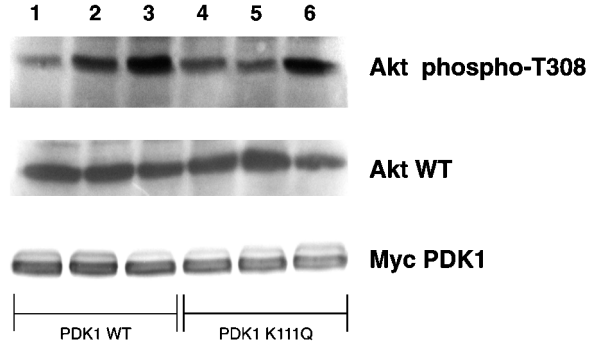

FIG. 6. **Elevated intracellular sphingosine levels increase endogenous Akt threonine 308 phosphorylation in a PDK1-dependent manner.** COS-7 were transfected with PDK1 WT (*lanes 1–3*) or PDK1 K111Q (*lanes 4–6*), allowed to express for 30 h, and then serum starved for 18 h. *Top panel,* cells were incubated with Me_2SO (*lanes 1* and *4*), with sphingomyelinase (1 unit/ml) (*lanes 2* and *5*) for 30 min, or IGF-1 (100 ng/ml) (*lanes 3* and *6*) for 10 min at 37 °C. The protein in the total cell lysate was quantitated by BCA assay (Pierce, Rockford, IL), and equal portions were separated on 12% SDS-polyacrylamide gels and blotted with phospho-Akt Threonine 308 antibodies (Upstate Biochemicals). *Middle panel,* immunoblot showing equal amounts of endogenous Akt detected in the COS-7 cells under each experimental condition. *Bottom panel,* immunoblot showing equal expression of PDK1 WT and PDK1 K111Q in transfected cells.

DISCUSSION

Until recently, PDK1 activity was thought to be constitutive. However, it was recently shown that PDK1 activity could be stimulated modestly (~2-fold) by PtdIns 3,4,5 P_3 in the presence of PDK1-interacting factor (14). In this study, we show that sphingosine acts independently of PtdIns 3,4,5 P_3 to stimulate PDK1 autophosphorylation 25-fold and increases phosphate incorporation into known and novel PDK1 substrates. These findings suggest that regulatory events other than localization of PDK1 and its substrates by PtdIns 3,4,5 P_3 may be required for efficient signal transduction. Localization of PDK1 to the membranes by increased PtdIns 3,4,5 P_3 levels is an important mechanism for bringing the enzyme in proximity to substrates. In this study, we show that increased activity of PDK1 in the absence of localization also contributes to phosphorylation of substrate. Overexpression of wild type or catalytically inactive (K111Q) PDK1 results in a ubiquitous distribution of the protein in COS-7 cells, as determined by immunofluorescence, probably placing potential substrates within close proximity to the expressed PDK1. Treatment of these cells with sphingomyelinase increases intracellular sphingosine levels but not PtdIns 3,4,5 P_3 levels. Under these conditions, activation of PDK1 and phosphorylation of Akt at threonine 308 are observed.

Although we showed that increases in intracellular sphingosine levels can result in the phosphorylation of Akt in a PDK1-dependent manner, the question remains whether these sphingosine levels can be reached during cellular activation. Resting intracellular sphingosine levels have been estimated to be in the low micromolar range but could be much higher in microdomains within the cell or after a stimulatory event (17). Elevation of intracellular sphingosine levels were shown to increase endogenous Akt phosphorylation at threonine 308 by a process that requires wild type PDK1 but not catalytically inactive PDK1. These data suggest that intracellular concentrations of sphingosine generated in response to stimuli are sufficient to activate PDK1. Previous work in our laboratory (9) has shown that sphingosine levels increase significantly upon addition of sphingomyelinase or other sphingosine elevating agents in COS-7 cells. Other studies have implicated elevated intracellular sphingosine levels on activation of phosphatidylinositol

4-kinase (18) and other unidentified kinases (19), as well as the inhibition of protein kinase C (20). The levels of sphingosine required for these events to occur are in the same range as the levels required for PDK1 activation.

During the preparation of this report, Casamayor *et al.* (21) identified five PDK1 autophosphorylation sites. One serine residue (serine 241), the activation loop serine, was identified as a constitutively phosphorylated site. Our two-dimensional phosphopeptide maps also identify a constitutively phosphorylated residue (Fig. 4, *arrow*) that may represent this site. Casameyer *et al.* (21) also identified two autophosphorylation sites within the serine-rich region (serine 393 and 396). Our data clearly show three autophosphorylation sites within this region, suggesting that autophosphorylation of PDK1 after stimulation with sphingosine results in the appearance of at least one novel autophosphorylation site. It is unclear at this time whether the autophosphorylation sites identified by Casamayor *et al.* (21) differ from the sites we saw after stimulation. Studies are currently under way to determine which sites within this region are the sites of autophosphorylation.

Based on the data presented here, localization and activation of PDK1 may require two separate lipid signals: PtdIns 3,4,5 P_3, which is necessary for localization of PDK1 to membranes, where it colocalizes with substrates, and sphingosine, which is required for full activation of the enzyme. Such regulation might be pertinent during signal transduction through growth factor receptors, such as the platelet-derived growth factor receptor. Platelet-derived growth factor is known to up-regulate many different signal transduction pathways, including synthesis of PtdIns 3,4,5 P_3 through phosphatidylinositol 3-OH-kinase (22) and elevation of intracellular sphingosine (23). It is possible that coordination of these two lipid signals in cells is required for maximal activity of PDK1 toward substrates.

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