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 isomers and stearylamine also increased PDK1 autophosphorylation. However, C10-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 C10-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) 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 3,4,5 bisphosphate, and phosphatidylinositol 3,4,5 trisphosphate (PtdIns 3,4,5 P3), 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, p70S6 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-GTPyS. 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 C10-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 phosphate; 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 were 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 GTG AAG-3'; reverse primer, 5'-GCC GTG CCT ACG CAA CCA CTT GTG TTC CCA TCC-3'. The primers for Akt kinase subdomain VIII were as follows: forward primer, 5'-GCC GTG CCT AGG GGA TCA AGG GT-3'; reverse primer, 5'-GGC CAG TCG CCG TAG TCG TTG GCC TCC-3'. Underlined base pairs indicate restriction sites.) The PAK1 kinase subdomain VIII polymerase chain reaction product was cut with BamHI-SalI and inserted into BamHI-SalI cut pGEX-2T vector (Amerham Pharmacia Biotech). The Akt kinase subdomain VIII polymerase chain reaction product was cut with BamHI-EcoRI and inserted into BamHI-EcoRI cut pGEX-T3 vector (Amerham 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, Amerham Pharmacia Biotech).

COS-7 cells were maintained in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) containing 10% fetal bovine 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% CO2. For transient transfection experiments, COS-7 cells (seeded at 1.2 × 105 cells/100-mm dish) were harvested and lysed, or they were starved and incubated with Me2SO, or they were starved and incubated with Me2SO, saline and lysed, or they were starved and incubated with Me2SO, or they were starved and incubated with Me2SO, saline and lysed, or they were starved and incubated with Me2SO, or they were starved and incubated with Me2SO, saline and lysed, or they were starved and incubated with Me2SO, or they were starved and incubated with Me2SO, saline and lysed, or they were starved and incubated with Me2SO, or they were starved and incubated with Me2SO, saline and lysed, or they were starved and incubated with Me2SO, or they were starved and incubated with Me2SO, saline and lysed, or they were starved and incubated with Me2SO, or they were starved and incubated with Me2SO, saline and lysed, or they were starved and incubated with Me2SO, or they were starved and incubated with Me2SO, saline and lysed, or they were starved and incubated with Me2SO, or they were starved and incubated with Me2SO, saline and lysed, or they were starved and incubated with Me2SO, or they were starved and incubated with Me2SO, saline and lysed, or they were starved and incubated with Me2SO, or they were starved and incubated with Me2SO, saline and lysed, or they were starved and incubated with Me2SO, or they were starved and incubated with Me2SO, saline and lysed, or they were starved and incubated with Me2SO, or they were starved and incubated with Me2SO, saline and lysed, or they were starved and incubated with Me2SO, or they were starved and incubated with Me2SO, saline and lysed, or they were starved and incubated with Me2SO, or they were starved and incubated with Me2SO, saline and lysed, or they were starved and incubated with Me2SO, or they were starved and incubated with Me2SO, saline and lysed, or they were starved and incubated with Me2SO, or they were starved and incubated with Me2SO, saline and lysed, or they were starved and incubated with Me2SO, or they were starved and incubated with Me2SO, saline and lysed, or they were starved and incubated with Me2SO, or they were starved and incubated with Me2SO, saline and lysed, or they were starved and incubated with Me2SO, or they were starved and incubated with Me2SO, saline and lysed, or they were starved and incubated with Me2SO, or they were starved and incubated with Me2SO, saline and lysed, or they were starved and incubated with Me2SO, or they were starved and incubated with Me2SO, saline and lysed, or they were starved and incubated with Me2SO, or they were starved and incubated with Me2SO, saline and lysed, or they were starved and incubated with Me2SO, or they were starved and incubated with Me2SO, saline and lysed, or they were starved and incubated with Me2SO, or they were starved and incubated with Me2SO, saline and lysed, or they were starved and incubated with Me2SO, or they were starved and incubated with Me2SO, saline and lysed, or they were starved and incubated with Me2SO, or they were starved and incubated with Me2SO, saline and lysed, or they were starved and incubated with Me2SO, or they were starved and incubated with Me2SO, saline and lysed, or they were starved and incubated with Me2SO, or they were starved and incubated with Me2SO, saline and lysed, or they were starved and incubated with Me2SO, or they were starved and incubated with Me2SO, saline and lysed, or they were starved and incubated with Me2SO, or they were starved and incubated with Me2SO, saline and lysed, or they were starved and incubated with Me2SO, or they were starved and incubated with Me2SO, saline and lysed, or they were starved and incubated with Me2SO, or they were starved and incubated with Me2SO, saline and lysed, or they were starved and incubated with Me2SO, or they were starved and incubated with Me2SO, saline and lysed, or they were starved and incubated with Me2SO, or they were starved and incubated with Me2SO, saline and lysed, or they were starved and incubated with Me2SO, or they were starved and incubated with Me2SO, saline and lysed, or they were starved and incubated with Me2S

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-GTP/γS or sphingosine. Wild type PDK1 incorporated a basal level of [32P]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[32P]ATP alone, as measured by PhosphoImager 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 of PDK1 was decreased significantly in the presence of Cdc42-GTP/γS, suggesting that PDK1 activity may be negatively regulated by Cdc42-GTP/γS. PDK1 incubated with GDPγS-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.

The ability of sphingosine to stimulate PDK1 autophosphorylation and substrate phosphorylation was further examined. Fig. 2A 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 ED50 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 Cβ (PKCβII), and threo-

Table I

| Kinase | Amino acid sequence |
|--------|---------------------|
| PAK-1  | K R S T M V G T P Y W M A E V V |
| PKA    | R T W L C G T P E Y L A P E I I |
| PKCβII | T T R T F C G T P D Y I A P E I I |
| Akt    | T M K R F C G T P E Y L A P E V L |
| P70S6  | V H T F C G T I E Y M A P E I L |

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phosphothreonine at amino acid residue 500 in the activation loop and not some other. Both PKC and PKA immunoblots with this antibody showed an increase in phosphate shown to cross-react with phosphothreonine 197 in PKA. Previously (5), this antibody was shown are representative of five separate experiments. The bottom blot shows the levels of PAK1 K299A or PKD1 WT as determined by immunoblotting with the anti-Myc monoclonal antibody.

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$_9$- or C$_6$-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 (Table II), suggesting that a protonable site is important for sphingosine activation of PDK1. This experiment is representative of at least three performed.
activate the enzyme (2). PtdIns 3,4,5 P3-dependent activation has been shown to localize PDK1 to the membrane, but does not lead to serine or phosphatidylcholine lipid vesicles containing sphingosine stimulation of PDK1 in the presence of PtdIns 3,4,5 P3. Binding has been shown to bind PtdIns 3,4,5 P3. Binding has recently been described in the presence of a PDK1-interacting factor (14). We wanted to test whether 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 P3. Binding has been shown to localize PDK1 to the membrane, but does not activate the enzyme (2). PtdIns 3,4,5 P3-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 P3-containing lipid vesicles could enhance PDK1 auto-phosphorylation (Fig. 3 and Table II). Consistent with previously reported data, PtdIns 3,4,5 P3-containing vesicles were unable to induce PDK1 autophosphorylation or substrate phosphorylation over incubation with ATP alone. Phosphatidylserine or phosphatidyicholine lipid vesicles containing sphingosine in the presence or absence of PtdIns 3,4,5 P3-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 PIP3 on PDK1 activation by sphingosine.

### Table II

| Lipid                  | Increase in PDK1 Autophosphorylation | n  |
|------------------------|--------------------------------------|----|
| Sphingosine            | Yes                                  | 35 |
| C16:0-sphingosine      | No                                   | 3  |
| PtdIns 3,4,5 P3-linked | No                                   | 7  |
| C2-ceramide            | No                                   | 7  |
| C12-ceramide           | No                                   | 3  |
| Δ-6-Erythro-sphingosine| Yes                                  | 3  |
| l-erythro-sphingosine  | Yes                                  | 3  |
| Δ-Three-sphingosine    | Yes                                  | 3  |
| Δ, l-Dihydroxy-sphingosine| Yes                                 | 3  |
| Δ, l-Erythro-dihydroxy-sphingosine| Yes                              | 3  |
| Phytopsphingosine      | Yes                                  | 3  |
| Octylamine             | No                                   | 7  |
| Stearylamine           | Yes                                  | 7  |
| Stearic acid           | No                                   | 7  |
| PtdCho/Ser+            | No                                   | 3  |
| PtdIns 3,4,5 P3        | No                                   | 4  |
| PtdIns 4,5 P3          | No                                   | 4  |
| PtdIns 3,4,5 P3 +      | No                                   | 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 PtdCho/Ser, phosphatidylcholine and serine.

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 P3. Binding has been shown to localize PDK1 to the membrane, but does not activate the enzyme (2). PtdIns 3,4,5 P3-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 P3-containing lipid vesicles could enhance PDK1 auto-phosphorylation (Fig. 3 and Table II). Consistent with previously reported data, PtdIns 3,4,5 P3-containing vesicles were unable to induce PDK1 autophosphorylation or substrate phosphorylation over incubation with ATP alone. Phosphatidylserine or phosphatidyicholine lipid vesicles containing sphingosine in the presence or absence of PtdIns 3,4,5 P3-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 PIP3 on PDK1 activation by sphingosine.

### Sphingosine-stimulated PDK1 Autophosphorylation 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. 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 indicate 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...
from the original peptide (5375.9) by a multiple of 80 Da. In the inset did not correspond to any PDK1 mass. In the peak corresponding to a PDK1 mass could be identified. The other peak (*) represented PDK1 was incubated alone or with sphingosine (400 μM) or with absence of sphingosine (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 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 P3 in the presence of PD1K-interacting factor (14). In this study, we show that sphingosine acts independently of PtdIns 3,4,5 P3 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 P3 may be required for efficient signal transduction. Localization of PDK1 to the membranes by increased PtdIns 3,4,5 P3 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 P3 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

**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 trypsic digest representing the carboxy-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.

**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 MeSO (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 expression of PDK1 WT and PDK1 K111Q in transfected cells.
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. Casamayor 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 P3, 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 events, such as the platelet-derived growth factor receptor. Platelet-dependent growth factor receptor. Platelet-derived growth factor is known to up-regulate many different signal transduction pathways, including synthesis of PtdIns 3,4,5 P3 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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