A Novel Sphingosine-dependent Protein Kinase (SDK1) Specifically Phosphorylates Certain Isoforms of 14-3-3 Protein*

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Protein kinases activated by sphingosine or N,N-di-methylsphingosine, but not by other lipids, have been detected and are termed sphingosine-dependent protein kinases (SDKs). These SDKs were previously shown to phosphorylate endogenous 14-3-3 proteins (Megidish, T., White, T., Takio, K., Titani, K., Igarashi, Y., and Hakomori, S. (1995) Biochem. Biophys. Res. Commun. 216, 739–747). We have now partially purified one SDK, termed SDK1, from cytosol of mouse Balb/c 3T3(A31) fibroblasts. SDK1 is a serine kinase with molecular mass 50–60 kDa that is strongly activated by N,N'-dimethylsphingosine and sphingosine, but not by ceramide, sphingosine 1-phosphate, or other sphingo-, phospho-, or glycerolipids tested. Its activity is inhibited by the protein kinase C activator phorbol ester. Activity of SDK1 is clearly distinct from other types of serine kinases tested, including casein kinase II, the α and ζ isoforms of protein kinase C, extracellular signal-regulated mitogen-activated protein kinase 1 (Erk-1), Erk-2, and Raf-1. SDK1 specifically phosphorylates certain isoforms of 14-3-3 (α, β, γ) but not others (α, γ). The phosphorylation site was identified as Ser* in the sequence Arg-Arg-Ser-Ser*-Trp-Arg in 14-3-3 β. The α and γ isoforms of 14-3-3 lack serine at this position, potentially explaining their lack of phosphorylation by SDK1. Interestingly, the phosphorylation site is located on the dimer interface of 14-3-3 phosphoproteins. Phosphorylation of this site by SDK1 was studied in 14-3-3 mutants. Mutation of a lysine residue, located 9 amino acids N-terminal to the phosphorylation site, abolished 14-3-3 phosphorylation. Furthermore, co-immunoprecipitation experiments demonstrate an association between an SDK and 14-3-3 in situ. Exogenous N,N'-dimethylsphingosine stimulates 14-3-3 phosphorylation in Balb/c 3T3 fibroblasts, suggesting that SDK1 may phosphorylate 14-3-3 in situ. These data support a biological role of SDK1 activation and consequent phosphorylation of specific 14-3-3 isoforms that regulate signal transduction. In view of the three-dimensional structure of 14-3-3, it is likely that phosphorylation by SDK1 would alter dimerization of 14-3-3, and/or induce conformational changes that alter 14-3-3 association with other kinases involved in signal transduction.

1 The abbreviations used are: Sph, sphingosine; Ab, antibody; AEBSF, 4-(2-aminoethyl)-benzenesulfonfyl fluoride; CKII, casein kinase II; DAG, diacylglycerol; DMS, N,N'-dimethylsphingosine; PK, protein kinase A; PKC, protein kinase C; PKM, catalytic domain of protein kinase C; PS, phosphatidyl-serine; SDK, sphingosine-dependent protein kinase; Sph-1-P, sphingosine 1-phosphate; TPA, 12-O-tetradecanoylphorbol-13-acetate; PAGE, polyacrylamide gel electrophoresis; DMEM, Dulbecco's modified Eagle's medium; MOPS, 4-morpholinopropanesulfonic acid.

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number, identities, and substrates of these SDKs are unclear. Using amino acid sequencing, we have identified several cellular proteins that are phosphorylated in vitro by SDKs including the 14-3-3 isoforms β, ε, and τ (21).

14-3-3 proteins comprise a family with a remarkable evolutionary conservation extending to lower eukaryotes and plants. There are seven distinct mammalian isoforms (β, γ, ε, η, τ, and σ). 14-3-3 isoforms appear to modulate a large variety of functional proteins and enzymes, including lipid-activated kinases (22–24) such as PKC, Raf-1, and phosphatidylinositol 3-kinase, and other kinases or phosphatases involved in cell cycle, cell death, and mitogenesis (25–27). 14-3-3 proteins are thought to function as adaptor proteins that allow interaction between signaling proteins that do not associate directly with each other (25). The association of 14-3-3 with different kinases in cytosol and membrane may contribute to kinase activation during intracellular signaling (28, 29).

Here we report purification of one type of cytosolic SDK (SDK1) which phosphorylates only certain isoforms (β, η, and ε) but not other isoforms (σ and τ) of 14-3-3, or other proteins known to be substrates of other SDKs. We also identified the site at which SDK1 phosphorylates 14-3-3. Although the physiological significance of 14-3-3 phosphorylation is unknown, there is an interesting possibility that activation of SDK1 and consequent phosphorylation of 14-3-3, in combination with an increase in the intracellular levels of Sph or DAMPS, promotes 14-3-3 dimerization, through which 14-3-3 activity is regulated.

**Experimental Procedures**

**Materials and Reagents**

v-erythro-Sph (31), and N,N'-dimethyl-v-erythro-Sph (7) were synthesized chemically as described. Protein disulfide isomerase (PDI), TPA and phorbol 12-myristate 13-acetate (PMA) were purchased from Calbiochem (San Diego, CA). Columns with Q-Sepharose Fast Flow HR 10/10, Mono-S (HR 5/5), Mono-Q (HR 5/5), Sephacryl 200 (HR 10/30; for gel filtration), Q-Sepharose Fast Flow HR 10/10, Mono-S, Mono-Q (HR 5/5), and Mono-XL, and contributing to cell survival (30). These agonists also increase Sph level, suggesting a link between 14-3-3 and Sph signaling. The diverse effects of Sph and its derivatives on cell cycle progression, cell death, and mitochondria, and the similarity between Sph biological effects and 14-3-3 phosphorylation site (21). Platelet-derived growth factor and insulin-like growth factor 1 are known to be involved in cell cycle, cell death, and mitogenesis (25–27). 14-3-3 proteins are thought to function as adaptor proteins that allow interaction between signaling proteins that do not associate directly with each other (25). The association of 14-3-3 with different kinases in cytosol and membrane may contribute to kinase activation during intracellular signaling (28, 29).

**Purification of SDK1**

All purification procedures were carried out at 0–4 °C, and buffers used were filtered through membrane and degassed under vacuum immediately prior to column chromatography. The following buffer systems were used. Buffer A consisted of 20 mM Tris buffer (pH 8.5), 1 mM EDTA. Buffer B was Buffer A plus 10 mM DTT, 10 mM sodium fluoride, and 0.1 mg/ml AEBSF. Buffer C was Buffer A plus 10 mM DTT, 10 mM sodium fluoride, and 1 mM KCl. Buffer D consisted of Buffer A with pH adjusted to 5.8–6.0, plus 10 mM DTT, 10 mM sodium fluoride, and 0.2% n-octyl-β-D-glucoside. Buffer E was Buffer D with pH adjusted to 7.5 and Buffer F consisted of Buffer E plus 100 mM NaCl.

**Step 1: Preparation of Cell Extract—**Aliquots of packed frozen cells (2.5 ml, 100 mg) were thawed on ice and suspended in 45 ml of homogenizing buffer consisting of 20 mM Tris (pH 7.5), 0.5 mM EDTA, 0.5 mM EGTA, 10 mM sodium fluoride, 10 mM β-mercaptoethanol, 10 µM leupeptin, 10 µM meprin inhibitor, 10 µM phosphatase inhibitor cocktails. Homogenization was performed by 40 strokes in an ice-cooled, tight-fitting Dounce homogenizer. The homogenate was centrifuged at 500 × g for 10 min, and the supernatant was centrifuged at 100,000 × g for 60 min. The supernatant (cytosol) was subjected to further purification of SDK1, and activity was tested as described under "Standard SDK1 Activity Assay."

**Step 2: Q-Sepharose Column Chromatography—**45 ml of cytosolic extract was mixed with 2.5 ml of packed Sphingosine-dependent Protein Kinase 1 (SDK1) protein. The homogenate was centrifuged at 4°C for 1 h, and immediately loaded on a Q-Sepharose column (1 × 10.5 cm) pre-equilibrated with Buffer B and washed with 4 volumes of Buffer B. In order to separate SDK1 activity from other kinases, proteins were then eluted by a combination of stepwise changes and linear gradient changes of NaCl concentration (0 to 1.0 M), as indicated by a dotted line in Fig. 1A. The linear gradient was performed from 0 to 0.2 M NaCl for 15 min, 0.2–0.35 M for 85 min, constant at 0.35 M for 33 min, 0.35–1 M for 24 min, and finally constant at 1 M for 18 min. Elution was performed at a flow rate of 0.5 ml/min, and 1.5-ml fractions were collected and subjected to standard SDK1 assay. Fractions containing SDK1 activity were pooled and dialyzed 4–6 h against 4 liters of Buffer A containing 10 mM β-mercaptoethanol.

**Step 3: Phenyl-Sepharose Column Chromatography—**A phenyl-Sepharose column was pre-equilibrated with Buffer C. KCl (final concentration 1 M) was added to the pooled fractions showing SDK1 activity at step 2 (after Q-Sepharose column chromatography). The pooled fractions were loaded on the phenyl-Sepharose column in Buffer C and washed with 4 column volumes of Buffer C. Proteins were eluted at a flow rate of 0.5 ml/min by decreasing KCl. The concentration was decreased from 1 to 0.5 M for 20 min, maintained at 0.5 M for 14 min, decreased from 0.5 to 0 M for 56 min (Fig. 1C), and 1-ml fractions were collected. Flow-through (FT) (unbound) fractions were pooled and dialyzed against 4 liters of Buffer A containing 10 mM β-mercaptoethanol for at least 6 h.

**Step 4: Mono-S Column Chromatography—**Dialyzed FT and bound phenyl-Sepharose fractions (diluted 4 times in buffer D) were passed through a Mono-S column (1 × 10.5 cm) pre-equilibrated in Buffer D containing 10 mM β-mercaptoethanol and 1.5 ml fractions were collected and subjected to standard SDK1 activity assay. Fractions containing SDK1 activity were pooled and dialyzed 4–6 h against 4 liters of Buffer A containing 10 mM β-mercaptoethanol.
separately through Mono-S column. SDK1 activity from both pools was eluted in FT fraction, and minimal SDK1 activity was bound to Mono-S.

**Step 5: Mono-Q Column Chromatography—**Mono-S FT fractions of both pools were subjected to Mono-Q ion-exchange chromatography. A Mono-Q (HR 5/5) column was equilibrated with buffer E, and proteins were loaded at a flow rate of 1 ml/min. The column was washed with 6 ml of buffer E. In order to separate SDK1 activity from major PKC activity, elution was performed by applying a combined stepwise and linear gradient of NaCl (0 to 1.0 M) at a flow rate of 0.25 ml/min as indicated by the dotted line in Fig. 1D. The linear gradients were from 0 to 0.2 M NaCl for 10 min, constant at 0.2 M for 10 min, from 0.2 to 0.35 M NaCl for 30 min, constant at 0.35 M for 20 min, from 0.35 to 1 M for 10 min, and finally constant at 1 M for 30 min. Fractions (0.65 ml) were collected, and active fractions were pooled. Obtained from the phenyl-Sepharose-bound pool is shown in Fig. 1D. A similar pattern was obtained from the phenyl-Sepharose-unbound pool (data not shown).

**Step 6: Size Exclusion Chromatography—**In order to estimate the Mr of SDK1, a sample (0.5 ml) obtained from a Mono-Q column of phenyl-Sepharose-unbound pool was analyzed by gel filtration. The Sephacryl S-200 column (1 × 30 cm; total column volume 28 ml) was eluted with buffer F, which was equilibrated with Buffer F. The sample was loaded at a flow rate of 0.5 ml/min for 3 min and eluted at a flow rate of 0.1 ml/min, and 0.5-ml fractions were collected. Standard SDK1 assay was performed, except that reactions were performed for 30 min in the presence of 1 μM ATP (1.4 × 10^5 cpm/pmol). Gel-filtration protein standards β-amylose (200 kDa), bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa), and cytochrome c (12.4 kDa) were fractionated immediately after SDK1 fractionation, and the Mr of SDK1 was estimated (Fig. 1E).

**Standard SDK1 Activity Assay**

Sph and DMS both activate SDK1; however, DMS was used for detection of SDK1 activity during purification since it cannot be metabolized to either ceramide or Sph-1-P. SDK1 activity during purification was estimated by standard assay utilizing 0.1 μM of 14-3-3 β and 100 μM DMS in 0.5% octyl-β-glucoside. SDK1 activity required Mg2+ with optimal concentration of 15 mM (Mn was ineffective), and decreased as ionic strength increased. An IC50 of 150 μM NaCl was defined at 60% for 45 min in 0.1% trifluoroacetic acid, utilizing a Gilson auto-EL system (France). Purity and authenticity of the peptide was checked by reverse phase high performance liquid chromatography and electrospray ionization (ESI) mass spectrometry (ESI voltage −4.5 kV, scan 200–2000 m/z) and subjected to autoradiography. Purity and authenticity of the peptide was checked by reverse phase high performance liquid chromatography and electrospray ionization (ESI) mass spectrometry (ESI voltage −4.5 kV, scan 200–2000 m/z) and subjected to autoradiography. The method described previously (36) using a CKII assay kit (Amersham) with CKII specific substrate, a synthetic peptide. Samples (5 μl) were incubated in the presence of 250 μM peptide and 150 mM NaCl in 16 ml MOPS (pH 7.2), and reactions were initiated by addition of a mixture of 200 μM ATP, 10 mM MgCl2, and 0.2 μM of β[ γ-32P]ATP in 10 ml HEPES pH 7.5. After a 30-min incubation at 30 °C, radiolabeled peptide was separated from unincorporated 32P by binding to affinity phosphocellulose paper. Degree of phosphorylation was determined by liquid scintillation counting. Enzyme activity was calculated from the amount of 32P incorporated into the peptide, taking into account radiolotope specific activity and reaction time.

**Chemical Synthesis of Hexadecapeptide Containing Phosphorylation Site**

The peptide KVNGVARRSWVIRT was synthesized by the Fmoc/PyBop method in 15 μmol scale utilizing a Shimadzu PSSM-8 synthesizer (Shimadzu Corp., Kyoto, Japan). Generated peptides were purified by preparative reversed-phase high performance liquid chromatography on a C18 column (20 × 250 mm, Nakarai Tesque, Japan) at a flow rate of 10 ml/min, with a linear gradient of acetonitrile (0 to 60% for 45 min) in 0.1% trifluoroacetic acid, utilizing a Gilson auto-EL system (France). Purity and authenticity of the peptide was checked by reverse phase high performance liquid chromatography and electrospray ionization (ESI) mass spectrometry (ESI voltage −4.5 kV, scan 200–2000 m/z) and subjected to autoradiography. The method described previously (36) using a CKII assay kit (Amersham) with CKII specific substrate, a synthetic peptide. Samples (5 μl) were incubated in the presence of 250 μM peptide and 150 mM NaCl in 16 ml MOPS (pH 7.2), and reactions were initiated by addition of a mixture of 200 μM ATP, 10 mM MgCl2, and 0.2 μM of β[ γ-32P]ATP in 10 ml HEPES pH 7.5. After a 30-min incubation at 30 °C, radiolabeled peptide was separated from unincorporated 32P by binding to affinity phosphocellulose paper.

**Phosphoamino Acid Analysis and Two-dimensional Phosphopeptide Mapping of 14-3-3 β**

One μg of 14-3-3 β was phosphorylated by SDK1 as described under “Standard SDK1 Activity Assay,” except that the specific activity of β[ γ-32P]ATP was 6.6 × 106 cpm/pmol. Reactions were continued for 90 min, and the products were separated on SDS-PAGE and transferred to NC membranes. Proteins were visualized by staining with Amido Black 10B. The products were subjected to 2D gel electrophoresis (4% methanol/acetic acid, 45:10) and subjected to autoradiography. The phosphorylated 14-3-3 band was excised, treated with 1% polyvinylpyrrolidone-40 in 100 mM acetic acid at 37 °C for 1 h, washed 5 times with water, and digested with 10 μg of trypsin, endo-arginylpeptidase (ArgC), or endo-lysylpeptidase (LysC) in 50 mM ammonium bicarbonate at 37 °C for 16–24 h. After the first 2 h, an additional 10 μg of protease was added to ensure complete cleavage. Peptides were recovered from NC membrane by washing in 20% acetonitrile, dried, washed with water, and dried 4 times to remove ammonium bicarbonate. 32P recovered in the supernatants from these digestions was 80, 70, or 50% for the three enzymes, respectively. For phosphoamino acid analysis, tryptic peptides were hydrolyzed in 6 N HCl at 110 °C for 2 h, and analyzed (37).

The digested peptides were subjected to two-dimensional phosphopeptide mapping (37). Briefly, the peptides were separated according to charge by electrophoresis at pH 1.9 at 1.2 kV for 25 min in the first dimension, followed by separation according to hydrophobicity by chromatography in 65% isobutyric buffer (10 h) in the second dimension. After visualization by autoradiography (Bio-Max film), 32P-labeled phosphopeptides were extracted from the cellulose plates in 200 μl (pH 9.9) of buffer by vortex, followed by centrifugation. Phosphopeptides in
the supernatant were dried, washed 3 times with water, and subjected to either a second analytical digestion or manual sequencing by Edman degradation (37). The degradation products were resolved at pH 3.5 at 1.3 kV for 16 min, and visualized by autoradiography. From the data of protein acid digestion, performic acid was prepared by mixing 10 μl of H2O2, (30% in 90 μl of HCOOH (98%). The solution was incubated on ice for 1 h before use. Digested phosphopeptides were recovered from NC membrane, dried once, incubated in H2O/HCOOH on ice for 2 h, washed, and analyzed as described above for unoxidized peptides.

In vitro Phosphorylation of Synthetic Peptide by PKM of PKCα

1 μl of solution containing 5.4 ng of PKM (specific activity, 800 fmol/min/ng) was incubated with 1 μm synthetic peptide in 14 μl of kinase buffer containing 50 mM Tris-HCl (pH 7.5), 1 mM peptide, 3 mM DTT, 1 mM CaCl2, 0.4% DMSO dissolved in 0.3% Triton X-100 (8 mol% of Triton X-100), 2 μg/ml TPA, 15 mM magnesium acetate, and 1 μM ATP (containing 2 μCi of [γ-32P]ATP) for 1 h at 30 °C. The synthetic peptide was omitted in control reaction. The phosphorylated peptide was separated from uncleaved peptide by thin layer electrophoresis at pH 3.5, 1.6 kV, for 16 min. After visualization by autoradiography at room temperature for 10 min, phosphopeptides were extracted from the plate in pH 1.9 buffer and dried in a Speed Vac. The purified phosphopeptide was digested with trypsin as described for phosphorylated 14-3-3 proteins.

Western Blotting with Isoform-specific Anti-14-3-3 Abs

Cell homogenates or samples obtained from column fractions were resolved on SDS-PAGE and transferred to NC membranes. The membranes were incubated at room temperature in 20 mM Tris-HCl (pH 7.4), 200 mM NaCl, 3% bovine serum albumin, and 10% horse serum for 30 min, incubated with specific antiserum for 1–2 h, and washed. The blot was incubated with secondary Ab for 45 min, and developed for enhanced chemiluminescence. The Pan Ab was raised to a peptide found in all 14-3-3 isoforms, KSELVQKAKLAEQAERYDD, corresponding to amino acids 134–149 of human 14-3-3-ζ. The rabbit Ab was raised to peptide CAGDDKKGIVDQSQQAY, corresponding to amino acids 280–300 of 14-3-3-α. Mouse 14-3-3 was identical to human 14-3-3-ζ, and therefore these terms are used interchangeably in the literature (21, 38, 39); we will refer to them as α. For Western blot analysis, 14-3-3 Abs and CKII Abs were used at 1:2000 dilution, kinase suppressor of ras (KSR) and PKC Abs at 1:1000 dilution, and Raf, Erk1, and Erk2 Abs at 1:500 dilution.

Immunoprecipitation and in Vitro Kinase Assay

Cells were homogenized with 40 strokes in Dounce homogenizer in lysis buffer containing TEN buffer (50 mM Tris, pH 7.5, 5 mM EDTA, 100 mM NaCl, and 50 mM sodium fluoride) plus 30 μg/ml leupeptin, 30 μg/ml pepstatin, 50 mM AEBSF, 0.5% Triton X-100. Proteins were solubilized by incubation at 4 °C for 30 min. Lysates were centrifuged in Eppendorf centrifuge at 4 °C for 15 min. The supernatant (6 mg) was diluted 1:2 in lysis buffer without Triton X-100 and 1 μg of anti-14-3-3 Ab was added at 4 °C. After 1 h, Protein A-agarose was added for 10–14 h. Immunoprecipitates were washed 2 times with TEN buffer containing 0.1% Triton X-100 at room temperature, 1 time with the TEN buffer, and then washed for 5 min at room temperature in TEN buffer containing 0.5% n-octyl-β-glucoside and washed (this last step was repeated). The total wash was with 50 μM Tris and 50 μM NaCl. Pellets were resuspended in 150 μl of 50 mM Tris (pH 7.5) and 6 mM DTT. The suspension (15 μl) was subjected to standard SDK1 assay, except that DTT was not added, and reactions were performed in the presence of 1 μM ATP (1.4 × 105 cpm/μmol) for 30 min.

Metabolic 32P Labeling and Immunoprecipitation

3T3(A31) fibroblasts were incubated in phosphate-free DMEM containing dialyzed 10% fetal bovine serum for 3 h. The cells were labeled in serum-free, phosphate-free DMEM containing [32P]orthophosphate (0.045 mCi/ml) for 9 h. During the last 20 min cells were challenged with [32P]orthophosphate in the presence of 0.1% ethanol or with 0.1% ethanol dissolved in 1% ethanol, or with 200 mM TPA. Cells were washed with phosphate-buffered saline without Ca2+ and Mg2+ and harvested with 0.5 mM EDTA at 4 °C. Cell viability was not affected under these conditions. Cells were sonicated in lysis buffer (10 mM Tris, pH 7.5, 5 mM EDTA, 50 mM sodium fluoride, 50 μg/ml leupeptin, 50 μg/ml aprotinin, 50 μg/ml trypsin inhibitor, 0.1 μg/ml AEBSF, 3 μg mercaptoethanol, 1% Triton X-100), and incubated at 4 °C for 1 h for protein solubilization. Lysates were pelleted in an Eppendorf centrifuge at 4 °C for 15 min. Samples were denatured at 70 °C for 30 min, centrifuged, and supernatants were diluted 1:4 in the above buffer containing 0.2 mM NaCl, without Triton X-100. PAN anti-14-3-3 Ab complexed with immobilized Protein A/G (Santa Cruz) was added, and 14-3-3 was immunoprecipitated at 4 °C for 3 h. The immunoprecipitates were washed 3 times with TEN buffer containing 0.05% Tween 20 and once with buffer containing 50 mM Tris (pH 7.5) and 100 mM NaCl. Samples were resuspended in 2 times Laemmli sample buffer, separated by SDS-PAGE, and transferred to a NC membrane. Equal recovery of 14-3-3 proteins was confirmed by probing the filter with 14-3-3 Ab and enhanced chemiluminescence. Chemiluminescent substrate was removed by washing in 50 mM Tris (pH 7.5) and 100 mM NaCl, and by exposure to light for 1 h. The NC membrane was subjected to autoradiography to detect radioactive 14-3-3.

RESULTS

Purification and Separation of SDK1-containing Fraction—SDK1 was purified by sequential column chromatography, and detected using 100 μM DMS as activator and 14-3-3 β as substrate (Fig. 1). SDK1 activity was eluted at 260–350 mM NaCl from a Q-Sepharose column at purification step 2 (Fig. 1A). SDK1 phosphorylated 14-3-3-β, ζ, η, and an endogenous 28-kDa protein, which was eluted between 300 and 350 mM NaCl (Fig. 1B). The 28-kDa protein most likely consists of 14-3-3 isoforms, since it was detected with PAN 14-3-3 Ab by Western blotting (data not shown). In some cases a minor SDK1 activity peak was eluted from the column at 0.1 mM NaCl, without change in the elution pattern of the major SDK1 peak, which was purified further.

SDK1 activity was separated from some endogenous 14-3-3 by fractionation of the major SDK1 activity containing fractions on phenyl-Sepharose. SDK1 with low hydrophobicity, found in unbound phenyl-Sepharose pool, was separated from SDK1 with high hydrophobicity, bound to phenyl-Sepharose (Fig. 1C, fractions 11–17). Biochemical properties of SDK1 activities in both pools were examined by further purification on Mono-S followed by Mono-Q ion exchange chromatography. SDK1 activity that bound phenyl-Sepharose was eluted at 280–300 mM NaCl (fractions 9–16) (Fig. 1D). SDK1 activity obtained from unbound phenyl-Sepharose pools was eluted at 330–350 mM NaCl (data not shown). The kinase was finally isolated from minor contaminants by gel filtration (step 6). Purified SDK1 showed a sharp elution peak with molecular mass of 50–60 kDa on a gel filtration column (Fig. 1E).

Sph and DMS Are the Only Sphingolipids That Activate SDK1—The effects of various concentrations of Sph and DMS delivered in ethanol or n-octyl-β-glucoside, and their ability to activate purified SDK1 and consequently phosphorylate 14-3-3-ζ, were examined. Purified SDK1 was capable of phosphorylating 14-3-3 in the presence of Sph or DMS, but not in the presence of ethanol or n-octyl-β-glucoside alone. Other controls lacking kinase or 14-3-3 failed to activate phosphorylation of 14-3-3 (data not shown). Very low concentrations (5 μM Sph or 25 μM DMS) were required for maximum SDK1 activation when ethanol was used as vehicle (Fig. 2A). A sigmoidal kinetic curve was observed when Sph or DMS were solubilized in ethanol for both 14-3-3-ζ and β assay (data not shown). However, this phenomenon disappeared when the vehicle was changed from ethanol to n-octyl-β-glucoside, and higher concentrations of Sph or DMS (100 μM) were necessary for SDK1 activation (Fig. 2B). The fact that 14-3-3 phosphorylation reached a plateau in the presence of n-octyl-β-glucoside implies that this detergent prevents secondary effects, such as 14-3-3 dimerization or 14-3-3 oligomerization during SDK1 reaction. n-Octyl-β-glucoside also increased SDK1 activity 5-fold relative to ethanol. These results suggest that Sph is the primary activator of SDK1 and that this activation is not mediated by a detergent effect. In contrast, SDK1 was not activated by other...
Fig. 1. Sequential purification and chromatographic separation of SDK1 from PKC and CKII. Panel A, separation pattern of SDK1, PKC, and CKII activities through Q-Sepharose ion-exchange column. This panel represents step 2 of the purification procedure. Proteins in cytosolic extract solubilized with Triton X-100 were loaded on Q-Sepharose column (1 × 10.5 cm), washed with Buffer B, and eluted at a flow rate of 0.5 ml/min with 0–1 M NaCl (combined linear and stepwise gradient). 5-μl aliquots of representative fractions were analyzed for SDK1, PKC, and CKII activities, and the amount of 32P incorporated into the substrates was quantified as described under “Experimental Procedures.” Left ordinate, SDK1 activity (fmol/min). Near right ordinate, PKC and CKII activities (pmol/min). Dotted line, change in NaCl concentration (M; far right ordinate) during gradient elution. ■ SDK1 activity tested with 120 nM (0.1 μg/ml) 14-3-3 β as substrate and 100 μM DMS in 0.5% octyl-β-glucoside as lipid activator. □ SDK1 activity toward 28-kDa protein identified as endogenous 14-3-3, tested without addition of 14-3-3 substrate. ●, PKC activity tested in the presence of myelin basic protein peptide substrate, PS/TPA, and Ca2⁺. ○, PKC activity tested in the absence of PS/TPA and Ca2⁺. Abscissa, selected chromatography fractions. Panel B, elution profile of SDK1 activity from Q-Sepharose column, indicated by autoradiogram of SDS-PAGE gel. The cytosolic extract (step 1) was treated before fractionation with 0.5% octyl-β-glucoside instead of Triton X-100. The sample pH was increased to pH 8.5, and the sample was fractionated on a Q-Sepharose column equilibrated in buffer B containing 0.5% octyl-β-glucoside (pH 8.5). Phosphorylation by the indicated fractions was analyzed in the presence of 120 nM 14-3-3 β, η, and ζ, or without addition of exogenous 14-3-3 (“endogenous substrates”). Dotted lines shown above the gels indicate change in NaCl concentration (M) during elution. Panel C, phenyl-Sepharose column chromatography of SDK1 activity. The salt concentration in SDK1 peak (31 ml) in panel A was adjusted to 1 M KCl, and proteins were loaded on a phenyl-Sepharose column (step 3). Bound proteins were eluted by decreasing the concentration of KCl from 1 to 0 M (dotted line; scale shown on right ordinate). ■, SDK1 activity (fmol/min; scale shown on left ordinate). Right panel, levels of SDK1 activity toward human recombinant 14-3-3 β in the flow-through (lane FT) and in column fractions (5 μl) are shown in the autoradiogram of phosphorylation pattern induced in the presence of SDK1 and DMS. Arrow, position of 14-3-3 β. These results are a representative example from at least four separate experiments. Panel D, separation of SDK1 activity from PKC by Mono-Q ion-exchange chromatography. The eluate from phenyl-Sepharose (step 3) was diluted 4 times and fractionated on Mono-S (step 4) followed by Mono-Q (step 5) equilibrated in Buffer E. Bound proteins were eluted by increasing the concentration of NaCl. Dotted line, change in NaCl concentration (M, far right ordinate) during gradient elution. 5-μl aliquots of representative fractions were used for standard SDK1 activity assay and for PKC activity assay. ■, SDK1 activity (fmol/min; scale shown on left ordinate). ●, PKC activity with complete assay system. ○, PKC activity without PS/TPA and Ca2⁺ (indicated on near right ordinate in pmol/min). Right panel, autoradiogram of SDS-PAGE showing phosphorylated 14-3-3 β induced by SDK1 present in fraction before purification (load) and in representative eluted fractions (5 μl) in the presence of DMS. Activity in the absence of DMS (--) is shown for fraction 17. Panel E, estimated M, of SDK1 by gel filtration analysis. A sample (0.5 ml) obtained from the Mono-Q column of phenyl-Sepharose-unbound pool was analyzed by gel filtration. Fractions (0.5 ml) were collected and SDK1 activity was assayed in aliquots (10 μl) of representative fractions. ■, SDK1 activity tested for 30 min. Scale (ordinate) is fmol. Arrows, positions of molecular weight standards (200, 66, 29, and 12.4 kDa). Right panel, autoradiogram of 12% SDS-PAGE of phosphorylated 14-3-3 β induced by SDK1 present in fraction before gel filtration (load) and in eluted fractions (5 μl) in the presence of DMS. Positions of molecular weight markers are indicated at right.

closely related sphingolipids such as Sph-1-P, C15- ceramide, PS, lyso-G_{M2}, and oleoyl-lysophosphatidic acid (Fig. 2C).

Substrate Specificity of SDK1—SDK1 was activated by DMS, resulting in phosphorylation of 14-3-3 β, η, or ζ, but not of α or τ (Fig. 3A). This trend was already evident in crude cytosolic fraction, and was unchanged after sequential chromatography
including S200 column (purification step 6) (Fig. 3B). For the η isoform, the DMS requirement was less stringent than for β or ζ, as seen in various steps of purification (Fig. 3B). These results suggest that 14-3-3 η is phosphorylated by a kinase less sensitive to DMS, and that this kinase was removed by gel filtration on S200 column (step 6).

PDI and calreticulin were identified as substrates for cytosolic and membrane-bound SDK, respectively. 3 However, SDK1 failed to phosphorylate PDI or calreticulin under the same conditions whereby 14-3-3 β was phosphorylated (Fig. 3C).

SDK1 is a Kinase Distinct from CKII, PKCs, Erks, Raf, and Ca2+-dependent Kinases—To determine whether SDK1 corresponds to known protein kinases, the elution profile of known kinases determined by either immunoblotting or specific kinase assays was compared to the elution profile of SDK1. The results of these experiments indicated that SDK1 is a kinase distinct from CKII, PKCs, Erks, Raf, and Ca2+-dependent kinases.

3 T. Megidish, K. Takio, K. Titani, Y. Igarashi, A. Helenius, and S. Hakomori, manuscript in preparation.
 kinase assay was compared with the elution profile of SDK1 activity. Ca2+- and phospholipid-dependent PKC activity (mostly PKCa) and CKII activity were measured in fractions obtained by Q-Sepharose ion-exchange chromatography. CKII activity was completely separated from SDK1 activity by fractionation on a Q-Sepharose column (Fig. 1A). PKC activity partially overlapped SDK1 activity, but was completely removed after Mono-Q chromatography (Fig. 1D). Abs to PKC a, Raf, and KSR did not recognize any proteins in SDK1 peak (data not shown). We further confirmed these results for SDK1 purified from cells before treatment with TPA. The elution pattern of SDK1 activity from the Q-Sepharose column was compared with elution of PKC c, Erk1, Erk2, CKII, and Raf proteins. Western blot analysis utilizing PKC c, Erk1 and Erk-2, and CKII Ab (labeled at left) was performed on the fractions labeled in the top panel. Arrows, positions of kinases Erk-1 (44 kDa), Erk-2 (42 kDa), and CKII (two subunits with molecular mass 28 and 43 kDa). Downward arrow indicates SDK1-containing pooled fractions 11–18. Note that SDK1-containing fractions 11–18 do not contain any of these kinases. Panel B, PS is an inhibitor of SDK1. Partially purified SDK1 from TPA-treated cells (purification step 3, described in Fig. 1) was incubated in the absence (−) or presence (+) of 50 μM DMS delivered in 1% ethanol (lanes 1 and 2). The effects of Ca2+ (lanes 3 and 4), Ca2+ plus PS/TPA (lanes 5 and 6), PS (lanes 7 and 8), and DAG (lanes 9 and 10) on SDK1 activity were tested. PS or DAG were delivered in 1% ethanol while PS/TPA were dispersed in 0.3% Triton X-100. Arrow, position of 14-3-3 β. Positions of molecular weight markers are indicated at left.

The SDK1 activity peak partially overlapped Ca2+-phospholipid-dependent PKC activity on Q-Sepharose or phenyl-Sepharose, but was separated on Mono-Q. To evaluate the possibility that SDK1 activity results from other Ca2+-phospholipid-independent PKC or Ca2+-dependent kinase activity, we studied the effect of the PKC activators PS, TPA, DAG, and Ca2+ on activation of SDK1. PS inhibited activation of SDK1 by DMS in the absence or presence of PKC activators Ca2+ and TPA (Fig. 4B). In contrast, DAG, Ca2+ (Fig. 4B), and TPA (data not shown) did not inhibit SDK1 activation in the presence of DMS. Taken together, these results indicate that (i) SDK1 activity is clearly distinguishable not only from PKC a, PKC c, CKII, Raf-1, Erk-1, Erk-2, and KSR, but also from Ca2+-dependent kinases; (ii) SDK1 is distinct from all PKC isoforms since its activity is inhibited by PS but not stimulated by PKC activators TPA, DAG, and Ca2+.

**Kinetics of SDK1 Activity**—Reactions with various concentrations of 14-3-3 ζ and SDK1 revealed a linear relationship between velocity of phosphorylation and quantity of both 14-3-3 ζ and SDK1. The velocity of phosphorylation was positively correlated with substrate concentration (60–480 nM), without a plateau. However, when substrate concentration was 60 nM, the reaction was linear for higher concentrations of kinase (0–1000 ng) (Fig. 5A). Phosphorylation of 14-3-3 ζ was very low at 44 °C and optimal at 30 °C (Fig. 5B). The velocity of phosphorylation was linear and positively correlated with reaction time (Fig. 5B). Stoichiometric analysis of 14-3-3 ζ phosphorylation activated by SDK1 was performed using a large quantity of substrate.
of substrate, higher concentration of ATP, and prolonged incubation period (2–4 h). Approximately 55 and 60 pmol of 32P was incorporated into 60 pmol of 14-3-3 z during 2 and 4 h incubation, respectively, and phosphorylation was completed after 4 h (Fig. 5C). Similar results were obtained in four separate experiments. These findings indicate that SDK1 phosphorylates only a single Ser residue in 1 mol of 14-3-3 monomer.

Identification of Phosphorylation Site—In order to understand the molecular basis of SDK1 specificity toward particular 14-3-3 isoforms, we identified the phosphorylation site on 14-3-3 b. 14-3-3 b was phosphorylated by crude SDK1 (purification step 2), digested with trypsin, and acid hydrolyzed, and PAA analysis on two-dimensional electrophoresis was performed as described under “Experimental Procedures.” The positions of PAA were determined by separation on cellulose thin-layer plates according to charge at pH 1.9 (horizontal axis) and pH 3.5 (vertical axis). The positions of phosphoryserine (S), phosphorythreonine (T), and phosphotyrosine (Y) standards were visualized with ninhydrin staining. Note that only phosphoryserine was 32P-labeled. +, origin. Panels B–D, two-dimensional phosphopeptide mapping of 32P-labeled 14-3-3 b digested with trypsin (panel B) or endoarginylpeptidase (ArgC) (panel C). 14-3-3 b was 32P-phosphorylated by purified SDK1 (step 5, Fig. 1E) and analyzed as described under “Experimental Procedures.” Panel D, labeled components 1, 2, 3, and 4 derived from performic acid oxidation of the single tryptic peptide indicated by arrow in panel B. In panels B–D, phosphopeptides were separated by electrophoresis at pH 1.9 (horizontal axis) and by chromatography (vertical axis) in hydrophobic buffer (85% isobutyric acid). Panel E, change of electrophoretic mobility of 14-3-3 b tryptic phosphopeptide and release of free phosphate upon the third cycle of manual Edman degradation. The 32P-labeled phosphopeptide was eluted from the TLC plate, subjected to manual Edman degradation, and products were separated by thin layer electrophoresis at pH 3.5 and visualized by autoradiography (Bio-Max film). Basic residue was released from the phosphopeptide upon the first cycle of Edman degradation, and a free phosphate (P) was released upon the third cycle. The origin is indicated by a line.
amphipathic groove in ligand binding. Lys$^{49}$-Glu mutation disrupted ligand association but Arg$^{60}$-Glu had only a small effect (32). To test whether SDK1 also uses this amphipathic groove of 14-3-3 for substrate recognition, we examined the effect of the Lys$^{49}$-Glu mutation on phosphorylation of 14-3-3 by activated SDK1. The Lys$^{49}$-Glu mutation abolished the ability of 14-3-3 to serve as a SDK1 substrate, while the Arg$^{60}$-Glu mutation reduced phosphorylation only by about 50% (Fig. 8A, upper panel). Equivalent quantities of 14-3-3 were used for each reaction (Fig. 8A, lower panel). These results suggest that the requirements for SDK1 recognition of 14-3-3 are the same as those for ligand binding to 14-3-3 (32). It is interesting that the mutation in Arg$^{60}$ in position 2+ relative to the phosphorylation site, had much less effect than the Lys$^{49}$-Glu mutation in position −9. These findings suggest that direct binding of SDK1 via the amphipathic groove in 14-3-3 is required for subsequent phosphorylation at Ser$^{58}$.

An SDK Is Associated with 14-3-3 and Is Activated by Exogenous DMS. Leading to 14-3-3 Phosphorylation in Situ—14-3-3 proteins were purified together with SDK1 at the first purification step on ion-exchange chromatography. To confirm the interaction between 14-3-3 and SDK1, we tested the ability of various 14-3-3 Abs to precipitate SDK activity from cytosolic extract. The PAN, $\zeta$, and $\tau$ Abs raised against different epitopes of 14-3-3 isoforms were utilized. These Abs detected endogenous 14-3-3 $\beta$, $\zeta$, and $\tau$ found in 3T3 cell lysates (data not shown). SDK activity was co-immunoprecipitated by PAN Ab or $\zeta$ Ab as indicated by phosphorylation of exogenous 14-3-3 $\zeta$ in the presence of DMS (Fig. 9A). In contrast, $\tau$ Ab did not precipitate SDK activity together with 14-3-3, although Western blot analysis revealed a similar level of 14-3-3 proteins in all immunoprecipitates (data not shown). These results indicate a specific association of an SDK with certain isoforms of 14-3-3. It is reasonable to conclude that SDK1 is probably associated with 14-3-3 $\zeta$ in situ, since the $\zeta$ Ab is isoform-specific and cross-reacts only with 14-3-3 $\zeta$ but not with 14-3-3 $\beta$, $\eta$, or $\tau$.

In order to investigate whether SDK1 is activated by DMS and subsequently phosphorylates 14-3-3 in situ, 14-3-3 proteins were immunoprecipitated from DMS-treated fibroblasts. Cells were labeled with $^{32}$Porthophosphate in serum-free medium, and challenged with 0, 2, or 10 $\mu$M DMS in ethanol, or 200 nM TPA, for 20 min. These treatments did not affect cell viability. Phosphorylated 14-3-3 proteins were immunoprecipitated with PAN Ab. Western blotting analysis indicated equal recovery of 14-3-3 proteins after all treatments (Fig. 9B, right, lanes 2–5). Autoradiography of the same Western blot clearly revealed DMS-induced phosphorylation of a 28-kDa band corresponding to 14-3-3 (Fig. 9B, left, lanes 3 and 4). In contrast, 14-3-3 was not phosphorylated in cells treated with vehicle control or TPA (Fig. 9B, left, lanes 2 and 5, respectively). These results indicate that DMS activates an SDK, leading to phosphorylation of 14-3-3 in situ. In summary, an SDK is associated with 14-3-3 $\zeta$ in situ, and is activated by DMS to subsequently phosphorylate 14-3-3 in intact cells. We propose that the SDK activated in situ to phosphorylate 14-3-3 is most likely SDK1, since this is the only SDK known to phosphorylate 14-3-3 in vitro.

**DISCUSSION**

We partially purified a sphingosine-dependent protein kinase, SDK1, that is activated by Sph or DMS but not by other lipids, and specifically phosphorylates certain isoforms of 14-3-3 protein in vitro. Sph-dependent phosphorylation of endogenous 14-3-3 proteins was previously detected in a crude system, suggesting that 14-3-3 proteins are major substrates for
Fig. 9. Association of 14-3-3 with SDK activity, and induction of in situ 14-3-3 phosphorylation in Balb/c 3T3(A31) cells incubated with DMS. Panel A, cytosolic extracts derived from 3T3(A31) cells were immunoprecipitated with PAN Ab which detects all 14-3-3 isoforms (lanes 1–4), α Ab specific to 14-3-3 ζ (lanes 5–8), or γ Ab which detects 14-3-3 ζ and γ (lanes 9–12). SDK1 activity in the resulting immunocomplexes was determined in the absence (−) or presence (+) of 120 nM 14-3-3 ζ, and of 100 μM DMS delivered in 0.5% octyl-β-glucoside as indicated at the top, and in the presence of [γ-32P]ATP as described under "Experimental Procedures." Reaction products were analyzed by SDS-PAGE followed by autoradiography. Arrow, position of 14-3-3 ζ. Positions of molecular weight markers are indicated at left. Panel B, effect of exogenous DMS on phosphorylation of endogenous 28-kDa protein corresponding to 14-3-3. Confluent Balb/c 3T3(A31) fibroblasts were metabolically labeled with [32P]orthophosphate in serum-free medium and challenged with DMS or TPA as described under "Experimental Procedures." Cells were challenged with vehicle 0.02% ethanol (lanes 1 and 2) without DMS as negative control, 2 μM DMS (lane 3), 10 μM DMS (lane 4), or 200 μM TPA for 20 min. Cytosolic extracts were prepared from these cells, and 14-3-3 proteins were immunoprecipitated with PAN Ab. The immunoprecipitated fractions were resolved on SDS-PAGE followed by Western blotting utilizing PAN Ab followed by autoradiography. Lane 1, cell extract without immunoprecipitation. Panel B, right, Western blot of the immunoprecipitated fractions. The strong heterogeneous bands in lanes 2–5 were immunoglobulin detected by secondary Ab after blotting. Panel B, left: autoradiography of the Western blot shown in panel B, right.

Fig. 10. Crystal structure of 14-3-3. Panel A, a ribbons representation of 14-3-3 ζ dimer viewed down the molecular 2-fold axis and rotated 90° with 9 anti-parallel helices as cylinders that form a ligand binding groove. Helix 3 of one monomer (blue) interacts with helix 1 of the second monomer (gray). SDK1 phosphorylation site is localized on the longest helix, helix 3, of one monomer (blue); Ser28 (red) is indicated. Two point mutations located on the opposite face of the helix, Arg60-Glu (green) at position +2 and Lys49-Glu (yellow) at position −9, are indicated. Panel B, 90° rotated view with enlargement of dimer interface showing that SDK1 phosphorylation site Ser28 is localized on the longest helix, helix 3, on one monomer (blue). Note that Ser28 is buried in the dimer interface forming the shortest distance from helix 1 of the opposite (gray) molecule by association with Arg18 which contributes salt bridges on the periphery.

cellular SDK activities. Addition of DMS to Balb/c 3T3(A31) fibroblasts stimulates phosphorylation of 14-3-3 proteins, and SDK activity is associated with endogenous 14-3-3 ζ; therefore, SDK1 may also phosphorylate 14-3-3 proteins in intact cells. The failure of SDK1 to phosphorylate PDI and calreticulin, endogenous substrates of other types of SDKs, indicates the existence of several other SDKs with distinct substrate specificity. Thus, several different kinases, including SDK1, other SDKs, and certain tyrosine kinases, may mediate signal transduction induced by Sph and DMS (2, 17, 18, 20).

SDK1 phosphorylates the η, β, and ζ isoforms of 14-3-3 at Ser adjacent to Trp in the sequence Arg-Arg-Ser-Trp-Arg. 14-3-3 proteins have consensus sequences for phosphorylation by several protein kinases (41). The site phosphorylated by SDK1 fits the consensus motif for phosphorylation by PKA and PKC (41); however, PKA fails to phosphorylate 14-3-3-3 (40), and PKC phosphorylates a different site that is conserved in all isoforms of 14-3-3 (η, β, ζ, γ, and σ) (40, 42). 14-3-3 β and ζ isolated from sheep brain are phosphorylated on Ser184 in the site Ser-Pro-Glu-Lys (44), a consensus sequence motif of proline-dependent protein kinase (41). Casein kinase I phosphorylates 14-3-3 ζ and γ on residue 233 (45, 46). A breakpoint cluster region protein (Bcr) phosphorylates Ser, and Bcr-Abl phosphorylates Ser and Tyr, on 14-3-3 γ in vitro, but the phosphorylated residues have not been mapped (47).

The crystal structure of 14-3-3 at 2.9-Å resolution indicates a dimer, and each monomer contains nine helices (Fig. 10A) (43).
The longest helix, helix 3, interacts with helix 1 of the opposite monomer and contributes to the dimer interface as well as the ligand binding groove. Many residues located in this helix are invariant among vertebrates, yeast, and plants (43). Mutagenesis and structural studies indicate that the surface of helix 3 that faces helix 5 forms the pocket in 14-3-3 that binds to phosphopeptide ligands of the sequence Arg-Ser-X-pSer-X-Pro (32, 43, 48, 49). However, the residue (Ser58 of 14-3-3ζ) that is phosphorylated by SDK1 in helix 3 of one monomer faces away from the ligand-binding pocket and toward the other monomer in the 14-3-3 dimer (Fig. 10B; Ser shown in red). This Ser is buried in the dimer interface, lying just 5.8 Å from a conserved Arg (Arg18 in 14-3-3ζ) of the other monomer. After phosphorylation, a negative charge on phosphoserine 58 may interact with the positive charge on the side chain of Arg18, resulting in dimer stabilization. Thus, the newly phosphorylated 14-3-3 monomer may sequester other 14-3-3 monomers, preventing monomer functions such as interaction with inactive Raf (50). The possibility described here for 14-3-3ζ may also apply to 14-3-3β and η, because the key residues of the dimer interface are conserved across the 14-3-3 family (43, 49).

The location of the SDK1 phosphorylation site in the dimer interface of 14-3-3ζ also raises the question of how SDK1 phosphorylates this buried residue. Although the recombinant 14-3-3 used during SDK1 purification may be partly monomeric, the phosphorylation of endogenous 14-3-3 proteins suggests that SDK1 or Sph can promote either dimer dissociation or conformational changes that expose the phosphorylation site. PKC may not induce such conformational changes, because PKC fails to phosphorylate the SDK1 site in native 14-3-3 but does phosphorylate this site in a peptide substrate (40).

All protein kinases interact via their catalytic pocket with the phosphoaccepting hydroxyamino acid, as well as with several specificity conferring residues that flank the phosphoacceptor site (51). Substrate recognition by SDK1 was investigated using mutants Lys49-Glu (Fig. 10B, yellow) and Arg60-Glu (green). Lys49, which is located nine amino acids (2.5 helix turns) from the phosphorylation site, facing the opposite side of helix 3 (43), had a surprisingly large effect on phosphorylation by SDK1. On the other hand, Arg60-Glu mutation, located 2 amino acids C-terminal to the phosphorylation site, reduced phosphorylation by only 50%, suggesting that the positive charge of Arg60 has little effect on SDK1 activity. Lys49 faces the proposed 14-3-3 ligand binding groove (32). The requirement for a functional binding groove in 14-3-3, and the lack of a strong requirement for a residue close to the phosphorylation site, suggest that the molecular interactions mediating binding of SDK1 to 14-3-3 are not typical of most protein kinases and their substrates.

Sph is generated from sphingolipid degradation in the plasma membrane, and can be transferred to other intracellular membrane compartments. However, Sph traffic into the soluble phase of the cytoplasm has not been investigated. The majority of SDK1 activity is in the cytosol, but it is not clear whether the Sph that activates SDK1 is in the membrane or cytosol. SDK1 may be activated by translocation to the membrane, since 14-3-3ζ is associated with SDK1 in situ, and partially purified membrane SDKs phosphorylate 14-3-3ζ (21). In addition, 14-3-3 proteins, which are found in cytosol, are also associated with the plasma membrane (52), and can promote translocation of kinases to different cellular compartments upon stimulation with agonists. For example, serum stimulation translocates KSR-14-3-3ζ complex from cytosol to membrane, resulting in association with Raf-1 and activation (29). In contrast, the η and τ isoforms promote translocation from cytoskeleton to cytosol of A20 and PKC θ, respectively (53, 54).

The τ isoform inhibits PKC θ by preventing its translocation to membrane (54). Because SDK1 is associated with 14-3-3, association of 14-3-3 with membrane could also provide a means for activation of SDK1 by membrane-bound Sph.

We used DMS to demonstrate Sph-dependent phosphorylation of 14-3-3 in situ, since in fibroblasts this Sph derivative cannot be phosphorylated or modified to create other potential kinase activators. DMS and Sph are found naturally in cells, and enzymes that convert Sph to DMS have been detected in tissues, suggesting that DMS is a physiological activator (15, 16, 55). A relatively low concentration of exogenous DMS (2 μM) induced rapid phosphorylation of 14-3-3 in intact cells. The rapid penetration of Sph or DMS into platelets (56) and the fact that 80% of DMS is incorporated in A31 cells within 5 min (data not shown) suggest an intracellular activation of SDK1 by Sph or DMS. Extracellular agonists such as platelet-derived growth factor and insulin-like growth factor, which increase Sph levels within the cell, may also stimulate 14-3-3 phosphorylation. Further studies are needed to correlate phosphorylation of η, β, and ζ isoforms of 14-3-3 (SDK1 substrates) with the increase of intracellular Sph levels induced by growth factors. Such studies would shed light on the regulatory role of Sph and SDK1 activity in intracellular signaling via modulation of the function of specific 14-3-3 isoforms.

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REFERENCES

1. Hakomori, S. (1990) J. Biol. Chem. 265, 18713–18716
2. Spiegel, S., and Milstein, S. (1995) J. Membr. Biol. 146, 225–237
3. Hannun, Y. A., and Linardie, C. M. (1993) Biochem. Biophys. Acta 1154, 223–236
4. Merrill, A. H., and Stevens, V. L. (1989) Biochim. Biophys. Acta 1010, 131–139
5. Spiegel, S., Foster, D., and Kolesnick, R. N. (1996) Curr. Opin. Cell Biol. 8, 159–167
6. Hannun, Y. A., Loomis, C. R., Merrill, A. H., Jr., and Bell, R. M. (1986) J. Biol. Chem. 261, 12894–12899
7. Igarashi, Y., Hakomori, S., Toyokuni, T., Dean, B., Fujita, S., Sugimoto, M., Ogawa, T., El-Ghendy, K., and Racker, E. (1989) Biochemistry 28, 6796–6800
8. Jefferson, A. B., and Schulman, H. (1988) J. Biol. Chem. 263, 15241–15244
9. Arnold, R. S., and Newton, A. C. (1991) Biochemistry 30, 7747–7754
10. McDonald, O. B., Hannun, Y. A., Reynolds, C. H., and Sahyoun, N. (1991) J. Biol. Chem. 266, 21773–21776
11. Igarashi, Y., Kitamura, K., Toyokuni, T., Dean, B., Fenderson, B., Ogawa, T., and Hakomori, S. (1990) J. Biol. Chem. 265, 5385–5389
12. Abdel-Ghany, M., Osauky, M., Igarashi, Y., Hakomori, S., Shalloway, D., and Racker, E. (1992) Biochim. Biophys. Acta 1137, 349–355
13. Coroneos, E., Martinez, M., McKenna, S., and Kester, M. (1995) J. Biol. Chem. 270, 23305–23309
14. Ohita, H., Sweeney, E. A., Masamune, A., Yatomi, Y., Hakomori, S., and Igarashi, Y. (1995) Cancer Res. 55, 691–697
15. Igarashi, Y., and Hakomori, S. (1989) Biochem. Biophys. Res. Commun. 164, 1411–1416
16. Mano, N., Oda, Y., Yamada, K., Asakawa, N., and Katayama, K. (1997) Anal. Biochem. 244, 291–300
17. Seufferlein, T., and Rozengurt, E. (1994) J. Biol. Chem. 269, 27610–27617
18. Blakesley, V. A., Beiter-Johnson, D., Van Brocklyn, J. R., Rani, S., Shen-Orr, Z., Stannard, B. S., Spiegel, S., and LeRoith, D. (1997) J. Biol. Chem. 272, 16211–16215
19. Zhang, H., Buckley, N. E., Gibson, K., and Spiegel, S. (1990) J. Biol. Chem. 265, 76–81
20. Pushkareva, M. Yu., Khan, W. A., Aleskenso, A. V., Sahyoun, N., and Hannun, Y. A. (1992) J. Biol. Chem. 267, 15246–15251
21. Meguidish, T., White, T., Takio, K., Titani, K., Igarashi, Y., and Hakomori, S. (1992) Biochem. Biophys. Res. Commun. 187, 619–628
22. Morrison, D. (1995) Science 266, 56–57
23. 9. Arnold, R. S., and Newton, A. C. (1991) Biochim. Biophys. Acta 1137, 619–628
30. Datta, S. R., Dudek, H., Tao, X., Masters, S., Fu, H., Gotoh, Y., and Greenberg, M. E. (1997) *Cell* **91**, 231–241
31. Radunz, H.-E., Devant, R. M., and Eiermann, V. (1988) *Liebigs Ann. Chem.* 1103–1105
32. Zhang, W., Wang, H., Liu, D., Liddington, R., and Fu, H. (1997) *J. Biol. Chem.* **272**, 13717–13724
33. Rodriguez-Pena, A., and Rozengurt, E. (1984) *Biochem. Biophys. Res. Commun.* **120**, 1053–1059
34. Lavie, Y., Blusztajn, J. K., and Liscovitch, M. (1994) *Biochim. Biophys. Acta* **1220**, 1053–1059
35. Hannun, Y. A., Loomis, C. R., and Bell, R. M. (1985) *J. Biol. Chem.* **260**, 10039–10043
36. Litchfield, D. W., Lozeman, F. J., Piening, C., Sommercorn, J., Takio, K., Walsh, K. A., and Krebs, E. G. (1990) *J. Biol. Chem.* **265**, 7638–7644
37. Boyle, W. J., van der Geer, P., and Hunter, T. (1991) *Methods Enzymol.* **201**, 110–149
38. Watanabe, M., Isobe, T., Ichimura, T., Kuwano, R., Takahashi, Y., Kondo, H., and Inoue, Y. (1994) *Mol. Brain Res.* **25**, 113–121
39. Perego, L., and Berruti, G. (1997) *Mol. Reprod. Dev.* **47**, 370–379
40. Toker, A., Sellers, L. A., Amess, B., Patel, Y., Harris, A., and Aitken, A. (1992) *Eur. J. Biochem.* **206**, 453–461
41. Kennelly, P. J., and Krebs, E. G. (1991) *J. Biol. Chem.* **266**, 15555–15558
42. Aitken, A., Howell, S., Jones, D., Madrazo, J., Martin, H., Patel, Y., and Robinson, K. (1995) *Mol. Cell. Biochem.* **149/150**, 41–49
43. Liu, D., Bienkowski, J., Petosa, C., Collier, R. J., Fu, H., and Liddington, R. (1995) *Nature* **376**, 191–194
44. Aitken, A., Howell, S., Jones, D., Madrazo, J., and Patel, Y. (1995) *J. Biol. Chem.* **270**, 5706–5709
45. Rommel, C., Radziwill, G., Lovric, J., Noeldeke, J., Heinicke, T., Jones, D., Aitken, A., and Moelling, K. (1996) *Oncogene* **12**, 609–619
46. Dubois, T., Rommel, C., Howell, S., Steinhussen, U., Soneji, Y., Morrice, N., Moelling, K., and Aitken, A. (1997) *J. Biol. Chem.* **272**, 28882–28888
47. Reuther, G. W., Fu, H., Cripe, L. D., Collier, R. J., and Pendergast, A. M. (1994) *Science* **266**, 129–133
48. Yaffe, M. B., Rittinger, K., Volinia, S., Caron, P. R., Aitken, A., Jeffers, H., Gamblin, S. J., Smerdon, S. J., and Cantley, L. C. (1997) *Cell* **91**, 961–971
49. Xiao, B., Smerdon, S. J., Jones, D. H., Dodson, G. G., Soneji, Y., Aitken, A., and Gamblin, S. J. (1995) *Nature* **376**, 188–191
50. Luo, Z., Zhang, X., Rapp, U., and Avruch, J. (1995) *J. Biol. Chem.* **270**, 23681–23687
51. Taylor, S. S., Radzio-Andzelm, E., and Hunter, T. (1995) *FASEB J.* **9**, 1255–1266
52. Martin, H., Rostas, J., Patel, Y., and Aitken, A. (1994) *J. Neurochem.* **63**, 2229–2235
53. Vincenz, C., and Dixit, V. M. (1996) *J. Biol. Chem.* **271**, 20029–20034
54. Miller, N., Liu, Y.-C., Collins, T. L., Bonnefoy-Berard, N., Baier, G., Isakov, N., and Altman, A. (1996) *Mol. Cell. Biol.* **16**, 5782–5791
55. Kobayashi, T., Mitrauf, K., and Goto, I. (1988) *Eur. J. Biochem.* **172**, 747–752
56. Yatomi, Y., Ozaki, Y., Satoh, K., Kume, S., Ruan, F., and Igarashi, Y. (1997) *Biochem. Biophys. Res. Commun.* **231**, 848–851