Wheeler R. B., Sharp J. D., Schultz R. A., Joslin J. M., Williams R. E. and Mole S. E. (2002) The gene mutated in variant late-infantile neuronal ceroid lipofuscinosis (*CLN6*) and in nclf mutant mice encodes a novel predicted transmembrane protein. *Am. J. Hum. Genet.* **70**, 537–542.

Yasuda Y., Kageyama T., Akamine A., Shibata M., Kominami E., Uchiyama Y. and Yamamoto K. (1999) Characterization of new fluorogenic substrates for the rapid and sensitive assay of cathepsin E and cathepsin D. *J. Biochem.* **125**, 1137–1143.

Journal of Biomolecular Techniques
14:191–196 © 2003 ABRF

Improvement of Automatic In-Gel Digestion by *in Situ* Alkylation of Proteins

Takeshi Yokono,[a] Reiko Mineki,[b] Hikari Taka,[b] Hiroto Kotaniguchi,[a] Kimie Murayama[b]

[a]M & S Instruments Trading Inc., Technical Division; [b]Juntendo University School of Medicine, Central Laboratory of Medical Science, Tokyo, Japan

We have recently improved the automation of an in-gel digestion system, DigestPro 96, using *in situ* alkylation of proteins with acrylamide, conducted during one-dimensional (1D) SDS-PAGE. The improved method included the processes of destaining, dehydration, trypsin digestion, and extraction but excluded the reduction and alkylation steps following staining of proteins with CBB. The extracted peptide mixtures were directly loaded onto a micro C18 LC column of the mass spectrometer. The resultant spectra were processed with "Mascot" search engine to estimate the sequence coverage of the bovine serum albumin (BSA). The original method, designed for Laemmli 1D SDS gel applications, consisted of reduction and post-alkylation with iodoacetamide, which produced carboxyamidemethyl (CAM; $-S-CH_2CONH_2$) derivatives. The original method also included a desalting step essential for mass spectrometry, especially matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. We compared the original and improved methods using BSA (3 pmol loaded to the gel, one third of digested peptide mixture injected into LC-MS). The original method yielded both CAM and propionicamide (PAM; $-S-CH_2CH_2CONH_2$) derivatives. The source of PAM derivatives is the unpolymerized acrylamide formed during electrophoresis. The sequence coverage of CAM derivatives of BSA by the original method was 10% with desalting and 19% without desalting. The sequence coverage of PAM derivative by the improved method was 32%. Our results clearly show the advantage of our improved automated in-gel digestion method for *in situ* PAM alkylated protein with respect to peptide recovery, compared with the original method with CAM post-alkylation.

KEY WORDS: proteomics, protein, in-gel digestion, *in situ* alkylation, automation.

ADDRESS CORRESPONDENCE AND REPRINT REQUESTS TO: Takeshi Yokono, Technical Division, M&S Instruments Trading Inc., 113, Yarai-cho, Shinjuku-ku, Tokyo, 162-0805, Japan (email: tyokono@technosaurus.co.jp).

In-gel digestion of protein is a widely used procedure in proteomics studies. However, the procedure is tedious and susceptible to contamination. Automation of the procedure[1,2] has simplified the assay and reduced potential contamination. The automated protocol comprises several steps including destaining, reduction, alkylation (using $-S-CH_2CONH_2$ [carboxyamidemethyl; CAM]), digestion and extraction of the tryptic peptides. Among the steps of the automated protocol, reduction and alkylation are performed so that the cysteinyl peptide yields CAM derivatives. The cysteinyl residues, however, could also react with unpolymerized acrylamide monomer present in the electrophoresis gel. The reaction occurs during the electrophoresis run and produces propionic amide (PAM) derivatives, which are stable byproducts. The formation of PAM sometimes hinders fragment search procedure that expects CAM derivatives to be present. Mineki et al.[3] have considered this by-product (PAM derivative) to be the major and sole "S"-containing peptide for mass spectrometry (MS) analysis. The digestion method described by Mineki et al.[3] does not include reduction, alkylation, and desalting procedures. In this article we report improvement of the automated in-gel digestion protocol.

MATERIALS AND METHODS

Material

Molecular marker proteins of a wide molecular mass range (6.5–200 kDa) were obtained from Bio-Rad (Hercules, CA). The marker was run with one-dimensional sodium dodecyl sulfate (1D SDS) electrophoresis so that a band of 3 pmol of bovine serum albumin (BSA) was present in one lane.

In Situ Alkylation and 1D SDS-PAGE

Except for testing the protocol recommended by the supplier of the instrument, the protein was *in situ* alkylated during the electrophoresis run using the method described by Mineki et al.[3] Briefly, sample were added to sample buffer containing 10% glycerol, 2% SDS, 0.1 M dithioerythritol (DTE) and 0.0025% bromphenol blue. The sample was then incubated at 95°C for 3 min. After cooling the sample to room temperature, a 4.7-μL aliquot of 30% acrylamide solution (20 μmol) was added. Then, the sample solution was loaded onto a gel and run.

A 12-lane, 130 × 130 × 1-mm SDS gel was prepared with 10% separation gel and 4% stacking gel acrylamide containing 0.87% and 0.35% piperazine diacrylamide, respectively. The gel was allowed to stand overnight at 4°C, and then pre-run at 20 mA for 1 h before loading the sample. After loading, proteins were concentrated in the stacking gel at 5 mA for 50 min and were resolved in the separation gel at 20 mA for 2 h. Staining was performed using CBB R-350 (Pharmacia & Upjohn Diagnostics, Uppsala, Sweden) or Plus One silver nitrate reagent free of glutardialdehyde (Pharmacia & Upjohn) according to the method of Mineki et al.[3]

In-Gel Digestion

The BSA band was cut and processed by an automatic in-gel digestion instrument (DigestPro96, Intavis AG, Koeln, Germany). The original method supplied by the manufacturer is shown in Figure 1. A gel run by Laemmli's method[4] was used for this protocol. The improved method for CBB-stained gel is shown in Figure 2, and the modified method of silver-stained gel in Figure 3. The manual digestion method employed was the one described by Mineki et al.[3]

Mass Spectrometry Analysis

Mass spectrometry analysis was performed with AB-QSTAR pulsar *i* hybrid (Applied Biosystems, Fram-

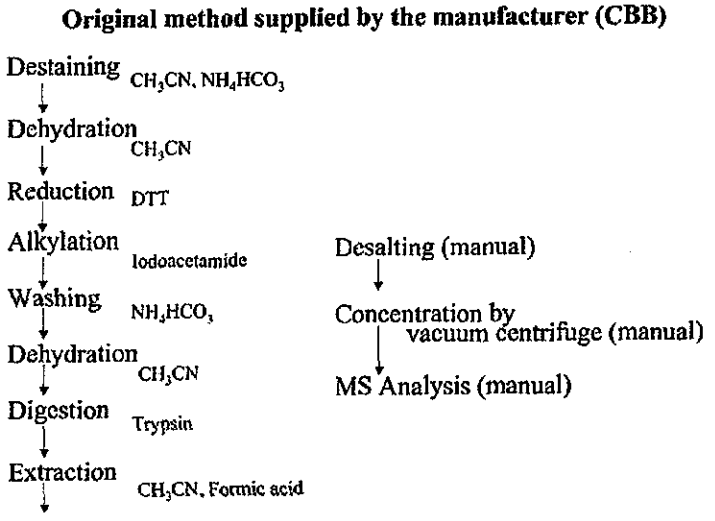

FIGURE 1

Cysteinyl residues of the protein were reduced and alkylated with CAM before it was digested with trypsin. The original protocol (Digest1.xcp) includes reduction and alkylation processes. The method is for CBB-stained gel.

ingham, CA) combined with a microliquid chromatograph (Magic 2002; Michrom Bioresources, Abum, CA) equipped with 0.2-mm ID × 50 mm *Magic C18* column. In order to evaluate the improved method of *DigestPro96*, the sequence coverage of BSA by MS/MS was obtained with automatic search using "Mascot" software (Matrix Science, London, UK). The sequence coverage was calculated in the "Mascot" software as (total number of the amino acid residues of identified sequences) / (total number of amino acid residues of BSA).

RESULTS AND DISCUSSION

Comparison of Sequence Coverage of BSA Between the Original and Improved Methods on 1D SDS-PAGE

The BSA sequence coverage analysis by Mascot software was performed using CAM derivatives and also using passively coexisting PAM derivatives. The coverage was 10% for CAM derivatives and 16% for PAM derivatives. PAM derivatives were formed during electrophoresis and are shown in Table 1. The sequence coverage of the method, which did not include desalting with Zip Tip, was 19% with CAM derivatives and 27% with PAM derivatives. These results indicate that the recovery of peptides from the desalting column was 50–59%. Small-sized hydrophilic peptides seemed to pass through the Zip Tip while the large hydrophobic peptides were retained on the column (Figures. 4 and 5). This resulted in a marked decrease in the recovery of peptides.

With regard to the improved method using *in situ* alkylation with acrylamide, the sequence of BSA identified by Mascot software was limited to PAM derivative. The method provided 32% coverage of BSA. Compared with the original method, three processes were omitted in the improved method: reduction, alkylation of stained proteins before in-gel trypsin digestion, and desalting before mass spectrometric analysis.

Comparison of Sequence Coverage of BSA Between Manual and Automated Protocol (Performed by DigestPro96)

In the next step, we compared the BSA sequence coverage obtained by the manual protocol with that obtained by the improved (automated) method. Both protocols employed *in situ* alkylation with acrylamide during electrophoresis. The sequence coverage of the manual protocol was 48% and that of the automated instrument was 32%. The latter represents

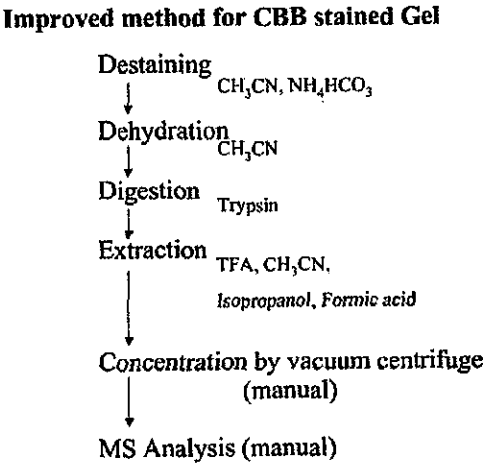

FIGURE 2

Cysteinyl residues of protein were alkylated *in situ* with acrylamide during electrophoresis. In this automated protocol (improved method) for proteins stained by CBB, the reduction and alkylation processes were omitted. The desalting step was also omitted because of the deletion of the alkylation process.

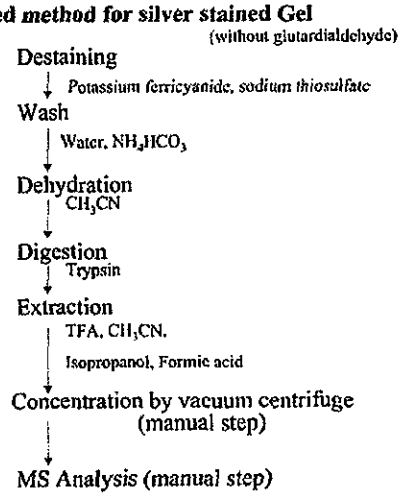

FIGURE 3

Cysteinyl residues of protein were alkylated during the electrophoresis run. In the automated protocol (improved method) for proteins stained by silver nitrate, the reduction and alkylation processes were deleted. The desalting step was also omitted because of the deletion of the alkylation process.

66% of the sequence coverage of the manual protocol. Taking into consideration the relatively small amount of protein (3 pmol) that was digested, our results indicate a significant improvement by the automated protocol.

Differences in Sequence Coverage of BSA (3 pmol) Between CBB and Silver Nitrate Staining

Next we compared the sequence coverage of BSA stained with CBB with the coverage obtained with sil-

TABLE 1

Comparison of Original Method and Improved Method

	Original (Post-electrophoresis alkylation)		Improved (*In situ* alkylation)
	With desalting	No desalting	
CAM derivatives	10%	19%	—
PAM derivatives	16%	27%	32%

Data are sequence coverage of 3 pmol BSA stained with CBB. One third of the extract was injected for mass spectrometry.

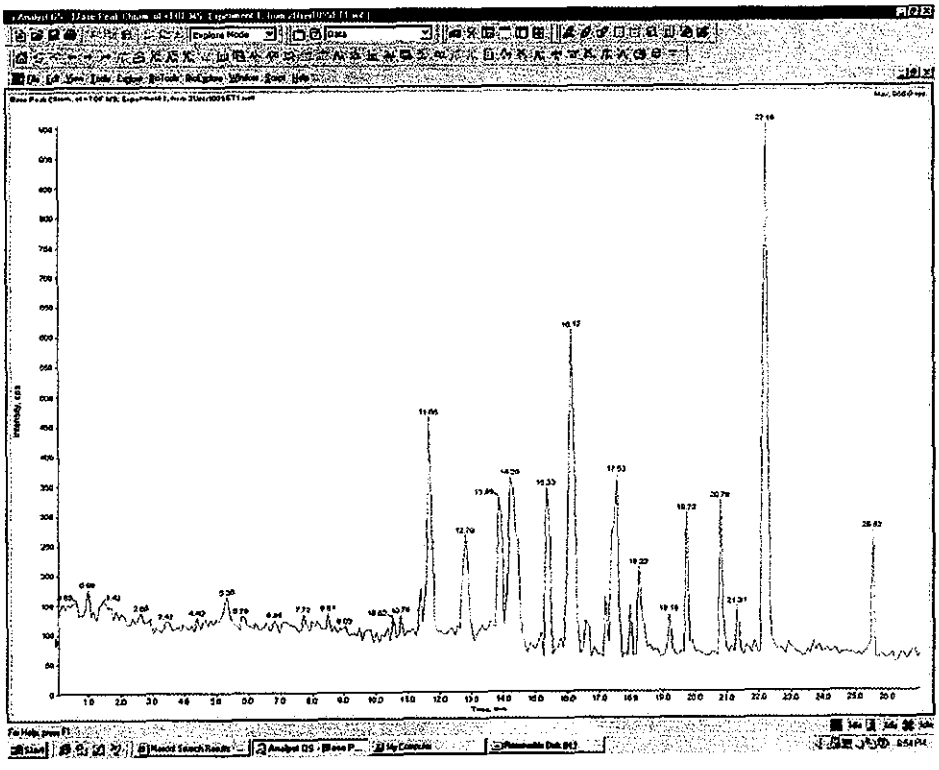

FIGURE 4

Mass spectrometric total ion current of the sample processed through assays that included desalting. The samples were processed by the original method shown in Table 1.

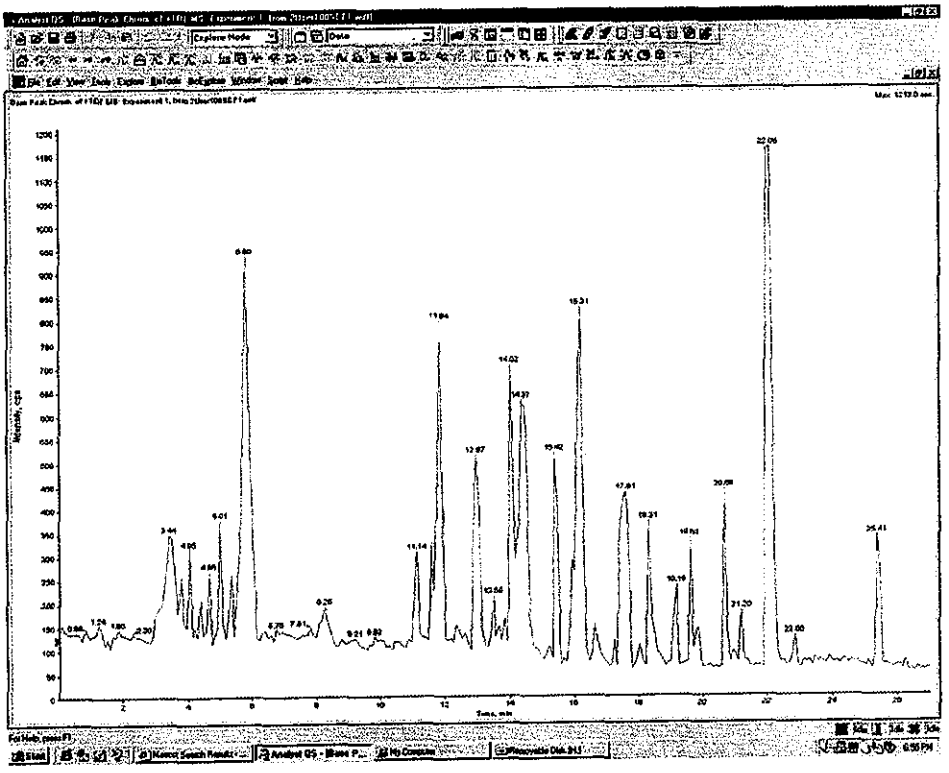

FIGURE 5

Mass spectrometric total ion current of the sample processed through assays that included no desalting. The samples were processed by the original method shown in Table 1.

ver nitrate reagent (Table 2). The protocol used in both studies was the automated protocol employing *in situ* alkylated proteins.

The sequence coverage of BSA stained with silver nitrate was 21%, which represented 66% of the coverage of BSA stained with CBB. Although the sequence coverage on silver staining was lower, the

TABLE 2

Comparison of BSA Sequence Coverage and Detection Limits Between CBB-Stained and Silver-Stained (Without Glutardialdehyde) Gel in the Automated Protocol

	Loaded amount to gel	Loaded amount to MS[a]	BSA sequence coverage
SEQUENCE COVERAGE			
CBB stain (n = 5)	3 pmol	1 pmol	32%
Silver stain (n = 2)	3 pmol	1 pmol	21%
DETECTION LIMIT FOR SILVER STAINED GEL			
Silver stain (n = 2)	3 pmol	1.5 pmol	21%
Silver stain (n = 2)	750 fmol	750 fmol	11%
Silver stain (n = 2)	375 fmol	375 fmol	Not detected

[a]Expressed as amount of protein loaded to gel.

improved protocol with silver staining was still considered to be usable.

We also analyzed the detection limit of the modified automated protocol for silver staining (Table 2). The detection limit was 750 fmol with a BSA sequence coverage of 11%.

CONCLUSION

In this report, we describe the use of a modified automated in-gel digestion protocol for proteins using *in situ* alkylation with acrylamide. The sequence coverage of BSA using the modified protocol was 32%, which was threefold higher than that of the original method (10%). The improvement is probably due to PAM derivatization during electrophoresis, elimination of reduction and alkylation processes before in-gel digestion with trypsin, and deletion of the desalting process before mass spectrometric analysis. The sequence coverage of BSA by the improved protocol exhibited 66% value of the corresponding manual protocol in the digestion of the 3-pmol sample. Thus, the automated protocol seems to be useful for routine protein digestion. The protocol of the silver-stained gel showed 21% sequence coverage, which corresponded to 66% of the value of CBB-stained gel. The detection limit of the protocol was 750 fmol.

REFERENCES

1. Houthaeve T, Gausepohl H, Ashman K, Nillson T, Mann M. Automated protein preparation techniques using a digest robot. *J Protein Chem* 1997;16:343–348
2. Ashman K, Houthaeve T, Clayton J, et al. The application of robotics and mass spectrometry to the characterisation of the *Drosophila melanogaster* indirect flight muscle proteome. *Lett Peptide Sci* 1997;4:57–65
3. Mineki R, Taka H, Fujimura T, Kikkawa M, Shindo N, Murayama K. *In situ* alkylation for identification of cysteinyl residues in proteins during one- and two-dimensional sodium dodecyl sulphate–polyacrylamide gel electrophoresis. *Proteomics* 2002;2:1672–1681
4. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970;227:680–685.

Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical and Biophysical Research Communications 307 (2003) 589–594
BBRC
www.elsevier.com/locate/ybbrc

A sphingosine-dependent protein kinase that specifically phosphorylates 14-3-3 (SDK1) is identified as the kinase domain of PKCδ: a preliminary note☆,☆☆

Akikazu Hamaguchi,[a,1,2] Erika Suzuki,[a,1] Kimie Murayama,[b,1] Tsutomu Fujimura,[b] Toshiyuki Hikita,[a,3] Kazuhisa Iwabuchi,[a,4] Kazuko Handa,[a] Donald A. Withers,[a] Shane C. Masters,[c] Haian Fu,[c] and Senitiroh Hakomori[a,*]

[a] *Division of Biomembrane Research, Pacific Northwest Research Institute, and Department of Pathobiology, University of Washington, Seattle, WA, USA*
[b] *Central Laboratory of Medical Science, Juntendo University Medical School, Tokyo, Japan*
[c] *Department of Pharmacology, Emory University School of Medicine, Atlanta, GA, USA*

Received 19 May 2003

Abstract

A specific protein kinase that phosphorylates Ser60, Ser59, or Ser58 of 14-3-3β, η, or ζ, respectively, only in the presence of sphingosine (Sph) or *N,N*-dimethyl-Sph (DMS), was termed "sphingosine-dependent protein kinase-1" (SDK1) [J. Biol. Chem. 273(34) (1998) 21834]. We have now identified SDK1 as a protein having the same amino acid sequence as in the C-terminal-half kinase domain of PKCδ, with ~40 kDa molecular mass, based on large-scale purification of a protein from rat liver, and partial sequence using three different combinations of LC-MS or LC-MS/MS with respective search engine. PKCδ did not display any SDK1 activity and PKCδ activity was inhibited by Sph and DMS. However, strong SDK1 activity, only in the presence of Sph or DMS, became detectable when PKCδ was incubated with caspase-3, which releases the ~40 kDa kinase domain.

Keywords: LC-MS/MS; Amino acid sequence; Sphingosine; *N,N*-Dimethylsphingosine; Sphingosine-dependent protein kinase; 14-3-3; Protein kinase Cδ; Caspase-3; Catalytic domain; Phosphorylation at dimer interface

Although sphingosine (Sph) [1–3] and *N,N*-dimethyl-Sph (DMS) [3] were originally found as inhibitors of conventional protein kinase C, the majority of recent studies on effects of sphingolipids on cellular function have focused on ceramide (Cer) (e.g. [4,5]) or Sph-1-phosphate (Sph-1-P) (e.g. [6,7]). However, we found previously that several proteins acting as chaperones of key signal transducers, or as modulators of ER protein function, are phosphorylated only in the presence of Sph or DMS, but not Cer or Sph-1-P [8]. Such Sph- or DMS-dependent factors include a protein kinase involved in phosphorylation of 14-3-3 at Ser60 in β, Ser59 in η, and

☆ This investigation was supported by Grant R01 CA82167 from the National Cancer Institute, National Institutes of Health (to S.H.), and by R01 GM53165 from the National Institute of General Medical Sciences, NIH (to H.F.).

☆☆ *Abbreviations:* a.a., amino acid; Cer, ceramide; DLU, digital light units; DMS, *N,N*-dimethyl-D-erythro-sphingosine; LC, liquid chromatography; MS, mass spectrometry; SDK, sphingosine-dependent protein kinase; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; Sph, D-erythro-sphingosine; Sph-1-P, D-erythro-sphingosine-1-phosphate.

* Corresponding author. Fax: 1-206-726-1212.
E-mail address: hakomori@u.washington.edu (S. Hakomori).
[1] These three authors were equal, major contributors to this study.
[2] Present address: Department of Urology, Shiga Medical University, Shiga 520-0232, Japan.
[3] Present address: Department of Pediatrics, Teikyo University School of Medicine, Tokyo 173-8605, Japan.
[4] Present address: Department of Biochemistry II, Juntendo University School of Medicine, Tokyo 113-8421, Japan.

0006-291X/03/$ - see front matter © 2003 Elsevier Science (USA). All rights reserved.
doi:10.1016/S0006-291X(03)01070-2

Ser58 in ζ isoforms, termed "sphingosine-dependent protein kinase-1" (SDK1) [9].

The chaperone or modulatory protein, 14-3-3, has received increasing attention for its interaction with and functional modification of key molecules involved in signal transduction associated with various cellular processes including proliferation, differentiation, and apoptosis [10]. SDK1 specifically phosphorylates 14-3-3 β, η, and ζ only in the presence of Sph and DMS, but not 16 other lipids tested (including Cer and Sph-1-P) [11]. However, biochemical properties of SDK1 have not yet been clearly elucidated.

Here, we describe purification of SDK1 and identify it as the C-terminal-half kinase domain of PKCδ, distinguishable from PKCδ per se.

Materials and methods

Procedure for purification of SDK1 from rat liver. One hundred fifty grams of Sprague–Dawley rat liver at one time was cut into small pieces and homogenized in Waring blender (30 s at "hi" speed, three times) in ice in a cold room, with 1000 ml of ice-cold homogenizing buffer containing 20 mM Tris–HCl, 0.5 mM EDTA, 0.5 mM EGTA, 12 mM NaF, 5% glycerol, 3 μg/ml leupeptin, 5 μg/ml aprotinin, 3 μg/ml chymostatin, 1 mM PMSF, and 1 mM DTT, pH 8.0. The homogenate was centrifuged for 30 min at 4000 rpm. The supernatant was adjusted to pH 8.0 and centrifuged for 1 h at 100,000*g*. The supernatant was filtered with 0.5 μm filter and used for six steps of chromatographic purification, guided by SDK1 activity, as indicated in Table 1.

Determination of PKCδ and SDK1 activities. PKCδ and common PKC peptide substrate RFARKGSLRQKNV were from Panvera (Madison, WI). PKCδ assay was performed according to the manufacturer's protocol. ^{32}P incorporation into the peptide was determined by adsorption on phosphocellulose membrane (P81 paper; Upstate Biotechnology, Waltham, MA), followed by washing and counting.

SDK1 activity was determined using 14-3-3β as substrate by the procedure described previously [9,12] with minor modification, including quantitative comparison of activities by use of "Phosphoimager" (Cyclone Storage Phosphor Screen; Packard Biosci., Meridian, CT). Intensities of each band were quantified by OptiQuant software, subtracted by the value for a control ^{14}C marker, and compared in terms of digital light units (DLU). Alternatively, activity of ^{32}P-phosphorylated 14-3-3β separated on SDS–PAGE was quantitated by Scion Image analysis.

Cleavage of PKCδ by caspase-3, determination of SDK1 activity in the released catalytic domain, and effect of PKCδ inhibitor. PKCδ was incubated with caspase-3 as described previously [13]. Briefly, 200 ng human recombinant PKCδ was added in the following mixture with total reaction volume 20 μl: 2 U active human recombinant caspase-3 (Gene Therapy Systems, San Diego, CA) in 25 mM Hepes, pH 7.4, 250 mM sucrose, 1 mM EDTA, and 2.5 mM DTT. Cleavage was confirmed by Western blot, using a polyclonal antibody against the carboxy terminus of PKCδ (Santa Cruz Biotech, Santa Cruz, CA).

For SDK1 activity determination of caspase-3-treated PKCδ, aliquots of the above mixture (e.g., 8.5 μl containing 85 ng PKCδ with 0.85 U caspase-3), or aliquots of untreated full-length PKCδ (8.5 μl containing 85 ng PKCδ) were incubated with 14-3-3β, Sph or DMS, and [γ-^{32}P]ATP, and quantity of ^{32}P-phosphorylated 14-3-3 was determined as described in Fig. 2 legend. In some incubation mixtures, 1–10 μM GF109203X (Alexis Biochemicals, San Diego, CA) was added as PKCδ inhibitor, as described in Fig. 4 legend.

Determination of partial amino acid sequence of SDK1 protein. As an example, 500 μl of Fr. 18 from Step 6 (MonoQ chromatography), showing high SDK1 activity, was precipitated with equal volume of 20% trichloroacetic acid, centrifuged, and washed three times with cold ethylether with centrifugation to eliminate trichloroacetic acid. The final precipitate was subjected to 1D SDS–PAGE, and the ~40 kDa band separated was manually excised by razor blade and subjected to *in gel* trypsinization, by essentially the same procedure described previously [14]. *In gel* digested products were extracted with 15% isopropanol/20% formic acid/25% CH_3CN/40% H_2O, and finally with 80% CH_3CN. All the extracts were successively dried in a single Eppendorf tube (by Speed Vac) and the residue was dissolved in 6 L ultrapure water. Three aliquots (2 μl each) were used, respectively, for amino acid sequencing by three different combinations of LC-MS or LC-MS/MS with search engine, i.e., A.i, A.ii, and B below.

A. API QSTAR Pulsar hybrid mass spectrometer system (Foster City, CA) with a micro liquid chromatograph (Magic 2002; Michrom BioResource, Auburn, CA). Detailed conditions will be described elsewhere (Hamaguchi A. et al., in prep.).

i. Micro-LC/MS (QSTAR) using the PROWL (ProFound) search engine (http://prowl.rockefeller.edu/cgi-bin.ProFound) and public domain database (NCBI) available on the Internet [15,16].

ii. The major ion peaks of the total ion chromatogram were further analyzed by LC-MS/MS using the Mascot search engine (www.matrixscience.com) [15,17,18] under the same conditions (QSTAR with Magic micro-LC) and the same database (second third of the same sample used).

B. LCQ-DECA XP (ThermoFinnigan, San Jose, CA) with a Magic micro-LC was also used with MS/MS mode (third third of the sample used). Identification of probable sequence from MS/MS data was performed using "TurboSequest" search engine (http://fields.scripps.edu/sequest) [15,19,20] and the NCBI database (in house).

Inhibition of SDK1 activity of the kinase domain released from PKCδ, by 14-3-3 peptide. Aliquots of caspase-3-released kinase domain from 85 ng PKCδ were incubated (5 min, 30 °C) with various concentrations of 14-3-3 icosapeptide YKNVVGARRSSWRVISSIEQ, previously identified as the SDK1-dependent phosphorylation site.

Table 1
Protein quantity, specific SDK1 activity, and fold purification at various steps of purification

Step		Protein (mg)	Volume (ml)	Specific SDK1 activity (DLU/mg protein)	Fold purification
0	Homogenate	69,174	1620	1.4×10^7	1
1	QFF	2720	640	1.2×10^8	8.8
2	Hydrophobic	1820	860	1.5×10^8	10.5
3	Heparin–Sepharose	498	360	4.4×10^8	31.6
4	Hydroxyapatite	23.4	64	2.9×10^9	205
5	Chromatofocusing	2.14	24	1.3×10^{10}	966
6	MonoQ	0.01	3	7.5×10^{11}	53,348

Expressed as digital light units (DLU) from "Phosphoimager" analysis (see text).

Control peptide (17 a.a.) with the sequence (Ac-YGGSA-ESS(Aib)KSEASSK(Aib)SA-CONH$_2$) (Aib, aminoisobutyric acid) was synthesized in the laboratory of Dr. Tomikazu Sasaki (Department of Chemistry, University of Washington) and kindly donated to us. The incubation mixture was assayed for SDK1 activity with 14-3-3β as substrate and DMS as activator.

Results

Purification of proteins with SDK1 activity

The yield of proteins, volume, specific SDK1 activity, and fold purification, at each step of purification, taken from one typical experiment, are shown in Table 1. About 53,000-fold purification was obtained at the MonoQ step, in comparison to initial extract; specific SDK1 activity was increased to a similar degree. However, a large number of protein bands were still present, separated by SDS–PAGE, even at the MonoQ step.

The chromatography fractions showing highest SDK1 activity by MonoQ column (Fr. 18, 19, or 20, depending on batch) showed a band with molecular mass ~40 kDa on SDS–PAGE, which was absent in other fractions having no or minimal SDK1 activity. The same molecular mass with high SDK1 activity was also shown by gel filtration (Fig. 1). Therefore, amino acid sequence analysis was performed for this band, as described in Materials and methods.

Partial sequence of the ~40 kDa band present in the fraction separated by MonoQ chromatography (Step 6)

Results of three methods with different search engines, (i) QSTAR MS with "Prowl"; (ii) QSTAR MS/MS with "Mascot"; and (iii) LCQ-DECA XP MS/MS with "TurboSequest," indicated a common sequence as shown in bold letters in Table 2. This sequence aligned with ~53% of that of PKCδ catalytic domain [21]. Non-bold letters indicate probable sequence of catalytic domain, initiated from caspase-3-cleavable site (a.a. 1–346; corresponding to a.a. 328–673 of PKCδ). The only protein having high homology with this sequence, based on search of the NCBI database with the BLAST algorithm (www.ncbi.nlm.nih.gov/blast), was rat PKCδ,

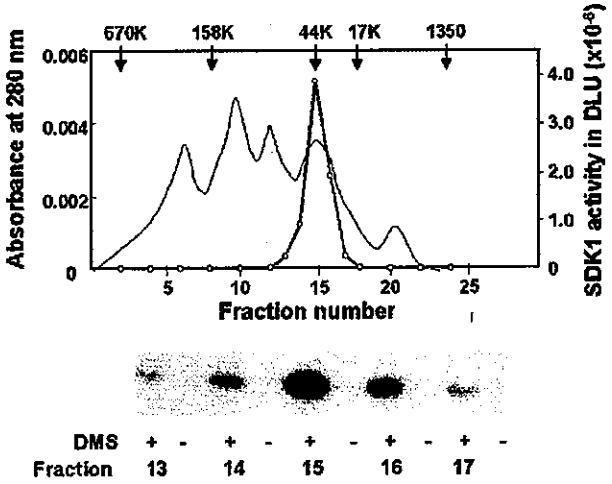

Fig. 1. Gel filtration pattern of MonoQ fraction showing major SDK1 activity. Upper panel: MonoQ chromatography fraction from step 6 of purification, showing very high SDK1 activity (either 18 or 19, depending on batch), was subjected to gel filtration through Superdex 200 10/30 column in SmartSystem (Pharmacia, Piscataway, NJ). Thin line, protein with absorbance at 280 nm. Thick line with circle, SDK1 activity determined by Phosphoimager, expressed as DLU. Lower panel: SDK1 activity of fractions 13–17 in terms of phosphorylation pattern of 14-3-3β in the absence (–) or presence (+) of DMS.

Table 2
Overall sequence of SDK1 protein identified by mass spectrometry

```
1                                                          60
NNGTYGKIWE GSNRCRLENF TFQKVLGKGS FGKVLLAELK GKERYFAIKY LKKDVVLIDD
                                *
61                                                         120
DVECTMVEKR VLALAWENPF LTHLICTFQT KDHLFFVMEF LNGGDLMFHI QDKGRFELYR
121                                                        180
ATFYAAEIIC GLQFLHGKGI IYRDLKLDNV MLDKDGHIKI ADFGMCKENI FGENRASTFC
                          **
181                                                        240
GTPDYIAPEI LQGLKYSFSV DWWSFGVLLY EMLIGQSPFH GDDEDELFES IRVDTPHYPR
241                                                        300
WITKESKDIM EKLFERDPAK RLGVTGNIRL HPFFKTINWN LLEKRKVEPP FKPKVKSPSD
301                                                346
YSNFDPEFLN EKPQLSFSDK NLIDSMDQTA FKGFSFVNPK YEQFLE
```

The sequence is aligned with 53 letters indicating probable sequence of catalytic domain, initiated from caspase-3-cleavable site (a.a. 1–346; corresponding to a.a. 328–673 of PKCd). Sequences underlined by single (*) denote ATP binding region signature; or double underlined by (**) denote Ser/Thr protein kinase active site signature.

although the homology was restricted to C-terminal-half (catalytic domain) (see Discussion).

Absence of SDK1 activity in full-length PKCδ, and its appearance associated with release of C-terminal-half kinase domain by caspase-3

Full-length PKCδ showed novel PKCδ activity with common PKC substrate requiring diacylglycerol, phosphatidylserine, and Mg^{2+}, but not Ca^{2+}. However, the kinase activity of PKCδ with the common PKC substrate RFARKGSLRQKNV was inhibited by Sph or DMS, as in conventional PKCα and β (data not shown).

Strong SDK1 activity, highly dependent on the presence of Sph or DMS, was found when PKCδ was treated with caspase-3 (Fig. 2). Full-length PKCδ did not phosphorylate 14-3-3 in the presence of DMS (Fig. 3A-a), i.e., did not show SDK1 activity. In contrast, PKCδ treated with caspase-3 showed strong SDK1 activity (Fig. 3A-b) and release of a ~40 kDa band was indicated by Western blot with antibody directed to C-terminal region of PKCδ (Fig. 3B). A similar strong SDK1 activity was observed for rat liver ~40 kDa SDK1 separated by MonoQ column chromatography, without caspase-3 treatment (see below; cf. Fig. 4A).

Inhibition of SDK1 activity and its susceptibility to PKCδ inhibitor and icosapeptide

SDK1 activities of both ~40 kDa protein present in rat liver MonoQ Fr. 18–19 and ~40 kDa protein released from PKCδ by caspase-3 were inhibited by GF109203X (general inhibitor for PKC) (Figs. 4A and B).

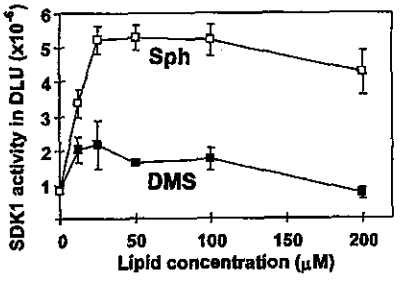

Fig. 2. Dose-dependent promoting effect of DMS and Sph on SDK1 activity of caspase-3-cleaved PKCδ, using 14-3-3β as substrate. Aliquots of incubation mixture containing 85 ng PKCδ with 0.85 U caspase-3 (or 85 ng untreated PKCδ as control), 1 μg 14-3-3β, various concentrations of DMS or Sph (shown on abscissa) (in final solvent concentration 1% ethanol, 0.05 M NaCl), 3 mM DTT, 25 μM ATP, 45 mM MgAc, 2.5 μCi [γ-^{32}P]ATP, and 26.7 mM Tris, pH 7.3 (final volume 30 μl) were incubated for 15 min at 30 °C. Concentrations of DMS or Sph in the incubation mixture are indicated on the abscissa (0–200 μM). The reaction was stopped by addition of Laemmli's buffer (4× concentrated) and heating at 95 °C for 5 min. 14-3-3 phosphorylation was analyzed by 10–12% SDS–PAGE. 14-3-3β phosphorylation was quantitated by Phosphoimager and expressed as DLU. Mean values of three experiments; SD shown by bar.

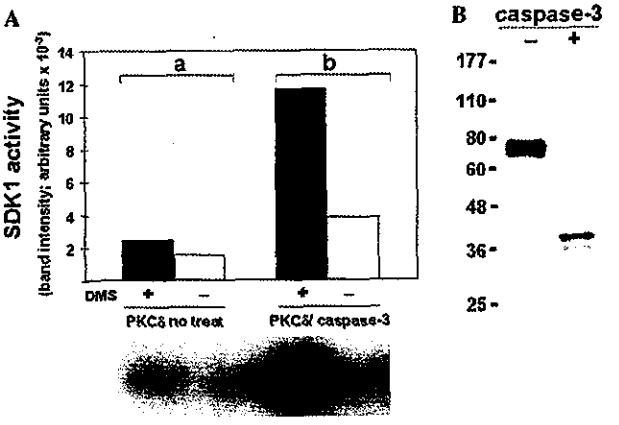

Fig. 3. Full-length and caspase-3-treated PKCδ, and their SDK1 activity in the presence vs. absence of DMS. (A) SDK1 activity of full-length PKCδ ("a") and of caspase-3-treated PKCδ ("b"), using 14-3-3β as substrate, in the presence (+; black bar) vs. absence (–; white bar) of DMS. Quantities of PKCδ and caspase-3 in the reaction mixture were as described in Fig. 2 legend. Bottom: 14-3-3β phosphorylation pattern. Bar graph at top: intensity of 14-3-3β phosphorylation, determined by Scion Image analysis. For "a" and "b," data are shown from one typical experiment; total number of separate experiments, showing essentially the same results, was 4 and 6, respectively. (B) Western blot of PKCδ (~30 ng) without (–) and with (+) caspase-3 (~0.3 U) incubation. Blotting was made with antibody directed to C-terminal region of PKCδ.

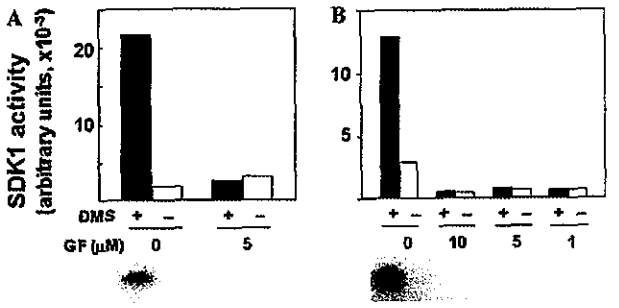

Fig. 4. Inhibition of SDK1 activity by PKC inhibitor GF109203X. (A) SDK1 activity of ~40 kDa SDK1 protein from rat liver fraction and its inhibition by 5 μM GF109203X. Quantity of ~40 kDa protein, and composition and conditions of reaction mixture used for this assay, were approximately the same as in (B) below, but without inclusion of caspase-3. (B) Dose-dependent inhibitory effect of GF109203X on SDK1 activity of caspase-3-released catalytic domain of PKCδ. About 85 ng PKCδ with 0.85 U caspase-3 was incubated and subjected to SDK1 activity assay as described in Fig. 2 legend. The incubation mixture included 1, 5, or 10 μM GF109203X. Bar graphs in both (A) and (B): SDK1 activities in the presence and absence of DMS, determined by Scion Image analysis, are shown, respectively, as black bars (+) and white bars (–).

Since the SDK1 phosphorylation site was identified previously [9], SDK1-dependent phosphorylation of 14-3-3 may be inhibitable by the icosapeptide (20-a.a. peptide) that includes the phosphorylation site. We therefore tested the effect of this peptide on the SDK1 activity of caspase-3-released fragment of PKCδ. The

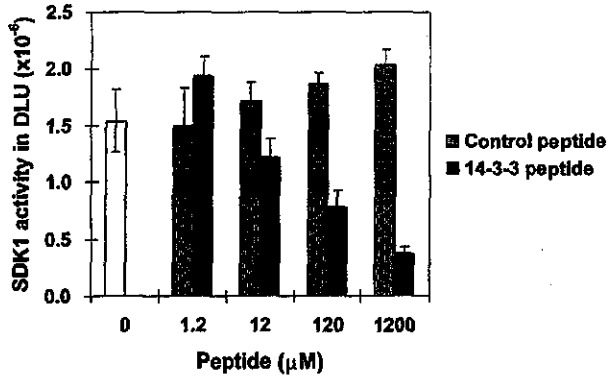

Fig. 5. Inhibitory effect of 14-3-3β icosapeptide on caspase-3-released SDK1 activity from PKCδ SDK1 activity of PKCδ treated with caspase-3 (quantities same as in Figs. 2 and 4) was determined in the presence of various concentrations (abscissa) of 14-3-3β icosapeptide and control peptide, as described in Materials and methods. SDK1 activity in the presence of 14-3-3β icosapeptide (black bar) was strongly inhibited, whereas control peptide (gray bar) had no effect.

SDK1 activity was completely blocked by this peptide at 120 μM concentration, while control peptide had no effect (Fig. 5).

Discussion

We previously found several protein kinases whose activity is greatly enhanced by Sph or DMS but not by Cer, Sph-1-P, or other sphingolipids or phospholipids. These were termed "sphingosine-dependent protein kinases" (SDKs) [8,9,11]. One SDK that catalyzes specific phosphorylation of 14-3-3β, η, and ζ at Ser60, Ser59, and Ser58, respectively, was termed "SDK1" [9].

We have now purified a protein fraction guided by SDK1 activity through large-scale purification from rat liver with six steps of chromatography. A protein with molecular mass ~40 kDa, assumed to have high SDK1 activity, was enriched in the final step (MonoQ chromatography). This band was absent in fractions without SDK1 activity at the MonoQ chromatography step. The ~40 kDa band separated on SDS–PAGE was excised, *in gel* digested, and the sequence of peptide fragments was identified using three different combinations of LC-MS or LC-MS/MS with respective search engine. Thus, the ~40 kDa band was identified as having ~53% of the same a.a. sequence as found in the kinase domain of PKCδ. However, there is no indication that ~40 kDa SDK1 protein occurs naturally, based on NCBI database search with the BLAST algorithm. It is unclear at this time whether the ~40 kDa SDK1 is present independently of PKCδ, or is a cleavage product of PKCδ. Studies by mRNA analysis with possible differential splicing are underway.

14-3-3 has been suggested to promote cell survival through its interaction with multiple pro-apoptotic proteins such as Bad and Bax [22,23]. SDK1, in the presence of Sph or DMS, phosphorylates 14-3-3 at the dimer interface. We propose that SDK1-dependent phosphorylation may interfere with 14-3-3 binding to diverse ligands and neutralize its apoptosis-inhibiting function, thus promoting apoptosis.

Sph has been suggested to be a "second messenger," since its low level is greatly increased upon stimulation of cells. For example, platelet-derived growth factor and insulin-like growth factor cause activation of ceramidase that converts Cer to Sph and fatty acid [24,25]. Increase of Sph may be due in part to synthesis from serine and palmitoyl CoA, the classic synthetic system for Sph [26], although de novo synthesis in response to cell stimulation has not been clarified. Treatment of HL60 cells by phorbol ester increased Sph level 3-fold, whereby the cells differentiated into macrophages. Treatment of the same cells by exogenous Sph caused apoptosis [27]. DMS is considered to be derived from Sph by methylation through *N*-methyltransferase [28]. The chemical level of cellular Cer is much higher than that of Sph and the level of DMS is much lower than that of Sph. For example, quantities of Cer, Sph, Sph-1-P, and DMS in 1×10^6 HL60 cells, determined by LC-MS analysis, were ~12.07 μg, 0.61 ng, 0.048 ng, and 2.49 pg, respectively [29]. The low levels of Sph and DMS, and their increase in response to stimulation of cells, are consistent with the hypothesis that they function as second messengers.

Sph may be short-lived because it is readily converted to Cer or Sph-1-P. A minor quantity of DMS, if synthesized, is inhibitory to Sph kinase [30] and is absolutely stable. Thus, Sph in combination with DMS may be an effective inducer of various kinases, including SDK1 as well as other SDKs [8] whose biochemical properties remain to be elucidated.

Acknowledgments

We thank Dr. Tomikazu Sasaki (Dept. of Chemistry, Univ. of Washington) for his kind donation of control 17-a.a. peptide, and Dr. Stephen Anderson for preparation of the manuscript and figures.

References

[1] Y.A. Hannun, R.M. Bell, Functions of sphingolipids and sphingolipid breakdown products in cellular regulation, Science 243 (1989) 500–507.
[2] A.H.J. Merrill, S. Nimkar, D. Menaldino, Y.A. Hannun, C.R. Loomis, R.M. Bell, S.R. Tyagi, J.D. Lambeth, V.L. Stevens, R. Hunter, D.C. Liotta, Structural requirements for long-chain (sphingoid) base inhibition of protein kinase C in vitro and for the cellular effects of these compounds, Biochemistry 28 (1989) 3138–3145.
[3] Y. Igarashi, S. Hakomori, T. Toyokuni, B. Dean, S. Fujita, M. Sugimoto, T. Ogawa, K. El-Ghendy, E. Racker, Effect of chemically well-defined sphingosine and its *N*-methyl derivatives on protein kinase C and src kinase activities, Biochemistry 28 (1989) 6796–6800.

[4] R.N. Kolesnick, M. Kronke, Regulation of ceramide production and apoptosis, Annu. Rev. Physiol. 60 (1998) 643–665.
[5] B.J. Pettus, C.E. Chalfant, Y.A. Hannun, Ceramide in apoptosis: an overview and current perspectives, Biochim. Biophys. Acta 1585 (2002) 114–125.
[6] S. Spiegel, D. English, S. Milstien, Sphingosine 1-phosphate signaling: providing cells with a sense of direction, Trends Cell Biol. 12 (2002) 236–242.
[7] T. Sano, D. Baker, T. Virag, A. Wada, Y. Yatomi, T. Kobayashi, Y. Igarashi, G. Tigyi, Multiple mechanisms linked to platelet activation result in lysophosphatidic acid and sphingosine 1-phosphate generation in blood, J. Biol. Chem. 277 (2002) 21197–21206.
[8] T. Megidish, K. Takio, K. Titani, K. Iwabuchi, A. Hamaguchi, Y. Igarashi, S. Hakomori, Endogenous substrates of sphingosine-dependent kinases (SDKs) are chaperone proteins: heat shock proteins, glucose-regulated proteins, protein disulfide isomerase, and calreticulin, Biochemistry 38 (1999) 3369–3378.
[9] T. Megidish, J. Cooper, L. Zhang, H. Fu, S. Hakomori, A novel sphingosine-dependent protein kinase (SDK1) specifically phosphorylates certain isoforms of 14-3-3 protein, J. Biol. Chem. 273 (1998) 21834–21845.
[10] H. Fu, R.R. Subramanian, S.C. Masters, 14-3-3 Proteins: structure, function, and regulation, Annu. Rev. Pharmacol. Toxicol. 40 (2000) 617–647.
[11] T. Megidish, T. White, K. Takio, K. Titani, Y. Igarashi, S. Hakomori, The signal modulator protein 14-3-3 is a target of *sphingosine*- or *N,N*-dimethylsphingosine-dependent kinase in 3T3(A31) cells, Biochem. Biophys. Res. Commun. 216 (1995) 739–747.
[12] T. Megidish, A. Hamaguchi, K. Iwabuchi, S. Hakomori, Assays of sphingosine-dependent kinase for 14-3-3 protein, Methods Enzymol. 312 (2000) 381–387.
[13] M.F. Denning, Y. Wang, S. Tibudan, S. Alkan, B.J. Nickoloff, J.-Z. Qin, Caspase activation and disruption of mitochondrial membrane potential during UV radiation-induced apoptosis of human keratinocytes requires activation of protein kinase C, Cell Death Differ. 9 (2002) 40–52.
[14] R. Mineki, H. Taka, T. Fujimura, M. Kikkawa, N. Shindo, K. Murayama, In situ alkylation with acrylamide for identification of cysteinyl residues in proteins during one- and two-dimensional sodium dodecyl sulphate–polyacrylamide gel electrophoresis, Proteomics 2 (2002) 1672–1681.
[15] P.R. Graves, T.A.J. Haystead, Molecular biologist's guide to proteomics, *Microbiol. Mol. Biol. Rev.* 66 (2002) 39–63.
[16] W. Zhang, B.T. Chait, ProFound: an expert system for protein identification using mass spectrometric peptide mapping information, Anal. Chem. 72 (2000) 2482–2489.
[17] D.N. Perkins, D.J. Pappin, D.M. Creasy, J.S. Cottrell, Probability-based protein identification by searching sequence databases using mass spectrometry data, Electrophoresis 20 (1999) 3551–3567.
[18] J.S. Choudhary, W.P. Blackstock, D.M. Creasy, J.S. Cottrell, Matching peptide mass spectra to EST and genomic DNA databases, Trends Biotechnol. 19 (2001) S17–S22.
[19] J.K. Eng, A.L. McCormack, J.R. Yates, An approach to correlate tandem mass-spectral data of peptides with amino-acid sequences in a protein database, J. Am. Soc. Mass Spectrom. 5 (1994) 976–989.
[20] W.S. Hancock, S.-L. Wu, P. Shieh, The challenges of developing a sound proteomics strategy, Proteomics 2 (2002) 352–359.
[21] Y. Ono, T. Fujii, K. Ogita, U. Kikkawa, K. Igarashi, Y. Nishizuka, The structure, expression, and properties of additional members of the protein kinase C family, J. Biol. Chem. 263 (1988) 6927–6932.
[22] J. Zha, H. Harada, E. Yang, J. Jockel, S.J. Korsmeyer, Serine phosphorylation of death agonist BAD in response to survival factor results in binding to 14-3-3 not BCL-X_L, Cell 87 (1996) 619–628.
[23] H. Yang, S.C. Masters, H. Wang, H. Fu, The proapoptotic protein Bad binds the amphipathic groove of 14-3-3 zeta, Biochim. Biophys. Acta 1547 (2001) 313–319.
[24] S. Spiegel, S. Milstien, Sphingolipid metabolites: members of a new class of lipid second messengers, J. Membr. Biol. 146 (1995) 225–237.
[25] E. Coroneos, M. Martinez, S. McKenna, M. Kester, Differential regulation of sphingomyelinase and ceramidase activities by growth factors and cytokines: implications for cellular proliferation and differentiation, J. Biol. Chem. 270 (1995) 23305–23309.
[26] E.E. Snell, S.J. Dimari, R.N. Brady, Biosynthesis of sphingosine and dihydrosphingosine by cell-free systems from *Hansenula ciferri*, Chem. Phys. Lipids 5 (1970) 116–138.
[27] H. Ohta, E.A. Sweeney, A. Masamune, Y. Yatomi, S. Hakomori, Y. Igarashi, Induction of apoptosis by sphingosine in human leukemic HL-60 cells: a possible endogenous modulator of apoptotic DNA fragmentation occurring during phorbol ester-induced differentiation, Cancer Res. 55 (1995) 691–697.
[28] Y. Igarashi, S. Hakomori, Enzymatic synthesis of *N,N*-dimethylsphingosine: demonstration of the sphingosine:*N*-methyltransferase in mouse brain, Biochem. Biophys. Res. Commun. 164 (1989) 1411–1416.
[29] N. Mano, Y. Oda, K. Yamada, N. Asakawa, K. Katayama, Simultaneous quantitative determination method for sphingolipid metabolites by liquid chromatography/ionspray ionization tandem mass spectrometry, Anal. Biochem. 244 (1997) 291–300.
[30] L.C. Edsall, J.R. Van Brocklyn, O. Cuvillier, B. Kleuser, S. Spiegel, *N,N*-dimethylsphingosine is a potent competitive inhibitor of sphingosine kinase but not of protein kinase C: modulation of cellular levels of sphingosine 1-phosphate and ceramide, Biochemistry 37 (1998) 12892–12898.

THE JOURNAL OF BIOLOGICAL CHEMISTRY

Vol. 278, No. 42, Issue of October 17, pp. 41557–41565, 2003
Printed in U.S.A.

Sphingosine-dependent Protein Kinase-1, Directed to 14-3-3, Is Identified as the Kinase Domain of Protein Kinase Cδ*

Received for publication, May 20, 2003, and in revised form, June 17, 2003
Published, JBC Papers in Press, July 10, 2003, DOI 10.1074/jbc.M305294200

Akikazu Hamaguchi‡§¶, Erika Suzuki‡§, Kimie Murayama§‖, Tsutomu Fujimura‖, Toshiyuki Hikita‡, Kazuhisa Iwabuchi‡ ‡‡, Kazuko Handa‡, Donald A. Withers‡, Shane C. Masters§§, Haian Fu§§, and Senitiroh Hakomori‡¶¶**

From the ‡Division of Biomembrane Research, Pacific Northwest Research Institute, and the Department of Pathobiology, University of Washington, Seattle, Washington 98122-4302, the ‖Central Laboratory of Medical Science, Juntendo University School of Medicine, Tokyo 113-8421, Japan, and the §§Department of Pharmacology, Emory University School of Medicine, Atlanta, Georgia 30322

Some protein kinases are known to be activated by D-*erythro*-sphingosine (Sph) or *N,N*-dimethyl-D-*erythro*-sphingosine (DMS), but not by ceramide, Sph-1-P, other sphingolipids, or phospholipids. Among these, a specific protein kinase that phosphorylates Ser^{60}, Ser^{59}, or Ser^{58} of 14-3-3β, 14-3-3η, or 14-3-3ζ, respectively, was termed "sphingosine-dependent protein kinase-1" (SDK1) (Megidish, T., Cooper, J., Zhang, L., Fu, H., and Hakomori, S. (1998) *J. Biol. Chem.* 273, 21834–21845). We have now identified SDK1 as a protein having the C-terminal half kinase domain of protein kinase Cδ (PKCδ) based on the following observations. (i) Large-scale preparation and purification of proteins showing SDK1 activity from rat liver (by six steps of chromatography) gave a final fraction with an enhanced level of an ~40-kDa protein band. This fraction had SDK1 activity ~50,000-fold higher than that in the initial extract. (ii) This protein had ~53% sequence identity to the Ser/Thr kinase domain of PKCδ based on peptide mapping using liquid chromatography/mass spectrometry and liquid chromatography/tandem mass spectrometry data. (iii) A search for amino acid homology based on the BLAST algorithm indicated that the only protein with high homology to the ~40-kDa band is the kinase domain of PKCδ. The kinase activity of PKCδ did not depend on Sph or DMS; rather, it was inhibited by these sphingoid bases, *i.e.* PKCδ did not display any SDK1 activity. However, strong SDK1 activity became detectable when PKCδ was incubated with caspase-3, which releases the ~40-kDa kinase domain. PKCδ and SDK1 showed different lipid requirements and substrate specificity, although both kinase activities were inhibited by common PKC inhibitors. The high susceptibility of SDK1 to Sph and DMS accounts for their important modulatory role in signal transduction.

Although D-*erythro*-sphingosine (Sph)[1] (1–3) and *N,N*-dimethyl-D-*erythro*-sphingosine (DMS) (3) were originally found as inhibitors of conventional protein kinase C (PKCα or PKCβ), the majority of recent studies on effects of sphingolipids on cellular function have focused on ceramide (Cer) (*e.g.* Refs. 4–9) or Sph-1-P (*e.g.* Refs. 10–15). However, we found previously that several proteins acting as chaperones of key signal transducers or as modulators of endoplasmic reticulum protein function are phosphorylated only in the presence of Sph or DMS, but not Cer or Sph-1-P (16, 17). Such Sph- or DMS-dependent factors are as follows: (i) a protein kinase involved in phosphorylation of 14-3-3 at defined phosphorylation sites (Ser^{60} in 14-3-3β, Ser^{59} in 14-3-3η, and Ser^{58} in 14-3-3ζ), termed "sphingosine-dependent protein kinase-1" (SDK1) (16); (ii) protein-disulfide isomerase, essential for maintaining disulfide-dependent secondary structure of proteins, termed "SDK2"; and (iii) another protein kinase closely associated with and modulating the function of glucose-regulated proteins (*e.g.* GRP105 and GRP94) or heat-shock proteins (*e.g.* HSP84, HSP86, and HSP90), termed "SDK3" (17).

The chaperone or modulatory protein 14-3-3 has received increasing attention for its interaction with and functional modification of various key molecules involved in signal transduction, transport, cell proliferation, and apoptosis (18). SDK1 specifically phosphorylates 14-3-3β, 14-3-3η, and 14-3-3ζ only in the presence of Sph and DMS, but not 16 other lipids tested (including Cer and Sph-1-P). The phosphorylation takes place at Ser^{60}, Ser^{59}, or Ser^{58}, located in helix 3, which may interfere with interaction between two molecules of 14-3-3 to form a dimer. Non-phosphorylated 14-3-3 prefers to form a dimer and may function as a chaperone or modulator of major signal transducers such as Raf1, Bad, and Ber. SDK1-dependent phosphorylation may counteract this effect and thereby modulate signal transduction to induce cell proliferation, differentiation, or apoptosis. However, the molecular identity and biochemical properties of SDK1 have not yet been clearly elucidated. Here, we describe the characterization of SDK1 and identify it as the C-terminal half kinase domain of PKCδ, distinguishable from PKCδ *per se*.

* This work was supported by NCI Grant R01 CA82167 (to S. H.) and NIGMS Grant R01 GM53165 (to H. F.) from the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ These authors contributed equally to this work.

¶ Present address: Dept. of Urology, Shiga Medical University, Shiga 520-0232, Japan.

** Present address: Dept. of Pediatrics, Teikyo University School of Medicine, Tokyo 173-8605, Japan.

‡‡ Present address: Dept. of Biochemistry II, Juntendo University School of Medicine, Tokyo 113-8421, Japan.

¶¶ To whom correspondence should be addressed: Pacific Northwest Research Inst., 720 Broadway, Seattle, WA 98122-4302. Tel.: 206-726-1244; Fax: 206-309-0368; E-mail: hakomori@u.washington.edu.

[1] The abbreviations used are: Sph, D-*erythro*-sphingosine; DMS, *N,N*-dimethyl-D-*erythro*-sphingosine; PKC, protein kinase C; Cer, ceramide; SDK, sphingosine-dependent protein kinase; HPLC, high-performance liquid chromatography; Aib, aminoisobutyric acid; DTT, dithiothreitol; MES, 4-morpholineethanesulfonic acid; BisTris, 2-[bis(2-hydroxyethyl)-amino]-2-(hydroxymethyl)propane-1,3-diol; PS, phosphatidylserine; MS, mass spectrometry; LC, liquid chromatography; MS/MS, tandem mass spectrometry.

EXPERIMENTAL PROCEDURES

Materials

Frozen rat livers (from 7–8-week-old Sprague-Dawley rats, both genders) were purchased from Pel-Freez Biologicals (Rogers, AR) and used as a source for large-scale preparation of SDK1. For all experiments, water used as solvent was Millipore-filtered and double-distilled from a glass still. Specific column contents for HPLC were from Amersham Biosciences, except for heparin-Sepharose, which was prepared in the laboratory of Dr. Kazuo Fujikawa (Department of Biochemistry, University of Washington). HPLC with Q-Sepharose Fast Flow, phenyl-Sepharose, heparin-Sepharose, hydroxylapatite, and chromatofocusing was performed in a HPLC Fast Flow assembly (Amersham Biosciences). HPLC with Mono Q and gel filtration (Superdex 200) was performed in a SmartSystem assembly (Amersham Biosciences). PKCδ was from Panvera Corp. (Madison, WI). Caspase-3 was from Gene Therapy Systems (San Diego, CA). [γ-^{32}P]ATP was from PerkinElmer Life Sciences. Sph, Sph-1-P, and DMS were from Matreya, Inc. (Pleasant Gap, PA). C_2-Cer and C_{16}-Cer were from Sigma. Polyclonal antibodies against the C-terminal domain of PKCδ were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Various other reagents and solvents were from Sigma.

The icosapeptide (20-amino acid peptide) YKNVVGARRSSWRVISSIEQ, which is present in helix 3 of 14-3-3 and includes the phosphorylation site (Ser next to Trp), was synthesized in the laboratory of K. M. at Juntendo University. The control peptide (17 amino acids) with the sequence Ac-YGGSAESS(Aib)KSEASSK(Aib)SA-CONH$_2$) was synthesized in the laboratory of Dr. Tomikazu Sasaki (Department of Chemistry, University of Washington) and kindly donated to us.

Procedure for Purification of SDK1 from Rat Liver

150 g of rat liver at one time was thawed, cut into small pieces, and homogenized in a Waring blender (30 s at high speed, three times) in ice in a cold room with 1000 ml of ice-cold homogenization buffer containing 20 mM Tris-HCl, 0.5 mM EDTA, 0.5 mM EGTA, 12 mM NaF, 5% glycerol, 3 μg/ml leupeptin, 5 μg/ml aprotinin, 3 μg/ml chymostatin, 1 mM phenylmethylsulfonyl fluoride, and 1 mM dithiothreitol (DTT) (pH 8.0). The homogenate was centrifuged for 30 min at 4000 rpm in Beckman centrifuge. The supernatant was adjusted to pH 8.0 and ultracentrifuged for 1 h at 100,000 × *g*. The supernatant was filtered through a 0.5-μm filter and used for six steps of chromatographic purification, guided by SDK1 activity.

Step 1: Q-Sepharose Fast Flow Chromatography—The pH of the filtered extract described above was adjusted to 8.0 and applied to a Q-Sepharose Fast Flow XK 26/20 column (Amersham Biosciences) pre-equilibrated with Buffer A (20 mM Tris-HCl, 1 mM EDTA, 10 mM NaF, and 10 mM DTT (pH 8.0)) as described previously (16). Elution was performed by a combination of stepwise and linear gradient changes of the NaCl concentration (0–1.0 M) under conditions of 4 ml/min and 8 ml/fraction. 10 μl of each fraction was analyzed for SDK1 activity using 14-3-3β as substrate (see below). The activity was consistently found between fractions 22 and 31 (see Fig. 1*A*).

Step 2: Hydrophobic Interaction Chromatography—Fractions showing SDK1 activity from Step 1 were pooled, added with powdered KCl to give a final concentration of 1 M, filtered through a 0.5-μm membrane, and applied to a phenyl-Sepharose 6 Fast Flow column (Amersham Biosciences). All SDK1 activity was recovered in the flow-through fraction with increased specific activity (see "Results").

Step 3: Cation-exchange Chromatography on a Heparin-Sepharose Column—The flow-through fraction (15 ml) from Step 2 was subjected to buffer exchange by pass-through a HiPrep 26/10 desalting column (Amersham Biosciences) and eluted with 2 column volumes (100 ml) of 50 mM MES (pH 6.7), 1 mM EDTA, and 10 mM NaF. Proteins were eluted with 15–20 ml of MES buffer prior to elution of KCl. The buffer-exchanged sample was applied to a heparin column pre-equilibrated with the same MES buffer and subjected to gradient elution with the same buffer containing increasing NaCl concentrations under conditions of 1.5 ml/min and 4.5 ml/fraction. High SDK1 activity was consistently found between fractions 15 and 21 (see Fig. 1*B*).

Step 4: Hydroxylapatite Chromatography—The fraction with SDK1 activity resulting from Step 3 was buffer-exchanged to 10 mM KH_2PO_4. Elution was performed with increasing KH_2PO_4 concentrations under conditions of 1.0 ml/min and 2 ml/fraction. Peak SDK1 activity was observed between fractions 10 and 20 (see Fig. 1*C*).

Step 5: Chromatofocusing—The SDK1 active fraction from Step 4 was buffer-exchanged to 25 mM BisTris (pH 7.3), applied to a Mono P HR 5/20 column (4-ml bed volume; Amersham Biosciences), and subjected to gradient elution with Polybuffer 74 (pH 4.3) (Amersham Biosciences) under conditions of 0.5 ml/min and 2 ml/fraction. Peak SDK1 activity was observed between fractions 16 and 18 (see Fig. 1*D*).

Step 6: Mono Q Chromatography—The SDK1 active fraction from Step 5 was buffer-exchanged to Buffer A (pH 8.0), placed on a Mono Q HR 5/5 column (1-ml bed volume; Amersham Biosciences) pre-equilibrated with Buffer A, and subjected to gradient elution with 0.15–0.5 M NaCl under conditions of 0.25 ml/min and 0.5 ml/fraction. High SDK1 activity was observed in fractions 17–21; the maximal peak was in fraction 18, 19, or 20 depending on the sample (in one case, shown in Fig. 1*E*, the peak was in fraction 20). A band with molecular mass of ~40 kDa was detected in the SDK1 active fractions. This band, separated by SDS-PAGE, was therefore subjected directly to sequence analysis after in-gel digestion.

Step 7: Size-exclusion Chromatography—The SDK1 active fraction from Step 6 was centrifuged at 10,000 × *g* for 10 min to eliminate impurities, subjected to gel filtration through a Superdex 200 HR 10/30 column (24-ml bed volume; Amersham Biosciences), and eluted with 50 mM Tris-HCl (pH 7.0) and 0.15 M NaCl under conditions of 500 μl/min and 500 μl/fraction.

Determination of PKCδ Activity

PKCδ activity with the common PKC peptide substrate RFARKGSLRQKNV (where S denotes the phosphorylation site; Panvera Corp.) was determined by incubating the following mixture (50-μl total volume): 2 μl of 0.5 M HEPES (pH 7.4), 5 μl of 100 mM $MgCl_2$, 5 μl of 1 mM EGTA, 0.5 μl of 100 μM peptide substrate, 0.5 μCi of [γ-^{32}P]ATP, 6.7 μl of 750 μM ATP, 5 μl 10× lipid mixture (2 mg/ml phosphatidylserine (PS), 200 μg/ml diacylglycerol, 20 mM HEPES (pH 7.4), and 0.3% Triton X-100), and 5 μl containing 25 ng of PKCδ in 10 mM HEPES (pH 7.4), 5 mM DTT, and 0.01% Triton X-100. The reaction mixture was incubated for 10 min at 30 °C. The reaction was stopped by spotting 25 μl of this mixture onto phosphocellulose membrane (P-81 paper, Upstate Biotechnology, Inc., Lake Placid, NY). The membranes were washed three to five times with 0.5% phosphoric acid and counted in a scintillation counter. PKCδ activity was quantified as the amount of ^{32}P-phosphorylated peptide trapped on the membrane as measured in the scintillation counter.

Determination of SDK1 Activity in Chromatographic Fractions from Rat Liver

This was performed by a modification of the previously described method (16, 19), including quantitative comparison of activities using a PhosphorImager (Cyclone storage phosphor screen, PerkinElmer Life Sciences). Briefly, 10 μl of sample solution, *i.e.* aliquot of the chromatographic fraction, was mixed with 3 μl of 50 mM Tris (pH 7.5) containing 1 μg of 14-3-3β (1 μl of a 1 μg/μl solution) and 0.15 μl of 10 mM DMS in Me_2SO (final DMS concentration of 50 μM in the incubation mixture). The reaction was initiated by addition of 10 μl of ATP/Mg^{2+} solution (75 μM unlabeled ATP and 2.5 μCi of [γ-^{32}P]ATP in Tris-HCl (pH 7.5), 3 mM DTT, and 45 mM MgAc) and H_2O to adjust the total volume to 30 μl. The reaction mixture was incubated for 15 min at 30 °C, and the reaction was stopped by addition of 10 μl of 4× concentrated Laemmli sample buffer and heating for 3 min at 100 °C. Phosphorylated 14-3-3β was separated by 12% SDS-PAGE and transferred to a polyvinylidene difluoride membrane. The membrane was air-dried, and the activity of phosphorylated 14-3-3β band was determined by contact of the membrane with the screen of the PhosphorImager. The intensities of each band were quantified by OptiQuant software, subtracted by the value for a control ^{14}C marker, and compared in terms of digital light units.

Cleavage of PKCδ by Caspase-3, Determination of SDK1 Activity in the Released Catalytic Domain, and Effect of PKCδ Inhibitors

PKCδ was incubated with caspase-3 as described previously (20). Briefly, 200 ng of human recombinant PKCδ was added to the following mixture with a total reaction volume of 20 μl: 2 units of active human recombinant caspase-3 in 25 mM HEPES (pH 7.4), 250 mM sucrose, 1 mM EDTA, and 2.5 mM DTT. Cleavage was confirmed by Western blotting using a polyclonal antibody against the carboxyl terminus of PKCδ.

For SDK1 activity determination of caspase-3-treated PKCδ, 8.5 μl of the above mixture (containing 85 ng of caspase-3-treated PKCδ) or of untreated full-length PKCδ was incubated for 15 min at 30 °C with 1 μg of 14-3-3β, 50 μM DMS (at a final solvent concentration of 1% ethanol and 0.05 M NaCl), 3 mM DTT, 25 μM ATP, 45 mM MgAc, 2.5 μCi of [γ-^{32}P]ATP, and 26.7 mM Tris (pH 7.3) at a final volume of 30 μl. Controls were carried out in the absence of DMS. The reaction was stopped by addition of Laemmli buffer (4× concentrated) and heating at 95 °C for 5 min. 14-3-3 phosphorylation was analyzed by 10–12% SDS-PAGE. Gels were subjected to autoradiography using an intensifier

screen (exposure time of 2 h at −80 °C) or a PhosphorImager. In some reactions, 5–150 μM rottlerin (Calbiochem) or 1–10 μM GF109203X (Alexis Biochemicals, San Diego, CA) was used as a PKCδ inhibitor. Other lipids (Sph-1-P, C_2-Cer, and C_{16}-Cer) were also tested to verify SDK1 activity.

Determination of a Partial Amino Acid Sequence of SDK1

One-dimensional SDS-PAGE—500 μl of fraction 18, associated with very high SDK1 activity from Step 6 (Mono Q), was precipitated with an equal volume of 20% trichloroacetic acid, centrifuged at 16,000 × *g* for 30 min, washed with 1 ml of cold ethyl ether, and centrifuged at 16,000 × *g* for 10 min. Washing with cold ethyl ether was repeated three times to eliminate trichloroacetic acid. The final precipitate was dissolved in 10 μl of sample buffer (62.5 mM Tris-HCl (pH 6.8) containing 10% glycerol, 2% SDS, 5% mercaptoethanol, and 0.0025% bromphenol blue). The resulting mixture was loaded onto a gel and separated by one-dimensional SDS-PAGE. The conditions were as follows: gel size of 130 × 130 × 1 mm; 4 and 10% acrylamide containing 2.6% piperazine diacrylamide/acrylamide (Bio-Rad) for the stacking and running gels, respectively; and running buffer consisting of 25 mM Tris and 192 mM glycine (pH 8.45) containing 0.1% SDS. Proteins were concentrated at 5 mA/gel for 70 min in stacking gel and run at 20 mA/gel for 2 h. Gels were stained with 0.1% Coomassie Blue R-350 (Bio-Rad) dissolved in 10% acetic acid and 30% methanol and washed with 7% acetic acid and 30% methanol for 20 min (three times) and with 10% acetic acid for 20 min (three times). The protein profile was analyzed by a *Master Scan* image analyzer (Scanlytics, Billerica, MA). Molecular masses of bands were calculated based on molecular mass standard proteins (broad-range; Bio-Rad).

In-gel Digestion with Trypsin and Extracted Peptides—The ~40-kDa band was subject to in-gel digestion as described previously (21). Briefly, the band was excised manually using a razor blade, placed in an Eppendorf tube, washed with H_2O (10 min, 37 °C, five times), and destained in 100 μl of 50% CH_3CN and 100 mM ammonium carbonate (pH 8.5) for 10 min at 37 °C until colorless. The gel was dehydrated in 100 μl of CH_3CN in an Eppendorf tube for 10 min at 37 °C and dried in Speed-Vac™ for 5 min. The dried residue was immersed in 50 μl of 0.001% trypsin (10 ng/μl) in 100 mM ammonium carbonate (pH 8.5) and incubated overnight at 37 °C. The original trypsin solution was 20 μg/20 μl of 50 mM acetic acid. The incubation mixture in the Eppendorf tube was centrifuged, and the residue was extracted with 50% CH_3CN and 0.1% trifluoroacetic acid and centrifuged again. The residue was further extracted with 15% isopropyl alcohol, 20% formic acid, 25% CH_3CN, and 40% H_2O and finally with 80% CH_3CN. All of the extracts were successively dried in a single Eppendorf tube, and the residue was dissolved in 6 μl of ultrapure water. Aliquots (2 μl) were used for protein identification and for amino acid sequencing by mass spectrometry (MS).

Peptide Sequencing—Peptide mapping was performed using the API QSTAR pulsar hybrid mass spectrometer system with a micro-liquid chromatograph (Magic 2002, Michrom BioResource, Auburn, CA). Conditions of micro-LC were as follows: Magic C18 column (0.2 mm, inner diameter × 50 mm) and elution with 0.1% formic acid (solvent A) and 0.1% formic acid in 90% CH_3CN (solvent B) using a program of 3% solvent B for 2 min, gradient at 2.1%/min for 45 min, 100% solvent B for 5 min, and a flow rate of 2.5 μl/min. The QSTAR pulsar hybrid mass spectrometer system consists of nanoelectrospray ionization as the ionization source and quadrupole time-of-flight MS. Mass accuracy was ±0.1 mass unit. Conditions of MS were as follows: ion spray voltage of 3.0–3.8 kV, voltage for the electron multiplier of 2200 V, nitrogen 10 curtain gas for MS and MS/MS analyses, and nitrogen 10 collision gas and collision energy of 20–55 eV for MS/MS analysis. Conditions of micro-LC were as follows: Magic C18 column (0.2 mm, inner diameter × 50 mm), elution with solvent A and solvent B using a program of 3% solvent B for 2 min, gradient at 2.1%/min for 45 min, 100% solvent B for 5 min, and a flow rate of 2.5 μl/min.

To identify protein in the ~40-kDa band, peptide mapping of one-third of the in-gel digested product was performed by micro-LC/MS (QSTAR) using the PROWL (*ProFound*) search engine[2] and the NCBI Database (22, 23). The major ion peaks of the total ion chromatogram were further analyzed to obtain the amino acid sequences of the tryptic peptides by LC/MS/MS using the Mascot search engine (22, 24, 25)[3] under the same conditions (QSTAR with a Magic micro-liquid chromatograph) and the same data base (one-third of the same sample used).

LCQ-DECA XP (ThermoFinnigan, San Jose, CA) with a Magic micro-liquid chromatograph was also used for determination of the amino acid sequences of the tryptic peptides using the MS/MS mode (one-third of the sample used). The conditions of MS were as follows: ion spray voltage of 1.8 kV, transfer tube temperature of 250 °C for MS and MS/MS analyses, helium collision gas, and normalized collision energy of 35% for MS/MS analysis. Identification of probable sequences from MS/MS data was performed using the TurboSequest search engine (22, 26, 27)[4] and the NCBI Database (in house).

Inhibition of the SDK1 Activity of Caspase-3-treated PKCδ by the 14-3-3 Peptide

Aliquots of the caspase-3-released kinase domain from 85 ng of PKCδ were incubated (5 min, 30 °C) with various concentrations of the 14-3-3 icosapeptide, previously identified as the SDK1-dependent phosphorylation site, or of the unrelated control peptide (17 amino acids) with the sequence described under "Materials." The incubation mixture was assayed for SDK1 activity with 14-3-3β as substrate, DMS as activator, and [γ-^{32}P]ATP, followed by determination of phosphorylated 14-3-3β separated by SDS-PAGE as described above.

RESULTS

Purification of Proteins with SDK1 Activity—The results of the chromatography steps (Q-Sepharose Fast Flow, heparin-Sepharose, hydroxylapatite, chromatofocusing, Mono Q, and gel filtration pattern of the fraction obtained by Mono Q) are shown in Fig. 1 (*A–F*, respectively). The results of purification by phenyl-Sepharose (Step 2) are essentially the same as described previously (16) and therefore are not shown. The SDK1 activity of each fraction at each chromatography step, expressed as digital light units, is also indicated in each panel of Fig. 1. The yield of proteins, their SDK1 activities, specific activities (SDK1 activity/mg of protein), and -fold purification are shown in Table I. An ~55,000-fold purification was obtained at the Mono Q step in comparison with the initial extract; specific SDK1 activity was increased to a similar degree. However, a large number of protein bands were still present, separated by SDS-PAGE, even at the Mono Q step. Perhaps some of these proteins were present as complexes and were extremely difficult to separate into components, even after many chromatography steps. Further separation was achieved only by SDS-PAGE, whereby SDK1 activity was lost because of denaturation.

The chromatographic fractions showing the highest SDK1 activity on the Mono Q column (fraction 18, 19, or 20, depending on the batch) showed a band with a molecular mass ~40 kDa upon SDS-PAGE, which was absent in other fractions with no or minimal SDK1 activity. Therefore, amino acid sequence analysis was performed for this band as described under "Experimental Procedures."

The ~40-kDa Protein from a Fraction Containing SDK1 Activity Has the Same Sequence Found in the C-terminal Half Domain of PKCδ—Based on procedures described above, the in-gel digested product of the ~40-kDa band was analyzed by micro-LC/MS and MS/MS combined with QSTAR or LCQ-DECA XP, and the probable peptide sequence was studied using three online search engines (PROWL, Mascot, and TurboSequest) as described under "Experimental Procedures." The probable sequences of peptide fragments as determined by these three methods are indicated in Table II. The overlap from the three methods confirms the reality of a common sequence as shown in Table III. The sequence so far determined covered ~53% of that of the C-terminal half domain (catalytic domain) of PKCδ.

SDK1 Activity Is Harbored in the Catalytic Domain of PKCδ Released by Caspase-3—Based on the sequence data in Tables II and III, we searched the NCBI Database with the BLAST algorithm.[5] The only protein with high homology to the sequence in Table II was rat PKCδ (28), although the homology

[2] Available at prowl.rockefeller.edu/cgi-bin.ProFound.
[3] Available at www.matrixscience.com.
[4] Available at fields.scripps.edu/sequest.
[5] Available at www.ncbi.nlm.nih.gov/blast.

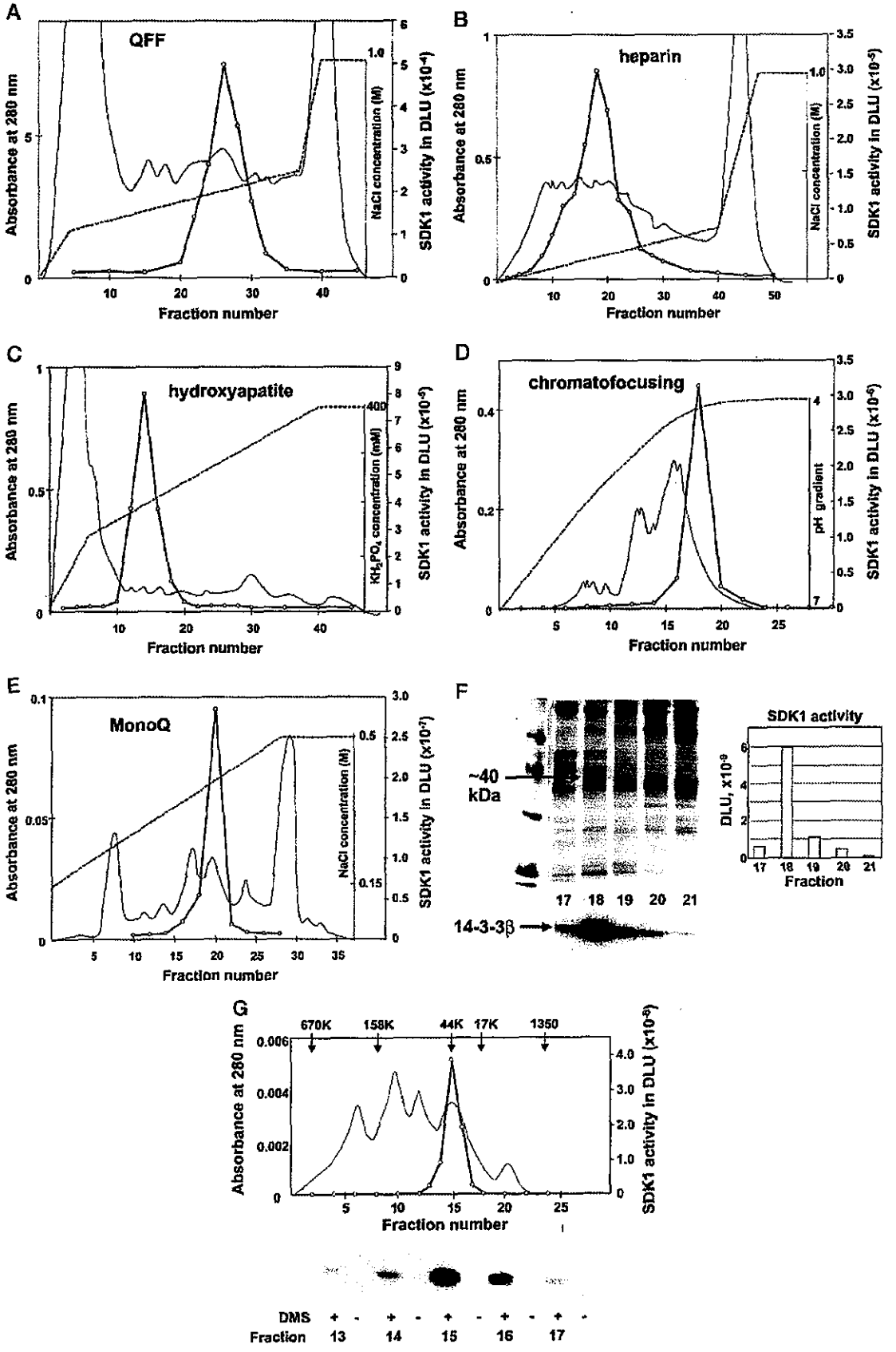

FIG. 1. **Purification from a Sprague-Dawley rat liver extract of a protein fraction showing SDK1 activity by six steps of chromatography.** *A–E*: Q-Sepharose Fast Flow (*QFF*; Step 1), heparin (Step 3), hydroxylapatite (Step 4), chromatofocusing (Step 5), and Mono Q column (Step 6) chromatography patterns, respectively. Step 2 (phenyl-Sepharose column chromatography) is not shown because the pattern was

TABLE I
Protein quantity, total SDK1 activity, and specific SDK1 activity at various steps of purification

Step	Protein	Volume	Total SDK1 activity[a]	Specific SDK1 activity	Yield	Purification
	mg	*ml*	*DLU*	*DLU/mg protein*	*%*	*fold*
0. Homogenate	69,174	1620	9.7×10^{11}	1.4×10^{7}	100.0	1
1. QFF[b]	2720	640	3.3×10^{11}	1.2×10^{8}	34.4	8.8
2. Hydrophobic	1820	860	2.7×10^{11}	1.5×10^{8}	27.5	10.5
3. Heparin-Sepharose	498	360	2.2×10^{11}	4.4×10^{8}	22.7	31.6
4. Hydroxylapatite	23.4	64	6.7×10^{10}	2.9×10^{9}	20.2	205
5. Chromatofocusing	2.14	24	2.9×10^{10}	1.3×10^{10}	10.8	966
6. Mono Q	0.01	3	7.5×10^{9}	7.5×10^{11}	3.4	53,348

[a] Expressed as digital light units (DLU) from PhosphorImager analysis (see "Determination of SDK1 Activity in Chromatographic Fractions from Rat Liver").
[b] Q-Sepharose Fast Flow.

TABLE II
Peptide fragment sequences from the SDK1 active ~40-kDa protein obtained by LC/MS or LC/MS/MS with various peptide search engines

SDK1 residues	Sequence	QSTAR PROWL MS	QSTAR Mascot MS/MS	LCQ-DECA XP TurboSequest MS/MS
8–14	IWEGSNR	×		×
17–24	LENFTFQK	×	×	×
25–33[a]	**VLGKGSFGK**	×	×	
29–40	**GSFGKVLLAELK**	×		
34–40	**VLLAELK**	×	×	×
45–52	**YFAIKYLK**	×		
45–49	**YFAIK**	×	×	×
50–53	YLKK	×		
54–69	DVVLIDDDVECTMVEK	×		
116–120	FELYR	×	×	×
139–143[b]	**GIIYR**	×	×	
144–146	**DLK**	×		
144–154	**DLKLDNVMLDK**	×		
147–154	**LDNVMLDK**	×	×	×
147–159	**LDNVMLDKDGHIK**	×		
160–167	IADFGMCK	×		
168–175	ENIFGENR	×		
233–240	VDTPHYPR	×	×	×
241–244	WITK	×		×
248–252	DIMEK	×		
253–256	LFER	×	×	
253–260	LFERDPAK	×	×	×
261–269	RLGVTGNIR	×		
262–269	LGVTGNIR	×	×	×
270–275	LHPFFK	×		×
276–284	TINWNLLEK		×	×
286–294	KVEPPFKPK	×		
287–292	VEPPFK	×		
287–294	VEPPFKPK	×		×
321–332	NLIDSMDQTAFK	×	×	×
321–340	NLIDSMDQTAFKGFSFVNPK	×		
341–346	YEQFLE	×	×	

[a] Residues 25–49 (shown in boldface) correspond to the ATP-binding region signature.
[b] Residues 139–159 (shown in boldface) correspond to the Ser/Thr protein kinase active-site signature.

was restricted to the C-terminal half (catalytic domain). Full-length PKCδ showed novel PKCδ activity with the common PKC substrate, requiring diacylglycerol, PS, and Mg^{2+}, but not Ca^{2+}. However, the kinase activity of PKCδ with the common PKC substrate (RFARKGSLRQKNV) was inhibited by Sph or DMS (Fig. 2*A*). Strong SDK1 activity, highly dependent on the presence of Sph or DMS, was found when PKCδ was treated with caspase-3 (Fig. 2*B*). Full-length PKCδ did not phosphoryl-

essentially the same as described previously (16). For detailed procedures of each step, see "Experimental Procedures." In each panel, the *thin solid line* indicates the elution pattern of the protein determined by UV adsorption at 280 nm (*left ordinate*). The *dashed line* indicates the change in the elution medium (*e.g.* concentration of NaCl or KH_2PO_4 or pH) as indicated by *inset* on the *right ordinate*. The *thick solid line* indicates SDK1 activity expressed as digital light units (*DLU*; see "Determination of SDK1 Activity in Chromatographic Fractions from Rat Liver") (units on the *main right ordinate*). *F*: *upper left panel*, fractions 17–21 obtained from Mono Q ion-exchange chromatography were separated by SDS-PAGE and silver-stained. Note that the ~40-kDa band was enriched in fraction 18 in this case. *Left lower panel*, SDK1 activity of fractions 17–21 indicated by the 14-3-3β phosphorylation pattern obtained with a PhosphorImager. *Right panel*, SDK1 activity of fractions 17–21 quantitated with a PhosphorImager. All data were from the same 10-μl aliquots of the respective fractions. For details on the procedure for determination of SDK1 activity using the PhosphorImager, see "Determination of SDK1 Activity in Chromatographic Fractions from Rat Liver." *G*: *upper panel*, gel filtration patterns of the Mono Q fraction showing major SDK1 activity through a Superdex 200 HR 10/30 column in SmartSystem (see "Experimental Procedures"). The *thin solid line* indicates the elution pattern of the protein determined by UV adsorption at 280 nm (*left ordinate*). The *thick solid line* indicates SDK1 activity expressed as digital light units (*right ordinate*). *Lower panel*, SDK1 activity of fractions 13–17 in terms of the phosphorylation pattern of 14-3-3β in the absence (−) or presence (+) of 50 μM DMS using Me_2SO as vehicle (see "Determination of SDK1 Activity in Chromatographic Fractions from Rat Liver").

TABLE III

Overall sequence of the SDK1 protein identified by mass spectrometry

Sequences indicated in boldface are from fragments shown in Table II. The sequence is aligned with 53% of the catalytic domain sequence of PKCδ (28). Lightface letters indicate probable sequence of the catalytic domain, initiated from the caspase-3-cleavable site (amino acids 1–346, corresponding to amino acids 328–673 of PKCδ). The ATP-binding region signature is underlined; the Ser/Thr protein kinase active-site signature is double-underlined.

[1]NNGTYGKIWE	GSNRCRLENF TFQKVLGKGS FGKVLLAELK GKERYFAIKY LKKDVVLIDD[60]
[61]DVECTMVEKR	VLALAWENPF LTHLICTFQT KDHLFFVMEF LNGGDLMFHI QDKGRFELYR[120]
[121]ATFYAAEIIC	GLQFLHGKGI IYRDLKLDNV MLDKDGHIKI ADFGMCKENI FGENRASTFC[180]
[181]GTPDYIAPEI	LQGLKYSFSV DWWSFGVLLY EMLIGQSPFH GDDEDELFES IRVDTPHYPR[240]
[241]WITKESKDIM	EKLFERDPAK RLGVTGNIRL HPFFKTINWN LLEKRVEPP FKPKVKSPSD[300]
[301]YSNFDPEFLN	EKPQLSFSDK NLIDSMDQTA FKGFSFVNPK YEQFLE[346]

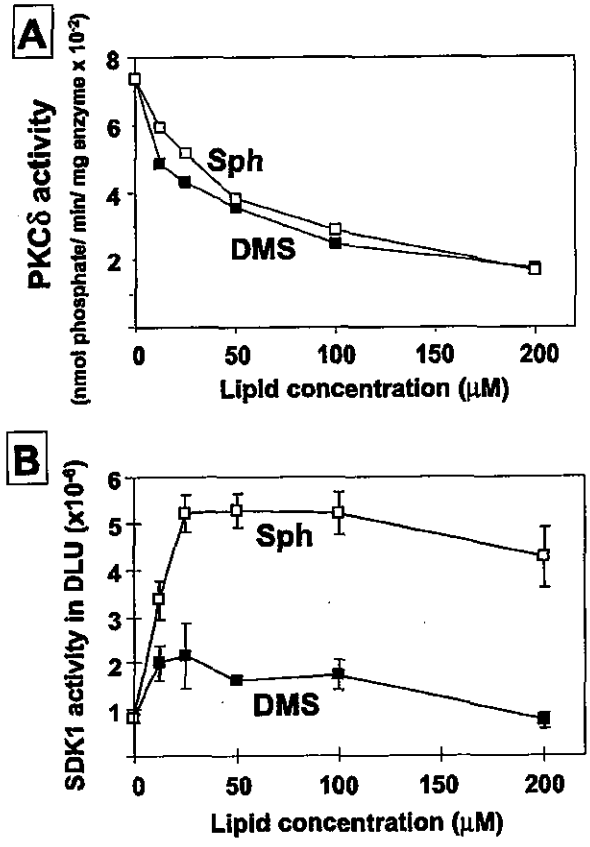

FIG. 2. **Inhibitory effect of Sph and DMS on human recombinant PKCδ activity and their promoting effect on SDK1 activity following caspase-3 cleavage.** *A*, dose-dependent inhibitory effect of DMS (■) and Sph (□) on human recombinant PKCδ activity using the conventional PKC peptide substrate (RFARKGSLRQKNV). *PKCδ* activity was calculated as nmol of phosphate/min/mg of enzyme based on ^{32}P bound to the peptide substrate adsorbed on phosphocellulose membrane (see "Determination of SDK1 Activity in Chromatographic Fractions from Rat Liver"). Values are the means of two experiments; variation was very small (<5%). *B*, dose-dependent promoting effect of DMS (■) and Sph (□) on the SDK1 activity of caspase-3-treated PKCδ (aliquots of ~85 ng of PKCδ incubated with ~0.85 units of caspase-3) using 1 μg of 14-3-3β as substrate. 14-3-3β phosphorylation was quantified with a PhosphorImager and expressed as digital light units (*DLU*; see "Determination of SDK1 Activity in Chromatographic Fractions from Rat Liver"). Values are the means of triplicate experiment; S.D. values are shown by the *error bars*. In both panels, Sph and DMS were given with a final concentration of 1% ethanol in 0.05 M NaCl as vehicle.

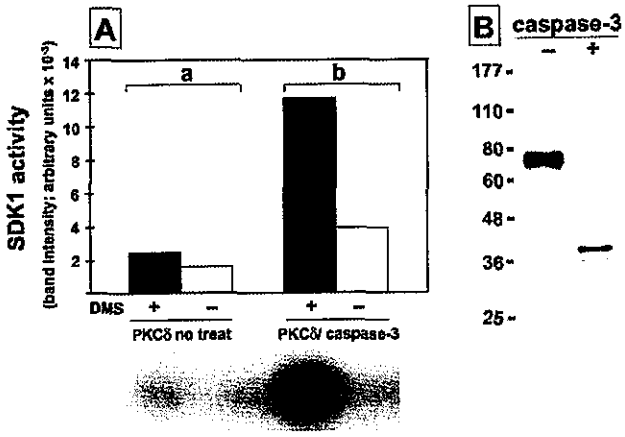

FIG. 3. **Full-length PKCδ and caspase-3-treated PKCδ and their SDK1 activities in the presence *versus* absence of DMS.** *A*, SDK1 activities of PKCδ (*group a*) and of caspase-3-treated PKCδ (*group b*) using 14-3-3β as substrate in the presence (+; *black bars*) *versus* absence (−; *white bars*) of DMS. The phosphorylation pattern of 1 μg of 14-3-3β was determined as described under "Cleavage of PKCδ by Caspase-3, Determination of SDK1 Activity in the Released Catalytic Domain, and Effect of PKCδ Inhibitors" using ~85 ng of PKCδ incubated with ~0.85 units of caspase-3. The autoradiography pattern of 14-3-3β is shown at bottom. The bar graph shows the intensity of 14-3-3β phosphorylation as determined by Scion Image analysis. Data shown in *groups a* and *b* are typical results of five separate experiments. The ratios of band intensity of caspase-3-treated *PKCδ versus* full-length PKCδ in the presence of DMS (*i.e. black bar* in *group b* compared with *black bar* in *group a*) were 3.5 and 3.7 in two separate side-by-side *group a* and *b* experiments, respectively. Higher ratios (*i.e.* greater difference between *group a* and *b black bars*) were seen in the other three experiments. The statistical significances of differences in the values from the five experiments were as follows: $p = 0.002$ for caspase-3-treated PKCδ with DMS *versus* full-length PKCδ with DMS, $p = 0.0005$ for caspase-3-treated PKCδ with DMS *versus* without DMS, and $p = 0.18$ (not significant) for full-length PKCδ with DMS *versus* without DMS. *B*, Western blot of PKCδ (~30 ng) without (−) and with (+) caspase-3 (~0.3 units) incubation. Blotting was performed with antibody directed to the C-terminal region of *PKCδ*.

ate 14-3-3 in the presence of DMS (Fig. 3*A*, *group a*), *i.e.* did not show typical SDK1 activity. In contrast, *PKCδ* treated with caspase-3 showed strong SDK1 activity (Fig. 3*A*, *group b*), and release of an ~40-kDa band was indicated by Western blotting with antibody directed to the C-terminal region of PKCδ (Fig. 3*B*). A similar strong SDK1 activity was observed for rat liver ~40-kDa SDK1 separated by Mono Q column chromatography without caspase-3 treatment (see below).

SDK1 Activities of the Rat Liver Fraction and of the Caspase-3-released Domain of PKCδ, Their Lipid Requirements, and Their Susceptibility to PKCδ Inhibitors and the 14-3-3 Icosapeptide—Over half (53%) of the amino acid sequence of the ~40-kDa protein present in SDK1 active fraction 18 (or 19) from the Mono Q column chromatography of rat liver extract (Fig. 1*E*) aligned identically with the C-terminal half kinase domain of the rat liver PKCδ protein. The fraction showed strong SDK1 activity, similar to that of the caspase-3-released domain of PKCδ, and both of these activities were greatly enhanced by DMS and inhibited by PKC inhibitors (see below).

Lipid requirements for SDK1 activity were determined using the caspase-3-released domain of PKCδ. The activity was detectable only in the presence of Sph, DMS, or *N,N,N*-trimethylsphingosine, but not Sph-1-P, C_2-Cer, or C_{18}-Cer, similar to

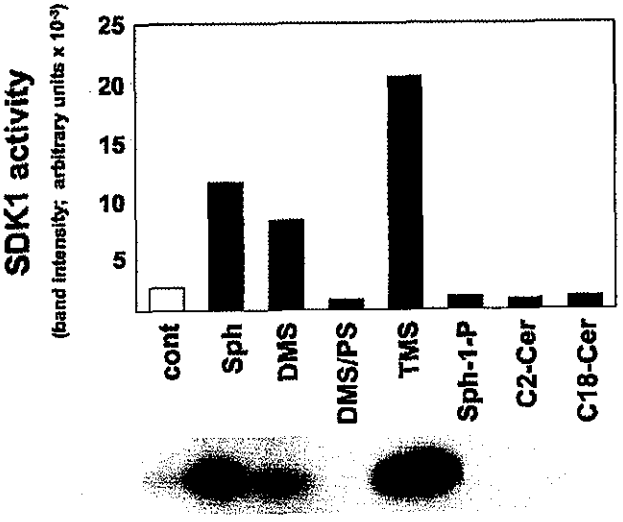

FIG. 4. **Lipid requirements for the SDK1 activity of caspase-3-treated PKCδ.** Aliquots (85 ng) of PKCδ were treated with caspase-3 (0.85 units) and subjected to SDK1 assay using 1 μg of 14-3-3β as substrate in the presence of each of various sphingolipids (50 μM; *abscissa*). The intensity of phosphorylated 14-3-3β was determined by Scion Image analysis and is shown as a bar graph. *DMS/PS* is a mixture of 50 μM DMS and 50 μM PS. *TMS*, *N,N,N*-trimethylsphingosine. SDK1 activity as indicated by degree of 14-3-3β phosphorylation is shown from one experiment; a second experiment gave very similar results (~5% variability). Sph, DMS, and other lipids were given with a final concentration of 1% ethanol in 0.05 M NaCl as vehicle. *cont*, control.

reported results for SDK1 of 3T3-A31 cells (16, 29). The SDK1 activity of the kinase domain determined in the presence of DMS was strongly inhibited by PS (Fig. 4), in contrast to PKCδ activity (with PKC substrate), which was enhanced by PS. The contrasting properties of SDK1 and PKCδ are listed in Table IV. The SDK1 activities of both the ~40-kDa protein present in rat liver Mono Q fraction 18 or 19 (Fig. 5*A*) and the ~40-kDa protein released from PKCδ by caspase-3 (Fig. 5*B*) were inhibited by rottlerin (preferential inhibitor of PKCδ) and by GF109203X (general inhibitor of PKC).

SDK1 Activity of the ~40-kDa Kinase Domain of PKCδ Is Inhibited by the 14-3-3β Peptide—SDK1 activity was originally identified to phosphorylate Ser^{60} in 14-3-3β, Ser^{59} in 14-3-3η, and Ser^{58} in 14-3-3ζ (16). We therefore tested the activity of the kinase domain released from PKCδ by caspase-3 using an icosapeptide (20-amino acid peptide) that includes the phosphorylation site (S) of 14-3-3, *i.e.* YKNVVGARRSSWRVISSIEQ. Phosphorylation failed to occur in the peptide under the same conditions used to test the SDK1 activity of 14-3-3. However, the SDK1 activity of the catalytic domain released from PKCδ was completely inhibited by this peptide at 120 μM. In contrast, a control peptide with the unrelated amino acid sequence Ac-YGGSAESS(Aib)KSEASSK(Aib)SA-$CONH_2$ had no inhibitory effect on SDK1 activity (Fig. 6).

TABLE IV
Contrasting properties of PKCδ and SDK1 activities

Property	PKCδ	SDK1
Lipid requirement		
Diacylglycerol	Yes	No
PS	Yes	No; inhibitory
Sph	No; inhibitory	Yes
DMS	No; inhibitory	Yes
N,N,N-Trimethylsphingosine	No; inhibitory	Yes
Cer	No	No
Sph-1-P	No	No
Bivalent cation requirement		
Ca^{2+}	No	No
Mg^{2+}	Yes	Yes
Substrate	Unidentified[a]	14-3-3[b]

[a] The real physiological substrate is unknown. The conventional substrate for the PKC assay is RFARKGSLRQKNV.
[b] The phosphorylation site is **Ser** next to Trp of YKNVVGARRSS-WRVISSIEQ in the helix 3 domain of 14-3-3.

DISCUSSION

The Ser protein kinase that catalyzes Sph- or DMS-dependent phosphorylation of 14-3-3 at a defined site is termed SDK1 (16). SDK1 has been considered as a modulator of functional membrane proteins through 14-3-3 phosphorylation, which may affect dimer formation; however, the kinase involved has not been identified. We previously tried to perform affinity purification of this kinase employing the 14-3-3 icosapeptide that includes the phosphorylation site, but without success. We then initiated large-scale purification from rat liver as described in this study. The major difficulty was that the number of protein bands separated by SDS-PAGE was not diminished after many steps of chromatography, even though total or specific SDK1 activity increased progressively at each step of purification. This suggests that multiple protein complexes were present and that their components were not separable by the various types of chromatography we employed. The components were separable only by SDS-PAGE, but SDK1 activity was thereby lost. The presence of an ~40-kDa band associated with high SDK1 activity and the absence of such band in fractions without SDK1 activity were noticed at the Mono Q chromatography step. Finally, the ~40-kDa band was identified as having >53% of the same amino acid sequence found in the kinase domain of PKCδ. The ~40-kDa band was presumed to be SDK1, and this was confirmed by several lines of study as described here. However, there is no indication that the ~40-kDa SDK1 protein occurs naturally, based on an NCBI Database search with the BLAST algorithm. Thus, it is unclear at this time whether the ~40-kDa SDK1 protein is present independently of PKCδ or is a cleavage product of PKCδ. Studies by mRNA analysis with possible differential splicing are under way.

We have now identified SDK1 as being closely associated with PKCδ, although PKCδ *per se* has no SDK1 activity. Many recent studies suggest that PKCδ translocated to the mitochondrial membrane may be involved in the apoptotic process, but a definitive mechanism is still under debate (Ref. 30 and references therein). PKCδ activity requires PS and diacylglycerol and is inhibited by Sph or DMS, in striking contrast to SDK1 activity, which requires Sph or DMS and is inhibited by PS (Fig. 4 and Table IV). The C-terminal half kinase domain of PKCδ is now identified as displaying all properties of SDK1, *i.e.* high dependence on Sph or DMS and no dependence on Cer or Sph-1-P. Sph at low concentration (5 μM) showed a maximal effect when low levels of the kinase and 14-3-3 were used with ethanol as vehicle (16). The Sph or DMS concentration required for the maximal effect on SDK1 activity depended on the vehicle used (ethanol, Me_2SO, or octyl glucose), quantity of the kinase and of the 14-3-3 substrate, and composition of the reagents in the reaction mixture. The effect of these variable factors on SDK1 activity was much reduced when a higher concentration of Sph or DMS (50 μM) was used. DMS is a stronger base than Sph, and its enhancement of SDK1 activity was more consistent than that of Sph. Therefore, 50 μM DMS was used in the assay system for technical convenience. The enhancing effect of Sph was highly stereospecific because L-*threo*-Sph, Cer, and Sph-1-P had no effect on SDK1 activity. Likewise, L-*threo*-DMS, stearylamine, and hexadecyltrimethylammonium bromide have no effect on SDK1 activity despite their cationic aliphatic structures (17). These findings (high susceptibility to Sph and DMS under certain conditions and