

## Sphingosine Kinase Type 2 Is a Putative BH3-only Protein That Induces Apoptosis\*

Received for publication, April 29, 2003, and in revised form, June 18, 2003  
Published, JBC Papers in Press, June 30, 2003, DOI 10.1074/jbc.M304455200

Hong Liu‡, Rachele E. Toman‡§, Sravan K. Goparaju‡, Michael Maceyka‡, Victor E. Nava‡, Heidi Sankala‡, Shawn G. Payne‡, Meryem Bektas‡, Isao Ishii\*\*, Jerold Chun‡‡, Sheldon Milstien‡, and Sarah Spiegel‡§§

From the ‡Department of Biochemistry, Medical College of Virginia Campus, Virginia Commonwealth University, Richmond, Virginia 23298-0614, §Interdisciplinary Program in Neuroscience and Department of Biochemistry, Georgetown University Medical Center, Washington, D. C. 20007, ¶Laboratory of Pathology, NCI, National Institutes of Health, Bethesda, Maryland 20892, \*\*Department of Molecular Genetics, National Institute of Neuroscience, Tokyo 187-8502, Japan, ‡‡Department of Molecular Biology, The Scripps Research Institute, La Jolla, California 92037, ‡‡‡Laboratory of Cellular and Molecular Regulation, National Institute of Mental Health, Bethesda, Maryland 20892

**There are two isoforms of sphingosine kinase (SphK) that catalyze the formation of sphingosine 1-phosphate, a potent sphingolipid mediator. Whereas SphK1 stimulates growth and survival, here we show that SphK2 enhanced apoptosis in diverse cell types and also suppressed cellular proliferation. Apoptosis was preceded by cytochrome *c* release and activation of caspase-3. SphK2-induced apoptosis was independent of activation of sphingosine 1-phosphate receptors. Sequence analysis revealed that SphK2 contains a 9-amino acid motif similar to that present in BH3-only proteins, a pro-apoptotic subgroup of the Bcl-2 family. As with other BH3-only proteins, co-immunoprecipitation demonstrated that SphK2 interacted with Bcl-x<sub>L</sub>. Moreover, site-directed mutation of Leu-219, the conserved leucine residue present in all BH3 domains, markedly suppressed SphK2-induced apoptosis. Hence, the apoptotic effect of SphK2 might be because of its putative BH3 domain.**

Sphingosine kinase (SphK),<sup>1</sup> a highly conserved enzyme found in organisms as diverse as plants, yeast, worms, flies, and mammals, catalyzes the phosphorylation of sphingosine to form sphingosine 1-phosphate (S1P), a bioactive sphingolipid metabolite (1). As a specific ligand for a family of five G protein-coupled receptors (S1P<sub>1-5</sub>) (2), which are ubiquitously expressed and couple to various G proteins (3–5), S1P regulates a wide variety of important cellular processes, including cytoskeletal rearrangements and cell movement (6–10), angiogenesis and vascular maturation (6, 11–14), and heart development (15). An important function for S1P in lymphocytes and immune responses emerged from studies with the immunosuppressive drug FTY720, a sphingosine analogue, that is phos-

phorylated by SphK1 (16). Phosphorylated FTY720 is a potent agonist of all S1PRs, except S1P<sub>2</sub> (17), and modulates chemotactic responses and lymphocyte trafficking (16, 17).

Although there is no doubt that S1P acts extracellularly, several studies suggest that this important bioactive lipid, like its precursors sphingosine and ceramide (*N*-acylsphingosine), may also have intracellular functions important for calcium homeostasis (18), cell growth (19, 20), and suppression of apoptosis (21–23). Because intracellular targets of S1P have not yet been identified, its intracellular function is still a matter of debate (5).

Like other signaling molecules, S1P levels in cells are low and tightly regulated in a spatial-temporal manner, and SphK is a central regulating enzyme (1). Two distinct isoforms of SphK, designated SphK1 and SphK2, have been cloned (24, 25). Although highly similar in amino acid sequence and possessing five conserved catalytic domains related to that of the diacylglycerol kinase family (26, 27), SphK2 diverges in its amino terminus and central region. These two isoenzymes have different kinetic properties and also differ in developmental expression (25), implying that they may have distinct physiological functions.

Recent studies suggest that SphK1 and formation of S1P are linked to cell growth and survival (1). Diverse external stimuli, particularly growth and survival factors, stimulate SphK1, and intracellularly generated S1P has been implicated in their mitogenic and anti-apoptotic effects. Tumor necrosis factor- $\alpha$  stimulates SphK1 leading to activation of ERK1/2 (26) and of NF- $\kappa$ B, critical for prevention of apoptosis (28). Similar to platelet-derived growth factor (20), the potent angiogenic vascular endothelial growth factor can also stimulate SphK1, producing S1P, which mediates vascular endothelial growth factor-induced activation of Ras and, consequently, ERK signaling and cell growth (29). Indeed, Ras transformation requires SphK1 activation (30). Moreover, expression of SphK1 enhanced proliferation and growth in soft agar, promoted the G<sub>1</sub>-S transition, protected cells from apoptosis (20, 23), and induced tumor formation in mice (30, 31).

In contrast to SphK1, despite its ubiquitous expression (25), nothing is yet known about the functions of SphK2. Here, we examined the biological functions of SphK2 and discovered its Janus face. Rather than promoting growth and survival, SphK2 suppressed growth and also markedly enhanced apoptosis that was independent of S1PRs. Our results suggest that the apoptotic activity of SphK2 might be related to its putative BH3 motif.

\* This work was supported by National Institutes of Health Grants CA61774 (to S. S.) and in part by MH01723 (to J. C.). 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.

§§ To whom correspondence should be addressed. Tel.: 804-828-9330; Fax: 804-828-8999; E-mail: sspiegel@vcu.edu.

<sup>1</sup> The abbreviations used are: SphK, sphingosine kinase; BrdUrd, bromodeoxyuridine; GFP, green fluorescent protein; GPCR, G protein-coupled receptor; S1P, sphingosine 1-phosphate; ERK, extracellular signal-regulated kinase; HEK, human embryonic kidney; MEF, mouse embryonic fibroblasts; PBS, phosphate-buffered saline; NGF, nerve growth factor; BH3, Bcl-2 homology 3; Ac-DEVD-AMC, acetyl-Asp-Glu-Val-Asp-aminomethylcoumarin.

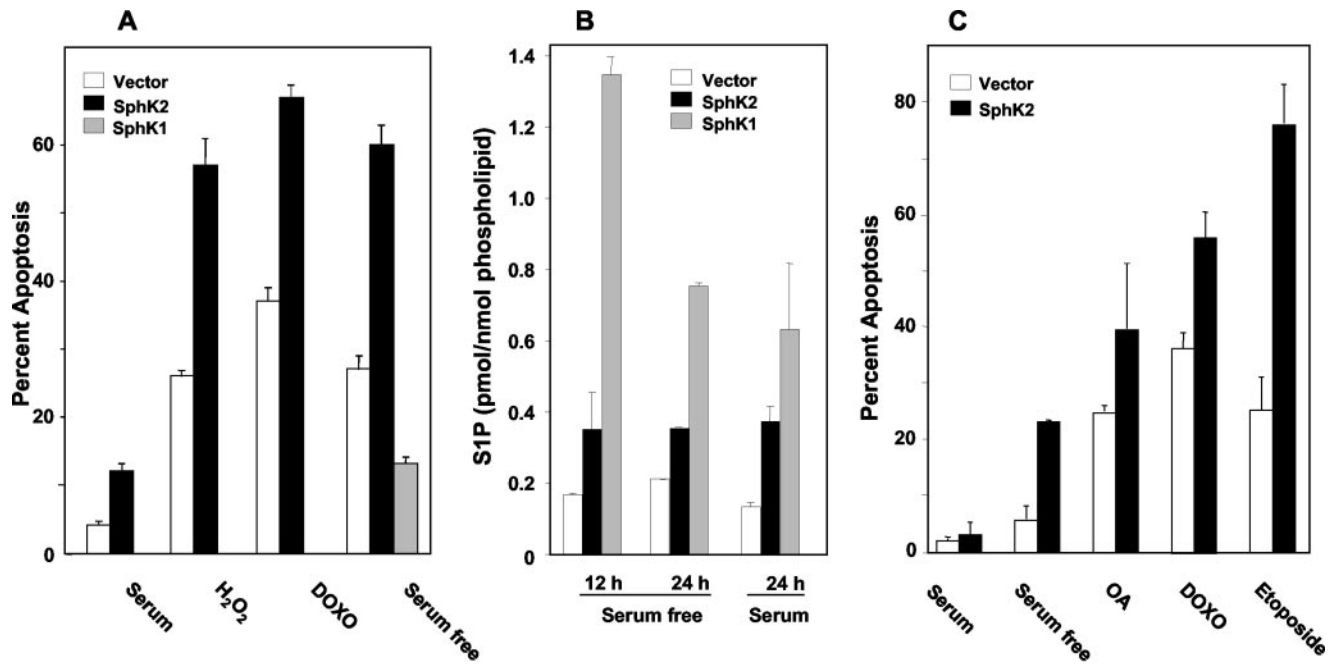


FIG. 1. **SphK1 and SphK2 have opposite effects on apoptosis.** A, NIH 3T3 fibroblasts were transfected with vector, SphK2, or SphK1 together with GFP at 5:1 ratio. Cells were cultured in 10% serum in the absence or presence of doxorubicin (DOXO, 1  $\mu$ g/ml), H<sub>2</sub>O<sub>2</sub> (100  $\mu$ M), or in serum-free medium. After 24 h, cells were fixed and the nuclei stained with Hoechst. Total GFP-expressing cells and GFP-expressing cells displaying condensed, fragmented nuclei indicative of apoptosis were scored. The results are from a representative experiment in duplicate, and data are averages  $\pm$  S.D. At least 300 cells were scored in a double-blind manner. For all treatments,  $p \leq 0.01$  for SphK2-induced apoptosis compared with vector transfected cells. Similar data were obtained in three independent experiments. B, NIH 3T3 fibroblasts were transfected with vector, SphK2, or SphK1. Cells were cultured in the absence or presence of 10% serum for the indicated times. Lipids were extracted and S1P levels were measured as described under "Experimental Procedures."  $p \leq 0.01$  and  $p \leq 0.005$  for SphK1 and SphK2, respectively, compared with vector transfected cells. C, TSU-Pr1 prostate cancer cells stably expressing vector (open bars) or SphK2 (filled bars) were cultured in serum-free media in the absence or presence of serum (fetal bovine serum, 10%), doxorubicin (DOXO, 1  $\mu$ g/ml), okadaic acid (OA, 30 nM), or etoposide (200  $\mu$ M) and apoptosis was determined after 46 h. For all treatments except okadaic acid,  $p \leq 0.01$  for SphK2-induced apoptosis compared with vector transfected cells.

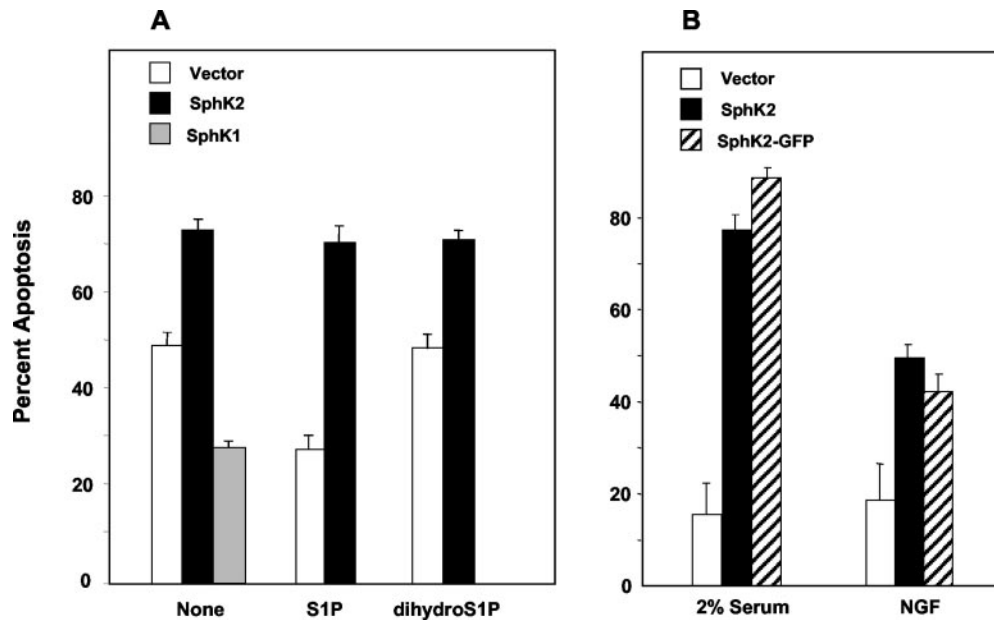


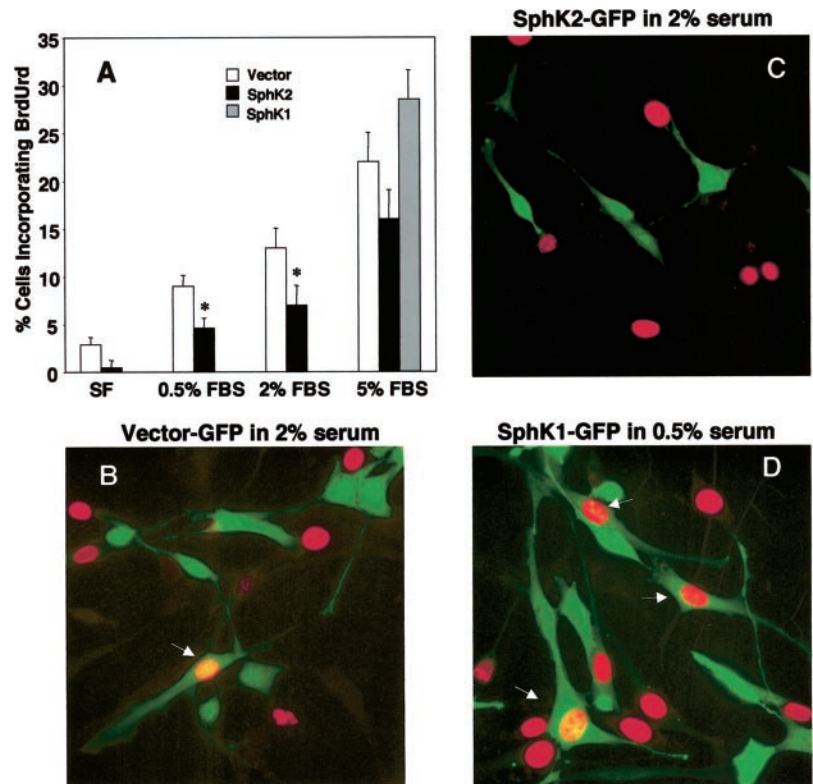
FIG. 2. **SphK2 induces apoptosis in PC12 cells even in the presence of NGF.** A, PC12 cells were transfected with vector, SphK2, or SphK1 together with GFP at 5:1 ratio and then cultured in serum-free media for 24 h in the absence or presence of 100 nM S1P or 100 nM dihydro-S1P. Apoptosis was determined as described under "Experimental Procedures." B, PC12 cells were transfected with vector or SphK2 together with GFP at 5:1 ratio or with SphK2-GFP and then cultured in the presence of 2% serum or 100 ng/ml NGF, and apoptosis of transfected cells was scored. For all treatments,  $p < 0.01$  for SphK2-induced apoptosis compared with vector transfected cells. Similar data were obtained in three independent experiments.

#### EXPERIMENTAL PROCEDURES

**Cell Culture and Transfections**—NIH 3T3 fibroblasts, human embryonic kidney (HEK 293) cells, PC12 pheochromocytoma cells, MCF-7 human breast cancer cells stably expressing Bcl-x<sub>L</sub>, and TSU-Pr1 human prostate cancer cells were cultured and transfected as described (20, 23, 32, 33), except for PC12 cells, which were transfected with

TransFast (Promega). Typical transfection efficiencies were 40, 70, and 15% for NIH 3T3, HEK 293, and PC12 cells, respectively. Where indicated, non-clonal pools of stable transfectants selected in medium containing 1 g/liter G-418 were used to avoid clonal variability. Mouse embryonic fibroblasts (MEFs) were derived from embryonic day 14 embryos generated by the wild type or knock-out double intercrosses as

**FIG. 3. Expression of SphK2 suppresses BrdUrd incorporation into nascent DNA.** A, NIH 3T3 fibroblasts were transiently transfected with GFP-vector (*open bars*), SphK2-GFP (*black bars*), or SphK1-GFP (*gray bar*). Cells were serum-starved for 8 h and incubated in serum-free medium (*SF*) or in the presence of the indicated concentration of serum. After 16 h, BrdUrd was added for an additional 3 h. Double immunofluorescence was used to visualize transfected cells and BrdUrd incorporation, and the proportion of cells incorporating BrdUrd among total transfected cells (expressing the GFP) was determined. Data are means  $\pm$  S.D. of duplicate cultures from a representative experiment. At least 10 different fields with a minimum of 10–50 cells were scored. Asterisks indicate  $p \leq 0.05$  compared with vector transfectants. Similar results were obtained in two independent experiments. B–D, representative images of NIH 3T3 cells transfected with vector-GFP (B), SphK2-GFP (C) cultured in 2% serum, or SphK1-GFP transfectants (D) cultured in 0.5% serum visualized by double immunofluorescence. Arrows indicate cells that are positive for both BrdUrd and GFP. FBS, fetal bovine serum.



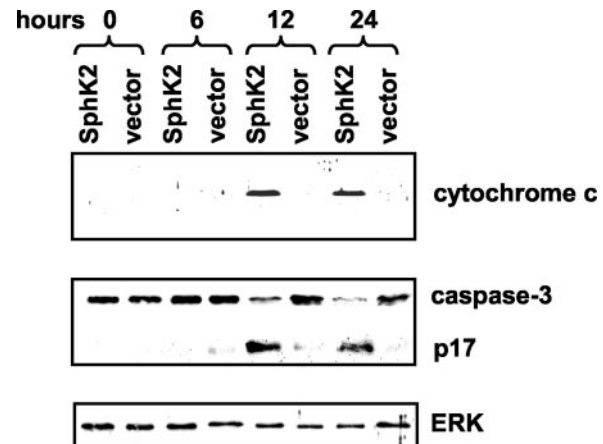
described previously (34). MEFs were cultured in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum and antibiotics.

**Sphingosine Kinase Mutants**—Mammalian expression constructs of cDNAs for SphK1 and SphK2 were described previously (20, 25). Murine SphK2 (25) was cloned into the *Bgl*II site of pSG5-HA vector. The QuikChange site-directed mutagenesis kit (Stratagene) was used to prepare HA-SphK2-L219A (forward primer, 5'-GCTTTACGAGGTGCGAATGGGCTCCTTG-3', and reverse primer, 5'-CAAGGAGCCCATTCGCCACCTCGTAAAGC-3'). The mutation was confirmed by sequencing.

The following PCR primers were used to construct expression vectors for amino- and carboxyl-terminal fragments of mSphK2: forward, 5'-T-GGAATTCTGGCCCCACCACCCTACTGCCAGT-3'; reverse, 5'-CTC-TCAGTCTGGCCGATCAAGGAG-3'; forward, 5'-AACATGGAGGATGCCGTGCGGATG-3', reverse, 5'-TGGTTCACCAACTCGCCATGCTT-3', respectively. PCR products were subcloned into pcDNA3.1/V5-His Topo (Invitrogen).

**Sphingosine Kinase Assay and Measurement of S1P**—SphK activity in cytosol and membrane fractions was determined in the presence of sphingosine complexed with 4 mg/ml bovine serum albumin and [ $\gamma$ -<sup>32</sup>P]ATP in buffer containing 200 mM KCl as described previously (25). Specific activity is expressed as picomoles of S1P formed/min/mg of protein. For mass measurements of S1P, cells were washed with PBS and scraped in 1 ml of methanol containing 2.5  $\mu$ l of concentrated HCl. Lipids were extracted by adding 2 ml of chloroform, 1 M NaCl (1:1, v/v) and 100  $\mu$ l of 3N NaOH, and phases were separated. The basic aqueous phase containing S1P, and devoid of sphingosine, ceramide, and the majority of phospholipids, was transferred to a siliconized glass tube. The organic phase was re-extracted with 1 ml of methanol, 1 M NaCl (1:1, v/v) plus 50  $\mu$ l of 3N NaOH, and the aqueous fractions were combined. S1P in the aqueous phase and total phospholipids in the organic phase were measured as described (20).

**Immunoprecipitation**—MCF-7 cells stably expressing Bcl-x<sub>L</sub> and transfected with HA-SphK2 were lysed in buffer A (10 mM HEPES, pH 7.2, 142.5 mM KCl, 5 mM MgCl<sub>2</sub>, 1 mM EGTA, 0.2% Nonidet P-40, and protease inhibitors). Lysates were incubated on ice for 45 min and then centrifuged at 10,000  $\times$  g for 5 min. Equal amounts of proteins (500  $\mu$ g) were pre-cleared by incubating with 10  $\mu$ l of protein A/G-Sepharose at 4  $^{\circ}$ C for 1 h. After removal of the Sepharose by centrifugation, the pre-cleared lysates were incubated with anti-Bcl-x<sub>L</sub> antibody at 4  $^{\circ}$ C for 2 h, followed by incubation with 20  $\mu$ l of protein A/G-Sepharose. After 1 h at 4  $^{\circ}$ C, the Sepharose beads were washed three times with lysis buffer, boiled in SDS-PAGE sample buffer, electrophoresed, transferred



**FIG. 4. SphK2-induced apoptosis proceeds through cytochrome c release and activation of caspase-3.** NIH 3T3 vector or SphK2 transfectants were deprived of serum for the indicated times. Cytosolic proteins were resolved by SDS-PAGE and analyzed by immunoblotting with antibodies against cytochrome c and caspase-3 and subsequently with anti-ERK2 antibody to show equal loading. p17 is the active cleaved fragment of caspase-3.

to nitrocellulose, and immunoblotted with anti-HA antibody.

**Western Analysis**—Unless otherwise indicated, cells were washed with ice-cold PBS and scraped in 500  $\mu$ l of buffer B (50 mM HEPES, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 2 mM sodium orthovanadate, 4 mM sodium pyrophosphate, 100 mM NaF, and 1:500 protease inhibitor mixture (Sigma)). For analysis of cytochrome c and caspase-3, cytosolic fractions were prepared by resuspending cells in buffer B containing 75 mM NaCl, 1 mM NaH<sub>2</sub>PO<sub>4</sub>, 8 mM Na<sub>2</sub>HPO<sub>4</sub>, 1 mM EDTA, 250 mM sucrose, and 700  $\mu$ g/ml digitonin. Lysates were then centrifuged at 14,000  $\times$  g for 15 min. Equal amounts (20  $\mu$ g) of proteins were separated by SDS-PAGE and then transblotted to nitrocellulose. Anti-cytochrome c, anti-HA, anti-ERK2 (Santa Cruz Biotechnology), and anti-caspase-3 (Stressgen) were used as primary antibodies. Proteins were visualized by ECL (Pierce) with anti-rabbit horseradish peroxidase-conjugated IgG (Jackson ImmunoResearch Laboratories Inc.).

**Staining of Apoptotic Nuclei**—Cells were fixed by addition of an

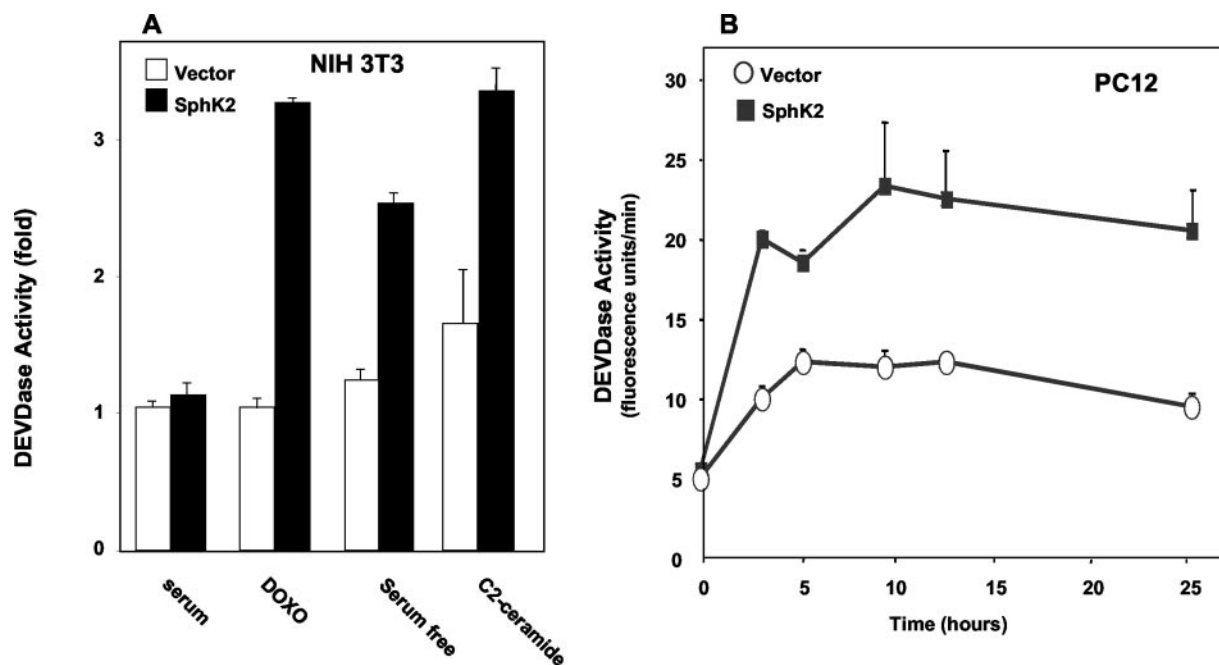
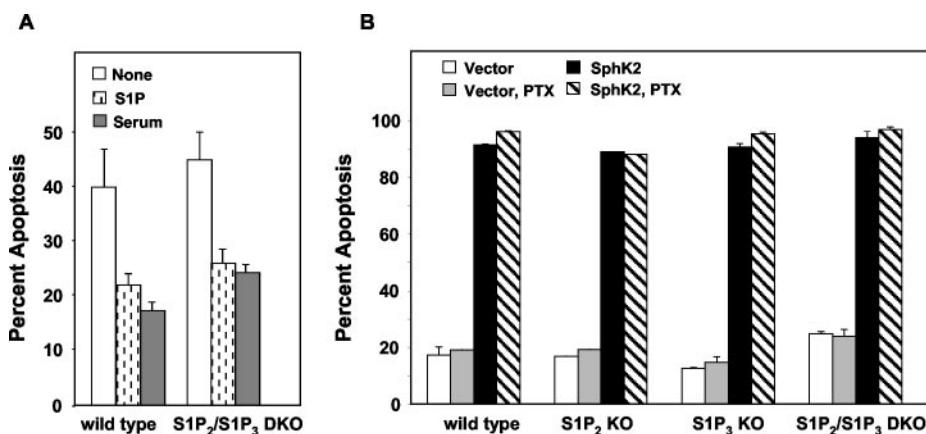


FIG. 5. **SphK2 enhances caspase activation because of trophic factor withdrawal.** A, NIH 3T3 fibroblasts stably expressing empty vector (open bars) or SphK2 (filled bars) were cultured for 24 h in 10% fetal bovine serum in the absence or presence of doxorubicin (DOXO, 1  $\mu$ M), or in serum-free media in the absence or presence of C<sub>2</sub>-ceramide (5  $\mu$ M). Activation of DEVD-specific caspases was measured by the cleavage of the fluorogenic substrate Ac-DEVD-AMC. For all treatments except serum,  $p \leq 0.01$  for SphK2 transfectants compared with vector transfected cells. B, extracts from vector (open circles) and SphK2 (filled squares) PC12 cell transfectants were prepared at the indicated times after trophic factor withdrawal, and DEVDase activity was measured.

#### FIG. 6. S1PRs are dispensable for apoptosis induced by SphK2.

A, exogenous S1P suppresses apoptosis. Wild type or S1P<sub>2</sub>/S1P<sub>3</sub> double knock-out MEFs were cultured in serum-free medium for 24 h, without or with S1P (10  $\mu$ M) or 10% serum, and the percent of apoptotic cells was determined. B, wild type, S1P<sub>2</sub> or S1P<sub>3</sub> single knock-out or S1P<sub>2</sub>/S1P<sub>3</sub> double knock-out MEFs were transfected with GFP vector or GFP-SphK2 and cultured for 24 h in 10% serum without or with pertussis toxin (20 ng/ml) as indicated. The percentage of apoptotic nuclei in cells expressing GFP fluorescence was determined.



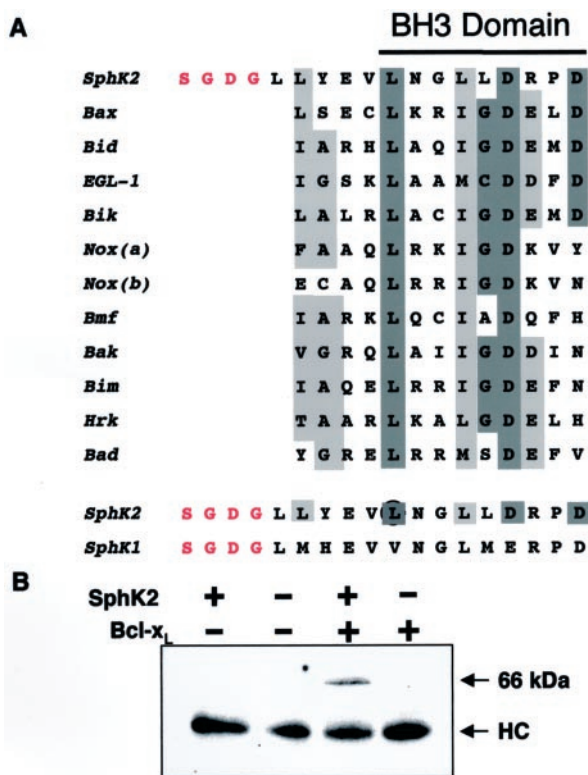
equal volume of PBS containing 4% paraformaldehyde, 4% sucrose, and apoptosis was assessed by staining with 4  $\mu$ g/ml Hoechst 33342. Cells expressing GFP were examined with an inverted fluorescence microscope, and apoptotic cells were distinguished by condensed, fragmented nuclear regions (20). The percentage of intact and apoptotic nuclei in cells expressing GFP fluorescence was determined. A minimum of 300 cells was scored in a double-blind manner.

**Incorporation of Bromodeoxyuridine**—24 h after transfection, NIH 3T3 cells were serum-starved in Dulbecco's modified Eagle's medium supplemented with 2  $\mu$ g/ml insulin, 2  $\mu$ g/ml transferrin, and 20  $\mu$ g/ml bovine serum albumin and then stimulated with serum. After 16 h, cells were incubated for 3 h with bromodeoxyuridine (BrdUrd, 10  $\mu$ M) and then fixed in 4% paraformaldehyde containing 5% sucrose (pH 7.0) for 20 min at room temperature. After washing with PBS, cells were incubated in permeabilization buffer (0.5% Triton/PBS, pH 7.4, containing 10 mg/ml bovine serum albumin) for 20 min at room temperature and then incubated for 1 h at room temperature with monoclonal anti-BrdUrd antibody in the presence of DNase (50 units/ml). After being washed with PBS, cells were stained with Texas Red-conjugated anti-mouse antibody in 5% bovine serum albumin/PBS for 1 h, washed with PBS, and then photographed using a Nikon Eclipse TE200 inverted fluorescence microscope connected to a CoolSnap digital camera (Roper). Cells expressing GFP with positive BrdUrd staining were counted. At least 10 different fields with a minimum of 10–50 cells were scored per field.

**Fluorogenic DEVD Cleavage Enzyme Assays**—Enzyme reactions were performed in 96-well plates with 20  $\mu$ g of cytosolic proteins and a final concentration of 20  $\mu$ M Ac-DEVD-AMC substrate as described previously (35).

## RESULTS

**Opposite Actions of SphK1 and SphK2 in Apoptosis**—SphK1 and concomitant formation of S1P has generally been associated with growth and survival (20, 23, 30). In agreement, overexpression of SphK1 in NIH 3T3 fibroblasts almost completely prevented apoptosis induced by serum starvation (Fig. 1A). Surprisingly, expression of SphK2 markedly enhanced apoptosis of serum-starved NIH 3T3 fibroblasts (Fig. 1A), where shrinkage and condensation of nuclei were clearly evident. Although S1P has been shown to protect cells against anticancer drug-induced apoptosis (22), SphK2 unexpectedly increased doxorubicin- and oxidation-induced cell death (Fig. 1A). Expression of SphK2 in HEK 293 cells has previously been shown to increase cellular S1P (25). It was of interest to examine S1P levels in SphK1 and SphK2 transfectants in serum-free conditions where expression of SphK1 suppresses and SphK2 enhances apoptosis. As shown in Fig. 1B, S1P levels were



**FIG. 7. SphK2 is a putative member of the BH3-only pro-apoptotic protein family.** *A*, alignment of the putative BH3 domain of SphK2 with those of pro-apoptotic BH3-only subfamily proteins is shown. *Dark shaded* boxes indicate identical amino acids, and *gray* boxes indicate very similar residues. Alignment of SphK1 and SphK2 is shown below these. The *circled* residue was mutated in this study. The *red* color indicates residues that form part of the SphK nucleotide binding motif (SGDGX<sub>17-21</sub>K). *B*, SphK2 associates with Bcl-x<sub>L</sub>. MCF-7 cells stably expressing Bcl-x<sub>L</sub> were transfected with vector or HA-SphK2. Cell lysates were immunoprecipitated with anti-Bcl-x<sub>L</sub> antibody and were immunoblotted with anti-HA antibody. *HC* and *66 kDa* indicate the migration of heavy chains and HA-SphK2, respectively. Results are representative of two independent experiments.

significantly increased by overexpression of SphK2 even in the absence of serum, albeit to a lesser extent than SphK1 (Fig. 1B).

Inhibition of SphK1 and decreased S1P has previously been shown to sensitize TSU-Pr1 prostate cancer cells to apoptotic anticancer therapies (33). Yet, overexpression of SphK2 in these cells also markedly increased cell death because of serum withdrawal and the phosphatase inhibitor okadaic acid, as well as the DNA-damaging drugs doxorubicin and etoposide (Fig. 1C).

S1P and activation of SphK1 have also been implicated in NGF-mediated neuronal survival (23). Similar to previous studies (23, 36), overexpression of SphK1, or addition of S1P, but not dihydro-S1P, suppressed apoptosis of PC12 pheochromocytoma cells induced by trophic factor withdrawal (Fig. 2A). In sharp contrast, expression of SphK2 enhanced apoptosis induced by serum deprivation (Fig. 2A). Even in the presence of 2% serum or nerve growth factor, which protect naïve PC12 cells from serum withdrawal-induced apoptosis, ectopic expression of SphK2 was still strongly apoptotic (Fig. 2B). Moreover, SphK2 fused to GFP, which has the same sphingosine phosphorylating activity as wild type SphK2 (data not shown), also induced apoptosis to a similar extent of PC12 cells cultured in the presence of 2% serum or NGF (Fig. 2B).

Because overexpression of SphK1 markedly enhanced growth of many types of cells and increased the proportion of cells in the S-phase of the cell cycle (20, 28–30), we compared the effects of transfection of SphK2 to SphK1 on cellular pro-

liferation. In agreement with previous reports (20, 30), transient expression of SphK1-GFP in NIH 3T3 fibroblasts increased DNA synthesis, as measured by incorporation of BrdUrd into nascent DNA (Fig. 3, A and D). In contrast however, transfection of SphK2 not only reduced DNA synthesis in serum-starved cells, it also significantly reduced DNA synthesis induced by several concentrations of serum (Fig. 3, A, B, and C).

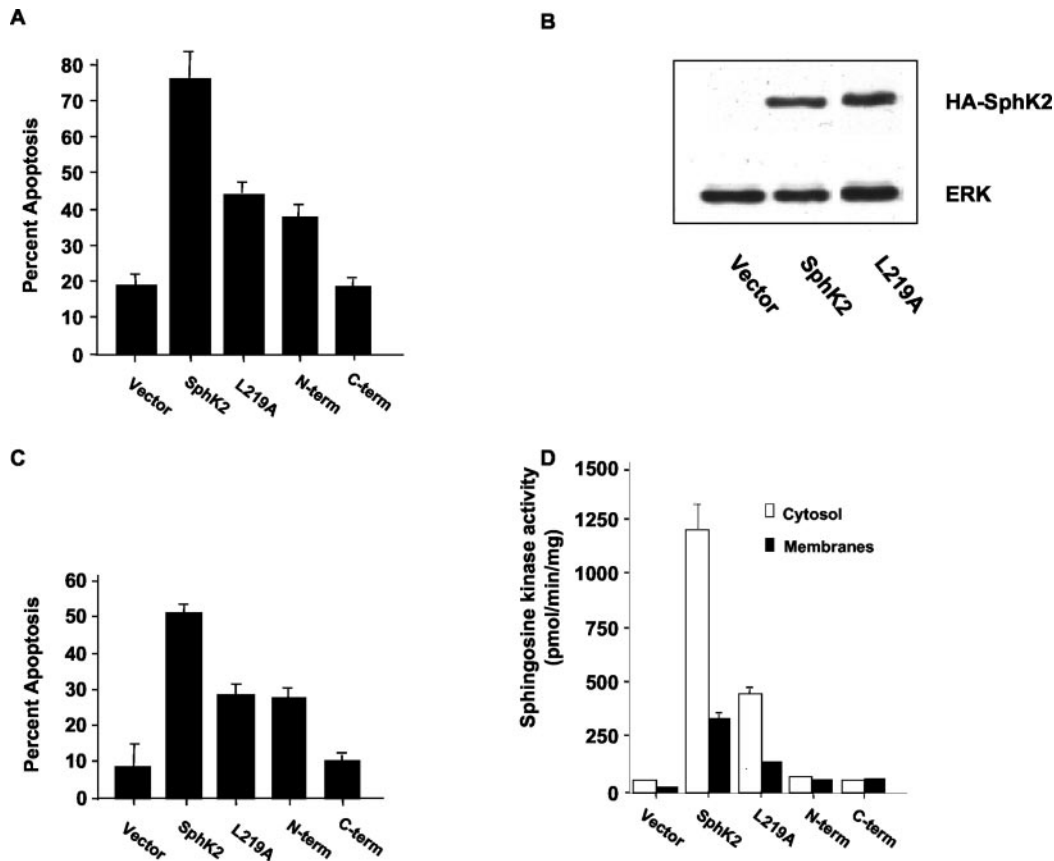
*SphK2-induced Apoptosis Is Independent of S1PR Activation*—Mitochondria are a key regulatory element of stress-induced apoptosis as release of cytochrome *c* and its subsequent complex with cytosolic Apaf-1 leads to activation of caspase-9 that in turn activates caspase-3 (37). SphK2 increased release of cytochrome *c* from the mitochondria into the cytosol and activated caspase-3 as determined by processing to the p17 form (Fig. 4), which preceded the appearance of fragmented nuclei after 24 h. Moreover, SphK2 expression induced a time-dependent increase of caspase activity, as measured with the fluorogenic substrate Ac-DEVD-AMC, in NIH 3T3 cells after serum deprivation or treatment with C<sub>2</sub>-ceramide or doxorubicin (Fig. 5A) and in PC12 cells after serum withdrawal (Fig. 5B).

Because the most well known functions of S1P, the product of SphK2, are elicited by binding to S1P<sub>1-5</sub>, it was of interest to examine whether they are involved in SphK2-mediated apoptosis. To this end, we utilized mouse embryonic fibroblasts (MEFs) isolated from single or double S1P<sub>2</sub>/S1P<sub>3</sub> knock-out mice (34). Serum deprivation-induced apoptosis of MEFs was clearly evident after 24 h, and deletion of S1P<sub>2</sub>, S1P<sub>3</sub>, or both, did not have a significant effect (Fig. 6, A and B). In agreement with previous results with wild type MEFs (9), addition of micromolar concentrations of exogenous S1P (Fig. 6A) suppressed apoptosis in these S1P<sub>2</sub>/S1P<sub>3</sub>-null fibroblasts. Once more, SphK2 induced apoptosis of these cells even in the presence of 10% serum (Fig. 6B). In contrast to wild type MEFs, which express S1P<sub>1-3</sub>, S1P<sub>2</sub>/S1P<sub>3</sub> nulls only express S1P<sub>1</sub> (34), which is coupled solely to G<sub>i</sub> (5). Treatment with pertussis toxin to inactivate G<sub>i</sub>, which completely blocked S1P-induced ERK1/2 activation and DNA synthesis (data not shown), did not abrogate the ability of SphK2 to induce apoptosis even in these MEFs devoid of functional S1PRs (Fig. 6B).

*How Does SphK2 So Profoundly Regulate Apoptosis?*—We were intrigued by the unexpected finding that SphK2 promotes cell death whereas its close relative SphK1 enhances survival. Sequence analysis revealed that both human and mouse SphK2, but not their close SphK1 relatives, contain a 9-amino acid sequence reminiscent of the Bcl-2 homology 3 (BH3) domain present in “BH3 domain-only” proteins (Fig. 7A) (38, 39). These pro-apoptotic members of the Bcl-2 family have sequence homology only within this amphipathic  $\alpha$ -helical segment that allows their interaction with pro-survival Bcl-2 family members to trigger apoptosis (38, 39).

Similar to association of other BH3-only proteins with the pro-survival Bcl-x<sub>L</sub>, we found that SphK2 immunoprecipitated with co-expressed Bcl-x<sub>L</sub>, confirming that SphK2 has a functional BH3 domain (Fig. 7B). Because substitution of the highly conserved leucine residue in the BH3 domain of these proteins has been shown to interfere with their function (38), a similar substitution was introduced in SphK2. This L219A mutation diminished the apoptosis-inducing effect of SphK2 in both NIH 3T3 and PC12 cells (Fig. 8, A and C). Nonetheless, SphK2-L219A still retained significant kinase activity albeit less than the wild type SphK2 (Fig. 8D), although both were expressed similarly (Fig. 8B).

To examine further whether the BH3 domain of SphK2 itself can induce apoptosis, we expressed truncated forms of SphK2



**FIG. 8. SphK2-induced apoptosis requires the BH3 domain.** NIH 3T3 (A and B) and PC12 cells (C) were co-transfected with vector, SphK2, SphK2-L219A, or the N- and C-terminal truncations of SphK2 together with GFP at 5:1 ratio. NIH 3T3 cells were then cultured in serum-free medium (A) and PC12 cells in 2% serum without NGF (C) for 24 h and fixed, and the nuclei were stained with Hoechst. Total GFP-expressing cells and GFP-expressing cells displaying condensed nuclei indicative of apoptosis were scored as described in the legend to Fig. 1. Except for the C-terminal fragment, apoptosis induced by all other constructs was statistically different from the vector control ( $p \leq 0.01$ ). B, equal expression of wild type and SphK2-L219A was confirmed by Western blotting. Lysates from duplicate cultures of NIH 3T3 fibroblasts were separated by SDS-PAGE and transferred to nitrocellulose. After blotting with anti-HA antibody to detect HA-tagged SphK2 proteins, the blot was stripped and reprobbed with anti-ERK antibody as a loading control. D, sphingosine kinase activity of the various SphK2 constructs was measured in cytosol (open bars) and membrane fractions (filled bars) as described previously (25).

split after its BH3 domain into a 227-amino acid N-terminal fragment and a 391-amino acid C-terminal fragment. Because this also fragments the five conserved catalytic domains, it was expected that both N- and C-terminal constructs would lack kinase activity (Fig. 8D). Only the N-terminal fragment, which contains the BH3 domain, induced apoptosis of NIH 3T3 and PC12 cells, whereas the C-terminal fragment did not (Fig. 8, A and C) suggesting that SphK2 may contain a functional BH3 domain.

#### DISCUSSION

We have discovered that in contrast to the pro-survival effects of SphK1, SphK2 enhances apoptosis in diverse cell types induced by a variety of stress stimuli. Apoptosis induced by SphK2 proceeded via the intrinsic mitochondrial death pathway as evidenced by release of cytochrome *c* and subsequent activation of the caspase cascade. Although SphK2, similar to SphK1, increased cellular S1P levels even under serum-free conditions, several lines of evidence suggest that the apoptotic effect of SphK2 does not require expression of the cell surface receptors for S1P. First, the apoptotic effects of SphK2 were observed in diverse cell types that have completely different patterns of S1P receptor expression. Second, in contrast to SphK2, addition of exogenous S1P, which activates S1P receptors, suppressed rather than enhanced apoptosis. Third, SphK2 still induced apoptosis even in S1P<sub>2</sub>/S1P<sub>3</sub> double knock-out MEFs treated with pertussis toxin, which then have no func-

tional S1PRs. Moreover, pertussis toxin did not block SphK2-induced apoptosis (data not shown). These observations are in sharp contrast to inhibition by pertussis toxin of numerous other biological responses of S1P, including its mitogenic effects (1, 5, 34). Taken together, these results suggest that S1PRs are dispensable for the apoptotic effect of SphK2 and provide some support for intracellular actions of S1P.

Similar to our study demonstrating opposing functions of SphK1 and SphK2, the yeast homologues Lcb4 and Lcb5, also appear to have different functions in yeast. Deletion of *LCB4* but not *LCB5* prevented growth inhibition and cell death when both S1P lyase and S1P phosphatase activities were eliminated (40). In contrast, Lcb5, but not Lcb4, plays a role in heat stress resistance during induction of thermotolerance (41). Intriguingly, recent studies demonstrate that Lcb4 and Lcb5 not only differ in membrane association properties but they also have different subcellular localization (42). Lcb4, but not Lcb5, is present in the endoplasmic reticulum, and it has been suggested that membrane-associated and cytosolic Lcb4s play distinct roles to differentially generate biosynthetic and signaling pools of phosphorylated long chain sphingoid bases in yeast (42).

The BH3-only proteins are pro-apoptotic members of the Bcl-2 family that share with their relatives only the short 9-amino acid amphipathic helix BH3 domain, which allows their interaction with pro-survival Bcl-2 family members to

trigger apoptosis (38, 43). Genetic experiments have demonstrated that BH3-only proteins are essential for initiation of programmed cell death throughout evolution (38). Experiments with gene knock-out mice have shown that pathways to apoptosis resulting from different damage signals require distinct BH3-only proteins for initiation (38, 43). Our results indicate that SphK2 has a putative BH3 motif (Fig. 7) and also demonstrate that similar to other BH3-only proteins, SphK2 binds to the pro-apoptotic Bcl-x<sub>L</sub>. Moreover, mutations of the critical upstream leucine residues present in all BH3 domains (44, 45) render them inactive. Similarly, mutation of the equivalent leucine residue (Leu-219) in SphK2 sharply decreased its ability to induce apoptosis. However, the BH3 motif of SphK2 is somewhat unusual. Whereas most BH3 proteins have a glycine prior to the highly conserved aspartic acid, at least three, Bnip3, Egl-1, and Bak, do not, and SphK2 is the only one with a leucine in this position. In addition, the BH3 domains of SphK2 and Nox(a) are the only known BH3 motifs that have positively charged amino acids, arginine and lysine, respectively, immediately preceding the conserved aspartic acid.

BH3-only proteins act at an upstream point in an apoptotic signal transduction cascade by directly or indirectly activating Bax and Bak to induce their oligomerization resulting in permeabilization of the outer mitochondrial membrane, cytochrome *c* release, and activation of caspases (reviewed in Refs. 38, 43, and 46). Death signals can engage 2 distinct classes of BH3-only proteins to integrate diverse apoptotic stimuli into a common cell death pathway governed by Bcl-2 and its multi-BH domain relatives (47). BID-like "activators" directly bind and activate mitochondrial-localized BAK and BAX, triggering their oligomerization and apoptosis. Anti-apoptotic Bcl-2 proteins sequester BID-like proteins preventing their interaction with BAK/BAX. BAD-like "enabler" proteins sensitize apoptosis by binding anti-apoptotic Bcl-2 family proteins and thus preventing the sequestration of BID-like BH3 activators (47). It is tempting to speculate that overexpression of SphK2 or, in response to stress, the putative single BH3 domain of SphK2 allows it to interact with pro-survival Bcl-2 proteins, such as Bcl-x<sub>L</sub>, and induce apoptosis by the sensitizing mechanism. Because the N-terminal fragment of SphK2, which contains the BH3 domain, induced apoptosis to a lesser extent than the holoprotein, it is also possible that SphK2 has additional apoptosis inducing activity that has not yet been uncovered.

**Acknowledgments**—We thank Samantha Poulton for technical assistance and Dr. J. Marie Hardwick for pSG5-HA.

#### REFERENCES

- Spiegel, S., and Milstien, S. (2002) *J. Biol. Chem.* **277**, 25851–25854
- Chun, J., Goetzl, E. J., Hla, T., Igarashi, Y., Lynch, K. R., Moolenaar, W., Pyne, S., and Tigyi, G. (2002) *Pharmacol. Rev.* **54**, 265–269
- Goetzl, E. J., and An, S. (1998) *FASEB J.* **12**, 1589–1598
- Pyne, S., and Pyne, N. J. (2000) *Biochem. J.* **349**, 385–402
- Hla, T., Lee, M. J., Ancellin, N., Paik, J. H., and Kluk, M. J. (2001) *Science* **294**, 1875–1878
- Wang, F., Van Brocklyn, J. R., Hobson, J. P., Movafagh, S., Zukowska-Grojec, Z., Milstien, S., and Spiegel, S. (1999) *J. Biol. Chem.* **274**, 35343–35350
- Okamoto, H., Takuwa, N., Yokomizo, T., Sugimoto, N., Sakurada, S., Shigematsu, H., and Takuwa, Y. (2000) *Mol. Cell. Biol.* **20**, 9247–9261
- Lee, M., Thangada, S., Paik, J., Sapkota, G. P., Ancellin, N., Chae, S., Wu, M., Morales-Ruiz, M., Sessa, W. C., Alessi, D. R., and Hla, T. (2001) *Mol. Cell* **8**, 693–704
- Rosenfeldt, H. M., Hobson, J. P., Maceyka, M., Olivera, A., Nava, V. E., Milstien, S., and Spiegel, S. (2001) *FASEB J.* **15**, 2649–2659
- Graeler, M., Shankar, G., and Goetzl, E. J. (2002) *J. Immunol.* **169**, 4084–4087
- Lee, M. J., Thangada, S., Claffey, K. P., Ancellin, N., Liu, C. H., Kluk, M., Volpi, M., Sha'afi, R. I., and Hla, T. (1999) *Cell* **99**, 301–312
- Liu, Y., Wada, R., Yamashita, T., Mi, Y., Deng, C. X., Hobson, J. P., Rosenfeldt, H. M., Nava, V. E., Chae, S., Lee, M. J., Liu, C. H., Hla, T., Spiegel, S., and Proia, R. L. (2000) *J. Clin. Investig.* **106**, 951–961
- English, D., Welch, Z., Kovala, A. T., Harvey, K., Volpert, O. V., Brindley, D. N., and Garcia, J. G. (2000) *FASEB J.* **14**, 2255–2265
- Garcia, J. G., Liu, F., Verin, A. D., Birukova, A., Dechert, M. A., Gerthoffer, W. T., Bamberg, J. R., and English, D. (2001) *J. Clin. Investig.* **108**, 689–701
- Kupperman, E., An, S., Osborne, N., Waldron, S., and Stainier, D. Y. (2000) *Nature* **406**, 192–195
- Brinkmann, V., Davis, M. D., Heise, C. E., Albert, R., Cottens, S., Hof, R., Bruns, C., Prieschl, E., Baumrucker, T., Hiestand, P., Foster, C. A., Zollinger, M., and Lynch, K. R. (2002) *J. Biol. Chem.* **277**, 21453–21457
- Mandala, S., Hajdu, R., Bergstrom, J., Quackenbush, E., Xie, J., Milligan, J., Thornton, R., Shei, G. J., Card, D., Keohane, C., Rosenbach, M., Hale, J., Lynch, C. L., Rupprecht, K., Parsons, W., and Rosen, H. (2002) *Science* **296**, 346–349
- Meyer zu Heringdorf, D., Lass, H., Alemany, R., Laser, K. T., Neumann, E., Zhang, C., Schmidt, M., Rauen, U., Jakobs, K. H., and van Koppen, C. J. (1998) *EMBO J.* **17**, 2830–2837
- Van Brocklyn, J. R., Lee, M. J., Menzeleev, R., Olivera, A., Edsall, L., Cuvillier, O., Thomas, D. M., Coopman, P. J. P., Thangada, S., Hla, T., and Spiegel, S. (1998) *J. Cell Biol.* **142**, 229–240
- Olivera, A., Kohama, T., Edsall, L. C., Nava, V., Cuvillier, O., Poulton, S., and Spiegel, S. (1999) *J. Cell Biol.* **147**, 545–558
- Cuvillier, O., Pirianov, G., Kleuser, B., Vanek, P. G., Coso, O. A., Gutkind, S., and Spiegel, S. (1996) *Nature* **381**, 800–803
- Morita, Y., Perez, G. I., Paris, F., Miranda, S. R., Ehleiter, D., Haimovitz-Friedman, A., Fuks, Z., Xie, Z., Reed, J. C., Schuchman, E. H., Kolesnick, R. N., and Tilly, J. L. (2000) *Nat. Med.* **6**, 1109–1114
- Edsall, L. C., Cuvillier, O., Twitty, S., Spiegel, S., and Milstien, S. (2001) *J. Neurochem.* **76**, 1573–1584
- Kohama, T., Olivera, A., Edsall, L., Nagiec, M. M., Dickson, R., and Spiegel, S. (1998) *J. Biol. Chem.* **273**, 23722–23728
- Liu, H., Sugiura, M., Nava, V. E., Edsall, L. C., Kono, K., Poulton, S., Milstien, S., Kohama, T., and Spiegel, S. (2000) *J. Biol. Chem.* **275**, 19513–19520
- Pitson, S. M., Moretti, P. A., Zebol, J. R., Xia, P., Gamble, J. R., Vadas, M. A., D'Andrea, R. J., and Wattenberg, B. W. (2000) *J. Biol. Chem.* **275**, 33945–33950
- Liu, H., Chakravarty, D., Maceyka, M., Milstien, S., and Spiegel, S. (2002) *Prog. Nucleic Acid Res.* **71**, 493–511
- Xia, P., Wang, L., Moretti, P. A., Albanese, N., Chai, F., Pitson, S. M., D'Andrea, R. J., Gamble, J. R., and Vadas, M. A. (2002) *J. Biol. Chem.* **277**, 7996–8003
- Shu, X., Wu, W., Mosteller, R. D., and Broek, D. (2002) *Mol. Cell. Biol.* **22**, 7758–7768
- Xia, P., Gamble, J. R., Wang, L., Pitson, S. M., Moretti, P. A., Wattenberg, B. W., D'Andrea, R. J., and Vadas, M. A. (2000) *Curr. Biol.* **10**, 1527–1530
- Nava, V. E., Hobson, J. P., Murthy, S., Milstien, S., and Spiegel, S. (2002) *Exp. Cell Res.* **281**, 115–127
- Cuvillier, O., Nava, V. E., Murthy, S. K., Edsall, L. C., Levade, T., Milstien, S., and Spiegel, S. (2001) *Cell Death Differ.* **8**, 162–171
- Nava, V. E., Cuvillier, O., Edsall, L. C., Kimura, K., Milstien, S., Gelmann, E. P., and Spiegel, S. (2000) *Cancer Res.* **60**, 4468–4474
- Ishii, I., Ye, X., Friedman, B., Kawamura, S., Contos, J. J., Kingsbury, M. A., Yang, A. H., Zhang, G., Brown, J. H., and Chun, J. (2002) *J. Biol. Chem.* **277**, 25152–25159
- Cuvillier, O., Rosenthal, D. S., Smulson, M. E., and Spiegel, S. (1998) *J. Biol. Chem.* **273**, 2910–2916
- Edsall, L. C., Pirianov, G. G., and Spiegel, S. (1997) *J. Neurosci.* **17**, 6952–6960
- Reed, J. C., and Green, D. R. (2002) *Mol. Cell.* **9**, 1–3
- Huang, D. C., and Strasser, A. (2000) *Cell* **103**, 839–842
- Cheng, E. H., Wei, M. C., Weiler, S., Flavell, R. A., Mak, T. W., Lindsten, T., and Korsmeyer, S. J. (2001) *Mol. Cell* **8**, 705–711
- Kim, S., Fyrst, H., and Saba, J. (2000) *Genetics* **156**, 1519–1529
- Ferguson-Yankey, S. R., Skrzypek, M. S., Lester, R. L., and Dickson, R. C. (2002) *Yeast* **19**, 573–586
- Funato, K., Lombardi, R., Vallée, B., and Riezman, H. (2003) *J. Biol. Chem.* **278**, 7325–7334
- Korsmeyer, S. J., Wei, M. C., Saito, M., Weiler, S., Oh, K. J., and Schlesinger, P. H. (2000) *Cell Death Differ.* **7**, 1166–1173
- Zha, J., Harada, H., Osipov, K., Jockel, J., Waksman, G., and Korsmeyer, S. J. (1997) *J. Biol. Chem.* **272**, 24101–24104
- Puthalakath, H., Villunger, A., O'Reilly, L. A., Beaumont, J. G., Coultas, L., Cheney, R. E., Huang, D. C., and Strasser, A. (2001) *Science* **293**, 1829–1832
- Chittenden, T. (2002) *Cancer Cell* **2**, 165–166
- Letai, A., Bassik, M., Walensky, L., Sorcinelli, M., Weiler, S., and Korsmeyer, S. (2002) *Cancer Cell* **2**, 183–192