SphK1 and SphK2, Sphingosine Kinase Isoenzymes with Opposing Functions in Sphingolipid Metabolism*  

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The potent sphingolipid metabolite sphingosine 1-phosphate is produced by phosphorylation of sphingosine catalyzed by sphingosine kinase (SphK) types 1 and 2. In contrast to pro-survival SphK1, the putative BH3-only protein SphK2 inhibits cell growth and enhances apoptosis. Here we show that SphK2 catalytic activity also contributes to its ability to induce apoptosis. Overexpressed SphK2 also increased cytosolic free calcium induced by serum starvation. Transfer of calcium to mitochondria was required for SphK2-induced apoptosis, as cell death and cytochrome c release was abrogated by inhibition of the mitochondrial Ca2+ transporter. Serum starvation increased the proportion of SphK2 in the endoplasmic reticulum and targeting SphK1 to the endoplasmic reticulum converted it from anti-apoptotic to pro-apoptotic. Overexpression of SphK2 increased incorporation of [3H]palmitate, a substrate for both serine palmitoyltransferase and ceramide synthase, into C16-ceramide, whereas SphK1 decreased it. Electrospray ionization-mass spectrometry/mass spectrometry also revealed an opposite effect on ceramide mass levels. Importantly, specific down-regulation of SphK2 reduced conversion of sphingosine to ceramide in the recycling pathway and conversely, down-regulation of SphK1 increased it. Our results demonstrate that SphK1 and SphK2 have opposing roles in the regulation of ceramide biosynthesis and suggest that the location of sphingosine 1-phosphate production dictates its functions.

The sphingolipid metabolite, sphingosine 1-phosphate (S1P),3 a ligand for a family of five specific G protein-coupled receptors, and regulates many important cellular processes including growth, survival, differentiation, cytokine receptor arranagements, motility, angiogenesis, and immunity (reviewed in Refs. 1–3). Although there is no doubt that S1P acts extracellularly, several studies suggest that this potent lipid, like its precursors sphingosine (4) and ceramide (N-acylsphingosine) (5–7), may also have intracellular functions important for calcium homeostasis (8), cell growth (9, 10), and suppression of apoptosis (11–14).

Like other lipid mediators, S1P levels are tightly regulated by the balance between synthesis, catalyzed by sphingosine kinase (SphK), irreversible cleavage by S1P lyase, and reversible dephosphorylation to sphingosine by specific S1P phosphatases. Two distinct SphK isoforms, SphK1 and SphK2, have been cloned and characterized (15, 16). Diverse external stimuli, particularly growth and survival factors, stimulate SphK1 and intracellularly generated S1P has been implicated in their mitogenic and anti-apoptotic effects (10, 13, 17–24). Expression of SphK1 enhanced proliferation and growth in soft agar, promoted the G1-S transition, protected cells from apoptosis (10, 14, 17), and induced tumor formation in mice (17, 18). However, although SphK1 and intracellularly generated S1P can signal “inside-out” to regulate cytoskeletal rearrangements and cell movement, remarkably, cell growth stimulation and suppression of apoptosis induced by SphK1 are not mediated by S1P receptors (14).

In contrast to SphK1, much less is known about SphK2. Although highly similar in amino acid sequence and possessing five evolutionarily conserved domains found in all SphKs (25), SphK2 diverges in its amino terminus and central region. These two isoenzymes have different kinetic properties and also differ in developmental and tissue expression (16), implying that they may have distinct physiological functions. Indeed, rather than promoting growth and survival, SphK2 suppressed growth and enhanced apoptosis that was preceded by cytochrome c release and activation of caspase-3 (26, 27). Moreover, SphK2-induced apoptosis was independent of activation of S1P receptors (26). Sequence analysis revealed that SphK2 contains a 9-amino acid motif similar to that present in Bcl2 homology domain 3 (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-xL (26). However, mutation of the conserved leucine residue present in all BH3 domains (28) only partially reduced apoptosis induced by SphK2 (26), suggesting that the SphK2 protein possesses additional determinants important for this function. In this study, we found that the catalytic activity of SphK2 and its localization to the ER during stress contribute to its apoptosis-inducing properties. Our results provide clues to how SphK1 and SphK2, two very closely related enzymes that use the same substrate and produce the same product, have opposite effects on cell survival and reveal their opposing functions in regulation of sphingolipid metabolism.

**Experimental Procedures**

Materials—[γ-32P]ATP, [3H]palmitic acid, and L-3-[3H]serine were purchased from Amersham Biosciences. S1P and sphingosine were obtained from Biomol (Plymouth Meeting, PA). The internal standards for quantitation of the sphingolipids by ESI-MS/MS were obtained from.

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3 The abbreviations used are: S1P, sphingosine 1-phosphate; BAPTA-AM, 1,2-bis(2-aminophenoxy)ethane-N,N’,N’-tetraacetic acid tetrasodium salt; BHC, Bcl-2 homology domain 3; DKO, double knockout; ESI-MS/MS, electrospray tandem mass spectrometry; SM, sphingomyelin; SphK, sphingosine kinase; ER, endoplasmic reticulum; ERK, extracellular signal-regulated kinase; HA, hemagglutinin; PBS, phosphate-buffered saline.
Avanti Polar Lipids (Alabaster, AL). Serum and medium were from BioFluids (Rockville, MD). G418 was from Invitrogen. Monoclonal anti-HA antibody (3F10) was from Roche (Indianapolis, IN). Antibodies to myc (9E10), cytochrome c, ERK2, and HA (rabbit) were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibody to mitochondrial pyruvate dehydrogenase E1α was from Molecular Probes (Eugene, OR). Polyclonal rabbit antibodies to SphK2 were raised against a unique peptide sequence (QALH1QRLPKPKEARR) as previously described (29). Caspase-12 antibody was kindly provided by Dr. J. Yuan (30). Horseradish peroxidase-conjugated and highly cross-adsorbed fluorophore conjugated anti-rat, mouse, and rabbit secondary antibodies were from Jackson ImmunoResearch (West Grove, PA). Antibodies to calreticulin and calnexin were from StressGen Biotechnologies (San Diego, CA).

Cells and Plasmids—NIH 3T3 fibroblasts, HEK 293 cells, and wild type and has−/−/bac−/− mouse embryonic fibroblasts (a kind gift of Dr. H. Harada) were cultured and transfected as described (26). Mammalian expression constructs for SphK1, SphK1-G82D, SphK2, and SphK2-L219A were described previously (10, 26, 31, 32). The QuikChange site-directed mutagenesis kit (Stratagen) was used to prepare the G213-L219A SphK2 double mutant with the G213 primers using SphK2-L219A as template. SphK1 and SphK1-G82D were cloned into pCMV/myc/ER (Invitrogen) to generate constructs with ER target-ting sequences. Sequences of all constructs were confirmed by direct DNA sequencing.

Reverse Transcriptase-PCR—Total RNA was isolated with TRIzol reagent (Invitrogen) and was reverse transcribed with Superscript II (Invitrogen). Real-time PCR was performed with pre-mixed primer-probe sets obtained from Applied Biosystems (Foster City, CA).

Sphingosine Kinase Activity—SphK2 activity in cytosol and 100,000 × g membrane fractions was determined in the presence of sphingosine complexed with 4 mg/ml bovine serum albumin and [γ-32P]ATP in buffer containing 1 mM KCl as previously described (16), a condition in which SphK2 activity is optimal and SphK1 is inhibited. Specific activity is expressed as picomoles of S1P formed per min/mg of protein.

Intracellular Calcium Measurements—Cells plated on glass coverslips were loaded with 1 μM Fura-2 acetoxymethyl ester (Fura-2AM; Molecular Probes) in buffer containing 116.8 mM NaCl, 1.8 mM CaCl2, 5 mM HEPES, 11.1 mM glucose, and 10 mM HEPES (pH 7.4) for 20 min at 37 °C (41). Coverslips were broken, shards placed in a perfusion chamber of an inverted fluorescence microscope (IX-50, Olympus), and perfused at 1 ml/min with the same buffer. The ratio of fluorescence emission (510 nm) of Ca2+-bound Fura-2 excitation at 340 nm to unbound Fura-2 excitation at 380 nm) was measured in at least 25 fields of 1–5 cells each using a xenon arc excitation source, a galvanometer-driven mirror to select between 340 and 380 nm, a photomultiplier, and a photon counter (IonOptix, Milton, MA). Data were acquired and analyzed with Ion wizard software and [Ca2+]i was calculated from a cell-free calibration curve. In some experiments, [Ca2+]i was also measured with a SLM/Aminco fluorescence spectrophotometer (model AB2).

Immunoblotting Analysis—Unless otherwise indicated, cells were washed with ice-cold PBS and scraped in 500 μl of buffer containing 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, 1,500 protease inhibitor mixture (Sigma). For analysis of cytochrome c, cytosolic fractions were prepared by resuspending cells in lysis buffer containing 75 mM NaCl, 1 mM Na2HPO4, 8 mM Na2HPO4, 1 mM EDTA, 250 mM sucrose, and 700 μg/ml digitonin. Lysates were then centrifuged at 14,000 × g for 15 min. Equal amounts (20 μg) of proteins were separated by SDS-PAGE, blotted to nitrocellulose, and immunopositive bands visualized by enhanced chemiluminescence (Pierce Chemical Co.). Where indicated, blots were scanned and densitometric analysis was performed with AlphaEaseFC software (Alpha Innotec, San Leandro, CA).

Apoptosis Assays—Apoptosis was measured by staining nuclei with the Hoechst dye bisbenzimide and apoptotic cells were distinguished by condensed, fragmented nuclear regions exactly as described (26). Alternatively, apoptosis was measured with the Cell Death Detection ELISA+PLUS kit (Roche Applied Science) that determines cytoplasmic histone-associated DNA fragments.

Immunofluorescence and Confocal Microscopy—Transfectants grown on glass coverslips were treated as indicated in the figure legends. Cells were washed with PBS, fixed for 20 min at room temperature with 3.7% formalin, and permeabilized with 0.5% Triton X-100 in PBS for 3 min. Alternatively, cells were fixed in methanol at −20 °C for 5 min, then blocked for 1 h with 4% bovine serum albumin. After washing, cells were incubated for 30–45 min with primary antibodies in PBS containing 1% bovine serum albumin, then for 30 min with the corresponding secondary antibodies. As the calreticulin antibody stained poorly after methanol fixation, calnexin was used instead as a marker for the ER in these experiments. Coverslips were mounted on glass slides, and images were collected either on a Nikon TE300 fluorescence microscope with a ×60 objective and a CoolSnap CCD run with MetaMorph software or on a Zeiss LSM 510 laser confocal microscope. There was no fluorescence crossover between the channels, and images were collected separately using the appropriate laser excitation and then merged.

Subcellular Fractionation—Cells were suspended in buffer containing 20 mM HEPES (pH 7.4), 10 mM KCl, 2 mM MgCl2, 1 mM EDTA, 10 μg/ml each of aprotinin and leupeptin, 1 mM phenylmethylsulfonyl fluoride, and 0.25 mM sucrose. Subcellular fractionation was performed by sequential centrifugation, essentially as described (32). The postnuclear supernatants were centrifuged for 10 min at 5,000 × g to generate the heavy membrane mitochondria fraction. The supernatants were then centrifuged at 17,000 × g for 15 min to obtain the light membrane fraction containing ER and Golgi. Alternatively, postnuclear supernatants were directly centrifuged at 17,000 × g for 15 min to obtain the intracellular membrane fraction containing mitochondria, ER, and Golgi. The remaining supernatants were centrifuged at 100,000 × g for 1 h to obtain cytosol (S) and pelleted plasma membrane fractions.

Labeling with 1,3-[3H]Serine and [3H]Palmitic Acid—Cells were serum-starved for 18 h and then incubated without or with sphingosine (5 μM) in the presence of [3H]palmitic acid (10 μCi/ml) or 1,3-[3H]serine (30 μCi/ml). After 6 h, cells were scraped in 2 × 600 μl of methanol/concentrated HCl (200:2, v/v). Extracts were sonicated, 600 μl of chloroform and 500 μl of H2O were added and vortexed, and phases were separated by addition of 600 μl of 2 mM KCl and 600 μl of chloroform with vigorous vortexing. For determination of ceramide, aliquots of the organic phases (corresponding to 5 × 10⁶ cpm) were separated by TLC on silica gel plates developed in chloroform/acetic acid (9:1, v/v) (33). Labeled sphingolipids were visualized by autoradiography after spraying with En3Hance (DuPont), and ceramides were quantified with a Bioscan 2000 radiochromatogram scanner. Labeled phospholipids were also resolved by TLC with chloroform, methanol, 20 mM CaCl2 (60:20:4, v/v), as the developing solvent. To analyze sphingolipids, an aliquot of the lipid extract (5 × 10⁶ cpm) was subjected to alkaline hydrolysis in 0.2 M KOH in methanol for 2 h at 37 °C to degrade glycerolipids prior to TLC analysis.

Mass Spectrometry of Sphingolipids and Metabolites—Cells were serum-starved for 18 h and incubated without or with sphingosine (5 treatment.

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**FIGURE 1.** SphK2-induced apoptosis requires its BH3 domain and catalytic activity. A–C, NIH 3T3 cells were transfected with empty vector, SphK2, SphK2-L219A, SphK2-G213E, or SphK2-G213E/L219A as indicated and SphK activity was measured in cytosol (A) and membrane (B) fractions. C, protein expression was determined by immunoblotting with anti-HA antibody. Blots were stripped and re-probed with ERK2 antibody to demonstrate equal loading. D, NIH 3T3 cells were co-transfected with green fluorescent protein and empty vector, SphK2, SphK2-L219A, SphK2-G213E, or SphK2-G213E/L219A at a ratio of 1:5 as indicated. Cells were serum starved for 24 h, fixed, and nuclei were stained with Hoechst. Apoptosis was determined by scoring the fraction of green fluorescent protein-expressing cells displaying fragmented, condensed nuclei indicative of apoptosis compared with the total number of green fluorescent protein-expressing cells (26). A minimum of 300 cells was scored in a double-blind manner. Similar results were obtained in at least three independent experiments. **, p < 0.01; *, p < 0.05.

**RESULTS**

**Apoptosis Induced by SphK2 Requires Its Catalytic Activity—**Many studies have shown that SphK1 promotes cell growth and inhibits apoptosis (10, 17, 19, 20, 22–24). In stark contrast, overexpression of SphK2 does the opposite, suppressing cell growth and inducing apoptosis (26, 27). Previously we have shown that mutation of leucine 219 in its putative BH3 domain, a highly conserved leucine present in all BH3 domains (28), reduced its ability to induce apoptosis (26) (Fig. 1D). Because SphK2-L219A with a mutated BH3 domain also had residual SphK activity (Fig. 1, A and B), we examined whether its enzymatic activity and formation of S1P was also important for its apoptotic effects. All members of the SphK superfamily have the ATP binding sequence (SGDGX₁₇₋₂₁K(R)) present within the conserved C2 domain (35) and a single point mutation of the second conserved glycine residue to asparagine (SGDGGX₁₇₋₂₁K(R)) present within the conserved C2 domain (35) and a single point mutation of the second conserved glycine residue to asparagine has been used to prepare a catalytically inactive SphK1 (35, 36). Similarly, site-directed mutagenesis of the equivalent residue in SphK2 (G213E) resulted in a complete loss of sphingosine phosphorylating activity (Fig. 1, A and B). This catalytically inactive mutant slightly enhanced apoptosis in serum-starved NIH 3T3 fibroblasts, albeit much less than wild type SphK2 (Fig. 1D). The double G213E/L219A mutation not only eliminated the enzymatic activity of SphK2 (Fig. 1, A and B), it totally abrogated its apoptotic activity (Fig. 1D). These effects did not result from differential expression, as the levels of the mutant SphK2 proteins were essentially the same as the wild-type enzyme (Fig. 1C).

**Serum Starvation Increases SphK2 in the ER—**Although SphK1 and SphK2 have opposite effects on apoptosis, surprisingly, when overexpressed, the enzymatic activities were mainly cytosolic (10, 16). Confocal microscopy revealed that, similar to SphK1, epitope-tagged SphK2 is distributed diffusely throughout the cytoplasm of NIH 3T3 fibroblasts cultured in the presence of serum (Fig. 2A). However, we noticed that in the absence of serum, a fraction of SphK2 appeared to co-localize with the ER marker, calnexin (Fig. 2B), but not the mitochondria marker pyruvate dehydrogenase E1α. The catalytically inactive mutant SphK2-G213E also was cytosolic when cells were cultured in the presence of serum (Fig. 2A) and co-localization with calnexin was enhanced upon serum withdrawal (Fig. 2B), suggesting that the catalytic activity of SphK2 was dispensable for translocation to the ER. Moreover, SphK2-L219A also appeared to co-localize with the ER marker after serum starvation (Fig. 2D), albeit to a lesser extent than wild-type SphK2. This suggests that SphK2 can be localized to the ER in BH3-dependent and independent manners. This is not so surprising as SphK2 also contains numerous putative transmembrane domains and has also been shown to interact with other membrane proteins, including the interleukin-12 receptor β1 (37).

Because the yeast homologue of SphK2, Lcb4, is found in internal membranes, including ER, Golgi, and probably endosomes (38, 39), SphK2 localization was examined in more detail. Lysates were subcellularly fractionated by differential centrifugation and expression of SphK2 was determined by Western blotting. Specific enzyme markers were used to substantiate and define purity of the different compartments. In NIH 3T3 cells transfected with V5-SphK2, SphK2 expression was detected not only in the cytosol, but in all membranes fractions including internal membranes containing the ER and in the plasma membrane fraction (Fig. 4A). Moreover, serum deprivation for 24 h reduced expression of SphK2 in all subcellular fractions, particularly...
FIGURE 2. Translocation of SphK2 to the ER by serum starvation. NIH 3T3 cells grown on coverslips were transfected with HA-tagged SphK2. After 48 h, cells were cultured in the presence (A) or absence (B) of serum for 24 h, fixed with methanol, and immunostained for SphK2 with antibodies to HA (left panels), calnexin (ER), or pyruvate dehydrogenase E1a (Mitochondria). Cells were visualized by confocal microscopy. Right-hand panels show the superimposed merged pictures, the yellow color indicates colocalization of SphK2 with the ER marker calnexin. Representative cells of ~100 cells examined are shown.

FIGURE 3. Localization of SphK2 mutants. NIH 3T3 cells grown on coverslips were transfected with HA-tagged SphK2-G213E (A and B) and SphK2-L219A (C and D). After 48 h, cells were cultured for 24 h in the absence or presence of serum, fixed, and stained with HA antibody (SphK2) or calnexin antibody (ER). Cells were visualized by confocal microscopy. Right panels show the superimposed merged pictures, the yellow color indicates colocalization. Representative cells of ~100 cells examined are shown.

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noticeable in the cytosol and plasma membrane fractions. This might explain the apparent greater localization of SphK2 in internal membranes of serum-starved cells detected by confocal microscopy (Fig. 2B). Endogenous SphK2 was also present in internal membranes, plasma membranes, and cytosol (Fig. 4A). After serum starvation, endogenous SphK2, like the overexpressed protein, decreased in the plasma membrane and cytosol and a much greater proportion was associated with internal membranes.

Next, it was of interest to determine whether the different SphK2 mutants differ in their localizations and whether the BH3 motif plays a role in membrane targeting. Expression of all of the mutants was distributed between membrane fractions and the cytosol similar to wild type SphK2 (Fig. 4B). As with wild type SphK2, culturing for 24 h in the absence of serum reduced their expression in the cytosol and a larger proportion appeared to be associated with membranes.

In HEK 293 cells, ectopically expressed SphK2 was also present in all membrane fractions and in the cytosol, and serum starvation also reduced its level in the cytosol of these cells (Fig. 4C). Endogenous SphK2 is expressed in the plasma membrane and in internal membranes of HEK 293 cells and at much lower levels in the cytosol (Fig. 4C). After serum starvation, total levels of SphK2 were reduced. However, a larger proportion of the SphK2 was now present in light membrane fractions containing the ER and Golgi (Fig. 4, C and D).

Targeting SphK1 to the ER Promotes Apoptosis—Interestingly, many previous studies have shown that endogenous SphK1 is mainly cytosolic (10, 23, 36, 38). In contrast to SphK2, serum starvation had no effect on localization of SphK1, which remained predominantly cytosolic (Fig. 5A). The observation that expression of SphK2 in the ER increases upon serum deprivation (Figs. 2 and 3) and disappears from the cytosol suggests that its apoptotic effects might be related to production of S1P in the ER. To examine this possibility, we generated SphK1 and catalytically inactive SphK1-G82D mutants targeted to the ER. Both ER-SphK1 (Fig. 5B) and ER-SphK1-G82D (Fig. 5C) co-localized with the ER marker calnexin. ER targeted SphK1 had similar SphK activity as wild type cytosolic SphK1 (Fig. 6A), whereas ER-SphK1-G82D had no enzymatic activity, as expected (Fig. 6A). In agreement, when ER-SphK1 or SphK2 overexpressing HEK 293 cells were treated with sphingosine, there was a 3–4-fold increase in S1P levels, whereas levels were not significantly increased by transfection with ER catalytically inactive SphK1 (Fig. 6C). Although both ER-SphK1 and ER-SphK1-G82D were now membrane-associated proteins, only wild-type ER-SphK1 had significant enzymatic activity, which was almost as high as SphK2 (Fig. 6D). These results indicate that SphK1 retains its catalytic activity when targeted to the ER. Intriguingly, targeting SphK1 to the ER converted it from anti-apoptotic into an apoptogenic protein (Fig. 6B), which induced apoptosis to a similar extent as SphK2. In contrast, ER-targeted catalytically inactive SphK1-G82D only induced minimal apoptosis (Fig. 6B), possibly because of accumulation of misfolded protein in the ER. Collectively, these results suggest that the differences between pro-survival SphK1 and pro-apoptotic SphK2 are related in part to distinct subcellular localizations and spatially restricted production of S1P.

SphK2-induced Apoptosis Depends on Ca2+ Release from Intracellular Stores—The role of S1P as a ligand of S1P receptors is well established (1, 40). Much less is known of its intracellular actions, although several studies have proposed that this bioactive sphingolipid may serve as a second messenger to mobilize calcium from internal sources in an inositol trisphosphate-independent manner (8, 41, 42). BH3-only pro-
It was thus important to determine whether this increase in \([\text{Ca}^{2+}]\), was involved in propagation of the apoptotic cascade. Treatment of cells with BAPTA-AM, a cell-permeant calcium chelator, slightly enhanced apoptosis of vector transfectants, while markedly reducing apoptosis induced by SphK2 in NIH 3T3 cells (Fig. 7B). Although the main intracellular Ca\(^{2+}\) store is the ER, mitochondria also take up and release calcium very efficiently and are strategically located close to calcium sources allowing mitochondria to shape calcium signals (44, 45). Because calcium uptake by mitochondria is important for cytochrome c release during apoptosis, particularly that induced by the pro-apoptotic Bcl-2 protein Bax (46), we examined the effect of Ru-360, an inhibitor of the mitochondrial calcium antiporter (47). Ru-360 drastically reduced SphK2-induced cell killing of serum-starved NIH 3T3 cells (Fig. 7B). As previously reported (26), SphK2 expression caused the release of cytochrome c from the mitochondria of cells cultured in the absence of serum (Fig. 7C). Treatment with Ru-360 markedly reduced this cytochrome c release, suggesting that mitochondrial calcium uptake may be important for SphK2-induced apoptosis.

Multiple death signals converge at mitochondria to release cytochrome c and initiate the caspase cascade (48). Previous studies have suggested the existence of a novel apoptotic pathway in which caspase-12 is specifically activated by ER stresses, particularly because of perturbation of calcium homeostasis (30). Consistent with its effects on calcium, SphK2 activated pro-caspase-12 as shown by its processing (Fig. 7D), which preceded the appearance of fragmented nuclei. BAX and BAK are two pro-apoptotic proteins known to be involved in ER stress-induced calcium release, caspase-12 activation, and apoptosis (43, 49). To examine their involvement, we utilized embryonic fibroblasts from bax\(^{-/-}\)/bak\(^{-/-}\) double knock-out (DKO) mice. These DKO cells do not have defects in the handling of mitochondrial calcium but...
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cannot deliver enough calcium to the mitochondria because their [Ca\(^{2+}\)]\(_{\text{ER}}\) is too low and thus are resistant to a wide range of apoptotic stimuli (50) and many ER stresses, including H\(_2\)O\(_2\), arachidonic acid, and ceramide (43). In agreement with our previous results (26), SphK2 expression markedly induced apoptosis of wild type mouse embryonic fibroblasts, especially in the absence of serum (Fig. 7E). However, the BAX/BAK DKO cells transfected with SphK2 were almost completely resistant to apoptosis induced by serum withdrawal (Fig. 7E).

SphK2 Increases, while SphK1 Decreases Intracellular Ceramide—Many studies have linked an increase in ceramide to cell growth arrest and induction of apoptosis (5–7), whereas elevation of S1P has been shown to play a cytoprotective role (1), particularly in opposing ceramide-mediated apoptosis (5–7), whereas elevation of S1P has been shown to play a cytoprotective role (1), particularly in opposing ceramide-mediated apoptosis (5–7). In agreement with our previous results (26), SphK2 markedly increased incorporation of radioactivity into ceramide (Fig. 8, B and C). SphK1 expression decreased \[^{3}H\]palmitate incorporation into ceramides relative to the vector transfectants by 40% (Fig. 8, B and C). SphK2 expression also changed the relative ratio of the long chain to very long chain ceramides, with long chain ceramides incorporating significantly more radioactivity than very long chain ceramides (Fig. 8B).

Expression of either SphK1 or SphK2 had no discernable effect on \[^{3}H\]palmitate labeling of glycerophospholipids or sphingomyelin (SM) (Fig. 8D). However, treatment with sphingosine also increased the incorporation of \[^{3}H\]palmitate primarily into the lower migrating monohexosylceramide band (containing long chain ceramides) in vector and SphK2 transfectants but not in SphK1 transfectants (Fig. 8E).

Because targeting SphK1 to the ER induced apoptosis similarly as overexpression of SphK2, the effects of ER-SphK1 on ceramide levels were examined. In agreement with the observation that SphK2 expression increased ceramide levels, ER-SphK1 also increased ceramide (Fig. 8F). Collectively, these results suggest that SphK1 reduces and SphK2 increases ceramide, consistent with their opposite roles in apoptosis and cell growth regulation.

SphK1 and SphK2 Alter the Acyl Chain Distribution of Ceramides—We next examined the effects of SphK1 and SphK2 on sphingolipid species measured by high performance liquid chromatography ESI-MS/MS, as this is the best method to simultaneously determine the degree of saturation of the sphingoid base and the chain length of the fatty acids (34, 54). ESI-MS/MS can also simultaneously determine the mass levels of sphingolipids derived from ceramide, including SM and monohexosylceramides, as well as their various chain length species.

In agreement with previous studies (52, 55), the most abundant ceramide species in HEK 293 cells was 16:0, followed by 24:1, 24:0, and 18:0 (Fig. 9A). Expression of SphK1 reduced levels of all of these ceramide species, whereas SphK2 increased ceramide mass levels (Fig. 9, A and B). Exogenous sphingosine increased the mass of all ceramide acyl chain species, most obviously enhancing the C16 and C18 species (Fig. 9A), and as suggested from the labeling experiments (Fig. 8B), expression of SphK1 reduced, whereas SphK2 increased all ceramide species (Fig. 9A). It is also evident that when the subspecies distributions are compared in pie diagrams, treatment with sphingosine increased the proportion of long-chain ceramides, particularly C16 and C18 (Fig. 9B).

The increased ceramide levels caused by SphK2 were reflected in an increase of the mass of ceramide-based sphingolipids, including monohexosylceramides (Fig. 10A) and SM (Fig. 10B). Conversely, not only did SphK1 reduce ceramide levels, it also reduced the mass levels of monohexosylceramides (Fig. 10A). Intriguingly, treatment with sphingosine increased the monohexosylceramides (Fig. 10A), but not SM (Fig. 10B), and the monohexosylceramide levels were consistently higher in SphK2 and lower in SphK1 expressing cells relative to vector transfectants (Fig. 10A).

ESI-MS/MS also enabled us to accurately determine levels of sphingoid bases and their phosphorylated counterparts (54). In agreement with a previous report (53), C18-sphingosine was the predominant band). These two bands co-migrated with authentic standards (data not shown). SphK1 expression inhibited total incorporation of serine into ceramides, whereas SphK2 increased it (Fig. 8A). Cells also make ceramide and sphingolipids in a salvage pathway by re-utilizing sphingosine from degraded sphingolipids, and these ceramides are not labeled with serine. Therefore, cells were next labeled with \[^{3}H\]palmitate, which serves as a substrate for both serine palmitoyltransferase and ceramide synthase. Again, we observed that SphK1 decreased labeling of ceramide, whereas SphK2 increased it (Fig. 8A). When cells were treated with sphingosine, which is readily converted to ceramide in the salvage pathway, expression of SphK2 markedly increased incorporation of radioactivity into ceramide (Fig. 8, B and C). SphK1 expression decreased \[^{3}H\]palmitate incorporation into ceramides relative to the vector transfectants by 40% (Fig. 8, B and C). SphK2 expression also changed the relative ratio of the long chain to very long chain ceramides, with long chain ceramides incorporating significantly more radioactivity than very long chain ceramides (Fig. 8B).

Expression of either SphK1 or SphK2 had no discernable effect on \[^{3}H\]palmitate labeling of glycerophospholipids or sphingomyelin (SM) (Fig. 8D). However, treatment with sphingosine also increased the incorporation of \[^{3}H\]palmitate primarily into the lower migrating monohexosylceramide band (containing long chain ceramides) in vector and SphK2 transfectants but not in SphK1 transfectants (Fig. 8E).

Because targeting SphK1 to the ER induced apoptosis similarly as overexpression of SphK2, the effects of ER-SphK1 on ceramide levels were examined. In agreement with the observation that SphK2 expression increased ceramide levels, ER-SphK1 also increased ceramide (Fig. 8F). Collectively, these results suggest that SphK1 reduces and SphK2 increases ceramide, consistent with their opposite roles in apoptosis and cell growth regulation.
sphingoid base present, with much lower levels of dihydrosphingosine (TABLE ONE). C20-sphingosine, which only occurs in central nervous system gangliosides (56), was not detected. Interestingly, expression of SphK1 markedly increased dihydrosphingosine, and as a consequence, there was a large increase in dihydro-S1P (TABLE ONE). SphK2 only modestly increased dihydro-S1P. However, in the presence of sphingo-
FIGURE 9. Effects of SphK1 and SphK2 on ceramide species. A, HEK 293 cells expressing vector, SphK1, or SphK2 were serum-starved for 18 h, then treated without or with 5 μM sphingosine (Sph) for 6 h as indicated. Lipids were extracted and ceramide species were determined by ESI-MS/MS. Data from A are presented in “pie” charts showing a proportion of each indicated ceramide species. Numbers indicate chain length followed by the number of double bonds in the fatty acid. Data are averages of triplicate determinations and are expressed as picomole of lipid/mg of protein. Similar results were found in two additional experiments.
SphK Isoenzymes in Sphingolipid Metabolism

FIGURE 10. Effects of SphK1 and SphK2 on monohexosylceramide and sphingomyelin species. HEK 293 cells expressing vector, SphK1, or SphK2 were serum-starved for 18 h, then treated without or with 5 μM sphingosine (Sph) for 6 h as indicated. Lipids were extracted and monohexosylceramides (A) and sphingomyelin (B) species were determined by ESI-MS/MS. Data are averages of triplicate determinations and are expressed as picomole of lipid/mg of protein. Similar results were found in two independent experiments.

TABLE ONE
Effect of SphK1 and SphK2 on levels of free sphingoid bases and phosphorylated forms

|                | Dihydro-Sph | Dihydro-S1P | Sph | S1P  |
|----------------|-------------|-------------|-----|------|
| Vector         | 68 ± 5      | 1 ± 0.1     | 290 ± 17 | 17 ± 2 |
| SphK1          | 419 ± 27    | 22 ± 3      | 263 ± 25 | 21 ± 2 |
| SphK2          | 96 ± 4      | 5 ± 1       | 316 ± 11 | 15 ± 4 |
| Vector+Sph     | 168 ± 19    | 2 ± 0.1     | 1844 ± 106 | 93 ± 7 |
| SphK1+Sph      | 505 ± 41    | 50 ± 3      | 1596 ± 86 | 397 ± 12 |
| SphK2+Sph      | 208 ± 31    | 11 ± 2      | 2253 ± 242 | 250 ± 121 |

sine, both SphK1 and SphK2 markedly increased S1P levels (4- and 2.5-fold, respectively).

Opposing Functions of Endogenous SphK1 and SphK2 in Ceramide Formation—Consistent with the decreased ceramide levels induced by overexpression of SphK1, it has been shown that SphK1 activity inversely correlates with ceramide levels (10, 17–24). Because ectopically expressed proteins may not always exactly mimic the localization and functions of their endogenous counterparts, it was important to establish the involvement of endogenous SphK1 and SphK2 in regulation of ceramide levels. To this end, we utilized small interfering RNA targeted to a specific sequence of SphK1 that has been successfully used to down-regulate SphK1 protein and activity (19, 32, 57–59). As expected, down-regulation of SphK1 with small interfering RNA significantly reduced SphK1 protein levels, mRNA, and enzymatic activity (Fig. 11A and data not shown). SphK1 down-regulation enhanced incorporation of [3H]palmitate into ceramide when cells were treated with sphingosine (Fig. 11A), consistent with the inhibitory effect of overexpression of SphK1 (Fig. 8C).

In agreement with our recent study (32), pSilencer targeted to SphK2 reduced SphK2 mRNA by 70% by quantitative PCR analysis, without affecting expression or activity of SphK1 (data not shown). SphK2 protein, determined by immunoblotting with a specific SphK2 antibody (Fig. 11B), and enzymatic activity (Fig. 11C) were also markedly reduced. In agreement, siSphK2 also slightly decreased both basal S1P levels and the increased S1P resulting from treatment with sphingosine (Fig. 11E). Importantly, sphingosine-induced incorporation of [3H]palmitate into ceramide (Fig. 11D) and sphingosine-induced increases in the major ceramide species were sharply curtailed by down-regulation of SphK2 (Fig. 11F), further suggesting a role for SphK2 in reutilization of sphingosine and its conversion to ceramide.

DISCUSSION

It is now well established that S1P produced by SphK1 promotes cell growth and inhibits apoptosis, in part because of antagonism of ceramide-induced apoptosis (reviewed in Refs. 5, 7, and 60). Thus, it was surprising to discover that overexpression of the second isozyme, SphK2, was associated with inhibition of cell growth and was pro-apoptotic (26, 27). This raised the question: how can two enzymes that catalyze the same reaction and produce the same product, S1P, have opposite effects on cell growth and apoptosis? A possible explanation for the opposing effects of SphK1 and SphK2 is that the two proteins are located in, or translocated to, different compartments within the cell, and that localized production of S1P has distinct functions. Conflicting data exists on the localization of ectopically expressed SphK2. Igarashi and co-workers (27) reported that when SphK2 was transiently expressed in HeLa and COS7 cells, it was localized in the nuclei, whereas in HEK 293 cells it was localized to the cytosol. In contrast, overexpressed SphK2 was found to associate with the interleukin-12 receptor-β1 at the plasma membrane in HEK 293 cells and T cell hybridomas (37). However, using a specific antibody, we
were unable to detect nuclear expression of endogenous SphK2 in either HEK 293 or MDA-MB-453 cells (32). In agreement, in this study, we found that endogenous SphK2 in HEK 293 cells was mainly localized to the plasma membrane and internal membranes, and was present at much lower levels in the cytosol.

SphK1, on the other hand, has been shown to be predominantly cytosolic, and several growth factors induce its translocation to the plasma membrane (1), possibly because of phosphorylation by protein kinase C or ERK2 (19, 23, 36). Moreover, S1P produced by translocation of SphK1 to the plasma membrane has been implicated in transactivation of cell surface S1P receptors (29, 31, 61). Nevertheless, S1P made by SphK2 does not transactivate S1P receptors because neither treatment with pertussis toxin, which inhibits the G_{i1} only coupled S1P_{1} receptor, nor deletion of S1P_{2} or S1P_{3}, interfered with the ability of SphK2 to induce apoptosis (26).

Unlike SphK1, we found that the proportion of SphK2 localized to the ER increased several hours after serum withdrawal. The ER localization of SphK2 was partly dependent on its BH3 domain, consistent with other studies showing that pro-apoptotic BH3-only proteins also localize to the ER via their BH3 domains. For example, the BH3-only protein BIK functions at the ER to stimulate cytochrome c release from mitochondria (62). However, we found that mutation of the conserved

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**FIGURE 11. Effects of down-regulation of SphK1 and SphK2 on ceramide.** A, HEK 293 cells transfected with control small interfering RNA or small interfering RNA targeted to SphK1 were serum-starved for 18 h, then incubated for 6 h with [3H]palmitate (10 μCi/ml) in the absence or presence of 5 μM sphingosine. [3H]Ceramide was resolved by TLC and radioactivity quantified with a radiochromatogram scanner. Inset, duplicate cultures were lysed and equal amounts of proteins (20 μg) were immunoblotted with anti-SphK1. Blots were stripped and reprobed with anti-tubulin to confirm equal loading. B–F, HEK 293 cells were transfected with either pSilencer control small interfering RNA (pSil-Vec) or pSilencer directed against SphK2 (pSil-K2). B, cell lysates were separated into supernatant and 100,000 × g pellets, and expression of SphK2 protein was examined by immunoblot analysis with specific SphK2 antibodies. Blots were stripped and re-probed with anti-calnexin or anti-tubulin antibodies as loading controls. C, in duplicate cultures, SphK2 activity was determined in the presence of 1 μM KCl. D, HEK 293 cells expressing pSilencer vector or pSilencer-SphK2 were serum-starved for 18 h, then incubated for 6 h with [3H]palmitate (10 μCi/ml) in the absence or presence of 5 μM sphingosine. [3H]Ceramide was resolved by TLC and radioactivity quantified with a radiochromatogram scanner. E and F, duplicate cultures were serum starved for 18 h, then treated without or with 5 μM sphingosine for 6 h. Lipids were extracted and S1P levels (E) and ceramide species (F) were determined by ESI-MS/MS. Similar results were obtained in three independent experiments.
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**FIGURE 12. Proposed model for opposing functions of SphK1 and SphK2 in sphingolipid metabolism.** Our results support the notion that SphK2, similar to yeast membrane-associated Lcb4 (39), might play a role in the sphingosine salvage pathway of mammalian cells, acting in concert with S1P phosphatase (SPPase) to convert S1P back to sphingosine and then to ceramide. Ceramide generated in the ER has been linked to increased calcium release, leading to apoptosis. Moreover, cytosolic S1P formed by SphK1 inhibits de novo ceramide biosynthesis as a cellular sensing mechanism to minimize unneeded biosynthesis of ceramide. For more details, see text. The locations of the sphingolipid metabolizing enzymes depicted in this scheme are not meant to indicate topology as the active site topologies have not all been unequivocally identified.

leucine in the SphK2 BH3 domain did not abolish its localization to the ER, suggesting that its putative transmembrane domains might also contribute to its association with internal membranes. Alternatively, SphK2 may interact with other membrane proteins (37). Consistent with the notion that localization of SphK2 to the ER and internal membranes is important for its function, targeting cytosolic SphK1, but not catalytically inactive SphK1, to the ER converted it from anti-apoptotic to pro-apoptotic. These results provide further support for the importance of the sphingosine phosphorylating activity of SphK2 for its ability to induce apoptosis. More significantly, they suggest that the difference between the effects of SphK1 and SphK2 on apoptosis may be linked to the location at which S1P is produced.

How does SphK1 decrease and SphK2 increase ceramide levels? One of the most intriguing results of this study is that these two isoenzymes that catalyze the same reaction have opposite effects on ceramide and sphingolipid levels. It should be noted that sphingosine is not produced by enzymes that catalyze the same reaction have opposite effects on ceramide and sphingolipid levels. It should be noted that sphingosine is not produced by enzymes that catalyze the same reaction. Rather, it is derived from cleavage of ceramide by ceramidases in the sphingolipid degradative pathway, and can then be re-utilized for ceramide and complex sphingolipid synthesis or phosphorylated by SphKs to form S1P (Fig. 12). S1P can be irreversibly cleaved to ethanolamine phosphate and hexadecenal by S1P lyase or dephosphorylated back to sphingosine by specific S1P phosphatases. Cells can conserve resources by reutilizing sphingosine, minimizing the de novo synthesis of ceramide. Previously, it has been suggested that externally supplied sphingoid bases may be phosphorylated after their entry into cells and require dephosphorylation before they can be used for ceramide synthesis (33, 39, 63, 64). In support of this idea, the major yeast SphK, Lcb4, but not Lcb5, is localized to the cytosolic side of internal membranes, including ER, Golgi, and probably endosomes (38, 39), and is required for ceramide synthesis from exogenously added sphingoid bases (39). Indeed, we found that when SphK2 transfectants were labeled with [3H]palmitate, addition of sphingosine caused a 3-fold increase in incorporation of radioactivity into long-chain ceramides, whereas down-regulation of SphK2 decreased incorporation by 50% and also reduced the mass level of ceramides determined by mass spectrometry. Thus, this is the first evidence in support of the notion that SphK2, similar to yeast membrane-associated Lcb4, might play a role in a sphingosine salvage pathway of mammalian cells, acting in concert with S1P phosphatase to convert S1P back to sphingosine and then to ceramide (Fig. 12). It has been reported previously that a specific S1P phosphohydrolase, S1P phosphohydrolase-1, is localized to the ER (33, 65, 66). Indeed, in agreement with our hypothesis, overexpression of S1P phosphohydrolase-1 increases ceramide levels and induces apoptosis (33). Conversely, down-regulating this phosphatase increases S1P, reduces sphingosine levels, and abrogates apoptosis (65). The significance of the phosphorylation/dephosphorylation of added sphingoid bases is unclear, but it has recently been suggested that this might be a mechanism to differentiate between sphingoid bases arising from de novo synthesis and recycling, possibly for regulation of sphingolipid biosynthesis or signaling (67).

In contrast, cytosolic S1P formed by SphK1 inhibits ceramide biosynthesis possibly as a cellular sensing mechanism to regulate levels of ceramide (Fig. 12). SphK1 can reduce ceramide levels by routing sphingosine, the substrate for ceramide synthesis, to phosphorylation and irreversible degradation by S1P lyase. Alternatively, it is also possible that S1P formed by SphK1 negatively regulates ceramide synthesis as it has been suggested that phosphorylated long-chain sphingoid bases may inhibit serine palmitoyltransferase activity and de novo ceramide biosynthesis (68). Interestingly, we found that overexpressed SphK1 increases dihydrocerphosphogolipidid and as a consequence dihydro-S1P, yet reduces ceramide levels. This raises another provocative possibility that cytosolic S1P formed by SphK1 might negatively regulate (dihydro)-ceramide synthase. Collectively, our results suggest that cytosolic S1P formed by SphK1 has a different function than S1P produced at the ER and/or other membranes by SphK2. Similarly, in yeast, membrane-associated and cytosolic Lcb4 play distinct roles to differentially generate biosynthetich and signaling pools of phosphorylated long chain sphingoid bases (39).

Increases in cytosolic free calcium have been intimately linked to both survival and apoptosis; whereas calcium is necessary for cell growth and survival, inappropriately increased intracellular levels may result in cell death (44). Both ER ceramide (69) and BH3-only proteins have been connected to the ER-calcium apoptotic gateway (43). Several studies have shown that [Ca^{2+}]{\text{ER}} is an important determinant of the response of cells to stresses, including increased ceramide (43), and that the balance between anti- and pro-apoptotic Bel-2/BAX family members regulates [Ca^{2+}]{\text{ER}} (46). For example, BAK and BAX directly affect [Ca^{2+}]{\text{ER}} with subsequent sensitization of mitochondria to calcium-mediated fluxes and cytochrome c release (46). Moreover, ceramide itself causes the release of calcium from the ER and thus increases both cytosolic and mitochondrial calcium. The reduction of [Ca^{2+}]{\text{ER}} or buffering of cytoplasmic calcium changes, can prevent mitochondrial damage and protect cells from apoptosis induced by ceramide (69). In yeast, calcium influx induced by ER stress is mediated by calcium channels that are similar to voltage-gated calcium channels. This calcium influx, important for replenishment of secretory organelles with calcium, was stimulated by intracellular S1P accumulation and required both Lcb4 and Lcb5 (70). Our results strengthen the notion that S1P may also contribute to regulation of calcium homeostasis in mammalian cells, particularly in response to stress, reminiscent of its function in yeast (70) and plants (71) that lack S1P receptors.
