

Involvement of Sphingosine Kinase 2 in p53-Independent Induction of p21 by the Chemotherapeutic Drug Doxorubicin

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Abstract

Sphingosine-1-phosphate is a potent lipid mediator formed by phosphorylation of sphingosine, a metabolite of sphingolipids, catalyzed by two sphingosine kinase (SphK) isoenzymes, SphK1 and SphK2. Expression of SphK2, which is enriched in the nucleus of MCF7 human breast cancer cells, increased expression of the cyclin-dependent kinase inhibitor p21 but had no effect on p53 or its phosphorylation. The anticancer drug doxorubicin is known to increase p21 via p53-dependent and p53-independent mechanisms. Down-regulation of endogenous SphK2 with small interfering RNA targeted to unique mRNA sequences decreased basal and doxorubicin-induced expression of p21 without affecting increased expression of p53. Down-regulation of SphK2 also decreased G₂-M arrest and markedly enhanced apoptosis induced by doxorubicin. Moreover, siSphK2 reduced doxorubicin-induced p21 expression in p53-inactivated MCF7 cells. Likewise, in human wild-type p53- and p21-expressing HCT116 colon carcinoma cells, as well as in p53-null counterparts, down-regulation of SphK2 markedly reduced p21 induction by doxorubicin. Knockdown of SphK2 sensitized HCT116 cells to apoptosis induced by doxorubicin with concomitant cleavage of poly(ADP-ribose) polymerase. Collectively, our results show that endogenous SphK2 is important for p53-independent induction of p21 expression by doxorubicin and suggest that SphK2 may influence the balance between cytostasis and apoptosis of human cancer cells. [Cancer Res 2007;67(21):10466–74]

Introduction

Sphingosine-1-phosphate (S1P) is now emerging as a vital lipid mediator that regulates a wide variety of physiologic and pathologic processes (reviewed in refs. 1–3). S1P is the ligand for five specific G protein-coupled receptors (GPCR; named S1P₁₋₅). These receptors are coupled to distinct G protein signaling pathways, enabling S1P to regulate numerous physiologic processes particularly important for cancer, including growth, survival, migration and invasion, and angiogenesis and vascular maturation (reviewed in refs. 4, 5). In addition, evidence suggests that S1P may also have intracellular functions important for calcium homeostasis, cell growth, and suppression of apoptosis (1).

S1P is produced in cells by phosphorylation of sphingosine, the backbone of sphingolipids, catalyzed by sphingosine kinase (SphK). Two isoforms of mammalian SphK (SphK1 and SphK2) have been cloned and characterized (5). Both have five conserved domains and are highly homologous, but SphK2 has an extended NH₂ terminus and a different central region. SphK1 and SphK2 have distinct localizations (6, 7), kinetic properties, and developmental and tissue expression (8), suggesting that they have different functions. Unlike SphK1, SphK2 has a somewhat lower substrate specificity and also can phosphorylate the prodrug FTY720 (9–11), producing an immunosuppressant that induces lymphopenia.

Numerous external stimuli, including growth factors, ligands for GPCRs, proinflammatory cytokines, and cross-linking of immunoglobulin receptors (reviewed in refs. 1, 2), activate SphK1. To date, only epidermal growth factor (EGF; ref. 12) and FcεRI triggering (13) have been shown to stimulate SphK2. Many studies have shown that SphK1 is critical for growth, metastasis, and chemoresistance of human breast cancers. For example, MCF7 cells overexpressing SphK1 are more resistant to apoptosis induced by doxorubicin (one of the most active antineoplastic agents used in clinical oncology) and, when injected into mammary fat pads of ovariectomized nude mice, produce larger tumors with higher microvessel density in the periphery (14). Moreover, in these cells, estrogen transactivates EGF receptor by stimulating SphK1 (15). Down-regulation of SphK1 in MCF7 cells reduced EGF- and serum-stimulated growth and enhanced sensitivity to doxorubicin (16). SphK1 is overexpressed in breast tumors and SphK1 inhibitors are antiproliferative toward a panel of breast cancer cell lines, including those with the multidrug resistance phenotype, and suppressed growth of mammary adenocarcinoma tumors in mice (17).

Much less is known of the functions of SphK2. Its overexpression has been shown to suppress cell growth and enhance apoptosis (8, 18). The nuclear localization sequence of SphK2 was required for inhibition of DNA synthesis when overexpressed in NIH 3T3 fibroblasts (18). Induction of apoptosis by overexpressed SphK2 was independent of activation of S1P receptors but required its putative Bcl-2 homology domain 3 (BH3) and estrogen receptor localization (19, 20). A recent study showed an essential requirement for SphK2 in a sphingolipid apoptosis pathway activated by FTY720 analogues (21). It has been suggested that SphK1 and SphK2 have opposing roles in the regulation of salvage of sphingosine and its reuse for ceramide biosynthesis (20), indicating that the location of S1P production dictates its functions. However, mice with a deficiency of either SphK1 or SphK2 develop normally, yet the double knockout is embryonically lethal due to severely abnormal neurogenesis and angiogenesis, and massive apoptosis in the brain (7). Therefore, SphK1 and SphK2 might have overlapping and/or complementary physiologic functions. In agreement, both SphK1 and SphK2 were required for EGF-induced migration of MDA-MB-453 breast cancer cells (12).

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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In this study, we investigated the functions of endogenous SphK2 in carcinoma cells and how it influences the balance between cyto-stasis and apoptosis in response to the chemotherapeutic anthra-cyclin doxorubicin. We found that endogenous SphK2 is critical for p53-independent induction of p21 expression by doxorubicin and its apoptotic actions in human breast and colon cancer cells.

Materials and Methods

Reagents. Doxorubicin was purchased from Sigma, reconstituted in molecular biology grade water, and stored protected from light. Serum and medium were from Biofluids. Antibodies to p21, lamin A/C, caspase-7, phosphorylated p53 (Ser¹⁵), and poly(ADP-ribose) polymerase (PARP) were purchased from Cell Signaling. Anti-p53 antibody was from Oncogene. Anti-underphosphorylated Rb antibody was from Transduction Laboratories. Anti-protein disulfide isomerase antibody was from Stressgen Biotechnologies. Antitubulin antibody was from Santa Cruz Biotechnology. SphK2 rabbit polyclonal antiserum raised against a unique SphK2 peptide sequence (QALHIQRLRPKPEARPR) was purified as described (12). Secondary antibodies were from Jackson ImmunoResearch.

cDNA cloning and expression vectors. Construction of human SphK2 (UniProt: Q9NRA0-2) expression vectors has been described previously (8, 12). SphK2 with an NH₂-terminal extension of 36 amino acids (SphK2-L,

UniProt: Q9NRA0-1) was amplified from cDNA isolated from HEK 293 cells by PCR using Platinum Taq Polymerase High Fidelity (Invitrogen) with 5'-CACCATGAATGGACACCTTGAA-3' and 5'-TCCGGGCTCCCGCCCCG-3' sense and antisense primers, respectively. The PCR product was cloned into pcDNA 3.1 V5-His vector (Invitrogen). All sequences were verified by DNA sequencing and protein expression after transfection of HEK 293 cells was confirmed by Western blotting with anti-SphK2 and anti-V5 antibodies (12).

Cell culture and transfection. MCF7 cells were cultured in phenol red-free improved minimum essential medium supplemented with 0.25% glucose and 10% fetal bovine serum (FBS). MCF7 cells infected with pLXSN vector only (MCF7/Vec) and infected with human papillomavirus-16 (HPV-16) E6 (MCF7/E6) were described previously (22). HCT116 wild-type (WT), p21^{-/-}, and p53^{-/-} cells were cultured in DMEM supplemented with 10% FBS. MCF7 cells were transfected with LipofectAMINE Plus (Invitrogen) and cultured overnight. SphK2 expression in MCF7 and HCT116 cells was down-regulated by transfection with sequence-specific small interfering RNA (siRNA) for human SphK2 (sense, 5'-GGAUUGCGCUCGU-CGCUUUCAU-3'; antisense, 5'-AUGAAAGCGAGCGCAAUCCTG-3'; Ambion) and control siRNA (Ambion) using Oligofectamine (Invitrogen). In addition, ON-TARGETplus SMARTpool siRNA against SphK2 and control siRNA from Dharmacon were used to confirm lack of off-target effects. In some experiments, siRNA targeted to another human SphK2 sequence (5'-GCTGGGCTGTCTTCAACCT-3'; Qiagen) and control siRNA (Qiagen) were used.

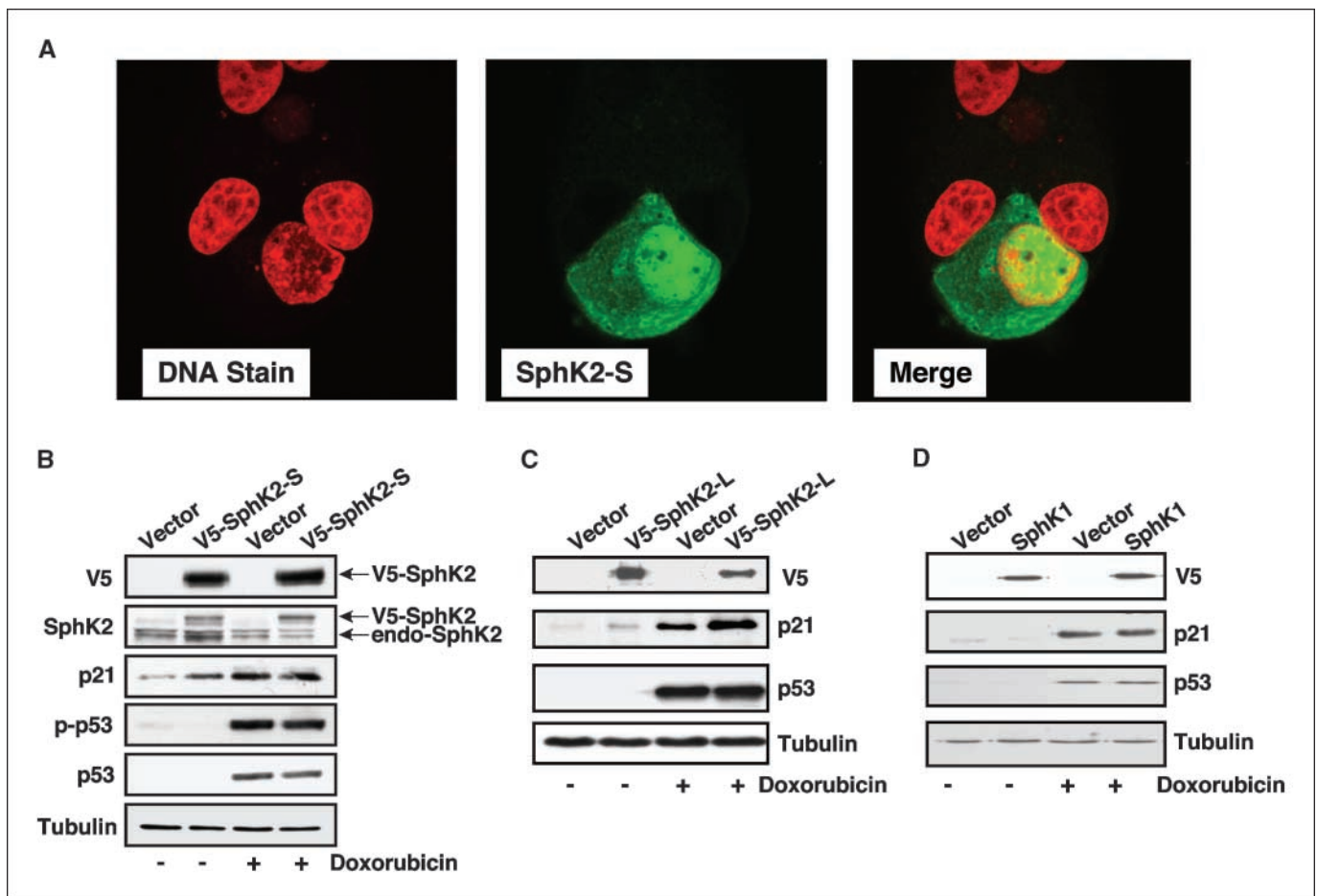


Figure 1. SphK2-S and SphK2-L increase p21 expression without affecting p53. *A*, MCF7 cells grown on coverslips were transiently transfected with V5-SphK2-S and subsequently cultured for 24 h in the presence of serum, fixed, and immunostained with anti-V5 antibody followed by FITC-conjugated secondary antibody. Nuclei were also stained with Hoechst 33342 and colocalization (yellow) was shown in the merged pictures. Cells were visualized by confocal fluorescence microscopy. Representative cells of ~100 cells examined are shown. *B* to *D*, MCF7 cells transiently transfected with vector, V5-SphK2-S (*B*), V5-SphK2-L (*C*), or V5-SphK1 (*D*) were cultured in the absence or presence of doxorubicin (1 μ g/mL) for 24 h. Equal amounts of cell lysate proteins were separated by SDS-PAGE and then immunoblotted with SphK2, V5, p21, p53, or phosphorylated p53 (Ser¹⁵; p-p53) antibodies as indicated. Membranes were subsequently probed with tubulin antibody to show equal loading. Similar results were obtained in two additional experiments.

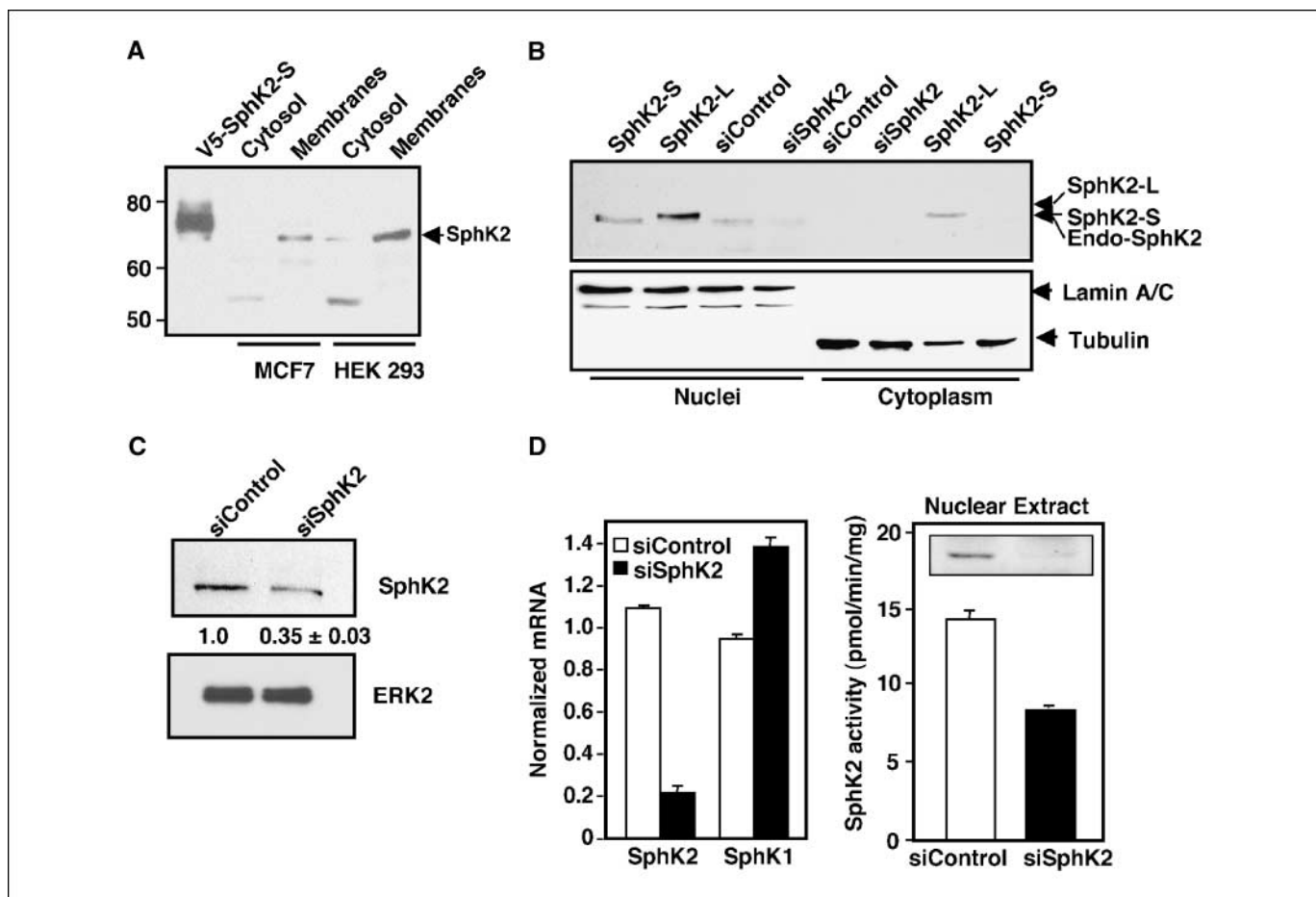


Figure 2. SphK2 is mainly a nuclear protein in MCF7 cells. *A*, cytosol and membrane fractions from naive MCF7 and HEK 293 cells were prepared by centrifugation at $100,000 \times g$. Equal amounts of proteins were separated by SDS-PAGE and immunoblotted with anti-SphK2 antibody. Cell lysate from MCF7 cells transiently transfected with V5-tagged SphK2-S was included as a positive control. *B*, MCF7 cells were transfected with control siRNA or siRNA targeted to SphK2. After 48 h, nuclear and cytosolic fractions were prepared using NE-PER cytoplasm and nuclear extraction reagents. Equal amounts of proteins were separated by SDS-PAGE and immunoblotted with anti-SphK2. Antibodies against lamin A/C and tubulin were used as nuclei and cytoplasm markers, respectively. Cell lysates from MCF7 cells transiently transfected with untagged SphK2-S or untagged SphK2-L were included to indicate the molecular weight of these proteins. Similar results were obtained in two additional experiments. *C* and *D*, down-regulation of SphK2 with siRNA. MCF7 cells were transfected with control siRNA (*white columns*) or siRNA targeted to SphK2 (*black columns*). *C*, after 48 h, equal amounts of cell lysate proteins were separated by SDS-PAGE and immunoblotted with anti-SphK2 antibody. Blots were stripped and probed with extracellular signal-regulated kinase 2 (*ERK2*) antibody as a loading control. SphK2 levels were normalized to total ERK2, and the ratio relative to siControl is indicated. *Columns*, mean of three independent experiments; *bars*, SD. *, $P < 0.05$. *D*, RNA was isolated from duplicate cultures and mRNA levels of SphK1 and SphK2 and 18s RNA were determined by quantitative real-time PCR. Nuclear fractions were also isolated and SphK2 activity was measured. *Inset*, equal amounts of nuclear proteins were separated by SDS-PAGE and immunoblotted with anti-SphK2 antibody.

Western blot analysis. Unless otherwise indicated, cells were lysed in buffer containing 50 mmol/L HEPES (pH 7.4), 150 mmol/L NaCl, 0.1% Triton X-100, 1.5 mmol/L MgCl₂, 1 mmol/L EDTA, 2 mmol/L sodium orthovanadate, 4 mmol/L sodium pyrophosphate, 100 mmol/L NaF, and 1:500 protease inhibitor mixture (Sigma). Equal amounts of proteins were separated by SDS-PAGE and transblotted to nitrocellulose, blocked with 5% nonfat dry milk for 1 h at room temperature, and then incubated with primary antibodies overnight. Appropriate horseradish peroxidase-conjugated secondary antibodies were added in TBS containing 5% nonfat milk. Immunoreactive signals were visualized by enhanced chemiluminescence (Pierce Chemical Co.) and blots were exposed to Kodak X-Omat film. Where indicated, relative amounts of immunoreactive protein in each band were determined by scanning densitometric analysis of the X-ray films with the AlphaEase FC 4.0 (Innotech) program and normalized to the appropriate loading control.

Immunofluorescence and confocal microscopy. MCF7 cells were grown on four-chambered slides (Nalge Nunc) and transfected with V5-tagged SphK2-S or SphK2-L. Subcellular localization studies using confocal microscopy were done as described previously (20). Briefly, cells were washed with PBS, fixed for 20 min at room temperature with 4% para-

formaldehyde, and permeabilized with 0.1% Triton X-100 in PBS for 5 min. After washing, cells were incubated for 45 min with anti-V5 antibody in PBS containing 0.1% bovine serum albumin (BSA) and then for 45 min with secondary antibody conjugated with FITC. Coverslips were mounted on glass slides using an antifade kit and examined with a Zeiss LSM 510 laser confocal microscope.

Nuclear extracts. Cytoplasmic and nuclear fractions were isolated from MCF7 cells using the NE-PER nuclear and cytoplasmic isolation kit (Pierce Chemical) according to the manufacturer's instructions.

Quantitative PCR. Total RNA was isolated with Trizol reagent (Invitrogen). RNA was reverse transcribed with SuperScript II (Invitrogen). For real-time PCR, premixed primer-probe sets were purchased from Applied Biosystems and cDNA was amplified with ABI 7900HT.

SphK2 activity measurements. SphK2 activity was determined with sphingosine added as a complex with 4 mg/mL BSA and [γ -³²P]ATP in the presence of 1 mol/L KCl, conditions in which SphK2 activity is optimal and SphK1 is strongly inhibited (8) exactly as described (12).

Cell cycle analysis. Cells were trypsinized, washed, fixed, and stained with propidium iodide (0.05 mg/mL in 3.8 μ mol/L sodium citrate, 0.1% Triton X-100, and 7 kilounits/mL RNase B) for 2 h and analyzed on a

Beckman Coulter XL-MCL flow cytometer. Cell cycle profiles were evaluated using ModFit LT 3.0 (Verity Software House).

Cell death assays. Apoptotic cell death was measured by staining cell nuclei with the Hoechst dye bisbenzimidazole and apoptotic cells were identified by condensed, fragmented nuclear regions as described previously (20). A minimum of 300 cells was scored. PARP cleavage and activation of caspase-7 were also determined with antibodies that recognize cleaved products as described previously (16). Cytochrome *c* release from mitochondria into the cytosol was determined by immunoblotting with anti-cytochrome *c* as described previously (20).

Statistical analysis. Experiments were repeated at least thrice with consistent results. For each experiment, the data from triplicate samples were calculated and expressed as mean \pm SD. Differences between groups were determined with Student's *t* test or a one-way ANOVA with a Tukey post hoc test, and $P < 0.05$ was considered significant.

Results

SphK2 up-regulates p21 in MCF7 cells independently of p53.

SphK1 is mainly cytosolic and several growth factors induce its translocation to the plasma membrane (23–25), which is important for its oncogenic functions (26). In contrast, the localization of endogenous SphK2 is still unclear as it may be different in different cell types (6, 12, 18, 20, 27). Although possessing a putative nuclear localization signal, overexpressed SphK2 was mainly cytosolic in HEK 293 cells but was localized to the nucleus of HeLa cells (18) and in endothelial cells was present in the cytosol, in perinuclear structures, and in the nucleus (27). In agreement, both SphK2-S and SphK2-L were expressed mainly in the nucleus of MCF7 cells determined by confocal fluorescence microscopy (Fig. 1A; Supplementary Fig. S1A). In contrast to HEK 293 cells (6, 20) but similar to HeLa cells (18), the localization of either splice variant was not affected by serum withdrawal (Supplementary Fig. S1A). Western blot analysis revealed major protein bands with the predicted molecular masses (Supplementary Fig. S1B) and lysates from these

cells had significantly increased sphingosine phosphorylating activity (Supplementary Fig. S1C).

Previous studies have shown that overexpression of SphK2 in contrast to SphK1 reduces cell growth (6, 18–20). However, it is not known how SphK2 inhibits cell proliferation. p21^{WAF1/CIP1} (hereafter referred to as p21), a member of a family of cyclin-dependent kinase inhibitors, is an important negative regulator of cell progression and prevents aberrant mitosis leading to cell death (28). Therefore, it was of interest to examine the effects of SphK2 expression on p21. SphK2-S increased levels of p21 compared with vector-transfected cells (Fig. 1B). Similarly, SphK2-L also increased p21 (Fig. 1C). In agreement with numerous reports (29–31), treatment with the DNA-damaging agent doxorubicin markedly increased levels of p21 and its transcriptional activator p53 (Fig. 1B and C). In the presence of doxorubicin, overexpression of SphK2-S did not significantly increase p21 further (Fig. 1B), whereas SphK2-L slightly increased it (Fig. 1C). However, neither SphK2-S nor SphK2-L expression altered p53 levels. Moreover, neither SphK2 splice form influenced p53 levels induced by doxorubicin (Fig. 1B and C). Among the multiple modification sites on p53, phosphorylation at Ser¹⁵ in response to DNA damage correlates with both the accumulation of total p53 protein and its functional activation (32). As expected, doxorubicin induced phosphorylation of p53 at Ser¹⁵. However, SphK2 expression had no significant effects on this phosphorylation (Fig. 1B). Doxorubicin also slightly decreased the level of endogenous SphK2 (Fig. 1B). In contrast to SphK2, expression of SphK1 had no effect on p21 expression (Fig. 1D).

Endogenous SphK2 is predominantly localized to the nucleus in MCF7 cells. Although all experiments with overexpression of SphK2 were restricted to moderate increases in SphK2 expression (Fig. 1A), it was important to examine localization and functions of endogenous SphK2. To this end, we used

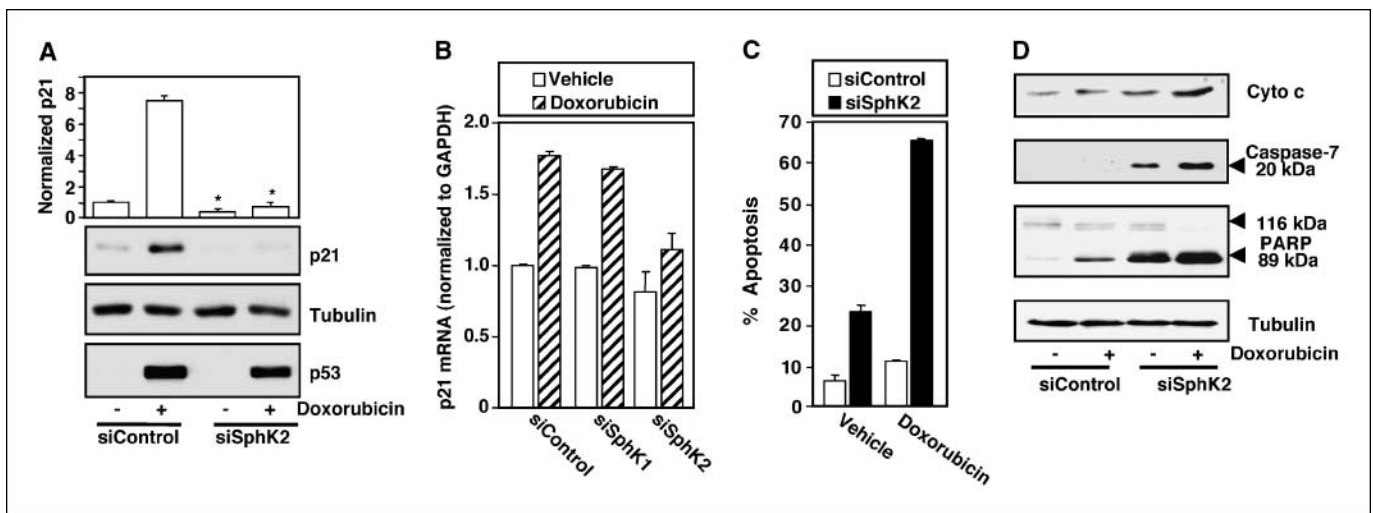


Figure 3. Down-regulation of SphK2 reduces expression of p21 induced by doxorubicin and sensitizes MCF7 cells to apoptosis. **A**, MCF7 cells transfected with siRNA directed to SphK2 or control siRNA were cultured in the absence or presence of doxorubicin (1 μ g/mL) for 24 h. Equal amounts of cell lysate proteins were separated by SDS-PAGE and immunoblotted with p21 or p53 antibodies. Equal loading was verified with antitubulin antibody. Similar results were obtained in three additional experiments. Relative p21 levels were normalized to tubulin. **Columns**, mean fold change relative to siControl from three independent experiments; **bars**, SD. *

$P < 0.05$. **B**, MCF7 cells transfected with siControl, siSphK1, or siSphK2 were cultured in the absence (*white columns*) or presence (*hatched columns*) of doxorubicin (1 μ g/mL) for 24 h. RNA was isolated and mRNA levels of p21 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were determined by quantitative real-time PCR. **C**, MCF7 cells transfected with siControl (*white columns*) or siSphK2 (*black columns*) were cultured in the absence or presence of doxorubicin (1 μ g/mL) for 48 h, and nuclei were stained with Hoechst. Apoptosis was determined by scoring the percentage of cells displaying fragmented, condensed nuclei indicative of apoptosis. A minimum of three different fields was analyzed, scoring a minimum of 300 cells. **D**, proteins from duplicate cultures were resolved by SDS-PAGE and analyzed by immunoblotting with antibodies against cytochrome *c* (Cyto *c*), cleaved caspase-7, and cleaved PARP. Blots were stripped and reprobbed with tubulin antibody to show equal loading. **Arrows**, active p20 subunit of caspase-7 and p116 full-length PARP and its p89 fragment.

rabbit polyclonal anti-SphK2 antibodies, which we previously used to identify endogenous SphK2 in HEK 293 cells by Western blotting analyses (12). In MCF7 cells, an immunoreactive band with the same apparent molecular mass as the endogenous SphK2 previously detected in HEK 293 and MDA-MB-453 cells was present mainly in the cellular membrane fraction (Fig. 2A). Based on initial studies with subcellular fractions prepared by differential centrifugation, we observed that, in contrast to HEK 293 and MDA-MB-453 cells where the majority of endogenous SphK2 was in the plasma membrane fraction (12), endogenous SphK2 in MCF7 cells was readily detected in the nuclear fraction and barely detectable in other subcellular fractions (data not shown). Because the nuclear proteins are very dilute after subcellular fractionation, more concentrated nuclear and cytoplasmic fractions were prepared with NE-PER reagents. In agreement with confocal microscopy (Fig. 1A), endogenous SphK2 was clearly localized in the nuclei of MCF7 cells (Fig. 2B).

It has recently been suggested that SphK2-L is the predominant splice variant in several human cell lines and tissues (6). However, although mRNA for SphK2-L could be detected by quantitative PCR, endogenous nuclear SphK2 in MCF7 cells had similar electrophoretic mobility as untagged SphK2-S rather than untagged SphK2-L (Fig. 2B). To further examine the localization and function of endogenous SphK2 in MCF7 cells, its expression was down-regulated by a siRNA targeted to a specific SphK2 sequence. siRNA directed toward SphK2 significantly decreased SphK2 protein (Fig. 2B and C) and mRNA (Fig. 2D) without changing the level of S1P₂, S1P₃, or S1P₅ mRNA (data not shown). It should be noted that reduction of SphK2 protein levels in the nucleus with siSphK2 was also accompanied by a decrease in nuclear SphK2 activity (Fig. 2D). Collectively, these data suggest that the predominant splice variant of SphK2 in MCF7 cells, which is expressed in the nucleus, is the short form.

Involvement of endogenous SphK2 in doxorubicin-induced up-regulation of p21 and apoptosis. To explore the role of endogenous SphK2 in responses of MCF7 cells to doxorubicin, we examined the effect of SphK2 knockdown on doxorubicin-induced p21 and p53 expression. Decreasing endogenous levels of SphK2 using siSphK2 decreased induction of p21 on exposure to doxorubicin but had no effect on doxorubicin-induced p53 expression (Fig. 3A). To exclude nonspecific off-target effects, SphK2 expression was also down-regulated with siRNA targeted to two other regions of the SphK2 sequence. These siSphK2s, but not scrambled siRNA controls, also markedly reduced expression of SphK2 mRNA and protein. They also almost completely abolished p21 expression induced by doxorubicin without significantly altering expression of p53 (Fig. 3A; data not shown). As expected (30, 33), doxorubicin increased transcription of p21 (Fig. 3B). Down-regulation of SphK2 but not SphK1 markedly reduced the effect of doxorubicin on p21 mRNA expression (Fig. 3B).

The involvement of endogenous SphK2 in doxorubicin-induced MCF7 cell cycle checkpoints was next examined by flow cytometry analysis. In agreement with several previous reports (29, 30, 34), doxorubicin produced a marked increase in the proportion of cells in the G₂-M phase of the cell cycle (from 23.1 ± 1.6% to 53.4 ± 2.3%). The fraction of siSphK2-transfected cells in G₂-M phase of the cell cycle was 21.2 ± 1.8% and 26.8 ± 2.2% in the absence or presence of doxorubicin, respectively. Thus, down-regulation of SphK2 prevented the doxorubicin-induced G₂-M arrest.

Because down-regulating SphK2 reduced p21 (Fig. 3A), and inhibiting p21 expression influences the outcome of a p53 response

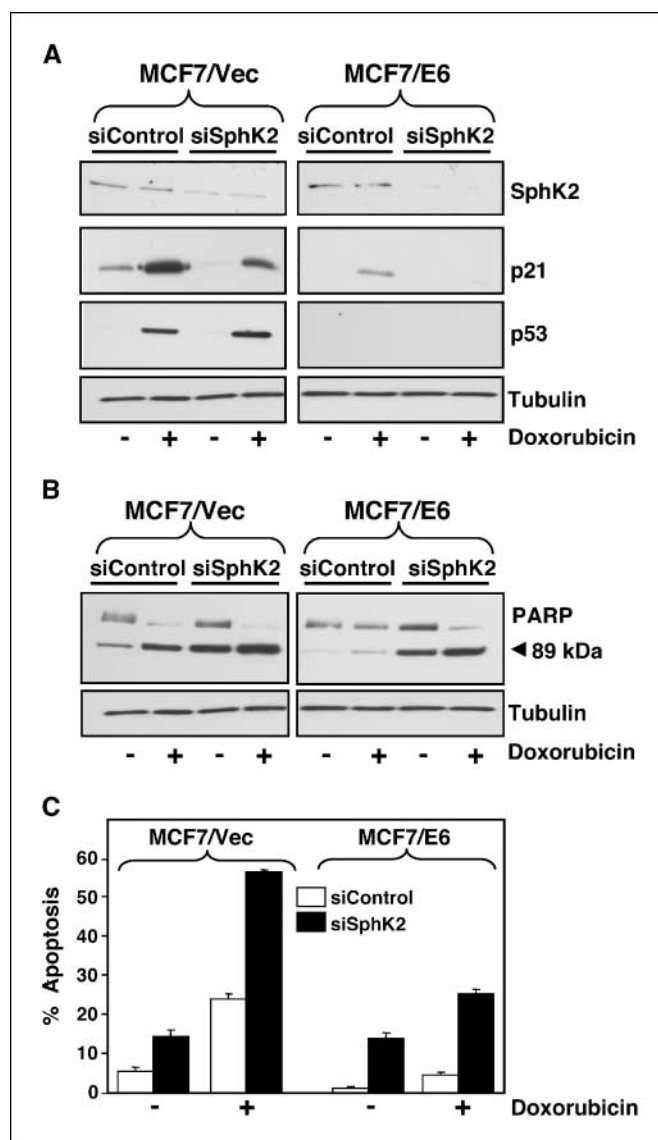


Figure 4. Down-regulation of SphK2 decreases doxorubicin-induced up-regulation of p21 in MCF7 cells lacking p53. MCF7 cells infected with vector pLXSN only (MCF7/Vec) or HPV-16 E6 (MCF7/E6) were transfected with siControl or siSphK2 and cultured in the absence or presence of doxorubicin (1 μ g/mL) for 24 h. A, equal amounts of cell lysate proteins were separated by SDS-PAGE and immunoblotted with antibodies against SphK2, p53, and p21. Blots were stripped and reprobed with tubulin antibody to show equal loading. B, proteins from duplicate cultures were resolved by SDS-PAGE and analyzed by immunoblotting with antibodies against cleaved PARP. Blots were stripped and reprobed with tubulin antibody to show equal loading. C, cells were treated as above and apoptosis was determined by scoring the percentage of cells displaying fragmented, condensed nuclei indicative of apoptosis. A minimum of three different fields and 300 cells was analyzed.

to doxorubicin in favor of cell death (30, 35), we next examined the effect of siSphK2 on doxorubicin-induced apoptosis. Exposure of MCF7 cells to doxorubicin for 24 h only induced minimal apoptosis (Fig. 3C), in agreement with previous studies (16, 34). Although down-regulation of SphK1 did not induce apoptotic cell death of MCF7 cells (16), siSphK2, but not siControl, induced apoptosis (Fig. 3C). Moreover, siSphK2 sensitized MCF7 cells to apoptosis induced by doxorubicin (Fig. 3C). In agreement, down-regulation of SphK2 induced apoptotic traits, including activation of caspase-7, the main effector caspase in MCF7 cells (36), and

cleavage of PARP, a substrate for caspase-mediated proteolysis during apoptosis (Fig. 3D). Moreover, down-regulation of SphK2 further enhanced doxorubicin-induced PARP cleavage, caspase-7 activation, and increased cytochrome *c* release to the cytosol (Fig. 3D), suggesting that loss of SphK2 enhances sensitivity to chemotherapy.

Involvement of endogenous SphK2 in doxorubicin-induced up-regulation of p21 and apoptosis in MCF7 cells lacking p53.

To further substantiate the notion that endogenous SphK2 is important for p53-independent induction of p21 by doxorubicin, MCF7 cells were infected with HPV-16 E6, which targets p53 protein for ubiquitination and degradation, thereby inactivating p53 function (37). In agreement with previous studies (22), there was no induction of p53 by doxorubicin in these cells (Fig. 4A). Nevertheless, doxorubicin slightly increased p21 in MCF7/E6 cells, and as expected, this response was greatly attenuated compared with MCF7/Vec cells infected with pLXSN. Down-regulation of SphK2 in MCF7/E6 cells also abolished the increase in p21 induced by doxorubicin (Fig. 4B). Reduction of SphK2 also sensitized these p53-deficient MCF7 cells to apoptosis induced by doxorubicin, as determined by cleavage of PARP and appearance of condensed nuclei (Fig. 4B and C), although apoptosis was somewhat attenuated compared with the p53 counterparts (Fig. 4C).

SphK2 is also involved in doxorubicin-induced up-regulation of p21 in a p53-independent manner in HCT116 human colon carcinoma cells. It was of interest to examine the involvement of SphK2 in regulation of p21 expression by doxorubicin in another type of cancer cell. Human HCT116 colon carcinoma cells were used for several reasons: SphK2 is also present in the nucleus of these cells (Fig. 5A); siRNA targeted to SphK2 markedly reduced its expression without affecting expression of SphK1 determined by

real-time PCR (Fig. 5B); and finally, isogenic cell lines derived from human HCT116 colon carcinoma cells in which p53 and p21 were inactivated by targeted homologous recombination have been extensively studied to examine the roles of these proteins in apoptosis in response to doxorubicin (29, 35, 38, 39). As in MCF7 cells, decreasing endogenous levels of SphK2 with siSphK2 decreased induction of p21 on exposure to doxorubicin but had no significant effect on doxorubicin-induced p53 expression in WT HCT116 cells (Fig. 5C). As expected, the doxorubicin-induced increase in p21 in HCT116 p53^{-/-} cells was greatly attenuated compared with WT cells (Fig. 5C). Down-regulation of SphK2 in HCT116 p53^{-/-} cells markedly reduced this small increase in p21 induced by doxorubicin (Fig. 5C). Knockdown of SphK2 did not significantly influence the increase in p53 levels in response to doxorubicin in either WT or p21^{-/-} cells.

Down-regulation of SphK2 sensitized WT HCT116 cells to apoptosis induced by doxorubicin and concomitantly increased PARP cleavage (Fig. 6A and B). In agreement with previous studies (29, 30, 35, 40), death induced by doxorubicin was increased in p21^{-/-} cells (Fig. 6B). Reduction of SphK2 expression only marginally increased doxorubicin-induced apoptosis in these p21-null cells (Fig. 6B). A higher level of cleavage of PARP and apoptosis was also observed in SphK2 down-regulated HCT116 p53^{-/-} cells treated with doxorubicin (Fig. 6B). Collectively, these results support the notion that p21 expression regulated by endogenous SphK2 impedes the progression of the apoptotic pathway.

Discussion

It is now well established that SphK1 promotes cell growth, inhibits apoptosis induced by anticancer agents, enhances

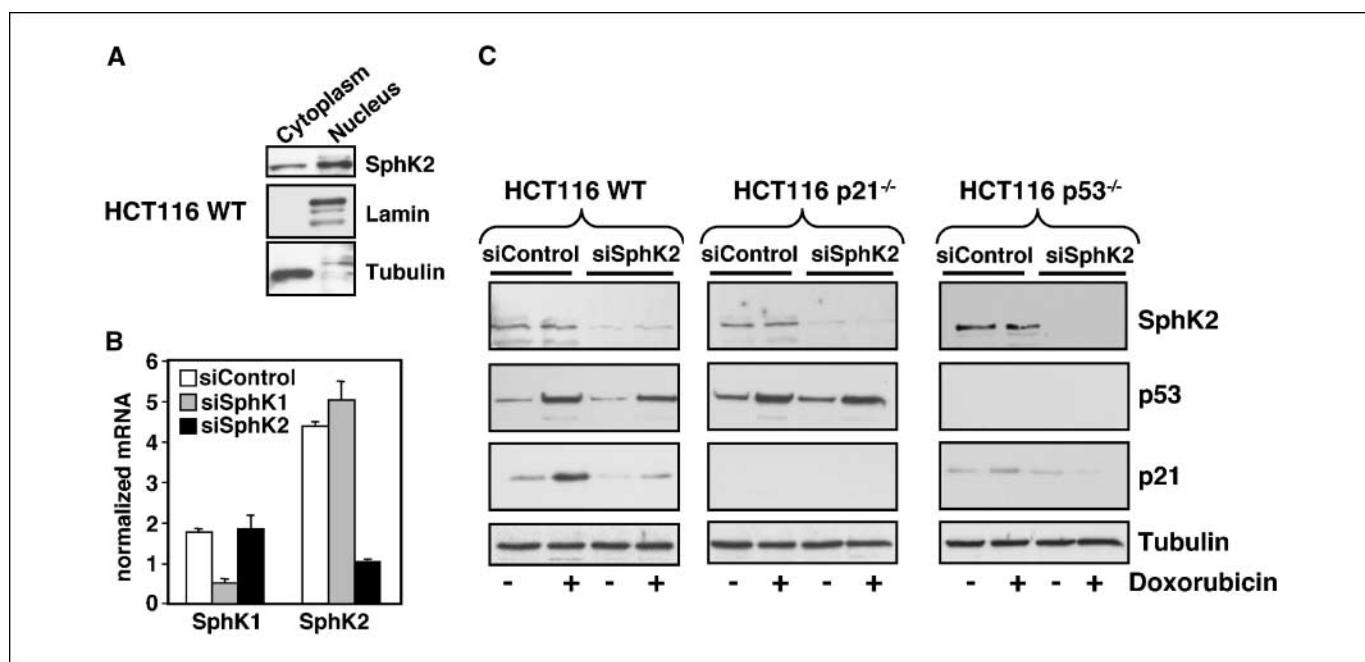


Figure 5. Down-regulation of SphK2 inhibits p21 induction by doxorubicin in HCT116 human colon carcinoma cells. **A**, nuclear and cytosolic fractions were prepared from HCT116 cells using NE-PER cytoplasm and nuclear extraction reagents. Equal amounts of proteins were separated by SDS-PAGE and immunoblotted with anti-SphK2. Antibodies against lamin A/C and tubulin were used as nuclei and cytoplasm markers, respectively. **B**, HCT116 cells were transfected with control siRNA or siRNA targeted to SphK2 or SphK1. After 48 h, RNA was isolated and mRNA levels of SphK1 and SphK2 and 18s RNA were determined by quantitative real-time PCR. **C**, HCT116 WT, p21^{-/-}, and p53^{-/-} were transfected with siControl or siSphK2 and then cultured in the absence or presence of doxorubicin (1 μ mol/L) for 18 h. Equal amounts of cell lysate proteins were separated by SDS-PAGE and immunoblotted with antibodies against SphK2, p53, and p21. Blots were stripped and reprobed with tubulin antibody to show equal loading. Similar results were obtained in three additional experiments.

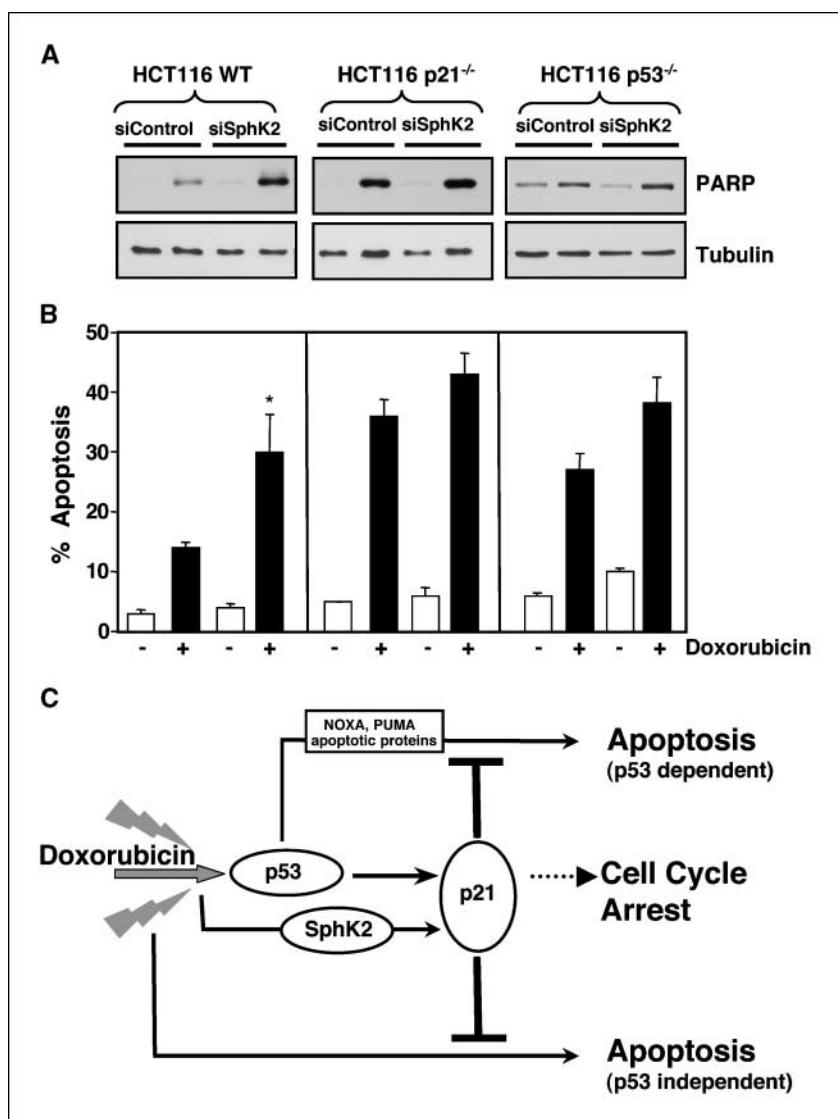


Figure 6. Down-regulation of SphK2 sensitizes HCT116 cells to doxorubicin-induced apoptosis. *A*, HCT116 WT, p21^{-/-}, and p53^{-/-} cells were transfected with siControl or siSphK2 and then cultured in the absence or presence of doxorubicin (1 μ mol/L) for 18 h. Equal amounts of cell lysate proteins were separated by SDS-PAGE and immunoblotted with anti-PARP antibody. Blots were stripped and reprobbed with tubulin antibody to show equal loading. *B*, nuclei of duplicate cultures were stained with Hoechst. Apoptosis was determined by scoring the percentage of cells displaying fragmented, condensed nuclei indicative of apoptosis. A minimum of three different fields was analyzed, scoring a minimum of 300 cells. *, $P < 0.05$. *C*, proposed model depicting the involvement of SphK2 in doxorubicin-induced expression of p21 and apoptosis. Doxorubicin induces expression of p53 leading to induction of proapoptotic factors, such as BAX, NOXA, and PUMA, and cell death and also induces p21. Doxorubicin can also enhance expression of p21 in a p53-independent manner. SphK2 contributes to induction of p21 independently of p53. Down-regulation of SphK2 prevents induction of p21 and removes p21-mediated protection against apoptosis, enabling cell death.

tumorigenicity in animal models, is up-regulated in many human cancers, and correlates with poor prognosis (4, 5). However, very few studies have examined the functions of endogenous SphK2 and it is still not clear whether SphK1 and SphK2 have redundant, overlapping, complementary, or antagonistic functions in human cancer cells. In MDA-MB-453 human breast cancer cells, SphK1 and SphK2 have overlapping and/or complementary functions in regulation of EGF-induced migration. In contrast, only SphK1, but not SphK2, was required for migration of HEK 293 cells toward EGF (12).

Although overexpression of SphK2 induces growth arrest and enhances apoptosis (6, 18–20), surprisingly, decreasing SphK2 expression inhibited glioblastoma cell proliferation and enhanced apoptosis more potently than did SphK1 knockdown (41). Similarly, we found that loss of SphK2 greatly sensitized MCF7 cells to mitochondrial-dependent apoptosis in the presence of doxorubicin as shown by increased release of cytochrome *c* from the mitochondria into the cytosol and activated caspase-7 and PARP cleavage, suggesting that endogenous SphK2 may regulate sensitivity to chemotherapy. Loss of SphK2 also sensitized HCT116 cells to apoptosis. Interestingly, although neither SphK1- nor SphK2-null mice have an obvious phenotype, the double knock-

outs have a dramatic increase in apoptosis in the developing nervous system (7).

Collectively, these results raise the conundrum of how overexpression of SphK2 decreases cell growth and enhances apoptosis (6, 18–20), whereas its down-regulation sensitizes cells to apoptosis. A simple answer may be provided by our results and those of others showing that, when SphK2 is overexpressed, not all of the overexpressed protein has the same subcellular distribution as endogenous SphK2 and may cause nonphysiologic effects. SphK2 contains a putative α -helical BH3 domain analogous to that present in other BH3-only proapoptotic proteins (19), such as BIM and BID. As short BH3 domain peptides can induce oligomerization of BAK and BAX releasing cytochrome *c* to sensitize mitochondrial apoptosis (42), it is possible that proteolysis of overexpressed SphK2 might induce apoptosis due to liberation of its BH3 peptide domain. Finally, it has been suggested that the molecular balance between apoptosis and cell cycle arrest is determined in part by the level of p21. In case of a high level of p21 expression, cells are arrested in G₂, whereas blockage of p21 expression directly leads to apoptosis (30, 35, 39, 43, 44).

Two SphK2 splice variants have been described, the originally reported 68-kDa form, designated SphK2-S (8), and the NH₂-terminal extended 72-kDa SphK2-L (6, 10). Based mainly on real-time quantitative PCR of mRNA using primer sets that differentiate between SphK2-S and SphK2-L, it has been suggested that SphK2-L is the predominant form in human cell lines and tissues (6). However, the presence of endogenous SphK2 protein forms was not directly examined in these studies. Using Western blotting with an antibody directed against a peptide sequence present in both SphK2-S and SphK2-L, we detected an immunopositive band that was down-regulated by siSphK2, which had a similar electrophoretic mobility as SphK2-S rather than SphK2-L. Our results clearly indicate that the endogenous form of SphK2 expressed in MCF7 cells, which is particularly enriched in the nucleus, is not SphK2-L but rather SphK2-S.

Although the function of SphK2 in the nucleus is not clear, we found that endogenous SphK2 is important for doxorubicin-induced expression of p21 independently of p53. Several lines of evidence support this notion. First, overexpression of SphK2 increased expression of p21 in MCF7 cells without affecting p53 levels or its activation. In contrast, overexpression of SphK1 had no effect on either p21 or p53 expression. Second, down-regulation of endogenous SphK2 markedly reduced basal and doxorubicin-induced p21 without affecting p53 expression in MCF7 breast cancer cells and HCT116 colon cancer cells. Finally, in MCF7 cells that lack functional p53 or in p53-null HCT116 cells, down-regulation of SphK2 completely abrogated doxorubicin-induced expression of p21.

p21 seems to be a major determinant of cell fate in response to anticancer therapy as it plays an essential role in growth arrest after DNA damage (28, 45) and inhibits p53-dependent and p53-independent apoptosis (29). Thus, repression of p21 by gene targeting, overexpression of c-Myc, or chemical p21 inhibitors all sensitize tumor cells to apoptosis by anticancer drugs (30, 39, 46). Indeed, down-regulation of SphK2 inhibited doxorubicin-induced

G₂ checkpoint arrest, consistent with the failure of these cells to up-regulate p21 in response to doxorubicin. It also sensitized them to apoptosis induced by doxorubicin. Collectively, our results suggest that SphK2 may be one of the gatekeepers that influence the balance between cytostasis and apoptosis in response to doxorubicin (Fig. 6C). According to this idea, doxorubicin-induced expression of p53, which in turn can induce p21, leads to cell cycle arrest or induction of proapoptotic factors, including PUMA and NOXA, leading to cell death (28, 30, 39). However, it has been suggested that the presence of these proapoptotic factors induced by p53 in response to DNA damage is not sufficient to initiate apoptosis unless these events are coupled with improper cell cycle progression due to the absence of p21 (28, 35, 39). Our results indicate that SphK2 is required for maximal increases in p21 (independently of p53), which enables cell cycle arrest, DNA repair, and prevention of execution of the cell death program. Down-regulation of SphK2 expression represses p21 and switches the response from cell cycle arrest to apoptosis (Fig. 6C), suggesting that SphK2 may influence the balance between cytostasis and apoptosis of human cancer cells. It is tempting to speculate that tumors that are highly responsive to doxorubicin might have lower levels of SphK2. Moreover, targeting SphK2 to decrease p21 expression may have the potential to improve the action of anticancer drugs.

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Involvement of Sphingosine Kinase 2 in p53-Independent Induction of p21 by the Chemotherapeutic Drug Doxorubicin

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