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14. ABSTRACT

The sphingolipid metabolite sphingosine-1-phosphate (S1P) is the ligand for a family of five specific G protein-coupled receptors (S1P1-5)that regulate a wide variety of important cellular functions, including growth, survival, cytoskeletal rearrangements, and cell motility. However, whether it also has an intracellular action is still a matter of debate. S1P is formed by the ATP-dependent phosphorylation of sphingosine catalyzed by types 1 and 2 sphingosine kinase (SphK). Many studies have shown that SphK1 stimulates cell proliferation and protects cells from apoptosis. Recently, we reported that in contrast, expression of SphK2 inhibited growth and enhanced apoptosis independently of S1P receptor activation (1). In this study, we investigated the role of SphK2 in human breast carcinoma MCF7 cells in response to the DNA damaging agent doxorubicin. Down-regulation of endogenous SphK2 in MCF7 cells with siRNA decreased doxorubicin-induced expression of p21 without affecting p53. Exposure of MCF7 breast tumor cells to doxorubicin produces a marked increase in the G2/M and S phase populations and a corresponding decrease in the G0/G1 population. Down regulation of SphK2 resulted in decreased G2/M and S phase populations and an increased G0/G1 population. Our results suggest that SphK2 might be involved in increases in p21WAF1 induced by doxorubicin in a p53-independent manner.

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INTRODUCTION

Sphingolipid metabolism is a dynamic and tightly regulated process resulting in the formation of a number of bioactive metabolites, including ceramide, sphingosine and S1P, which have all been implicated as important components of cell fate decisions (2,3). Ceramide and sphingosine are usually associated with apoptosis and negative effects on cell growth and survival, whereas S1P, formed by SphK1 opposes these effects. Increased ceramide and sphingosine levels have been shown to induce apoptosis in many cell types: for example, ceramide and sphingosine enhance apoptosis of radiation resistant prostate and breast cancer cells (4). In contrast, it has been shown that exogenous addition of S1P protects oocytes *in vivo* from radiation-induced apoptosis (5). This has led to the proposal that the balance between the cellular concentrations of ceramide and sphingosine versus S1P, the "sphingolipid rheostat", is important in determining whether cells survive or die (6). In agreement, studies from our lab show that sphingosine is involved in mitochondria-mediated apoptotic signaling induced by doxorubicin in human breast cancer cells (7). Whereas, in sharp contrast, S1P, formed by phosphorylation of sphingosine protects against ceramide-mediated apoptosis and promotes estrogen-dependent tumorogenesis of MCF7 cells (8). Recently, another mammalian isoform of SphK (SphK2) was cloned and characterized in our laboratory (9). Although highly similar in amino acid composition and sequence, SphK2 is much larger than SphK1 and diverges in its amino terminus and central region. Although little is yet known about the functions of SphK2, these distinct differences imply that SphK1 and SphK2 may have different physiological functions (9). Indeed, rather than promoting growth and survival, SphK2 expression in fibroblasts or PC12 cells suppressed growth and enhanced apoptosis that was preceded by cytochrome c release and activation of caspase-3 (9) (10). Currently little information exists regarding the effect of SphK2 expression in breast cancer cell lines in response to anti-cancer agents. The anthracyclin doxorubicin, a major anti-tumor agent used for the treatment of a variety of human cancers, is known to cause cellular damage through a number of mechanisms including free radical formation and inhibition of topoisomerase II (11). p21, a member of a family of cyclin dependent kinase (cdk) inhibitors, which also include p27 and p53, appears to be a major determinant of cell fate in response to anticancer therapy (12). p21 plays an essential role in growth arrest after DNA damage (13) (14) and overexpression leads to G1 and G2 (15) or S-phase arrest (16). Both p53-dependent and –independent pathways of p21 induction in cells exposed to DNA damaging and maturation/differentiation inducing agents have been described (17-19). Currently, two forms of SphK2 have been described, the N-terminal-extended SphK2 (Sphk2-L) (20) (21) and the originally reported form (SphK2-S) (9) (10) lacking exon 1 with the first initiation codon for translation being within exon 2. The goal of the present investigation was to determine the effect of SphK2 expression in MCF7 cells in response to doxorubicin. Our results indicate that SphK2 is predominantly localized to the nucleus of MCF7 cells. In contrast to doxorubicin, which increases the cyclin-dependent kinase inhibitor p21 and p53 levels, SphK2 expression increased p53-independent expression of p21 and hypophosphorylation of pRb. Importantly, down-regulation of endogenous SphK2 protected MCF7 cells from doxorubicin-induced apoptosis and its effects on p21 without affecting p53.

BODY

Specific Aim 1. To examine the role of the BH3 domain of SphK and its interactions with Bcl-2 family members in human breast cancer cells.

Previous studies demonstrate that in contrast to SphK1, overexpression of SphK2 suppresses growth and enhances apoptosis (1,10,21,22). Surprisingly, however, RNA interference to knockdown SphK2 expression inhibited glioblastoma cell proliferation more potently than did SphK1 knockdown (23). Similarly, downregulation of SphK2 in MCF7 cells with siSphK2 (see results for specific aim 4 for more information on siSphK2) reduced their growth when cultured in the presence of serum (Fig. 1A). Because downregulating SphK2 also reduced p21 (specific aim 4), and inhibiting p21 expression influences the outcome of a p53 response to doxorubicin in favor of cell death (24), 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. 1B). Surprisingly, although downregulation of SphK1 itself did not induce apoptotic cell death of MCF7 cells (25), siSphK2, but not siControl, significantly induced apoptosis (Fig. 1C). Moreover, siSphK2 sensitized MCF7 cells to apoptosis induced by 24 h treatment with doxorubicin (Fig. 1B). In agreement, downregulation of SphK2 induced apoptotic traits including, activation of caspase-7, (the main effector caspase in MCF7 cells), and cleavage of poly(ADP-ribose) polymerase (PARP), a marker of caspase-mediated proteolysis during the apoptotic response. Downregulation of SphK2 further enhanced doxorubicin-induced PARP cleavage, caspase-7 activation, and increased cytochrome c release to the cytosol (Fig. 1B), suggesting that loss of SphK2 enhances sensitivity to chemotherapy. As part of this proposed study, it was reported previously that overexpression of SphK2 in increased apoptosis in MCF7 cells. Our results therefore present a conundrum. One possible explanation is that it is proteolysis of overexpressed SphK2 might induce apoptosis due to liberation of its BH3 domain.

Specific Aim 2. To determine the role of caspase cleavage of the DxxD motif of SphK2.

Previously truncated forms of SphK2 split after its BH3 domain into a 227 amino acid N-terminal fragment and a 391 amino acid C-terminal fragment in NIH 3T3 cells. Only the N-terminal fragment, which contains the putative BH3 domain induced apoptosis while the C-terminal fragment did not. Interestingly, these fragments would potentially be produced by caspase cleavage at the $D^{224}XXD^{227}$caspase recognition sequence in SphK2. Additionally, upon treatment with doxorubicin (1μg/ml) initial results show that endogenous SphK2 levels are reduced in MCF7 cells leading to the hypothesis that SphK2 is cleaved (Fig. 2). The effects of caspase inhibitors, proteosome inhibitors and calpeptin (a calpepin inhibitor) on SphK2 cleavage will be investigated.

Specific Aim 3. To examine the role of the catalytic activity of SphK2 in apoptosis of breast cancer cells.

As part of specific aim 3 it was proposed to examine the localization of SphK2 by confocal microscopy and subcellular fractionation. SphK1 is mainly cytosolic and several growth factors induce its translocation to the plasma membrane (26-28), which is important for its oncogenic functions (29). In contrast, the localization of SphK2 is still unclear as it is different in different cell types (10,21,22,30). 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 (10). A recent report suggested that the

predominant form of SphK2 in HEK 293 cells, SphK2-L, was also cytosolic and translocated to the nucleus during serum starvation (21). To examine their localization in MCF7 cells, cells were transiently transfected with V5 tagged SphK2-S or SphK2-L. Western blot analysis of lysates from these cells with anti-SphK2 antibody detected major protein bands (Fig. 3A). Transient transfection with SphK2-S and SphK2-L resulted in significantly increased sphingosine phosphorylating activity (Fig. 3B). Both SphK2-S and SphK2-L were expressed mainly in the nucleus of MCF-7 cells when examined by confocal fluorescence microscopy (Fig. 3C). In contrast to HEK 293 cells, but similar to HeLa cells (10,21), their localization was not affected by serum withdrawal (Fig. 3B).

Ectopically expressed proteins do not always have the same subcellular localization and functions of their endogenous counterparts. Although all experiments with overexpression of SphK2 were restricted to moderate increases in SphK2 expression (Fig. 3B), it was important to examine localization and functions of endogenous SphK2. To this end, we utilized rabbit anti-SphK2 antibody. In MCF7 cells, endogenous SphK2 was clearly predominantly localized in the nuclei of MCF7 cells (Fig. 3D). Although the function of SphK2 in the nucleus is not clear, we previously reported that overexpression of SphK2 increased p53-independent expression of p21.

Recent studies suggest that SphK2-L is the predominant splice variant in several human cell lines and tissues (21). This conclusion was based mainly on real-time quantitative PCR of mRNA using primer sets that differentiate between the SphK2-S and SphK2-L. However, the presence of these proteins was not determined. Therefore, it was important to determine which splice variant of SphK2 was expressed in MCF-7 cells. Because anti-SphK2 antibody cross-reacted with several proteins on western blots, to unequivocally identify endogenous SphK2, it was necessary to downregulate its expression with specific siRNA and compare it with lysates from MCF7 cells expressing either untagged SphK2-S or untagged SphK2-L. The immunopositive band in nuclei of MCF7 cells that was downregulated by siSphK2 had the same apparent size as SphK2-S whereas, untagged SphK2-L migrated as a slightly higher molecular weight species. It should be noted that reduction of SphK2 protein levels in the nucleus with siSphK2 was also accompanied by a decrease in SphK2 activity (Fig. 3E). This data suggests that the predominant form of SphK2 in MCF7 cells is SphK2-S, which is expressed mainly in the nucleus.

Specific Aim 4. To determine the role of endogenous SphK2 on apoptosis of breast cancer.

To examine the function of endogenous SphK2 in MCF-7 cells, its expression was downregulated by siRNA targeted to a specific SphK2 sequence. This siRNA decreased SphK2 mRNA by more than 80% (Fig. 4A). siSphK2 also significantly reduced SphK2 protein levels (Fig. 4B). However, interestingly siSphK2 increased SphK1 protein and mRNA levels (Fig. 4A, B), possibly due to a compensatory effect. In agreement with previous reports, SphK1 is mainly cytosolic with a molecular mass of 42.5 kDa (Fig. 4C). In contrast, SphK2, which is predominantly localized in membranes, was markedly reduced by siSphK2 but not by siControl (Fig. 4C).

To further understand the importance of endogenous SphK2 in tumor cell function, we examined the effect of SphK2 knockdown on doxorubicin-induced p21 and p53 expression in MCF7 cells. Decreasing endogenous levels of SphK2 using siSphK2 resulted in a decrease in p21 protein levels in MCF7 cells upon exposure to doxorubicin (Fig. 5A). siSphK2 had no effect on doxorubicin induced p53 expression.

To exclude nonspecific off-target effects, SphK2 expression was also down-regulated with siRNA targeted to another region of the SphK2 sequence. This siSphK2 also markedly reduced expression of SphK2 mRNA and protein (data not shown), it almost completely abolished p21 expression induced by doxorubicin, and also did not alter expression of p53 (Fig. 5A). These results suggest that SphK2 might be involved in increases in p21 induced by doxorubicin in a p53-independent manner.

To characterize the involvement of endogenous SphK2 in doxorubicin-activated MCF7 cell cycle checkpoints, we examined the effect of downregulating its expression on the cell cycle profile by flow cytometry analysis. Downregulation of SphK2 only had a small effect, slightly reducing the percentage of cells in S and G2/M phases while increasing the percentage of cells in G0/G1 phase (Fig. 5B). In agreement with several previous reports (24,31,32), doxorubicin produced a marked increase in the proportion of cells in G2/M phase with a corresponding decrease in G0/G1 (Fig. 5B). siSphK2, but not siControl, blunted these effects of doxorubicin.

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 (Rockville, MD). Antibodies to p21, lamin A/C, caspase 7 and PARP were purchased from Cell Signaling (Beverly, MA). Anti-p53 antibody was from Oncogene (San Diego, CA). Anti-under-phosphorylated Rb antibody was from Transduction Laboratories (San Diego, CA). Anti-PDI antibody was from Stressgen Biotechnologies (San Diego, CA). Anti-tubulin antibody was from Santa Cruz Biotechnology (Santa Cruz, CA). SphK2 rabbit polyclonal antibody raised against a unique SphK2 peptide sequence (QALHIQRLRPKPEARPR) was purified as described (30). Secondary antibodies were from Jackson Immunoresearch (West Grove, PA).

cDNA Cloning and Expression Vectors

Construction of human SphK2 (UniProt: Q9NRA0-2) expression vectors have been described previously (9) (30). SphK2 with an N-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'-TCCGGGCTCCCGCCCCGG-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 or anti-V5 antibody (30).

Cell Culture and Transfection

MCF7 cells were cultured in phenol-red free IMEM supplemented with 0.25% glucose and 10% heat-inactivated FBS. Cells were transfected with Lipofectamine Plus (Invitrogen) and cultured overnight**.** SphK2 expression was down-regulated by transfecting with sequence specific siRNA for human SphK2 (sense, 5'-GGAUUGCGCUCGUCGCUUUCAU-3'; antisense, 5'-AUGAAAGCGAGCGCAAUCCTG-3', Ambion) and control siRNA (Ambion) using OligofectAMINE (Invitrogen). In some experiments, siRNA targeted to another human SphK2 sequence (5'-GCTGGGCTGTCCTTCAACCT-3', Qiagen) and control siRNA (Qiagen) was utilized.

Nuclear Extract Isolation

Cytoplasmic and nuclear fractions were isolated from MCF7 cells using the NE-PER nuclear and cytoplasmic isolation kit (Pierce) according to the manufacturers instructions.

Western Analysis.
Cells were scraped in lysis buffer containing 50 mM Hepes (pH 7.4), 150 mM NaCl, 0.1% Triton X-100, 1.5 mM $MgCl_2$, 1 mM EDTA, 2 mM sodium orthovanadate, 4 mM sodium pyrophosphate, 100 mM NaF, 1:500 protease inhibitor mixture (Sigma). Equal amounts of proteins were separated by SDS-PAGE and transblotted to nitrocellulose, blocked with 5% non-fat dry milk for 2 h at room temperature and then incubated with primary antibodies overnight. Appropriate horseradish peroxidase-conjugated secondary antibodies were added in Tris-buffered saline containing 5% milk and immunoreactive signals were visualized by enhanced chemiluminescence (Pierce) and exposed to Kodak X-omat film.

Immunofluorescence and Confocal Microscopy
MCF7 cells were grown on four-chambered slides (Nalge/Nunc) and transfected with V5 tagged SphK2-S or SphK2-L plasmids. Subcellular localization studies using confocal microscopy were performed as described previously with anti-V5 antibodies as a primary antibody and rabbit-FITC conjugate as a secondary antibody (22). Briefly, cells were washed with PBS, fixed for 20 min at room temperature with 4% paraformaldehyde, and permeabilized with 0.1% Triton X-100 in PBS for 5 min. After washing, cells were incubated for 45 min with primary antibodies in PBS containing 0.1% BSA, and then for 45 min with the corresponding secondary antibodies conjugated with FITC. Coverslips were mounted on glass slides using an Anti-Fade kit and examined by confocal with a Zeiss LSM 510 laser confocal microscope.

Quantitative PCR
Total RNA was isolated with TRIzol Reagent (Life Technologies, Gaithersburg, MD). RNA was reverse transcribed with Superscript II (Life Technologies, Gaithersburg, MD). For real-time PCR, pre-mixed primer-probe sets were purchased from Applied Biosystems (Foster City, CA) and cDNA amplified with ABI 7900HT.

Sphingosine Kinase Assay
Cells were lysed by freeze-thawing in SphK buffer (20 mM Tris (pH 7.4), 20% glycerol, 1 mM 2-mercaptoethanol, 1 mM EDTA, 5 mM sodium orthovanadate, 40 mM b-glycerophosphate, 15 mM NaF, 10 μg/ml leupeptin, aprotinin, and soybean trypsin inhibitor, 1 mM phenylmethylsulfonyl fluoride, and 0.5 mM 4-deoxypyridoxine). SphK2 activity was determined with sphingosine added as a complex with 4 mg/ml BSA and [γ-^{32}P]ATP in the presence of 1 M KCl, conditions in which SphK2 activity is optimal and SphK1 strongly inhibited (9). Labeled S1P was extracted and separated by TLC with chloroform/acetone/methanol/acetic acid/H2O (10:4:3:2:1, v/v). Radioactive bands corresponding to S1P were quantified with a FX Molecular Imager (Bio-Rad). Specific activity is expressed as picomoles of S1P formed/min/mg of protein.

Cell Cycle Analysis.
Cells were trypsinized, washed, fixed, and stained with propidium iodide (0.05 mg/ml in 3.8 μM sodium citrate, 0.1% Triton X-100, and 7 kU/ml RNase B) for 2 h and separated on a Beckman-Coulter XL-MCL flow cytometer (Hialeah, FL). Cell cycle was analyzed using Mod Fit LT 3.0 (Verity Software House; Topsham, ME).

Cell Death and Proliferation Assays.
Apoptotic cell death was measured by staining cell nuclei with the Hoechst dye bisbenzimide and apoptotic cells were identified by condensed, fragmented nuclear regions as previously described (33). A minimum of 300 cells were scored. Cell proliferation was determined with the WST-1 reagent (Roche, Indianapolis, IN) as described (25). Absorbance was measured at 450 nm with background subtraction at 630 nm.

FIGURES

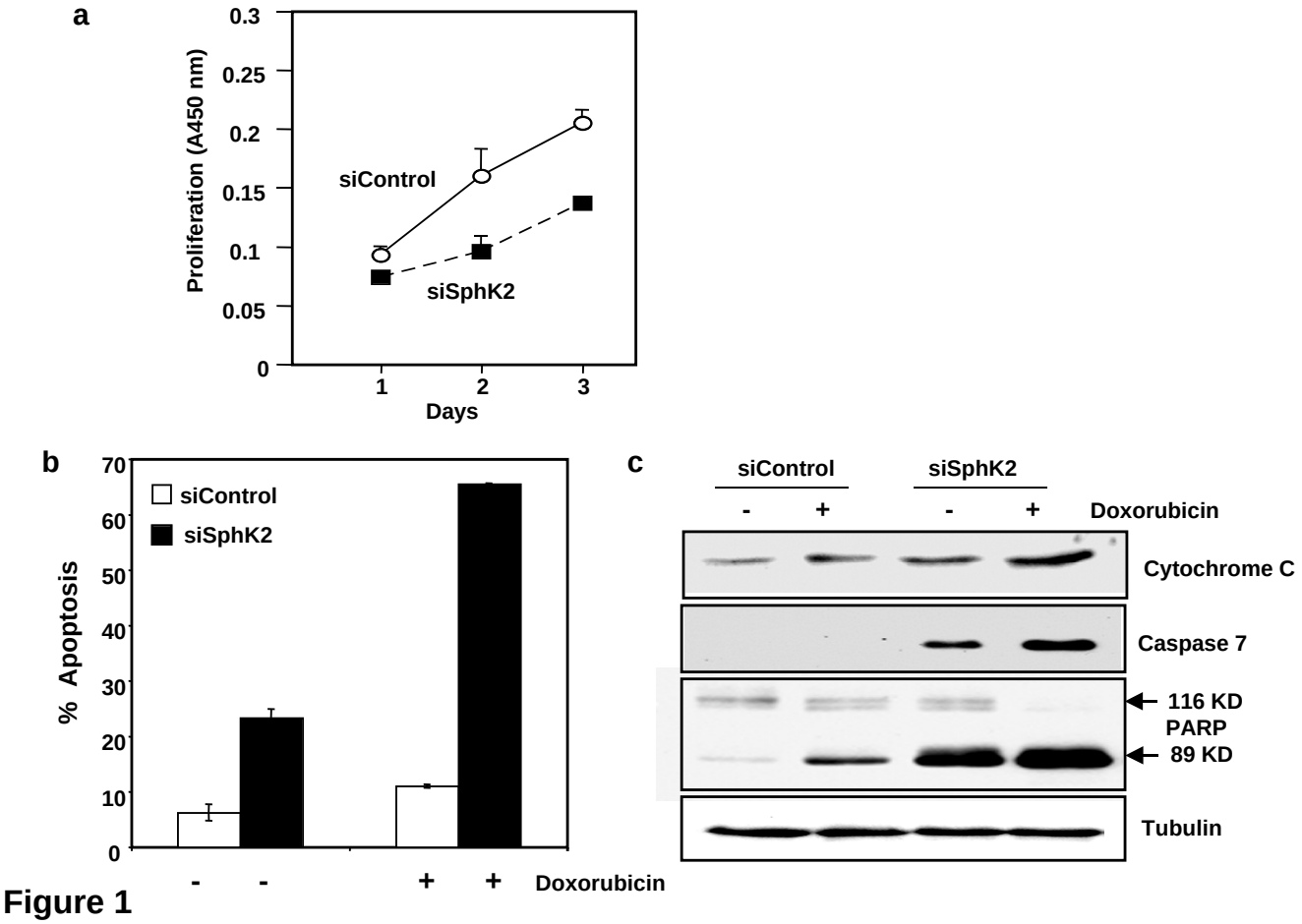

Figure 1

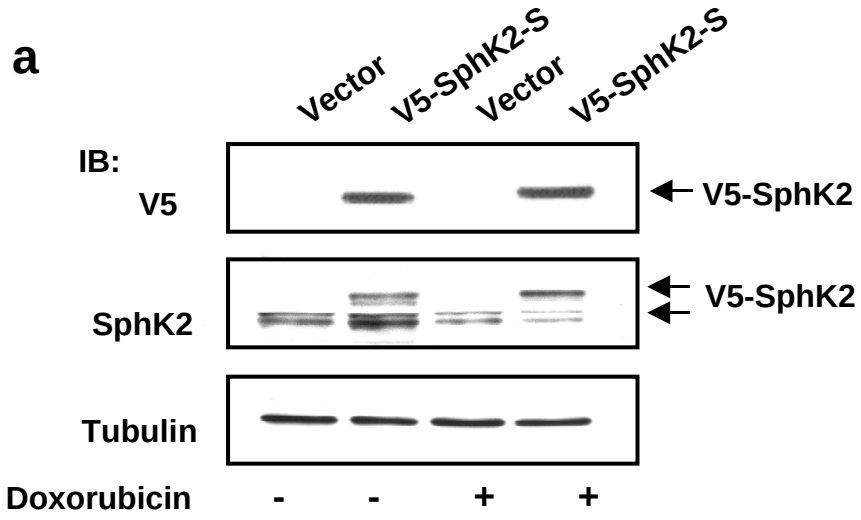
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Figure 2

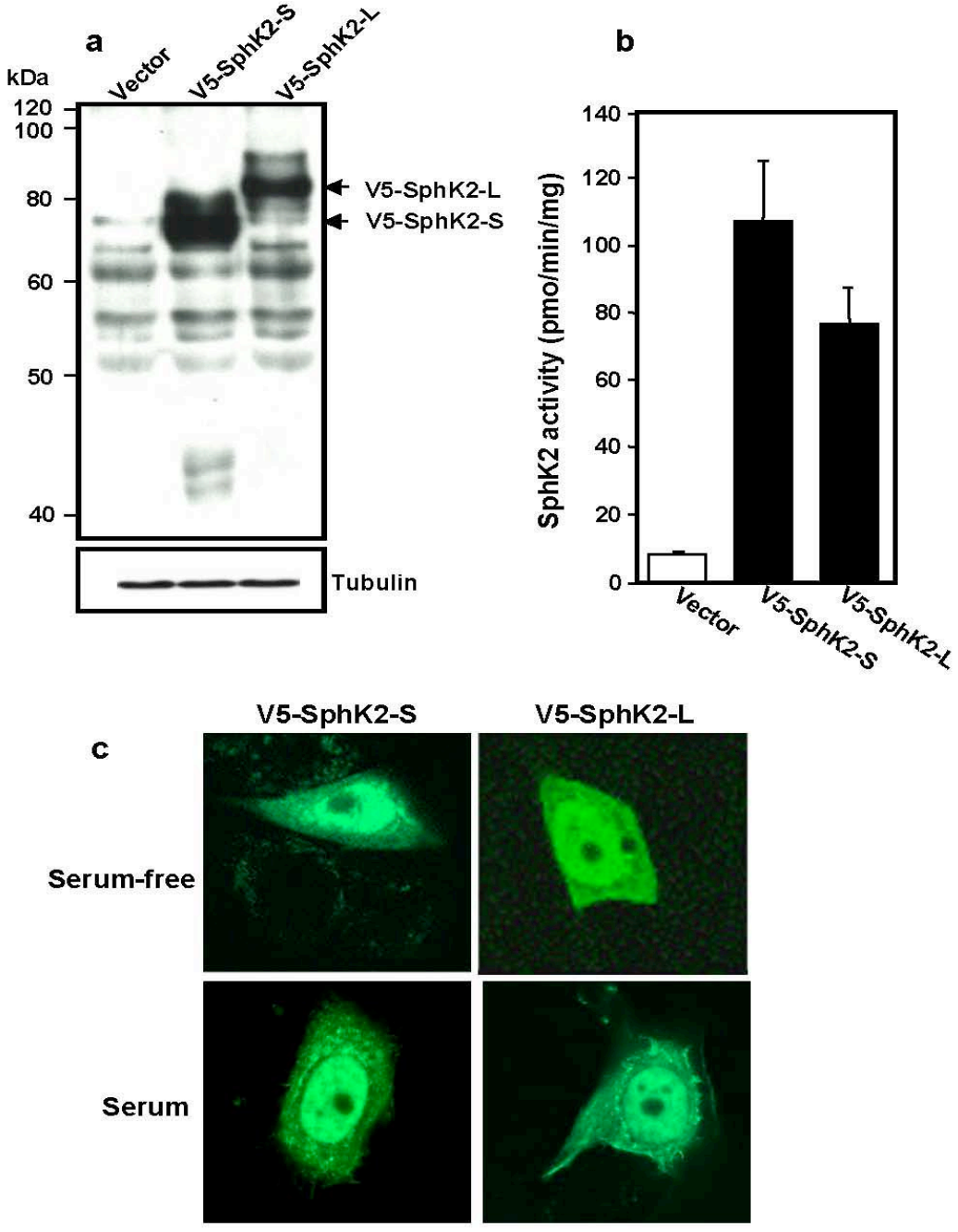
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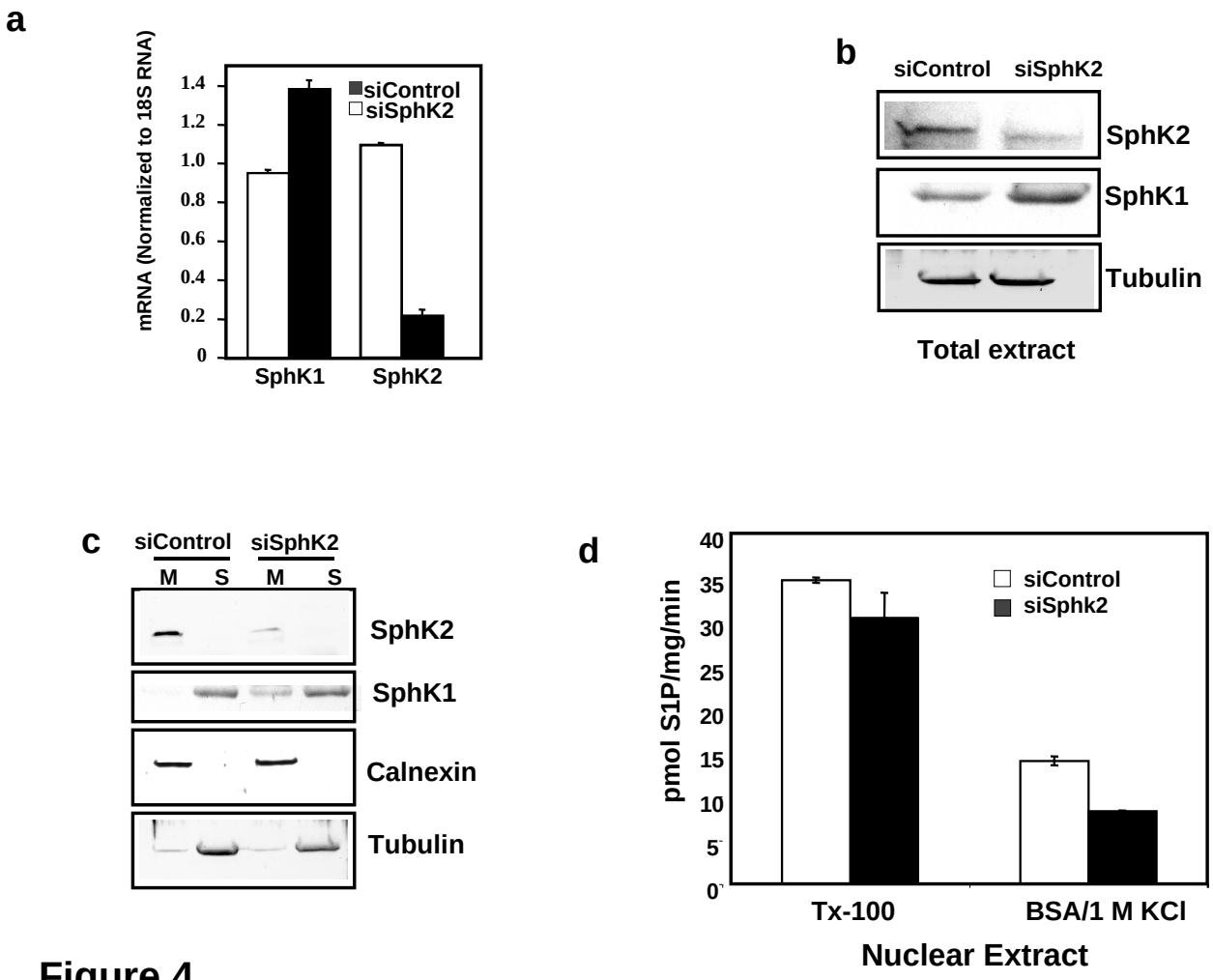
FIGURE 3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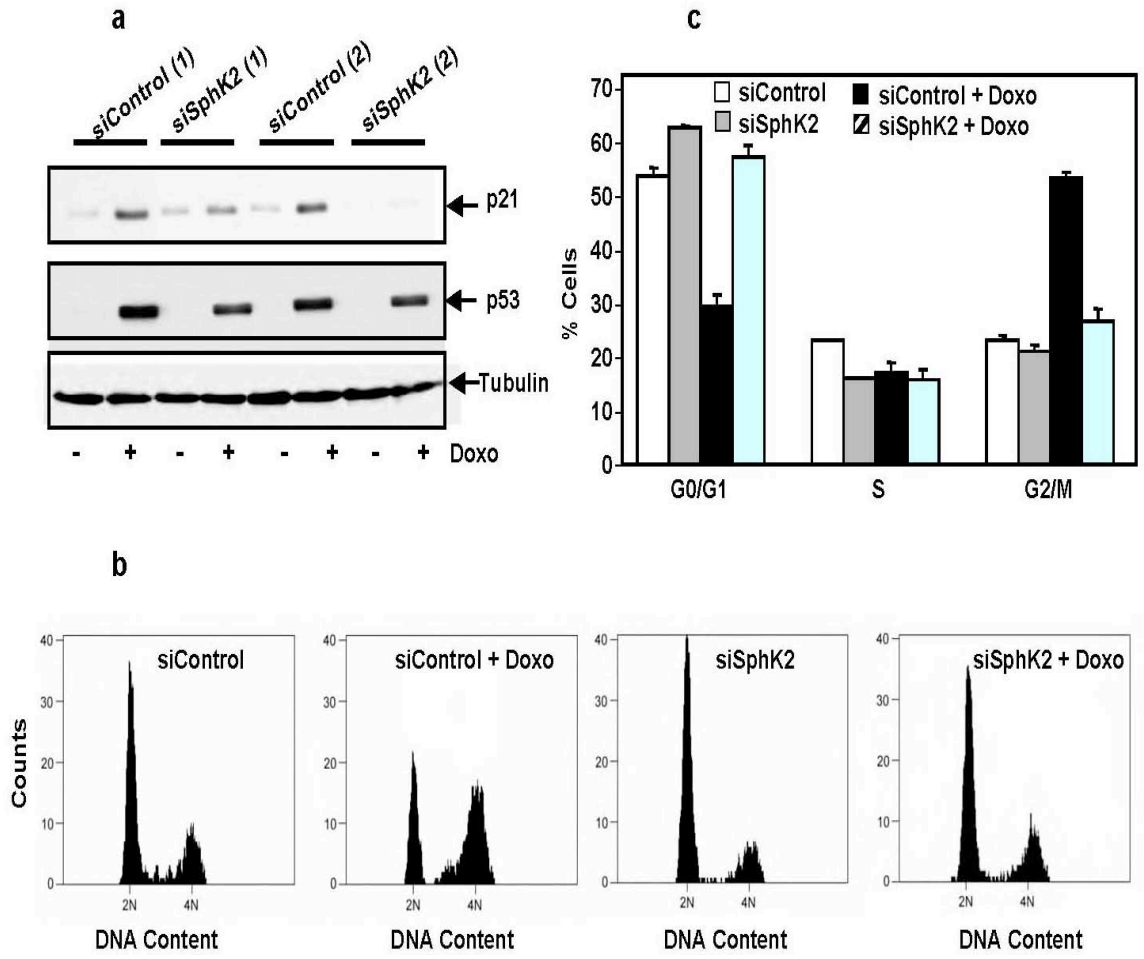
Nuclear Extract

Figure 4

Figure 5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S
G2/M
b
Counts
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siControl + Doxo
siSphK2
siSphK2 + Doxo
0 10 20 30 40
2N 4N
DNA Content

FIGURE LEGENDS

Figure 1. Downregulation of SphK2 reduces growth and sensitizes MCF7 cells to doxorubicin-induced apoptosis. (a) MCF7 cells were transfected with siControl or siSphK2 and after 48 h, plated in 24-well tissue culture dishes. Cells were cultured in media containing 5% serum and at the indicated times, proliferation was determined with the WST-1 reagent. Data are means ± S.D. of triplicate determinations. (b) MCF7 cells transfected with siControl or siSphK2 were cultured in the absence or presence of doxorubicin (1 μg/ml) for 48 h, fixed, and nuclei 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 were analyzed, scoring a minimum of 300 cells. (c) Cytosolic proteins from duplicate cultures were resolved by SDS-PAGE and analyzed by immunoblotting with antibodies against cytochrome *c*, cleaved caspase-7, and cleaved PARP. Blots were stripped and re-probed with tubulin antibody to demonstrate equal loading. Arrows indicate active p20 subunit of caspase-7 and p116 full length PARP and its p89 fragment.

Figure 2. MCF7 cells transiently transfected with vector, V5-SphK2-S were cultured in the absence or presence of doxorubicin (1 μg/ml) for 24 h. Equal amounts of cell lysate proteins were separated by SDS-PAGE and then immunoblotted with SphK2 antibody as indicated. Membranes were subsequently probed with tubulin antibody to show equal loading. Similar results were obtained in two additional experiments.

Figure 3 Expression and localization of SphK2 in MCF-7 Cells. (a) MCF7 cells were transiently transfected with vector, V5-SphK2-S, or V5-SphK2-L. Cell lysate proteins were separated by SDS-PAGE and then immunoblotted with anti-SphK2 antibody. Blots were stripped and re-probed with tubulin antibody to demonstrate equal loading. (b) SphK2 activity was measured in cell lysates prepared from duplicate cultures with sphingosine added as a BSA complex in the presence of 1 M KCl. Data are means ± S.E. of triplicate determinations. (c) MCF7 cells grown on coverslips were transiently transfected with vector, V5-SphK2-S or V5-SphK2-L and subsequently cultured for 24 h in the absence or presence of serum, fixed with methanol, and immunostained with anti-V5 antibody followed by FITC-conjugated secondary antibody. Cells were visualized by confocal fluorescence microscopy. Representative cells of approximately 100 cells examined are shown. (d) Nuclear and cytosolic fractions were prepared from MCF7 cells using NE-Per cytoplasm and nuclear extraction reagents and equal amounts of proteins were separated by SDS-PAGE and immunoblotted with anti-SphK2. Antibodies against lamin A/C and protein-disulfide isomerase (PDI) were used as nuclear and cytoplasmic markers, respectively.

Figure 4 Downregulation of SphK2 with siRNA. (a) MCF7 cells were transfected with control siRNA (open bars) or siRNA targeted to SphK2 (filled bars). After 48 h, RNA was isolated and mRNA levels of SphK1, SphK2, and 18s RNA were determined by quantitative real-time PCR. (b) MCF7 cells were transfected with control siRNA or siRNA targeted to SphK2. Cell lysate proteins were separated by SDS-PAGE and then immunoblotted with anti-SphK2 or anti-SphK1 antibodies. Blots were stripped and re-probed with tubulin antibody to demonstrate equal loading. (c) Cytosolic (S) and membrane fractions (M) from MCF7 cells transfected with siControl or siSphK2 were prepared by centrifugation at 100,000 x g. Equal amounts of proteins were resolved by SDS-PAGE and then immunoblotted with anti-SphK2 or anti-SphK1 antibodies. Calnexin and tubulin antibodies were used as membrane and cytosolic markers, respectively. (d) MCF-7 cells were transfected with control siRNA or siRNA targeted to

SphK2. After 48 h nuclear fractions were isolated and SphK2 activity was measured. Insert: Equal amounts of nuclear proteins were separated by SDS-PAGE and immunoblotted with anti-SphK2 antibody.

Figure 5 Effect of downregulation of SphK2 on doxorubicin-induced increases of p21 and cell cycle arrest. (a) MCF7 cells transfected with siRNA directed at two different SphK2 sequences and two different control siRNAs, as indicated, were cultured in the absence or presence of doxorubicin (1 μg/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 anti-tubulin antibody. Similar results were obtained in two additional experiments. (b) Cellular DNA content of duplicate cultures was analyzed by flow cytometry of cells after staining with propidium iodide. Each plot represents the analysis of 5,000 events. (c) Quantification of the percentage or cells in each cell cycle phase. Data are means ± S.D. of triplicate determinations. Similar results were obtained in two additional experiments.

KEY RESEARCH ACCOMPLISHMENTS

(1) Two forms of SphK2 (short and long) transcripts are present in MCF7 cells. Confocal studies show that overexpressed SphK2-S and SphK2-L is enriched in the nucleus of MCF7 cells.

(2) Western analysis showed that endogenous SphK2 is enriched in the nucleus of MCF7 cells.

(3) siSphK2 reduces G2/M arrest and downregulates p21 independently of p53 in MCF7 cells.

(4) siSphK2 induces apoptosis, breakdown of PARP, caspase 7 cleavage, and cytochrome c release.

REPORTABLE OUTCOMES

Abstracts and Poster Presentations

Sankala H, Hait NC, Paugh SW, Milstien S, Spiegel S. Sphingosine kinase 2 regulates doxorubicin-induced cell cycle arrest and p21 independently of p53 in MCF7 cells. 22nd Annual Daniel T. Watts Research Poster Symposium, Virginia Commonwealth University, Richmond, VA. October 25-26, 2005.

Sankala H, Hait NC, Paugh S, Milstien S, Spiegel S. The role of sphingosine kinase 2 in doxorubicin-induced cell cycle arrest in MCF7 cells. Integrative Cellular and Molecular Signaling Symposium, Virginia Commonwealth University, Richmond, VA. October 28-29, 2005.

Sankala H, Spiegel S. The functions of sphingosine kinase 2 in apoptosis. 33rd Annual John C. Forbes Graduate Student Honors Colloquium, Virginia Commonwealth University, Richmond, VA. May 2005.

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CONCLUSIONS

It is now well established that S1P produced by SphK1 promotes cell growth and inhibits apoptosis, in part due to antagonism of ceramide-induced apoptosis. In contrast little information exists regarding the effect of SphK2 expression in response to anti-cancer agents. The goal of the present investigation was to determine the effect of SphK2 expression in MCF7 cells in response to the anthracyclin doxorubicin. Our results indicate SphK2 may be one of the gatekeepers that influences the balance between cytostasis and apoptosis in response to doxorubicin. According to this idea, SphK2 is required for maximal increases in p21 (independently of p53), enabling cell cycle arrest. Downregulation of SphK2 expression represses p21 and switches the response from cell cycle arrest to apoptosis. In addition it has also been reported that SphK2 knockdown strongly decreased growth in the glioblastoma muliforme cell lines U-87 MG and U-1242 MG and also resulted in a small increase in apoptosis of U-87 MG cells (23). Our laboratory recently observed that downregulation of SphK2 reduced formation of ceramide in the sphingolipid salvage pathway (22). Evidence has been provided for the ability of ceramide to regulate both p21 and pRb (34) (35). Interestingly, it was recently shown in murine oocytes that sphingolipids such as S1P and ceramide modulate doxorubicin actions by, respectively, altering its intracellular trafficking, or by sustaining the drugs contact with DNA (36).

Two forms of SphK2 have been described, the N-terminal-extended SphK2 (Sphk2-L) (20,21) and the originally reported form (SphK2-S) (9,10) lacking exon 1 with the first initiation codon for translation being within exon 2. It has been suggested that SphK-L is the predominant form in human cell lines and tissues (21). However, the presence of endogenous SphK2 protein forms was not directly examined in these studies. Using western blot analysis with an antibody directed against a peptide sequence present in both SphK2-S and SphK2-L, we detected an immunopositive band that was downregulated by siSphK2, which had a similar electrophoretic mobility as SphK2-S rather than SphK2-L.

It has recently been proposed that p21 might provide a novel therapeutic target in cancer treatment and the search for pharmacological agents able to impair its expression in anti-cancer regimen has been urged because loss of p21 usually increases sensitivity of tumor cells to apoptosis targeting SphK2 to eliminate p21 expression may improve the action of anticancer drugs. Advancement in defining sphingolipid-dependent signaling pathways, derived from establishing a large body of biochemical, genetic, and physiological data, may provide new targets for modulation of anti-cancer drug responses with important potential for clinical applications in breast cancer.

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