Sphingosine kinase 1 (SK1), a key enzyme in sphingosine 1-phosphate (S1P) synthesis, regulates various aspects of cell behavior, including cell survival and proliferation. DNA damaging anti-neoplastic agents have been shown to induce p53, ceramide levels, and apoptosis; however, the effects of anti-neoplastic agents on SK1 have not been assessed. In this study, we investigated the effects of a DNA damaging agent, actinomycin D (Act D), on the function of sphingosine kinase (SK1). Act D caused a reduction in the protein levels of SK1, as indicated by Western blot analysis, with a concomitant decrease in SK activity. The down-regulation was posttranscriptional, because the mRNA levels of SK1 remained unchanged. Similar decreases in SK1 protein were observed with other DNA damaging agents such as doxorubicin, etoposide, and γ-irradiation. ZVAD, the pancaspase inhibitor, and Bcl-2 annulled the effect of Act D on SK1, demonstrating a role for cysteine proteases downstream of Bcl-2 in the down-regulation of SK1. Inhibition of caspases 3, 6, 7, and 9 only partially reversed Act D-induced SK1 loss. Inhibition of cathepsin B, a lysosomal protease, produced a significant reversal of SK1 decline by Act D, suggesting that a multitude of ZVAD-sensitive cysteine proteases downstream of Bcl-2 mediated the SK1 decrease. When p53 up-regulation after Act D treatment was inhibited, SK1 down-regulation was rescued, demonstrating p53 dependence of SK1 modulation. Treatment of cells with S1P, the product of SK1, partially inhibited Act D-induced cell death, raising the possibility that a decrease in SK1 may be in part necessary for cell death to occur. Furthermore, the knockdown of SK1 by small interfering RNA in MCF-7 cells resulted in a significant reduction in cell viability. These studies demonstrate that SK1 is down-regulated by genotoxic stress, and that basal SK1 function may be necessary for the maintenance of tumor cell growth.

Sphingolipids have received major attention over the past two decades as important mediators of signal transduction and cell regulation. Key lipids in the sphingolipid pathway are ceramide, sphingosine, and sphingosine 1-phosphate (S1P). Ceramide has been shown to mediate responses to a variety of stressors such as tumor necrosis factor, DNA damage, and FasL (1). The ceramide-mediated responses are growth suppressive, through the processes of differentiation, senescence, cell cycle arrest, or apoptosis (2–6). Sphingosine is also a growth inhibiting messenger, although its role as a mitogenic lipid has also been proposed (7). S1P, on the other hand, has emerged as a growth promoting lipid (8). It has been shown to regulate cell proliferation, survival, migration, and angiogenesis. Furthermore, S1P can attenuate ceramide-induced apoptosis (9). The differential roles of these bioactive lipids have made it essential to decipher the mechanisms that regulate their relative levels in cells. From this aspect, it is crucial to understand the regulation of the enzymes that mediate biosynthesis and breakdown of these three bioactive lipids.

DNA damage is a major stressor that cells are exposed to in the development of cancer and in chemotherapy-based treatment (10). Cell death following DNA damage has been shown to occur via the mitochondrial (intrinsc) pathway of apoptosis. When cells are committed to die by the intrinsic pathway, mitochondrial function is disrupted, resulting in the release of cytochrome c from the intermembrane space and leading to the activation of downstream effector caspases 3, 6, and 7. The latter execute the program of cell death by cleaving key proteins that maintain cellular integrity and function. The sequence of events from DNA damage to the disruption of the mitochondrion is poorly characterized, but p53 has been suggested to be involved. The classical response of the cell to genotoxic stress is to activate the tumor suppressor protein p53, which is often regarded as the guardian of the genome. The common absence of p53 in tumors allows them to circumvent the apoptotic responses that normal cells launch when they are exposed to DNA damaging stimuli.

Ceramide, a key lipid in the sphingolipid pathway, can accumulate following exposure of cells to various DNA damaging chemotherapeutic agents (11). Because of its apoptosis-inducing effects in cancer cells, ceramide has been termed the “tumor suppressor lipid” (12). Ceramide accumulation can occur via: (i) activation of de novo synthesis mediated by serine palmitoyltransferase and ceramide synthase; (ii) activation of sphingomyelin hydrolysis; (iii) inhibition of ceramide hydrolysis; and/or (iv) stimulation of glucosylceramide hydrolysis or inhibition of
its synthesis (11). Some agents activate a multitude of these pathways. The buildup of ceramide following DNA damage has recently been shown to occur in a p53-dependent manner (13), because cells that did not up-regulate p53 following genotoxic stress also failed to accumulate ceramide; however, the mechanism of p53-mediated ceramide induction was not investigated in that study.

Despite the immense literature examining the effects of anticancer agents on ceramide metabolizing enzymes, very few studies have examined the regulation of sphingosine kinase (SK) following challenge of cells by chemotherapeutic agents. SK is a key enzyme in sphingolipid metabolism, because it serves the dual function of modulating ceramide and S1P levels. Enhanced SK activity reduces ceramide by driving ceramide catabolism and enhances the synthesis of sphingoid base phosphates, which have also been shown to engage in negative feedback inhibition on serine palmitoyltransferase (14), the rate-limiting enzyme of ceramide biosynthesis.

Given the significance of sphingosine kinase in regulating ceramide and S1P levels, we evaluated the possible regulation of SK following chemotherapeutic agents. Actinomycin D (Act D) is a well known anticancer agent and topoisomerase II modulator utilized for the treatment of carcinomas, sarcomas, and leukemias, and previous work has implicated ceramide as a downstream effector of Act D (13).

In this study, we demonstrate that DNA damaging agents regulate the down-regulation of the SK1 protein and SK activity in Molt-4 cells. This effect is dependent on ZVAD-sensitive proteases, and is accompanied by a significant increase in sphingosine and several ceramide species. Moreover, the down-regulation of SK1 occurs only in cells expressing functional p53, strongly suggesting that DNA damage-mediated SK1 decline is p53 dependent. Our results also show that endogenous SK1 function is necessary for tumor cell viability.

MATERIALS AND METHODS

Chemicals and Reagents—Actinomycin D was purchased from Sigma. Caspase inhibitors Z-VAD-fmk, Z-DEVD-fmk, Z-VEID-fmk, and Z-LEHD-fmk were purchased from R&D Systems. The cathepsin B inhibitor CA-074-Me and the calpain inhibitor calpeptin were purchased from Calbiochem. S1P was obtained from the Lipidomics Core Facility at the Department of Biochemistry and Molecular Biology at Medical University of South Carolina.

Cell Culture—Human T-cell Molt-4 leukemia cells and MCF-7 cells were grown in RPMI 1640 supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 µg/ml streptomycin. Molt-4 LXSN and Molt-4 E6 cells were generated by retroviral mediated gene delivery as described previously (15). These cells were maintained in the same media as above, supplemented with 250 µg/ml G418. Molt-4 vector-transfected and Bcl-2-transfected cells generated previously (16) were maintained in RPMI 1640 supplemented with 100 µg/ml hygromycin B1 (Calbiochem).
The aqueous layer was aspirated, and 250 μl of H9262/H9262 gen) according to the manufacturer instructions. Lipids were resolved on silica TLC plates (Whatman) using 1-butanol/°H9262/°H9262° solvent.

Cells were collected by centrifugation at 42 °C for 5 min. Then, 4 μl of 5× First Strand buffer, 2 μl of 0.1 μM dithiothreitol, and 1 μl of RNase Out (Invitrogen) were added, and the mixture was incubated at 42 °C for 2 min. Next, 1 μl of Superscript II reverse transcriptase (Invitrogen) was added and the mixture was kept on ice for 5 min. It was stopped by the addition of 20 μl of NaCl and 900 μl of chloroform/methanol/HCl (100:200:1). After letting the samples sit at room temperature for 10 min, 240 μl of chloroform and 240 μl of 2 μM potassium chloride were added, and the samples were centrifuged at 3000 x g for 5 min. The aqueous layer was aspirated, and 250 μl of the organic layer were transferred to new glass tubes. The samples were dried in a SpeedVac and then resuspended in chloroform/methanol/HCl (100:200:1). Lipids were resolved on silica TLC (BDH Whatman) using 1-butanol/methanol/acetic acid/water (80:20:10:20) as a solvent system. Labelled S1P was visualized by autoradiography.

Reverse Transcription—RNA was isolated using the RNeasy kit (Qiagen) according to the manufacturer’s instructions. Isolated RNA (1 μg) was added to 4 μl of 2.5 mM dNTP and 1 μl of 0.5 mg/ml oligo(dT) and brought to a volume of 12 μl with ddH2O. The mixture was incubated at 65 °C for 5 min. Then, 4 μl of 5× First Strand buffer, 2 μl of 0.1 M dithiothreitol, and 1 μl of RNase Out (Invitrogen) were added, and the contents were incubated at 42 °C for 2 min. Next, 1 μl of Superscript II reverse transcriptase (Invitrogen) was added and the mixture was kept on ice for 5 min.

The data are presented as C, values, which represent the number of cycles required to reach the threshold fluorescence. The C value is inversely proportional to the amount of mRNA in a particular sample. The experiment was repeated three separate times, and performed in triplicate each time. Data are presented as the mean ± S.E.
Changes in the sphingolipid profile following Act D treatment

Analysis of sphingosine and the different ceramide species produced in Molt-4 cells following a 24-hr treatment with Act D (10 ng/ml). The results are expressed as picomole of lipid/mg of protein and they represent the mean ± S.D. for three independent experiments. The differences between the vehicle and the Act D-treated samples are all statistically significant at the 95% confidence level. The abbreviations used are: Sph, sphingosine; Cer, ceramide; dh-Cer: dihydroceramide.

|        | Sph  | C14-Cer | dhC16-Cer | C16-Cer | C18-1-Cer | C18-Cer | C20-Cer | C24:1-Cer | C24-Cer | Total Cer |
|--------|------|---------|-----------|---------|-----------|---------|---------|-----------|---------|-----------|
| Molt-4 vehicle | 15.2 ± 1.2 | 22.4 ± 0.1 | 12.3 ± 1.5 | 366.6 ± 5.8 | 10.9 ± 0.1 | 34.6 ± 2.6 | 17.5 ± 0.5 | 54.3 ± 0.5 | 62.7 ± 2.4 | 581.3 ± 8.0 |
| Molt-4 Act D  | 35.2 ± 4.6 | 69.2 ± 10.1 | 44.0 ± 5.4 | 1594.4 ± 197.5 | 25.6 ± 3.3 | 53.3 ± 3.1 | 30.7 ± 0.4 | 116.3 ± 9.4 | 122.8 ± 4.2 | 2056.4 ± 218.2 |

RESULTS

DNA Damage Down-regulates SK1—To determine the effect of DNA damage on the regulation of SK, we used Act D, a widely utilized anti-neoplastic agent that intercalates with DNA and interferes with topoisomerase II function. This agent is indicated in several pediatric tumors, and because leukemias are among the most common tumors in children, we chose Molt-4, a leukemia T-cell line, for our studies. Act D was used at 10 ng/ml, a concentration previously shown to be effective at inducing p53, ceramide accumulation, and poly(ADP-ribose) polymerase cleavage (13). Treatment of Molt-4 cells with Act D

Table II

|        | vehicle | ZVAD | Act D | ZVAD + Act D |
|--------|---------|------|------|-------------|
| SK Activity (% Control) | 100% | 50% | 25% | 12.5% |

Fig. 2. Down-regulation of SK1 by ZVAD-sensitive proteases downstream of Bcl-2. A, Molt-4 cells were pretreated with ZVAD (20, 50, or 100 μM) for 1 h followed by Act D (10 ng/ml) for 24 h. Cells were then harvested for SK1 analysis by immunoblotting. B, Molt-4 cells were preincubated with ZVAD (20 μM) for 1 h followed by Act D (10 ng/ml) for 24 h. Cells were harvested for determination of SK activity as described under “Materials and Methods.” C and D, vector-transfected or Bcl-2-transfected Molt-4 cells were treated with Act D for 24 h and subjected to Western blotting (C) or assayed for SK activity (D). Data are representative of three separate experiments and D shows the mean values of three independent experiments reported with S.E. Asterisk (*) represents a significant decrease in SK activity of the Act D-treated sample compared with the vehicle-treated sample, whereas the number sign (#) is indicative of a significant difference between the Act D-treated and the Act D + ZVAD-treated samples.

Data Analysis—The β-actin gene was used as an internal reference control to normalize relative levels of gene expression. Real time RT-PCR results were analyzed using Q-Gene® software (18), which expresses data as mean normalized expression. Mean normalized expression is directly proportional to the amount of RNA of the target gene relative to the amount of RNA of the reference gene. Alternatively, the results are represented as Ct values for each gene. The higher the Ct value for a gene, the lower the level of expression of that gene, given that the expression level of the control housekeeping gene (β-actin in our studies) is unchanged. Relative gene expression levels between treated and untreated samples are obtained from the formula (1 + AE)ΔCt (19), where AE is the amplification efficiency of the gene under analysis (for the genes considered in this study, it is close to one), and ΔCt is the difference in Ct values between the treated and untreated samples. ΔCt is the difference between the Ct value of the gene under analysis and the Ct value of the control β-actin gene.

Mass Spectrometric Analysis of Lipids—These were performed using electrospray ionization MS/MS analysis on a Thermo Finnigan TSQ 7000 triple quadruple mass spectrometer, operating in multiple reaction monitoring positive ionization mode as described previously (20). SiRNA Treatments—MCF-7 cells were seeded in 6-well plates at a density of 50,000 cells per well the day before the experiment. Scrambled siRNA 5’-AATTCTCGAGAATGTACACGTTCAAGCT-3’ and 5’-ACGAGGACTGTACAGAAGCTGTTAAC-3’, and SK1-specific siRNA 5’-CAGCUCTGTCTTATGCACAGTATCG-3’ (Xeragon) were transfected into the cells using OligofectAMINE reagent (Invitrogen). MTT Assay—Cells were grown for 2 h in 0.5% fetal bovine serum and then treated with different agents. After 24 h, MTT (Sigma) was added to the cells and they were incubated at 37 °C for 4 h. Then, MTT solubilization solution (10% Triton X-100 in acidic isopropanol, 0.1 N HCl) was added to the cells overnight. For the siRNA experiments, cells were grown following the OligofectAMINE reagent protocol and MTT was added to the cells for 2 h prior to analysis. Colorimetric measurements were obtained in a microplate reader (Molecular Devices) at 562 nm and background was subtracted at 650 nm.

RESULTS

DNA Damage Down-regulates SK1—To determine the effect of DNA damage on the regulation of SK, we used Act D, a widely utilized anti-neoplastic agent that intercalates with DNA and interferes with topoisomerase II function. This agent is indicated in several pediatric tumors, and because leukemias are among the most common tumors in children, we chose Molt-4, a leukemia T-cell line, for our studies. Act D was used at 10 ng/ml, a concentration previously shown to be effective at inducing p53, ceramide accumulation, and poly(ADP-ribose) polymerase cleavage (13). Treatment of Molt-4 cells with Act D
FIG. 3. SK1 down-regulation is mediated by several proteases. Treatment of Molt-4 cells with the caspase inhibitors DEVD (50 μM) (A), VEID (20 or 50 μM) (C), or LEHD (100 μM) (E) only partially reversed the Act D-induced SK1 decrease in Molt-4 cells. SK activity was also only partially restored by DEVD (B) and VEID (D). VEID and DEVD remained inferior to ZVAD in their abilities to reverse SK1 decrease, even when used in combination (F). Calpeptin (50 μM), the calpain inhibitor, did not have a significant effect on Act D-induced SK1 decline (G), whereas the cathepsin B inhibitor CA-074-Me, significantly reversed the effects of Act D on SK1 protein down-regulation (H). Data are representative of three separate experiments and B and D show the mean values of three independent experiments reported with S.E. Asterisk (*) represents a significant decrease in SK activity of the Act D-treated sample compared with the vehicle-treated sample, whereas the number sign (#) is indicative of a significant difference between the Act D-treated and the Act D + DEVD- (B) or Act D + VEID (D)-treated samples.
for 24 h produced a substantial decrease in SK1 protein levels as demonstrated by Western blot analysis (Fig. 1A). This effect was dose-dependent, such that a concentration as low as 5 ng/ml resulted in a significant drop in the levels of the protein, and 10 ng/ml led nearly to a complete loss of the SK1 protein (Fig. 1B). The decrease in SK1 starts to occur at 14 h, and is most prominent at 24 h (data not shown).

Three other DNA damaging agents, etoposide, doxorubicin, and γ-irradiation produced a similar down-regulation of SK1 in Molt-4 cells (Fig. 1C). Etoposide also induced a decrease in SK1 protein in MCF-7 cells (Fig. 1D). These results indicate that SK1 is down-regulated by multiple DNA damaging stimuli and in other cells.

To demonstrate that the decrease in SK1 protein with Act D was accompanied by a decrease in SK activity, the effects of the DNA damaging agent on enzyme activity were determined. Indeed, SK activity, as measured by the incorporation of [32P]ATP into the sphingosine substrate, was markedly reduced in Molt-4 cells following Act D treatment as shown in Fig. 1E. This confirmed that the down-regulation of SK1 is mirrored by a significant decrease in activity; however, the decrease seen by Western blots was greater than 75%, whereas the decrease in activity was somewhat less pronounced. This could be in part because of sphingosine kinase activity from SK2.

Because Act D is a well known transcriptional inhibitor that blocks elongation by RNA polymerase (21), we evaluated if the Act D-induced decrease in SK1 is because of a general inhibition of gene expression. Thus, cells were treated with Act D, and the mRNA expression of several sphingolipid metabolizing enzymes was analyzed by real time RT-PCR. Table I shows that the different enzymes have variable sensitivities to inhibition by Act D. Sphingosine kinases 1 and 2, sphingosine phosphate phosphatase-1, mitochondrial ceramidase, acid ceramidase, and the sphingomyelinases all showed no transcriptional inhibitory effects at 10 ng/ml Act D. Alkaline ceramidase, sphingosine 1-phosphate lyase, and LCB1, a subunit of serine palmitoyltransferase, showed very subtle decreases (less than one Ct value that corresponds to changes less than 2-fold) in the mRNA levels. Only LCB2, the second subunit of serine palmitoyltransferase, showed a remarkable decrease in mRNA, as illustrated by the greater than two Ct, i.e. 4-fold difference, between the vehicle-treated and the 10 ng/ml Act D-treated samples. At a concentration of 100 ng/ml Act D, however, inhibition of transcription became a significant consideration for all the enzymes tested. In all subsequent studies, 10 ng/ml Act D was used, because this concentration had no effect on SK1 and SK2 gene expression.

Next, the effects of Act D on cellular sphingolipid levels were determined. Lipid analysis by positive mode ESI/MS/MS mass spectrometry showed an approximate 2-fold increase in sphingosine and a 3.5-fold increase in total ceramide following Act D treatment (Table II). C-16 ceramide showed the most pronounced increase (4-fold) among the ceramide species measured, followed by dihydro-C16 ceramide and C-14 ceramide (3-fold), then C-18:1, C-24, and C-24:1 ceramides (2-fold), then C-20 and C-18 ceramides (1.8- and 1.5-fold, respectively). The levels of S1P were below detection levels in the Molt-4 cell line. This lipid profile, however, is compatible with a decrease in SK activity.

_Down-regulation of SK1 Is Dependent on ZVAD-sensitive Proteases and Is Downstream of Bcl-2—In many cell systems, DNA damage is an apoptotic stimulus that activates the intrinsic pathway of apoptosis (24). Because caspases are implicated in mediating this pathway, their possible involvement in the down-regulation of SK1 was examined. Pretreatment of cells with a pancaspase inhibitor, ZVAD, for 1 h followed by Act D treatment for 24 h demonstrated a significant reversal of SK1 protein reduction (Fig. 2A), suggesting that caspases are involved in down-regulation of the protein after a DNA damaging stressor. This effect was seen at concentrations of ZVAD as low as 20 μM and was most pronounced at 100 μM. The activity of SK was also completely restored with 20 μM of the pancaspase inhibitor (Fig. 2B).

Because the Bcl-2 protein represents a major component of the intrinsic pathway of apoptosis, it was important to determine whether the down-regulation of SK1 occurs upstream or downstream of this protein. To test this, Bcl-2 stable transfectants of Molt-4 cells were treated with 10 ng/ml Act D for 24 h. As seen in Fig. 2, the decline in SK1 was almost completely reversed by Bcl-2 at the protein (Fig. 2C) and the activity level (Fig. 2D). These results suggest that the regulation of SK1 by Act D is mediated by a ZVAD-sensitive protease operating downstream of Bcl-2 in the apoptotic pathway.

_Multiple Proteases Are Involved in Act D-mediated SK1 Down-regulation—Act D treatment of Molt-4 cells produces significant poly(ADP-ribose) polymerase cleavage (13), which is a well identified target for three caspases downstream of Bcl-2 and the mitochondrion: caspases 3, 7, and 9, with caspase-3 being the major cleaving protein (25). Therefore, the effects of DEVD, a caspase-3 inhibitor, on SK1 down-regulation were assessed. Treatment with DEVD (50 μM) for 1 h prior to Act D addition for 24 h had a limited effect on reversing the loss in SK1 protein levels (Fig. 3A), indicating that caspase-3 is only partially involved in SK1 breakdown. Nevertheless, DEVD was significantly effective at reversing Act D-induced loss of SK activity, because Molt-4 cells were able to recover as high as 30% of their activity when treated with Act D in the presence of the caspase-3 inhibitor (Fig. 3B). It is noteworthy that DEVD
can also inhibit caspase-7 activity, and the fact that it partially reversed SK1 down-regulation indicates that caspase-7 may also be involved in Act D-driven SK1 decline.

Because caspases 3 and 7 did not seem to be the primary candidate proteases for SK1 down-regulation, the other caspases downstream of the mitochondrion were investigated; these included caspases 6 and 9. Because caspase-6 can be activated by chemotherapeutic agents (26), its role in Act D-mediated SK1 loss was tested. Pretreatment of Molt-4 cells with 20 μM VEID, the caspase-6 inhibitor, partially inhibited SK1 loss (Fig. 3C). In addition, SK activity was also restored up to 25% by the caspase-6 inhibitor (Fig. 3D). As with DEVD, the reversal was less pronounced than that seen with the pan-caspase inhibitor. Caspase 9 inhibition by LEHD also produced a comparable reversal of SK1 decline as compared with DEVD and VEID (Fig. 3E). When the caspase 3 and 6 inhibitors were used together, their effects were not additive and remained inferior to the ZVAD effect (Fig. 3F), suggesting the possibility that several ZVAD-sensitive proteases were involved in the down-regulation of SK1.

Recent studies have shown that ZVAD can inhibit non-caspase cysteine proteases such as cathepsins and calpains (27, 28). Therefore, the possible involvement of these proteases was tested. The calpain-specific inhibitor, calpeptin, did not show a
significant effect on SK1 protein levels (Fig. 3H), but the cathepsin B inhibitor, CA-074-Me, had a remarkable ability to reverse the Act D-induced SK1 decline (Fig. 3). These results suggested that a combination of ZVAD-fmk to protect against noncaspase proteases and p53 overexpression contributed to the down-regulation of SK1 by Act D in Molt-4 cells. Whether these proteases are directly or indirectly involved remains to be tested.

**Loss of SK1 by Act D Is p53 Dependent**—Genotoxic stress is known to be one of the most potent activators of p53, and many chemotherapeutic agents are able to up-regulate p53 in cells because of their DNA damaging capacity. Therefore, the requirement for p53 in Act D-mediated SK1 down-regulation was investigated. To examine this, we used a Molt-4 cell system expressing either the empty vector LXSN or the vector into which was inserted the E6 gene of human papillomavirus. The E6 protein has been shown to target p53 to ubiquitination and subsequent proteasomal degradation (29). Treatment of cells for 24 h with 10 ng/ml Act D showed a profound decrease in SK1 protein in vector control cells; in contrast, cells that overexpress E6 failed to show loss of SK1 in response to Act D (Fig. 4A). Of note, there was very significant induction of p53 in the LXSN cells that was not observed in the E6 expressing cells (Fig. 4A). In agreement with the protein levels, the activity of SK was also regulated by p53, where Act D produced a marked decrease (approximately 50%) in enzymatic activity in the control cells but not in the E6-transfected cells (Fig. 4B). Furthermore, baseline SK activity was higher in cells lacking p53 compared with those expressing it, suggesting that p53 may modulate baseline as well as stress-regulated SK activity (data not shown). These data strongly suggest that Act D-mediated SK1 loss in Molt-4 cells is dependent on p53.

**Loss of SK1 Contributes to Tumor Cell Death**—It was previously shown that Act D induces cell death in Molt-4 cells (13). Given the down-regulation of SK1 following DNA damage, it became important to determine whether the decrease in cell viability seen with Act D could be reversed by exogenous addition of S1P. Pretreatment of cells with S1P followed by Act D attenuated the Act D-mediated decrease in cell viability in a dose-dependent manner (Fig. 5A), with a significant reversal of up to 15% at 500 nM S1P. These effects were not simply because of the well established ability of S1P to induce proliferation in cells, because S1P, by itself, did not significantly increase cell viability (Fig. 5A, inset). These results suggest that the decrease in SK activity (and consequently S1P levels) may have a significant effect on Act D-mediated cell death.

To further understand the importance of endogenous SK1 in regulating tumor cell function, we examined the effect of SK1 knockdown on cell viability in MCF-7 cells, which, as shown in Fig. 1D, down-regulate SK1 in response to etoposide. These cells exhibited a gross morphological change upon treatment with SK1 siRNA (Fig. 5B), accompanied by a 40% reduction in cell viability (Fig. 5C). These data strongly suggest that SK1 is required for cell survival and that attenuation of SK1 may be important for the execution of cell death.

**DISCUSSION**

Although both ceramide and S1P have emerged as key and reciprocal regulators of anti-neoplastic action, studies examining the effects of DNA damaging chemotherapeutic agents on the key sphingolipid metabolizing enzymes sphingosine kinase are lacking. This work shows, for the first time, a regulated decline of SK1 protein and SK activity, events that are likely to be mediated in a p53-dependent manner by proteases downstream of Bcl-2, and that are accompanied by increases in sphingosine and ceramide.

The novel observation that SK1 is down-regulated is intriguing in light of the substantial body of literature on SK regulation, all of it relating to its activation (30). In addition, SK1 function has mostly been studied through overexpression of the enzyme in various cell lines. The understanding of endogenous SK1 regulation has been hampered by the lack of antibodies. Using a recently developed rabbit polyclonal antibody directed against human SK1 (31), we showed significant loss of SK1 protein in response to Act D. This occurred at concentrations of Act D (5–10 ng/ml) that were not inhibitory to SK gene expression. Indeed, SK mRNA levels, as measured by real-time PCR, did not change (Table I). Rather, SK1 loss was found to occur at the protein level and it appeared to be mediated by multiple proteases. In fact, this is the first report of the down-regulation of SK1 by a protease-dependent pathway. Whereas there is clearly a role for proteases in SK1 modulation, we do not know at this point whether such regulation occurs through an indirect mechanism or through a distinct proteolytic cleavage of the enzyme. It has been particularly difficult to identify cleavage fragments of SK1 on Western blots. The involvement of cathepsin enzymes in the pathway, however, allows the speculation that any possible cleavage of the enzyme may be so dramatic that identification of individual fragments by an antibody directed against one segment of the protein becomes very difficult. It has been previously suggested that sphingolipid metabolizing enzymes are regulated by proteases of the caspase family, although none of these studies show direct regulation, and all of them rely on the use of pharmacological inhibitors, which by no means are entirely specific. One study has shown that the viral protein CrmA, which inhibits caspase-8 activity, caused an inhibition of tumor necrosis factor-induced ceramide...
and response to therapy. Thus, several attempts are being made to restore p53 function in cancer cells, as to “discipline” their behavior (34). p53 has been shown to regulate many downstream mediators of cell signaling including transcription of Bax, p21, and Fas. The current data show failure to down-regulate SK1 in the absence of p53. This obviously has very significant implications, as virtually all tumors lack functional p53. The lack of p53 may remove the check on sphingosine kinase function in the cell, as cells lacking p53 show somewhat higher SK activity. Given the role of S1P as a growth promoting lipid, the uncontrolled increase in S1P, driven to a certain extent by lack of p53, may be a contributing factor to tumor cell behavior of enhanced proliferation, failure of apoptosis, and activation of angiogenesis.

In light of these results, we propose the following model for SK1 regulation by genotoxic stress (Fig. 6): DNA damage causes an up-regulation of the tumor suppressor protein p53, which then drives the classical mitochondrial pathway of apoptosis, leading to effector caspase activation and down-regulation of SK1; moreover, p53 activates a non-mitochondrial (possibly lysosomal) pathway, involving noncaspase proteases, such as cathepsins, which also contribute to SK1 decline following genotoxic insult. p53-mediated lysosomal activation has been proposed by Yuan et al. (35), so it is reasonable to speculate that a similar pathway may be operating in the Molt-4 system as well. In addition to the mechanistic aspects of SK1 regulation by DNA damage, this study also addressed the physiological relevance of such modulation. The roles of S1P, whether as an intracellular second messenger or an extracellular lipid ligand, require small concentrations of the molecule; therefore, even subtle changes in the levels of the lipid may have pronounced effects on cell physiology. In fact, as shown in Table I, the gene expression of the various S1P receptors was differentially regulated by Act D, indicating that they may or may not have a role in regulating cell viability by DNA damage. The significance of S1P in maintaining cell function is illustrated by the siRNA data, where knockdown of SK1 alone resulted in a major decrease in cell viability. Therefore, the basal endogenous function of SK1 may be essential for proper cell growth. This observation has interesting implications, particularly in the context of recent reports on SK1 elevation in cancerous tissues (36). Furthermore, S1P, the lipid product of SK1, attenuated the adverse effects of Act D on cell viability (Fig. 5), suggesting that S1P is a protective molecule against the DNA damaging stressor. Interestingly, the expression of S1P2 was up-regulated by Act D (Table I), which may impart enhanced sensitivity of Act D-treated Molt-4 cells to the pro-survival effects of S1P.

In conclusion, this study shows that SK1 is down-regulated by DNA damaging stressors in a p53-dependent manner in Molt-4 cells. The decline is mediated by multiple proteomes, some of which are the classical effector caspases of the mitochondrial apoptotic pathway, whereas others are part of a nonmitochondrial lysosomal pathway. Furthermore, our work illustrates the significance of SK1 (and S1P) in maintaining proper cell function, both basally and under genotoxic stress-induced conditions.

Acknowledgments—We thank members of the Lipidomics Core Facility in the Department of Biochemistry and Molecular Biology for the lipid analysis.

REFERENCES

1. Hannun, Y. A. (1996) Science 274, 1855–1859.
2. Hannun, Y. A., and Bell, E. M. (1989) Curr. Opin. Chem. Biol. 15, 335–345.
3. Jayadev, S., and Hannun, Y. A. (1996) J. Lipid Mediat. Cell Signal. 14, 295–301.
4. Jayadev, S., Liu, B., Bielawski, A. E., Lee, J. Y., Nazaire, F., Pushkareva, M., Obeid, L. M., and Hannun, Y. A. (1995) J. Biol. Chem. 270, 2047–2052.
5. Kolesnick, R., and Hannun, Y. A. (1999) Trends Biochem. Sci. 24, 224–225.
6. Hannun, Y. A., and Luberto, C. (2000) Trends Cell Biol. 10, 73–80.
7. Merrill, A. H., Jr., Schmelz, E. M., Dillehay, D. L., Spiegel, S., Shayman, J. A., Schroeder, J. J., Riley, R. T., Voss, K. A., and Wang, E. (1997) Toxicol. Appl. Pharmacol. 142, 208–225.
8. Spiegel, S., and Milstein, S. (2003) Nat. Rev. Mol. Cell. Biol. 4, 397–407.
9. Cuviller, O., Prianion, G., Kleuver, B., Vanek, P. G., Coo, O. A., Gutkind, S., and Spiegel, S. (1996) Nature 381, 869–873.
10. Petak, I., and Houghton, J. A. (2001) Pathol. Oncol. Res. 7, 95–106.
11. Radin, N. S. (2003) Biochem. J. 371, 241–256.
12. Hannun, Y. A. (1997) Adv. Exp. Med. Biol. 405, 305–312.
13. Dibaio, G. S., Pushkareva, M. Y., Rachid, R. A., Alter, N., Smyth, M. J., Obeid, L. M., and Hannun, Y. A. (1996) J. Clin. Invest. 102, 329–339.
14. van Echten-Deckert, G., Zschoche, A., Bar, T., Schmidt, R. R., Raths, A., Heinemann, T., and Sandhoff, K. (1997) J. Biol. Chem. 272, 15825–15833.
15. Foster, S. A., Demers, G. W., Etschis, B. G., and Galloway, D. A. (1994) J. Virol. 68, 5688–5705.
16. Zhang, J., Alter, N., Reed, J. C., Borner, C., Obeid, L. M., and Hannun, Y. A. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 5325–5328.
17. Oliveira, A., Barlow, K. D., and Spiegel, S. (2000) Methods Enzymol. 311, 215–223.
18. Muller, P. Y., Janovjak, H., Miserez, A. R., and Dobbie, W. E. (2001) BioTechniques 31, 1372–1374, 1376–1378.
19. Mitas, M., Mikhitarian, K., Walters, C., Baron, P. L., Elliott, B. M., Brothers, T. R., Robison, J. G., Meierkord, H., Zhang, Z., Gillanders, W. E., and Cole, D. J. (2001) Int. J. Cancer 93, 162–171.
20. Pettus, B. J., Bielawski, J., Porcelli, A. M., Reames, D. L., Johnson, K. R., Morrow, J., Chaffant, C. E., Obeid, L. M., and Hannun, Y. A. (2003) FASEB J. 17, 1411–1421.
21. Sobel, H. M. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 5328–5331.
22. Deleted in proof.
23. Deleted in proof.
24. Sellers, W. R., and Fisher, D. E. (1999) J. Clin. Investig. 104, 1655–1661.
25. Deckor, P., Isenberg, D., and Muller, S. (2000) J. Biol. Chem. 275, 9043–9046.
26. MacLachlan, T. K., and El-Deiry, W. S. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 9492–9497.
27. Foghsgaard, L., Wissing, D., Mauch, D., Lademann, U., Bastholm, L., Boes, M., Elling, P. E., Leist, M., and Iuattela, M. (2001) J. Cell Biol. 153, 999–1010.
28. Wolf, B. B., Goldstein, J. C., Stennicke, H. B., Beere, H., Amarante-Mendes, G. P., Salvesen, G. S., and Green, D. R. (1999) Blood 94, 1683–1692.
29. Kessis, T. D., Sieben, U. R., Nelson, W. G., Kastan, M. B., Plunkett, S. B., Han, S. M., Lorincz, A. T., Hedrick, I., and Chou, K. R. (1995) Proc. Natl. Acad. Sci. U. S. A. 90, 3988–3992.
30. Maeyaka, M., Payne, S. G., Milstein, S., and Spiegel, S. (2002) Biochim. Biophys. Acta 1585, 193–201.
31. Johnson, K. R., Becker, K. P., Facchinietti, M. M., Hannun, Y. A., and Obeid, L. M. (2002) J. Biol. Chem. 277, 35257–35262.
32. Dibaio, G. S., Perry, D. K., Gamard, C. J., Platt, R., Poirier, G. G., Obeid, L. M., and Hannun, Y. A. (1997) J. Exp. Med. 185, 481–490.
33. Bourtree, S., Hausser, A., Doppler, H. H., Hurn-Muller, J., Ropke, C., Schwarzmann, G., Pfenninger, K., and Muller, G. (1998) J. Biol. Chem. 273, 31240–31251.
34. Willis, A. C., and Chen, X. (2002) Curr. Med. Mol. Biol. 32, 329–345.
35. Yuan, X. M., Li, W., Dolen, H., Lotem, J., Kama, R., Sacho, L., and Bruenk, U. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 6286–6291.
36. French, K. J., Schrengass, R. S., Lee, B. D., Zhuang, Y., Smith, S. N., Eberly, J. L., Yun, J. K., and Smith, C. D. (2003) Cancer Res. 63, 5962–5969.
Down-regulation of Sphingosine Kinase-1 by DNA Damage: DEPENDENCE ON PROTEASES AND p53
Tarek A. Taha, Walid Osta, Lina Kozhaya, Jacek Bielawski, Korey R. Johnson, William E. Gillanders, Ghassan S. Dbaibo, Yusuf A. Hannun and Lina M. Obeid

J. Biol. Chem. 2004, 279:20546-20554.
doi: 10.1074/jbc.M401259200 originally published online February 26, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M401259200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 34 references, 16 of which can be accessed free at http://www.jbc.org/content/279/19/20546.full.html#ref-list-1