Disruption of Sphingosine 1-Phosphate Lyase Confers Resistance to Chemotherapy and Promotes Oncogenesis through Bcl-2/Bcl-xL Upregulation

Sandra Colié,1,2 Paul P. Van Veldhoven,4 Blandine Kedjouar,1,2 Carmen Bedia,1,2 Virginie Albinié,1,2 Sonia-Caroline Sorli,1,2 Virginie Garcia,1,2 Mojgan Djavaheri-Mergny,5 Chantal Bauvy,3 Patrice Codogno,5 Thierry Levade,1,2,3 and Nathalie Andrieu-Abadie1,2

1INSERM U858; 2Université Paul Sabatier Toulouse III, Institut de Médecine Moléculaire de Rangueil; 3Laboratoire de Biochimie Métabolique, CHU Toulouse, Toulouse, France; 4LIPT, Katholieke Universiteit Leuven, Leuven, Belgium; and 5INSERM U756, Chatenay-Malabry, France

Abstract

Sphingosine 1-phosphate (S1P) is a bioactive sphingolipid metabolite involved in cancer development through stimulation of cell survival, proliferation, migration, and angiogenesis. Irreversible degradation of S1P is catalyzed by S1P lyase (SPL). The human SGPL1 gene that encodes SPL maps to a region often mutated in cancers. To investigate the effect of SPL deficiency on cell survival and transformation, the susceptibility to anticancer drugs of fibroblasts generated from SPL-deficient mouse embryos (Sgpl1−/−) was compared with that of cells from heterozygous (Sgpl1+/−) or wild-type (Sgpl1+/+) embryos. First, loss of SPL caused resistance to the toxic effects of etoposide and doxorubicin. Interestingly, heterozygosity for the Sgpl1 gene resulted in partial resistance to apoptosis. Secondly, doxorubicin-induced apoptotic signaling was strongly inhibited in Sgpl1−/− cells (phosphatidylinerine externalization, caspase activation, and cytochrome c release). This was accompanied by a strong increase in Bcl-2 and Bcl-xL protein content. Whereas correction of SPL deficiency in Sgpl1−/− cells led to downregulation of antiapoptotic proteins, Bcl-2 and Bcl-xL small interfering RNA–mediated knockdown in SPL-deficient cells resulted in increased sensitivity to doxorubicin, suggesting that Bcl-2 upregulation mediates SPL protective effects. Moreover, SPL deficiency led to increased cell proliferation, anchorage-independent cell growth, and formation of tumors in nude mice. Finally, transcriptomic studies showed that SPL expression is downregulated in human melanoma cell lines. Thus, by affecting SIP metabolism and the expression of Bcl-2 members, the loss of SPL enhances cell resistance to anticancer regimens and results in an increased ability of cells to acquire a transformed phenotype and become malignant. [Cancer Res 2009;69(24):OF1–8]

Introduction

Sphingolipids are essential ubiquitous constituents of membranes and, notably, microdomains (1). However, like mediators derived from glycerophospholipids, sphingolipids are a source of an important family of bioactive signaling molecules that regulate numerous physiologic and pathologic processes (2). The importance of sphingolipids in human health and disease is illustrated by the dramatic consequences of inherited disruption of their normal metabolism (3) and also by their involvement as bioeffector molecules in cancer and hematologic malignancies (4–6). The best characterized sphingolipid mediators are ceramide and sphingosine 1-phosphate (S1P); whereas ceramide generally transduces antiproliferative responses, the lysolipid S1P has crucial roles in cell survival, cell migration, and angiogenesis and as well as immune responses (7).

S1P exerts its effects either through autocrine and paracrine actions as a ligand for a family of specific G protein–coupled receptors (S1P1–5) as well as by intracellular functions as a second messenger involved in the control of cell growth and death signaling pathways (8, 9). Both intracellular and circulating levels of S1P depend on the activity of some membrane transporters and of three classes of enzymes that control its metabolism. Whereas sphingosine kinases 1 (SK1) and 2 produce S1P by phosphorylating sphingosine, two S1P phosphatases dephosphorylate S1P to generate sphingosine, and S1P lyase (SPL) irreversibly degrades S1P to phosphoethanolamine and 2-trans-hexadecenal (10). The critical role played by SIP as a “tumor-promoting” agent has been mostly evidenced through manipulation of the expression and biological activity of SK1. Indeed, SK1 is overexpressed in multiple types of cancers and upregulation of SK1 has been associated with tumor angiogenesis and resistance to radiation and chemotherapy. Conversely, pharmacologic or genetic inhibition of SK1 activity in vitro on different cultured tumor cell models and in vivo on transplanted animals has been shown to enhance drug-induced lethality (11, 12). Furthermore, SIP-generating enzyme SK1 acts as an oncogene because NIH3T3 fibroblasts overexpressing SK1 acquired a transformed phenotype and the capability to form tumors in nude mice (13).

Of the enzymes involved in determining SIP abundance, SPL represents an undeniable candidate as potential regulator of cell’s fate in response to stress (14). SPL is a microsomal pyridoxal 5′-phosphate–dependent aldehyde-lyase that catalyzes the irreversible cleavage of S1P in the final step of sphingolipid catabolism (15, 16). In mammalian cells, overexpression of SPL was shown to sensitize cells to apoptotic stress such as serum deprivation and chemotherapy (17–19). SPL-induced apoptosis requires its enzyme activity and can be blocked by addition of exogenous SIP (17). In contrast, products of the lyase reaction had no effect on apoptosis. The cytotoxic effect of SPL appears to be dependent on the activation of proapoptotic signaling pathways involving
Materials and Methods

Cell culture and genotyping. Sgpl1+/− mice were generated from OST58278 (27) gene-trapped ES cells, bought from OmniBank, by Lexicon Genetics on a fee basis, and inbred into a C57BL/6 background as reported previously.6 Heterozygous mice were mated and murine embryonic fibroblasts (MEF) were prepared at embryonic day 16 from the skin of wild-type (Sgpl1+/+), SPL heterozygous (Sgpl1+/−), and SPL-deficient (Sgpl1−/−) embryos. By dilution culturing, spontaneously immortalized MEFs were obtained. Cells were grown in DMEM containing 10% FCS (Invitrogen) at 37°C in 5% CO2 humidified incubators. Total genomic DNA was extracted and quantified using the Nanodrop ND-1000. Fragments derived from the mSPL-Trap gene trap and genotype of wild-type (+/+), SPL heterozygous (+/−), and SPL-deficient (−/−) cells. Genomic DNA was extracted and amplified using one set of primers for wild-type mSPL (499-bp PCR product) and another set of primers for lacZ (667-bp PCR product). Western blot analysis of mSPL expression in wild-type, heterozygous, and SPL-deficient MEFs. 

Vectors and cell transfections. The cDNA encoding mSPL (29) was amplified by PCR using Phusion polymerase (Finnzymes) and oligonucleotide primers matching the first and last bases, respectively, of the mSPL-Trap-r sequence: MmSPL-Trap-r 5′-CgAATACCTgTTCCgTCATAgC, and MmSPL-Trap-r 5′-CTAgAAGAgCAAAACTgCCTTg. Amplification was carried out for 35 cycles using an annealing temperature of 60°C. DNA fragments were analyzed by electrophoresis on a 1.5% agarose gel.

MEF were transfected with the pcDNA5TO-SGPL1 sequence was amplified by PCR using Phusion polymerase (Finnzymes) and oligonucleotide primers: MmSPL-Trap-r 5′-CgAATACCTgTTCCgTCATAgC, and MmSPL-Trap-r 5′-CTAgAAGAgCAAAACTgCCTTg. Amplification was carried out for 35 cycles using an annealing temperature of 60°C. DNA fragments were analyzed by electrophoresis on a 1.5% agarose gel.

Cell viability, flow cytometry, and morphologic analyses. For cytotoxicity assays, cells were seeded in flat-bottomed 24-well plates (4 × 105 per well). After 24 h, the medium was replaced with fresh medium containing 5% FCS and etoposide or doxorubicin, and cells were incubated at 37°C for different times. Cell viability was assessed using the MTT assay (Euromedex). Phosphatidylserine externalization was evaluated by flow cytometry after labeling with Annexin V-FITC (250 ng/mL) and propidium iodide (12.5 μg/mL; AbCys) using a FACScan (BD Biosciences) cytometer.

p38 mitogen-activated protein kinase, p53, caspase-2, and caspase-3 (19). However, whereas SK1 overexpression was associated with upregulation of the antiapoptotic protein Bcl-2 (20, 21), the connection between SPL with Bcl-2 family members has never been investigated. Interestingly, the SGPL1 gene, which encodes SPL, has been reported to be significantly downregulated in human colon cancer tissues compared with normal tissues (22). Moreover, the human SGPL1 gene maps to chromosomal region 10q21 (23), which is deleted or mutated in many human tumor types (24–26). Altogether, these observations suggest that loss of SPL expression might potentiate cancer cell proliferation and contribute to tumorigenesis; this, however, remains to be demonstrated.

Here, we report that the lack of SPL leads to upregulation of the antiapoptotic proteins Bcl-2 and Bcl-xL and consequently protects against apoptosis induced by chemotherapeutic agents but not autophagy. Moreover, SPL-deficient cells isolated from Sgpl1−/− mouse embryos showed an increase in cell growth rate in culture, colony formation in soft agar, and tumor progression in nude mice compared with cells isolated from wild-type embryos. Collectively, these observations suggest that loss of SPL expression might potentiate cancer cell proliferation and contribute to tumorigenesis; this, however, remains to be demonstrated.
For analysis of cytochrome c release, cells seeded on glass coverslips at 2 × 10^5/mL were incubated with doxorubicin, fixed with formaldehyde (4%), washed with PBS, permeabilized with Triton X-100 (0.1%), and stained with a monoclonal anti-cytochrome c antibody (BD Pharmingen) and a secondary Alexa 488-labeled antibody (Molecular Probes/Invitrogen).

DEVD cleavage enzyme assay. Cell lysates were incubated for 30 min with Ac-Asp-Glu-Val-Asp-aminomethylcoumarin (Alexis). The amount of the released fluorescent product was determined fluorometrically at 351 and 430 nm for the excitation and emission wavelengths, respectively. Protein concentrations were determined according to the Bradford method.

Western blot analyses. Equal amounts of proteins were electrophoresed on a 10% to 15% SDS-polyacrylamide gel, transferred to a nitrocellulose membrane (Perkin-Elmer), and blotted with monoclonal anti-Bax and anti-poly(ADP-ribose) polymerase, and anti-β-actin antibodies (Cell Signaling Technology). Polyclonal anti-Bax and anti-Bcl-2 antibodies were from BD Pharmingen. Proteins were detected using an enhanced chemiluminescence detection system (Pierce). SPL was detected using a rabbit polyclonal anti-β-actin antibody (BD Pharmingen) and a second-ary Alexa 488-labeled antibody (Molecular Probes/Invitrogen).

SIP measurements. The amount of cellular SIP was evaluated as reported (31) using d-erythro-[4,5-3H]dihydro-S1P (ARC/Isobio) as described (30).

Anchorage-independent growth assay. Six-well plates were precoated with 2 mL DMEM containing 10% FCS and 0.6% agar. DMEM (2 mL) containing 2 μCi/mL; 1.5 μmol/L; Perkin-Elmer).

Figure 2. Resistance of SPL-deficient cells to anticancer agents. Wild-type, heterozygous, and SPL-deficient MEFs were incubated for 24 h in medium containing 5% FCS in the absence or presence of the indicated concentrations of etoposide (A) or doxorubicin (B). Alternatively, cells were incubated for the indicated times in the absence or presence of 1 μmol/L doxorubicin (C). Viability was assessed using the MTT test and is expressed as a percentage of the value determined at the corresponding time in the absence of the drug. Mean ± SE of four to eight independent experiments done in triplicate. D, wild-type MEFs were transfected with control (Ctrl) or SPL targeting siRNAs. SPL expression was evaluated by Western blotting (top). After 48 h of transfection with the siRNA and 24 h treatment with doxorubicin (1 μmol/L), cell viability was evaluated using the MTT test. Mean ± SE of three independent experiments.
Results

Gene dosage effect of Sgpl1 on cell death induced by chemotherapeutic drugs. To address the effect of SPL deficiency on anticancer drug–induced cell death, we first used a genetic approach by testing mutant MEFs derived from heterozygous (Sgpl1+/−) and homozygous (Sgpl1−/−) SPL-null mouse embryos. Sgpl1+/− and Sgpl1−/− cells carry a β-galactosidase-neomycin fusion gene trap cassette within the second intron of the Sgpl1 gene (Fig. 1A). Only in these mutant cells bacterial β-galactosidase activity was measurable (Supplementary Fig. S1). Moreover, Western blot analysis using an antibody against the murine SPL protein (Fig. 1B) and SPL enzymatic assay (Fig. 1C) confirmed that SPL expression and activity were decreased in Sgpl1+/− cells and abolished in Sgpl1−/− cells compared with wild-type cells. Disruption of Sgpl1 gene was accompanied by an intracellular increase of S1P levels (Fig. 1D; Supplementary Table S1).

The importance of SPL in stress-induced cell death was investigated by treating wild-type and SPL mutant cells with various concentrations of etoposide (Fig. 2A) and doxorubicin (Fig. 2B). These agents led to a dose- and time-dependent reduction in the viability of Sgpl1+/− cells (Fig. 2C; Supplementary Fig. S2A). Under all concentrations tested, Sgpl1+/− MEFs were significantly less sensitive to the lethal effects of the drugs than their normal counterparts, whereas Sgpl1−/− MEFs were highly resistant. Interestingly, the chemoresistance of heterozygous and homozygous cells was also observed in primary MEFs (Supplementary Fig. S3) as well as SV40-transformed MEFs (data not shown). Furthermore, nuclear condensation observed in doxorubicin-treated Sgpl1+/− cells by fluorescence microscopy was reduced in Sgpl1+/− and completely abolished in Sgpl1−/− cells (Supplementary Fig. S2B). Finally, Sgpl1+/− MEFs transfected with a siRNA targeting SPL, which reduced SPL protein content by ~50%, became partially resistant to doxorubicin (Fig. 2D). Similar observations were made on NIH3T3 fibroblasts treated with mSPL siRNA (data not shown). These data point to a strong correlation between SPL expression and cell sensitivity to chemotherapy.

SPL is required for apoptosis induced by doxorubicin. To substantiate the importance of SPL in anticancer drug–induced cell death, the apoptotic cascade activated by doxorubicin in Sgpl1−/− cells was analyzed. Of note, intracellular uptake of doxorubicin, analyzed by fluorometry, was similar in Sgpl1+/− and Sgpl1−/− cells (data not shown but illustrated in Fig. 3D) by the red fluorescence of the nuclei). In wild-type but not in SPL-deficient cells, doxorubicin led to an increased proportion of Annexin V–positive and propidium iodide–negative cells, with externalized phosphatidylserine (Fig. 3A). These results are consistent with the absence of morphologic alterations such as nuclear condensation observed in mutant cells compared with control cells (Supplementary Fig. S2B) and suggest that apoptosis is impaired in SPL-deficient cells.

To test whether caspase activation was altered in SPL mutant cells upon doxorubicin treatment, we analyzed caspase activity by measuring cleavage of the fluorogenic tetrapeptide substrate Ac-Asp-Glu-Val-Asp-aminomethylcoumarin. Caspase activity was measured (mean ± SE of four independent experiments) of propidium iodide–negative/Annexin V–positive cells are indicated. B and C, wild-type and SPL-deficient cells were incubated with doxorubicin (1 μmol/L) for the indicated times. B, effector caspase (DEVDase) activity was measured (mean ± SE of three independent experiments). C, cell lysates (30 μg protein) were subjected to SDS-PAGE and Western blotted with anti-caspase-9, caspase-7, caspase-3, poly(ADP-ribose) polymerase (PARP), and β-actin antibodies. Representative of at least three independent experiments. D, effector caspase activity was assessed in wild-type and SPL-deficient MEFs grown on glass coverslips were incubated for 8 h in the absence or presence of doxorubicin (1 μmol/L) and then stained using anti–cytochrome c antibody. The red staining corresponds to the fluorescence emitted by doxorubicin.

Figure 3. Doxorubicin-induced apoptosis is impaired in SPL-deficient cells. A, wild-type and SPL-deficient cells were incubated for 16 h in medium containing 5% FCS in the absence or presence of doxorubicin (1 μmol/L). Cells were labeled with Annexin V-FITC and propidium iodide and analyzed by flow cytometry. Percentages (mean ± SE of three independent experiments) of propidium iodide–negative/Annexin V–positive cells are indicated. B and C, wild-type and SPL-deficient cells were incubated with doxorubicin (1 μmol/L) for the indicated times. B, effector caspase (DEVDase) activity was measured (mean ± SE of four independent experiments). C, cell lysates (30 μg protein) were subjected to SDS-PAGE and Western blotted with anti-caspase-9, caspase-7, caspase-3, poly(ADP-ribose) polymerase (PARP), and β-actin antibodies. Representative of at least three independent experiments. D, wild-type and SPL-deficient MEFs grown on glass coverslips were incubated for 8 h in the absence or presence of doxorubicin (1 μmol/L) and then stained using anti–cytochrome c antibody. The red staining corresponds to the fluorescence emitted by doxorubicin.

Figure 3. Doxorubicin-induced apoptosis is impaired in SPL-deficient cells. A, wild-type and SPL-deficient cells were incubated for 16 h in medium containing 5% FCS in the absence or presence of doxorubicin (1 μmol/L). Cells were labeled with Annexin V-FITC and propidium iodide and analyzed by flow cytometry. Percentages (mean ± SE of three independent experiments) of propidium iodide–negative/Annexin V–positive cells are indicated. B and C, wild-type and SPL-deficient cells were incubated with doxorubicin (1 μmol/L) for the indicated times. B, effector caspase (DEVDase) activity was measured (mean ± SE of four independent experiments). C, cell lysates (30 μg protein) were subjected to SDS-PAGE and Western blotted with anti-caspase-9, caspase-7, caspase-3, poly(ADP-ribose) polymerase (PARP), and β-actin antibodies. Representative of at least three independent experiments. D, wild-type and SPL-deficient MEFs grown on glass coverslips were incubated for 8 h in the absence or presence of doxorubicin (1 μmol/L) and then stained using anti–cytochrome c antibody. The red staining corresponds to the fluorescence emitted by doxorubicin.

Figure 3. Doxorubicin-induced apoptosis is impaired in SPL-deficient cells. A, wild-type and SPL-deficient cells were incubated for 16 h in medium containing 5% FCS in the absence or presence of doxorubicin (1 μmol/L). Cells were labeled with Annexin V-FITC and propidium iodide and analyzed by flow cytometry. Percentages (mean ± SE of three independent experiments) of propidium iodide–negative/Annexin V–positive cells are indicated. B and C, wild-type and SPL-deficient cells were incubated with doxorubicin (1 μmol/L) for the indicated times. B, effector caspase (DEVDase) activity was measured (mean ± SE of four independent experiments). C, cell lysates (30 μg protein) were subjected to SDS-PAGE and Western blotted with anti-caspase-9, caspase-7, caspase-3, poly(ADP-ribose) polymerase (PARP), and β-actin antibodies. Representative of at least three independent experiments. D, wild-type and SPL-deficient MEFs grown on glass coverslips were incubated for 8 h in the absence or presence of doxorubicin (1 μmol/L) and then stained using anti–cytochrome c antibody. The red staining corresponds to the fluorescence emitted by doxorubicin.
Autophagy is not altered in Sgpl1−/− cells. As the increase of S1P levels after SK1 overexpression was shown to stimulate autophagy and to protect cells from apoptotic death during nutrient starvation (33), we examined the effect of SPL deficiency on this phenomenon. As illustrated in Supplementary Fig. S4, starvation-induced apoptosis was reduced in Sgpl1−/− compared with Sgpl1+/+ cells. However, autophagy, monitored by measuring proteolysis of long-lived proteins, formation of LC3-II or LC3 translocation from the cytosol to aggregates into autophagic vacuoles, which are prerequisites for autophagosome formation and are regarded as autophagy markers (34, 35), was similar in wild-type and mutant cells. These data suggest that, under nutrient starvation, the resistance of SPL-deficient cells to apoptosis is not associated with alterations in autophagy.

Upregulation of the expression of antiapoptotic Bcl-2 family members contributes to the chemoresistance of SPL-deficient cells. Overexpression of prosurvival Bcl-2 proteins occurs in numerous tumors and correlates with resistance to antineoplastic drugs (36). To determine whether resistance to doxorubicin of SPL-deficient cells was accompanied with an alteration of the balance between proapoptotic and antiapoptotic members of the Bcl-2 family, the expression pattern of these proteins was investigated. A comparative Western blot analysis revealed that Sgpl1−/− cells displayed higher levels of both Bcl-2 and Bcl-xL than Sgpl1+/+ cells, whereas Bid and Bax were not modified, indicating a possible contribution of Bcl-2 antiapoptotic members to the chemoresistance of SPL-deficient cells (Fig. 4A; Supplementary Fig. S3C). To test the latter hypothesis, we investigated the expression of Bcl-2 and Bcl-xL in Sgpl1−/− cells transiently transfected with a cDNA encoding mSPL. As shown in Fig. 4B, SPL-corrected Sgpl1−/− cells displayed lower levels of both Bcl-2 and Bcl-xL than mutant cells transiently transfected with an empty vector. We next employed a knockdown approach to deplete Bcl-2 and Bcl-xL either individually or in combination in Sgpl1−/− cells. Effective downregulation of the target protein Bcl-2 or Bcl-xL was confirmed by Western blot analysis (Fig. 4C). Sgpl1−/− cells transfected with both Bcl-2 and Bcl-xL siRNA displayed enhanced sensitivity to doxorubicin compared with their control counterparts (Fig. 4D). Collectively, these data point to a tight regulation between the expression of Bcl-2 antiapoptotic proteins and SPL in the control of sensitivity to chemotherapeutic drugs.

Lack of SPL expression leads to increased cell proliferation in vitro and oncogenesis in vivo. The S1P-generating enzyme SK1 behaves as an oncogene when overexpressed in nontransformed NIH3T3 fibroblasts (13). To investigate the effect of S1P-degrading SPL on cell transformation, we analyzed the growth properties of Sgpl1−/− and Sgpl1+/+ cells. We found that, in serum-free medium or in the presence of 1% serum, SPL-deficient cells exhibited higher rates of proliferation than their wild-type counterparts. In S1P-containing medium, full serum, both cell lines grew at identical rates (Fig. 5A). However, using the scratch wound closure assay, we observed that cell migration was not altered in SPL-deficient cells (data not shown). We then tested whether increased growth of Sgpl1−/− cells was accompanied by the ability to form colonies in
soft agar. As illustrated in Fig. 5B and Supplementary Table S2, SPL-deficient cells gave rise to numerous colonies, whereas wild-type cells showed much less anchorage-independent growth. To evaluate the in vivo transforming properties of SPL, we tested the ability of Sgpl1+/+ and Sgpl1−/− cells to grow in immunocompromised mice. When injected s.c. in female athymic nude mice, SPL-deficient cells induced rapidly growing tumors at the site of inoculation within 15 days, whereas mice grafted with Sgpl1+/+ cells did not develop tumors even 40 days postgraft (Fig. 5C). In all tumor-bearing mice sacrificed after 40 days, the bacterial β-galactosidase carried in the gene trap was fully active (Supplementary Fig. S5A). Histologic analysis showed that all tumors presented high cellularity and consisted of spindle cells, some with atypical nuclei, forming fascicles highly suggestive of a fibrosarcoma (Supplementary Fig. S5B). Altogether, these data indicate that expression of a functional SPL is required for inhibition of tumorigenesis.

**Downregulation of SPL expression in melanoma cells.** Mammalian genes frequently present allelic variants that differ in their expression levels and that, in the case of tumor suppressor genes, can be of relevance for cancer susceptibility. Because the human

---

**Figure 5.** Increased growth, colony, and tumor formation of SPL-deficient cells. A, wild-type and SPL-deficient cells were seeded in 24-well plates at 3 × 10^4/mL. After 16 h (time 0), the medium was removed and replaced by fresh medium containing 5%, 1%, or 0% FCS, and cells were further incubated for the indicated times. MTT assay was used to monitor growth. Representative of at least three independent experiments done in triplicate. B, wild-type and SPL-deficient cells were cultured on soft agar and fed with medium containing 10% FCS. Colonies were stained with MTT and photographed after 2 wk of incubation. C, wild-type and SPL-deficient cells were injected s.c. into nude mice (12 per group) and tumor growth was monitored regularly for 40 d.

**Figure 6.** SGPL1 expression in melanoma. SGPL1 mRNA levels in human melanoma cell lines were quantified by real-time quantitative PCR. Data are mRNA fold change compared with adult (NHEM-M2) or juvenile (NHEM-f) normal human melanocytes. Mean ± SE of two independent experiments done in duplicate.
**SGPL1** gene maps to chromosomal 10q21 and that alteration of chromosome 10 is common in human cancers including melanoma (37), we evaluated the expression of SPL in a series of invasive or metastatic melanoma cells and compared with that of melanocytes derived from healthy individuals. As shown in Fig. 6, **SGPL1** expression was significantly reduced in most melanoma cell lines tested, suggesting that this gene is downregulated during melanoma tumorigenesis.

**Discussion**

SIP is considered as a tumor-promoting agent for its role in the regulation of cancer cell growth, survival, adhesion, migration, metastatic potential, and angiogenesis (2, 7). Several antitumoral therapeutic approaches targeting SIP formation and signaling have been developed including the synthesis of sphingosine kinase inhibitors (38), the targeting of SIP receptors with the immunosuppressant FTY720 (39), and the use of an anti-SIP antibody (40). By controlling SIP catalysis, SPL emerges as a novel target in the generation of sphingolipid-based therapeutics (14).

Analysis of SPL-deficient genetic models revealed that SPL is crucial for mammalian survival because SPL knockout mice displayed severe lymphopenia and developed myeloid cell hyperplasia and also significant lesions in several nonlymphoid organs (28, 41). Here, using fibroblasts generated from SPL-deficient mouse embryos, we show that complete loss of SPL activity confers resistance to apoptosis in response to chemotherapeutic agents. Of particular interest was the finding that a gene dosage effect exists in the sensitivity to anticancer drugs because the loss of a single SPL allele (as seen in heterozygous cells) resulted in partial resistance to genotoxic stress. This observation was corroborated by the fact that siRNA-mediated knockdown of SPL in control fibroblasts also led to a partial resistance to doxorubicin-induced cell death. SPL deficiency blocked apoptotic events such as the cleavage of effector caspases and the caspase substrate poly(ADP-ribose) polymerase in accordance with data showing that caspase-3 activity was increased in SPL-overexpressing cells (17, 19). Moreover, we found that the release of mitochondrial cytochrome c into the cytosol and the cleavage of initiator caspase-9 were impaired in **Sgpl1**−/− cells, suggesting that SPL likely acts upstream of mitochondria and triggers the intrinsic apoptotic pathway.

Several Bcl-2 family proteins insert into mitochondrial membranes operating as guardians of these organelles (42). Here, we show that whereas loss of SPL activity contributes to upregulation of the oncoproteins Bcl-2 and Bcl-xL but not Bax and Bid, its restoration in SPL-deficient cells was able to diminish their expression. Moreover, double knockdown of Bcl-2 and its homologue Bcl-xL could sensibilize **Sgpl1**−/− cells to the cytotoxic effect of doxorubicin. These findings identify SPL as an upstream regulator of the oncoproteins Bcl-2 and Bcl-xL that control the mitochondria cell death pathway. They are consistent with the notion that SIP metabolism is tightly connected to the expression of Bcl-2 members. Indeed, whereas the prosurvival effect of SIP-generating enzyme SK1 has been associated with upregulation of antiapoptotic Bcl-2 proteins (21, 43), its pharmacologic inhibition resulted in cleavage of Bcl-2, a response linked to mitochondria-dependent apoptosis (38).

Bcl-2-family proteins are also capable of regulating macroautophagy by interacting with the autophagy protein Beclin 1 (44). In **Sgpl1**−/− cells, neither the expression of Beclin 1 (data not shown) nor nutrient starvation-induced autophagy were altered. These results suggest that Bcl-2 overexpression does not blunt the autophagic response in **Sgpl1**−/− cells and that autophagy cannot account for the resistance of **Sgpl1**−/− cells to starvation-induced apoptosis.

All antiapoptotic Bcl-2 family members are oncoproteins and the oncogenic potential of Bcl-2 was first illustrated through its transcriptional deregulation in human follicular lymphoma (45). However, although high levels of Bcl-2 or Bcl-xL are often associated with a more aggressive malignant phenotype and mice bearing a modified Bcl-2 transgene develop spontaneous lymphoid tumors, Bcl-2 overexpression on its own is not highly oncogenic but requires synergistic pretumorigenic lesions to increase the likelihood of malignancy (46). The present study provides evidence that combined effects of SPL loss and Bcl-2 overexpression in **Sgpl1**−/− immortalized cells contribute to their ability to form colonies in soft agar and to generate solid tumors when implanted into nude mice. SPL expression may also repress cellular transformation and antagonize the oncogenic effect of the SIP-generating enzyme SK1 (13). However, whether loss of SPL affects the expression of other oncoproteins cannot be excluded. For instance, it has been shown that SPL promotes apoptosis via a p53-dependent pathway (19). The tumor suppressor p53 has been also associated to Bcl-2 because it could inactivate its antiapoptotic functions by promoting its phosphorylation (47). Moreover, p53 is inactivated in many cancers mostly through missense mutations that result in the abrogation of its activity and the acquisition of oncogenic functions (48).

SIP metabolism has also been linked to the oncogenic Ras-mediated transformation because the SIP-generating enzyme SK1 was activated in NIH3T3 cells transfected with an active mutant of Ras, whereas the transforming ability of Ras was inhibited in cells cotransfected with a dominant-negative mutant of SK1 (13). Interestingly, activation of the Ras pathway resulted in the rapid upregulation of Bcl-2 and Bcl-xL (49), and Bcl-2 and Ras mutant cooperated to give rise to malignant disease (50). Future studies are necessary to determine whether SPL loss and Bcl-2 overexpression are causally related to the Ras pathway or the expression of other oncoproteins.

Our data show that reduced SPL activity results in both resistance to apoptosis and oncogenesis, suggesting that SPL could restrict tumor development. The reduced expression of SPL in solid cancers would go along such a tumor suppressor role. Indeed, we observed that **SGPL1** expression is downregulated in human melanoma. This finding is consistent with the diminished SPL expression reported on human colorectal carcinomas (19). A more in-depth analysis of the molecular mechanisms that regulate SPL expression and its effects are required to better understand its role in SIP-mediated oncogenesis.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Acknowledgments**

Received 6/17/09; revised 9/22/09; accepted 9/27/09; published OnlineFirst 11/24/09.

**Grant support:** INSERM and Paul Sabatier University, MENESR fellowship (S. Colin), and Ligue Nationale Contre le Cancer fellowship (B. Kedjouar). Chimeric mice were generated by Lexicon Genetics on a fee basis, which was covered by FWO-Vlaanderen grants G.0405.02 and G.0581.09 (P.F. Van Veldhoven).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Drs. M. Ikeda and A. Kihara (Hokkaido University) for providing the mSPL cDNA and polyclonal antisera against mSPL and Dr. A. Brouchet, S. Carpenter, J.C. Thiers, and the staff of Plateforme Anexplo Toulouse (S. Le Gouidec, A. Desquesnes, and A. Estival) for advice and technical support.
References

Disruption of Sphingosine 1-Phosphate Lyase Confers Resistance to Chemotherapy and Promotes Oncogenesis through Bcl-2/Bcl-xL Upregulation

Sandra Colié, Paul P. Van Veldhoven, Blandine Kedjouar, et al.

Cancer Res  Published OnlineFirst November 24, 2009.

Updated version  Access the most recent version of this article at:
doi:10.1158/0008-5472.CAN-09-2198

Supplementary Material  Access the most recent supplemental material at:
http://cancerres.aacrjournals.org/content/suppl/2009/11/24/0008-5472.CAN-09-2198.DC1

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, use this link
http://cancerres.aacrjournals.org/content/early/2009/11/24/0008-5472.CAN-09-2198.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.