Phosphorylation of Serine Palmitoyltransferase Long Chain-1 (SPTLC1) on Tyrosine 164 Inhibits Its Activity and Promotes Cell Survival**

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Background: Increasing evidence points toward the role of tyrosine phosphorylation in the regulation of ER homeostasis.

Results: BCR-ABL-mediated tyrosine phosphorylation of SPTLC1 at Tyr164 attenuates SPT activity, thus preventing ceramide-dependent cell death.

Conclusion: Tyrosine phosphorylation is critical for regulating SPT activity in the ER.

Significance: This is a novel mechanism of oncogenic protein kinase-dependent control of sphingolipid metabolism.

In BCR-ABL-expressing cells, sphingolipid metabolism is altered. Because the first step of sphingolipid biosynthesis occurs in the endoplasmic reticulum (ER), our objective was to identify ABL targets in the ER. A phosphoproteomic analysis of canine pancreatic ER microsomes identified 49 high scoring phosphotyrosine-containing peptides. These were then categorized in silico and validated in vitro. We demonstrated that the ER-resident protein serine palmitoyltransferase long chain–1 (SPTLC1), which is the first enzyme of sphingolipid biosynthesis, is phosphorylated at Tyr164 by the tyrosine kinase ABL. Inhibition of BCR-ABL using either imatinib or shRNA-mediated silencing led to the activation of SPTLC1 and to increased apoptosis in both K562 and LAMA-84 cells. Finally, we demonstrated that mutation of Tyr164 to Phe in SPTLC1 increased serine palmitoyltransferase activity. The Y164F mutation also promoted the remodeling of cellular sphingolipid content, thereby sensitizing K562 cells to apoptosis. Our observations provide a mechanistic explanation for imatinib-mediated cell death and a novel avenue for therapeutic strategies.

Recent advances in the treatment of chronic myeloid leukemia have typically relied on imatinib to induce apoptosis in BCR-ABL-positive cells (1). Interestingly imatinib-induced cell death was associated with the activation of an ER3 stress response (2) and the production of C18-ceramide (3). These observations were supported by reports showing that cell death activation was secondary to the alteration of sphingolipid levels in BCR-ABL-positive cells (4). Further studies to investigate the mechanisms underlying imatinib toxicity revealed an unexpected cytoprotective role for cABL coupled to the ER stress response (5). These observations led us to investigate how ABL signaling may serve as a cytoplasmic rheostat for lipotoxicity, thereby allowing leukemia cells to adjust the level of prosurvival ceramides and ceramide-induced ER stress and apoptosis.

The ER is a multifunctional organelle that controls important cellular processes, including Ca2+ and lipid homeostasis, secretory protein synthesis and folding, protein trafficking, and apoptosis (6–9). Upon alteration of its homeostasis, the ER triggers an adaptive response referred to as the unfolded protein response (6). ER stress is induced by an increasing number of anti-cancer agents, including imatinib (2, 10, 11). Several studies have shown that imatinib-induced apoptosis is coupled to the unfolded protein response, which, therefore, has been identified as a downstream target of BCR-ABL (12). Moreover, imatinib has been reported to affect ER homeostasis in particular by reducing glucose uptake and ceramide content through the inhibition of BCR-ABL (13).

In an attempt to identify new ABL substrates in the ER that could account for the role of BCR-ABL in ER stress signaling, we developed an integrated strategy of ER phosphopeptide enrichment, mass spectrometry-based analysis, and computational predictions. This approach led to the identification of an unexpected functional pair composed of the BCR-ABL kinase and SPTLC1 (serine palmitoyltransferase long chain-1). Our
study reveals that imatinib prevents tyrosine phosphorylation of SPTLC1 at Tyr164 by inhibition of BCR-ABL, induces the serine palmitoyltransferase (SPT) activity, and is significantly associated with apoptosis initiation. This study identifies SPTLC1 as a potential therapeutic target that could be selectively triggered to alleviate imatinib resistance in BCR-ABL-positive leukemia cells.

MATERIALS AND METHODS

Cells and Reagents—K562 and LAMA-84 cells were cultured in 10% fetal bovine serum/RPMI 1640 medium, 10 mM HEPES, 100 units/ml penicillin, and 50 µg/ml streptomycin in a humidified atmosphere containing 5% (v/v) CO2 at 37 °C. Exponentially growing cells were used in all experiments. Imatinib mesylate (Gleevec®, Novartis, Basel, Switzerland) was dissolved in DMSO at a stock concentration of 250 mM, stored at −20 °C, and subsequently diluted with serum-free RPMI medium prior to use. Down-regulation of ABL expression in K562 cells was obtained by lentiviral expression of shRNA as described previously (14). Transduction efficiency was verified by FACS monitoring of GFP expression.

ER Microsome Phosphoproteome—Dog pancreatic microsomes were prepared as described previously (15). Total membranes were resolved by SDS-PAGE and Coomassie Blue R-250-stained or in vitro phosphorylated as described previously (15) or immunoblotted using an anti-phosphotyrosine antibody (PY-20). For MS analysis, bands were excised from the SDS-polyacrylamide gels, dehydrated in acetonitrile, and washed by two cycles of 10 min each in 100 mM (NH4)2CO3 before the addition of an equal volume of acetonitrile. The destained gel slices were then treated for 30 min with 10 mM diithiothreitol to reduce cysteine residues and for 20 min with 55 mM iodoacetamide to effect alkylation. After an additional round of (NH4)2CO3 and acetonitrile washes, the gel slices were excised with acetonitrile at 37 °C and then incubated with trypsin (6 ng/µl in 50 mM (NH4)2CO3) for 5 h at 37 °C. The resulting peptides were extracted in 1% formic acid, 2% acetonitrile followed by two additional extractions with acetonitrile. Purified peptides were then analyzed by tandem mass spectrometry. The MS/MS data peak list (MassLynx; MicroMass) was submitted to Mascot (MatrixScience) for database search analysis against the NCBI mammalian non-redundant database.

Database Search, Data Filtering, and Site Localization—Data were analyzed for homologies using BLAST and Blink as well as NCBI Gene Ontology programs. Potential transmembrane domains were identified using the TMHMM Server version 2.0, and molecular masses were computed using the Compute pi/Mw tool at the ExPaSy SIB Bioinformatics Research Portal. In an attempt to predict general or kinase-specific phosphorylation sites, phosphopeptide sets were submitted to the NetPhos 2.0 server (16). Further database searches within PhosphoBase 6.0 (17) were also carried out to confirm the phosphorylation of identified peptides and the exact position of known phosphorylation motifs. Scansite 2.0 (18) was used to search for potential Ser/Thr or Tyr kinases by analyzing specific motifs within the phosphorylated peptide. The data set was also analyzed using Networkin (19), a program that predicts in vivo kinase-substrate relationships including STRING context.

Motif Analysis—For prediction of in vivo kinase-substrate relationships, network maps, including STRING context (1067 phosphopeptide sequences) were extracted from the Networkin database (19) and submitted to the Motif-X algorithm (20). The human protein database was used as a background. Only those sites with Ascore values of at least 13 were used. For single phosphorylation motifs, sequences were centered on each phosphorylation site and extended to 13 amino acids (±6 residues). The Motif-X algorithm excluded sites that could not be extended because of their localization to the N or C terminus. In addition, a minimum occurrence of 20 was required to derive a significant consensus sequence. We performed the residue-specific approach on all class I sites using the entire human proteome as a background model.

In Vitro ABL Kinase Assay—AlphaScreen® assays (21) were performed in Optiplate 384-well microplates in a final reaction volume of 25 µl. The biotinylated peptides were serially diluted in kinase buffer (50 mM Tris, pH 7.5, 10 mM MgCl2, 10 mM ATP) and incubated with ABL kinase (50 nM; Enzo Life science) for 2 h at room temperature. Anti-Tyr(P) antibody PT66-coated acceptor beads (0.025 mg/ml per well) were then added to the reaction mixture and incubated for 45 min at room temperature. Following centrifugation for 90 s at maximal speed, the supernatant was discarded, and the beads were suspended in reaction buffer (50 mM HEPES, pH 7.4, 100 mM NaCl, 5 mM MgCl2, 1 mM DTT, 1 mg/ml BSA) containing streptavidin donor beads at a final concentration of 0.025 mg/ml. Laser excitation of the beads was carried out at 680 nm, and after 1 h of incubation, signal intensities were read at 520–620 nm using an Envision reader (PerkinElmer Life Sciences). ABL substrate peptide sequences used were as follows: EAIYAAPFKKK (ABLTIDE) (22); CYP2E1, DRQEMPYMDAVVH; CYP2A1, ILEEAGYLIKTQ; SPTLC1, TEEAIYYSFGST; PDIA3, RGF-PTIFYSPANK; CACGN5, TKDAETFYNYKG; INSR, YASS-NPEYLSASDV. SPTLC1 peptide sequences were as follows: SPTLC1V1N4T, Biotin-TEEAIYYSFGST; SPTLC1Y164F, Biotin-TEEAIYYSFGST; SPTLC1Y166D, Biotin-TEEAIYYSFGST.

Quantitative RT-PCR—mRNA was extracted with TRIzol reagent (Invitrogen) and transcribed to cDNA using oligo(dT) primer and Maxima® reverse transcriptase (Thermo Scientific) according to the manufacturer’s instructions. The following specific primers (0.2 µM) for the three subunits of SPT were used: SPTLC1fw, 5′-GCCAGGGGATACCTGCTTCTTCA-3′; SPTLC1rev, 5′-TTTGTCGCCACTTCTTCTCTC-3′; SPTLC2fw, 5′-CCTGTCACGGCATCTACCA-3′; SPTLC2rev, 5′-TTGCGCAACAGACCTC-3′; SPTLC3fw, 5′-TATTCCCAGCACAAAGACT-3′; SPTLC3rev, 5′-TGTGTGACCTCTAG-GACCCAGA-3′. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the reference gene for quantification. StepOne Plus™ PCR was performed with a DNA SYBR Green kit according to the manufacturer’s instructions (Roche Applied Science). Amplification was carried out for each SPT subunit: 40 cycles, each consisting of 15 s at 95 °C, 30 s at 60 °C, and 20 s at 72 °C.

Immunoprecipitation and Immunoblot—To assess the physical interaction between ABL and SPTLC1, K562 cells (3×105)
were lysed with radioimmune precipitation assay buffer (50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS), supplemented with Complete™ protease inhibitor (Roche Applied Science) and 0.1 μM sodium orthovanadate. Pre cleared lysates (1 mg of total protein) were incubated with 2 μg of rabbit anti-SPTLC1 IgG or an equivalent amount of normal rabbit IgG. Immune complexes were captured with Protein G-Sepharose beads for 45 min at 4 °C, and the immune complex-containing beads were washed extensively. The samples were then subjected to SDS-PAGE followed by immunoblot analysis. Bands on the blot were visualized using Clean-Blot IP Detection Reagent (HRP) (Pierce).

**Site-directed Mutagenesis**—The human cDNA encoding SPTLC1 (gift from Dr. T. Hornemann, Zurich, Switzerland) was cloned into pcDNA3.1. Site-directed mutagenesis was performed using the QuikChange XL mutagenesis kit (Stratagene) according to the manufacturer’s recommendations. Oligonucleotide primers used were as follows: SPTLC1WTf, 5′-TGAAT-CCACATGGCAGCGCACGGACGCTGTTTCCC-3′; SPTLC1WTrev, 5′-TGGTGGCAAATCCATATGATTCTATACCATAG-3′; SPTLC1Y164Efw, 5′-GACAGAAGAAGCCATTA-GCTTCTTCTGTC-3′; SPTLC1Y164Erev, 5′-GGCAAATCCATATGATTCTATACCATAG-3′; SPTLC1Y166Ffw, 5′-TGGTGGCAAATCCATATGATTCTATACCATAG-3′; SPTLC1Y166Frev, 5′-GACAGAAGAAGCCATTA-GCTTCTTCTGTC-3′; SPTLC1Y164Ffw, 5′-GACAGAAGAAGCCATTA-GCTTCTTCTGTC-3′; SPTLC1Y164Frev, 5′-GGCAAATCCATATGATTCTATACCATAG-3′; SPTLC1Y166Ffw, 5′-TGGTGGCAAATCCATATGATTCTATACCATAG-3′; SPTLC1Y166Frev, 5′-GACAGAAGAAGCCATTA-GCTTCTTCTGTC-3′; SPTLC1Y164Efw, 5′-GACAGAAGAAGCCATTA-GCTTCTTCTGTC-3′. The resulting plasmids were verified by sequencing.

**Transient Transfection**—cDNAs encoding full-length wild type (WT), phosphorylation-defective (Y164F and Y166F), and phosphomimic (Y164E) SPTLC1 were subcloned into pcDNA3 containing the V5 tag. K562 cells (10⁶ cells/condition) were transiently transfected with WT or mutant SPTLC1 cDNA containing the V5 tag. K562 cells (10⁶ cells/condition) were transiently transfected with WT or mutant SPTLC1 cDNA using the Amaxa Nucleofector Device (program T-16) with the cell line-specific Nucleofector kit V (Amaxa GmbH, Germany).

**SPT Activity**—Assays were performed according to Rütti et al. (23) with slight modifications. Briefly, ~300 μg of total K562 cell extract was incubated for 30 min at 37 °C in a 100-μl final reaction mixture volume containing 50 mM HEPES (pH 8.0), 1 mM EDTA, 0.5 mM MnCl₂, 0.5 mM L-serine, 0.1% sucrose monolaurate, 0.05 mM palmitoyl-CoA, 50 μM pyridoxal 5′-phosphate, 1 μCi of [1-U-3H]serine (PerkinElmer Life Sciences). Control samples included myriocin (40 μM) to specifically inhibit SPT activity. The reaction was stopped by adding 400 μl of 0.5 x N H₄OH, followed by the addition of 2.25 ml of chloroform/methanol (1:2). Long chain bases were extracted by adding 0.75 ml of N H₄OH and 0.75 ml of chloroform, vortexing, and centrifuging briefly. The upper aqueous layer was aspirated, and the lower organic layer was washed twice with 2 ml of 0.1 M KCl. The organic phase was then removed and counted for radioactivity.

**Ceramide Mass Measurement**—Lipids were extracted, and ceramide mass was determined as reported previously (24). Radioactive ceramide 1-phosphate was isolated by TLC using chloroform/acetone/methanol/acetic acid/water (50:20:15:10:5, v/v/v/v/v) and then scraped before counting radioactivity by liquid scintillation.

**Sphingolipid Treatment**—Ceramides were obtained from Avanti Polar Lipids. Stock solutions of ceramides were prepared in ethanol at a concentration of 50 mM (25) and stored at 4 °C. Working solutions of ceramides were prepared as 1:1 complexes with 1 mM bovine serum albumin (BSA) in phosphate-buffered saline (PBS) and stored at 4 °C for up to 3 weeks. Prior to each experiment, ceramides were incubated at 37 °C for 8 h and sonicated for 2 h at room temperature to ensure the formation of ceramide-BSA complexes.

**Indirect Immunofluorescence Analyses**—Twenty-four hours post-transfection, K562 cells were cytopun and fixed with 4% PFA at room temperature for 1 h, permeabilized with 0.1% Triton X-100 on ice for 5 min, and blocked with 3% BSA in immunofluorescence buffer (0.15 M NaCl, 2 mM EGTA, 1 mM MgCl₂, and 10 mM PIPES-Na, pH 7.2) for 30 min at room temperature. The cells were then incubated for 1 h with primary antibody (mouse anti-SPTLC1 (1:200), rabbit anti-calnexin (1:500), or mouse anti-V5 (1:100)) followed by secondary antibody (Alexa-488-labeled anti-mouse IgG or Alexa-568-labeled anti-rabbit IgG (1:250), Invitrogen) for 1 h. Nuclei were stained for 15 min using Hoechst 33342 (Invitrogen). The coverslips were then mounted onto microscope slides and observed with a Leica TCS SP5 confocal microscope.

**Apoptosis Assay**—Flow cytometry-based analysis of cell apoptosis was performed following staining of the cells with annexin V-FITC and propidium iodide using the annexin V-FITC kit (Beckman Coulter). The extent of apoptosis was quantified as the percentage of annexin V-positive cells. The extent of imatinib-induced apoptosis was assessed using the formula, percentage of specific apoptosis = (test − control) / 100/(100 − control). Cell death was assessed using annexin V-fluorescein isothiocyanate-propidium iodide (annexin-V FITC apoptosis kit, Beckman Coulter) according to the manufacturer’s protocol.

**Statistical Analyses**—Data are presented as mean ± S.E. of three separate experiments and compared using one-way analysis of variance followed by Dunnett’s multiple comparison tests. The level of significance was set at p < 0.05. All statistical analyses were performed using GraphPad Prism (version 5) statistical software (GraphPad Software, San Diego, CA).

**RESULTS**

**ER Phosphoproteome Analysis Reveals a High Phosphoryrosine Protein Content**—To identify potential tyrosine kinase substrates in the ER, we used highly purified dog pancreatic rough ER microsomes. The purity of the microsomes was assessed by electron microscopy (Fig. 1A) and immunoblotting using antibodies against the ER marker calnexin, the Golgi marker MG-160, and the mitochondrial marker Tom20 (Fig. 1B). In vitro phosphorylation of the microsomes revealed 21 discrete bands corresponding to ³²P-labeled phosphoproteins (Fig. 1C, lane 1), whereas 22 phosphoryrosine-containing bands were observed in the microsomal fraction by immunoblotting using anti-Tyr(P) antibodies (Fig. 1C, lane 2). These results suggested that both Ser/Thr kinase and tyrosine kinase activities...
were present in the microsomal fraction. To identify the phosphoproteins present in this fraction, microsomal proteins were purified using Zn- or Ga-immobilized metal affinity chromatography. Mass spectrometry analysis of the purified phosphoproteins revealed that 25.2% of the total peptides contained Tyr(P), whereas peptides containing serine/threonine represented 73.4% of the total phosphopeptide population (supplemental Fig. S1, A and B). Phosphopeptide annotation using the Gene Ontology database (version 2008-04) revealed that 32% belonged to cell growth/maintenance and 12% belonged to signaling processes (Fig. 1D).

Tyrosine Kinase/Substrate Pair Predictions Identify ER-resident Candidate Substrates for ABL—Phosphotyrosine-containing peptides identified by MS/MS were first subjected to localization analysis using the Uniprot database and peptides that did not belong to ER-resident proteins (70%) were discarded. This led to the selection of 59 Tyr(P)-containing peptides (supplemental Table S1). These peptides were further analyzed for their sites of phosphorylation by combining the information available in the Phospho-ELM and Phosphobase databases using the NetPhos tool. This analysis revealed 49 phosphopeptides for which data were found in at least one of the above mentioned databases. The sequences of these 49 peptides were then processed using both Scansite and Networkin to predict the potential kinases responsible for their phosphorylation. This led to the characterization of four peptide families. The phosphopeptides were then analyzed using the STRING program to include known functional interactions with known kinases. Because our objective was to characterize ER-resident substrates for ABL, four Tyr(P)-containing peptides were iden-
for the WT and Y166F peptides showed no signal, whereas increased phosphorylation levels were detected for the preferred substrates for phosphorylation by ABL. Mutant peptide Y164F was also assessed by comparing the phosphorylation of SPTLC1-derived peptides using various concentrations of ABL. As expected, increasing concentrations of the WT SPTLC1 peptide led to an increased AlphaScreen signal in the presence of a constant concentration of ABL (50 nM). An increased signal was also observed for the Y166F mutant peptide but not for the Y164F mutant peptide, thereby indicating that ABL-dependent phosphorylation most likely occurred at Tyr\(^{164}\), as predicted from the consensus sequence analysis (supplemental Fig. S1B). These results were confirmed in assays in which the peptide concentration was kept constant and the ABL concentration increased (Fig. 2C) or when both peptide and ABL concentrations were kept constant in a time course experiment (Fig. 2D). These results supported the phosphoproteomics analysis carried out with canine pancreatic rough microsomes and identified as potential ABL substrates (supplemental Fig. S2A), namely SPTLC1 (serine palmitoyltransferase long chain-1), PDIA3 (protein-disulfide isomerase A3), CYP2A1 (cytochrome P450 2A1), and CYP2E1 (cytochrome P450 2E1). To extend our \textit{in silico} data, we extracted the complete list of ABL kinase substrates (1085 phosphopeptides) from the Networkin database and subjected them to analysis using Motif-X (20) to find overrepresented motifs. By matching sequence motifs obtained from this analysis, a minimal ABL kinase phosphorylation sequence was proposed (supplemental Fig. S2B).

\textit{In Vitro} ABL-mediated Tyrosine Phosphorylation of SPTLC1—To confirm the validity of the potential ABL substrates identified in the proteomics analysis, we developed a novel \textit{in vitro} ABL kinase assay using the AlphaScreen\textsuperscript\textregistered technology (21) (see supplemental Fig. S3A and “Materials and Methods”). We tested ABL-mediated tyrosine phosphorylation of the SPTLC1-, PDIA3-, CYP2A1-, or CYP2E1-derived peptides (supplemental Fig. S3B) that contained the putative phosphorylation sites identified in the proteomics analysis. We used the ABL substrate ABLTIDE and a peptide derived from the insulin receptor (INSR) as positive controls and a peptide derived from calcium channel, voltage-dependent, \(\gamma\) subunit 5 (CACNG5) as a negative control (see “Materials and Methods”). As expected, the ABLTIDE substrate showed maximal ABL-mediated tyrosine phosphorylation at 1 \(\mu\text{M}\), whereas CACNG5 did not reveal any detectable phosphorylation (Fig. 2A, \textit{closed rectangles} and \textit{dashed line}, respectively). The positive control (INSR) showed marked tyrosine phosphorylation when used at a concentration of 1 \(\mu\text{M}\) but to a lower intensity than that observed for the ABLTIDE (Fig. 2A, \textit{closed rectangles}). Among the four predicted peptides tested, three did not display any ABL-mediated tyrosine phosphorylation (Fig. 2A, PDIA3, CYP2A1, and CYP2E1, \textit{open symbols}). In contrast, the SPTLC1-derived peptide displayed tyrosine phosphorylation with a maximum signal obtained at 10 nM (Fig. 2A, \textit{closed circles}) and was therefore an \textit{in vitro} substrate for the ABL kinase.

Based on the sequence of the SPTLC1 peptide used in our assay, there was a potential ambiguity as to which tyrosine residue was phosphorylated by ABL. To clarify this, we used SPTLC1 mutant peptides in the AlphaScreen\textsuperscript\textregistered assay (Fig. 2B). As expected, increasing concentrations of the WT SPTLC1 peptide led to an increased AlphaScreen\textsuperscript\textregistered signal in the presence of a constant concentration of ABL (50 nM). An increased signal was also observed for the Y166F mutant peptide but not for the Y164F mutant peptide, thereby indicating that ABL-dependent phosphorylation most likely occurred at Tyr\(^{164}\), as predicted from the consensus sequence analysis (supplemental Fig. S1B). These results were confirmed in assays in which the peptide concentration was kept constant and the ABL concentration increased (Fig. 2C) or when both peptide and ABL concentrations were kept constant in a time course experiment (Fig. 2D). These results supported the phosphoproteomics analysis carried out with canine pancreatic rough microsomes and identi-
fied a novel kinase/substrate pair of potential physiological relevance. Therefore, we next investigated the functional relationship between SPTLC1 and the ABL kinase in a physiologically relevant model.

**ABL-mediated Phosphorylation of SPTLC1 Affects Its ER Localization**

We first examined whether the ABL kinase inhibitor imatinib might inhibit SPTLC1 peptide phosphorylation in the *in vitro* ABL kinase assay (Fig. 3A), imatinib was effective inhibitor for both the WT peptide (closed squares) and the Y166F mutant peptide (closed circles) with an IC₅₀ of ~1 μM, further confirming that ABL-mediated phosphorylation of SPTLC1 was at Tyr164.

We next further tested whether imatinib affected SPTLC1 tyrosine phosphorylation in chronic myeloid leukemia cell lines. K562 and LAMA-84 cells were treated with increasing concentrations of imatinib (0.5–10 μM) for 2 h. The cells were then lysed, and the precleared lysates were immunoprecipitated with anti-SPTLC1 antibody. Immunoblot analysis of the anti-SPTLC1 immune complexes using anti-SPTLC1, anti-ABL, or anti-Tyr(P) antibodies (Fig. 3B) revealed that SPTLC1 co-immunoprecipitated with ABL, and that SPTLC1 was tyrosine-phosphorylated under basal conditions. Moreover, SPTLC1 tyrosine phosphorylation was abrogated in the presence of increasing concentrations of imatinib. These results indicated that SPTLC1 is phosphorylated by an imatinib-sensitive kinase both in K562 and LAMA-84 cells (Fig. 3B).

Remarkably, in imatinib-resistant LAMA-84 cells (LAMAr), SPTLC1 phosphorylation still occurred in the presence of imatinib although to a lesser extent than in the absence of imatinib. (Fig. 3B). Moreover, SPTLC1 that was dephosphorylated by treatment with alkaline phosphatase (Fig. 3C, lane 1) could be *de novo* phosphorylated upon incubation with ABL and ATP (Fig. 3C, lane 3). These results strongly suggested an active endogenous tyrosine phosphorylation of SPTLC1.

To assess whether BCR-ABL was the kinase responsible for tyrosine phosphorylation of SPTLC1 *in vivo*, ABL was silenced in K562 cells using shRNA-mediated RNA silencing. Immunoblot analysis revealed that knockdown of ABL decreased BCR-
ABL expression (as well as ABL; not shown) in K562 cells (Fig. 3D, top), and also decreased the level of SPTLC1 tyrosine phosphorylation observed in the anti-SPTLC1 immunoprecipitate of ABL-silenced cells (Fig. 3D, middle). To verify that the tyrosine-phosphorylated protein observed in the anti-SPTLC1 immunoprecipitates was indeed SPTLC1, siRNA-mediated silencing of SPTLC1 was performed in K562 cells for 24 and 48 h followed by immunoprecipitation of the cell lysate using anti-SPTLC1 antibodies. Immunoblot analysis revealed reduced levels of SPTLC1 in the immune complexes from cells treated with SPTLC1 siRNA as compared with control siRNA, and this correlated with a decreased Tyr(P) signal (Fig. 3E). These results demonstrated that ABL and/or BCR-ABL were able to phosphorylate SPTLC1 at Tyr164, but the functional significance of this phosphorylation remained unclear.

We next evaluated whether inhibition of the BCR-ABL kinase in K562 cells affected SPTLC1 and SPTLC2 expression. Imatinib treatment of the cells in either a time- or dose-dependent manner had little effect on the expression levels of SPTLC1 (Fig. 4A and B) and SPTLC2 (Fig. 4C and D). SPTLC3 expression could not be detected in K562 cells (supplemental Fig. S4A). Interestingly, the expression of SPTLC1 was much lower in imatinib-resistant K562 cells (K562R) than in imatinib-sensitive cells (K562S), thereby suggesting that a functional relationship exists between BCR-ABL and SPTLC1.

Because imatinib did not affect SPTLC1 expression, we next evaluated the effect of ABL-mediated tyrosine phosphorylation of SPTLC1 on its subcellular localization in K562 cells. K562 cells were treated with 5 μM imatinib for 3 or 12 h and stained with antibodies against SPTLC1 or calnexin (as an ER marker) or co-stained with antibodies to SPTCL1 and either anti-VCP/P97 or giantin (as a Golgi marker). In the absence of imatinib treatment, similar staining patterns were observed in the cells using anti-SPTLC1 (Fig. 5A, No treatment), anti-calnexin (Fig. 5A, inset, No treatment) and anti-PDI (data not shown) antibodies, thereby indicating that SPTLC1 localized in the ER under basal conditions. Interestingly, SPTLC1 translocated to the Golgi complex following a 3-h imatinib treatment (Fig. 5A, + imatinib) as assessed by co-staining with either anti-VCP/P97 or anti-giantin antibodies. In addition, imatinib induced the translocation of SPTLC1 to the Golgi complex in imatinib-sensitive LAMA-84 cells (LAMA84s), but this phenomenon was significantly reduced (p < 0.001) in imatinib-resistant (LAMA84r) cells (Fig. 5B and C). This suggested that translocation of SPTLC1 to the Golgi complex is dependent on inhibition of ABL kinase activity.

Y164F Mutation Affects SPTLC1 Subcellular Localization, Induction of Apoptosis, and Cell Sensitivity to Imatinib—To further validate the hypothesis that ABL-mediated tyrosine phosphorylation of SPTLC1 could affect its subcellular localization, Tyr164 in SPTLC1 was mutated to either the phospho-mimic amino acid glutamic acid (Y164E) or the non-phosphorylable amino acid phenylalanine (Y164F). WT SPTLC1 or its variants contained the V5 tag at their C terminus. We first verified that the three recombinant proteins were expressed ectopically in K562 cells by transfecting these cells with SPTLC1 siRNA as compared with control siRNA, and this correlated with a decreased Tyr(P) signal (Fig. 3E). These results demonstrated that ABL and/or BCR-ABL were able to phosphorylate SPTLC1 at Tyr164, but the functional significance of this phosphorylation remained unclear.

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Y164F Mutation Affects SPTLC1 Subcellular Localization, Induction of Apoptosis, and Cell Sensitivity to Imatinib—To further validate the hypothesis that ABL-mediated tyrosine phosphorylation of SPTLC1 could affect its subcellular localization, Tyr164 in SPTLC1 was mutated to either the phospho-mimic amino acid glutamic acid (Y164E) or the non-phosphorylatable amino acid phenylalanine (Y164F). WT SPTLC1 or its variants contained the V5 tag at their C terminus. We first verified that the three recombinant proteins were expressed ectopically in K562 cells by transfecting these cells with an empty vector or the same vector containing the WT or variant SPTLC1 cDNAs. Twenty-four hours post-transfection, the cells were lysed and immunoprecipitated using anti-SPTLC1 antibodies followed by immunoblotting using anti-V5, anti-SPTLC1, or anti-Tyr(P) antibodies (Fig. 6A). All of the recombinant forms of SPTLC1 were expressed in K562 cells with similar expression levels. Furthermore, a decrease in SPTLC1 tyrosine phosphorylation was only observed in lysates from
Interestingly, overexpression of the SPTLC1 mutants Y164E and Y164F increased the expression of SPTLC2 (supplemental Fig. S4B), most likely as an adaptive mechanism to maintain active SPT subunit stoichiometry.

To investigate the effects of these mutations on SPTLC1 subcellular localization, each protein was transiently expressed in K562 cells and followed by 5 μM imatinib treatment for 24 h. Cells were then stained with anti-V5 and giantin antibodies or anti-V5 and calnexin antibodies. In the presence of imatinib, SPTLC1 WT and Y164E localized to both the Golgi complex and to the ER (Fig. 6B, left and middle panels). In contrast, the SPTLC1Y164F mutant localized exclusively to the Golgi complex (Fig. 6B, right panels). We next examined whether ABL-mediated phosphorylation of SPTLC1 affected imatinib-induced cell death in K562 cells. Flow cytometry analysis of non-transfected K562 cells stained with annexin V and propidium iodide showed that apoptosis was significantly induced (24%, p < 0.001) upon imatinib treatment (5 μM, 24 h) (Fig. 6C).

Next, K562 cells were transfected with WT or mutant plasmids for 24 h and lysed, and SPT activity was assayed as described previously (23). The highest SPT activity was found with the plasmid SPTLC1Y164F (8.5 ± 0.45 pmol/min/mg of proteins) compared with empty vector (3.8 ± 0.05 pmol/min/mg of proteins), SPTLC1Y164E (3.49 ± 0.35 pmol/min/mg of proteins), or SPTLC1WT (5.27 ± 0.25 pmol/min/mg of proteins) (Fig. 7C). No signal was detected in the presence of myriocin (Myr), an inhibitor of SPT activity (Fig. 7C). We then investigated if transfected K562 cells with SPTLC1Y164F presented an increased ability to generate normal or altered cer-

**FIGURE 5. Inhibition of SPTLC1 tyrosine phosphorylation upon imatinib treatment leads to its translocation to the Golgi apparatus.** A, immunofluorescence analysis of K562 cells before (left, No treatment) or after (middle and right) imatinib treatment. Cytopsins of the untreated and imatinib-treated K562 cells were immunostained with either anti-SPTLC1 or anti-calnexin (CNX, inset) antibodies. Double immunostaining of cytopsins from imatinib-treated K562 cells was performed using anti-SPTLC1 and anti-VCP antibodies or anti-SPTLC1 and anti-giantin antibodies, as indicated. Nuclei were counterstained with Hoechst 33258. Bar, 25 μm. B, immunofluorescence analysis of LAMA-84 imatinib-resistant (r) or -sensitive cells (s) before (left panels) or after (right panels) imatinib treatment. C, quantification of giantin and SPTLC1 co-localization using ImageJ. The average pixel intensity was determined and statistically analyzed. GC, Golgi complex. Error bars, S.E.

K562Y164F cells (Fig. 6A). Interestingly, overexpression of the SPTLC1 mutants Y164E and Y164F increased the expression of SPTLC2 (supplemental Fig. S4B), most likely as an adaptive mechanism to maintain active SPT subunit stoichiometry.

To investigate the effects of these mutations on SPTLC1 subcellular localization, each protein was transiently expressed in K562 cells and followed by 5 μM imatinib treatment for 24 h. Cells were then stained with anti-V5 and giantin antibodies or anti-V5 and calnexin antibodies. In the presence of imatinib, SPTLC1 WT and Y164E localized to both the Golgi complex and to the ER (Fig. 6B, left and middle panels). In contrast, the SPTLC1Y164F mutant localized exclusively to the Golgi complex (Fig. 6B, right panels). We next examined whether ABL-mediated phosphorylation of SPTLC1 affected imatinib-induced cell death in K562 cells. Flow cytometry analysis of non-transfected K562 cells stained with annexin V and propidium iodide showed that apoptosis was significantly induced (24%, p < 0.001) upon imatinib treatment (5 μM, 24 h) (Fig. 6C). We then investigated whether ABL-mediated SPTLC1 phosphorylation affected the induction of apoptosis in K562 cells transfected with SPTLC1 mutants under basal conditions or upon imatinib treatment. Overexpression of SPTLC1Y164F led to a significant induction of apoptosis in K562 cells under basal conditions when compared with SPTLC1WT (p = 0.0011) or SPTLC1Y164E (p = 0.0057) (Fig. 6D, closed histograms). This indicated that overexpression of SPTLC1Y164F was sufficient to induce apoptosis in K562 cells independently of ABL signaling. Imatinib treatment only induced apoptosis in ~30% of SPTLC1 WT- and Y164E-expressing cells (Fig. 6D, open histograms). In contrast, imatinib triggered apoptosis in more than 70% of SPTLC1 Y164F-expressing cells (p = 0.0049) (Fig. 6D, open histogram). These results highlight the significant contribution of SPTLC1 Tyr164 phosphorylation inhibition in imatinib-mediated K562 cell death.

**SPTLC1 Phosphorylation Status on Tyr164 Modifies SPT Activity—**SPTLC1 is an ER transmembrane subunit of the SPT complex that is responsible for the first step in sphingolipid biosynthesis predominantly composed of 18-carbon chain length sphingoid bases in most mammals (26). Following imatinib treatment of K562 cells, SPT activity increased in a time- and dose-dependent manner (Fig. 7A and B, respectively). Next, K562 cells were transfected with WT or mutant plasmids for 24 h and lysed, and SPT activity was assayed as described previously (23). The highest SPT activity was found with the plasmid SPTLC1Y164F (8.5 ± 0.45 pmol/min/mg of proteins) compared with empty vector (3.8 ± 0.05 pmol/min/mg of proteins), SPTLC1Y164E (3.49 ± 0.35 pmol/min/mg of proteins), or SPTLC1WT (5.27 ± 0.25 pmol/min/mg of proteins) (Fig. 7C). No signal was detected in the presence of myriocin (Myr), an inhibitor of SPT activity (Fig. 7C). We then investigated if transfected K562 cells with SPTLC1Y164F presented an increased ability to generate normal or altered cer-
amide content. The ceramide content was evaluated in cells transfected with the indicated constructs 24 h after transfection. Transfected K562 cells synthesized significantly more ceramides than mock-transfected cells (368 ± 49 nmol/mg protein). A significant difference in ceramide content between SPTLC1Y164F and SPTLC1Y164E was also found (p < 0.05) (Fig. 7D).

SPTLC1 Translocation to the Golgi Complex Is Associated with Ceramide Concentration in K562 Cells—In the present study, we have shown that ABL-mediated phosphorylation of SPTLC1 on Tyr164 decreased SPTLC1 activity, whereas imatinib treatment affected the localization of SPTLC1 to the Golgi complex. These observations seemed counterintuitive because ceramides are synthesized in the ER and then transported to the Golgi complex. Therefore, we hypothesized that increased concentrations of sphingoid bases/ceramides in the ER might lead to the relocalization of the SPT complex as either a negative feedback mechanism or as an alteration of ER-to-Golgi trafficking. To test this, K562 cells were exposed to various sphingolipid analogs (C2, C6, C16, and C18) that had been preincubated with 2 μM BSA for 1 h. The localization of SPTLC1 was then determined using indirect immunofluorescence microscopy. When K562 cells were exposed to the C16 and C18 analogs, SPTLC1 co-localized with giantin to the Golgi complex (Fig. 8A), which coincided with attenuated staining in the ER (Fig. 8B). This phenomenon was not observed in cells treated with the shorter analogs C2 and C6. Notably when K562 cells were exposed to the ceramide analogs for 24 h, Hoechst staining revealed the presence of pycnotic nuclei (data not shown). These data confirm previous reports that cellular increases in long chain ceramides perturb ER-to-Golgi trafficking (27) and, in our experiments, appeared to be sufficient to induce the translocation of SPTLC1 to the Golgi complex. Taken together, our data support a model whereby ABL kinase inhibition mediates an increase in SPTLC1 activity, resulting in SPTLC1 translocation to the Golgi complex.

DISCUSSION

In the present study, we have characterized a functional interaction between BCR-ABL signaling and sphingolipid biosynthesis pathways. This occurred through the specific ABL-mediated phosphorylation of SPTLC1 on Tyr164, leading to the attenuation of its activity.

Our initial goal was to identify tyrosine-phosphorylated proteins in the ER using canine rough ER microsomes as a source of highly pure mammalian rough ER. MS analysis of the microsomes identified 222 high confidence phosphopeptides, of which 49 contained phosphotyrosine residues. Among these, our analysis revealed that the best ABL in vitro substrate was a peptide belonging to the SPTLC1 protein, which is a subunit of the SPT

FIGURE 6. Phosphorylation of SPTLC1 on Tyr164 regulates its subcellular localization and affects KS62 cell imatinib sensitivity. A, lysates from SPTLC1wt (WT)-, SPTLC1Y164F (Y164F)-, SPTLC1Y166F (Y166F)-, or empty pCDNA3.1 (EV)-expressing KS62 cells were immunoprecipitated (Ip) using SPTLC1 antibody 24 h post-transfection and blotted using anti-V5, anti-SPTLC1, or anti-phosphotyrosine antibodies. Ib, immunoblot. B, KS62 cells transfected with WT or mutant SPTLC1 were exposed to 5 μM imatinib treatment or vehicle. The cells were then cytopsin and fixed in 4% PFA followed by immunostaining with the indicated antibodies as described under “Materials and Methods.” CNX, calnexin. C, imatinib-treated (IM+) or -untreated (IM−) KS62 cells were stained with annexin V-FITC/propidium iodide and analyzed by flow cytometry according to the manufacturer’s instructions (BD Biosciences). D, KS62 cells transfected with an empty vector (EV) or SPTLC1 WT, Y164E (YE), or Y164E (YF) cDNAs were incubated in the absence (IM−) or presence (IM+) of 5 μM imatinib. Apoptosis measurements were then carried out as described in C. The results are representative of three separate experiments. Differences were considered as significant if p was <0.05. Error bars, S.E.
Indeed, biochemical and cell-based analysis revealed that ABL phosphorylates SPTLC1 at Tyr164, a site that has not been previously described as phosphorylated (Figs. 1 and 2). Moreover, our data showed that this phosphorylation event contributed to the regulation of SPTLC1 activity and the subsequent sphingolipid cellular content and was also involved in maintaining the balance between cellular prosurvival and pro-apoptotic signals (Fig. 6). Distinct sphingolipid species may differ in their subcellular compartmentalization as a means to accomplish specific signaling roles. Our work also provides new insight into the role of SPT activity in imatinib-mediated cell death. Indeed, our work points to a central role for SPTLC1 in the control of a “sphingolipid rheostat” and strongly suggests that increased SPT activity may play a major role in cell death as a response to a wide spectrum of anti-cancer drugs, such as imatinib. Notably, we observed that increased SPT activity correlated with SPTLC1 tyrosine dephosphorylation, and this preceded imatinib-induced cell death, confirming previous observations that BCR-ABL played a major role in ceramide-induced apoptosis resistance (28).

The increase in SPT activity upon imatinib treatment was regulated in a time-dependent manner in K562 cells. Consistent with this observation, a previous study showed that treatment with imatinib increased the generation of endogenous ceramide (mainly C18) in imatinib-sensitive K562 cells but not in the imatinib-resistant subclones (3). In our model, 2 h of imatinib treatment led to a 3-fold increase in SPT activity in K562 cells compared with untreated cells (Fig. 7B).

Ceramide accumulation upon inhibition of BCR-ABL by imatinib treatment results in part from activation of de novo synthesis pathway via SPTLC1 dephosphorylation and upon inhibition of sphingomyelin synthase 1, as was recently reported (29). Indeed, several enzymes implicated in lipid metabolism seem to be under the control of BCR-ABL tyrosine kinase activity. Accumulation of endogenous ceramide (3) and decrease of both antiapoptotic sphingosine 1-phosphate (4) and diacyl-glycerol (29) are consistent with the inhibition of cell proliferation observed upon treatment with imatinib. Our study provides new insight into the fine tuning regulation of lipid metabolism by the BCR-ABL oncogene. We found that SPT activity is regulated by BCR-ABL tyrosine kinase activity in K562 cells toward modulating bioactive lipids influencing cell fate decisions. This pathway offers interesting targets to modulate many different aspects of leukemogenesis and impact both cell survival and death mechanisms.

Sphingolipid enzymes and metabolites are differentially expressed or generated in cancer, and most chemotherapeutic drugs have been shown to increase the level of ceramides (30). Ceramides activate tyrosine kinase signaling, including SRC (31) and LCK (32), and alter ER-to-Golgi trafficking. Our results could provide a mechanistic explanation for the apoptosis resistance phenotype observed in BCR-ABL-expressing cells through an alteration of the ceramide pathway (3, 4). Moreover, Tamehiro et al. proposed that SPTLC1 phosphorylation might represent a mean to control SPT activity and consequently the de novo synthesis of sphingolipids (33). This could occur for...
instance through the regulation of the SPTLC1/SPTLC2 interaction. Indeed, SPT shares significant structural and mechanistic homology with members of the pyridoxal phosphate-dependent ω-oxoamine synthase subfamily (34, 35). The homology was observed at a conserved motif around the lysine residue that is responsible for formation of a Schiff’s base with the pyridoxal 5′-phosphate present in SPTLC2 but not in SPTLC1. Structural studies also showed that the catalytic site is formed at the interface between the subunits SPTLC1 and SPTLC2 (36); hence, both Cys133 and Val144 of SPTLC1 were predicted to be spatially close to the pyridoxal phosphate binding site of SPTLC2 (supplemental Fig. S2). In this configuration, one could postulate that Tyr164, which is located in close proximity to Cys133 and Val144, might be involved in formation of the catalytic site, and phosphorylation of Tyr164 would presumably contribute to the regulation of the active catalytic site. Consequently, the phosphorylation of SPTLC1 described in the present report could represent an attractive explanation for imatinib-induced cell death via modulation of SPT activity.

In summary, our study reveals an important role for BCR-ABL-mediated SPTLC1 phosphorylation in modulating imatinib-induced cell death in leukemia cells. Because a crucial role for ceramide metabolism in mediating the anti-leukemic effects of various chemotherapeutic agents has been reported previously (37), our data demonstrate that in addition to inactivation of the BCR-ABL pathway, imatinib could reactivate ceramide-mediated toxicity in BCR-ABL-positive cells, thereby providing a potentially efficient second line approach for the development of therapeutics to overcome imatinib resistance (Fig. 9).

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