Essential Requirement for Sphingosine Kinase 2 in a Sphingolipid Apoptosis Pathway Activated by FTY720 Analogues*

Received for publication, September 26, 2006, and in revised form, March 29, 2007 Published, JBC Papers in Press, March 30, 2007, DOI 10.1074/jbc.M609124200

Anthony S. Don1, Carolina Martinez-Lamenca5, William R. Webb9, Richard L. Proia1, Ed Roberts9, and Hugh Rosen1,2

From the Departments of 1Immunology and 5Chemistry, The Scripps Research Institute, La Jolla, California 92037 and the 4NIDDK, National Institutes of Health, Bethesda, Maryland 20892

The clinical immunosuppressant FTY720 is a sphingosine analogue that, once phosphorylated by sphingosine kinase 2 (Sphk2), is an agonist of multiple receptor subtypes for sphingosine 1-phosphate. Short exposures to FTY720 afford long term protection in lymphoproliferative and autoimmune disease models, presumably by inducing apoptosis in subsets of cells essential for pathogenesis. Sphingosine itself is pro-apoptotic, and apoptosis induced with FTY720 or sphingosine is thought to proceed independently of their phosphorylation. Following chemical mutagenesis of Jurkat cells we isolated mutants that are selectively resistant to FTY720 analogue AAL(R), as well as natural sphingolipid bases, including sphingosine. Cells lacking functional Sphk2 were resistant to apoptosis induced with AAL(R), indicating that apoptosis proceeds through AAL(R) phosphorylation. Phosphorylation of AAL(R) was also required for induction of lymphocyte apoptosis in mice, as apoptosis was not induced with the non-phosphorylatable chiral analogue, AAL(S). Apoptosis was induced in the spleen but not the thymus of mice administered 1 mg/kg AAL(R), correlating with levels of AAL(R)-phosphate (AFD(R)) in organ extracts. AFD(R) did not induce apoptosis when added to the cell culture medium, indicating that it induces apoptosis through an intracellular target. NBD-labeled AAL(R) localized to the endoplasmic reticulum, and AAL(R) treatment resulted in elevated cytosolic calcium, Bax redistribution from cytosol to mitochondrial and endoplasmic reticulum membranes, and caspase-independent mitochondrial redistribution from cytosol to mitochondrial and endoplasmic reticulum membranes, and caspase-independent mitochondrial permeabilization in Jurkat cells. We therefore describe an apoptotic pathway triggered by intracellular accumulation of sphingolipid base phosphates and suggest that sphingoid base substrates for Sphk2 acting intracellularly could be useful in the treatment of lymphoproliferative diseases.

The sphingolipids are a class of lipids characterized by a serine head group with one or two fatty acyl tails. The common constituent of all sphingolipids is the long chain base (LCB),3 and the most commonly occurring LCBs in mammals are sphingosine (Fig. 1A) and dihydrosphingosine. Sphingosine may be phosphorylated by sphingosine kinases 1 and 2 (Sphk1 and Sphk2), yielding sphingosine 1-phosphate (S1P), which has mitogenic and pro-survival properties (1, 2). Ceramides are the N-acylated precursors of sphingosine, generated either through the breakdown of the abundant membrane lipid sphingomyelin, or through de novo synthesis of sphingolipids from palmitoyl coenzyme A and serine. Ceramides have been widely implicated in apoptosis, leading to the proposal of a sphingolipid rheostat, with pro-survival S1P at one end and pro-apoptotic ceramide at the other (1). Sphingosine itself is pro-apoptotic when added exogenously to cells, and its levels are elevated in response to a number of pro-apoptotic stimuli, including anti-Fas, tumor necrosis factor, and dexamethasone (3, 4).

S1P is secreted from cells, following phosphorylation of sphingosine on internal membranes, and may act in an autocrine or a paracrine fashion to affect a diverse array of physiological processes, including maturation and survival of the vasculature, trafficking of lymphocytes through blood, lymph, and secondary lymphoid organs, inflammation, and cell transformation (2, 5). These effects of S1P are mediated in large part through the activation of a family of five G-protein-coupled receptors, termed S1P1–S1P5. A number of effects have also been attributed to S1P acting through unidentified intracellular targets (2). Release of caged S1P inside the cell was shown to induce calcium release from endoplasmic reticulum (ER) stores, an effect that was not seen with S1P released on the outside of the cell (6). Using S1P receptor-deficient fibroblasts in combination with inhibition of G-protein signaling, Olivera et al. (7) also found that Sphk1 promotes proliferation and survival independently of the S1P receptors. Furthermore, whereas overexpression of Sphk1 enhances cell proliferation and survival, overexpression of Sphk2 has been shown to inhibit pro-

3 The abbreviations used are: LCB, long chain base; AAL(R), 2-amino-4-(4-hexyloxyphenyl)-2-methylbutanolate; ER, endoplasmic reticulum; LC-MS, liquid chromatography mass spectrometry; MEF, murine embryonic fibroblast; S1P, sphingosine 1-phosphate; Sphk, sphingosine kinase; Sphk1, sphingosine kinase 1; Sphk2, sphingosine kinase 2; EGFP, enhanced green fluorescent protein; FS, frameshift; Ctrl, control; MTI, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NBD, 12-(N-methyl-N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)); SBR, sphingoid base resistant; Z, benzyloxy carbonyl; fmk, fluoromethyl ketone.
liferation and enhance apoptosis in response to a number of pro-apoptotic agents or serum withdrawal (8–11).

The sphingosine analogue FTY720 is a promising novel immunosuppressant that is currently in Phase III clinical trials for the treatment of multiple sclerosis. Phosphorylation of FTY720 by Sphk2 in vivo yields the bioactive derivative, FTY720-phosphate, which inhibits lymphocyte egress from lymph nodes and thymus into the bloodstream (12–16). FTY720-phosphate is a high potency agonist of four of the five S1P receptors: S1P1, S1P3, S1P4, and S1P5, and immunosuppression is the result of this compound’s binding to S1P1 (5, 17). FTY720 showed considerable promise in the prevention of transplant rejection, is effective in a number of autoimmune diseases, and is in clinical trials for the treatment of multiple sclerosis (12).

In addition to its immediate immunosuppressive effects, a short course of FTY720 affords long term protection against the lymphoproliferative and autoimmune disease caused by Fas deficiency in MRL-lpr/lpr mice (18, 19). FTY720 induces apoptosis of human multiple myeloma cells at concentrations considerably lower than those required to induce apoptosis of normal peripheral blood mononuclear cells (20), and inhibits the growth of androgen-independent prostate carcinoma (21), hepatocellular carcinoma (22), and breast cancer (23) xenografts in mice. The efficacy of FTY720 against solid tumors and lymphoproliferative diseases is most likely the result of an induction of apoptosis in the target cells.

The precise molecular basis for the induction of apoptosis with FTY720 remains unknown, although a number of mechanisms have been suggested. Several reports have described induction of the mitochondrial permeability transition and consequent activation of caspases by FTY720, with modulation of these processes by the mitochondrial gatekeeper Bcl-2 family proteins (20, 24, 25). Other reports have described a down-modulation of pro-survival mitogen-activated protein kinase and phosphatidylinositol 3-kinase/Akt pathways, and up-regulation of stress-activated kinases such as p38 (26, 27). Effects on modulation of stress-activated kinases such as p38 (26, 27). Effects on the cytoskeleton and integrin-dependent adhesion have also been described for both FTY720 and sphingosine (23, 28). Protein kinase C is an established target for inhibition by sphingosine (29, 30), whereas a number of protein kinases, including the “sphingosine dependent kinases” are activated by sphingosine but not S1P or ceramide (30, 31). A direct intracellular target for FTY720 has not been identified.

In this study we have used a combination of genetic and chemical biology approaches to investigate the molecular basis for induction of apoptosis with the FTY720 analogue 2-amino-4-(4-heptyloxyphenyl)-2-methylbutanol (AAL(R)). We show that phosphorylation of AAL(R) by Sphk2 is an absolute requirement for its induction of apoptosis in vitro and in vivo, that inactivation of Sphk2 reduces the apoptotic potency of endogenous LCs including sphingosine, and that apoptosis is driven by intracellular LC phosphates, most likely formed at the ER. We therefore describe an apoptotic pathway in mammalian cells, with potential in the treatment of cancer, that is triggered by phosphorylation of long chain bases.

### EXPERIMENTAL PROCEDURES

**Cell Culture and Transfection**—Jurkat and HeLa cells were cultured in RPMI1640 supplemented with 10% fetal bovine serum (HyClone), penicillin/streptomycin, and 2 mM l-glutamine. Cells were transfected with Lipofectamine 2000, using 4 µg of DNA and 10 µl of Lipofectamine to transfect 10⁶ cells. Jurkat cells transfected with the pIRES-EGFP vector were selected with 0.8 mg/ml G418 for 2–3 weeks, after which the cells were sorted for EGFP expression by flow cytometry. Assays were performed with cells sorted for equal EGFP expression. Cell culture reagents were purchased from Invitrogen.

**Mutagenesis**—Jurkat cells were mutagenized at a density of 5 × 10⁶ cells/ml, by treatment with 4 µg/ml ICR191 (Sigma) for 48 h (32). The medium was replaced and the cells were allowed to recover for 5–6 days before the next round of mutagenesis. Following three rounds of mutagenesis, the cells were treated for 48 h with 10 µM AAL(R), then plated into 96-well plates to isolate surviving clonal lines.

**PCR and Vector Construction**—Primers AGCTCTGGTGA-GATCTATTCTATGTTACCGGCGGG (forward) and GCTTGCCGGCGAATTCTCATAGGGCTCTTCGCGG-GTGG (reverse), and TCCCGTTGAAATTCAAGCAGCA-GGACCAGCAGATGAAT (forward) and CGACCGGGATC-CAGGCTTTGCATTTTCGAGCTTCCC (reverse) were used for amplification of SPHK1 and SPHK2, respectively, from human endothelial cDNA. PCR products were cloned into the pCR-TOPO 2.1 vector (Invitrogen) and sequenced, before sub-cloning into the pIRES-EGFP expression vector (Clontech). Internal SPHK2 sequence primers TGTCTTGCTCCGAGGAGGAC-TGCCA and ACGGGTGAGCTAGAGCTGCCT were used for sequencing of SPHK2.

**Detection of Frameshift Mutations in SPHK2**—SPHK2 cDNA was amplified from SBR1 cells and sequenced. Two frameshifting mutations were identified as single base insertions that caused a mixed signal. To confirm that these mutations occurred on opposite alleles of the SBR1 SPHK2 gene, a PCR assay was employed. The following primers were used: 5’ control (Ctrl), CCATGCCCCTGCCCCCA; 5’ frameshifted (FS), CATGGCCCGCCCCCACA; 3’ Ctrl, TCTGTGCCCCTGAGC-GCCCAT; 3’ FS, GTGCCCCTGACGGGCCC. SPHK2 was amplified from cDNA with 36 cycles, using 62°C annealing for the 5’/3’ Ctrl primer pair, and 64°C for the 5’/3’ Ctrl/3’ FS, 5’/3’ FS primer pairs. SPHK2 could be amplified from SBR1 cDNA using only the 5’/3’ FS primer pairs, whereas only the 5’/3’ FS control pair amplified SPHK2 from control Jurkat cDNA.

**Cell Viability/Apoptosis Assays**—For MTT and caspase-3/7 assays, Jurkat cells were seeded at 5 × 10⁵ cells/well into a 96-well plate, and treated for 18 h with compounds. MTT assays were performed with an ATCC assay kit, exactly as described in the product information. Caspase-3/7 assays were performed with a Caspase-Glo 3/7 assay kit (Promega), according to the product information. AAL(R) and AAL(S) (kindly provided by Dr. V. Brinkmann, Novartis, Basel) were dissolved at 20 mg/ml in dimethyl sulfoxide, then diluted in water for addition to cells. Sphingosine, dihydrosphingosine, and phytosphingosine (Avanti Polar Lip-
ids) were dissolved in dimethyl sulfoxide at 2 mM, and delivered in dimethyl sulfoxide, to a final concentration of 0.5% dimethyl sulfoxide. S1P (Biomol) and AFD(R) (V. Brinkmann) were dissolved in methanol at 1 mM, and added to the cells as a complex with 0.1% fatty acid-free bovine serum albumin (Sigma).

Measurement of Apoptosis in Cultured Splenocytes and in Vivo—MEF Cells—Splenocytes were isolated by grinding spleens between frosted glass slides, followed by a single round of red blood cell lysis with 0.17 M NH₄Cl for 5 min on ice. Cells were resuspended in RPMI 10% fetal bovine serum, treated for 18 h with compounds at 37 °C, then incubated on ice with 1 μg/ml propidium iodide and immediately analyzed by flow cytometry. Viability was scored as the proportion of cells excluding propidium iodide, then normalized to 100% for the vehicle-treated cells. Sphk1<sup>−/−</sup> (33) and Sphk2<sup>−/−</sup> (34) mice were on a mixed Sv/C57BL6 background.

AAL(R) and AAL(S) were administered by intraperitoneal injection in 2% dimethyl sulfoxide to 3-month-old male MRL/Mpl-lpr/lpr mice. Spleen, inguinal lymph nodes, and thymus were homogenized and filtered through 40-μm sieves. Spleen was subjected to red blood cell lysis as above. Lymphocytes were resuspended in 0.1 ml of 20 mM Hepes, pH 7.4, 150 mM NaCl, 2.5 mM CaCl₂, and incubated for 15 min with 1:50 annexin V-fluorescein isothiocyanate (BD Pharmingen) and 1 μg/ml propidium iodide, after which the volume was increased to 0.3 ml and the cells were analyzed with an LSRII cytometer (BD Biosciences), and FlowJo software (Treestar).

WT (+/+) and knockout (−/−) MEF cells were derived from day 13.5 embryos of a single Sphk2<sup>+/−</sup> female bred with a Sphk2<sup>−/−</sup> male. Head and liver were removed, and the remaining tissue was digested in 0.25% trypsin/EDTA solution (Invitrogen) on ice for 6 h, then at 37 °C for 30 min, after which the embryonic cells were dissociated by pipetting, and plated in RPMI1640 supplemented with 10% fetal bovine serum. Mice were genotyped as described (34).

Measurement of Lipids by Liquid Chromatography Mass Spectrometry (LC-MS)—Cells were incubated at a density of 2.5 x 10⁶ cells/ml with 5 μM AAL(R) for 4 h or 5 μM sphingosine for 2 h at 37 °C. AFD(R) or S1P formation was determined to be linear with respect to time over this time frame in Jurkat cells. Cells were washed once with phosphate-buffered saline, then extracted into 0.1 ml of ice-cold methanol. Extracts were cleared at 21,800 x g for 15 min and run over a Zorbax SB-C18 column (Agilent) at 0.35 ml/min, loading in 0.1% formic acid, 15% acetonitrile, and increasing to 98% acetonitrile over 15 min. An Agilent 1100 single quadrupole mass spectrometer was used. To extract AAL(R) and AFD(R) from spleen and thymus, the organs were homogenized with a Biospec Products Mini-BeadBeater in 0.5 ml of cold methanol. Extracts were incubated for 20 min at 4 °C on a platform rocker, then cleared at 21,800 x g for 20 min. Spiked controls were used to estimate compound recovery from spleen and thymus.

Sphingosine Kinase Assays—Use of NBD-sphingosine to assay sphingosine kinase activity has been described previously (35). Cells were lysed with a single freeze-thaw in 50 mM Hepes, pH 7.4, 10 mM KCl, 15 mM MgCl₂, 0.1% Triton X-100, 20% glycerol, 2 mM orthovanadate, 2 mM dithiothreitol, 10 mM NaF, 1 mM deoxyxypiridine, and EDTA-free complete protease inhibitor (Roche). Lysates were cleared at 21,800 x g for 15 min. Total sphingosine kinase activity was measured in 50 mM Hepes, pH 7.4, 15 mM MgCl₂, 10 mM KCl, 10% glycerol, 2 mM ATP, 5 mM NaF, and 1 mM deoxyxypiridine, to which was added 10 μM NBD-sphingosine as substrate. Sphk1 activity was measured in 50 mM Hepes, pH 7.4, 15 mM MgCl₂, 0.5% Triton X-100, 10% glycerol, 2 mM ATP; and Sphk2 activity in 50 mM Hepes, pH 7.4, 15 mM MgCl₂, 0.5 mM KCl, 10% glycerol, 2 mM ATP (36). Reactions were started with the addition of 25 μg of Jurkat lystate protein. The 50-μl reactions were extracted with the addition of 50 μl of 1 M potassium phosphate, pH 8.5, followed by 250 μl of chloroform/methanol (2:1), then cleared at 15,000 x g for 1 min. NBD fluorescence was read using 100 μl of upper aqueous phase, combined with 100 μl of dimethylformamide. Reactions containing no enzyme were used for blanks, and fluorescence units were converted to nanomoles of S1P using NBD-S1P standards extracted as described above. NBD-sphingosine and NBD-S1P were from Avanti Polar Lipids. To assay inhibition of Sphk1 in vitro, HEK293 lysates were prepared 48 h after transfection with Sphk1 expression plasmid.

Expression and Purification of Recombinant Sphk2—Sphk2, full-length or truncated, was subcloned into the pMAL-c2E vector (New England Biolabs). Maltose-binding protein-Sphk2 (MBP-Sphk2) fusion proteins were produced from 200 ml of bacterial cultures, as described in the pMAL-c2E product manual. Maltose-binding protein-Sphk2 fusion protein was soluble, and stable when stored at −20 °C in the presence of 50% glycerol. Activity was determined in Sphk2 selective assay buffer (as above), using 2 μg of recombinant protein/30-min reaction.

Synthesis of AAL(R)-NBD—AAL(R)-NBD was synthesized as described (37), with the following modification: the key phenolic intermediate was alkylated with 1-azido-7-iodoheptane (80% yield), followed by quantitative reduction of the azide group to the primary amine, which was then reacted with NBD-Cl. The t-butoxy carbonyl group was cleaved to afford the NBD-labeled (R)-AAL derivative.

Immunofluorescence—HeLa cells, seeded overnight on glass coverslips, were incubated for 1 h with 10 μM NBD-AAL(R). The cells were then washed once and incubated in fresh growth medium for 1 h, with ER Tracker Red (2 μM), Lysotracker Red (75 nM), or Mitotracker Deep Red (50 nM) (all from Molecular Probes) added for the final 30 min. The cells were washed once more, and immediately imaged (in growth medium at room temperature) with an Olympus FV500 confocal microscope.

Western Blotting and Subcellular Fractionation—Cells were treated at a density of 2.5 x 10⁶ cells/ml, then washed with phosphate-buffered saline, and either lysed directly in Laemmli buffer, or resuspended in fractionation buffer: 200 mM mannitol, 68 mM sucrose, 10 mM KCl, 2 mM MgCl₂, 0.5 mM EGTA, 10 mM Hepes, pH 7.4, and complete protease inhibitor mixture (Roche). Cells were broken with 50 passes through a 27-gauge needle, then centrifuged for 10 min at 800 x g to pellet nuclei and unbroken cells, followed by 4,000 and 22,000 x g spins to pellet mitochondrial and microsomal fractions. Proteins were resolved on 4–12% NU-PAGE gels (Invitrogen). Antibodies used included rabbit anti-Bax NT (Upstate), mouse anti-calnexin and mouse anti-TOM20 (BD Biosciences), and rabbit anti-PARP1 (Novus).
Intracellular Ca²⁺ Measurement—Jurkat cells were treated for 8 h with 5 μM AAL(R) or AAL(S), incubated for 30 min with 5 μM Fluo-3 (Molecular Probes) in phosphate-buffered saline, 1% bovine serum albumin, 0.02% Pluronic F127 (Molecular Probes), then washed and incubated for a further 30 min in phosphate-buffered saline, 1% bovine serum albumin at 37 °C, after which the cells were analyzed by flow cytometry. Geometric mean Fluo-3 fluorescence was determined for viable cells only, gated on the basis of light scatter.

RESULTS

Isolation of AAL(R)-resistant Mutants—Jurkat cells were mutagenized with ICR191, then treated with the FTY720 analogue, AAL(R) (Fig. 1B), for 48 h to isolate resistant cells. Nine AAL(R)-resistant cell lines were derived from two distinct mutagenized pools. As we aimed to isolate mutants that were resistant to AAL(R) but not to all apoptosis inducing agents, the mutant cell lines were then tested by MTT assay for resistance to the DNA damaging agent etoposide, and anti-Fas antibody. Five mutants were found to be resistant to AAL(R) but not etoposide or anti-Fas antibody. The viability curves for three such mutants, designated SBR1–3 (sphingoid base resistant), are shown in Fig. 2, A–C.

Induction of Apoptosis with AAL(R) Requires Phosphorylation by Sphk2—These cell lines were assayed for their capacity to phosphorylate AAL(R) in culture, using LC-MS to detect AAL(R) and the phosphorylated derivative AFD(R). Mutant SBR1 was unable to phosphorylate AAL(R) (Fig. 2D). Several publications have reported that FTY720 is phosphorylated efficiently by Sphk2, but not by Sphk1 (15, 16, 38). Lysates of SBR1 cells were deficient in Sphk2 activity, but not Sphk1 activity (Fig. 3A and B). Exogenous expression of Sphk2 restored the capacity for SBR1 to phosphorylate AAL(R) in culture (Fig. 3C), and restored the sensitivity of the SBR1 cell line to AAL(R) (Fig. 3D).

Real time PCR indicated that Sphk2 mRNA was reduced by 33% in the SBR1 cells compared with the parent Jurkat cells.
AAL(R)/Sphingosine Kinase 2 Apoptotic Pathway

This decrease was not sufficient to account for the complete loss of Sphk2 activity. We were unable to detect endogenous Sphk2 by Western blotting of cell lysates. Sequencing of the Sphk2 cDNA from SBR1 cells uncovered two frameshifting mutations: one allele carrying a cytosine insertion 80 nucleotides into the open reading frame, which is expected to result in the synthesis of a non-functional 83-amino acid peptide; the other allele carrying a guanine insertion 80 nucleotides from the 3' end of the open reading frame. This is expected to result in the synthesis of a 715-amino acid protein, in which the final 25 amino acids of the native Sphk2 protein are altered (native human Sphk2 contains 654 amino acids). The last 25 amino acids of Sphk2 are outside (3') of any previously identified conserved domains in the sphingosine kinase family (2). To prove that these residues are important to the function of the protein, we generated truncation mutants, in which the final 25 or 16 amino acids were deleted. Sphk2(full-length), Sphk2(Δ630–654), and Sphk2(Δ639–654) were expressed in Escherichia coli as C-terminal fusions to maltose-binding protein, and purified on amylose resin. Whereas all three proteins were expressed, only the full-length Sphk2 possessed catalytic activity, phosphorylating sphingosine-NBD with $K_m$ 7.6 $\mu M$ and $V_{max}$ 58.5 nmol of S1P-NBD/mg/min.

Phosphorylation Is Required for in Vivo Induction of Apoptosis with AAL(R)—We also tested primary splenocytes derived from Sphk1 or Sphk2 knock-out mice for sensitivity to AAL(R) (Fig. 4A). In agreement with the above results, Sphk2- but not Sphk1-deficient splenocytes were resistant. The chiral analogue to AAL(R), AAL(S) (Fig. 1B), is not a substrate for Sphk2 (38). Brinkmann et al. (38) reported that lymphocyte cell death was induced equally with AAL(R) or AAL(S), suggesting that phosphorylation of AAL(R) is not required for induction of apoptosis with this compound. In contrast to their results, and in further support of our findings with Sphk2-deficient cells, we found that AAL(R) but not AAL(S) induced cell death in both primary splenocytes (Fig. 4B) and Jurkat cells (data not shown).

A single 1 mg/kg dose of AAL(R) administered to MRL/MpJ-lpr/lpr mice significantly increased the proportion of apoptotic lymphocytes in the spleen and lymph nodes, but not in the thymus (Fig. 4C). At this dose, AFD(R) was found in the spleen at levels above those required to induce apoptosis of splenocytes in vitro (Fig. 4D). AFD(R) levels were lower in the thymus, suggesting that the concentration achieved in the thymus was not sufficient to trigger apoptosis. As was seen previously (39), the conversion of AAL(R) to AFD(R) was rapid in vivo, with AAL(R) levels in the spleen and
**AAL(R)/Sphingosine Kinase 2 Apoptotic Pathway**

**TABLE 1**

| Strain    | AAL(R) Mean IC<sub>50</sub> | Sphingosine Mean IC<sub>50</sub> | Dihydrosphingosine Mean IC<sub>50</sub> | Phytosphingosine Mean IC<sub>50</sub> |
|-----------|-----------------------------|----------------------------------|----------------------------------------|----------------------------------------|
| Jurkat    | 2.32 ± 0.39                 | 2.01 ± 0.52                      | 3.53 ± 1.26                            | 3.75 ± 0.71                            |
| SBR1      | 8.72 ± 1.09                 | 3.48 ± 0.42                      | 1.7                                     | 1.4                                    |
| SBR2      | 7.72 ± 0.45                 | 2.88 ± 0.24                      | 1.4                                     | 1.3                                    |
| SBR3      | 7.12 ± 0.3                  | 3.38 ± 0.31                      | 1.7                                     | 1.8                                    |

**FIGURE 5. Phosphorylation of sphingosine by Sphk1 or Sphk2 promotes apoptosis.** A, parent Jurkat (■), or SBR1 (□) cells were treated for 18 h with sphingosine and viability was assessed by MTT assay. B, phosphorylation of sphingosine (5 μM) in culture by parent Jurkat, or SBR1 cells stably expressing Sphk1, Sphk2, or empty pIRE-EGFP vector, as determined by LC-MS. Phosphorylation efficiency is expressed as the molar ratio of S1P:sphingosine in the cell pellet. C, MTT assay was used to determine the viability of SBR1 cells stably expressing Sphk1 (□), Sphk2 (×), or vector only (■), following 18 h sphingosine treatment. D, phosphorylation of AAL(R) in culture, determined by LC-MS. E, MTT assay for viability of SBR1 cells stably expressing Sphk1 (□), Sphk2 (×), or vector only (■), following 18 h AAL(R) treatment. F and G, MTT assay for viability of Sphk2<sup>−/−</sup> (□) or Sphk2<sup>−/−</sup> SFB cells, following 20 h AAL(R) (F) or sphingosine (G) treatment. MTT results are the mean ± S.E. of triplicate treatments, whereas LC-MS results are mean and range of duplicate treatments. All results are representative of two or more experiments. H, total Sphk activity in cell lysates. I, Sphk activity was assayed using lysates of Sphk1-expressing HEK293 cells, in the presence of the indicated concentrations of AFD(R) (□), AAL(R) (●), or AAL(S) (×). Results are mean ± S.E. for triplicate assays.

SBR Mutants Are Resistant to Endogenous Long Chain Bases—We sought to distinguish a mechanism of cell death in which AAL(R) inhibited an enzyme of normal sphingolipid metabolism from a mechanism of apoptosis shared with naturally occurring LCBs. Welsch et al. (40) showed that *Saccharomyces cerevisiae* cells respond similarly to FTY720 and phytosphingosine treatment, using single gene deletants and transcriptional profiling. Phytosphingosine is phosphorylated by Sphk2 but not Sphk1, whereas sphingosine and dihydrosphingosine are substrates for both Sphk1 and Sphk2 (2, 36). We tested the resistance of the SBR mutants to sphingosine, dihydrosphingosine, and phytosphingosine (Table 1). All three mutants showed some resistance to natural LCBs, although the resistance to AAL(R) was more pronounced. Sphk2 loss of function mutant SBR1 showed stronger resistance to phytosphingosine than the other two mutants.

The resistance of SBR1 cells to sphingosine (Table 1 and Fig. 5A) correlated with a deficiency in their capacity to phosphorylate sphingosine in culture (Fig. 5B). Exogenous expression of either Sphk1 or Sphk2 in SBR1 cells enhanced cellular sphingosine kinase activity, and restored the capacity for sphingo- sine to induce apoptosis (Fig. 5C). Overexpression of Sphk1 also partially restored AAL(R) phosphorylation by SBR1 cells (Fig. 5D), resulting in a partial restoration of apoptosis in response to AAL(R) (Fig. 5E). Thus, an increase in the intracellular S1P or AFD(R) concentration mediated through either Sphk1 or Sphk2 triggers an apoptotic response.

Cells were treated with compounds for 18 h, and cell viability was determined by MTT assay. An IC<sub>50</sub> (μM) was determined for each of three experiments, as the concentration at which 50% MTT reduction, relative to the vehicle treatment, was measured. IC<sub>50</sub> shown is the mean ± S.D. of three experiments, each with triplicate treatments. -Fold resistance was derived by dividing the mean IC<sub>50</sub> for each mutant by that of the parent Jurkat cells.

Cells were treated with compounds for 18 h, and cell viability was determined by MTT assay. An IC<sub>50</sub> (μM) was determined for each of three experiments, as the concentration at which 50% MTT reduction, relative to the vehicle treatment, was measured. IC<sub>50</sub> shown is the mean ± S.D. of three experiments, each with triplicate treatments. -Fold resistance was derived by dividing the mean IC<sub>50</sub> for each mutant by that of the parent Jurkat cells.

The resistance of SBR1 cells to sphingosine (Table 1 and Fig. 5A) correlated with a deficiency in their capacity to phosphorylate sphingosine in culture (Fig. 5B). Exogenous expression of either Sphk1 or Sphk2 in SBR1 cells enhanced cellular sphingosine kinase activity, and restored the capacity for sphingosine to induce apoptosis (Fig. 5C). Overexpression of Sphk1 also partially restored AAL(R) phosphorylation by SBR1 cells (Fig. 5D), resulting in a partial restoration of apoptosis in response to AAL(R) (Fig. 5E). Thus, an increase in the intracellular S1P or AFD(R) concentration mediated through either Sphk1 or Sphk2 triggers an apoptotic response.

thymus below 0.2 pmol/mg of tissue after only 1 h. AAL(S) at 1 mg/kg did not induce lymphocyte apoptosis in the spleen (Fig. 4E), thus confirming that phosphorylation is required for induction of apoptosis with AAL(R) in vivo.
We also measured the resistance of murine embryonic fibroblast (MEF) cells, derived from Sphk2 knock-out mice, to AAL(R) and sphingosine (Fig. 5, F and G). As expected, the Sphk2−/− cells were resistant to AAL(R), although the extent of resistance was relatively less than was seen with the Sphk2-deficient Jurkat cells and primary splenocytes. These cells were not resistant to sphingosine. The discrepancy between the Jurkat cells and the MEF cells can be explained in terms of their total Sphk activity, and the extent to which Sphk2 contributes to the total Sphk activity in these cells: total Sphk activity was 12-fold higher in MEF cells, compared with Jurkat cells (Fig. 5), and loss of Sphk2 had less of an effect on the total Sphk activity of MEF cells, as Sphk activity was 28-fold higher in the Sphk2−/− MEF cells, when compared with SBR1 cells.

**Apoptosis Is Triggered by Intracellular AFD(R)**—To test whether the apoptosis induced with AAL(R) was mediated through intracellular targets, or through secretion of AFD(R) coupled to activation of plasma membrane S1P receptors, cells were treated for 18 h with AAL(R), AFD(R), or S1P and cell survival assessed (Fig. 6A). Due to their zwitterionic head group, AFD(R) and S1P do not readily cross the plasma membrane. As expected, AFD(R) and S1P did not induce cell death, indicating that these compounds induce apoptosis through an intracellular target. Similarly, AAL(R), but not AFD(R) or S1P, induced cell death in cultured primary splenocytes (Fig. 6B).

FTY720 has previously been shown to inhibit Sphk1 (41). We have found that both AAL(R) and AAL(S) are partial Sphk1 inhibitors, at relatively high micromolar concentrations, whereas AFD(R) does not inhibit the enzyme (Fig. 5f). Together with the observation that Sphk1 overexpression in SBR1 cells enhanced rather than reduced their sensitivity to AAL(R), we conclude that Sphk1 inhibition is not the means through which AAL(R) induces cell death.

**AAL(R) Shows an ER Localization Pattern**—To gain further insight into the mechanism by which AAL(R) induces apoptosis, we synthesized AAL(R) coupled to an NBD fluorescent group (Fig. 1B). AAL(R)-NBD localized to perinuclear reticular membranes, with a varying number of intense fluorescent spots (Fig. 7A). This labeling pattern persisted for at least 4 h after washout of the compound, and longer incubation times resulted in a loss of fluorescence, presumably due to secretion and degradation of the compound. Co-labeling of the cells with ER Tracker Red and Mitotracker Deep Red revealed strong co-localization of AAL(R)-NBD with the ER, but little or no co-localization with the mitochondria (Fig. 7A). The intense fluorescent spots of AAL(R)-NBD co-localized with Lysotracker Red (Fig. 7B). Deacidification of the lysosomes with bafilomycin A eliminated the punctate lysosomal localization of AAL(R)-NBD (Fig. 7C), but did not inhibit the apoptosis induced with AAL(R) in either Jurkat cells (Fig. 7D) or HeLa cells (data not shown). In the presence of bafilomycin A, the AAL(R)-NBD remained essentially in the ER (Fig. 7C).

**Apoptotic Events following AAL(R) Treatment**—Effector caspases (DEV-Dase activity) were activated by AAL(R) treatment in parent Jurkat, but not SBR1 cells (Fig. 8A), and cleavage of the caspase substrate PARP1 was apparent 16 h after treatment of Jurkat cells with 5 μM
AAL(R), but not AAL(S) (Fig. 8, panel i). PARP1 cleavage occurred to a lesser extent in SBR1 cells (Fig. 8B, panel ii). Nagahara et al. (25) reported that caspase activity is not required for mitochondrial permeabilization in response to FTY720. In agreement with their results, we found that inhibition of caspases with Z-VAD-fmk blocked loss of mitochondrial membrane potential (Fig. 8, panel iii). Western blot for cleaved (85 kDa) poly-ADP ribose polymerase 1 in lysates of Jurkat cells treated for the indicated times with 5 μM AAL(R) or AAL(S) (i), or lysates of Jurkat or SBR1 cells treated for 16 h with 0, 2, or 5 μM AAL(R) (ii). C and D, MTT assay on Jurkat cells following 18 h AAL(R) (C) or etoposide (D) treatment, with (C) or without (D) a 30-min pre-treatment with 50 μM Z-VAD-fmk. E, blots for Bax, calnexin (as a marker for ER), and TOM20 (as a marker for mitochondria), at 4,000 × g (mito), 22,000 × g (micro), or cytosolic fractions of Jurkat (i) or SBR1 (ii) cells treated for 16 h with 0, 2, or 5 μM AAL(R). Results are representative of three fractionation experiments. F, cytosolic Ca²⁺ was measured with Fluo-3, following 8 h treatment with 5 μM AAL(S) or AAL(R). Results are mean ± S.E. of triplicate treatments, representative of two experiments.

**DISCUSSION**

It has recently been shown that Sphk2 is essential to lymphopenia induced with FTY720, mediated through agonism of the S1P₁ receptor by FTY720-phosphate (15, 16). Until now, apoptosis in response to FTY720 was thought to occur independently of its phosphorylation (38, 43). We have isolated a set of three Jurkat cell mutants that are resistant to the FTY720 analogue AAL(R), as well as naturally occurring LCBs, and have shown for the first time that phosphorylation of AAL(R) by Sphk2 is essential to its induction of apoptosis. Functional Sphk2 was not required for apoptosis induced with the DNA damaging agent etoposide or through activation of the Fas death receptor, but Jurkat cells lacking functional Sphk2 were more resistant to naturally occurring sphingoid bases, including sphingosine. MEF cells lacking Sphk2 were resistant to AAL(R) but not to sphingosine. This difference between the Jurkat and MEF cells is probably a function of the greater role played by Sphk2 in Jurkat cells, as the MEF cells possessed a high level of Sphk activity even in the absence of Sphk2. The importance of the Sphk2 isoform to cellular Sphk activity in Jurkat cells, a lymphoid cell line, may help to explain the lymphoid selectivity of AAL(R) in vivo.

The membrane impermeable phosphate esters AFD(R) and S1P did not cause a loss of viability when added directly to cultured cells, indicating that apoptosis is mediated through intracellular targets for these compounds. Taken together with recent findings that Sphk2 can act as a pro-apoptotic protein (8–10), and that increased intracellular LCB phosphates kill S. cerevisiae cells (44), our results provide further evidence for a pro-apoptotic pathway activated by intracellular accumulation of LCB phosphates. AAL(R) is a more potent and more specific activator of this pathway than sphingosine, which is most likely the result of three properties of AAL(R): 1) it is efficiently phosphorylated by Sphk2; 2) it is more soluble than sphingosine; and 3) it is not readily incorporated into sphingolipid biosynthetic pathways (16, 45). FTY720 is not a substrate for S1P lyase (46), and could be quantitatively recovered as the phosphate ester when incubated with blood in vitro (14).

AAL(R) was phosphorylated with poor efficiency by Sphk1, but this low rate of phosphorylation was enough to partially restore the apoptotic response in SBR1 cells. The observation that apoptosis can proceed through phosphorylation of AAL(R) or sphingosine by either sphingosine kinase does not contradict the well established paradigm that Sphk1 overexpression enhances proliferation and protects against apoptosis (7, 11).
Under physiological conditions, sphingosine and other sphingolipids are enriched in the plasma membrane, and present at much lower levels in the ER and mitochondria (47). In our system, we are adding exogenous ligand, whose phosphorylation in the ER is quite distinct from the mitogenic phosphorylation of sphingosine catalyzed by Sphk1 at the plasma membrane (11). Enforced localization of Sphk1 to the ER has been shown to promote apoptosis (10).

Whereas effector caspases are activated in response to AAL(R), our results and those of others (25) indicate that loss of mitochondrial function proceeds independently of caspase activation. We show that AAL(R)-NBD co-localizes with ER-tracker and Lysotracker. Lysosomal accumulation of AAL(R)-NBD is most likely the result of its weakly basic nature, as it could be blocked by deacidification of lysosomes with bafilomycin A. Apoptosis induced with AAL(R) was not inhibited by bafilomycin A, suggesting that AAL(R) phosphorylated at the ER by Sphk2 transmits a caspase-independent permeabilization signal to the mitochondria. We show that Bax relocates from the cytosol to mitochondria- and ER-enriched subcellular fractions in response to AAL(R) treatment. It is now well established that the balance between pro-apoptotic and anti-apoptotic Bcl-2 family members at the ER influences the control of intracellular Ca\(^{2+}\) stores and ER-dependent apoptosis (42, 48).

Furthermore, intracellular S1P has been implicated in the release of Ca\(^{2+}\) from intracellular stores (6, 10). We found that AAL(R) treatment elevates the cytosolic Ca\(^{2+}\) concentration, suggesting that excessive intracellular AFD(R) levels cause apoptosis through ER stress and Ca\(^{2+}\) release. Release of calcium from the ER can directly trigger the mitochondrial permeability transition, thus initiating apoptosis independently of caspase activation (48, 49). Finally, we have seen no evidence for the induction of a conventional ER stress/unfolded protein response with AAL(R), after looking into the expression level of several ER stress markers, including GADD153, GRP78, calnexin, and calreticulin.

Maximal lymphopenia is induced with a plasma concentration of 20 \(\mu\)M AFD(R) (0.2 mg/kg AAL(R)) in mice (39), well below the threshold for induction of apoptosis. However, the treatment of cancer xenografts in mice (21–23) and the long term protection seen against lymphoproliferative and autoimmune diseases with FTY720 (12, 18, 19) cannot be explained by a transient and reversible lymphopenia. Lymphocyte apoptosis would contribute to long term protection by reducing the pathogenic lymphocyte burden, thus turning back the disease clock. Rats given a 2–3-week course of FTY720 at 0.1 or 1 mg/kg exhibit a prolonged decrease in the total lymphocyte count, especially pronounced in the peripheral lymphoid organs (13). Apoptosis of circulating T-cells was previously observed in mice treated with 5 mg/kg FTY720 (50). We have consistently observed increased T- and B-cell apoptosis in the peripheral lymphoid organs of mice treated with a single 1 mg/kg dose of AAL(R). If we assume that 1 g of tissue equates to a 1-ml volume, then the concentration of AFD(R) achieved in the spleen between 1 and 8 h after dosing is around 5 \(\mu\)M (Fig. 4D). The AFD(R) concentration in the thymus was below the apoptotic threshold, reflecting the greater vascularity of the spleen compared with the thymus. Therefore apoptosis is more likely to impact on the numbers of mature T-cells, although having less impact on developing T-cells.

FTY720 is currently in Phase III clinical trials for the treatment of multiple sclerosis. Given this, and in light of the efficacy of FTY720 at killing human multiple myeloma cells (20) and inhibiting lymphoproliferative and autoimmune diseases in mice, an understanding of the mechanism by which the compound induces apoptosis is important. The future identification of the mutations that confer resistance to AAL(R) in SBR2 and SBR3 will unveil other key players in the LCB apoptosis pathway, and may afford insights into the normal role for intracellular S1P.

Acknowledgments—We thank Nora Leaf, David Marsolais, Aaron Semena, and Jeremy Verango for expert technical assistance.

REFERENCES

1. Ogretmen, B., and Hannun, Y. A. (2004) Nat. Rev. Cancer 4, 604–616
2. Taha, T. A., Hannun, Y. A., and Obeid, L. M. (2006) J. Biochem. Mol. Biol. 39, 113–131
3. Cuvillier, O. (2002) Biochim. Biophys. Acta 1585, 153–162
4. Lepine, S., Lakatos, B., Courageot, M. P., Le Stunff, H., Sulpice, J. C., and Giraud, F. (2004) J. Immunol. 173, 3783–3790
5. Rosen, H., and Goetzl, E. J. (2005) Nat. Rev. Immunol. 5, 560–570
6. Meyer Zu Heringdorf, D. (2004) J. Cell. Biochem. 92, 937–948
7. Olivera, A., Rosenfeldt, H. M., Bekta, M., Wang, F., Ishii, L., Chun, J., Milstien, S., and Spiegel, S. (2003) J. Biol. Chem. 278, 46452–46460
8. Igarashi, N., Okada, T., Hayashi, S., Fujita, T., Jahanseer, S., and Nakamura, S. (2003) J. Biol. Chem. 278, 46832–46839
9. Liu, H., Toman, R. E., Goparaju, S. K., Maceyka, M., Nava, V. E., Sankala, H., Payne, S. G., Bekta, M., Ishii, L., Chun, J., Milstien, S., and Spiegel, S. (2003) J. Biol. Chem. 278, 40330–40336
10. Maceyka, M., Sankala, H., Hait, N. C., Le Stunff, H., Liu, H., Toman, R., Collier, C., Zhang, M., Satin, S. L., Merrill, A. H., Jr., Milstien, S., and Spiegel, S. (2005) J. Biol. Chem. 280, 37118–37129
11. Pitson, S. M., Xia, P., Declercq, T. M., Moretti, P. A., Zebol, J. R., Lynn, H. E., Wattenberg, B. W., and Vadas, M. A. (2005) J. Exp. Med. 201, 49–54
12. Chiba, K. (2005) Pharmacol. Ther. 108, 308–319
13. Chiba, K., Yanagawa, Y., Katoaka, H., Kawaguchi, T., Ohtsuki, M., and Hoshino, Y. (1999) Transplant. Proc. 31, 1230–1233
14. Mandal, S., Hajdu, R., Bergstrom, J., Quackenbush, E., Xie, J., Milligan, J., Thornton, R., Shei, G. J., Card, D., Keohane, C., Rosenbach, M., Hale, J., Lynch, C. L., Rupprecht, K., Parsons, W., and Rosen, H. (2002) Science 296, 346–349
15. Kharel, Y., Lee, S., Snyder, A. H., Sheasley-O’Neill, S. L., Morris, M. A., Setlady, Y., Zhu, R., Zagler, M. A., Burcin, T. L., Ley, K., Tung, K. S., Engelhard, V. H., Macdonald, T. L., Pearson-White, S., and Lynch, K. R. (2005) J. Biol. Chem. 280, 36865–36872
16. Zemann, B., Kinzel, B., Muller, M., Reuschel, R., Mechtcheriakova, D., Urtz, N., Bornancin, F., Baumrucker, T., and Blichec, A. (2005) Blood 107, 1454–1458
17. Matloubian, M., Lo, C. G., Cinamon, G., Lesnieski, M. J., Xu, Y., Brinkmann, V., Allende, M. L., Proia, R. L., and Cyster, J. G. (2004) Nature 427, 355–360
18. Suzuki, S., Li, X. K., Shimonoya, T., Enosawa, S., Amemiya, H., Amari, M., and Naoe, S. (1997) Clin. Exp. Immunol. 107, 103–111
19. Okazaki, H., Hirata, D., Kamimura, T., Sato, H., Iwamoto, M., Yoshio, T., Masuyama, J., Fujimura, A., Kobayashi, E., Kano, S., and Minota, S. (2002) J. Rheumatol. 29, 707–716
20. Yasui, H., Hideshima, T., Raje, N., Roccaro, A. M., Shiraiashi, N., Kumar, S., Hamasaki, M., Ishitsuku, K., Tai, Y. T., Podar, K., Catley, L., Mitsiades, C. S., Richardson, P. G., Albert, R., Brinkman, V., Chauhan, D., and Anderson, K. C. (2005) Cancer Res. 65, 7478–7484
21. Chua, C. W., Lee, D. T., Ling, M. T., Zhou, C., Man, K., Ho, J., Chan, F. L.,...
Wang, X., and Wong, Y. C. (2005) *Int. J. Cancer* 117, 1039–1048
22. Ho, J. W., Man, K., Sun, C. K., Lee, T. K., Poon, R. T., and Fan, S. T. (2005) *Mol. Cancer Ther.* 4, 1430–1438
23. Azuma, H., Takahara, S., Ichimaru, N., Wang, J. D., Itoh, Y., Otsuki, Y., Morimoto, J., Fukui, R., Hoshiga, M., Ishihara, T., Nonomura, N., Suzuki, S., Okuyama, A., and Katsuoka, Y. (2002) *Cancer Res.* 62, 1410–1419
24. Nagahara, Y., Ikekita, M., and Shinomiya, T. (2002) *Br. J. Pharmacol.* 137, 953–962
25. Nagahara, Y., Ikekita, M., and Shinomiya, T. (2000) *J. Immunol.* 165, 3250–3259
26. Matsuoka, Y., Nagahara, Y., Ikekita, M., and Shinomiya, T. (2003) *Br. J. Pharmacol.* 138, 1303–1312
27. Permpongkosol, S., Wang, J. D., Takahara, S., Matsumiya, K., Nonomura, N., Nishimura, K., Tsujimura, A., Kongkanand, A., and Okuyama, A. (2002) *Int. J. Cancer* 98, 167–172
28. Suzuki, E., Handa, K., Toledo, M. S., and Hakomori, S. (2004) *Proc. Natl. Acad. Sci. U. S. A.* 101, 14788–14793
29. Megidish, T., Takio, K., Titani, K., Hamaguchi, A., Igarashi, Y., and Hakomori, S. (1999) *Biochemistry* 38, 3369–3378
30. Cvijic, M. E., Xiao, G., and Sun, S. C. (2003) *J. Immunol. Methods* 278, 293–304
31. Allende, M. L., Sasaki, T., Kawai, H., Olivera, A., Mi, Y., van Echten-Deckert, G., Hajdu, R., Rosenbach, M., Keohane, C. A., Mandalà, S., Spiegel, S., and Proia, R. L. (2004) *J. Biol. Chem.* 279, 52487–52492
32. Mizugishi, K., Yamashita, T., Olivera, A., Miller, G. F., Spiegel, S., and Proia, R. L. (2005) *Mol. Cell. Biol.* 25, 11113–11121
33. Billich, A., and Ettmayer, P. (2004) *Anal. Biochem.* 326, 114–119
34. Liu, H., Sugiiura, M., Nava, V. E., Edsall, L. C., Kono, K., Poulton, S., Mili-stien, S., Kohama, T., and Spiegel, S. (2000) *J. Biol. Chem.* 275, 19513–19520
35. Ettmayer, P., Baumruker, T., Guerini, D., Mechtcheriakova, D., Nussbaumer, P., Streiff, M. B., and Billich, A. (2006) *Bioorg. Med. Chem. Lett.* 16, 84–87
36. Liu, H., Sugiura, M., Nava, V. E., Edsall, L. C., Kono, K., Poulton, S., Mili-stien, S., Kohama, T., and Spiegel, S. (2000) *J. Biol. Chem.* 275, 19513–19520
37. Brinkmann, V., Davis, M. D., Heise, C. E., Albert, R., Cottens, S., Hof, R., Bruns, C., Prieschl, E., Baumruker, T., Hiestand, P., Foster, C. A., Zollinger, M., and Lynch, K. R. (2002) *J. Biol. Chem.* 277, 21453–21457
38. Rosen, H., Alfonso, C., Surh, C. D., and McHeyzer-Williams, M. G. (2003) *Proc. Natl. Acad. Sci. U. S. A.* 100, 10907–10912
39. Welsch, C. A., Roth, L. W., Goetschy, J. F., and Movva, N. R. (2004) *J. Biol. Chem.* 279, 36720–36731
40. Lee, W. J., Yoo, H. S., Suh, P. G., Oh, S., Lim, J. S., and Lee, Y. M. (2004) *Exp. Mol. Med.* 36, 420–427
41. Boyce, M., and Yuan, J. (2006) *Cell Death Differ.* 13, 363–373
42. Brinkmann, V., Wilt, C., Kristofic, C., Nikolova, Z., Hof, R. P., Chen, S., Albert, R., and Cottens, S. (2001) *Transplant. Proc.* 33, 3078–3080
43. Zhang, X., Skrzypek, M. S., Lester, R. L., and Dickson, R. C. (2001) *Curr. Genet.* 40, 221–233
44. Chiba, K., Hoshino, Y., Ohtsuki, M., Kataoka, H., Maeda, Y., Matsuyuki, H., Sugahara, K., Kuchi, M., Hirose, R., and Adachi, K. (2005) *Transplant. Proc.* 37, 102–106
45. Bandhuvula, P., Tam, Y. Y., Oskouian, B., and Saba, J. D. (2005) *J. Biol. Chem.* 280, 33697–33700
46. van Meer, G., and Lisman, Q. (2002) *J. Biol. Chem.* 277, 25855–25858
47. Scorrano, L., Oakes, S. A., Opferman, J. T., Cheng, E. H., Sorcinelli, M. D., Pozzan, T., and Korsmeyer, S. J. (2003) *Science* 300, 135–139
48. Szabadkai, G., and Rizzuto, R. (2004) *FEBS Lett.* 567, 111–115
49. Nagahara, Y., Enosawa, S., Ikekita, M., Suzuki, S., and Shinomiya, T. (2000) *Immunopharmacology* 48, 75–85