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A novel immunosuppressive agent FTY720 induced Akt dephosphorylation in leukemia cells

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1 Our previous studies revealed that the immunosuppressive agent, FTY720, mainly induces mitochondria-involved apoptosis in some types of cancer cells, since Bcl-2 overexpression prevents the FTY720-induction of apoptotic stimuli. Furthermore, FTY720 induces G0/G1 cell cycle arrest. The present study further examines the correlation between intracellular signaling kinases with FTY720-induced mitochondria-involved apoptosis.

2 Human T cell leukemia Jurkat was exposed to FTY720. Dephosphorylation of Akt occurred in a time- and concentration-dependent manner. FTY720 also induced Bad (Ser^{136}) and ribosomal p70S6 kinase ($p70^{S6k}$) (Thr^{389}) dephosphorylation.

3 FTY720-induced Akt dephosphorylation was not because of Akt upstream phosphatidylinositol 3′-kinase (PI 3-kinase) pathway inhibition.

4 FTY720 also induced Akt dephosphorylation in human B cell leukemia BALL-1. BALL-1 cells were resistant to FTY720-induced apoptosis.

5 Okadaic acid (OA) inhibited the FTY720-induced dephosphorylation of Akt and $p70^{S6k}$, suggesting that FTY720 promotes Ser/Thr protein phosphatase (PP) activity.

6 OA partially inhibited FTY720-induced caspase-3 activation.

7 PP2A or PP2A-like phosphatase was temporarily activated in cells exposed to FTY720. In addition, FTY720 activated purified PP2A (ABC).

8 Overall, the results suggest that FTY720 activated PP2A or PP2A-like phosphatase and dephosphorylated Akt pathway factors resulting in the enhancement of apoptosis via mitochondria.

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Keywords: FTY720; Akt; PKB; $P70^{S6k}$; Bad; PP2A; apoptosis; leukemia cells

Abbreviations: CDK, cyclin-dependent kinase; MAPK, mitogen-activated protein kinase; MEK, MAPK kinase; OA, okadaic acid; $p70^{S6k}$, ribosomal p70 S6 kinase; PDGF, platelet-derived growth factor; PDK, phosphoinositide-dependent kinase; PDK1, phosphoinositide-dependent kinase 1; phospho-, phosphorylated; $PI(3,4)P_2$, phosphatidylinositol 3,4-diphosphate; $PI(3,4,5)P_3$, phosphatidylinositol 3,4,5-triphosphate; PI 3-kinase, phosphatidylinositol 3′-kinase; $PI(4,5)P_2$, phosphatidylinositol 4,5-diphosphate; PP, protein phosphatase; pRb, retinoblastoma protein; SGK, serum and glucocorticoid-inducible kinase; $\Delta\Psi_m$, mitochondrial membrane potential

Introduction

PI 3-kinase is an upstream effector of Ser/Thr kinase Akt (also known as protein kinase B), which is the major mediator of survival signals that protect cells from undergoing apoptosis (Toker, 2000). Two phosphoinositides produced by PI 3-kinase, phosphatidylinositol 3,4,5-triphosphate ($PI(3,4,5)P_3$) and phosphatidylinositol 3,4-diphosphate ($PI(3,4)P_2$), bind to the PH domain of Akt, which in turn leads to a conformational change that exposes Thr^{308} and Ser^{473} to phosphoinositide-dependent kinase 1 (PDK1) (Vanhaesebroeck & Alessi, 2000). Activated PDK1 allows for the phosphorylation of Akt at Thr^{308} and enables the activation of Akt by triggering autophosphorylation on Ser^{473} (Toker & Newton, 2000). Another signaling factor $p70^{S6k}$, which is also located downstream of PI 3-kinase, is activated by PDK1 and is believed to be an important factor regulating the translation of a set of mRNAs that encode ribosomal proteins and translation elongation factors; this regulatory function is important for cell cycle progression through the G1 phase (Shima *et al*., 1998; Balendran *et al*., 1999). The protooncogene Bcl-2 family of proteins promotes either cell survival (e.g., Bcl-2 and Bcl-X_L) or cell death (e.g., Bad and Bax). Bad is usually phosphorylated at Ser^{136} by Akt, and at Ser^{112} by mitogen-activated protein kinase (MAPK) (Bonni *et al*., 1999; Scheid *et al*., 1999). Dual dephosphorylated Bad associates with Bcl-2 and Bcl-X_L (Zha *et al*., 1997). PI 3-kinase activity is opposed by the dual-specificity phosphatase PTEN (also known as MMAC1 and TEP1), which acts against position 3 of the inositol ring (Wen *et al*., 2001). Mutations of *PTEN* have been demonstrated in many types of cancer and, in particular, in leukemia cells (Liu *et al*., 2000), suggesting that PI 3-kinase and Akt are critical factors in the genesis of cancer (Simpson & Parsons, 2001). Thus, antiapoptotic kinases in cellular signal

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transduction pathways play a central role in the survival of cancer cells.

The activation of antiapoptotic signaling proteins proceeds as described above. The inactivation of phosphorylated proteins is regulated by Ser/Thr protein phosphatases (PP1, PP2A, PP2B, and PP2C). Reversible phosphorylation is believed to be a mechanism involved in the rapid regulation of critical cell functions including metabolism, signal transduction, cell division, and growth (Zolnierowicz & Bollen, 2000). This involvement suggests that aberrations of intracellular signaling pathways via protein phosphatases contribute to human carcinogenesis.

The immunosuppressant FTY720 (2-amino-2-[2-(4-octylphenyl) ethyl]-1,3-propanediol hydrochloride) was originally screened from synthesized analogs of ISP-1, which is an immunosuppressive metabolite of *Isaria sinclairii* (Fujita *et al*., 1996). The administration of FTY720 prolongs allograft survival (Suzuki *et al*., 1996a). This agent specifically induces apoptosis in peripheral blood lymphocytes, especially in T cells *in vivo* and *in vitro* (Suzuki *et al*., 1996a; Nagahara *et al*., 2000a). The ability of FTY720 to initiate apoptosis in some leukemia cell lines and normal lymphocytes has been investigated. FTY720 induces apoptosis via caspase-3 activation in the human prostate cancer cell line DU145 (Wang *et al*., 1999). Moreover, FTY720 induces apoptosis through mitochondria and is inhibited by Bcl-2, a process that does not occur via the Fas pathway (Suzuki *et al*., 1996b, 1997). Bcl-2 inhibits the loss of mitochondrial membrane potential ($\Delta\Psi_m$) from apoptotic stimuli; Bcl-2 also blocks the cytochrome *c* release that is required for caspase-9 activation in cooperation with the mammalian Ced-4 homolog Apaf-1 (Wolf *et al*., 2001). This mitochondria-involved apoptotic mechanism is also supported by the FTY720-induced reduction of $\Delta\Psi_m$ and the release of cytochrome *c* from mitochondria (Nagahara *et al*., 2000b). Moreover, FTY720 induces G0/G1 cell cycle arrest in HL-60RG and Jurkat cells, involving the dephosphorylation of retinoblastoma protein (pRb) (Nagahara *et al*., 2001).

However, FTY720 may also inhibit further upstream signaling pathways. We therefore examined the effects of FTY720 on the PI 3-kinase/Akt pathway and found that phosphorylation levels of Akt, $p70^{S6k}$, and Bad were decreased. Furthermore, an examination of enzymatic phosphorylation and dephosphorylation activities revealed a novel mechanism of FTY720 action.

Methods

Reagents

FTY720 synthesized and supplied in powder form by Taito (Tokyo, Japan), in cooperation with Mitsubishi Pharma (Osaka, Japan), was dissolved in 150 mM NaCl (1 mM). Wortmannin, PD98059 and OA were obtained from Sigma (St. Louis, MO, U.S.A.) and dissolved in DMSO. Cells were unaffected by only the vehicles throughout the present studies. Platelet-derived growth factor (PDGF) was from Upstate Biotechnology (Lake Placid, NY, U.S.A.). Bovine PP2A trimer (ABC) purified from kidney as described by Damuni *et al*. (1989) was a gift from T. Honjo (Morinaga Institute of Biological Science, Yokohama, Japan). PP2A dimer (AC) was purchased from Upstate Biotechnology. PP1α was purchased from Calbiochem (San Diego, CA, U.S.A.). Anti-Akt (sc-1618 and C-20), anti-$p70^{S6k}$ (C-18) and anti-Bcl-2 antibodies, and S6 peptide were from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Antiphosphorylated (phospho-) Akt (Ser^{473}/Thr^{308}), antiphospho-$p70^{S6k}$ (Thr^{389}), anti-Bad and antiphospho-Bad (Ser^{112}/Ser^{136}) antibodies were from New England Biolabs (Berverly, MA, U.S.A.). Anti-PI 3-kinase-p85 antibody was from Upstate Biotechnology.

Cells

Human monoblastic leukemia U937 cells were a gift from the Cell Resource Center for Biomedical Research, Tohoku University (Miyagi, Japan). Mouse fibroblast 3T3 cells and human acute lymphoblastic leukemia BALL-1 were obtained from the Japanese Collection of Research Bioresources (Tokyo, Japan). The Jurkat human T cell line was obtained from Dainippon Pharmaceutical (Tokyo, Japan). Cells cultured in RPMI-1640 medium supplemented with 10% fetal calf serum and 75 mg/l-kanamycin, were maintained at 37°C in a humidified chamber under a 95% air and 5% CO_2 atmosphere.

DNA fragmentation analysis

DNA fragmentation (2×10^6 cells lane^{-1}) was analyzed by 1.8% agarose gel electrophoresis (Wyllie, 1980; Shinomiya *et al*., 1997).

Immunoblotting

Cells were washed and suspended at a density of 2×10^5 cells ml^{-1} in fresh culture medium. Cells were then incubated with 8 μM FTY720 for various periods in the presence or absence of 1 μM wortmannin or 50 μM PD98059. Cells were collected, washed with PBS and placed on ice for 20 min in lysis buffer (50 mM Tris-HCl, pH 7.5, 1% Nonidet P-40, 150 mM NaCl, 50 mM NaF, 1 mM EDTA, 10 μg ml^{-1} leupeptin, 10 μg ml^{-1} aprotinin, 0.5 mM phenylmethylsulfonylfluoride and 0.1 mM sodium orthovanadate). Cell lysates were centrifuged at 4°C for 15 min at $15{,}000 \times g$. The protein concentration of the supernatant was determined using the BCA protein assay (Pierce, Rockford, IL, U.S.A.). Cell lysates were mixed with an equivalent volume of sample application buffer (4% SDS, 125 mM Tris-HCl, pH 6.8, 10% glycerol, 0.02 mg ml^{-1} bromophenol blue, 10% 2-mercaptoethanol), placed in boiling water for 3 min, then loaded onto 4–20% gradient polyacrylamide gel (Iwaki, Tokyo, Japan). Proteins (15 μg lane^{-1}) were separated by electrophoresis, transferred to PVDF membranes (Millipore, Bedford, MA, U.S.A.) and incubated overnight with specific antibodies against Akt, phospho-Akt, phospho-$p70^{S6k}$, $p70^{S6k}$, Bad, phospho-Bad, and Bcl-2. The membranes were then incubated with horseradish peroxidase-conjugated anti-goat IgG or horseradish peroxidase-conjugated anti-rabbit IgG as second antibodies. Positive bands were visualized on X-ray film using enhanced chemical luminescence (Amersham Life Science, Buckinghamshire, U.K.).

Caspase-3 activity assay

Cells (1×10^7) were lysed in RIPA buffer (25 mM Tris, pH 7.4, 150 mM KCl, 5 mM EDTA, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS), and cell extracts were obtained by centrifugation at $10{,}000 \times g$ for 5 min at 4°C. The protein concentration was determined using the BCA protein assay. Cell extracts were incubated in 250 μl of caspase buffer (50 mM Hepes, pH 7.4, 100 mM NaCl, 1 mM EDTA, 0.1% Chaps, 10% sucrose, 5 mM DTT) containing 80 μM substrate, Ac-DEVD-MCA, with or without 1 μM caspase inhibitor, Ac-DEVD-CHO. After an incubation at 37°C for 30 min, 250 μl of stop solution (0.2 M glycine-HCl, pH 2.7) was added. The mixture was centrifuged, and the release of 7-amino-4-methyl-coumarin in the supernatant was measured using the spectrofluorometer at excitation and emission wavelengths of 380 and 460 nm, respectively.

Akt, p70^{S6k}, PI 3-kinase, and PDK assays

Cells were harvested and lysed on ice as described above. Akt was immunoprecipitated from cell lysates using 4 μg of anti-Akt and Protein A-Sepharose beads. Akt immunoprecipitates were washed twice with lysis buffer, twice with LiCl buffer (500 mM LiCl, 100 mM Tris-HCl, pH 7.5, 1 mM EDTA) and once with kinase buffer (50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 1 mM DTT). Beads were resuspended in kinase buffer containing 2.5 μg histone 2B (H2B) (Roche Diagnostics GmbH, Mannheim, Germany), 0.5 μM heat-stable inhibitor of cAMP-dependent protein kinase, 50 μM ATP, and 100 kBq [γ-^{32}P] ATP (specific activity 111 TBq mmol^{-1}) and incubated at 30°C for 30 min. Reactions were stopped by adding two volumes of SDS-PAGE sample buffer and placing the mixture in boiling water for 5 min. Samples were resolved by SDS electrophoresis SDS-PAGE and transferred to PVDF membranes that were cut in half. One-half was immunoblotted for Akt, and the other was visualized by autoradiography. ^{32}P-H2B was quantified on the autoradiograms using a imaging analyzer (BAS 2000, Fuji film, Tokyo, Japan).

To measure p70^{S6k} activity, p70^{S6k} was immunoprecipitated from cell lysates using 2 μg of anti-p70^{S6k} and Protein A-Sepharose beads. p70^{S6k} immunoprecipitates were washed twice with lysis buffer, twice with lysis buffer containing 0.5 M NaCl and once with p70^{S6k} kinase buffer (20 mM Hepes, pH 7.2, 10 mM $MgCl_2$, 0.1 mg ml^{-1} bovine serum albumin, 3 mM 2-mercaptoethanol). Beads were resuspended in kinase buffer containing 25 μg S6 peptide, 20 μM ATP and 370 kBq [γ-^{33}P] ATP and incubated at 30°C for 30 min. Reactions were stopped by adding 250 mM EDTA and placing the mixture in boiling water for 5 min. ^{33}P-S6 peptide samples were spotted onto P81 paper (Upstate Biotechnology) and quantified by scintillation counter (LSC-3050, Aloka, Tokyo, Japan).

To measure PI 3-kinase activity, semiconfluent mouse fibroblast 3T3 cells were cultured in serum-free RPMI 1640 medium overnight, then stimulated with 50 ng ml^{-1} PDGF for 5 min. Whole cell extracts were incubated with anti-PI 3-kinase-p85 antibody and Protein A-Sepharose for 2 h at 4°C under constant agitation. PI 3-kinase was subsequently assayed, as described (Gutkind *et al*., 1990), by evaluating the ability of the immunoprecipitates to phosphorylate PI to yield PI 3-phosphate. Following thin-layer chromatography, ^{32}P-phospholipids were detected by autoradiography.

To measure PDK1 activity, PDK1 was immunoprecipitated with 4 μg of sheep anti-PDK1 antibody and Protein G-Sepharose. PDK1 kinase activity was assayed using serum and glucocorticoid-inducible kinase (SGK)/Akt substrate peptide (after lysing cells as described above) using a PDK1 kinase assay kit from Upstate Biotechnology according to the manufacturer's protocol.

Preparation of cellular protein phosphatase

Cells (1×10^7) were rinsed with wash buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1 mM EDTA). Extracts were prepared by disruption in 200 μl of ice-cold homogenization buffer (50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.1 mM EGTA, 0.5% Triton X-100, 1 mM benzamidine, 1 mM DTT, protease inhibitor cocktail (Sigma) for 10 min followed by repeated aspiration through a 22-gauge needle (15 strokes), and centrifugation at $200 \times g$ to pellet the nuclei. The supernatant was separated by centrifugation at $100{,}000 \times g$ for 30 min at 4°C. The protein concentration was determined using the Bradford reagent (Bio-Rad; Richmond, CA, U.S.A.) and 0.1 μg of cytosolic protein was used for the assay.

Assay of protein phosphatases

PP1 and PP2A activities were determined using a protein phosphatase assay system (Life Technologies, Gaithersburg, MD, U.S.A.). Reactions were carried out in buffer A (10 mM imidazole-HCl, pH 7.63, 0.1% 2-mercaptoethanol, 0.1 mM EDTA, 1 mg ml^{-1} BSA) and measured as the release of trichloroacetic acid soluble phosphate from ^{32}P-phosphorylase α. One unit of phosphatase activity corresponds to the release of 1.0 nmol phosphate min^{-1} at 30°C. Extracts were preincubated with Mn^{2+} to measure cation-dependent protein phosphatase.

Statistical analysis

All statistical analyses were performed using Bonferroni/Dunn test. Significance was established at the $P < 0.05$ level.

Results

FTY720 decreases phospho-Akt and induces apoptotic cell death

In this study, Jurkat cells were used to determine the effects of FTY720. A recent study had already shown that tumor cells with mutant forms of PTEN contained a high level of $PI(3,4,5)P_3$, and $PI(3,4)P_2$, and high levels of endogenous Akt phosphorylation (Bruni *et al*., 2000; Gottschalk *et al*., 2001). The defective expression of the PTEN protein in Jurkat T-cell lines resulted in the accumulation of $PI(3,4,5)P_3$, and were thus responsible for the constitutive phosphorylation of Akt (Shan *et al*., 2000). Therefore, Jurkat cells are considered to have a high level of phosphorylated Akt at a steady state. Changes in the phospho-Akt level in response to FTY720 were analyzed by Western blotting using specific antibodies against phospho-Akt. Exposure of Jurkat cells to FTY720 caused a significant time- and concentration-dependent decrease in both phospho-Akt (Ser^{473}) and phospho-Akt (Thr^{308}) (Figure 1a)

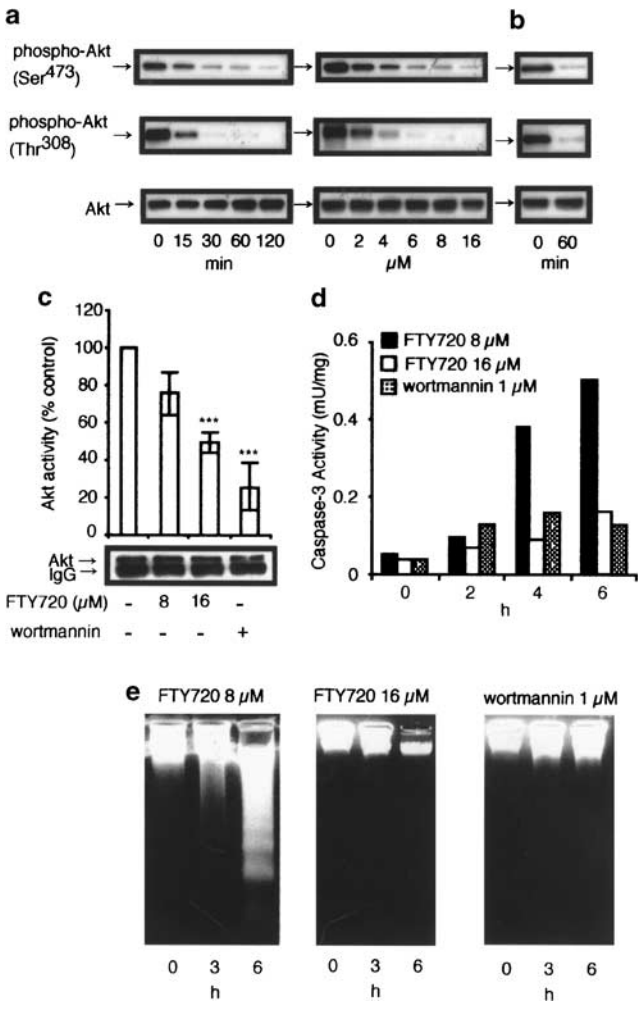

Figure 1 FTY720 reduces phospho-Akt. (a) Jurkat cells were incubated with 8 μM FTY720 for the indicated periods of time (left panels), or with the indicated concentrations of FTY720 for 45 min (right panels). Cytosolic fractions were prepared as described under "Methods", and were fractionated by SDS-PAGE. Western blots were probed with antibodies specific for phospho-Akt (Ser^{473}), phospho-Akt (Thr^{308}), and Akt. (b), Jurkat cells were exposed or not exposed to 1 μM wortmannin for 60 min. The presence of the indicated proteins was determined as in (a). (c) Kinase assays of Akt immunoprecipitated from the lysates of FTY720- or 100 nM wortmannin-stimulated Jurkat cells at 60 min, using the anti-Akt (C-20) antibody. Akt kinase activity was determined using H2B as a substrate, as described under "Methods". Bars in the graph show the standard deviation obtained from three independent experiments. $***P<0.001$, compared to the control. Akt levels were determined by anti-Akt antibody immunoblotting (lower panel). Immunoblots are representative of three independent experiments with similar results. (d) Caspase-3 activity was measured by 7-amino-4-methyl-coumarin fluorescence, as described under "Methods". Data are representative of three independent experiments. (e) Jurkat cells were incubated with reagents for the indicated periods of time, and then DNA fragmentation was assayed.

that reached approximately 1–5% of the control level by 120 min at both phosphorylated sites in the presence of 8 μM FTY720. To determine whether or not Akt dephosphorylation is regulated by the inhibition of PI 3-kinase, cells were incubated with the PI 3-kinase inhibitor wortmannin. The addition of 1 μM wortmannin completely decreased phospho-Akt (Ser^{473}) and phospho-Akt (Thr^{308}) levels (Figure 1b), demonstrating that PI 3-kinase is required for Akt phosphorylation in Jurkat cells. Similarly, 100 nM wortmannin partially decreased phospho-Akt levels (data not shown). Moreover, the results obtained with another PI 3-kinase inhibitor, LY294002, were identical (data not shown). In contrast, the total Akt amounts were almost identical under each of the present conditions (Figure 1a and b). We then examined intracellular Akt kinase activity in more detail. Cells were incubated with FTY720 or wortmannin under the same conditions as described in Figure 1a and b. Akt was immunoprecipitated and enzymatic activity was measured as described under "Methods". Figure 1c shows that Akt activity was decreased by FTY720 in a concentration-dependent manner, and inhibited in the presence of 100 nM wortmannin. We also simultaneously examined cell apoptosis by FTY720. Executer apoptosis protease, caspase-3, was activated time-dependently by 8 μM FTY720 treatment, and genomic DNA fragmentation was detected after incubating the cells with 8 μM FTY720 for 3 h (Figure 1d and e). However, a higher concentration (16 μM) of FTY720 induced necrosis such that only a slight activation of caspase-3 was detected, and smeared DNA was observed at 6 h treatment. Microscopic observation revealed that a marked number of cells were destroyed after 4 h treatment of 16 μM FTY720 because of FTY720-induced necrotic cell death (data not shown). Moreover, 1 μM wortmannin induced a slight activation of caspase-3 and DNA fragmentation. Similar results were previously obtained by Fujita *et al*., that is, that 1 μM wortmannin activated caspase-3 and fragmented DNA after a relatively long treatment period (Fujita *et al*., 1996).

FTY720 decreases phospho-Bad and phospho-p70^{S6k}

Phospho-Bad (Ser^{136}) is mediated by Akt and Bad (Ser^{112}) and is phosphorylated by an MEK-dependent signal transduction pathway (Bonni *et al*., 1999; Scheid *et al*., 1999). To assess the effects of FTY720 on phospho-Bad, the lysates of FTY720-treated Jurkat cells were separated by SDS-PAGE and were Western blotted with two specific antibodies against phospho-Bad (Ser^{136}) and phospho-Bad (Ser^{112}). FTY720 reduced the phospho-Bad (Ser^{136}) level in a time-dependent manner, although levels of phospho-Bad (Ser^{112}) were not reduced in this manner (Figure 2a). The decreased intensity of phospho-Bad (Ser^{136}) exactly paralleled the reduction in the amount of phospho-Akt. To test Bad (Ser^{112}) phosphorylation with MAPK kinase (MEK), Jurkat cells were incubated with the MEK inhibitor PD98059. In these Jurkat cells, we investigated that 1 h treatment of 50 μM PD98059 sufficed to induce dephosphorylation of one of the MAPKs, ERK1/2 (data not shown). A 1 h exposure to PD98059 produced no reduction in the levels of either phospho-Bad (Ser^{112}) or phospho-Bad (Ser^{136}) in Jurkat cells (Figure 2b). Incubation with 1 μM wortmannin for 1 h induced slight dephosphorylation of Bad (Ser^{136}), thus confirming that Bad (Ser^{136}) is downstream of PI 3-kinase (Figure 2b). The fact that FTY720 decreased phospho-Bad (Ser^{136}) but did not affect the level of phospho-Bad (Ser^{112}) demonstrates that FTY720 mediates signal transduction through an Akt pathway, and not via an MAPK pathway.

Both $p70^{S6k}$ /$p85^{S6k}$ isoforms are mitogen-activated Ser/Thr protein kinases that lie on a mitogen-activated signaling pathway downstream of PI 3-kinase (Berven & Crouch,

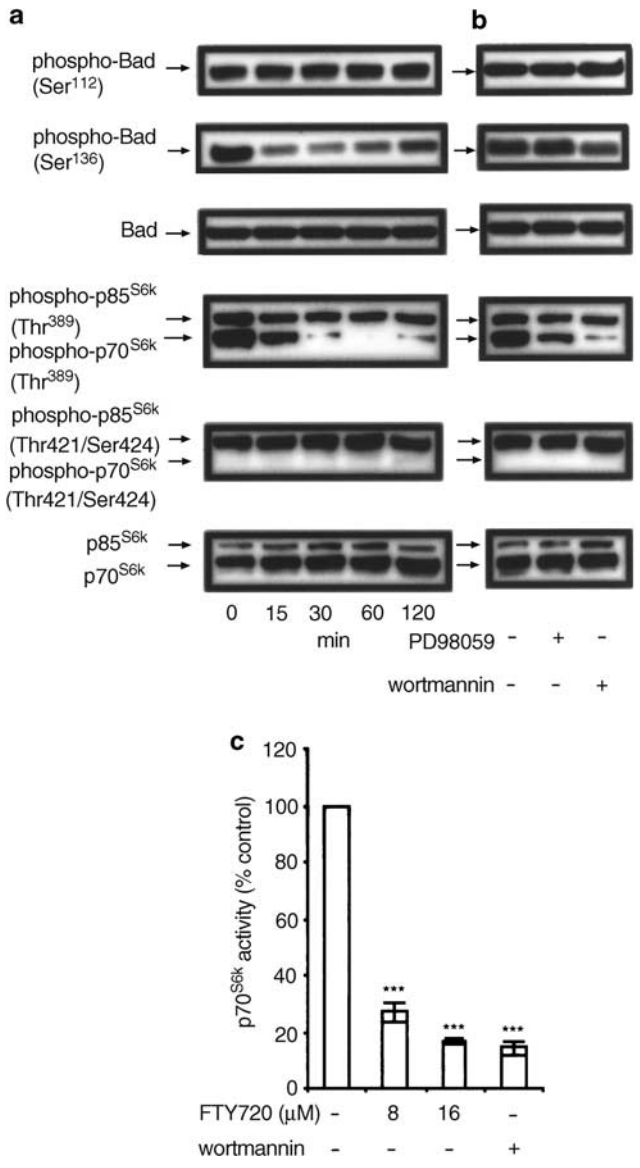

Figure 2 FTY720 reduces phospho-Bad (Ser^{136}) and phospho-$p70^{S6k}$/$p85^{S6k}$ (Thr^{389}). (a) Jurkat cells were incubated with 8 μM FTY720 for the indicated periods of time. Cytosolic fractions were prepared as described under ''Methods'' and fractionated by SDS-PAGE. Western blots were probed with antibodies specific for phospho-Bad (Ser^{112}), phospho-Bad (Ser^{136}), Bad, phospho-$p70^{S6k}$/$p85^{S6k}$ (Thr^{389}), phospho-$p70^{S6k}$/$p85^{S6k}$ (Thr^{421}/ Ser^{424}), and $p70^{S6k}$/$p85^{S6k}$. (b) Jurkat cells were incubated with 50 μM PD98059 or 1 μM wortmannin for 60 min. The presence of the indicated proteins was determined as in (a). Immunoblots are representatives of three independent experiments with similar results. (c) Kinase assays of $p70^{S6k}$ immunoprecipitated from the lysates of FTY720- or 1 μM wortmannin-stimulated Jurkat cells at 60 min, using the anti-$p70^{S6k}$ (C-18) antibody. $p70^{S6k}$ kinase activity was determined using the S6 peptide as a substrate, as described under ''Methods''. Bars in the graph show the standard deviation obtained from three independent experiments. $***P<0.001$, compared to the control.

2000). Activation of $p70^{S6k}$ appears to be first mediated by Thr^{389} phosphorylation, and is then regulated by wortmannin-sensitive and PI 3-kinase-dependent input, possibly through Akt (Pullen *et al*., 1998). We examined whether or not FTY720 affects the phospho-$p70^{S6k}$ level, especially at Thr^{389}. Figure 2a shows that FTY720 induced phospho-$p70^{S6k}$ (Thr^{389}) dephosphorylation in a time-dependent manner and slightly reduced the rate of dephosphorylation of phospho-$p85^{S6k}$ (Thr^{389}). To confirm that $p70^{S6k}$/$p85^{S6k}$ (Thr^{389}) phosphorylation proceeds through PI 3-kinase, Jurkat cells were incubated with 1 μM wortmannin for 1 h, and then were Western blotted. Figure 2b shows reduced levels of wortmannin-induced phospho-$p70^{S6k}$/$p85^{S6k}$ (Thr^{389}), indicating that $p70^{S6k}$/$p85^{S6k}$ (Thr^{389}) is downstream of the PI 3-kinase pathway. The protein $p70^{S6k}$ has four clustered phosphorylation sites at the carboxyl-terminal domain (Ser^{411}, Ser^{418}, Thr^{421}, and Ser^{424}), which are phosphorylated by an array of proline-directed protein kinases, including MAPK (Weng *et al*., 1998; Berven & Crouch, 2000). In contrast to phospho-$p70^{S6k}$ (Thr^{389}), phosphorylation of $p70^{S6k}$ was undetectable at Thr^{421}/Ser^{424} at the carboxyl-terminal domain (Figure 2a). Although phosphorylation of $p85^{S6k}$ at Thr^{421}/Ser^{424} was detectable, FTY720 did not simply induce dephosphorylation at these sites (Figure 2a). Our data indicated that the MAPK inhibitor PD98059 reduced phospho-$p70^{S6k}$/$p85^{S6k}$ at Thr^{389} but not at Thr^{421}/Ser^{424} (Figure 2b), suggesting that MAPK pathways regulate the phosphorylation of $p85^{S6k}$ at Thr^{389} rather than at Thr^{421}/Ser^{424} in Jurkat cells. Figure 2c shows that $p70^{S6k}$ activity was decreased by FTY720 in a concentration-dependent manner; this finding was similar to the results regarding Akt activity decrease by FTY720 (Figure 1c).

Effect of FTY720 on Akt, PI 3-kinase, and PDK kinase activity

Dephosphorylation of both Akt and $p70^{S6k}$ induced by FTY720 may be because of the direct inhibition of Akt, PI 3-kinase, and PDK1. To clarify the mechanism whereby FTY720 decreases phospho-Akt and phospho-$p70^{S6k}$, we examined the effects of FTY720 on PI 3-kinase survival pathways. We initially investigated whether or not FTY720 exerts a direct effect on the enzymatic activity of Akt immunoprecipitated from Jurkat cells. The results of cell extract immunocomplex kinase assays demonstrated that FTY720 did not immediately reduce basal Akt activity (Figure 3a). We subsequently confirmed that FTY720 disrupts the upstream signaling of Akt by measuring the activity of PI 3-kinase in response to FTY720. We stimulated 3T3 fibroblasts with PDGF for 5 min, then lysed and immunoprecipitated them with an 85-kDa subunit (p85) of PI 3-kinase antibody. Figure 3b shows that activated PI 3-kinase did not decrease in response to FTY720, whereas the potent PI3-kinase inhibitor, wortmannin, abolished PI 3-kinase activity as potently as did LY294002 (data not shown).

PDK1 is required for Akt and $p70^{S6k}$ activation (phosphorylation), and it is located in a region downstream of PI 3-kinase and upstream of Akt. To further explore the notion that FTY720 affects the upstream region of Akt, Jurkat cell extracts were immunoprecipitated with a PDK1-specific antibody and the PDK1 kinase activity was assayed with SGK/Akt substrate peptide in the presence of FTY720. Figure 3c reveals that FTY720 did not inhibit the immunoprecipitated PDK1 enzymatic activity of these cell extracts. The results of an assay using immunoprecipitated PDK1 from FTY720-treated Jurkat cells were similar (Figure 3d). At concentrations higher than 12 μM, PDK1 activity was decreased by 20–30% of the controls. However, PDK1 activity was not affected at the concentration (4–8 μM) used to induce

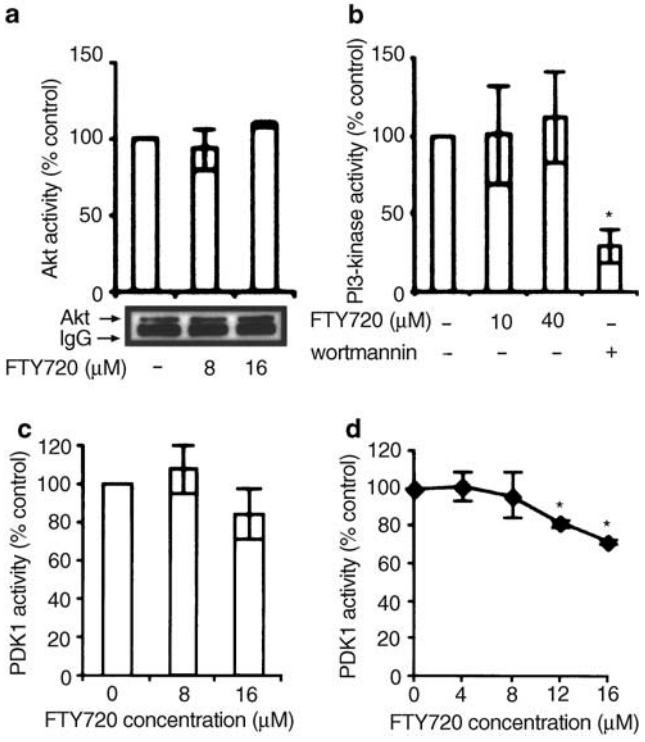

Figure 3 Inability of FTY720 to directly inhibit Akt, PI 3-kinase, and PDK1 kinase activities. (a) Kinase assays of Akt immunoprecipitated from lysates of Jurkat cells using anti-Akt (C-20) antibody. Akt kinase activity was determined using H2B as a substrate in the absence or presence of FTY720, as described under ''Methods''. The basal level of Akt activity was equal to the result obtained in the absence of FTY720. Bars in the graph show the standard deviation obtained from three independent experiments. The Akt level was determined by immunoblotting against anti-Akt antibody (lower panel). Immunoblots are representative of three independent experiments with similar results. (b) Effects of FTY720 on PDGF-stimulated PI 3-kinase activity in 3T3 cells. PI 3-kinase activity was assayed in immune complexes prepared with anti-PI 3-kinase-p85 antibody from 3T3 cell extracts stimulated with 50 ng ml^{-1} PDGF. PI 3-kinase activity was determined after a 30-min incubation at 30°C using PI as a substrate in the absence or presence of the agents. Assay mixtures containing the indicated concentrations of FTY720 or 100 nM wortmannin were separated on thin-layer chromatography plates and 3′ phosphorylated lipids were detected by autoradiography. Results are shown as percent mean ± s.d. from three independent experiments. *$P<0.05$, compared to the control. (c) PDK1 kinase assays of immunoprecipitates from untreated-Jurkat cell extracts, using anti-PDK1 antibody. PDK1 kinase activity was determined in the presence of the indicated concentrations of FTY720. (d) PDK1 kinase assays of immunoprecipitates obtained from intact Jurkat cells incubated with FTY720, using the anti-PDK1 antibody. Jurkat cells were incubated with the indicated concentrations of FTY720 for 30 min. PDK1 kinase activity was determined as described under ''Methods''. Results are shown as the percent means ± s.d. from three independent experiments. *$P<0.05$, compared to the control.

the dephosphorylation of Akt in this study; high concentrations (i.e., those over 12 μM) of FTY720 induced necrotic cell death rather than apoptotic cell death. It appears that the effects of PDK1 activity at higher concentrations of FTY720 do not reflect the appropriate intracellular response. Constitutive PDK1 phosphorylation (activation) in intact cells may be gradually abolished in response to unusual intracellular conditions induced by FTY720, especially at higher concentrations. We concluded that FTY720 does not affect the inhibition of Akt, PI 3-kinase, or PDK1 enzymatic activities.

Effects of FTY720 on other leukemia cell lines

We investigated whether or not the effects of FTY720 on phospho-Akt levels were similar between two leukemia cell lines, namely, BALL-1 and U937 cells. FTY720 reduced phospho-Akt (Ser^{473}) in the acute lymphoblastic leukemia line BALL-1 (Figure 4a). However, the effect of FTY720 in U937 was unclear. The results at Thr^{308} were identical to those described above (data not shown). Figure 4b shows the endogenous phosphorylated Akt levels in Jurkat, BALL-1, and U937 cells at the same protein concentrations. The level of Akt phosphorylation followed the order of Jurkat > BALL-1 (Figure 4b). However, in U937 cells, the phospho-Akt level was consistently low or undetectable, and it was therefore difficult to detect a difference after exposure to FTY720 (Figure 4a and b). Recent data published by Dahia *et al.* demonstrated that U937 cells have very low PTEN transcript levels and low expression levels of phospho-Akt (Dahia, 2000). These findings suggested that in U937 cells, phosphorylation did not sharply decrease because of the lack of sufficient phospho-Akt (Figure 4b). Figure 4b also shows endogenous antiapoptotic protein, and Bcl-2 levels of Jurkat, BALL-1, and U937 cells. The expression level of Bcl-2 revealed that BALL-1

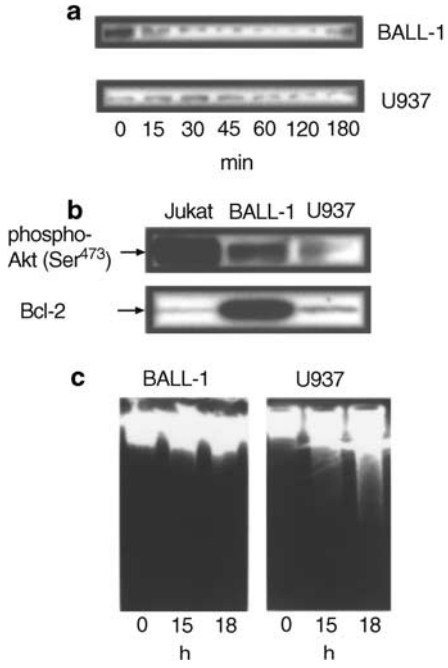

Figure 4 Difference of Akt dephosphorylation with FTY720 in various cells. (a) BALL-1 and U937 cells were incubated with 8 μM FTY720 for the indicated periods. Lysates were fractionated by SDS-PAGE and immunoblotted with antiphospho-Akt (Ser^{473}) antibody. Samples were exposed to X-ray films for 1 and 5 min, respectively. (b) Jurkat, BALL-1, and U937 cell lysates were fractionated by SDS-PAGE and immunoblotted with antiphospho-Akt (Ser^{473}) and anti-Bcl-2 antibodies. Samples were exposed to X-ray films for 1 and 0.5 min, respectively. Results are representative of three independent experiments. (c) BALL-1 and U937 cells were incubated for the indicated periods with 8 μM FTY720, and then DNA fragmentation was assayed.

possessed a significant amount of Bcl-2 among these cell lines. In addition, we examined the cytotoxic effects of FTY720. Figure 4c shows the time-dependent effect of 8 μM FTY720 on the cytotoxicity of U937 after 18 h. A ladder was undetectable in BALL-1 cells. Previous study revealed that FTY720-induced apoptosis is inhibited by Bcl-2 overexpression (Suzuki *et al*., 1996b). Thus, BALL-1 cells were able to avoid FTY720-induced apoptosis because of the presence of a significant amount of Bcl-2.

Synergistic effects of FTY720 and OA on Akt, p70 ^{S6k}, and caspase-3

One cause of the dephosphorylation of Akt and p70^{S6k}/p85^{S6k} induced by FTY720 may be the activation of Ser/Thr protein phosphatases, including PP2A, which dephosphorylate both kinases (Hemmings, 1997). To investigate this issue, cells were incubated in the presence or absence of the PP1 and PP2A inhibitor, OA, for 30 min. Thereafter, FTY720 was added and the cells were incubated for an additional 30 min. Western blots of cells incubated with various concentrations (25, 50, and 250 nM) of OA showed that the immunoreactivity of phospho-Akt and phospho-p70^{S6k}/p85^{S6k} was slightly higher than that of untreated cells (Figure 5a). Incubating the cells with 250 nM OA did not enhance the reduction of phospho-Akt (Ser^{473}), phospho-Akt (Thr^{308}), or phospho-p70^{S6k}/p85^{S6k} (Thr^{389}) in response to FTY720 (Figure 5a). The effects of FTY720 were gradually and concentration-dependently abolished by prior exposure to OA. In contrast, no significant inhibition was observed using the PP2B inhibitor cyclosporine A (data not shown). These data suggested that PP1- or PP2A-like protein phosphatase activity is associated with the dephosphorylation effects of FTY720 on phospho-Akt and phospho-p70^{S6k}/p85^{S6k}. Figure 5b shows the effects of OA on FTY720-induced caspase-3 activity. Treatment with OA alone did not activate caspase-3. In all, 4-h treatment with FTY720 activated caspase-3 to the level of a 15-fold increase. However, preincubation of OA partially inhibited FTY720-induced caspase-3 activation.

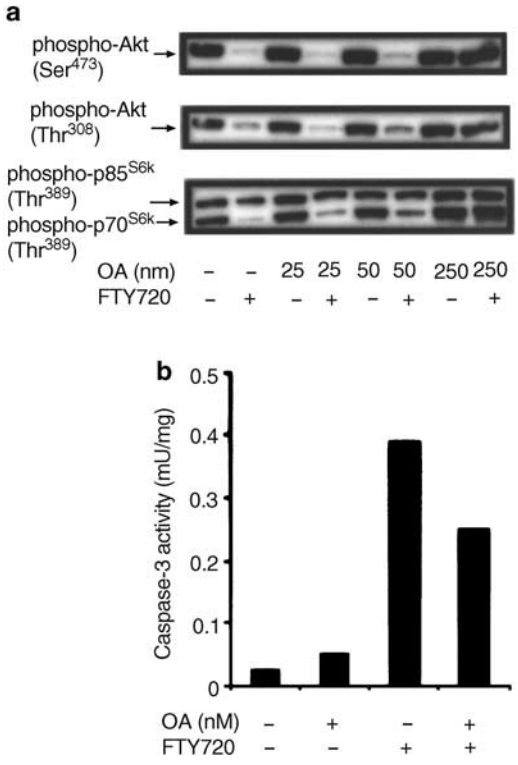

Figure 5 OA restores FTY720 reduction of phospho-proteins and activation of caspase-3. (a) Jurkat cells were incubated with the indicated concentrations of OA for 30 min, then with 8 μM FTY720 for 30 min. Lysates were fractioned by SDS-PAGE and probed using antibodies to phospho-Akt (Ser^{473}), phospho-Akt (Thr^{308}), and phospho-p70^{S6k}/p85^{S6k} (Thr^{389}). Data are representative of three independent experiments. (b) Jurkat cells were incubated with or without 250 μM OA for 1 h, followed by incubation with or without 8 μM FTY720 for 4 h. Caspase-3 activity was measured by 7-amino-4-methyl-coumarin fluorescence, as described under "Methods". Data are representative of three independent experiments.

FTY720 affects cytosolic protein phosphatase

To test the Ser/Thr protein phosphatase activities of FTY720-treated Jurkat cells, we analyzed the cells with an apparent inhibitor. The activities of PP were measured by a phosphatase assay using ^{32}P-phosphorylase α as the classic substrate for type 1 and 2A phosphatase. Both PP1 and PP2A activities are known to be active in this assay and they account for 100% of the cellular activity (Cohen *et al*., 1989). The inhibition assay with OA showed an intermediate plateau of inhibition at about 0.1–1 nM (Figure 6a). The PP2A activity corresponded to the phosphatase activity most sensitive to OA ($\leqslant$1 nM), and PP1 activity corresponded to the least OA-sensitive activity ($\geqslant$1 nM) (Figure 6a). Cell extracts from controls and FTY720-treated cells incubated with 8 μM FTY720 revealed maximal activation in a time-dependent manner after 45 min (Figure 6b). To examine whether or not OA affects FTY720-activated phosphatase, Jurkat cell extract was incubated with 1 nM OA. Protein phosphatase activity in the cell extract at the indicated times was almost totally abolished by 1 nM OA (Figure 6b), indicating that FTY720 stimulated cellular PP2A-like activities. PP2A activity was calculated by subtracting the activity measured in the presence of OA from that measured in the absence of OA. Figure 6c shows that FTY720 activated PP2A to 183% of the control level.

Effect of FTY720 on purified PP1 and PP2A

We further examined whether or not FTY720 activates purified PP1α, heterodimeric PP2A (AC), and heterotrimeric PP2A (ABC). It was found that FTY720 did not have any effect on phosphate release from the substrate. The results shown in Figure 7 indicate that 10 μM FTY720 activated PP2A (ABC) by 175% and PP2A (AC) by 120%. Furthermore, it was shown that FTY720 did not significantly respond to PP1.

Discussion

The ability of FTY720 to initiate apoptosis in some leukemia cell lines has been investigated. Although FTY720 activates proapoptotic death signaling proteins, such as c-Jun NH_2-terminal kinase and p38/CSBP (Matsuda *et al*., 1999), no studies have demonstrated its effect on signaling proteins as regards antiapoptotic survival. Here, we demonstrated that FTY720 decreased the activation of Akt, which promotes cell survival, growth, migration, and protein synthesis in various

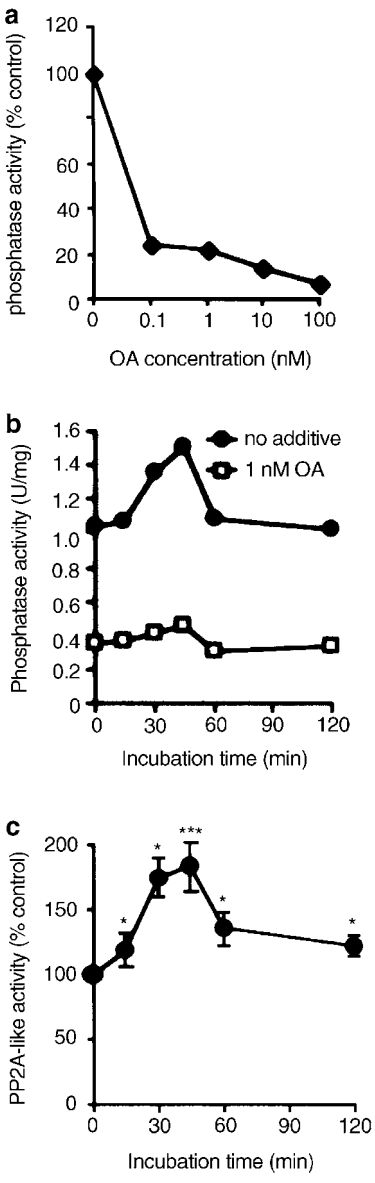

Figure 6 Effects of FTY720 on cellular protein phosphatases. (a)–(c) Jurkat cells were incubated with FTY720 for 0, 15, 30, 45, 60, and 120 min. FTY720-stimulated protein phosphatase activity was determined with or without inhibitors and 0.1 μg of cytosolic protein in 20 μl of buffer A. Reactions were initiated by adding 10 μl of 15 μM ^{32}P-phosphorylase α, and then were terminated after 10 min at 30°C with 90 μl 20% TCA. (a) Inhibition of the protein phosphatase activity by increasing concentrations of OA. Results are representative of five independent experiments. (b) FTY720-activated protein phosphatase in Jurkat cells. Protein phosphatase activity in cell extracts was determined without (●), or with 1 nM OA (□). One unit corresponds to a release of 1.0 nmol phosphate/min at 30°C. Results are representative of five independent experiments. (c) PP2A activity (% control) was calculated by subtracting the activity measured in the presence of OA from that measured in the absence of OA. A total of 100% activity was determined by subtraction at 0 min of incubation. Results are expressed as% initial activity in the absence of FTY720, and are the percent mean ± s.d. from five independent experiments. $*P<0.05$, $***P<0.001$, compared to the control.

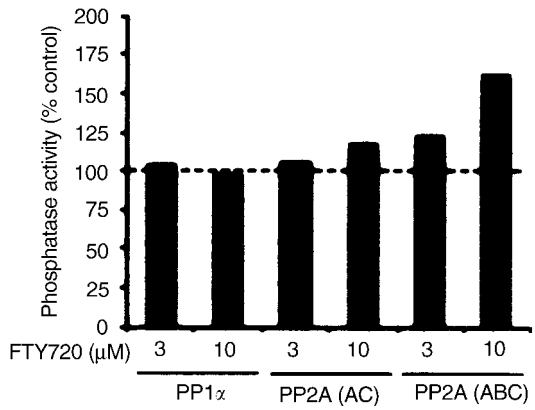

Figure 7 Effects of FTY720 on purified PP1 and PP2A protein phosphatases. Protein phosphatase activity was determined in the presence of the indicated concentrations of FTY720 and 1 mU of PP1α, 1.5 mU of PP2A (AC), or 0.3 μg of PP2A (ABC), respectively, in 20 μl of buffer A. Reactions were initiated by adding 10 μl of 15 μM ^{32}P-phosphorylase α, then terminated after 10 min at 30°C with 90 μl 20% TCA. Results are expressed as% initial activity in absence of FTY720 and are representative of three independent experiments.

cancer cells (Toker, 2000). FTY720 reduced both phosphorylated sites on Akt, at the Ser^{473} and Thr^{308} amino-acid residues (Figure 1a). Akt was dephosphorylated prior to FTY720-induced and apoptosis-related functions, for example, the activation of caspase-3 and DNA fragmentation. Moreover, a downstream proapoptotic protein, Bad, was also dephosphorylated and activated (Figure 2a), suggesting that FTY720 cut off the Akt survival pathway and promoted Bad activation. When preventing FTY720-induced Akt dephosphorylation by OA, caspase-3 activation was partially attenuated (Figure 5b). In U937 cells, Akt dephosphorylation was not as clearly observed with that in Jurkat cells, and apoptosis was more weakly induced in U937 cells than in Jurkat cells (Figure 4). This finding may be the result of the inactivation of the Akt/Bad pathway, thereby leading to the attenuation of FTY720-induced apoptosis. Moreover, recent studies by Wen *et al.* revealed that U937 cells expressed higher levels of an antiapoptotic protein, Survivin, than did Jurkat cells (Wen *et al.*, 2000). This postmitochondrial antiapoptotic protein might protect FTY720-induced apoptotic stimuli in U937 cells.

However, although FTY720 decreased phospho-Akt in a time-dependent manner in BALL-1 cells, apoptosis was not induced. BALL-1 cells have a higher level of endogenous Bcl-2 than do Jurkat cells (Figure 4). Since Bcl-2 is downstream of Akt/Bad, it overrides the Akt/Bad effect. The induction of Bad-dependent apoptosis might clearly be inhibited by increased levels of Bcl-2. The results obtained from Jurkat cells that overexpressed Bcl-2 were similar (data not shown).

FTY720 reduced the level of phospho-p70^{S6k}/p85^{S6k} (Thr^{389}), which was also sensitive to wortmannin and PD98059, whereas the level of phospho-p70^{S6k}/p85^{S6k} (Thr^{421}/Ser^{424}) of the four clustered phosphorylation sites was not reduced by PD98059 (Figure 2b). These data indicated that the reduction of phospho-p70^{S6k} (Thr^{389}) is based on a decrease in phospho-Akt, which might be dependent on both the MAPK and PI 3-kinase pathways in Jurkat cells. The function of p70^{S6k} /p85^{S6k} during the G1 phase is important for cell growth and cell cycle progression (Shima *et al.*, 1998; Balendran *et al.*, 1999). We found that FTY720 induced cell arrest and tumor cytotoxicity at the G1 phase (Nagahara *et al.*, 2001). The observed

dephosphorylation of $p70^{S6k}$ (Thr^{389}) by FTY720 may support these findings.

We have addressed the observation that FTY720-induced Akt dephosphorylation is neither because of the direct inhibition of Akt (Figure 3a), nor because of the inhibition of the Akt upstream kinases, PI 3-kinase and PDK1 (Figure 3b and c). $PI(3,4)P_2$ and $PI(3,4,5)P_3$, produced from PI(4)P and $PI(4,5)P_2$ by PI 3-kinase, bind to the PH domain of Akt, and then translocate to the plasma membrane, which in turn causes a conformational change that exposes Thr^{308} and Ser^{473} to the membrane-associated and activated upstream kinase, PDK1 (Vanhaesebroeck & Alessi, 2000). Therefore, to confirm whether the FTY720-induced dephosphorylation of Akt was because of FTY720-resolved $PI(3,4)P_2$ and $PI(3,4,5)P_3$, we examined the hypothesis that FTY720 inhibits the phosphoinositide generation pathway. We performed an anion-exchange HPLC and found that wortmannin decreased the $PI(3,4)P_2$ and $PI(3,4,5)P_3$ levels, whereas FTY720 did not change these levels as compared with those of the control cells (data not shown). This result indicates FTY720 does not inhibit intracellular PI-3 kinase activity, and that the reduction of phospho-Akt was not because of a decrease in phosphoinositides.

The mechanisms by which FTY720 decreases phospho-Akt and phospho-$p70^{S6k}$ remain to be clarified. Although several possibilities appear plausible (as described above), we suspect that FTY720 activates phosphatases, which then dephosphorylate Ser/Thr protein kinases. Akt and $p70^{S6k}$ are inactivated (dephosphorylated) by PP2A (Meier *et al*., 1998). Based on the observation that OA abolished the effects of FTY720 and restored the amount of phospho-Akt (Thr^{308}/Ser^{473}) and phospho-$p70^{S6k}$ (Thr^{389}) decreased by FTY720 (Figure 5a), we suggest here that FTY720 accelerates Akt- and $p70^{S6k}$-dephosphorylation. The finding that FTY720-treated cells activated Ser/Thr protein phosphatase, and that this effect was abolished by treatment with 1 nM OA, indicates that FTY720 activates a cellular PP2A-like protein phosphatase (Figure 6). Akt and $p70^{S6k}$/$p85^{S6k}$ dephosphorylation is probably promoted by FTY720-activated PP2A or a PP2A-like protein phosphatase. However, we were only able to detect indirect effects of FTY720 on cellular PP2A-like protein phosphatase, as shown in Figure 6. Cell extracts were diluted (and the FTY720 concentration was also reduced) when the cellular protein phosphatase was prepared, resulting in the abolition of FTY720 and PP2A-like phosphatase direct interactions. Since our findings using purified PP1 and PP2A revealed that 10 μM of FTY720 directly increased the activity of PP2A but not that of PP1 (Figure 7), these data indicate that FTY720 could directly activate intracellular PP2A. It is suggested that both indirect and direct pathways might exist in the case of FTY720-induced PP2A-like phosphatase activation. This FTY720-induced intracellular activation of PP2A or of a PP2A-like protein phosphatase mechanism remains to be elucidated in future studies. PP2A regulates cell-cycle progression, in particular that occurring during mitosis, through the regulation of pRb phosphorylation and cyclin-dependent kinase (CDK)1, CDK2, and CDC2 activities (Breeden, 2000). These results also support the finding that FTY720-induced cell cycle arrest and pRb dephosphorylation (Nagahara *et al*., 2001) are caused by PP2A or by PP2A-like phosphatase activation.

In summary, the present study reveals that an Akt/Bad-dependent mitochondrion-related apoptosis pathway, as well as an FTY720-direct mitochondrion-related apoptosis pathway, coordinate FTY720-induced apoptosis. A coordinated balance between the cut-off mechanisms of the Akt survival pathway and direct effects on the mitochondria may result in cell-specific apoptotic death by FTY720. Understanding how apoptosis may be differentially regulated by effector/secondary messenger molecules may provide insights into novel clinical strategies aimed at defeating leukemia cells.

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References

BALENDRAN, A., CURRIE, R., ARMSTRONG, C.G., AVRUCH, J. & ALESSI, D.R. (1999). Evidence that 3-phosphoinositide-dependent protein kinase-1 mediates phosphorylation of p70 S6 kinase *in vivo* at Thr-412 as well as Thr-252. *J. Biol. Chem.,* **274,** 37400–37406.

BERVEN, L.A. & CROUCH, M.F. (2000). Cellular function of $p70^{S6K}$: a role in regulating cell motility. *Immunol. Cell Biol.,* **78,** 447–451.

BONNI, A., BRUNET, A., WEST, A.E., DATTA, S.R., TAKASU, M.A. & GREENBERG, M.E. (1999). Cell survival promoted by the Ras-MAPK signaling pathway by transcription-dependent and -independent mechanisms. *Science,* **286,** 1358–1362.

BREEDEN, L.L. (2000). Cyclin transcription: Timing is everything. *Curr. Biol.,* **10,** R586–R588.

BRUNI, P., BOCCIA, A., BALDASSARRE, G., TRAPASSO, F., SANTORO, M., CHIAPPETTA, G., FUSCO, A. & VIGLIETTO, G. (2000). PTEN expression is reduced in a subset of sporadic thyroid carcinomas: evidence that PTEN-growth suppressing activity in thyroid cancer cells mediated by $p27^{kip1}$. *Oncogene,* **19,** 3146–3155.

COHEN, P., KLUMPP, S. & SCHELLING, D.L. (1989). An improved procedure for identifying and quantitating protein phosphatases in mammalian tissues. *FEBS Lett.,* **250,** 596–600.

DAHIA, P.L. (2000). PTEN, a unique tumor suppressor gene. *Endocr. Relat. Cancer,* **7,** 115–129.

DAMUNI, Z., AMICK, G.D. & SNEED, T.R. (1989). Purification and properties of a distinct protamine kinase from the cytosol of bovine kidney cortex. *J. Biol. Chem.,* **264,** 6412–6416.

FUJITA, E., KOUROKU, Y., MIHO, Y., TSUKAHARA, T., ISHIURA, S. & MOMOI, T. (1998). Wortmannin enhances activation of CPP32 (Caspase-3) induced by TNF or anti-Fas. *Cell Death Diff.,* **5,** 289–297.

FUJITA, T., HIROSE, R., YONETA, M., SASAKI, S., INOUE, K., KIUCHI, M., HIRASE, S., CHIBA, K., SAKAMOTO, H. & ARITA, M. (1996). Potent immunosuppressants, 2-alkyl-2-aminopropane-1,3-diols. *J. Med. Chem.,* **39,** 4451–4459.

GOTTSCHALK, A.R., BASILA, D., WONG, M., DEAN, N.M., BRANDTS, C.H., STOKOE, D. & HAAS-KOGAN, D.A. (2001). $p27^{Kip1}$ is required for PTEN-induced G1 growth arrest. *Cancer Res.,* **61,** 2105–2111.

GUTKIND, J.S., LACAL, P.M. & ROBBINS, K.C. (1990). Thrombin-dependent association of phosphatidylinositol-3 kinase with $p60^{c\text{-}src}$ and $p59^{fyn}$ in human platelets. *Mol. Cell. Biol.,* **10,** 3806–3809.

HEMMINGS, B.A. (1997). Akt signaling: linking membrane events to life and death decisions. *Science,* **275,** 628–630.

LIU, T.C., LIN, P.M., CHANG, J.G., LEE, J.P., CHEN, T.P. & LIN, S.F. (2000). Mutation analysis of PTEN/MMAC1 in acute myeloid leukemia. *Am. J. Hematol.,* **63,** 170–175.

MATSUDA, S., MINOWA, A., SUZUKI, S. & KOYASU, S. (1999). Differential activation of c-Jun NH_2-terminal kinase and p38 pathways during FTY720-induced apoptosis of T lymphocytes that is suppressed by the extracellular signal-regulated kinase pathway. *J. Immunol.,* **162,** 3321–3326.

MEIER, R., THELEN, M. & HEMMINGS, B.A. (1998). Inactivation and dephosphorylation of protein kinase Bα (PKBα) promoted by hyperosmotic stress. *EMBO J.,* **17,** 7294–7303.

NAGAHARA, Y., ENOSAWA, S., IKEKITA, M., SUZUKI, S. & SHINOMIYA, T. (2000a). Evidence that FTY720 induces T cell apoptosis *in vivo*. *Immunopharmacology,* **48,** 75–85.

NAGAHARA, Y., IKEKITA, M. & SHINOMIYA, T. (2000b). Immunosuppressant FTY720 induces apoptosis by direct induction of permeability transition and release of cytochrome *c* from mitochondria. *J. Immunol.,* **165,** 3250–3259.

NAGAHARA, Y., MATSUOKA, Y., SAITO, K., IKEKITA, M., HIGUCHI, S. & SHINOMIYA, T. (2001). Coordinate involvement of cell cycle arrest and apoptosis strengthen the effect of FTY720. *Jpn. J. Cancer Res.,* **92,** 680–687.

PULLEN, N., DENNIS, P.B., ANDJELKOVIC, M., DUFNER, A., KOZMA, S.C., HEMMINGS, B.A. & THOMAS, G. (1998). Phosphorylation and activation of $p70^{s6k}$ by PDK1. *Science,* **279,** 707–710.

SCHEID, M.P., SCHUBERT, K.M. & DURONIO, V. (1999). Regulation of bad phosphorylation and association with Bcl-x_L by the MAPK/Erk kinase. *J. Biol. Chem.,* **274,** 31108–31113.

SHAN, X., CZAR, M.J., BUNNELL, S.C., LIU, P., LIU, Y., SCHWARTZBERG, P.L., WANGE & R.L. (2000). Deficiency of PTEN in Jurkat T cells causes constitutive localization of Itk to the plasma membrane and hyperresponsiveness to CD3 stimulation. *Mol. Cell. Biol.,* **20,** 6945–6957.

SHIMA, H., PENDE, M., CHEN, Y., FUMAGALLI, S., THOMAS, G. & KOZMA, S.C. (1998). Disruption of the $p70^{s6k}$/$p85^{s6k}$ gene reveals a small mouse phenotype and a new functional S6 kinase. *EMBO J.,* **17,** 6649–6659.

SHINOMIYA, T., LI, X.K., AMEMIYA, H. & SUZUKI, S. (1997). An immunosuppressive agent, FTY720, increases intracellular concentration of calcium ion and induces apoptosis in HL-60. *Immunology,* **91,** 594–600.

SIMPSON, L. & PARSONS, R. (2001). PTEN: life as a tumor suppressor. *Exp. Cell Res.,* **264,** 29–41.

SUZUKI, S., ENOSAWA, S., KAKEFUDA, T., SHINOMIYA, T., AMARI, M., NAOE, S., HOSHINO, Y. & CHIBA, K. (1996a). A novel immunosuppressant, FTY720, with a unique mechanism of action, induces long-term graft acceptance in rat and dog allotransplantation. *Transplantation,* **61,** 200–205.

SUZUKI, S., LI, X.K., ENOSAWA, S. & SHINOMIYA, T. (1996b). A new immunosuppressant, FTY720, induces bcl-2-associated apoptotic cell death in human lymphocytes. *Immunology,* **89,** 518–523.

SUZUKI, S., LI, X.K., SHINOMIYA, T., ENOSAWA, S., AMEMIYA, H., AMARI, M. & NAOE, S. (1997). The *in vivo* induction of lymphocyte apoptosis in MRL-$^{pr/lpr}$ mice treated with FTY720. *Clin. Exp. Immunol.,* **107,** 103–111.

TOKER, A. (2000). Protein kinases as mediators of phosphoinositide 3-kinase signaling. *Mol. Pharmacol.,* **57,** 652–658.

TOKER, A. & NEWTON, A.C. (2000). Akt/protein kinase B is regulated by autophosphorylation at the hypothetical PDK-2 site. *J. Biol. Chem.,* **275,** 8271–8274.

VANHAESEBROECK, B. & ALESSI, D.R. (2000). The PI3K-PDK1 connection: more than just a road to PKB. *Biochem. J.,* **346Pt3,** 561–576.

WANG, J.D., TAKAHARA, S., NONOMURA, N., ICHIMARU, N., TOKI, K., AZUMA, H., MATSUMIYA, K., OKUYAMA, A. & SUZUKI, S. (1999). Early induction of apoptosis in androgen-independent prostate cancer cell line by FTY720 requires caspase-3 activation. *Prostate,* **40,** 50–55.

WEN, J., RAMADEVI, N., NGUYEN, D., PERKINS, C., WORTHINGTON, E. & BHALLA, K. (2000). Antileukemic drugs increase death receptor 5 levels and enhance Apo-2L-induced apoptosis of human acute leukemia cells. *Blood,* **96,** 3900–3906.

WEN, S., STOLAROV, J., MYERS, M.P., SU, J.D., WIGLER, M.H., TONKS, N.K. & DURDEN, D.L. (2001). PTEN controls tumor-induced angiogenesis. *Proc. Natl. Acad. Sci. U.S.A.,* **98,** 4622–4627.

WENG, Q.P., KOZLOWSKI, M., BELHAM, C., ZHANG, A., COMB, M.J. & AVRUCH, J. (1998). Regulation of the p70 S6 kinase by phosphorylation *in vivo*. Analysis using site-specific anti-phosphopeptide antibodies. *J. Biol. Chem.,* **273,** 16621–16629.

WOLF, B.B., SCHULER, M., LI, W., EGGERS-SEDLET, B., LEE, W., TAILOR, P., FITZGERALD, P., MILLS, G.B. & GREEN, D.R. (2001). Defective cytochrome *c*-dependent caspase activation in ovarian cancer cell lines due to diminished or absent apoptotic protease activating factor-1 activity. *J. Biol. Chem.,* **276,** 34244–34251.

WYLLIE, A.H. (1980). Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation. *Nature,* **284,** 555–556.

ZHA, J., HARADA, H., OSIPOV, K., JOCKEL, J., WAKSMAN, G. & KORSMEYER, S.J. (1997). BH3 domain of BAD is required for heterodimerization with BCL-X_L and pro-apoptotic activity. *J. Biol. Chem.,* **272,** 24101–24104.

ZOLNIEROWICZ, S. & BOLLEN, M. (2000). Protein phosphorylation and protein phosphatases. De Panne, Belgium, September 19–24, 1999. *EMBO J.,* **19,** 483–488.

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