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Mitochondrial targeting of JNK/SAPK in the phorbol ester response of myeloid leukemia cells

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Abstract

Treatment of human U-937 myeloid leukemia cells with 12-O-tetradecanoylphorbol-13-acetate (TPA) is associated with activation of the stress-activated protein kinase (SAPK) and induction of terminal monocytic differentiation. The present studies demonstrate that TPA targets SAPK to mitochondria by a mechanism dependent on activation of protein kinase C (PKC)β. Translocation of SAPK to mitochondria in response to TPA is associated with release of cytochrome *c*, caspase-3 activation and induction of apoptosis. The results show that TPA induces the association of SAPK with the mitochondrial anti-apoptotic Bcl-x_L protein. Overexpression of Bcl-x_L attenuated the apoptotic response to TPA treatment. Moreover, expression of Bcl-x_L mutated at sites of SAPK phosphorylation (Thr-47, -115) was more effective than wild-type Bcl-x_L in abrogating TPA-induced cytochrome *c* release and apoptosis. By contrast, expression of Bcl-x_L had little effect on induction of the monocytic phenotype. These findings indicate that myeloid leukemia cells respond to TPA with targeting of SAPK to mitochondria and that this response contributes to terminal differentiation through the release of cytochrome *c* and induction of apoptosis. *Cell Death and Differentiation* (2001) **8,** 794–800.

Keywords: terminal differentiation; TPA; protein kinase Cβ; stress-activated protein kinase; mitochondria; Bcl-x_L

Abbreviations: TPA, 12-O-tetradecanoylphorbol-13-acetate; PKC, protein kinase C; SAPK/JNK, stress-activated protein kinase/c-Jun NH2-terminal kinase; PI, propidium iodide; DAPI, 4,6-diamino-2-phenylindole; HSP, heat shock protein; PCNA, proliferating cell nuclear antigen; PDGF, platelet-derived growth factor; ERK, extracellular signal regulated kinase; ER, endoplasmic reticulum

Introduction

Human U-937 and HL-60 myeloid leukemia cell lines proliferate autonomously in the absence of exogenous hematopoietic growth factors.[1,2] These cells, however, respond to 12-O-tetradecanoylphorbol-13-acetate (TPA) and other agents that activate protein kinase C (PKC) with induction of a differentiated monocytic phenotype and apoptosis.[1–3] Resistance of human myeloid leukemia cells to TPA is associated with down-regulation or functional defects in PKCβ expression.[4–9] Moreover, induction of PKCβ expression by retinoic acid treatment or transfection of the PKCβ gene restores TPA-induced differentiation.[7,10] These findings have indicated that factor-independent growth of myeloid leukemia cells is reversible by activation of PKCβ-mediated signals.

Other studies have shown that TPA treatment of myeloid leukemia cells is associated with induction of the stress-activated protein kinase (JNK/SAPK) and that this response is dependent on PKCβ expression.[11] SAPK is a serine/threonine kinase that is related to the MAPK family and is induced by diverse types of stress.[12,13] SAPK phosphorylates the N-terminus of c-Jun and thereby activates the c-Jun transcription function.[12,13] The ATF2 and Elk1 transcription factors are also phosphorylated by SAPK.[14–16] In concert with these results, TPA-induced activation of SAPK in myeloid leukemia cells is associated with induction of the c-*jun* gene.[17–19] The early growth response 1 (EGR1) gene is also activated during TPA-induced monocytic differentiation and is necessary for appearance of the monocytic phenotype.[20–22] These findings have supported a model in which TPA treatment of myeloid leukemia cells is associated with activation of PKCβ and thereby the induction of SAPK activity and early response gene expression.

The present studies demonstrate that TPA-induced activation of PKCβ targets SAPK to mitochondria. The results show that SAPK interacts with the anti-apoptotic Bcl-x_L protein and induces the release of cytochrome *c*. The findings support a role for mitochondrial targeting of SAPK in the apoptotic response of myeloid leukemia cells to TPA.

Results and Discussion

To assess the effects of TPA on the subcellular distribution of SAPK, intracellular fluorescence was measured with a high sensitivity CCD camera and image analyzer. Examination of the distribution of fluorescence markers in control U-937 cells showed distinct patterns for anti-SAPK (red signal) and the mitochondrion-selective dye Mitotracker (green signal) (Figure 1A). By contrast, treatment with TPA was associated with a change in fluorescence signals (red and green→yellow/orange) that supported translocation of SAPK to mitochondria

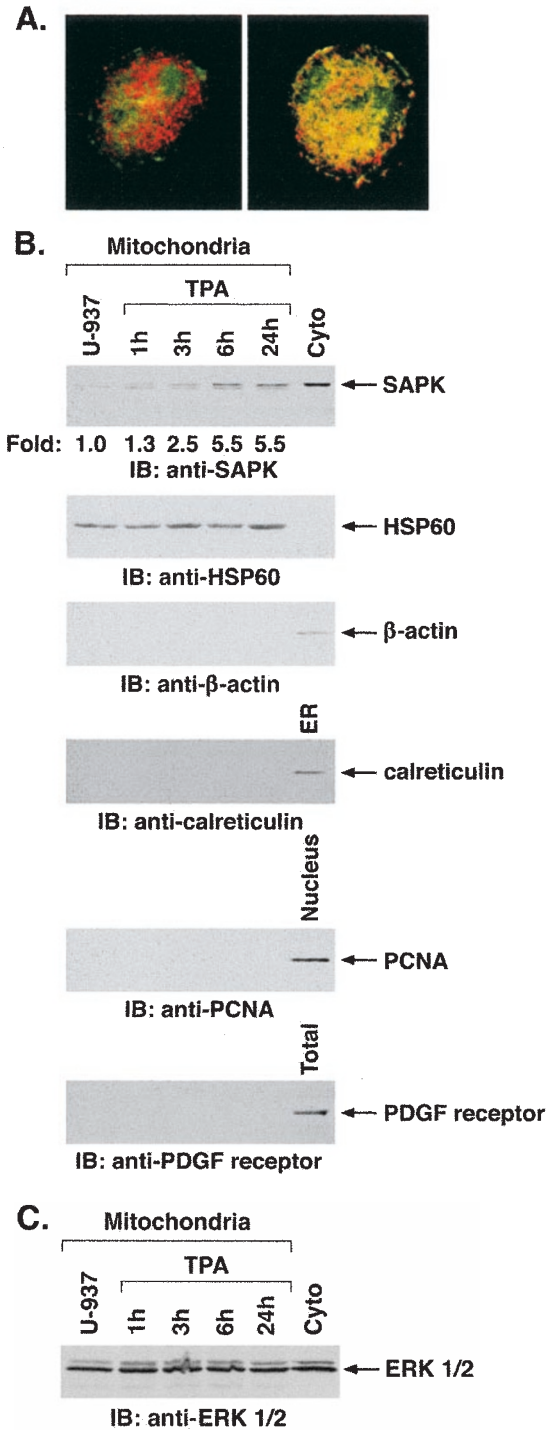

Figure 1 Translocation of JNK/SAPK to mitochondria in response to TPA. (**A**) U-937 cells were treated with 50 nM TPA for 6 h. After washing, the cells were immobilized on slides, fixed, and incubated with anti-SAPK antibody followed by Texas Red-conjugated goat anti-rabbit IgG. Mitochondria were stained with the mitochondrial-selective dye Mitotracker Green. Nuclei were stained with DAPI. The slides were visualized using a fluorescence microscope coupled to a high sensitivity CCD camera and image analyzer. Red signal, JNK/SAPK; green signal, Mitotracker; yellow/orange signals, co-localization of SAPK and Mitotracker. (**B**) U-937 cells were treated with TPA and harvested at the indicated times. Mitochondrial fractions were isolated and subjected to immunoblotting with anti-SAPK, anti-HSP60, anti-β-actin, anti-calreticulin, anti-PDGF receptor or anti-PCNA. Cytoplasmic (Cyto), endoplasmic reticulum (ER), nuclear fractions and total cell lysates (total) were used as controls. Signal intensities were analyzed by densitometric scanning. The intensity of the SAPK signals was compared to that of the untreated control. (**C** U-937 cells were treated with TPA for the indicated times. Mitochondrial fractions were subjected to immunoblotting with anti-ERK 1/2

(Figure 1A). To confirm these finding, mitochondrial lysates were subjected to immunoblotting with anti-SAPK. The results demonstrate that TPA induces over a fivefold increase in mitochondrial levels of SAPK (Figure 1B). Equal loading of the lanes was confirmed by immunoblot analysis of the mitochondrial HSP60 protein (Figure 1B). Purity of the mitochondrial fraction was assessed by immunoblotting with antibodies against the cytoplasmic β-actin protein, the ER-associated calreticulin, the nuclear PCNA protein and the cell membrane PDGF receptor (Figure 1B). Moreover, the selectivity of TPA-induced translocation of SAPK was supported by the absence of detectable changes in mitochondrial levels of ERK 1/2 (Figure 1C). These findings demonstrate that TPA induces SAPK to translocate to mitochondria.

Previous work has demonstrated that the TPA-resistant U-937 clone, designated TUR, is defective in TPA-induced activation of SAPK.[11] To determine whether induction of SAPK activity is necessary for translocation to mitochondria, TUR cells were treated with TPA and mitochondrial lysates were analyzed by immunoblotting with anti-SAPK. The results demonstrate little effect of TPA on mitochondrial levels of SAPK (Figure 2A). Mitochondrial lysates from TPA-treated U-937 and TUR cells were also subjected to immunoprecipitation with anti-SAPK. Analysis of the precipitates for phosphorylation of GST–Jun demonstrated induction of SAPK activity in TPA-treated U-937, but not TUR, cells (Figure 2B). To assess whether PKCβ is required for TPA-induced translocation of SAPK to mitochondria, TUR cells were studied that stably express the PKCβ gene.[11] The results show that TPA treatment of TUR/PKCβ cells is associated with translocation of SAPK to mitochondria (Figure 2C). To provide further support for PKCβ-mediated translocation of SAPK to mitochondria, U-937 cells were pretreated with the PKC inhibitor, calphostin C. The results demonstrate that calphostin C blocks TPA-induced targeting of SAPK to mitochondria (Figure 2D). Other studies have demonstrated that okadaic acid induces U-937 cell differentiation by a PKCβ-independent mechanism.[9] In contrast to TPA, okadaic acid had little if any effect on mitochondrial SAPK levels (Figure 2E). These findings support a model in which TPA-induced activation of the PKCβ→SAPK pathway is necessary for translocation of SAPK to mitochondria.

U-937 cells respond to TPA with the induction of differentiation and apoptosis.[3] To determine whether targeting SAPK to mitochondria is associated with apoptosis, U-937, TUR and TUR/PKCβ cells were studied for TPA-induced release of mitochondrial cytochrome *c* and activation of caspase-3. The results demonstrate that U-937, but not TUR, cells respond to TPA with release of cytochrome *c* to the cytosol (Figure 3A). Moreover, the TUR/PKCβ cells responded to TPA with cytochrome *c*

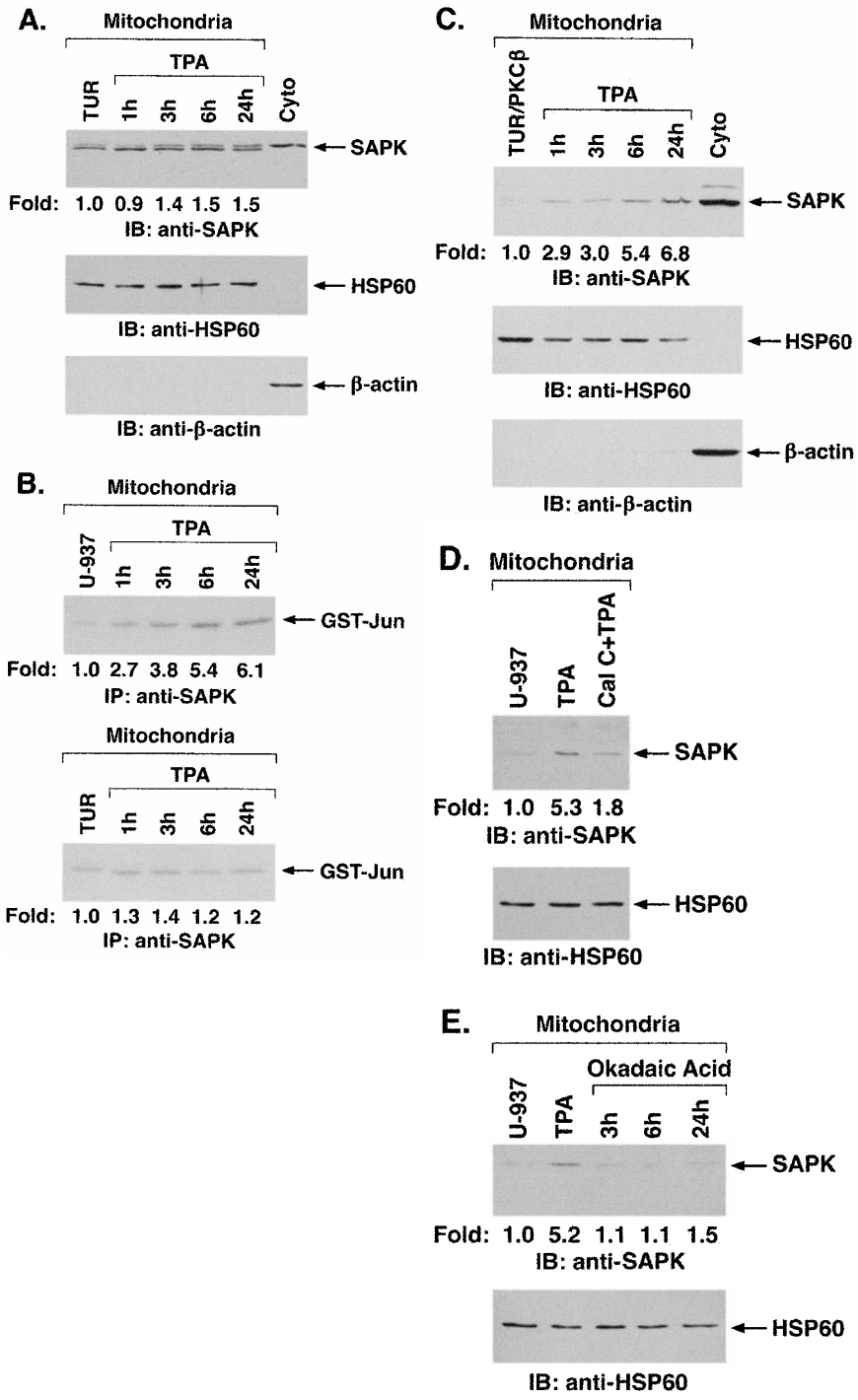

Figure 2 PKCβ expression is required for TPA-induced translocation of JNK/SAPK to mitochondria. (**A**) TUR cells were treated with TPA and harvested at the indicated times. Mitochondrial fractions were subjected to immunoblotting with anti-SAPK (upper panel), anti-HSP60 (middle panel) or anti-β-actin (lower panel). Cytoplasmic (Cyto) fraction was used as a control. Intensity of the SAPK signals was compared to that of the control. (**B**) U-937 (upper panel) and TUR (lower panel) cells were treated with TPA for the indicated times. Mitochondrial fractions were subjected to immunoprecipitation with anti-SAPK. The precipitates were analyzed in an immune complex kinase assay using GST-Jun as substrate. (**C**) TUR/PKCβ cells were treated with TPA for the indicated times. Mitochondrial fractions were subjected to immunoblotting with anti-SAPK (upper panel), anti-HSP60 (middle panel) or anti-β-actin (lower panel). Cytoplasmic (Cyto) fraction was used as a control. (**D**) U-937 cells were pretreated with 100 nM calphostin C (Cal C) for 1 h and then incubated with TPA for 6 h. Mitochondrial fractions were subjected to immunoblotting with anti-SAPK (upper panel) and anti-HSP60 (lower panel). (**E**) U-937 cells were treated with TPA for 6 h or with 50 nM okadaic acid for the indicated times. Mitochondrial fractions were subjected to immunoblotting with anti-SAPK (upper panel) and anti-HSP60 (lower panel). Intensity of the SAPK signals was compared to that of the untreated control

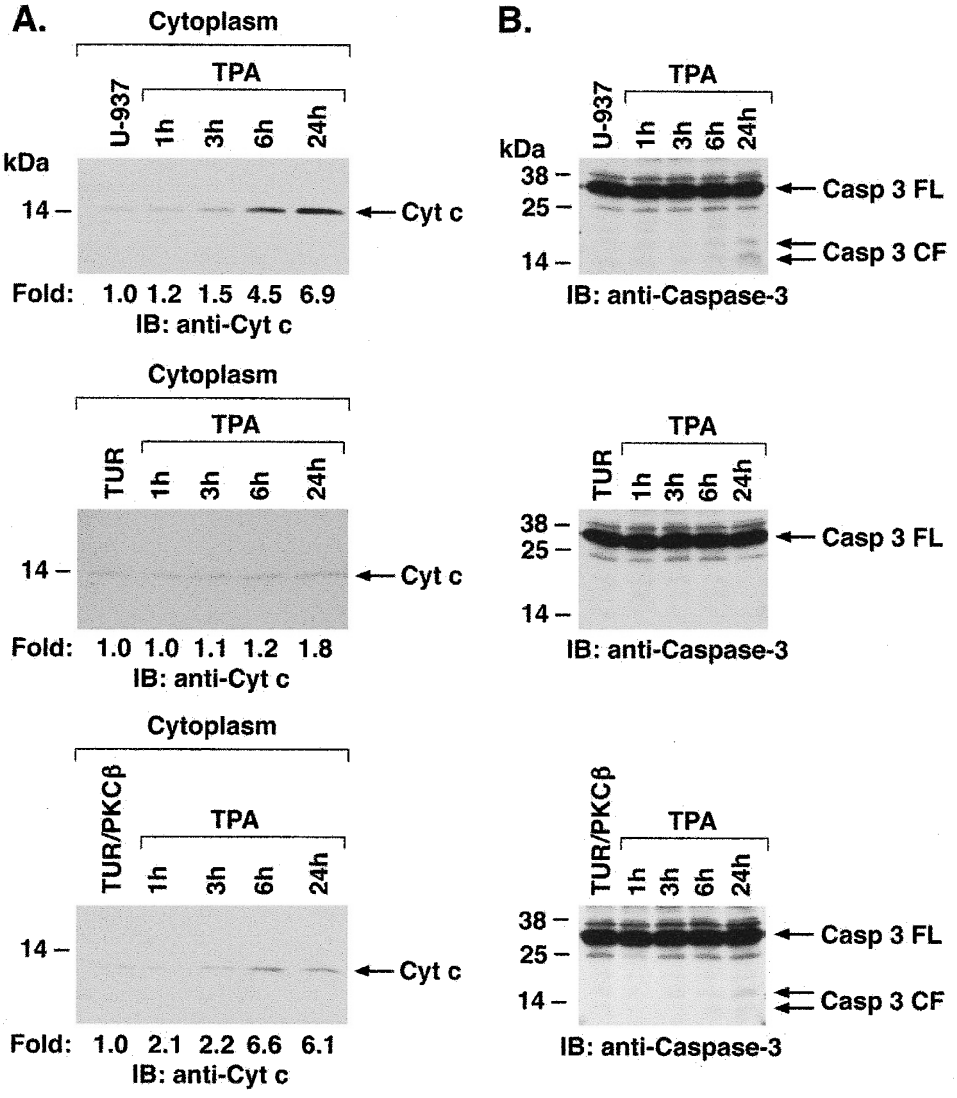

Figure 3 TPA induces cytochrome *c* release and caspase-3 activation and apoptosis by a PKCβ-dependent mechanism. (**A**) U-937 (upper panel), TUR (middle panel) and TUR/PKCβ (lower panel) cells were treated with TPA and harvested at the indicated times. Cytoplasmic fractions were subjected to immunoblotting with anti-cytochrome *c* (Cyt *c*). Intensity of the cytochrome *c* signals was compared to that of the control. (**B**) Total cell lysates were analyzed by immunoblotting with anti-caspase-3 (Casp 3). FL, full length; CF, cleaved fragment

release (Figure 3A). Cytosolic cytochrome *c* binds to Apaf-1, induces the autoprocessing of caspase-9 and thereby the activation of caspase-3.[23,24] In concert with TPA-induced release of cytochrome *c*, U-937 and TUR/PKCβ, but not TUR, cells responded to TPA with activation of caspase-3 (Figure 3B). These findings indicated that TPA-induced activation of the PKC$\beta\rightarrow$SAPK pathway is associated with the induction of cytochrome *c* release and caspase-3 activation.

Previous work has shown that SAPK phosphorylates the mitochondrial Bcl-x_L protein on Thr-47 and Thr-115.[25] To determine whether Bcl-x_L regulates TPA-induced signaling, studies were performed on U-937 cells stably overexpressing Bcl-x_L (U-937/Bcl-x_L).[26] Bcl-x_L expression had no detectable effect on TPA-induced translocation of SAPK to mitochondria (Figure 4A, left panel). Moreover, TPA-treatment induced the association of SAPK and Bcl-x_L (Figure 4A, right panel). By contrast, TPA-induced release of cytochrome *c* was attenuated in the U-937/Bcl-x_L cells (Figure 4A). Bcl-x_L also had no effect on TPA-induced cell adhesion, a characteristic of monocytic differentiation[11] (Figure 4B). To extend the analysis, other studies were performed on U-937 cells stably overexpressing Bcl-x_L in which Thr-47 and Thr-115 were mutated to alanines (U-937/Bcl-x_L (A-47, -115)).[25] Overexpression of Bcl-x_L (A-47, -115) had no effect on TPA-induced translocation of SAPK,

but blocked cytochrome *c* release compared to that obtained with U-937 and U-937/Bcl-x_L cells (Figure 4C). Bcl-x_L (A-47, -115) also had no effect on TPA-induced cell adhesion (Figure 4D). These results support a role for targeting of SAPK to mitochondria in the regulation of Bcl-x_L function and the release of cytochrome *c*.

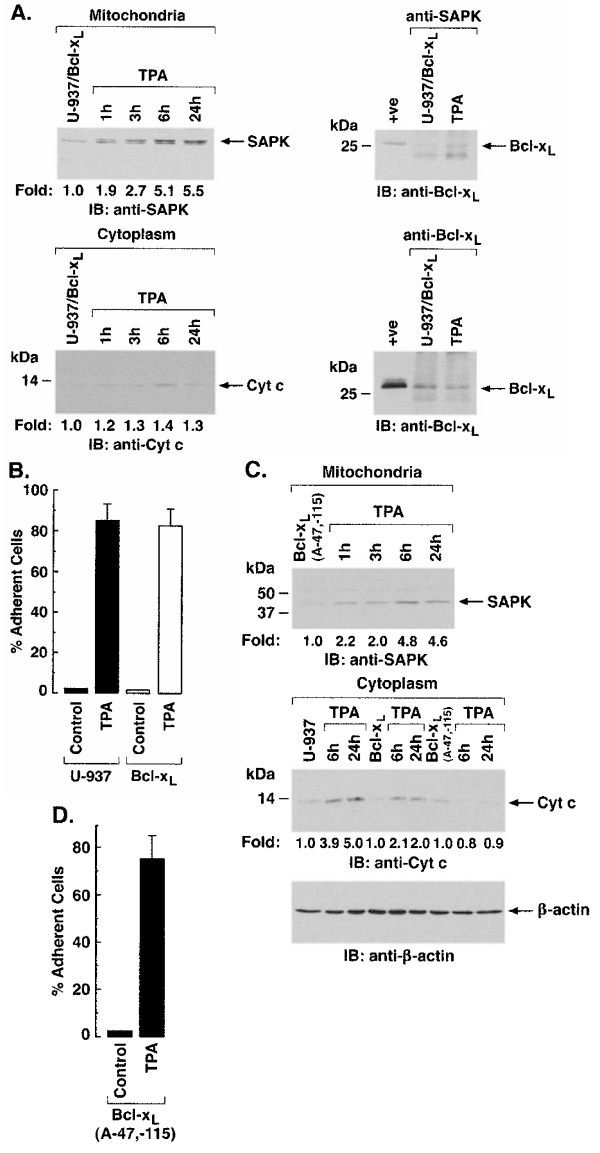

Figure 4 Functional interaction between SAPK and Bcl-x_L in mitochondria. (**A**) U-937/Bcl-x_L cells were treated with TPA and harvested at the indicated times. Mitochondrial and cytoplasmic fractions were subjected to immunoblotting with anti-SAPK (left upper panel) or anti-cytochrome *c* (left lower panel). U-937/Bcl-x_L cells were treated with TPA for 6 h. Mitochondrial fractions were subjected to immunoprecipitation with anti-SAPK or anti-Bcl-x_L. The immunoprecipitates were analyzed by immunoblotting with anti-Bcl-x_L (right panels). (**B**) U-937 or U-937/Bcl-x_L cells were treated with TPA for 24 h. The percentage of adherent cells are expressed as the mean ± S.D. from three independent experiments performed in duplicate for the U-937 (solid bar) and Bcl-x_L (open bar) cells. (**C**) U-937, U-937/Bcl-x_L and U-937/Bcl-x_L (A-47, -115) cells were treated with TPA and harvested at the indicated times. Mitochondrial and cytoplasmic fractions were subjected to immunoblotting with anti-SAPK (upper panel), anti-cytochrome *c* (middle panel) or anti-β-actin (lower panel). (**D**) U-937/Bcl-x_L (A-47, -115) cells were treated with TPA for 24 h. The percentage of adherent cells is expressed as the mean ± S.D. from three independent experiments each performed in duplicate. Intensity of the SAPK and cytochrome *c* signals was compared to that of the untreated control

In concert with the demonstration that TPA targets SAPK to mitochondria and thereby release of cytochrome *c*, U-937, and not TUR, cells responded to TPA with induction of apoptosis (Figure 5). Dependence of the apoptotic response on PKCβ was supported by the finding that TUR/PKCβ cells also responded to TPA with induction of apoptosis (Figure 5). TPA-induced apoptosis was slower and less pronounced than that achieved when U-937 cells were treated with ionizing radiation (i.e., 40% apoptosis at 24 h).[25] Indeed, other studies have demonstrated that ionizing radiation and TPA activate SAPK by distinct mechanisms.[11,27] The present results also demonstrate that expression of Bcl-x_L blocks TPA-induced apoptosis (Figure 5). Moreover, the Bcl-x_L (A-47, -115) mutant was more effective in blocking the apoptotic response to TPA treatment (Figure 5). These results provide further support for targeting of SAPK to mitochondria as a pro-apoptotic signal.

SAPK is activated in the TPA response of myeloid leukemia cells and is required for the induction of terminal monocytic differentiation.[11] The present studies demonstrate that TPA targets SAPK to the mitochondria by a PKCβ-dependent mechanism. The results support a role for mitochondrial targeting of SAPK in a signaling cascade that

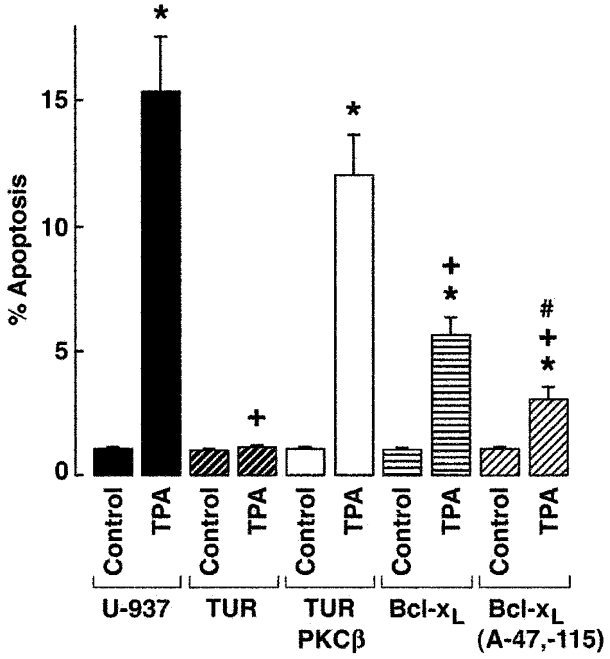

Figure 5 TPA-induced apoptosis is PKCβ-dependent and blocked by Bcl-x_L. U-937, TUR, TUR/PKCβ, U-937/Bcl-x_L and U-937/Bcl-x_L (A-47, -115) cells were treated with TPA for 24 h. After fixing, cells were stained with propidium iodide and sub-G_1 DNA content was assayed by FACScan. The percentage of apoptotic cells with sub-G_1 DNA content is expressed as the mean ± S.D. from three independent experiments each performed in duplicate. As determined by *t*-test, the significance of the following comparisons is: $^*P<0.05$, *versus* control; $+P<0.05$, *versus* U-937/TPA; $\#P<0.05$, U-937/Bcl-x_L/TPA *versus* U-937/Bcl-x_L (A-47, -115)/TPA

confers TPA-induced apoptosis. In this context, SAPK interacts with the mitochondrial Bcl-x_L protein and overexpression of Bcl-x_L attenuates TPA-induced cytochrome *c* release. Moreover, expression of Bcl-x_L mutated at sites of SAPK phosphorylation (Thr-47, -115) was more effective than wild-type Bcl-x_L in abrogating TPA-induced cytochrome *c* release and apoptosis. Bcl-x_L regulates cytochrome *c* release by the mitochondrial voltage dependent anion channel.[28,29] Thr-47 resides in a 60-residue loop that is non-essential for the anti-apoptotic activity of Bcl-x_L, while Thr-115 is adjacent to the α3 helix which may be structurally important in the formation of ion channels.[30,31] Importantly, overexpression of Bcl-x_L had little if any effect on monocytic differentiation. These findings support a model in which TPA-induced activation of the PKCβ→SAPK pathway contributes to terminal monocytic differentiation by targeting SAPK to Bcl-x_L in mitochondria and inducing cytochrome *c* release, caspase-3 activation and apoptosis.

Materials and Methods

Cell culture and reagents

Human U-937 and TUR,[9] TUR/PKCβ,[11] U-937/Bcl-x_L[26] and U-937/Bcl-x_L (A-47, -115) cells were myeloid leukemia cells were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine. Cells were treated with 50 nM TPA (Sigma Chemical Co.).

Immunofluorescence microscopy

Cells were fixed onto poly D-lysine coated glass coverslips with 3.7% formaldehyde/PBS (pH 7.4) for 10 min, washed with PBS, permeabilized with 0.2% Triton X-100 for 10 min, washed again and incubated for 30 min in complete medium. The coverslips were then incubated with 5 μg/ml of anti-SAPK polyclonal antibody (Santa Cruz) for 1 h followed by Texas Red-goat anti-rabbit IgH (H+L) conjugate (Molecular Probes, Eugene, OR, USA). Mitochondria were stained with 100 nM Mitotracker Green FM (Molecular Probes, OR, USA). Nuclei were stained with 4,6-diamino-2-phenylindole (DAPI; 1 μg/ml in PBS). Coverslips were mounted onto slides with 0.1 M Tris (pH 7.0) in 50% glycerol. Cells were visualized by digital confocal immunofluorescence and images were captured with a cooled CCD camera mounted on a Zeiss Axioplan 2 microscope. Images were deconvolved using Slidebook software (Intelligent Imaging Innovations, Inc., Denver, CO, USA).

Isolation of mitochondrial, cytoplasmic and ER fractions

Cells were washed twice with PBS, and cell fractionation was performed as described.[28,32,33]

Immunoprecipitation and immunoblot analysis

Soluble proteins were subjected to immunoprecipitation with anti-SAPK as described.[25] Immunoblot analysis was performed with anti-SAPK (Santa Cruz), anti-HSP60 (StessGen, Victoria, British Columbia), anti-β-actin (Sigma), anti-calreticulin (StessGen), anti-PDGF receptor (Oncogene), anti-PCNA (Calbiochem), anti-ERK 1/2 (Santa Cruz) and anti-cytochrome *c*.[34]

Immune complex kinase assays

Soluble proteins were incubated with anti-SAPK (Santa Cruz) for 1 h and precipitated with protein A-Sepharose for an additional 1 h. The resulting immune complexes were analyzed for phosphorylation of GST-Jun (2–100) as described.[11]

Apoptosis assays

Cells were washed with PBS, fixed with 80% ethanol and incubated with 2.5 μg/ml propidium iodide and 50 μg/ml RNAse. FACScan (Becton Dickinson) was used to assess cells with sub-G1 DNA content.

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