

JNK- and p38 Kinase-mediated Phosphorylation of Bax Leads to Its Activation and Mitochondrial Translocation and to Apoptosis of Human Hepatoma HepG2 Cells*

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Bong-Jo Kim[‡], Seung-Wook Ryu[§], and Byoung-Joon Song^{‡1}

From the [‡]Laboratory of Membrane Biochemistry and Biophysics, National Institute on Alcohol Abuse and Alcoholism, and the [§]Biochemistry Section, Surgical Neurological Branch, National Institute of Neurological Disorders and Stroke, National Institutes of Health, Bethesda, Maryland 20892-9410

Mitochondrial translocation of pro-apoptotic Bax prior to apoptosis is well established after treatment with many cell death stimulants or under apoptosis-inducing conditions. The mechanism of mitochondrial translocation of Bax is, however, still unknown. The aim of this work was to investigate the mechanism of Bax activation and mitochondrial translocation to initiate apoptosis of human hepatoma HepG2 and porcine kidney LLC-PK1 cells exposed to various cell death agonists. Phosphorylation of Bax by JNK and p38 kinase activated after treatment with staurosporine, H₂O₂, etoposide, and UV light was demonstrated by the shift in the pI value of Bax on two-dimensional gels and confirmed by metabolic labeling with inorganic [³²P]phosphate in HepG2 cells. Specific inhibitors of JNK and p38 kinase significantly inhibited Bax phosphorylation and mitochondrial translocation and apoptosis of HepG2 cells. A specific small interfering RNA to MAPKK4 (the upstream protein kinase of JNK and p38 kinase) markedly decreased the levels of MAPKK4 and MAPKK3/6, blocked the activation of JNK or p38 kinase, and inhibited Bax phosphorylation. However, the negative control small interfering RNA did not cause these changes. Confocal microscopy of various Bax mutants showed differential rates of mitochondrial translocation of Bax before and after staurosporine treatment. Among the Bax mutants, T167D did not translocate to mitochondria after staurosporine exposure, suggesting that Thr¹⁶⁷ is a potential phosphorylation site. In conclusion, our results demonstrate, for the first time, that Bax is phosphorylated by stress-activated JNK and/or p38 kinase and that phosphorylation of Bax leads to mitochondrial translocation prior to apoptosis.

Programmed cell death or apoptosis is an important cellular process that eliminates unwanted cells during normal development or damaged cells after removal of trophic factors or exposure to toxic chemicals. Recent studies have demonstrated that

a variety of apoptosis-stimulating agents cause translocation of pro-apoptotic Bax and BH3 (Bcl-2 homology 3)-only proteins such as Bim and truncated Bid to mitochondria from the cytoplasm to initiate mitochondrion-dependent apoptosis through changing mitochondrial permeability (1–3). Apoptosis is reported to be stimulated by staurosporine (STS)² (4–6); irradiation (4); dexamethasone (4); removal of interleukin-3 (7), interleukin-7 (8), or nerve growth factor (9); vitamin E succinate (10); various chemotherapeutic agents such as etoposide (11) and camptothecin (12); ethanol combined with tumor necrosis factor (13); and others. In contrast, treatment with cell survival factors such as interleukin-7 (8), cAMP (9), and granulocyte/macrophage colony-stimulating factor (14) prevents Bax translocation to mitochondria and the subsequent apoptosis, possibly through activation of the phosphatidylinositol 3-kinase- and Akt/protein kinase B-related cell survival pathway (15). This pathway was recently shown to promote phosphorylation of Bax at Ser¹⁸⁴, followed by its retention in the cytoplasm, thus preventing Bax translocation to mitochondria (14, 16). These results and other reports described below indicate that Bax and other pro-apoptotic proteins may be retained by their interacting proteins present in the cytoplasm in normal physiological states. Bax-interacting proteins identified so far are Bcl-2 and its homologous proteins (17, 18), adenine nucleotide translocator (19), voltage-dependent anion channel protein (20), humanin (21), Ku70 (22), 14-3-3 (23, 24), heat shock protein Hsp60 (25), cofilin (26), Mcl-1 (27), protein kinase Cε (28), Asc (29), etc. Despite the characterization of the Bax-retaining proteins (17–29), it is largely unknown how Bax is activated and then translocated to mitochondria to initiate apoptosis after exposure to cell death stimulants. The aim of this study was to investigate the mechanism of activation and mitochondrial translocation of pro-apoptotic Bax to initiate apoptosis. Our results provide a novel mechanism by which JNK- and p38 mitogen-activated protein kinase (p38 kinase)-mediated phosphorylation of Bax leads to its activation prior to mitochondrial translocation and induction of apoptosis upon exposure to cell death stimulants such as STS and H₂O₂.

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¹ To whom correspondence should be addressed: Laboratory of Membrane Biochemistry and Biophysics, National Institute on Alcohol Abuse and Alcoholism, 9000 Rockville Pike, Bethesda, MD 20892-9410. Tel.: 301-496-3985; Fax: 301-594-3113; E-mail: bjs@mail.nih.gov.

² The abbreviations used are: STS, staurosporine; JNK, c-Jun N-terminal kinase; mAb, monoclonal antibody; MAPKK, mitogen-activated protein kinase kinase; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; DAPI, 4',6-diamidino-2-phenylindole dihydrochloride; XTT, 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide sodium salt; siRNA, small interfering RNA; PVDF, polyvinylidene difluoride; GFP, green fluorescent protein; MEF, mouse embryonic fibroblast.

EXPERIMENTAL PROCEDURES

Materials—Mouse anti-Bax monoclonal antibody (mAb) B9, anti-Hsp60 mAb, rabbit polyclonal antibody specific to MAPKK3/6 (catalog no. sc-13069), major upstream kinases of p38 kinase, and horseradish peroxidase-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology, Inc. Anti-Bax mAbs 6A7 and 2D2 (which recognize activated and total (native + activated) Bax, respectively), CHAPS, STS, 4',6-diamidino-2-phenylindole dihydrochloride (DAPI), Me₂SO, Hoechst 33342, and 2,3-bis(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide sodium salt (XTT; catalog no. Tox-2) were obtained from Sigma. Antibody to cytochrome *c* was from BD Biosciences. A specific small interfering RNA (siRNA) to MAPKK4 (SEK1; catalog no. 51333), the upstream protein kinase of JNK and p38 kinase (30), and a nonspecific negative control No. 1 siRNA (catalog no. 4635) were obtained from Ambion, Inc. (Austin, TX). Other materials not listed here were as described previously (31–34).

Cell Culture, Treatments, and Measurement of Cell Viability—Human hepatoma HepG2 cells and porcine kidney LLC-PK1 cells (purchased from American Type Culture Collection, Manassas, VA) were maintained in minimal essential medium with Earle's salts, 10% (v/v) heat-inactivated fetal bovine serum, 2 mM glutamine, and antibiotics as described (31–34). LLC-PK1 or HepG2 cells (grown in 96-well plates at 2×10^4 cells/well for 1 day) were incubated with 1 μ M or 2 μ M STS, respectively, in minimal essential medium with Earle's salts and 1% fetal bovine serum for the indicated times. Cell viability was determined by XTT reduction. Alternatively, cell death rates were determined by staining with DAPI or Hoechst 33342 (32–34). More than 300 cells in three different areas for each sample were counted by fluorescence microscopy. Data are presented as the means \pm S.D. of four measurements for each group, repeated three times (unless indicated otherwise). Another batch of HepG2 cells was exposed to UV light at 8 or 16 J/m² using a Stratalinker 1800 UV cross-linker (Stratagene) and incubated for an additional 24 or 48 h before cell harvest. Transient transfection of HepG2 cells with each siRNA (50 nM) was performed as described previously (33, 34).

Immunoprecipitation and Two-dimensional Gel Analysis—Freshly harvested HepG2 and LLC-PK1 cells were homogenized with hypotonic buffer (50 mM Tris-HCl (pH 7.4), 1 mM NaF, 100 μ M sodium orthovanadate, and protease inhibitor mixture) with 1% CHAPS. Anti-Bax antibody B9 or 2D2 was used to immunoprecipitate Bax. CHAPS-solubilized proteins (1 mg/sample) were initially incubated with 50 μ l of protein G-agarose (50% suspension) to remove nonspecific binding proteins by following a previously published method (21). The remaining proteins were then incubated with 3 μ g of anti-Bax mAb B9 or 2D2 for 2 h with constant agitation. Protein G-agarose was added to each tube and further incubated for an additional 1 h to facilitate immunoprecipitation. Proteins bound to protein G-agarose were washed three times with 1 \times phosphate-buffered saline and 1% CHAPS, which does not cause conformational change (4), to remove nonspecifically bound proteins. After the final centrifugation, the proteins bound to anti-Bax antibodies and agarose beads were dissolved in two-

dimensional gel buffer (8 M urea, 50 mM dithiothreitol, 2% CHAPS, and 0.5% immobilized pH gradient buffer (pH 3–10)) 30 min before isoelectrofocusing on dry immobilized pH gradient strips (nonlinear gradient of pH 3–10) as described (31) and subjected to immunoblot analysis.

Preparation of Subcellular Fractions and Immunoblot Analysis—Freshly harvested HepG2 or LLC-PK1 cells were used to determine the relative distribution of Bax or cytochrome *c* in the cytosol and mitochondria prepared by differential centrifugation (31). Cells pretreated with STS or H₂O₂ (0.3 mM) were washed twice with 1 \times phosphate-buffered saline, resuspended in isotonic STE buffer (0.25 M sucrose, 50 mM Tris-HCl (pH 7.4), 1 mM EDTA, 1 mM NaF, 0.1 mM sodium orthovanadate, and protease inhibitor mixture) on ice for 10 min, and homogenized, and cell debris and nuclear fractions were removed by centrifugation at $700 \times g$ for 5 min. The supernatant fraction was then subjected to centrifugation at $16,100 \times g$ for 20 min to prepare the cytosolic and mitochondrial fractions. The mitochondrial pellets were washed once with STE buffer to minimize contamination of the cytosolic proteins. After centrifugation of the washed pellets, the mitochondrial pellets were dissolved in hypotonic buffer (50 mM Tris-HCl, pH 7.4) containing 1.0% CHAPS. Cytosolic and solubilized mitochondrial proteins were separated on SDS-polyacrylamide gels, transferred to Immobilon polyvinylidene difluoride (PVDF) membranes, and then incubated with specific antibody to each target protein. The antigen detected by the primary antibody was visualized with the appropriate secondary antibody conjugated to horseradish peroxidase for enhanced chemiluminescence detection (31–34).

Metabolic Labeling and Autoradiography—Intact HepG2 cells grown in culture dishes (150-mm diameter) were metabolically labeled overnight with [³²P]orthophosphoric acid (1 mCi/culture dish; specific activity of >9000 Ci/mmol; PerkinElmer Life Sciences) in phosphate-free Dulbecco's modified Eagle's medium (catalog no. 11971, Invitrogen) with 5% fetal bovine serum and antibiotics. HepG2 cells were then treated with STS (2 μ M) for an additional 8 h before cell harvest and quick freezing in dry ice. Frozen cells were extracted with hypotonic buffer with 1% CHAPS. CHAPS-solubilized proteins (1 mg of protein each) were subjected to immunoprecipitation with anti-Bax mAb B9 or 2D2 for further two-dimensional gel analyses, transfer to Immobilon PVDF membranes, and autoradiography.

Site-directed Mutagenesis and Confocal Microscopic Analysis—Based on the structural prediction of phosphorylation sites in Bax, we prepared various Bax mutants using the QuikChange[®] site-directed mutagenesis kit (Stratagene) following the manufacturer's direction. In addition, some Bax mutants were prepared on a contract basis (GenScript Corp., Piscataway, NJ). The correct nucleotide sequence of each Bax mutant was confirmed by DNA sequencing (data not shown) prior to further analysis. Green fluorescent protein (GFP)-fused wild-type Bax (5) was kindly provided by Dr. Richard J. Youle (National Institutes of Health, Bethesda, MD) and used as a positive control. The plasmid for GFP-P168A Bax (35) was kindly provided by Dr. Christoph Borner (Institute for Molecular Medicine and Cell Research, Freiburg, Germany) and used as a negative control. Mouse embryonic fibroblast (MEF) cells

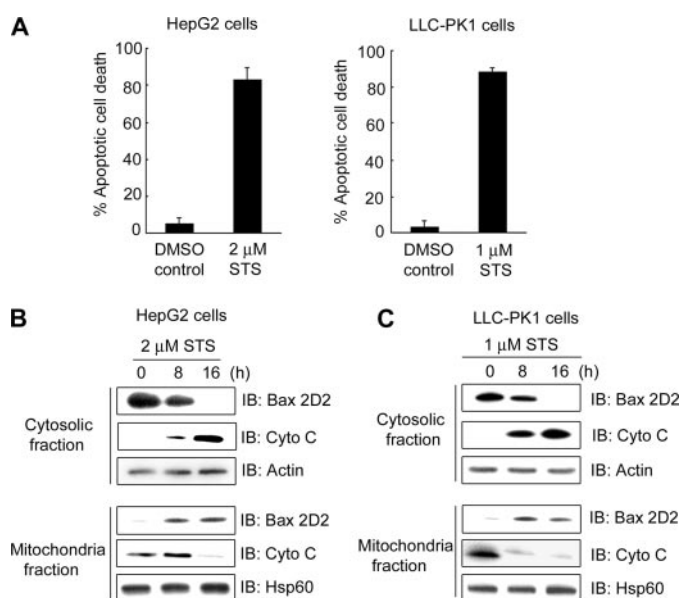


FIGURE 1. Increased rate of cell death and translocation of activated Bax to mitochondria after exposure to STS. HepG2 and LLC-PK1 cells (grown in 96-well microtiter plates) were treated with STS for 24 h before cell death rates were determined by XTT reduction (A). Relative cell death rates are presented. HepG2 (B) and LLC-PK1 (C) cells (grown in large culture dishes) were exposed to 2 and 1 μM STS, respectively, for the indicated times before cell harvest and subcellular fractionation. Equal amounts of cytosolic and mitochondrial proteins (20 μg/well) isolated from HepG2 cells and LLC-PK1 cells were separated on 12% SDS-polyacrylamide gels and then subjected to immunoblot (IB) analysis for various antigens as indicated. DMSO, Me₂SO; Cyto C, cytochrome c.

from Bax/Bak double knock-out mice (36), kindly provided by Dr. Craig B. Thompson (University of Pennsylvania, Philadelphia, PA), were grown in Dulbecco's modified Eagle's medium with 10% fetal bovine serum and transfected with each mutant cDNA. Confocal microscopy of transfected MEF cells with various Bax mutants was performed before and after treatment with STS for 2 or 4 h by the method as described (5).

Statistical Analysis—Most of the experimental data shown were repeated more than three times, unless indicated otherwise. Statistical analysis was performed by Student's *t* test, and *p* < 0.05 was considered significant.

RESULTS

Mitochondrial Translocation of Bax and Apoptosis upon Exposure to STS—We treated human hepatoma HepG2 or porcine kidney LLC-PK1 cells with STS as a cell death stimulant to study the mechanism for translocation of Bax to mitochondria and apoptosis. Consistent with the previous reports (4–6), our results showed that STS caused apoptosis of both HepG2 and LLC-PK1 cells. The morphology of STS-treated HepG2 and LLC-PK1 cells showed cell shrinkage, rounding, and partial detachment, with the lobulated appearance of apoptotic cells and condensed DNA stained with DAPI dye (data not shown). STS significantly increased the rates of cell death determined at 24 h in both cells (Fig. 1A). Translocation of Bax to mitochondria (detected by anti-Bax mAb 2D2) and release of mitochondrial cytochrome *c* to the cytoplasm started to take place at 8 h after STS exposure, whereas cytosolic Bax detected by mAb 2D2 began to decline (Fig. 1, B and C). Increased translocation

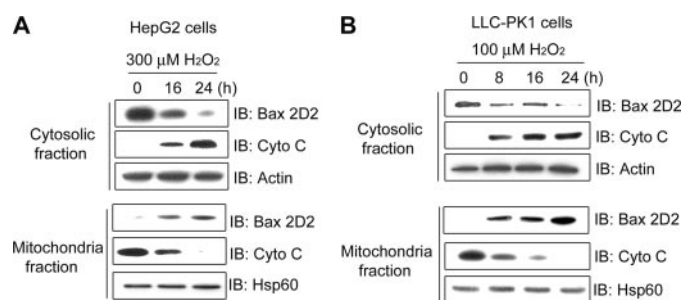


FIGURE 2. Increased translocation of activated Bax to mitochondria after exposure to H₂O₂. HepG2 (A) and LLC-PK1 (B) cells (grown in culture dishes 150 mm in diameter) were exposed to 0.3 and 0.1 mM H₂O₂, respectively, for the indicated times before cell harvest. Equal amounts of cytosolic and mitochondrial proteins (20 μg/well) isolated from HepG2 and LLC-PK1 cells were separated on 12% SDS-polyacrylamide gels and then subjected to immunoblot (IB) analysis for various target proteins. Cyto C, cytochrome c.

of Bax to mitochondria and release of mitochondrial cytochrome *c* were evident in HepG2 cells at 16 h, whereas the levels of actin and Hsp60 (used as loading controls for the cytoplasm and mitochondria, respectively) were comparable in all samples analyzed. We also observed similar patterns of mitochondrial translocation of Bax and cytochrome *c* release from mitochondria to the cytoplasm after HepG2 and LLC-PK1 cells were treated with H₂O₂ for different times (Fig. 2).

JNK- or p38 Kinase-dependent Bax Translocation and Cytochrome *c* Release after STS Treatment—Because of the well established role of JNK and p38 kinase in apoptosis caused by various cell death stimuli (32–34, 37–40), we investigated whether STS activates these cell death-related protein kinases in HepG2 cells. Although STS is a potent broad-spectrum inhibitor of protein kinases, including protein kinase C (41), it activated cell death-related JNK and p38 kinase in a time-dependent manner. The activities of both JNK and p38 kinase, determined by immunoblot analysis using specific antibodies to phosphorylated (activated) forms of both kinases, were increased at 4 and 8 h after STS treatment, whereas the levels of non-phosphorylated (inactive) JNK and p38 kinase were comparable in all samples (Fig. 3A). We used SP600125 (1,9-pyrazoloanthrone), a specific inhibitor of JNK, and SB203580 (4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-1H-imidazole), a selective inhibitor of p38 kinase, to effectively inhibit the activation of JNK and p38 kinase, respectively. When these inhibitors were used at 5 or 10 μM, we observed only partial inhibition of the respective kinase in HepG2 cells. However, nearly complete inhibition of each kinase was observed in the presence of the respective inhibitor at 20 μM or above. Therefore, in subsequent studies, we used inhibitors at 20 μM. This concentration is similar to or less than that used by other investigators (40, 42–44). Both SP600125 and SB203580 at 20 μM effectively inhibited the activation of JNK and p38 kinase, respectively (Fig. 3B). Under our conditions, SP600125 did not inhibit p38 kinase activity (*i.e.* little reduction in the level of phospho-p38 kinase). In addition, SB203580 did not inhibit JNK activity (Fig. 3B). We then studied the effects of selective inhibitors of JNK and p38 kinase on Bax activation and mitochondrial translocation. After exposure to STS, the level of Bax in the cytosol was markedly decreased, whereas the mitochondrial content of Bax was increased (Fig. 3C, upper panels

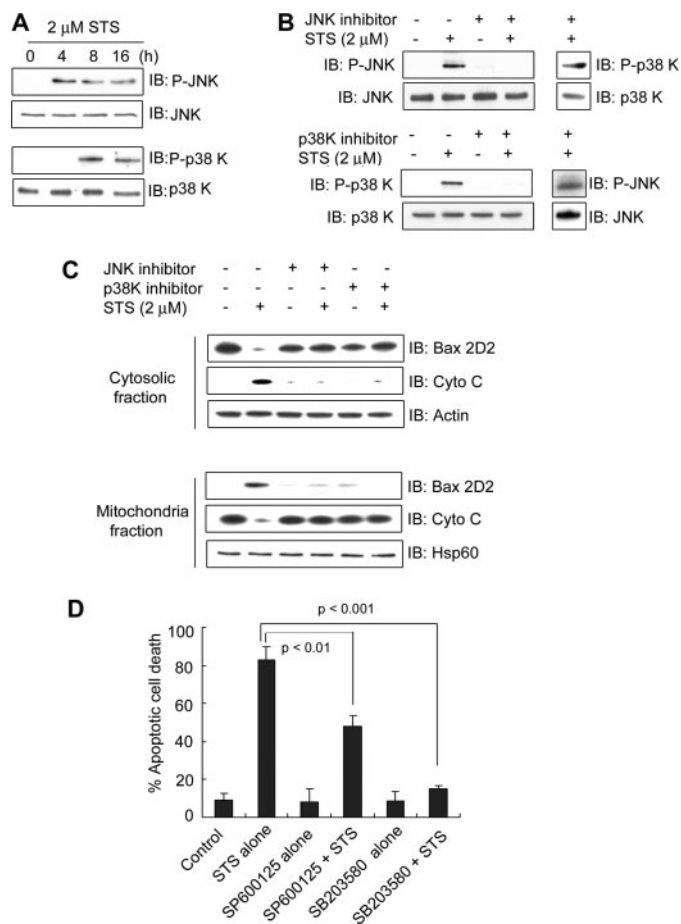


FIGURE 3. Time-dependent activation of JNK and p38 kinase and their roles in Bax translocation in HepG2 cells after exposure to STS. *A*, cytosolic proteins (30 $\mu\text{g}/\text{lane}$) from HepG2 cells treated with 2 μM STS for the indicated times were separated on 12% SDS-polyacrylamide gels and subjected to immunoblot (*IB*) analysis for each target protein as indicated: phospho-JNK (*P-JNK*), JNK, phospho-p38 kinase (*P-p38 K*), or p38 kinase (*p38 K*). *B*, cytosolic proteins (30 $\mu\text{g}/\text{lane}$) from HepG2 cells treated with 2 μM STS in the absence or presence of 20 μM SP600125 or SB203580 were separated on SDS gels and subjected to immunoblot analysis for the antigen indicated. *C*, cytosolic proteins (20 $\mu\text{g}/\text{lane}$) and mitochondrial proteins (30 $\mu\text{g}/\text{lane}$) isolated from HepG2 cells treated for 16 h with different agents as indicated were separated on 12% SDS-polyacrylamide gels and subjected to immunoblot analysis for Bax, cytochrome *c* (*Cyto C*), actin, and Hsp60. *D*, HepG2 cells (grown in 96-well microtiter plates) were treated with 2 μM STS in the absence or presence of 20 μM SP600125 or SB203580 for 24 h before cell death rates were determined by XTT reduction.

for both the cytosolic and mitochondrial fractions, *second lanes*). In contrast, the levels of cytochrome *c* in the cytosol and mitochondria were inversely related to those of Bax after STS treatment (*middle panels, second lanes*). Pretreatment of cells with SP600125 or SB203080 efficiently blocked translocation of Bax and cytochrome *c* release induced by STS (*fourth and sixth lanes, respectively*). In addition, these inhibitors significantly reduced the STS-mediated cell death rates (Fig. 3*D*), although the p38 kinase inhibitor ($p < 0.001$) was more efficient in reducing the rates of STS-mediated cell death than the JNK inhibitor ($p < 0.01$). Similar patterns of JNK- and p38 kinase-mediated translocation of Bax and cytochrome *c* release were also observed in LLC-PK1 cells treated with STS (Fig. 4). These results confirm that both JNK and p38 kinase, activated by STS, are involved in the increased mitochondrial translocation of

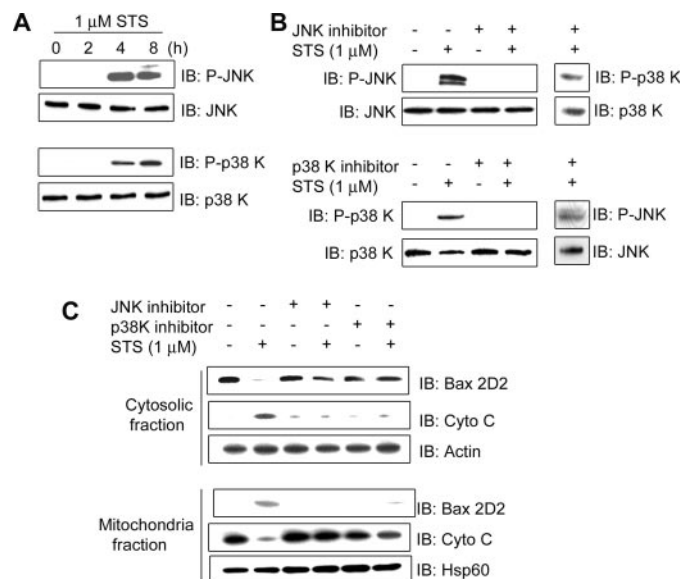


FIGURE 4. Time-dependent activation of JNK and p38 kinase and their roles in Bax translocation in LLC-PK1 cells after STS exposure. *A*, cytosolic proteins (30 $\mu\text{g}/\text{lane}$) from LLC-PK1 cells treated with 1 μM STS for the indicated times were separated on 12% SDS-polyacrylamide gels and subjected to immunoblot (*IB*) analysis for each target protein as indicated: phospho-JNK (*P-JNK*), JNK, phospho-p38 kinase (*P-p38 K*), or p38 kinase (*p38 K*). *B*, cytosolic proteins (30 $\mu\text{g}/\text{lane}$) from LLC-PK1 cells treated with 1 μM STS in the absence or presence of 20 μM SP600125 or SB203580 were separated on SDS gels and subjected to immunoblot analysis for the antigen indicated. *C*, cytosolic proteins (20 $\mu\text{g}/\text{lane}$) and mitochondrial proteins (30 $\mu\text{g}/\text{lane}$) isolated from LLC-PK1 cells treated for 16 h with different agents as indicated were separated on 12% SDS-polyacrylamide gels and subjected to immunoblot analysis for Bax, cytochrome *c* (*Cyto C*), actin, and Hsp60.

Bax, followed by the release of cytochrome *c* to the cytoplasm and cell death.

Evidence for Phosphorylation of Bax after Exposure to STS and Two Other Apoptosis-stimulating Agents—Because of the significant role of JNK and p38 kinase in Bax activation and translocation, we further studied the effect of JNK or p38 kinase on the potential phosphorylation of Bax prior to its mitochondrial translocation. CHAPS-solubilized whole cell homogenates of HepG2 cells treated with either Me_2SO (vehicle control) or STS for 8 h were subjected to immunoprecipitation with anti-Bax mAb B9. Immunoprecipitated Bax was separated on two-dimensional gels and then subjected to immunoblot analysis with anti-Bax mAb 2D2. The immunoblot results revealed that the pI of Bax in Me_2SO -treated control cells was ~ 5.1 (Fig. 5*A, upper panel*), but that the pI shifted to ~ 4.0 without a change in the molecular mass (21 kDa) after STS exposure (*lower panel*), suggesting that Bax might be covalently modified (phosphorylated). Phosphorylation of Bax was confirmed by autoradiography of two-dimensional gels after HepG2 cells were metabolically labeled with [^{32}P]orthophosphoric acid, followed by exposure to Me_2SO (control) or STS for an additional 8 h (Fig. 5*B*). In Me_2SO -treated control cells, no radiolabeled Bax protein was detected (*upper panel*), whereas one ^{32}P -labeled Bax spot (with a pI of 4.0 at 21 kDa) was detected after exposure to STS (*lower panel*). To determine whether Bax phosphorylation by apoptotic inducers is universal, we also studied the effects of two other cell death stimulants, H_2O_2 and etoposide, because these agents are also known to activate JNK and/or p38 kinase (11, 38, 39). The shift in the pI value of Bax

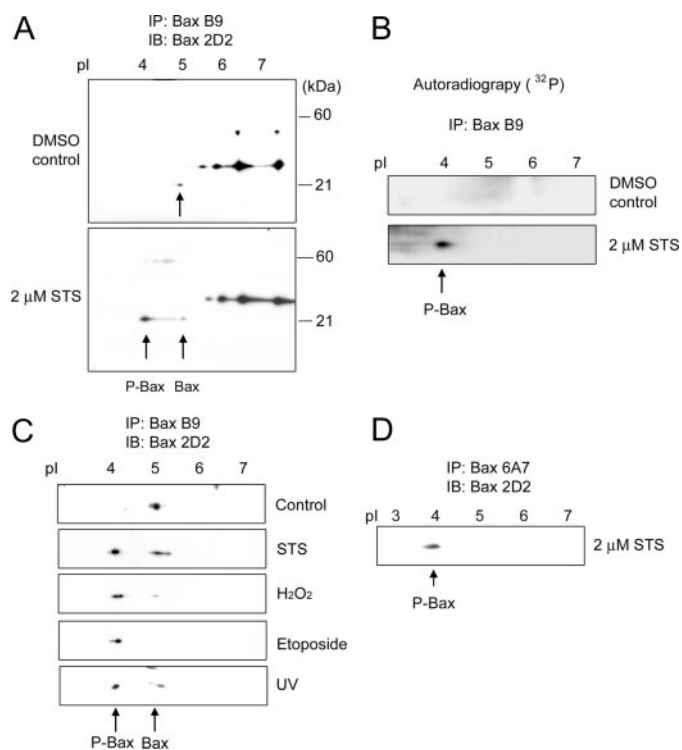


FIGURE 5. Phosphorylation of Bax after exposure to STS, H₂O₂, etoposide, and UV light. *A*, cytosolic proteins (1 mg/sample) from Me₂SO (*DMSO*)-treated control or STS-treated HepG2 cells were incubated with anti-Bax mAb B9. Immunoprecipitated (*IP*) Bax was separated on two-dimensional gels, transferred to Immobilon PVDF membranes, and then subjected to immunoblot (*IB*) analysis with anti-Bax mAb 2D2. Spots representing Bax and phospho-Bax (*P-Bax*) are marked with arrows. Typical pI values are shown at the top, and the molecular masses of marker proteins are indicated on the right. *B*, HepG2 cells (grown in culture dishes 150 mm in diameter) were labeled with [³²P]orthophosphoric acid (1 mCi/150-mm culture dish) in phosphate-free Dulbecco's modified Eagle's medium for 16 h and then exposed to Me₂SO (negative control) or 2 μM STS for an additional 8 h. Harvested HepG2 cells were resuspended in hypotonic buffer with 1% CHAPS. Solubilized proteins (1 mg/sample) were incubated with anti-Bax mAb B9 for 2 h prior to protein G-agarose addition. After three cycles of washing with 1 × phosphate-buffered saline and 1% CHAPS, proteins bound to agarose were analyzed on two-dimensional gels, transferred to Immobilon PVDF membranes, and exposed to x-ray films for autoradiography. The spot for ³²P-labeled phospho-Bax is designated with an arrow. *C*, another batch of HepG2 cells was exposed to Me₂SO (vehicle control), 2 μM STS, 0.3 mM H₂O₂, or 50 μM etoposide for 8 h or to UV light (8 or 16 J/m²) before cell harvest. Cytosolic proteins (1 mg/sample) from each sample were subjected to immunoprecipitation with anti-Bax mAb 2D2, followed by two-dimensional gel analysis and immunoblot analysis. Spots representing Bax and phospho-Bax are marked with arrows. *D*, cytosolic proteins (1 mg protein) from HepG2 cells treated with STS for 16 h were subjected to immunoprecipitation with anti-Bax mAb 6A7, followed by two-dimensional gel analysis and immunoblot analysis with anti-Bax mAb 2D2.

was also observed in extracts of HepG2 cells pretreated with these two toxic compounds as well as UV exposure (Fig. 5C), suggesting a universal phenomenon of Bax phosphorylation by JNK and/or p38 kinase in apoptosis. Activated Bax in STS-treated cells was immunoprecipitated with anti-Bax mAb 6A7, followed by two-dimensional gel analysis and immunoblot analysis with anti-Bax mAb 2D2. A strong signal of activated Bax was detected in the pI 4.0 region after treatment with 2 μM STS for 16 h (Fig. 5D), suggesting that phosphorylation of Bax leads to a conformational change with exposure of the Bax N terminus (activation). In contrast, very small amounts of activated Bax were immunoprecipitated with anti-Bax mAb 6A7 in the Me₂SO-treated cells (data not shown).

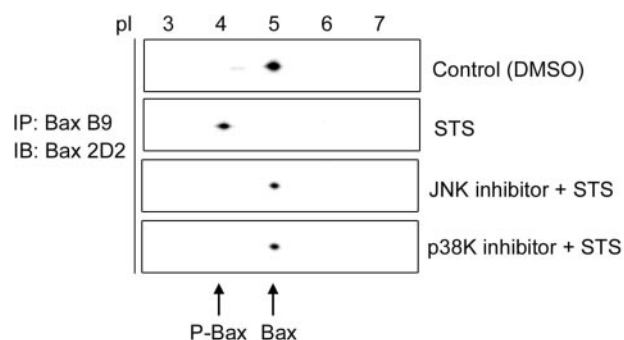


FIGURE 6. JNK- and p38 kinase-mediated phosphorylation of Bax in HepG2 cells. HepG2 cells were treated with Me₂SO (*DMSO*; control) or 2 μM STS in the absence or presence of 20 μM SP600125 or SB203580 for 8 h before harvest. Cytosolic proteins (1 mg/sample) were subjected to immunoprecipitation (*IP*) with anti-Bax mAb B9, followed by two-dimensional gel electrophoresis and immunoblot (*IB*) analysis with anti-Bax mAb 2D2. Spots representing Bax and phospho-Bax (*P-Bax*) are marked with arrows.

JNK- and p38 Kinase-mediated Phosphorylation of Bax—To better understand the phosphorylation and translocation of Bax upon exposure to STS, we studied the direct effect of JNK or p38 kinase on phosphorylation of Bax in intact cells. Consistent with the results in Fig. 5, Bax was not phosphorylated in Me₂SO-treated control cells (pI 5.1) (Fig. 6A, first panel). HepG2 cells treated with STS for 8 h exhibited phosphorylated Bax with its pI shifted to 4.0 (second panel). However, simultaneous incubation with either SP600125, a JNK inhibitor, or SB203580, a p38 kinase inhibitor, effectively blocked the phosphorylation of Bax and its pI shift (third and fourth panels) even though Bax was detected in the region at pI 5.1, similar to that in the Me₂SO-treated control cells.

It is possible that specific chemical inhibitors of JNK or p38 kinase may inhibit the activities of other protein kinases in a nonspecific manner especially at high concentrations (45). However, this was not the case under our conditions (Fig. 3B) because little inhibition of JNK was observed after treatment with the specific inhibitor of p38 kinase and vice versa. To further establish the critical role of JNK and p38 kinase in Bax phosphorylation, we also performed experiments by transfecting HepG2 cells with a specific siRNA to MAPKK4 (SEK1), an upstream protein kinase of JNK and p38 kinase (30), or a negative control siRNA. The specific siRNA to MAPKK4 (SEK1) significantly reduced the level of MAPKK4 at 24 or 48 h (Fig. 7A, upper panel, third and fourth lanes). The negative control siRNA did not change the MAPKK4 (SEK1) level at 24 h (second lane), whereas the levels of actin (used as a loading control) were comparable in all samples (lower panel). The specific siRNA to MAPKK4 also significantly reduced the levels of MAPKK3 and MAPKK6, the major upstream kinases of p38 kinase (Fig. 7A), leading to suppression of p38 kinase signaling. In addition, the specific siRNA to MAPKK4 (SEK1) prevented activation (phosphorylation) of JNK (Fig. 7B, upper panel, sixth lane) and p38 kinase (lower panel, sixth lane) even after exposure to STS. Similar levels of JNK and p38 kinase were detected in all samples (Fig. 7B, upper and lower panels). In contrast, the negative control siRNA did not block phosphorylation of JNK (Fig. 7B, upper panel, fourth lane) and p38 kinase (lower panel, fourth lane) activated by STS treatment. Consistent with these results, phosphorylation of Bax, as determined by the pI shift

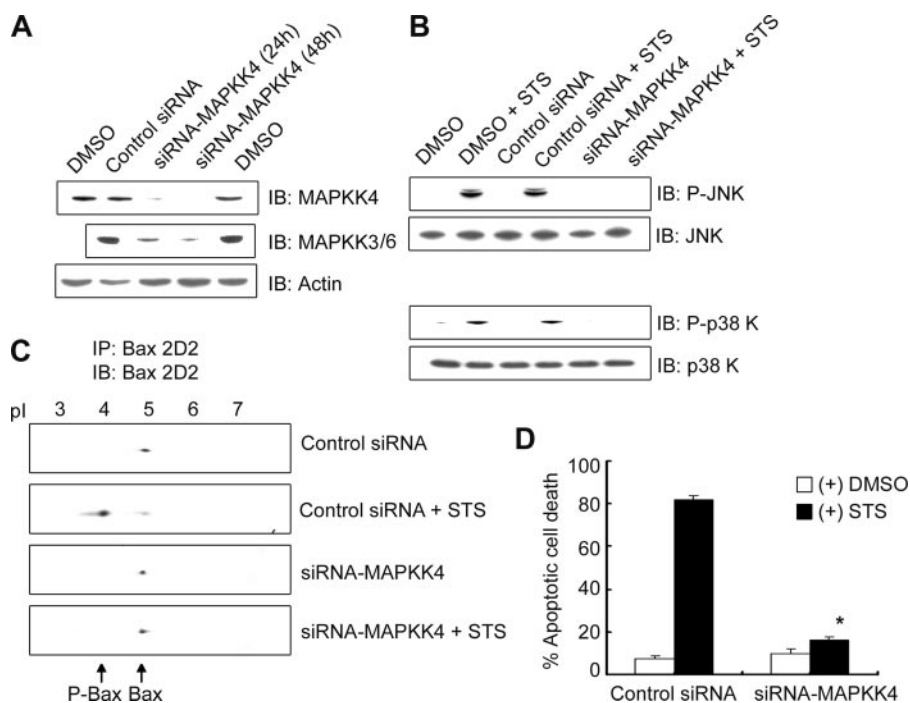


FIGURE 7. Effect of a specific siRNA to MAPKK4 on JNK- and p38 kinase-mediated phosphorylation of Bax in HepG2 cells. *A*, HepG2 cells were transfected with a specific siRNA to MAPKK4 (50 nM) or a nonspecific negative control siRNA and incubated for an additional 24 or 48 h. Transfected HepG2 cells were then treated with Me₂SO (DMSO; vehicle control) or 2 μM STS for an additional 8 h before harvest. Cytosolic proteins (30 μg/lane) from the siRNA-transfected HepG2 cells treated with Me₂SO or STS were separated on 12% SDS-polyacrylamide gels and subjected to immunoblot (IB) analysis for MAPKK4 (SEK1), MAPKK3/6, and actin as indicated. *B*, the same cytosolic proteins (30 μg/lane) were subjected to immunoblot analysis for the respective antigen: phospho-JNK (P-JNK), JNK, phospho-p38 kinase (P-p38 K), or p38 kinase (p38 K). *C*, the same cytosolic proteins (1 mg/sample) were incubated with anti-Bax mAb 2D2 in the presence of protein G-agarose. Immunoprecipitated (IP) Bax was separated on two-dimensional gels, transferred to Immobilon PVDF membranes, and then subjected to immunoblot analysis with anti-Bax mAb 2D2. Spots representing Bax and phospho-Bax (P-Bax) are marked with arrows. Typical pI values are shown at the top. *D*, HepG2 cells were transfected with each siRNA as indicated and treated with Me₂SO (open bars) or STS (closed bars) for an additional 24 h. Cell death rates were then determined by counting >300 cells for each sample after staining with DAPI and are presented as the means ± S.D. *, significantly different compared with the negative control siRNA ($p < 0.0001$). Similar results were also observed upon staining with 2 μM Hoechst 33342 (within 15 min at room temperature). The results represent a typical pattern from two independent experiments.

from 5.1 to 4.0, was blocked by the specific siRNA to MAPKK4 (SEK1) even in the presence of STS (Fig. 7C, fourth panel). The negative control siRNA did not prevent the pI shift of phosphorylated Bax after exposure to STS (second panel). The effects of the transfected siRNAs on cell death rates were then determined by staining with DAPI or Hoechst 33342 with and without STS treatment for 24 h. Approximately 82% of the cells died after STS exposure when HepG2 cells were transfected with the negative control siRNA. However, 16% of the cells died when they were transfected with the specific siRNA to MAPKK4. This value is significantly different from that with the negative control siRNA (Fig. 7D). These results obtained from the siRNA experiments (Fig. 7) are therefore consistent with those obtained from the experiments with specific inhibitors of JNK and p38 kinase (Figs. 3, 4, and 6). All these results demonstrate the important role of JNK or p38 kinase in phosphorylation of Bax prior to its mitochondrial translocation and apoptosis.

Confocal Microscopy of Various Bax Mutants—It is well established that stress-activated JNK and p38 kinase are proline-directed serine/threonine kinases. Therefore, Ser⁸⁷-Pro⁸⁸ and/or Thr¹⁶⁷-Pro¹⁶⁸ of Bax may be a prime candidate for phosphorylation by JNK and/or p38 kinase. Based on this prediction, we

replaced Ser⁸⁷ or Thr¹⁶⁷ of GFP-Bax with Ala by site-directed mutagenesis for confocal microscopy. MEF cells from Bax/Bak double knock-out mice were transfected with each mutant DNA, and the rates of mitochondrial translocation of Bax were determined by confocal microscopy before and after STS treatment for 2 or 4 h. Considerable amounts of transfected wild-type Bax (GFP-Bax), S87A mutant Bax (Fig. 8A), or T167A mutant Bax (data not shown) were associated with mitochondria even without STS treatment. However, the amounts of transfected Bax in mitochondria were significantly increased after STS exposure (see below). Based on these initial results with the Ala mutants and re-examination of the modeled structure of the Bax C terminus (35), we hypothesized that hydrogen bonding between Thr¹⁶⁷ and Trp¹⁷⁰ of Bax may be important in keeping Bax in the cytoplasm by preventing the exposure of the C-terminal hydrophobic transmembrane domain. To test this hypothesis, we replaced Thr¹⁶⁷ with Asp, hoping that the hydroxyl group in the additional carboxyl group of Asp would interact with Trp¹⁷⁰ of the transmembrane domain, thus preventing mitochondrial translocation of this Bax mutant. Confocal microscopy consistently showed that the levels of mitochon-

drial Bax in the S87A mutant and wild-type Bax were significantly increased in STS-treated MEF cells compared with those without STS treatment (Fig. 8, B and C). However, the T167D mutant, which could not be phosphorylated by any protein kinase, was quite resistant to mitochondrial translocation, and the majority of cell bodies were still intact even after treatment with STS (Fig. 8B). In fact, the mitochondrial contents of Bax in the T167D mutant were not significantly increased by STS treatment. This result was similar to that observed with the P168A mutant (Fig. 8C). Furthermore, two-dimensional gel analysis did not show any acidic shift in the T167D and P168A mutants even after STS treatment (Fig. 8D). In contrast, wild-type Bax and the S87A mutant were phosphorylated after STS exposure and thus detected in the acidic pI region (Fig. 8D). All these results strongly indicate that Thr¹⁶⁷ is a key amino acid potentially phosphorylated by stress-activated JNK and p38 kinase.

DISCUSSION

It is well established that pro-apoptotic Bax is activated with a conformational change prior to being translocated to mitochondria and initiation of the mitochondrion-dependent cell death process after exposure to various cell death stimulants,

including STS (1–7). Biochemical, mutational, and structural analyses also suggested that the N- and C-terminal domains of Bax interact with each other and thus prevent its mitochondrial translocation in unstimulated states (46–48). However, it is still unknown how Bax is activated and then translocated to mitochondria after cells are exposed to various cell death stimulants. Several mechanisms for activation of Bax prior to its translocation have been proposed. First, an internal pH change may be responsible for Bax activation and conformational change, followed by mitochondrial translocation after treatment with cell death agonists (8, 49). Second, caspase-dependent activation of Bax may lead to Bax translocation (23, 46). Third, rapid degradation of Bax-retaining proteins such as Ku70 (22) and Mcl-1 (27) may cause Bax release and translocation to mitochondria. Fourth, interaction with truncated Bid or Bim may be responsible for activation of Bax (36, 50). Fifth, suppression of the phosphatidylinositol 3-kinase- and Akt/protein kinase B-related cell survival pathway causes Bax translocation, as demonstrated recently (51, 52). Sixth, JNK-mediated phosphorylation of Bax-retaining proteins causes Bax release, as demonstrated with phosphorylated 14-3-3 (24). Finally, reduction or degradation of β -tubulin content may cause Bax translocation after treatment with specific antisense oligonucleotides (53) or vincristine (54). Each of these hypotheses has a considerable amount of supporting evidence, but is not always observed in different model systems. For instance, little change in Bax conformation despite pH fluctuation (47) and caspase-independent Bax translocation (9, 13) have been reported. We have also observed that STS, alcohol, or acetaminophen did not reduce the Ku70 level in ethanol-sensitive E47 HepG2 cells with transduced human CYP2E1 (13, 31), although Bax translocation to mitochondria, followed by apoptosis, was clearly observed.³ These results suggest that the mechanism of Bax activation and translocation depends largely on the cell types studied, the microenvironment of the cells, the cell death agonist used in each experiment, or a combination of these factors. Furthermore, our results provide evidence for another mechanism of Bax activation through JNK- or p38 kinase-mediated phosphorylation of Bax accompanied by exposure of its N terminus.

JNK and p38 kinase are proline-directed serine/threonine kinases. These protein kinases are frequently activated after cells are exposed to toxic chemicals and/or environmental stress such as UV exposure and γ -ray irradiation or after removal of trophic factors. This signaling cascade has been proposed as a mediator of stress-induced cell death (32–34, 37–39, 55–57). An earlier study suggested that Bax itself is not phosphorylated before and after its translocation to mitochondria (47). However, recent data strongly suggest that Bax may be phosphorylated by JNK for its activation because Bax was not activated in JNK-deficient fibroblasts upon exposure to environmental stress (38). Alternatively, Bax may be activated through p38 kinase-mediated phosphorylation because its mitochondrial translocation and cell death rate were significantly reduced by the specific inhibitor of p38 kinase, whereas

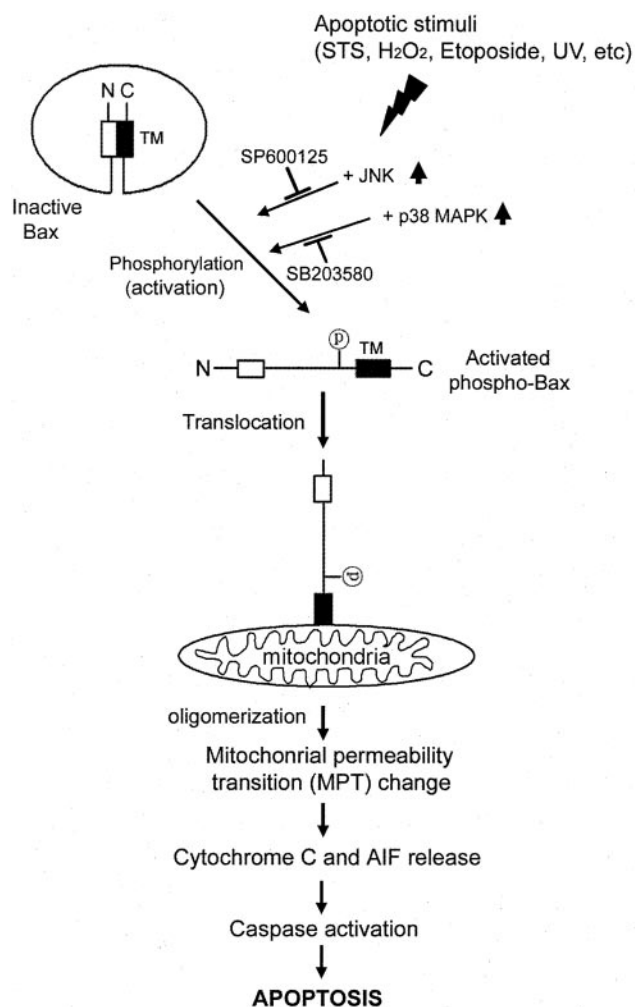


FIGURE 9. Proposed model for mitochondrion-dependent apoptosis through JNK- and p38 kinase-mediated phosphorylation of Bax. In normal physiological states, inactive Bax stays alone or is retained by its binding proteins in the cytoplasm. After treatment with STS or other cell death stimulants, Bax is phosphorylated by stress-activated JNK and/or p38 kinase, leading to exposure of its N terminus (activation) and conformational change. The subsequent availability of its C-terminal hydrophobic transmembrane (TM) domain likely results in translocation of activated Bax to mitochondria to initiate mitochondrion-dependent apoptosis. MAPK, mitogen-activated protein kinase.

they were promoted by transfection of MAPKK3, a major upstream kinase of p38 kinase (40). In contrast, Akt-mediated phosphorylation of Bax at Ser¹⁸⁴ caused its retention in the cytoplasm after cells were treated with granulocyte/macrophage colony-stimulating factor (14) or nicotine (16). Despite these conflicting reports, there were no follow-up studies on phosphorylation of Bax by JNK or p38 kinase and its role in Bax activation prior to mitochondrial translocation and apoptosis. Our results provide evidence that various apoptosis-inducing agents such as STS, H₂O₂, etoposide, and UV exposure promote Bax phosphorylation through activation of JNK and/or p38 kinase (Figs. 3–7). Phosphorylation of Bax, confirmed by metabolic labeling with [³²P]orthophosphoric acid, caused a marked pI shift after exposure to various cell death stimulants. The pI shift (via phosphorylation) of Bax was blocked by the specific inhibitor of JNK or p38 kinase and the specific siRNA to MAPKK4. Furthermore, confocal microscopy of various Bax

³ Y. M. Lee, B. J. Kim, and B. J. Song, unpublished data.

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mutants, including the T167D mutant (Fig. 8), provided direct evidence for potential phosphorylation of Bax by stress-activated protein kinases prior to its mitochondrial translocation. This post-translational modification was observed only by two-dimensional gel analyses because the mobility of phosphorylated Bax on one-dimensional SDS gels was not altered, unlike that of phosphorylated Mcl-1 (55) and Bcl-2 (56), which migrate slower than their non-phosphorylated counterparts on one-dimensional SDS-polyacrylamide gels. Our results also show that phosphorylation of Bax leads to a conformational change (activation) with exposure of its N-terminal domain, which is then recognized and immunoprecipitated by anti-Bax mAb 6A7. Our results regarding phosphorylation of Bax by stress-activated protein kinases likely represent a novel mechanism by which Bax is activated prior to mitochondrial translocation and apoptosis. Furthermore, our results are consistent with many previous results and thus help to explain the molecular basis for the important role of JNK and/or p38 kinase in translocation of Bax to mitochondria and the subsequent apoptosis (10, 13, 37–39, 57).

Recent reports have shown that Akt-mediated phosphorylation of Bax at Ser¹⁸⁴ is important in the cytosolic retention of Bax following treatment of cells with anti-apoptotic agents (14, 16). In addition, Ser¹⁶³ of Bax was reported to be phosphorylated by glycogen synthase-3 β during neuronal apoptosis (58). Because JNK and p38 kinase are proline-directed Ser/Thr protein kinases, we believe that the specific amino acid residues that are phosphorylated by JNK and/or p38 kinase must be different from Ser¹⁸⁴ or Ser¹⁶³. Analysis using the NetPhos software program (available at www.cbs.dtu.dk/services/NetPhos) suggested that various Bax Ser and Thr residues, including Ser⁸⁷-Pro⁸⁸ and/or Thr¹⁶⁷-Pro¹⁶⁸, may be good candidates for phosphorylation by JNK and/or p38 kinase. This prediction is consistent with recent results regarding various Bax mutants (Pro¹⁶⁸ replaced with Ala, Gly, or Glu), which were not translocated to mitochondria (35). Confocal microscopy of the various Bax mutants, including T167D and P168A (Fig. 8), provided evidence that Thr¹⁶⁷ is a critical amino acid and can be phosphorylated by stress-activated JNK and/or p38 kinase.

On the basis our current results and thoughts, we propose the following model of Bax activation and mitochondrial translocation prior to apoptosis after STS treatment (Fig. 9). In the unstimulated state, Bax is likely to stay alone or to be retained by its binding proteins in the cytoplasm (22–29). Bax is phosphorylated by stress-activated JNK and/or p38 kinase when cells are exposed to STS or other cytotoxic agents. Phosphorylation of Bax leads to a conformational change with exposure of its N terminus (activation). Phosphorylation and activation of Bax with exposure of its N terminus are likely to disturb the previous interaction between the N and C termini of Bax (46–48), resulting in the exposure of the C-terminal transmembrane domain needed for mitochondrial translocation. After activated Bax is translocated to mitochondria, it is oligomerized with itself (homo-oligomerization) and Bcl-2 or other family members such as Bak (hetero-oligomerization), making a larger permeability transition pore. This leads to changes in mitochondrial permeability transition prior to the release of mitochondrial cytochrome *c*, apoptosis-inducing factor, and other

proteins to the cytoplasm (1–3, 59). Increased amounts of cytochrome *c* and apoptosis-inducing factor in the cytoplasm activate various caspases. Following activation of caspases, cells become committed to terminal apoptotic processes.

In conclusion, to our knowledge, this is the first report providing direct evidence that JNK- and p38 kinase-mediated phosphorylation of Bax leads to its activation with the exposure of its N terminus and possibly its C-terminal transmembrane domain. This leads to mitochondrial translocation of activated Bax and initiation of mitochondrion-dependent apoptosis of cells treated with a variety of cell death stimulants.

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