(JBC in press: Epub as of May 18, 2006 as manuscript M510644200)

JNK- and p38 Kinase-Mediated Phosphorylation of Bax Leads to its Activation, Mitochondrial Translocation and Apoptosis of Human Hepatoma HepG2 Cells

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Running title: Phosphorylation and activation of Bax prior to apoptosis

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Mitochondrial translocation of proapoptotic 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 current research is to investigate the mechanism of Bax activation and mitochondrial translocation to initiate apoptosis in human hepatoma HepG2 and porcine kidney LLC-PK1 cells exposed to various cell death agonists. Phosphorylation of Bax, through JNK and p38 kinase activated after exposure to staurosporine, H_2O_2, etoposide and UV, was demonstrated by the shift in the pI value of Bax on 2-D gels and confirmed by metabolic labeling with [^{32}P]-inorganic phosphate in HepG2 cells. Specific inhibitors of JNK and p38 kinase significantly inhibited Bax phosphorylation, mitochondrial translocation of Bax, and apoptosis in HepG2 cells. A specific siRNA to mitogen activated protein kinase kinase isoform 4 (MAPKK4), upstream 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 siRNA did not cause these changes. Confocal microscopy of various Bax mutants showed differential rates of mitochondrial translocation of Bax before and after STS treatment. Among the Bax mutants, Thr167Asp mutant did not translocate to mitochondria after STS exposure, suggesting that Thr^{167} is a potential phosphorylation site. In conclusion, our current 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 to eliminate unwanted cells during normal development or damaged cells after removal of trophic factors or exposure to toxic chemicals. Recent studies demonstrate that a variety of apoptosis-stimulating agents cause translocation of proapoptotic Bax and BH3-only proteins such as Bim or truncated Bid to mitochondria from cytoplasm to initiate mitochondria-dependent apoptosis through changing mitochondrial permeability (1-3). Apoptosis is reported to be stimulated

by: staurosporine (STS) (4-6); irradiation (4); dexamethasone (4); removal of IL-3 (7), IL-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 IL-7 (8), cAMP (9) and granulocyte-macrophage colony stimulating factor (14) prevented Bax translocation to mitochondria and subsequent apoptosis, possibly through activation of phosphatidylinositol-3-kinase (PI-3-kinase) and Akt/protein kinase B (PKB)-related cell survival pathway (15). This pathway was recently shown to promote phosphorylation at Ser^{184} of Bax followed by its retention in 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 under 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 60 (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 proapoptotic Bax to initiate apoptosis. Our results provide a novel mechanism by which c-Jun *N*-terminal protein kinase (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 H_2O_2.

EXPERIMENTAL PROCEDURES

Materials - Mouse anti-Bax B9 monoclonal antibody (mAb), anti-Hsp60 mAb, rabbit polyclonal antibody specific to MAPKK3/6 (catalog no. sc-13069), major upstream kinases of p38 kinase, and horse radish peroxidase (HRP)-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology. Anti-Bax mAb 6A7 and mAb 2D2, which recognize the activated and total (native + activated) Bax, respectively, 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), STS, 4',6-diamidino-2-phenylindole dihydrochloride (DAPI), dimethyl sulfoxide (DMSO), Hoechst 33342, and 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide sodium salt (XTT, catalog number X-4751) were obtained from Sigma Chemicals (St. Louis, MO). Antibody to cytochrome c was from BD Bioscience. A specific siRNA to mitogen activated protein kinase kinase isoform 4 (MAPKK4; SEK1; catalog no. 51333), upstream protein kinase of JNK and p38 kinase (30), and a non-specific negative control #1 siRNA (catalog no. 4635) were obtained from Ambion (Austin, Texas). Other materials not listed here were as same as previously described (31-34).

Cell Culture, Treatments, and Measurement of Cell Viability - HepG2 human hepatoma cells and LLC-PK1 porcine kidney cells, purchased from American Type Culture Collection

(Manassas, VA), were maintained in minimal essential media with Earl's salts (MEM), 10% (v/v) heat-inactivated fetal bovine serum (FBS), 2 mM glutamine, and antibiotics, as described (31-34). HepG2 cells or LLC-PK1 cells, grown in 96-well plates (2 x 10^4 cells/well) for 1 day, were incubated with 1 μM (LLC-PK1 cells) or 2 μM STS (HepG2 cells) in MEM with 1% FBS for indicated times. Cell viability was determined with 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 under fluorescent microscopy. Data are presented with averages with standard deviation (four measurements for each group and repeated three times, unless otherwise stated). Another batch of HepG2 cells was exposed to UV light at 8 or 16 Joule/m^2 by using a Stratalinker 1800 device (Stratagene) and incubated for additional 24 h or 48 h before cell harvest. Transient transfection of HepG2 cells with 50 nM of each siRNA was performed, as previously described (33).

Immunoprecipitation and 2-D Gel Analysis - Freshly harvested HepG2 and LLC-PK1 cells were homogenized with a hypotonic buffer (50 mM Tris-HCl, pH 7.4, 1 mM NaF, 100 μM sodium orthovanadate, and protease inhibitor cocktail) with 1% CHAPS. Specific anti-Bax antibodies B9 or 2D2 were used to immunoprecipitate Bax. CHAPS-solubilized proteins (1 mg/sample) were initially incubated with 50 μl of protein G-agarose (50% suspension) to remove non-specific binding proteins by following the published method (21). The remaining proteins were then incubated with 3 μg 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 additional 1 h to facilitate immunoprecipitation. Proteins bound to protein G-agarose were washed three times with 1 x phosphate buffered saline (PBS) buffer and 1% CHAPS, which does not cause conformational change (4), to remove non-specifically bound proteins. After the final centrifugation, the proteins bound to anti-Bax antibodies and agarose beads were dissolved in the 2-D gel buffer (8 M urea, 50 mM DTT, 2% CHAPS, 0.5% immobilized pH gradient (IPG) buffer, pH 3 – 10) 30 min before the isoelectrofocusing on dry IPG strips (non-linear pH gradient of 3 – 10), as reported (31) and subjected to immunoblot analysis.

Preparation of Subcellular Fractions and Immunoblot Analysis - Freshly harvested HepG2 or LLC-PK1 cells were used to determine relative distribution of Bax or cytochrome c in cytosol and mitochondria prepared by differential centrifugation (31). Cells pretreated with STS or H_2O_2 (0.3 mM) were washed twice in 1 x PBS, resuspended in isotonic STE buffer (0.25 M sucrose, 50 mM Tris-HCl, pH 7.4 and 1 mM EDTA, 1 mM NaF, 0.1 mM sodium orthovanadate and protease inhibitor cocktails) on ice for 10 min, homogenized, cell debris and nuclear fraction removed by centrifugation at 700 x *g* for 5 min. The supernatant fraction was then subjected to centrifugation at 16,100 x *g* for 20 min to prepare the cytosolic and mitochondrial fractions. The mitochondrial pellets were washed once with the extraction buffer to minimize contamination of the cytosolic proteins. After centrifugation of the washed pellets, the mitochondrial pellets were dissolved in the 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 PVDF-Immobilon 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 with HRP for enhanced chemiluminescence (ECL) detection (31-34).

Metabolic Labeling and Autoradiography - Intact HepG2 cells grown in culture dishes (diameter 150 mm) were metabolically labeled with 1 mCi/culture dish of [^{32}P]ortho-phosphoric acid (specific activity >9,000 Ci/mmol from Perkin-Elmer, Boston, MA) overnight in phosphate-free Dulbecco's modified Eagle medium (DMEM) (Invitrogen catalog #11971) with 5% FBS and antibiotics. HepG2 cells were then treated with STS (2 μM) for additional 8 h before cell harvest and quick freeze in dry ice. Frozen cells were extracted with the hypotonic buffer with 1% CHAPS. CHAPS-solubilized proteins (1 mg protein each) were used for immunoprecipitation with anti-Bax mAb B9 or 2D2 for further 2-D gel analyses, transfer to PVDF-Immobilon 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 by using QuikChange® site-directed mutagenesis kit following the manufacturer's direction (Stratagene) or custom-made at Genscript Corp (Piscataway, New Jersey). The correct nucleotide sequence of each Bax mutant was confirmed by DNA sequencing (data not shown) prior to further analysis. The wild type Bax-conjugated with green fluorescence protein (GFP) (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-Pro168Ala Bax mutant (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 fibroblasts (MEF) from Bax/Bak double knockout mice (36), kindly provided by Dr. Craig B. Thompson, University of Pennsylvania (Philadelphia, PA), were grown in DMEM with 10% FBS 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 otherwise indicated. 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. 1*A*). Translocation of Bax to mitochondria, detected by anti-Bax mAb 2D2 and release of mitochondrial cytochrome c to cytoplasm started to take place while cytosolic Bax detected by mAb 2D2 began to decline at 8 h after STS exposure (Figs. 1*B* and 1*C*). Increased translocation of Bax to mitochondria and release of mitochondrial cytochrome c were evident in HepG2 cells at 16 h while the levels of actin and Hsp60, used as loading controls for 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 cytoplasm after HepG2 and LLC-PK1 cells were treated with H_2O_2 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 activated 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 while the levels of the non-phosphorylated (inactive) JNK or p38 kinase were comparable in all samples (Fig. 3*A*). We used SP600125, a specific inhibitor of JNK, and SB203580, 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 only observed partial inhibition of the respective kinase in HepG2 cells. However, near 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 20 μM inhibitors. This concentration is similar 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. 3*B*). Under our conditions, SP600125 did not inhibit the p38 kinase activity (i.e. little reduction of the level of phospho-p38 kinase). In addition, SB203580 did not inhibit the JNK activity (Fig. 3*B*). 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 while the mitochondrial content of Bax was increased (Fig. 3*C*, lane 2, top panels for both cytosol and mitochondrial fractions). In contrast, the levels of cytochrome c in the cytosol and mitochondria were inversely related to those of Bax after STS treatment (lane 2, middle panels). Pretreatment of cells with SP600125 or SB203080 efficiently blocked translocation of Bax and cytochrome c release induced by STS (lanes 4 and 6, respectively). In addition, these inhibitors significantly reduced the STS-mediated cell death rates (Fig. 3*D*), although the p38 kinase inhibitor was more efficient ($p<0.001$) 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 support that both JNK and p38 kinase, activated by STS, were involved in the increased mitochondrial translocation of Bax followed by the release of cytochrome c to 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 potential phosphorylation of Bax prior to its mitochondrial translocation. CHAPS-solubilized whole cell homogenates of HepG2 cells, treated with either DMSO (vehicle control) or STS for 8 h, were subjected to immunoprecipitation with anti-Bax mAb B9. Immunoprecipitated Bax was separated on 2-D gels and then subjected to immunoblot analysis with anti-Bax mAb 2D2. Immunoblot results revealed that pI of Bax in DMSO-treated control cells was approximately 5.1 (Fig. 5*A*, top) but shifted to approximately 4.0 without changes in its molecular mass (21 kDa) after STS exposure (Fig. 5*A*, bottom), suggesting that Bax might be covalently modified (phosphorylated). Phosphorylation of Bax was confirmed by autoradiography of 2-D gels after HepG2 cells were metabolically labeled with [^{32}P]ortho-phosphoric acid followed by exposure to DMSO (control) or STS for additional 8 h (Fig. 5*B*). In DMSO-treated control cells, no radiolabeled Bax protein was detected (top panel) while one ^{32}P-labeled Bax spot (with a pI of 4.0 and 21 kDa) was detected after exposure to STS (bottom 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 since these agents are also known to activate JNK and/or p38 kinase (11, 38, 39). The shift in the pI value of Bax was also observed in extracts of HepG2 cells pretreated with these two toxic compounds as well as UV exposure (Fig. 5*C*), suggesting a universal phenomenon of Bax phosphorylation by JNK and/or p38 kinase in apoptosis. The activated Bax in STS-treated cells was immunoprecipitated with anti-Bax mAb 6A7 followed by 2-D gel analysis and immnublot analysis with anti-Bax 2D2 antibody. A strong signal of activated Bax was detected in the pI 4.0 region after treatment with 2 μM STS for 16 h (Fig. 5*D*), suggesting that phosphorylation of Bax leads to a conformational change with an exposure of the Bax *N*-terminus (activation). In contrast, very little amounts of activated Bax were immunoprecipitated with anti-Bax 6A7 antibody in the DMSO-treated cells (data not shown).

JNK and p38 Kinase-Mediated Phosphorylation of Bax - In order to better understand 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 DMSO-treated control cells (pI 5.1, Fig. 6*A*, top 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 at the region of pI 5.1, similar to that in the DMSO-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 non-specific manner especially at high concentrations (45). However, this was not the case under our conditions (Fig. 3B), since 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 on Bax phosphorylation, we also performed experiments by transfecting HepG2 cells with a specific siRNA to MAPKK4 (SEK1), 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, top panel, lanes 3 and 4). The negative control siRNA did not change the MAPKK4 (SEK1) level at 24 h (lane 2) while the levels of actin used as a loading control were comparable in all samples (bottom panel). The specific siRNA to MAPKK4 also significantly reduced the levels of MAPKK3/6, the major up-stream kinases of p38 kinase (Fig. 7A), leading to suppression of the p38 kinase signaling. In addition, the specific siRNA to MAPKK4 (SEK1) prevented activation (phosphorylation) of JNK (Fig. 7B, top panel, lane 6) and p38 kinase (bottom panel, lane 6) even after exposure to STS. Similar levels of JNK or p38 kinase were respectively detected in all samples (Fig. 7B, top and bottom panels). In contrast, the negative control siRNA did not block phosphorylation of JNK (Fig. 7B, top panel, lane 4) and p38 kinase (Fig. 7B, bottom panel, lanes 4) activated by STS treatment. Consistent with these results, phosphorylation of Bax, as determined by the pI shift 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 (Fig. 7C, 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 cells died after STS exposure when HepG2 cells were transfected with the negative control siRNA. However, 16% of 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 (Fig. 7) with the siRNA experiments are therefore consistent with those of 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 Ser^{87}-Pro and/or Thr^{167}-Pro of Bax may be prime candidates for phosphorylation by JNK and/or p38 kinase. Based on this prediction, we replaced Ser^{87} or Thr^{167} of GFP-Bax wild type with Ala by site-directed mutagenesis for confocal microscopy. MEF cells from Bax/Bak double-knockout mice were transfected with each mutant DNA and the rates of mitochondrial translocation of Bax determined by confocal microscopy

before and after STS treatment for 2 h or 4 h. Considerable amounts of the transfected wild type Bax (GFP-Bax), Ser87Ala mutant (Fig. 8A), or Thr167Ala mutant (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 Bax C-terminus (35), we hypothesized that hydrogen bonding between Thr^{167} and Trp^{170} of Bax may be important in keeping Bax in the cytoplasm by preventing the exposure of the C-terminal hydrophobic transmembrane (TM) domain. To test this hypothesis, we replaced Thr^{167} with Asp, hoping that the hydroxyl group in the additional carboxyl group of Asp, may interact with Trp^{170} of the TM domain, thus preventing mitochondrial translocation of this Bax mutant. Confocal microscopy consistently showed that the levels of mitochondrial Bax in the Ser87Ala mutant and wild type Bax were significantly increased in STS-treated MEF cells, compared with those without STS (Fig. 8B and 8C). However, the Thr167Asp mutant, which can not be phosphorylated by any protein kinase, was quite resistant to mitochondrial translocation and the majority of cell bodies still intact even after treatment with STS (Fig. 8B). In fact, the mitochondrial contents of Bax in the Thr167Asp mutant were not significantly increased by STS treatment. This result was similar to that observed with the Pro168Ala mutant (Fig. 8C). Furthermore, 2-D gel analysis did not show any acidic shift in the Thr167Asp and Pro168Ala mutants even after STS treatment (Fig. 8D). In contrast, the wild type Bax and the Ser87Ala mutant were phosphorylated after STS exposure and thus identified in the acidic pI region (Fig. 8D). All these results strongly indicate that Thr^{167} is a key amino acid potentially phosphorylated by stress-activated JNK and p38 kinase.

DISCUSSION

It is well established that proapoptotic Bax is activated with a conformational change prior to being translocated to mitochondria and initiation of mitochondria-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*-terminal and *C*-terminal domains of Bax interact with each other and thus prevent its mitochondrial translocation in un-stimulated 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 PI-3 kinase and Akt/PKB-related cell survival pathway causes Bax translocation, as recently demonstrated (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 anti-sense 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 instances, 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, 32), although Bax translocation to mitochondria followed by apoptosis was clearly observed (unpublished results). These results suggest that the mechanism of Bax activation and translocation depends largely on cell types studied, microenvironment of the cells, and cell death agonist used in each experiment or combination of these factors. Furthermore, our current 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 gamma ray irradiations or after removal of trophic factors. This signaling cascade has been proposed as a mediator of stress-induced cell death (33,34,37-39,55-57). An earlier study suggested that Bax itself was 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, since its mitochondrial translocation and cell death rate were significantly reduced by the specific inhibitor of p38 kinase while they were promoted by transfection of MAPKK3, a major upstream kinase of p38 kinase (40). In contrast, Akt-mediated phosphorylation of Ser^{184} of Bax 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 current results provide evidence that various apoptosis-inducing agents such as STS, H_2O_2, 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 [^{32}P]ortho-phosphoric acid, causes 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. Furthermore, confocal microscopy of various Bax mutants including the Thr167Asp 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 only

observed by 2-D gel analyses, because the mobility of phosphorylated Bax on 1-D SDS gels was not altered, unlike those of the phosphorylated Mcl-1 (55) and Bcl-2 (56) that migrated slower than their un-phosphorylated counterparts on 1-D SDS-polyacrylamide gels. Our results also showed 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 6A7 mAb. Our results about phosphorylation of Bax by stress-activated protein kinases are likely to 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 subsequent apoptosis (10,13,37-39,57).

Recent reports have shown that Akt-mediated phosphorylation of Bax at Ser^{184} is important in cytosolic retention of Bax after treating cells with anti-apoptotic agents (14, 16). In addition, Ser^{163} of Bax was reported to be phosphorylated by glycogen synthase - 3β during neuronal apoptosis (58). Because JNK and p38 kinase are proline-directed Ser/Thr protein kinases, we believe that specific amino acid residues that are phosphorylated by JNK and/or p38 kinase must be different from Ser^{184} or Ser^{163}. Analysis through the NetPhos software program (www.cbs.dtu.dk/services/NetPhos) suggests that various Ser and Thr residues including Ser^{87}-Pro and/or Thr^{167}-Pro of Bax may be good candidates for phosphorylation by JNK and/or p38 kinase. This prediction is consistent with the recent results about the various Bax mutants (Pro^{168} replaced with Ala, Gly, or Glu), which was not translocated to mitochondria (35). Confocal microscopy of the various Bax mutants including the Thr167Asp and Pro168Ala mutants (Fig. 8) provide evidence that Thr^{167} is a critical amino acid, which can be phosphorylated by stress-activated JNK and/or p38 kinase.

Based on 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 (AIF) and other proteins to cytoplasm (1-3, 59). Increased amounts of cytochrome c and AIF in cytoplasm activate various caspases. Following activation of

caspases, cells become committed to terminal apoptosis processes.

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

ACKNOWLEDGMENTS

We are grateful to Drs. I. James Lee and James P. Hardwick for critical reading of this manuscript. We thank Drs. Richard J. Youle, Christoph Borner, and Craig B. Thompson for providing GFP-Bax wild type, Pro168Ala Bax mutant, and MEF cells from Bax/Bak double-knockout mice, respectively, used in our study. We also appreciate Drs. Norman Salem, Jr. and Richard J. Youle for their support. This study was supported by the Intramural Research Program of the National Institute on Alcohol Abuse and Alcoholism and the Gift Fund from SK Chemicals in Korea.

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FOOTNOTES

The abbreviations used are: AIF, apoptosis inducing factor; CHAPS, 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate; DAPI, 4',6-diamidino-2-phenylindole dihydrochloride; DMEM, Dulbecco's modified Eagle medium; DMSO, dimethyl sulfoxide; ECL, enhanced chemiluminescence; FBS, fetal bovine serum; HRP, horse radish peroxidase; Hsp, heat shock protein; IPG, immobilized pH gradient; JNK, c-Jun *N*-terminal protein kinase; mAb, monoclonal antibody; MAPKK3/6, mitogen activated protein kinase kinase isoform 3 and 6; MAPKK4, mitogen activated protein kinase kinase isoform 4; MEF, mouse embryonic fibroblasts; MEM, minimal essential media with Earl's salts; p38 kinase; p38 mitogen activated protein kinase; pAb, polyclonal antibody; PBS, phosphate buffered saline; PI-3-kinase, phosphatidylinositol-3-kinase; SB203580, 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1H-

imidazole; SP600125, 1,9-pyrazoloanthrone; STS, staurosporine; TM, transmembrane; XTT, 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide sodium salt.

FIGURE LEGENDS

Fig. 1. Increased rate of cell death and translocation of activated Bax to mitochondria after exposure to STS. *A*, 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 the method of XTT reduction. Relative cell death rates are presented. (*B*) HepG2 cells and (*C*) LLC-PK1 cells, grown in large culture dishes, were exposed to 2 and 1 μM STS, respectively, for indicated times before cell harvest and subcellular fractionation. Equal amounts of cytosolic and mitochondrial proteins (20 μg/well) isolated from HepG2 cells (*B*) and LLC-PK1 cells (*C*) were separated on 12% SDS-polyacrylamide gels and then subjected to immunoblot analysis for various antigens as indicated.

Fig. 2. Increased translocation of activated Bax to mitochondria after exposure to H_2O_2. HepG2 and LLC-PK1 cells, grown in culture dishes (150 mm diameter), were exposed to 0.3 and 0.1 mM H_2O_2, respectively, for indicated times before cell harvest. Equal amounts of cytosolic and mitochondrial proteins (20 μg/well) isolated from HepG2 cells (*A*) or LLC-PK1 cells (*B*) were separated on 12% SDS-polyacrylamide gels and then subjected to immunoblot analysis for various target proteins.

Fig. 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 μg/lane) from HepG2 cells treated with 2 μM STS for indicated times were separated by 12% SDS-polyacrylamide gels and subjected to immunoblot analysis for each target protein as indicated: phospho-JNK, JNK, phospho-p38 kinase, or p38 kinase. (*B*) Cytosolic proteins (30 μg/lane) from HepG2 cells, treated with 2 μM STS in the absence or presence of 20 μM of SP600125 or SB203580, were separated on SDS gels and subjected to immunoblot analysis for the antigen indicated. (*C*) Cytosolic proteins (20 μg/lane) and mitochondrial proteins (30 μg/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 : Bax, cytochrome c, actin, or 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.

Fig. 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 μg/lane) from LLC-PK1 cells treated with 1 μM STS for indicated times were separated by 12% SDS-polyacrylamide gels and subjected to immunoblot analysis for each target protein as indicated: phospho-JNK, JNK, phospho-p38 kinase, or p38 kinase. (*B*) Cytosolic proteins (30 μg/lane) from LLC-PK1 cells treated with 1 μM STS in the absence or presence of 20 μM of SP600125 or SB203580 were separated on SDS gels and subjected to immunoblot analysis for the antigen indicated. (*C*) Cytosolic proteins (20 μg/lane) and

mitochondrial proteins (30 μg/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: Bax, cytochrome c, actin, or Hsp60.

Fig. 5. Phosphorylation of Bax after exposure to STS, H_2O_2, etoposide, and UV exposure. (*A*) Cytosolic proteins (1 mg/sample) from DMSO-treated control or STS-treated HepG2 cells were incubated with anti-Bax mAb B9. The immunoprecipitated Bax was separated on 2-D gels, transferred to PVDF-Immobilon membranes, and then subjected to immunoblot analysis with anti-Bax mAb 2D2 antibody. Spots representing Bax and phospho-Bax are marked with arrows. Typical pI values are shown at the top and molecular weights (kDa) of marker proteins are indicated on the right. (*B*) HepG2 cells, grown in culture dishes (150 mm diameter), were labeled with [^{32}P]ortho-phosphoric acid (1 mCi/150 mm culture dish) in phosphate-free DMEM for 16 h and then exposed to DMSO (negative control) or 2 μM STS for additional 8 h. Harvested HepG2 cells were resuspended in a 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 x PBS and 1% CHAPS, proteins bound to agarose were analyzed on 2-D gels, transferred to PVDF-Immobilon membranes, and exposed to x-ray films for autoradiography. The spot for ^{32}P-labeled phospho-Bax is designated with an arrow. (*C*) Another set of HepG2 cells was exposed to DMSO (vehicle control), 2 μM STS, 0.3 mM H_2O_2, 50 μM etoposide for 8 h, or UV exposure (8 or 16 Joule/m^2) before harvesting cells. Cytosolic proteins (1 mg/sample) from each sample were subjected to immunoprecipitation with anti-Bax mAb 2D2 followed by 2-D gel analysis and immunoblot analysis. Spots representing Bax and phospho-Bax are marked with arrows. (*D*) Cytosolic proteins (1 mg protein) from STS-treated HepG2 cells for 16 h were subjected to immunoprecipitation with anti-Bax mAb 6A7 followed by 2-D gel analysis and immunoblot analysis with anti-Bax mAb 2D2.

Fig. 6. JNK- and p38 kinase-mediated phosphorylation of Bax in HepG2 cells. HepG2 cells were treated with 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 with anti-Bax mAb B9, 2-D gel electrophoresis, and immunoblot analysis with anti-Bax mAb 2D2. Spots representing Bax and phospho-Bax are marked with arrows.

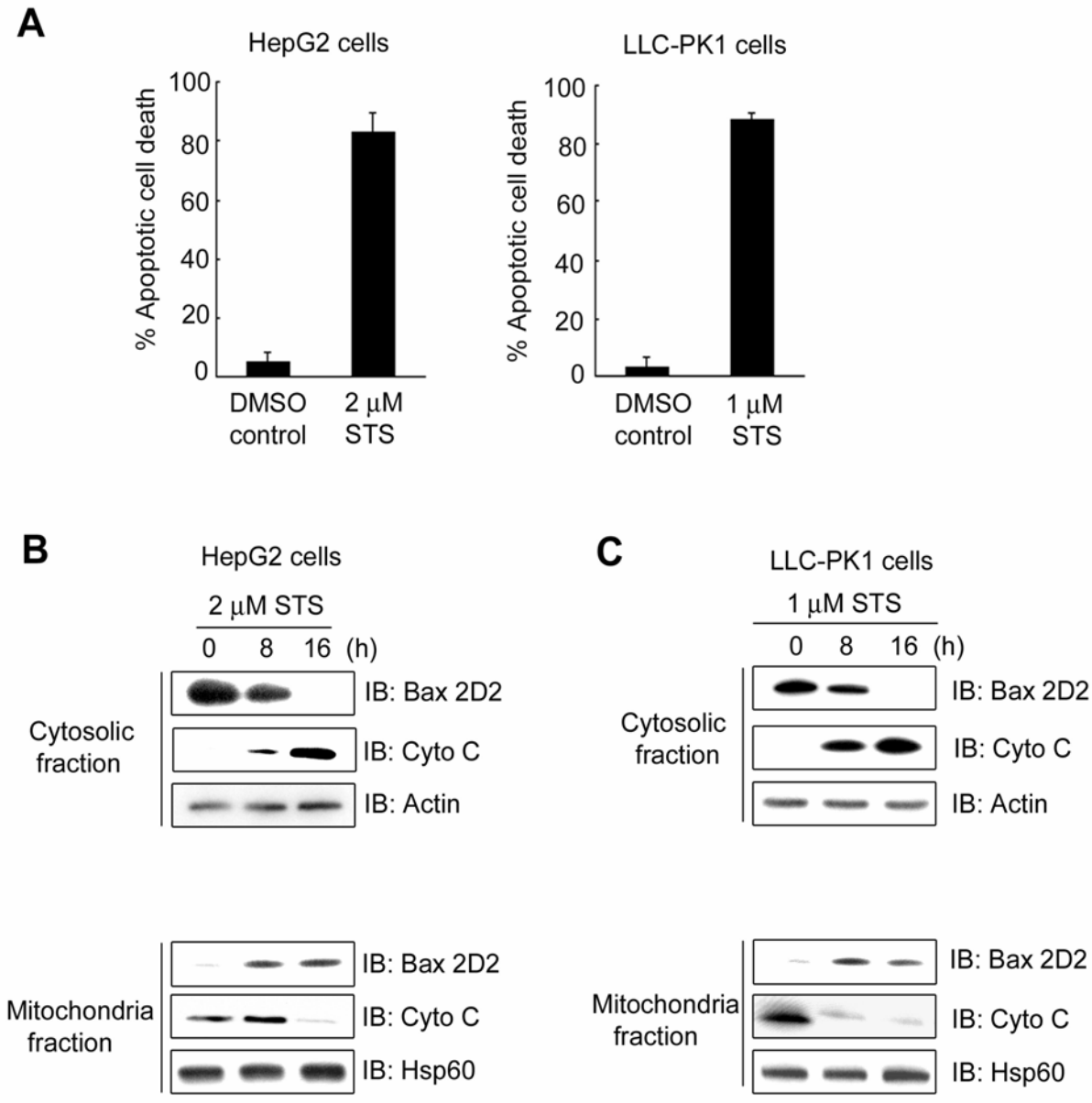
Fig. 7. Effect of a specific siRNA to MAPKK4 on JNK- and p38 kinase-mediated phosphorylation of Bax in HepG2 cells. (*A*) HepG2 cells were transfected with 50 nM of a specific siRNA to MAPKK4 or a non-specific negative control siRNA and incubated for additional 24 or 48 h. Transfected HepG2 cells were then treated with DMSO (vehicle control) or 2 μM STS for additional 8 h before harvest. (*A*) Cytosolic proteins (30 μg/lane) from the siRNA-transfected HepG2 cells treated with DMSO or STS were separated by 12% SDS-polyacrylamide gels and subjected to immunoblot analysis for MAPKK4 (SEK1), MAPKK3/6, or actin, as indicated. (*B*) The same cytosolic proteins (30 μg/lane) were subjected to immunoblot analysis for the respective antigen: phospho-JNK, JNK, phospho-p38 kinase, or p38 kinase. *(C)* The same cytosolic proteins (1 mg/sample) were incubated with anti-Bax mAb 2D2 in the presence of protein G agarose.

The immunoprecipitated Bax was separated on 2-D gels, transferred to PVDF-Immobilon membranes, and then subjected to immunoblot analysis with anti-Bax mAb 2D2 antibody. Spots representing Bax and phospho-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 for DMSO (open) or STS (closed) for additional 24 h. Cell death rates were then determined by counting more than 300 cells for each sample after staining with DAPI and presented with the average ± SD. *, Significantly different from that with the negative control siRNA ($p<0.0001$). Similar results were also observed with staining with 2 μM Hoechst 33342 (within 15 min at room temperature). These results in this figure represent a typical pattern from two independent experiments.

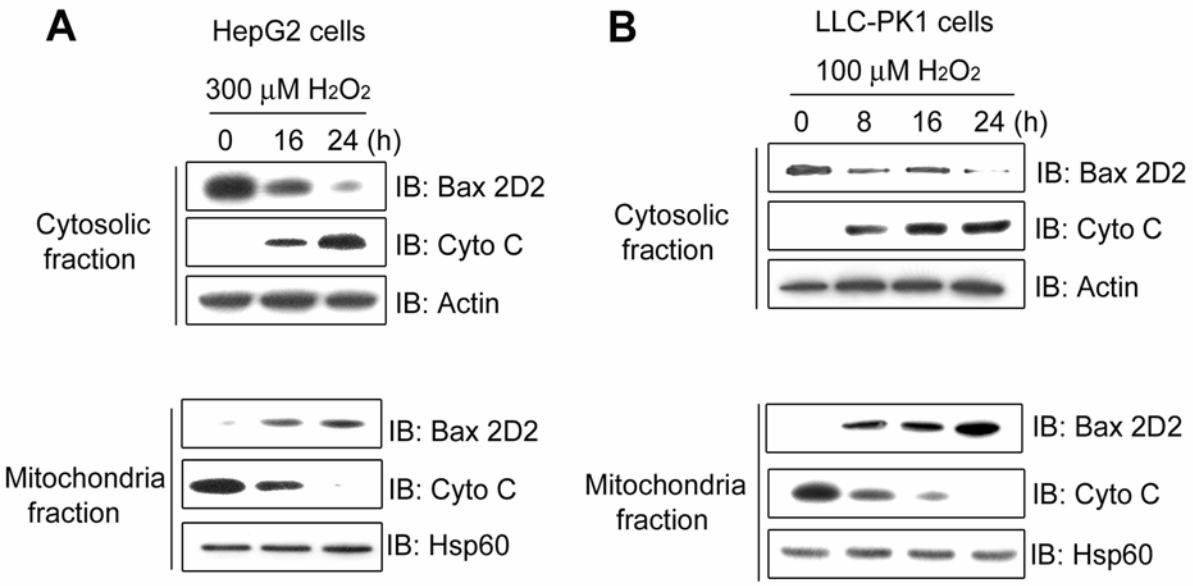
Fig. 8. Confocal microscopy for mitochondrial translocation of various Bax mutants in the absence and presence of STS. MEF cells from Bax/Bak double-knockout mice were grown in DMEM and transfected with each mutant cDNA (1 μg each/chamber) as indicated: wild type GFP-Bax (Wt) used as a positive control, GFP-Pro168Ala mutant (P168A) used as a negative control, Ser87Ala (S87A), and Thr167Asp (T167D). The confocal microscopy was performed before *(A)* and after *(B)* treatment with 2 μM STS. Mitochondria were visualized with *Discosoma sp*-red fluorescent protein (DsRed-Mito) shown in the third column. *(C)* The rates of mitochondrial translocation of various Bax mutants in the absence or presence of 2 μM STS were estimated by confocal microscopy image, as described (5). This result represents a typical data of three independent experiments which showed similar patterns. *(D)* CHAP-solubilized proteins (0.5 mg/sample) from MEF cells, transfected with different Bax cDNA as indicated and treated with 2 μM STS for 4 h, were immunoprecipiated with anti-Bax 2D2 antibody in the presence of protein G agarose. The immunoprecipitated Bax was separated on 2-D gels and subjected to immunoblot analysis as described in Fig. 7 legend.

Fig. 9. Proposed model for mitochondria-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. Subsequent availability of its C-terminal hydrophobic transmembrane (TM) domain is likely to result in translocation of activated Bax to mitochondria to initiate mitochondria-dependent apoptosis.

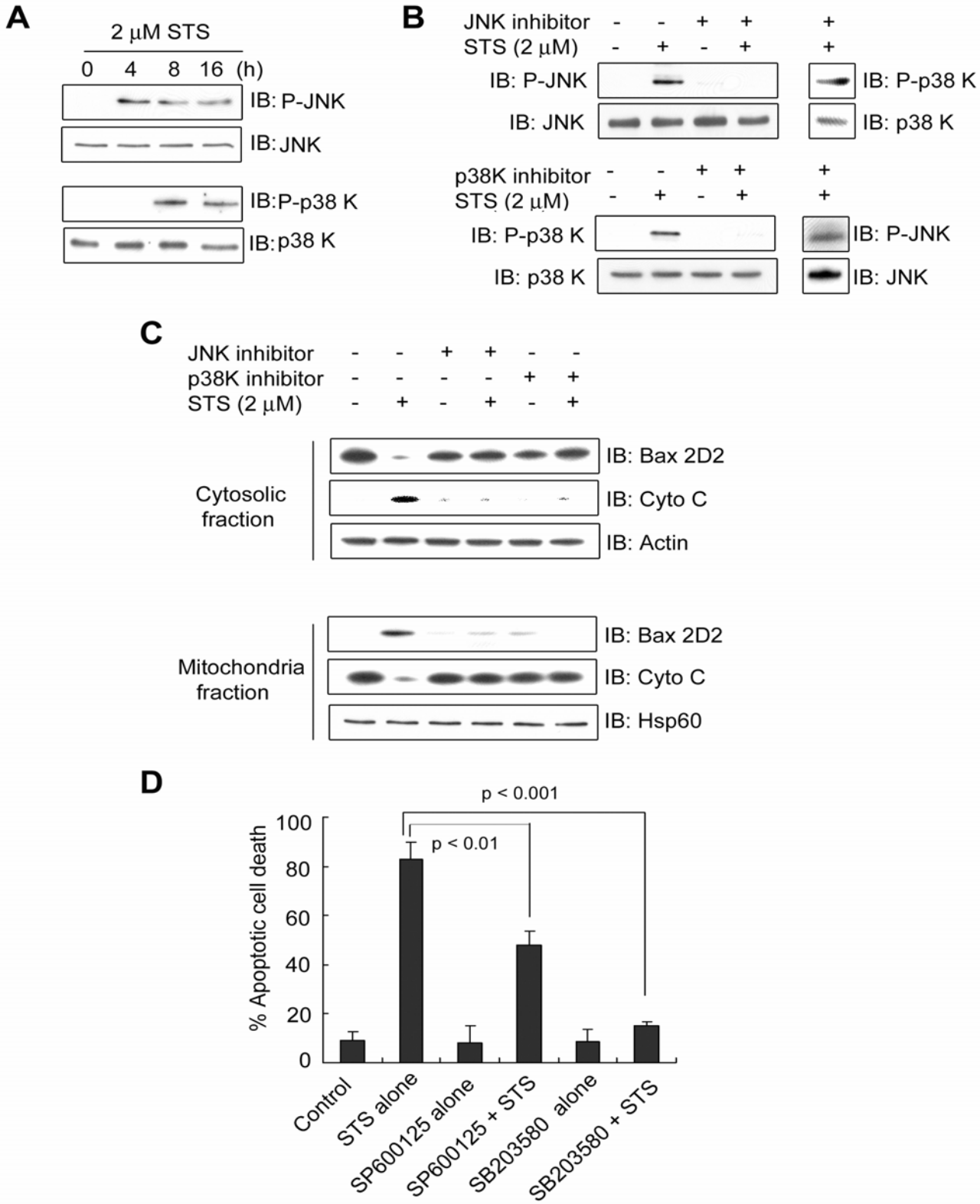
Kim et al, Fig. 1



Kim et al, Fig. 2



Kim et al, Fig. 3



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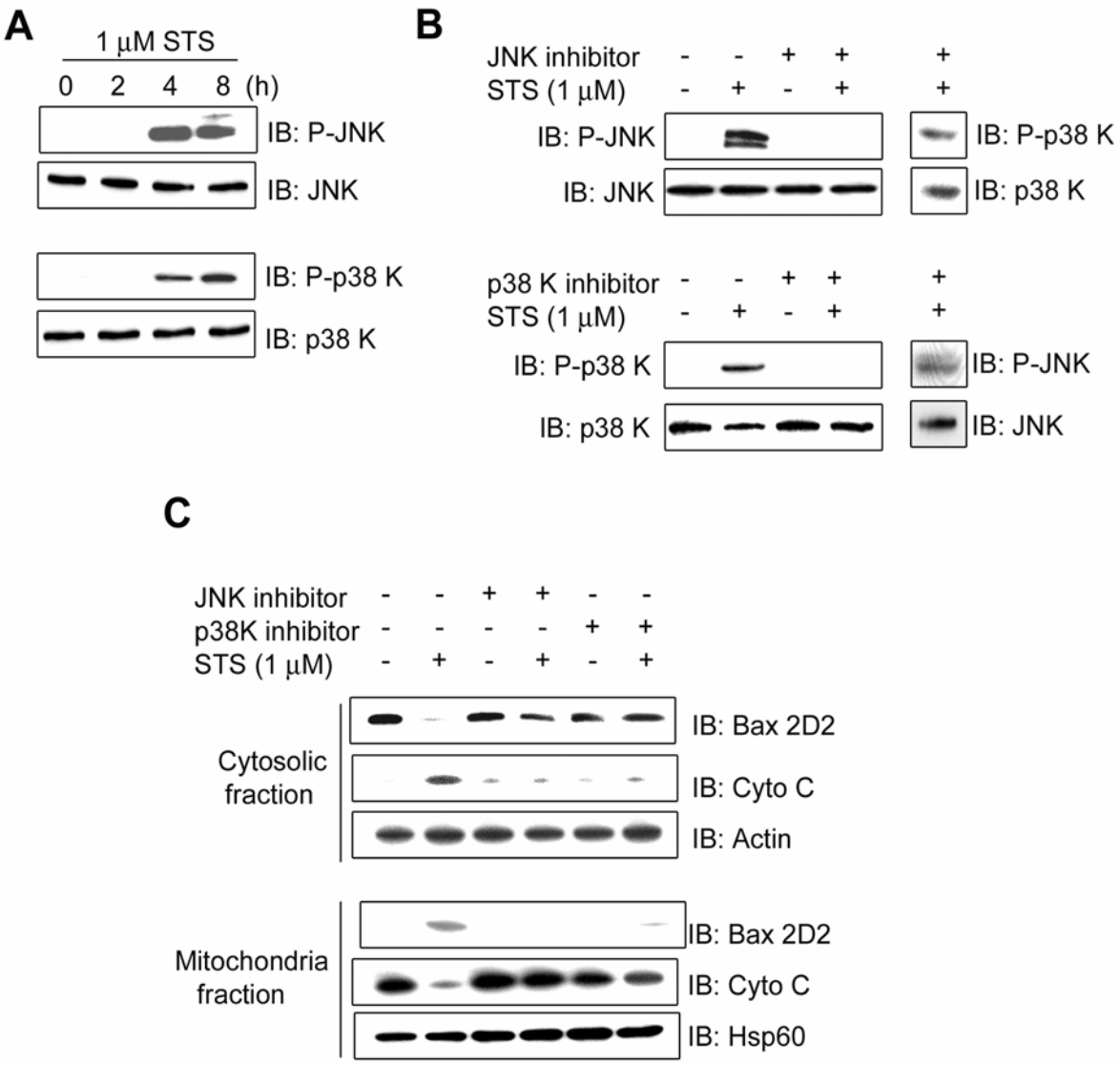

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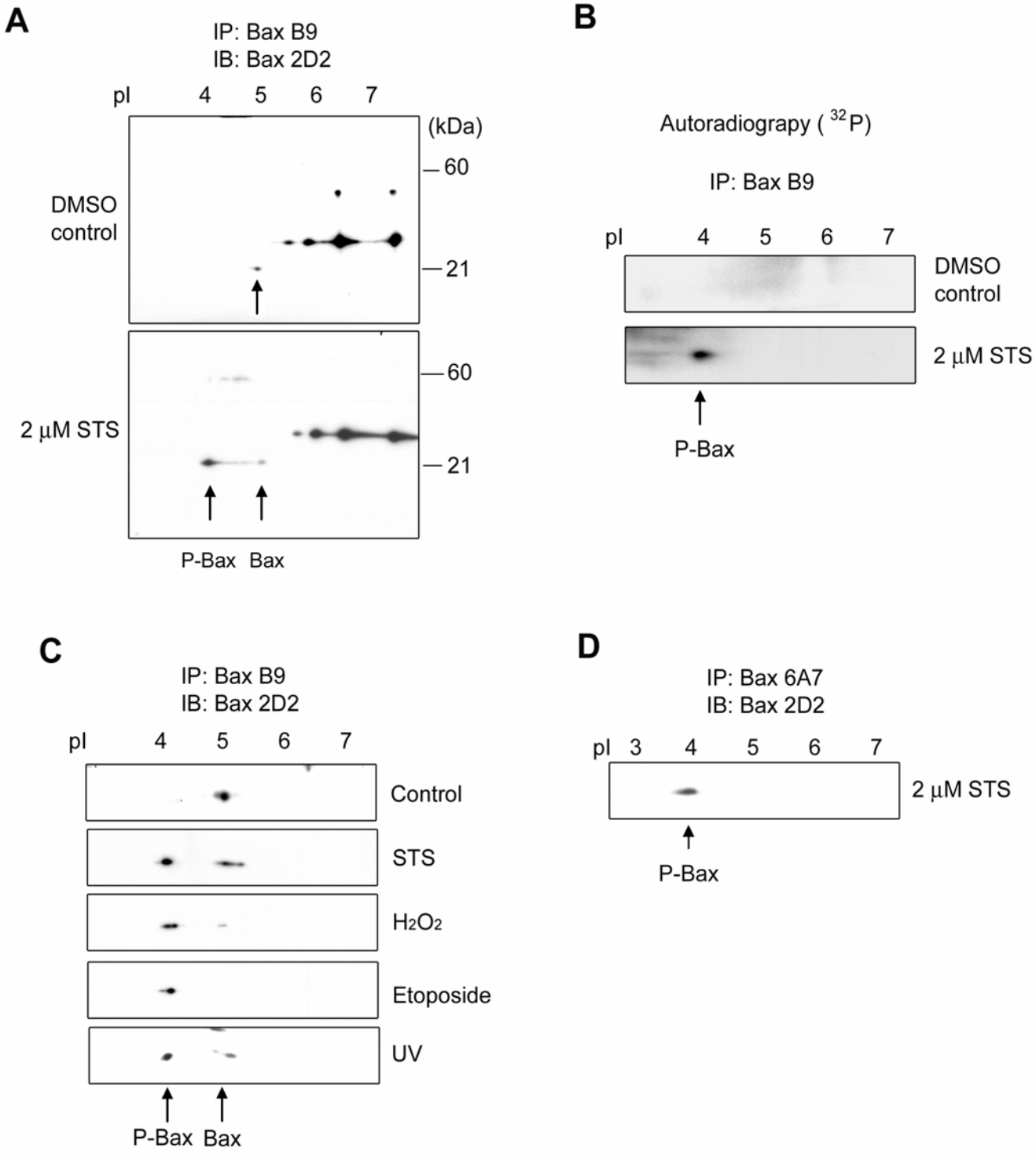

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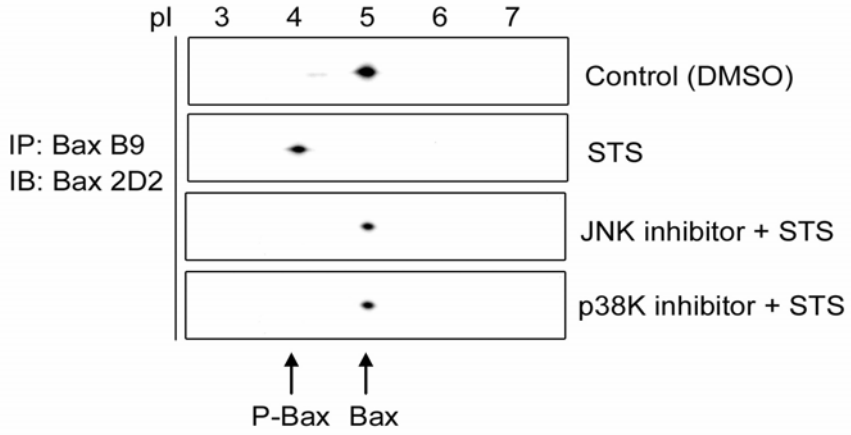

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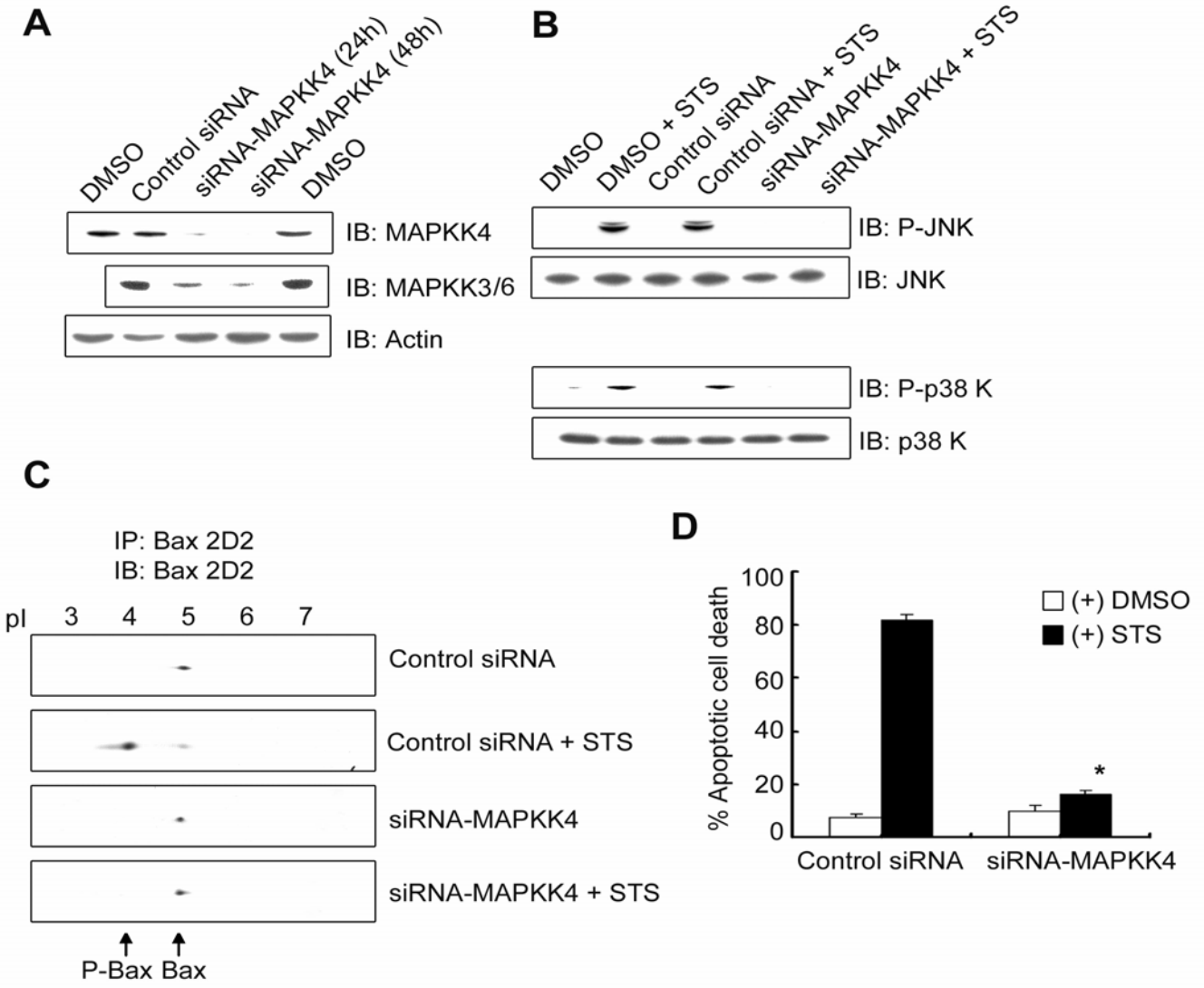

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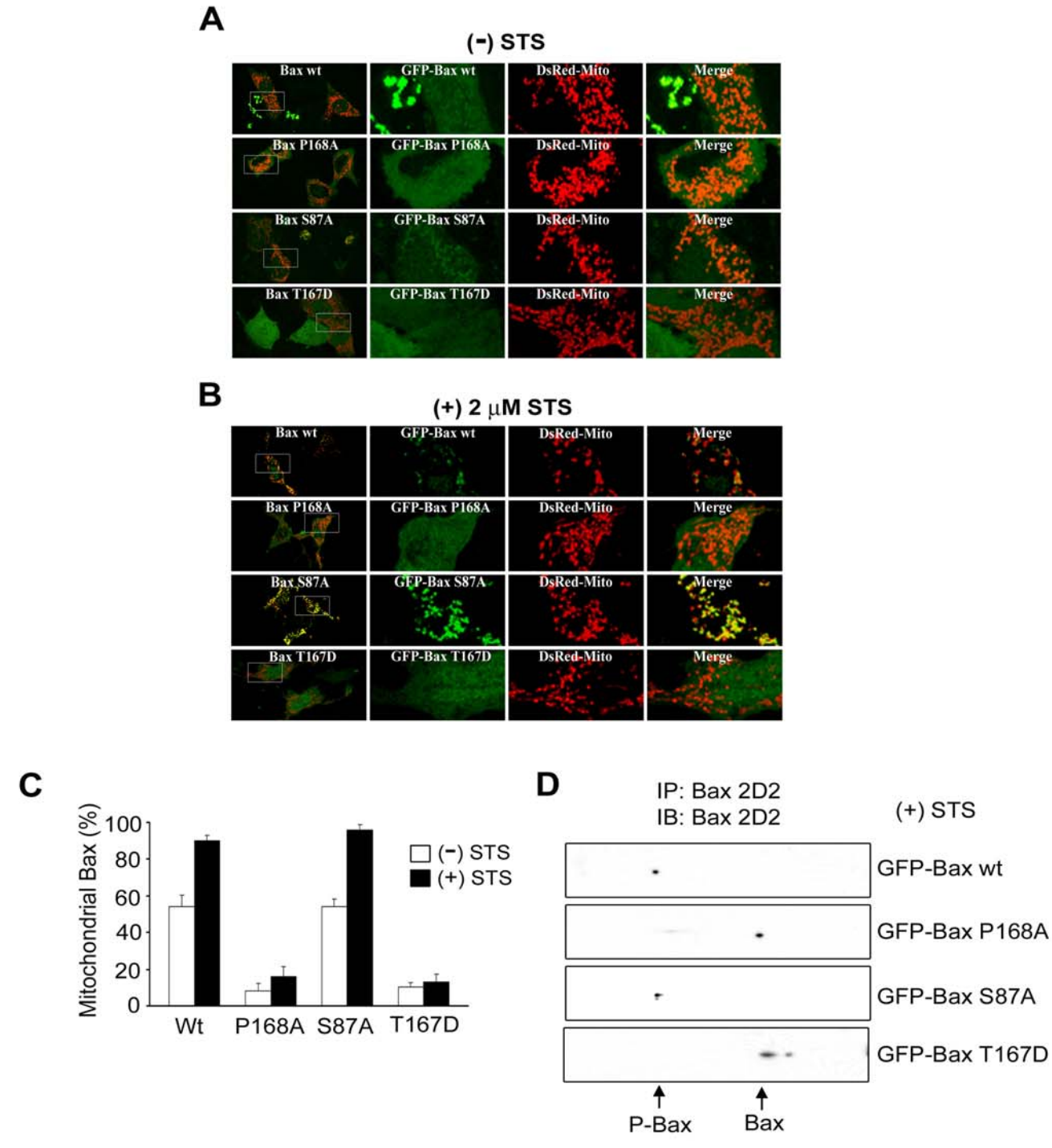

Kim et al, Fig. 9