Translocation of SAPK/JNK to Mitochondria and Interaction with Bcl-xL in Response to DNA Damage*

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The stress-activated protein kinases (SAPKs), also known as c-Jun amino-terminal kinases (JNKs) and p38 MAP kinase, are activated in response to diverse stimuli including DNA damage, heat shock, interleukin-1, tumor necrosis factor α, and Fas (12–22). Recent studies have shown that activation of c-Abl by exposure to IR contributes to the induction of Jun kinase and p38 MAPK (14, 15, 22). Activation of SAPK and p38 MAPK pathways has been associated with induction of apoptosis (23–25). However, the mechanisms involved in SAPK/JNK-induced apoptosis are presently unclear. Recent studies have demonstrated that mitochondria play a central role in inducing apoptosis by releasing cytochrome c (26, 27). The demonstration that the anti-apoptotic Bcl-2 family of proteins is expressed in the mitochondrial membrane has also implicated mitochondria in the induction of apoptosis (28, 29). Importantly, we and others have shown that overexpression of Bcl-2 or the related Bcl-xL blocks the release of cytochrome c from mitochondria, which otherwise occurs when cells are signaled to undergo apoptosis (30–32).

The present studies demonstrate that exposure of U-937 cells to IR is associated with translocation of active SAPK to mitochondria and its association with the anti-apoptotic protein Bcl-xL. The results also demonstrate that SAPK phosphorylates Bcl-xL on threonines 47 and 115, and overexpression of mutant Bcl-xL (A-47, A-115) causes a more potent inhibition of IR-induced apoptosis.

MATERIALS AND METHODS

Cell Culture and Reagents—Human U-937, U-937/Bcl-xL, and U-937/Bcl-xL(A-47,-115) myeloid leukemia cells were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM l-glutamine. Rat-1/myc, Rat-1/myc/Bcl-xL, and Rat-1/myc/Bcl-xL(A-47,-115) cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal bovine serum and antibiotics. IR was performed using a γ-ray source (Cs 173, Gamma Cell 1000, Atomic Energy of Canada, Ontario) at a fixed dose rate of 13 Gy/min. Cells were also treated with bleomycin (Sigma).

Immunofluorescence Microscopy—Control and IR-treated U-937 cells immobilized on slides were fixed (3.7% formaldehyde), permeabilized (0.2% Triton X-100), and incubated with 20 ng/ml of anti-SAPK polyclonal antibody (Santa Cruz Biotechnology), followed by Texas Red-conjugated goat anti-rabbit IgG (Southern Biotechnology Associates, Inc.). Mitochondria were stained with 0.006 ng/ml of Mitotracker Green FM (Molecular Probes). Nuclei were stained with 4,6-diamidino-2-phenylindole (1 μg/ml in phosphate-buffered saline). The slides were analyzed using a Zeiss Axioshot fluorescence microscope coupled to a CCD camera and a Power Macintosh 8100. Image analysis was performed using the IPLab Spectrum 3.1 software (Signal Analytics).

Isolation of Cytoplasmic and Mitochondrial Fractions—Cells were washed twice with phosphate-buffered saline, and cell fractionation was performed as described (30).

Immunoprecipitation and Immunoblot Analysis—Immunoprecipita-
tion was performed as described (33). In brief, soluble proteins were incubated with anti-SAPK (Santa Cruz) antibody for 1 h and precipitated with protein A-Sepharose for an additional 30 min. The resulting immune complexes were analyzed by immunoblotting with anti-Bcl-xL antibody (34). U-937/Bcl-xL cells were exposed to 20 Gy IR and harvested 3 h later. Total cell lysates were subjected to immunoprecipitation with anti-Bcl-xL and the precipitates were analyzed by immunoblotting with anti-Bax (Santa Cruz) antibody.

For Western Analysis—Column-purified Hr-Bcl-x47, Bcl-x47p, or H6 proteins were resolved by SDS-PAGE and transferred to a nitrocellulose filter. The filters were incubated with purified GST-SAPK for 1 h, and immunoblot analysis was performed as described (30). Purified SAPK and H6-SHPTP1 proteins were resolved by SDS-PAGE and transferred to nitrocellulose filters. The filters were incubated with Hr-Bcl-x47 or H6 proteins and analyzed by immunoblotting with anti-Bcl-xL antibody.

**Immune Complex Kinase Assays—GST-SAPK or anti-SAPK immunoprecipitates from cells were incubated with Hr-Bcl-x47, Bcl-x47p, Hr-Bcl-x47(A-47,-115), or GST-Jun (35), and in vitro immune complex kinase assays were performed as described (15).**

**Phosphopeptide Mapping and Phosphoamino Acid Analysis—** Labeled Bcl-xL recovered from unfixed dried SDS-polyacrylamide gels by homogenizing gel slices in the presence of trichloroacetic acid was oxidized with performic acid, resuspended in 25 mM NH4HCO3, and digested overnight at 37 °C with 10 µg of tosylphenylalanyl chloromethyl ketone-treated trypsin (36). After digestion, samples were repeatedly lyophilized. Trypsin-digestive products of the phosphorylated Bcl-xL and Bcl-xL(A-47) were subjected to electrophoresis using pH 1.9 buffer (15% acetic acid, 5% formic acid) followed by second chromatography with Scheidemann buffer (isobutyric acid:pyridine:acetic acid:butanol:water (65:5:3:2:29)) as described (37). Phosphopeptides were visualized by autoradiography. Individual spots were scraped from the cellulose plates, and phosphopeptides were eluted with pH 1.9 buffer. After lyophilization, phosphopeptides were subjected to partial acid hydrolysis, and hydrolyzed products were subjected to two-dimensional electrophoresis.

**Phosphorylation of Bcl-xL by SAPK in Vivo—** 293T cells were transiently cotransfected with HA-Bcl-xL and MEKK-1 or MEKK-1 K-K by LipofectAMINE (Life Technologies, Inc.). At 36 h after transfection, cells were labeled with [32P]orthophosphate (0.5 mCi/ml) in a phosphate-free media for 3 h. Total cell lysates were then subjected to immunoprecipitation with anti-HA antibody. Immunopurified labeled HA-Bcl-xL under conditions of active or inactive MEKK-1 were separated by SDS-PAGE and analyzed by autoradiography. Anti-HA immunoprecipitates were also analyzed by immunoblotting with anti-HA. As a control, total cell lysates from transfected but unlabeled 293T cells were subjected to immunoprecipitation with anti-SAPK, and in vitro immune complex kinase assays were performed using GST-Jun as substrate as described above.

**Generation of Bcl-xL and Bcl-xL Mutants—** Mutation in full-length (Bcl-xL) or a truncated (Bcl-xL(T)) and generation of various plasmids (HA-Bcl-xL) were performed as described (30).

**Cell Survival and Apoptosis Assays—** U-937, U-937/Bcl-xL, or U-937/Bcl-xL(A-47,-115) cells were treated with 20 Gy IR and harvested at the indicated times. Terminal deoxynucleotidyltransferase-mediated UTP end-labeling (TUNEL) assays were performed as described (38). Numbers of cells with sub-G1 DNA content were determined with a ModFit LT program (Verity Software House) (38). Rat-1/myc, Rat-1/myc/Bcl-xL, Rat-1/myc/Bcl-xL(A-47,-115), or GST-Jun (35) and in vitro immune complex kinase assays were performed as described (15).

**RESULTS AND DISCUSSION**

The stress-activated protein kinase (SAPK/JNK) is induced by IR and other genotoxic agents (13, 14, 16, 41–43). Other studies support a role for SAPK in induction of apoptosis (23, 24). Translocation of activated SAPK to the nucleus has been found in hypoxic cells and after treatment with ultraviolet radiation (44, 45). Here we investigated subcellular localization of SAPK in response to genotoxic stress by measuring intracellular fluorescence with a high sensitivity CCD camera and image analyzer. Examination of the distribution of fluorescence markers in control U-937 cells showed distinct patterns for anti-SAPK (red signal) and a mitochondrion-selective dye (Mitotracker; green signal) (Fig. 1A). By contrast, exposure to IR was associated with a dramatic change in fluorescence signals (red and green → yellow/orange), supporting translocation of SAPK to mitochondria (Fig. 1A). To confirm these findings, cytoplasmic and mitochondrial fractions were subjected to immunoblotting with anti-SAPK. Consistent with the immunofluorescence data, IR exposure induced translocation of SAPK to mitochondria (Fig. 1B). Purity of the mitochondrial and cytoplasmic fractions was confirmed by reprobing the blot with an antibody against the mitochondrial-specific HSP60 protein (Fig. 1B). These findings demonstrate that IR induces SAPK to translocate to mitochondria.

Bcl-xL is predominantly a mitochondrial protein (29, 46). To determine if SAPK associates with Bcl-xL following translocation to mitochondria, we studied co-localization of these proteins using immunofluorescence microscopy. In unirradiated cells, staining patterns of SAPK (green signal) and Bcl-xL (red signal) were mainly distinct (Fig. 2A). Importantly, IR exposure was associated with apparent co-localization of these proteins, as evident from the marked changes of fluorescence signals to yellow/orange (Fig. 2A). To determine biochemically whether SAPK forms a complex with Bcl-xL, we subjected anti-SAPK immunoprecipitates to immunoblotting with anti-Bcl-xL. Cytoplasmic fractions from control and irradiated cells demonstrated little if any association of SAPK and Bcl-xL (Fig. 2B); however, a similar analysis of the mitochondrial fraction demonstrated that IR induces binding of SAPK to Bcl-xL (Fig. 2B). To address whether Bcl-xL interacts directly with SAPK, we prepared a truncated His-tagged Bcl-xL protein lacking 21 amino acids from the carboxyl terminus to avoid aggregation and precipitation in in vitro reactions (H6-Bcl-xL(T)) (30). GST-SAPK fusion protein was resolved by gel electrophoresis and transferred to a nitrocellulose filter. Two identical filters were prepared. Filters were separately incubated either with Hr-Bcl-xL or H6 proteins and analyzed by immunoblotting with anti-Bcl-xL. As a control, purified H6-SHPTP1 protein was also
resolved by SDS-PAGE and transferred to nitrocellulose filters. After incubation with H\textsubscript{6}-Bcl-x\textsubscript{L}, the filters were probed with anti-Bcl-x\textsubscript{L}. By contrast to SHPTP1, reactivity of anti-Bcl-x\textsubscript{L} at the position corresponding to GST-SAPK (80 kDa) indicated direct interaction between Bcl-x\textsubscript{L} and SAPK (Fig. 2C). In the reciprocal experiment, Bcl-x\textsubscript{LT} and Bcl-x\textsubscript{ST} were resolved by gel electrophoresis and transferred to filters. After incubation with recombinant GST-SAPK, the filters were probed with anti-SAPK. The results confirmed direct binding of SAPK to Bcl-x\textsubscript{L} and not Bcl-x\textsubscript{S} (Fig. 2D).

SAPK phosphorylates c-Jun in response to genotoxic stress. To determine whether Bcl-x\textsubscript{LT} is also a substrate for SAPK
phosphorylated H$_6$-Bcl-xLT (Fig. 3A). As SAPK preferentially phosphorylates serine and/or threonine residues that are followed by prolines, we asked if the two Thr-Pro (amino acids 47, 48 and 115, 116) and/or one Ser-Pro (amino acids 62, 63) sites in Bcl-x$_L$ are phosphorylated by SAPK. Phosphopeptide mapping studies of Bcl-x$_L$ phosphorylated by SAPK demonstrated the presence of two $^{32}$P-labeled peptides (Fig. 3B). Phosphoamino acid analysis of these peptides revealed phosphorylation on Thr residues (Fig. 3B). These studies identified Thr-47 and Thr-115 as SAPK phosphorylation sites. Accordingly, there was no detectable SAPK-mediated phosphorylation of a Bcl-x$_{1,T}$ mutant in which these sites had been mutated to alanines (Ala-47,Ala-115) (Fig. 3C).

Since activation of SAPK contributes to induction of apoptosis (23, 24), we asked if the Bcl-x$_L$(A-47,-115) mutant is functional in regulating the apoptotic response. Sub-G$_1$ DNA content in propidium iodide-stained cells was assessed as a measure of apoptosis. Although exposure of U-937 cells to IR increased the proportion of cells with sub-G$_1$ DNA, expression of the Bcl-x$_L$(A-47,-115) mutant resulted in a smaller sub-G$_1$ peak and increased resistance to apoptosis compared with that in U-937 cells expressing wild-type Bcl-x$_L$ (Fig. 5A). To extend these findings, we used Rat-1 cells transformed with c-myc (Rat-1/myc) that respond to genotoxic stress with induction of apoptosis (46). Overexpression of wild-type Bcl-x$_L$ blocked bleomycin-induced cell death of Rat-1/myc cells (Fig. 5B). Significantly, overexpression of the Bcl-x$_L$(A-47,-115) mutant was more effective than Bcl-x$_L$ in blocking induction of apoptosis (Fig. 5B). Thus, interaction between SAPK and Bcl-x$_L$ is functionally
important in induction of apoptosis in different cell types treated with diverse genotoxic agents.

Previous studies have shown that Bcl-x₅ heterodimerizes with Bax (52). Other studies have demonstrated that Bad selectively forms heterodimers with Bcl-x₅ (53). When Bad heterodimerizes with Bcl-x₅ in mammalian cells, it displaces Bax from Bcl-x₅ and promotes cell death (53). To determine whether phosphorylation of Bcl-x₅ by SAPK affects the interaction of Bax with Bcl-x₅, U-937 cells overexpressing Bcl-x₅ were exposed with IR, and total cell lysates were subjected to immunoprecipitation with anti-Bcl-x₅. The protein precipitates were then analyzed by immunoblotting with anti-Bax. The results demonstrate that SAPK-mediated phosphorylation of Bcl-x₅ has no detectable effect on its association with Bax (data not shown).

SAPK is activated by genotoxic stress (13, 14, 16, 41–43) and functions upstream to induction of apoptosis in response to DNA damage (23, 24). The present work demonstrates for the first time that genotoxic stress induces translocation of SAPK to mitochondria. Recent studies have supported the release of cytochrome C from mitochondria to the cytosol in the apoptotic response to DNA damage (30). However, the initial signal that triggers mitochondrial changes in response to apoptotic stimuli is presently not known. The findings that Bcl-2 and Bcl-x₅ block release of cytochrome C and activation of caspases has further supported the importance of mitochondria in induction of apoptosis (30–32). In this context, our finding that SAPK associates with Bcl-x₅ in mitochondria provides a potential link between mitochondrial translocation of SAPK and apoptosis. The results demonstrate that SAPK phosphorylates Bcl-x₅ at Thr-Pro sites. Thr-47 resides in a 60-residue loop that is non-essential for anti-apoptotic activity (54, 55), whereas Thr-115 is adjacent to the α3 helix, which may be important structurally for formation of ion channels. The Bcl-x₅(A-47,-115) mutant was more effective than wild-type Bcl-x₅ in blocking apoptosis. Thus, the Bcl-x₅ mutant may, as a defective substrate, promote formation of stable SAPK complexes that would otherwise dissociate after phosphorylation of wild-type Bcl-x₅. In this regard, our results demonstrate that the Thr to Ala mutant of Bcl-x₅ associates with SAPK with higher affinity than that obtained with wild-type Bcl-x₅ (data not shown). Whereas SAPK activation is necessary for induction of apoptosis (23, 24), sequestration of SAPK in mitochondria by Bcl-x₅(A-47,-115) could abrogate other functions of SAPK, particularly in the cytoplasm or nucleus, that are required for the apoptotic response.

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FIG. 5. DNA damage-dependent inhibition of apoptosis in cells stably overexpressing Bcl-x₅(A-47,-115). A, U-937, U-937/Bcl-x₅, or U-937/Bcl-x₅(A-47,-115) cells were treated with 20 Gy IR and harvested at the indicated times. After fixing, cells were stained with propidium iodide, and sub-G₁ DNA content was measured using FACScan (upper panel). The percentage of apoptotic cells with sub-G₁ DNA content is expressed as the mean ± S.D. from three independent experiments performed in duplicate for the U-937 (hatched bar), U-937/Bcl-x₅ (open bar), and U-937/Bcl-x₅(A-47,-115) (solid bar) cells (lower panel). The percentage of the following cells in G₀/G₁, S, G₂/M phases for were: U-937, 72.5, 25.2, 2.2%; U-937/Bcl-x₅, 57.4, 37.0, 5.5%; U-937/Bcl-x₅(A-47,-115), 61.3, 32.6, 6.1%. B, Rat-1/myc (wild-type (wt)), Rat-1/myc/Bcl-x₅, or Rat-1/myc/Bcl-x₅(A-47,-115) (four independently selected clones) cells were plated in 96-well tissue culture dishes and treated 12 h later with bleomycin. After 3 days, the percentage apoptotic cells were determined. Results are expressed as the mean ± S.D. from three independent determinations each done in triplicate.
