Journal of Cellular Biochemistry 92:1257–1270 (2004)

Daxx Deletion Mutant (Amino Acids 501–625)-Induced Apoptosis Occurs Through the JNK/p38-Bax-Dependent Mitochondrial Pathway

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Abstract Death-associated protein (Daxx) deletion mutant (aa 501–625) has been known to be an inducer of apoptosis. In this study, we observed that the Bax-dependent mitochondrial death signaling pathway plays an important role in Daxx501–625-induced apoptosis. Daxx fragment-induced activation of caspase-9 and -3 was mediated through the apoptosis signal-regulating kinase 1 (ASK1)–MEK–c-Jun-N-terminal kinase (JNK)/p38–Bax pathway. By over-expressing JNK-binding domain (JBD) of JIP1, a JNK-inhibitory protein, and treatment with SB203580, a specific p38 inhibitor, DU-145 cells were made resistant to Daxx501–625-induced apoptosis. Capase-3 deficiency, Bax deficiency, or overexpression of a dominant-negative caspase-9 mutant prevented apoptosis, even though the Daxx501–625 fragment still activated the ASK1–MEK–MAPK pathway. Interestingly, Daxx501–625-induced Bcl-2 interacting domain (Bid) cleavage was suppressed in the dominant-negative caspase-9 mutant cells, whereas Bim was still phosphorylated in these cells. These results suggest that cleavage of Bid occurs downstream of caspase-9 activation. In contrast, phosphorylation of Bim is upstream of caspase-9 activation. Taken together, our results suggest that Daxx501–625-induced apoptosis is mediated through the ASK1–MEK–JNK/p38–Bim–Bax-dependent caspase pathway. J. Cell. Biochem. 92: 1257–1270, 2004. © 2004 Wiley-Liss, Inc.

Key words: Daxx; ASK1; JNK; Bax; Bim; cytochrome c; caspase

Death-associated protein (Daxx) is a nuclear protein. It interacts with the promyelocytic leukemia protein (PML) and localizes to PML oncogenic domains (PODs) [Li et al., 2000]. Daxx represses basal transcription, likely by recruiting histone deacetylase, and its activity is regulated by interaction with PML [Li et al., 2000]. Daxx is also localized to the cytoplasm and associates with cytoplasmic and cell surface molecules including transforming growth factor-β (TGF-β) and Fas [Yang et al., 1997; Perlman et al., 2001]. The cytoplasmic Daxx binds to apoptosis signal-regulating kinase 1 (ASK1) and enhances Fas-induced apoptosis by promoting the c-Jun-N-terminal kinase (JNK) cascade [Chang et al., 1998; Ko et al., 2001]. In particular, a deletion mutant of Daxx (aa 501–625) contains cell death activity [Yang et al.,

Abbreviations used: Bid, Bcl-2 interacting domain; Daxx, death-associated protein; ASK1, apoptosis signal-regulating kinase 1; JNK, c-Jun-N-terminal kinase; JBD, JNK binding domain; JSAP1, JNK/SAPK-associated protein 1; DMEM, Dulbecco's modified Eagle medium; PCR, polymerase chain reaction; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulphate; PAGE, polyacrylamide gel electrophoresis; ECL, enhanced chemiluminescence; EDTA, ethylenediaminetetraacetic acid; EGTA, ethyleneglycol-bis-(β-amino ethyl ether)-tetraacetic acid; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; GST, glutathione S-transferase; ATP, adenosine triphosphate; PVDF, polyvinylidine difluoride; SEK1, stress-activated protein kinase/extracellular-signal regulated kinase; TGF-β, transforming growth factor-β; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-fluorescein nick-end labeling; PARP, poly(ADP-ribose) polymerase.

Grant sponsor: NCI; Grant numbers: CA95191, CA96989; Grant sponsor: Competitive Medical Research Fund of the UPMC Health System; Grant sponsor: Oral Cancer Center Grant Fund; Grant sponsor: Elsa U. Pardee Foundation; Grant sponsor: Pittsburgh Foundation; Grant sponsor: DOD Prostate Program Fund; Grant number: PC020530; Grant sponsor: DOD Prostate Cancer Traineeship.

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Received 23 February 2004; Accepted 20 April 2004

DOI 10.1002/jcb.20155

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1997]. However, the mechanism by which Daxx501–625 promotes cell death is poorly understood. In this study, we hypothesize that Daxx501–625-induced apoptosis is mediated through the caspase family of proteases, and that the Bax-dependent mitochondrial machinery and JNK/p38 activation are responsible for caspase activation.

Caspases are a family of specific cysteine proteases that cleave protein substrates after aspartic acids, and their activation is critical in the intracellular execution of programmed cell death [Henkart, 1996; Cohen, 1997; Cryns and Yuan, 1999]. Among the more than ten members that have been identified, caspase-3 is a major player in the effector phase of apoptosis induced by a variety of stimuli [Salvesen and Dixit, 1997; Jänicke et al., 1998; Woo et al., 1998]. Previous studies illustrate that caspase-3 activation can be mediated through two different pathways, mitochondria-independent and mitochondria-dependent pathways. Initiator caspase-8 can directly activate downstreameffector caspase-3 [Cohen, 1997]. Caspase-8 also cleaves Bcl-2 interacting domain (Bid) and triggers mitochondrial damage that in turn leads to cytochrome c release [Li et al., 1998; Schendel et al., 1999]. Cytochrome c in the cytoplasm binds to Apaf-1, which then permits recruitment of procaspase-9. Caspase-9 cleaves and activates procaspase-3 [Slee et al., 1999].

Bax is a protein, which belongs to the Bcl-2 family [Oltvai et al., 1993]. Its apoptotic activation mechanism begins with a conformational change followed by oligomerization. The oligomerized Bax integrates in the outer mitochondrial membrane, where it triggers cytochrome c release [Desagher et al., 1999; Eskes et al., 2000; Korsmeyer et al., 2000]. The conformational change of Bax by truncated Bid, a BH3 only molecule, has been extensively reported [Desagher et al., 1999; Korsmeyer et al., 2000]. JNK is probably involved in Bid cleavage [Tournier et al., 2000]. In addition to Bid, Bim, another BH3-only molecule, directly binds and activates Bax to trigger apoptosis [Letai et al., 2002; Marani et al., 2002]. Bim is essential for JNK-dependent apoptosis [Lei et al., 2002]. Recent studies demonstrate that Bim links JNK and Bax-dependent mitochondrial apoptotic machinery [Lei and Davis, 2003].

JNK and p38 are key mediators of the inflammatory response and are activated by cytokines, growth factors, and a variety of cellular stresses including UV, ionizing radiation, hyperosmolarity, and heat shock [Chen et al., 1996; Kyriakis and Avruch, 1996; Ip and Davis, 1998; Roulston et al., 1998; Yuasa et al., 1998]. JNK/p38 activation is mediated through dual phosphorylation on Thr and Tyr by two MAPK kinases (MKKs); MKK4 and MKK7 are responsible for JNK activation, and MKK3 and MKK6 are associated with phosphorylation of p38 [Davis, 2000]. Several researchers have reported that activation of JNK/p38 leads to apoptotic death [Kawakami et al., 1997; Kawasaki et al., 1997]. However, contradictory findings have also been documented. For example, no association between p38 MAPK activity and cell death was found in WEHI-231 and NIH3T3 cells [Sutherland et al., 1996; Molnar et al., 1997]. The role of JNK activation in apoptosis is also highly controversial, being suggested to have a pro-apoptotic, an anti-apoptotic or no role at all in this process [Nishina et al., 1997; Ameyar et al., 1998; Herr et al., 1999a,b; Tournier et al., 2000; Lin, 2002]. Even in the case of the pro-apoptotic role, the connection between JNK activation and caspase-associated apoptotic death seems to be dependent upon the stimulus. Induction of caspase activity by the anti-cancer drugs etoposide and camptothecin is mediated through JNK1 activation [Seimiya et al., 1997], whereas doxorubicin- or death ligand-induced apoptosis and caspase activation appear to be independent of the JNK pathway [Herr et al., 1999a]. JNK activation has also been reported to occur downstream of caspase activation based on studies with caspase inhibitors [Cahill et al., 1996; Lenczowski et al., 1997; Ozaki et al., 1999]. Thus, the relationship between these two pathways remains obscure [Mandlekar et al., 2000].

Apoptosis signal-regulating kinase (ASK) 1 is a member of the MAPKKK group that activates MKK4/MKK7–JNK and MKK3/MKK6–p38 signaling cascades [Ichijo et al., 1997; Tobiume et al., 2001]. JSAP1 (also named JIP3; JNK/SAPK-associated protein 1), a JNK-binding protein [Ito et al., 1999], can be phosphorylated by ASK1, and its phosphorylation facilitates the recruitment of stress-activated protein kinase/extracellular-signal regulated kinase (SEK1), MKK7, and JNK into the JSAP1–ASK1 signaling complex [Matsuura et al., 2002]. The binding between JSAP1 and JNKs (including JNK1,

2, 3) is specific, while there is no or very low binding of ERK2 and p38α to JSAP1 [Ito et al., 1999].

In this study, we observed that Daxx501–625-induced apoptosis was related with the ASK1–MEK–MAPK signal transduction pathway. JNK/p38-associated caspase activation is mediated through the Bax-dependent mitochondrial signal transduction pathway. Although JNK does not directly cleave Bid, it is involved in the cleavage of Bid through activation of caspase-9. JNK/p38-induced phosphorylation of Bim may link JNK/p38 activation with the Bax-dependent mitochondrial apoptotic machinery.

MATERIALS AND METHODS

Cell Culture and Survival Determination

Human prostate adenocarcinoma (DU-145) cells were cultured in Dulbecco's modified Eagle medium (DMEM) with 10% fetal bovine serum (Gibco-BRL, Gathersburg, MD) and 26 mM sodium bicarbonate for monolayer cell culture. The cells were maintained in a humidified atmosphere containing 5% CO_2 and air at 37°C. For trypan blue exclusion assay, trypsinized cells were pelleted and resuspended in 0.2 ml of medium, 0.5 ml of 0.4% trypan blue solution, and 0.3 ml of phosphate-buffered saline solution (PBS). The samples were mixed thoroughly, incubated at room temperature for 15 min, and examined by microscopy.

Reagents and Antibodies

Polyclonal anti-caspase-3 and anti-Bax antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho-Bim EL (Ser55) from Upstate Biotechnology (Lake Placid, NY), anti-phospho JNK from Promega (Madison, WI), anti-phospho-ATF-2, anti-p38, and anti-Bid from Cell Signaling Technology (Beverly, MA) were used. Monoclonal antibodies were purchased from the following companies: anti-caspase-8 and anti-caspase-9 from Upstate Biotechnology, anti-HA (clone 3F10) from Roche Diagnostics (Mannheim, Germany), anti-Flag from Sigma-Aldrich (St. Louis, MO), anti-actin from ICN (Costa Mesa, CA), anti-cytochrome c from PharMingen (San Diego, CA), and anti-poly (ADP-ribose) polymerase (PARP) from Biomol Research Laboratory (Plymouth Meeting, PA). Other chemicals were purchased from Sigma-Aldrich.

Shuttle Vector Construction

pFlag/CMV2-Daxx was kindly provided by Dr. Horikoshi (Washington University, St. Louis, MO). pAdlox/Flag-Daxx was made by inserting the *Spe* I/*BamH* I fragment from pFlag/CMV2-Daxx into *Spe* I/*BamH* I-cut pAdlox shuttle vector [Hardy et al., 1997]. Daxx deletion mutant Flag-tagged at its N-terminal and restriction enzyme recognition sites at the flanking sides (5′, *EcoR* I; 3′, *BamH* I) was produced by polymerase chain reaction (PCR). For Daxx501–625 (amino acids 501–625), sense primer was 5′-GCTCGAATTCAATCTCCAATGAAAAGAACCTGG-3′ and anti-sense primer was 5′-CTGCGGATCCCTAACCAGAATCTCCCCAGTTGTG-3′. pcDNA3/HA-ASK1 was kindly provided by Dr. Ichijo (Tokyo Medical and Dental University, Tokyo, Japan). pAdlox/HA-ASK1 was made by inserting *Spe* I/*Xba* I fragment from pcDNA3/HA-ASK1 into *Xba* I-cut pAdlox. pAdlox/Flag-JNK binding domain (JBD) was made by inserting *Spe* I/*Xba* I fragment from pcDNA3/Flag-JBD into *Spe* I/*Xba* I-cut pAdlox. pAdlox-Flag-JSAP1 was made by inserting PCR product of Flag-JSAP1 digested with *Sph* I/*Acc* I to *Sph* I/*Acc* I-cut pAdlox. Sense primer was 5′-CTGCGCATGCTGATGGACTACAAAGACGATGACGACAAGCT-3′ introducing *Sph* I site and anti-sense primer was 5′-CATCTAGTCGACTCACTCAGGGGTGTAGGACACCTGCC-3′ introducing *Acc* I. pcDNA3-Flag-JSAP1 was kindly provided by Dr. Yoshioka (Kanazawa University, Kanazawa, Japan).

Adenoviral Vector Construction

All recombinant adenoviruses were constructed by employing the *Cre-lox* recombination system [Hardy et al., 1997]. The selective cell line CRE8 has an β-actin-based expression cassette driving a Cre recombinase gene with an N-terminal nuclear localization signal stably integrated into 293 cells. Transfections were done by using Lipofectamine Reagent (Invitrogen, Carlsbad, CA). Cells (5×10^5) were split into a 6-well plate 1 day before transfection. For the production of recombinant adenovirus, 2 μg of *Sfi* I/*Apa* I-digested Adlox/HA-ASK1 fragment or *Sfi* I/*Apa* I-digested Adlox/Flag-JBD or *Sfi* I-digested Adlox/Flag-Daxx including Daxx deletion mutants (501–625) or

Adlox/Flag-JSAP1 and 2 μg of Ψ5 viral genomic DNA were co-transfected into CRE8 cells. The recombinant adenoviruses were generated by intermolecular homologous recombination between the shuttle vector and Ψ5 viral DNA. The new virus has an intact packaging site and carries a recombinant gene. Plaques were harvested, analyzed and purified. The insertion of HA-ASK1 or Flag-Daxx (including 501–625 Daxx) or Flag-JBD or Flag-JSAP1 to adenovirus was confirmed by Western blot analysis, after infection of corresponding recombinant adenovirus into DU145 cells.

In Vivo Binding of Daxx501–625 With ASK1 and JSAP1

To examine the interaction between Daxx501–625 and ASK1/JSAP1, DU-145 cells in 10 cm culture plates were infected with adenovirus of Flag-tagged Daxx501–625 (Ad.Flag-Daxx501–625) at an MOI of 30 and adenovirus of HA-tagged ASK1 (Ad.HA-ASK1) or Flag-tagged JSAP1 (Ad.Flag-JSAP1) at an MOI of 10. For immunoprecipitation, after 48 h of infection, cells were lysed in buffer containing 150 mM NaCl, 20 mM Tris-HCl (pH 7.5), 10 mM ethylenediaminetetraacetic acid (EDTA), 1% Triton X-100, 1% deoxycholate, 1 mM phenylmethylsulfonyl fluoride (PMSF), 80 μM aprotinin, and 2 mM leupeptin. The lysates were incubated with 3 μg of anti-Flag M2 mouse IgG1 (Sigma-Aldrich) or 0.5 μg of anti-HA (clone 3F10, Roche, Mannheim, Germany) for 2 h. After the addition of protein G agarose (Santa Cruz Biotechnology), the lysates were incubated for an additional 2 h. The beads were washed three times with the lysis buffer, separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE), and immunoblotted with mouse anti-Flag or mouse anti-HA (clone 12CA5, Roche, Mannheim, Germany). Proteins in the membranes were then visualized using the enhanced chemiluminescence (ECL) reagent as recommended by the manufacturer (Amersham Pharmacia Biotech, Arlington Heights, IL).

In Vitro Kinase Assay

For the immune complex kinase assay, DU-145 cells were infected with Ad.HA-ASK1 and Ad.Flag-Daxx501–625 at an MOI of 10 and 50, respectively. After 48 h of infection, cells were lysed in a buffer solution containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 5 mM ethyleneglycol-bis-(β-amino ethyl ether)-tetraacetic acid (EGTA), 10 mM NaF, 1% Triton X-100, 0.5% deoxycholate, 2 mM dithiothreitol (DTT), 1 mM sodium orthovanadate, 1 mM PMSF, and protein inhibitor cocktail solution (Sigma-Aldrich). Cell extracts were clarified by centrifugation, and the supernatants were immunoprecipitated with rat anti-HA antibody (clone 3F10) for 2 h. After the addition of protein G-agarose (Santa Cruz Biotechnology), the beads were washed twice with a solution containing 150 mM NaCl, 20 mM Tris-HCl (pH 7.5), 5 mM EGTA, 2 mM DTT, 1 mM sodium orthovanadate, 1 mM PMSF, and protein inhibitor cocktail solution, and washed once with the kinase buffer solution, and then they were subjected to kinase assays. To measure the ASK1 activity, 0.5 μg of glutathione S-transferase (GST)-SEK1 [Song et al., 2002] was incubated with the immune complexes for 30 min at 30°C in a final volume of 25 μl in kinase buffer containing 20 mM Tris-HCl (pH 7.5), 20 mM $MgCl_2$, 1 mM sodium orthovanadate, 2 mM DTT, 20 μM adenosine triphosphate (ATP), and 100 μCi/ml [γ-^{32}P]ATP. Finally, the reaction was stopped by adding 2× Laemlli buffer.

Immunoblot Analysis

Cell lysates were subjected to electrophoresis on 10% polyacrylamide gels containing SDS under reducing conditions, and the proteins in the gels were transferred onto a polyvinylidine difluoride (PVDF) membrane. The membranes were incubated with 7% (w/v) skim milk in PBST (PBS containing 0.1% Tween-20, v/v) and then reacted with primary antibodies (diluted according to the manufacturer's instructions). After washing three times with PBST, the membranes were incubated with horseradish peroxidase-conjugated anti-IgG. Proteins in the membranes were then visualized using the ECL reagent (Amersham Pharmacia Biotech) as recommended by the manufacturer.

Terminal Deoxynucleotidyl Transferase (TdT)-Mediated dUTP-Fluorescein Nick-End Labeling (TUNEL) Assay

TUNEL staining was performed according to the manufacture's instructions (R&D Systems, Minneapolis, MN). Briefly, DU145 cells were grown on Lab-Tek chamber slides (Nalge Nunc, Naperville, IL), and infected with Ad/Flag-Daxx501–625 at an MOI of 50 for various times. Cells were fixed with 3.7% formaldehyde

solution in PBS for 10 min at room temperature and then permeabilized with cytonin for 30 min after rehydration. The cells were then exposed to the TUNEL reaction mixture containing the TdT enzyme, followed by fluorescein detection.

Cytochrome c Release

Cell growing in 100 mm dishes were trypsinized and collected by centrifugation. The cell pellets were washed twice with PBS and resuspended in buffer containing 250 mM sucrose, 20 mM HEPES, pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, and 0.1 mM PMSF, and protease inhibitor cocktail solution. The cells were lysed by forcing them through a 27-G needle 15–20 times. The lysate was centrifuged at 15,000 rpm for 15 min and the supernatant was subjected to immunoblotting for the detection of cytochrome c.

RESULTS

Daxx Fragment (501–625) Induces Apoptosis and Activates JNK/p38

To investigate the effect of Daxx fragment (501–625) on cell death, human prostate adenocarcinoma DU-145 cells were infected with Ad.Flag-Daxx501–625 at an MOI of 50 and incubated for various times (0–48 h). Data from TUNEL assays show that apoptotic cell death occurred in the Ad.Flag-Daxx-infected, but not Ad.cont, cells (Fig. 1A). Uninfected control or control vector infected cells showed very limited fluorescent labeling, whereas Daxx501–625-expressed cells showed increasing fluorescent labeling with increasing time post-infection. This observation was consistent with the result from the trypan blue exclusion assay (Fig. 1B). It is well known that apoptotic death is mediated through a caspase cascade. To confirm that Daxx501–625-induced cell death is associated with caspase activation, activation of caspases was examined by Western blot analysis. Figure 2A shows that procaspase-8 (54/55 kDa), procaspase-9 (46 kDa), and procaspase-3 (32 kDa) were cleaved to yield fragments 41/43, 37, and 17 kDa, respectively (Fig. 2A). Caspase activation was dependent upon MOI and was associated with PARP cleavage, a hallmark feature of apoptosis (Fig. 2A). Interestingly, caspase-9 was the most prominently cleaved among the three caspases. We also observed the reduction of Bid in Daxx501–625 expressing cells (Fig. 2A). Because JNK/p38 has been implicated in mediating apoptosis, we further examined whether there is a correlation of induction of apoptosis with the activation of the JNK/p38 pathways. As shown in Figure 2B, JNK/p38 was activated in proportion to the expression of Daxx501–625.

Both JNK and p38 Activation Are Required for Apoptosis Induced by Daxx Fragment (501–625)

Data from Figure 2 illustrate that Daxx fragment-induced apoptotic signals would be related to JNK and p38 activation. To examine whether the activation of JNK/p38 acts as a trigger of a caspase cascade, JBD, a JNK inhibitor and SB203580, a specific p38 inhibitor were used to inhibit JNK and p38 activation (Fig. 3A–D). When JBD was overexpressed in Daxx501–625-expressing cells, the hallmark features of apoptosis such as PARP cleavage, caspase activation and Bid cleavage were not altered even though activation of JNK, but not p38, was inhibited (Fig. 3A,B). Similar results were observed with SB203580. SB203580 inhibited Daxx501–625-induced phosphorylation of p38 substrate molecule, ATF2, but not activation of JNK (Fig. 3C). However, Daxx501–625-induced apoptotic signaling pathways were suppressed when cells were treated with SB203580 in combination with JBD expression (Fig. 3D). These results suggest that Daxx501–625-associated apoptosis requires both JNK and p38 activation.

Daxx501–625 Increases ASK1 Activity and the Binding of ASK1 to JSAP1

Based on our previous observations, we postulate that Daxx501–625-induced JNK/p38 activation is mediated through ASK1 activation. Figure 4A,B show that ASK1 bound Daxx501–625 and its interaction induced ASK1 activation, which was measured by detecting phosphorylation of GST-SEK1 as a downstream substrate [Song et al., 2002]. Interestingly, Daxx501–625 stimulated the association of ASK1 with JSAP1, a scaffolding protein (Fig. 4C). These results suggest that interaction between Daxx501–625 and ASK facilitates the binding of ASK1 to JSAP1.

JNK/p38 Activation Does Not Require Caspase-3 or -9 Activity

To determine whether JNK/p38 activation is upstream or downstream of the caspase

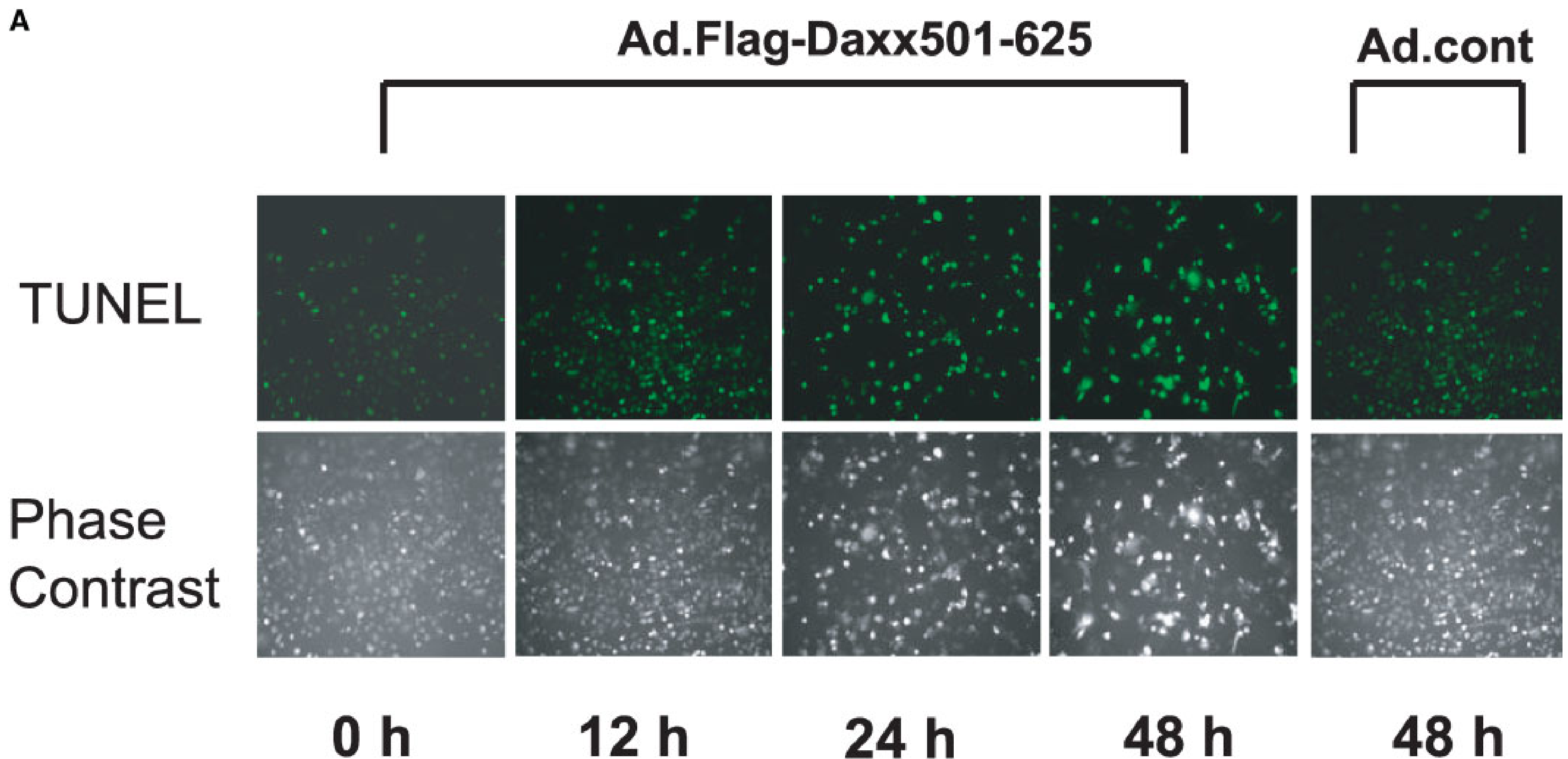


Fig. 1. Death-associated protein (Daxx)501–625-induced apoptosis in human prostate adenocarcinoma DU-145 cells. DU-145 cells were infected with adenoviral vector containing Daxx501–625 (Ad.Flag-Daxx501–625) or control adenoviral vector (Ad.cont) at an MOI of 50. Cytotoxicity was determined by terminal deoxynucleotidyl transferase-mediated dUTP-fluorescein nick-end labeling (TUNEL) assay (**A**) or trypan blue exclusion assay (**B**) various times (0–48 h) after infection. A: **Upper panels** are TUNEL assay showing apoptotic death. **Lower panels** are phase-contrast micrographs. B: Cytotoxicity was determined by the trypan blue exclusion assay. Error bars represent standard error of the mean from three separate experiments.

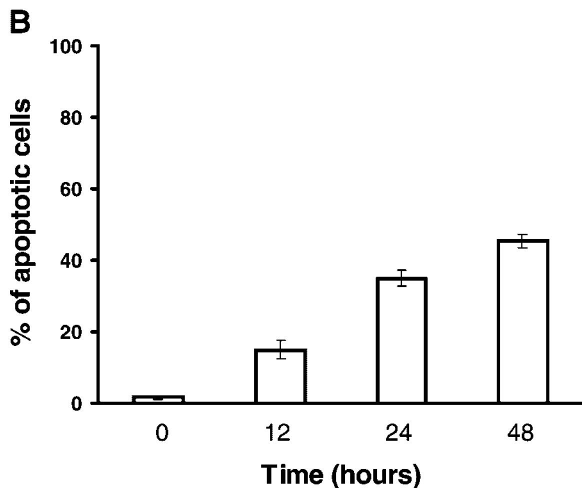


Fig. 1. (*Continued*)

pathway, we employed the MCF-7 cell line, a caspase-3-deficient cell line, and a caspase-9-dominant negative MCF-7 cell line. If JNK/p38 activation requires caspase-3 activation, JNK/p38 activation should be significantly diminished in caspase-3-deficient (Casp3−/−) cells

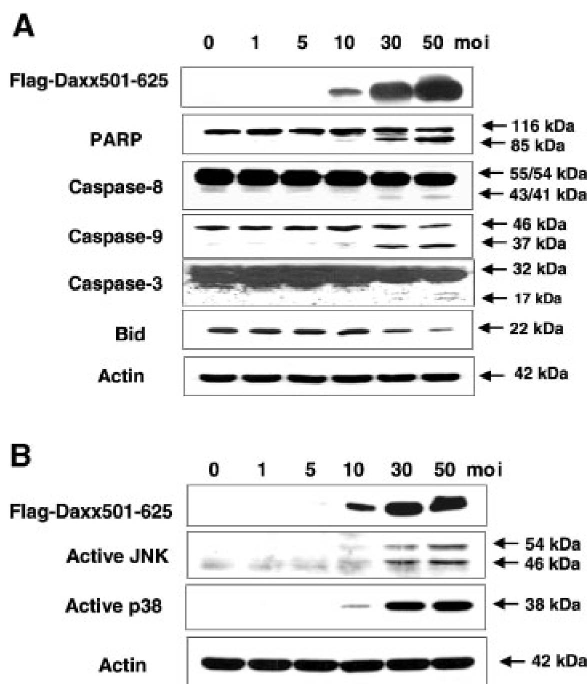


Fig. 2. Daxx501−625-induced apoptotic signal pathway. DU-145 cells were infected with Ad.Flag-Daxx501−625 at various MOIs. After 48 h incubation, cell lysates containing equal amounts of protein (20 μg/ml) were separated by SDS−PAGE and immunoblotted with (**A**) anti-actin, anti-Bcl-2 interacting domain (Bid), anti-Flag, anti-poly(ADP-ribose) polymerase (PARP), anti-caspase-8, 9, or 3 antibodies, (**B**) anti-actin, anti-Flag, anti-phospho JNK, or anti-phospho p38 antibodies.

expressing Daxx501−625. However, JNK/p38 was still activated in caspase-3-deficient (Casp3−/−) cells expressing Daxx501−625 (Fig. 5A), and cleavage of PARP, caspase 9 and Bid also occurred (Fig. 5B). Interestingly, overexpression of ASK1 alone induced JNK activation. To examine whether JNK/p38 activation is upstream of caspase-9 activation, MCF-7 cells were stably transfected with caspase-9-dominant negative mutant (C287A) cDNA. Figure 5C shows that JNK/p38 activation still occurred in caspase-9 dominant negative-MCF-7 cells during expression of Daxx501−625. Taken together, these results suggest that JNK/p38 activation by Daxx501−625 is upstream of caspase activation.

Bid Cleavage Requires Caspase-9 Activation

Bid, a proapoptotic member of the Bcl-2 family containing only a BH3 domain [Wang et al., 1996; Luo et al., 1998], was cleaved even in caspase-3-deficient MCF-7 cells (Fig. 5B), while it was not processed in caspase-9 dominant negative MCF-7/Cas9DN cells (Fig. 5C). These results suggest that, unlike the classical pathway of Bid cleavage in Fas-mediated apoptosis, Bid cleavage is dependent on caspase-9 activation when Daxx501−625 is present.

Daxx501−625-Induced Bim Phosphorylation Is Upstream of Caspase-9 Activation

Recent studies demonstrate that JNK phosphorylates Bim and phosphorylation of Bim may play an important role in Bax-dependent apoptosis. We further examined whether Bim phosphorylation is dependent on caspase-9 activation during Daxx501−625 expression. Figure 6 shows that expression of Daxx501−625 induced JNK/p38 activation as well as Bim phosphorylation. Interestingly, Daxx501−625-induced phosphorylation of Bim was not suppressed in MCF-7/Cas9DN cells. Therefore, Bim phosphorylation does not require caspase-9 activation. Rather, its phosphorylation occurs prior to caspase-9 activation.

Cytochrome c Release Is Upstream of Caspase-9 (or -3) Activation

From the previous observations, we postulate that caspase-9 activation is essential for Daxx501−625-induced apoptosis and caspase-9 activation is mediated through mitochondrial cytochrome c release. Figure 7 shows that cytochrome c was released in DU-145 and MCF-7/

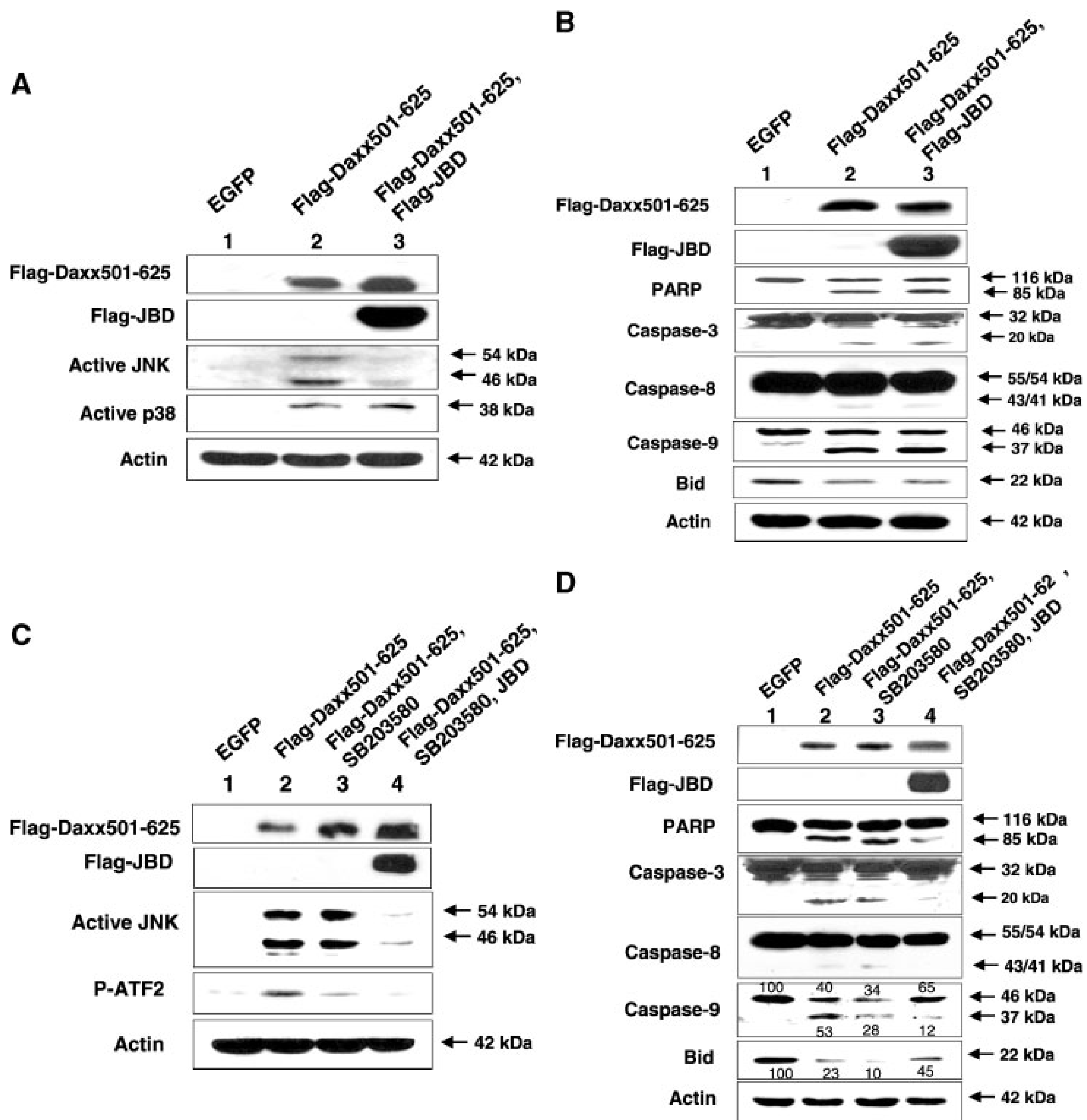


Fig. 3. Effect of JNK binding domain (JBD) or SB203580 on Daxx501–625-induced JNK/p38 activation and apoptotic signal pathway. **A**, **B**: DU-145 cells were infected with Ad.Flag-Daxx501–625 alone at an MOI of 50 or co-infected with Ad.Flag-Daxx501–625/Ad.Flag-JBD at the same MOI (MOI = 50). After 48 h incubation, cell lysates were separated by SDS–PAGE and immunoblotted as described in Figure 2. **C**, **D**: DU-145 cells were infected with Ad.Flag-Daxx501–625 at an MOI of 50 and treated with 10 μM SB203580. After 48 h incubation, cell lysates were separated by SDS–PAGE and immunoblotted as described in Figure 2. Active p38 activity was measured by manufacturer's protocol (p38 MAP kinase assay kit; Cell Signaling Technology). The relative intensity of each band was shown as numbers.

cas9DN cells, when they were infected with Ad.Daxx501–625. These results clearly demonstrate that cytochrome c release is upstream of caspase-9 (or -3) activation.

Bax Is Required for Daxx501–625-Induced Apoptosis

Next, we investigated whether the Bax molecule is involved in cytochrome c release during Daxx501–625 expression. Recent studies reveal that Bax is an initial molecule activating the mitochondrial cell death pathway by JNK/p38 through Bim phosphorylation [Lei and Davis, 2003]. To test this possibility, we employed Bax−/− HCT116 cells. Our results show that Daxx501–625-induced cytochrome c release, as well as caspase-9 activation, were suppressed in Bax−/− cells, even though JNK and p38 were

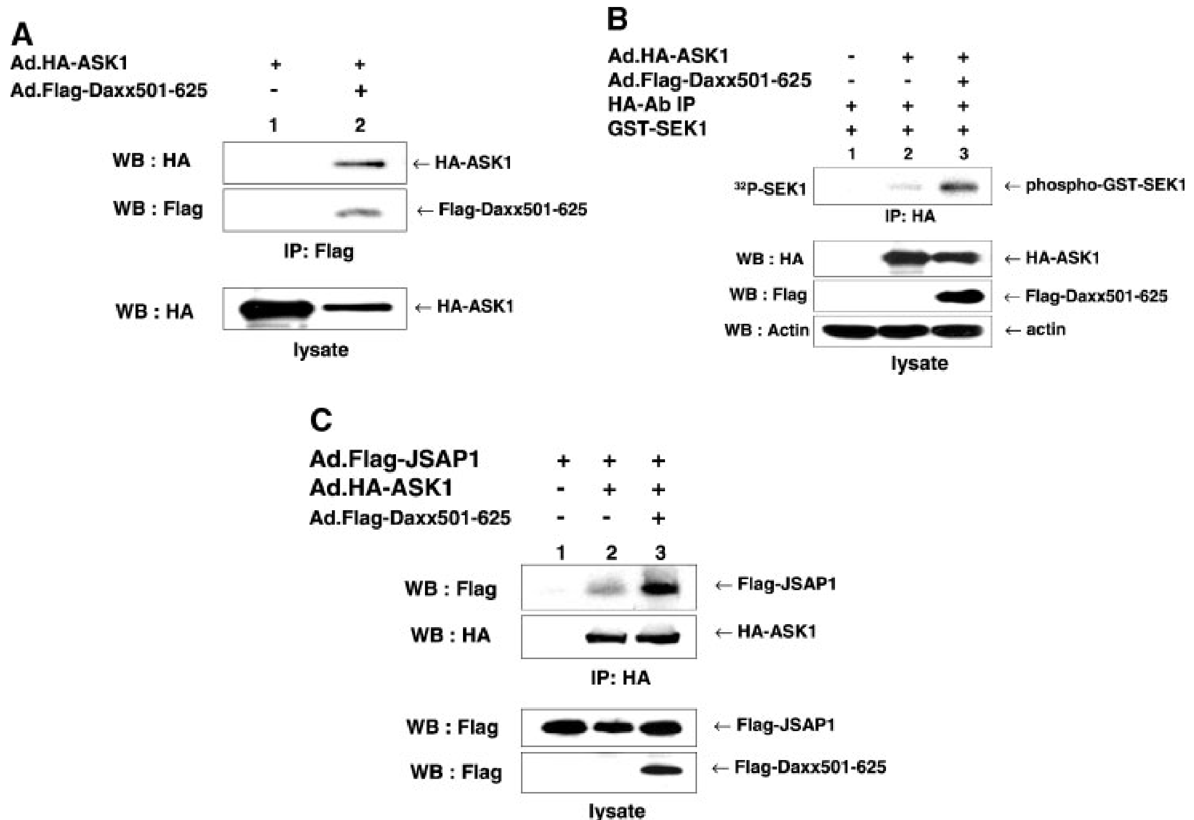


Fig. 4. Daxx501–625-induced apoptosis signal-regulating kinase 1 (ASK1) activation and association of Daxx501–625 with ASK1 and JNK/SAPK-associated protein 1 (JSAP1). **A**: DU-145 cells were co-infected with Ad.Flag-Daxx501–625 and Ad.HA-ASK1 at an MOI of 30 and 10, respectively. After 48 h infection, lysates were immunoprecipiated with anti-Flag antibody, and immunoblotted with anti-HA antibody or anti-Flag antibody (**upper**). The presence of HA-ASK1 in the lysates was verified by immunoblotting with anti-HA antibody (**lower**). **B**: DU-145 cells were co-infected with Ad.Flag-Daxx501–625 and Ad.HA-ASK1 at an MOI of 30 and 10, respectively. After 48 h infection, lysates were immunoprecipiated with anti-HA antibody. The immune complex was incubated with glutathione S-transferase (GST)-stress-activated protein kinase/extracellular-signal regulated kinase (SEK1). Phosphorylated GST-SEK1 was autoradiographed (**upper**). The presence of HA-ASK1, Flag-Daxx501–625, and actin in the lysates was verified by Western blot (**lower**). **C**: DU-145 cells were co-infected with Ad.Flag-JSAP1 at an MOI of 10, Ad.HA-ASK1 at an MOI of 10, or Ad.Flag-Daxx501–625 at an MOI of 30. After 48 h infection, lysates were immunoprecipitated with anti-HA antibody, and immunoblotted with anti-Flag or anti-HA antibody (**upper**). The presence of Flag-JSAP1 and Flag-Daxx501–625 in the lysates was verified by immunoblotting with anti-Flag antibody (**lower**).

still activated (Fig. 8A,B). These results suggest that Bax links the JNK/p38 signal transduction pathway with mitochondria-dependent apoptotic cell death.

Schematic Diagram of Apoptosis Pathways Induced by Daxx501–625

Our experimental data are summarized in Figure 9. Our previous studies and the literature reveal that the Daxx501–625 fragment induces apoptosis through the Bax-dependent mitochondrial pathway. First, Daxx501–625 binds and activates the ASK1 signal transduction pathway. Activation of JNK/p38 induces caspase activation through the Bax-dependent mitochondrial signaling pathway. Bim probably links JNK/p38 to the Bax-dependent mitochondrial apoptotic machinery.

DISCUSSION

In the present study, we observed that the Daxx501–625 fragment induces apoptosis through mitochondria-dependent caspase activation and its activation is mediated through the ASK1–JNK/p38 signal transduction pathway. Our results also support the previous report that Daxx association with ASK1 provides a mechanism for caspase-independent activation of JNK [Chang et al., 1998].

We previously observed that wild-type Daxx, which contains two nuclear localization signals,

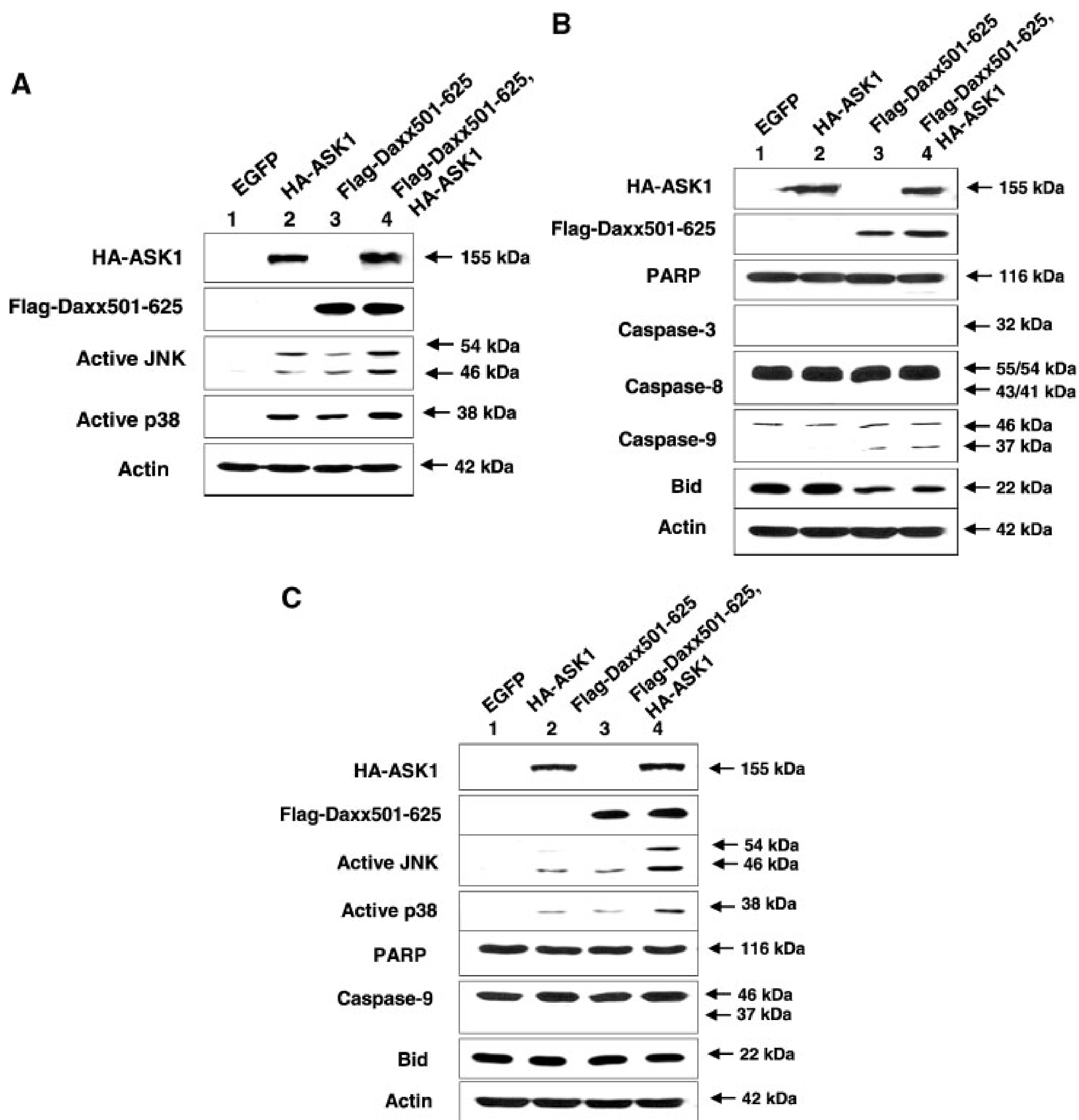


Fig. 5. Role of caspase-3 and caspase-9 in Daxx501–625-induced apoptosis. **A**, **B**: Casapse-3-deficient MCF-7 cells were co-infected with Ad.HA-ASK1 and Ad.Flag-Daxx501–625 at an MOI of 30 and 50, respectively. After 48 h incubation, cell lysates were separated by SDS–PAGE and immunoblotted as described in Figure 2. **C**: MCF-7 cells were stably transfected with a dominant-negative caspase-9 mutant (MCF-7/Cas9DN) and then co-infected with Ad.HA-ASK1 and Ad.Flag-Daxx501–625 at an MOI of 30 and 50, respectively. After 48 h incubation, cell lysates were separated by SDS–PAGE and immunoblotted as described in Figure 2.

is mainly located in the nucleus [Song and Lee, 2003]. In contrast, the Daxx501–625 fragment is mainly located in the cytoplasm [Song and Lee, 2003]. Wild-type Daxx relocalizes to the cytoplasm and interacts with ASK1 during metabolic oxidative stress [Song and Lee, 2003]. In contrast, Daxx501–625 associates with ASK1 in both stressed and unstressed cells, activating the ASK1–MEK–MAPK signal transduction pathway (Figs. 3 and 4). The physical interaction between Daxx and ASK1 leads to ASK1 oligomerization [Song and Lee, 2003]. This oligomerization probably maintains the activated ASK1–MEK–MAPK signal. Our results are consistent with previous observations that ASK1 is required for initiating

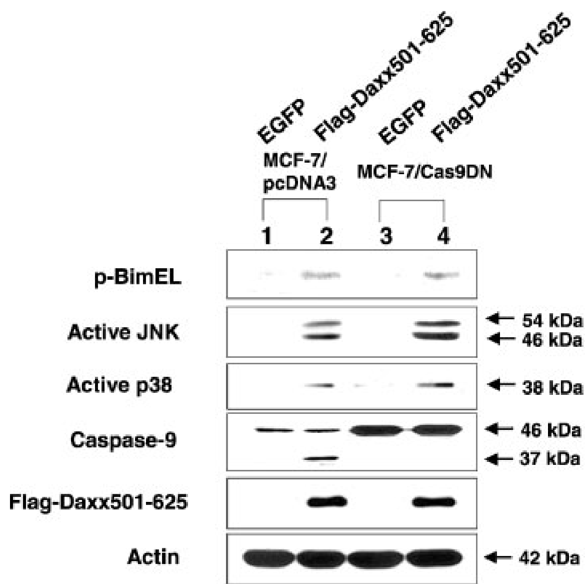


Fig. 6. Role of caspase-9 in Daxx501–625-induced phosphorylation of Bim. MCF-7/Cas9DN and MCF-7/pcDNA3 cells were infected with either Ad.EGFP or Ad.Flag-Daxx501–625 at an MOI of 50. After 48 h incubation, cell lysates were separated by SDS–PAGE and immunoblotted for p-BimEL, active JNK, active p38, caspase 9, actin, and Flag Daxx501–625.

activation of JNK/p38 and subsequently sustaining the signals [Saitoh et al., 1998; Tobiume et al., 2001]. Previous studies suggest that sustained activation of the JNK/p38 signal causes apoptosis [Tobiume et al., 2001]. A fundamental question is how Daxx501–625-induced JNK/p38 activation leads to caspase activation and consequently apoptotic death. Previous studies suggest that Bid is a target for

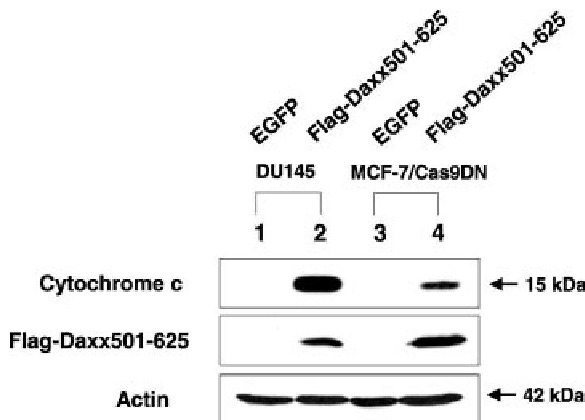
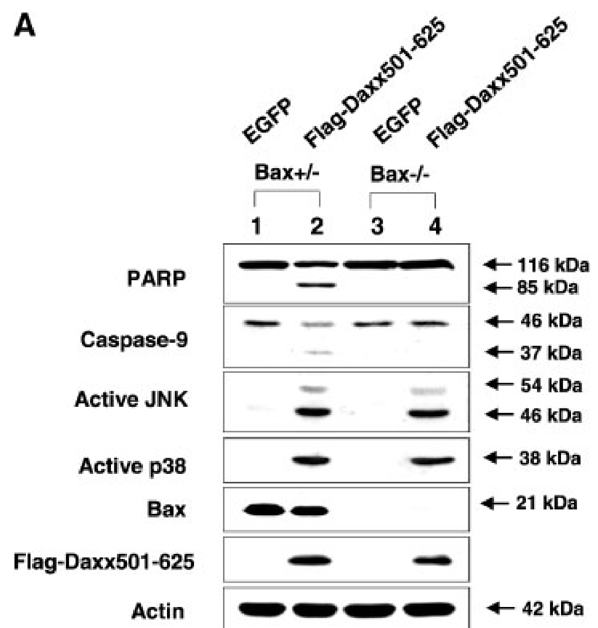


Fig. 7. Daxx501–625-induced cytochrome c release in DU145 or MCF-7/Cas9DN cells. DU145 or MCF-7/Cas9DN cells were infected with Ad.Flag-Daxx501–625 at an MOI of 50. After 48 h incubation, cells were lysed and the cytoplasmic fraction was subjected to immunoblotting for cytochrome c.



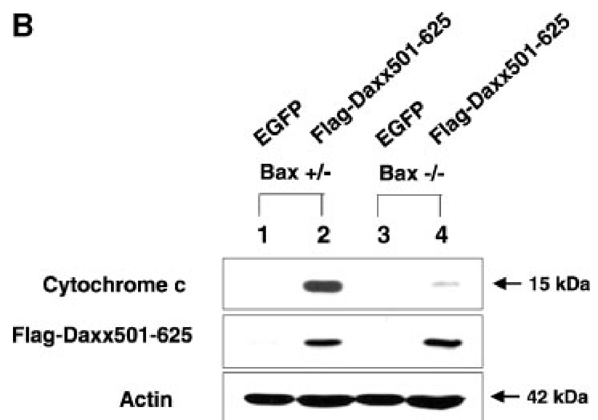


Fig. 8. Role of Bax in Daxx501–625-induced apoptosis. HCT116 Bax−/− or Bax+/− cells were infected with Ad.Flag-Daxx501–625 at an MOI of 50. After 48 h incubation, cell lysates were subjected to immunoblotting for active JNK, active p38, PARP, caspase-9, Bax, actin (**A**), and cytochrome c (**B**).

JNK signaling [Tournier et al., 2000]. Truncated Bid is associated with Bax and subsequently induces oligomerization of Bax. The oligomerized Bax translocates from the cytosol to mitochondria and then integrates into the outer mitochondrial membrane, where it triggers cytochrome c release [Eskes et al., 2000]. However, our data from Figure 5 clearly demonstrate that activated JNK/p38 does not directly cleave Bid. JNK/p38-induced activation of caspase-9, but not caspse-8, is required for Bid cleavage and its cleavage occurs without the presence of caspase-3 (Fig. 5B). Although several researchers previously reported that

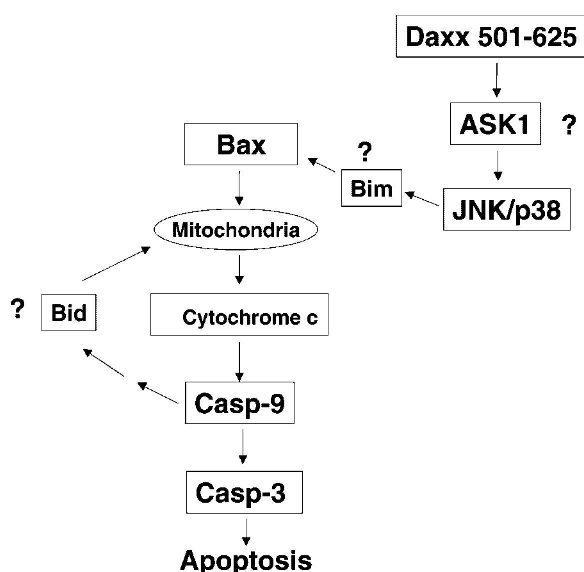


Fig. 9. A model for the mechanism by which Daxx 501–625 amino acid fragment-induced apoptosis.

caspase-8 cleaves Bid and triggers mitochondrial damage which in turn leads to cytochrome c release [Li et al., 1998; Luo et al., 1998], we observed that the cleavage of caspase-8 was of very low efficiency while Daxx501–625 fragment induced apoptosis (Fig. 2A). These results indicate that the Daxx501–625 fragment-induced apoptotic death is mainly mediated through the mitochondria-dependent pathway by activating caspase-9. Several researchers reported that activated caspase-9 cleaves and activates procaspase-3, an executor for apoptotic death. Figure 5B showed that the activation of caspase-3 is probably essential for apoptosis, even though the cleavage of caspase-3 occurs at somewhat low efficiency. Taken together, we postulate that the cleavage of caspase-9 and -3, but not caspase-8 and Bid, is essential for Daxx501–625-induced apoptotic death. Unlike Bid, Daxx501–625-induced phosphorylation of Bim does not require activated caspase-9. Recent studies show that JNK-mediated Bim phosphorylation potentiates Bax-dependent apoptosis [Putcha et al., 2001; Lei and Davis, 2003]. Thus, our results suggest that Bim acts as a molecular link between the JNK/p38 signal transduction pathway and the Bax-dependent mitochondrial apoptotic machinery.

Bim is a member of the BH-3 only proapoptotic subfamily of the Bcl-2 protein family. Alternative splicing generates three major Bim isoforms: BimS, BimL, and BimEL [O'Reilly et al., 2000]. Even though all three isoforms are potent inducers of apoptosis, the short isoform (BimS) is more cytotoxic than BimL or BimEL. BimS is normally only transiently expressed during apoptosis. The longer isoforms (BimL and BimEL) are expressed in normal cells, but the apoptotic activity of these proteins is suppressed by sequestration to the microtubule-associated dynein motor complex via an interaction with dynein light chain LC8/DLC1 [Puthalakath et al., 1999]. BimL and BimEL, but not BimS, contain a short peptide motif (DKSTQTP), which mediates the binding of Bim to DLC1. Lei and Davis [2003] reported that phosphorylation by JNK disrupts the function of the DLC binding motif and consequently may cause the release of Bim from dynein motor complexes. The activated Bim may directly or indirectly activate the Bax-dependent mitochondrial apoptotic pathway [Putcha et al., 2001]. Indeed, we observed that Daxx501–625 expression leads to Bim phosphorylation and its phosphorylation is upstream of caspase-9 activation (Fig. 6). At the present time, however, we cannot rule out another possibility. The Bim-related molecule Bmf also contains a similar motif (DKATQTLSP) that binds DLC2, a component of the myosin V motor complex [Puthalakath et al., 2001]. Exposure of cells to stresses such as UV radiation causes the release of Bmf from sites of sequestration by dissociation of Bmf from myosin V motor complexes [Puthalakath et al., 2001]. Thus, Daxx501–625-induced ASK1–MEK–MAPK signaling may cause the phosphorylation of Bmf and subsequently lead to the release of Bmf from these complexes. The activated Bmf may also directly activate Bax and Bak or may indirectly activate Bax and Bak by binding anti-apoptotic Bcl-2 family proteins such as Bcl-2 and Bcl-X_L. These mechanisms may lead to mitochondria-dependent apoptotic death.

In this paper, we present the possibility that the ASK1–MEK–MAPK–Bim signaling pathway and the Bax-dependent mitochondrial apoptotic pathway are linked. We believe that this model provides a framework for future studies into the nature of that linkage.

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