JNK promotes Bax translocation to mitochondria through phosphorylation of 14-3-3 proteins

Fuminori Tsuruta[1], Jun Sunayama[1], Yasunori Mori[1], Seisuke Hattori[2], Shigeomi Shimizu[3], Yoshihide Tsujimoto[3], Katsuji Yoshioka[4], Norihisa Masuyama[1] and Yukiko Gotoh[1,5,]*

[1]Institute of Molecular and Cellular Biosciences, University of Tokyo, Yayoi, Bunkyo-ku, Tokyo, Japan, [2]Division of Cellular Genome Proteomics, Institute of Medical Science, University of Tokyo, Shirokanedai, Minato-ku, Tokyo, Japan, [3]Department of Post-Genomics & Disease, Osaka University Graduate School of Medicine, Yamadaoka, Suita, Osaka, Japan, [4]Division of Cell Cycle Regulation, Cancer Research Institute, Kanazawa University, Takara-machi, Kanazawa, Japan and [5]PRESTO Research Project, Japan Science and Technology Corporation, Tokyo, Japan

Targeted gene disruption studies have established that the c-Jun NH2-terminal kinase (JNK) is required for the stress-induced release of mitochondrial cytochrome *c* and apoptosis, and that the Bax subfamily of Bcl-2-related proteins is essential for JNK-dependent apoptosis. However, the mechanism by which JNK regulates Bax has remained unsolved. Here we demonstrate that activated JNK promotes Bax translocation to mitochondria through phosphorylation of 14-3-3, a cytoplasmic anchor of Bax. Phosphorylation of 14-3-3 led to dissociation of Bax from this protein. Expression of phosphorylation-defective mutants of 14-3-3 blocked JNK-induced Bax translocation to mitochondria, cytochrome *c* release and apoptosis. Collectively, these results have revealed a key mechanism of Bax regulation in stress-induced apoptosis.

Introduction

Apoptosis is essential for normal development and maintenance of tissue homeostasis. Disruption of the equilibrium between pro- and anti-apoptotic factors results in pathological conditions such as cancer, autoimmune disease and neurodegenerative disorders (Krammer, 2000; Yuan and Yankner, 2000). The loss of mitochondrial membrane integrity and the consequent release of cytochrome *c* into the cytosol are important events during apoptosis and are regulated by the Bcl-2 family of proteins (Wang, 2001). As the members of the Bcl-2 family exert their actions mostly at the level of mitochondria and reside upstream of the onset of irreversible cellular damage, they play a pivotal role in determining whether a cell will live or die (Gross *et al*, 1999; Tsujimoto and Shimizu, 2000). All Bcl-2 family members possess at least one of four conserved motifs known as Bcl-2 homology domains (BH1–BH4). Most anti-apoptotic members, including Bcl-2 and Bcl-x_L, contain all the four domains. Pro-apoptotic members such as Bax and Bak lack the BH4 domain, whereas other pro-apoptotic members, the so-called BH3 domain-only proteins that include Bid, Bim and Bad, contain only the BH3 domain (Gross *et al*, 1999; Tsujimoto and Shimizu, 2000).

Recently, it has been demonstrated that Bax plays an essential role in inducing apoptosis in response to stress stimuli, as revealed by gene disruption of Bax and of Bax and Bak (Knudson *et al*, 1995; Lindsten *et al*, 2000; Wei *et al*, 2001; Zong *et al*, 2001). Bax is localized mostly in the cytoplasm, but redistributes to mitochondria in response to stress stimuli (Hsu *et al*, 1997; Wolter *et al*, 1997). After translocation to mitochondria, Bax induces cytochrome *c* release either by forming a pore by oligomerization in the outer mitochondrial membrane, or by opening other channels (Shimizu *et al*, 1999; Saito *et al*, 2000; Kuwana *et al*, 2002). The mechanisms underlying Bax translocation, however, are not fully understood.

Recent studies have shown that several proteins including 14-3-3 proteins prevent apoptosis through sequestration of Bax (Samuel *et al*, 2001; Guo *et al*, 2003; Nomura *et al*, 2003; Sawada *et al*, 2003). 14-3-3 proteins, which include seven isoforms in humans, are also implicated in antagonizing apoptotic signals through association with other pro-apoptotic proteins such as Bad, FKHRL1, ASK1 and Nur77 (Zha *et al*, 1996; Datta *et al*, 1997; Brunet *et al*, 1999; Zhang *et al*, 1999; Masuyama *et al*, 2001; van Hemert *et al*, 2001; Yaffe, 2002). Most 14-3-3 target proteins require phosphorylation to interact with 14-3-3, and consensus phosphoserine containing 14-3-3 binding motifs (RSXpSXP and RXXXpSXP) have been defined (Muslin *et al*, 1996; Yaffe *et al*, 1997). On the other hand, there are several examples of proteins containing dramatic variations from these motifs, including some that do not even require phosphorylation for binding, such as exoenzyme S, the platelet glycoprotein IB-IX-V complex, the plasma membrane H$^+$-ATPase AHA2 and *Drosophila* PAR-1 (Du *et al*, 1996; Masters *et al*, 1999; Svennelid *et al*, 1999; Benton *et al*, 2002). Bax also interacts with 14-3-3 proteins in a phosphorylation-independent manner (Nomura *et al*, 2003). A substantial proportion of Bax molecules is bound to 14-3-3 proteins in the cytosol of healthy cells; in response to stress stimuli, however, Bax dissociates from 14-3-3 and redistributes to mitochondria (Nomura *et al*, 2003). Moreover, caspases activated by stress stimuli cleave 14-3-3θ within its COOH-terminal region and promote its dissociation from Bax (Nomura *et al*, 2003). These observations are consistent with previous results showing that disruption of the 14-3-3σ gene promotes Bax translocation to mitochondria in response to cellular stresses (Samuel *et al*, 2001). However,

*Corresponding author. Institute of Molecular and Cellular Biosciences, University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-0032, Japan. Tel.: +81 3 5841 8473 or 7859; Fax: +81 3 5841 8472; E-mail: ygotoh@iam.u-tokyo.ac.jp

the translocation of Bax to mitochondria occurs independently of caspase activation in a number of systems (Putcha *et al*, 1999; Gilmore *et al*, 2000; Tsuruta *et al*, 2002), suggesting the existence of another mechanism responsible for the dissociation of Bax from 14-3-3 proteins.

The c-Jun N-terminal kinase (JNK) represents a group of mitogen-activated protein kinases (MAPKs), which is activated when cells are exposed to environmental stresses (Davis, 2000). In all, 10 members of the JNK family are generated by the alternative splicing of transcripts derived from the JNK1, JNK2 and JNK3 genes. The studies of JNK gene disruption in mice have confirmed that JNK contributes to stress responses (Davis, 2000). JNK3 is essential for apoptosis of hippocampal neurons following exposure to excitotoxic stresses (Yang *et al*, 1997). JNK1 and JNK2 are required for apoptosis of thymocytes in response to ligation of the T-cell receptor and of neurons in the developing hindbrain (Kuan *et al*, 1999; Sabapathy *et al*, 2001). Furthermore, JNK1 and JNK2 double-knockout cells are resistant to apoptosis induced by UV, anisomycin or DNA damage (Tournier *et al*, 2000). Moreover, recent studies have shown that Bax and Bak are required for JNK-induced apoptosis, and that Bax remains inactive upon exposure of JNK-deficient fibroblasts to environmental stress (Lei *et al*, 2002). How then does JNK activate Bax?

In this study, we show that 14-3-3ζ and 14-3-3σ are direct targets of JNK and that phosphorylation of 14-3-3 proteins by JNK results in dissociation of Bax from 14-3-3 proteins, leading to apoptosis. This novel function of JNK may provide the missing link between the stress-activated kinase cascade and Bax translocation to mitochondria, a critical step in the regulation of apoptosis.

Results

Active JNK promotes Bax translocation to the mitochondria

We first examined whether JNK activation is sufficient to promote Bax translocation to the mitochondria in COS-1 cells, since Bax has been reported to be required for JNK-induced apoptosis in mouse embryonic fibroblasts (Lei *et al*, 2002). To this end, we utilized a green fluorescent protein (GFP)–Bax fusion construct to monitor Bax localization in real time (Tsuruta *et al*, 2002). In all the experiments with GFP–Bax, p35, a pan-caspase inhibitor, was co-transfected to block caspase activation induced by overexpression of GFP–Bax. As a constitutively active form of JNK, we used a fusion protein (MKK7–JNK) in which MKK7α1 and JNK1β1 are connected by the Flag epitope tag (Figure 1A); in this construct, which is similar to the one used by Lei *et al* (2002), MKK7 phosphorylates and activates JNK1 intramolecularly (K Yoshioka, unpublished data). We found that expression of MKK7–JNK (wild type, WT) promoted GFP–Bax translocation to the mitochondria (Figure 1B and C). In contrast, the fusion construct of MKK7α1 and a kinase negative JNK (MKK7–JNK (KN)) had no effect on GFP–Bax localization (Figure 1B and C), suggesting that JNK promotes Bax translocation by phosphorylation of some target(s). To determine if JNK activity triggers translocation of the endogenous Bax protein, the distribution of endogenous Bax was assessed by subcellular fractionation after transfection of COS-1 cells with the MKK7–JNK constructs. The amount of endogenous Bax

detected in the mitochondrial fraction was increased, and that detected in the cytosolic fraction was decreased, by expression of MKK7–JNK(WT) but not by that of MKK7–JNK(KN). On the other hand, the abundance of the mitochondrial marker F_0F_1-ATPase subunit α (or that of the cytosolic marker α-tubulin) in the corresponding fraction was unaffected by the expression of either construct (Figure 1D). Although expression of MKK7–JNK(WT) resulted in the activation of caspase-3 in COS-1 cells (Supplementary Figure 1) (Lei *et al*, 2002), coexpression of p35, which prevented caspase-3 activation by MKK7–JNK(WT) (Supplementary Figure 1), failed to inhibit Bax translocation to the mitochondrial fraction (Figure 1D), suggesting that MKK7–JNK(WT) promoted the translocation of endogenous Bax to mitochondria independently of caspase activation.

To determine whether JNK activity is required for stress-induced translocation of Bax to the mitochondria, we examined the effect of SP600125, a JNK inhibitor, on GFP–Bax redistribution induced by the protein synthesis inhibitor anisomycin. Inhibition of protein synthesis by anisomycin triggers apoptosis in a broad variety of cells. Treatment of COS-1 cells with anisomycin resulted in phosphorylation of the JNK substrate c-Jun, and pretreatment with SP600125 blocked this effect of anisomycin (Figure 2A). Anisomycin also induced a gradual redistribution of GFP–Bax to mitochondria, and this effect of anisomycin was inhibited by SP600125 (Figure 2B). We also measured Bax translocation to the mitochondria using subcellular fractionation and found that SP600125 inhibited the translocation of endogenous Bax to mitochondria in response to treatment with anisomycin (Supplementary Figure 2). The concentration of SP600125 required for the inhibition of Bax translocation was the same as that required for the inhibition of c-Jun phosphorylation (data not shown). To verify that the effect of SP600125 was specific for JNK, we also used the JNK-binding domain peptide (JBD) (Dickens *et al*, 1997) and a dominant-negative (DN) JNK to block JNK activity. The JBD blocked anisomycin-induced GFP–Bax translocation (Figure 2C) and expression of DN form of JNK blocked GFP–Bax translocation induced by staurosporine (Supplementary Figure 3), another agent known to induce apoptosis. Taken together, these results suggest that JNK activity is required for Bax translocation to the mitochondria induced by anisomycin or by staurosporine.

JNK can regulate Bax localization independently of c-Jun, Akt and Bim

We first tested the ability of JNK to phosphorylate Bax and found that Bax is not phosphorylated by JNK in an *in vitro* kinase assay (Supplementary Figure 4). We therefore hypothesized that there is another JNK target that regulates Bax localization in response to stress stimuli. Apoptosis induced by neurotrophic factor deprivation in sympathetic neurons is mediated by the JNK-catalyzed phosphorylation of c-Jun (Harris and Johnson, 2001; Putcha *et al*, 2001; Whitfield *et al*, 2001). We examined whether c-Jun phosphorylation is required for Bax translocation to the mitochondria. MKK7–JNK(WT) phosphorylated c-Jun and induced activation of c-Jun-dependent transcription, as measured by a luciferase reporter gene controlled by an AP-1-dependent promoter (which monitors the activity of the Jun–Fos complex).
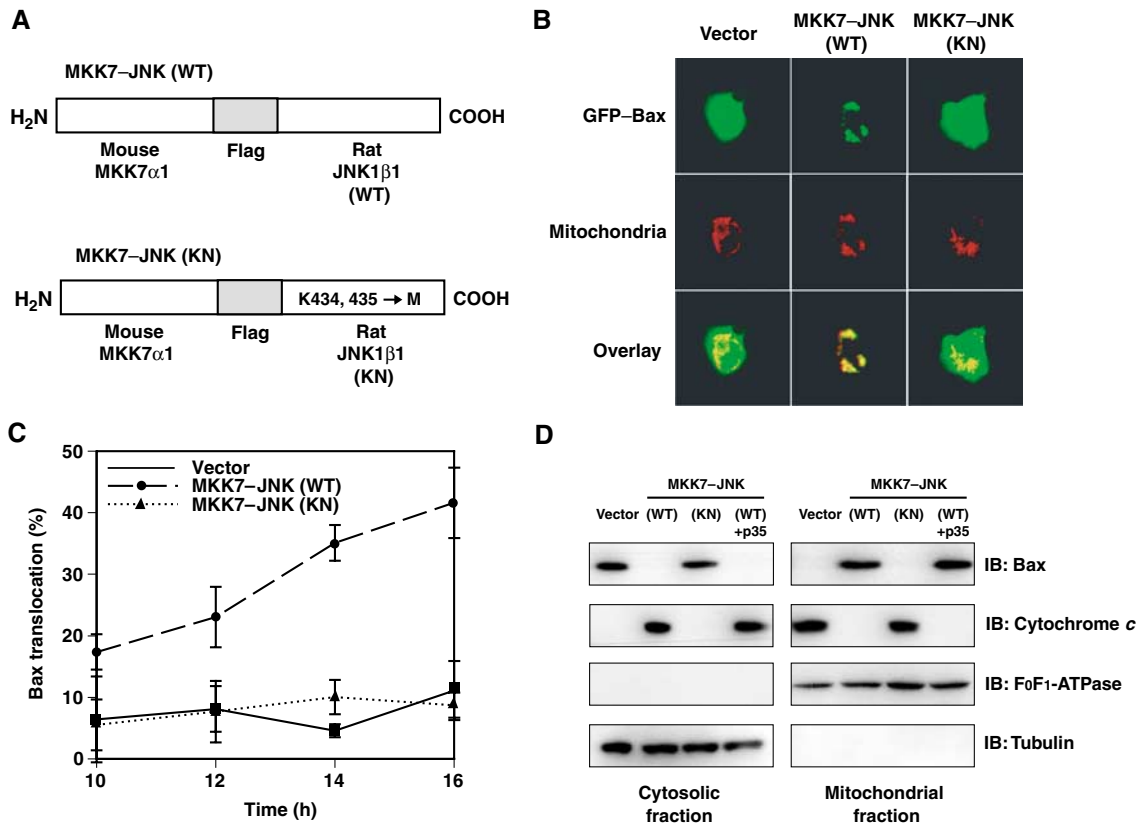
Figure 1 Expression of active JNK promotes Bax translocation to the mitochondria. (**A**) Schematic representation of the structure of the MKK7–JNK fusion proteins (WT or KN), which comprise mouse MKK7α1 linked to rat JNK1β1 by the Flag epitope sequence. The kinase-negative mutant of JNK1 was generated by replacement of Lys[434] and Lys[435] with methionines. (**B**) COS-1 cells were transfected for 13 h with expression vectors for GFP–Bax, the mitochondrial marker DsRed-Mito and the caspase inhibitor p35, together with a vector for MKK7-JNK (WT or KN) or the corresponding empty vector, as indicated. They were then examined for the distribution of GFP–Bax (green) and mitochondria (red) by fluorescence microscopy; the two separate images for each representative cell are also shown superimposed (overlay). (**C**) COS-1 cells were transfected for the indicated times as in (B), and the percentage of cells exhibiting GFP–Bax localization to mitochondria was determined. Data are means ± s.d. of values obtained from five fields of 30–150 cells in each of three independent experiments. (**D**) COS-1 cells were transfected for 20 h with expression vectors for GFP and MKK7–JNK(WT or KN) in the absence or presence of a vector for p35, as indicated. The transfection efficiency was ~75%, as determined by monitoring the expression of GFP. The cells were then subjected to subcellular fractionation, and the amount of endogenous Bax and that of cytochrome *c* in the mitochondrial and cytosolic fraction were assessed by immunoblot analysis (IB) with antibodies specific for these proteins. The amounts of the mitochondrial marker F_0F_1-ATPase and cytosolic marker α-tubulin were similarly assessed as an internal standard.

Activation of c-Jun-dependent transcription was blocked by coexpression of a DN form of c-Jun (Figure 3A). However, expression of DN c-Jun did not inhibit the translocation of GFP–Bax to mitochondria induced by MKK7–JNK(WT) (Figure 3B). The MKK7–JNK(WT)-induced increase in the amount of endogenous Bax in the mitochondrial fraction was also unaffected by DN c-Jun (Supplementary Figure 5). These results therefore suggest that JNK induces Bax translocation to mitochondria independently of the transcriptional activity of c-Jun.

Since previous reports have shown that Bax translocation to the mitochondria in response to apoptotic stimuli is suppressed by the PI3K–Akt pathway (Yamaguchi and Wang, 2001; Tsuruta *et al*, 2002; Molton *et al*, 2003), we examined the involvement of the PI3K–Akt pathway in JNK-mediated Bax translocation. We found that expression of MKK7–JNK(WT) or of MKK7–JNK(KN) had no effect on the EGF-induced activation and phosphorylation of Akt at Ser-473 in COS-1 cells (Supplementary Figure 6). Moreover, expression of constitutively active or DN Akt constructs did not affect the level of JNK phosphorylation (Tsuruta *et al*,

2002) or the JNK-induced GFP–Bax translocation to the mitochondria (Supplementary Figure 7). These results suggest that JNK does not induce Bax translocation by inhibiting the PI3K–Akt pathway.

The Bcl-2 family member Bim can regulate Bax translocation and has been reported to be a target of JNK (Lei and Davis, 2003; Putcha *et al*, 2003). We therefore examined whether Bim is phosphorylated by JNK under the conditions used in this study and whether Bim phosphorylation correlates with Bax translocation to mitochondria. Bim phosphorylation was monitored by a shift in its electrophoretical mobility on SDS–PAGE as described (Lei and Davis, 2003; Putcha *et al*, 2003). Immunoblot analysis showed a mobility shift of Bim in response to anisomycin treatment. The JNK inhibitor SP600125, however, had little effect on the aniso-mycin-induced mobility shift of Bim, whereas it effectively blocked phosphorylation of c-Jun (Supplementary Figure 8). Importantly, expression of MKK7–JNK(WT) promoted Bax translocation to mitochondria, but did not induce the mobility shift of Bim (Supplementary Figure 8). These data suggest that a kinase or kinases other than JNK are activated by
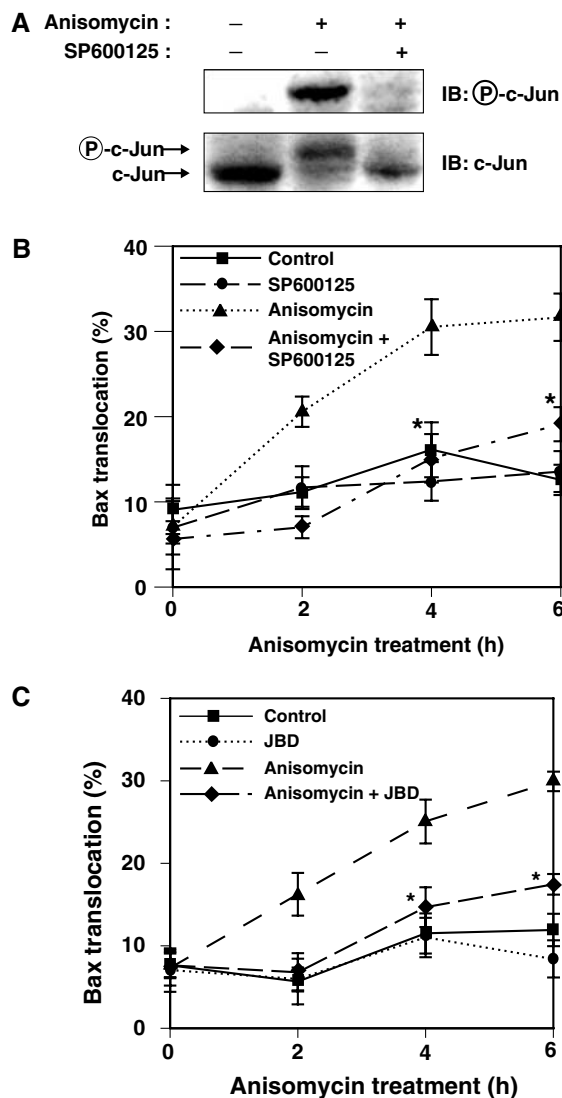
A

| Anisomycin : | – | + | + |
| SP600125 : | – | – | + |

IB: (P)-c-Jun

(P)-c-Jun→
c-Jun→

IB: c-Jun

B

C

Figure 2 JNK is required for stress-induced translocation of Bax to the mitochondria. (**A**) COS-1 cells were incubated first for 30 min with or without 20 μM SP600125 and then for 1 h in the presence or absence of anisomycin (10 μg/ml). Cell lysates were then subjected to immunoblot analysis with antibodies to phosphorylated c-Jun or to c-Jun. The anisomycin-induced shift in the electrophoretic mobility of the band detected by the antibody to c-Jun reflects phosphorylation of c-Jun. (**B**) COS-1 cells were transfected for 11 h with expression vectors for GFP–Bax and p35, were pretreated with or without 20 μM SP600125 for 30 min, and were then incubated for the indicated times in the presence or absence of anisomycin (10 μg/ml). The percentage of cells in which GFP–Bax was localized to mitochondria was then determined. Data are means ± s.d. of values obtained from five fields of 30–150 cells in each of three independent experiments (*$P < 0.0005$ as compared with the anisomycin group). (**C**) COS-1 cells were transfected for 11 h with expression vectors for GFP–Bax and p35, together with a vector for JBD or the corresponding empty vector, as indicated. The cells were then incubated for the indicated times in the presence or absence of 10 μg/ml anisomycin, after which the percentage of cells exhibiting GFP–Bax localization to mitochondria was determined. Data are means ± s.d. of values obtained from five fields of 30–150 cells in each of two independent experiments (*$P < 0.0005$ as compared with the anisomycin group).

anisomycin and phosphorylate Bim, and that JNK induces Bax translocation independently of Bim phosphorylation at least in this system.
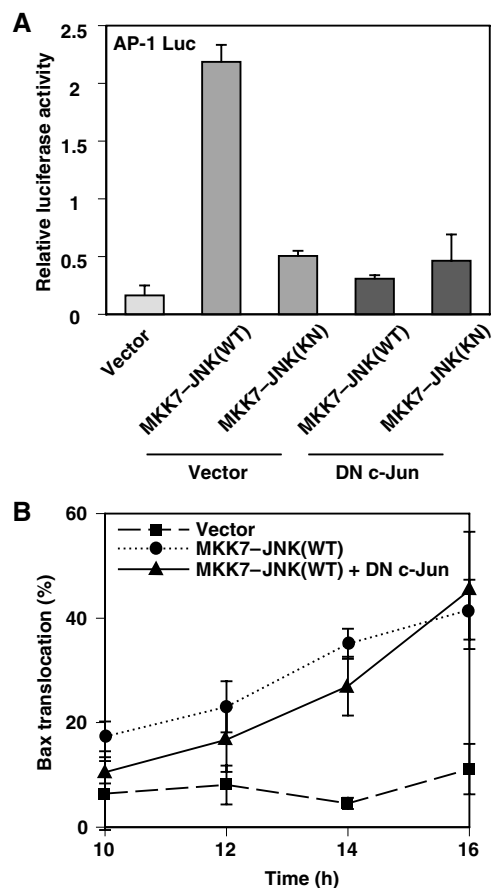
A

B

Figure 3 JNK induces Bax translocation to the mitochondria independently of c-Jun. (**A**) COS-1 cells were transfected for 1 day with an AP-1-luciferase reporter plasmid and expression vectors for MKK7–JNK(WT or KN) and a DN form of c-Jun or the corresponding empty vectors, as indicated. The normalized luciferase activity of cell lysates was then determined. Data represent the means ± s.d. of triplicate determinations from three independent experiments. (**B**) COS-1 cells were transfected for the indicated times with expression vectors for GFP–Bax and p35 together with vectors for MKK7–JNK(WT or KN) and DN c-Jun, as indicated. The percentage of cells exhibiting GFP–Bax localization to mitochondria was then determined. Data are means ± s.d. of values obtained from five fields of 30–150 cells in each of two independent experiments.

JNK phosphorylates 14-3-3ζ at Ser-184 and 14-3-3σ at Ser-186 in vitro

Under resting conditions, 14-3-3 proteins function as cytoplasmic anchors of Bax and prevent Bax from translocating to the mitochondria (Nomura et al, 2003). Consistent with this observation, targeted disruption of the 14-3-3σ gene was shown to accelerate Bax translocation to the mitochondria in human HCT116 cells (Samuel et al, 2001). These findings prompted us to investigate the possibility that JNK promotes Bax translocation to mitochondria by regulating the interaction between Bax and 14-3-3 proteins. Previous reports have shown that several isoforms of 14-3-3 are phosphorylated in vivo at a serine residue in the region between α-helices 7 and 8 (Ser-186 of 14-3-3β and Ser-184 of 14-3-3ζ), although the kinase or kinases responsible for this phosphorylation were not identified (Aitken et al, 1995). Interestingly, this residue conforms to the consensus sequence for JNK phosphorylation (Ser-Pro), and resides within the putative Bax-binding region (Figure 4A) (Nomura et al, 2003). We thus
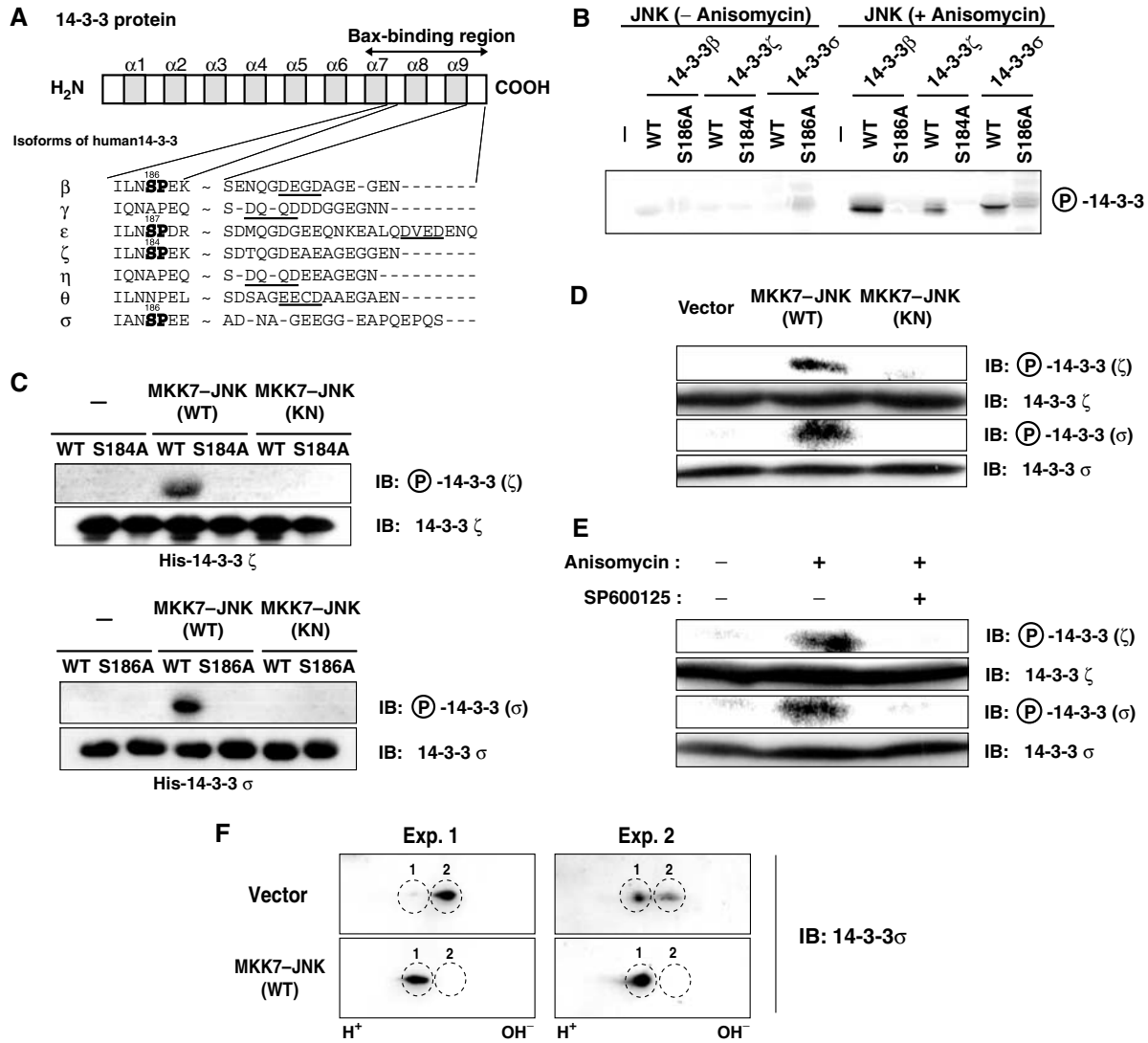
Figure 4 JNK phosphorylates 14-3-3ζ and 14-3-3σ both *in vitro* and *in vivo*. (**A**) Schematic representation of the structure of human 14-3-3 proteins and the amino-acid sequences surrounding putative JNK phosphorylation sites (SP, shown in bold) and caspase-3 cleavage sites ((D/E)XXD, underlined) in the Bax-binding region of the indicated isoforms. The numbers indicate the position of putative phosphorylation sites. (**B**) COS-1 cells were transfected for 24 h with an expression vector for Flag-tagged JNK and then incubated for 1 h in the absence or presence of anisomycin (10 μg/ml). Flag–JNK was immunoprecipitated from cell lysates with antibodies to Flag and incubated with the indicated recombinant His_6-tagged 14-3-3 proteins (WT or Ser→Ala mutants) in the presence of $[\gamma\text{-}^{32}P]ATP$. The amount of JNK immunoprecipitated from anisomycin-treated cell extracts was comparable to that from untreated cell extracts under the conditions used in this *in vitro* kinase assay (data not shown). Phosphorylation of 14-3-3 proteins was detected by electrophoresis and autoradiography. (**C**) Recombinant His_6-tagged 14-3-3 proteins (WT or mutant) were incubated with or without recombinant MKK7–JNK(WT or KN) in the presence of ATP and were then subjected to immunoblot analysis with antibodies specific for 14-3-3ζ or 14-3-3ζ phosphorylated on Ser^{184} (upper panels) or for 14-3-3σ or 14-3-3σ phosphorylated on Ser^{186} (lower panels). (**D**) HCT116 cells were transfected for 1 day with an expression vector for MKK7–JNK(WT or KN) or the corresponding empty vector, after which cell lysates were subjected to immunoblot analysis with the antibodies described in (C). (**E**) HCT116 cells were incubated first for 30 min with or without 20 μM SP600125 and then for 3 h in the presence or absence of anisomycin (10 μg/ml). Cell lysates were then subjected to immunoblot analysis as described in (D). (**F**) HCT116 cells were transfected for 1 day with an expression vector for MKK7–JNK(WT) or the corresponding empty vector, after which cell lysates were subjected to two-dimensional gel electrophoresis and immunoblot analysis with anti-14-3-3σ antibody. Two typical results (Exp. 1 and Exp. 2) are shown. The ratio between Spot 1 and Spot 2 in control cells varied among experiments, but expression of MKK7–JNK (WT) repeatedly increased Spot 1 and abolished Spot 2.

first examined whether JNK is capable of phosphorylating 14-3-3 proteins. Active JNK immunoprecipitated from anisomycin-treated COS-1 cell extracts efficiently phosphorylated recombinant His-tagged 14-3-3β, 14-3-3ζ and 14-3-3σ in an *in vitro* kinase assay (Figure 4B). JNK immunoprecipitates prepared from untreated cells did not phosphorylate recombinant 14-3-3 proteins, confirming that the kinase activity of JNK was responsible for this phosphorylation. Mutant pro-

teins in which Ser-184 of 14-3-3ζ or Ser-186 of 14-3-3β or 14-3-3σ was replaced with alanine were not phosphorylated by the active JNK immunoprecipitated from anisomycin-treated cells (Figure 4B), indicating that these serine residues are the JNK-mediated phosphorylation sites *in vitro*.

To further indicate that Ser-184 of 14-3-3ζ and Ser-186 of 14-3-3σ are phosphorylated by JNK, we prepared antibodies to phosphopeptides corresponding to these phosphorylation
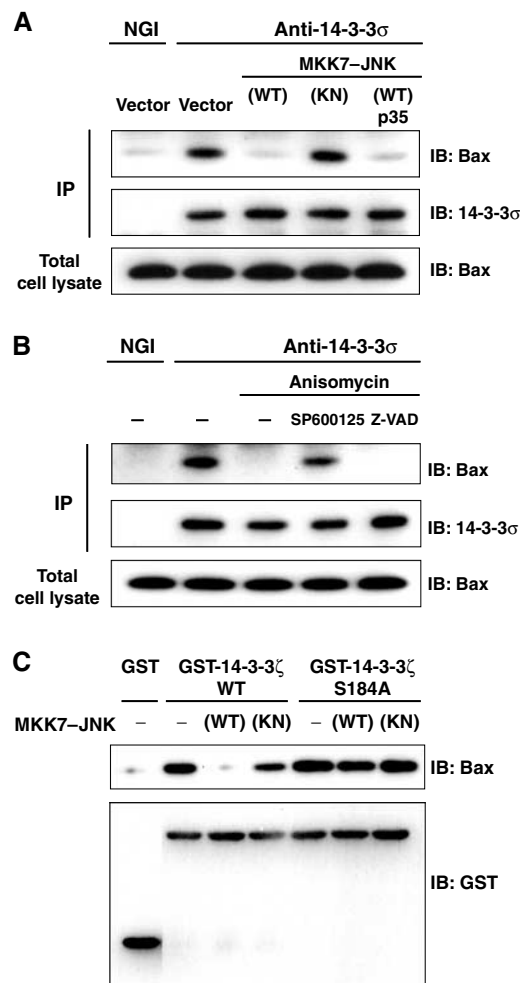
Figure 5 JNK promotes dissociation of Bax from 14-3-3 proteins. (**A**) HCT116 cells were transfected for 24 h with expression vectors for GFP, MKK7–JNK(WT or KN) and p35, as indicated. Cell lysates were then subjected either to immunoblot analysis with antibodies to Bax or to immunoprecipitation (IP) with antibodies to 14-3-3σ (or to normal goat IgG (NGI) precipitation); the resulting precipitates were subjected to immunoblot analysis with antibodies to 14-3-3σ and to Bax. (**B**) HCT116 cells were incubated first for 30 min with or without 20 μM SP600125 or 100 μM Z-VAD-CH₂DCB and then for 3 h in the presence or absence of anisomycin (10 μg/ml). Cell lysates were then subjected to immunoprecipitation and immunoblot analysis as described in (A). (**C**) Equal amounts of recombinant GST–14-3-3ζ (WT or Ser^{184}→Ala mutant) or GST alone were incubated with or without recombinant MKK7–JNK(WT or KN) in the presence of ATP for 30 min, and the GST proteins were precipitated with glutathione-sepharose beads. The beads were then incubated with HeLa cell lysates, and subjected to immunoblot analysis with antibodies to Bax or to GST.

Figure 6 14-3-3 mutants inhibit JNK-induced Bax translocation to mitochondria. (**A**) COS-1 cells were transfected for 16 h with expression vectors for GFP–Bax and p35 together with those for MKK7–JNK(WT) and either 14-3-3ζ or 14-3-3σ. The percentage of cells exhibiting GFP–Bax localization to the mitochondria was then determined. Data are means ± s.d. of values obtained from five fields of 30–150 cells in each of three independent experiments (*P < 0.0005). (**B**) COS-1 cells were transfected for 11.5 h with expression vectors for GFP–Bax, p35 and either 14-3-3ζ or 14-3-3σ, and were incubated for 6 h in the presence or absence of anisomycin (10 μg/ml). The percentage of cells in which GFP–Bax was localized to the mitochondria was then determined and shown as in (A) (*P < 0.0005 and **P < 0.005).

sites. The anti-phospho-Ser^{184}-14-3-3ζ and anti-phospho-Ser^{186}-14-3-3σ antibodies recognized recombinant 14-3-3 proteins (14-3-3ζ and σ) that had been phosphorylated by recombinant MKK7–JNK(WT), but not those incubated with MKK7–JNK(KN) (Figure 4C). Moreover, these antibodies did not react with the Ser→Ala (SA) mutants of the corresponding 14-3-3 proteins, confirming that they specifically recognize phosphorylated 14-3-3 proteins at these serine residues.

JNK phosphorylates 14-3-3ζ at Ser-184 and 14-3-3σ at Ser-186 *in vivo*
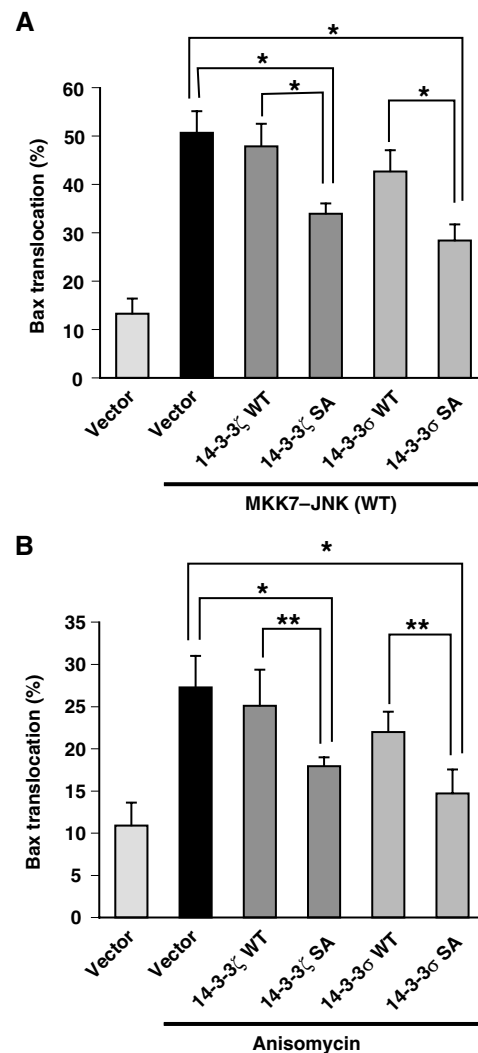To determine whether JNK also phosphorylates 14-3-3 *in vivo*, we examined the phosphorylation status of 14-3-3

proteins with the anti-phospho-14-3-3 antibodies. We used HCT116 cells in which ζ and σ isoforms of 14-3-3 are expressed endogenously. Expression of MKK7–JNK(WT), but not MKK7–JNK(KN), in HCT116 cells increased the bands reactive to the antibodies raised against phosphopeptides for 14-3-3ζ at Ser-184 and 14-3-3σ at Ser-186 (Figure 4D), suggesting that endogenous 14-3-3 proteins became phosphorylated upon JNK activation. We then addressed whether the phosphorylation of 14-3-3 is induced by cellular stresses. Exposure of HCT116 cells to anisomycin induced the phosphorylation of 14-3-3 proteins, revealed by the antibodies for 14-3-3ζ at Ser-184 and 14-3-3σ at Ser-186 (Figure 4E). The concentration of anisomycin required for phosphorylation of 14-3-3 was similar to that required for
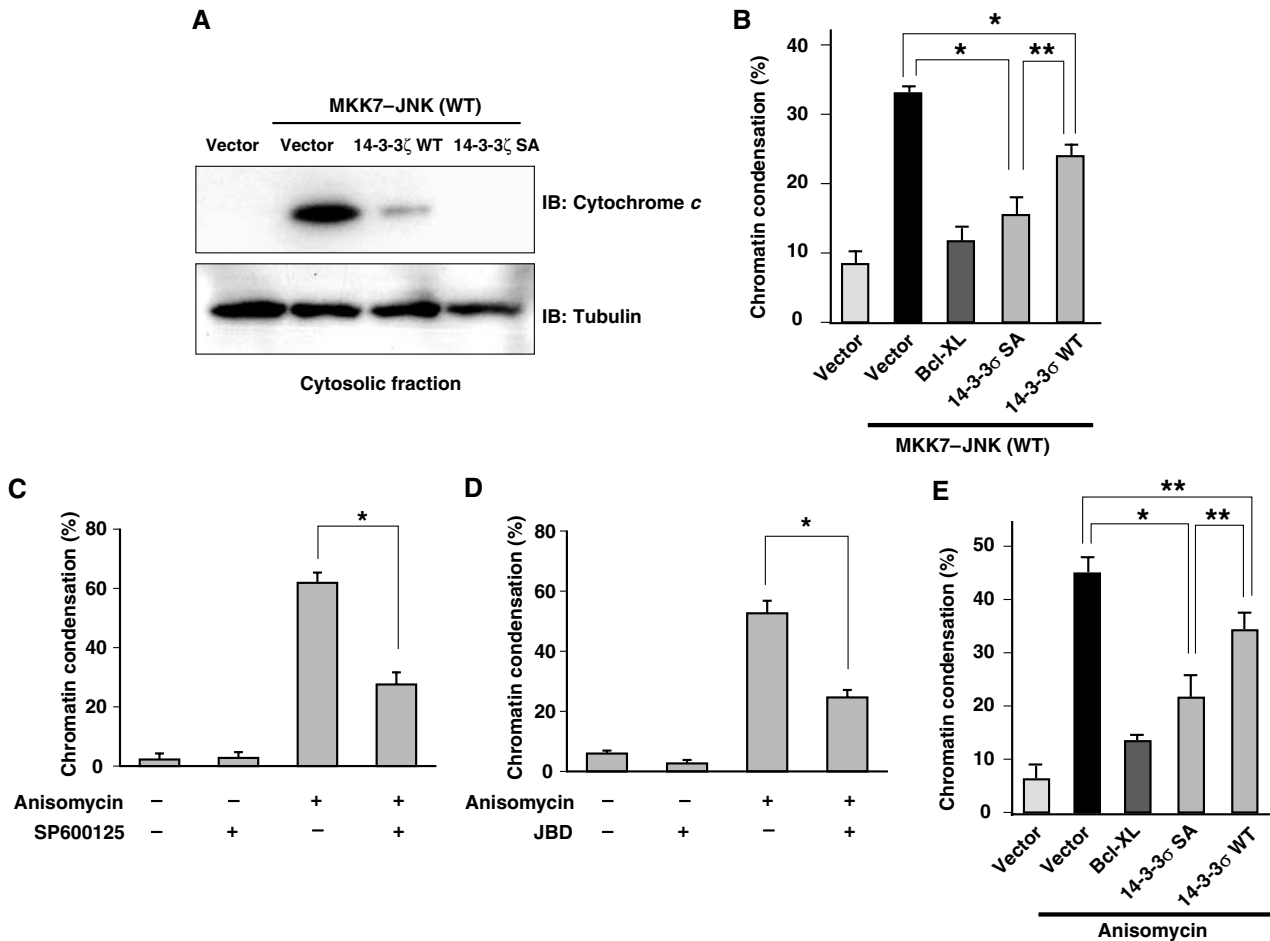
Figure 7 14-3-3 mutants inhibit JNK-induced cell death. (**A**) COS-1 cells were transfected for 20 h with expression vectors for GFP, MKK7–JNK(WT), and either 14-3-3ζ WT or 14-3-3ζ S184A, as indicated, and were then subjected to subcellular fractionation. The amounts of endogenous cytochrome *c* and α-tubulin (internal control) in the cytosolic fraction were determined by immunoblot analysis with specific antibodies. (**B**) COS-1 cells were transfected for 20 h with expression vectors for GFP, MKK7–JNK(WT) and either Bcl-XL, 14-3-3σ S186A or the corresponding WT proteins, as indicated. They were then stained with Hoechst 33342 (6.7 μg/ml) for 10 min, and the percentage of GFP-positive cells with pyknotic nuclei was determined. Data are means ± s.d. of values obtained from three fields of 200–300 cells in each of three independent experiments (*$P < 0.0005$ and **$P < 0.005$). (**C**) HeLa cells were incubated first for 30 min with or without 20 μM SP600125 and then for 3 h in the presence or absence of anisomycin (10 μg/ml). They were then stained with Hoechst 33342 (6.7 μg/ml) for 10 min, and the percentage of cells with pyknotic nuclei was determined. Data are means ± s.d. of values obtained from three fields of 100–200 cells in each of two independent experiments (*$P < 0.0005$). (**D**) HeLa cells were transfected with expression vectors for GFP and JBD and then for 3 h in the presence or absence of anisomycin (10 μg/ml). They were then stained with Hoechst 33342 (6.7 μg/ml) for 10 min, and the percentage of GFP-positive cells with pyknotic nuclei was determined. Data are means ± s.d. of values obtained from three fields of 100–150 cells (*$P < 0.0005$). (**E**) HeLa cells were transfected with expression vectors for GFP and either Bcl-XL, 14-3-3σ S186A or the corresponding WT protein, and then for 3 h in the presence or absence of anisomycin (10 μg/ml). They were then stained with Hoechst 33342 (6.7 μg/ml) for 10 min, and the percentage of GFP-positive cells with pyknotic nuclei was determined and shown as in (C) (*$P < 0.0005$ and **$P < 0.005$).

JNK activation (data not shown). Furthermore, anisomycin-induced phosphorylation of 14-3-3 proteins (14-3-3ζ and 14-3-3σ) was inhibited by pretreatment of cells with the JNK inhibitor SP600125 (Figure 4E), suggesting that JNK activation is required for anisomycin-induced 14-3-3 phosphorylation *in vivo*.

To further examine phosphorylation of 14-3-3 upon JNK activation *in vivo*, we carried out two-dimensional gel electrophoresis to separate hyper- and hypophosphorylated forms of 14-3-3. We detected two spots of 14-3-3σ in control (vector-transfected) HCT116 cells. Expression of MKK7–JNK(WT) (Figure 4F) or treatment with anisomycin (data not shown) resulted in a marked increase in the spot with greater negative charge (Spot 1) and a corresponding decrease in the other spot (Spot 2). We confirmed that Spot 1 is

a phosphorylated form of Spot 2, as alkaline phosphatase treatment of MKK7–JNK(WT)-expressing cell extracts reduced Spot 1 and increased Spot 2 (data not shown). These results suggest that a high proportion of 14-3-3σ becomes phosphorylated upon JNK activation.

JNK promotes dissociation of Bax from 14-3-3 proteins

We next asked whether JNK-mediated phosphorylation of 14-3-3 affects the interaction between Bax and 14-3-3. Immunoprecipitation of 14-3-3σ from HCT116 cell lysates resulted in the co-precipitation of endogenous Bax (Figure 5A). Expression of MKK7–JNK(WT), but not that of MKK7–JNK(KN), resulted in a marked decrease in the amount of Bax that co-precipitated with 14-3-3σ. This effect of MKK7–JNK(WT) was not affected by coexpression of p35,

suggesting that JNK promotes the dissociation of Bax from 14-3-3 independently of caspase activation.

Exposure of HCT116 cells to anisomycin also reduced the amount of Bax that co-immunoprecipitated with 14-3-3σ (Figure 5B). Pretreatment of cells with the caspase inhibitor Z-VAD-CH$_2$DCB had no effect on the anisomycin-induced dissociation of Bax from 14-3-3, whereas pretreatment with the JNK inhibitor SP600125 blocked this effect of anisomycin. These results thus suggest that JNK mediates the anisomycin-induced dissociation of Bax from 14-3-3.

To examine further whether JNK-induced dissociation of Bax from 14-3-3 is dependent on the phosphorylation status of 14-3-3, we performed glutathione *S*-transferase (GST) pull-down assays with a GST–14-3-3ζ fusion protein (Figure 5C). Incubation of GST–14-3-3ζ, but not that of GST alone, with HeLa cell lysates resulted in the co-precipitation of Bax by glutathione-Sepharose. However, prior phosphorylation of GST-14-3-3ζ by MKK7–JNK(WT) led to a marked decrease (approximately 90% reduction) in the amount of Bax that co-precipitated with GST-14-3-3ζ. The S184A mutant of GST-14-3-3ζ precipitated similar amounts of Bax, regardless of whether or not it had been preincubated with MKK7–JNK(WT). These results are thus consistent with the notion that JNK-mediated phosphorylation of 14-3-3ζ at Ser184 reduces its affinity for Bax.

14-3-3 mutants inhibit Bax translocation, cytochrome c release and cell death

If 14-3-3 is a major target for JNK in induction of Bax translocation, one might expect that expression of a phosphorylation site mutant of 14-3-3 would block JNK-induced Bax translocation. Indeed, expression of either 14-3-3ζ S184A or 14-3-3σ S186A mutant inhibited MKK7–JNK- and anisomycin-induced GFP–Bax translocation to mitochondria (Figure 6A and B and Supplementary Figure 9). In addition, when distribution of endogenous Bax was assessed by subcellular fractionation, expression of 14-3-3ζ S184A mutant inhibited the increase of Bax protein in the mitochondrial fraction induced by MKK7–JNK (Supplementary Figure 10). Importantly, in both assays, 14-3-3 WT was less effective than its SA mutant in suppression of JNK-induced Bax translocation (Figure 6A and B and Supplementary Figure 10), suggesting strongly that phosphorylation of 14-3-3 at this Ser residue is critical for Bax translocation to mitochondria upon JNK activation.

We next examined whether 14-3-3 mutants inhibited JNK-induced cytochrome *c* release and cell death. The amount of endogenous cytochrome *c* detected in the cytosolic fraction was increased when MKK7–JNK(WT), but not MKK7–JNK(KN), was expressed in COS-1 cells (Figure 7A and data not shown). In contrast, co-expression of 14-3-3ζ S184A mutant, and that of the corresponding 14-3-3 WT to a lesser extent, inhibited MKK7–JNK (WT)-induced cytochrome *c* release, whereas the amount of the cytosolic marker α-tubulin in the cytosolic fraction was unaffected by MKK7–JNK or the 14-3-3 mutants (Figure 7A). Expression of MKK7–JNK (WT) in COS-1 cells induced chromatin condensation, an indication of cell death (Figure 7B). However, expression of 14-3-3σ SA mutant rendered the cells resistant to this effect of MKK7–JNK (WT) (Figure 7B). Moreover, 14-3-3σ WT was less effective in protecting cells from MKK7–JNK (WT)-induced cell death, compared to the 14-3-3σ SA

mutant (Figure 7B). We next asked whether the 14-3-3σ mutant can inhibit anisomycin-induced cell death. Treatment of HeLa cells with anisomycin resulted in cell death, and pretreatment with SP600125 or coexpression of JBD or DN JNK partially blocked this effect (Figure 7C and D and Supplementary Figure 11), suggesting that JNK is required for anisomycin-induced cell death in HeLa cells. We found that expression of 14-3-3σ SA mutant blocked anisomycin-induced cell death, and 14-3-3σ WT was less effective compared to the 14-3-3σ SA mutant (Figure 7E), supporting the notion that phosphorylation of 14-3-3 by JNK plays an important role in JNK-mediated cell death.

Discussion

Recent studies have demonstrated that JNK plays a pivotal role in activation of the intrinsic apoptotic pathway that is mediated by mitochondria in response to cellular stress (Davis, 2000). Although Bax has been shown to be necessary for this action of JNK, the nature of the functional relation between these two proteins has been unclear. We now provide several lines of evidence that demonstrate that JNK-mediated phosphorylation of 14-3-3 induces the release of Bax from 14-3-3 and triggers its translocation to the mitochondria: (1) expression of an active form of JNK promoted Bax translocation to mitochondria; (2) inhibition of JNK (either by a chemical inhibitor or by a DN mutant) reduced the extent of Bax translocation to mitochondria in response to cellular stress; (3) JNK phosphorylated 14-3-3ζ at Ser-184 and 14-3-3σ at Ser-186 both *in vitro* and *in vivo*, and such phosphorylation reduced the affinity of 14-3-3 proteins for Bax; (4) expression of active JNK or treatment of cells with anisomycin induced the dissociation of Bax from 14-3-3 proteins; and (5) expression of phosphorylation site mutants of 14-3-3 proteins inhibited Bax translocation to mitochondria. These results strongly indicate that JNK regulates the activity of Bax by phosphorylating 14-3-3 proteins.

The initial studies of the role of JNK in apoptotic signals were performed by investigating neuronal cell death in response to neurotrophic factor withdrawal (Xia *et al*, 1995). The role for JNK in stress-induced neuronal cell death was confirmed by the studies of mice with a targeted disruption of the neuronal gene JNK3 (Yang *et al*, 1997). Although the JNK3 knockout mice are developmentally normal, they are resistant to excitotoxins. A similar resistance was observed in mice with a germ-line mutation in the c-Jun gene that replaced the JNK phosphorylation sites with alanine (Behrens *et al*, 1999). These data suggested that JNK mediates transcription-dependent apoptotic signals in neurons. In contrast, in non-neuronal cells, the JNK-mediated cytochrome *c* release and apoptosis induced by UV do not require *de novo* gene expression (Tournier *et al*, 2000). It is thus likely that relevant targets of JNK required for cytochrome *c* release and apoptosis are already present in these cells. Potential targets of JNK that may regulate cytochrome *c* release and apoptosis include members of the Bcl-2 family. The anti-apoptotic members Bcl-2, Bcl-x$_L$ and Mcl-1, and the pro-apoptotic members Bad, Bim and Bmf are phosphorylated by JNK, although the significance of these phosphorylation events remains unclear (Ito *et al*, 1997; Maundrell *et al*, 1997; Kharbanda *et al*, 2000; Donovan *et al*, 2002; Inoshita *et al*, 2002; Lei and Davis, 2003; Putcha *et al*, 2003). We have

now shown that expression of phosphorylation site mutants of 14-3-3, and 14-3-3 WT to a lesser extent, suppressed JNK-induced Bax translocation, mitochondrial cytochrome *c* release and subsequent cell death. We confirmed that the 14-3-3 mutant did not hamper JNK-mediated phosphorylation of other targets such as c-Jun (F Tsuruta and Y Gotoh, unpublished results). These results strongly suggest that 14-3-3 protein is a major target of JNK in induction of these apoptotic events.

The JNK-mediated phosphorylation of 14-3-3β and 14-3-3ζ was found to occur at the previously identified phosphorylation sites (Aitken *et al*, 1995); 14-3-3β and 14-3-3ζ phosphorylated at these sites were named 14-3-3α and 14-3-3δ, respectively. It remains to be determined how such phosphorylation leads to the dissociation of Bax from 14-3-3. However, since the JNK phosphorylation sites reside within the domain responsible for the interaction of 14-3-3 with Bax (Nomura *et al*, 2003), it is likely that phosphorylation impairs the interaction at the binding interface. Alternatively, although the overall structure of 14-3-3 proteins is thought to be relatively rigid (Liu *et al*, 1995; Xiao *et al*, 1995), it is possible that phosphorylation might change the global conformation of 14-3-3 and thereby induce the dissociation of Bax.

On the Bax side, the 14-3-3-interacting domains are in the NH_2- and COOH-terminal regions (Nomura *et al*, 2003). Interestingly, previous studies have proposed that these regions are masked when Bax is in the inactive form and are exposed upon activation. Indeed, antibodies against certain epitopes at the NH_2-terminal region (cf. 6A7 monoclonal antibody) can react only with active Bax (Nechushtan *et al*, 1999; Lei *et al*, 2002). It is thus possible that 14-3-3 proteins physically conceal the NH_2- and COOH-terminal domains of Bax and thereby maintain it in its inactive form, and that dissociation of Bax from 14-3-3 may be essential for its activation in addition to the conformational change of Bax. According to this scenario, our observation that phosphorylation of 14-3-3 by JNK releases Bax from 14-3-3 may explain how JNK activates Bax. Consistent with this notion, targeted disruption of JNK genes has been shown to prevent the activation of Bax in response to cellular stresses, as judged by the lack of reactivity toward the 6A7 antibody (Lei *et al*, 2002).

Another proposed mechanism of Bax activation is a conformational change due to acidification or alkalization of the cytoplasmic pH (Khaled *et al*, 1999; Nomura *et al*, 2003). However, we could not detect a significant change in the signal level of a pH indicator incorporated into the cytoplasm in the presence or absence of activated JNK in our prelimin-

ary experiments (F Tsuruta and Y Gotoh, unpublished results), suggesting that it is unlikely that JNK promotes Bax activation indirectly through regulation of cytoplasmic pH.

Whereas we have shown that three 14-3-3 isoforms (β, ζ and σ) serve as JNK targets, some isoforms (γ, η and θ) do not possess a corresponding JNK phosphorylation site. One of these isoforms (θ) has been reported to be cleaved by caspase, resulting in dissociation of Bax (Nomura *et al*, 2003). Our results indicate that caspase activity is dispensable for the JNK-dependent dissociation of Bax from 14-3-3, indicating the existence of two independent mechanisms to regulate their dissociation. Since caspase is activated downstream of JNK, it is possible that phosphorylation-dependent release of Bax from 14-3-3 takes place first, and the caspase-dependent release of Bax subsequently amplifies the apoptotic signal through positive feedback.

Recently, it has been reported that Ku70 and Humanin also prevent apoptosis through sequestration of Bax (Guo *et al*, 2003; Sawada *et al*, 2003). However, Sawada *et al* (2003) have shown that the absence of Ku70 is not sufficient for inducing the apoptotic level of Bax translocation to mitochondria in the absence of apoptotic stimuli. Guo *et al* (2003) have also demonstrated that elimination of endogenous Humanin does not induce Bax translocation to mitochondria and apoptosis in healthy cells. It is thus likely that several cytoplasmic anchors for Bax work in parallel to sequester Bax from mitochondria, and dissociation of Bax from all or some of these cytoplasmic anchors may be required for initiation of Bax translocation to mitochondria.

In conclusion, we have demonstrated that JNK promotes Bax translocation to mitochondria through phosphorylation of 14-3-3 proteins. This finding may account at least in part for the apoptosis-inducing activity of the JNK and shed light on the mechanism of stress-induced apoptosis.

Materials and methods

The Materials and methods used in this study are described in Supplementary data.

Supplementary data
Supplementary data are available at *The EMBO Journal* Online.

Acknowledgements

References

Aitken A, Howell S, Jones D, Madrazo J, Patel Y (1995) 14-3-3 alpha and delta are the phosphorylated forms of raf-activating 14-3-3 beta and zeta. *In vivo* stoichiometric phosphorylation in brain at a Ser-Pro-Glu-Lys MOTIF. *J Biol Chem* **270:** 5706–5709

Behrens A, Sibilia M, Wagner EF (1999) Amino-terminal phosphorylation of c-Jun regulates stress-induced apoptosis and cellular proliferation. *Nat Genet* **21:** 326–329

Benton R, Palacios IM, Johnston DS (2002) Drosophila 14-3-3/PAR-5 is an essential mediator of PAR-1 function in axis formation. *Dev Cell* **3:** 659–671

Brunet A, Bonni A, Zigmond MJ, Lin MZ, Juo P, Hu LS, Anderson MJ, Arden KC, Blenis J, Greenberg ME (1999) Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. *Cell* **96:** 857–868

Datta SR, Dudek H, Tao X, Masters S, Fu H, Gotoh Y, Greenberg ME (1997) Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. *Cell* **91:** 231–241

Davis RJ (2000) Signal transduction by the JNK group of MAP kinases. *Cell* **103:** 239–252

Dickens M, Rogers JS, Cavanagh J, Raitano A, Xia Z, Halpern JR, Greenberg ME, Sawyers CL, Davis RJ (1997) A cytoplasmic inhibitor of the JNK signal transduction pathway. *Science* **277:** 693–696

Donovan N, Becker EB, Konishi Y, Bonni A (2002) JNK phosphorylation and activation of BAD couples the stress-activated

signaling pathway to the cell death machinery. *J Biol Chem* **277:** 40944–40949

Du X, Fox JE, Pei S (1996) Identification of a binding sequence for the 14-3-3 protein within the cytoplasmic domain of the adhesion receptor, platelet glycoprotein Ib alpha. *J Biol Chem* **271:** 7362–7367

Gilmore AP, Metcalfe AD, Romer LH, Streuli CH (2000) Integrin-mediated survival signals regulate the apoptotic function of Bax through its conformation and subcellular localization. *J Cell Biol* **149:** 431–446

Gross A, McDonnell JM, Korsmeyer SJ (1999) BCL-2 family members and the mitochondria in apoptosis. *Genes Dev* **13:** 1899–1911

Guo B, Zhai D, Cabezas E, Welsh K, Nouraini S, Satterthwait AC, Reed JC (2003) Humanin peptide suppresses apoptosis by interfering with Bax activation. *Nature* **423:** 456–461

Harris CA, Johnson Jr EM (2001) BH3-only Bcl-2 family members are coordinately regulated by the JNK pathway and require Bax to induce apoptosis in neurons. *J Biol Chem* **276:** 37754–37760

Hsu YT, Wolter KG, Youle RJ (1997) Cytosol-to-membrane redistribution of Bax and Bcl-X(L) during apoptosis. *Proc Natl Acad Sci USA* **94:** 3668–3672

Inoshita S, Takeda K, Hatai T, Terada Y, Sano M, Hata J, Umezawa A, Ichijo H (2002) Phosphorylation and inactivation of myeloid cell leukemia 1 by JNK in response to oxidative stress. *J Biol Chem* **277:** 43730–43734

Ito T, Deng X, Carr B, May WS (1997) Bcl-2 phosphorylation required for anti-apoptosis function. *J Biol Chem* **272:** 11671–11673

Khaled AR, Kim K, Hofmeister R, Muegge K, Durum SK (1999) Withdrawal of IL-7 induces Bax translocation from cytosol to mitochondria through a rise in intracellular pH. *Proc Natl Acad Sci USA* **96:** 14476–14481

Kharbanda S, Saxena S, Yoshida K, Pandey P, Kaneki M, Wang Q, Cheng K, Chen YN, Campbell A, Sudha T, Yuan ZM, Narula J, Weichselbaum R, Nalin C, Kufe D (2000) Translocation of SAPK/JNK to mitochondria and interaction with Bcl-x(L) in response to DNA damage. *J Biol Chem* **275:** 322–327

Knudson CM, Tung KS, Tourtellotte WG, Brown GA, Korsmeyer SJ (1995) Bax-deficient mice with lymphoid hyperplasia and male germ cell death. *Science* **270:** 96–99

Krammer PH (2000) CD95's deadly mission in the immune system. *Nature* **407:** 789–795

Kuan CY, Yang DD, Samanta Roy DR, Davis RJ, Rakic P, Flavell RA (1999) The Jnk1 and Jnk2 protein kinases are required for regional specific apoptosis during early brain development. *Neuron* **22:** 667–676

Kuwana T, Mackey MR, Perkins G, Ellisman MH, Latterich M, Schneiter R, Green DR, Newmeyer DD (2002) Bid, Bax, and lipids cooperate to form supramolecular openings in the outer mitochondrial membrane. *Cell* **111:** 331–342

Lei K, Davis RJ (2003) JNK phosphorylation of Bim-related members of the Bcl2 family induces Bax-dependent apoptosis. *Proc Natl Acad Sci USA* **100:** 2432–2437

Lei K, Nimnual A, Zong WX, Kennedy NJ, Flavell RA, Thompson CB, Bar-Sagi D, Davis RJ (2002) The Bax subfamily of Bcl2-related proteins is essential for apoptotic signal transduction by c-Jun NH(2)-terminal kinase. *Mol Cell Biol* **22:** 4929–4942

Lindsten T, Ross AJ, King A, Zong WX, Rathmell JC, Shiels HA, Ulrich E, Waymire KG, Mahar P, Frauwirth K, Chen Y, Wei M, Eng VM, Adelman DM, Simon MC, Ma A, Golden JA, Evan G, Korsmeyer SJ, MacGregor GR, Thompson CB (2000) The combined functions of proapoptotic Bcl-2 family members bak and bax are essential for normal development of multiple tissues. *Mol Cell* **6:** 1389–1399

Liu D, Bienkowska J, Petosa C, Collier RJ, Fu H, Liddington R (1995) Crystal structure of the zeta isoform of the 14-3-3 protein. *Nature* **376:** 191–194

Masters SC, Pederson KJ, Zhang L, Barbieri JT, Fu H (1999) Interaction of 14-3-3 with a nonphosphorylated protein ligand, exoenzyme S of *Pseudomonas aeruginosa*. *Biochemistry* **38:** 5216–5221

Masuyama N, Oishi K, Mori Y, Ueno T, Takahama Y, Gotoh Y (2001) Akt inhibits the orphan nuclear receptor Nur77 and T-cell apoptosis. *J Biol Chem* **276:** 32799–32805

Maundrell K, Antonsson B, Magnenat E, Camps M, Muda M, Chabert C, Gillieron C, Boschert U, Vial-Knecht E, Martinou JC, Arkinstall S (1997) Bcl-2 undergoes phosphorylation by c-Jun

N-terminal kinase/stress-activated protein kinases in the presence of the constitutively active GTP-binding protein Rac1. *J Biol Chem* **272:** 25238–25242

Molton SA, Todd DE, Cook SJ (2003) Selective activation of the c-Jun N-terminal kinase (JNK) pathway fails to elicit Bax activation or apoptosis unless the phosphoinositide 3′-kinase (PI3K) pathway is inhibited. *Oncogene* **22:** 4690–4701

Muslin AJ, Tanner JW, Allen PM, Shaw AS (1996) Interaction of 14-3-3 with signaling proteins is mediated by the recognition of phosphoserine. *Cell* **84:** 889–897

Nechushtan A, Smith CL, Hsu YT, Youle RJ (1999) Conformation of the Bax C-terminus regulates subcellular location and cell death. *EMBO J* **18:** 2330–2341

Nomura M, Shimizu S, Sugiyama T, Narita M, Ito T, Matsuda H, Tsujimoto Y (2003) 14-3-3 interacts directly with and negatively regulates pro-apoptotic Bax. *J Biol Chem* **278:** 2058–2065

Putcha GV, Deshmukh M, Johnson Jr EM (1999) BAX translocation is a critical event in neuronal apoptosis: regulation by neuroprotectants, BCL-2, and caspases. *J Neurosci* **19:** 7476–7485

Putcha GV, Le S, Frank S, Besirli CG, Clark K, Chu B, Alix S, Youle RJ, LaMarche A, Maroney AC, Johnson Jr EM (2003) JNK-mediated BIM phosphorylation potentiates BAX-dependent apoptosis. *Neuron* **38:** 899–914

Putcha GV, Moulder KL, Golden JP, Bouillet P, Adams JA, Strasser A, Johnson EM (2001) Induction of BIM, a proapoptotic BH3-only BCL-2 family member, is critical for neuronal apoptosis. *Neuron* **29:** 615–628

Sabapathy K, Kallunki T, David JP, Graef I, Karin M, Wagner EF (2001) c-Jun NH2-terminal kinase (JNK)1 and JNK2 have similar and stage-dependent roles in regulating T cell apoptosis and proliferation. *J Exp Med* **193:** 317–328

Saito M, Korsmeyer SJ, Schlesinger PH (2000) BAX-dependent transport of cytochrome *c* reconstituted in pure liposomes. *Nat Cell Biol* **2:** 553–555

Samuel T, Weber HO, Rauch P, Verdoodt B, Eppel JT, McShea A, Hermeking H, Funk JO (2001) The G2/M regulator 14-3-3sigma prevents apoptosis through sequestration of Bax. *J Biol Chem* **276:** 45201–45206

Sawada M, Sun W, Hayes P, Leskov K, Boothman DA, Matsuyama S (2003) Ku70 suppresses the apoptotic translocation of Bax to mitochondria. *Nat Cell Biol* **5:** 320–329

Shimizu S, Narita M, Tsujimoto Y (1999) Bcl-2 family proteins regulate the release of apoptogenic cytochrome *c* by the mitochondrial channel VDAC. *Nature* **399:** 483–487

Svennelid F, Olsson A, Piotrowski M, Rosenquist M, Ottman C, Larsson C, Oecking C, Sommarin M (1999) Phosphorylation of Thr-948 at the C terminus of the plasma membrane H(+)-ATPase creates a binding site for the regulatory 14-3-3 protein. *Plant Cell* **11:** 2379–2391

Tournier C, Hess P, Yang DD, Xu J, Turner TK, Nimnual A, Bar-Sagi D, Jones SN, Flavell RA, Davis RJ (2000) Requirement of JNK for stress-induced activation of the cytochrome *c*-mediated death pathway. *Science* **288:** 870–874

Tsujimoto Y, Shimizu S (2000) Bcl-2 family: life-or-death switch. *FEBS Lett* **466:** 6–10

Tsuruta F, Masuyama N, Gotoh Y (2002) The phosphatidylinositol 3-kinase (PI3K)-Akt pathway suppresses Bax translocation to mitochondria. *J Biol Chem* **277:** 14040–14047

van Hemert MJ, Steensma HY, van Heusden GP (2001) 14-3-3 proteins: key regulators of cell division, signalling and apoptosis. *BioEssays* **23:** 936–946

Wang X (2001) The expanding role of mitochondria in apoptosis. *Genes Dev* **15:** 2922–2933

Wei MC, Zong WX, Cheng EH, Lindsten T, Panoutsakopoulou V, Ross AJ, Roth KA, MacGregor GR, Thompson CB, Korsmeyer SJ (2001) Proapoptotic BAX and BAK: a requisite gateway to mitochondrial dysfunction and death. *Science* **292:** 727–730

Whitfield J, Neame SJ, Paquet L, Bernard O, Ham J (2001) Dominant-negative c-Jun promotes neuronal survival by reducing BIM expression and inhibiting mitochondrial cytochrome *c* release. *Neuron* **29:** 629–643

Wolter KG, Hsu YT, Smith CL, Nechushtan A, Xi XG, Youle RJ (1997) Movement of Bax from the cytosol to mitochondria during apoptosis. *J Cell Biol* **139:** 1281–1292

Xia Z, Dickens M, Raingeaud J, Davis RJ, Greenberg ME (1995) Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. *Science* **270:** 1326–1331

Xiao B, Smerdon SJ, Jones DH, Dodson GG, Soneji Y, Aitken A, Gamblin SJ (1995) Structure of a 14-3-3 protein and implications for coordination of multiple signalling pathways. *Nature* **376:** 188–191

Yaffe MB (2002) How do 14-3-3 proteins work? Gatekeeper phosphorylation and the molecular anvil hypothesis. *FEBS Lett* **513:** 53–57

Yaffe MB, Rittinger K, Volinia S, Caron PR, Aitken A, Leffers H, Gamblin SJ, Smerdon SJ, Cantley LC (1997) The structural basis for 14-3-3:phosphopeptide binding specificity. *Cell* **91:** 961–971

Yamaguchi H, Wang HG (2001) The protein kinase PKB/Akt regulates cell survival and apoptosis by inhibiting Bax conformational change. *Oncogene* **20:** 7779–7786

Yang DD, Kuan CY, Whitmarsh AJ, Rincon M, Zheng TS, Davis RJ, Rakic P, Flavell RA (1997) Absence of excitotoxicity-induced apoptosis in the hippocampus of mice lacking the Jnk3 gene. *Nature* **389:** 865–870

Yuan J, Yankner BA (2000) Apoptosis in the nervous system. *Nature* **407:** 802–809

Zha J, Harada H, Yang E, Jockel J, Korsmeyer SJ (1996) Serine phosphorylation of death agonist BAD in response to survival factor results in binding to 14-3-3 not BCL-X(L). *Cell* **87:** 619–628

Zhang L, Chen J, Fu H (1999) Suppression of apoptosis signal-regulating kinase 1-induced cell death by 14-3-3 proteins. *Proc Natl Acad Sci USA* **96:** 8511–8515

Zong WX, Lindsten T, Ross AJ, MacGregor GR, Thompson CB (2001) BH3-only proteins that bind pro-survival Bcl-2 family members fail to induce apoptosis in the absence of Bax and Bak. *Genes Dev* **15:** 1481–1486