hsp70-DnaJ chaperone pair prevents nitric oxide- and CHOP-induced apoptosis by inhibiting translocation of Bax to mitochondria

T Gotoh*,ˡ, K Terada[1], S Oyadomari[1] and M Mori[1]

[1] Department of Molecular Genetics, Graduate School of Medical Sciences, Kumamoto University, Honjo, Kumamoto, Japan

* Corresponding author: T Gotoh, Department of Molecular Genetics, Graduate School of Medical Sciences, Kumamoto University, Honjo 1-1-1, Kumamoto 860-8556, Japan. Tel: +81-96-373-5140; Fax: +81-96-373-5145; E-mail: tomomi@gpo.kumamoto-u.ac.jp

Abstract

We reported that the endoplasmic reticulum (ER) stress pathway involving CHOP, a member of the C/EBP transcription factor family, plays a key role in nitric oxide (NO)-mediated apoptosis of macrophages and pancreatic β cells. We also showed that the cytosolic chaperone pair of hsp70 and dj1 (hsp40/hdj-1) or dj2 (HSDJ/hdj-2) prevents NO-mediated apoptosis upstream of cytochrome *c* release from mitochondria. To analyze roles of the chaperone pair in preventing apoptosis, RAW 264.7 macrophages stably expressing hsp70 and dj1 or dj2 were established. The chaperone pair prevented LPS/IFN-γ-induced and NO-mediated apoptosis downstream of CHOP induction. hsp70 mutant protein lacking the ATPase domain or the C-terminal EEVD sequence were not effective in preventing CHOP-induced apoptosis. A mutant dj2 lacking the C-terminal prenylation CaaX motif, was also not effective. When wild-type RAW 264.7 cells were treated with LPS/IFN-γ, NO-mediated apoptosis was induced, and proapoptotic Bcl-2 family protein Bax was translocated from cytosol to mitochondria. This translocation was prevented in cells stably expressing hsp70/dj2, and in CHOP knockout cells. Overexpression of CHOP in wild-type cells also induced translocation of Bax and this translocation was prevented in cells expressing hsp70/dj2. CHOP-induced apoptosis was prevented by Bax knock-down. Coimmunoprecipitation experiments showed that Bax interacts with both hsp70 and dj1/dj2. ATPase domain of hsp70 was necessary for the binding with Bax. These findings indicate that CHOP-induced apoptosis is mediated by translocation of Bax from the cytosol to the mitochondria, and hsp70/dj1 or dj2 chaperone pair prevents apoptosis by interacting with Bax and preventing translocation to the mitochondria.

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Introduction

Nitric oxide (NO) is a multifunctional biomolecule involved in a variety of physiological and pathological processes.[1] Excess NO production is toxic to the host, and is considered to cause diseases such as septic shock, autoimmune diseases, cerebral infarction, and diabetes mellitus. NO-mediated apoptosis is often observed in such cases.[2–4] NO has cytotoxic effects, including reactions with proteins and nucleic acids. NO-induced apoptosis was generally considered to be mediated by DNA or mitochondrial damages; however, the cascade of the cell death has not been clarified. We recently found that the endoplasmic reticulum (ER) stress pathway involving CHOP plays a key role in NO-mediated apoptosis in pancreatic β cell,[5] microglia,[6] and macrophages.[7] We also showed that p90ATF6, an ER membrane-bound transcription factor involved in ER stress response, is cleaved to its active soluble form p50ATF6, which is transported to the nucleus and activates transcription of the CHOP gene in LPS/IFN γ-induced apoptosis.[7] We showed that a dominant-negative form of CHOP prevents p50ATF6- and LPS/IFNγ-induced apoptosis, and that NO-induced apoptosis is prevented in CHOP knockout cells.[5,7] These results show that ATF6 and CHOP are involved in NO- and LPS/TFNγ-induced apoptosis.

CHOP, also known as GADD153, is a member of the C/EBP family that heterodimerizes with other members of the C/EBP transcription factor family. CHOP is involved in the process of apoptosis associated with ER stress.[8–12] In ER stress, the activated ATF6 induces ER chaperones, CHOP, and another bZIP-type transcription factor XBP1 via direct binding to the ERSE (ER stress-responsive element).[13–15] The spliced form of XBP1, produced from induced XBP1 mRNA by the action of an activated ER-membrane protein IRE1α, further enhances transcription of the ER chaperone genes via direct binding to the ERSE. On the other hand, Fawcett *et al.*[16] reported that the CHOP gene can be activated by ATF4, another bZIP-type transcription factor, via its binding to the C/EBP-ATF site present in the CHOP promoter region that is distinct from the ERSE. In addition, Harding *et al.*[17] reported that translation of ATF4 is selectively increased during ER stress and that this translational induction is mediated by PERK, a type 1 transmembrane protein kinase in the ER, which senses ER stress and transmits signals by phosphorylating the α subunit of eukaryotic initiation factor 2.

CHOP induces apoptosis in some cell types.[9] Embryonic fibroblasts derived from CHOP-knockout mice exhibit

significantly less apoptosis compared with wild-type cells, when challenged with ER stress-inducing reagents.[9] CHOP was found to induce apoptosis in M1 myeloblastic leukemia cells in a p53-independent manner and Bcl-2 delayed this process.[18] Because CHOP functions as a transcription factor, there must be a target gene(s), the transcription of which is activated by CHOP, and whose product(s) functions in the apoptosis signal cascade. Wang *et al.*[19] found candidate target genes of the CHOP protein when using representational difference analysis. However, these genes are distinct from known factors involved in the ER stress response and apoptosis. McCullough *et al.*[20] reported that CHOP expression results in downregulation of Bcl-2 expression, depletion of cellular glutathione, and exaggerated production of reactive oxygen species. However, the precise apoptosis cascade downstream of CHOP is unknown.

Heat-shock proteins (hsps) induced in response to various stresses afford protection.[21,22] The major hsps can be divided into several groups based on both size and function.[23] Some constitutively expressed hsps act as molecular chaperones. Members of the hsp70 family are involved in the folding and intracellular transport of newly synthesized proteins.[24,25] To protect cells from various stresses, hsp70s bind to damaged and misfolded proteins, and either facilitate their refolding or target severely damaged proteins for degradation.[21] hsp70 also protects cells from apoptosis induced by various stresses and agents, including NO, oxidative stress, tumor necrosis factor, anticancer drugs, ceramide and radiation.[21] There are several reports concerning the antiapoptotic effects of hsp70. However, the mechanism of antiapoptotic effects of hsp70 is not fully understood. DnaK, the hsp70 homolog of *Escherichia coli*, is regulated by partner chaperones, DnaJ and GrpE.[25,26] Several mammalian DnaJ homologs (hsp40 family members) have been identified,[27] and it seems likely that hsp70 family members are regulated by these homologs.[28] Major DnaJ homologs in mammalian cytosol are dj1 (DNAJB1: hsp40/hdj-1) and dj2 (DNAJA1: HSDJ/hdj-2). We reported that dj1-hsp70 and dj2-hsp70 chaperone pairs are both effective in preventing NO-mediated apoptosis upstream of both caspase 3 activation and cytochrome *c* release from the mitochondria.[29]

Here we report that CHOP induces apoptosis in RAW 264.7 macrophages through translocation of a proapoptotic Bcl-2 family protein Bax from the cytosol to the mitochondria. We also found that the hsp70/DnaJ chaperone pair prevents CHOP-mediated apoptosis by interacting with Bax and by preventing its translocation from the cytosol to the mitochondria.

Results

hsp70/DnaJ chaperone pairs prevent NO-mediated apoptosis downstream of CHOP induction in RAW 264. 7 cells

When mouse macrophage-like RAW 264.7 cells were treated with *E. coli* lipopolysaccharide (LPS) and interferon-γ (IFN-γ), inducible NO synthase (iNOS) is highly induced and a large amount of NO is produced. Under these conditions, apoptosis occurs.[30] We reported that the hsp70/DnaJ chaperone pairs

prevent this apoptosis upstream of the cytochrome *c* release from the mitochondria.[29] To clarify the precise antiapoptotic mechanism of hsp70/DnaJ chaperone pairs, we established stably transformed RAW 264.7 cell lines expressing hsp70/dj1 or dj2 (Figure 1a,b). Expression levels of hsp70, dj1, and dj2 in the stable transformed cells were similar to those in heat-treated RAW 264.7 cells. NO production and iNOS induction by LPS/IFN-γ treatment were unchanged by the expression of hsp70 or hsp70/DnaJ (Figure 1c,d).

We next asked if expression of hsp70/dj1 or hsp70/dj2 would prevent NO-mediated apoptosis (Figure 2). Formation of the DNA ladder, characteristic of apoptotic cells, was observed when wild-type cells were treated with LPS/IFN-γ (Figure 2a). This same formation was also observed in hsp70-expressing cells. In contrast, cell lines expressing hsp70/dj1 or hsp70/dj2 did not show ladder formation in case of LPS/IFN-γ treatment. There are several subgroups in caspases, among which, caspase 3 activation is positioned downstream of the caspase activation cascade, and is thought to be the point of no return in the process of apoptosis. Caspase 3 activation was prevented by the coexpression of hsp70 and dj1 or dj2 (Figure 2b). Loss of mitochondrial membrane potential, also characteristic of apoptosis, was prevented by the coexpression of hsp70 and dj1 or dj2 (Figure 2c). Figure 2d shows morphology of cells treated with LPS/IFN-γ. When wild-type cells were treated with LPS/IFN-γ, chromatin condensation and nuclear fragmentation were seen in Hoechst dye 33258 staining. Similar apoptotic changes were observed in cells expressing hsp70 and such changes were strongly prevented by the coexpression of hsp70/dj1 or hsp70/dj2. These findings support our data obtained when doing transient expression experiments.[29]

As NO-induced apoptosis in RAW 264.7 cells is mediated by induction of CHOP,[7] we determined if the induction of CHOP would be prevented by expression of hsp70/dj1 or hsp70/dj2. Figure 3 shows immunoblot analysis of nuclear extracts for CHOP. CHOP, barely detectable before LPS/IFN-γ treatment, was induced after treatment in wild-type cells and this induction was not suppressed in cells expressing hsp70/dj1 or hsp70/dj2. Thus, the hsp70/DnaJ chaperone pairs prevent LPS/IFN-γ-induced apoptosis downstream of CHOP induction in RAW 264.7 cells.

Apoptosis induced by CHOP is prevented by hsp70/DnaJ chaperone pairs in COS-7 cells

We reported that overexpression of CHOP or p50ATF6 (active form of ATF6) induces apoptosis in RAW 264.7 cells and COS-7 cells.[7] Expression of the transfected CHOP gene was barely detected in RAW 264.7 cells. Therefore, we used COS-7 cells, in which higher levels of expression can be obtained, in the following experiments. We examined whether hsp70/DnaJ chaperone pairs would prevent CHOP-induced apoptosis in COS-7 cells (Figure 4). Cells were cotransfected with an EGFP expression plasmid and various combinations of expression plasmids for CHOP, hsp70, dj1, dj2, and Bcl-2. When cells were transfected with only the EGFP plasmid, many cells became fluorescent (Figure 4a). Cotransfection with the CHOP plasmid markedly reduced the number of

EGFP-positive cells, because of cell death.[7] Transfection of a plasmid for hsp70, dj1, or dj2 alone did not prevent CHOP-induced apoptosis. In contrast, the reduced number of EGFP-positive cells by CHOP expression was markedly prevented by cotransfection with the plasmids for hsp70/dj1 or hsp70/
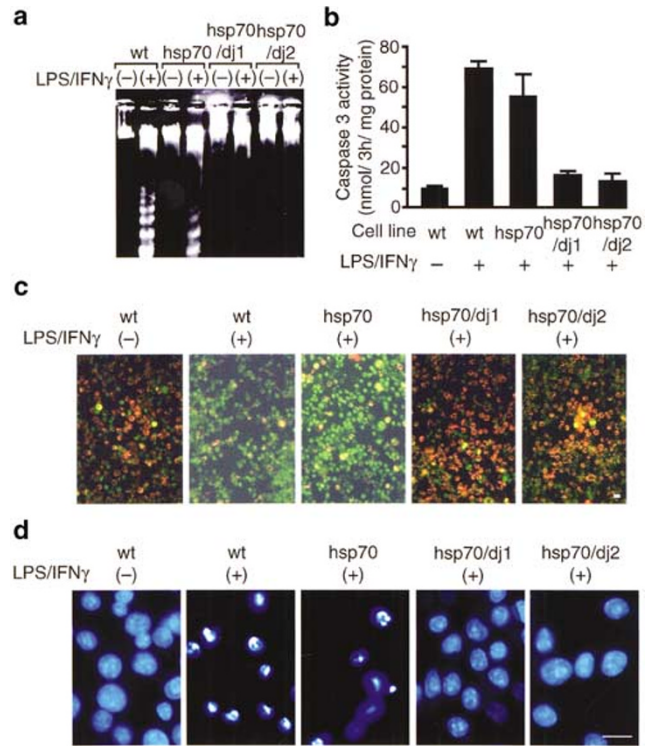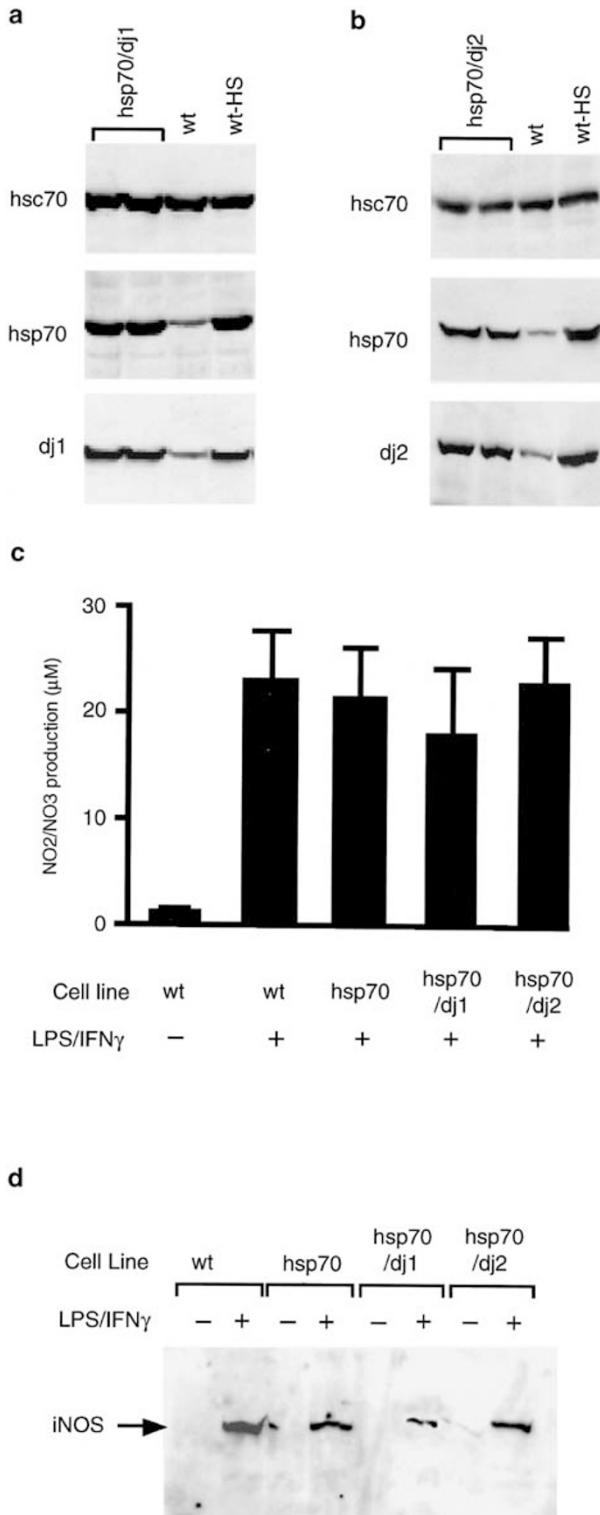

Figure 1 Establishment of stable RAW 264.7 cell lines expressing hsp70 and dj1 or dj2. (**a**) Cell extracts (20 μg of protein) of stably transformed RAW 264.7 cell lines expressing hsp70 and dj1, wild-type RAW 264.7 cell line (wt) and RAW 264.7 cells treated with heat shock (wt-HS) were subjected to immunoblot analysis for hsc70, hsp70, and dj1 proteins. (**b**) Cell extracts (20 μg of protein) of stably transformed RAW 264.7 cell lines expressing hsp70 and dj2, wild-type RAW 264.7 cell line and RAW 264.7 cells treated with heat shock (wt-HS) were subjected to immunoblot analysis for hsc70, hsp70, and dj2 proteins. (**c**) Wild type and stable transformed RAW 264.7 cell lines expressing hsp70, stably transformed RAW 264.7 cell lines expressing hsp70/dj1 or hsp70/dj2 were treated with or without 150 μg/ml LPS and 100 U/ml IFN-γ for 10 h. Then, NO$_2^-$ plus NO$_3^-$ in the medium was measured. The results are shown as means $\pm$ S.D. for three dishes. (**d**) Wild-type and stably transformed RAW 264.7 cell lines expressing hsp70, hsp70/dj1, or hsp70/dj2 were treated with or without 150 μg/ml LPS and 100 U/ml IFN-γ for 10 h as indicated on the top, and cell extracts (50 μg of protein) were subjected to immunoblot analysis for inducible type NO synthase (iNOS)

Figure 2 The hsp70/DnaJ stable transformant is resistant to NO-mediated apoptosis. (**a**) Wild-type and stably transformed RAW 264.7 cell lines expressing hsp70, hsp70/dj1, or hsp70/dj2 were treated with or without 150 μg/ml LPS and 100 U/ml IFN-γ for 12 h as indicated on the top. DNAs were isolated, resolved on an agarose gel, stained with ethidium bromide, and visualized for DNA fragmentation by UV transillumination. (**b**) Wild-type and stably transformed RAW 264.7 cell lines expressing hsp70, hsp70/dj1, or hsp70/dj2 were treated with or without 150 μg/ml LPS and 100 U/ml IFN-γ for 10 h as indicated, extracts were prepared, and caspase 3 activity was measured, as described in Materials and Methods. The results are shown by means $\pm$ S.D. for three dishes. (**c**) Wild-type and stably transformed RAW 264.7 cell lines were treated with or without 150 μg/ml LPS and 100 U/ml IFN-γ for 10 h as indicated on the top, and cells were stained with a mitochondrial membrane potential-dependent dye DePsipher, as described in Materials and Methods. The red–orange fluorescent aggregates represent the dye aggregates formed depending on the mitochondrial membrane potential. If the potential is disrupted, the dye remains in its green fluorescent form in the cytosol. Original magnifications: $\times$ 400. Bar, 10 μm. (**d**) Wild-type and stably transformed RAW 264.7 cell lines were treated with or without 150 μg/ml LPS and 100 U/ml IFN-γ for 18 h as indicated. After fixation, the cells were stained with Hoechst dye 33258, and observed by fluorescence microscopy. Original magnifications: $\times$ 400. Bar, 10 μm. A portion of the cells were detached from coverslips by treatment with LPS/IFN-γ
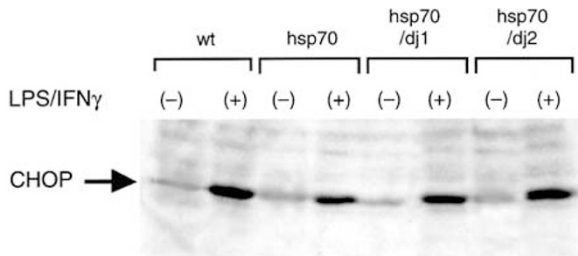
Figure 3 Expression of hsp70/DnaJ chaperone pair does not prevent NO-induced CHOP induction. Wild-type and stably transformed RAW 264.7 cell lines expressing hsp70, hsp70/dj1, or hsp70/dj2 were treated with or without 150 μg/ml LPS and 100 U/ml IFN-γ for 8 h as indicated on the top. Nuclear extracts (30 μg of protein) were subjected to immunoblot analysis for CHOP protein
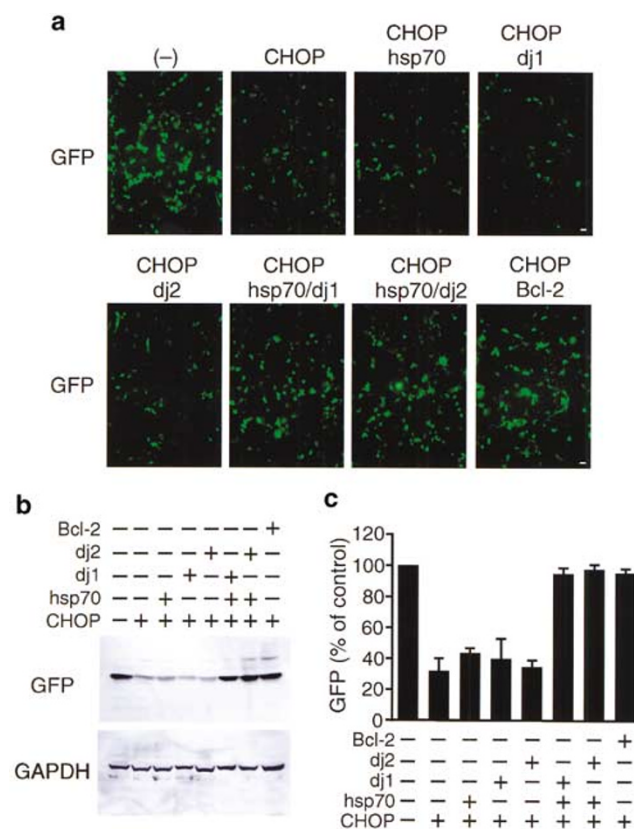

Figure 4 Coexpression of hsp70, and dj1 or dj2 protected COS-7 cells from CHOP-induced apoptosis. (**a**) Cells were cotransfected with an EGFP expression plasmid and various combinations of expression plasmids for CHOP, hsp70, dj1, dj2, or Bcl-2 as indicated on the top. After 24 h, cells were observed using fluorescence microscopy. Original magnifications: $\times$ 200. Bars, 10 μm. (**b**) Cells were treated as in (**a**) and cell extracts (20 μg of protein) were subjected to immunoblot analysis for GFP and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). (**c**) The results in (**b**) were quantified, and are shown by means $\pm$ S.D. for three dishes

dj2. Transfection with a plasmid, for an antiapoptotic protein Bcl-2, also prevented the reduction in numbers of EGFP-positive cells induced by CHOP. This result shows CHOP induce apoptosis through mitochondria pathway. This finding agrees with our previous observations that hsp70/DnaJ chaperone pairs prevent NO-induced and CHOP-mediated apoptosis upstream of cytochrome *c* release from the

mitochondria.[29] Therefore, we conclude that hsp70/DnaJ chaperone pairs exert their antiapoptotic effects downstream of CHOP induction, and upstream of cytochrome *c* release from mitochondria. Expression of EGFP was quantified by immunoblot analysis, and the results are shown in Figure 4b, c. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was also measured as control.

Apoptosis induced by the active form of ATF6 is prevented by hsp70/DnaJ chaperone pairs in COS-7 cells

ATF6 exists constitutively as a transmembrane protein p90ATF6 in the ER under nonstressed conditions.[31] ER stress induces proteolysis of p90ATF6 and releases a soluble transcription factor p50ATF6 (active form) which is transported into the nucleus, binds to the ER stress-responsive element (ERSE) of the CHOP and ER chaperone genes, and activates their transcription. ATF6 is activated in LPS/IFN-γ-induced apoptosis and functions as a transactivator of the CHOP gene.[7] Therefore, we asked if the apoptosis induced by the overexpression of p50ATF6 would be prevented by transfection with expression plasmids for hsp70/dj1 or hsp70/dj2 in COS-7 cells (Figure 5). When cells were cotransfected with the EGFP plasmid and the plasmid for p50ATF6, the amount of EGFP was reduced because of cell death, and this reduction was prevented by transfection with the plasmids for hsp70/dj1 or hsp70/dj2. Transfection with the plasmid for hsp70, dj1, or dj2 alone had little effect. Transfection with a plasmid for Bcl-2 prevented the p50ATF6-induced reduction of EGFP, suggesting that this cell death is mediated by the mitochondrial apoptosis pathway.

ATPase domain and the C-terminal portion of hsp70, and farnesylation of dj2 are necessary for antiapoptotic effect of hsp70/dj2 pair in CHOP-induced apoptosis

ATP which binds the N-terminal domain of hsp70 drives conformational changes in the C-terminal peptide binding domain that alters its affinity for substrates.[23] The ATP-bound state of hsp70 shows low affinity and fast exchange rates for substrates, whereas the ADP-bound state shows high affinity and slow exchange rates for substrates. Therefore, the ATPase domain of hsp70 is crucial for its chaperone activity. On the other hand, the C-terminal EEVD motif of hsp70 is responsible for binding with co-chaperone hop and chip.[32,33]

We investigated whether the ATPase domain and C-terminal motif are essential for antiapoptotic function of hsp70 (Figure 6). We constructed expression plasmids for hsp70 mutant forms, lacking the ATPase domain or the C-terminal EEVD motif. Plasmids for these hsp70 mutants in combination with the dj2 expression plasmid, were not effective in preventing CHOP-induced apoptosis (Figure 6a, b). Therefore, ATPase activity and the C-terminal EEVD motif of hsp70 are essential for its antiapoptotic activity. Figure 6c shows the expression of transfected genes.
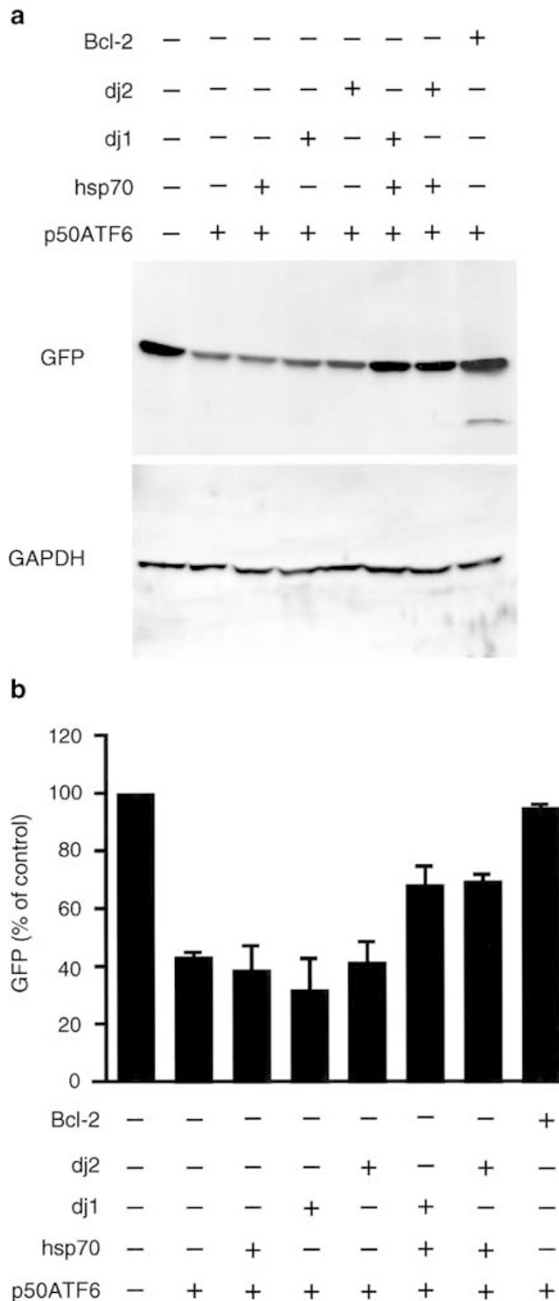
a

b

Figure 5 Coexpression of hsp70, and dj1 or dj2 protected COS-7 cells from p50ATF6-induced apoptosis. (**a**) Cells were cotransfected with an EGFP expression plasmid and various combinations of expression plasmids for p50ATF6, hsp70, dj1, dj2, and Bcl-2 as indicated on the top. After 24 h, cell extracts (20 μg of protein) were subjected to immunoblot analysis for GFP and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). (**b**) The results in (**a**) were quantified, and are shown by means $\pm$ S.D. for three dishes

dj2 has the CaaX prenylation motif at its C-terminal and undergoes farnesylation.[34] We asked whether this modification is crucial for anti-apoptotic effect of dj2 (Figure 7). We constructed an expression plasmid for unprenylated dj2 mutant C394S, in which cysteine of the 'CaaX box' was mutated to serine. Cotransfection with this mutant plasmid in combination with hsp70 was partly, but not fully effective in
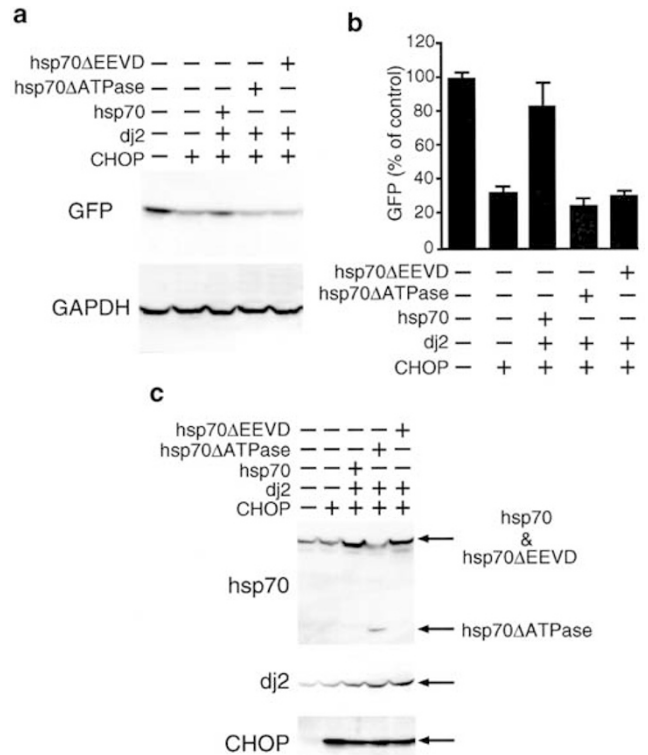
a

b

c

Figure 6 Mutant hsp70 does not protect COS-7 cells from CHOP-induced apoptosis. (**a**) Cells were cotransfected with an EGFP expression plasmid and various combinations of expression plasmids for CHOP, wild-type hsp70, ATPase domain-deleted hsp70 (hsp70ΔATPase), C-terminal sequence EEVD-deleted hsp70 (hsp70ΔEEVD) or dj2 as indicated on the top. After 24 h, cell extracts (20 μg of protein) were subjected to immunoblot analysis for GFP and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). (**b**) The results in (**a**) were quantified, and are shown by means $\pm$ S.D. for three dishes. (**c**) Cells were treated as in (**a**), and cell extracts (20 μg of protein) were subjected to immunoblot analysis for hsp70, dj2, and CHOP

preventing the CHOP-induced reduction of EGFP expression (Figure 7a, b). This indicates that prenylation is important, but not essential in the antiapoptotic effect of dj2. Figure 7c shows the expression of transfected genes.

CHOP induces translocation of Bax from the cytosol to the mitochondria in NO-mediated apoptosis, and this translocation is prevented by the hsp70/dj2 chaperone pair

Bax is a proapoptotic Bcl-2 family protein.[35] Translocation of Bax from the cytosol to the mitochondria occurs in the apoptosis induced by various stresses and agents, and is thought to cause cytochrome *c* release from mitochondria. Therefore, we asked whether translocation of Bax takes place in LPS/IFN-γ-induced apoptosis, and whether this translocation is prevented by the expression of hsp70/dj2 chaperone pair (Figure 8a). After LPS/IFN-γ treatment, wild-type and hsp70/dj2-transformed RAW 264.7 cells were fractionated into the soluble fraction and the particulate fraction containing mitochondria, using digitonin, and immunoblot analysis was performed. In both wild-type and transformed cells,
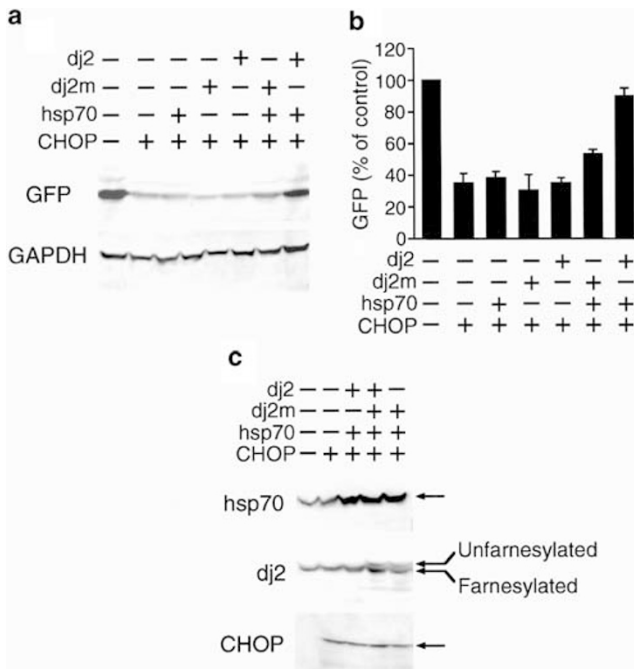
Figure 7 Farnesylation of dj2 is important for antiapoptotic effect in CHOP-induced apoptosis. (**a**) COS-7 cells were cotransfected with an EGFP expression plasmid and various combinations of expression plasmids for CHOP, hsp70, dj2, or unfarnesylated mutant form of dj2 (dj2 m) as indicated on the top. After 24 h, cell extracts (20 μg of protein) were subjected to immunoblot analysis for GFP and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). (**b**) The results in (**a**) were quantified, and are shown by means ±S.D. for three dishes. (**c**) COS-7 cells were treated as in (**a**), and cell extracts (20 μg of protein) were subjected to immunoblot analysis for hsp70, dj2, and CHOP

cytochrome *c* was recovered exclusively in the particulate fraction, and Bax was recovered exclusively in the soluble fraction before the treatment. When wild-type cells were treated with LPS/IFN-γ, cytochrome *c* was recovered exclusively in the soluble fraction, whereas Bax was recovered mostly in the particulate fraction. In contrast, when transformed cells were treated with LPS/IFN-γ, cytochrome *c* was almost completely recovered in the particulate fraction, and Bax was recovered mostly in the soluble fraction. Under these conditions, a mitochondrial matrix protein hsp60 was recovered exclusively in the particulate fraction, whereas a cytosolic protein GAPDH was recovered exclusively in the soluble fraction. Therefore, we conclude that translocation of Bax in LPS/IFN-γ-induced apoptosis is prevented by the hsp70/dj2 chaperone pair in RAW 264.7 cells. This was confirmed by double immunostaining of RAW 264.7 cells (Figure 8b). Particulate structures in the cytoplasm were stained with an hsp60 antibody, this reflects the mitochondria localization of this protein. In untreated wild-type cells, the cytoplasm was diffusely positive for Bax immunostaining, whereas particulate structures similar to those positive for hsp60 immunostaining were observed in the cytoplasm after LPS/IFN-γ treatment, thus indicating translocation of Bax to the mitochondria. In contrast, when transformed cells were treated with LPS/IFN-γ, the cytoplasm was still diffusely positive for Bax immunostaining, thereby indicating prevention of Bax translocation by hsp70/dj2.

We next asked whether Bax translocation to mitochondria by NO is dependent on CHOP, using primary-cultured peritoneal macrophages from CHOP knockout mice (Figure 8c). As shown in Figure 8b, Bax was localized in cytosol under nonapoptotic conditions. When apoptosis-inducing stress or reagent is added, Bax conformation change to the proapoptotic state takes place and Bax is translocated to mitochondria.[36] Bax antibody (N-20) is specific to the proapoptotic state,[36] and Bax antibody (Δ21) reacts with any conformation of Bax. When untreated peritoneal macrophages from wild-type or CHOP knockout mice were immunostained with Bax antibodies, the cytoplasm was diffusely stained with the Δ21 antibody, whereas no immuno-fluorescence was detected with the N-20 antibody. In contrast, when peritoneal macrophages from wild-type mice were treated with SNAP (1.5 mM) for 10 h, chromatin condensation was seen in Hoechst dye 33258 staining, indicating apoptosis. In Bax immunostaining using the Δ21 antibody or N-20 antibody, particulate structures were observed in the cytoplasm, indicating translocation of Bax to mitochondria. Positive staining with the N-20 antibody shows the conformation change of Bax. In contrast, when peritoneal macrophages from CHOP knockout mice were treated with SNAP for 10 h, apoptotic change was barely detected in Hoechst dye 33258 staining. The cytoplasm was diffusely stained with the Δ21 antibody and little immunostaining was observed with the N-20 antibody. These results show that translocation of Bax to mitochondria and conformation change of Bax induced by NO is CHOP dependent.

We then asked whether CHOP-induced apoptosis is dependent on Bax (Figure 8d–f). Hela cells were cotransfected with an EGFP expression plasmid, an inducible CHOP expression plasmid and siRNA for Bax knock down. At 48 h after transfection, IPTG was added to induce CHOP expression, and cells were incubated further for 24 h. When CHOP was induced, the number of EGFP-positive cells was reduced indicating apoptosis. This reduction was not prevented by mouse Bax sequence-specific siRNA (RNAiMBax), used as control. In contrast, when human Bax sequence-specific siRNA (RNAiHBax), was transfected, reduction of EGFP-positive cells were prevented, same as in the case of hsp70/dj2 chaperone pair expression. Expression of Bax protein, which did not change by control siRNA (RNAiMBax), was reduced by transfection of human Bax sequence-specific siRNA (RNAiHBax) (Figure 8g). Expression of CHOP protein, which was not detected in control cells, was induced by IPTG addition, and did not change by control or human Bax-specific siRNA. These results show that CHOP-induced apoptosis is dependent on Bax.

We next asked whether the translocation of Bax is induced by CHOP expression, and whether this translocation is prevented by the hsp70/dj2 chaperone pair in COS-7 cells (Figure 9a, b). Cells were cotransfected with an EGFP-Bax fusion plasmid and various combinations of plasmids for CHOP, hsp70, dj1, dj2, and Bcl-2. When cells were transfected with an EGFP-Bax plasmid alone, cells were diffusely positive for EGFP. In contrast, when COS-7 cells were cotransfected with plasmids for EGFP-Bax and CHOP, small particulate structures in the cytoplasm were positive for EGFP, thus reflecting the mitochondrial localization of Bax. In
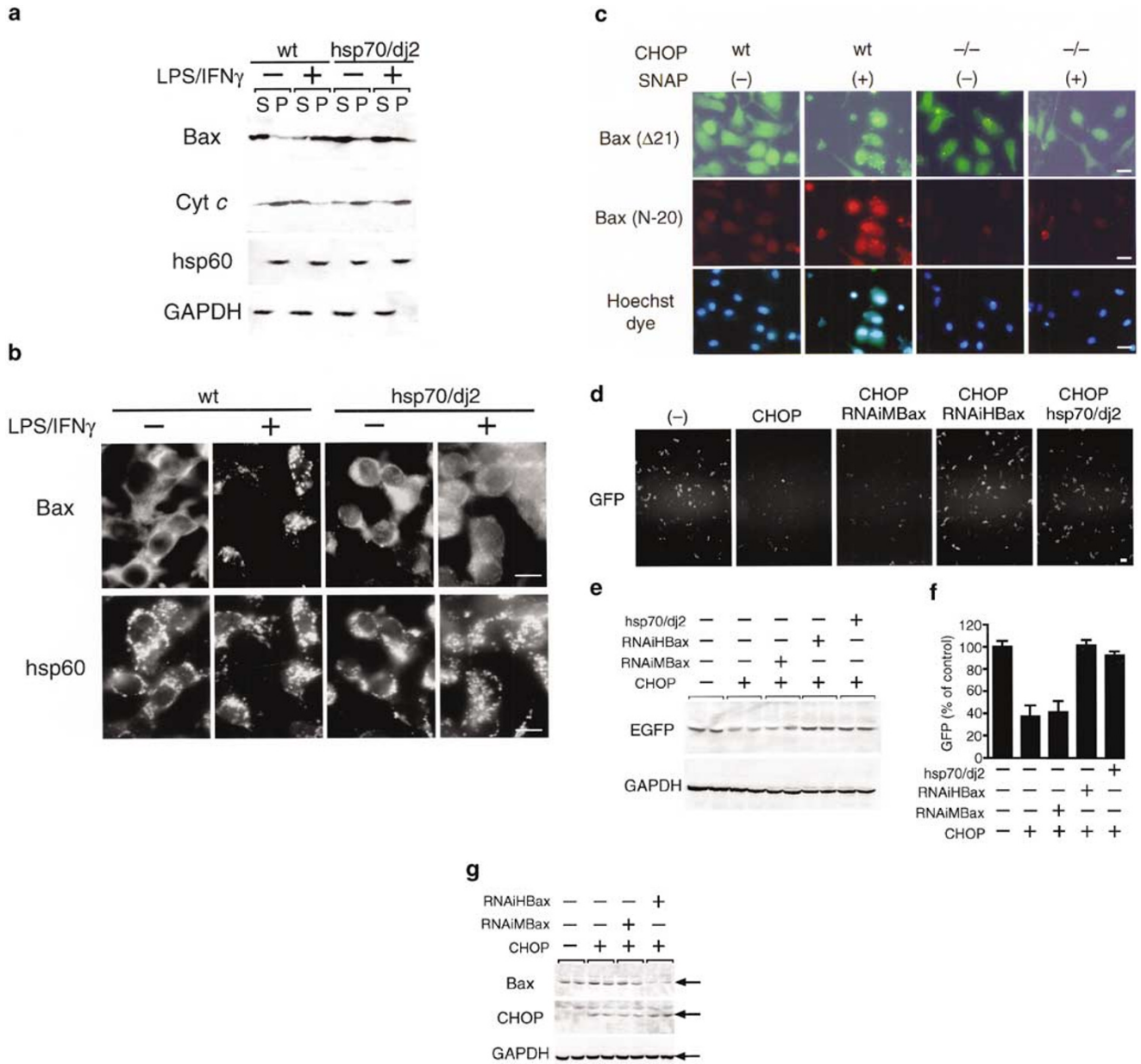
Figure 8 hsp70/dj2 chaperone pair prevent NO and CHOP-induced Bax translocation. (**a**) Wild-type and hsp70/dj2-stable transformant RAW 264.7 cell lines were treated with LPS (150 μg/ml) plus IFNγ (100 U/ml) for 10 h and fractionated into the soluble fraction (S) and the particulate fraction (P), as described under Materials and Methods. Distribution of total protein in the soluble fraction and the particulate fraction was 65 and 35%, respectively. The fractions (10 μg of protein) were subjected to immunoblot analysis for Bax, cytochrome *c* (Cyt *c*), hsp60, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). (**b**) Wild-type and hsp70/dj2-stable transformant RAW 264.7 cell lines were treated as in (**a**). After fixation, the cells were double-immunostained with antibodies against Bax and mitochondria chaperone hsp60 as described under Materials and Methods. Original magnifications: ×1000. Bars, 10 μm. (**c**) Peritoneal macrophages were prepared from wild-type (wt) and CHOP knockout mice (−/−). Cells were treated with 1.5 mM NO-donor SNAP for 10 h. The cells were then fixed and double-immunostained with a monoclonal antibody against Bax lacking the carboxy terminal 21 amino-acid residues (Bax (Δ21)) or a polyclonal antibody against the N-terminal portion of Bax (Bax (N20)), stained with Hoechst dye 33258 and observed by fluorescence microscopy. Original magnifications: ×400. Bars, 10 μm. (**d**) Hela cells were cotransfected with an EGFP expression plasmid, a CHOP-inducible expression plasmid, and a p3′SS lac repressor-expressing vector. siRNAs for mouse Bax (RNAiMBax) or human Bax (RNAiHBax), and expression plasmids for hsp70 and dj2 were also cotransfected as indicated on the top. After 48 h, IPTG (5 mM) was added to induce CHOP as indicated on the top. The cells were further cultured for 24 h, and observed by fluorescence microscopy. Original magnifications: ×200. Bar, 10 μm. (**e**) Hela cells were treated as in (**d**), and cell extracts (20 μg of protein) were subjected to immunoblot analysis for GFP and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). (**f**) The results in (**e**) were quantified, and are shown by means ± S.D. for three dishes. (**g**) Hela cells were treated as in (**d**), and cell extracts (60 μg of protein) were subjected to immunoblot analysis for Bax, CHOP, and GAPDH

addition, cells became condensed and round-shaped, reflecting apoptotic changes. Cotransfection with a plasmid for hsp70, dj1, or dj2 alone did not affect these changes in EGFP

localization and of the cell shape induced by CHOP. These changes were canceled out by cotransfection with plasmids for hsp70/dj1 or hsp70/dj2. Expression of Bcl-2 was also

effective. This was confirmed by cell fractionation (Figure 9b). COS-7 cells were fractionated into the soluble fraction containing cytosol and the particulate fraction containing mitochondria, and immunoblot analysis was performed. When EGFP-Bax alone was expressed, it was recovered exclusively in the soluble fraction. When EGFP-Bax and CHOP were coexpressed, EGFP-Bax was recovered in the particulate fraction, and this translocation of EGFP-Bax by CHOP was prevented by coexpression of hsp70/dj2.

Translocation of EGFP-Bax from the cytosol to the mitochondria was also confirmed by cotransfection with a plasmid for another fluorescent protein DsRed containing mitochondrial targeting signal (DsRed-Mit) (Figure 9c). When COS-7 cells were cotransfected with plasmids for DsRed-Mit and EGFP, DsRed fluorescence was detected in small particulate structures in the cytoplasm, thereby reflecting the mitochondrial localization of DsRed. EGFP was diffuse in the cytoplasm. On the other hand, EGFP-Bcl-2 fusion protein was detected in mitochondria. EGFP-Bax was detected in the cytoplasm without CHOP. When the CHOP expression plasmid was cotransfected, EGFP-Bax was colocalized to mitochondrial DsRed. Therefore, CHOP induces the translocation of Bax from the cytosol to the mitochondria and this translocation is prevented by the hsp70/dj2 chaperone pair.
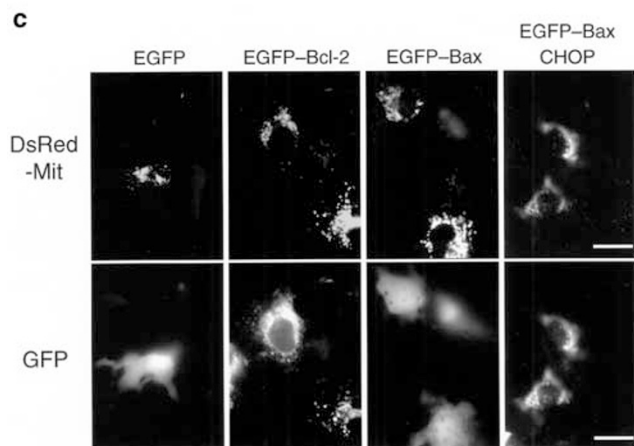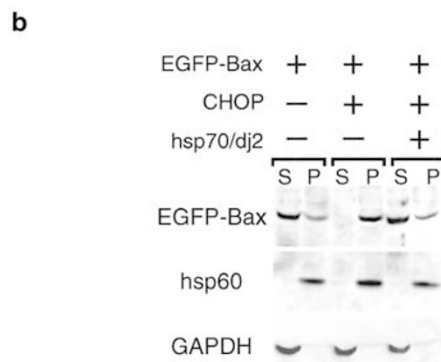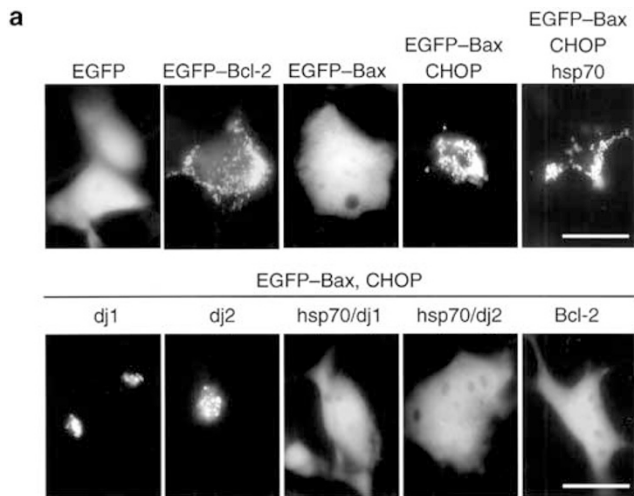
Bax interacts with hsp70 and dj1/dj2

We next asked whether Bax interacts with hsp70 and DnaJ chaperones (Figure 10a). RAW 264.7 cells were heat treated to induce hsp70, dj1, and dj2. This treatment protects RAW 264.7 cells from NO-induced apoptosis.[29] Then cell extracts were subjected to immunoprecipitation with an anti-Bax antibody. When we used extracts of heat-treated cells, hsp70, hsc70, dj1, and dj2 were co-precipitated. When extracts from nontreated RAW 264.7 cells were used, the precipitated hsp70, dj1, and dj2 were reduced, reflecting low expression of chaperones in untreated cells (Figure 1). While expression of hsc70 was not changed by heat treatment (Figure 1), precipitation of hsc70 in extracts from nontreated cells was a little increased. This is probably because of the lack of competition with hsp70 for binding with Bax in nontreated cell extract. These results indicate that Bax interacts with hsp70, dj1, and dj2 in heat-treated cells, and suggest that this association prevents Bax translocation to the mitochondria. In nontreated cells, hsc70 interacts with Bax, but this is not enough for preventing the translocation of Bax.

Binding of hsp70 mutants with Bax is shown in Figure 10b. When hsp70 and dj2 were coexpressed with Bax in COS-7 cells, both hsp70 and dj2 were co-precipitated with Bax. In contrast, when ATPase domain-deleted hsp70 was expressed, this mutant was not co-precipitated with Bax and dj2. We speculated that under these conditions dj2 was co-precipitated with endogenous hsp70 and hsc70, together with Bax. These results together with the results in Figure 6 show

Figure 9 Coexpression of hsp70, and dj1 or dj2 protected CHOP-induced EGFP-Bax translocation to mitochondria in COS-7 cells. (**a**) Cells were transfected with various combinations of expression plasmids for EGFP, EGFP-Bcl-2, EGFP-Bax, CHOP, hsp70, dj1, dj2, or Bcl-2 as indicated on the top. After 24 h, cells were observed by fluorescence microscopy. Original magnifications: × 1000. Bars, 10 μm. (**b**) Cells were transfected with an EGFP-Bax expression plasmid and indicated combinations of expression plasmids for CHOP, hsp70, or dj2. After 24 h, cells were fractionated into the soluble fraction (S) and the particulate fraction (P), as described under Materials and Methods. Distribution of total protein in the soluble fraction and the particulate fraction was 62 and 38%, respectively. The fractions (10 μg of protein) were subjected to immunoblot analysis for Bax (EGFP-Bax), hsp60 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). (**c**) Cells were cotransfected with an DsRed-Mit expression plasmid and an expression plasmid for EGFP, EGFP-Bcl-2, EGFP-Bax, or EGFP-Bax plus CHOP. After 24 h, cells were observed by fluorescence microscopy. DsRed images (upper panels) and EGFP images (lower panels) of the same fields are shown. Original magnifications: × 1000. Bars, 10 μm
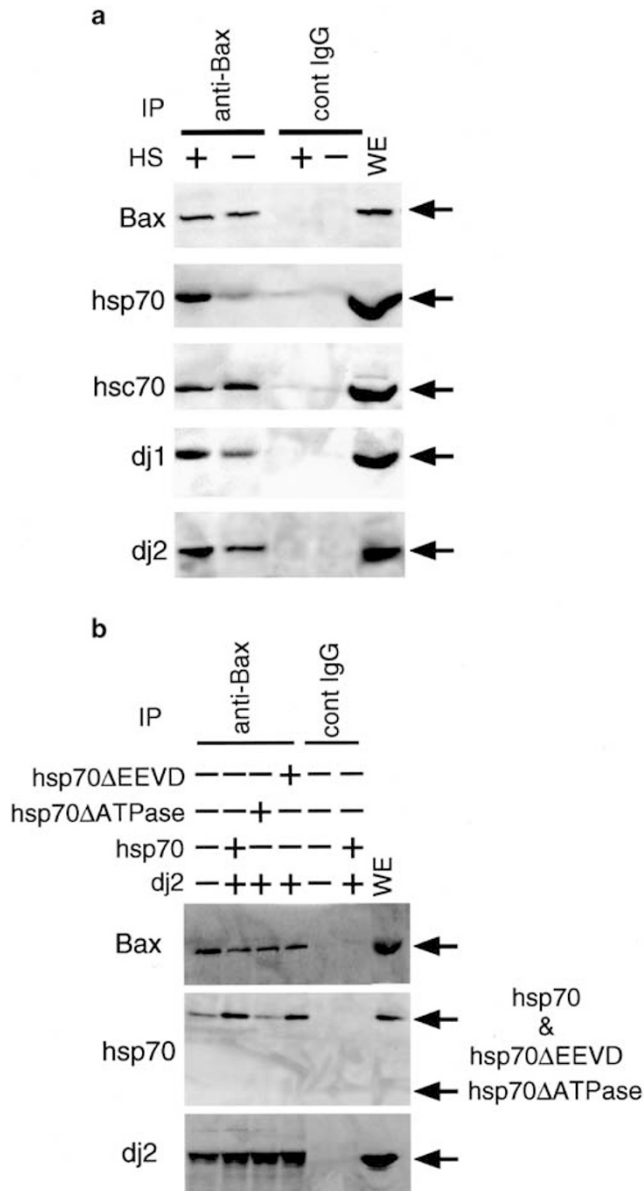
Figure 10 Bax interacts with hsp70, dj1, and dj2. (**a**) RAW 264.7 cells were heat treated at 42°C for 2 h and returned to 37°C for 6 h (HS). Cell extracts (300 µg of protein) were subjected to immunoprecipitation using an anti-Bax polyclonal antibody or control IgG (nonimmune rabbit IgG) as described in Materials and Methods. The immunoprecipitates were subjected to immunoblot analysis for the indicated proteins. WE, whole cell extract of heat-treated RAW 264.7 cells (50 µg of protein). (**b**) COS-7 cells were transfected with a Bax expression plasmid and various combinations of expression plasmids for wild-type hsp70, ATPase domain-deleted hsp70 (hsp70ΔATPase), C-terminal sequence EEVD deleted hsp70 (hsp70ΔEEVD) or dj2 as indicated on the top. After 24 h, cell extracts (300 µg of protein) were subjected to immunoprecipitation using an anti-Bax polyclonal antibody or control IgG (cont IgG, nonimmune rabbit IgG) as described in Materials and Methods. The immunoprecipitates were subjected to immunoblot analysis for the indicated proteins. WE, whole cell extract of COS-7 cells (30 µg of protein) transfected with expression plasmids for Bax, hsp70, and dj2

that the ATPase domain of hsp70 is necessary both for antiapoptotic effect and binding with Bax. On the other hand, the C-terminal EEVD motif is necessary for antiapoptotic effect, but not for binding with Bax. As already mentioned, the

C-terminal EEVD motif of hsp70 is responsible for binding with co-chaperones including hop and chip. Therefore, we speculate that hop and chip are involved, not in the binding with Bax, but the following antiapoptotic events.

Discussion

CHOP induce apoptosis through Bax translocation to mitochondria in NO-mediated apoptosis in macrophages

NO induces apoptosis through the ER stress pathway involving CHOP induction in some cell types.[6,7] We previously showed that pancreatic islets and peritoneal macrophages from CHOP-knockout mice are more resistant to NO-induced apoptosis.[5,7] However, the precise mechanism of CHOP-induced apoptosis was not fully elucidated. Here we found that CHOP induce apoptosis through Bax translocation from cytosol to mitochondria. We also showed that overexpression of Bcl-2 prevents CHOP-induced apoptosis. This agrees well with our previous findings that NO induces apoptosis through a mechanism involving cytochrome *c* release from mitochondria.[29] Bcl-2 family members are key regulators that control release of cytochrome *c* and other apoptosis-promoting factors from mitochondria.[35,37,38] Bcl-2 family proteins are separated into three groups. Group I members including Bcl-2 and Bcl-X$_L$, have four conserved Bcl-2 homology (BH) domains (BH1-BH4), and antiapoptotic function. Group II members including Bax and Bak, lack the N-terminal BH4 domain, and function as proapoptotic molecules. Group III members including Bid and Bim, contain only a single BH3 domain, and function as proapoptotic molecules.

Bax is mainly located in the cytosol under nonapoptotic conditions and translocates to the mitochondria in response to apoptotic stimuli. Once the BH3-only proteins bind to the mitochondria, they cooperate with other proapoptotic Bcl-2 family proteins to induce the release of apoptogenic proteins. The proapoptotic group II Bcl-2 family members, such as Bax and Bak, are likely to be mediators for BH3-only proteins. The important role of these proteins in apoptosis was demonstrated using *Bax* and *Bak* double knockout mice.[39] MEF cells lacking *Bax* and *Bak* are resistant to multiple apoptotic stimuli, including ultraviolet irradiation, growth factor deprivation, treatment with a variety of drugs, and the overexpression of BH3-only proteins tBid, Bim, and Bad. Concurrent with the translocation of the BH3-only proteins to the mitochondria, Bax undergoes conformational changes and oligomerization, and is inserted into the outer membrane of the mitochondria.[36,40,41] Enforced dimerization of Bax results in translocation to the mitochondria and apoptosis.[42] The oligomerized Bax and Bak may form a pore for apoptogenic proteins, or may interact with the mitochondrial outer membrane voltage-dependent anion channel (VDAC) to induce a change of VDAC permeability to allow apoptogenic proteins to pass through.[43] However, the mechanism of Bax-induced cytochrome *c* release from the mitochondria is not fully understood.

Other remaining questions to be clarified are what drives the oligomerization of Bax and its insertion. In this study, we showed that excess NO and CHOP expression induces Bax

translocation to the mitochondria. NO and NO-related species were reported to induce apoptosis through activation of p38 and JNK MAP kinase.[4,44,45] JNK activation induces cytochrome c release from the mitochondria. The molecular mechanism of this release is unclear, but may involve regulation of expression and phosphorylation of the Bcl-2 family proteins. It was shown that cells lacking both *Bax* and *Bak* become resistant to the apoptosis induced by ER stress.[11,46] Thus, Bax is crucial in ER stress-induced apoptosis. Several pathways of ER stress-induced apoptosis have been reported, in addition to the CHOP pathway.[11] Therefore, these results do not mean that CHOP-induced apoptosis is mediated solely by Bax. However, our results do show that CHOP expression is able to induce Bax translocation and apoptosis. We also showed that Bax knock down at least partially prevents CHOP-induced apoptosis. The precise mechanism of CHOP-induced Bax translocation remains to be clarified. Recently, Scorrano et al.[47] reported that Bax and Bak operate at ER as well as mitochondria to maintain homeostasis of ER Ca^{2+}. We reported that excess NO increases Ca^{2+} in cytosol, presumably due to the release from ER.[5] Therefore, it also remains to be clarified whether there is any interaction between Bax and NO at ER.

hsp70/DnaJ chaperone pair prevents CHOP-induced apoptosis through the inhibition of Bax translocation

Stress proteins or hsps are a set of conserved proteins the expression of which is induced by various stresses. They have cytoprotective effects and behave as molecular chaperones for cellular proteins.[21,22,48,49] Under normal conditions, hsp70 family members function as ATP-dependent molecular chaperones by assisting in folding and assembly of newly synthesized polypeptides and their transport to various organelles. Under stress conditions, hsp70 (inducible form) is induced and protects cells from apoptosis. Although the antiapoptotic effects of hsp70 have been noted in several systems, molecular mechanisms are unclear. In heat-induced cell death, hsp70 was reported to inhibit the activation of the stress-activated protein kinase SAPK/JNK (c-Jun N-terminal kinase), the release of cytochrome c and the processing of caspase 3.[50,51] Gabai et al.[52] reported that hsp70 specifically interferes with the Bid-dependent apoptotic pathway via inhibition of JNK in tumor necrosis factor-α (TNFα)-induced apoptosis. On the other hand, the site of hsp70 action was suggested to be downstream of caspase 3 in TNFα-induced apoptosis.[53] In addition, the overexpression of hsp70 prevented heat-induced apoptosis, but not Fas-mediated apoptosis in a Jurkat T cell line.[54] Using *in vitro* systems, hsp70 was found to prevent apoptosome formation[55] and caspase 3 activation.[56] In these studies, however, participation of a DnaJ family member(s) was not given attention. Using *in vitro* systems, King et al.[57] showed that the hsc70/dj1 chaperone pair bind to p53 and may regulate the stabilization and localization of p53. We previously found that hsp70-dj1 or dj2 pairs prevent NO-mediated apoptosis upstream of cytochrome c release from the mitochondria in RAW 264.7 cells.[29] In the present study, we found that hsp70/DnaJ chaperone

pairs prevent LPS/IFN-γ-induced apoptosis downstream of CHOP induction and upstream of Bax translocation from the cytosol to the mitochondria. We also found that Bax interacts with both hsp70 and dj2. These results suggest that hsp70/ DnaJ chaperone pairs protect cells from apoptosis by binding to Bax and prevent its translocation. As already mentioned, Bax undergoes conformation change before being inserted into mitochondrial outer membrane.[36,40,41] hsp70/DnaJ pair may prevent Bax from changing into the proapoptotic conformation.

The ATPase domain and C-terminal EEVD motif of hsp70 are essential for chaperone activity.[32] We report here that the ATPase domain and C-terminal EEVD motif of hsp70 are crucial for antiapoptotic activity in CHOP-induced apoptosis. Similar results were noted in case of TNFα-induced apoptosis,[52] and in heat-shock-induced apoptosis.[51] These results suggest that the chaperone activity of hsp70 is indispensable in its antiapoptotic activity. We also found that unfarnesylated dj2 mutant decreases its antiapoptotic activity. The role of farnesylation for dj2 chaperone activity remains unknown.

Materials and Methods

Plasmids

pcDNA3.1-hsp70, a mammalian expression plasmid for human hsp70, was constructed by inserting the full-length human hsp70 cDNA (GenBank accession number NM_005346) into the *Eco*RI site of a mammalian expression plasmid pcDNA3.1(+) (Invitrogen, Carlsbad, CA, USA) that harbors the G418 resistant gene after linker attachment. The mammalian expression plasmids for mutant hsp70 were constructed by inserting the mutated hsp70 cDNA into the *Eco*RI site of a mammalian expression plasmid pcDNA3.1(+) after linker attachment. pcDNA3.1-hsp70ΔAT-Pase express mutant human hsp70 that defect the N-terminal ATPase domain (amino acids 1–380). pcDNA3.1-hsp70ΔEEVD express mutant human hsp70 that defect the C-terminal EEVD sequence. The mammalian expression plasmids for human dj1 and dj2 were constructed by inserting the full-length human dj1 or dj2 cDNA into the *Eco*RI site of a mammalian expression plasmid pcDNA3.1(+)/Hyg (Invitrogen) that harbors the hygromycin-resistant gene after linker attachment, yielding pcDNA3.1/ Hyg-dj1 and pcDNA3.1/Hyg-dj2. The mammalian expression plasmids for C394S mutant of human dj2 has been described elsewhere.[34] pcDNA3.1-mCHOP, a mammalian expression plasmid for mouse CHOP, was also described previously.[7] The LacSwitch-inducible mammalian expression system (Stratagene, La Jolla, CA, USA) was used for conditional expression of CHOP. pOPRSVI-CHOP, an IPTG-inducible CHOP expression plasmid, was described.[18] pUSEamp-Bcl-2 and pUSEamp-Bax, the mammalian expression plasmids for mouse Bcl-2 and Bax, respectively, were obtained from Upstate Biotechnology (Lake Placid, NY, USA). pEGFP-C1 and pDsRed-N1, a mammalian expression plasmids for enhanced GFP and DsRed, respectively, were obtained from Clontech Laboratories, Inc. (Palo Alto, CA, USA). The expression plasmid for DsRed with the mitochondria import signal (pDsRed-Mit) was constructed by replacing the EYFP gene of pEYFP-Mito (Clontech Laboratories, Inc.) with DsRed gene. The mammalian expression plasmid for the human active form ATF6 (p50ATF6) was as described.[31] The mammalian expression plasmids for EGFP-Bcl-2 or EGFP-Bax fusion proteins were constructed by inserting mouse Bcl-2 or Bax full-length cDNA just downstream of the EGFP gene of the plasmid pEGFP-C1 after linker attachment.

Materials

Monoclonal antibodies against human/mouse hsc70 and Bax were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Monoclonal antibodies against human hsp70 and human hsp60 were obtained from StressGen Biotechnologies Corp. (Victoria, Canada). Monoclonal antibody against human dj2 was obtained from NeoMarkers (Union City, CA, USA). Polyclonal anti-human dj1 and dj2 antibodies were those described elsewhere.[28] Monoclonal antibody against mouse iNOS was obtained from Transduction Laboratories (Lexington, KY, USA). A polyclonal antibody against mouse CHOP and polyclonal and monoclonal antibodies against human Bax were obtained from Santa Cruz Biotechnology Inc. Polyclonal antibody against GFP was as described elsewhere.[58] Monoclonal antibodies against human/mouse cytochrome *c* and rabbit GAPDH were obtained from R & D Systems Inc. (Minneapolis, MN, USA) and CHEMICON International Inc. (Temecula, CA, USA), respectively.

Cell culture and transfection

Mouse macrophage-like RAW 264.7 cells were grown in Eagle's minimal essential medium supplemented with 10% fetal calf serum. COS-7 cells and human cervical cancer Hela cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Mouse peritoneal macrophages were prepared and grown in RPMI 1640 medium supplemented with 10% fetal calf serum as described.[59] CHOP knockout mice were described.[5] Transfection of cells with siRNA and/or plasmids was carried out using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the protocol provided by the manufacturer. In each experiment, the same total amounts of plasmids were transfected by adding insert-less expression plasmids. To establish stably transformed RAW 264.7 cell line, RAW 264.7 cells were screened with hygromycin (250 μg/ml) and/or G418 (500 μg/ml) after transfection of plasmids harboring hygromycin or the G418-resistant gene. The sequence of siRNA for human Bax knock down are:

$$5' - \quad \text{GGUGCCGGAACUGAUCAGAdTdT} \quad -3'$$
$$3' - \text{dTdTCCACGGCCUUGACUAGUCU} \quad -5'$$

The sequence of siRNA for negative control (mouse Bax sequence) are:

$$5' - \quad \text{GGCCUCCUCUCCUACUUCGdTdT} \quad -3'$$
$$3' - \text{dTdTCCGGAGGAGAGGAUGAAGC} \quad -5'$$

Treatment of RAW 264.7 cells and mouse peritoneal macrophages

RAW 264.7 cells were heated at 42°C for 2 h and allowed to recover at 37°C for 6 h then the cell extracts were used as heat-shock-treated extracts. To induce iNOS and NO-mediated apoptosis in RAW 264.7 cells, cells were treated with *E. coli* LPS (serotype 0127:B8, Sigma Chem. Co., St. Louis, MO, USA, 150 μg/ml) plus mouse IFNγ (100 U/ml) for the indicated periods. To induce NO-mediated apoptosis in mouse peritoneal macrophages, cells were treated with a NO donor *S*-nitroso-*N*-acetyl-DL-penicillamine (SNAP, 1.5 mM) for the indicated periods.

Immunoblot analysis

RAW 264.7 cells, COS-7 cells or Hela cells were homogenized in 20 mM HEPES-KOH (pH 7.5) containing 1% Triton X-100, 20% glycerol and 1 mM dithiothreitol. After centrifugation, the supernatants were used as whole cell extracts for immunoblot analysis. Nuclear extracts from RAW 264.7 cells were prepared, as described,[7] and used for immunoblot analysis. The cell extracts were then subjected to SDS-polyacrylamide gel electrophoresis, and proteins were electrotransferred to nitrocellulose membranes. Immunodetection was performed using the ECL kit (Amersham, Buckinghamshire, UK) according to the protocol provided by the manufacturer.

Measurement of NO production

Concentration of NO_2^- plus NO_3^- in culture supernatants was measured, as described.[29]

Detection of apoptosis

RAW 264.7 cells or Hela cells were treated as described above. Detection of DNA ladder formation was done, as described.[29] Caspase 3 activity was measured, as described.[29] To detect mitochondrial membrane potential disruption, cells were stained with a membrane potential-dependent dye DePsipher (Trevigen Inc., Gaithersburg, MD, USA), as described.[5] To analyze morphological changes in nuclei, the cells were fixed, stained with Hoechst dye 33258 (8 μg/ml) for 5 min, and washed with phosphate-buffered saline. The stained cells were then observed under a fluorescence microscope.

Subcellular fractionation

Cell fractionation was done, as described.[29] Briefly, the cell suspension (about 5×10^7 cells/ml) was mixed with an equal volume of digitonin (1 mg/ml) in phosphate-buffered saline (PBS), incubated at 25°C for 5 min, then kept on ice for 5 min then centrifuged at $15\,000 \times g$ for 2 min. The supernatant served as the soluble fraction. The pellet containing mitochondria was dissolved in 0.5% Triton X-100 in PBS, insoluble material was removed by centrifugation, and the supernatant served as the particulate fraction.

Immunocytochemical staining

RAW 264.7 cells and mouse peritoneal macrophages were cultured on coverslips and treated as indicated. The cells were fixed with 4% paraformaldehyde for 40 min at room temperature and treated with phosphate-buffered saline containing 1% Triton X-100. The cells were incubated with first antibodies, and then with second antibodies conjugated with Alexa Fluor (Molecular Probes, Eugene, OR, USA). The stained cells were observed using a fluorescence microscope.

Co-immunoprecipitation of Bax and hsp70-dj2

Raw 264.7 cells or COS-7 cells were lysed in CelLytic MT Mammalian Tissue Lysis/extraction Reagent (Amersham). The cell lysates were centrifuged at $25\,000 \times g$ for 15 min at 4°C. The supernatant fractions (1 ml, 300 μg of protein) were incubated with an anti-Bax polyclonal antibody or control IgG (nonimmune rabbit IgG) for 1 h at 20°C then 100 μl of a 12% suspension of protein A-Sepharose (Amersham) was added. After mixing for 1 h at 20°C, the resin was collected by centrifugation and washed three times with wash buffer (50 mM Tris-HCl (pH 7.5) containing 150 mM NaCl, 5 mM EDTA, 0.1% Triton X-100). Proteins were extracted and subjected to immunoblot analysis, using monoclonal antibodies against Bax, hsp70, hsc70, dj1, or dj2.

Acknowledgements

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