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Coupling endoplasmic reticulum stress to the cell-death program: a novel HSP90-independent role for the small chaperone protein p23

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Abstract

The endoplasmic reticulum (ER) is the principal organelle for the biosynthesis of proteins, steroids and many lipids, and is highly sensitive to alterations in its environment. Perturbation of Ca^{2+} homeostasis, elevated secretory protein synthesis, deprivation of glucose or other sugars, altered glycosylation and/or the accumulation of misfolded proteins may all result in ER stress, and prolonged ER stress triggers cell death. Studies from multiple laboratories have identified the roles of several ER stress-induced cell-death modulators and effectors through the use of biochemical, pharmacological and genetic tools. In the present work, we describe the role of p23, a small chaperone protein, in preventing ER stress-induced cell death. p23 is a highly conserved chaperone protein that modulates HSP90 activity and is also a component of the steroid receptors. p23 is cleaved during ER stress-induced cell death; this cleavage, which occurs close to the carboxy-terminus, requires caspase-3 and/or caspase-7, but not caspase-8. Blockage of the caspase cleavage site of p23 was associated with decreased cell death induced by ER stress. Immunodepletion of p23 or inhibition of p23 expression by siRNA resulted in enhancement of ER stress-induced cell death. While p23 co-immunoprecipitated with the BH3-only protein *PUMA* (p53-upregulated modulator of apoptosis) in untreated cells, prolonged ER stress disrupted this interaction. The results define a protective role for p23, and provide further support for a model in which ER stress is coupled to the mitochondrial intrinsic apoptotic pathway through the activities of BH3 family proteins.
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Keywords: endoplasmic reticulum; ER stress; p23; HSP90; caspase; apoptosis

Abbreviations: ER, endoplasmic reticulum; pcd, programmed cell death; RNAi, RNA interference

Introduction

An imbalance between the load of newly synthesized proteins in the endoplasmic reticulum (ER) and the organelle's protein-folding capacity triggers ER stress. ER stress may also result from alterations in Ca^{2+} homeostasis, viral infections, hypoxia, ischemia, mutations that impair client protein folding and accumulation of misfolded proteins (either within or outside the ER itself).[1,2] Prolonged ER stress impairs the protective mechanisms designed to promote correct folding and degrade faulty proteins, ultimately leading to organelle dysfunction and programmed cell death (pcd).[2–5] Studies from multiple laboratories have disclosed roles for several ER stress-induced cell-death modulators and effectors.[6] There is a growing realization that some such mediators may be specific for a given inducer or set of inducers.[7]

In our earlier study, we used a set of complementary approaches including two-dimensional gel electrophoresis coupled with MALDI-TOF mass spectrometry and nano-LC-ESI-MS/MS (matrix-assisted laser desorption ionization-time of flight mass spectrometry and nano-liquid chromatography-electrospray ionization mass spectrometry with tandem mass spectrometry), RNA interference (RNAi), co-immunoprecipitation, immunodepletion of candidate proteins, and reconstitution studies, and identified the calcium-binding protein apoptosis-linked gene-2 (ALG-2), and valosin-containing protein (VCP), as mediators of ER stress-induced pcd.[7] In the present report we describe the role of p23, a small chaperone protein, in preventing ER stress-induced cell death. Specifically, p23 seems to function as an antiapoptotic protein by interacting with the BH3-only proapoptotic protein PUMA, and to exert a protective effect that is abrogated following ER stress-induced caspase-dependent cleavage near its carboxy-terminus, associated with the release of PUMA and the availability of PUMA to interact with Bax.

Results

Evaluation of HSP90 and p23 in ER stress-induced cell death

We previously generated a list of 40 candidate ER-apoptosomal mediators of ER stress-induced apoptosis,[7] and described in detail the roles of apoptosis-linked gene-2 (ALG-2) and VCP in mediating ER stress-induced pcd.[7] As part of our continuing investigation of these candidates, we assessed the role of HSP90, which showed at least a 1.5-fold increase in ER-stressed HEK 293T cells, as determined by two-dimensional IEF/SDS-PAGE patterns (data not shown). Further analysis of cell-free cytosolic extracts revealed different

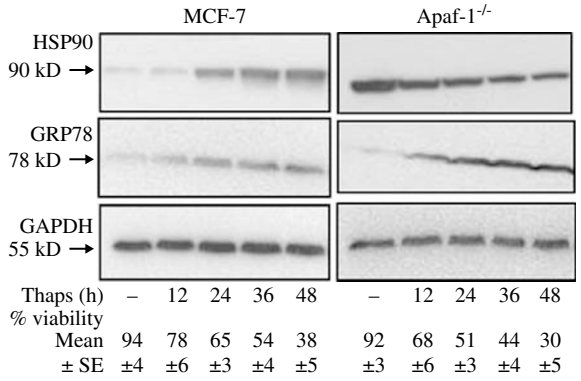

Figure 1 HSP90 protein expression during ER stress. HSP90 protein expression in MCF-7 cells and Apaf-1$^{-/-}$ fibroblasts treated with 2.0 and 0.5 μM thapsigargin, respectively, for different time periods. Cell extracts (150 μg protein) were then analyzed by Western blot analysis for HSP90 expression. Increased levels of GRP78 protein expression in similar extracts indicate ER stress response. Surviving *versus* apoptotic cells were quantified as described in the Materials and Methods. Data (mean $\pm$ S.E.) are from more than three independent experiments. Each figure is representative of at least three independent experiments

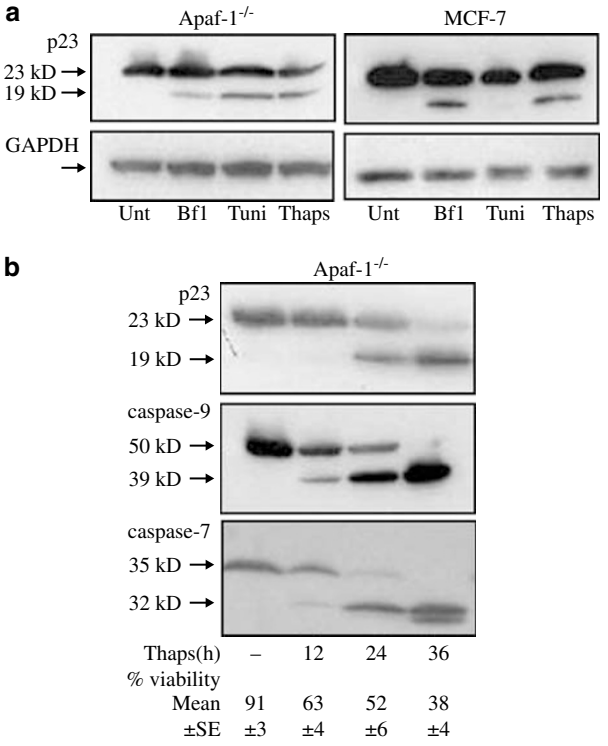

Figure 2 p23 protein expression during ER stress. p23 protein expression in Apaf-1$^{-/-}$ fibroblasts and MCF-7 cells treated with various ER stress inducers for 24 h. (**a**) Apaf-1$^{-/-}$ fibroblasts were treated with 1.0 μM brefeldin-A, 2.0 μg/ml tunicamycin, and 0.5 μM thapsigargin. MCF-7 cells were treated with 2.0 μM brefeldin-A, 2.0 μg/ml tunicamycin, and 2.0 μM thapsigargin. Cell extracts (150 μg protein) were then analyzed by Western blot analysis for p23 expression. MCF-7 cells do not elicit an ER stress response when cells are exposed to tunicamycin treatment. Cell extracts were also probed with GAPDH as a loading control. (**b**) p23 cleavage is accompanied by caspase processing. Cell extracts (150 μg of protein) were prepared from cells treated with 0.5 μM thapsigargin for various time periods. p23 is cleaved to a low Mw species (approximately 19 kD) and thapsigargin-induced p23 cleavage is also accompanied by caspase-9 and -7 processing. Surviving *versus* apoptotic cells were quantified as described in Materials and Methods. Data are from three independent experiments

HSP90 protein expression patterns in different cell lines: while cell extracts from MCF-7 or 293T cells treated with thapsigargin showed increased HSP90 expression, Apaf-1$^{-/-}$ cells showed decreased HSP90 expression with time of thapsigargin treatment (Figure 1). Similar HSP90 expression profiles were also observed in cells undergoing ER stress triggered by brefeldin-A (data not shown). Evidence of ER stress was the high level of GRP78 protein expression[2,3,8] following thapsigargin treatment of both Apaf-1$^{-/-}$ and MCF-7 cells (Figure 1). Since HSP90 protein expression was not induced consistently in different cell types following ER stress, we turned our attention to p23, an HSP90-associated chaperone protein. p23 is a widely expressed protein, and a subpopulation of p23 exists in complex with HSP90. The p23–HSP90 protein complex is involved in the assembly of steroid hormone receptor and telomerase complexes, and also regulates the binding, folding and processing of proteins.[9,10]

We assessed the expression of p23 in cell extracts isolated from untreated and ER stress-induced cells. As shown in Figure 2, p23 is a low molecular weight (23 kD) chaperone protein whose expression is detectable both in MCF-7 and Apaf-1$^{-/-}$ fibroblasts. Treatment with ER stress-inducing agents resulted in the cleavage of p23 to a species of lower molecular mass (approximately 19 kD) in both cell types (Figure 2a). (It should be noted that MCF-7 cells have been shown not to mount an ER stress response when exposed to tunicamycin treatment,[11] and this may explain the lack of cleavage of p23 in tunicamycin-treated MCF-7 cells (Figure 2a)). Processing of p23 to the 19 kD band was also seen in primary neuronal cells exposed to hypoxia (data not shown). This was also associated with a high level of GRP78 protein expression in cells, indicative of hypoxia-induced ER stress (data not shown).

Based on our earlier studies on the involvement of various caspases in ER stress-induced cell death, we proceeded to determine whether or not p23 cleavage was accompanied by caspase processing. As shown in Figure 2b, thapsigargin-induced p23 cleavage was accompanied by caspase-9 and -7 processing. The anti-caspase-9 antibody recognized the full-length 50 kD band and the amino-terminal-cleaved product (39 kD, prodomain + large subunit) of caspase-9.[12] As shown in Figure 2b, complete processing of the proform to the 39 kD product occurred by 36 h of treatment. Similarily, we assessed the expression of caspase-7, a caspase activated by ER stress.[12,13] Procaspase-7 (35 kD) is first converted to a 32 kD intermediate (prodomain + large subunit), which is further processed into active subunits consisting of 20 and 11 kD forms.[12] Similarly, ER stress-induced cell death was accompanied by processing of caspases-12 and -3 in Apaf-1$^{-/-}$ fibroblasts and caspase-7 in MCF-7 cells (data not shown).

Mutation of caspase cleavage site prevents processing of p23

We next sought to determine whether ER stress-induced cleavage of p23 is a caspase-dependent phenomenon. As

shown in Figure 3a, while the addition of purified active caspase-8 to cell extracts failed to cleave p23, addition of purified active caspase-3 or -7 resulted in the cleavage of p23 protein and appearance of a 19 kD band. The p23 cleaved fragments arising from thapsigargin and brefeldin-A treatment closely matched the fragment sizes generated from caspase-3 and -7 cleavage of p23. Appearance of the proteolytic fragment of p23 triggered by brefeldin-A treatment was also blocked by the pan caspase inhibitor zVAD-fmk (Figure 3b), suggesting that caspases are likely to cleave p23.

The antibody used to detect p23 was raised against the N-terminal 94 amino-acid residues of mouse p23 protein. Since the antibody was capable of detecting the 19 kD cleaved fragment of p23, it suggested that the p23 cleavage site occurred at the carboxy-terminus. The C-terminal sequence of p23 contains eight potential caspase cleavage sites (Figure 3c). We therefore generated several single, double and triple mutants of p23. The mutant proteins were expressed in 293T cells. Cellular extracts were incubated with recombinant purified caspase-3 or -7. As shown in Figure 3d, except for D142N, none of the other mutations (D145N, D150N, D153N, D150/153N or D145/150/153N) blocked cleavage of p23 by caspases-3 and -7. A single mutation at D142, however, prevented the protein from being cleaved by recombinant caspases.

We next determined whether or not ER stress-mediated cell death is blocked by p23 caspase mutants. MCF-7 cells were either transfected with a mock vector, WT p23, p23D142N or p23D150/153N mutant constructs. At 24 h after transfection, thapsigargin (2 μM) or brefeldin-A (2 μM) was added to cells. Cell viability was measured 24 h after treatment, as described earlier.[8,12] While WTp23 suppressed to some extent ER stress induced by brefeldin-A or thapsigargin, the expression of p23D142N mutant significantly suppressed cell death induced by both agents. Both the mock vector and the p23D150/153N mutant construct failed to block cell-death induction by thapsigargin or brefeldin-A. Similarly, expression of the p23D142N mutant also suppressed cell death induced by non-ER stress agents including etoposide (25 μM; data not shown). This suggested that p23D142 is a critical site that is

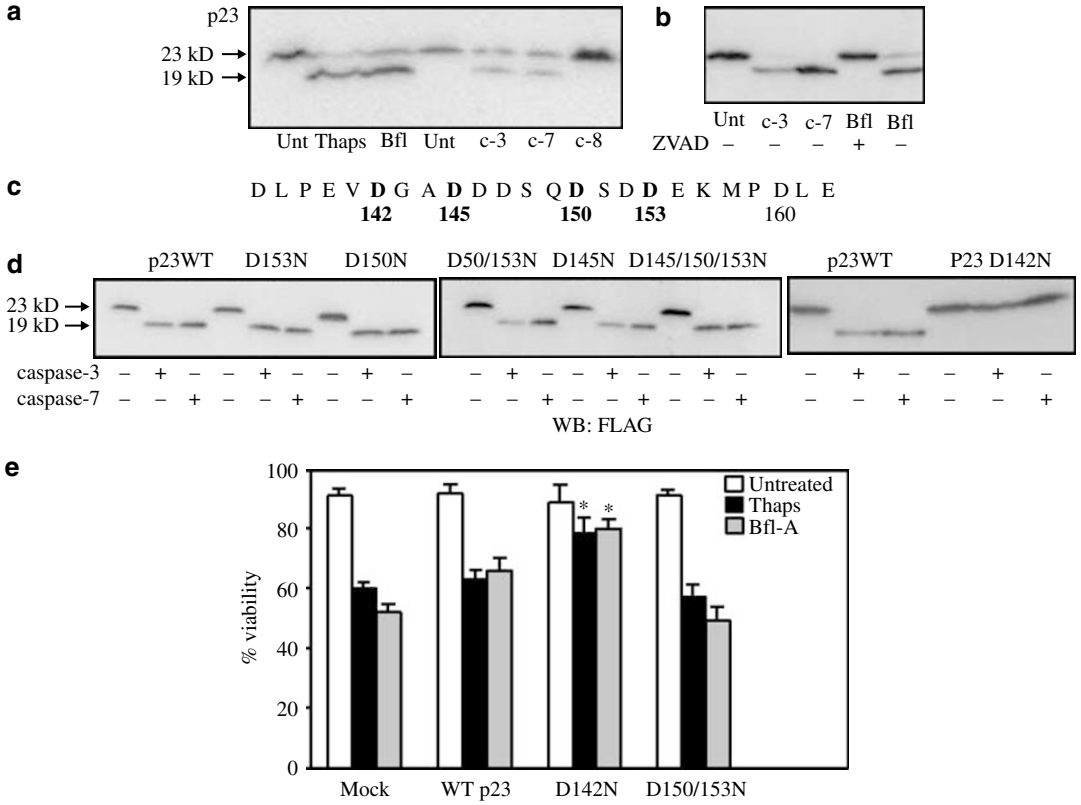

Figure 3 ER stress-induced cleavage of p23 is a caspase-dependent phenomenon. (**a** and **b**) p23 protein expression in Apaf-1$^{-/-}$ fibroblasts treated with 0.5 μM thapsigargin or 1.0 μM brefeldin-A in the presence or absence of ZVAD-fmk. Purified active caspase-3 (50 ng/ml), caspase-7 (100 ng/ml) or caspase-8 (1.0 μg/ml) was added to cell-free cytosolic extracts (150 μg protein) made from Apaf-1$^{-/-}$ fibroblasts (lanes 5–7). Extracts were incubated at 37°C for 1 h, separated on SDS-PAGE gels, transferred to PVDF membrane and incubated with p23 antibody. p23 is cleaved to a low Mw (19 kD) species (**a**) that is blocked by ZVAD-fmk (**b**). (**c**) Potential caspase cleavage sites in p23. A total of eight 'D' (aspartate) residues exist in the carboxy-terminus that are potential sites for caspase activity. (**d**) p23 has several potential caspase sites at the carboxy-terminus. Site-directed mutagenesis of several of these sites were carried out as described in Materials and Methods. Cell-free extracts were made from 293T cells transfected with wild-type Flagp23, D142N, D145N, D150N, D153N, D150/153N and D145/150/153N constructs. Samples (150 μg protein) were incubated with 50 ng/ml caspase-3 or 100 ng/ml caspase-7 at 37°C for 1 h. Samples were analyzed by immunoblotting with anti-FLAG antibody to detect p23 and its cleavage product (19 kD). (**e**) p23 caspase mutant inhibits ER stress-induced cell death. MCF-7 cells were transfected with 6 μg of pcDNA3, pcDNAp23, p23D142N or p23D150/153N mutant constructs. After 24 h, cells were treated with 2.0 μM thapsigargin (Thaps) or 2.0 μM brefeldin-A (Bfl-A) for 24 h. Cells were gently lifted and washed once with PBS at room temperature. Surviving *versus* apoptotic cells were quantified as described in the Materials and Methods. *$P < 0.05$

susceptible to caspase cleavage, and cleavage at this site may lead to a loss of the antiapoptotic function of p23, both in ER stress and non-ER stress insults.

Subcellular localization of p23

p23 is a widely expressed chaperone protein that is involved in the binding, folding and processing of proteins, as well as the assembly of steroid hormone receptor and telomerase complexes (these events are closely associated with the ER).[9,10] While caspase-3 is predominantly cytosolic, we and others have shown that caspase-7 is associated with ER during ER stress.[8,13] Since ER stress-induced cell death results in caspase-7- and casapse-3-mediated cleavage of p23, it prompted us to examine the subcellular localization of p23.

To determine the localization of p23, we performed immunocytochemistry on untreated and hypoxic cortical neuronal cells using antibodies specific to p23 and calreticulin. The antibody to p23 stained within the cytosolic compartment with a granular pattern in the perinuclear region (Figure 4a, green). Calreticulin, a marker protein for ER, demonstrated robust, punctate staining predominantly in the ER (Figure 4a, red). Approximately half the green fluorescent volume (p23) in the untreated sample was found to colocalize with the calretriculin channel, with no change in the overall fraction of colocalization following 24 or 48 h of hypoxia.

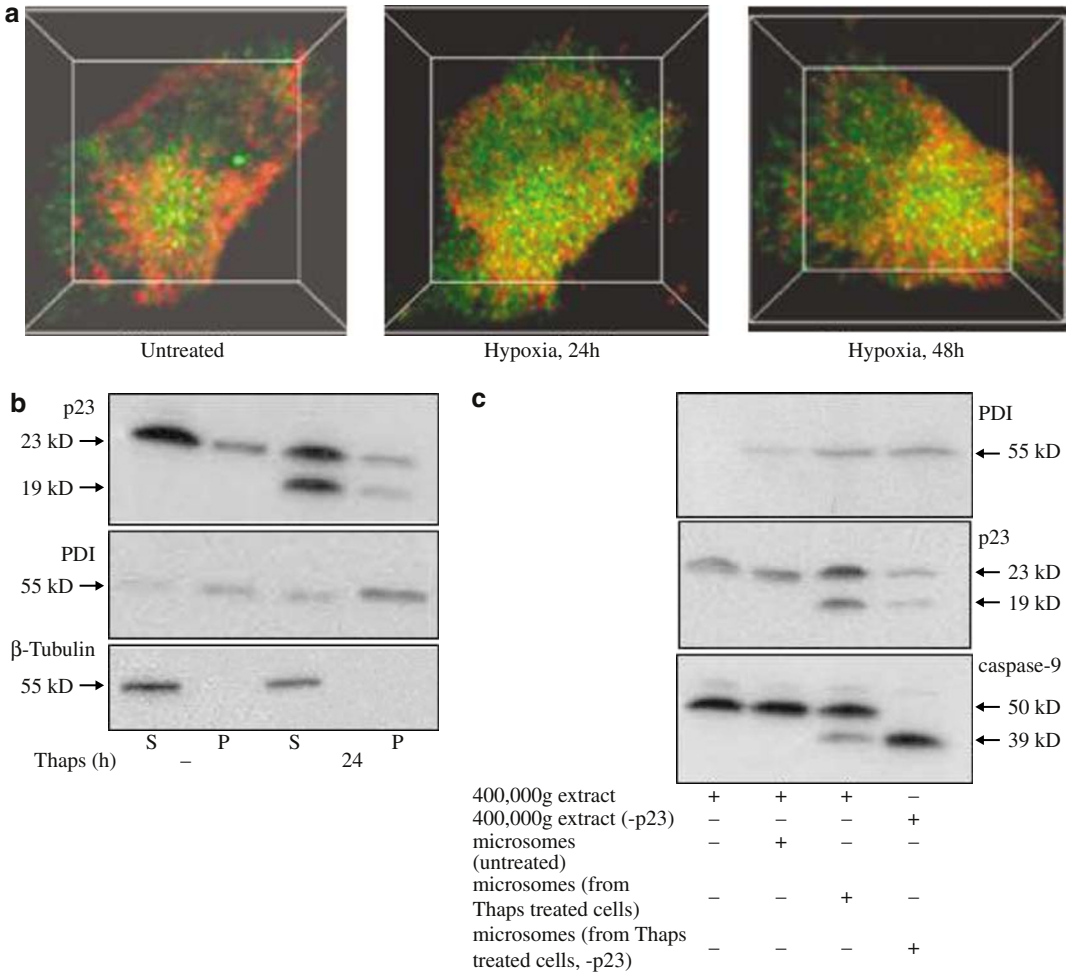

Figure 4 Subcellular localization of p23. (**a**) Immunofluorescent staining for p23 before and after subjecting cortical neurons to hypoxia. For the detection of p23, cells were stained with anti-p23 monoclonal antibody as the primary antibody and 488-conjugated donkey anti-mouse IgG as the secondary antibody (green). Cells were also stained with anti-calreticulin polyclonal antibody and 555-conjugated donkey anti-rabbit IgG (red) as the secondary antibody. (**b**) Microsomes were isolated from cell-free extracts prepared from untreated or thapsigargin-treated Apaf-1$^{-/-}$ fibroblasts (24 h) as described in Materials and Methods. A total of 50 μg of protein from the 400 000 $\times$ *g* supernatant (S) and microsomal (P) fractions was subjected to SDS-PAGE and Western blotting. Membranes were probed with anti-p23 monoclonal antibody. Cellular fractions were also probed with anti-PDI (protein disulfide isomerase) antibody and anti-β-tubulin as standard marker proteins for ER and cytosol, respectively. (**c**) p23 is required for the processing of caspase-9 in response to thapsigargin-induced ER stress. Untreated cell extracts and microsomes isolated from 24 h thapsigargin-treated cell extract were immunodepleted of p23 using the p23 monoclonal antibody. Samples were spun briefly to pellet the protein A/G-Sepharose conjugate. The supernatant was collected, subjected to SDS-PAGE, and probed for (1) p23 that would indicate the extent of immunodepletion and (2) PDI as ER marker protein to serve as loading controls. Additionally, a caspase-9 processing assay was carried out by incubating 100 μg of the supernatant protein at 37°C for 1 h with untreated cell extracts or extracts that had been immunodepleted of p23. Following the reactions, samples were analyzed by SDS-PAGE and Western blotting. Membranes were probed with anticaspase-9 antibody. Note that the reduction of p23 was approximately 75%

p23 localization was also determined in cellular extracts isolated from cells before and/or after ER stress. Apaf-1$^{-/-}$ fibroblasts were either left untreated or treated with 0.5 μM thapsigargin. Cell-free cytosolic extracts that lack whole cells, nuclei, and mitochondria were recentrifuged at 400 000 $\times$ *g* for 1 h for the preparation of microsomes, as described under Materials and Methods. Microsomes (400 000 $\times$ *g* pellet (P)) and the 400 000 $\times$ *g* supernatant (S) were both subjected to SDS-PAGE and Western blot analysis. As shown in Figure 4b, p23 was predominantly present in the supernatant fractions, with a lower expression in the microsomal fractions. Treatment with thapsigargin resulted in the appearance of the 19 kD cleaved band. There was a decreased expression of both the 23 and 19 kD bands in the microsomal fraction isolated from thapsigargin-treated cells. A similar pattern of expression was also seen in cellular extracts isolated from MCF-7 cells treated with 2.5 μM brefeldin (data not shown). While the data indicate that p23 is predominantly a cytosolic protein with a subpopulation of p23 also associated with the ER compartment, comparison of the immunochemical staining with the fractionation studies indicates that localization of p23 may also depend on the cell type.

To determine whether p23 was required for the processing of caspases, we immunodepleted p23 from untreated cell extracts and fragmented (sonicated) microsomes isolated from thapsigargin-treated Apaf-1$^{-/-}$ cells as described earlier.[7,12] Since there was a greater population of p23 in the supernatant fraction, it was decided to immunodeplete both the microsomal and supernatant fractions. Immunodepletion resulted in a decrease of p23 protein by approximately 70–80%. The immunodepleted microsomal fraction was incubated with immunodepleted cytosolic extracts and the effects compared to those extracts that had not been immunodepleted. As shown in Figure 4c, immunodepletion of p23 enhanced the degree of cleavage of procaspase-9 (lane 4). While the results do not reveal the specific contribution of microsomal *versus* cytosolic p23 to caspase processing, the data do suggest that p23 (alone and/or in combination with other molecule(s)) may play a role in blocking ER stress-induced caspase activation (at least in this model system).

Effect of RNAi on ER stress-induced caspase activation and cell death

To complement the results obtained from immunodepletion studies and to evaluate the effects of reducing p23 concentration in cells (as opposed to extracts), we used RNAi to reduce the expression of p23. Small interfering RNAs (siRNAs) were designed to target two regions of the p23 gene, based on predicted accessible (loop) and unique (specific) regions. As shown in Figure 5a, siRNA-targeting nucleotides 419–429 and 446–466 were very effective in reducing the expression of p23 protein (by approximately 50 and 70%, respectively). A combination of these two siRNAs nearly eliminated the expression of p23 (approximately 90% reduction, Figure 5a, comb). The siRNAs did not affect the expression of HSP90.

The effect of RNAi on ER stress-induced caspase activation and cell death was studied by treating Apaf-1$^{-/-}$ cells with thapsigargin after transfection with the p23 siRNAs. As shown in Figure 5b, inhibition of p23 expression by siRNA resulted in an enhancement of thapsigargin-induced caspase-9 and -7 processing. Similarly, inhibition of p23 expression by siRNA

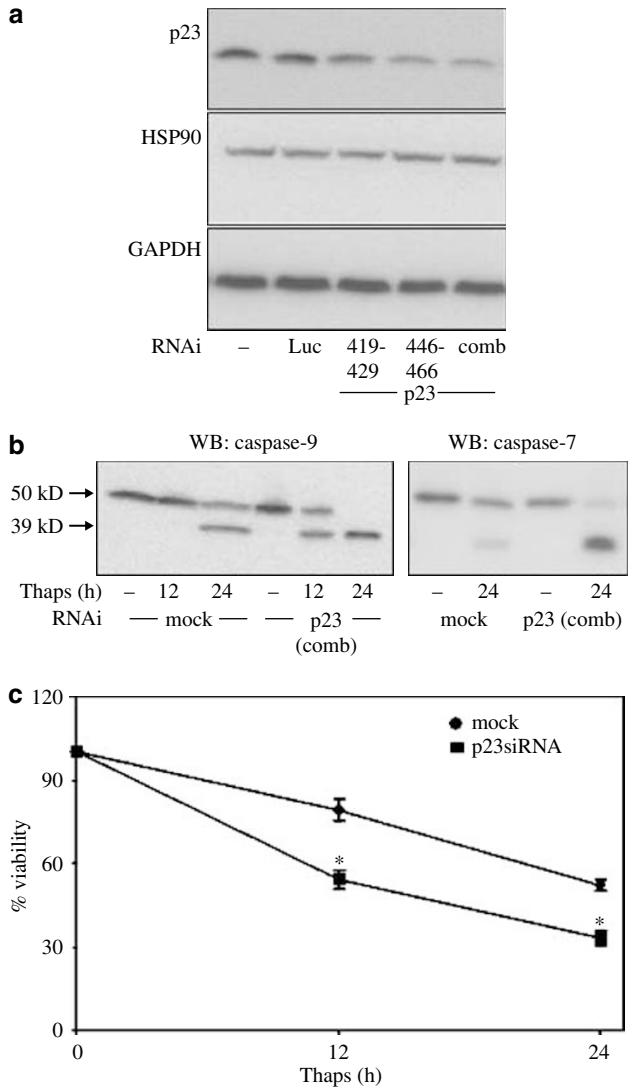

Figure 5 Transfection of siRNA-targeting p23. (**a**) Small interfering RNAs (siRNAs) were designed to target two regions each for p23. Apaf-1$^{-/-}$ cells were first transfected with 80 nM siRNA as described in Materials and Methods. To estimate the efficiency of transfection, fluorescently labeled siRNA targeting the luciferase gene was used. Cells were gently lifted 36 h after siRNA transfection and washed once with PBS at room temperature. Cell lysates were immunoblotted with anti-p23 and anti-HSP90 antibodies. None of the siRNAs crossinhibited the expression of HSP90. Cell extracts were also probed with anti-GAPDH as a loading control. (comb) indicates cotransfection of the two siRNAs designed to target the p23 gene transcript (nucleotides 419–429 and 446–466). (**b**) Effect of RNA interference on ER stress-induced caspase activation and cell death. Apaf-1$^{-/-}$ cells were first transfected with 80 nM siRNA as described in Materials and Methods. A combination of both siRNAs (nucleotides 419–429 and 446–466) was transfected to optimize the decrease of expression of p23 (Figure 5a, comb). At 24 h after transfection, cells were exposed to 50 nM thapsigargin (Thaps) for 12 and 24 h. Cells were gently lifted and washed once with PBS at room temperature. Cell extracts were subjected to SDS-PAGE and Western blotting. Membranes were probed with anti-caspase-9 or -7 antibody. (**c**) Thapsigargin-induced cell death was quantified by the Trypan blue procedure as described in Materials and Methods. $^{*}P < 0.05$

significantly rendered cells more susceptible to pcd induced by thapsigargin (Figure 5c). Similar results to those of thapsigargin were also obtained with brefeldin-A (data not shown). The results support the notion that p23 may play a protective role during ER stress; and either prolonged ER stress (resulting in p23 cleavage), or downregulation of p23 expression, renders cells more susceptible to pcd induced by ER stress.

Interaction of p23, PUMA and Bax

Recent studies have disclosed the roles of several ER stress-induced cell-death effectors, including members of the Bcl-2 family,[6] and p53-dependent gene products like PUMA/Bbc3 and scotin.[6,14,15] All of these gene products have been shown to link ER stress to the mitochondrial apoptotic pathways.

To complement the functional studies of p23 in ER stress-induced apoptosis, we asked whether any of the above effector molecules associate with p23. We performed co-immunoprecipitation of endogenous proteins and repeated these studies following vector-driven expression of Flag-p23. We did not observe co-immunoprecipitation of p23 with Bik, Bak or scotin before or after ER stress (data not shown). However, endogenous p23, Flag-p23 and the caspase cleavage site mutant protein p23D142N co-immunoprecipitated with endogenous PUMA in untreated MCF-7 cells, providing evidence of interaction between p23 and PUMA (Figure 6a and b). The interaction of p23 and PUMA was also evaluated in cells treated with thapsigargin and brefeldin-A for different time periods. As shown in Figure 6b, while PUMA associated strongly with the caspase mutant protein p23D142N before and after ER stress, WTp23 interaction with PUMA decreased with time of treatment (Figure 6a). The interaction of these proteins was weak at 24 h, and more so at 36 h of treatment. This is the time point at which WTp23 undergoes caspase-dependent cleavage (Figure 2).

In order to exclude the possibility that the decrease in p23-PUMA co-immunoprecipitation was simply due to a decrease in PUMA expression during prolonged ER stress, we assessed the expression of PUMA in cell extracts isolated from untreated and ER stress-induced cells. As shown in Figure 6c, ER stress-induced induction of PUMA occurred independently of p53 regulation, and PUMA did not undergo degradation with prolonged ER stress. Thus, the most likely explanation for the weak interaction of PUMA and WTp23 during ER stress may be cleavage of p23 and a resultant failure to maintain PUMA binding.[16] This may also explain the strong interaction of the uncleavable mutant protein p23D142N with PUMA, which was irrespective of ER stress.

Since earlier reports suggested that p23-associated HSP90 may define a subpopulation of HSP90 that is involved in protein binding, folding or processing of steroid receptors,[9,17] we asked whether the association of p23 with PUMA is direct or is dependent on HSP90 binding. As shown in Figure 7a, while p23 associated with HSP90 before and after ER stress, there was no evidence of interaction between PUMA and HSP90 (Figure 7a). Similar results were obtained when we performed reciprocal immunoprecipitations (data not shown). The results suggest that, despite being considered a

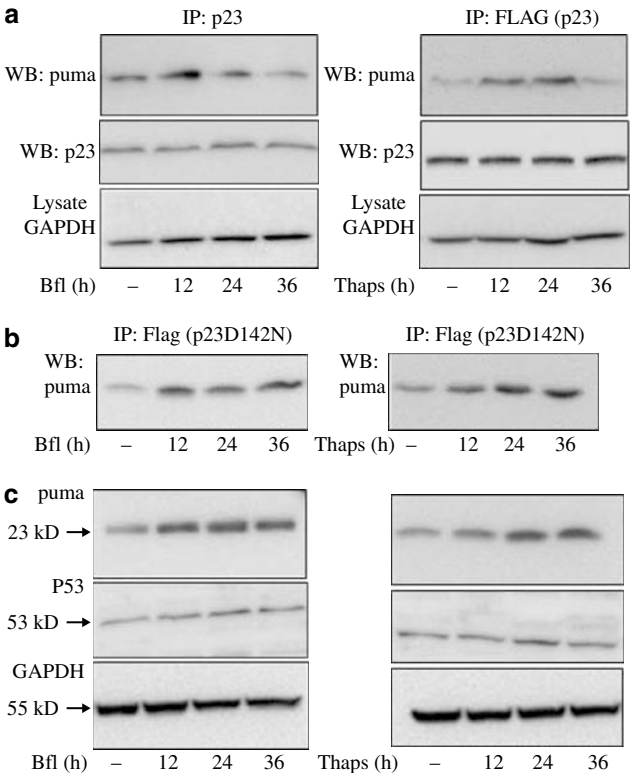

Figure 6 Co-immunoprecipitation of p23, and PUMA (**a** and **b**) Co-immunoprecipitation was carried out in untransfected MCF-7 cells and cells transfected with 6 μg of WTp23FLAG cDNA (**a**) or FLAGp23D142N (**b**). Transfection was allowed to proceed for 24 h before the addition of ER stress inducers. ER stress was induced by the addition of 2.0 μM brefeldin-A (Bfl) or 2.0 μM thapsigargin (Thaps) for different time periods. Cells were gently lifted and washed once with PBS at room temperature. Immunoprecipitations were performed with anti-p23 antibody or anti-Flag monoclonal antibody (for Flag-tagged p23), and the resulting immunoprecipitates were analyzed by immunoblotting using antisera specific for PUMA or p23. (**c**) Cellular extracts were prepared from MCF-7 cells treated with 2.0 μM brefeldin-A (Bfl) or 2.0 μM thapsigargin (Thaps) for different time periods. Cells were gently lifted washed once with PBS at room temperature. Cell extracts were subjected to SDS-PAGE and Western blotting. Membranes were probed with anti-PUMA antibody, anti-P53 antibody and anti-GAPDH antibody

cochaperone protein of HSP90, there exists a pool of p23 that interacts with PUMA to the exclusion of HSP90, arguing for additional functions of p23 that are independent of HSP90.[17] The results underscore the importance of a p23–PUMA interaction that is independent of HSP90 binding, and the subsequent release of PUMA from the p23–PUMA complex in association with the triggering of the cell death process. Since p23D142N blocked both caspase cleavage and release of PUMA and inhibits apoptosis, the release of PUMA from the p23–PUMA complex may be an important event in ER stress-induced apoptosis. Furthermore, PUMA−/− cells are resistant to ER-stress induced cell death based on an earlier report.[15] The extent of protection seen in PUMA KO cells following ER stress is less than that afforded by uncleavable p23.[15]

PUMA induces a conformational modification of Bax, resulting in its accumulation on the mitochondria and triggering the release of cytochrome *c* to activate the final

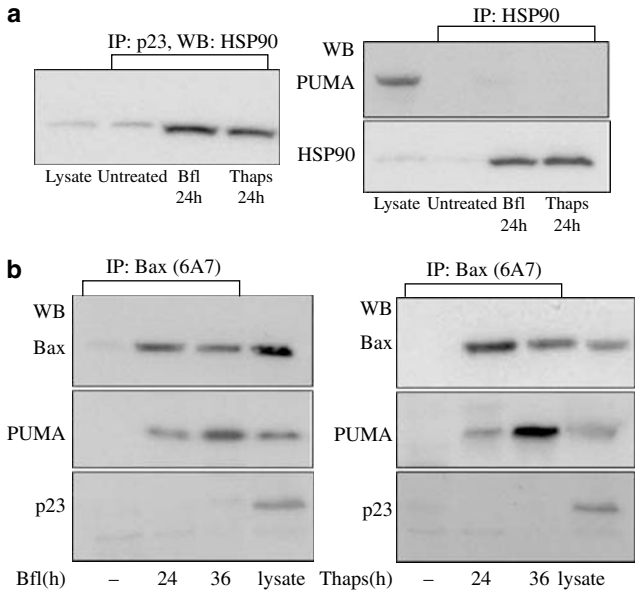

Figure 7 Co-immunoprecipitation of p23, HSP90, PUMA and Bax. (**a**) Co-immunoprecipitation was carried out in untransfected MCF-7 cells. ER stress was induced by the addition of 2.0 μM brefeldin-A (Bfl) or 2.0 μM thapsigargin (Thaps) for 24 h. Cells were gently lifted and washed once with PBS at room temperature. Immunoprecipitations were performed with anti-p23 antibody or anti-HSP90 antibody and the resulting immunoprecipitates were analyzed by immunoblotting using antisera specific for HSP90 or PUMA. (**b**) Co-immunoprecipitation was carried out in untransfected MCF-7 cells. ER stress was induced by the addition of 2.0 μM brefeldin-A (Bfl) or 2.0 μM thapsigargin (Thaps) for 24 h. Cells were gently lifted washed once with PBS at room temperature. Immunoprecipitations were performed with anti-Bax6A7 antibody that recognizes conformationally changed Bax, and the resulting immunoprecipitates were analyzed by immunoblotting using antisera specific for Bax (anti-Bax N20), PUMA and p23 antibody

steps of pcd.[15,18] BH3-only proteins like PUMA and Bid have been shown to interact with the first helix of Bax, induce a change in its conformation and stimulate its mitochondrial activity leading to apoptotic cell death.[19] ER stress inducers also trigger the Bax conformational change and mitochondrial translocation, leading to the release of Smac and Omi and activation of pcd.[20,21] In order to define further the functional implication of the p23–PUMA interaction, we examined the ability of p23 and PUMA to associate with Bax before and after ER stress. Immunoprecipitation was performed with the anti-Bax6A7 antibody which recognizes conformationally altered Bax.[19,22] We did not observe co-immunoprecipitation of p23 with Bax either before or after ER stress (Figure 7b). While there was no evidence of interaction between Bax and PUMA in untreated MCF-7 cells (Figure 7b), PUMA associated strongly with Bax only following ER stress. The interaction of these two proteins was evident at 24 and 36 h of treatment. These are also the time points corresponding to caspase-dependent cleavage of p23 and dissociation of PUMA from the p23–PUMA complex. The results indicate that prolonged ER stress triggers caspase-dependent cleavage of p23 followed by disruption of the PUMA–p23 complex and subsequent association of PUMA and Bax to trigger mitochondrial-dependent pcd.

Discussion

The ER is extremely sensitive to changes that affect its structure, integrity and function.[2,3] Changes in calcium homeostasis, inhibitors of protein glycosylation, viral infections, hypoxia, ischemia, and growth factor depletion can all disrupt protein synthesis and folding, resulting in unfolded or misfolded proteins. Accumulation of unfolded/misfolded proteins creates an imbalance between the synthesis of new proteins and the ER's ability to process newly synthesized proteins, resulting in 'ER stress'.[1,2] Prolonged ER stress overwhelms cellular protective mechanisms, ultimately triggering cell death. ER stress-induced cell death is coupled to specific independent death pathways, as well as demonstrating crosstalk with the intrinsic and extrinsic apoptotic pathways.[7,8,12,23,24]

We and others have identified several ER stress-induced cell death modulators that include members of the Bcl-2 family, p53-dependent gene products and caspases.[4–7] Although we identified HSP90 by two-dimensional gel electrophoresis coupled with MALDI-TOF mass spectrometry and nano-LC-ESI-MS/MS, its cochaperone protein p23 displayed a more consistent profile in ER stress-induced cell death. Therefore, the present studies were undertaken to define the role of this small chaperone protein. p23 is a highly conserved and widely expressed chaperone protein involved in the assembly of protein complexes, such as steroid hormone receptor and telomerase complexes.[9,25,26] The chaperone activity of p23 prevents aggregation of denatured proteins. p23 is also highly expressed in mammary carcinomas.[27]

We report here that p23 is cleaved to yield a 19 kD product during ER stress-induced cell death triggered by various agents including tunicamycin, brefeldin-A and thapsigargin. Primary neuronal cells subjected to hypoxia were also susceptible to ER stress and p23 cleavage. p23 was processed by caspases-7 and -3 but not caspase-8. A recent report also indicated that p23 undergoes a caspase-dependent cleavage in chemotherapy-induced apoptosis,[27] suggesting that cleavage of p23 may be an indication of cells undergoing some form of stress. To our knowledge, p23 is the first chaperone protein to be identified as a caspase substrate.

Despite generating several single, double and triple caspase mutant sites, caspase-3- and caspase-7-dependent cleavage of p23 was prevented only by the D142N mutation. Our results are at odds with the report by Gausdal *et al.*,[27] who demonstrated that p23 is cleaved at D142 and D145. While these authors used an *in vitro* reticulocyte lysate ^{35}S-p23 cleavage assay, we incubated recombinant purified caspases with extracts isolated from cells transfected with the p23 mutants. The most plausible explanation for this discrepancy is that, in the cellular extracts, p23 may be bound to other proteins (or be phosphorylated or otherwise modified), and therefore expose only the D142 site for cleavage, as opposed to ^{35}S-p23 in the reticulocyte lysate that may have more than one site accessible to caspase proteolysis. Mutation of the p23D142N site not only blocked the cleavage by caspases and formation of the 19 kD product, but also attenuated the cell-death process, suggesting that ER stress-induced cell

death may proceed through caspase-7- and casapse-3-mediated cleavage of p23.

Using complementary approaches, including immunodepletion and downregulation of the expression of p23 by RNAi, resulted in enhanced ER stress-induced apoptosis, suggesting a role for p23 as an antiapoptotic protein. While the increase in apoptosis seen after downregulating p23 by siRNA is significant but in and of itself not very impressive, this is just one piece of data that, taken with the transfection studies, effects of uncleavable p23, and interaction studies, supports the conclusion that p23 and its cleavage represent mediators of ER stress-induced apoptosis. It has been observed that the entire carboxy-terminus of p23 is required for its chaperone activity and to enhance the assembly of protein complexes.[16,17] Removal of the C-terminal 30 amino acids abolishes its chaperone activity.[16] Similarly, it is possible that ER stress-induced cleavage of p23 at the D142 site, resulting in the 19 kD product, abolishes the ability of p23 to act as an antiapoptotic protein, thus rendering cells more susceptible to pcd. It is also possible that the 19 kD cleaved product may display proapoptotic activity analogous to proteins such as Bcl-2 and Bid.[28,29]

PUMA/Bbc3 is a BH3-only proapoptotic gene that is significantly upregulated after prolonged ER stress triggered by tunicamycin and thapsigargin.[15] Induction of Bbc3/PUMA is also observed in primary neuron cultures and in response to cerebral ischemia *in vivo*, demonstrating the involvement of PUMA in ER stress-induced cell death.[15,30] While PUMA was previously identified as a p53 target gene,[31–33] induction of PUMA has also been shown to involve p53-independent regulation.[15,30,31] PUMA links the ER stress response to the mitochondrial apoptosis pathway independently of p53 expression.[15,30]

Our results are compatible with a p53-independent increased expression of PUMA in brefeldin- and thapsigargin-treated cells. Our studies demonstrating the interaction of an HSP90-independent subpopulation of p23 with PUMA provide a potential mechanism by which p23 may regulate ER stress-induced cell death. Prolonged ER stress triggers caspase-mediated cleavage of p23 that in turn abolishes the interaction of p23 and PUMA. This release of PUMA from p23 may increase the PUMA available for interaction with Bax and the triggering of apoptosis. Our results demonstrating the interaction of Bax and PUMA are in agreement with recent work by Cartron *et al.*[19] showing the interaction of Bax, Bid and PUMA, allowing Bid and PUMA to function as death agonists of Bax. These studies have reported that PUMA binds to the first helix of Bax and triggers a conformational modification of Bax, resulting in its accumulation on the mitochondria and inducing the release of cytochrome *c* to activate the final steps of pcd.[15,18,19] These reports argue that the current 'dogma,' that is, that BH3-only proteins such as PUMA act as antagonists of antiapoptotic Bcl-2 family members such as Bcl-xL [34,35] but not as direct interactors with Bax or Bak, may turn out to be an overly simplistic model, and that, instead, at least some of these proteins may interact with Bax (or with both Bax and Bcl-2). Similarly, Reimertz *et al.*[15] recently demonstrated colocalization of Bax and PUMA in SH-SY5Y cells during activation of the mitochondrial apoptotic pathway triggered by tunicamycin.

It is interesting to note that, despite being a cochaperone of HSP90, p23 binds to PUMA independently of HSP90. It has been postulated that p23 may exhibit chaperone activity in the absence of HSP90.[17,26,36] This suggests that there may be several pools of p23, one of which is closely associated with HSP90 and involved in binding misfolded proteins[37] and a second pool of p23 that binds specifically to PUMA (and possibly other molecules). Interestingly, while prolonged ER stress abolishes the interaction of p23 and PUMA, it does not affect the p23–HSP90 interaction (Figure 6d). Earlier results demonstrated that removal of the C-terminal tail of p23 disrupts the chaperone activity of p23 but does not affect heterocomplex formation with HSP90.[10,16,17]

Earlier reports demonstrated that activation of apoptosis by ER stress features an Apaf-1 and cytochrome *c*-independent pathway.[12,38] It is possible that prolonged ER stress may simultaneously trigger more than one cell-death pathway, some of which feature molecules that link to the mitochondria and others that activate pathways that trigger caspase activation independent of mitochondria. In summary, the present work suggests that the activation of apoptosis by ER stress features p23, a small chaperone protein. In addition to its chaperone function, p23 may also function independently of HSP90 as an antiapoptotic protein. Further work will be required to determine whether p23 plays additional roles in ER stress-induced cell death. Studies are also ongoing to determine whether PUMA functions with or without the p23-cleaved product in eliciting ER stress-induced cell death.

Materials and Methods

Cells, culture conditions, plasmids and transfection

Apaf-1$^{-/-}$ immortalized mouse embryonic fibroblasts, human embryonic kidney 293T cells and MCF-7 breast cancer cells were cultured in DMEM containing 10% FBS and 1% penicillin/streptomycin. Apaf-1$^{-/-}$ fibroblasts were derived by retroviral-driven T-antigen expression in Apaf-1 null mouse embryonic fibroblasts and were provided by Dr. Peter Gruss, Max Planck Institute for Biophysical Chemistry, Göttingen, Germany. These fibroblasts are relatively resistant to cell death initiated by specific proapoptotic agents that utilize the intrinsic (mitochondrial dependent) pathway (directly or indirectly), including Fas (in type II cells) and ceramide-mediated pathways.[7,12] However, the exposure of these Apaf-1-null cells to ER stress-inducing agents has been shown to result in a time-dependent decrease in cell viability.[12] MCF-7 breast cancer cells are caspase-3 deficient.[39]

Cortical neuron cultures were prepared from 16-day Charles River CD1 mouse embryos. Experiments were carried out at an AAALAC-accredited site, performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, approved by local committee (IACUC) review, and every effort was made to minimize animal suffering and reduce the number of animals used. Cerebral hemispheres were removed aseptically, freed of meninges, olfactory bulbs, basal ganglia and hippocampi, and incubated at 37°C in Ca^{2+}- and Mg^{2+}-free Earle's balanced salt solution containing 0.25% trypsin for 2 min. Then, 10% horse serum and 10% fetal bovine serum were added and cells were suspended by trituration. After allowing undispersed tissue to settle for 3 min, the supernatant was transferred to a 15-ml centrifuge tube and centrifuged for 1 min at 200 × *g*. Cells were resuspended in Neurobasal medium

containing 2% B27 supplement, 2 mm glutamine, 20 ng/ml fibroblast growth factor, 1% penicillin and streptomycin (Life Technology, Rockville, MD, USA). Cell suspensions were filtered through a 70-μm Falcon nylon cell strainer and seeded at 3×10^5 cells per well on 24-well Corning culture dishes coated with 100 μg/ml of poly-D-lysine. Cultures were incubated at 37°C in humidified 95% air/5% CO_2 for 4 days, and then one-half of the medium was replaced with Neurobasal medium containing 2% B27. Experiments were conducted at 8 days *in vitro*, when $>$99% of cells were neurons, as determined by immunoreactivity for the microtubule-associated protein MAP2 but not glial fibrillary acidic protein.

Human p23 cDNA was generously provided by Dr. David Toft (Mayo Clinic, Rochester, MN, USA). N-terminal Flag-p23 was subcloned into a pcDNA3 expression vector (Invitrogen) by PCR-amplification of p23. Using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA), the following potential caspase cleavage site mutants were generated: D142N, D145N, D150N, D153N, D150/153N and D145/150/153N. The sequences of all constructs were confirmed by DNA sequencing, and Western blot analyses were performed to verify protein expression. Transient transfections of MCF-7 cells with Superfect (Qiagen) or 293T cells with Lipofectamine 2000 (Invitrogen) were performed as described earlier[8,12] and according to the manufacturer's instructions. Typically, 2×10^6 cells were seeded into 10 cm dishes, respectively, and transfected a day later with 6 μg of the specified construct using a ratio of 1 μg DNA: 5 μl of transfection reagent. The transfection efficiency using these conditions was approximately 65–70%.

Hypoxia

To induce hypoxia, cultures were placed in modular incubator chambers (Billups-Rothenberg, Del Mar, CA, USA) for 0–24 h at 37°C, in humidified 95% air/5% CO_2 (control) or humidified 95%N_2/5% CO_2 (hypoxia). Cultures were then returned to normoxic conditions for the remainder (if any) of 24 h.

Cell-free extracts, cell fractionation and Western blotting

Cell-free cytoplasmic extracts and microsomes were prepared as previously described.[8,12] The 20 000 $\times$ *g* (14 000 rpm, Eppendorf Centrifuge, 5417R) supernatant that lacks whole cells, nuclei and mitochondria was recentrifuged at 400 000 $\times$ *g* (100 000 rpm Beckman Coulter Optima TLX Ultracentrifuge) for 1 h for the preparation of microsomes. The resulting supernatant contains the soluble cytosolic fraction (S) and the microsomal pellet (P) represents ER membrane and lumen proteins, as well as Golgi membranes. Cellular fractions were probed with anti-PDI (protein disulfide isomerase) antibody or anti-GRP78 antibody and anti-β-tubulin as standard marker proteins for ER and cytosol, respectively. SDS-PAGE and Western blot analyses were performed as described earlier.[7,8,12,24] Membranes were probed with a 1 : 500 dilution of a mouse-specific anticaspase-9 polyclonal antibody (Cell Signaling Laboratories), a 1 : 500 dilution of anti-PDI monoclonal antibody, a 1 : 500 dilution of anti-β-tubulin monoclonal antibody, a 1 : 1000 dilution of anti-p23 monoclonal antibody (all from BD Biosciences), a 1 : 1000 dilution of anti-FLAG monoclonal antibody, 1 : 500 dilution of anti-PUMA polyclonal antibody, 1 : 500 dilution of anti-Bax (6A7) monoclonal antibody (all from Sigma), 1 : 500 dilution of anti-PUMA polyclonal antibody from (Calbiochem PC686), 1 : 500 dilution of anti-Bax (N-20) antibody (Santa Cruz Biotechnology), 1 : 500 dilution of anti-GRP78 polyclonal antibody, a 1 : 500 dilution of anti-Hsp90 monoclonal antibody (all from Stressgen Bioreagents), and a 1 : 50 000 dilution of anti-GAPDH rabbit polyclonal antibody (Research Diagnostics Inc.). The pan-caspase inhibitor (ZVAD-fmk) was obtained from Calbiochem and dissolved in dimethyl formamide as a 10 mM stock solution.

Immunofluorescence staining

Cortical neuronal cells grown on poly-D-lysine-coated culture slides (Becton Dickinson Labware, Bedford, MA, USA) were washed with phosphate-buffered saline and fixed with 4% paraformaldehyde in phosphate-buffered saline for 20 min. For the detection of p23, cells were stained with anti-p23 monoclonal antibody (1 : 100 dilution, BD Transduction labs) as primary antibody and 488-conjugated donkey anti-mouse IgG (1 : 250 dilution, Molecular Probes) as the secondary antibody. Cells were also stained with anti-calreticulin polyclonal antibody (1 : 250 dilution, Stressgen) and 555-conjugated donkey anti-rabbit IgG (1 : 500 dilution, Molecular Probes) as the secondary antibody. Stained cells were examined using a Nikon PCM 2000 confocal mounted on Eclipse-E800 microscope equipped with appropriate filters. Z-stacks of the cells were acquired with voxel size of $0.048 \times 0.048 \times 0.25\ \mu m^3$ using PCI-Imaging software. In Bitplane Imaris 4.1.3 Suite, individual cells were cropped out of stacks. Subsequently, each cell's data set was run through Huygens deconvolution software applying Classic MLE and using constant parameters for like primary/secondary combinations. Colocalization statistics were generated in Imaris with each ROI set for the calretriculin (ER) channel and employing automatic threshholding.

2D gel electrophoresis protocol for the identification of microsomal proteins

Two-dimensional gel electrophoresis and analysis of the bioinformatics database system were performed as described earlier.[7]

Immunoprecipitations and immunodepletions

Transient transfection, cell lysis and immunoprecipitation were performed as previously described.[7,8,24] To understand protein–protein interaction, we performed co-immunoprecipitation of endogenous proteins and in some instances repeated these studies following vector-driven expression of Flag-p23. Immunoprecipitation of conformationally changed Bax with the anti-Bax 6A7 antibody was as described.[22] For immunodepletion and caspase-processing assays, microsomes were isolated from cell extracts prepared from thapsigargin/brefeldin-A-treated cells as previously described.[7,12] The microsomal pellet was washed twice with 10 mM Tris-Cl/NaCl pH 7.4, reconstituted in the same buffer and briefly sonicated to disperse the pellet fraction. A total of 200 μg protein from the cell extract or the microsomal pellet was subjected to immunodepletion using mouse anti-p23 antibody. Following an overnight incubation at 4°C with the antibodies, protein A/G-Sepharose was added to the samples and incubated at 4°C for an additional 6 h. Samples were spun briefly to pellet the protein A/G-Sepharose conjugate. The supernatant was subjected to SDS-PAGE and probed for (a) p23 protein that would indicate the extent of immunodepletion, and (b) probed for PDI as an ER marker protein.

Caspase-processing assay

Caspase-processing assays were carried out according to the manufacturer's instructions by incubating 50–100 μg of the supernatant protein with purified active caspase-3 (50 ng/ml), caspase-7 (100 ng/ml) or

(1.0 μg/ml) caspase-8 (all from BD Pharmingen) at 37°C for 1 h. Following the reactions, samples were analyzed by SDS-PAGE and Western blotting. Membranes were probed with anti-p23 antibody or anti-FLAG antibody for detection of p23 protein.

siRNA synthesis and transfection

siRNA were generated by *in vitro* transcription using the Silencer siRNA Construction Kit from Ambion as described earlier.[7] siRNAs were designed to target two or more regions for p23 based on predicted accessible (loop) and unique (specific) regions. The following siRNA sequences were designed to specifically target the mouse p23 gene: p23 (GenBank: AF153479) regions 419–439 (5′ AAGTAGATGGAGCAGAT GATG) and 446–466 (5′AAGACAGTGATGATGAAAAGA). Apaf-1$^{-/-}$ cells (2.5×10^5 cells/10 cm dish) were grown in DMEM (Life Technologies) supplemented with 10% fetal bovine serum (Sigma) with no antibiotics for 16 h. Transfection of siRNA specific for each target was carried out as described earlier[7] using Ambion's siPORT Amine transfection reagent according to the manufacturer's instructions. To estimate the efficiency of the transfections, fluorescently labeled siRNAs targeting the luciferase gene (region 152–173) from the luciferase-expressing vector pGL2-control (Promega) was also used. At 36 h after p23 siRNA transfection, cell extracts were prepared and subjected to SDS-PAGE and Western blot analysis.

In order to study the effect of ER stress on cells after reducing the p23 concentration, cells were transfected with p23 siRNA. At 24 h after transfection, cells were exposed to 50 nM thapsigargin (Thaps) for 12 or 24 h. Thapsigargin-induced cell death was quantified and cell extracts were also analyzed by SDS-PAGE and Western blot for caspase-9 processing.

Evaluation of apoptosis

Assessment of cell death was carried out by pelleting floating and adherent cells (after trypsinization) as previously described.[7,12] The pellet was resuspended in $1 \times$ PBS/0.4% Trypan blue and cells were counted using a hemocytometer. Cell death was determined as the percentage of dead cells over the total number of cells. Statistical significance was determined by two-way analysis of variance (ANOVA) analysis. Differences were considered statistically significant for $P < 0.05$.

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