Cytosolic Accumulation of HSP60 during Apoptosis with or without Apparent Mitochondrial Release

EVIDENCE THAT ITS PRO-APOPTOTIC OR PRO-SURVIVAL FUNCTIONS INVOLVE DIFFERENTIAL INTERACTIONS WITH CASPASE-3

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Most heat shock proteins (HSPs) have pro-survival functions. However, the role of HSP60, a mitochondrial matrix protein, is somewhat controversial with both pro-survival and pro-apoptotic functions reported. Here we show that in numerous apoptotic systems HSP60 protein accumulates in the cytosol. In BMD188-induced cell death, HSP60 accumulates in the cytosol with significant mitochondrial release. In contrast, in apoptosis induced by multiple other inducers, the cytosolic HSP60 accumulates without an apparent mitochondrial release. The short interfering RNA-mediated knockdown experiments revealed that in BMD188-induced apoptosis, HSP60 has a pro-death function and that the pro-death role of HSP60 seems to involve caspase-3 maturation and activation in the cytosol. In contrast, HSP60 appears to play a pro-survival role in other apoptotic systems where there is no apparent mitochondrial release as its knockdown promotes cell death. In these latter apoptotic systems HSP60 does not associate with active caspase-3. In both cases, HSP60 does not appreciably interact with Bax. Taken together, our results suggest the following: 1) cytosolic accumulation of HSP60 represents a common phenomenon during apoptosis induction; 2) cytosolic HSP60 accumulation during apoptosis occurs either with or without apparent mitochondrial release; and 3) the cytosolically accumulated HSP60 possesses either pro-survival or pro-death functions, which involves differential interactions with caspase-3.

The HSP family consists of high and low molecular weight chaperones. They were initially identified as proteins whose expression is induced upon heat treatment. Subsequent to their discovery, they have been shown to be induced not only by elevated temperature but also by a wide array of stresses and to play critical pro-survival functions in “stressed” cells (1). Upon heat or other stress stimulation, HSPs are induced by the transcription factor heat shock factor 1 (2).

Most HSPs play a protective role and hence promote cell survival during apoptosis (3). Apoptosis is governed by two major pathways, the intrinsic pathway that involves the mitochondria and the extrinsic pathway that involves death receptors. The final outcome is the activation of procaspases and cleavage of caspase substrates resulting in the characteristic membrane blebbing, chromatin condensation, and DNA fragmentation. HSP27, -70, and -90 have been shown to promote survival of the cells stimulated by UV radiation (4), DNA damage (5), death receptor ligation (6), heat shock (7), nutrient withdrawal (8), ceramide (9), reactive oxygen species (10), endoplasmic reticulum stresses, proteasome inhibition, and cytoskeletal perturbation (1). In many of these cases, these HSPs inhibit caspase activation or the subsequent cleavage of their substrates (1). They work by either inhibiting the release of apoptotic factors like cytochrome c, apoptosis-inducing factor (AIF), or Smac from the mitochondria or by inhibiting the activated caspases. Consistent with their purported pro-survival functions, many HSPs are overexpressed in various cancers, including prostate cancer (11), breast cancer (12), Hodgkin lymphoma (13), acute myeloid and lymphoblastic leukemia (14), and esophageal squamous cell carcinoma (15).

Unlike HSP27, -70, and -90 with obvious pro-survival functions, HSP60 appears to have both pro-survival and pro-death functions. HSP60 is a resident of the mitochondrial matrix, and it facilitates the folding of newly translated mitochondrial proteins transported into the mitochondrial matrix via the outer membrane. These interactions are presumably mediated by the mitochondrial matrix HSP60.

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5 The abbreviations used are: HSP, heat shock protein; AIF, apoptosis-inducing factor; BMD188, hydroxamic acid compound; Cox II, subunit II of cytochrome c oxidase; DAPI, 4,6-diamidino-2-phenylindole; Dox, doxorubicin; LDH, lactate dehydrogenase; mAb, monoclonal antibody; siRNA, short interfering RNA; Smac, second mitochondria-derived activator of caspases; STS, staurosporine; VP16, etoposide; VDAC, voltage-dependent anion channel; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; pAb, polyclonal antibody; IP, immunoprecipitation; RT, reverse transcription; AFC, 7-amo-4-trifluoromethylcoumarin.
and inner mitochondrial membrane translocation machinery. It is a homologue of the prokaryotic chaperone GroEL and complexes with HSP10, a homologue of the prokaryotic GroES (16). In complex with HSP10, it provides a sealed environment optimal for the unfolded or misfolded protein intermediates to return to their native conformation. Several experiments have implicated HSP60 in protecting cardiac myocytes during hypoxia possibly by complexing with Bax and preventing its translocation to the outer mitochondrial membrane (17, 18). HSP60 also protects epithelial cells from stress-induced death through activation of extracellular signal-regulated kinase (ERK) and inhibition of caspase 3 (19). Recently, HSP60 has been shown to confer neuroprotection (20–22). Another piece of evidence supporting a potential pro-survival role of HSP60 is its up-regulation in several cancers, including prostate (11), colorectal (23), and cervical cancer (24), Hodgkin lymphoma (13), as well as in cisplatin-resistant A431 carcinoma cells (25).

On the other hand, data exist supporting a death-promoting function for HSP60. Studies in Jurkat and HeLa cells demonstrated that HSP60 complexes with procaspase-3 and serves to accelerate the maturation of procaspase-3 by upstream activator caspases during apoptosis (26, 27). In addition, positive HSP60 expression in esophageal squamous cell carcinoma (15) as well as ovarian cancer (28) correlates with good prognosis for the patients. By contrast, loss of HSP60 expression is associated with the risk of developing an infiltrating recurrent bladder cancer (29). These observations suggest a pro-apoptotic and beneficial effect of HSP60 in these cancer cells in vivo. Thus the role of HSP60 during apoptosis might vary with different tissues, cell types, and apoptotic inducers. Here we study subcellular changes of HSP60, its potential roles, and potential underlying mechanisms for its involvement in apoptosis.

MATERIALS AND METHODS

Cells and Reagents—PC3 and LNCaP (prostate), GM701 (fibroblast), MDA-MB231 (breast), and HCT116 (colon) cancer cells were cultured as described previously (30, 31). Antibodies used include HSP60 (mAb; Chemicon), cytochrome c (mAb; BD Biosciences), holocytochrome c (mAb; R & D Systems), Smac (rabbit pAb; ProSci), caspase 3 (rabbit pAb; Biomol), AIF (mAb; Santa Cruz Biotechnology), Cox II (mAb; Molecular Probes), VDAC (rabbit pAb; Calbiochem), LDH (goat pAb; Chemicon), and Bax (rabbit pAb; BD Biosciences). Primary antibodies used for immunoprecipitation include HSP60 (goat pAb; StressGen), caspase 3 (mAb; BD Transduction Laboratories), Bax (rabbit pAb) (all from Upstate Biotechnology, Inc.). The anti-Fas cross-linking antibody (clone CH11) was purchased from Upstate Biotechnology, Inc. Secondary antibodies and ECL Plus reagents were acquired from Amersham Biosciences. Fluorescein isothiocyanate-conjugated goat anti-mouse or -rabbit secondary antibodies were obtained from Jackson ImmunoResearch. Fluorogenic caspase substrate DEVD-7-amino-4-trifluoromethyl-coumarin was purchased from Biomol. Recombinant HSP60 and (pro)caspase-3 were obtained from Stress-Gen and Biovision, respectively. All chemicals were obtained from Sigma unless specified otherwise.

Subcellular Fractionation, Preparation of Whole Cell Lysate, and Western Blotting—Mitochondria were prepared using differential centrifugation and characterized as described previously (30–33). To prepare whole cell lysate, cells were treated, harvested with trypsin-EDTA, washed twice with cold phosphate-buffered saline, and then resuspended in RIPA buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.25% sodium deoxycholate, 10 mM EDTA) containing 1% Triton X-100 and the protease inhibitor mixture. After 30 min of incubation on ice with intermitten vortexing, cleared whole cell lysate (i.e. the supernatant) was obtained by centrifugation at 10,000 × g for 2 min.

For Western blotting, 35 μg of proteins was loaded in each lane of a 15% SDS-polyacrylamide gel. After electrophoresis and protein transfer, the membrane was probed and reprobed with various primary and corresponding secondary antibodies. For immunoprecipitation samples, Western blotting was performed using the TrueBlot System (eBioscience). Briefly, TrueBlot secondary antibodies were used at 1:1000 for detection of primary antibody with minimal nonspecific binding to the heavy and light chains of the antibodies used in immunoprecipitation.

Quantification of Apoptotic Nuclei Using DAPI Staining of Live Cells—Cells were plated on glass coverslips, treated with various inducers, and incubated live with DAPI (31, 34). The percentage of cells exhibiting apoptotic nuclei, as judged by chromatin condensation or nuclear fragmentation, was assessed by fluorescence microscopy. An average of 600–700 cells were counted for each condition.

DEVDase Activity Measurement—Cells were lysed and incubated with fluorogenic peptide substrate, DEVD-AFC, and reduction of AFC was monitored in a spectrofluorimeter (32, 33).

Immunofluorescence Analysis of HSP60 Subcellular Distribution during Apoptosis—Cells grown on coverslips were treated and then fixed, permeabilized, and immunolabeled for HSP60, using protocols previously described (30, 32, 33).

Real Time PCR Analysis of HSP60 mRNA Expression—Total RNA was isolated from control or treated cells using the RNeasy mini kit from Qiagen. 600 ng of total RNA from each sample was used for reverse transcription using the High Capacity cDNA Archive kit (Applied Biosystems). 1 μl of the 20-μl cDNA reaction was utilized for real time PCR ran on the 7900HT real time PCR system (Applied Biosystems). Taqman gene expression assays (Applied Biosystems) for human HSP60 (assay ID, Hs99999905_m1) with 6-carboxy-fluorescein-labeled probes were employed in separate reactions to determine HSP60 expression compared with GAPDH expression in each sample. The 114-bp amplicon for HSP60 lies on chromosome 12 between 198176816 and 198177428 within exon 3 of the gene, whereas the 122-bp amplicon of GAPDH lies on chromosome 12 between 6515921 and 6516020 within exon 3 of the gene. The results were then processed, and HSP60 gene expression was normalized first to GAPDH expression within each sample and then to the no-treatment control of each group.

siRNA Down-regulation of HSP60—siGENOME SMARTpool for HSP60 (catalog number M-010600-02, which contains four siRNA down-regulation of HSP60—siGENOME SMARTpool for HSP60 (catalog number M-010600-02, which contains four...
siRNAs targeting hsp60 mRNA) and siCONTROL nontargeting siRNA *(i.e. cont-siRNA)* were obtained from Dharmacon. Cells, plated a day earlier on 6-well culture dishes to achieve 50–60% confluence, were transfected with HSP60-siRNAPool or cont-siRNA (100 nm each), using DharmaFECT reagent 1 for GM701, reagent 2 for PC3, and reagent 3 for LNCaP cells. HCT116 cells were transfected with Lipofectamine. Forty eight h after transfection, cells were treated with various apoptotic inducers as indicated. At the end of treatment, cells were harvested for apoptosis analysis.

**Gel Filtration Analyses**—PC3 and HCT116 cells were treated with BMD188 and anti-Fas antibody, respectively. At the end of treatment, cells were lysed and soluble fractions loaded onto a Superose 6 and a Superdex 200 HR10/30 column, respectively (Amersham Biosciences) (31, 34). Fractions were collected and analyzed by Western blotting.

**Immunoprecipitation**—Basic protocol was detailed previously (31, 34). GM701, PC3, LNCaP, or HCT116 cells (treated or untreated) were lysed, precleared with mouse, rabbit, or goat (depending on the primary antibodies used) IgG-conjugated agarose beads, and incubated with primary antibodies against HSP60, Bax, or caspase 3, or RglG (as control), followed by addition of goat, rabbit, or mouse IgG beads. Finally, the beads were pelleted, washed thoroughly, boiled in SDS sample buffer, and analyzed by TrueBlot Western blotting system (eBioscience).

**Cell-free Reconstitution Experiments Using Recombinant HSP60**—The basic protocol was described by others (26). For pulldown assays, recombinant HSP60 (1 µM) and procaspase-3 (90 nm) were incubated in homogenizing buffer (20 mM HEPES, pH 7.5, 10 mM KCl, 1.5 mM MgCl2, 1 mM EDTA, 100 mM EGTA, 250 mM sucrose, 1 mM dithiothreitol, and 1× protease inhibitor mixture) for 2 h at 37 °C. For maturation studies, reconstitution experiments were performed in 30 µl of reaction buffer containing 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 10 mM dithiothreitol, 5% glycerol. Purified recombinant procaspase-3 (Biomol (30)) was incubated alone or in different combinations with recombinant Hsp60 in the presence of 1.0 mM ATP at 37 °C for 20 min followed by addition of caspases-9 and incubated for an additional 2 h at 37 °C (26) (see figure legends for details). At the end, samples were used for DEVDase assay and Western blot.

**RESULTS**

In *BMD118*-induced PC3 and GM701 Cell Apoptosis, HSP60 Rapidly Accumulates in the Cytosol Accompanied by Mitochondrial Release—Our laboratory has been routinely utilizing three apoptotic systems, *i.e.* various cells treated with BMD188, LNCaP prostate cancer cells deprived of serum, and MDA-MB231 breast cancer cells treated with etoposide, to dissect the molecular mechanisms dictating the pro-survival versus pro-death fate of the stimulated cells (30–37). In a previous study (32), we observed that Hsp60 accumulated in the cytosol of cells early during apoptosis induction. This prompted us to determine here whether this might represent a general phenomenon during apoptosis.

We started by treating PC3 cells (prostate cancer; Fig. 1A) or GM701 (transformed human fibroblast; Fig. 1B) with BMD188. BMD188 is a fatty acid-containing hydroxamic acid that
induces potent cell death at low micromolar concentrations (35, 36). This compound was initially synthesized as a 12-lipoxygenase inhibitor; however, its apoptosis-inducing effect is independent of 12-lipoxygenase and seems to involve mitochondrial activation (35, 36). The exact mechanisms of action for the BMD188 compound remains to be characterized. In our experiments involving BMD188, cells were generally treated with 40 μM BMD188 and then harvested and fractionated into the cytosol and mitochondria. As before (30–33), we first characterized the relative purities of both fractions. The results revealed that the cytosolic marker lactate dehydrogenase (LDH) was detected only in the cytosolic preparations, and the mitochondrial marker cytochrome c oxidase subunit II (Cox II) was detected only in the mitochondrial preparations (Fig. 1, A and B). Another mitochondrial marker, voltage-dependent anion channel (VDAC) (31), was also detected exclusively in the mitochondria (see Fig. 2A). These results demonstrate that our subcellular fractions are relatively pure without significant cross-contamination.

We then focused on the changes of HSP60 in BMD188-treated cells and also compared changes in HSP60 with alterations of several other mitochondrial proteins. Both PC3 (Fig. 1A) and GM701 cells (Fig. 1B) normally expressed HSP60 mainly in the mitochondria. Treatment with BMD188 for as short as 5–30 min resulted in a prominent decrease in the mitochondrial HSP60 with a concomitant increase in the cytosolic HSP60 (Fig. 1, A and B), suggesting that BMD188 induced HSP60 protein release from the mitochondria to the cytosol. By 15 min in PC3 and 4 h in GM701 cells, there was very little HSP60 remaining in the mitochondria (Fig. 1, A and B), whereas the cytosolic amount of HSP60 leveled out. In PC3 cells, alterations in subcellular distribution of HSP60 temporally coincided with those of cytochrome c, a mitochondrial inter-membrane space protein, which also showed rapid translocation to the cytosol (Fig. 1A). By contrast, Smac, a protein also localized in the inter-membrane space, showed minimal changes in its subcellular distribution in BMD188-treated PC3 cells (Fig. 1A). AIF was localized exclusively in the mitochondria in PC3 cells, and BMD188 stimulation did not induce its release (Fig. 1A). The translocation of HSP60 from the mitochondria to the cytosol in BMD188-treated PC3 cells was observed much earlier than obvious cell death, which occurred around 1–2 h after stimulation as revealed by caspase-3 activation (i.e. the appearance of active caspase-3 cleavage product, p20 and p17) (31–33) and apoptotic nuclei (Fig. 1A, % apoptosis at the bottom). Interestingly, different from PC3 cells, GM701 cells had significant levels of procaspase-3 and prominent caspase-3 activation in the mitochondria as we observed previously (33), which might help explain why GM701 cells were more sensitive than PC3 cells to BMD188 (compare the % apoptosis in Fig. 1, A versus B).

HSP60 release in BMD188-treated GM701 cells took place temporally faster than cytochrome c release (Fig. 1B) suggesting that the released HSP60 might initiate events upstream of cytochrome c-triggered apoptosome activation. Note that in the majority of our Western blot experiments in this study, we utilized a monoclonal antibody that detects both apocytochrome c and the mitochondria-specific holo-cytochrome c, the latter of which contains the prosthetic heme group and is the pro-apoptotic form of cytochrome c (32). In all these experiments, the cytochrome c band detected was labeled simply as Cyto-c. In some experiments such as in Fig. 1B, we also re-probed the blots with a holo-cytochrome c (Holocyto-c)-specific monoclonal antibody (32), only to “confirm” the redistribution of the mitochondria-specific cytochrome c. We observed previously (32, 37) that in GM701 cells, BMD188 transcriptionally up-regulates several mitochondrial respiratory chain proteins, including cytochrome c and Cox II. We found here that BMD188 treatment of GM701 cells also resulted in increased protein levels in four mitochondrial proteins, i.e. cytochrome c, Cox II, Smac, and AIF (Fig. 1B). For Smac, AIF, and holo-cytochrome c, we observed increased protein levels in both cytosolic and mitochondrial fractions (Fig. 1B), suggesting that these proteins were up-regulated at the transcriptional/post-transcriptional levels and at the same time were also released from the mitochondria to the cytosol, as observed previously for cytochrome c (33). Unlike Smac, AIF, and cytochrome c, the up-regulated Cox II was not released into the cytosol throughout the treatment course (Fig. 1B).

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The BMD188-induced HSP60 release from the mitochondria in PC3 (Fig. 1A) and GM701 cells (Fig. 1B) was confirmed by immunofluorescence microscopy (data not shown). For example, by 30 min post-stimulation, many GM701 cells showed partial or complete HSP60 release, and by 2 h the majority of cells showed complete HSP60 release (data not shown). To determine whether HSP60 mRNA levels also changed in these two apoptotic systems, we carried out real time RT-PCR analyses. As shown in Fig. 1C, the HSP60 mRNA levels in PC3 cells treated with BMD188 decreased in a time-dependent manner. By contrast, in BMD188-treated GM701 cells, the HSP60 mRNAs gradually increased (Fig. 1D). An obvious increase in HSP60 mRNA levels was not observed until 2 h after stimulation (Fig. 1D), which was much later than the HSP60 release into the cytosol (i.e. 30 min; Fig. 1B), suggesting that HSP60 release occurs earlier than its mRNA up-regulation.

Altogether, the results in Fig. 1 suggest that in PC3 or GM701 cells stimulated by BMD188, HSP60 protein is rapidly released from the mitochondria to the cytosol and that this release is unrelated to its mRNA changes. BMD188 also induced HSP60 release into the cytosol in LNCaP prostate cancer cells (not shown).

In PC3 and GM701 Cell Apoptosis Induced by Several Other Stimuli, HSP60 Accumulates in the Cytosol Without Apparent Mitochondrial Release—To determine whether other apoptotic stimuli also cause HSP60 accumulation in the cytosol with concomitant release from the mitochondria, we treated PC3 or GM701 cells with several other apoptotic stimuli, including staurosporine (STS; a general protein kinase inhibitor), doxorubicin (Dox; a DNA-damaging agent), or Taxol (which disrupts the microtubule network by stabilizing the polymers). Surprisingly, although all these death stimuli caused increased HSP60 in the cytosol, in every case there was no apparent mitochondrial release (Fig. 2). LDH was detected only in cytosolic preparations (Fig. 2A), whereas VDAC (Fig. 2A) and Cox II (Fig. 2, B–D) were detected only in mitochondrial preparations, suggesting that there was little cross-contamination between the mitochondrial and cytosolic fractions. STS treatment of PC3 cells increased cell death within 6 h without significant release of cytochrome c or Smac (Fig. 2A). Nevertheless, caspase-3 was activated in this apoptotic system (Fig. 2A) (31). Upon STS treatment, increased HSP60 protein in the cytosol was observed at 1.5 h when only low levels of apoptosis were observed (Fig. 2A). The increase in cytosolic HSP60 in PC3 cells treated with STS, although not as prominent as in PC3 cells treated with BMD188 (Fig. 1A), was nonetheless consistently observed in several repeat experiments. Interestingly, the increase in cytosolic HSP60 in STS-stimulated PC3 cells was not accompanied by a significant decrease in the mitochondrial HSP60 protein levels (Fig. 2A). Similarly, when PC3 cells were treated with Dox, caspase-3 was activated at 12 h, and slightly increased cytochrome c release was observed at 6 h (Fig. 2B). Increased cytosolic HSP60 was also observed 6 h after the treatment; however, there was no significant mitochondrial HSP60 release (Fig. 2B).

When GM701 cells were treated with STS (Fig. 2C), caspase-3 was activated around 6 h when increased cytochrome c release was observed. By 12 h, cytosolic HSP60 accumulation was obvious but without a corresponding decrease in the mitochondrial HSP60 (Fig. 2C). Similar results were obtained in GM701 cells treated with Dox (Fig. 2D). When GM701 cells were treated with Taxol, increased cytochrome c was seen in both cytosol and mitochondria (Fig. 2D). Caspase-3 was activated, although this activation was not as obvious as in GM701 cells treated with STS or Dox. Again, increased cytosolic HSP60 was revealed without apparent mitochondrial release (Fig. 2D).

In Several Other Apoptotic Systems, HSP60 Also Accumulates in the Cytosol Without Apparent Mitochondrial Release—Next, we examined the changes of HSP60 in serum-starved LNCaP cells or etoposide (VP16)-stimulated MDA-MB231 cells, two apoptotic systems we have extensively studied (30–33, 35, 37). A major feature of these two apoptotic systems, like GM701 cells stimulated with BMD188 (Fig. 1B), is the early mitochondrial activation represented by prominent up-regulation of multiple mitochondrial respiratory chain components such as cytochrome c and Cox subunits (32, 33, 35, 37). Indeed, in our current experiments, cytochrome c was up-regulated in deprived LNCaP cells (evidenced by the increased levels in both mitochondrial and cytosolic fractions; Fig. 3A), and both cytochrome c and Cox II were up-regulated in VP16-treated MDA-MB231 cells (Fig. 3B). In addition, like in BMD188-stimulated GM701 cells (Fig. 1B), LNCaP cells following starvation also showed increased levels of Smac in both compartments (Fig. 3A).

HSP60 accumulated in the cytosols of serum-starved LNCaP (Fig. 3A) or etoposide-treated MDA-MB231 (Fig. 3B) cells without a concomitant decrease in the mitochondrial HSP60 protein levels. In serum-starved LNCaP cells, HSP60 accumulated in the cytosol 2 days after starvation, which occurred prior to increased cell death and caspase-3 activation (Fig. 3A). Interestingly, cytosolic accumulation of cytochrome c and Smac plateaued at earlier time points, whereas HSP60 levels kept increasing in serum-starved LNCaP cells (Fig. 3A). In VP16-treated MDA-MB231 cells, both HSP60 and cytochrome c levels in the cytosol kept increasing, and there was no obvious Smac accumulation in the cytosol (Fig. 3B). Although the cytosolic levels of HSP60 increased, the mitochondrial HSP60 levels remained constant (Fig. 3B).

We then further examined HSP60 alterations in several other apoptotic systems. We treated HCT116 colon cancer cells with STS, Fas ligation with a cross-linking antibody, or VP16, all of which induced cell death in HCT116 and some other cells within 24–48 h (31, 37). As shown in Fig. 3C, all three treatments resulted in increased cytosolic HSP60 without obvious loss of the mitochondrial HSP60. Similar observations were made in HCT116 cells treated with herbimycin A (Fig. 3D, left), a protein-tyrosine kinase inhibitor, PC3 cells treated with rotenone, a mitochondrial complex I inhibitor (35, 36) (Fig. 3D, right), and in LNCaP cells treated with Dox (not shown).

The time-dependent increase of HSP60 in the cytosol without concomitant decrease in the mitochondria suggests that cytosolically accumulated HSP60 in these apoptotic systems results from transcriptional and/or post-transcriptional mechanisms. To explore this suggestion, we performed real time RT-PCR analysis of HSP60 in serum-starved or Dox-treated LNCaP cells and in VP16-treated MDA-MB231 cells. The results...
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revealed that, surprisingly, HSP60 mRNA in serum-starved LNCaP cells gradually declined within the first 24 h (supplementary Fig. S1A). Thereafter, the levels started to bounce back (possibly because of autocrine activities), and by 5 days the HSP60 protein levels returned to basal levels (i.e. time 0) (supplementary Fig. S1A). In Dox-stimulated LNCaP cells, the HSP60 mRNA slightly increased at 6 h and then declined at 12–24 h (supplemental Fig. S1B). In MDA-MB231 cells stimulated by VP16, there was no change at all in HSP60 mRNA levels. These results together suggest that at least in these three apoptotic systems, the increased HSP60 protein accumulation in the cytosol probably results from post-transcriptional mechanisms.

Collectively, the results in Figs. 1–3 reveal the following: 1) BMD188 seems to be unique in inducing rapid mitochondrial release resulting in increased HSP60 accumulation in the cytosol, and 2) in contrast, most other apoptotic stimuli cause cytosolic HSP60 protein accumulation without obvious mitochondrial release.

**siRNA-mediated Knockdown of HSP60 Inhibits the BMD188-induced Cell Death**—The common phenomenon in all the above apoptosis models (Figs. 1–3) is accumulation of HSP60 in the cytosol, which appears to have resulted from two distinct mechanisms, i.e. with or without mitochondrial release. We first determined the potential role of HSP60 in BMD188-treated GM701 or PC3 cells. We transfected GM701 cells with an siRNA pool that contains four nonredundant siRNAs that specifically target four distinct regions in the HSP60 transcript. As shown in Fig. 4A, the Hsp60 siRNA pool completely knocked out the endogenous HSP60 protein in untreated cells and also effectively prevented HSP60 expression in BMD188-treated cells. Remarkably, knockdown of HSP60 in GM701 cells significantly delayed the time course of BMD188-triggered cell death (Fig. 4B). In control siRNA-transfected cells, cell rounding and death were evident starting from 15 min and, by 4 h after BMD188 treatment, most cells were completely apoptotic (Fig. 4B), and caspase-3 activation was obvious (Fig. 4C). In contrast, in HSP60 siRNA-transfected cells, only slightly increased cell death and caspase-3 activation were noticed at 4 h post-stimulation (Fig. 4B and C). Cell death and caspase-3 activation became obvious only at 24 h upon BMD188 treatment (data not shown). Similar inhibition of the apoptotic process by siRNA-mediated HSP60 knockdown was also observed in PC3 cells treated with BMD188 (Fig. 4D and data not shown). Taken together, the results in Fig. 4 suggest that in BMD188-treated GM701 and PC3 cells, inhibition of HSP60 retards the apoptotic process.

**Gel Filtration Analysis Suggests That Cytosolic HSP60 in BMD188-treated PC3 Cells Co-elutes and May Interact with Pro-caspase-3**—To understand how HSP60 might play a pro-death role in BMD188-treated cells, we freshly prepared both cytosol and mitochondria from PC3 cells treated with BMD188 for either 30 min or 2 h and subjected both fractions to gel filtration analysis. As shown in Fig. 5, in control PC3 cells, HSP60 was localized mainly in the mitochondria (consistent with earlier observation; Fig. 1A), which eluted in fractions 13–20 (molecular mass, ~60 to ~600 kDa). Thus, in native conditions, the mitochondrial HSP60 existed in both monomeric (i.e. ~60 kDa) and oligomeric forms or in complex with other proteins within the mitochondrial matrix. In control PC3

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**FIGURE 3. Cytosolic accumulation of HSP60 in the absence of obvious mitochondrial release.** LNCaP cells were subjected to serum starvation (A) or MDA-MB231 cells were treated with VP16 (B) for the time intervals indicated. Equal amounts of cytosol (Cyto) and mitochondria (Mito) (35 µg/lane) were used in Western blotting. C and D, HCT116 cells were treated with STS (0.5 µM), Fas ligation (CH11 cross-linking antibody; 0.6 µg/ml) (31), or VP16 (50 µM), or herbimycin A (HA, D, left), or PC3 cells were treated with rotenone (Rot; D, right) for the indicated time intervals. Equal amounts of cytosol and mitochondria (35 µg/lane) were used in Western blotting.
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To determine whether HSP60 released into the cytosol might be interacting with some apoptosis-related proteins such as (pro)caspase 3 and Bax, which had previously been proposed to interact with HSP60 (17, 21, 26, 27), we re-probed our gel filtration membrane blots for these two proteins. As control, we also probed for cytochrome c, which is not known to have any interaction with HSP60. As shown in Fig. 5, procaspase-3 was expressed mostly in the cytosol with much lower levels in the mitochondria of untreated PC3 cells, consistent with earlier observations (Fig. 1A). In both mitochondria and cytosol of untreated PC3 cells, procaspase-3 was found in fractions 17–20 (molecular mass, <67 to ~400 kDa), suggesting that procaspase-3 existed as monomers and also as oligomers or in protein complexes. As expected, the p20 and p17 active caspase-3 fragments were absent in control cytosol and mitochondria (Fig. 5). Interestingly, although the mitochondrial procaspase-3 was detected as a single ~32-kDa protein, the procaspase-3 in the cytosol, like HSP60 protein, was detected as several bands (Fig. 5), which might also represent the protein at different stages of maturation. Thirty min post-BMD188

cell cytosol, we did detect low levels of HSP60 on gel filtration (Fig. 5), although little HSP60 in untreated PC3 cell cytosol was detected on regular denaturing Western blot (Fig. 1A). This slight discrepancy could potentially be explained by the fact that, depending on the cell cycle status of cells, cytosolically synthesized HSP60 may not all be imported into the mitochondria (38). Interestingly, several bands were observed that reacted with the anti-HSP60 monoclonal antibody only in control cytosol but not in the mitochondria (Fig. 5). Some of these bands might represent HSP60 proteins at different maturation stages as the cytoplasmic HSP60 contains a 26-amino acid signal sequence at the N terminus, which is highly degenerate and susceptible to proteolysis (38). Upon importing into the mitochondria, the N terminus signal sequence is cleaved out (38), which explains why in the mitochondria we only detected one HSP60 band (Fig. 5). Thirty min and 2 h after BMD188 treatment, most mitochondrial HSP60 was released into the cytosol (Fig. 5). However, there was no significant shift in the overall sizes of the HSP60-containing complexes 30 min and 2 h after BMD188 treatment, both in the cytosol and mitochondria (Fig. 5).

Figure 4: siRNA-mediated knockdown of HSP60 reveals its pro-death functions in BMD188-treated cells. A and B, GM701 cells were transfected with either control (ctl) or HSP60 siRNA pool. Forty-eight h post-transfection, cells were treated with BMD188. A, whole cell lysates were prepared, and equal amounts of protein (35 μg/lane) were used in Western blotting. B, cells were imaged at the indicated time points. C and D, GM701 (C) or PC3 (D) cells were transfected with either control (ctl) or HSP60 siRNA pool (100 nm each). Forty-eight h post-transfection, cells were treated with BMD188. Whole cell lysates were prepared, and equal amounts of protein (35 μg/lane) were used in Western blotting for caspase 3. DEVDase activity was indicated below. NS, nonspecific band.

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that HSP60 might be in complex with (pro)caspase-3, in particular, in the cytosol.

In control PC3 cells, Bax (~22 kDa) was found in both cytosol and mitochondria mainly in fractions 20 and 21, corresponding to monomeric Bax protein (Fig. 5). Interestingly, like HSP60 and procaspase-3, the Bax protein in the control cytosol but not the control mitochondria was detected as two bands (Fig. 5), which again might represent the Bax protein at different stages of maturation. No changes in Bax were observed in all stages of maturation. No changes in Bax were observed in the control cytosol.

Co-immunoprecipitation (IP) Experiments Reveal HSP60 Interaction with (Pro)caspase-3 and Its Role in Caspase-3 Activation in BMD188-treated Cells—The observation that BMD188-induced (pro)caspase-3-containing complexes significantly overlapped the HSP60-containing protein complexes (Fig. 5) suggests that HSP60 might be in complex with (pro)caspase-3, particularly in the cytosol. To test this possibility directly, we took the gel filtration fraction 18 samples (Fig. 5) and carried out IP experiments using the monoclonal anti-HSP60 antibody and the TrueBlot system, which significantly reduces background binding by inhibiting nonspecific Ig binding (see “Materials and Methods”). As shown in Fig. 6A, HSP60 IP of fraction 18 did not pull down any (pro)caspase-3 in untreated cytosol (top, lane 1), probably because of the use of fractionated (i.e. very diluted) samples. 30 min upon BMD188 treatment, HSP60 IP pulled down faint ~32-kDa procaspase-3 and obvious 30-kDa caspase-3 that might have undergone initial cleavage at its pro-domain (Fig. 6A, top, lane 2). These results were fully consistent with the gel filtration studies (Fig. 5). By 2 h when most 30-kDa caspase-3 had been converted into p20/p17 active fragments that were present in much larger protein complexes (Fig. 5), HSP60 in fraction 18 at 2 h after treatment mainly associated with the 32-kDa procaspase-3, and no obvious HSP60 association with either p30 or p20/p17 caspase-3 was observed (Fig. 6A, top, lane 3). The inability to detect interaction between HSP60 and active caspase-3 was not because of an inability of the antibody to recognize the p20/p17 bands, because the same anti-caspase-3 antibody readily detected these cleaved bands in HCT116 cells treated with Fas cross-linking antibody (Fig. 6A, top, lane 12). The HCT116 results will be discussed in detail below. The co-IP experiments using the fraction 18 mitochondrial samples did not reveal significant interactions between HSP60 and (pro)caspase-3 (Fig. 6A, top, lanes 4–6).

HSP60 in other cell systems has been shown to interact with Bax (17, 21). We therefore reprobed the same co-IP blot with an anti-Bax antibody. Although anti-Bax antibody successfully immunoprecipitated Bax in HCT116 cells (Fig. 6A, bottom, lanes 16–18), no Bax protein was detected in HSP60 co-IP products (Fig. 6A, bottom, lanes 1–6).

To further determine whether HSP60 interacts specifically in the cytosol with (pro)caspase-3 but not with Bax, we carried out co-IP experiments in GM701 cytosolic fraction upon BMD188 treatment (3 h). As shown in Fig. 6B, the anti-HSP60 antibody pulled down procaspase-3 but not the cleaved fragments. Reciprocal IP using the anti-caspase-3 antibody also pulled down HSP60 and both pro- and active caspase-3 (Fig. 6B). In contrast, the anti-Bax antibody did not pull down HSP60, and the anti-HSP60 antibody did not pull down Bax (Fig. 6B), therefore confirming that in BMD188-treated PC3 (Fig. 6A) and GM701 (Fig. 6B) cells, HSP60 interacts with caspase-3 but not Bax.

The failure to detect obvious interaction between HSP60 and active caspase-3 fragments (Fig. 6, A and B) might be related to fewer amounts of samples used (e.g. fraction 18) and also to the fact that the low molecular weight proteins are generally harder to detect on Western blot. Consequently, we carried out yet another set of co-IP experiments using freshly purified...
BMD188-treated GM701 cytosol and mitochondria. The HSP60 antibody pulled down significantly increased amounts of HSP60 in the cytosol (Fig. 6C, compare lane 1 versus 2), consistent with its release into the cytosol (Fig. 1B). The anti-caspase-3 antibody also pulled down some HSP60 in control cytosol (Fig. 6C, lane 5) and more HSP60 in control mitochondria (Fig. 6C, lane 7), suggesting that in GM701 cells HSP60 endogenously interacts with pro-caspase-3. When reciprocal experiments were performed, HSP60 antibody also pulled down procaspase-3 in both control cytosol (Fig. 6C, lane 9) and mitochondria (Fig. 6C, lane 11). Upon BMD188 treatment increased association of HSP60 with procaspase-3 was observed (Fig. 6C, lanes 10 and 12). Importantly, low levels of p17 active caspase-3 fragment were also detected in the HSP60 immunoprecipitates (Fig. 6C, lane 10 and 12). IP using anti-caspase-3 antibody, as expected, immunoprecipitated both procaspase-3 and the p20/p17 cleavage products in BMD188-treated cytosol and mitochondria (Fig. 6C, lanes 14 and 16).

Finally, we carried out cell-free reconstitution experiments using recombinant HSP60, based on the protocols developed by Xanthoudakis et al. (26). Pulldown assays using recombinant HSP60 and procaspase-3 proteins revealed that the two proteins interacted directly (Fig. 6D, lanes 2 and 3). Importantly, recombinant HSP60 was found to promote procaspase-3 maturation, i.e. cleavage to generate active caspase-3 bands (Fig. 7A) and enhanced caspase-3 activity (i.e. DEVDase activity) (Fig. 7B), both of which could be abrogated by anti-HSP60 antibody (Fig. 7, A and B).

Taken together, these results (Fig. 6 and Fig. 7, A and B) indicate that HSP60 endogenously associates with procaspase-3 (at least in GM701 cells) and that BMD188 stimulation results in HSP60 association with either the 30-kDa procaspase-3 initial cleavage product (in PC3 cells) or p17 active caspase-3 fragment (in GM701 cells). Association of HSP60 with procaspase-3 promotes caspase-3 maturation and activation.

**Knocking Down HSP60 in Apoptotic Systems in Which HSP60 Accumulates in the Cytosol without Mitochondrial Release Reveals Its Pro-survival Function, No Interaction with the 30-kDa Active Caspase-3 during Apoptosis Induction—** Preceding studies in PC3 or GM701 cells treated with BMD188, in which HSP60 accumulates in the cytosol with a concomitant mitochondrial release, demonstrate that HSP60 possesses a pro-apoptotic function. In this set of
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FIGURE 7. HSP60 enhances procaspase-3 maturation and down-regulation of HSP60 in HCT116 cells enhances Fas antibody-induced caspase activation. A and B, recombinant HSP60 enhances procaspase-3 maturation and activation. Recombinant procaspase-3 (C3; 40 nm) preincubated either alone or in various combinations with HSP60 (HSP; 660 nm) for 20 min. At the end, 80 nm of procaspase-9 (C9) was added to the reaction mixture and incubated for 2 h at 37 °C (26). In one condition, the monoclonal anti-HSP60 antibody (anti-HSP) was added to the reaction mixture. At the end, 10 μl of the reaction mixture was used for caspase-3 Western blot (A) or DEVDase assays (B). B, bars represent mean ± S.D. of three independent experiments. C, HCT116 cells were transfected with either control (ctl) or HSP60 siRNA pool. 48 h post-transfection, cells were treated with Fas cross-linking antibody for the time intervals indicated and then harvested for DEVDase activity measurement. D, HCT116 cells were treated with either vehicle (Cont.) or Fas-cross-linking antibody (Fas) for 12 h. At the end of treatment, whole cell lysates were obtained and fractionated on a Sephadex 200 column. Fractions were collected, and 20 μl of fractions 3–28 was analyzed by Western blotting for the molecules indicated.

experiments, we studied the role of HSP60 in several apoptotic systems in which HSP60 accumulates in the cytosol without apparent mitochondrial release. We first utilized Fas-activated HCT116 cells, in which HSP60 accumulates in the cytosol at 12–24 h upon stimulation (see Fig. 3C). Transfection of HCT116 cells with HSP60 siRNA pool enhanced caspase activation induced by Fas ligation, at both 2 and 6 h (Fig. 7C) as well as cell death (not shown), suggesting that HSP60 in this apoptotic system has a pro-survival function.

To understand the potential mechanisms, we carried out gel filtration analyses (Fig. 7D) and co-IP experiments (Fig. 6A, lanes 7–18) by focusing, again, on its potential interactions with (pro)caspsae-3 and Bax. As shown in Fig. 7D, in control cells, HSP60 was present as both monomers and oligomers. 12 h Fas ligation induced an up-regulation of HSP60 protein as evidenced by the increase in monomeric HSP60 (Fig. 7D, fractions 12–17, demarcated by 2 vertical lines), whereas it did not affect the status of the oligomerized HSP60 (Fig. 7D). Bax existed mostly as monomers in control HCT116 cells (Fig. 7D, fractions 15–19). Fas activation resulted in Bax oligomerization (fractions 3–12) with a corresponding decrease in monomeric Bax (Fig. 7D). The up-regulated HSP60 (mainly monomeric) and the monomeric Bax in the cytosol showed little co-elution on gel filtration chromatography (Fig. 7D, fractions 12–19), but it did co-fractionate with procaspase-3 (Fig. 7D).

Like in other systems (Fig. 5), HSP60 protein did not co-fractionate with cytochrome c, which existed as monomers in both control and Fas-treated cells (Fig. 7D).

Co-IP using anti-HSP60 antibody pulled down low levels of procaspase-3 in control HCT116 cytosol (Fig. 6A, lane 7), which did not change upon Fas activation (Fig. 6A, lane 8). No association with procaspase-3 was observed in either control or stimulated mitochondrial fractions (Fig. 6A, lanes 9 and 10). Likewise, no association of HSP60 with Bax was detected (Fig. 6A, lanes 7–10), although the anti-Bax antibody successfully pulled down the ~22-kDa Bax in the cytosols (Fig. 6A, lanes 15–16), which underwent increased oligomerization in the mitochondria upon Fas activation (Fig. 6A, lane 18 versus 17).

In LNCaP cells treated with Dox, transfection of LNCaP cells with the HSP60 siRNA down-regulated the protein expression (Fig. 8A) and also enhanced Dox-induced caspase activation (Fig. 8B) as well as cell death (not shown), again suggesting...
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DISCUSSION

In this comprehensive study on HSP60 involvement in apoptosis, we have made several novel findings. First, in essentially every apoptotic system analyzed, we have observed cytosolic accumulation of HSP60 during apoptosis induction. Second, remarkably, there appear to be two distinct mechanisms leading to the accumulation of HSP60 in the cytosol. In BMD188-induced cell death, the increase in cytosolic HSP60 results from clear-cut mitochondrial release. In contrast, in apoptosis induced by many other stimuli (Figs. 2 and 3), the cytosolically accumulated HSP60 does not involve apparent mitochondrial release. Interestingly, in the latter apoptotic systems such as LNCaP cells subjected to serum starvation or Dox treatment, or MDA-MB231 cells stimulated by VP16, the accumulated HSP60 does not seem to result from increased hsp60 mRNA levels. Similar increases in cytoplasmic HSP60 protein levels without a corresponding increase in its mRNA levels have been previously observed in water-restricted rat kidney cortex cells (38), although the underlying mechanisms remain unclear. It will be very interesting in the future to determine how HSP60 protein levels are up-regulated in these apoptotic systems.

Comparison of BMD188 with many other apoptotic stimuli suggests that BMD188 may be unique in inducing rapid and significant HSP60 release from the mitochondria to the cytosol. Among a dozen or so stimuli we have studied here, BMD188 is the only one that triggers dramatic HSP60 release, although all other stimuli cause HSP60 accumulation in the cytosol. The BMD188 effect on HSP60 does not seem to be cell type-dependent because we have observed HSP60 release in BMD188-treated PC3 and GM701 cells (Fig. 1) as well as in LNCaP and mouse embryonic fibroblast cells. It is presently unclear how BMD188 induces apoptosis. BMD188 is a fatty acid-containing hydroxamic acid that was initially synthesized as a 12-lipoxygenase inhibitor (35, 36). Our previous studies show that its apoptosis-inducing effect is independent of 12-li-

Bax only in the cytosol, which did not undergo changes during doxorubicin treatment.

Finally, we pretreated PC3 cells with HSP60 siRNA followed by treatment with STS, which caused HSP60 accumulation in the cytosol without apparent mitochondrial release (Fig. 2A). As shown in Fig. 9A, HSP60 siRNA down-regulated HSP60 in PC3 cells and also significantly increased STS-induced caspase activation in PC3 cells. In contrast, pretreatment of LNCaP cells with HSP60 siRNA, which reduced HSP60 protein levels, inhibited caspase activation by BMD188 (Fig. 9B), which induced HSP60 release in LNCaP cells (not shown), just like its effect in PC3 and GM701 cells (Fig. 1).

FIGURE 8. Down-regulation of HSP60 in LNCaP cells enhances doxorubicin-induced caspase activation. A, representative data showing hsp60 siRNA-mediated knockdown of HSP60 in LNCaP cells 48 h after transfection. B, LNCaP cells were transfected with either control (ctl) or HSP60 siRNA pool. 48 h post-transfection, cells were treated with doxorubicin (Doxo, 20 μM) for the time intervals indicated and then harvested for Western blotting of caspase-3 and for DEVDase activity measurement. NS, nonspecific band (loading control). C, LNCaP cells were treated with Doxo for 24 h; cytosol (Cyto) and mitochondria (Mito) were obtained; IP was performed using anti-HSP60, anti-caspase-3, anti-Bax antibodies, or rabbit IgG (RbIgG), and the pulldown fractions were analyzed by Western blotting for the molecules indicated as described in Fig. 6A.
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The compound itself does not directly target the mitochondria (35, 36); therefore, the BMD188-induced mitochondrial HSP60 release is unlikely to be caused by any potential direct mitochondrial toxicity. In support, rotenone, a mitochondrial respiration poison, causes cytosolic HSP60 accumulation without inducing apparent mitochondrial release (Fig. 3D). How BMD188 induces HSP60 release from the mitochondria also remains to be elucidated, although it may share mechanisms responsible for cytochrome c release (Fig. 1, A and B). Indeed, our preliminary studies using cell-free reconstitution experiments, BH3 peptides, and Bax and/or Bak-deficient mouse embryonic fibroblasts suggest that tBid/Bax (but not Bim) seems to be involved in HSP60 release.  

Third, we have observed that increased HSP60 derived from two sources, i.e. with or without mitochondrial release, seems to play opposite biological functions. Specifically, the siRNA-mediated knockdown of HSP60 in PC3 and GM701 (Fig. 4) or LNCaP (Fig. 9) cells treated with BMD188 reveals the pro-death function of HSP60. Mechanistic studies indicate that the pro-death functions of the mitochondrially derived HSP60 may be mediated through its interaction with procaspase-3 and subsequent induction of the caspase-3 maturation and activation. Although direct proof that the mitochondrially released HSP60  

7 G. Choy and D. G. Tang, unpublished observations.
treated with Dox (Fig. 8B), or PC3 cells treated with STS (Fig. 9A), in which cytosolic HSP60 accumulates without obvious mitochondrial release, enhances caspase activation and cell death, thus supporting a pro-survival role for HSP60. In all these apoptosis systems, HSP60 is associated with procaspase-3 but not 30-kDa or active caspase-3. HSP60 accumulated in the cytosol in these systems, perhaps like most other HSPs, may promote cell survival by functioning as a “classic” molecular chaperone in facilitating normal protein folding and degradation of severely misfolded proteins. Exactly how HSP60 promotes caspase-3 activation and cell death in BMD188-treated cells but not in others remains an active subject of our ongoing research. It is interesting to note that in apoptotic systems where there is apparent HSP60 release, cells generally die fast (Fig. 1, A and B), which is consistent with the idea that the rapidly released HSP60 plays a pro-apoptotic role. In contrast, in apoptotic systems where HSP60 also accumulates in the cytosol but without apparent mitochondrial release, apoptosis generally proceeds more slowly (Figs. 2 and 3), consistent with a pro-survival role of HSP60 in these latter settings. Although several previous studies suggest that HSP60 interaction with Bax and subsequent prevention of Bax translocation to the mitochondria might underlie the pro-survival function of HSP60 (17, 21), our present results indicate that in all apoptotic systems we have studied there is no significant interaction between HSP60 and Bax, and Bax forms high molecular weight oligomers in the mitochondria regardless of the source of HSP60. Therefore, the pro-survival functions of HSP60 in our experimental systems are unlikely due to HSP60 interaction with Bax.

In conclusion, HSP60 can play both a pro-death and a pro-survival role depending on apoptotic context. The dual role of HSP60 and potential underlying mechanisms reported here clarifies certain controversy and confusion reported in the literature. The opposing functions of HSP60 might be exploited in different clinical settings, for example, to preserve the survival of infarcted cardiac myocytes by promoting the pro-survival functions of HSP60 and to help eradicate cancer cells by exploiting its pro-death functions.

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