

Hsp27 Inhibits Cytochrome *c*-Mediated Caspase Activation by Sequestering Both Pro-caspase-3 and Cytochrome *c*

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Mitochondrial cytochrome *c* release in response to pro-apoptotic signals leads to the formation of a cytochrome *c*/Apaf-1/procaspase-9 complex (the apoptosome) and resultant activation of caspase-9 and caspase-3. Here we demonstrate that the molecular chaperone, Hsp27, inhibits this cytochrome *c*-mediated activation of caspase-3. Immunodepletion of Hsp27 from cytochrome *c*-activated cytosols resulted in decreased caspase activity. Furthermore, immunoprecipitation of Hsp27 resulted in the coprecipitation of both cytochrome *c* and procaspase-3. In reciprocal experiments, immunoprecipitation of both procaspase-3 and cytochrome *c* resulted in coprecipitation of Hsp27, indicating two independent interactions. These results point to Hsp27 mediating its inhibition of procaspase-3 activation through its ability to sequester both cytochrome *c* and procaspase-3, and thus prevent the correct formation/function of the apoptosome complex.

Apoptosis Caspase Cytochrome *c* Hsp27 Stress

APOPTOSIS is a highly regulated form of cell death involving condensation of nuclear chromatin, cytoplasmic shrinkage, membrane blebbing, nuclear fragmentation, and, finally, formation of apoptotic bodies [for review see (18)]. Apoptosis is associated with activation of the caspase family of proteases, which consists of at least 14 caspases in mammalian cells. Caspases are synthesized as inactive zymogens and are converted to their active form through specific proteolytic cleavage [for review see (24)]. Upon activation they effect the demise of a cell through cleavage of specific target substrates leading to the highly regulated biochemical and morphological characteristics of apoptotic cell death [for review see (23)]. One of the mechanisms by which caspases are activated requires the release of cytochrome *c* from the mitochondria (8,26). Upon receiving an appropriate apoptotic signal cytochrome *c* escapes from the mitochondrial intermembrane space to the cytosol where it

binds to Apaf-1 and promotes its oligomerization (9,11). Pro-caspase-9 is then recruited to the complex, resulting in its autoprocessing, followed by caspase-9-mediated cleavage and activation of procaspase-3 and the eventual demise of the cell (27,28). The caspase activating complex consisting of Apaf-1/cytochrome *c*/dATP/procaspase-9 has been termed the apoptosome (28).

The molecular pathways leading to caspase activation during apoptosis are tightly regulated. Emerging evidence has pointed to a role in this regulation for a family of proteins, termed heat shock proteins (Hsps), whose expression is associated with induced thermotolerance [for review see (5,19,25)] and inhibition of apoptosis (6,13,20). One of the major mammalian Hsps is a 27-kDa protein (Hsp27), a member of the family of small heat shock proteins displaying an *in vitro* ATP-independent molecular chaperone activity (7). Hsp27 is transiently induced in response to heat

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shock and other sources of stress and has been shown to play a role in the development of thermotolerance (21) and to protect cells from the deleterious effects induced by stress stimuli (1). Further studies have revealed that overexpression of Hsp27 can render cells resistant to apoptosis induced by a variety of stimuli including anti-Fas mAb, staurosporine, etoposide, actinomycin-D, and camptothecin (3,12,20). The mechanism by which Hsp27 inhibits stress-induced apoptosis is not fully understood. It has been reported that Hsp27 counteracts the stress-induced disruption of the actin microfilament network (10). Preville et al. (17) have demonstrated an ability to modulate the redox parameters within cells by increasing and upholding glutathione in a reduced form. Although these may account partially for the ability of Hsp27 to increase cellular resistance, they do not account for its ability to prevent the activation of caspases during apoptosis. Our group and others have observed an effect by Hsp27 on caspase activation downstream of cytochrome *c* release from mitochondria (3,22). However, recent evidence from our groups suggests that Hsp27 exerts its antiapoptotic effect primarily through prevention of mitochondrial cytochrome *c* release, at least in thermotolerant cells (22). In the present study we examined the mechanism by which Hsp27 affects caspase activation downstream of cytochrome *c* release from mitochondria in an *in vitro* system.

MATERIALS AND METHODS

Cell Culture, Heat Shock Conditions, and Cell Transfection

Jurkat cells were grown in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum, 2 mM glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin in a humidified atmosphere of 5% CO₂ in air at 37°C. For heat shock, cell numbers were determined using a Neubauer hemocytometer and the density was adjusted to 10⁶ cells/ml. The required numbers of cells were placed in culture flasks, which were sealed by wrapping parafilm around their lids. The flask was immersed in a water bath at the indicated temperatures ($\pm 0.5^\circ\text{C}$) for 1 h. After the incubation period, cells were resuspended in fresh medium and incubated at 37°C for various times. Cell viability was assessed by the ability of cells to exclude trypan blue dye. Cell morphology was evaluated by staining cytospin preparations and apoptotic/necrotic cells were scored as described previously (20). Cell transfection was performed according to the protocol for DOTAP liposomal transfection reagent (Boehringer Mannheim). Cells were transfected

with pSFFV expression plasmid (Clontech) containing the entire coding sequence of hsp27, or a corresponding control vector.

Western Blotting

Protein samples were subjected to SDS polyacrylamide gel electrophoresis and the proteins were transferred onto a nitrocellulose membrane. Western blotting was performed using mouse monoclonal antibodies to Hsp27 (StressGen Biotechnologies Corp.), PARP (BIOMOL), cytochrome *c* (a gift from Dr. R. Jemmerson), and rabbit polyclonal antibodies to caspase-3 (p17 antibody, a gift from Dr. D. W. Nicholson).

Immunoprecipitation

Samples containing 50 μg of protein in 100 μl of immunoprecipitation buffer (100 mM sucrose, 1 mM EGTA, 20 mM MOPS, pH 7.4, a cocktail of protease inhibitors, 0.5% Triton X-100, and 0.5% NP-40) were centrifuged at 20,000 $\times g$ for 15 min to remove particulate material. The supernatant was then incubated with anti-Hsp27 (1:200, StressGen), anti-caspase-3 (1:500, CPP32, Transduction Laboratories), anti-caspase-9 (1:500, StressGen), or cytochrome *c* (1:500, gift from Dr. R. Jemmerson) with constant agitation at 4°C for 1 h. This was followed by the addition of 25 μl of 50% protein A-Sepharose and incubation with constant agitation at 4°C for a further 1 h. The pellet was collected after five washes in immunoprecipitation buffer and prepared for Western blotting.

*Preparation of the Cytosolic Fraction and In Vitro Caspase Activation With Cytochrome *c* and dATP*

Cells were washed in PBS and resuspended in S-100 buffer (100 mM sucrose, 1 mM EGTA, 20 mM MOPS, pH 7.4, 10 μM aprotinin, 10 μM pepstatin A, 10 μM leupeptin, calpain inhibitor I, and 1 mM PMSF). After 10-min incubation on ice, cells were centrifuged at 900 $\times g$ for 5 min, followed by a further centrifugation of the supernatant at 100,000 $\times g$ for 45 min to obtain the cytosolic fraction. For *in vitro* caspase activation, 30 μl of the cytosolic fraction (2 mg/ml) was incubated with cytochrome *c* and dATP for 60 min at 30°C.

Caspase Assay

The activity of group II caspases, DEVDases, was determined fluorometrically using a modified version of the method developed by Nicholson et al. (14). Briefly, lysate from 1×10^6 cells and substrate (DEVD-AMC, 50 μM) were combined in reaction

buffer [100 mM HEPES, 10% sucrose, 5 mM dithiothreitol (DTT), 0.0001% NP-40, and 0.1% 3-[(3-cholamidopropyl) dimethylammonio] propane-1-sulphonic acid (CHAPS), pH 7.25] and added in triplicate to a microtiter plate. Cleavage of the fluorogenic peptide substrate DEVD-AMC to liberate AMC in a fluorimeter using 355 nm excitation and 460 nm emission wavelengths. Fluorescence was measured at 70-s intervals over a 30-min period and fluorescence units were converted to pmol of AMC using a standard curve generated with free AMC. Data from triplicate samples were then analyzed by linear regression.

RESULTS

Induction of Hsp27 and Thermotolerance Is Associated With Inhibition of Caspase-3

In order to investigate the effect of Hsp27 induction on apoptosis, Jurkat cells were either heat shocked at 42°C for 1 h or maintained in culture at 37°C for the same period. After sufficient recovery time (12 h) apoptosis was induced by a subsequent heat shock at 44°C for 1 h. As demonstrated in Figure 1A, heat shocking at 42°C was associated with the induction of Hsp27. Subsequent Western blot analysis after induction of apoptosis demonstrated the cleavage of the caspase substrate PARP in cells maintained at 37°C, but not in those rendered thermotolerant by heat shock at 42°C, indicating that the thermotolerant cells were resistant to apoptosis and that caspases were inhibited. In order to further characterize this resistance, S-100 cytosols were prepared from thermotolerant and control cells and subsequently activated by adding cytochrome *c*/dATP. The addition of cytochrome *c*/dATP to cytosols from thermotolerant cells was associated with decreased caspase-3 activity in contrast to control cells (Fig. 1B). This result suggests that induction of Hsp27 is associated with inhibition of cytochrome *c*-dependent activation of caspase-3.

Hsp27 Prevents Caspase-3 Activity by Interfering With Apoptosome Formation

To further investigate the role of Hsp27 in inhibiting apoptosis, Jurkat cells were transiently transfected with either the Hsp27 gene (Jurkat-Hsp27) or an empty vector as control (Jurkat-neo). Western blot analysis revealed that the levels of Hsp27 expressed in the transfected cells was comparable with those levels expressed after heat shock, but transfection with empty vector had no effect on Hsp27 expression levels (Fig. 2A). To assess if the transfected cells were as efficient in inhibiting caspase-3 activity, we

measured cleavage of the peptide substrate DEVD-AMC after addition of cytochrome *c*/dATP to cytosols prepared from thermotolerant and transfected cells. As demonstrated by Figure 2B, the transfected cells were equipotent to thermotolerant cells in their ability to inhibit caspase-3 activity. To assess the mechanism of this inhibition, Hsp27 was immunodepleted from S-100 cytosol of Jurkat-Hsp27 cells (data not shown) before and after the addition of cytochrome *c*/dATP. When Hsp27 was immunodepleted prior to the addition of cytochrome *c*/dATP a decrease in DEVD-AMC cleavage was observed in the cytosolic fraction from Jurkat-Hsp27 cells (Fig. 2C, left panel). However, the decrease in caspase activity was much more significant when Hsp27 was immunodepleted after the addition of cytochrome *c*/dATP (Fig. 2C, right panel). Taken together, these findings support a model that Hsp27 mediates its caspase inhibition by interacting with either cytochrome *c*/dATP, but also through an interaction with a component of the apoptosome other than cytochrome *c*/dATP.

Coimmunoprecipitation of Hsp27 With Cytochrome c and Procaspase-3

A recent study demonstrated an interaction of Hsp27 with procaspase-3, preventing its processing into active caspase-3 (15). This interaction could account partially for our observation of the decrease in caspase-3 activity when Hsp27 is immunodepleted. However, this interaction would not account for the more significant decrease in caspase-3 activity when Hsp27 is immunodepleted after the addition of cytochrome *c*/dATP. To further characterize this inhibitory effect, immunoprecipitation studies were carried out with the different components of the apoptosome. Western blot analysis clearly demonstrates that Hsp27 coprecipitated with both cytochrome *c* and caspase-3 (Fig. 3A). In the reciprocal experiment, immunoprecipitation of Hsp27 resulted in the coprecipitation of cytochrome *c* and procaspase-3 (Fig. 3B). We did not detect an interaction between Hsp27 and Apaf-1 (data not shown), which is in agreement with a previous finding (15). These results are indicative of Hsp27 having two independent interactions with components of the apoptosome. This is substantiated by the finding that immunoprecipitation of cytochrome *c* does not result in the coprecipitation of procaspase-3 and vice versa (Fig. 3B).

DISCUSSION

The release of cytochrome *c* from the intermembrane space of mitochondria results in the formation

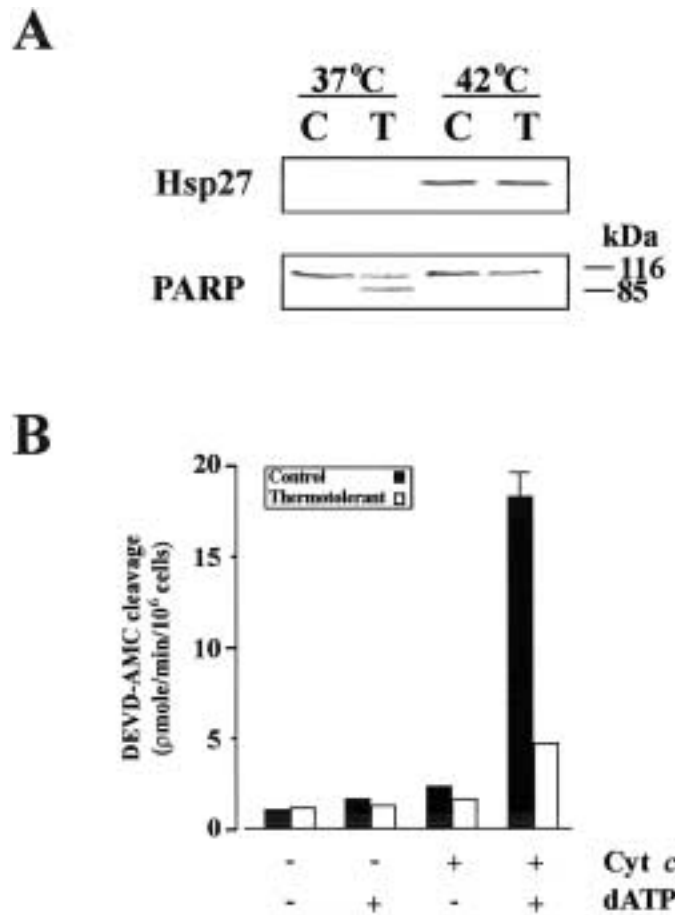


Figure 1. Induction of Hsp27 is associated with inhibition of cytochrome *c*-mediated activation of caspase-3. (A) Cells were maintained at 37°C or heat shocked at 42°C to induce Hsp27. After recovery cells were either further heat shocked at 44°C to induce apoptosis (T) or retained as controls (C). Equal amounts of protein were subjected to SDS-PAGE followed by Western blot analysis with specific antibodies against Hsp27 and PARP. (B) Cytosols prepared from control and thermotolerant cells were measured for DEVDase activity after the addition of cytochrome *c*/dATP.

of the apoptosome complex and the subsequent activation of caspase-3. Hsp27, a molecular chaperone, has been demonstrated to function both *in vitro* and *in vivo* as an endogenous inhibitor of apoptosis and caspases (3,15,22). However, the molecular mechanism of this inhibition is currently poorly understood. It has been reported that the protective effect of Hsp27 occurs after the release of cytochrome *c* (4) and prior to the activation of procaspase-9 (3). A more recent study has demonstrated an interaction between Hsp27 and procaspase-3 downstream of cytochrome *c* (15). The same study, and findings from our own group, also concluded that Hsp27 may act both upstream and/or downstream of cytochrome *c* release in a stimulus-dependent manner (15,22).

In this article we have presented data demonstrating the ability of Hsp27 to prevent the activation of procaspase-3 in an *in vitro* system. We have shown that immunoprecipitation of Hsp27 from the cyto-

solic fraction of cells transiently transfected with Hsp27 results in decreased caspase-3 activity following stimulation with cytochrome *c*/dATP. Our results are indicative of an interaction between Hsp27 and components of the apoptosome. Accordingly, in search for Hsp27 interactions with components of the apoptosome, immunoprecipitation studies revealed that Hsp27 can interact independently with both cytochrome *c* and procaspase-3. Interestingly, although the location of Hsp27 was previously thought to be mainly cytosolic, recent evidence has demonstrated a significant pool of Hsp27 located in the mitochondrial fraction of thermotolerant Jurkat cells where it is seen to block the loss of $\Delta\Psi_m$ and the subsequent release of cytochrome *c*. This was confirmed by transfection of cells with *hsp27* antisense, which prevented Hsp27 induction and failed to block the release of cytochrome *c* (22). It could be suggested that Hsp27 localized to mitochondria binds to cytochrome

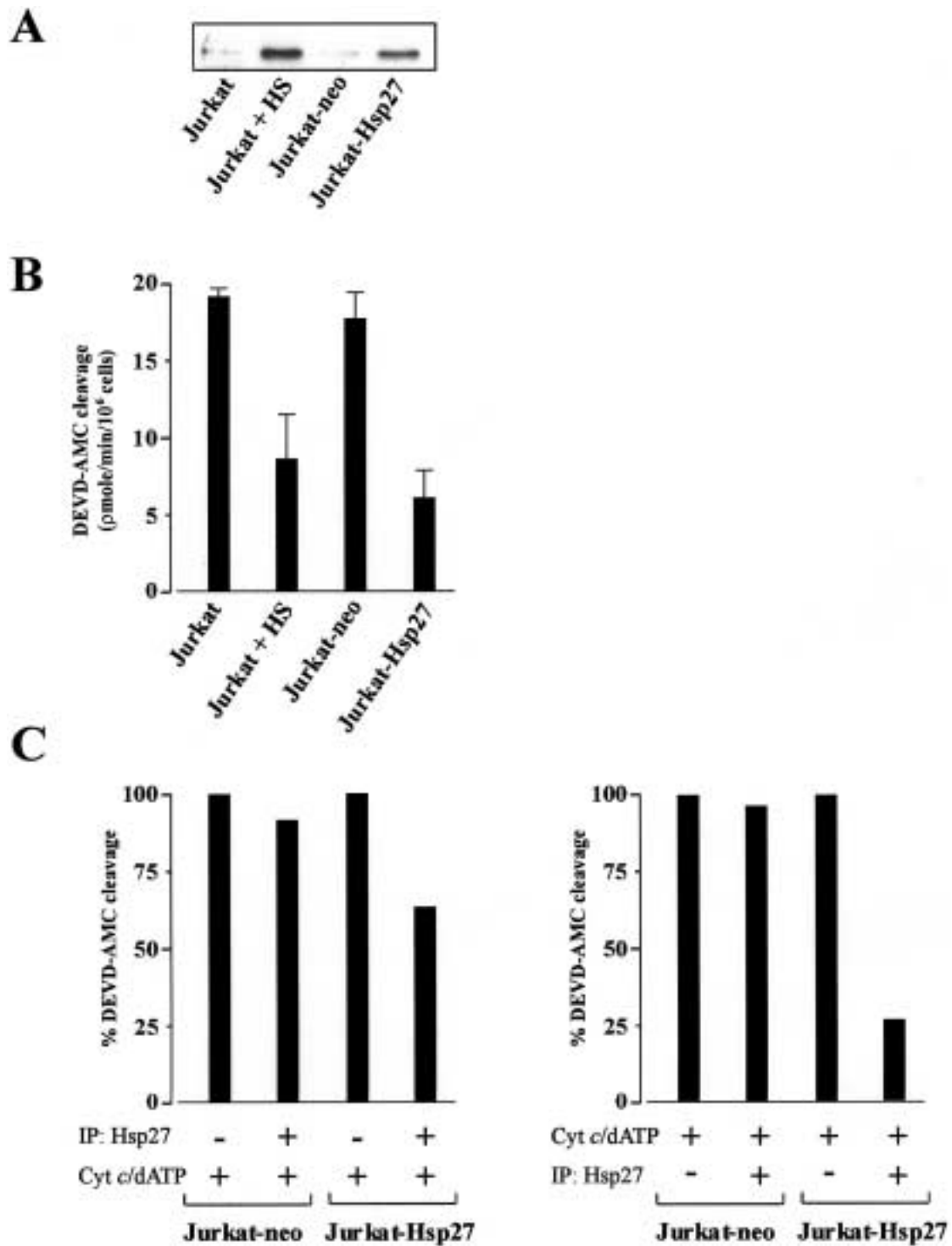


Figure 2. Hsp27 interferes with the formation of the apoptosome complex. (A) Western blot analysis of Hsp27 in thermotolerant and cells transiently transfected with Hsp27 gene. (B) Cytosols from thermotolerant and transfected cells were assayed for cleavage of DEVD-AMC after the addition of cytochrome *c*/dATP. (C) Jurkat-neo or Jurkat-Hsp27 cell cytosols were immunodepleted of Hsp27 prior to the addition of cytochrome *c*/dATP (left panel) or after the addition of cytochrome *c*/dATP (right panel). The immunodepleted extracts were assayed for cleavage of the fluorogenic substrate DEVD-AMC.

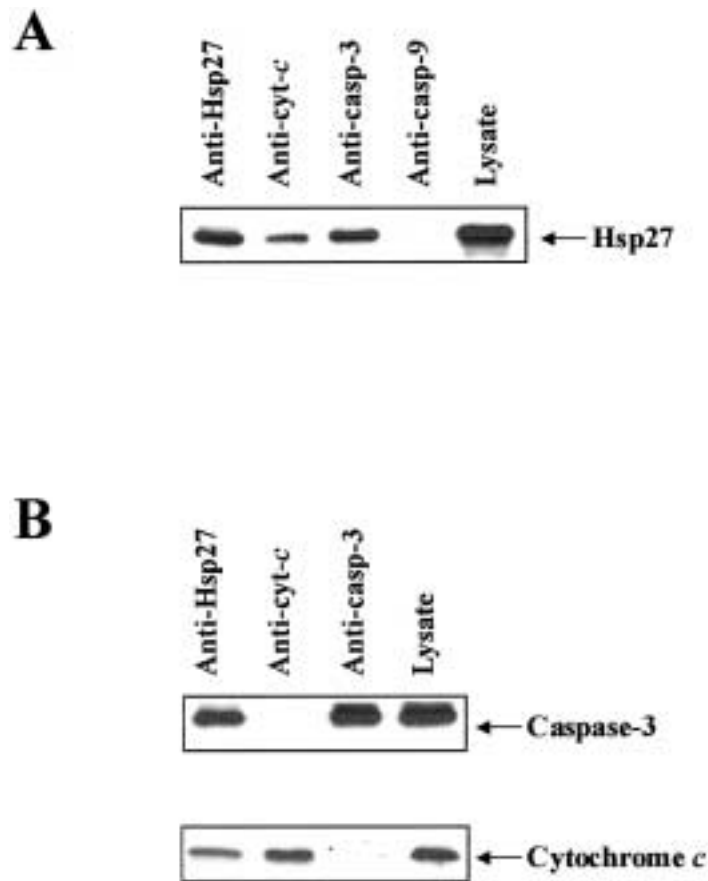


Figure 3. Hsp27 associates with both procaspase-3 and cytochrome *c*. (A) Jurkat-Hsp27 cell lysates were immunoprecipitated with anti-Hsp27, anti-cytochrome *c*, anti-caspase-3 (CPP32), or anti caspase-9, followed by Western blotting using antibody to Hsp27. (B) Western blot analysis using antibodies against caspase-3 (p17) and cytochrome *c*, of proteins immunoprecipitated from cell lysate with anti-Hsp27, anti-cytochrome *c*, or anti-caspase-3.

c, preventing its release into the cytosol and the subsequent activation of the apoptosome. This suggestion may be further substantiated by evidence that mitochondria are targets for the protective effects of thermotolerance against subsequent oxidative injury (16). Moreover, a recent study identified a novel set of sHsps that can associate with mitochondria of murine PC12 cells during heat stress and protect against subsequent injury (2). Although the idea that Hsp27 acts at the level of the mitochondrion is contrary to other reports (3,4), it could be argued that this effect may only be seen when Hsp27 is expressed under physiological conditions (i.e., during thermotolerance), but not when overexpressed.

The interaction of Hsp27 with procaspase-3 was demonstrated by immunoprecipitation studies. This interaction is therefore in agreement with another report, demonstrating that this interaction is with the procaspase-3 molecule, and not the active form, to

prevent its processing into active caspase-3 (15). In conclusion, our data demonstrate that Hsp27 inhibits cytochrome *c*-mediated caspase activation, by formation of two independent complexes, which sequester away two critical components of the apoptosome, cytochrome *c* and procaspase-3, thus preventing its correct formation.

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