Hsp72 Inhibits Apoptosis Upstream of the Mitochondria and Not through Interactions with Apaf-1*

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Hsp72 protects cells against apoptosis in response to various stresses. By simultaneously measuring cytochrome c localization and nuclear morphology in mouse embryo fibroblasts, we have shown that Hsp72 blocks cytochrome c release from mitochondria in response to cytotoxic stress and that permeabilization of the outer mitochondrial membrane is the critical point in deciding the fate of the cell. Hsp72 did not inhibit apoptosis in mouse embryo fibroblasts once cytochrome c had been released from the mitochondria. Recent reports have claimed that Hsp72 can prevent caspase activation by inhibiting the oligomerization of Apaf-1 in the presence of cytochrome c and dATP. We now show that this apparent function of recombinant Hsp72 is due to the presence of salt in the Hsp72 preparation and that the same response can be achieved by the addition of heat-denatured Hsp72 in the same high salt buffer or by the high salt buffer alone. Hsp72 expressed in a range of different cell lines had no inhibitory effect on cytochrome c-stimulated caspase activity of cytosolic extracts. We conclude that the protective effect of Hsp72 occurs upstream of the mitochondrion and not through the inhibition of the apoptosome.

Hsp72 belongs to an ancient family of proteins that play a role in many different cellular processes, including the folding of newly synthesized proteins, protein trafficking across cellular membranes, and the assembly/disassembly of protein complexes (1, 2). Less well understood is the ability of Hsp72 to protect cells from cell death. Hsp72 has been shown to protect cells from necrosis and is capable of protecting and repairing important cell metabolic processes (3–6). It has also been shown to inhibit apoptosis, whereby cells actively engineer their own destruction in response to extracellular signals and intracellular damage (7–11). In contrast to necrosis, which is caused by metabolic failure leading to cell swelling and the eventual rupture of the cell membrane, apoptosis requires active cell metabolism to degrade the nuclear DNA, divide the cell into smaller membrane-bound vesicles, and mark these for phagocytosis by surrounding healthy cells (12, 13).

Apoptosis commonly involves the activation of caspases, a family of intracellular proteases normally present as zymogens (14). These caspases commit a cell to apoptosis by activating proteins directly involved in the apoptotic process, or inactivating anti-apoptotic proteins and those responsible for normal homeostasis and repair. Apoptosis through extracellular signaling (the extrinsic pathway) and in response to intracellular damage (the intrinsic pathway) are both dependent on caspase activation but differ in how that activation is achieved. In the case of the extrinsic pathway, membrane-bound receptors are oligomerized in response to ligand binding and directly activate the initiator caspases, caspase-8 and -10 (15). These initiator caspases are then responsible for activating the effector caspases 3, 6, and 7 (16). In some cells (known as type I cells) this is sufficient to induce apoptosis. In type II cells death is dependent on the release of cytochrome c and other pro-apoptotic factors from the mitochondria in response to caspase-8 activity (17). This mitochondrial pathway is the same pathway activated in the cell in response to intracellular damage. Cytochrome c is released from the intermembrane space of the mitochondria and is bound by Apaf-1 along with ATP/dATP to form a large protein complex known as the apoptosome (18–21). This complex then binds and activates caspase-9, which is responsible for activating the effector caspases in a process analogous to that achieved by caspase-8 (21).

A number of proteins are known to inhibit apoptosis and, in particular, by preventing the activation of the caspases. The best known of these are the anti-apoptotic homologs of Bcl-2 that act by inhibiting the release of cytochrome c from the mitochondria (22). Another family of apoptotic inhibitors is the IAPs that directly inhibit caspase activity by binding to the active site of the enzyme (23, 24). Hsp72 also inhibits apoptosis but the mechanism by which this is achieved remains unclear. Two groups have proposed that Hsp72 prevents the formation of an active apoptosis by binding directly to Apaf-1 (25, 26). Additionally, it has been shown that Hsp72 functions upstream of the caspase cascade by inhibiting the release of cytochrome c from the mitochondria (27–29). This inhibition of cytochrome c release may be achieved by the ability of Hsp72 to prevent Bax translocation in response to stress (30). Two other reports have shown that Hsp72 failed to inhibit cytochrome c release but functioned either by inhibiting the activation of caspase-3 in cytosols incubated with dATP (31) or by acting downstream of the caspase cascade and down-regulating cytosolic phospholipase A2 activity (11). It has also been proposed that Hsp72 might inhibit apoptosis by targeting the JNK/SAPK/jp pathway.

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1 The abbreviations used are: JNK, c-Jun NH₂-terminal kinase; SAPK, stress-activated protein kinase; MEF, mouse embryo fibroblast; PBS, phosphate-buffered saline; TNFα, tumor necrosis factor α; Bcl, benzoylexycarbonyl; fmk, fluoromethyl ketone; DTT, dithiothreitol; BSA, bovine serum albumin; RCMLA, reduced carboxymethylated α-lactalbumin; FACS, fluorescence-activated cell sorting; UVC, ultraviolet C; PMSF, phenylmethylsulfonyl fluoride.

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(9, 32). Finally, it has been shown that Hsp72 binds to apoptosis-inducing factor, another apoptogenic factor released from the mitochondria, thereby preventing the chromatin condensation and apoptosis that result from apoptosis-inducing factor release (33).

This study endeavors to rationalize some of the many and often contradictory claims of Hsp72 function. We demonstrate that Hsp72 inhibits apoptosis in MEF by preventing the release of cytochrome c from the mitochondria and not through interactions with the apoptosome or other downstream points in the caspase-dependent apoptotic pathway. In addition, the inability of Hsp72 to inhibit caspase activation by the apoptosome is confirmed in a range of fibroblast, epithelial, and lymphoid cells.

**EXPERIMENTAL PROCEDURES**

**Cell Cultures and Reagents**

Mouse embryo fibroblasts (MEFs) derived from BALB/c mice were minimally transformed with E1A and Ras as described previously (34) and were kindly provided by Dr. Amato Giaccia (Stanford University). MEF, MDA-MB-231, and 293 cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum and 1% penicillin-streptomycin. All cultures were maintained at 37 °C in a humidified 5% CO₂ atmosphere. MEF constitutively expressing Hsp72 were prepared as previously described (8). MEFs expressing Bcl-2 were generated with a retroviral vector kindly provided by Dr. Andreas Strasser (Walter and Eliza Hall Institute, Australia).

Cells were heated by immersion in a circulating water bath calibrated to 44 ± 0.1 °C for 15–60 min. For UV treatment, the culture medium was removed and the cells were washed once in PBS before irradiation with 7 J/m² UVC in a CL-1000 UV Cross-linker (UVP). Cells were treated with lyophilized mouse TNF (Sigma) and etoposide (ETOPOPHOS, Bristol-Myers Squibb Oncology). Recombinant Hsp72 (Stressgen NSP555 and SPP755) was purchased from Stressgen Biotechnologies (Canada). Where indicated, cells were treated with 25–50 μM z-VAD-fmk or z-FA-fmk (Enzyme Systems Products) dissolved in Me₂SO.

**Apoptosis Assays**

Cells were stained with propidium iodide, and nuclear morphology was assessed by fluorescence microscopy as described previously (8). At least 200 cells were counted in each experiment, and the data are expressed as the mean ± S.D. of at least three independent experiments.

**Long Term Survival Assays**

Long term survival was assessed in MEFs by clonogenic assays. Briefly, dilutions containing known numbers of cells (1–10,000) were plated in Petri dishes at 37 °C for 7–10 days. Clones (>50 cells) derived from cells that survived the stress were stained with 0.1% crystal violet in 50% methanol and counted.

**Sulfur Rhodamine B Proliferation Assay**

Cells (3000 per well) were plated in 96-well plates 24 h prior to treatment. Cells were supplemented with 25 μM z-VAD-fmk immediately after heating and again 24 h later to ensure the drug was not depleted with time. The cells were allowed to recover at 37 °C and harvested at the specified time points by fixing with 10% trichloroacetic acid. The wells were then stained with 0.4% sulfur rhodamine B dissolved in 1% acetic acid. After extensive washing the immobilized dye was dissolved in 10 mM Tris, and the absorbance was measured at 550 nm.

**Western Analysis**

Samples containing equal amounts of protein were separated by SDS-PAGE and transferred to nitrocellulose membranes. Hsp72 expression in cells was detected using a mouse monoclonal antibody specific for human Hsp72 (N15) or with an antibody that detects both mouse and human Hsp72 and Hsp73 (N6, both kind gifts from Dr. W. Welch, University of California, San Francisco, CA). Human Apaf-1 expression was detected using a rat monoclonal antibody (2E12; kind gift from Dr L. O'Reilly, Walter and Eliza Hall Institute, Australia). Mouse Hsp60 was detected with a rabbit antibody (kind gift from Prof. N. Hoogenrad, La Trobe University, Australia).

**Preparation of Cytosolic Fractions**

For assay of caspase-3 activity, ~10⁷ MEF or 4 × 10⁷ 293 cells were harvested in PBS/0.1% EDTA, washed once in PBS and once in cytosol preparation buffer, CPB (20 mM Heps, pH 7.4, 250 mM sucrose, 10 mM KCl, 1.5 mM MgCl₂, 1 mM EDTA, 1 mM DTT, and 0.1 mM PMSF) and resuspended in 0.4 ml of CPB. Cells were incubated on ice for 15 min then disrupted using 15 strokes (20 strokes for 293 cells) of a Dounce homogenizer. Lysates were centrifuged at 16,000 × g for 10 min at 4 °C to remove organelles, including mitochondria. The protein concentration of the supernatant was determined by the Bradford assay.

For blue native electrophoresis, frozen cells were lysed by resuspending in 4 volumes of hypotonic lysis buffer (20 mM Tris, pH 7.5, 20 mM KCl, 2 mM MgCl₂, 0.1 mM EDTA, 1 mM DTT, 0.1 mM PMSF) and incubated on ice for 20 min before centrifugation for 10 min at 16,000 × g. Cytosols contained no detectable amounts of cytochrome c as determined by Western blotting.

**In Vitro Caspase-3 Activity Assays**

Caspases were activated by the addition of dATP and bovine cytochrome c (Sigma) to aliquots of cytosol and incubated for 30 min at 37 °C. Caspase-3 activity was measured by the rate of cleavage of DEVD-pNA colorimetric substrate (BioMol) according to the manufacturer’s instructions. The effect of Hsp72 on caspase-3 activity was determined by adding a 1/10th volume of the following to the cytosol and incubating for 10 min at 37 °C before the addition of cytochrome c and dATP-bovine serum albumin (BSA) or recombinant human Hsp72 (Stressgen NSP555) in phosphate-buffered saline (PBS) (2.7 mM KCl, 1.5 mM KH₂PO₄, 137 mM NaCl, 8.1 mM Na₂HPO₄); BSA or recombinant human Hsp72 (Stressgen SPP755) in 50 mM Tris, pH 7.5, 100 mM NaCl, 1 mM DTT, 0.1 mM PMSF; BSA or Hsp72 in PBS dialyzed against 20 mM Tris, pH 7.5, 20 mM NaCl, 1 mM DTT. Hsp72 was inactivated by heating at 95 °C for 10 min. Final concentrations of exogenous Hsp72 were 100 ng/μl or 1.4 μM. The effect of NaCl concentration on caspase-3 activity was determined by preincubation with the specified concentration of NaCl. Caspase-3 activity was measured in MEF, L929, MDA-MB-231, Skut1B, Jurkat, and CEM cells using the same method.

**Blue Native Gels**

Blue native electrophoresis was performed using a modification of the procedure of Schagger (35). Cell cytosols were activated by incubating with 2 mM dATP and 20 μg/ml cytochrome c for 30 min at 30 °C. Samples were resolved on a 4–13% linear gradient polyacrylamide gel and 2.5% polyacrylamide loading gel. The cathode buffer contained 15 μg/ml Coomassie G-250. Proteins were transferred to polyvinylidene difluoride membrane and the membranes immunoblotted as described above.

**Cytochrome c Release Assays**

**Mechanical Cell Disruption**—Cells were prepared as described previously, except lysates were centrifuged at 100 × g for 10 min at 4 °C to remove nuclei and intact cells. The supernatant was subsequently centrifuged at 16,000 × g at 4 °C for 10 min to pellet remaining organelles, including mitochondria. Proteins from the pellets and supernatants were resolved by 13% SDS-PAGE.

**Cytochrome c Detection by Immunofluorescence**—Cytochrome c release was measured using a modification of the method of Waterhouse and Trapani (36). Briefly, ~10⁶ MEFs were harvested and incubated for 10 min on ice in PBS containing 100 μg/ml digitonin and 100 mM KCl before being fixed in 4% paraformaldehyde. Effective permeabilization of cells by digitonin was confirmed by trypan blue staining. The cells were incubated with an anti-cytochrome c monoclonal antibody (BD Biosciences), an Alexa488-conjugated anti-mouse secondary antibody (Molecular Probes), followed by propidium iodide. The cells were subjected to FACS analysis on a BD Biosciences FACSCalibur. FACS analysis was performed using WinMDI 2.8 software. Confocal microscopy was performed on a Bio-Rad MRC 1024 equipped with a krypton/argon laser and attached to a Leica DMIREB microscope.

**RESULTS**

The Expression of Hsp72 Inhibits Apoptosis in Response to Heat, UVC Radiation, and TNFa but Not to Etoposide—As shown earlier (8), Hsp72 expression protects mouse embryonic fibroblasts (MEFs) from apoptosis induced by heat shock, TNFa, and UVC radiation (Fig. 1A). Hsp72 did not protect MEFs from two DNA-damaging stresses: etoposide (Fig. 1A) and ionizing radiation (8). The level of Hsp72 expression in two MEF lines infected with Hsp72 retrovirus, MEF-H2 and MEF-H3, is within the normal physiological range, because it has been shown previously that exogenous Hsp72 expression in
MEF-H2 (Fig. 2) development of apoptotic nuclear morphology in both MEF and UVC radiation and TNF-α. The Me2SO carrier affected the growth rate of unheated cells but not from etoposide. z-VAD-fmk was administered immediately after heating effectively inhibited the apoptosis, not just a delay in apoptosis, as shown by increased cleonogenic survival (Fig. 1B). MEF-H3 was less resistant to stress than MEF-H2 consistent with the lower expression of Hsp72 compared with MEF-H2 (Fig. 1C).

Apoptosis in MEFs Is Dependent on Caspase Activity—The addition of the broad-spectrum caspase inhibitor z-VAD-fmk (25 μM) immediately after heating effectively inhibited the development of apoptotic nuclear morphology in both MEF and MEF-H2 (Fig. 2A), suggesting that caspase activity is required for heat-induced apoptosis. z-FA-fmk at 50 μM failed to inhibit apoptosis under identical conditions. z-VAD-fmk was also found to inhibit apoptosis in response to UVC, etoposide, and TNF-α (data not shown). To determine if inhibition of caspase activity was sufficient to confer a long term survival advantage for MEF, a sulfur rhodamine B assay was employed to measure cell growth. As shown in Fig. 2B, MEF-H2 proliferated more rapidly than parental MEF after heat. By comparison both cell lines grew at similar rates when cultured at 37 °C. z-VAD-fmk at 25 μM did not confer any survival advantage to either cell line after heat treatment. x-VAD-fmk was administered immediately after heating and again 24 h later to ensure that the drug was not depleted over time. Neither x-VAD-fmk nor the Me2SO carrier affected the growth rate of unheated cells (data not shown). It has been shown previously that z-VAD-fmk will effectively inhibit apoptosis in response to cell stress, but that the damaged cells will continue to die by necrosis (37, 38). In contrast, 25 μM z-VAD-fmk did confer a long term survival advantage to MEFs cultured in the presence of TNFα, which acts through the extrinsic signaling pathway (Fig. 2C).

Given that Hsp72 inhibits apoptosis in response to heat, caspase activity in MEF was measured to determine if Hsp72 also functioned by inhibiting caspase-3 activity. Heat-induced caspase activity was found to be substantially less in MEF-H2 compared with parental MEFs (Fig. 2C). These results suggest that caspase activity is required for heat-induced apoptosis and that Hsp72 functions by inhibiting the ability of the cell to activate the caspase cascade.

Hsp72 Does Not Inhibit Cytochrome c Activation of Caspase Activity in an in Vitro System—It has been suggested that Hsp72 interacts directly with Apaf-1, inhibiting its ability to activate caspase-9 in response to cytochrome c and dATP, either by inhibiting the formation of the apoptosome, or by preventing the binding of pro-caspase-9 to this complex (25, 26). Thus, one would predict that cell cytosols derived from MEF-H2 would be more resistant to caspase activation in response to cytochrome c and dATP than cytosols derived from parental MEFs. However, as shown in Fig. 3 (A and B), the addition of cytochrome c and dATP induced at least as much, if not more caspase-3 activity in MEF-H2 cytosols compared with cytosols derived from parental MEF. This was demonstrated using a range of cytochrome c and dATP concentrations that induced caspase activity proportional to the dose. A similar result was found in the Hsp72-expressing cell lines derived from L929, MDA-MB-231, Skut1B, Jurkat, and CEM cells compared with base vector-transduced cells (data not shown). In all these cell lines Hsp72 is protective against various apoptotic stresses. The inability of constitutively expressed Hsp72 to inhibit caspase activation in cell cytosols in response to cytochrome c and dATP suggests that Hsp72 does not inhibit caspase activation through direct interaction with the apoptosome.

The Ability of Recombinant Hsp72 to Inhibit Caspase Activation in Cell Cytosols in the Presence of Cytochrome c and dATP Is Due to the Increased Salt Concentration—The lack of inhibition of caspase-3 activity in cytosols from Hsp72-expressing cells would not be predicted from the previously published results (25, 26). To investigate this anomaly, cytosols were prepared from 293 cells and incubated with cytochrome c/dATP in the presence or absence of the same recombinant human Hsp72 (Stressgen SPP755) as described in the previous publications. Recombinant Hsp72 was able to inhibit caspase activation (Fig. 3C, lanes 2 and 3). A similar result was obtained with another Hsp72 product (NSP555) from Stressgen (lane 5). Hsp72 (NSP555) was prepared in phosphate-buffered saline (PBS) instead of a Tris buffer containing 100 mM NaCl (SPP755). The reason for the reduced caspase activation upon addition of recombinant Hsp72 became apparent when cytosols were spiked with an equal volume of an identical buffer to that used in the Hsp72 preparation. BSA in PBS (lane 7), or PBS alone (data not shown), inhibited caspase activation to a similar extent as the recombinant Hsp72. Furthermore, Hsp72 that had been denatured by heating at 95 °C retained the ability to inhibit caspase activation (lanes 4 and 6). Similarly, recombinant Hsp72 dialyzed against 20 mM Tris pH 7.5, 20 mM NaCl, 1 mM DTT showed a reduced capacity to inhibit caspase activation (lane 8). BSA in PBS also lost its inhibitory capacity after dialysis in the same buffer (lane 9). The native conformation of dialyzed Hsp72 was confirmed by its ability to bind reduced carboxymethylated α-lactalbumin (RCMLA), a known binding substrate of Hsp70 (Fig. 3E) (39). The binding of RCMLA to Hsp72 was inhibited by 2 mM ATP confirming that both the C-terminal peptide binding domain and the N-terminal.

R. Steel, J. P. Doherty, K. Buzzard, N. Clemons, C. J. Hawkins, and Robin L. Anderson, unpublished data.
ATP binding domain were functional (39, 40). Heat-denatured Hsp72 was not detected by native gel electrophoresis, presumably because the protein was aggregated and failed to enter the gel (data not shown).

It is important to note that human 293 cells already express large quantities of the “inducible” Hsp72 in a constitutive fashion (Fig. 3F), a property that has been attributed to the presence of the E1A viral antigen in these cells (41, 42). Although 293 cells were used previously in this cytochrome c-activated caspase assay (25, 26), it was possible that the high endogenous levels of Hsp72 masked any potential function of the recombinant Hsp72. Hence, the experiment was repeated with cytosols derived from MDA-MB-231, which express Hsp72 at physiological levels (Fig. 3F) and with MEF cytosols (data not shown). Again, in MDA-MB-231 cytosols, both recombinant Hsp72 in PBS (NSP555) (Fig. 3D, lane 11) and heat-denatured NSP555 (lane 12) blocked caspase-3 activity while NSP555 dialyzed against a low salt buffer lost its activity (lane 13). Finally, increasing concentrations of NaCl were added to 293 cytosols incubated with cytochrome c and dATP to quantify the influence of salt. As reported previously (43, 44) NaCl was an efficient inhibitor of caspase activation. The NaCl concentration in the caspase activation assay was 14 mM (achieved by diluting Hsp72 NSP555 in PBS 1/10 in the cell cytosol). As shown in Fig. 3G NaCl is an efficient inhibitor of caspase activation at this concentration.

**Hsp72 Does Not Associate with Apaf-1 as Measured by Native Gel Electrophoresis**—Initial attempts to confirm the association of Hsp72 with Apaf-1 by co-immunoprecipitation proved problematic due to nonspecific interactions of Hsp72 and Apaf-1 with both the Sepharose beads and the tubes used in the procedure (data not shown). To avoid these problems, cytosols from 293 (Fig. 4A) and MDA-MB-231.Hsp72 cells (Fig. 4B) were resolved by blue native gel electrophoresis. This technique utilizes the binding of Coomassie Blue G-250 to proteins during electrophoresis, both to stabilize protein complexes and to increase their negative charge for improved resolution (35). The Hsp72 expression levels of 293 and MDA-MB-231.Hsp72 cells are shown in Fig. 3F. Caspase-3 is activated following the addition of cytochrome c and dATP and incubation at 30 °C for 30 min (Fig. 4A, A and B). Western blotting for Apaf-1 clearly shows the induction of the apoptosome (marked by arrow) under the same conditions that activate caspase-3. In the absence of cytochrome c, Apaf-1 migrates as a low molecular weight band that presumably represents the monomeric form. Hsp72 migrates predominantly as a single band with a higher electrophoretic mobility and does not co-migrate with Apaf-1, either in its monomeric form or following apoptosome formation. Although Hsp72 was possibly present in some minor higher molecular weight complexes, none suggested any co-association with Apaf-1 or the apoptosome. If Hsp72 was inhibiting Apaf-1/apoptosome func-
tion, it would be expected that Hsp72 would need to associate in a stoichiometric fashion with either monomeric Apaf-1 or with the apoptosome complex.

To demonstrate the detection and resolution of known Hsp72-protein complexes by blue native electrophoresis, an MDA-MB-231.Hsp72 cytosol was incubated with RCMLA, and the proteins were resolved on a 4–13% gradient gel. The binding of the 14-kDa RCMLA produced an identifiable gel shift in Hsp72 migration (Fig. 4A, lane 2), confirming the ability of this electrophoresis technique to resolve Hsp72-protein complexes. It is possible that the addition of 2 mM dATP or ATP could inhibit the binding of Hsp72 to Apaf-1 or to the apoptosome, because ATP is known to inhibit Hsp72-protein binding as shown in Fig. 4C, lane 3. However, the addition of cytochrome c alone to 293 cytosols was sufficient to induce some apoptosome formation (presumably assisted by endogenous ATP) (data not shown). Under these conditions there was no change to the pattern of Hsp72 migration even though Hsp72 was found to successfully bind RCMLA under identical conditions. Thus, association between Hsp72 and Apaf-1 could not be demonstrated by blue native gel electrophoresis.

**Hsp72 Inhibits the Release of Cytochrome c in Response to a Diverse Range of Apoptotic Stimuli**—Initial attempts to measure cytochrome c release from heated MEFs appeared to confirm previous reports that the ability of Hsp72 to inhibit apoptosis was independent of cytochrome c release (11, 29). Mechanical disruption of the cells revealed a delay in cytochrome c release in MEF-H2 cells compared with parental MEFs, but similar patterns of cytochrome c release were observed by 8 h after heating (Fig. 5A). However, when cytochrome c release was measured using a technique developed by Waterhouse and Trapani (36), a different result was obtained. Selective permeabilization of the plasma membrane by digitonin allows the release of soluble cytoplasmic proteins from the cell, whereas mitochondrial proteins are retained within the mitochondria. The cells are then fixed in paraformaldehyde, and cytochrome c is detected by immunofluorescence. As shown in Fig. 5B, FACS analysis of unheated digitonin-treated MEFs revealed a single population that reacted strongly with an anti-cytochrome c antibody. An IgG control revealed this fluorescence to be specific to the cytochrome c antibody (data not shown). In contrast, heated MEFs and
MEF-H2 developed a distinct population of cells with greatly reduced cytochrome c levels 12 h after heating. Cytochrome c release was maximal by 12 h after heating, but a substantial proportion of cells had undergone cytochrome c release and apoptosis after 8 h (data not shown). Cells not treated with digitonin displayed a small population of cells with low cytochrome c levels, probably due to the loss of cell membrane integrity associated with apoptosis. MEF-H2 expressing Hsp72 revealed a reduced proportion of cytochrome c negative cells after 12 h when compared with parental MEFs (Fig. 5B).

To investigate the link between cytochrome c release and apoptosis, the cells were additionally stained with propidium iodide and examined by confocal microscopy. Unheated MEFs possessed a diffuse and evenly stained nucleus with cytochrome c present in a punctate pattern indicative of a mitochondrial distribution (Fig. 5C). In cells that had been heated, two different cell morphologies were apparent (Fig. 5D). The cells either appeared similar to unheated cells or displayed a distorted and condensed nuclear morphology associated with apoptosis and a complete absence of cytochrome c. In 99% of all cells scored, the cells showed either a normal nucleus and retained cytochrome c or were apoptotic cells and lacked cytochrome c (data not shown). Apoptotic cells that had not been permeabilized by digitonin still retained cytochrome c that was evenly distributed around the cytoplasm rather than being restricted to the mitochondria (data not shown).

MEFs were also treated with UVC radiation (Fig. 5E), TNFα (Fig. 5F), and etoposide (Fig. 5G) and examined for cytochrome c release. In all cases, the development of an apoptotic nuclear morphology was associated with the complete loss of cytochrome c from the treated cells. Despite the different treatments, the appearance of all cells was similar.

**Cytochrome c Release Is the Critical Step in Determining the Fate of MEF Cells in Response to Apoptotic Signaling**—It has been reported previously that Hsp72 inhibits stress-induced cytochrome c release (27–29), but it has not been established if this is the critical point of Hsp72 function or whether Hsp72 also functions further downstream in the apoptotic pathway. To clarify this, parental MEF, MEF-H2, and MEF-H3 cells were assayed for stress-induced cytochrome c release and nuclear apoptosis by fluorescence microscopy (Fig. 6A). Hsp72 inhibited cytochrome c release in response to heat, UVC radiation, and TNFα but not etoposide, thus mirroring the results shown in Fig. 1A where nuclear morphology was scored. As in parental MEF, cytochrome c release in MEF-H2 and MEF-H3 continued to be associated with the development of a nuclear apoptotic morphology (data not shown). By way of comparison, MEF overexpressing Bcl-2, a known inhibitor of cytochrome c release, displayed reduced cytochrome c release in response to all stresses, including etoposide. If Hsp72 was directly inhibiting the activation of caspases, we predict there would be an increase in the number of cells exhibiting cytochrome c release but a normal nuclear morphology. This was not the case, and again 99% of the cells exhibited either a healthy or a classic

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**Fig. 4. Analysis of Hsp72 and Apaf-1 by blue native gel electrophoresis.** Cytosols were incubated with 20 µg/ml cytochrome c and 2 mM dATP at 30 °C for 30 min. All other samples were incubated on ice for corresponding amounts of time. Duplicate sets of activated and control cytosols were resolved on a single 4–13% polyacrylamide gel by blue native gel electrophoresis and transferred to polyvinylidene difluoride. This membrane was cut into two pieces and immunoblotted separately for Hsp72 and Apaf-1 before being carefully reassembled for ECL detection. This allows direct comparison of Hsp72 and Apaf-1 migration patterns. A, 293 cytosol; B, MDA-MB-231.Hsp72 cytosol. Arrows mark the migration patterns of the apoptosome (Apop) and Hsp72. Caspase-3 activity was measured in the same cytosols used for the gel electrophoresis. C, MDA-MB-231.Hsp72 cytosol was incubated with 20 µM RCMLA with or without 2 mM ATP at 30 °C for 30 min and resolved on a 4–13% gel by blue native gel electrophoresis.
apoptotic morphology. In comparison, the caspase inhibitor z-VAD-fmk completely inhibited nuclear condensation in response to heat (Fig. 5, H–J) but failed to inhibit cytochrome c release as published previously (45). In this particular experiment the cells were incubated with z-VAD-fmk prior to heating to preclude the possibility that caspases were required for early apoptotic signaling events. Although z-VAD-fmk failed to inhibit heat-induced cytochrome c release, it was found to inhibit cytochrome c release in response to TNFα/H9251 (data not shown).

**Hsp72 Can Inhibit Apoptosis Induced by Non-proteotoxic Stress**—It has been proposed that Hsp72 protects cells from stress by repairing damage to the protein machinery through its chaperone activity. However, we show here that Hsp72 can successfully inhibit apoptosis induced by UVC radiation and TNFα, two stresses not generally associated with protein damage. Damaged and unfolded proteins induce the expression of heat shock proteins in what is known as the heat shock response (46). Western blotting was used to assess if TNFα and UVC radiation induced the expression of endogenous Hsp72 as a marker of proteotoxic stress. Hsp72 was found to be strongly induced by heat but was not induced by the other stresses (Fig. 6B).

**DISCUSSION**

The role of Hsp72 in protecting cells from apoptosis has been under active investigation for a number of years and yet remains an enigma. Although understanding of the apoptotic process has progressed dramatically, the mechanism by which Hsp72 inhibits apoptosis in response to cellular insults and extracellular signaling is still largely one of speculation. Various targets for regulation by Hsp72 have been proposed, including JNK/SAPK, Bax, cytochrome c release, the formation of the apoptosome, and the inhibition of phospholipase A2 (9, 11, 25–29, 32). Part of this confusion could be due to the different experimental systems used to investigate the phenomenon, and it is possible that apoptotic pathways could differ between the different cell lines and species employed. There is also an apparent difference between systems employing inducible Hsp72 expression compared with those where Hsp72 is constitutively expressed (10). The variety of models has made it difficult for a strong consensus to be reached on the mechanism of Hsp72 action and for published results to be confirmed in other laboratories.

It has been widely accepted that Hsp72 can protect cells from apoptosis by inhibiting the function of the apoptosome, a protein complex comprising Apaf-1, cytochrome c, and dATP. This complex is responsible for activating the initiator caspase, caspase-9, which in turn cleaves and activates the downstream effector caspases. Indeed the data presented here confirm that the ability of Hsp72 to protect MEFs from stress-induced apoptosis is due to the inhibition of caspase activity. This model also predicts that Hsp72 should inhibit the in vitro activation of caspases by the addition of cytochrome c and dATP. However,
in the course of our investigations, we found that this was not the case and that cytochrome c/dATP induced caspase activity equally as effectively in cytosols from cells expressing Hsp72 compared with parental controls. The multiple cell lines used in these experiments were derived from fibroblast, epithelial, and lymphoid origins, suggesting that this failure of Hsp72 to inhibit caspase activation is a general phenomenon. However, this result is in direct opposition to that reported by Li et al. (31) using U937 cells expressing Hsp72, for reasons we cannot explain. In an effort to identify interactions between Hsp72 and Apaf-1, cell cytosols were subjected to blue native gel electrophoresis, but Hsp72 failed to co-migrate with either the monomeric or oligomeric forms of Apaf-1. In comparison, this gel technique was capable of resolving complexes between Hsp72 and RCMLA, a known binding partner for Hsp70 proteins.

Further studies investigated the ability of Hsp72 to inhibit cell death in vivo. Although Hsp72 was shown to confer a long term survival advantage for cells in response to heat, the caspase inhibitor z-VAD-fmk failed to promote continued cell growth despite being a much more efficient inhibitor of apoptosis than Hsp72. This failure of z-VAD-fmk to protect cells from stress-induced death, as opposed to caspase-dependent apoptosis, has been described previously in neuronal and lymphoid cells (37, 38) as well as in the fibroblasts used in this study. In contrast, z-VAD-fmk was able to promote a survival advantage and inhibit cytochrome c release in MEFs treated with TNFα, presumably through the inhibition of caspase-8, the apical caspase in the extrinsic signaling pathway. The extrinsic pathway leads to caspase activation as a direct result of ligand/receptor interactions, unlike the intrinsic pathway where caspase activation is a much later event. Although there have been reports of caspase-2 activity in the intrinsic apoptotic pathway upstream of mitochondrial permeabilization (47–49), recent reports have shown that caspase-2 is not an essential part of intrinsic apoptosis (50, 51). Because the pretreatment of MEFs with z-VAD-fmk failed to inhibit cytochrome c release in response to heat, it would appear that caspases do not play an important role in heat-induced apoptosis in MEFs until after cytochrome c release has occurred. Thus, in heat-induced apoptosis the inhibition of caspase-9 by z-VAD-fmk is functionally analogous to inhibition of the apoptosome and does not provide the long term cell survival that Hsp72 is observed to confer. Similarly, the putative inhibition of the apoptosome by Hsp72 would have little consequence for the long term survival of a cell. Supporting this argument, Ekert et al. (50) have reported that the loss of Apaf-1 from myeloid cells did not confer long term protection against growth factor withdrawal or cytotoxic drugs, despite the complete inhibition of apoptosis.

A novel method for measuring nuclear apoptosis and cytochrome c release was used to follow the fate of individual cells after exposure to a variety of apoptotic stimuli. The major advantage of this technique is the ability to simultaneously score cells for a mid term apoptotic event upstream of apoptosome function (cytochrome c release) and a late term apoptotic event dependent on caspase activation (nuclear condensation). It was found that almost all cells that developed an apoptotic nuclear morphology had also undergone cytochrome release. Heat-induced cytochrome c release was independent of caspase activity, because it was not inhibited by the caspase inhibitor z-VAD-fmk, which effectively blocked nuclear condensation. Hsp72 was found to be an effective inhibitor of cytochrome c release in response to heat, UVC radiation, and TNFα as previously published (27–29, 52). Hsp72 did not inhibit cytochrome c release or apoptosis in MEFs in response to the DNA-damaging drug etoposide. More importantly there was no sign that Hsp72 could inhibit nuclear apoptosis once cytochrome c release had occurred. If Hsp72 was acting on the apoptosome to inhibit caspase activation, or by directly inhibiting caspase activity, then one would have expected to see more cells exhibiting the cell morphology observed in the presence of z-VAD-fmk (cytochrome c release but normal nuclear
Stressgen Biotechnologies (Canada) and is produced in a buffer blocked caspase activation by cytochrome for cells exposed to intracellular stress. Dissolved was sufficient to inhibit caspase activation. The purified caspase from both MDA-MB-231 and from 293 cells that were described previously (25, 26). This effect was observed in cytosols maintained mitochondrial integrity, the act of measuring associated with effective mechanical disruption of cells while the same principle that should be adopted for the effective apoptotic stimulus is determined on a cell-by-cell basis and this is not induced proteotoxic stress, as shown in Fig. 6B. Further, it is conceivable that the ability of Hsp72 to inhibit apoptosis is separate and distinct from its ability to inhibit stress-induced necrosis (54). Protection from necrosis is thought to be achieved by the repair of the protein machinery through the chaperone function of Hsp72.

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Hsp72 Inhibits Apoptosis Upstream of the Mitochondria

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