Hsp72 Inhibits Fas-mediated Apoptosis Upstream of the Mitochondria in Type II Cells*

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Nicholas J. Clemons, Katherine Buzzard, Rohan Steel, and Robin L. Anderson‡

From the Cancer Biology Laboratory, Peter MacCallum Cancer Centre, St Andrew's Place, East Melbourne 3002, Victoria, Australia

Heat shock protein 72 (Hsp72) inhibits apoptosis induced by some stresses that trigger the intrinsic apoptosis pathway. However, with the exception of TNFα-induced apoptosis, a role for Hsp72 in modulating the extrinsic pathway of apoptosis has not been clearly established. In this study, it was demonstrated that Hsp72 could inhibit Fas-mediated apoptosis of type II CCRF-CEM cells, but not type I SW480 or CH1 cells. Similar results were obtained when Fas ligand or an agonistic Fas antibody initiated the Fas apoptosis pathway. In CCRF-CEM cells, Hsp72 inhibited mitochondrial membrane depolarization and cytochrome c release but did not alter surface Fas expression or processing of caspase-8 and Bid, indicating that Hsp72 acts upstream of the mitochondria to inhibit Fas-mediated apoptosis. Thus, the ability of Hsp72 to inhibit Fas-mediated apoptosis is limited to type II cells where involvement of the intrinsic pathway is required for efficient effector caspase activation.

Apoptosis is a tightly regulated, genetically controlled event crucial to normal development and tissue homeostasis (1, 2). Aberrations in the control of apoptosis can lead to a number of physiological disorders including cancer, where disruption of apoptosis confers a survival advantage to the tumorigenic cells. Deregulation of apoptosis is now viewed as a crucial step in tumorigenesis (3, 4). Apoptosis can be divided into two distinct but interconnecting pathways: the extrinsic pathway that is activated upon ligation of death receptors of the TNF receptor superfamily and the intrinsic pathway that is initiated by cellular stresses that activate pro-apoptotic members of the Bcl-2 family to target the mitochondria. Central to both pathways are the caspases, which cleave a specific set of target substrates leading to the classic hallmarks of apoptosis (5). Activation of caspases in the extrinsic pathway is mediated by formation of the death-inducing signaling complex (DISC) at the cytoplasmic death domains of ligated death receptor oligomers (6, 7). Formation of the DISC occurs via recruitment of the adaptor protein FADD and caspase-8zymogens, upon which caspase-8 is activated to cleave downstream effector caspases (8). In the intrinsic pathway, pro-apoptotic Bcl-2 proteins cause permeabilization of the mitochondria resulting in release of apoptogenic factors including cytochrome c from the mitochondrial intermembrane space (IMS) (9). Cytosolic cytochrome c triggers the formation of a multimeric complex called the apoptosome, which includes the cytosolic proteins Apaf-1 and pro-caspase-9 (10, 11). Caspase-9 is activated on the apoptosome in a dATP-dependent manner and subsequently activates downstream effector caspases.

Cross-talk between the extrinsic and intrinsic pathways exists through the cleavage of the pro-apoptotic Bcl-2 protein Bid, by caspase-8 (12, 13). The truncated form of Bid (tBid) activates the pro-apoptotic molecules Bax and Bak, which permeabilize the mitochondria and initiate the intrinsic pathway (14–16). In some cells (type II) but not others (type I) recruitment of the intrinsic pathway is required for efficient apoptosis (17), although this concept is controversial (18–20).

Apoptotic pathways are regulated by inhibitor of apoptosis (IAP) proteins that directly target the caspases (21), c-FLIP that can either promote or inhibit caspase activation at the DISC (22–24) and Bcl-2 proteins that target the mitochondria (25). In addition, the extrinsic pathway can be regulated by the relative expression of death and decoy receptors (26). Another class of apoptosis regulators, although poorly acknowledged, are the heat shock proteins (Hsps). While acting as molecular chaperones in unstressed cells, Hsps promote cell survival during periods of both acute and chronic stress (27). The ability of cells to develop thermostolerance, a state of transient resistance to severe stress, after a mild heat shock, is thought to be largely the responsibility of Hsp72 (28). In addition, expression of Hsp72 alone has been shown to inhibit apoptosis induced by a variety of stresses including TNFα, heat, UV radiation, ceramide, and a number of cytotoxic drugs (29–33). The significance of these findings is highlighted by studies that correlate high expression of Hsp72 in tumors with resistance to standard cancer therapies, increased metastasis to lymph nodes and a poor prognosis for disease free status and overall survival (34–36).

With the exception of TNFα-induced apoptosis, a role for Hsp72 in modulating apoptosis mediated through death receptors of the TNF receptor superfamily has not been clearly established. In this study we investigated the ability of Hsp72 to modulate Fas-induced apoptosis using the type II cell line CCRF-CEM and the type I cell lines SW480 and CH1. We found that Hsp72 inhibits Fas-induced apoptosis in the type II cells, acting downstream of Bid, but upstream of the mitochondria. In contrast, Hsp72 could not inhibit Fas-induced apoptosis in the type I cells indicating that Hsp72 acts only in the intrinsic pathway.

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‡ To whom correspondence should be addressed: Cancer Biology Laboratory, Peter MacCallum Cancer Centre, Locked Bag #1, A’Beckett St., Melbourne 8006, Victoria, Australia. Tel.: 61-3-9656-1284; Fax: 61-3-9656-1411; E-mail: robin.anderson@petermc.org.

The abbreviations used are: TNF, tumor necrosis factor; DISC, death-inducing signaling complex; TMRE, tetramethylrhodamine ethyl ester; z, benzoyloxycarbonyl; fmk, fluoromethylketone; Ab, antibody; GFF, green fluorescent protein; FITC, fluorescein isothiocyanate.
RESULTS

Hsp72 Inhibits Fas-mediated Apoptosis of Type II CCRF-CEM Cells—The effect of Hsp72 on Fas-mediated apoptosis was first examined in the type II cell line, CCRF-CEM. These cells have been shown previously to be dependent on the engagement of the intrinsic apoptotic pathway to undergo efficient apoptosis (17). Expression of Hsp72 was assessed in parental cells, vector control cells and Hsp72-transfected clonal populations (Fig. 1a). Clones 7 and 16 expressed high levels of Hsp72, while clone 18 had similar levels of Hsp72 to parental and vector only transfected cells. The functionality of Hsp72 in CCRF-CEM cells was demonstrated by its ability to inhibit apoptosis in vector only transfected cells (data not shown). Fas ligand (FasL)-induced apoptosis was also reduced in cells expressing Hsp72 compared with vector control cells (data not shown), confirming that Hsp72 can inhibit Fas-mediated apoptosis.

Nuclear morphology was assessed by fluorescence microscopy following propidium iodide (PI) staining as described previously (33). A cell was scored as apoptotic if it displayed one or more of the following: nuclear condensation, chromatin condensation, or formation of apoptotic bodies. At least 200 cells were counted in each experiment.

Long Term Survival Assay—Long term survival was assessed by clonogenic assay. For the suspension cell line, CCRF-CEM, a series of dilutions containing known concentrations of cells (5000 to 0.5 cells/ml) were prepared, placed into 96-well U-bottom plates and incubated at 37 °C for 10–12 days. Wells were scored as positive or negative for colony growth as described (37). For the adherent cell line SW480, dilutions containing known numbers of cells (1 to 10 000) were placed in Petri dishes at 37 °C for 10–12 days. Colonies were stained with 0.1% crystal violet in 50% methanol and counted. The fraction of surviving cells was calculated by comparison with untreated control plates.

Immunoblotting—Samples containing equal amounts of protein were separated by 13% SDS-PAGE and transferred to nitrocellulose membranes. Primary and secondary antibodies were added in 1% milk powder in PBS and incubated for 2 to 6 h at room temperature or overnight at 4 °C. HRP-conjugated and AP-conjugated secondary antibodies were detected by ECL (Lumilight, Roche Diagnostics), or colorimetric staining with naphtholphosphate (Sigma-Aldrich) and fast red TK (Bio-Rad), respectively.

Intracellular Membrane Depolarization—Mitochondrial membrane potential (ΔΨm) was assessed by uptake of the selective dye, TMRE. Cells were harvested, incubated with TMRE (final concentration 0.1 μM) for 5 min at room temperature and analyzed by flow cytometry on a FACS caliber. Ten thousand cells were counted for each sample.

Cytochrome c Release—Release of cytochrome c from cells was assessed by the method of Waterhouse and Trapani (38). Briefly, 2 to 106 SW480 cells or 5 to 105 CCRF-CEM cells were harvested and incubated on ice for 5 min on ice in phosphate-buffered saline containing 25 μg/ml digitonin and 100 mM KC1 before being fixed in 4% paraformaldehyde. Cells were immunostained with an anti-cytochrome c monoclonal antibody, followed by ALEXA Fluor® 488-conjugated anti-mouse secondary antibody. Ten thousand cells in each experiment were analyzed by flow cytometry on a FACS caliber. Ten thousand cells were counted for each sample.

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Hsp72 can provide long term protection, not just a delay in apoptosis, after initiation of Fas signaling in CCRF-CEM cells (Fig. 1e). Fas signaling was initiated by incubating cells with FasAb cross-linked with protein A for 16 h, prior to assessment of survival by clonogenic assay. Both Hsp72 expressing clones exhibited significantly enhanced clonogenic survival compared with parental or vector control cells. Approximately 60% of Hsp72 expressing cells survived long term after incubation with 15 ng/ml FasAb compared with 20% of parental or vector control cells.

**Hsp72 Cannot Inhibit Fas-mediated Apoptosis of the Type I Cell Lines SW480 and CH1**—We next assessed the ability of Hsp72 to inhibit apoptosis in the cell lines SW480 and CH1, which have type I characteristics (18, 39). Type I cells demonstrate efficient direct cleavage of caspase-3 by caspase-8 through the extrinsic pathway and are able undergo apoptosis independent of the intrinsic pathway (17).

A population of Hsp72-expressing SW480 cells was generated and Hsp72 expression confirmed by Western blotting (Fig. 2a). Initially, to ensure that the transfected Hsp72 was functional in these cells, we assessed long term survival of SW480 cells following heat stress by clonogenic assay (Fig. 2b). Hsp72-expressing cells exhibited a 10-fold increase in survival compared with vector only transfected cells after 90 min at 44 °C, indicating that the transfected Hsp72 was functional in these cells.

Fas-mediated apoptosis was induced in SW480 cells using both FasAb cross-linked with 100 ng/ml of protein A (Fig. 2c) and FasL cross-linked with 100 ng/ml of anti-FLAG antibody (Fig. 2d). In contrast to the type II CCRF-CEM cells, increased expression of Hsp72 did not protect SW480 cells from either FasL- or FasAb-induced apoptosis. Similar to CCRF-CEM cells, preincubation of SW480 cells with 50 μM z-VAD-fmk completely inhibited Fas-mediated apoptosis (data not shown).

Despite showing good expression of Fas on the cell surface (data not shown), SW480 cells required approximately ten times as much FasAb or three times as much FasL compared with CCRF-CEM cells to induce similar levels of apoptosis. Therefore, we examined the effect of Hsp72 expression on Fas-mediated apoptosis in another type I cell line, CH1, which is more sensitive to Fas-mediated apoptosis. A single cell clone of CH1 (CH1.14) and Hsp72-expressing clones derived from CH1.14 (clone D1 and clone H5) were generated and expression of Hsp72 confirmed by Western blotting (Fig. 2e). Approximately 50% of CH1.14 cells underwent apoptosis when incubated with only 10 ng/ml of anti-mouse Fas antibody (mFasAb), demonstrating that this cell line is highly sensitive to Fas-mediated apoptosis (Fig. 2f). Expression of Hsp72 in these cells...
had no effect on Fas-mediated apoptosis, confirming that Hsp72 cannot inhibit Fas-mediated apoptosis in type I cells.

**Bcl-2 Inhibits Fas-mediated Apoptosis in Type II CEM Cells but Not Type I SW480 Cells**—To confirm the characteristics of these cells as being either type I or type II, we examined the effect of Bcl-2 expression on Fas-mediated apoptosis in CEM and SW480 cells. Overexpression of Bcl-2 in CEM (Fig. 3a) and SW480 (Fig. 3c) compared with vector control cells was confirmed by Western blotting. Expression of Bcl-2 in CEM cells strongly inhibited both FasAb- and FasL-induced apoptosis (Fig. 3b). In contrast, expression of Bcl-2 in SW480 cells had no effect on FasAb-induced apoptosis (Fig. 3d). These results indicate that CEM cells require the intrinsic pathway for Fas-mediated apoptosis, and thus apoptosis is inhibited by the expression of Bcl-2. In contrast, Fas-mediated apoptosis of SW480 cells is unaltered by inhibition of the intrinsic pathway by Bcl-2. The expression of Hsp72 mirrors the ability of Bcl-2 to inhibit Fas-mediated apoptosis of type II but not type I cells, indicating that Hsp72 also acts in the intrinsic pathway to inhibit apoptosis. Thus, we next assessed the effect of Hsp72 expression on components of the intrinsic pathway during Fas-mediated apoptosis in CCRF-CEM cells.

**Hsp72 Inhibits Fas-mediated Cleavage of Caspase-9 and Downstream Effector Caspases in CCRF-CEM Cells**—The ability of Hsp72 to inhibit Fas-mediated cleavage of caspase-9 as well as the downstream effector caspases, caspase-3 and caspase-7, was assessed in CCRF-CEM cells by Western blotting (Fig. 4). The 35-kDa and 37-kDa cleavage products of caspase-3 were detected in vector control cells 6 h after the addition of anti-Fas antibody. The large (22 kDa) subunit and the small, fully active (17 kDa) subunit of caspase-3 were also detected at this time, while the active 21 kDa of caspase-7 was not detected until 12 h after addition of the antibody.

In contrast, cleavage products of caspase-9 were not detected in clone 7 or clone 16 lysates until 12 h or later after the addition of anti-Fas antibody and the levels of the 35- and 37-kDa fragments were substantially reduced compared with vector control cells. In addition, both caspase-7 and caspase-3 processing was inhibited in Hsp72-expressing clones. Even after 18 h, the fully active 17-kDa fragment of caspase-3 and the 21-kDa caspase-7 cleavage product were not detected in clone 16 cells. Cleavage products of caspase-3 and caspase-7 were detected at later time points in clone 7 cells, but again at lower levels compared with vector control cells. This is consistent with the findings that inhibition of Fas-mediated apoptosis was greater in clone 16 than clone 7. Thus, Hsp72 effectively inhibits Fas-mediated cleavage of caspase-9 and the effector caspases, caspase-3 and caspase-7.

**Hsp72 Inhibits Fas-mediated Mitochondrial Membrane Depolarization and Cytochrome c Release in CCRF-CEM Cells**—Recent data from our laboratory suggest that Hsp72 acts upstream of the mitochondria to inhibit apoptosis after certain stresses (40). To investigate the role of Hsp72 upstream of the mitochondria in Fas-mediated apoptosis, we examined mitochondrial membrane depolarization by using the fluorescent compound TMRE.

Cells were incubated with the indicated doses of anti-Fas antibody (FasAb) or Fas ligand (FasL) for 18 h, prior to harvest and addition of TMRE. Loss of mitochondrial membrane potential (ΔΨm) was assessed by flow cytometry (Fig. 5a). Activation of the Fas pathway effectively caused mitochondrial membrane depolarization of CCRF-CEM cells. Live cells with functional mitochondria selectively take up TMRE, but with increasing doses of FasAb, a second population of cells that does not take up TMRE appears, which represents cells with loss of ΔΨm. The proportion of cells that had lost ΔΨm was reduced in both clones overexpressing Hsp72 compared with vector control cells. Approximately 70% fewer clone 16 cells had lost ΔΨm compared with vector control cells after incubation with FasAb (Fig. 5b). Similar results were obtained when FasL was used instead of the antibody (Fig. 5c). Mitochondrial membrane depolarization was also significantly inhibited in clone 7, but to a lesser extent than in clone 16. The percentage of cells that had lost ΔΨm correlated closely with the percentage of cells that had undergone apoptosis as determined from earlier experiments (Fig. 5d).
potential membrane potential in CCRF-CEM cells. Polarization in vector control cells versus and vector control cells. Clone 18 cells responded in a similar manner to parental cells were also examined (Fig. 5d). Membrane depolarization times after 4 h.

The kinetics of Fas-mediated loss of mitochondrial membrane potential (Δψm) was assessed by FACS measurement of the uptake of the selective dye TMRE. a, representative histograms of an experiment analyzing the uptake of TMRE in cells following 18 h of incubation at 37 °C with the indicated doses of cross-linked FasAb. Cells were incubated with the indicated doses of cross-linked FasL (c) for 18 h at 37 °C. Kinetics of Fas-mediated loss of Δψm was assessed after incubation with 10 ng/ml of cross-linked FasAb (d). Cells were incubated with FasAb for the indicated time periods at 37 °C before staining with TMRE and measurement of Δψm. The mean and S.D. of at least three independent experiments are shown. Statistical analysis by Student’s t test; *, p < 0.05; **, p < 0.01.

1b). Clone 18 cells responded in a similar manner to parental and vector control cells.

The kinetics of Fas-mediated mitochondrial membrane depolarization in vector control cells versus Hsp72 expressing cells were also examined (Fig. 5d). Membrane depolarization became apparent between 4 and 8 h and significant inhibition of Δψm loss was observed in both Hsp72-expressing clones at times after 4 h.

Because Hsp72 could inhibit Fas-mediated Δψm loss in CCRF-CEM cells, we next examined the effect of Hsp72 expression on the release of cytochrome c. Cells were incubated for 18 h with 10 ng/ml or 15 ng/ml of FasAb, or 100 ng/ml of Fas ligand prior to harvest and assessment of residual mitochondrial cytochrome c as described (38).

FACS analysis of untreated cells revealed a single population of cells that reacted strongly with the anti-cytochrome c antibody (Fig. 6a). Cells treated with FasAb formed a second population with reduced fluorescence, indicating that in these cells, cytochrome c had been released from the mitochondria and had exited the cells after digitonin treatment. This second peak was significantly reduced in clones 7 and 16 compared with vector control cells. All cells that had released cytochrome c showed apoptotic morphology as assessed by staining with PI and examination by fluorescence microscopy (data not shown), providing a direct correlation between loss of cytochrome c and apoptosis in these cells. The combined data from three independent experiments revealed that the proportion of cells that had released cytochrome c following incubation with FasAb (Fig. 6b) or Fas ligand (Fig. 6c) was significantly reduced in Hsp72-expressing cells compared with vector control cells.

The ability of Bcl-2 to inhibit Fas-mediated cytochrome c release in CEM cells was also assessed (Fig. 6d). Bcl-2 dramatically inhibited cytochrome c release following incubation with either Fas ligand or FasAb, reflecting its effectiveness in inhibiting Fas-mediated apoptosis (Fig. 3b). In addition, cytochrome c release was completely inhibited by z-VAD-fmk (data not shown). Thus, as well as inhibiting loss of Δψm, Hsp72 can inhibit Fas-mediated cytochrome c release in CCRF-CEM cells. Taken together, these results demonstrate that Hsp72 acts upstream of the mitochondria to inhibit Fas-mediated apoptosis.

**Fas Expression Is Unchanged by Increased Expression of Hsp72**—Since Hsp72 inhibits Fas-mediated apoptosis upstream of the mitochondria, upstream components of the Fas pathway were examined to determine where Hsp72 is acting. Fas ligand and FasAb-induced apoptosis is initiated through cross-linking of the receptor Fas, a type I membrane protein. Thus, surface expression of Fas was examined to see if Hsp72 was inhibiting Fas-mediated apoptosis by diminishing receptor expression.

Vector control and Hsp72 expressing cells all showed detectable expression of Fas protein on the plasma membrane (Fig. 7a). To produce a quantitative comparison of Fas expression,
expression of the receptor was compared with that of the isotype control from three independent experiments (Fig. 7b). There was no significant difference in surface Fas expression between Hsp72 expressing cells and parental or vector control cells. Thus, Hsp72 inhibits Fas-mediated apoptosis in CCRF-CEM cells downstream of receptor expression.

**Hsp72 Does Not Inhibit Fas-mediated Caspase-8 and Bid Processing**—Ligation and oligomerization of the receptor brings together the cytoplasmic tails of Fas, which initiate formation of the death-inducing signaling complex (DISC) (7). This results in recruitment of pro-caspase-8 and its subsequent cleavage. Thus, the influence of Hsp72 on Fas-mediated caspase-8 processing was examined by Western blotting. Full-length caspase-8 is cleaved to 43 kDa and 41 kDa intermediate products and an 18 kDa active fragment. In vector control lysates, all three fragments were detected after 6 h of incubation with FasAb and increased progressively over time (Fig. 7c). Hsp72 expression did not inhibit Fas-mediated cleavage of caspase-8 as similar levels of the cleavage products were detected in clone 7 and clone 16 lysates. In fact, clone 7 cells had increased levels of the cleavage products by 18 h.

In type II cells such as CCRF-CEM, efficient apoptosis requires cross-talk with the intrinsic pathway, which is mediated through caspase-8 cleavage of the pro-apoptotic Bcl-2 family member, Bid. Although the cleavage product of Bid could not be detected with this antibody, a decrease in the amount of full-length Bid was observed (Fig. 7c). This was not changed in clone 7 and clone 16 lysates compared with vector control lysates, indicating that Hsp72 was acting downstream of Bid to inhibit Fas-mediated apoptosis. Attempts to visualize the translocation of tBid from the cytoplasm to the mitochondrial outer membrane by immunofluorescence were not successful, possibly due to the relatively few mitochondria present in CCRF-CEM cells and the rapid destruction of cell architecture once apoptosis has been initiated (data not shown).

**DISCUSSION**

It has been established previously that Hsp72 is capable of inhibiting apoptosis induced by intracellular damage, a process mediated by the so-called intrinsic signaling pathway (29–33). In this study we demonstrate that Hsp72 is also capable of inhibiting apoptosis induced by Fas signaling (an extrinsic signaling event), but only in Type II cells where recruitment of the intrinsic pathway is required for the successful initiation of apoptosis. Hsp72 not only provided a transient block of Fas-mediated apoptosis, but also increased long term survival after activation of Fas in the type II cell line, CCRF-CEM. Expression of Bcl-2 in CCRF-CEM cells inhibited apoptosis induced by both anti-Fas antibody and Fas ligand, demonstrating a requirement for recruitment of the intrinsic pathway in these cells. In contrast, expression of Hsp72 or Bcl-2 failed to protect against Fas-mediated apoptosis in two type I cell lines, SW480 and CH1. The functionality of Hsp72 was confirmed in both CCRF-CEM and SW480 cells by the increased heat resistance in Hsp72 expressing cells compared with base vector transduced controls. While CCRF-CEM cells were considerably more sensitive to heat than the SW480 cells, Hsp72 protected to a similar extent in both cell lines. In addition, the failure of Hsp72 to protect type I cells from Fas-induced apoptosis could not be attributed to differences in sensitivity to Fas signaling, as SW480 and CH1 cells were less and more sensitive respectively to Fas-induced apoptosis when compared with the CCRF-CEM cells.

Some controversy surrounds the use of anti-Fas antibodies to stimulate apoptosis, with suggestions that only multimeric forms of Fas ligand induce physiologically relevant apoptotic signaling through Fas (18, 19). A recent report demonstrated that *in vivo* administration of aggregated Fas ligand induced apoptosis in hepatocytes of *bcl-2* transgenic mice, while aggregated anti-Fas antibody did not (41). This suggests that anti-Fas antibody activates a type II like pathway while the more physiologically relevant Fas ligand activates a type I pathway. In our hands, expression of Bcl-2 in CEM cells inhibited apoptosis regardless of whether aggregated anti-Fas antibody or aggregated recombinant Fas ligand was used to initiate apoptosis. These findings are in direct contrast to those reported by Huang et al. (18) who demonstrated that Bcl-2 could not inhibit Fas-mediated apoptosis in CEM cells when co-cultured with cells expressing membrane bound Fas ligand. We are unable to explain these discrepancies. Nevertheless, we demonstrate here that Hsp72 can only inhibit Fas-mediated apoptosis in situations where Bcl-2 also inhibits. Taken together, these findings indicate that like Bcl-2, Hsp72 exerts its inhibitory function in the intrinsic pathway, but not the extrinsic pathway.

To confirm the ability of Hsp72 to inhibit the intrinsic pathway in Fas-mediated apoptosis of type II CCRF-CEM cells, we first examined the cleavage of caspase-9 and the downstream effector caspases, caspase-3 and -7. We found that cleavage of all of these caspases was inhibited by the expression of Hsp72. Further investigation revealed that Hsp72 inhibited Fas-mediated apoptosis upstream of the mitochondria in CCRF-CEM cells, as demonstrated by the inhibition of Δψm loss and cyto-
chromosome c release. Predictably, expression of Bel-2 also inhibited Fas-mediated cytochrome c release. In addition, inhibition by Hsp72 of both of these indicators of mitochondrial integrity correlated closely with inhibition of apoptosis in CCRF-CEM cells, demonstrating that the entire capacity of Hsp72 to inhibit Fas-mediated apoptosis could be attributed to its action upstream of the mitochondria. To further elucidate the point in the pathway at which Hsp72 was exerting its effect, we examined steps upstream of the mitochondria. We did not see any effect of Hsp72 on surface Fas expression, or caspase-8 and Bid processing, indicating that Hsp72 is acting at a point downstream of Bid to inhibit apoptosis. It is not surprising that Hsp72 did not alter surface expression of Fas or caspase-8 processing, as inhibition of these two signaling events would inhibit Fas-mediated apoptosis in all cell types. Cleavage of Bid results in activation and oligomerization of Bax and Bak in the outer mitochondrial membrane, causing permeabilization (14, 15). Thus, either protein is a likely anti-apoptotic target of Hsp72 as proposed recently by Gotot et al. (42) in the inhibition of nitric oxide (NO) and CHOP-induced apoptosis. Co-expression of Hsp72 and the co-chaperone Hdj-2 inhibited CHOP-induced translocation of Bax from the cytosol to the mitochondria during NO-induced apoptosis. Others have also reported the ability of Hsp72 to inhibit Bax translocation (43). Gotoh et al. (42) were able to co-immunoprecipitate Bax with Hsp72 and Hdj-1 and Hdj-2 in heat-treated cells, suggesting that Bax is the target of Hsp72 mediated inhibition of apoptosis. If correct, this is an attractive explanation for the ability of Hsp72 to inhibit Fas-mediated apoptosis of type II cells.

There is increasing evidence that Hsp72 inhibits apoptosis induced by a variety of stresses by acting upstream of the mitochondria in the intrinsic pathway. Others have reported Hsp72-mediated inhibition of cytochrome c release (44–47) and the mitochondrion was also shown to be the target of heat shock-mediated protection from oxidative stress (48). Recent work from our laboratory demonstrates that the entire effect of Hsp72 to inhibit apoptosis mediated by a number of stresses can be attributed to its ability to inhibit cytochrome c release (40).

It is important to note that although we found that Hsp72 blocks Fas-mediated cytochrome c release, blocking the release of cytochrome c per se may not be the critical factor in the inhibition of apoptosis. Rather, blocking the release of other apoptogenic factors from the mitochondria may also be important, particularly as it has been proposed that neither cytochrome c release (49) nor formation of the apoptosome are required for apoptosis (50). A number of other apoptogenic molecules are released from the mitochondria during apoptosis including apoptosis inducing factor (AIF), HtrA2/Omi, endonuclease G (endoG), and Smac/DIABLO (9). While AIF and endoG are involved in caspase-independent cell death, Smac/DIABLO contributes to caspase-dependent apoptosis. Smac/DIABLO is released along with cytochrome c from the mitochondria and functions by inhibiting the IAP family of proteins, thereby promoting caspase activation (51, 52). It has been reported that apoptosis induced by TRAIL, which appears to induce apoptosis in a similar fashion to Fas ligand, is dependent on Bax-mediated release of Smac/DIABLO from the mitochondria (53). In addition, others have suggested that type I and type II cells differ in the levels of XIAP and thus have differing requirements for release of Smac/DIABLO (54). Interestingly, we have found that pre-incubation with the caspase-9 inhibitor z-LEHD-fmk does not inhibit Fas-mediated apoptosis in CCRF-CEM cells. In light of these findings, it is possible that Hsp72 is inhibiting apoptosis in type II cells by inhibiting release of Smac/DIABLO rather than cytochrome c. Formal proof of this hypothesis remains to be established.

While, to the best of our knowledge, we are the first to report that Hsp72 can inhibit Fas-mediated apoptosis, an earlier report demonstrated that Hsp72 enhances Fas-mediated apoptosis of Jurkat T cells, while protecting the cells from heat-induced death (55). Liossis et al. (55) used solid-phase anti-Fas antibody (IgM) to initiate apoptosis, thus it would be interesting to see if similar results would be obtained if multimerized Fas ligand was employed. Apart from the use of a different cell line and different methods to evaluate apoptosis, we cannot explain the difference between our results and those of Liossis et al. (55). Others have reported that induction of thermotolerance does not affect Fas-induced apoptosis in the Jurkat line (56). Creagh and Cotter (56) also used an IgM anti-Fas antibody to induce apoptosis, however the effect of expressing Hsp72 alone on Fas-induced apoptosis was not investigated. Interestingly, it has been reported that hyperthermia can enhance Fas-mediated apoptosis (57, 58); however Tran et al. (58) concluded that this was due to heat-induced down-regulation of FLIP and was independent of heat-induced Hsp72 expression.

Our findings may have implications for Hsp72 in tumor progression. Increased expression of Hsp72 has been observed in some cancers (59–67). Its expression in breast cancer correlates with resistance to combination chemotherapy, radiation and hyperthermia, increased cell proliferation, poor differentiation, lymph node metastasis and a poor prognosis for disease-free status and overall survival (59, 65, 67–74). In addition, expression of Hsp72 increases the tumorigenic potential of rodent cells (75, 76) and transgenic mice expressing Hsp72 develop malignant T-cell lymphomas (77). Abrogation of Hsp72 expression with antisense technology inhibits proliferation and induces apoptosis, indicating that Hsp72 is required for tumor cell survival (78, 79). By combining our findings and the role of Fas signaling in tumor surveillance by immune cells (80), it is tempting to speculate that Hsp72 may, in part, be providing a growth advantage to tumor cells through inhibition of Fas-mediated apoptosis.

In summary, we have shown that inhibition of apoptosis by Hsp72 is not restricted to stresses that cause intracellular damage. Rather, Hsp72 can also inhibit apoptosis mediated by the death receptor Fas, but only in type II cells where apoptosis, while initiated by the extrinsic pathway, requires participation by the intrinsic signaling pathway for successful completion of the apoptotic program. Further, Hsp72 exerts its inhibitory effect by acting upstream of the mitochondria and downstream of Bid. Recent data from our laboratory have shown that Hsp72 also inhibits TNFα-induced apoptosis upstream of the mitochondria (40). Whether Hsp72 inhibits apoptosis through the inhibition of cytochrome c release, thereby inhibiting formation of the apoptosome, or by inhibiting the release of some other apoptogenic factor residing in the mitochondria, remains to be elucidated. Thus, while inhibition of apoptosis by Hsp72 is not restricted to stresses that cause intracellular damage, it is restricted to stresses that require the intrinsic pathway.

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