Heat-shock protein 70 antagonizes apoptosis-inducing factor

Luigi Ravagnan*, Sandeep Gurbuxani†, Santos A. Susin*, Carine Maisse*, Eric Daugas*‡, Naoufal Zamzami*, Tak Mak§, Marja Jäättelä¶, Josef M. Penninger§, Carmen Garrido†# and Guido Kroemer*#

*CNRS UMR 1599, Institute Gustave Roussy, Pavillon de Recherche 1, 39 rue Camille Desmoulins, 94805 Villejuif, France
†INSERM U-517, Faculty of Medicine and Pharmacy, 7 Boulevard Jeanne d'Arc, 21033 Dijon, France
‡Assistance Publique - Hôpitaux de Paris, Service de Néphrologie B, Hôpital Tenon, 20 rue de la Chine, F-75020 Paris, France
§The Amgen Institute and Ontario Cancer Institute, Department of Medical Biophysics and Immunology, University of Toronto, Toronto, Ontario M5G 2C1, Canada
¶Apoptosis Laboratory, Institute of Cancer Biology, Danish Cancer Society, Strandboulevarden 49,DK-2100 Copenhagen, Denmark
#These authors contributed equally to this work
**e-mail: kroemer@igr.fr*

Heat-shock protein 70 (Hsp70) has been reported to block apoptosis by binding apoptosis protease activating factor-1 (Apaf-1), thereby preventing constitution of the apoptosome, the Apaf-1/cytochrome *c*/caspase-9 activation complex[1,2]. Here we show that overexpression of Hsp70 protects Apaf-1$^{-/-}$ cells against death induced by serum withdrawal, indicating that Apaf-1 is not the only target of the anti-apoptotic action of Hsp70. We investigated the effect of Hsp70 on apoptosis mediated by the caspase-independent death effector apoptosis inducing factor (AIF), which is a mitochondrial intermembrane flavoprotein[3,4]. In a cell-free system, Hsp70 prevented the AIF-induced chromatin condensation of purified nuclei. Hsp70 specifically interacted with AIF, as shown by ligand blots and co-immunoprecipitation. Cells overexpressing Hsp70 were protected against the apoptogenic effects of AIF targeted to the extramitochondrial compartment. In contrast, an anti-sense Hsp70 complementary DNA, which reduced the expression of endogenous Hsp70, increased sensitivity to the lethal effect of AIF. The ATP-binding domain of Hsp70 seemed to be dispensable for inhibiting cell death induced by serum withdrawal, AIF binding and AIF inhibition, although it was required for Apaf-1 binding. Together, our data indicate that Hsp70 can inhibit apoptosis by interfering with target proteins other than Apaf-1, one of which is AIF.

Hsp70 is a prominent cytoprotective factor: its downregulation is sufficient to kill tumour cells or to facilitate the induction of apoptosis[5,6]. In contrast, its upregulation, as a consequence of either cellular stress or transfection, inhibits the induction of apoptosis by several insults[7] and may contribute to oncogenic transformation[8,9]. Expression levels of Hsp70 correlate negatively with response to chemotherapy, for example in breast cancer[10]. An almost universal feature of apoptosis is the activation of caspases, which, in the intrinsic pathway, is secondary to the mitochondrial release of cytochrome *c*. Once in the cytosol, cytochrome *c* triggers the oligomerization of Apaf-1, which in turn recruits pro-caspase-9 and pro-caspase-3 into the apoptosome — the caspase activation multiprotein complex. Hsp70 reportedly interacts with Apaf-1, thereby preventing its interaction with procaspase-9 (refs 1,2,7).

Apoptosis is also characterized by caspase-independent processes, which may be inhibited by Hsp70 (refs 11,12). Here we observed that Hsp70 overexpression (Fig. 1a) inhibited two signs of apoptosis, namely, the loss of the mitochondrial transmembrane potential ($\Delta\Psi_m$) (Fig. 1b, d) and plasma membrane permeabilization (Fig. 1b, c), in Apaf-1$^{-/-}$ mouse embryo fibroblasts (MEFs) cultured in the absence of serum — that is, in a system in which cell death is induced but caspases are not activated[13]. This clearly indicates that Apaf-1 (and by extension caspases) cannot be the only target(s) accounting for the anti-apoptotic effect of Hsp70.

Transfection with an Hsp70 anti-sense construct (which reduced the expression of Hsp70, Fig. 1a) sensitized MEF cells to serum-withdrawal-induced $\Delta\Psi_m$ loss (Fig. 1c), plasma membrane permeabilization (Fig. 1d) and cell loss (Fig. 1e), indicating that endogenous levels of Hsp70 may determine cell survival. Accordingly, as compared with the vector-only control, Hsp70 anti-sense cDNA reduced the clonogenic survival of Apaf-1$^{-/-}$ MEFs transiently exposed to serum withdrawal. In contrast, Hsp70 enhanced the clonogenicity of such cells (Fig. 1f). Thus, Hsp70 does not just retard cell death; instead, it provides true cytoprotection, even in the absence of Apaf-1.

Apoptosis inducing factor (AIF) is a caspase-independent death effector that, like cytochrome *c*, is released from mitochondria early in the apoptotic process[3]. Because AIF is essential for serum-withdrawal-induced apoptosis[4] (which is inhibited by Hsp70; Fig. 1), we investigated the possibility that Hsp70 might neutralize AIF. In a cell-free system, recombinant AIF protein causes isolated nuclei to undergo peripheral chromatin condensation, shrinkage and DNA loss[3]. We found that Hsp70 antagonized the apoptogenic effects of both recombinant AIF and of natural AIF released from the mitochondrial intermembrane space (Fig. 2a, b). In this cell-free system, AIF and Hsp70 were used at concentrations similar to those attained in the non-mitochondrial compartment of apoptotic or heat-shocked cells, as measured by quantitative immunoblots (data not shown). The constitutive Hsp70 homologue Hsc70 also neutralized AIF, whereas Hsp27 had no such effects (ref. 14; and Fig. 2b). Because pre-incubation of AIF with Hsp70 yielded a more efficient inhibition of apoptosis *in vitro* than pre-incubation of nuclei with Hsp70 (data not shown), we examined whether there is a direct interaction between Hsp70 and AIF. Among different inducible Hsps (Hsp10, -27, -60, -70), AIF only interacted with Hsp70, as determined by ligand blotting (Fig. 2c) and co-immunoprecipitation in U937 cells (Fig. 2d).

We next investigated whether Hsp70 would specifically antagonize the effect of AIF in intact cells. Overexpression of AIF by transient transfection induced cell death — an effect that was increased by deleting the amino-terminal mitochondrial localization sequence (AIFΔ1-100), which misdirects AIF to an ectopic (non-mitochondrial) localization (Fig. 3). The lethal action of AIF (or AIFΔ1-100) was reduced by stable overexpression of Hsp70 (Fig. 3a, b), correlating with an enhanced AIF–Hsp70 interaction (Fig. 3e). Conversely, downregulation of Hsp70 through stable transfection with an Hsp70 anti-sense construct increased the cytotoxic

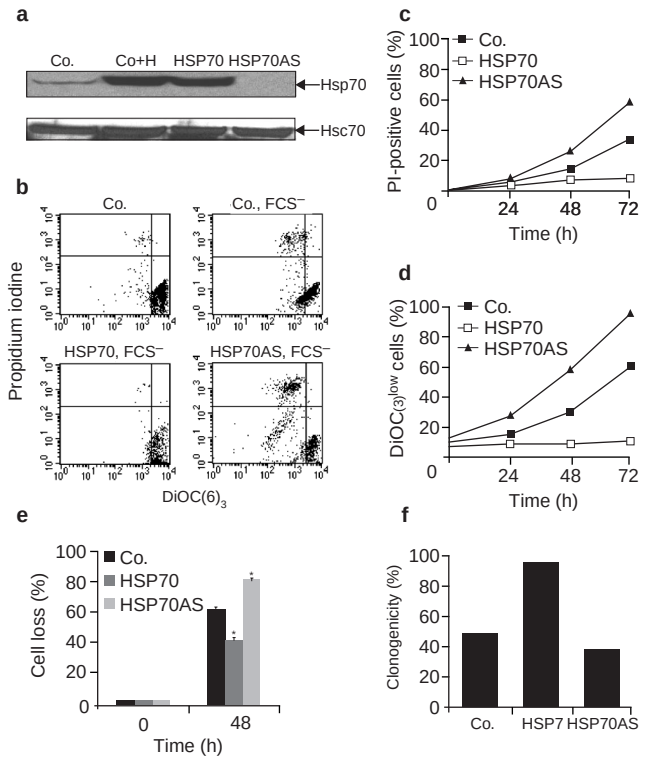

Figure 1 **Hsp70-mediated resistance of Apaf-1$^{-/-}$ MEFs to serum withdrawal.** **a**, Expression levels of Hsp70 in Apaf-1$^{-/-}$ MEF cells stably transfected with a control vector (Co.), *hsp70* (HSP70) or *hsp70* antisense (HSP70AS), as determined by immunoblotting. Control vector-transfected cells were heat-shocked (Co.+HS) for 1 h at 42 °C, and allowed to recover for 9 h at 37 °C, leading to an upregulation of Hsp70 similar to that obtained by *hsp70* transfection. The expression level of the constitutive Hsp, Hsc70, was detected as a loading control. **b**, Apaf-1$^{-/-}$ MEFs stably transfected with a control vector, *hsp70* or *hsp70* antisense were cultured for 48 h in the absence of FCS, stained with the $\Delta\Psi_m$-sensitive dye $DiOC_6(3)$ and the vital dye propidum iodide (PI) and subjected to FACS analysis. **c**, **d**, Kinetics of cell death (**c**) and the $\Delta\Psi_m$ dissipation (**d**) assessed in Apaf-1$^{-/-}$ MEFs transfected with control vector (filled squares), *hsp70* (open squares) or *hsp70* antisense (filled triangles) on different periods of serum withdrawal. **e**, Cell loss induced by serum withdrawal in MEF cells overexpressing *hsp70* or an *hsp70* antisense construct, as determined by crystal violet assay. **f**, Clonogenic survival of Apaf-1$^{-/-}$ MEF cells overexpressing *hsp70* or the *hsp70* antisense construct. Clonogenicity was determined after 48 h of serum deprivation. Results are means ± s.d. of triplicate measurements. Asterisks indicate significant differences with respect to values obtained for controls ($P < 0.01$). Experiments were repeated twice with similar results.

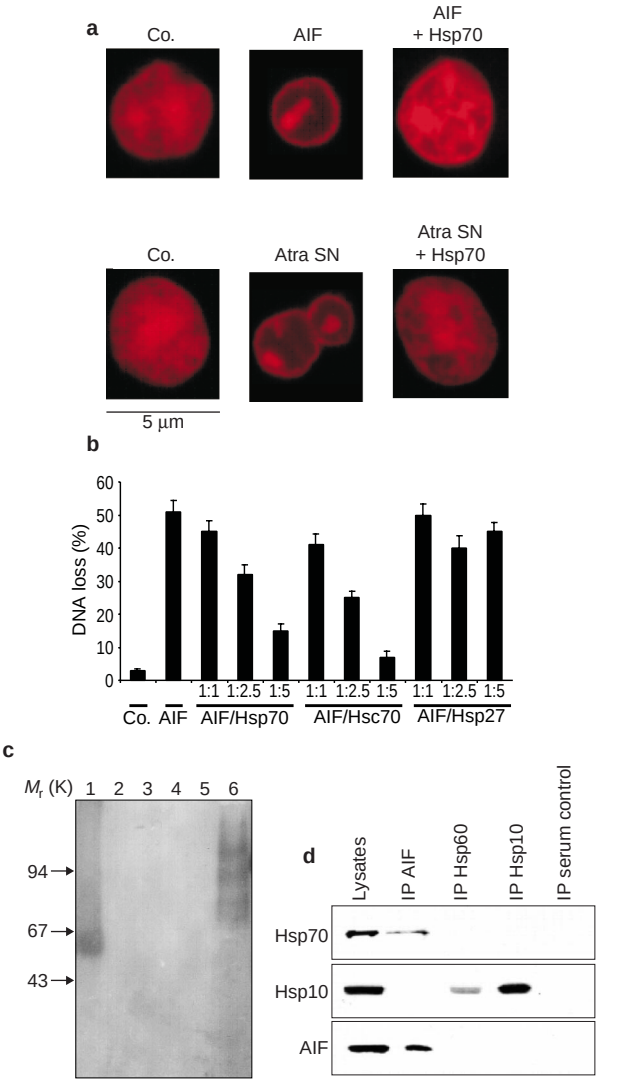

Figure 2 **Hsp70 effects on AIF activities determined in a cell-free system.** **a**, **b**, Effects of AIF and Hsp70 on nuclear morphology and DNA content. Purified HeLa nuclei were incubated with recombinant AIF (0.6 µM), the supernatant of atractyloside-treated mitochondria (Atra SN), Hsp70, Hsc70 or Hsp27 at the indicated molar ratios (1:5 in **a**), stained by PI, and then subjected to determination of chromatin condensation (**a**) or nuclear DNA loss (**b**). **c**, Interaction between AIF and Hsp70, determined by ligand blotting. Biotinylated AIFΔ1-120 (lane 1), AIFΔ1-120 (lane 2), BSA (lane 3), Hsp27 (lane 4), Hsp60 (lane 5) and Hsp70 (lane 6) were resolved by non-denaturating PAGE, transferred onto nitrocellulose membrane, subjected to ligand blotting with biotinylated AIFΔ1-120, and revealed with streptavidin-HRP. Note that streptavidin-HRP alone only reacted with immobilized biotinylated AIFΔ1-120 in lane 1 and not with other proteins (not shown). **d**, Co-immunoprecipitation of AIF and Hsp70 in U937 cell extracts.

potential of AIF and AIFΔ1-100 (Fig. 3a, b). All these effects of AIF and Hsp70 were observed both in wild-type MEFs (Fig. 3a, b) and in Apaf-1$^{-/-}$ MEFs (which failed to activate caspases[15,16]) (Fig. 3c, d), indicating that they occur independently of caspases.

To exclude the possibility that epigenetic effects or long-term changes in transcription profiles caused the cytoprotection conferred by Hsp70 (or the cytotoxicity of AIF and Hsp70 anti-sense cDNA), we microinjected recombinant AIF and/or Hsp70 into the cytoplasm of MEF cells. Microinjection of 2.5–7.5 fg of AIF per cell (endogenous AIF is ~3 fg per MEF cell) induces apoptosis rapidly (in 3 h; Fig. 3f) even in the absence of messenger RNA or protein synthesis[17]. Again, co-injection of 15–45 fg recombinant Hsp70 per cell (endogenous Hsp70 is ~100 fg per cell in heat-shocked cells and < 10 fg ml^{-1} in unstressed cells) (Fig. 3f, g left panel) or stable transfection with Hsp70 (Fig. 3g, right panel) prevented the nuclear chromatin condensation as well as the $\Delta\Psi_m$ dissipation triggered by AIF microinjection. No such effects were found for Hsp27 (Fig. 3g).

Like other chaperones, Hsp70 possesses both a docking site for interaction with a substrate (the peptide-binding domain; PBD), and an ATP-binding domain (ABD). We transfected human ME-180 cervix carcinoma cells either with wild-type Hsp70 or with Hsp70 deletion mutants in which the PBD or ABD had been removed (Fig. 4a). The ABD was not required for inhibiting AIF-induced cell death (Fig. 4b), in accord with the finding that Hsp70 does not require ATP to counteract AIF in the cell-free system

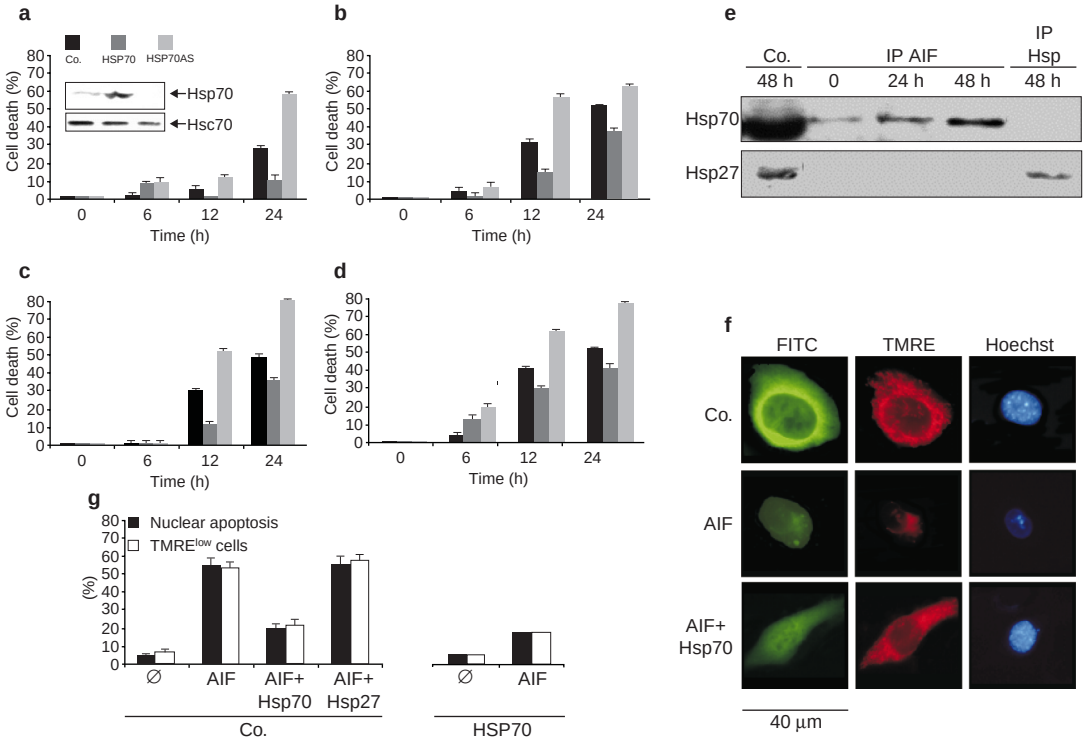

Figure 3 **Hsp70 antagonizes AIF cytotoxicity. a–d**, Cell death induced by AIF transfection. Wild-type (**a**, **b**) or Apaf-1$^{-/-}$ (**c**, **d**) MEFs stably transfected with Hsp70 or an Hsp70 antisense construct were transiently transfected with AIF (**a**, **c**) or AIF lacking the mitochondrial localization sequence (AIFΔ1-100) (**b**, **d**), and then their viability was determined. Inset in **a** shows an immunoblot of Hsp70 and Hsc70 in wild-type MEFs. **e**, Co-immunoprecipitation of AIF and Hsp70. MEFs overexpressing Hsp70 were transfected with AIFΔ1-100, and immunoprecipitation was performed at different intervals after transfection. **f**, Apoptosis features induced by AIF microinjection. Apaf-1$^{-/-}$ MEF cells were microinjected with dextran-FITC alone or in combination with AIF (9 µM), with or without Hsp70 (25 µM). Injected cells were identified by green fluorescence, $\Delta\Psi_m$ was measured with TMRE (red fluorescence), and nuclear apoptosis was measured with Hoechst dye (blue fluorescence). **g**, Microinjection of Apaf-1$^{-/-}$ MEF cells with AIF alone or in combination with Hsp70 or Hsp27 (Co., left) and Apaf-1$^{-/-}$ MEFs transfected with Hsp70 (HSP70, right). Nuclear apoptosis and $\Delta\Psi_m$ were measured as in **f**. ø, microinjection of buffer only.

(which has been performed in the absence of ATP; Fig. 2a,b).

In contrast, deletion of the PBD abolished the AIF-neutralizing action of Hsp70 (Fig. 4b). The cytoprotective potential of Hsp70 mutants correlated with AIF binding. Hsp70ΔABD co-immunoprecipitated with AIF to a similar extent as wild-type Hsp70 (Fig. 4c); in contrast, Hsp70ΔABD failed to interact with Apaf-1 (Fig. 4c). After serum withdrawal ME-180 cells died in a caspase-independent fashion, because cell death was not altered by adding the pan-caspase inhibitor Z-VAD.fmk (Fig. 4d). Hsp70ΔABD was as effective as Hsp70 in maintaining cell viability after serum withdrawal in the absence or presence of Z-VAD.fmk (Fig. 4d). Moreover, Hsp70ΔABD was as cytoprotective as Hsp70 when death was induced by menadione or staurosporine (Fig. 4e), and partially inhibited vinblastine-triggered cell death. In contrast to Hsp70, Hsp70ΔABD failed to confer protection against etoposide, *cis*-platin or doxorubicin. Neither Hsp70ΔABD nor Hsp70 prevented death induced by the ligation of death receptors by TRAIL, TNF-α or anti-CD95 (Fig. 4e).

Our data indicate that Hsp70 can bind and neutralize AIF. The physical interaction between Hsp70 and AIF has been revealed both in rather artificial settings, such as the ligand blot (Fig. 2c) and overexpression of AIF and Hsp70 (Fig. 3e, 4c), and in unmanipulated U937 cells (Fig. 2d). A functional interaction between Hsp70 and AIF occurred both in cell-free systems (Fig. 2a,b) and in intact cells microinjected with recombinant Hsp70 and/or AIF protein (Fig. 3f, g) or transfected with Hsp70 or AIF cDNA (Figs 3a–d, 4b). Hsp70 inhibited apoptosis induced by overexpressing both full-length AIF (which has to transit mitochondria to become apoptogenic[3,18]) and AIF lacking the mitochondrial localization sequence (AIFΔ1-100). Notably, endogenous levels of Hsp70 seem to be high enough to control AIF-mediated apoptosis, because downregulation of Hsp70 by an anti-sense cDNA construct increased the sensitivity of the cells to serum withdrawal (Fig. 1e, f) and AIF (Fig. 3a–d). The physical and functional interaction between AIF and Hsp70 does not require the ABD (Fig. 4), indicating that the chaperone function of Hsp70 (which relies on ATP binding and hydrolysis) may not be essential for its inhibitory effect on AIF.

AIF has been shown to act at the post-mitochondrial level (after mitochondrial membrane permeabilization; MMP) and at the (pre-)mitochondrial level (before or during MMP)[3,4,16–18]. Accordingly, the neutralization of AIF by microinjection of a specific antibody[17] or inactivation of the AIF gene[4] can prevent apoptotic MMP, whereas microinjection of AIF or transfection with AIF can trigger $\Delta\Psi_m$ loss[3,16], release of cytochrome *c*[17,18] or release of AIF itself[18] from mitochondria. The fact that Hsp70 can prevent MMP (ref. 19; and Fig. 1) thus might be attributed, at least in part, to its effect on AIF.

Hsp70 can reportedly retard cytolysis even under conditions of full activation of caspase[11]. In addition, Hsp70 may prevent cell death in conditions in which caspase activation does not occur, either through the addition of exogenous caspase inhibitors[12] or after inactivation of the *apaf-1* gene (Fig. 1). Notably, it seems that the structural features of Hsp70 required for inhibition of Apaf-1 and AIF inhibition are distinct. The *in vitro* interaction of Hsp70

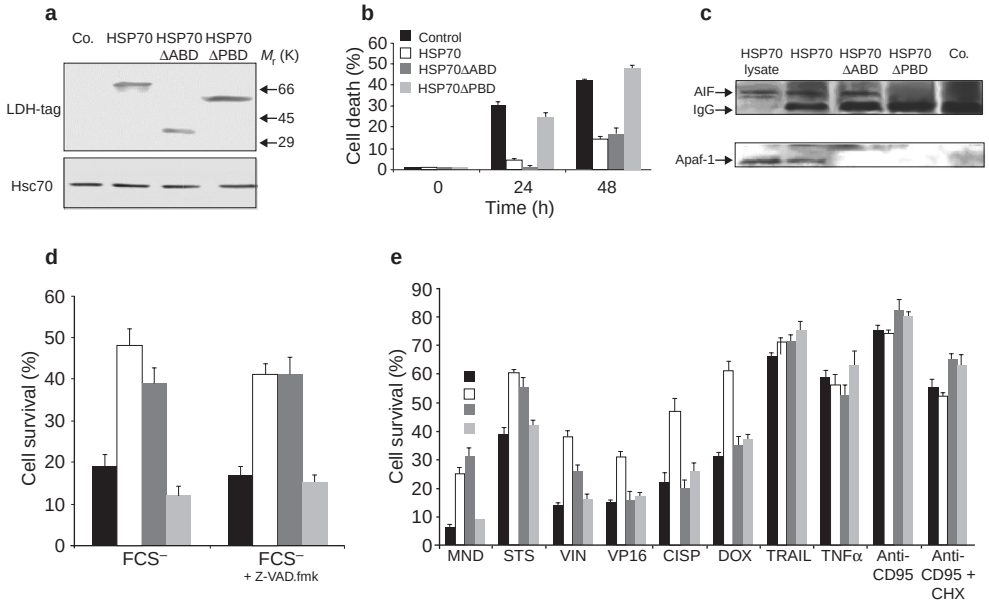

Figure 4 **Domains of Hsp70 required for AIF binding and inactivation.** **a**, Western blot of ME-180 cells stably transfected with LDH-tagged ME vector, Hsp70, Hsp70ΔABD or Hsp70ΔPBD. **b**, Cytotoxicity of AIF transfection. Cells were transiently transfected with AIF, and then assessed for cell survival at the indicated intervals. **c**, Co-immunoprecipitation of AIF or Apaf-1 with Hsp70 or Hsp70 mutants. Cells transfected with AIF as in **b** (at 48 h) were subjected to immunoprecipitation with an antiserum specific for the C-terminal LDH tag of Hsp70. Immunoblots were revealed with anti-AIF antibody or an anti-Apaf-1 antibody. **d**, Effect of Hsp70, Hsp70ΔABD, Hsp70ΔPBD and Z-VAD.fmk on cell death induced by serum withdrawal. Cells were cultured in the absence of serum and/or in the presence of Z-VAD.fmk (100 μM) for 48 h, and then assessed for cell survival. **e**, Effect of Hsp70, Hsp70ΔABD or Hsp70ΔPBD on cell death induced by different stimuli. ME-180 cells were cultured in the absence of serum or in the presence of menadione (MND, 150 μM), staurosporin (STS, 100 nM), vinblastine (VIN, 10 nM), etoposide (VP16, 100 μM), *cis*-platin (CISP, 2 μM), doxorubicin (DOX, 1 μM), TRAIL (100 ng ml^{-1}), TNFα (4 ng ml^{-1}), anti-CD95 (mAb CH11, 2 μg ml^{-1}), and/or cycloheximide (CHX, 3 μM) for 48 h. Results are means ± s.d. of triplicates, and have been reproduced twice.

and Apaf-1 relies on ATP hydrolysis[1], and transfection with Hsp70ΔABD fails to prevent caspase activation[20]. Accordingly, Hsp70ΔABD fails to co-immunoprecipitate with Apaf-1 (Fig. 4c) and fails to prevent (presumably Apaf-1- and caspase-dependent[15]) cell death induced by etoposide, *cis*-platin or doxorubicin (Fig. 4e). In strict contrast, Hsp70ΔABD does interact with AIF (Fig. 4c) and can protect cells against the overexpression of AIF (Fig. 4b), serum withdrawal (Fig. 4d), menadione, staurosporine (Fig. 4e), ultraviolet irradiation[21] or thermal stress[22]. This is in line with previous observations that AIF may be the rate-limiting factor in apoptosis induced by serum withdrawal, menadione[4] and staurosporine[3]. Stable binding of Hsp70 to apoptosis-regulatory proteins other than AIF (such as c-Jun N-terminal kinase[23] or topoisomerase I (ref. 24)) has also been reported to be ABD-independent. But, because these latter proteins are only involved in a limited set of apoptosis induction pathways, it is tempting to assume that the caspase-independent (ABD- and ATP-independent) anti-apoptotic action of Hsp70 can, at least in part, be attributed to its neutralizing interaction with AIF. □

Methods

Cells, plasmids, and transfections.

Wild-type MEF and Apaf-1$^{-/-}$ MEF cells[15] were stably transfected with a cDNA encoding Hsp70 in the pcDNA3-Neo vector (Invitrogen)[11]. The anti-sense Hsp70 construct was generated by inserting bases 475–974 of the published human Hsp70 sequence[25] in antisense orientation downstream of a CMV promoter of the same vector[11]. Cells were cultured in DMEM supplemented with L-glutamine, sodium pyruvate, HEPES buffer and penicillin/streptomycin, with or without 10% fetal calf serum (FCS). FACS analysis was performed on trypsinized cells stained with the $\Delta\Psi_m$-sensitive dye $DiOC_6(3)$ (Molecular Probes; 20 nM) and the vital dye propidium iodide (1 μg ml^{-1})[26]. We constructed pZEM-hsp70-tag by subcloning a 2.4-kilobase *Bam*HI/*Hin*dIII fragment from pSV-hsp70-tag, containing the entire reading frame of the human *hsp70* gene modified by the addition of sequence encoding the human testis-specific lactate hydrogenase (LDH) decapeptide immunotag into the carboxy terminus[11,25], downstream of metallothionin promoter in the pZEM-neo eukaryotic expression vector. To create expression plasmids encoding mutant Hsp70 proteins lacking the ATP-binding domain (pZEM-Hsp70-ΔABD-tag) or the peptide-binding domain (pZEM-Hsp70-ΔPBD-tag), plasmid pZEM-hsp70-tag was cleaved with *Bgl*II or *Sma*I restriction enzymes and re-ligated to give in-frame deletions of bases 842–1765 or 1797–2336, respectively, in the published Hsp70 sequence[25]. ME-180 human cervix carcinoma cells were transfected by electroporation (330 V, 960 μF, 1,000 Ω) with the empty pZEM-neo vector, pZEM-Hsp70-tag, pZEM-Hsp70-ΔABD-tag or pZEM-Hsp70-ΔPBD-tag plasmids[11]. We cloned G418-resistant cells by limiting dilution to create single cell clones named ME-vector, ME-Hsp70, ME-Hsp70-ΔABD and ME-Hsp70-ΔPBD.

Clonogenic survival assay.

Cells (50 per well) were seeded into 96-well plates 24 h before treatment. Cells were then washed three times with Ca^{2+}- and Mg^{2+}-free PBS, and cultured in complete medium (with 10% FCS) or serum-free medium for 48 h. We then cultured the cells in complete medium for 9 days to allow colony formation. Colonies were fixed in 70% ethanol, stained with methylene blue and counted.

AIF cytoxicity assay.

AIF toxicity was carried out after transient transfection of cells with the full-length AIF cDNA or AIFΔ1-100 cDNA[18]. We seeded 1×10^4 cells onto 96-well culture plates. Tansient transfection was performed using Superfect reagent (Quiagen). At different times after transfection, the number of adherent and surviving cells was determined by a crystal violet colorimetric test. Microinjection (5–15 fl per cell) of recombinant AIF (9 μM) and/or Hsp70 (25 μM), together with fluorescein isothiocyanate (FITC)-labelled dextran (0.25% w/v), was followed by cell staining with Hoechst 33342 (1 μg ml^{-1}) and the $\Delta\Psi_m$-sensitive dye TMRE (40 nM) for 15 min at 37 °C[3].

Cell-free system of apoptosis.

Purified HeLa cell nuclei were exposed for 120 min at 37 °C to supernatants from atractyloside-treated mouse liver mitochondria[27], and 600 nM recombinant AIF[3] either pre-incubated or not with Hsp70 or Hsp27 (Stressgen). For the assessment of chromatin condensation, nuclei were stained with propidium iodide (10 μg ml^{-1}, 15 min) and observed with a Leica DM IRB fluorescence microscope equipped with standard filters, as well as a Leica DC 200 colour video camera. We determined DNA content by FACS (Vantage, Becton-Dickinson).

Immunoprecipitation and ligand blots.

Cells were lysed in 900 μl of immunoprecipitation buffer (50 mM HEPES, pH 7.6, 150 mM NaCl, 5 mM EDTA, 0.1% NP-40). After centrifugation (10 min at 15,000*g*) to remove particulate material, the supernatant was incubated with constant agitation at 4 °C with either polyclonal antibodies (1:100) against AIF[3], Hsp60, Hsp27 or Hsp10 (Stressgen), or a rat polyclonal antibody specific for the LDH tag[28]. Immunocomplexes were precipitated with protein-A/sepharose. We washed the pellet five times and immunoblotted it with monoclonal antibodies directed against Apaf-1, Hsp10, Hsp27, Hsp60 or Hsp70, or with anti-AIF rabbit antiserum[3]. For ligand blots, 1 μg of recombinant Hsp70, HSP60,

HSP27, AIF, biotynilated AIF and bovine serum albumin (BSA) were resolved by PAGE in non-denaturing conditions, transferred to nitrocellulose membrane (90 V, 90 min) and exposed to biotinylated AIF (2 μg ml^{-1} in PBS with 5% BSA) revealed by a streptavidine-horseradish peroxidase (HRP) conjugate.

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Correspondence and requests for materials should be addressed to C.G. or G.K.