Negative regulation of the Apaf-1 apoptosome by Hsp70

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Release of cytochrome *c* from mitochondria by apoptotic signals induces ATP/dATP-dependent formation of the oligomeric Apaf-1–caspase-9 apoptosome. Here we show that the documented anti-apoptotic effect of the principal heat-shock protein, Hsp70, is mediated through its direct association with the caspase-recruitment domain (CARD) of Apaf-1 and through inhibition of apoptosome formation. The interaction between Hsp70 and Apaf-1 prevents oligomerization of Apaf-1 and association of Apaf-1 with procaspase-9. On the basis of these results, we propose that resistance to apoptosis exhibited by stressed cells and some tumours, which constitutively express high levels of Hsp70, may be due in part to modulation of Apaf-1 function by Hsp70.

Apoptosis is essential for cell development and tissue homeostasis in eukaryotic organisms[1–3]. The molecular machinery that drives the apoptotic programme consists of a family of cysteine proteases, the caspases that cleave their substrates after specific aspartic acid residues (reviewed in refs 4–8). In normal cells, caspases are constitutively expressed as inactive single polypeptide chains, known as procaspases, and their activation requires specific proteolytic cleavage[5–8]. Active caspases can typically amplify apoptotic events by their ability to cleave their own precursor forms as well as those of other caspases[9,10].

Mitochondria have a crucial function in initiating the cascade of caspase activation in response to different apoptotic signals[11]. Disruption of the outer mitochondrial membrane by apoptotic stimuli results in the release of cytochrome *c* into the cytoplasm[12]. Cytochrome *c* binds to the cytosolic apoptotic-protease-activating factor 1 (Apaf-1), thereby promoting Apaf-1-mediated activation of caspase-9 in an ATP/dATP-dependent manner[13–15]. Active caspase-9 can subsequently amplify the caspase cascade by its ability to process its own proenzyme as well as the effector caspase-3 (refs 13–15). Association of procaspase-9 with Apaf-1 is an essential step in its

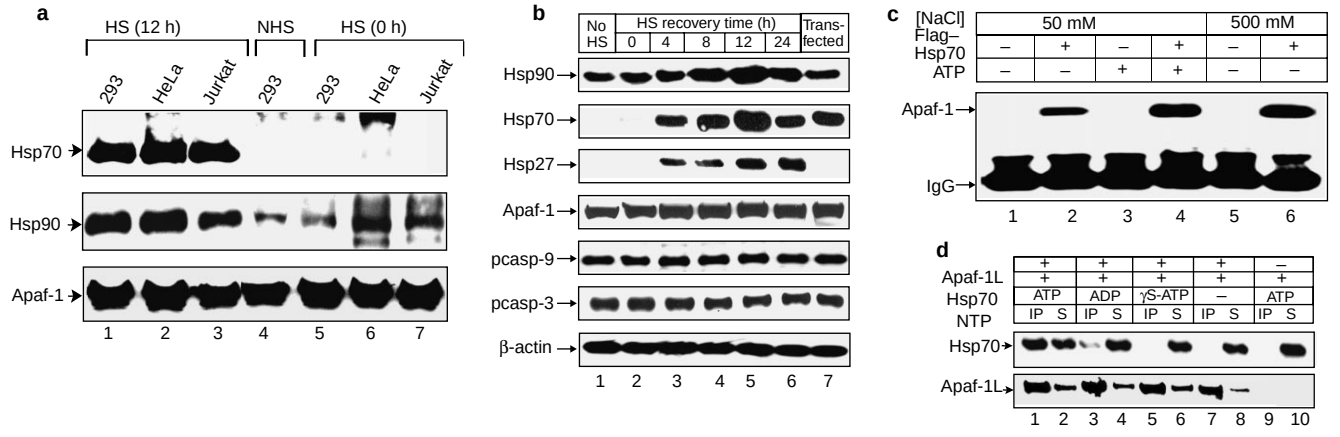

Figure 1 **Inducible Hsp70 associates with Apaf-1. a**, Whole-cell extracts (400 μg) obtained from 293, HeLa and Jurkat cells directly after heat-shock (HS) treatment (lanes 5–7) or 12 h after heat shock (lanes 1–3), or from non-heat-treated 293 cells (NHS), were incubated with 4 μg anti-Apaf-1 antibody coupled to cyanogenbromide-activated sepharose. Immunoprecipitates were fractionated on a 12% SDS–polyacrylamide gel and immunoblotted with antibodies against Hsp70, Hsp90 and Apaf-1. **b**, S100 extracts (120 μg) obtained from 293 cells before heat shock (No HS) or at the indicated time points (0–24 h) after heat shock, or from 293 cells transfected with *Flag–hsp70* (transfected), were analysed by western blotting with antibodies against Hsp90, Hsp70, Hsp27, Apaf-1, procaspase-9 and procaspase-3. The same membrane was reprobed for β-actin and served as a loading control. **c**, S100 extracts (350 μg) from 293T cells transfected with a control plasmid or with *Flag–hsp70* were incubated with or without ATP and then immunoprecipitated with an anti-Flag antibody after adjusting [NaCl] to 50 or 500 mM. Immunoprecipitates were analysed by western blotting with an anti-Apaf-1 antibody. The heavy chain of the Flag monoclonal antibody is labelled and served as a loading control. IgG, immunoglobulin G. **d**, Purified Apaf-1 and Hsp70 (4 μg each) were mixed and incubated with or without ATP, ADP or γ-S-ATP and then immunoprecipitated with an anti-Apaf-1 antibody. Immunoprecipitates (IP) and supernatants (S) were fractionated by SDS–PAGE and immunoprobed with anti-Hsp70 (upper panel) and anti-Apaf-1 (lower panel) antibodies.

initial activation and is mediated by the CARD sequence located at the amino termini of both proteins[16].

By homology with other caspase-activation events, it is thought that Apaf-1 induces caspase-9 activation by promoting clustering of this caspase[14]. In support of this model, we and others have shown that Apaf-1 forms a large heteromeric complex with cytochrome *c* in an ATP/dATP-dependent fashion and that only the oligomerized complex is capable of activating procaspase-9 (refs 15, 17, 18). On the basis of these findings, it is expected that modulation of Apaf-1 oligomerization and/or its association with procaspase-9 is the key to controlling activation of the caspase cascade triggered by the release of cytochrome *c* from mitochondria.

Several reports have provided direct evidence that the inducible heat-shock protein Hsp70 has a function in thermotolerance (reviewed in ref. 19). These studies demonstrated that suppression of Hsp70 expression or neutralization of its function renders cells extremely sensitive to heat. Further studies have shown that overexpression of Hsp70 in most cells provides efficient protection against cell death triggered by a variety of stimuli, including hyperthermia, oxidative stress, cytotoxicity induced by monocytes, many commonly used chemotheraputic agents and radiation[20–23]. In spite of these findings, however, the mechanism by which Hsp70 exerts its cytoprotective effect is not understood.

Here we examine the mechanisms through which Hsp70 regulates apoptosis and demonstrate that the anti-apoptotic effect of Hsp70 is mediated through its association with Apaf-1. Hsp70 interacts directly with the CARD of Apaf-1 in an ATP-dependent manner. Furthermore, we show that binding of Hsp70 to Apaf-1 inhibits both the cytochrome *c*/ATP-mediated oligomerization of Apaf-1 and its subsequent association with and activation of procaspase-9.

Results

Hsp70 associates with Apaf-1. To test the possibility that the protective effect of HSPs against apoptosis is mediated through inhibition of Apaf-1 function, we first investigated their potential association with Apaf-1 by immunoprecipitation assays of whole-cell extracts from 293, HeLa and Jurkat cells after mild heat shock. As shown in Fig. 1a, Hsp70 and Hsp90 were efficiently co-immunoprecipitated with Apaf-1 from the three cell lines 12 h after heat shock (lanes 1–3). Because Hsp70 is only present in heat-shocked or stressed cells (Fig. 1b), it was not detected in immunoprecipitates from the same cell lines 0 h after heat shock (Fig. 1a, lanes 5–7) or from non-heat-treated 293 cells (lane 4). In contrast, Hsp90, which is both constitutively expressed and heat-inducible (Fig. 1b), was detected in Apaf-1 immunoprecipitates from heat-treated (Fig. 1a, lanes 1–3) and non-heat-treated (lane 4) cells, although it was present at higher levels in heat-treated cells (lanes 1–3). Unlike Hsp70 or 90, Hsp27 was not detected in Apaf-1 immunoprecipitates from heat-treated or non-heat-treated cells (data not shown), although its expression was significantly increased after heat-shock treatment (Fig. 1b). Heat shock had no effect on levels of Apaf-1, procapase-9, procaspase-3 or β-actin (Fig. 1b).

To characterize further the specificity of the interaction between Apaf-1 and Hsp70 *in vivo*, we transfected 293T cells with a *Flag–hsp70* expression vector or a control vector, and determined the interactions by immunoprecipitation assays. Apaf-1 specifically associated with Flag–Hsp70 at low and high ionic concentrations (Fig. 1c, lanes 2, 4, 6). No Apaf-1-immunoreactive band was detected in immunoprecipitates from cells lacking Flag–Hsp70 (lanes 1, 3, 5), Interestingly, a threefold increase in the amount of co-immunoprecipitated Apaf-1 with Flag–Hsp70 was observed when the same immunoprecipitation reactions were carried out in the presence of ATP (compare lanes 2 and 4), indicating that the interaction between these two proteins may be, in part, ATP-dependent. The amount of ectopically expressed Flag–Hsp70 was roughly equal to the amount of endogenous Hsp70 detected 8 h after heat shock (Fig. 1b, lanes 4, 7). Furthermore, overexpression of Hsp70 did not affect expression of Hsp90, Hsp27, Apaf-1, procaspase-9 or procaspase-3 (Fig. 1b, lane 7).

To determine whether the association between Apaf-1 and Hsp70 is direct, as well as to investigate the requirement of ATP for this interaction, we incubated purified Apaf-1L (the long isoform of Apaf-1) and Hsp70 in the presence of buffer alone, or of buffer containing ATP, ADP or γ-S-ATP, and then carried out immunoprecipitations with an Apaf-1 antibody (Fig. 1d). Only in the presence of ATP was Hsp70 efficiently co-immunoprecipitated with Apaf-1 (lane 1). No Hsp70 was detected after immunoprecipitation in the absence of Apaf-1 (lane 9). These results confirm that the interaction between Apaf-1 and Hsp70 is direct and ATP-dependent.

Hsp70 inhibits Apaf-1-mediated procaspase-9 activation and apoptosis. To assess the functional significance of the interaction between Apaf-1 and Hsp70, we carried out a time-course analysis of procaspase-9 processing in S100 extracts from heat-shocked and *hsp70*-transfected cells. As shown in Fig. 2a, cytochrome *c*/ATP-

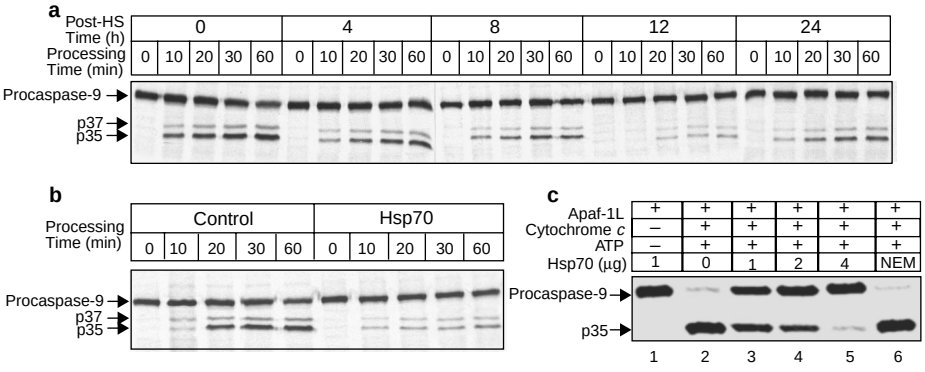

Figure 2 **Hsp70 prevents processing of procaspase-9 in an ATP-dependent manner. a**, ^{35}S-labelled procaspase-9, cytochrome *c* (10 μM) and ATP (2.5 mM) were incubated with 150 μg of S100 extracts obtained from 293 cells at the indicated time points after heat shock (post-HS). Processing of procaspase-9 was observed for 60 min in each case. Samples were analysed by SDS–PAGE and autoradiography. Procaspase-9 and the proteolytic fragments (p35 and p37) of mature caspase-9 are labelled. **b**, Similar procaspase-9-processing reactions were carried out with S100 extracts from 293T cells transfected with control vector or with *Flag–hsp70*. **c**, Processing of procaspase-9 by purified recombinant Apaf-1L (4 μg) in reaction mixtures with or without cytochrome *c*/ATP and Hsp70. The amount of Hsp70 added to each sample is indicated on the top of each lane. In lane 6, Hsp70 was pretreated with *N*-ethylmaleimide (NEM) before incubation with Apaf-1.

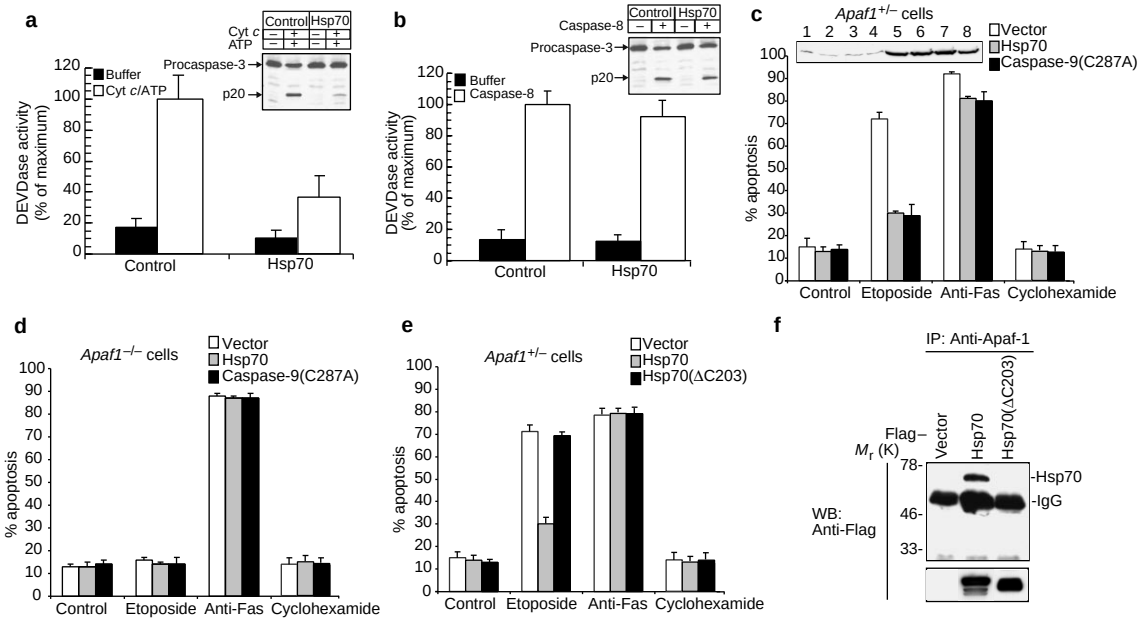

Figure 3 **Overexpression of Hsp70 inhibits cytochrome *c*/ATP-mediated activation of caspase-3 and apoptosis. a**, S100 extracts (150 μg) from 293T cells transfected either with a control vector (control) or with *Flag–hsp70* were incubated for 1.5 h at 37 °C in buffer alone or in buffer containing cytochrome *c* (Cyt *c*; 10 μM) and ATP (2.5 mM). They were then analysed for caspase-3 catalytic activity using the fluorogenic tetrapeptide substrate DEVD–AMC. Substrate hydrolysis was measured as a percentage of the maximum activity obtained with a control extract in the presence of cytochrome *c* and ATP. **b**, S100 extracts from control and *Flag–hsp70*-transfected cells were pre-incubated with or without recombinant caspase-8 (2.5 units), ATP (2.5 mM) and heat-denatured cytochrome *c* (10 mM), and then assayed for caspase-3 activity. Values are corrected to exclude fluorescence activity resulting from direct cleavage of the substrate by recombinant caspase-8 itself in a buffer control. Data are means from three independent experiments. Insets in **a** and **b** show western blots, with anti-caspase-3 antibody, of 100 μg protein from S100 extracts treated under similar conditions to those described in the fluorogenic assays. The positions of the M_r 32K precursor and the p20 cleavage product of caspase-3 are shown. **c**, **d**, *Apaf*$^{+/-}$ (**c**) and *Apaf*$^{-/-}$ (**d**) MEFs were transfected with empty vector, *Flag–hsp70*, or *procaspase-9(C287A)* in combination with a GFP–reporter plasmid, and then treated with etoposide or anti-Fas antibody plus cycloheximide for 10 h. Percentages of apoptotic cells were determined as described in Methods. Inset shows a western blot, with anti-Hsp70 antibody, of extracts from vector-transfected (lanes 1–4) or *hsp70*-transfected (lanes 5-8) *Apaf*$^{+/-}$ MEFs after treatment with etoposide (lanes 1, 5), anti-Fas antibody plus cycloheximide (lanes 2, 6) or cycloheximide (lanes 3, 7), or no treatment (lanes 4, 8). **e,** *Apaf*$^{+/-}$ MEFs were transfected with constructs encoding wild-type Hsp70 or truncated Hsp70 lacking the 203 C-terminal residues (Hsp70(ΔC203)), and then treated as above. **f**, 293T cells were transfected with empty vector, *Flag–hsp70* or *Flag–Hsp70(ΔC203)* and then lysed. Lysates were immunoprecipitated (IP) with a monoclonal antibody against Apaf-1 and immunoprecipitates were analysed by western blotting (WB) with anti-Flag antibody (upper panel). The heavy chain of the anti-Apaf-1 immunogobulin G (IgG) is labelled. Expression of Flag–Hsp70 and Flag–Hsp70(ΔC203) in lysates was determined by immunoblotting with anti-Flag antibody (lower panel). The M_r of Flag–Hsp70(ΔC203) is ~40K and it migrates between the 33K and 46K markers.

induced processing of procaspase-9 was gradually reduced after heat shock, reaching minimal levels in extracts obtained 12 h after heat shock. The reduction in procaspase-9 processing activity coincided with the increase in expression levels of Hsp70 and Hsp90 after heat shock (Fig. 1b). The partial recovery of procaspase-9 processing at 24 h after heat shock is also consistent with the decrease in the expression level of Hsp70 at this time point (Fig. 1b). Similarly, processing of procaspase-9 in a cytochrome *c*/ATP-dependent manner was also significantly reduced in S100 extracts from *hsp70*-transfected cells, compared with extracts from control-transfected cells (Fig. 2b).

To confirm that the inhibitory effect of Hsp70 on processing of procaspase-9 is directly mediated through its action on Apaf-1, we measured the effect of Hsp70 on cleavage of procaspase-9 using our previously described *in vitro* reconstituted system[15]. A purified Apaf-1L was capable of inducing maturation of procaspase-9 only in the presence of cytochrome *c* and ATP (Fig. 2c, lane 2). However, pre-incubation of Apaf-1L with purified recombinant Hsp70 in the presence of ATP inhibited processing of procaspase-9 in a dose-dependent manner (lanes 3–5). Inhibition of the ATP-binding activity of Hsp70 with *N*-ethylmaleimide[24] abrogated its ability to block Apaf-1L-dependent processing of caspase-9 (lane 6).

Overexpression of Hsp70 also inhibited procaspase-3 activation (Fig. 3a). The activity and processing of caspase-3 in the presence of cytochrome *c* and ATP was markedly reduced in S100 extracts containing Flag–Hsp70 compared with the activation in control extracts. In contrast, activation of procaspase-3 by caspase-8 in the same extracts was not affected (Fig. 3b), indicating that Hsp70 may function upstream of procaspase-3 and may not have a direct effect on the activity of mature caspase-3.

To determine whether Hsp70 selectively inhibits the Apaf-1 apoptotic pathway, we transfected *Apaf*$^{+/-}$ or *Apaf*$^{-/-}$ mouse embryonic fibroblasts (MEFs) with vectors expressing Hsp70 or the C287A dominant negative procaspase-9 mutant, and then treated these cells with etoposide or agonist Fas antibody. As shown in Fig. 3c, d, etoposide was able to induce apoptosis in empty-vector-transfected *Apaf*$^{+/-}$, but not *Apaf*$^{-/-}$, MEFs. In contrast, the agonist Fas antibody was able to induce apoptosis equally in both cell lines. This indicates that etoposide-induced, but not Fas-induced, apoptosis may be mediated by Apaf-1 in these cells. Expression of Hsp70 or the C287A dominant negative caspase-9 mutant significantly inhibited etoposide-induced, but not Fas-induced, apoptosis in *Apaf*$^{+/-}$ MEFs. In contrast, a carboxy-terminally truncated Hsp70 lacking the substrate-binding domain was unable to associate with Apaf-1 or to protect *Apaf*$^{+/-}$ MEFs against etoposide-induced apoptosis (Fig. 3e, f), indicating that this domain of Hsp70 is crucial for

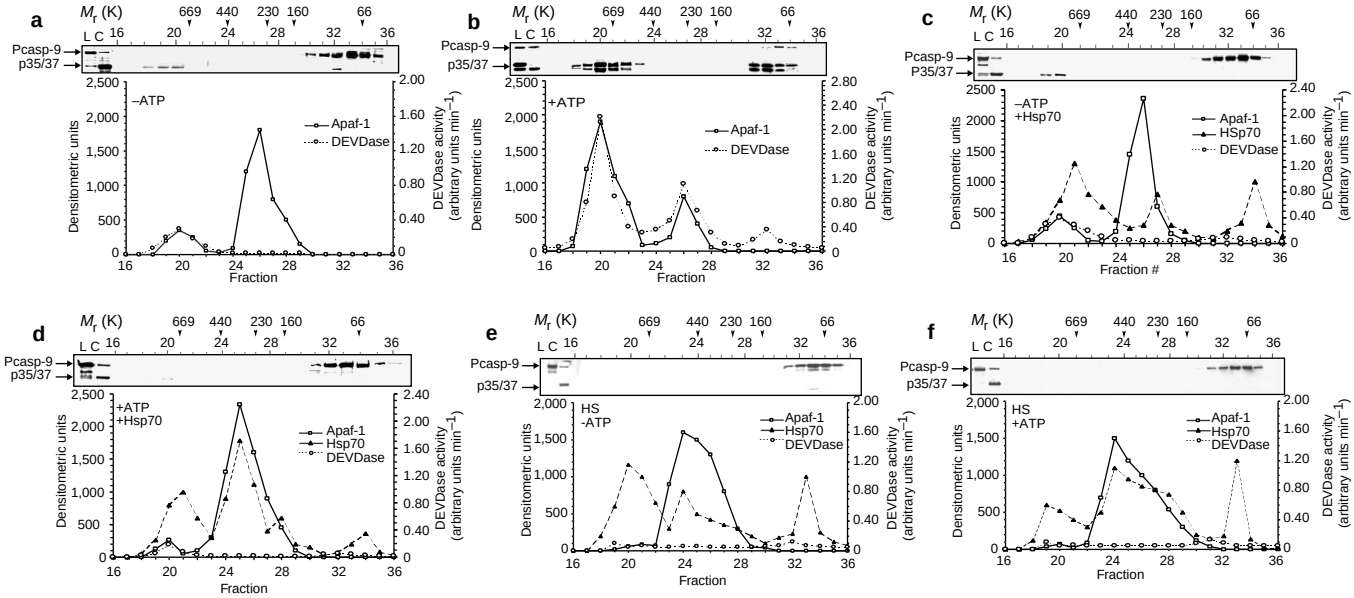

Figure 4 **Hsp70 inhibits formation of the Apaf-1 apoptosome in cell-free extracts.** Aliquots (1 ml) of S100 extracts (2.5 mg ml^{-1}) obtained from non-apoptotic HeLa cells were dialysed against oligomerization buffer II (see Methods) and fractionated by gel-filtration chromatography on a Superose 6 column before (**a**, **c**) or after (**b**, **d**) caspase activation with 2.5 mM ATP and 10 μM cytochrome *c* in the presence (**c**, **d**) or absence (**a**, **b**) of 15 μg ml^{-1} Hsp70. Proteins from 200 μl of the 500-μl fractions were precipitated with 10% trichloroacetic acid and analysed by western blotting for Apaf-1 (squares) and Hsp70 (triangles). Data in the graphs were obtained by densitometric scanning of immunoreactive bands on western blots. Similar gel-filtration experiments were carried out using S100 extracts 12 h after heat shock (HS; **e**, **f**). Insets above graphs show the same blots, probed with an anti-caspase-9 antibody that recognizes both the proform (M_r ~46K) and the processed p37 and p35 forms. L (load) represents 75 μg of the protein loaded onto the column. C (control) is a bacterially purified caspase-9 (Pcasp-9) used as a control for caspase-9-immunoreactive bands. Numbers above the lanes are the fraction numbers as eluted from the Superose 6 column. Sizes of calibration protein standards and their elution positions from the Superose 6 column are shown by arrowheads above the insets. Caspase-3 activity (circles) in 50 μl of the fractions was measured using the tetrapeptide substrate DEVD–pNA. Fractions 1–15 (void volume) did not exhibit Apaf-1, caspase-9, Hsp70 or DEVDase activity.

its ability to associate with Apaf-1 and inhibit Apaf-1-mediated apoptosis. Combined, these results indicate that Hsp70 may selectively inhibit Apaf-1-mediated, but not Fas-mediated, apoptosis through direct interaction with Apaf-1.

Mechanism of Hsp70's influence on cytochrome *c*/ATP-mediated procaspase-9 activation. The results described above indicate that the inhibitory effect of Hsp70 may occur at the level of Apaf-1-induced activation of procaspase-9. The effects of Hsp70 could be mediated through inhibition of cytochrome *c*/ATP-dependent oligomerization of Apaf-1, binding of procaspase-9 to the Apaf-1 apoptosome, or both. To examine the first possibility, we assessed the effect of purified Hsp70 on cytochrome *c*/ATP-induced oligomerization of Apaf-1 in cell-free extracts, using gel-filtration chromatography as described[15]. In the absence of ATP and Hsp70, the majority of Apaf-1 was eluted in a peak centered around fraction 26 (relative molecular mass ~300,000 (M_r ~300K)), whereas the majority of procaspase-9 was eluted predominantly as a protein of M_r ~80–90K (Fig. 4a). Following activation of the lysate with ATP and cytochrome *c*, most of the Apaf-1 was assembled into a large oligomeric complex of ~800–900K containing processed caspase-9 and exhibiting high DEVDase activity (Fig. 4b, fractions 18–23). Interestingly, addition of Hsp70 to the lysate before activation with cytochrome *c* and ATP prevented formation of the Apaf-1–caspase-9 oligomeric complex and induction of DEVDase activity (Fig. 4d). The majority of Apaf-1 and Hsp70 was co-eluted in a peak centered around fraction 25 (M_r ~350K, Fig. 4d). Hsp70 was also present in three other peaks centered around fractions 21, 28 and 34, indicating that it may also be associated with other cellular proteins. In the absence of ATP, Hsp70 was not co-eluted with Apaf-1 (Fig. 4c), providing further evidence that the association of Hsp70 with Apaf-1 is ATP-dependent.

Apaf-1 also failed to form the large oligomeric complex in S100 extracts obtained 12 h after heat shock in the presence and absence of ATP (Fig. 4e, f). In these extracts some of the endogenously expressed Hsp70 co-fractionated with Apaf-1 in a peak centered around fraction 24. However, after addition of ATP there was a roughly twofold increase in the amount of Hsp70 that was co-eluted with Apaf-1, indicating a possible increase in association between Apaf-1 and Hsp70. Moreover, all procaspase-9 was eluted independently of Apaf-1 in fractions 31–35 (Fig. 4e, f, inset), which was similar to the one in the unstimulated reaction. Together, these data clearly demonstrate that Hsp70 inhibits the formation of the large Apaf-1–caspase-9 apoptosome in cell-free extracts.

To confirm these observations, we carried out similar experiments with purified recombinant Apaf-1L (Fig. 5a). As expected, pre-incubation of Apaf-1L with Hsp70 in the presence of ATP for 30 min followed by addition of cytochrome *c* resulted in at least five-fold inhibition of Apaf-1L oligomerization (Fig. 5a, panel III). Most of the Apaf-1L protein was co-eluted with Hsp70 in a peak around fraction 27 (M_r ~250K), which also contained cytochrome *c* (data not shown). This indicates that the peak contains a heteromeric Apaf-1/cytochrome *c*/Hsp70 complex. In contrast, incubation of Hsp70 with Apaf-1L after addition of cytochrome *c* and ATP resulted in the formation of an Hsp70-free oligomeric complex with cytochrome *c*, which was eluted around fraction 17 (M_r ~1.4×10^6; Fig. 5a, panel IV). Most of the Hsp70 was eluted in a separate peak around fraction 33 (M_r ~80K), indicating that Hsp70 failed to interact with the oligomerized complex. Similarly, most Hsp70 failed to interact with monomeric Apaf-1 in the absence of ATP and the presence of cytochrome *c*, and was eluted in a separate peak around fraction 33 (Fig. 5a, panel I). In contrast, a large amount of Hsp70 was associated with monomeric Apaf-1L in the presence of ATP and the absence of cytochrome *c*, and was co-eluted with Apaf-1 around fraction 29 (M_r ~200K), indicating the formation of a

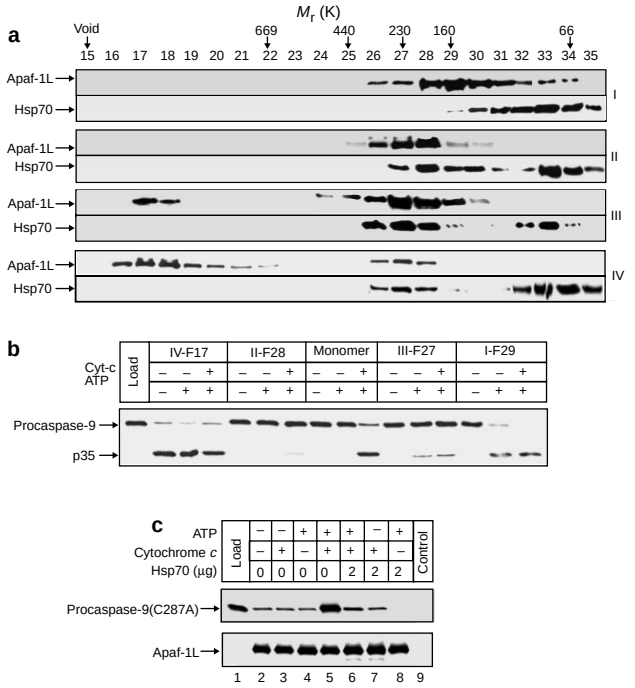

Figure 5 **Hsp70 inhibits oligomerization of Apaf-1L and Apaf-1–procaspase-9 interaction. a**, Purified Apaf-1L was mixed with recombinant Hsp70 and then incubated with cytochrome *c* (panel I), ATP (panel II) or cytochrome *c* and ATP (panel III) in oligomerization buffer I (see Methods). In panel IV, Apaf-1L was incubated with ATP and cytochrome *c* for 30 min before addition of Hsp70. After incubation, each sample was loaded onto a Superose 6 column and equal volumes of the column fractions were separated by SDS–PAGE and immunoblotted with anti-Apaf-1 and anti-Hsp70 antibodies. Fraction numbers are shown above panel I; sizes of the calibration protein standards and their elution positions from the Superose 6 column are indicated by vertical arrows. **b**, Procaspase-9 labelled with ^{35}S was incubated with a buffer control (load) or with equal amount of Apaf-1L aliquots of the peak fractions 17, 27, 28 and 29 from panels IV, III, II and I, respectively, in the presence or absence of cytochrome *c* and ATP as indicated. Samples were then analysed by SDS–PAGE and autoradiography. The positions of procaspase-9 and of the p35 fragment of mature caspase-9 are shown. Monomer represents the peak fraction of Apaf-1L from the Superose 6 column after incubation with ATP in the absence of Hsp70. **c**, Purified Apaf-1L was mixed with ^{35}S-labelled, C287A-mutant procaspase-9 in a buffer with (lanes 6–8) or without (lanes 2–5) Hsp70, and then incubated in the presence or absence of cytochrome *c* and ATP as indicated. The amount of Hsp70 added to each sample is indicated above the lanes. After 1 h incubation, Apaf-1L was immunoprecipitated from the samples and the presence of procaspase-9 (two-thirds of the sample amount, upper panel) was analysed by SDS–PAGE and autoradiography. Immunoprecipitates (one-third of the sample amount) were also analysed by western blotting for Apaf-1L (lower panel). Load (lane 1) represents one-third of the amount of the ^{35}S-labelled protein used in each sample. The control (lane 9) was similar to the immunoprecipitated sample in lane 6, but lacked Apaf-1L.

complex of Hsp70-bound monomeric Apaf-1L (Fig. 5a, panel II). These data indicate that Hsp70 may bind to the Apaf-1L monomer or to cytochrome *c*-bound monomeric Apaf-1L, and may inhibit the oligomerization process in an ATP-dependent manner.

Binding of Hsp70 to Apaf-1L inhibits activation of procaspase-9. To determine the activity of Apaf-1L in the four principal peak fractions of the gel-filtration experiments (Fig. 5a), we incubated samples of the peak fractions with purified ^{35}S-labelled procaspase-9 in the presence or absence of cytochrome *c*, ATP or both (Fig. 5b). Oligomeric Apaf-1L (trial IV, fraction 17) was capable of processing procaspase-9 without addition of cytochrome *c* and ATP (lanes 2–4). In contrast, the cytochrome *c*/Apaf-1L complex (trial I, fraction 29) was capable of efficiently processing procaspase-9 only when ATP was added (lanes 14–16). The procaspase-9-processing activity of the Hsp70/cytochrome *c*/Apaf-1L heteromeric complex (trial III, fraction 27) was markedly reduced in the presence of ATP (lanes 11–13) compared to the activity of the Apaf-1/cytochrome *c* complex (lanes 14–16). Furthermore, the Hsp70 complex with monomeric Apaf-1 (trial II, fraction 28; lanes 5–7) was active only in the presence of both ATP and cytochrome *c*, and this activity was substantially reduced in comparison with that of an equal amount of Hsp70-free monomeric Apaf-1L (lanes 8–10). These data demonstrate that binding of Hsp70 to Apaf-1 inhibits its ability to process procaspase-9.

Hsp70 binds to the Apaf-1 CARD and inhibits procaspase-9–Apaf-1L association. Binding of procaspase-9 to Apaf-1 is mediated through their CARD domains and is critical for maturation of the proenzyme by autocatalytic cleavage[14,16]. To determine whether the association of Hsp70 with Apaf-1 modulates the interaction between Apaf-1 and procaspase-9, we immunoprecipitated Apaf-1L from reaction mixtures containing a purified, ^{35}S-labelled version of the procaspase-9 C287A mutant in the presence and absence of Hsp70 (Fig. 5c). We used mutant proenzyme to avoid processing and the presumable release of active caspase-9 from Apaf-1 upon addition of cytochrome *c* and ATP. Pre-incubation of Apaf-1L and procaspase-9 with cytochrome *c* and ATP before immunoprecipitation resulted in a substantial increase in the level of procaspase-9 that was co-immunoprecipitated with Apaf-1L (lane 5), compared with pre-incubation in the presence or absence of either cytochrome *c* or ATP (lanes 2–4). This indicates that the affinity of oligomeric Apaf-1L for the proenzyme may be much higher than that of the monomeric form (lanes 2–4). In the presence of ATP and cytochrome *c*, Hsp70 reduced the association of procaspase-9 with Apaf-1L to background levels (lane 6). The small amount of procaspase-9 that was associated with monomeric Apaf-1L in the presence of ATP alone (lane 4) was reduced to undetectable levels in the presence of Hsp70 (lane 8). Combined, these results indicate that Hsp70 prevents the association of procaspase-9 with Apaf-1.

To determine whether Hsp70 associates with the CARD of Apaf-1, as well as to confirm that Hsp70 binds only to monomeric Apaf-1, we carried out affinity-binding assays for Hsp70 with Affigel-bound recombinant full-length Apaf-1L monomer, cytochrome *c*/Apaf-1L oligomer, Apaf-530 and the Apaf-1 CARD, in the presence or absence of ATP or ADP (Fig. 6a, upper panel). As expected, only in the presence of ATP was there a significant binding of Hsp70 to monomeric Apaf-1L. Interestingly, the binding affinity of Hsp70 for the Apaf-1 CARD (lane 1) was comparable to that for monomeric Apaf-1L (lane 4), indicating that the interaction between Apaf-1 and Hsp70 may be mediated through the CARD. The substantial reduction in the amount of Hsp70 associated with the Apaf-1 CARD or with monomeric Apaf-1L in the presence of ADP is consistent with the requirement for the ATP-binding activity of Hsp70 to facilitate the binding process (lanes 2 and 5, respectively).

Hsp70 also failed to associate significantly with cytochrome *c*/Apaf-1L oligomer (Fig. 6a, lane 7) and the spontaneously oligomerized Apaf-530 (lane 10), even in the presence of ATP, indicating that Hsp70 may specifically interact only with monomeric Apaf-1L. The latter finding explains the absence of Hsp70 from the M_r ~1.4×10^6 fraction containing Apaf-1L in the gel-filtration experiments (Fig. 5). A small amount of association that was observed in the case of oligomeric Apaf-1L and Apaf-530 in the presence of ATP could be a result of partial dissociation of the oligomeric complexes during their immobilization on Affigel or of the 2-h incubation period with Hsp70. The specificity of the association between Hsp70 and monomeric Apaf-1L or its CARD is also supported by the absence of such an interaction with Affigel–procaspase-9, Affigel-bound procaspase-9 CARD or Affigel–BSA (data not shown).

Immunoprecipitation experiments confirmed that Hsp70 interacts with the CARD of Apaf-1 in transfected 293T cells (Fig. 6b,

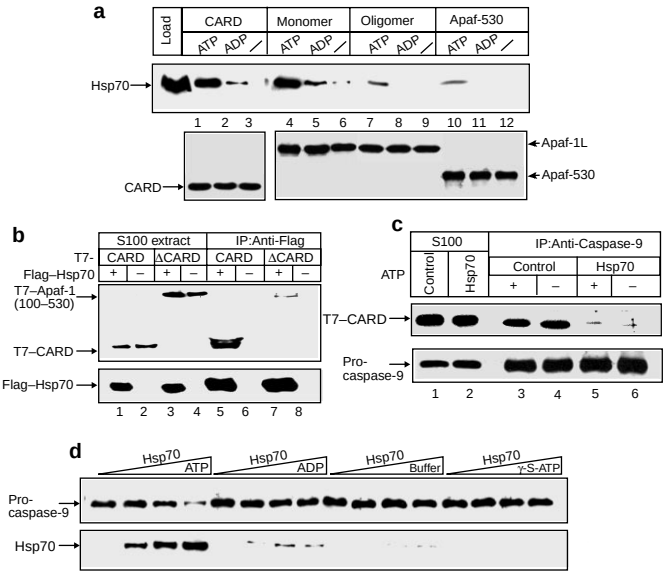

Figure 6 **Hsp70 associates with the CARD of monomeric Apaf-1 and modulates the CARD–CARD interaction between Apaf-1 and procaspase-9.**

a, Equal amounts (0.06 nmol) of Affigel-bound Apaf-1 CARD, Apaf-1L monomer, Apaf-1L/cytochrome *c* oligomer and Apaf-530 were incubated with Hsp70 (0.06 nmol) in buffer alone (–) or in buffer containing ATP or ADP. Associated Hsp70 was eluted from beads by addition of 2.5 M urea and analysed by SDS–PAGE and then by immunoblotting with anti-Hsp70 antibody (upper panel). Affigel-bound polypeptides were then eluted from the resin by boiling in 10 mM acetic acid and analysed by western blotting with anti-polyhistidine antibody (lower panels) after separation on 15% (lanes 1–3) or 11% (lanes 4–12) SDS–polyacrylamide gels. Load represents the amount of Hsp70 (5 μg) used in each sample. **b**, S100 extracts (400 μg) obtained from 293T cells co-transfected with either *T7–Apaf-1(CARD)* or *T7–Apaf-1*(100–530) (ΔCARD) and *Flag–hsp70* or control vector were incubated with anti-Flag antibody. The presence of T7-tagged polypeptides in the immunoprecipitates (IP) was assessed by western blotting with anti-T7 antibody (lanes 5–8, upper panel). Lanes 1–4 show expression of T7-tagged polypeptides in the different S100 extracts (50 μg protein). The same blot was reprobed with anti-Flag antibody to detect Hsp70 (lower panel). **c**, S100 extracts (1 mg) from 293T cells overexpressing the T7–Apaf-1(CARD) in the presence (Hsp70) or absence (control) of Flag–Hsp70 were immunoprecipitated with a polyclonal antibody against caspase-9. Immunoprecipitates were then analysed by western blotting with anti-T7 antibody (lanes 3–6, upper panel). Lanes 1, 2 show expression of T7–CARD in S100 extracts (150 μg protein). The same blot was reprobed with a monoclonal antibody against procaspase-9 (lower panel). **d**, Binding of procaspase-9 to the Affigel-bound Apaf-1 CARD in the presence of increased concentrations of Hsp70. Equal amounts of immobilized Apaf-1 CARD (0.06 nmol) were mixed with Ni^{2+}-purified procaspase-9(C287A) (0.06 nmol) in control buffer or in a buffer containing ATP, ADP or γ-S-ATP in the presence of 0, 0.03, 0.06 or 0.12 nmol of Hsp 70. After incubation, beads were washed with reaction buffer and bound proteins were eluted by urea and analysed by western blotting with anti-polyhistidine (procaspase-9, upper panel) or anti-Hsp 70 (lower panel) antibodies.

upper panel). Hsp70 efficiently immunoprecipitated the Apaf-1-CARD but not an Apaf-1 variant (Apaf-1(100–530)) lacking the CARD (lane 5). A small amount of Apaf-1(100–530) co-immunoprecipitated with Hsp70, possibly because of association of the Apaf-1(100–530) with endogenous Apaf-1 (see ref. 15). Hsp70 also significantly abolished association of Apaf-1 CARD with procaspase-9 in transfected 293T cells (Fig. 5c), indicating that binding of Hsp70 to the Apaf-1 CARD blocks the CARD–CARD interaction between Apaf-1 and procaspase-9.

We confirmed the ability of Hsp70 to block binding of procaspase-9 to the Apaf-1 CARD *in vitro*, using Affigel-bound Apaf-1 CARD and the purified procaspase-9 C287A mutant. As shown in Fig. 6d, Hsp70 inhibited the binding of procaspase-9 to Apaf-1-CARD only in the presence of ATP in a dose-dependent manner (that is, Hsp70 at double the molarity of CARD resulted in a 90% reduction in association of procaspase-9 with the immobilized polypeptide). Furthermore, the decrease in the amount of procaspase-9 associated with the Apaf-1 CARD was accompanied by an increase in Hsp70 binding to the Apaf-1 CARD (Fig. 6d, lower panel), providing further direct evidence that Hsp70 inhibits the CARD–CARD association between Apaf-1 and procaspase-9 by occupying the CARD of Apaf-1.

Discussion

One way in which Hsp70 could interfere with the apoptotic pathway is through inhibition of Apaf-1 function. Apaf-1 forms a large heteromeric complex with cytochrome *c* in an ATP/dATP-dependent manner after release of cytochrome *c* from mitochondria in response to apoptotic stimuli[11,12,15–18]. The oligomeric Apaf-1/cytochrome *c* complex can bind to, and induce autoproteolytic cleavage of, procaspase-9, indicating that oligomerization of Apaf-1 may be critical in promoting clustering of procaspase-9 and thereby inducing its activation. Our data clearly demonstrate that Hsp70 interferes specifically with the Apaf-1-mediated apoptotic pathway but not the Fas-mediated pathway. Although cell killing by Fas requires a functional Apaf-1 pathway in some cell types, such as hepatocytes[25], this is not the case with MEFs as *Apaf*$^{-/-}$ MEFs are sensitive to Fas-induced apoptosis (Fig. 3d).

Hsp70 associates directly and specifically with Apaf-1 and inhibits its activation of procaspase-9 and subsequently of caspase-3. This inhibitory effect is due in part to the ability of Hsp70 to modulate Apaf-1 oligomerization and thereby affect its interaction with procaspase-9. Immunoprecipitation and affinity-binding experiments showed that Hsp70 binds to the CARD sequence of Apaf-1 and is able to compete with procaspase-9 for binding to this domain. Although the CARD of Apaf-1 has not been shown to have a function in Apaf-1 oligomerization, it seems that binding of Hsp70 to this domain inhibits Apaf-1 oligomerization. Binding of Hsp70 to the Apaf-1 CARD could induce a conformational change in the adjacent ATP-binding pocket of Apaf-1, resulting in inhibition of Apaf-1 oligomerization. Another possibility is that association of Hsp70 with the CARD of Apaf-1 blocks oligomerization of Apaf-1 by steric hindrance.

The finding that the association of Hsp70 with Apaf-1 requires ATP indicates that the binding of ATP to Hsp70 may be critical for the association process. As predicted by this hypothesis, the interaction between these two proteins was substantially reduced in the presence of ADP and completely abolished in the presence of the nonhydrolyzable ATP analogue γ-S-ATP. Moreover, inhibition of the ATP-binding activity of Hsp70 by *N*-ethylmaleimide prevented the interaction between Hsp70 and Apaf-1, even in the presence of ATP (Fig. 2c and data not shown). These results are consistent with previous findings that the accessibility of the C-terminal substrate-binding domain of Hsp70 is conformation-dependent and is influenced by ATP binding to the N-terminal region of the protein[26]. This is also in line with the findings that deletion of the substrate-binding domain of Hsp70 prevents association with Apaf-1 and blocks the ability of Hsp70 to protect cells against etoposide- or heat-induced cell death (Fig. 3e and ref. 27).

We propose that Hsp70 indirectly inhibits cytochrome *c*/ATP-dependent activation of caspase-3 through its effect on Apaf-1-mediated activation of caspase-9. This is consistent with previous observations showing that constitutive expression of Hsp70 inhibits processing of procaspase-3 and subsequent cleavage of the common death substrate protein poly(ADP–ribose) polymerase (PARP)[22]. However, Hsp70 was unable to inhibit cleavage of PARP by recombinant mature caspase-3 *in vitro*, indicating that Hsp70 may function upstream of procaspase-3 activation. Similarly, it has

been demonstrated that overexpression of Hsp70 blocks PARP cleavage in some cells after heat shock and treatment with tumour necrosis factor-α (TNF-α)[28].

It has also been proposed that Hsp70 prevents apoptosis by other mechanisms. The anti-apoptotic effect of Hsp70 against TNFα and staurosporine is associated with inhibition of late caspase-dependent apoptotic events[28] (that is, after the release of cytochrome *c*, activation of caspase-3 and cleavage of death substrates), such as changes in the cytosolic phospholipase A2, which releases arachidonic acid from membrane phospholipids[29], and changes in nuclear morphology. Other data indicate that Hsp70 could inhibit the ATP depletion associated with cell death[30]. This raises the possibility that Hsp70 may protect mitochondrial membrane from damage and therefore prevent the efflux of cytochrome *c* to the cytoplasm[31]. However, further studies are needed to clarify both the importance of energy deprivation in the death process and the actual mechanism of action by which Hsp70 may influence it.

The ability of Hsp70 to prevent stress-induced apoptosis could limit the efficacy of cancer therapy. Expression of Hsp70 has been found to be an indicator of poor therapeutic outcome in breast cancer[32]. A function of Hsp70 in tumorigenesis has been proposed on the basis of the observation that many transformed cells exhibit elevated levels of Hsp70 (refs 32–35). Increased expression of Hsp70 can protect some cells from monocyte-induced and TNFα-induced cell death, and could therefore allow precancerous cells to escape immune surveillance[32,33]. This effect is probably a consequence of the ability of Hsp70 to prevent apoptosis, as targeted neutralization of Hsp70 expression in some cancerous cell lines promotes cell death[34–37]. The ability of Hsp70 to interfere with the mitochondrial pathway of apoptosis could provide a growth advantage to tumour cells and account for their resistance to cytotoxic treatments.

In summary, our results show that Hsp70 can prevent apoptosis by affecting signalling events upstream of procaspase-3 activation through inhibition of Apaf-1 function. The effect of Hsp70 on Apaf-1 probably accounts for its ability to provide resistance to the documented stress-induced apoptosis. Characterization of molecules that modulate the interaction between Apaf-1 and Hsp70 could be a first step towards effective antitumour therapy. □

Methods

Plasmid construction and transfection.

Complementary DNAs encoding Apaf-1 and caspase-9 variants were generated by polymerase chain reaction (PCR) and cloned in expression plasmids as described[14]. Inducible Hsp70 cDNA was cloned with an N-terminal Flag tag in pcDNA3. Transfections were carried out using standard procedures as described[14].

Purification of recombinant proteins.

Apaf-1L was expressed in Sf-9 cells with the baculovirus system. Recombinant monomeric Apaf-1 and oligomeric Apaf-1 complex were purified to homogeneity as described[15]. Apaf-530, the CARD of Apaf-1 and the procaspase-9 C287A mutant were expressed in bacteria as C-terminally His6-tagged proteins and purified to homogeneity on Ni^{2+}/nitrilotriacetic acid (NTA) and a Superose12 fast protein liquid chromatography (FPLC) column as described[14]. Purified recombinant Hsp70 (inducible form) was from Stressgen Biotechnologies (Victoria BC, Canada).

Heat-shock treatment.

293, Jurkat and HeLa cells were suspended at 1×10^6 cells per ml in closed polyethylene tubes (15 ml), heat shocked by incubation in a water bath for 1 h at 42 °C and allowed to recover for 12 h at 37 °C for maximum production of HSPs, as described[38].

Oligomerization of Apaf-1L protein.

All oligomerization reactions of Apaf-1L were carried out by incubating 4 μg Apaf-1 (~34 pmol) with 6 μg of purified recombinant Hsp70 (~80 pmol) in the presence or absence of cytochrome *c* (5 μg, ~0.4 nmol) and ATP (2.5 mM) at room temperature for 1 h in a final volume of 100 μl of oligomerization buffer I (25 mM HEPES pH 7.4, 50 mM NaCl, 10 mM KCl, 5 mM $MgCl_2$, 100 μg ml^{-1} BSA, 5% glycerol, and 0.1 mM dithiothreitol). After incubation, a further 100 μl of oligomerization buffer I was added to each sample and the reaction mixture was directly loaded onto a Superose 6 FPLC column at a flow rate of 0.2 ml min^{-1}. Aliquots containing 50 μl of the 500-μl fractions were fractionated by SDS–PAGE and analysed for the presence of Apaf-1L and Hsp70 by western blotting with their respective antibodies.

Fractionation of S100 extracts.

S100 extracts were prepared from HeLa cells as described[13] in a buffer containing 40 mM PIPES/KOH pH 7.0, 10 mM KCl, 2 mM EDTA, 3 mM dithiothreitol and protease inhibitors (20 μg ml^{-1} leupeptin, 10 μg ml^{-1} pepstatin, 10 μg ml^{-1} aprotinin and 2 mM PMSF). Extracts were then dialysed against the extraction buffer lacking KCl, but containing 5% glycerol and 0.1% (*w*/*v*) CHAPS (oligomerization buffer II). The dialysis step was essential to deplete ATP from the extracts. The protein concentration was adjusted to 2.5 mg ml^{-1} and stimulation of Apaf-1 oligomerization and activation of the caspases was carried out by addition of 2 mM $MgCl_2$, 10 μM cytochrome *c* and 2.5 mM ATP. Purified recombinant Hsp70 was added at 6 μg per mg protein of S100 extracts. This amount of Hsp70 is physiologically relevant to the endogenous expression of Hsp70 in HeLa cells at 12 h after heat shock, as measured by comparing the densities of known amounts of purified Hsp70 with different dilutions of stress-induced S100 extracts in western blots. In each reaction, 2.5 mg of S100 extract (1 ml) was loaded onto a Superose 6 column pre-equilibrated with oligomerization buffer II, and proteins were eluted from the column at a flow rate of 0.07 ml min^{-1}. From each of the 0.5-ml fractions, 0.2 ml were precipitated with 10% trichloroacetic acid and analysed by SDS–PAGE; this was followed by western blotting for Apaf-1, caspase-9 and Hsp70 using appropriate antibodies.

Caspase-3 activity was assessed in each of the column fractions by measuring the cleavage activity of the colorimetric substrate DEVD–para-nitroaniline (DEVD–pNA), using the ApoAlert CPP32 Assay Kit (Clontech). Cleavage activity in 100 μl of each fraction was observed for 5–15 min. One arbitrary unit of caspase-3 activity is defined as the amount of caspase-3 required to produce 1 pmol pNA per min at 30 °C, at a saturating substrate concentration.

Western blotting and immunoprecipitation.

Western blots and immunoprecipitations were carried out as described[14,15] using available commercial and non-commercial antibodies.

Affinity binding of Hsp70 to Apaf-1L and its CARD.

Purified versions (0.2 nmol) of monomeric Apaf-1L, oligomeric Apaf-1L, Apaf-530 and the CARD of Apaf-1 were individually coupled to 100 μl Affigel-10 (BioRad), according to the manufacturer's protocol. Each Affigel-coupled polypeptide (30 μl) was mixed with 4 μg of recombinant Hsp70 (~0.06 nmol) in oligomerization buffer I alone and in a buffer containing 2.5 mM ATP or ADP. Samples were incubated for 2 h at room temperature with continuous agitation. Beads were then washed with 5×1.5 ml of the oligomerization buffer to remove unbound proteins. Associated proteins were eluted from the Affigel-bound polypeptides with 2.5 M urea, and the presence of Hsp70 was detected by immunoblotting with its specific antibody. To verify that equal amounts of proteins were used in each of the affinity reactions, Affigel-bound polypeptides were eluted by boiling the beads in 10 mM acetic acid and then western blotting with an antibody against the polyhistidine tag. The same procedure was used to test the effect of increased concentrations of Hsp70 (0.03, 0.06 and 0.12 nmol) on the affinity binding of a recombinant procaspase-9 (0.06 nmol) to the Affigel-bound CARD of Apaf-1.

Apoptosis assays.

$Apaf^{+/-}$ or $Apaf^{-/-}$ cells (0.5×10^5 cells per well) in 12-well plates were transfected with 0.3 μg pEGFP–N1 reporter plasmid (Clontech), 1.2 μg of empty vector plasmid or plasmids encoding Flag–Hsp70 or the procaspase-9 C287A mutant, using the LipofectAMINE method. Cells were treated with etoposide (100 μM) or agonist Fas antibody (50 ng ml^{-1}) plus cycloheximide (1 μg ml^{-1}) for 10 h and then stained with Annexin V–PE (Pharmingen). Normal and apoptotic GFP-expressing cells were counted using fluorescence microscopy. The percentage of apoptotic cells in each experiment was expressed as the mean percentage of GFP-expressing cells exhibiting staining for Annexin V.

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