Heat-shock protein 70 inhibits apoptosis by preventing recruitment of procaspase-9 to the Apaf-1 apoptosome

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The cellular-stress response can mediate cellular protection through expression of heat-shock protein (Hsp) 70, which can interfere with the process of apoptotic cell death. Stress-induced apoptosis proceeds through a defined biochemical process that involves cytochrome *c*, Apaf-1 and caspase proteases. Here we show, using a cell-free system, that Hsp70 prevents cytochrome *c*/dATP-mediated caspase activation, but allows the formation of Apaf-1 oligomers. Hsp70 binds to Apaf-1 but not to procaspase-9, and prevents recruitment of caspases to the apoptosome complex. Hsp70 therefore suppresses apoptosis by directly associating with Apaf-1 and blocking the assembly of a functional apoptosome.

Apoptotic cell death is orchestrated by the activation of caspases, a family of cysteine proteases with specificity for aspartic acid residues, which cleave specific intracellular substrates to produce the characteristic features of this form of cell death[1]. In vertebrates, one of the principal mechanisms of caspase activation requires the release of cytochrome *c* from mitochondria[2], which then interacts with the CED-4 homologue Apaf-1 (ref. 3). In the presence of dATP/ATP, cytochrome *c* binds to, and triggers oligomerization of Apaf-1 (refs 4, 5). The resulting 'apoptosome' recruits and activates procaspase-9, which in turn recruits, cleaves and activates caspase-3 and caspase-7 (refs 6–8). These then mediate the execution of the cell through the selective proteolysis of key protein substrates[1].

Apoptotic cell death, which mediates the elimination of damaged or unwanted cells, seems to have evolved in the context of a far more ancient system designed to enhance cellular survival in response to adverse stress — the so called heat-shock protein (HSP) or stress response. HSPs are a family of highly conserved proteins, which are induced in both prokaryotes and eukaryotes in response to an array of physiological and environmental stresses[9]. Induction of HSPs in response to stress serves to protect against the initial insult, augment recovery, and produce a state of resistance to subsequent stress in the cell. This protective role of HSPs can be attributed to several functional properties, including prevention of protein aggregation and promotion of protein disaggregation by catalysing the refolding of damaged or denatured proteins[10–12].

The Hsp70 protein family is one of the most highly conserved and contains both constitutive and inducible forms that are localized to different intracellular organelles[9]. The heat-inducible form of Hsp70 is expressed at low basal levels but is rapidly induced by heat or other stresses and by cell-cycle progression[13]. Several recent studies have shown that Hsp70 functions to modulate the engagement and/or progression of apoptosis induced by a wide variety of stimuli[14]. Here we seek to determine the underlying mechanistic basis of Hsp70-mediated suppression of apoptosis.

Results

Stress-induced Hsp70 has been shown to block apoptosis induced by many different agents in a variety of cell types[14,15]. To investigate how this protein mediates its anti-apoptotic effects, we examined the effect of Hsp70 and a mutant, Hsp70AAAA (ref. 16) in an *in vitro* system of caspase activation[17,18]. As shown in Fig. 1a, addition of cytochrome *c* plus dATP to cytosolic extracts of Jurkat cells induced caspase activation, as determined by DEVDase activity using a synthetic substrate (Fig. 1a). Caspase activation involves cleavage of the proform of the enzymes, to generate proteolytically active subunits[1]. We used immunoblotting to demonstrate cleavage of procaspase-9 (Fig. 1b) and procaspase-3 (Fig. 1c) to their catalytically active subunits (p37/p35 and p20/p17, respectively), as induced by addition of cytochrome *c* (10 μM) and dATP (1 mM) to cytosolic extracts of Jurkat cells.

Addition of 0–4 μg of recombinant human Hsp70 (0–2.86 μM) to the extracts inhibited the ability of cytochrome *c* to induce caspase activation (Fig. 1a). In contrast, addition of Hsp70 to previously activated extracts (such as those from apoptotic cells) had no inhibitory effect on constitutive caspase activity (data not shown). Likewise, cleavage of procaspase-9 and procaspase-3 after addition of cytochrome *c* plus dATP was blocked by 5 μg Hsp70 (3.58 μM), as determined by immunoblotting (Fig. 1b, c). Similar observations were made when ATP was substituted for dATP (data not shown). Importantly, the concentration of Hsp70 required to block *in vitro* caspase activation was well within the range observed under physiological conditions, which can constitute as much as 5% of total cellular protein following heat stress. The level of Hsp70 in Jurkat cells is ~0.40 μM before stress and increases to 10 μM after heat shock (C. Schmidt and R. Morimoto, personal communication).

The extreme carboxy-terminal acidic motif of Hsp70 is vital for interaction with its target substrates as well as for functional co-operation with the regulatory ATPase domain and with co-chaperones[16]. Substitution of alanine residues for the four C-terminal amino acids (Hsp70AAAA) results in a protein with compromised ability to interact with target proteins and increased ATPase activity[16]. Furthermore, expression of Hsp70AAAA, in contrast to equivalent induction of the wild type, failed to provide resistance against heat-induced apoptosis in T cells (D.D.M., manuscript submitted). Using the cell-free system, we found that, contrary to the effects of wild-type Hsp70 (Fig. 1), addition of an equivalent concentration of Hsp70AAAA (3.58 μM) to extracts failed to suppress cytochrome *c*/dATP-mediated acti-

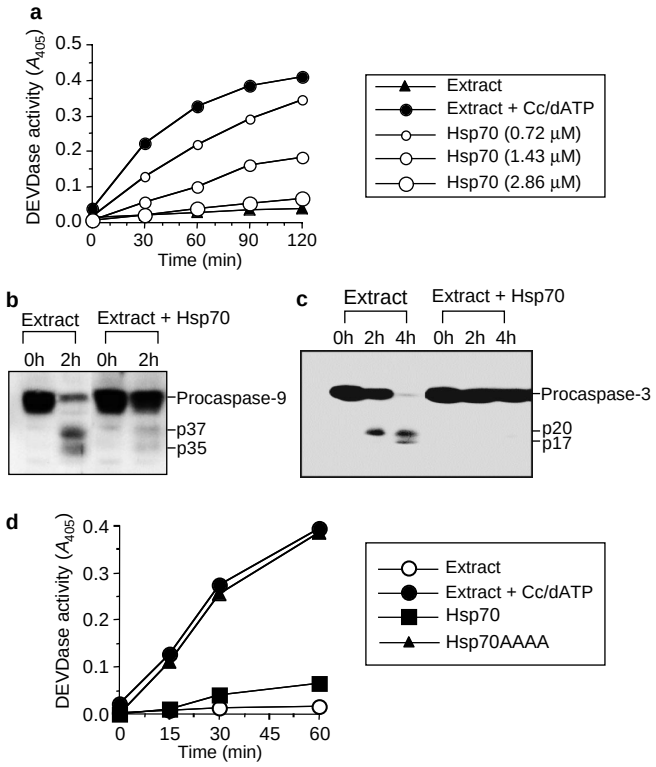

Figure 1 **Recombinant Hsp70, but not Hsp70AAAA, inhibits caspase processing *in vitro*. a**, Cytosolic extracts prepared from Jurkat T cells were incubated with cytochrome *c* (Cc; 10 μM) and dATP (1 mM) in the presence or absence of recombinant human Hsp70 (0–2.86 μM). Caspase activity was then assessed by spectrophotometric quantification of DEVD–pNA cleavage. **b**, **c**, Immunoblot analysis of procaspase-9 (**b**) and procaspase-3 (**c**) processing in extracts activated by addition of cytochrome *c* (10 μM) and dATP (1 mM) in the presence or absence of recombinant Hsp70 (3.58 μM). **d**, DEVDase activity was determined in Jurkat-cell extracts activated by exogenous addition of cytochrome *c* (10 μM) and dATP (1 mM) in the presence or absence of wild-type Hsp70 or Hsp70AAAA (3.58 μM), which lacks the ability to interact with its target substrates[16].

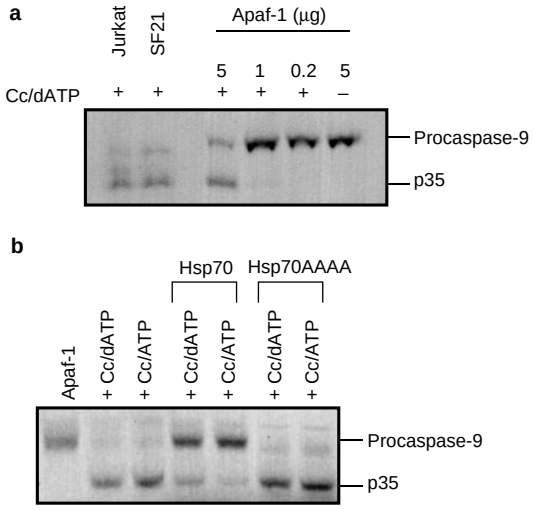

Figure 2 **Hsp70 inhibits activation of the recombinant apoptosome. a**, Recombinant Apaf-1 (5 μg), incubated in the presence of cytochrome *c* (Cc) and dATP, induces processing of [^{35}S]methionine-labelled procaspase-9 with similar efficiency to that seen in whole-cell extracts prepared from SF21 insect cells expressing Apaf-1 and from Jurkat T cells, each supplemented with 10 μM cytochrome *c* and 1 mM dATP. **b**, Co-addition of recombinant Hsp70 inhibits processing of procaspase-9 induced by either dATP or ATP in the presence of cytochrome *c*. However, Hsp70AAAA fails to inhibit the caspase-processing ability of the recombinant apoptosome.

vation of caspases, as determined by DEVDase activity (Fig. 1d). These results were confirmed by immunoblotting for caspase-3 and caspase-9 (data not shown). Suppression of caspase activation is therefore dependent upon the ability of Hsp70 to bind to target protein substrates.

Caspase activation involves assembly of the 'apoptosome' complex, which is initiated by association of cytochrome *c* with Apaf-1, to induce oligomerization of the latter protein in a dATP/ATP-dependent manner[4–8]. Caspase-9 is recruited to this partially formed complex where it is autoactivated[4,5,7,8] (a process that can be seen as cleavage of procaspase-9, although this may not be essential[19]). Activated caspase-9 then recruits, cleaves and activates procaspase-3 and procaspase-7 (refs 6–8). We therefore sought to determine whether the observed inhibitory effects of Hsp70 were the result of a direct effect on the apoptosome complex, and were not reliant on an intermediary target that may have been present in the cell extracts used for *in vitro* analysis of caspase activation. To do this, we reconstituted the apoptosome complex, using recombinant proteins as described[5].

As shown in Fig. 2a, Apaf-1 produced using the baculovirus system and then purified was capable of inducing activation and cleavage of procaspase-9 transcribed and translated *in vitro*, upon addition of cytochrome *c* (10 μM) and dATP (1 mM). In titrations with increasing amounts of Apaf-1, a minimum of 5 μg Apaf-1 was required to achieve procaspase-9 processing, as effectively as that observed in whole-cell extracts from SF21 insect cells expressing Apaf-1 and from Jurkat T cells, each supplemented with cytochrome *c* and dATP (Fig. 2a). We then evaluated the effects of Hsp70 and of Hsp70AAAA in the recombinant apoptosome. As illustrated in Fig. 2b, addition of 10 μg Hsp70 completely inhibited the processing of procaspase-9 by Apaf-1 and cytochrome *c* in the presence of dATP or ATP. In contrast, addition of an equivalent amount of Hsp70AAAA had no inhibitory effect on caspase-9 processing. We conclude that Hsp70 may exert its effect through a direct interaction with one or more members of the apoptosome complex.

We then sought to determine which component(s) of the apoptosome associate with Hsp70 to mediate the observed anti-apoptotic effects. In our initial experiments we used recombinant components to determine any direct interactions. We immobilized Apaf-1 on nickel beads and then incubated it in the presence or absence of cytochrome *c* and dATP, with or without Hsp70 or Hsp70AAAA. As a control, we incubated caspase-9, also immobilized on nickel beads, under identical experimental conditions. Immunoblotting for Hsp70 showed that Apaf-1 associated with Hsp70 in the presence or absence of cytochrome *c* and dATP, but did not associate with Hsp70AAAA protein in pull-down experiments (Fig. 3a). In contrast, we observed no binding of recombinant procaspase-9 to either Hsp70 or Hsp70AAAA under similar conditions. These results show that Apaf-1 and Hsp70 can bind effectively to each other in a specific manner (that is, non-specific binding by either Apaf-1 or Hsp70 is excluded under these conditions).

To examine the interaction of endogenous proteins at physiologically relevant levels, we compared the constitutive expression of Hsp70 with Hsp70 expression after transfection with an Hsp70–pcDNA3 expression construct in Jurkat T cells, MCF-7 breast-carcinoma cells and 293T cells (Fig. 3b, upper panel). Stripping and reprobing the blot with an anti-actin antibody confirmed equal protein loading (Fig. 3b, lower panel). Quantification of the amount of Hsp70 in each of these cell lines generated values ranging from 0.4

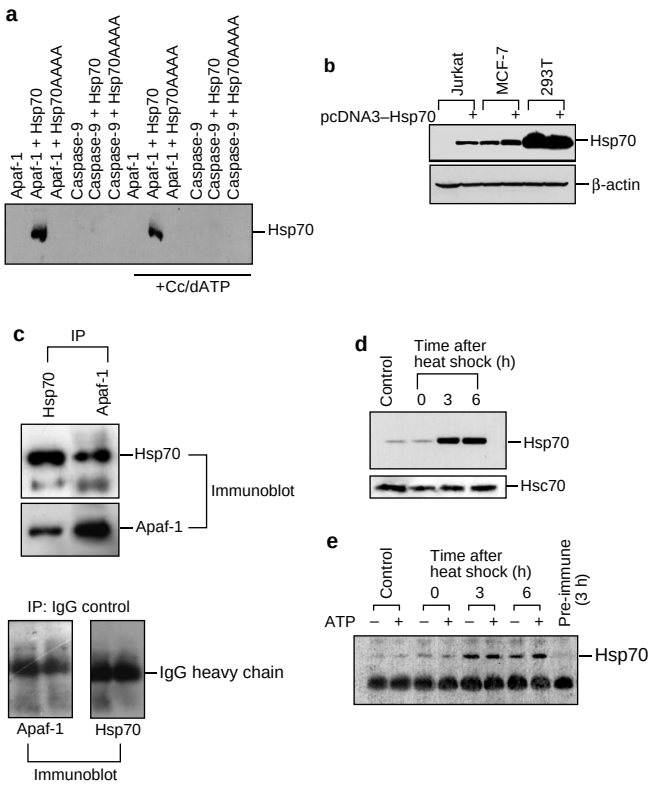

Figure 3 **Hsp70 directly and selectively associates with Apaf-1 but not with other apoptosome components. a**, Recombinant Apaf-1 or full-length caspase-9 (1 μg) were immobilized on Ni^{2+} and incubated with Hsp70 or Hsp70AAAA (2 μg) in the presence or absence of cytochrome *c* (Cc; 10 μM) and dATP (1 mM). Associated Hsp70 was detected by immunoblotting. Wild-type Hsp70 associated with recombinant Apaf-1 but not with caspase-9, whereas Hsp70AAAA associated with neither. **b**, Several cell lines were examined for Hsp70 before and after transfection with a construct expressing Hsp70 (5 μg; upper panel). β-actin served as a loading control (lower panel). **c**, Endogenous Apaf-1 was immunoprecipitated (IP) from 293T extracts using an antibody against part of the WD40 domain of Apaf-1xL and probed for associated proteins with an anti-Hsp70 antibody. Constitutive Hsp70 was immunoprecipitated using a monoclonal antibody against Hsp70 and probed for associated proteins with a monoclonal anti-Apaf-1 antibody (upper panels). The relevant immunoglobulin G (IgG) controls were used for immunoprecipitations and were probed for Hsp70 and Apaf-1 (lower panels). **d**, Immunoblot showing that Jurkat cells express increased levels of inducible Hsp70 after heat shock (45 min at 42 °C) and recovery at 37 °C for up to 6 h (upper panel). Hsc70, the non-inducible counterpart of Hsp70, served as a loading control (lower panel). **e**, Immunoblot showing that Hsp70 induced by heat stress co-precipitates with endogenous Apaf-1 immunoprecipitated from extracts of heat-shocked cells.

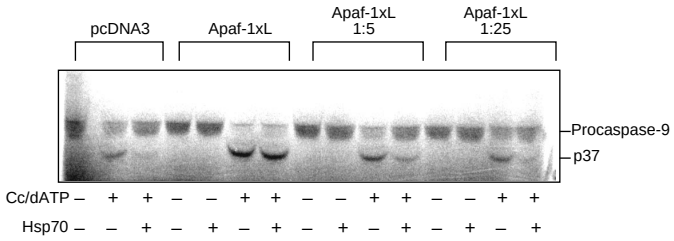

Figure 4 **Apaf-1 is the functional target of Hsp70 for inhibition of caspase activation.** Extracts were prepared from 293T cells 24 h after transfection with a construct expressing Apaf-1xL or with empty vector (pcDNA3; 5 μg total DNA per plate in each case). Extracts from cells overexpressing Apaf-1xL were serially diluted as indicated with vector-control extracts (to reduce the amount of Apaf-1xL) and the inhibitory effect of exogenous Hsp70 (5 μg) on caspase activation mediated by cytochrome *c* (Cc; 10 μM) and dATP (1 mM) was assessed by monitoring the processing of *in vitro*-translated procaspase-9.

μM in Jurkat cells and 4.5 μM in MCF-7 cells to 80 μM in 293 cells (C. Schmidt and R. Morimoto, personal communication). Given that 293T cells express large amounts of Hsp70 under constitutive conditions (Fig. 3b), as do many different tumour types[15], we did not need to increase levels of Hsp70 by transfection in order to study its interaction with target proteins in intact cells. Instead, taking advantage of the elevated level of endogenous Hsp70 in 293T cells, we immunoprecipitated endogenous Apaf-1 using an antiserum against the WD motif found in the functional Apaf-1xL isoform[20]. This demonstrated a co-association between Apaf-1 and endogenous Hsp70 (Fig. 3c, upper panels). The predominant band below Hsp70 corresponds to the antibody heavy chain. Conversely, immunoprecipitation of Hsp70 using a specific monoclonal antibody against the inducible form of Hsp70 effectively precipitated endogenous Apaf-1 in 293T extracts. No detectable caspase-9 was found after stripping and reprobing with an anti-caspase-9 antibody (data not shown), which is consistent with our results obtained using recombinant proteins (Fig. 3a). Relevant immunoglobulin G controls were also used in immunoprecipiations, and failed to co-associate with either Hsp70 or Apaf-1 (Fig. 3c, lower panels).

We confirmed the intracellular association of Apaf-1 and Hsp70 using Jurkat cells, which constitutively express minimal amounts of Hsp70 (~0.4 μM; Fig. 3b, d and data not shown), but which effectively increase their Hsp70 levels to ~10 μM following heat stress (42 °C for 45 min) and recovery at 37 °C (Fig. 3d, upper panel). In contrast, Hsc70, the constitutively expressed counterpart of Hsp70, was not induced by heat stress and served as a loading control (Fig. 3d, lower panel). Immunoprecipitation of Apaf-1 from extracts prepared at various times after heat shock precipitated Hsp70 from those extracts with elevated Hsp70 levels (Fig. 3e). Pre-immune serum failed to precipitate Hsp70 from extracts prepared 3 h after heat shock. These observations highlight the selective and specific interaction between Hsp70 and Apaf-1 at their physiologically expressed levels.

If Hsp70 suppresses caspase activation by targeting Apaf-1, then increasing the concentration of Apaf-1 in extracts should overwhelm the inhibitory effect of Hsp70. To test this argument, we transfected 293T cells with a construct expressing the functional isoform of Apaf-1 (Apaf-1xL–Myc) or with empty vector (pcDNA3). We prepared extracts 24 h after transfection, and determined their capacities to process *in vitro*-translated procaspase-9 in the presence of cytochrome *c* and dATP. Figure 4 shows that extracts prepared from cells overexpressing Apaf-1xL (confirmed by immunoblotting for the c-Myc tag; data not shown) exhibited an enhanced response to addition of cytochrome *c* and dATP. Exogenous addition of Hsp70 (3 μM) completely prevented procaspase-9 processing in vector-transfected extracts, but had no effect on the enhanced caspase activity in Apaf-1xL-transfected extracts. We then diluted the Apaf-1xL-overexpressing extract (which shows enhanced caspase activity and is refractory to inhibition by Hsp70 at levels sufficient to prevent cleavage of endogenous caspases by addition of cytochrome *c* and dATP) with extracts prepared from vector-transfected cells at Apaf-1/vector ratios of 1:5 and 1:25. Reducing the level of Apaf-1xL by dilution with control-transfected extract restored sensitivity to inhibition by exogenous addition of Hsp70. In contrast, we found that addition of higher concentrations of either cytochrome *c* or procaspase-9 to extracts had little or no effect on the inhibition of caspase activation by Hsp70 (data not shown). We conclude that Apaf-1 is the functional target of Hsp70 in the inhibition of caspase activation in this system.

The ability of Hsp70 to bind to Apaf-1 raised the possibility that inhibition of procaspase-9 activation is mediated by simple compe-

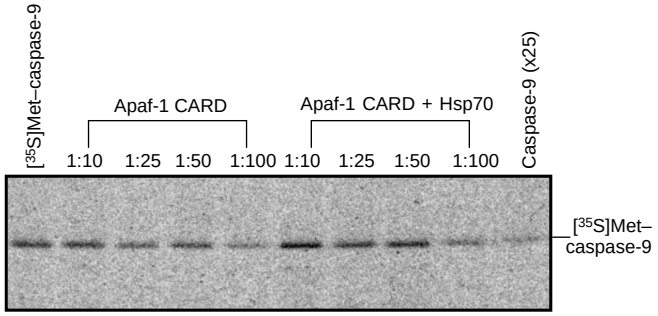

Figure 5 **Hsp70 does not compete with procaspase-9 for binding to the Apaf-1 CARD.** GST-tagged Apaf-1 CARD was immobilized on glutathione beads (2.5 mg ml^{-1}) and serially diluted as indicated to generate a range of 0.025–2.5 μg total GST–CARD per reaction. It was then incubated with *in vitro*-translated procaspase-9 in the presence or absence of Hsp70 (5 μg). Associated procaspase-9 was visualized by SDS–PAGE and autoradiography.

tition for Apaf-1. Apaf-1 and procaspase-9 interact through a region of each molecule called the caspase-recruitment domain (CARD). We therefore generated the Apaf-1 CARD as a recombinant protein tagged with glutathione-*S*-transferase (GST) and examined its binding to procaspase-9 transcribed and translated *in vitro*, under experimental conditions in which Hsp70 can inhibit activation of the caspase (the Apaf-1 CARD alone does not activate the caspase, but does bind to it). As shown in Fig. 5, the Apaf-1 CARD effectively bound to and precipitated procaspase-9. Addition of Hsp70, however, had no effect on this binding, even at concentrations of the Apaf-1 CARD that were limiting for detection of binding to caspase-9 (note that even at the highest amount of GST–CARD used, Hsp70 was in molar excess). Displacement of [^{35}S]methionine-labelled caspase-9 by a 25-fold excess of cold caspase-9 (translated *in vitro* in the absence of [^{35}S]methionine) demonstrated the potential for competition under these experimental conditions. We conclude, therefore, that Hsp70 does not inhibit Apaf-1 function simply by competing with caspase-9 for the CARD of Apaf-1.

As discussed, activation of caspases by cytochrome *c* involves induction of Apaf-1 oligomerization and recruitment of procaspases to the complex. We next sought to determine whether Hsp70 influences one or both of these events. Either situation could prevent the formation of a functional apoptosome and would be consistent with our data. To examine apoptosome assembly, we activated THP.1 extracts containing cytochrome *c* by addition of dATP in the presence or absence of Hsp70 or HspAAAA, and fractionated them by size-exclusion chromatography. We measured DEVDase activity fluorometrically using DEVD.AFC as a caspase substrate[6,7]. Figure 6a shows the fractionation of Apaf-1, procaspase-9 and procaspase-3 (detected by immunoblotting with specific antibodies) in non-activated cell extracts; Apaf-1 was eluted as a preapoptosome complex of relative molecular mass 200,000–300,000 (M_r 200–300K)[6,7]. No detectable DEVDase activity was observed (data not shown). However, addition of 2 mM dATP induced an apparent increase in the size of Apaf-1, as almost all of it was eluted as a M_r ~700K apoptosome complex (Fig. 6b). This observed increase represents oligomerization of Apaf-1 and is consistent with results from several previous studies[4–7]. Exogenous addition of Hsp70 (6 μM) had no effect on the formation of this multimeric Apaf-1 apoptosome complex (Fig. 6c).

Oligomerization of Apaf-1 is associated with sequential recruitment, activation and release of catalytically competent caspases, which can be observed as a redistribution of procaspase-3 and procaspase-9 and their active subunits after addition of dATP to cytosolic extracts[4–7]. We were able to detect both caspase-9 and caspase-3 in association with the apoptosome complex, and these

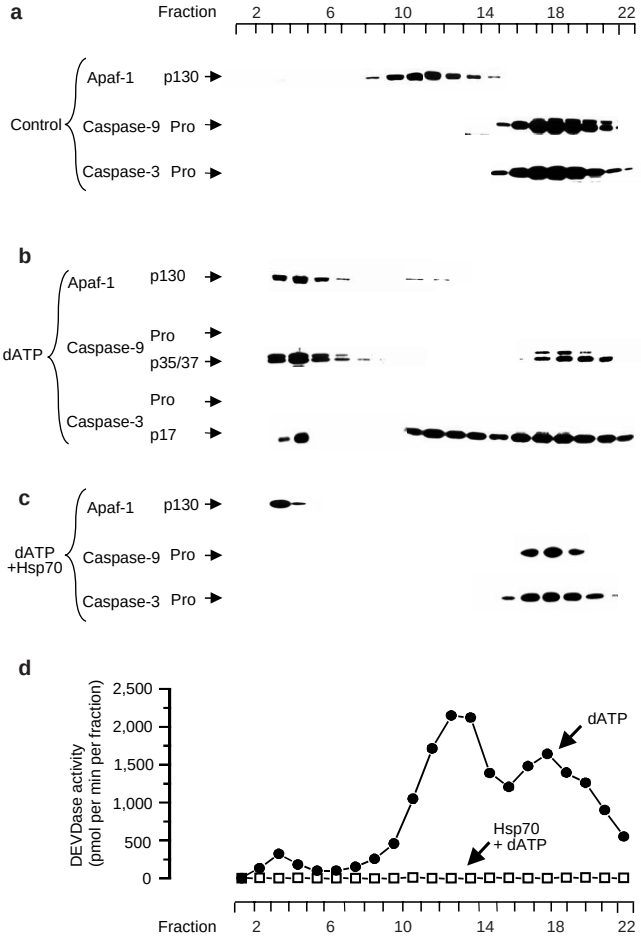

Figure 6 **Hsp70 inhibits dATP-dependent caspase activation by blocking recruitment of procaspase-9 to the apoptosome. a–c**, Control lysates (10 mg ml^{-1}) prepared from THP.1 cells[6,7] (**a**) were activated by addition of 2 mM dATP and incubation for 1 h at 37 °C (**b**), or were pre-incubated with recombinant Hsp70 (6 μM) for 30 min at 5 °C before activation with dATP (**c**). After treatment, lysates were fractionated by gel-filtration chromatography (see Methods) and Apaf-1, caspase-3 and caspase-9 were detected in the various fractions by immunoblotting. Pro, unprocessed procaspase. **d**, Caspase activity in each fraction was measured fluorimetrically using DEVD.AFC as a substrate. Data are expressed as pmol per min per fraction. In control lysates (**a**), Apaf-1 was eluted as a complex of M_r 200–300K (fractions 9–15), whereas procaspase-9 and procaspase-3 were eluted as free caspases (M_r 60–90K; fractions 16–22). After dATP activation (**b**), Apaf-1 was found in the apoptosome complex (M_r ~700K), which contained processed caspase-9 and caspase-3 (fractions 4–7). Processed caspase-3 was also detected as a M_r 200–300K microapoptosome complex (fractions 9–15) and as free active caspases (fractions 16–22, which also contained processed caspase-9). Hsp70 did not affect the oligomerization of Apaf-1 into the M_r ~700K complex but did block processing and recruitment of caspase-9 and caspase-3 to this complex (**c**). The profile of caspase activity in the dATP-activated extract correlated with the detection of 'recruited' or processed caspases (**d**, circles), which, consistent with Hsp70-mediated inhibition of caspase recruitment (**c**), was almost completely inhibited after pre-incubation of extracts with Hsp70 before addition of dATP (**d**, squares).

were cleaved into their active forms (p37/p35 and p17, respectively) after addition of dATP (Fig. 6b). The distribution of active caspase-3 was paralleled by the profile of DEVDase activity (Fig. 6d). Exogenous addition of Hsp70 completely blocked the recruitment of both procaspase-9 and procaspase-3 to the apoptosome

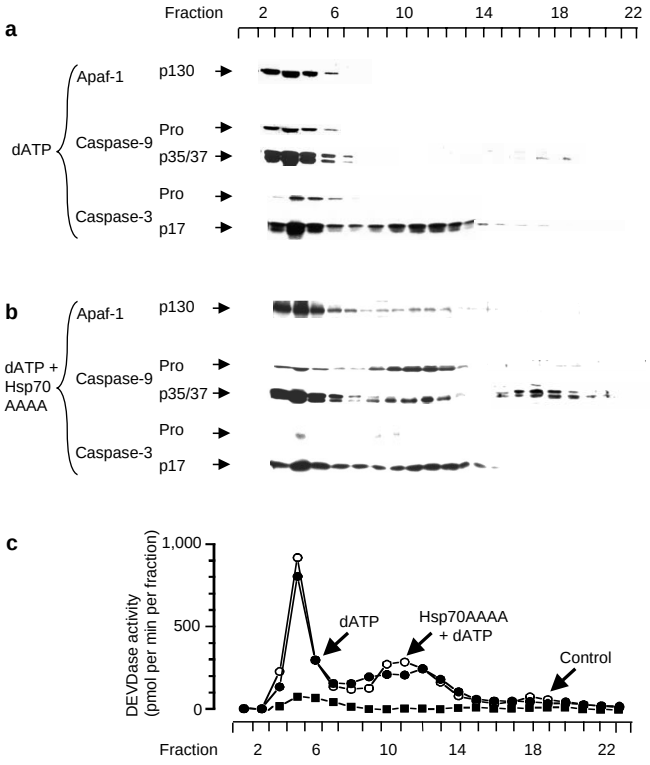

Figure 7 Hsp70AAAA does not inhibit recruitment of procaspases to the apoptosome or *in vitro* caspase processing. a, **b**, Immunoblotting of gel-fractionated cell lysates as in Fig. 6. In contrast to the effects of Hsp70 in preventing recruitment and activation of caspases (Fig. 6), Hsp70AAAA, which does not interact with Apaf-1 (Fig. 3a), failed to block both dATP-induced oligomerization of Apaf-1 and recruitment and processing of caspase-9 and caspase-3. Consistent with the idea that distribution of caspases is unchanged in the presence of Hsp70AAAA (**b**), induction of DEVDase activity by dATP (**c**, filled circles) was also completely unaffected by addition of Hsp70AAAA (**c**, open circles). Unactivated control extract showed no DEVDase activity (**c**, squares).

as well as their subsequent processing/activation (Fig. 6c), which was reflected by an almost complete absence of detectable DEVDase activity (Fig. 6d).

In contrast, Hsp70AAAA, which had no inhibitory effect on caspase activation *in vitro* (Figs 1d, 2), and no ability to bind to Apaf-1 (Fig. 3a) had no effect on either Apaf-1 oligomerization or recruitment and activation of the caspases (Fig. 7). Addition of dATP to the cytochrome *c*-containing extract induced oligomerization of Apaf-1 and recruitment and activation of caspases (Fig. 7a). Addition of Hsp70AAAA had no effect on the distribution of any of these components or on the activation of the caspases (Fig. 7b). The DEVDase profile after addition of dATP was unchanged by inclusion of Hsp70AAAA (Fig. 7c), and paralleled the immunoblot profile of active caspase-3 in each case.

Thus, by binding to Apaf-1, Hsp70 may prevent recruitment of procaspase-9 to the apoptosome complex (seen as a redistribution in the fractionation profiles). Although we detected Hsp70 in fractions containing oligomerized Apaf-1 after activation with dATP, it was also present in other fractions, which is consistent with its normal physiological function (data not shown).

Although Hsp70 allowed formation of the Apaf-1/cytochrome *c* aggregate, it prevented recruitment and activation of procaspase-9, which was dependent upon the ability of Hsp70 to interact with its target substrates (as Hsp70AAAA showed no binding or inhibition of caspase recruitment or activation). Recent studies have shown that recruitment of procaspase-3 to the apoptosome depends on recruitment of procaspase-9 (refs 6–8). Hsp70 is therefore able to block the pathway that leads to activation of caspase-3, providing a mechanism for its ability to inhibit apoptosis. This would help to explain how Hsp70 also blocks recruitment of procaspase-3 to the apoptosome complex and its activation of this caspase (Fig. 6c, d).

Discussion

The protective capacity of HSPs is classically defined by their ability to confer a state of 'thermotolerance', wherein exposure to a mild heat shock renders cells transiently resistant to subsequent, more damaging, high temperatures[11,12,21]. Furthermore, the acquisition of thermal tolerance, either by exposure of cells to a sublethal temperature or by overexpression of HSPs, specifically Hsp70 and Hsp27, is able to confer a more general resistance, by inhibiting apoptosis in response to a variety of stimuli[14,15]. These stimuli include increased temperature[22,23], chemotherapeutics[24], ultraviolet radiation[25] and tumour necrosis factor (TNF)[26]. However, an exception is provided by Fas/FasL-mediated apoptosis, which can proceed independently of the release of cytochrome *c* from mitochondria[27,28]. We have also found that heat-shock-induced thermotolerance of CEM cells (which induces high levels of Hsp70) protects these cells from stress-induced apoptosis (such as etoposide-induced apoptosis, which was inhibited by ~50%) but, if anything, slightly enhances cell death induced by anti-Fas antibody (apoptosis was increased by 20%).

The failure of Hsp70 to block Fas-induced death is consistent with our observations that Hsp70 inhibits apoptosis at the level of the apoptosome. Notably, Hsp27, which does not inhibit caspase activation in our cell-free system (data not shown), is reported to suppress apoptosis induced by Fas/FasL interaction[29]. These observations favour the idea that coordinated induction of HSPs in response to stress provides intervention at several points in the apoptotic pathway in order to maintain cellular survival.

Our results indicate that Hsp70 prevents cell death by interfering with the ability of cytochrome *c* and Apaf-1 to recruit procaspase-9. This is supported by the observation that the anti-apoptotic function of Hsp70 acts before processing and activation of procaspase-3 (refs 23, 30). In another study[31], despite increased clonogenic cellular survival, cells overexpressing Hsp70 showed no significant differences in mitochondrial release of cytochrome *c*. Our observations place one anti-apoptotic action of Hsp70 between these two events, that is, downstream of cytochrome *c* release and upstream of procaspase-3 activation.

Hsp70, but not Hsp70AAAA, binds to Apaf-1 (Fig. 3) and inhibits recruitment of procaspase-9 to the oligomerized Apaf-1 apoptosome (Fig. 6). Increasing the intracellular level of Apaf-1 by transfection overrides the inhibitory effect of exogenously added Hsp70, which is consistent with Apaf-1 being the functional target of the inhibitory effects of Hsp70 (Fig. 4). However, this does not seem to be the result of direct competition between Hsp70 and procaspase-9 for association with the Apaf-1 CARD (Fig. 5). Instead, it is possible that Hsp70 binds to Apaf-1 and inhibits a conformational change in the latter that would expose the CARD, an event that may be required for recruitment and activation of procaspase-9.

Many tumour lines constitutively express high levels of Hsp70, including 293T cells, which were used in some of our studies. As shown in Fig. 3b, immunoprecipitation of constitutively expressed Hsp70 from 293T extracts co-precipitated endogenous Apaf-1. Despite an increased constitutive level of Hsp70, some Apaf-1 function remained in the extract, as shown by the ability of cytochrome *c* plus dATP to activate caspase-9 processing (Fig. 4). However, addition of a relatively small amount (4μM) of Hsp70 to this extract completely inhibited any detectable Apaf-1 activity. It is therefore likely that a majority of the constitutively expressed Hsp70 in this cell is already committed to binding of other protein substrates and is unavailable

for interactions with new substrates. It is only upon further addition of Hsp70, either exogenously as in our experiment, or during recovery from heat shock, that the apoptotic machinery is completely bound and inhibited. It may not be possible to predict, solely from the levels of Hsp70 protein constitutively expressed in a transformed cell, whether the apoptotic pathway will necessarily be blocked.

However, Hsp70 may intervene at several points to halt progression of the apoptotic cascade. Other studies have indicated that at least some of the anti-apoptotic activity of Hsp70 can be attributed to its ability to suppress the activity of JUN-kinase[23,32]. Alternatively, Hsp70 may also act to modulate the involvement of mitochondria in apoptosis[33,34].

The ability of Hsp70 to block the recruitment and activation of procaspase-9 has implications for cellular survival in response to oncogenic signals that induce cell death. Transformation by c-Myc is offset by apoptosis[35] and involves release of cytochrome *c* and activation of caspases[36]. Fibroblasts lacking either Apaf-1 or procaspase-9, which show a reduced propensity to undergo apoptosis[37,38], exhibit enhanced survival and transformation by c-Myc and other oncogenes, even at low serum concentrations that favour cytochrome *c* release[39]. Likewise, the ability of Hsp70 to block formation of the Apaf-1/procaspase-9 complex may promote survival and transformation during oncogenesis. In fact, increased expression of Hsp70 is observed in a variety of tumour types and is linked to increased oncogenic potential *in vivo*[15].

The cellular-stress response seems to significantly predate the development of apoptosis, as the latter is seemingly restricted to metazoans (or at least eukaryotes). However, both mechanisms are engaged in response to stress (albeit one being protective and one potentially lethal). It is therefore likely that the apoptotic process evolved with an inherent susceptibility to intervention by one or more of the HSPs at different points in the apoptotic cascade. Potentially, then, this could result in a stress-response system that either directly activates cell death when it 'detects' that the damage can no longer be contained by its efforts[27,40], or intervenes to prevent the activation or progression of the apoptotic cascade. It seems likely that interplay between the stress response and apoptosis regulates cellular survival in response to damage. We have identified one such interface in the control of cytochrome *c*-induced formation of the apoptosome. However, given the complexity of each of the systems in question, there are probably many potential points of intervention that coordinate the survival or demise of cells in response to a given type and/or amount of damage. □

Methods

Purification of recombinant proteins.

An Apaf-1xL complementary DNA was obtained from G. Nunez[20] and subcloned into the pFASTBACHTb plasmid (Gibco). SF21 insect cells were then transfected with pFASTBACHTb–Apaf-1xL to produce recombinant baculovirus. SF21 cells were infected with Apaf-1xL baculovirus and recombinant Apaf-1 was purified using Ni^{2+}–sepharose 40–45 h after transfection. Purified protein was dialyzed against HEPES-buffered saline (HBS) containing 5% glycerol and 1 mM dithiothreitol and then used immediately for caspase-9-activation assays. Purified recombinant Hsp70 was from StressGen (Victoria BC, Canada) and Hsp 70AAAA was generated as described[16]. Caspase-9 and the Apaf-1 CARD were expressed in bacteria as fusion proteins with 6×-His and GST, respectively, and purified on nickel or glutathione–sepharose as described[41]. Procaspase-9 transcribed and translated *in vitro* was generated according to the manufacturer's instructions (Promega).

Transfection of 293T cells.

Cells (293T) were seeded onto 20-cm dishes (Becton Dickinson) before transfection the next day using a standard calcium-phosphate transfection procedure. Cells were transfected with pcDNA3 expression constructs containing either Apaf-1xL (5 μg) or empty vector.

Heat shock of Jurkat cells.

Jurkat cells (5×10^5–1×10^6 cells per ml) were sealed in 100-ml flasks and submerged in a preheated waterbath for 45 min at 42 °C. Cells were then allowed to recover at 37 °C for up to 6 h, during which time aliquots of cells were removed for preparation of cytosolic extracts required for immunoblotting and immunoprecipitation as indicated.

Immunoblotting and immunoprecipitation.

Antibodies used for immunoblotting and immunoprecipitation were as follows: specific monoclonal antibodies against Hsp70 and Hsc70 were from StressGen. Apaf-1 was immunoprecipitated using a polyclonal antiserum generated by immunizing rabbits with a peptide corresponding to the 13th WD repeat in Apaf-1xL (amino acids 823–865), and Apaf-1 was immunoblotted with a monoclonal antibody provided by Y. Lazebnik (Cold Spring Harbor Laboratory, New York)[42]. Caspase-3 (Pharmingen) and β-actin (ICN) were detected using commmercially available antibodies; caspase-9 was visualized using a rabbit polyclonal antibody against recombinant caspase-9 (ref. 43).

After SDS–PAGE and transfer of proteins to nitrocellulose (HighBond, Amersham), immunoblotting was carried out according to previously published methods[43] and proteins were visualized using enhanced chemiluminescence (ECL) according to the manufacturer's instructions (Amersham). Immunoprecipitation from extracts isolated from 293T or Jurkat cells used 300–500 μg of the total protein, to which either anti-Apaf-1 or anti-Hsp70 antibodies were added (previously immobilized on a 1:1 mixture of protein-A and protein-G beads (Pharmacia) by overnight incubation at 4 °C). Pre-immune serum was used as a control. Samples were incubated for 3–4 h at 4 °C before extensive washing of beads with HEPES-buffered saline (HBS). Precipitated proteins were analysed by SDS–PAGE and immunoblotting as described[43].

Quantification of Hsp70 levels.

Levels of Hsp70 were determined by quantitative western blotting. Whole-cell extracts were analysed by SDS–PAGE in which the adjacent lanes contained purified recombinant Hsp70 over a 100-fold range in concentration[16]. Immunoblotting was then carried out as described above. Chemiluminesence film was quantified by densitometric analysis and levels of Hsp70 were determined by interpolation relative to standards.

Preparation of cell extracts and measurement of *in vitro* caspase activity.

Cells (1×10^6–5×10^8) were collected and washed twice in ice-cold PBS and once in ice-cold cytoplasmic-extraction buffer (CEB; 50 mM PIPES pH 7.4, 50 mM KCl, 5 mM EGTA, 2 mM $MgCl_2$, 1 mM dithiothreitol, 10 μM cytochalasin B, 1 μg ml^{-1} leupeptin and 1 μg ml^{-1} aprotinin). The cell pellet was resuspended in an equal volume of CEB, incubated on ice for 15 min and lysed by ~25 strokes of a dounce homogenizer. Adequate cellular disruption to ensure complete release of nuclei was monitored by Hoechst staining and fluorescence microscopy. Lysates were then centrifuged for 15 min at 15000*g* and the supernatant was assessed for protein content (Pierce) and stored in aliquots at −70 °C.

Activation of endogenous caspases was induced by addition of 1 mM dATP/ATP and 10 μM horse-heart cytochrome *c* (Sigma) to cytosolic extracts (200-300 μg protein equivalent) in the presence of recombinant Hsp70 (StressGen) or Hsp70AAAA[16] where indicated, in a total volume of 20–100 μl, and incubation at 37 °C. In some experiments, 1–2 μl of *in vitro*-translated, [^{35}S]methionine-labelled procaspase-9 was also added. Protein aliquots (50 μg) were removed at various times for assessment of caspase processing by immunoblot analysis, SDS–PAGE and autoradiography to visualize cleavage of [^{35}S]methionine-labelled procaspase-9, or for quantification of DEVDase activity as follows: 10–50-μl aliquots of the reaction mix were brought up to 100 μl with DEVD assay buffer (20 mM PIPES pH 7.4, 100 mM NaCl, 1 mM EDTA, 10 mM dithiothreitol, 0.1% CHAPS and 10% sucrose), 0.2 mM DEVD–pNA colorimetric substrate (BioMol) was added and absorbance at 405 nm was monitored at 15–30-min intervals for 1–2 h.

In vitro reconstitution of the apoptosome using recombinant components.

Each batch of recombinant Apaf-1 prepared was titrated in the presence of cytochrome *c* (10 μM), dATP or ATP (1 mM) and [^{35}S]methionine-labelled procaspase-9 in DEVD assay buffer to determine the active concentration of the protein. Typically, 2–5 μg purified protein was sufficient to generate detectable cleavage of [^{35}S]methionine-labelled procaspase-9 under these conditions. The effect of added recombinant Hsp70 or Hsp70AAAA was then assessed.

In vitro association of recombinant apoptosome components.

Recombinant Apaf-1 or caspase-9 (~1 μg), previously immoblized on Ni^{2+} by incubation in HBS for 60 min at 4 °C, was incubated with or without cytochrome *c* (10 μM) and ATP (1 mM) in the presence or absence of Hsp70 (2 μg) or Hsp70AAAA (2 μg) for 2–3 h at 4 °C in HBS. Beads were washed extensively with HBS to remove non-specifically bound proteins and then analysed by SDS–PAGE and immunoblotted for Hsp70.

In vitro association of the Apaf-1 CARD with procaspase-9.

GST–CARD was prepared as described above and pre-immobilized on glutathione–sepharose overnight in HBS. After extensive washing in PBS/0.3% Tween to remove unbound protein, GST–CARD was incubated with 0.5 μl *in vitro*-translated, [^{35}S]methionine-labelled procaspase-9 at the indicated dilutions in a total volume of 200 μl, in the presence or absence of excess Hsp70 (5 μg) for 2 h at 4 °C. After extensive washing to remove any non-specific interactions, the remaining proteins were separated by SDS–PAGE and analysed by autoradiography.

Gel-filtration analysis of apoptosome assembly.

Lysates were prepared from THP.1 cells (10 mg ml^{-1}) and endogenous caspases were activated by addition of 2 mM dATP for 1 h at 37 °C, in the presence or absence of recombinant Hsp70 (6 μM) or Hsp70AAAA (6 μM; Hsp70 proteins were incubated for 30 min at 5 °C before dATP addition). Caspase activity was measured fluorimetrically using DEVD.AFC as a substrate[6,7]. After treatment, lysates were fractionated by gel-filtration chromatography using a HiPrep 16/60 S-300 Sephacryl high-resolution column and a fast protein liquid chromatography (FPLC) system (Amersham) as described[6,7]. The column was equilibrated with 5% (*w*/*v*) sucrose, 0.1% (*w*/*v*) CHAPS, 20 mM HEPES/NaOH and 5 mM dithiothreitol, pH 7.0, and eluted at a flow rate of 0.5 ml (5 °C); 2-ml fractions were then collected. Column calibration was carried out with an HMW gel-filtration protein standards kit (Amersham) and a Ni^{2+}-column-purified, His-tagged caspase-3. Appropriate fractions were concentrated fivefold in Vivaspin 4 (M_r 10K cutoff) concentrators (Vivascience, Stonehouse, UK); 30-μl aliquots were then analysed for Apaf-1, caspase-9 and caspase-3 by SDS–PAGE and immunoblotting.

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