

# 'The stress of dying': the role of heat shock proteins in the regulation of apoptosis

Helen M. Beere

La Jolla Institute for Allergy and Immunology, 10355 Science Center Drive, San Diego, CA 92121, USA  
(e-mail: hmbere@aol.com)

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## Summary

Heat shock proteins (Hsps) are a family of highly homologous chaperone proteins that are induced in response to environmental, physical and chemical stresses and that limit the consequences of damage and facilitate cellular recovery. The underlying ability of Hsps to maintain cell survival correlates with an inhibition of caspase activation and apoptosis that can, but does not always, depend upon their chaperoning activities. Several mechanisms proposed to account for these observations impact on both the 'intrinsic', mitochondria-dependent and the 'extrinsic', death-receptor-mediated pathways to apoptosis. Hsps can inhibit the activity of pro-apoptotic Bcl-2 proteins to prevent permeabilization of the outer mitochondrial membrane and release of apoptogenic

factors. The disruption of apoptosome formation represents another mechanism by which Hsps can prevent caspase activation and induction of apoptosis. Several signaling cascades involved in the regulation of key elements within the apoptotic cascade are also subject to modulation by Hsps, including those involving JNK, NF- $\kappa$ B and AKT. The coordinated activities of the Hsps thus modulate multiple events within apoptotic pathways to help sustain cell survival following damaging stimuli.

Key words: Heat shock protein (Hsp), Co-chaperones, Apoptosis, Apoptosome, Caspases, Death receptor, Mitochondria, Proteasome, NF- $\kappa$ B, Cytochrome *c*, Bcl-2

## Introduction

Paradoxically, damage to cells can engage one of two opposing responses: apoptosis, a form of cell death that removes damaged cells to prevent inflammation and the heat shock or stress response that prevents damage or facilitates recovery to maintain cell survival. Interactions between these two pathways determine the fate of a cell and, as such, have a profound effect on the biological consequences of stress.

Apoptosis is mediated by the activity of the aspartate-specific cysteine proteases – caspases (cysteiny, aspartate-specific proteases) – which cleave either to inactivate or activate target substrates (Wolf and Green, 1999). Caspases form a cascade in which 'initiator' caspases interact with specific adaptor molecules to facilitate their own autocatalytic processing. These, in turn, cleave and activate the downstream 'executioner' caspases that orchestrate the proteolytic dismantling of the cell (Thornberry, 1997; Thornberry, 1998; Thornberry and Lazebnik, 1998).

The sequence of events culminating in the activation of caspases can be broadly categorized into two pathways: the 'intrinsic' pathway (Fig. 1A,B) and the 'extrinsic' pathway (Fig. 2). The intrinsic pathway is characterized by the permeabilization of the outer mitochondrial membrane and the release of several pro-apoptotic factors into the cytosol. These include cytochrome *c* (Kluck et al., 1997; Yang et al., 1997a), Smac/Diablo (Du et al., 2000; Verhagen et al., 2000), AIF (Susin et al., 1999), EndoG (Li et al., 2001) and HtrA2/Omi (Suzuki et al., 2001). The precise mechanism of cytochrome *c* release remains unclear but is regulated by the antagonistic activities of the Bcl-2 family (Green and Reed, 1998; Willis et

al., 2003). Once released into the cytosol, cytochrome *c* binds to an adaptor protein, Apaf-1, which self-oligomerizes and recruits pro-caspase-9 to form the apoptosome complex (Zou et al., 1997; Zou et al., 1999). This promotes the auto-processing of pro-caspase-9 (Srinivasula et al., 1998), which in turn recruits and cleaves pro-caspase-3 that is then released into the cytosol to degrade target substrates proteolytically (Cain et al., 1999).

The extrinsic pathway can be initiated by one of several cell-surface death receptors when bound by the appropriate ligand (Locksley et al., 2001; Screaton and Xu, 2000). Tumor necrosis factor receptor 1 (TNFR1) and the Fas receptors contain death domains (DDs) and recruit the DD-containing adaptor molecules TNFR1-associated death domain (TRADD) and Fas-associated death domain (FADD), respectively. Homotypic interaction between the DDs of Fas and FADD induces the recruitment and self-activation of pro-caspase-8 (Chinnaiyan et al., 1995). In TNF signaling, TRADD recruits FADD following formation and release of a TNFR1 complex (Chinnaiyan et al., 1996; Hsu et al., 1996a; Hsu et al., 1996b; Micheau and Tschopp, 2003) to initiate pro-caspase-8 activation. The receptors for TNF-related apoptosis-inducing ligand (TRAIL), TRAIL-R1 (also known as death receptor 4) or TRAIL-R2 (also known as death receptor 5), also recruit and activate pro-caspase-8 (MacFarlane et al., 1997; Pan et al., 1997; Walczak et al., 1997) in a FADD-dependent manner (Schneider et al., 1997).

Cells respond to a variety of chemical and physiological stresses by rapidly synthesizing a group of highly conserved proteins known as heat shock or stress proteins (Hsps). These

proteins are broadly categorized according to their size and include the Hsp70, Hsp27, Hsp60, Hsp90 and Hsp100 families. Induction of the Hsps protects cells against the harmful consequences of a diverse array of stresses, including those imposed by heat shock (Hahn and Li, 1982; Li and Hahn, 1990), chemotherapeutic agents, nutrient withdrawal (Mailhos et al., 1993), ultraviolet (UV) irradiation (Simon et al., 1995), polyglutamine repeat expansion (Warrick et al., 1999) and TNF (Jaattela and Wissing, 1993; Van Molle et al., 2002). Historically, studies of the protective ability of the Hsps have focused largely on their role as chaperones to prevent misfolding of proteins and to accelerate their refolding and renaturation (Gething and Sambrook, 1992; Lindquist, 1986; Lindquist and Craig, 1988; Nollen and Morimoto, 2002; Parsell and Lindquist, 1990; Parsell and Lindquist, 1993; Parsell et al., 1993). However, more recently, the function of Hsps has been shown to be broader and encompass an anti-apoptotic role that can, but does not always, depend upon their chaperoning ability (Beere and Green, 2001; Parcellier et al., 2003a).

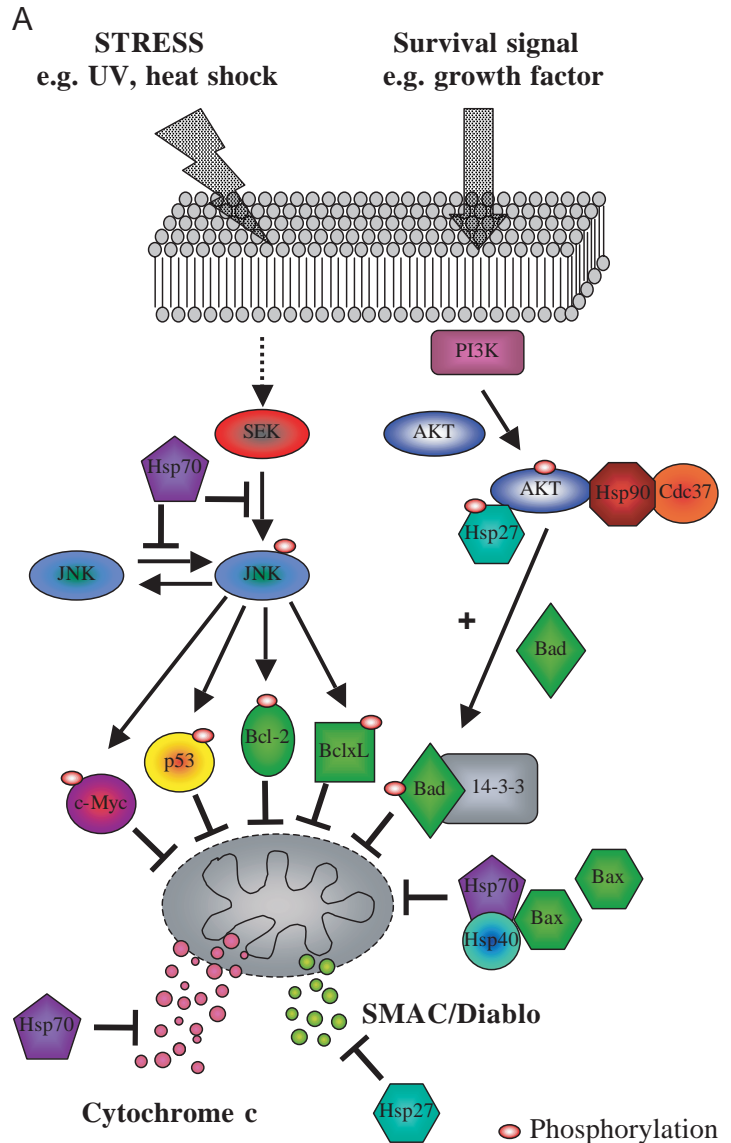
The regulatory role of Hsps depends on one fundamental property – their ability to interact with protein or polypeptide substrates (Georgopoulos and Welch, 1993). Hsp70 and Hsp90 proteins each comprise two domains: a highly conserved N-terminal ATPase domain (Flaherty et al., 1990) and a C-terminal domain that contains the polypeptide-binding site (Wang et al., 1993). The C-terminal four amino acids, EEVD, mediate inter-domain communication and peptide-binding capacity (Freeman et al., 1995), and are essential for regulation of protection against heat stress (Li et al., 1992). By contrast, Hsp27 lacks an ATPase domain and is instead regulated by mitogen-activated protein (MAP)-kinase-dependent phosphorylation and self-oligomerization (Garrido et al., 1999).

The chaperone activity of the Hsps is controlled by a reaction cycle of ATP binding, hydrolysis and nucleotide exchange to mediate a series of rapid association-dissociation cycles between the Hsp protein and its target polypeptide (Buchberger et al., 1995; McCarty et al., 1995; Rudiger et al., 1997). The ATP-bound form of an Hsp binds and releases peptides rapidly, resulting in low overall affinity, whereas the ADP-bound form binds peptides slowly but more stably (Palleros et al., 1994; Palleros et al., 1991; Schmid et al., 1994). Effective chaperoning activity is regulated by the binding of additional co-factors or co-chaperones that catalyze the inter-conversion between the ATP and ADP states. These include Hsp40 (HDJ-1 and HDJ-2) (Freeman et al., 1995), Hsc70-interacting protein (Hip) and Hsc70-Hsp90-organizing protein (Hop) (Frydman and Hohfeld, 1997).

Below, I discuss recent work that is beginning to reveal mechanisms by which the Hsps and their co-chaperones modulate specific elements of apoptotic signaling to alter the responses of cells to potential death-inducing stimuli.

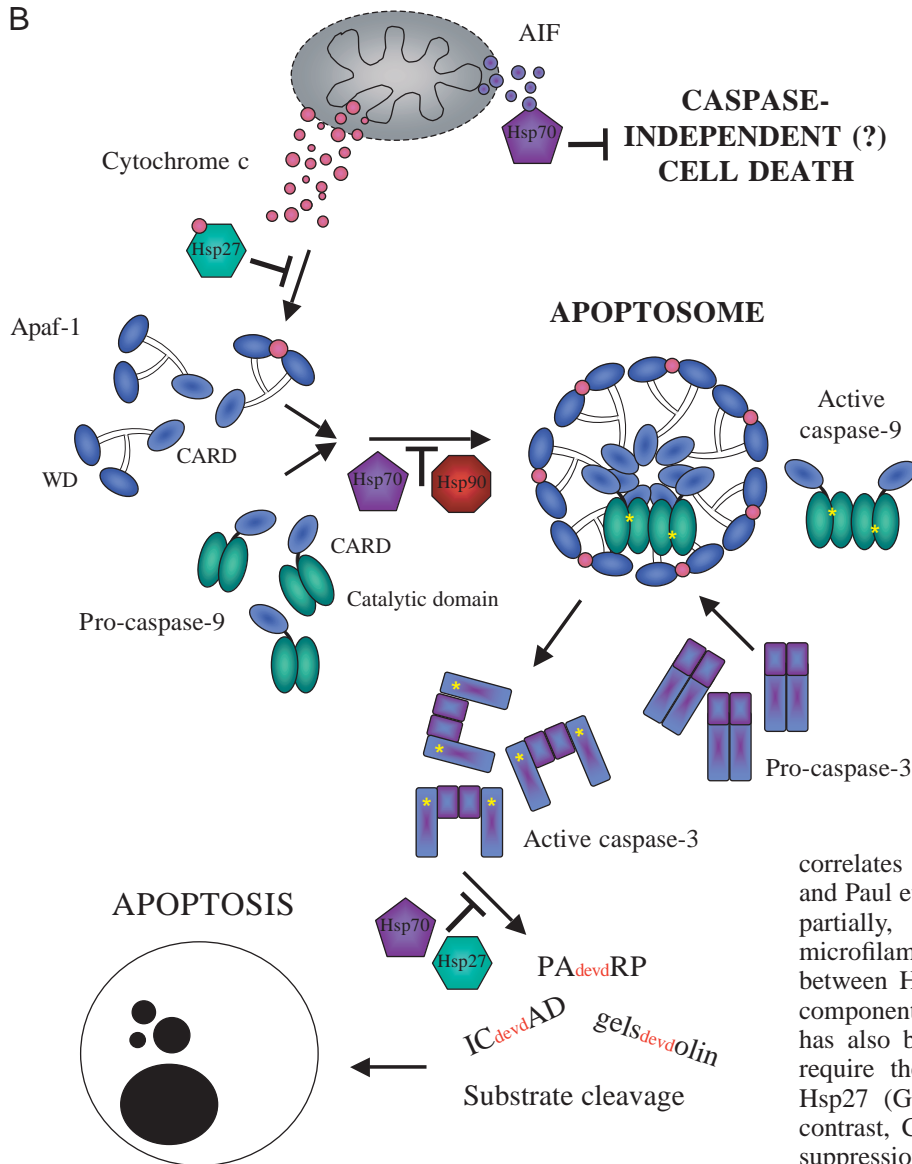
### Hsp-mediated inhibition of apoptosis

Numerous studies have attributed the survival-promoting effects of the Hsps to their ability to suppress the engagement



**Fig. 1.** Events regulated by Hsps in the mitochondrial or 'intrinsic' apoptotic pathway. Extracellular signals or stresses converge to regulate the mitochondria-mediated pathway to caspase activation and cell death. Heat shock proteins intervene at multiple points within this pathway both upstream (A) and downstream (B) of the associated mitochondrial changes to regulate the engagement and/or progression of apoptotic events. Hsp-mediated inhibition is indicated (T-bars) and Hsp-mediated potentiation of a signaling pathway is depicted as a direct interaction between the Hsp and its target (+).

of apoptosis in response to several stimuli, including heat, DNA damage and death receptor ligation. The unifying feature of these observations is seen as an inhibition of the proteolytic maturation and/or activity of caspases (Beere et al., 2000; Garrido et al., 1999; Mosser et al., 2000) and cleavage of its target substrates, including focal adhesion kinase (FAK) (Mao et al., 2003) and PARP (Garrido et al., 1999; Mosser et al., 2000). The activation of caspases represents the consequence of a series of signaling events resulting from cell damage and is the culminating feature of different apoptotic pathways. Therefore, the inhibition of caspase activation by Hsps could



indicate negative regulation at one or more points within multiple signaling cascades. Accordingly, several mechanisms have been ascribed to the anti-apoptotic role of the Hsps. These are discussed below.

### Modulation of the intrinsic pathway

Release of pro-apoptotic factors such as cytochrome *c* from mitochondria is a pivotal point within the intrinsic pathway that is regulated by Bcl-2 proteins. Hsps can regulate the release of pro-apoptotic factors from mitochondria following stress (Mosser et al., 2000). This might reflect a direct effect on the mitochondrion itself (He and Lemasters, 2003; Polla et al., 1996; Samali et al., 2001; Syken et al., 1999) or an indirect consequence of Hsp-mediated modification of events that lead to the release of these factors (Chauhan et al., 2003a; Gabai et al., 2002; Paul et al., 2002).

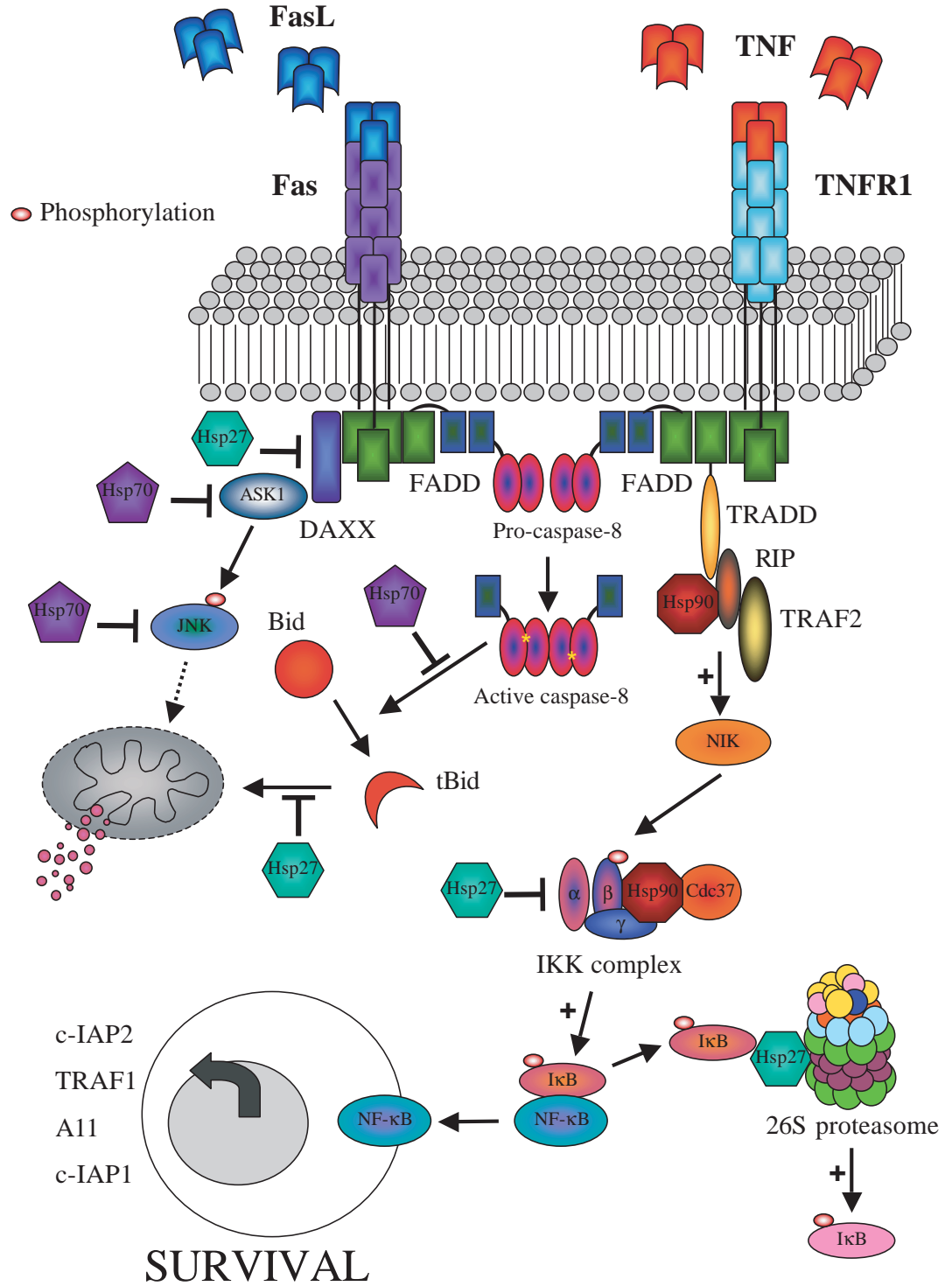
The Bcl-2 family includes pro-apoptotic members such as Bax, Bak, Bik, Bad and Bid, and anti-apoptotic proteins

including Bcl-2 and Bcl-x<sub>L</sub> (Green and Reed, 1998; Gross et al., 1999). A subset of the pro-apoptotic members, including Bim, Bid and Bad, contain only the Bcl-2-homology 3 (BH3) domain, which mediates homo/heterodimeric association of various family members (Bouillet and Strasser, 2002). Such BH3-only proteins facilitate the pro-apoptotic activities of Bax and Bak (Desagher et al., 1999; Eskes et al., 1998) and are targets for the pro-survival members Bcl-2 and Bcl-x<sub>L</sub> (Cheng et al., 2001; Vieira et al., 2002).

Bid is cleaved by caspase-8 to generate active truncated Bid (tBid), which leads to the Bax-dependent release of pro-apoptotic factors from mitochondria (Li et al., 1998; Luo et al., 1998). Consequently, this event integrates the extrinsic and intrinsic pathways (Fig. 2). Two recent studies have implicated both Hsp70 and Hsp27 in the modulation of Bid-dependent apoptosis (Gabai et al., 2002; Paul et al., 2002). Hsp27-mediated suppression of Bid

translocation to the mitochondria correlates with an inhibition of cytochrome *c* release, and Paul et al. have suggested that this reflects, at least partially, the ability of Hsp27 to stabilize actin microfilaments (Paul et al., 2002). The relationship between Hsp27-mediated stabilization of cytoskeletal components and the maintenance of cellular survival has also been reported previously and is thought to require the phosphorylation and oligomerization of Hsp27 (Guay et al., 1997; Huot et al., 1996). By contrast, Chauhan et al. have linked Hsp27 with the suppression of dexamethasone-induced apoptosis in myeloma cells through inhibition of Smac but not cytochrome *c* release (Chauhan et al., 2003a). Stress-

inducible Hsp70 is also reported to prevent cleavage and activation of Bid in response to TNF, and this effect is independent of its chaperoning ability (Gabai et al., 2002). This Hsp70 activity could reflect its capacity to suppress activation of the MAP kinase JNK (see below), which is part of a pro-apoptotic signaling cascade that modulates release of cytochrome *c* (Tournier et al., 2000) and Smac (Chauhan et al., 2003b) from mitochondria. Recent data have also implicated a cooperative role for Hsp70 and its co-chaperones Hsp40 (Hdj-1) or HSDJ (Hdj-2) in the inhibition of Bax translocation to the mitochondria to prevent nitric-oxide-induced apoptosis (Gotoh et al., 2004). This activity depends upon both the chaperoning and ATPase activities of Hsp70 and requires the C-terminal-prenylation CaaX motif of the Hdj-1 and Hdj-2 co-chaperone molecules (Gotoh et al., 2004). These observations indicate that, whereas Hsp proteins can function alone to inhibit apoptosis (also see below), cooperative interaction with their designated co-chaperone molecules is likely to enhance their anti-apoptotic activities.



**Fig. 2.** Events regulated by Hsps in the death receptor mediated or ‘extrinsic’ pathway to apoptosis. Ligation of cell surface death receptors, e.g. Fas and TNFR1, by the appropriate ligand engages multiple intracellular signals leading to caspase activation and cell death or NF-κB-mediated survival. Many elements of these pathways are regulated by the activities of Hsps to help maintain cellular survival following death receptor ligation. Hsp-mediated inhibition is indicated (T-bars) and Hsp-mediated potentiation of a signaling pathway is depicted as a direct interaction between the Hsp and its target (+).

**Disruption of apoptosome function**

Hsps might also modify apoptotic signaling downstream of mitochondria (Fig. 1B). Li et al. have found that the chaperoning activity of Hsp70 is essential for the suppression of caspase activation at some point downstream of cytochrome *c* release but upstream of caspase-3 activation (Li et al., 2000). This is consistent with several studies that have indicated that formation of the Apaf-1 apoptosome is a primary regulatory point for the anti-apoptotic effects of several Hsps. Hsp70 can

inhibit the formation of a functionally competent apoptosome by directly associating with Apaf-1 to prevent the recruitment and activation of the initiator caspase pro-caspase-9 (Beere et al., 2000; Saleh et al., 2000). Hsp70 might inhibit Apaf-1 oligomerization (Saleh et al., 2000) or maintain the oligomer in a conformation incompatible with pro-caspase-9 recruitment by preventing exposure of the Apaf-1 CARD domain (Beere et al., 2000). Although these findings might appear to contradict the idea of Hsp70-mediated suppression of



cytochrome *c* release (Mosser et al., 2000), both mechanisms might function in a complementary manner to ensure an effective, 'multi-hit' Hsp-mediated suppression of apoptosis. Hsp90 and Hsp27 are also reported to prevent Apaf-1 oligomerization by directly associating with Apaf-1 (Pandey et al., 2000b) and cytochrome *c*, respectively (Bruey et al., 2000). It remains to be determined whether co-chaperones can impact on the efficiency of Hsps to disrupt apoptosome formation.

### Modulation of other events downstream of mitochondria

Hsps might also disrupt caspase-independent cell death (Jaattela et al., 1998; Nylandsted et al., 2000) (discussed below), as well as being involved in a mechanism to suppress the activity of proteolytically mature caspases (Fig. 1B).

Pandey et al. have reported that Hsp27 binds to pro-caspase-3 to prevent its cleavage and activation by caspase-9 (Pandey et al., 2000a). By contrast, the small Hsp  $\alpha\beta$  crystallin, but not Hsp27 nor Hsp70, can suppress caspase-8- and cytochrome *c*-mediated autoactivation of caspase-3 through a direct interaction with caspase-3 to prevent its complete processing (Kamradt et al., 2001). Whether cell survival and/or proliferation can be maintained once caspases have been activated remains controversial. However, the apparent ability of Hsps to suppress caspase activity is reminiscent of the activity of the inhibitor of apoptosis proteins (IAPs), which can also inhibit the activity of proteolytically active caspases by binding to them (Salvesen and Duckett, 2002). Hsps might therefore function analogously to the IAPs.

Modulation of caspase activity might also represent a mechanism by which Hsps can enhance apoptosis (Galea-Lauri et al., 1996; Liossis et al., 1997). Xanthoudakis et al. have shown that Hsp60, as part of a multi-protein complex containing pro-caspases-3 and -6, enhances pro-caspase-3 activation by caspase-6, caspase-8 and, to a lesser extent, caspase-9 in an ATP-dependent manner (Xanthoudakis et al., 1999). Hsp60 can also form a complex with Hsp10 and pro-caspase-3 in mitochondria to promote the cytochrome *c*/ATP-dependent activation of pro-caspase-3 (Samali et al., 1999). Ordinarily, Hsp60 resides in the mitochondrial matrix as a homomultimer. Components of the mitochondrial matrix are generally not thought to have a vital role in cytochrome *c* release and caspase activation. However, 'late-stage' apoptosis might be accompanied by mitochondrial degeneration and release of matrix components, including Hsp60 (Samali et al., 1999), which could act as part of a feed-forward mechanism to optimize caspase activation.

Hsps also facilitate caspase activation in granzyme B-mediated apoptosis. Once released by cytotoxic T lymphocytes (CTLs) or natural killer (NK) cells into the target cell, granzyme B can directly cleave pro-caspase-3 or Bid to induce apoptosis (Barry and Bleackley, 2002; Pinkoski and Green, 2002; Trapani and Smyth, 2002). The accumulation of Hsp70 on the plasma membrane of tumor cells can render them more susceptible to immunological attack. The underlying mechanism might involve an enhanced uptake of granzyme B through its binding to Hsp70 (Gross et al., 2003), and could explain previous observations that Hsp70 enhances CTL-mediated killing (Dressel et al., 2000).

Hsp70 and its constitutively synthesized counterpart, Hsc70,

can enhance T-cell receptor (TCR)-mediated apoptosis by directly associating with caspase-activated DNase (CAD) to augment its activity (Liu et al., 2003). These observations might reflect the previously described role for Hsp70/Hsc70 and the co-chaperone Hsp40 in the co-translational folding of CAD and its inhibitor ICAD (Sakahira and Nagata, 2002). Interestingly, the peptide-binding domain of Hsp70 is both necessary and sufficient for its ability to enhance CAD activity (Liu et al., 2003). This contrasts with the survival-promoting effects of Hsp70, which appear to require both the C-terminal peptide-binding region and an intact ATPase domain (Mosser et al., 2000).

### Modulation of the extrinsic pathway

Hsps have been shown to modulate the signaling events engaged by the death receptors Fas, TNF and TRAIL. Fas-mediated induction of apoptosis is regulated by several Hsps, including Hsp70 and Hsp27 (Liossis et al., 1997; Mehlen et al., 1996b) (Fig. 2). Although Fas-induced apoptosis typically involves FADD and activation of caspase-8 (see above), an alternative pathway exists. In this case, recruitment of an alternative adaptor molecule, Daxx, leads to activation of the MAPKKK apoptosis-signal-regulated kinase (ASK-1) (Chang et al., 1998) to induce the activation of SAPK/JNK, leading to apoptosis (Yang et al., 1997b). Hsp27 and Hsp70 appear to suppress apoptosis by binding to and inhibiting Daxx and ASK-1, respectively (Charette et al., 2000; Park et al., 2002).

Several groups have also shown that Hsp27 and Hsp70 can effectively suppress TRAIL- (Ozoren and El-Deiry, 2003) and TNF-induced apoptosis in a variety of different cell types (Jaattela, 1993; Jaattela et al., 1992; Kim et al., 1997; Mehlen et al., 1995a; Mehlen et al., 1995b; Mehlen et al., 1996b), although the chaperone activity of Hsp70 might be dispensable for this function (Gabai et al., 2002). However, other studies contradict these findings, demonstrating that the expression of Hsp70 or Hsp90 can significantly enhance the susceptibility of cells to the death-inducing effects of TNF plus cycloheximide and Fas or TCR/CD3 ligation (Galea-Lauri et al., 1996; Liossis et al., 1997). The underlying mechanisms proposed for the inhibition of TNF-induced apoptosis by the Hsps include suppression of phospholipase A2 activation (Jaattela, 1993), inhibition of reactive oxygen species and concomitant increases in glutathione levels (Mehlen et al., 1996a), as well as regulation of intracellular calcium levels and phosphatase activity (Liossis et al., 1997). The difficulty in defining this particular role of Hsps might reflect the fact that TNF, although an extremely potent inducer of apoptosis and tissue damage, can also engage effective anti-apoptotic mechanisms through NF- $\kappa$ B (see below). The context and nature of the TNF signaling elicited might therefore determine how one or more of the Hsps ultimately influence susceptibility to TNF ligands (Fig. 2).

TNFR1 can engage apoptosis by recruiting FADD and stimulating caspase-8 autoactivation (see above) or promote survival through NF- $\kappa$ B-mediated induction of genes that encode anti-apoptotic factors including TRAF1 and TRAF2, c-IAP1 and c-IAP2 (Wang et al., 1998) and the Bcl-2 homolog A1 (Wang et al., 1999) (Fig. 2). A variety of extracellular stimuli, including TNF, induce the phosphorylation of the NF- $\kappa$ B inhibitor I $\kappa$ B, resulting in its ubiquitylation and

proteasome-dependent degradation (Chen et al., 1996). This releases NF- $\kappa$ B, allowing it to translocate to the nucleus and activate target genes. Phosphorylation of I $\kappa$ B is mediated by a 900 kDa I $\kappa$ B kinase (IKK) complex composed of a regulatory subunit IKK $\gamma$ /NEMO (Rothwarf et al., 1998) and two catalytic subunits, IKK $\alpha$  and IKK $\beta$  (DiDonato et al., 1997; Regnier et al., 1997; Zandi et al., 1997; Zandi et al., 1998).

Several recent studies have connected the anti-apoptotic activities of Hsps to the modulation of IKK complex stability and activity (Fig. 2). Chen et al. reported that IKK forms a complex with Hsp90 and Cdc37 (Chen et al., 2002) – a co-chaperone that binds cooperatively with Hsp90 to regulate signal transduction (Kimura et al., 1997; Septanova et al., 1996). Binding of Hsp90 to IKK $\gamma$  and to the kinase domains of IKK $\alpha$  and IKK $\beta$  is enhanced by Cdc37 and is absolutely essential for TNF-induced NF- $\kappa$ B activation because it targets the IKK complex to the membrane (Chen et al., 2002).

A protein related to Hsp90, TNFR-associated protein 1 (TRAP-1), binds directly to the intracellular region of TNFR1 (Song et al., 1995), which suggests a broader role for Hsp90 in the formation and/or maintenance of a functionally competent TNFR1-associated complex. Hsp90 associates directly with RIP, an essential component in TNF-induced activation of IKK that TRAF2 recruits to TNFR1 (Devin et al., 2000; Zhang et al., 2000). This maintains the stability of RIP and permits TNF-induced NF- $\kappa$ B activation (Chen et al., 2002; Lewis et al., 2000). The observation that the Hsp90 inhibitor geldanamycin enhances TNF-induced cell death in HeLa cells is consistent with the idea that Hsp90 promotes survival signaling by TNF over apoptotic signaling (Lewis et al., 2000).

Park et al. also observed an interaction between IKK $\beta$  and Hsp90 (but not Hsp70 or Hsc70), as well as association of Hsp27 with both IKK $\alpha$  and IKK $\beta$  (Park et al., 2003). The interaction between Hsp27 and IKK $\beta$ , but not that between Hsp27 and IKK $\alpha$ , is further enhanced by TNF-induced, MAP-kinase-dependent phosphorylation of Hsp27, which leads to an enhanced inhibition of IKK activity and consequent suppression of NF- $\kappa$ B activity (Park et al., 2003). The ability of Hsp27 to suppress NF- $\kappa$ B activation might be predicted to enhance TNF-induced apoptosis, although this would be at odds with the previous observation that Hsp27 can protect against TNF-induced apoptosis (Mehlen et al., 1995a; Mehlen et al., 1995b; Mehlen et al., 1996b).

Hsp70 does not appear to associate with the IKK complex, at least under those conditions in which interactions between Hsp90 or Hsp27 and IKK are detected (Lewis et al., 2000). However, several additional reports implicate Hsp70 in the protection against TNF-induced inflammation (Van Molle et al., 2002; Yoo et al., 2000), which might be associated with a suppression of NF- $\kappa$ B activation (Guzhova et al., 1997; Yoo et al., 2000).

In contrast to the study published by Park et al. (Park et al., 2003), another group demonstrated that Hsp27 can mediate enhancement of NF- $\kappa$ B activation and cell survival by promoting the proteasomal-dependent degradation of polyubiquitylated I $\kappa$ B (Parcellier et al., 2003b). This activity depends upon the chaperone function of Hsp27, which allows it to interact with phosphorylated I $\kappa$ B and the 26S proteasome (Parcellier et al., 2003b). These observations could prove particularly significant. Previous studies have demonstrated a

functional link between the ATP-dependent chaperones Hsp70 and Hsp90 and proteasome-mediated protein degradation via the cooperative activities of the ubiquitin-like co-factor BAG-1 and the E3 ligase C-terminal Hsp-interacting protein (CHIP) (Ballinger et al., 1999; Connell et al., 2001; Luders et al., 2000; Murata et al., 2001). Intriguingly, the ubiquitin-dependent turnover of several key regulators of the apoptotic pathway, including Bid (Breitschopf et al., 2000), Bcl-2 (Dimmeler et al., 1999) and Bim (Akiyama et al., 2003; Ley et al., 2003), has been described recently. It is tempting to speculate that the observations of Parcellier and colleagues (Parcellier et al., 2003b) herald the characterization of a novel pathway by which Hsps and their accessory molecules can modulate the sensitivity of cells to cell death by regulating the proteasome-dependent turnover of apoptotic proteins.

### Hsp-induced alteration of apoptotic signaling

The signals required to initiate apoptosis are necessarily complex, as are those that oppose activation of the cell death machinery. Several cascades have been implicated in promoting cell survival, and Hsps can regulate activation of those involving JNK, NF- $\kappa$ B (see above; Fig. 2), Ras/Raf and the kinase AKT (Beere, 2001) (Fig. 1A).

The precise roles of the stress kinases, including JNK, in the regulation of apoptosis remain controversial (Chen and Tan, 2000; Davis, 2000). Regardless of this, JNK activation is potently suppressed by Hsp70 (Gabai et al., 1997; Meriin et al., 1998; Mosser et al., 1997; Mosser et al., 2000; Park et al., 2001). This does not appear to require its ATPase activity (Mosser et al., 1997; Volloch et al., 1999) and probably reflects a direct effect on the kinase itself (Park et al., 2001; Yaglom et al., 1999). Hsp70 can prevent stress-induced inhibition of JNK dephosphorylation (Meriin et al., 1999), maintaining the levels of inactive dephosphorylated JNK or, alternatively, inhibiting its phosphorylation by SEK (Park et al., 2001). Either scenario leads to a block in JNK signaling and, therefore, under circumstances where JNK is required for apoptosis, Hsp70 helps to maintain survival.

JNK activity regulates several proteins involved in the apoptotic process, thereby providing an effective apical target for Hsp70 in the broader context of apoptotic regulation. JNK phosphorylates c-Myc and p53 (Fuchs et al., 1998a; Fuchs et al., 1998b; Noguchi et al., 1999), both of which have been implicated in the release of cytochrome *c* (Chipuk et al., 2003; Chipuk et al., 2004; Juin et al., 1999; Schuler et al., 2000). Likewise, JNK-mediated phosphorylation of Bcl-2 and Bcl-x<sub>L</sub>, which can antagonize the anti-apoptotic activities of these proteins (Fan et al., 2000; Maundrell et al., 1997; Yamamoto et al., 1999), could significantly alter the susceptibility of cells to damaging stimuli. Recent data also implicate JNK in the release of Smac/DIABLO from mitochondria (Chauhan et al., 2003b). The ability of Hsp70 to disrupt JNK signaling could therefore impact on multiple pathways in the apoptotic process and might represent the underlying mechanism of Hsp70-mediated suppression of cytochrome *c* release (Beere and Green, 2001; Mosser et al., 2000).

A variety of cytokines, including insulin-like growth factor 1 (IGF-1), nerve growth factor (NGF) and platelet-derived growth factor (PDGF), can sustain cell survival by stimulating signaling through phosphoinositide 3-kinase (PI3K) and its

downstream kinase Akt (also known as PKB) (Cantley, 2001; Datta et al., 1999). Akt phosphorylates several proteins involved in the regulation of apoptosis. For example, phosphorylation of the pro-apoptotic Bcl-2 protein Bad induces its dissociation from Bcl-x<sub>L</sub> and subsequent sequestration by cytosolic 14-3-3 proteins. This prevents its translocation to mitochondria and participation in the release of pro-apoptotic factors (Zha et al., 1996). Akt also regulates transcription factors that direct the expression of several cell death genes. Akt-mediated phosphorylation of forkhead (FKHRL1) prevents its translocation to the nucleus and therefore prevents the induced expression of its target genes including Fas ligand (FasL), IGF-1-binding protein (Brunet et al., 1999) and potentially Bim (Dijkers et al., 2000). AKT also phosphorylates IκB (Kane et al., 1999), promoting NF-κB-mediated transcription of genes that encode pro-survival proteins including the caspase inhibitors c-IAP1 (You et al., 1997) and c-IAP2 (Chu et al., 1997) and the Bcl-2 protein A1 (Zong et al., 1999).

Several studies have implicated both Hsp90 and Hsp27 in the maintenance of Akt activity, which could contribute to promotion of cell survival (Nakagomi et al., 2003; Rane et al., 2003; Sato et al., 2000) (Fig. 1B). The stability and activity of Akt is maintained when in complex with Hsp90 and Cdc37 (Basso et al., 2002; Sato et al., 2000), perhaps through inhibition of its dephosphorylation by the phosphatase PPA2 (Sato et al., 2000). Pharmacological disruption of the Akt survival pathway using the Hsp90 inhibitor geldanamycin sensitizes cells to the pro-apoptotic effects of taxol (Solit et al., 2003) and to the protein kinase C (PKC) inhibitor UCN-01 (Jia et al., 2003). One recent study also reported that geldanamycin-induced degradation of Akt triggers the Bax-dependent release of cytochrome *c* and Smac/DIABLO from mitochondria, which can be inhibited by Bcl-2 or Bcl-x<sub>L</sub> (Nimmanapalli et al., 2003). Hsp27 might also regulate Akt. Unlike Hsp70 and Hsp90, Hsp27 lacks an ATPase domain and is regulated primarily by p38-dependent, MAPK-activated protein kinase 2 (MAPKAPK-2)-mediated phosphorylation and oligomerization (Rouse et al., 1994). Akt can exist in a signaling complex with Hsp27, p38 MAPK and MAPKAPK-2 and is subject to MAPKAPK-2-dependent phosphorylation and activation (Rane et al., 2001). Furthermore, Akt-mediated phosphorylation of Hsp27 facilitates an interaction between these two proteins, which stabilizes Akt and so promotes cell survival (Rane et al., 2003).

### Hsp-mediated regulation of caspase-independent cell death

The precise nature of caspase-independent cell death remains controversial, although several mechanisms have been proposed (Jaattela and Tschopp, 2003; Lockshin and Zakeri, 2002), including roles for AIF (Cande et al., 2002) and RIP kinase (Holler et al., 2000). Antisense oligonucleotides directed against Hsp70 engage a form of cell death in MCF-7 breast carcinoma cells that is characterized by a morphology consistent with apoptosis but that is neither sensitive to inhibition by 'classical' caspase inhibitors such as zVAD and DEVD nor suppressed by Bcl-2 or Bcl-x<sub>L</sub> (Nylandsted et al., 2000). These findings are in keeping with a suggested role for Hsp70 in the inhibition of TNF-induced cell death that is

independent of any effect on caspase activation (Jaattela et al., 1998). Fas might also trigger a caspase-8-independent cell death pathway that requires RIP kinase (Holler et al., 2000), a known target of Hsps (see above) (Lewis et al., 2000; Vanden Berghe et al., 2003). Apaf-1-independent cell death represents another distinct form of cell death that may or may not involve caspase activation (Jaattela and Tschopp, 2003; Lockshin and Zakeri, 2002) and is also regulated by Hsp70 (Ravagnan et al., 2001). AIF, like cytochrome *c*, is released from mitochondria in response to apoptosis-inducing stimuli, after which it translocates to the nucleus to induce caspase-independent chromatin condensation and cell death (Susin et al., 1999). Hsp70 prevents nuclear import of AIF following its release from mitochondria and, as a consequence, neutralizes its death-inducing activity in Apaf-1-null cells (Gurbuxani et al., 2003; Susin et al., 1999). This has been attributed to a direct association between an N-terminal region in AIF (residues 150-228) and Hsp70 and does not require the ATPase domain in Hsp70 (Gurbuxani et al., 2003; Susin et al., 1999). However, recent observations indicate that the release of AIF from mitochondria requires caspase activation (Arnoult et al., 2003; Wang et al., 2002). If this is the case, AIF might not mediate caspase-independent cell death as previously suggested (Susin et al., 1999) and raises the possibility that Hsp70, by inhibiting caspase activation (see above), might indirectly prevent the release of AIF from mitochondria. This possibility has yet to be tested.

### Conclusion

The seemingly promiscuous and ubiquitous nature of the anti-apoptotic effects of multiple members of the Hsp protein family might be somewhat skeptically interpreted as a nonspecific effect that lacks true biological relevance. However, many of the key events necessary for the execution and regulation of the apoptotic program include alterations in protein conformation, multimerization of proteins, changes in protein location and turnover. All of these parameters are subject to regulation by the Hsps. Hsp-mediated regulation of the apoptotic pathways probably constitutes a fundamental protective mechanism that decreases cellular sensitivity to damaging events to allow cells to escape the otherwise inevitable engagement of apoptosis.

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