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Molecular chaperones and the stress of oncogenesis

Dick D Mosser*,[1] and Richard I Morimoto[2]

[1]*Department of Molecular Biology and Genetics, University of Guelph, Guelph, Ontario, Canada N1G 2W1;* [2]*Department of Biochemistry, Molecular Biology, and Cell Biology, Rice Institute for Biomedical Research, Northwestern University, 2153 North Campus Drive, Evanston, Il. 60208, USA*

Protein-damaging stresses induce the expression of 'heat-shock proteins', which have essential roles in protecting cells from the potentially lethal effects of stress and proteotoxicity. These stress-protective heat-shock proteins are often overexpressed in cells of various cancers and have been suggested to be contributing factors in tumorigenesis. An underlying basis of oncogenesis is the acquisition and accumulation of mutations that provide the transformed cell with the combined characteristics of deregulated cell proliferation and suppressed cell death. Heat-shock proteins with dual roles as regulators of protein conformation and stress sensors may therefore have intriguing and central roles in both cell proliferation and apoptosis. It has been established that heat-shock proteins exhibit specificity to particular classes of polypeptide substrates and client proteins *in vivo*, and that chaperones can stabilize mutations that affect the folded conformation. Likewise, overexpression of chaperones has also been shown to protect cells against apoptotic cell death. The involvement of chaperones, therefore, in such diverse roles might suggest novel anticancer therapeutic approaches targeting heat-shock protein function for a broad spectrum of tumor types.
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Molecular chaperones are key determinants of the cellular response to stress

Cell proliferation and cell death represent tightly regulated processes that are controlled by signal transduction pathways that transmit information from the environment to effector molecules. Cells under stress must react rapidly to prevent the inappropriate transmission of these death signals or to restrain passage through checkpoints leading to cell division. Protein-damaging stresses, typified by hyperthermia, activate ERK and JNK kinase pathways, each of which in particular settings are involved in controlling the cellular decision to proliferate, arrest or die. The response of cells to stress depends upon its severity, which is a product of intensity and duration. Whereas severe hyperthermia can inhibit all aspects of cellular metabolism including DNA replication, transcription, mRNA processing, and protein synthesis, transient exposure to elevated temperatures are tolerated provided that repair mechanisms accurately detect and respond to the level of protein damage. However, at a certain critical threshold of molecular damage, the cell will succumb to either apoptotic or necrotic cell death. The decision to live or die, therefore, appears to be made on the basis of the relative strengths of cellular survival and apoptotic signals.

Many key components of survival and apoptotic pathways are regulated by interactions with molecular chaperones. These proteins, primarily the Hsp70 family (Hsc70 (HS7C) and Hsp70 (HSPA1A), the Hsp90 family (Hsp90β (HSPCB) and Hsp90α (HSPCAL4), and Hsp27 (HSPB1) are essential for protein folding, translocation across cellular compartments, assembling and maintaining multiprotein complexes in activation competent states, preventing self-association, and directing misfolded and short-lived proteins to destruction by the proteasome (see Figure 1). Inappropriate activation of signaling pathways could occur during acute or chronic stress as a result of protein misfolding or disruption of regulatory complexes. The action of chaperones, through their properties in protein homeostasis, is thought to restore balance. For example, when normal growth conditions are restored following a severe exposure to elevated temperature, heat-damaged proteins are sequestered through interactions with chaperones and are then either refolded to the native state or transferred to the degradative machinery.

Cells recovering from stress contain elevated levels of heat-shock proteins and consequently are in a cytoprotected state against a subsequent exposure to a normally lethal stress exposure. This adaptive response, also known as induced thermotolerance, allows cells to rapidly respond during periods in which they are repeatedly challenged by diverse conditions of cell stress (Parsell and Lindquist, 1994). The final outcome for the cell depends upon the concentration of heat-shock proteins prior to exposure to stress. Cells that have been exposed to stresses that rapidly lead to the initiation of apoptosis will also induce the expression of heat-shock proteins and continue to express them

*Correspondence: DD Mosser or R Morimoto;
E-mail: rmosser@uoguelph.ca or r-morimoto@northwestern.edu

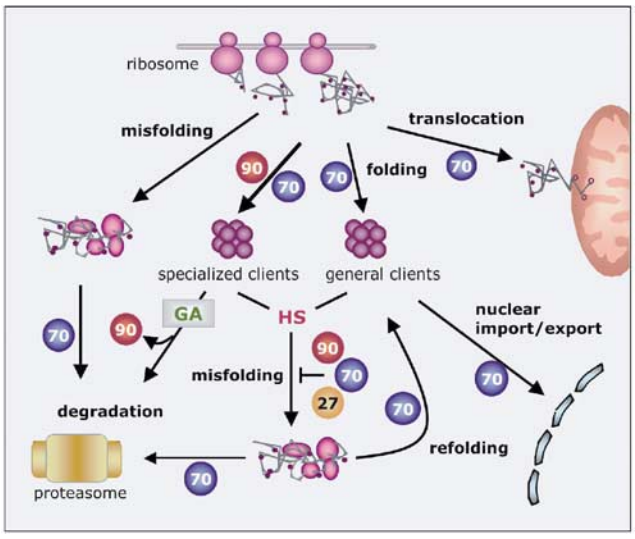

Figure 1 Chaperone-assisted protein folding. Hsp70 is required for the productive folding of newly synthesized proteins, their translocation across intracellular membranes (endoplasmic reticulum, mitochondria, lysosomes) and import into and export out of the nucleus. Specialized client proteins are transferred to hsp90 and held in activation-competent states. This includes signaling kinases (Raf-1, Akt, RIP), hormone receptors (glucocorticoid hormone receptor) and transcription factors (HSF1, HIF1α). Heat shock (HS) and other protein-damaging stresses cause protein misfolding and aggregation, which can be limited by the heat-shock proteins Hsp90, Hsp70 and Hsp27. These misfolded proteins can be rescued by the refolding activity of Hsp70. Proteins that cannot be productively refolded are targeted to the proteasome for degradation. The Hsp90-binding drug geldanamycin (GA) causes release of client proteins resulting in proteasomal degradation

until protein synthesis is completely arrested due to phosphorylation of eIF2α. In this circumstance, the induced expression of heat-shock proteins is futile since they accumulate to high levels after the decision to die has been made and the cell is already in the process of being destroyed. Whether the induced synthesis of heat-shock proteins under these conditions facilitates cell death is not known. Consequently, cell death results when the capacity of chaperones is overwhelmed by the excess quantity of damaged client proteins. Cell death can be triggered, for example, under conditions in which as little as 5% of the total cellular protein content has undergone aggregation (Lepock, 2003). Presumably, there are key heat-sensitive protein targets that upon inactivation trigger apoptosis although their identities remain elusive.

In this review, we examine the diverse roles that have been proposed for chaperones as sensors and regulators of stress-induced apoptosis. An emerging concept is that the stress response has evolved as a mechanism to prevent inappropriate activation of signaling pathways that lead to cell death. The heat-shock proteins serve as cellular safeguards to protect the network of protein–protein interactions that sense stress signals and relay them to the apoptotic machinery. As chaperones can act at multiple points in these pathways to ensure that stress-induced damage to the relay mechanism does not inappropriately trigger cell death, it has been difficult to understand whether heat-shock proteins have any specificity in this process. In some circumstances, the heat-shock proteins may prevent specific forms of cell death through a mechanism that is independent of their ability to refold stress-damaged proteins and in other situations heat-shock protein specificity may be determined by the presence of co-chaperones, by subtle differences in substrate affinities, and by the ability of chaperones to precisely yet subtlety influence conformational states. We suggest that the ability of heat-shock proteins to influence a cell's fate through modulation of numerous control points endows these proteins with the unusual capacity to contribute in a decisive way and at multiple points in the process of tumorigenesis.

Protein folding by Hsp70 is regulated by interactions with co-chaperones

Hsp70 influences the productive folding of proteins to the native state, and thus prevents protein misfolding and aggregation by binding to exposed stretches of hydrophobic amino acids in the substrate or client protein (reviewed in Hartl and Hayer-Hartl, 2002). This folding machine carries out cycles of substrate binding and release that are accelerated by ATP hydrolysis and modulated by association with regulatory co-chaperones (Hsp40s, Hip, Bag, Hop) that interact with Hsp70 and regulate its ATPase activity. The substrate-binding domain of Hsp70 is localized to a 25-kDa C-terminal region with substrate access controlled by a C-terminal lid that exposes the peptide-binding domain in the ATP-bound form and allows substrate binding to occur when Hsp70 is in the ADP-bound form. Opening and closing of the lid is governed by conformational changes associated with ATP binding and hydrolysis that occurs within the cleft of a 45 kDa N-terminal bilobed domain. Nucleotide exchange, which results in ATP binding in place of ADP, causes substrate release, thus allowing Hsp70 to enter a new round of substrate binding and release.

Hsp70 chaperone activity is enhanced by the co-chaperone Hsp40 (Hdj-1/Hdj-2) that binds to the C-terminus of Hsp70, stimulates its ATPase activity and thereby stabilizes the substrate-bound form (Freeman *et al*., 1995; Minami *et al*., 1996). Other co-chaperones including Hop and CHIP also bind to the C-terminal end of Hsp70. Hop is a tetratricopeptide repeat (TPR) domain-containing protein that couples the Hsp70 and Hsp90 chaperone machines via association through the C-terminus of Hsp90. Hop binding inhibits the ATPase activity of Hsp90 and this stabilizes its interaction with client proteins. CHIP, another TPR-containing protein, inhibits the Hsp40-stimulated ATPase activity of Hsp70 and has been suggested to reduce the ability of Hsp70 to refold certain denatured substrates (Ballinger *et al*., 1999). These results contrast with *in vitro* observations that transient overexpression of CHIP increases the

ability of Hsp70 to refold heat-denatured firefly luciferase (Kampinga *et al*., 2003). Binding of these TPR-containing co-chaperones to Hsp70 requires the C-terminal amino acids EEVD, which are conserved among all Hsp70 family members (Freeman *et al*., 1995). Deletion or substitution of these four amino acids impairs the ability of Hsp70 to bind to denatured substrates and therefore destroys its refolding activity. The co-chaperones Hip and Bag1 bind to the ATPase domain of Hsp70. Binding of Hip increases chaperone activity by stabilizing the ADP-bound state, whereas Bag1 accelerates nucleotide exchange causing premature release of the unfolded substrate (Hohfeld *et al*., 1995; Takayama *et al*., 1997; Bimston *et al*., 1998).

An important feature to achieve specificity in chaperone interactions with client proteins may derive from the level, role, and regulatory properties of each Hsp70 co-chaperone. Although the levels of a subset of co-chaperones are influenced by cell stress, nevertheless, their concentrations remain well below the levels of Hsp70 or Hsp90. Consequently, during each reaction cycle, it is possible that there may be only a limited probability of being influenced by a particular co-chaperone. Co-chaperone involvements, as well may also be substrate specific and required for specific tasks. For example, CHIP contains a U-box that imparts E3-ubiquitin ligase activity and overexpression of CHIP was shown to enhance degradation of CFTR (Jiang *et al*., 2001; Meacham *et al*., 2001). Bag1 has also been suggested to have roles in addition to acting as a negative regulator of Hsp70 activity (reviewed in Takayama and Reed, 2001; Townsend *et al*., 2003). Bag1 was initially identified as a Bcl-2-associated protein, and in combination with Bcl-2 inhibited apoptosis (Takayama *et al*., 1995). Bag1 also binds to hormone and growth factor receptors, the serine/threonine kinase Raf1, and the ubiquitin-conjugating factor Siah. Some of these interactions occur together with Hsp70, while others (Raf1) are inhibited in the presence of Hsp70 (Song *et al*., 2001). Other Bag family members, including Bag2, Bag3 (CAIR), and Bag4 also interact with Hsp70, suggesting a more expansive role for Bag family proteins as regulators of stress responsive pathways (reviewed in Nollen and Morimoto, 2002; Takayama and Reed, 2001).

Hsp70 and the inhibition of stress-induced apoptosis

Overexpression of Hsp70 can protect cells from the adverse effects of protein-damaging stresses. Likewise, overexpression of Hsp70 in mammalian cells suppresses heat-induced inactivation of firefly luciferase and facilitates its reactivation during recovery from heat shock (Michels *et al*., 1997). However, Hsp70-overexpressing cells do not show the same level of resistance as cells made thermotolerant by a preconditioning heat shock even though equivalent levels of Hsp70 are expressed (Nollen *et al*., 1999), which suggests that full heat resistance requires the assistance of co-chaperones or the activities of other chaperones. The ability of Hsp70 to protect firefly luciferase from heat inactivation in mammalian cells is enhanced by the coexpression of either Hsp40 or Hip (Michels *et al*., 1997; Nollen *et al*., 2001). Presumably this enhanced chaperone activity should also result in enhanced resistance to stress-induced apoptosis, although the role of co-chaperones in apoptosis-suppression has not been fully explored.

Many studies have attempted to correlate the cytoprotective and chaperoning functions of Hsp70 using various deletion mutants. For example, some level of heat resistance is observed by overexpression of Hsp70 lacking the ATPase domain, indicating that binding to misfolded proteins without refolding them is sufficient to provide resistance (Li *et al*., 1992). The ATPase-deleted protein, however, is 10-fold less effective than the full-length protein. Both full-length Hsp70 and Hsp70 lacking the ATPase domain are able to reduce the extent of heat-induced nuclear protein aggregation, although the ATPase-deleted protein is less effective (Stege *et al*., 1994). An important point that must be considered is that rather than simply observing a loss of function, the expression of mutant Hsp70 proteins could titrate co-chaperones and disturb the coordination of Hsp70-mediated protein folding events in the cell. For example, overexpression of the ATPase-deleted protein could diminish the amount of Hsp40 or Hop that is available for the endogenous Hsc70/Hsp70. These mutant proteins could also have dominant-negative effects by interfering with the productive formation of hsp70 and hsp90 chaperone machines.

The observation that thermotolerant cells resist stress-induced apoptosis suggests that, rather than simply preventing cellular inactivation, the heat-shock proteins have specific roles in the suppression of selective components of cell determined death pathways (Mosser and Martin, 1992). The tremendous advancement in our knowledge on apoptotic and other death pathways has provided a number of clues to examine how Hsp70 might confer its cytoprotective properties. Overexpression of Hsp70 alone can prevent stress-induced apoptotic death (Gabai *et al*., 1997; Mosser *et al*., 1997). Inhibition of stress-induced apoptosis by Hsp70 requires the chaperone function, since deletion of the ATPase domain or the C-terminal EEVD sequence or replacement of the EEVD sequence with alanine residues prevented its ability to block caspase activation and apoptosis in heat-shocked cells (Mosser *et al*., 2000). Hsp70 has been demonstrated to affect processes regulating apoptotic signaling, effector molecule activation, and events downstream of caspase activation (see Figure 2) (reviewed in Beere and Green, 2001). Processing of procaspases 9 and 3 is inhibited in cells overexpressing Hsp70, indicating that Hsp70 can exert its antiapoptotic effects upstream of caspase activation (Mosser *et al*., 1997; Buzzard *et al*., 1998). This is supported by the observation that purified Hsp70 did not affect the ability of activated caspase-3 to cleave poly(ADP-ribose)polymerase *in vitro* (Mosser *et al*., 1997). Hsp70 was also unable to prevent the cleavage of

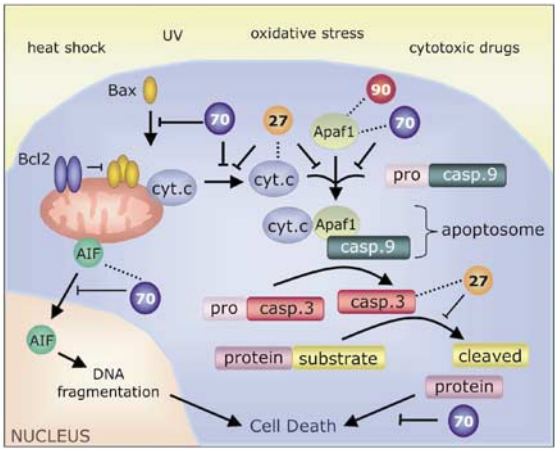

Figure 2 Influence of heat-shock proteins in suppressing stress-induced apoptosis. Hsp70 and Hsp27 prevent cytochrome *c* (cyt.*c*) release from mitochondria. Apoptosome formation is blocked by Hsp27 binding to cytochrome *c* and by Hsp90 and Hsp70 binding to Apaf-1. Hsp27 can prevent apoptosis downstream of caspase-3 (casp.3) activation by interacting with caspase-3. Hsp70 also prevents apoptosis downstream of caspase-3 activation by an unknown mechanism. Hsp70 prevents release of AIF from mitochondria and also prevents the nuclear import of released AIF. Dotted lines indicate interactions that have been demonstrated *in vitro*

caspase targets in cells expressing active caspase-3 by gene transfection (Jaattela *et al*., 1998). These cells nevertheless retained viability indicating that Hsp70 can also affect processes downstream of the caspases in providing cytoprotection. Hsp70 has also been reported to prevent the cleavage of a caspase-3 target (focal adhesion kinase) by directly interacting with this target protein (Mao *et al*., 2003).

Stress-induced caspase activation occurs as a result of cytochrome *c* release from mitochondria. Binding of cytochrome *c*, together with dATP, to the apoptotic protease-activating factor (Apaf-1) leads to Apaf-1 oligomerization and recruitment of procaspase-9, which is required for activation of procaspase-9 to its proteolytic active state. Apaf-1 oligomerization can be initiated *in vitro* by the addition of cytochrome *c* and dATP to cytosolic extracts, and the addition of purified Hsp70 to these extracts prior to the addition of cytochrome *c* prevented apoptosome formation (Saleh *et al*., 2000) and recruitment of procaspase-9 (Beere *et al*., 2000). A chaperone-defective mutant of Hsp70 in which the C-terminal EEVD sequence was mutated did not inhibit the cytochrome *c*–dATP-induced activation of procaspases 9 or recruitment of procaspases 9 to the apoptosome *in vitro* (Beere *et al*., 2000), suggesting that chaperone remodeling activity is important. Apaf-1 can be coimmunoprecipitated with Hsp70 although the proportion of total cellular Apaf-1 present in this inhibitory complex is not known. Apaf-1 oligomerization creates a template for procaspase-9 binding and dimerization between the hydrophobic interface of each monomer (Shi, 2002). The *in vitro* activation of Apaf-1 by cytochrome *c*/dATP could generate substrates for chaperones; however, whether these are bona fide substrates for heat-shock proteins in intact cells remains to be determined. For example, addition of purified Hsp70 lacking the ATPase domain to cell free extracts is equally effective as the full-length protein at preventing cytochrome c/dATP-mediated caspase-3 processing (Li *et al*., 2000); however, the ATPase-deleted protein does not protect cells from heat-induced apoptosis (Mosser *et al*., 2000).

The influence of Hsp70 can also be traced upstream of apoptosome formation. Overexpression of Hsp70 prevents the release of cytochrome *c* and apoptosis-inducing factor (AIF) from mitochondria and prevents the loss of mitochondrial transmembrane potential (Creagh *et al*., 2000; Mosser *et al*., 2000; Ravagnan *et al*., 2001). The AIF released from mitochondria binds to Hsp70 and this interaction prevents the nuclear import of AIF (Gurbuxani *et al*., 2003). Hsp70 binding and inhibition of AIF-induced cell death required only the peptide-binding domain of Hsp70 (Ravagnan *et al*., 2001). Some authors have proposed that the release of cytochrome *c* and AIF from mitochondria can occur through distinct mechanisms (Arnoult *et al*., 2002). Whereas stress-induced cytochrome *c* release is mediated by the translocation of Bax from the cytosol to mitochondria where its insertion causes the formation of pores that allow the loss of cytochrome *c* (Jurgensmeier *et al*., 1998), AIF release required caspase activation (Arnoult *et al*., 2002). Overexpression of Hsp70 was reported to have no effect on stress-induced cytochrome *c* release in transfected U937 cells, although it did prevent caspase-3 activation (Li *et al*., 2000). How stress leads to cytochrome *c* release is not understood other than the established roles for members of the Bcl-2 family in regulating this process (reviewed in Adams, 2003). Heat shock has been shown to cause Bax translocation to mitochondria and Hsp70 prevents this from occurring (Mosser *et al*., unpublished). Inhibition of Bax translocation and cytochrome *c* release requires the refolding activity of Hsp70 since deletion of the ATPase domain destroyed the ability of Hsp70 to prevent both of these effects. Placing Hsp70 both upstream and downstream of mitochondria in the stress-induced apoptotic pathway suggests a failsafe mechanism to ensure that death can be averted.

Hsp70 regulates stress-activated protein kinase signaling pathways

A variety of cellular stresses, including heat shock, oxidative stress, and UV exposure activate kinase signaling cascades wherein MAP3 kinases phosphorylate the MAP2 kinases MKK4/7 and MKK3/6, which in turn phosphorylate the MAP1 kinases JNK and p38 (reviewed in Johnson and Lapadat, 2002). Overexpression of a kinase-inactive mutant of SEK1 (MKK4) or a nonphosphorylatable dominant-negative mutant of c-jun blocked stress-induced apoptosis (Verheij *et al*.,

1996; Zanke *et al*., 1996). JNK activation is believed to be an essential mediator of stress-induced apoptosis. Mouse embryo fibroblasts with targeted disruption of JNK1 and JNK2 were found to be resistant to UV-induced apoptosis due to their failure to release cytochrome *c* (Tournier *et al*., 2000). For JNK to induce apoptosis, Bax must be expressed although it does not appear to be a substrate for this kinase (Lei *et al*., 2002). However, other Bcl2 family members, including the antiapoptotic proteins Bcl-2 and $BclX_L$ and the BH3-only proapoptotic proteins Bad and Bim are phosphorylated by JNK (Fan *et al*., 2000; Donovan *et al*., 2002; Lei and Davis, 2003). Phosphorylation inhibited the activity of Bcl-2/$BclX_L$ and activated Bad and Bim. Another target of JNK phosphorylation implicated in apoptosis regulation is p53. Phosphorylation of p53 by JNK prevents its ubiquitination and degradation leading to increased p53 levels (Fuchs *et al*., 1998). Other studies, however, suggest that JNK activation is not a requirement for stress-induced cell death and in certain situations and cell types may prevent apoptosis. For example, stress-induced apoptosis, including caspase-3 processing, can occur in the absence of JNK activation in *sek1*$^{-/-}$ *mkk7*$^{-/-}$ ES cells (Nishitai *et al*., 2004). As well, inhibition of JNK by either expression of a dominant negative version of SEK1 or by treatment with the specific JNK inhibitor SP600125 prevented early events in the apoptotic pathway (i.e., release of cytochrome *c*), yet activation of caspase-3 was only delayed and cell death was not prevented (Krilleke *et al*., 2003). The effects of JNK activation may be dependent upon the activation state of other survival and apoptotic signal transduction networks and is therefore likely to be cell type dependent.

Hsp70 has been shown to be a potent inhibitor of JNK and p38 activation in stressed cells (Gabai *et al*., 1997; Mosser *et al*., 1997) and appears to achieve this inhibition through a variety of mechanisms (see Figure 3) (reviewed in Gabai and Sherman, 2002). Heat shock, and other protein-damaging stresses activated JNK without increasing the activity of SEK1 (Meriin *et al*., 1999). Instead, the increased JNK activity resulted from the basal activity of SEK1 and heat-induced inhibition of a JNK phosphatase. Overexpression of Hsp70 is suggested to prevent the heat-induced inactivation of the JNK phosphatase as it has been shown that JNK phosphatase M3/6 is very sensitive to heat-induced inactivation (Palacios *et al*., 2001). Prior synthesis of heat-shock proteins by a conditioning heat treatment reduced the extent of M3/6 inactivation resulting in a reduced activation of JNK. Not all JNK activating stresses, however, cause protein damage. Exposure of cells to UV, osmotic shock, or interleukin-1 activates JNK by stimulating the upstream kinase SEK1. In each case, prior synthesis of Hsp70 blocked JNK but not SEK1 activation (Meriin *et al*., 1999; Yaglom *et al*., 1999). Hsp70 also prevents JNK activation in cells expressing constitutively active forms of MEKK1, a MAP3K, or its upstream activator cdc42. However, this did not occur through inhibition of SEK1 activity.

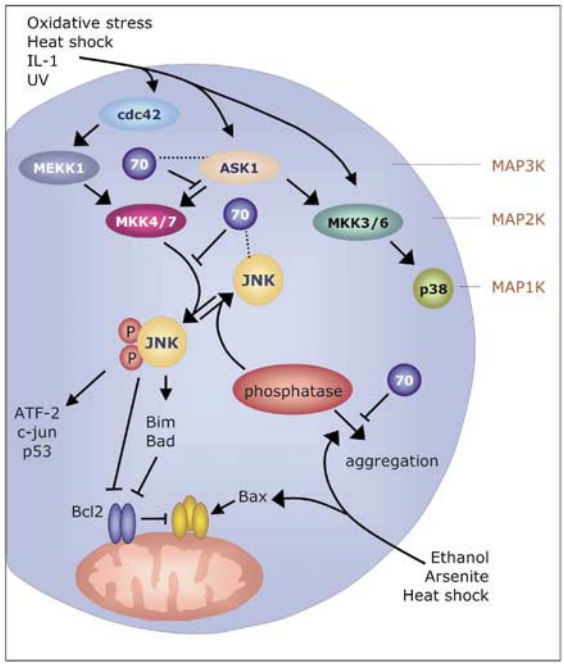

Figure 3 Inhibition of the stress-activated kinase pathway by Hsp70. Binding of Hsp70 to JNK prevents its phosphorylation by the MAP2 kinase MKK4 (SEK1). Hsp70 also prevents SEK1 activation through an interaction with the MAP3 kinase Ask1. Heat shock and other protein-damaging stresses inactivate a JNK phosphatase leading to increased JNK activity. Hsp70 can prevent JNK activation in heat-shocked cells by preventing the heat-induced aggregation of the phosphatase. Dotted lines indicate interactions that have been demonstrated *in vitro*

Presumably, this is due to an ability of Hsp70 to accelerate the activity of a JNK phosphatase (Yaglom *et al*., 1999). Inhibition in each of these cases could be accomplished by the C-terminal peptide-binding portion of Hsp70 and did not require the ATPase domain.

Hsp70 has also been demonstrated to suppress JNK activation by specifically associating with JNK and preventing it from being phosphorylated by SEK1 (Park *et al*., 2001). Overexpressed Hsp70 blocked JNK activation by UV treatment but did not prevent activation of the upstream kinases MEKK1 or SEK1. Hsp70 also blocked JNK activation and prevented apoptosis in cells expressing an active form of MEKK1 (ΔMEKK1). The addition of purified Hsp70 to *in vitro* kinase assays resulted in the inhibition of JNK but not MEKK1, SEK1, or p38. Hsp70 did not interact with the MAP2 kinases, MKK3 or MKK6. Hsp70 binding to JNK1 and inhibition of ΔMEKK1-induced apoptosis required the peptide-binding domain of Hsp70 but not the ATPase domain. Hsp70 can also block the stress signal upstream of the MAP2 kinases by interacting with the apoptosis signal-regulating kinase 1 (ASK1) (Park *et al*., 2002). ASK1 binding and kinase inhibition required the ATPase domain of Hsp70 and not the peptide-binding domain. Remarkably, expression of the ATPase domain alone was able to prevent apoptosis in cells expressing a constitutively active form of ASK1

(ASK1-ΔN). Hsp70 therefore appears to ensure that the JNK pathway is blocked by acting on both the proximal MAP3K, Ask1, and the distal MAP1K, JNK.

JNK activation is also an essential component of Fas- and TNF-induced apoptosis. Hsp70 overexpression can prevent TNFα-stimulated cell killing (Jaattela *et al*., 1992). Hsp70-mediated suppression of JNK activation in TNF-treated cells resulted in inhibited Bid cleavage (Gabai *et al*., 2002). A chaperone-defective mutant of Hsp70 (lacking the C-terminal EEVD sequence) was also effective in preventing TNF-induced JNK activation and Bid cleavage. This mutant form of Hsp70 did not protect cells from heat shock (Mosser *et al*., 2000; Gabai *et al*., 2002). Hsp70 does not appear to provide protection from Fas-induced apoptosis. In contrast, a prior heat shock or overexpression of Hsp70 sensitized cells to Fas killing (Liossis *et al*., 1997). Heat-induced sensitization is not due to altered JNK or ERK signaling and could not be prevented by Hsp70 (Tran *et al*., 2003). Rather, sensitization occurred as a result of down-regulated FLIP levels in the cells that were given a heat stress before Fas treatment.

Although suppression of JNK has been observed when Hsp70 is transiently overexpressed using tet-regulated expression in stable cell lines, adenovirus delivery, or after a mild heat treatment (Gabai *et al*., 1997; Mosser *et al*., 1997; Volloch *et al*., 1998), cell lines that were stably transfected with Hsp70 expression plasmids that provide constitutive high-level expression of Hsp70 do not always show JNK suppression (Mosser *et al*., 1997; Buzzard *et al*., 1998; Jaattela *et al*., 1998). JNK activation is an important component of growth factor signaling pathways and therefore the prolonged inhibition of JNK by Hsp70 may be incompatible with proliferation and could require compensatory adaptation in order for cells to grow in the presence of elevated levels of Hsp70. These contradictory results with stable versus induced expression of Hsp70 question whether inhibition of JNK by Hsp70 is essential for the prevention of apoptosis. In fact, Hsp70 mutants that lack the ATPase domain, or the C-terminal EEVD sequence are effective at blocking heat-induced JNK activation in lymphoid cells but do not prevent caspase activation or inhibit cell death (Mosser *et al*., 2000). However, in fibroblast cells, Hsp70 lacking the ATPase domain blocks JNK activation and provides resistance to heat shock (Yaglom *et al*., 1999). Does this mean that the chaperone activity of Hsp70 is dispensable for inhibition of apoptosis in fibroblasts and inhibition of JNK activation is sufficient? Heat-induced cell death is morphologically different in lymphocytes and fibroblasts. Heat-shock treatment (45°C, 60 min) of fibroblasts results in a nonapoptotic form of reproductive cell death that does not involve caspase activation. Some protection from this form of cell death might be achieved by the holding capability of Hsp70 molecules that contain only the peptide-binding domain and assisted by the newly synthesized Hsp70 that accumulates during recovery. In lymphoid cells, mild heat stress (43°C, 60 min) induces a rapid apoptotic program in which active caspases can be detected within 3–6 h. In this case, inhibition of the apoptotic program requires the refolding activity of intact Hsp70 regardless of whether JNK activation is blocked (Mosser *et al*., 2000).

In addition to stress-induced JNK activation, heat shock also activates kinase pathways that control proliferation and survival (ERK1/2, Akt) (see Figure 4). Activated Akt can prevent JNK activation, through its interaction with JIP1, and also phosphorylate and inactivate the proapoptotic protein Bad (Datta *et al*., 1997; Whitmarsh *et al*., 2001). Prevention of ERK1 or Akt activation during heat shock increases heat sensitivity, whereas overexpression of wild-type ERK1 protects cells from stress (Woessmann *et al*., 1999; Gabai *et al*., 2000; Ma *et al*., 2001). Activation of ERK during heat shock occurs through a combination of increased MEK1/2 activation and impaired ERK phosphatase activity. Although ERK1/2 activation enhances the survival of cells exposed to heat shock, surprisingly, cells containing elevated levels of Hsp70 have a reduced ability to activate ERK (Yaglom *et al*., 2003). This occurred due to an ability of Hsp70 to prevent the heat-induced insolubilization of the dual specificity phosphatases MKP-1 and MKP-3 (Yaglom *et al*., 2003). The chaperone-defective mutant of Hsp70 that lacks the C-terminal sequence EEVD was unable to protect these phosphatases from insolubilization. Inhibition of ERK activation by Hsp70 would appear to be counter-strategic since this would eliminate the cytoprotective effects that could be gained by activating these kinases. Activation of ERK1/2 or Akt is not

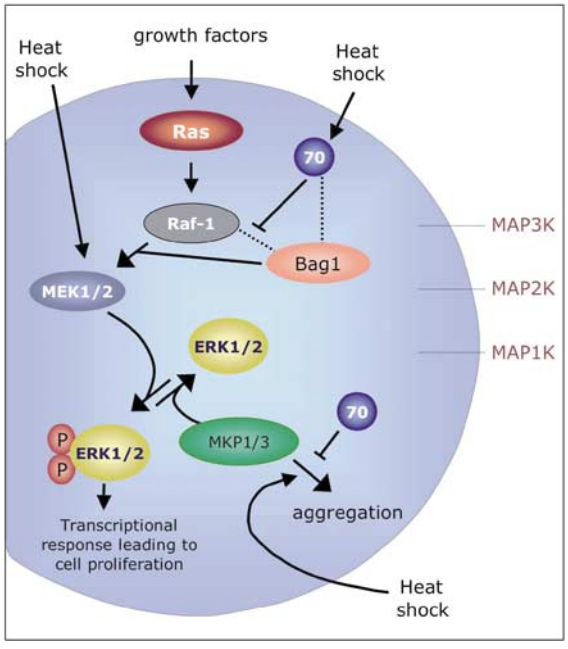

Figure 4 Influence of Hsp70 on the ERK1/2 signaling pathway. Hsp70 coordinates stress signaling to cell cycle arrest by binding to Bag1 and preventing Bag1 from associating with and activating the MAP3 kinase Raf-1. Heat shock causes the aggregation and inactivation of the ERK1/2 phosphatases MKP1 and MKP3, which can be prevented by Hsp70. Dotted lines indicate interactions that have been demonstrated *in vitro*

required for thermotolerance since their inhibition did not prevent it, whereas, inhibiting JNK does prevent the acquisition of thermotolerance (Gabai *et al*., 2000). Perhaps it is more beneficial for the cell to repress proliferation during heat stress, and prevention of ERK1/2 and Akt activation by Hsp70 averts these inappropriate proliferative signals.

ERK1/2 activation can be achieved by Ras-dependent and independent means. Bag1 can activate the ERK1/2 kinase Raf-1 independently of Ras through a direct interaction with the Raf-1 catalytic domain (Wang *et al*., 1996). The Raf-1 interaction domain on Bag1 overlaps with that of Hsp70 and the addition of Hsp70 to preformed Raf-1–Bag1 complexes displaces Raf-1 from Bag1 (Song *et al*., 2001). These results suggest that Hsp70 could transmit stress signals to proliferative signaling pathways through Bag1 as a way to inhibit cell cycle advance during periods of stress. In fact, cells expressing Bag1 mutants with single amino-acid substitutions that prevent their interaction with Hsp70 were unable to arrest DNA synthesis in response to heat shock. This result may also explain why overexpression of Hsp70 has been observed to have a negative effect on cell growth in stably transfected cell lines (Feder *et al*., 1992; Mosser *et al*., 2000).

Hsp90

The chaperone activities of Hsp90, as described for Hsp70, are also regulated by ATP binding and hydrolysis. However, unlike Hsp70, which has the ability to both hold unfolded polypeptides and refold protein substrates to the native state, Hsp90 appears to be more specialized to capture and hold client proteins in intermediate conformations with refolding of denatured substrates requiring transfer to the Hsp70/Hsp40 chaperone machine (Freeman and Morimoto, 1996) (see Figure 1). A role for Hsp90 in chaperoning heat-damaged proteins during stress *in vivo* has not been demonstrated. Inactivation of Hsp82 in *Saccharomyces cerevisiae* has no effect on induced thermotolerance although overexpression of Hsp90 in mammalian cells does provide some level of increased heat resistance (Cheng *et al*., 1992; Heads *et al*., 1995). As suggested, Hsp90 appears to have selective capacities to associate with client proteins involved in cell signaling including nuclear hormone receptors and protein kinases (reviewed in Pratt and Toft, 2003). In the case of the glucocorticoid receptor, the nascent receptor assembles into an Hsp90 complex via Hsp70/Hsp40 and association with Hop. The mature aporeceptor formed upon release of Hsp70/Hsp40/Hop and the binding of the Hsp90 co-chaperone p23 adopts a conformation competent for hormone binding. Likewise, for protein kinases, Hsp90 is required for maturation to the activation-competent state and for signal-induced conformational changes. The discovery that the benzoquinone ansamycin drug geldanamycin (GA) binds to the ATP-binding site of Hsp90 causing disruption of Hsp90-stabilized complexes has provided key insights into the roles of Hsp90 on signaling cascades and cell cycle regulation (Isaacs *et al*., 2003). GA treatment, for example, suppresses the Raf-1–MEK–ERK signaling pathway by disrupting the Raf-1–Hsp90 interaction leading to Raf-1 destruction (Schulte *et al*., 1996).

Stabilizing components of signal transduction pathways, in particular during exposure to cell stress, could therefore account for the cytoprotective properties of Hsp90. GA treatment disrupts Hsp90 interaction with the death domain kinase receptor interacting protein RIP leading to its degradation (Lewis *et al*., 2000). Since RIP binding to the tumor necrosis factor receptor 1 complex (TNFR1) provides a survival signal through activation of nuclear factor κB (NFκB) and inhibition of TNF-induced cell death, the degradation of RIP in GA-treated cells results in increased sensitivity to TNF. However, contrary to the cytoprotective role for Hsp90 in death-receptor mediated killing, overexpression of Hsp90 in a monoblastoid cell line was found to increase its sensitivity to TNF-induced cell killing (Galea-Lauri *et al*., 1996). Other roles for Hsp90 in the stress-induced apoptotic pathway have been suggested by the observation that Hsp90, like Hsp70, binds to Apaf-1 and prevents the cytochrome *c*/dATP-mediated oligomerization of Apaf-1 (Pandey *et al*., 2000b). One suggestion is that Hsp90 appears to compete with cytochrome *c* for binding to Apaf-1, consequently during stress, the amount of Hsp90 associated with Apaf-1 decreases as cytoplasmic levels of cytochrome *c* increase. This suggests that Hsp90 could prevent premature activation of Apaf-1, ensuring that apoptosome formation only occurs when Hsp90 is preoccupied by an abundance of misfolded proteins and the cytosol is flooded with cytochrome *c*.

Hsp27

Hsp27, in contrast to the other major chaperones, is ATP independent, yet can efficiently associate with unfolded proteins and maintain them in a folding-competent state. The chaperone activity of Hsp27 is regulated by heat-induced changes in phosphorylation and oligomerization (reviewed in Haslbeck and Buchner, 2002). Phosphorylation, which occurs during stress, is catalysed by MAPKAP kinase-2, a target of the p38 MAP kinase (Landry *et al*., 1992; Rouse *et al*., 1994). Reactivation of the Hsp27-bound intermediates involves association with the ATP-dependent Hsp70 folding machinery (Ehrnsperger *et al*., 1997). Hsp27, similar to other chaperones, is also a potent cell survival factor that contributes to thermotolerance (Landry *et al*., 1989). Heat-induced nuclear protein aggregation is resolved more rapidly during recovery from severe stress exposure in cells overexpressing Hsp27 (Kampinga *et al*., 1994). Hsp27 binds to F-actin and can prevent disruption of the cytoskeleton resulting from either heat stress or cytochalasin D-induced disruption of actin filaments (Guay *et al*., 1997).

Hsp27 has been shown to interact with and inhibit components of both stress and death-receptor induced apoptotic pathways. In cells expressing higher levels of Hsp27, caspase activation but not cytochrome *c* release was blocked following exposure to etoposide (Garrido *et al*., 1999). In extracts from these cells, the *in vitro* activation of procaspases 9 and 3 by cytochrome *c*–dATP was inhibited by Hsp27 and restored by immunodepletion of Hsp27 from the extracts (Garrido *et al*., 1999). Hsp27 has been shown to interact with released cytochrome *c*, thus providing an additional mechanism to interfere with binding of cytochrome *c* to Apaf-1 (Bruey *et al*., 2000). In contrast, Pandey *et al*. (2000a) have shown that Hsp27 blocked cytochrome *c*/dATP-induced caspase-3 activation without any apparent effect on apoptosome formation, since caspase-9 activation was not prevented. Hsp27 association with procaspase-3 has been suggested to be responsible for the inability of activated caspase-9 to process procaspase-3, although there are disparate observations regarding whether Hsp27 is stably associated with procaspase-3.

The small heat-shock protein family includes the α-crystallin proteins that also have chaperone activity and can protect cells from apoptosis (Kamradt *et al*., 2001). Addition of purified αB-crystallin to cell free extracts prevented procaspase-3 processing by either cytochrome *c*/dATP-mediated apoptosome assembly or by addition of caspase-8. Procaspase-3 processing was inhibited at a step after separation of the large and small subunits, leaving a partially processed p24 (p20) fragment, composed of the large subunit and the prodomain, which could be coimmunoprecipitated with αB-crystallin. Cleavage of procaspase-3 at $IETD^{175}$ is predicted to expose the hydrophobic *β*-sheets that interact to form the caspase-3 dimer and consequently this intermediate could be a substrate for Hsp27 *in vitro*. Whether Hsp27 traps this intermediate in living cells, however, has not been determined.

The relevance of Hsp27 interaction with cytochrome *c* has also been questioned. Although cytochrome *c* can be immunoprecipitated with Hsp27, the amount of cytochrome *c* that is bound to Hsp27 comprises only a very small fraction of the total cytochrome *c* released from mitochondria; therefore, it is unlikely that these events alone have a major inhibitory effect on apoptosome formation (Paul *et al*., 2002). Rather, Hsp27 has been found to act upstream of the apoptosome by preventing cytochrome *c* release (Paul *et al*., 2002; Samali *et al*., 2001). The mechanism responsible for this is not known, however, it has been suggested to involve Hsp27 effects on the cytoskeleton, since overexpression of Hsp27 prevented the F-actin depolymerizing effects of cytochalasin D and subsequent cytochrome *c* release (Paul *et al*., 2002).

In addition to the suggested roles for Hsp27 to associate with procaspases, Hsp27 also inhibits apoptosis by regulating upstream signaling pathways. Hsp27 binds Akt and this interaction is necessary for the activation of Akt in stressed cells (Konishi *et al*., 1997; Rane *et al*., 2003). Akt was shown to phosphorylate Hsp27 leading to the disruption of the Hsp27–Akt complex (Rane *et al*., 2003). When phosphorylated, the oligomeric structure of Hsp27 is shifted from high-molecular multimers to dimers (Lambert *et al*., 1999). The phosphorylated dimers interact with Daxx, preventing Daxx from activating Ask1 in FasR-stimulated apoptosis (Charette *et al*., 2000). This protective mechanism prevents the caspase-independent apoptosis that is mediated by Ask1 activation and not the Fas-induced caspase-dependent killing mediated by FADD-induced procaspase-8 processing.

Heat-shock proteins and cancer

Although heat-shock proteins are only induced transiently after periods of cell stress, they are often constitutively overexpressed in tumor cells. Elevated expression of Hsp90, Hsp70 and Hsp27 either individually or in combination has been widely reported in breast, uterine, renal, osteosarcoma and endometrial cancer, and various leukemias (reviewed in Jaattela, 1999; Helmbrecht *et al*., 2000; Jolly and Morimoto, 2000). The overabundance of these proteins in biopsy samples has been suggested to be of prognostic value in breast, renal and endometrial cancer and in some cases overexpression has been suggested to be an indicator of poor therapeutic outcome. For example, gene-expression profiling of lung cancer tumor samples placed *hsp70* among a group of tumor-specific genes whose expression was indicative of poor patient outcome (Beer *et al*., 2002). Proteome profiling has also revealed that several classes of heat-shock proteins are overexpressed and detected on the cell surface of tumor cells (Shin *et al*., 2003). Targeting heat-shock protein expression or function has been suggested as an effective anticancer strategy based on the speculation that higher levels of chaperones are protective against cell death and increase survival against modalities used in chemotherapy.

The molecular mechanisms responsible for overexpression of heat-shock proteins in cancer cells are not known but may be tumor specific. Transcription of the heat-shock genes is regulated by a transcription factor (HSF1) that senses protein damage and responds by forming homotrimers that possess sequence-specific DNA-binding ability. Transcriptional competence is further controlled by changes in HSF1 phosphorylation. The phosphorylation of S303 and S307 by GSK-3*β* and S363 by JNK inhibits transcriptional activity, whereas phosphorylation of S230 by CaMK II increases activity (Morimoto *et al*., 1992; Pirkkala *et al*., 2001). Variations in the basal level of HSF1 trimers exist among tumor cell lines, which could be the consequence of expression of mutant proteins in transformed cells (Mathew *et al*., 2001). It is not known whether HSF1 is present in an activated state in tumor samples, although the suboptimal tumor environment would be expected to trigger HSF1 activation. HSF1 protein levels were found to be elevated in prostate adenocarcinoma cell lines and were associated with elevated basal levels of

Hsp27 but not Hsp90 or Hsp70, which would be inconsistent with the expected coregulation of all chaperone genes (Hoang *et al*., 2000). In the human A431 carcinoma cell line, increased expression of Hsp90β is attributed to gene amplification and not related to HSF1 levels or activity (Jolly *et al*., 1997). In addition to being stress-responsive, the expression of heat-shock genes is highly regulated at specific periods in the cell cycle and in response to growth factors (Wu and Morimoto, 1985; Milarski and Morimoto, 1986; Ferris *et al*., 1988). In human cells, this contributes to a basal level of heat-shock gene expression that is mediated by interactions between general transcription factors and basal promoter elements (reviewed in Morimoto *et al*., 1992). More complex forms of Hsp70 gene transcription occur in response to the positive regulators c-*myc*, adenovirus E1a and SV40 T-antigen, which are associated with cellular transformation (Kingston *et al*., 1984; Kingston *et al*., 1986; Wu *et al*., 1986a, b, 1987; Williams *et al*., 1989).

The observation that tumor cells often have elevated levels of heat-shock proteins therefore raises intriguing questions regarding whether such cells have a selective prosurvival advantage that contributes to the process of tumorigenesis. Overexpression of Hsp70 or Hsp27 in transformed cell lines enhanced tumorigenic potential when the cells were transferred into syngenic mice (Jaattela, 1995; Garrido *et al*., 1998), and transgenic mice overexpressing Hsp70 were found to develop malignant T-cell lymphomas (Seo *et al*., 1996). Overexpression of Hsp70 in the immortalized Rat-1 cell line, by expression with a recombinant adenovirus, resulted in several features characteristic of oncogenic transformation including loss of contact inhibition, foci formation, growth on soft agar and tumor formation in nude mice (Volloch and Sherman, 1999). All of these features were reversed when expression of Hsp70 was repressed. Importantly, this phenotype was not observed when Hsp70 was overexpressed in primary fibroblasts indicating that Hsp70 could have affected the activity of a signaling pathway that is disrupted in the immortalized cell line. Consistent with the premise that Hsp70 overexpression is associated with tumorigenesis is the observation that reduction of Hsp70 levels in certain tumor cell lines results in growth inhibition and cell death (Wei *et al*., 1995; Nylandsted *et al*., 2000). How uncontrolled cell growth is enhanced by Hsp70 and Hsp90 is not well understood beyond lines of evidence that these chaperones associate with and control the activity of several cell cycle regulators and components of signal transduction pathways (Jolly and Morimoto, 2000); consequently, their association with mutant regulatory proteins could contribute to tumorigenesis by stabilizing active conformations.

In addition to the effects of various chaperones as regulators of signaling processes that influence cell growth, the same heat-shock proteins could as well contribute to tumorigenesis by blocking apoptosis. Oncogenic transformation occurs via a process of acquired mutations that endow cells with the characteristics of unrestrained cell division and suppressed apoptosis. The overabundance of heat-shock proteins in stressed and transformed cells could supply the antiapoptotic side of this equation. However, tumor cells that have high levels of heat-shock proteins are no more resistant to chemotherapeutic drugs than are tumor cells with low levels of heat-shock proteins (Volm *et al*., 1995; Hettinga *et al*., 1996). Unlike thermotolerant cells in which the transiently elevated levels of heat-shock proteins are freely available to interact with new substrates, the pool of heat-shock proteins in tumor cells could be engaged in chaperone complexes and unavailable for use in cytoprotection. Hsp90 has been reported to be present in multichaperone complexes in tumor cells but uncomplexed in normal cells (Kamal *et al*., 2003). Disrupting these complexes with the Hsp90 inhibitor 17-allylaminogeldanamycin (17-AAG) results in proteasomal degradation of Hsp90 client proteins and provides specific antitumor effects (Isaacs *et al*., 2003). The proapoptotic effect of geldanamycin treatment results from the enhanced degradation of prosurvival oncogenes, such as Raf1 and Akt. Reduction of heat-shock protein levels in tumor cells may induce cell death by a similar mechanism. Rather than diminishing an antiapoptotic potential afforded by the elevated expression of heat-shock proteins, reduction of heat-shock protein levels could uncover a phenotype that is incompatible with sustained viability. This might occur for example in growth-factor independent tumors where the oncogenic form of the component within the signal transduction pathway is dependent upon chaperone-mediated stabilization for functionality. Similar to the role postulated for Hsp90 in buffering phenotypic variation in the environment (Rutherford and Lindquist, 1998), heat-shock proteins may buffer the potentially life-threatening effects of the numerous mutant proteins that arise due to the genetic instability of tumor cells. Whether the Hsp70 or Hsp27 that is present in those tumor cells that contain elevated levels of these proteins are freely available to directly inhibit apoptosis or are similarly engaged in chaperoning complexes is not known. Although, overexpression of Hsp70 or Hsp27 can increase resistance to stress-induced apoptosis in cultured cell lines, it is unclear whether the elevated expression of heat-shock proteins in tumor cells contributes to tumorigenesis by preventing stress-induced apoptosis.

A plausible scenario is that oncogenic mutations create an increased demand for chaperone activity within cells expressing protein variants that possess less than optimal folding characteristics. This increased heat-shock protein expression is accomplished either by gain of function mutations in basal transcription factors that increase basal heat-shock promoter activity or by directly increasing the level of activated HSF. This cancer-specific stress response represents an adaptive process that allows cells to configure signal transduction pathways in such a way as to permit unrestrained proliferation. These oncogenic proliferative signals are tied to apoptotic processes that must be circumvented. This requirement may be met by the antiapoptotic function of heat-shock proteins. However, heat-shock

protein expression, in the context of a stress response, suppresses cell division. In part, this is mediated by the ability of Hsp70 to sequester Bag1 resulting in impaired Bag1-activated Raf1 kinase activity (Song *et al*., 2001). Therefore, tumor cells expressing high levels of Hsp70 must also acquire the ability to overcome the growth-inhibitory effect of continuously elevated levels of Hsp70.

The molecular mechanisms that are engaged as cells undergo apoptosis are impeded by multiple safeguards that act to prevent the inappropriate transmission of a death-inducing signal. This includes the antiapoptotic members of the Bcl-2 family that regulate the release of apoptotic mediators from the mitochondria and thereby prevent caspase activation, as well as the inhibitor of apoptosis proteins (IAP) that act as specific caspase inhibitors. The heat-shock proteins serve as another safety measure in apoptosis suppression. However, rather than targeted to specific points in the apoptotic pathway, they instead are able to act at multiple levels. Where they act may have much to do with the particular defect in the apoptotic pathway that is present in the tumor cell line in which their function is being studied since the abundance of various apoptotic regulators is often altered in tumor cells. The ability to stabilize potentially oncogenic conformations of signal transduction molecules and to impair the transmission of apoptotic signals is a powerful and potentially dangerous property for a class of proteins to possess. It is for this reason that their expression is tightly controlled. However, this property also reveals a vulnerability of cancer cells, since targeting chaperone protein function can be expected to disable a wide variety of tumor types regardless of the lesions responsible for the oncogenic phenotype.

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