Presence of a pre-apoptotic complex of pro-caspase-3, Hsp60 and Hsp10 in the mitochondrial fraction of Jurkat cells

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Activation of pro-caspase-3 is a central event in the execution phase of apoptosis and appears to serve as the convergence point of different apoptotic signaling pathways. Recently, mitochondria were found to play a central role in apoptosis through release of cytochrome *c* and activation of caspases. Moreover, a sub-population of pro-caspase-3 has been found to be localized to this organelle. In the present study, we demonstrate that pro-caspase-3 is present in the mitochondrial fraction of Jurkat T cells in a complex with the chaperone proteins Hsp60 and Hsp10. Induction of apoptosis with staurosporine led to the activation of mitochondrial pro-caspase-3 and its dissociation from the Hsps which were released from mitochondria. The release of Hsps occurred simultaneously with the release of other mitochondrial intermembrane space proteins including cytochrome *c* and adenylate kinase, prior to a loss of mitochondrial transmembrane potential. In *in vitro* systems, recombinant Hsp60 and Hsp10 accelerated the activation of pro-caspase-3 by cytochrome *c* and dATP in an ATP-dependent manner, consistent with their function as chaperones. This finding suggests that the release of mitochondrial Hsps may also accelerate caspase activation in the cytoplasm of intact cells.

Keywords: apoptosis/caspase/chaperone/Hsp/
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Introduction

Apoptotic cell death is a fundamental and indispensable process during normal embryonic development, tissue homeostasis and regulation of the immune system (Jacobson *et al.*, 1997). Deregulated apoptosis leading to insufficient or excessive cell death can contribute to human disease, including cancer or degenerative disorders (Thompson, 1995). All forms of apoptosis have a number of common characteristic structural features including a reduction in cell volume, membrane blebbing, chromatin condensation and nuclear DNA fragmentation (Kerr, 1971; Wyllie *et al.*, 1980). These observations suggest that the cell activates a death program, upon which diverse signal-transducing pathways converge (White, 1996).

Apoptosis is associated with the activation of an evolutionarily conserved family of aspartic acid-specific cysteine proteases (Nicholson and Thornberry, 1997), referred to as caspases (Alnemri *et al.*, 1996). There are at least 14 caspases identified in mammalian cells, which are synthesized as inactive precursor molecules (pro-caspases), and are converted by proteolytic cleavage to an active heterodimer (Thornberry and Lazebnik, 1998). Several substrates have been identified that are cleaved by caspases in apoptotic cells, including poly(ADP-ribose) polymerase (PARP) (Nicholson *et al.*, 1995; Tewari *et al.*, 1995), protein kinase C δ (Emoto *et al.*, 1995), the U1-associated 70 kDa protein (Casciola-Rosen *et al.*, 1996), sterol-regulatory element-binding protein (SREBPs) (Wang *et al.*, 1996), huntingtin (Goldberg *et al.*, 1996), the DNA fragmentation factor (DFF) (Liu *et al.*, 1997) and calpastatin (Porn-Ares *et al.*, 1998; Wang *et al.*, 1998). Cleavage of specific substrates has been proposed to either activate death effector molecules or trigger the structural changes characteristic of apoptotic cells.

Evidence is emerging that mitochondria play a key role in the activation or amplification of the caspase cascade via the release of cytochrome *c* from the mitochondrial intermembrane space (Kluck *et al.*, 1997a,b; Yang *et al.*, 1997). Wang and colleagues recently demonstrated that in the presence of dATP, cytochrome *c* participates in a protein–protein interaction with Apaf-1 which leads to the sequential activation of pro-caspases-9 and -3 (Kim *et al.*, 1996; Liu *et al.*, 1996; P.Li *et al.*, 1997; Zhou *et al.*, 1997). The molecular mechanism responsible for the translocation of cytochrome *c* from mitochondria to cytosol during apoptosis is unknown. However, a number of different models have been proposed to explain the mechanism of cytochrome *c* release from the intermembrane space of mitochondria during apoptosis, with some evidence to support each of them. These include selective disruption of the outer membrane as a result of mitochondrial matrix hyperpolarization/matrix swelling (Vander Heiden *et al.*, 1997), pore formation by proteins such as Bax (Manon *et al.*, 1997; Rosse *et al.*, 1998) or rapid loss of $\Delta\Psi_m$ following permeability transition (Kroemer *et al.*, 1998).

The anti-apoptotic properties of Bcl-2 and Bcl-x_L have been attributed to their ability to prevent translocation of cytochrome *c* to the cytosol and thereby interfere with the subsequent activation of cytosolic caspases and apoptosis (Kim *et al.*, 1997; Kluck *et al.*, 1997a; Yang *et al.*, 1997). However, recent studies have demonstrated that apoptosis triggered by microinjection of cytochrome *c* into various cell types is inhibited in cells overexpressing Bcl-x_L (F.Li *et al.*, 1997) or Bcl-2 (Zhivotovsky *et al.*, 1998). Furthermore, Bcl-2 also prolonged cell survival after Bax-induced translocation of cytochrome *c* into cytosol (Rosse *et al.*, 1998). These reports suggest that protection

by anti-apoptotic proteins may also occur downstream of cytochrome *c* release.

Apart from the well-studied regulators of apoptosis (e.g. Bcl-2 family members), heat shock proteins (Hsps) are also implicated in apoptosis (Mehlen *et al.*, 1996; Samali and Cotter, 1996; Gabai *et al.*, 1997; Mosser *et al.*, 1997; Arrigo, 1998; Jaatella *et al.*, 1998; Samali and Orrenius, 1998; Gorman *et al.*, 1999). Hsps are a set of highly conserved proteins, some of which are constitutively expressed in mammalian cells (e.g. Hsp90 and Hsp60), whereas others are induced in response to stress (e.g. Hsp72 and Hsp27). The inducible expression, or constitutive overexpression, of the latter group of Hsps is known to promote cell survival through inhibition of apoptosis (for a review see Samali and Orrenius, 1998). The constitutively expressed Hsps act as chaperones for other cellular proteins, binding to nascent polypeptides to prevent premature folding, to maintain enzymes in active conformation and to facilitate translocation of proteins into organelles (for a review see Lindquist and Craig, 1988). Overexpression of Hsp90, unlike that of Hsp72 or Hsp27, has been demonstrated to increase the rate of apoptosis in the monoblastoid cell line U937 following induction with tumor necrosis factor (TNF) and cycloheximide (Galea-Lauri *et al.*, 1996). However, little is known about the role of Hsp60 and its co-chaperone, Hsp10, in eukaryotic cell death. The Hsp60 and Hsp10 homologs, GroEL and GroES, have been implicated in bacterial cell survival (Volker *et al.*, 1992; Lund, 1995). The majority of mammalian Hsp60 and Hsp10 is localized to mitochondria, the organelles that are remnants of the symbiotic co-existence of bacteria-like organisms and primitive eukaryotes (Bukau and Horwich, 1998). However, Hsp60 is also detectable in extramitochondrial sites of some cells (Soltys and Gupta, 1996). The function of Hsp60 is similar to that of bacterial GroEL, in that it participates in the folding of mitochondrial proteins and facilitates proteolytic degradation of misfolded or denatured proteins (Bukau and Horwich, 1998). The functions of Hsp60 are strongly dependent upon Hsp10, which binds to the Hsp60 molecule to regulate its substrate binding and ATPase activity (Lindquist and Craig, 1988; Ryan *et al.*, 1997; Bukau and Horwich, 1998).

In the present study, we investigated the involvement of Hsp60 and Hsp10 in the regulation of apoptosis, particularly in the light of recent findings that pro-caspase-3 is present in mitochondria (Mancini *et al.*, 1998; Samali *et al.*, 1998). Here, we show that mitochondrial pro-caspase-3 is present in a complex with Hsp60 and Hsp10. Upon induction of apoptosis, pro-caspase-3 was activated and dissociated from the Hsp complex. This occurred simultaneously with the release of Hsps from mitochondria. Recombinant Hsp60 and Hsp10 accelerated pro-caspase activation *in vitro*, suggesting that these Hsps may serve as positive regulators of caspases during apoptosis.

Results and discussion

Subcellular localization of pro-caspase-3, Hsp60 and Hsp10

In order to determine the subcellular distribution of pro-caspase-3, Hsp60 and Hsp10 in the human Jurkat T lymphocytic cell line, cytosolic, nuclear, mitochondrial

Fig. 1. Subcellular localization and interaction of pro-caspase-3, Hsp60 and Hsp10. (A) Western blot analysis of pro-caspase-3, Hsp60 and Hsp10 in cytosolic, mitochondrial, nuclear and microsomal fractions from Jurkat cells (25 μ g of protein loaded per lane). (B) Co-immunoprecipitation of Hsp60, Hsp10 and pro-caspase-3 from Jurkat cell mitochondria. Immunoprecipitations were carried out using antibodies against Hsp60, pro-caspase-3 (CPP32) or Hsc70, followed by Western blotting using antibodies to Hsp60, Hsp10, caspase-3 (p17) or Hsc70. (C) Western blot analysis, using antibodies against caspase-3 (p17), of proteins immunoprecipitated from mitochondrial fractions of control or staurosporine-treated (1 μ M, 4 h) cells with anti-Hsp60.

and microsomal fractions were prepared. Examination of the intracellular distribution pattern of pro-caspase-3 by Western blotting with an anti-caspase-3 antibody (p17) demonstrated that the majority of pro-caspase-3 was localized to the cytosolic fraction but a sizeable amount was also present in the mitochondria (Figure 1A). In contrast, Hsp60 and Hsp10 were present primarily in mitochondria, with small amounts of Hsp60 present in all other fractions (Figure 1A). Pro-caspase-3, Hsp60 and Hsp10 were also detectable in the microsomal fraction. Mitochondria isolated from Jurkat cells exhibited minor contamination by other subcellular organelles, as revealed by assays for specific marker enzymes: lactate dehydrogenase (LDH) (cytosol, 2.3%), inosine diphosphatase (IDPase) (microsomes, 6.8%) and lamin B₁ (nuclei, not detectable). Light mitochondria, which co-sediment with microsomes, may be the source of the Hsps and pro-caspase-3 in this fraction. The presence of relatively high cytochrome *c* oxidase (COX) activity (12.1%) in the

microsomal fraction is indicative of contamination with light mitochondria (data not shown). The percentages were calculated from the total cellular activity for each enzyme.

Pro-caspase-3, Hsp60 and Hsp10 form a complex

Based on the above results, Hsp60 and Hsp10 were found to be localized mainly to mitochondria in the Jurkat cell line. In order to investigate the possibility of an interaction between these chaperones and mitochondrial pro-caspase-3, co-immunoprecipitation studies were performed on extracts from isolated mitochondria. Antibodies against pro-caspase-3 (CPP32) co-precipitated both Hsp60 and Hsp10 from mitochondrial extracts (Figure 1B). Similarly, immunoprecipitation of Hsp60 and Western blotting with antibodies to caspase-3 (p17) and Hsp10 also demonstrated an interaction between these proteins (Figure 1B). While the antibodies to pro-caspase-3 precipitated large amounts of Hsp60, anti-Hsp60 antibody precipitated only a fraction of pro-caspase-3. This observation suggests that most of the mitochondrial pro-caspase-3 was complexed with Hsp60 and Hsp10, but not all of the Hsp60 was involved in this complex, consistent with a relative abundance of Hsp60 in this organelle (~1% of total mitochondrial protein) (Lindquist and Craig, 1988). We also used antibodies directed to Hsc70, a constitutively expressed cytosolic member of the Hsp70 family, as a negative control in our immunoprecipitation studies. Hsc70 which also interacts with outer mitochondrial membrane was detectable in the mitochondrial fraction of Jurkat cells. However, the anti-Hsc70 antibody did not co-precipitate Hsp60, Hsp10 or pro-caspase-3. This suggests that the interaction between Hsp60, Hsp10 and pro-caspase-3 is specific and not due to the 'promiscuity' of Hsps in interacting with a large number of substrates.

To determine the fate of the pro-caspase-3-chaperone complex upon induction of apoptosis, we immunoprecipitated Hsp60 from the mitochondrial fraction of untreated or staurosporine-treated cells. Western blot analysis demonstrated that although most of the Hsp10 and pro-caspase-3 was still co-immunoprecipitated with Hsp60, the active caspase fragments p20 and p17 were not part of this complex (Figure 1C). These results suggest that once activated, caspase-3 dissociates from the Hsps. Such dissociation may be a result of the significant conformational changes that occur in the caspase molecule during its maturation.

Changes in the distribution pattern of Hsp60 and Hsp10 during apoptosis

The release of cytochrome *c* from the mitochondrial intermembrane space during staurosporine-induced apoptosis has been well documented in several cell systems (Bossy-Wetzel *et al.*, 1997; Kim *et al.*, 1997; Kluck *et al.*, 1997a; Yang *et al.*, 1997). Apart from cytochrome *c*, which participates in caspase activation, mitochondrial pro-caspase-3 is also reported to be present in this sub-compartment (Mancini *et al.*, 1998; Samali *et al.*, 1998). Therefore, we hypothesized that the complex of pro-caspase-3 with Hsp60 and Hsp10 should also be localized to this compartment. If that is the case, then one may be able to detect the translocation of Hsp60 and Hsp10 into the cytosol during apoptosis. In order to test this hypothesis, the distribution pattern of Hsp60/10 upon induction

of apoptosis was next studied with respect to other mitochondrial marker proteins. Western blot analysis of the cytosolic fraction prepared from staurosporine-treated cells (1–4 h) demonstrated a release of Hsp60 and Hsp10 into the cytosolic fraction which increased as a function of time (Figure 2A). The release of Hsps coincided with release of cytochrome *c* and adenylate kinase (AK), both of which are localized to the intermembrane space (Figure 2A). Since we detected the release of a large number of proteins from mitochondria, it was necessary to determine whether the release was due to total disintegration of mitochondria or to a selective disruption of the outer mitochondrial membrane. A biochemical study of marker enzymes of the mitochondrial intermembrane space, inner membrane and matrix [AK, COX and citrate synthase (CTS), respectively] in the above samples demonstrated an increased activity of AK in the cytosolic fraction and a concomitant decrease in the pellet. In contrast, the activity of COX and CTS remained relatively unchanged in both fractions (Figure 2B). Taken together, these results suggest that Hsp60, Hsp10, cytochrome *c* and AK are released simultaneously from mitochondria. These findings are in agreement with a recent report showing the simultaneous release of AK and cytochrome *c* during apoptotic death of Jurkat cells (Single *et al.*, 1998; Kohler *et al.*, 1999). Taken together, these observations strongly suggest that the release of intermembrane proteins from mitochondria is likely to be the result of permeabilization of the outer mitochondrial membrane, which initially leads to release of intermembrane space proteins only.

Recently, Babor and colleagues reported the lack of cytochrome *c* release during CD95-mediated apoptosis (Adachi *et al.*, 1998). They suggested that the reason for the apparent differences between their finding and those of others may be due either to short incubation times with anti-CD95 antibody, or that cytochrome *c* release observed by others may be an artifact of cytosol preparation and damage to mitochondria. To eliminate this possibility, we also carried out immunocytochemical studies of cytochrome *c*, AK and Hsp60 distribution in untreated or staurosporine-treated Jurkat cells. The MitoTracker Red CMXRos staining of untreated or staurosporine-treated cells clearly indicated the localization site of mitochondria (data not shown). The immunostaining of untreated cells demonstrated a punctate pattern for cytochrome *c*, AK-2 and Hsps consistent with their mitochondrial localization (Figure 2C, control). Upon induction of apoptosis, cytochrome *c* and AK lost their punctate distribution and a diffuse staining was seen throughout the cell (Figure 2C, staurosporine). The immunostaining of Hsp60 in staurosporine-treated cells revealed a punctate localization also during apoptosis, probably due to the fact that the majority of mitochondrial Hsps are localized to the matrix. These data further strengthen the contention that there is a selective release of mitochondrial intermembrane space proteins during apoptosis.

Release of mitochondrial proteins occurs prior to the loss of $\Delta\Psi_m$

A number of different models have been proposed to explain the mechanism of cytochrome *c* release from the intermembrane space of mitochondria during apoptosis (Manon *et al.*, 1997; Reed, 1997; Vander Heiden *et al.*,

Fig. 2. Release of mitochondrial intermembrane space proteins and changes in $\Delta\Psi_m$. Jurkat cells were treated with 1 μ M staurosporine and the cytosolic fraction was separated from mitochondria at the indicated time points. (A) Western blot analysis of cytosolic proteins (30 μ g) using antibodies against cytochrome *c*, AK-2, Hsp60, Hsp10 or caspase-3. (B) Distribution of mitochondrial marker enzymes AK (circle), COX (square) and CTS (diamond) in the cytosolic fraction (open symbols) and the pellet (filled symbols) after treatment with staurosporine. (C) Confocal microscopy of control and staurosporine-treated cells (4 h) labeled with antibodies against Hsp60, AK-2 and cytochrome *c*.

Fig. 3. Release of mitochondrial proteins into the cytosol occurs prior to loss of $\Delta\Psi_m$. Changes in $\Delta\Psi_m$ in staurosporine-treated (1–4 h) Jurkat cells were measured using JC-1 uptake by flow cytometry in both green (FL-1) and red (FL-3) channels. The cells with high $\Delta\Psi_m$ are seen as a shift to stronger JC-1 fluorescence.

1997; Kroemer *et al.*, 1998; Rosse *et al.*, 1998). To determine which, if any, of the already proposed models apply to our system, alterations in the $\Delta\Psi_m$ were studied using JC-1 dye. The staurosporine-treated cells demonstrated an increase in both green and red fluorescence of JC-1, indicating increased dye uptake as a result of mitochondrial hyperpolarization and/or matrix swelling (Figure 3). However, the red fluorescence was reduced after 6 h (data not shown), probably due to disruption of the mitochondrial inner membrane and concomitant loss of $\Delta\Psi_m$. This pattern of early increase and late decrease in $\Delta\Psi_m$ was not peculiar to JC-1 as similar data were obtained using TMRE (data not shown). These observations suggest that the release of mitochondrial intermembrane space proteins into the cytosol occurs prior to the loss of $\Delta\Psi_m$ and that the latter is a late event during apoptosis in staurosporine-treated cells. Several other groups have also reported that $\Delta\Psi_m$ remained stable in staurosporine-treated HL-60, CEM and HeLa cells for several hours after cytochrome *c* was released from mitochondria (Bossy-Wetzel *et al.*, 1997; Kim *et al.*, 1997; Yang *et al.*, 1997). Based on the results presented here, it appears that the model proposed by Thompson and co-workers (Vander Heiden *et al.*, 1997), which suggests that permeabilization of the outer mitochondrial membrane occurs after a period of hyperpolarization and prior to loss of $\Delta\Psi_m$, is most consistent with our data.

Concomitant activation of mitochondrial pro-caspase-3 and the release of intermembrane space proteins during apoptosis

To determine the relationship between mitochondrial pro-caspase-3 activation and the release of mitochondrial proteins upon induction of apoptosis, we established an

Fig. 4. Concomitant activation of mitochondrial caspase-3 and release of intermembrane space proteins. S-100 cytosolic fractions were prepared from untreated or anti-CD95-treated (200 ng/ml for 30 min) cells (lanes 1 and 3). Aliquots of these S-100 cytosols were then incubated with freshly isolated rat thymus mitochondria for 60 min at 30°C, after which the cytosolic fraction from untreated (lane 2) and treated (lane 4) cells were separated from the respective mitochondria (lanes 5 and 6). The samples were then prepared for (A) Western blot analysis of cytochrome *c*, Hsp60 and Hsp10; and (B) caspase activity (DEVDase) assay.

in vitro assay. This assay was based on incubation of isolated rat thymus mitochondria with the S-100 cytosolic fraction from untreated (inactive) or anti-CD95-treated (activated) Jurkat cells. Anti-CD95 treatment was chosen since it results in the direct activation of caspases via receptor trimerization without the early release of mitochondrial cytochrome *c* into the cytosol. It should be noted that the activated S-100 cytosols did not contain significant levels of cytochrome *c* although high levels of active caspase were present (Figure 4A and B). After 1 h co-incubation at 30°C, mitochondrial and cytosolic fractions were separated. The pre- and post-incubation cytosols along with the mitochondria that were incubated with either of these cytosols were then analyzed for the presence of cytochrome *c*, Hsp60, Hsp10 or active caspase-3. The results clearly demonstrated that the cytosolic fraction from activated cells induced release of cytochrome *c*, Hsp60 and Hsp10 from the rat thymus mitochondria (Figure 4A). A study of the level of pro-caspase-3 in mitochondrial fractions (Figure 4A, lanes 5 and 6) demonstrates that the mitochondria incubated with the activated cytosolic fraction contained considerably lower amounts of pro-caspase-3 (lane 6) as compared with mitochondria incubated with the inactive fraction (lane 5). The reduction of pro-caspase-3 levels in this lane coincided with the appearance of the cleavage products p20 and p17 (Figure 4A, lane 6). The reduction in the levels of pro-caspase-3 may be a result of the release of the pro-enzyme into the

Fig. 5. Acceleration of pro-caspase-3 activation by recombinant Hsp60 and Hsp10 *in vitro*. S-100 cytosolic fractions were incubated with cytochrome *c* (300 nM) and dATP (900 nM), in the presence or absence of recombinant Hsp60 and Hsp10 (200 ng), for 1 h at 30°C. The caspase-3-like DEVDase activity was measured fluorometrically using DEVD-AMC. Where indicated, the reaction mixture was also supplemented with 1 mM ATP.

cytosol, along with other intermembrane space proteins. An alternative and more plausible explanation may be that the pro-caspase-3 was processed to yield the fragments p20 and p17 in the mitochondria, both of which appeared in this fraction simultaneously with a reduction in pro-caspase-3 levels. However, the possibility of contamination with the cytosolic caspase-3 cannot be ruled out completely. The presence of catalytically active caspase-3 in the mitochondrial fraction was confirmed by fluorometric activity assay using the caspase substrate, DEVD-AMC. The results demonstrated that the caspase-3-like activity in the mitochondrial fraction was significantly increased after incubation with the activated cytosol (Figure 4B). Taken together, these results suggest that the cleavage and activation of mitochondrial pro-caspase-3 and the release of intermembrane space proteins occur concomitantly.

Recombinant Hsps stimulate *in vitro* activation of pro-caspase-3

In order to determine the effect of Hsp60 and Hsp10 on caspase activation, we performed an *in vitro* caspase activation assay using the S-100 cytosolic fraction from Jurkat cells which can be activated with exogenous cytochrome *c* and dATP (Hampton *et al.*, 1998). The addition of the recombinant human Hsp60 and Hsp10 (1:1) to the S-100 fraction did not cause any DEVD-AMC cleavage; however, Hsps had a stimulatory effect on the rate of caspase activation by cytochrome *c* and dATP (Figure 5). The stimulation of DEVDase activity by Hsps occurred in an energy-dependent manner in that addition of ATP further stimulated the process. These results suggest that Hsp60 and Hsp10 may regulate pro-caspase-3 activation in their capacity as molecular chaperones, possibly via folding/maintaining pro-caspase-3 in a protease-sensitive conformation in an ATP-dependent manner.

Fig. 6. Subcellular localization of pro-caspase-9. Western blot analysis of pro-caspase-9 in mitochondrial, cytosolic, microsomal and nuclear fractions from Jurkat cells; 25 µg of protein was loaded per lane.

Pro-caspase-9 is localized to both cytosol and mitochondria

The positive regulation of pro-caspase-3 by Hsps appears to require other proteolytic events, e.g. pro-caspase-9 cleavage at Apaf-1, since Hsp60 and Hsp10 had no significant effect on caspase activation on their own. In this regard, one has to keep in mind that pro-caspase-3 is not prone to autoactivation and, therefore, requires a first step cleavage by an IETDase caspase (e.g. caspase-6, -8 or -9) or by another mitochondrial protease. Amongst these caspases, pro-caspase-9, unlike pro-caspase-8, does not require receptor-mediated oligomerization. The Hsp acceleration/facilitation of caspase-9-dependent caspase-3 activation by cytochrome *c* and dATP raised the possibility that this pro-caspase may also be localized to the mitochondrial fraction. As demonstrated in Figure 6, pro-caspase-9 was found in both the cytosolic and mitochondrial fraction of Jurkat cells. A similar finding was reported by Kroemer and co-workers (Susin *et al.*, 1999), which is consistent with the data presented in the current study. Therefore, mitochondrial caspase-9 would be a candidate for the activation of mitochondrial pro-caspase-3. The possible presence in mitochondria of Apaf-1, which mediates cytochrome *c*-dependent caspase activation in the cytosol, remains to be determined.

Conclusions

The current study was aimed at investigating the involvement of Hsp60 and Hsp10 in the regulation of apoptosis with respect to caspase activation. We have demonstrated that the 'mitochondrial chaperones' Hsp60 and Hsp10 are part of a complex involving pro-caspase-3, which is localized to the intermembrane space of mitochondria. This interaction was specific to these chaperones since antibodies to Hsc70 did not co-precipitate pro-caspase-3. The complex of Hsps with pro-caspase-3 dissociates during apoptosis upon processing of pro-caspase-3 to the mature enzyme. The fact that the caspase processing was not hindered by the presence of Hsps implies that either the Hsps act as docking molecules with a passive role in the maturation process, or that they have an active but subtle chaperone function in the process. In *in vitro* experiments, recombinant Hsp60 and Hsp10 demonstrated a stimulatory effect on caspase activation by cytochrome *c* and dATP in S-100 cytosol. This phenomenon was energy-dependent in that the addition of ATP further stimulated caspase activation. The ATP dependency of the process suggests that Hsp60 and Hsp10 may regulate pro-caspase-3 activation in their capacity as molecular chaperones, possibly via folding/maintaining pro-caspase-3 in a protease-sensitive conformation. The maturation of pro-caspase-3 requires conformational changes in the protein molecule that may reduce its affinity for binding to Hsps. Loss of affinity for p17 and p12 subunits of active caspase-3 may explain their dissociation from the Hsps.

The dissociation of Hsps from active caspase-3 co-

incided with the release from mitochondria of Hsps and other intermembrane space proteins including cytochrome *c* and AK. The release of cytochrome *c* has been well documented in several cell systems (Bossy-Wetzel *et al.*, 1997; Kim *et al.*, 1997; Kluck *et al.*, 1997a; Yang *et al.*, 1997) and the release of AK was recently reported to occur simultaneously with that of cytochrome *c* (Single *et al.*, 1998; Kohler *et al.*, 1999). However, the significance of AK release or its contribution to the apoptotic process, if any, is not clear. The only conclusion that may be drawn from this observation at the moment is that the release of intermembrane space proteins is not selective for cytochrome *c*.

Regarding the function of Hsp60 and Hsp10 in this pre-apoptotic complex, the *in vitro* studies were indicative of an auxiliary role for Hsp60 and Hsp10 in caspase activation by cytochrome *c*. Therefore, it is likely that dissociation of Hsps from active mitochondrial caspase-3 and their redistribution may facilitate their recycling through binding to free cytosolic pro-caspase-3 upon release from mitochondria. In our experiments, Hsps alone were not able to activate pro-caspase-3 and required cytochrome *c* and dATP to initiate the process. However, the rapid activation of caspases by the optimal concentrations of cytochrome *c* and dATP used in the present study may partially mask a more significant role for these chaperones in caspase processing *in vivo*. Under the present conditions, Hsp60 and Hsp10 demonstrate an auxiliary or secondary role in caspase activation and, as demonstrated by Nicholson and co-workers (Xanthoudakis *et al.*, 1999), Hsp60 may play a more significant role in caspase activation independently of the cytochrome *c* system. Therefore, the possibility of direct chaperone-mediated caspase activation under physiological conditions cannot be ruled out completely, and an alternative, more efficient mechanism for caspase activation by Hsps may exist.

How mitochondrial caspases are activated or if the activation occurs in intact mitochondria is not clear. We have shown here that incubation of CD95-activated S-100 cytosol with isolated mitochondria is sufficient to induce processing of mitochondrial pro-caspase-3. This process occurred simultaneously with the release of intermembrane space proteins, suggesting that disruption of the outer mitochondrial membrane may allow a cytosolic factor to activate mitochondrial pro-caspases. The fact that cytochrome *c*, pro-caspase-3, pro-caspase-9 and Hsps are all present in mitochondria suggests that the presence of Apaf-1 alone may initiate the activation process. Nevertheless, whether Apaf-1 is present in this organelle is currently unknown. The release of intermembrane space proteins in CD95-mediated apoptosis is thought to be via cleavage of Bid by caspase-8 (Li *et al.*, 1998; Luo *et al.*, 1998). The early disruption of the outer mitochondrial membrane which leads to release of intermembrane space proteins may also make the mitochondrial caspases accessible to cytosolic Apaf-1.

Of the different proposed mechanisms for cytochrome *c* release during apoptosis, the model by Thompson and co-workers (Vander Heiden *et al.*, 1997) is the one most consistent with the data from staurosporine-induced apoptosis we have presented here. The early increase in JC-1 uptake observed in this study occurred concomitantly with the loss of cytochrome *c* and caspase processing;

however, a drop in $\Delta\Psi_m$ was observed later in the apoptotic process (after 6 h). The lack of a decrease in $\Delta\Psi_m$ of staurosporine-treated cells observed in this study is in agreement with previous reports (Bossy-Wetzel *et al.*, 1997; Kim *et al.*, 1997; Vander Heiden *et al.*, 1997; Yang *et al.*, 1997).

In summary, we have demonstrated that mitochondrial pro-caspase-3 is present in a complex with the regulatory chaperones Hsp60 and Hsp10. These chaperones appear to make pro-caspase-3 more susceptible to cytochrome *c*/dATP activation. Induction of apoptosis resulted in the activation of mitochondrial pro-caspase-3, which led to the dissociation of mature caspase-3 from Hsps. The Hsps were then released from the mitochondrial intermembrane space with cytochrome *c* and AK prior to the loss of $\Delta\Psi_m$. The dissociation of the complex occurred concomitantly with the release of mitochondrial proteins. Since mitochondria contain high concentrations of these chaperones, we propose that one of their functions may be to regulate pro-caspase-3 activation in mitochondria and that the release of these Hsps from mitochondria may participate in the amplification of the caspase cascade in the cytosol.

Materials and methods

Reagents

Anti-adenylate kinase antibody (AK-2) against the intermembrane space isoform of the enzyme was a gift from Takafumi Noma. Anti-caspase-9 was a gift from Xiaodong Wang. Anti-caspase-3 (CPP32) antibodies were purchased from Transduction Laboratories. Anti-cytochrome *c* antibody was a gift from Ronald Jemmerson. Anti-Fas antibodies were from MBL. Anti-lamin B₁ was from NovaCosta Laboratories. Anti-p17 (caspase-3) antibodies were a gift from Donald W. Nicholson. Anti-Hsp60, -Hsp10 (Cpn10) and -Hsc70 antibodies and the recombinant Hsps were purchased from StressGen Biotechnologies Corp. Horseradish peroxidase (HRP)-conjugated secondary antibodies were from Pierce. Fluorescein isothiocyanate (FITC)-labeled goat anti-mouse antibodies were from Sigma, and the ECL detection kit was obtained from Amersham. DEVD-AMC was from Enzyme Systems Products, and protein A-Sepharose was purchased from Pharmacia. MitoTracker Red CMXRos, TMRE and JC-1 were purchased from Molecular Probes. All other reagents were purchased from Sigma.

Cell culture and induction of apoptosis

The Jurkat T cell line, from the European Tissue and Cell Collection, was cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM glutamine and 1% penicillin/streptomycin in a humidified atmosphere of 5% CO₂ in air at 37°C. Cells were treated with 1 μ M staurosporine (1–4 h) to induce apoptosis.

Preparation of subcellular fractions

Cells were washed in buffer A (100 mM sucrose, 1 mM EGTA, 20 mM MOPS, pH 7.4) and resuspended in buffer B [buffer A plus 5% Percoll, 0.01% digitonin and a cocktail of protease inhibitors: 10 μ M aprotinin, 10 μ M pepstatin A, 10 μ M leupeptin, 25 μ M calpain inhibitor I and 1 mM phenylmethylsulfonyl fluoride (PMSF)]. After 15 min incubation on ice, unbroken cells and nuclei were pelleted by centrifugation at 2500 *g* for 10 min. The supernatant was centrifuged at 15 000 *g* for 15 min to pellet mitochondria which were resuspended in buffer C (300 mM sucrose, 1 mM EGTA, 20 mM MOPS pH 7.4 and the cocktail of protease inhibitors). The supernatant was centrifuged further at 100 000 *g* for 1 h. The resultant supernatant and the pellet were designated as the cytosolic and microsomal fractions, respectively. The nuclear pellet was resuspended in 10 mM Tris–HCl, pH 7.5, 2.5 mM KCl, 2.5 mM MgCl₂ and isolated after centrifugation at 90 000 *g* for 30 min through 2.1 M sucrose in 50 mM Tris–HCl pH 7.5, 5 mM MgCl₂.

Characterization of subcellular fractions

Each fraction was tested for cross-contamination with others, using LDH (Farrell *et al.*, 1990), COX (Sottocasa *et al.*, 1967) and IDPase (Nilsson

and Dallner, 1975) activities as specific markers of cytosol, mitochondria and microsomes, respectively. Enzyme activities were determined per mg of protein for each fraction. The level of cross-contamination was calculated as the percentage of the total cellular enzyme activity in the different fractions. Nuclear contamination of the other fractions was tested by Western blotting using anti-lamin B₁ antibody (1:1000).

Electrophoresis and Western blot analysis

Equal amounts of protein were resuspended in SDS–PAGE buffer (62.5 mM Tris–HCl, pH 6.8, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 1 mM PMSF) and boiled in a water bath for 5 min. Lysates were stored at –20°C until further analysis. Proteins were separated under reducing conditions for 2 h at 120 V in 12% SDS–polyacrylamide gels. Gels were transblotted onto nitrocellulose membranes at 100 V for 2 h. Membranes were blocked overnight in high salt buffer (50 mM Tris base, 500 mM NaCl, 0.05% Tween-20) containing 5% bovine serum albumin and then incubated for 1 h with anti-p17 (1:5000), -caspase-9 (1:2000), -cytochrome *c* (1:2500), -AK-2 (1:1000), -Hsp60 (1:1000) and -Hsp10 (1:2000) or -Hsc70 (1:5000), followed by incubation with HRP-conjugated secondary antibodies (Pierce), goat anti-mouse or anti-rabbit (1:10 000). Protein bands were visualized by ECL.

Immunoprecipitation

Samples containing 50 μ g of protein in 100 μ l of immunoprecipitation buffer (100 mM sucrose, 1 mM EGTA, 20 mM MOPS pH 7.4, the cocktail of protease inhibitors, 0.5% Triton X-100 and 0.5% NP-40) were centrifuged at 20 000 *g* for 15 min to remove particulate material. The supernatant was then incubated with anti-Hsp60 (1:100), -caspase-3 (CPP32) (1:500) or -Hsc70 (1:500) with constant agitation at 4°C for 1 h. This was followed by the addition of 25 μ l of 50% protein A-Sepharose and incubation with constant agitation at 4°C for a further 1 h. The pellet was collected after five washes in immunoprecipitation buffer and was prepared for Western blotting.

Rapid preparation of cytosolic fraction

Cells were washed and resuspended in 100 μ l of mitochondrial buffer (70 mM Tris base, 0.25 M sucrose and 1 mM EDTA, pH 7.4). An equal volume of digitonin (2 mg/ml dissolved in MES buffer: 19.8 mM EGTA, 19.8 mM EDTA, 0.25 M D-mannitol and 19.8 mM MES, pH 7.4) was added to the samples until ~90% of cells were permeabilized, as determined by trypan blue uptake. After centrifugation at 900 *g* for 2 min, the supernatant was centrifuged further at 20 000 *g* for 5 min to obtain the cytosolic fraction. The pellet (mitochondria-enriched fraction) was resuspended in the mitochondrial buffer. Samples were then prepared for Western blotting or assays for the measurement of mitochondrial marker enzymes.

Mitochondrial marker enzymes

The activities of AK, COX and CTS, markers for the mitochondrial intermembrane space, inner membrane and matrix, respectively, were assayed both in cytosolic fraction and mitochondria-enriched pellets. AK activity was measured spectrophotometrically as previously described (Sottocasa *et al.*, 1967; Scarlett and Murphy, 1997). COX activity was measured as described above, and CTS was assayed according to Stere's protocol (Scarlett and Murphy, 1997).

Changes in mitochondrial transmembrane potential

To measure $\Delta\Psi_m$, control or staurosporine-treated cells were incubated with 2 μ g/ml JC-1 (Molecular Probes) added to the culture media for 30 min at 37°C. Cells were then washed in phosphate-buffered saline (PBS) and analyzed on a FACScan flow cytometer (Becton Dickinson) using both the green (FL-1) and red (FL-3) channels. At least 10 000 events were collected per sample.

Immunofluorescence microscopy

Jurkat cells were cultured under normal conditions. Apoptosis was induced by treatment with 1 μ M staurosporine. Four hours later, the cells were rinsed once with PBS and then fixed in 2% formaldehyde for 5 min at room temperature. Cells were then permeabilized with 100% methanol (–20°C) for 5 min, followed by incubation in blocking buffer (2% horse serum/0.5% Triton X-100 in PBS) for 20 min. Incubations with primary antibodies (anti-Hsp60 at 1:60, anti-cytochrome *c* 1:40 and anti-AK-2 at 1:60) were carried out for 45 min at room temperature in a humidified chamber. Excess antibody was removed by washing the coverslips with PBS (three times, 5 min). This was followed by incubation with appropriate FITC-conjugated secondary antibodies for 45 min at room temperature, with protection against light. Images were collected

on a confocal system (Bio-Rad 1024) with the built-in three-channel imaging settings. Labeling of mitochondria within intact cells was also performed with MitoTracker Red CMXRos according to the manufacturer's protocol.

In vitro system for detection of release of mitochondrial proteins

S-100 cytosolic fractions from untreated or anti-CD95-treated (200 ng/ml, 30 min) cells were prepared as previously described (Hampton *et al.*, 1998) and incubated with freshly isolated rat thymus mitochondria for 60 min at 30°C. After two rounds of centrifugation (at 20 000 *g* for 5 min), the mitochondrial and the cytosolic fractions were separated, and the mitochondria were washed twice in mitochondrial buffer and prepared for Western blotting or caspase assay.

DEVD-AMC assay

Cells were collected by centrifugation at 1000 *g* for 5 min and the medium was removed. After washing once with PBS at 4°C, the cell pellet was resuspended with 25 µl of PBS. The cells (2–5 × 10⁶ in 25 µl) were frozen rapidly by transferring them directly into a 96-well plate which was floating on liquid nitrogen. The cells were stored at –80°C until required. Measurement of DEVD-AMC cleavage was modified from Nicholson *et al.* (1995) as previously described (Hampton *et al.*, 1998). Immediately before the assay, 50 µl of the buffer [100 mM HEPES (pH 7.25), 10% sucrose, 0.1% CHAPS, 5 mM dithiothreitol (DTT), 10% NP-40] containing 50 µM DEVD-AMC was added to each well. Substrate cleavage leading to the release of free AMC (excitation 355 nm, emission 460 nm) was monitored with time at 37°C. Fluorescent units were converted to pmoles of AMC released using a standard curve generated with free AMC.

In vitro caspase activation

S-100 cytosolic fractions from Jurkat cells were incubated with cytochrome *c* (300 nM) and dATP (900 nM) at 30°C for 60 min, to activate pro-caspase-3. To determine the effects of recombinant Hsp60 and Hsp10 on pro-caspase-3 activation, 200 ng/ml of these proteins were included in the incubation mixture in the presence of 1 mM ATP.

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